

Cannabinoid WIN 55,212-2 Regulates TRPV1 Phosphorylation in Sensory Neurons*

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Nathaniel A. Jeske[‡], Amol M. Patwardhan[§], Nikita Gamper^{¶1}, Theodore J. Price^{§2}, Armen N. Akopian[‡], and Kenneth M. Hargreaves^{‡53}

From the Departments of [‡]Endodontics, [§]Pharmacology, and [¶]Physiology, University of Texas Health Science Center, San Antonio, Texas 78229-3900

Cannabinoids are known to have multiple sites of action in the nociceptive system, leading to reduced pain sensation. However, the peripheral mechanism(s) by which this phenomenon occurs remains an issue that has yet to be resolved. Because phosphorylation of TRPV1 (transient receptor potential subtype V1) plays a key role in the induction of thermal hyperalgesia in inflammatory pain models, we evaluated whether the cannabinoid agonist WIN 55,212-2 (WIN) regulates the phosphorylation state of TRPV1. Here, we show that treatment of primary rat trigeminal ganglion cultures with WIN led to dephosphorylation of TRPV1, specifically at threonine residues. Utilizing Chinese hamster ovary cell lines, we demonstrate that Thr¹⁴⁴ and Thr³⁷⁰ were dephosphorylated, leading to desensitization of the TRPV1 receptor. This post-translational modification occurred through activation of the phosphatase calcineurin (protein phosphatase 2B) following WIN treatment. Furthermore, knockdown of TRPA1 (transient receptor potential subtype A1) expression in sensory neurons by specific small interfering RNA abolished the WIN effect on TRPV1 dephosphorylation, suggesting that WIN acts through TRPA1. We also confirm the importance of TRPA1 in WIN-induced dephosphorylation of TRPV1 in Chinese hamster ovary cells through targeted expression of one or both receptor channels. These results imply that the cannabinoid WIN modulates the sensitivity of sensory neurons to TRPV1 activation by altering receptor phosphorylation. In addition, our data could serve as a useful strategy in determining the potential use of certain cannabinoids as peripheral analgesics.

Cannabinoids have been shown to exert anti-inflammatory and anti-hyperalgesic effects via peripheral site(s) of action in several pain models (1–5). These effects are thought to be medi-

ated by cannabinoid type 1 (CB1)⁴ and/or 2 (CB2) receptor activation, both peripherally and centrally (4–7). Cannabinoids could exert their effects by acting on CB1/CB2 receptors located on sensory neurons and/or other peripheral cells influencing sensory neuronal function (8). However, there is a <5–10% co-localization of metabotropic CB1/CB2 receptors with nociceptive neuronal markers such as TRPV1 (transient receptor potential subtype V1) and calcitonin gene-related peptide in trigeminal and dorsal root ganglion neurons (9–11), suggesting that cannabinoids could act on nociceptors through non-CB1/CB2 receptor mechanism(s). Certain cannabinoids have been shown to activate channels such as TRPV1, including arachidonyl-2-chloroethylamide (ACEA) (12), *N*-arachidonyldopamine (13), and anandamide (14), as well as TRPA1 (transient receptor potential subtype A1), including Δ^9 -tetrahydrocannabinol (15). In addition, the synthetic cannabinoid *R*(+)-WIN 55,212-2 (WIN) has demonstrated non-CB1/CB2 receptor activities in trigeminal ganglia (11). The results from these studies suggest that cannabinoids may activate calcium channel function similar to non-cannabinoid transient receptor potential agonists, including the ability to desensitize channel activity.

The transient receptor potential channel TRPV1 is a nonselective cation channel that responds to various stimuli, including heat (>42 °C), protons, capsaicin, and certain cannabinoids (14, 16–19). TRPV1 is principally expressed in C-type nociceptive afferent neurons throughout the periphery and has been demonstrated to play a critical role in the induction of thermal hyperalgesia in inflammatory pain models (16, 20, 21). There is general agreement that TRPV1 controls nociceptor sensitization to thermally noxious stimuli by inflammation-induced post-translational modifications, including phosphorylation (22, 23). Conversely, dephosphorylation of TRPV1 can lead to pharmacological desensitization of its activation by chemical stimuli (24–26).

The desensitizing effect of channel activation has been utilized clinically to reduce the afferent transmission of painful stimuli (27). Repeated activation of TRPV1 by chemical stimuli results in calcium-dependent desensitization of the receptor

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¹ Present address: Inst. of Membrane and System Biology, University of Leeds, Leeds LS2 9JT, UK.

² Present address: Dept. of Anesthesia, McGill University, Montreal, Quebec H3G 1Y6, Canada.

³ To whom correspondence should be addressed: Dept. of Endodontics, University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX 78229-3900. Tel.: 210-567-3388; Fax: 210-567-3389; E-mail: Hargreaves@UTHSCSA.edu.

⁴ The abbreviations used are: CB1, cannabinoid type 1; CB2, cannabinoid type 2; ACEA, arachidonyl-2-chloroethylamide; WIN, WIN 55,212-2; CHO, Chinese hamster ovary; TG, trigeminal ganglion; ANOVA, analysis of variance; PBS, phosphate-buffered saline; siRNA, small interfering RNA; TRITC, tetramethylrhodamine isothiocyanate; HPLC, high performance liquid chromatography; GFP, green fluorescent protein; PLC β , phospholipase C β ; PHD, pleckstrin homology domain; *I*_{CAP}, inward capsaicin current.

(24). Specifically, capsaicin has been shown to lead to dephosphorylation of TRPV1, thereby desensitizing the receptor (25). As the receptor ion channel is activated, calcium ions enter the cell and stimulate calcium-dependent signaling mechanisms, including calcineurin-dependent dephosphorylation of TRPV1 (26). Coincidentally, calcium-dependent sensitization of the receptor can also occur through activation of Ca^{2+} /calmodulin-dependent kinase II (28) and protein kinase C (29). The balance between calcium-stimulated kinase and phosphatase activities results in a tightly regulated system responsible for modulating TRPV1 activity.

In this study, we examined whether certain cannabinoids can regulate the phosphorylation state of TRPV1, resulting in modulation of receptor activities. Furthermore, we demonstrate that treatment with the cannabinoid WIN results not only in calcineurin activation and dephosphorylation of the TRPV1 receptor at Thr¹⁴⁴ and Thr³⁷⁰, but does so in a manner dependent upon TRPA1 coexpression.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection of cDNA—Trigeminal ganglia were removed bilaterally from male Sprague-Dawley rats (200–250 g; Charles River Laboratories, Wilmington, MA) and dissociated by treatment with collagenase (Worthington) for 30 min, followed by treatment with trypsin (Sigma) for 15 min and DNase I (Roche Applied Science) for 5 min. Cells were centrifuged and resuspended between each treatment with Pasteur pipettes. Cells were centrifuged; aspirated; resuspended in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum (Invitrogen), 250 ng/ml nerve growth factor (Harlan Sprague Dawley, Inc., Indianapolis, IN), 1% 5-fluorodeoxyuridine (Sigma), 1% penicillin/streptomycin (Invitrogen), and 1% L-glutamine (Sigma); and then plated onto plates coated with poly-D-lysine. Cultures were maintained at 37 °C and 5% CO₂ and grown in 10-cm plates for 5–7 days for phosphorylation experiments. Chinese hamster ovary (CHO) cells were utilized for heterologous expression of cDNA constructs. They were maintained at 37 °C and 5% CO₂ and transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Trigeminal ganglion (TG) neurons were transfected using a PDS-1000/He biolistic system (Bio-Rad) according to the manufacturer's instructions.

cDNA Constructs and Site-directed Mutagenesis—Rat TRPV1 cDNA was kindly provided by Dr. David Julius (University of California, San Francisco, CA), and mouse TRPA1 cDNA was kindly provided by Dr. Ardem Patapoutian (Scripps Research Institute, San Diego, CA). The entire coding sequence of mouse TRPA1 (30), apart from the start codon, was used to generate a Myc-tagged mouse TRPA1 construct in pCMV-Myc (Clontech). pEGFP-N1 cDNA was purchased from Clontech, and bradykinin type 2 and muscarinic type 1 receptor cDNAs were purchased from the University of Missouri cDNA Resource Center (Rolla, MO). Site-directed mutagenesis was performed using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. Rat TRPV1(T144A) cDNA was kindly provided by Dr. Carla Nau (Friedrich-Alexander University, Erlangen, Germany). To create rat TRPV1(T370A), the forward primer used

was 5'-CCAGGAAGTTCGCCGAATGGGCCTATGGG. To create rat TRPV1(T704A), the forward primer used was 5'-GCAGAGAGCCATCGCCATCCTGGATACAG. All mutations were confirmed by sequencing at the Advanced Nucleic Acids Core Facility of the University of Texas Health Science Center at San Antonio.

Immunoprecipitation and Western Blot Analysis—For each experimental condition, cells were treated with the appropriate compounds and harvested as described previously (31). Protein determination was completed using the Bradford assay (Bio-Rad) as recommended by the manufacturer. For radioactivity experiments, 10-cm plates of trigeminal ganglia were incubated with 1 mCi of [³²P]orthophosphate (PerkinElmer Life Sciences), and 6-cm plates of CHO cells were incubated with 125 μCi for 4 h at 37 °C in phosphate-free Dulbecco's modified Eagle's medium. Following harvesting, cleared lysates were immunoprecipitated with 1 μg of anti-TRPV1 antiserum (Ab-2, Calbiochem), resolved on 15% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). Western blots were either exposed overnight to film at –80 °C for autoradiography or blocked in 5% bovine serum albumin in Tris-buffered saline/Tween 20 and visualized using anti-TRPV1 (Ab-1, Calbiochem), anti-phosphoserine (Calbiochem), or anti-phosphothreonine (Calbiochem) antibody, followed by the appropriate horseradish peroxidase-conjugated secondary antisera and enhanced chemiluminescence (GE Healthcare) following the manufacturer's instructions.

Other antibodies used in these experiments included rabbit anti-TRPA1 polyclonal antibody, which recognizes an N-terminal epitope (COOH-KRSLRRVLRPEERKE), and anti-FKBP12 antibody (Affinity BioReagents, Golden, CO). Fig. 1 (A–G) illustrates the specificities of the anti-TRPV1 and anti-TRPA1 antibodies used.

Autoradiography and Western blot results were scanned and quantified using NIH Image Version 1.62. Background optical densities were subtracted from band densities to calculate accurate optical measurements of band intensity. All autoradiographic and phospho-specific bands were normalized to values obtained from total immunoprecipitated TRPV1. Results are representative of three to five independent experiments, and statistical significance was determined using two-way analysis of variance (ANOVA) or a paired *t* test as appropriate.

Electrophysiology—All recordings were made in a perforated patch voltage-clamp configuration at a holding potential of –60 mV. Recordings were carried out at 22–24 °C from transiently transfected CHO cells (48 h post-transfection) using an Axopatch 200B amplifier and pCLAMP 9.0 software (Axon Instruments, Union City, CA). Cells were transfected with the indicated cDNAs along with the pEGFP-N1 vector for identification of channel-expressing cells. Data were filtered at 0.5 kHz, and samples were filtered at 2 kHz. Borosilicate pipettes (Sutter Instrument Co., Novato, CA) were polished to resistances of 4–7 megaohms in perforated patch pipette solution. If necessary, access resistance was compensated by 40–80% to 10–15 megaohms.

All recordings are made in the presence of 2 mM Ca²⁺ in external solution. Standard external solution contained 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM D-glucose,

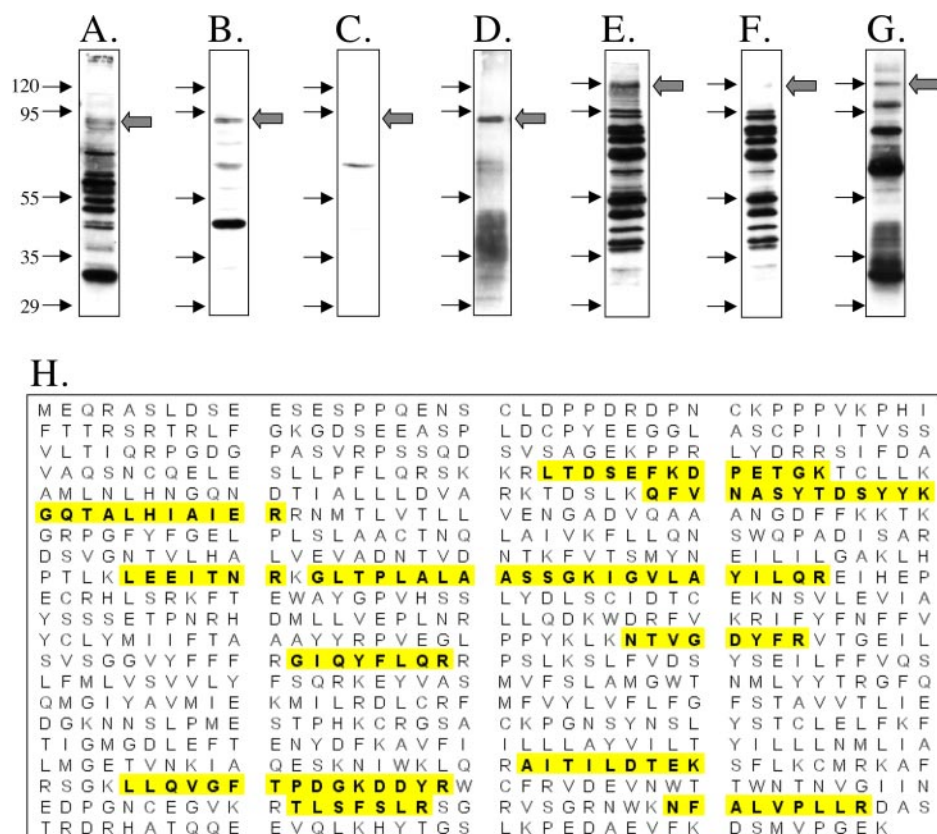


FIGURE 1. Western blot and mass spectrometric data illustrating the specificity of the antibodies used to identify TRPV1 and TRPA1. Western blot data were generated utilizing several antibodies and sources, including anti-TRPV1 Ab-2 on 50 μ g of TG cell lysate (A), anti-TRPV1 Ab-1 on 50 μ g of TG cell lysate (B), anti-TRPV1 Ab-1 preincubated with 25 μ g of antigenic peptide (Calbiochem) on 50 μ g of TG cell lysate (C), anti-TRPV1 Ab-1 on 25 μ g of cell lysate from CHO cells rat transiently transfected with TRPV1 (D), antibody against the TRPA1 N terminus on 50 μ g of TG cell lysate (E), antibody against the TRPA1 N terminus preincubated with 100 μ g of antigenic peptide on 50 μ g of TG cell lysate (F), and antibody against the TRPA1 N terminus on 25 μ g of cell lysate from CHO cells transiently transfected with mouse TRPA1 (G). Black arrows indicate size markers (in kilodaltons), and gray arrows indicate the immunoreactive band of interest. Western blot results are representative of two independent experiments. TRPV1 was immunoprecipitated from 500 mg of TG cell lysate, resolved on 15% SDS-polyacrylamide gel, stained with Coomassie Blue, excised from the gel, and analyzed for trypsin digestion by mass spectrometry. 13 unique peptides were identified (H, highlighted in yellow), providing 15% coverage of the amino acid sequence for rat TRPV1 (GenBank™ accession number AF029310). Mascot analytical software made a 100% match between the cumulative peptides and rat TRPV1.

and 10 mM HEPES (pH 7.4). The pipette solution for the perforated patch consisted of 110 mM potassium methanesulfonate, 30 mM KCl, 1 mM MgCl₂, 10 mM HEPES (pH 7.3), and 250 μ g/ml amphotericin B (Sigma). Drugs were applied using a computer-controlled pressure-driven eight-channel system (ValveLink8, AutoMate Scientific, Inc, San Francisco, CA).

Ca²⁺ and Fluorescence Imaging in TG Neurons—To measure intracellular Ca²⁺ levels, the dye Fura-2 acetoxymethyl ester (2 μ M; Molecular Probes, Carlsbad, CA) was loaded for 30 min at 37 °C into cells in the presence of 0.05% PLURONIC F-127 (Calbiochem). Fluorescence was detected with a Nikon Eclipse TE2000-U microscope fitted with a \times 40/1.30 numerical aperture Fluor objective. Fluorescence images from excitation wavelengths were collected and analyzed with MetaFluor software (MetaMorph imaging system, Universal Imaging Corp., Downingtown, PA). The net change in Ca²⁺ was calculated by subtracting the basal Ca²⁺ level (mean value collected for 60 s prior to agonist addition) from the peak Ca²⁺ level achieved after exposure to the agonists. Ratiometric data were converted

to [Ca²⁺]_i (in micromolar) using the following equation: [Ca²⁺]_i = K*(R - R_{min})/(R_{max} - R), where R is the 340/380 nm fluorescence ratio. R_{min}, R_{max}, and K* (0.1, 1.6, and 0.65 μ M, respectively) were measured according to a previously described method (32).

Calcineurin Activity Assay—Cultured TG neurons (BIOMOL International, L.P., Plymouth Meeting, PA) were grown for 5 days and harvested following the manufacturer's instructions. Cells were rinsed twice with 1 \times phosphate-buffered saline (PBS) at 4 °C and harvested in 50 mM Tris (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.2% Nonidet P-40 with protease inhibitor mixture. Cells were gently triturated via 10 passes through a 20-gauge needle and lysed via three freeze/thaw cycles (45 s of liquid N₂ and 45 s of a 30 °C water bath). Cell lysates were centrifuged at 1000 \times g to remove nuclei and unlysed cells. The ensuing supernatant was retained, and protein determination was completed using the Bradford assay. Lysates were purged of free phosphates by gel filtration, and calcineurin dephosphorylation of RII phosphopeptide substrate (BIOMOL International, L.P.) was monitored colorimetrically (A_{620 nm}) on a Versa Max microplate reader (Molecular Devices, Sunnyvale, CA).

Small Interfering RNA (siRNA) Transfection—siRNAs directed against rat TRPA1 (A-1, GGAACUGCAUACCAAC-UUdTdT, sense) and *Drosophila* TRPA1 (Adr-1, GCAAUGU-CATCGAUUAUCAdTdT, sense) were custom-synthesized by Dharmacon RNA Technologies (Chicago, IL). Silencer negative control 1 siRNA (Ambion, Inc., Austin, TX) was used as the scrambled negative control. For transfection, 20 μ l of HiPerFect transfection reagent (Qiagen Inc., Valencia, CA) and 625 ng of siRNA were combined in nutrient mixture F12 (Invitrogen) and incubated at 25 °C for 15 min. TG neurons were cleared of all cell debris and incubated overnight with siRNA/HiPerFect/nutrient mixture F-12 at 37 °C. Post-transfection, TG neurons were moved to normal medium (see above) and incubated as described above. This procedure was conducted twice (on days 2 and 4, with cells collected on day 6 for experimental procedures).

Immunocytochemistry—Cultured TG cells were grown on poly-D-lysine-coated coverslips for 5–7 days in normal medium. Coverslips were rinsed with PBS and fixed with 4%

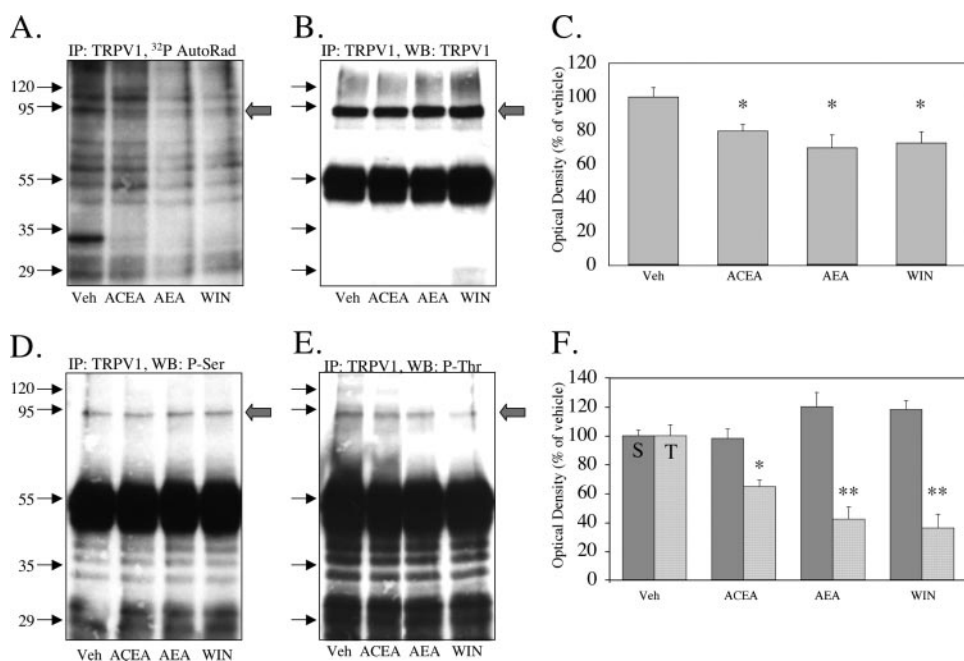


FIGURE 2. **Cannabinoids reduce TRPV1 phosphorylation.** TG neurons were treated with ACEA (25 μ M), anandamide (AEA; 25 μ M), and WIN (25 μ M) and analyzed by SDS-PAGE and Western blotting (WB) for [³²P]phosphate incorporation by TRPV1 (A). Autoradiographic (AutoRad) results were normalized to total immunoprecipitated (IP) TRPV1 (B), with band densities quantified and expressed as a percentage of vehicle (Veh)-treated cells (C). *, $p < 0.05$ (ANOVA; $n = 3$). TG neurons were treated with ACEA (25 μ M), anandamide (25 μ M), and WIN (25 μ M), and immunoprecipitated TRPV1 was analyzed for phosphoserine (D) and phosphothreonine (E) immunoreactivities. Black arrows indicate size markers (in kilodaltons), and gray arrows indicate the immunoreactive band of interest. Western blot results are representative of three to four independent trials. Phospho results were normalized to total immunoprecipitated TRPV1, with band optical densities quantified and expressed as a percentage of vehicle-treated cells (F). S, serine phosphorylation; T, threonine phosphorylation. *, $p < 0.05$; **, $p < 0.01$ (significant compared with vehicle; ANOVA; $n = 4$).

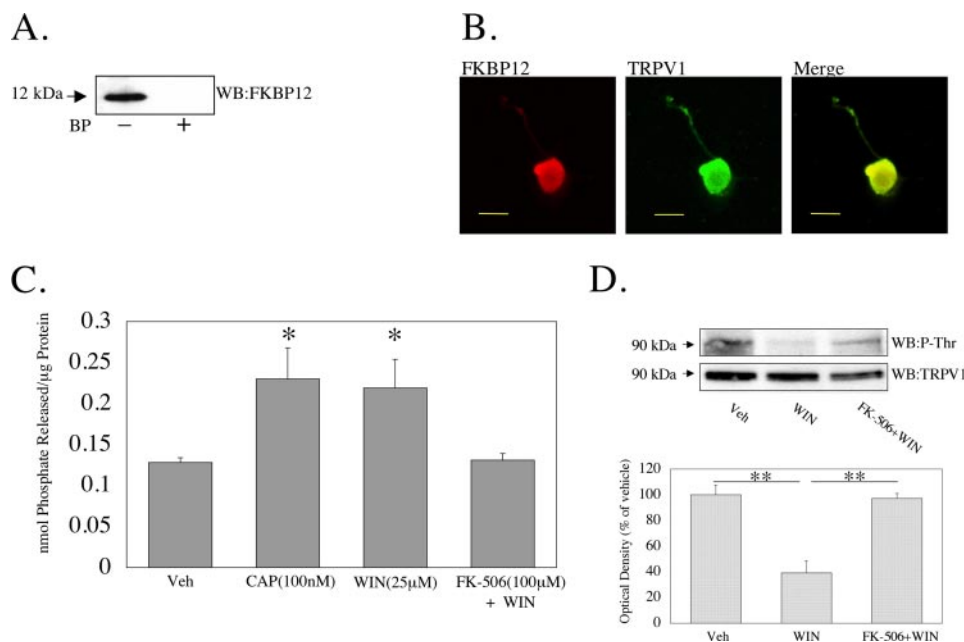


FIGURE 3. **WIN dephosphorylates TRPV1 through calcineurin.** A, FKBP12 expression in TG neurons was analyzed by Western blotting (WB) in the absence and presence of antibody-blocking peptide (BP). The results are representative of three independent trials. B, FKBP12 expression was analyzed by immunofluorescence in a TG neuron coexpressing TRPV1. The results are representative of two independent trials. Scale bars = 25 μ m. C, calcineurin activity from TG neurons treated with Me₂SO vehicle (Veh), capsaicin (CAP; 100 nM), WIN (25 μ M), and FK506 (100 μ M) and WIN was analyzed by RII phosphopeptide assay. *, $p < 0.05$ (ANOVA and one-tailed t test; $n = 3$). D, TG neurons were treated with WIN (25 μ M) or FK506 (100 μ M) and WIN, and immunoprecipitated TRPV1 was analyzed for phosphothreonine immunoreactivity. Phosphothreonine results were normalized to total immunoprecipitated TRPV1, with band optical densities quantified and expressed as a percentage of vehicle-treated cells. **, $p < 0.01$ (ANOVA; $n = 4$). The results are representative of four independent trials.

paraformaldehyde for 10 min at 25 °C. Following fixation, coverslips were rinsed twice with PBS and incubated with 5% normal goat serum and 0.5% Triton X-100 in PBS for 30 min at 25 °C. Coverslips were then incubated overnight at 4 °C with antisera directed specifically toward FKBP12 (1:500 dilution) and guinea pig TRPV1 (1:500 dilution; NeuroMics, Edina, MN). Coverslips were then rinsed three times and incubated for 1 h at 25 °C with the appropriate secondary antibodies (TRITC-labeled, 1:250 dilution; fluorescein isothiocyanate-labeled, 1:250 dilution). Following rinsing three times with PBS, coverslips were attached to microscope slides and dried overnight. For double-label immunofluorescence, coverslipped images were acquired using a $\times 40$ objective lens mated to a Nikon E600 microscope equipped with a Photometrics SensiSys digital CCD camera (Roper Scientific, Inc., Tucson, AZ) connected to a computer equipped with MetaMorph Version 4.1 image analysis software.

Mass Spectrometry—Immunoprecipitated TRPV1 was resolved by 15% SDS-PAGE, and protein bands were stained with Coomassie Blue. The band of interest was excised and digested *in situ* with trypsin (modified; Promega Corp., Madison, WI). The resulting digests were subjected to capillary HPLC/electrospray ionization tandem mass spectrometry on a Thermo Electron LTQ linear ion trap mass spectrometer used with an Eksigent NanoLC-2D micro-HPLC system. On-line capillary HPLC separation of the tryptic peptides was accomplished under the following conditions: PicoFrit™ column (75- μ m inner diameter; New Objective, Inc.) packed to 10 cm with C₁₈ adsorbent (218MSB5, 5 μ m, 300 Å; Vydac); mobile phase A, 0.5% acetic acid and 0.005% trifluoroacetic acid; mobile phase B, 90% acetonitrile, 0.5% acetic acid, and 0.005% trifluoroacetic acid; linear gradient of 2–72% mobile phase B over 30 min; and flow rate of 0.4 μ l/

min. A data-dependent acquisition protocol was employed in which the seven most intense ions in a survey scan were sequentially fragmented in the ion trap by collision-induced dissociation using an isolation width of 3.0 and a relative collision energy of 35%. Uninterpreted tandem mass spectra were analyzed by Mascot (Matrix Science, London, UK). Further assessment of probabilities of protein identification was performed using Scaffold (Proteome Software Inc., Portland, OR). The probability of identification of rat TRPV1 (GenBank™ accession number AF029310) by cross-correlation of the search results from Mascot and X! Tandem was 100% (13 unique peptides, 55% sequence coverage), as shown in Fig. 1H. Mass spectrometric analyses were carried out in the Mass Spectrometry Laboratory of the University of Texas Health Science Center at San Antonio.

RESULTS

Cannabinoids Dephosphorylate Threonine Residues of TRPV1 in TG Neurons—The cannabinoids ACEA, anandamide, and WIN were evaluated in this individual study because all have been shown to result in significant peripheral anti-hyperalgesia and/or antinociception (3, 4, 33). As TRPV1 has been shown to control peripheral inflammatory thermal hyperalgesia (20, 21), it is hypothesized that certain cannabinoids may affect TRPV1 phosphorylation, which is known to regulate channel activity (22, 24, 25, 34–36). To test this hypothesis, we examined the phosphorylation status of the TRPV1 receptor immunoprecipitated from TG neurons following cannabinoid treatments.

Cultured TG neurons were loaded with [³²P]orthophosphate and then treated with cannabinoids for 10 min to analyze receptor incorporation of phosphate groups. Fig. 2A illustrates a significant reduction in [³²P]phospho labeling of the immunoprecipitated TRPV1 receptor following treatment with ACEA (25 μM), anandamide (25 μM), or WIN (25 μM) in comparison with treatment with vehicle. ACEA, anandamide, and WIN treatment led to 20.1 ± 3.8, 30.1 ± 7.6, and 27.2 ± 6.5% reductions ($p < 0.05$; $n = 3$) in TRPV1 phosphorylation, respectively, compared with vehicle treatment. It is important to note that the concentrations of cannabinoids used in these experiments were between 1000- and 10,000-fold higher than those concentrations initially characterized to activate the cannabinoid G-protein-coupled receptors CB1 and CB2 (37, 38), consistent with the involvement of non-CB1/CB2 receptors. Both serine and threonine residues have been reported as potential phosphorylation sites for various kinases that serve to regulate channel activity via post-translational modifications of the TRPV1 receptor channel (25, 36, 39). To determine whether serine or threonine residues of the TRPV1 amino acid sequence were modified following cannabinoid treatments, equal immunoprecipitates from treated TG neurons were probed with antibody specific for phosphoserine or phosphothreonine. Cannabinoid treatment produced a significant reduction in the phosphothreonine immunoreactivity of TRPV1 immunoprecipitated from TG neurons compared with vehicle-treated neurons (Fig. 2B). In contrast, cannabinoid treatment produced no significant change in phospho-

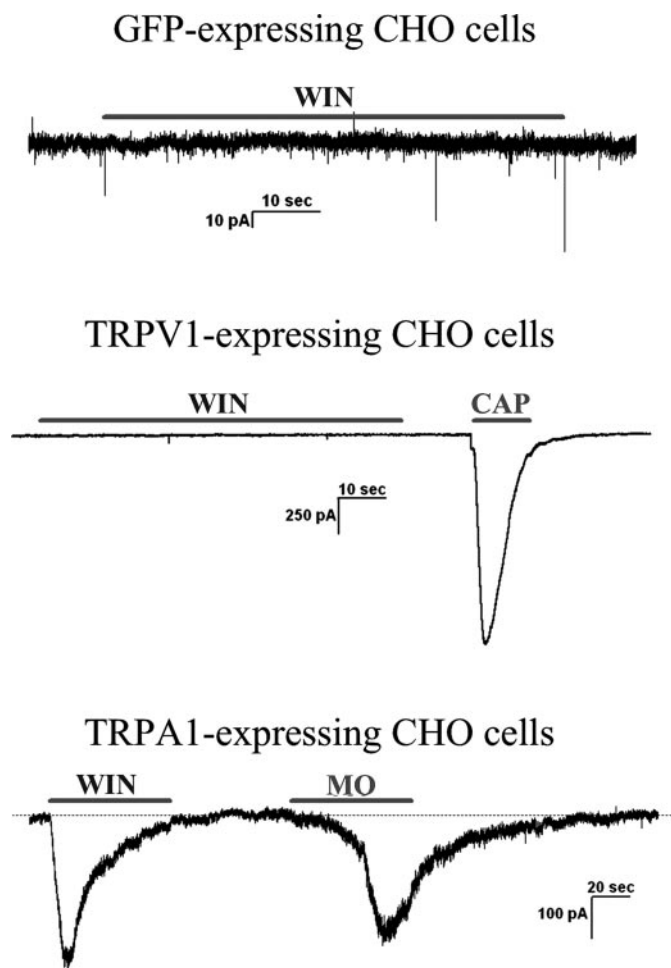


FIGURE 4. Current traces from transfected CHO cells. CHO cells were transfected with GFP, TRPV1, or TRPA1 vector and treated with WIN (25 μM) with or without capsaicin (100 nM) or mustard oil (MO; 20 μM). Recordings were made in the perforated patch voltage-clamp configuration. Cells were analyzed for currents, and the results are representative of five to nine trials. Drug application durations are indicated by horizontal bars.

serine immunoreactivity (1.7 ± 6.5% reduction for ACEA ($p = 0.75$), 20.3 ± 9.9% increase for anandamide ($p = 0.16$), and 18.3 ± 6.2% increase for WIN ($p = 0.09$), all with $n = 4$). ACEA, anandamide, and WIN treatment led to 35.2 ± 4.7, 57.8 ± 8.7, and 63.9 ± 9.5% reductions ($p < 0.05$, $n = 4$) in TRPV1 threonine phosphorylation, respectively, compared with vehicle treatment. These results suggest that cannabinoid treatment produces a significant dephosphorylation of threonine residues in TRPV1.

WIN-induced TRPV1 Dephosphorylation Is Dependent on Calcineurin Activation—TRPV1 channel activity has been reported to be sensitive to dephosphorylation by Ca²⁺-dependent phosphatases such as calcineurin (25, 26). To evaluate whether calcineurin may subserve cannabinoid-mediated TRPV1 dephosphorylation, we examined TG neurons for expression of the endogenous calcineurin inhibitor FKBP12. The immunosuppressant drug FK506 is known to complex with FKBP12 and calcineurin when administered *in vivo*, effectively inhibiting calcineurin phosphatase activity (40). We investigated the expression of FKBP12 in cultured TG neurons and found that it was expressed at the correct size

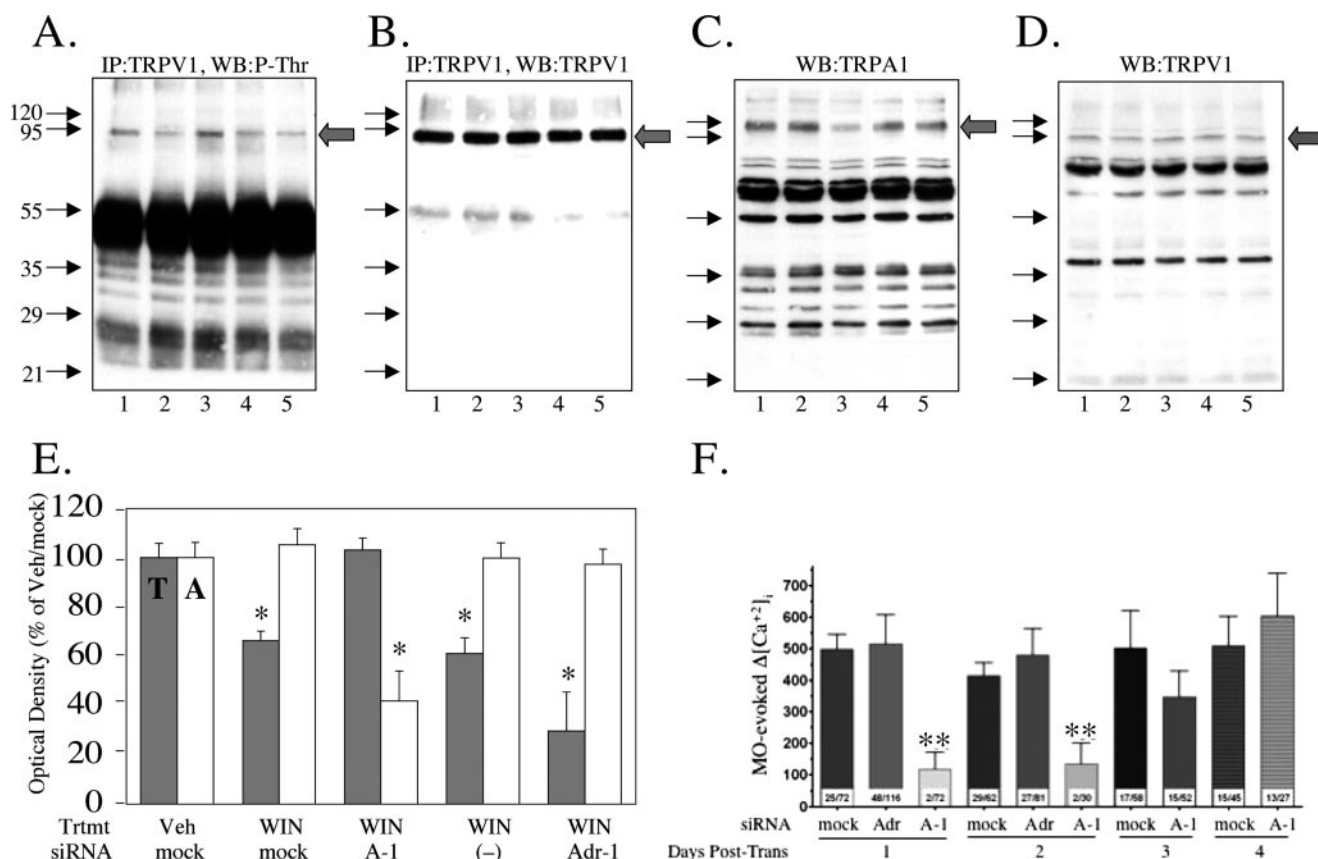


FIGURE 5. TRPA1 mediates WIN-induced dephosphorylation of TRPV1 in TG neurons. TG neurons were transfected with siRNA directed against TRPA1 (A-1), *Silencer* negative siRNA (-), siRNA directed against *Drosophila* TRPA1 (Adr-1), or no siRNA (mock) and analyzed for TRPV1 phosphothreonine by Western blotting (WB) following WIN (25 μ M) treatment (Trtmt). Lanes 1, mock transfection, Me₂SO vehicle (Veh) treatment; lanes 2, mock transfection, WIN treatment; lanes 3, A-1 transfection, WIN treatment; lanes 4, *Silencer* negative siRNA control, WIN treatment; lanes 5, Adr-1 control, WIN treatment. Phospho results (A) were normalized to total immunoprecipitated (IP) TRPV1 (B), and TRPA1 expression (C) was normalized to TRPV1 expression (D), with band optical densities quantified and expressed as a percentage of vehicle-treated cells (E). *, $p < 0.05$ (significant compared with vehicle/mock transfection). Gray bars indicate threonine phosphorylation (T), and white bars indicate TRPA1 protein expression (A) by Western blotting. Black arrows indicate size markers (in kilodaltons), and gray arrows indicate the immunoreactive band of interest. The results are representative of four independent trials. F shows mustard oil (MO; 20 μ M)-evoked calcium imaging of mock-, Adr-1 (fluorescein isothiocyanate-labeled)-, and A-1-transfected TG cultures over a 4-day period of post-transfection (Post-Trans). **, $p < 0.01$ (ANOVA; $n = 4$).

(~12 kDa) (Fig. 3A) and with TRPV1-positive neurons (Fig. 3B). *In vitro* analysis of calcineurin activity from cultured TG neurons revealed a significant increase in phosphatase activity following WIN treatment that was sensitive to FK506 pretreatment (Fig. 3C). Furthermore, dephosphorylation of threonine residues in TRPV1 from TG neurons following WIN treatment was reversed following FK506 pretreatment ($p < 0.05$, $n = 4$) (Fig. 3D), suggesting a role for calcineurin in TRPV1 modulation by WIN. Dose-response experiments conducted previously (41) were utilized to determine the optimal concentration of WIN used in these and the following studies.

TRPA1 Is Necessary for WIN-induced TRPV1 Dephosphorylation—CB1/CB2 receptor expression patterns in sensory neurons have been characterized predominantly in non-nociceptive afferent neurons (9, 10); and therefore, it has been postulated that non-CB1/CB2 receptor mechanisms of peripheral cannabinoid actions on nociceptive neurons could exist (42–44). A recent report (and this study) also indicated that inhibition of TRPV1 by WIN is G-protein-independent, occurs at low micromolar concentrations of WIN, and is Ca²⁺-dependent

(41). Moreover, TRPA1 was revealed as a candidate receptor activated by WIN.⁵

Therefore, we investigated the role of TRPA1 in WIN-induced dephosphorylation of TRPV1 in TG neurons. First, TRPA1 or TRPV1 was expressed in CHO cells and probed with WIN, capsaicin (a TRPV1-specific agonist) (20), and mustard oil (a TRPA1-specific agonist) (45). Fig. 4 illustrates that application of 25 μ M WIN, unlike that of 100 nM capsaicin, did not gate a current in CHO cells transfected with TRPV1. In contrast, TRPA1-transfected CHO cells robustly responded to application of WIN as well as mustard oil (20 μ M). Control CHO cells expressing green fluorescent protein (GFP) did not generate currents upon WIN application. Thus, WIN selectively gates TRPA1, but not TRPV1. To verify the dependence of TRPA1 expression for WIN-induced dephosphorylation of TRPV1, siRNA directed against TRPA1 was utilized to knock down receptor channel expression in TG neurons. In neurons

⁵ Ruparel, N. B., Akopian, A. N., Patwardhan, A. M., Jeske, N. A., and Hargreaves, K. M., Society for Neuroscience 35th Annual Meeting, Washington, D. C., November 12–16, 2005, Abstr. 622.3.

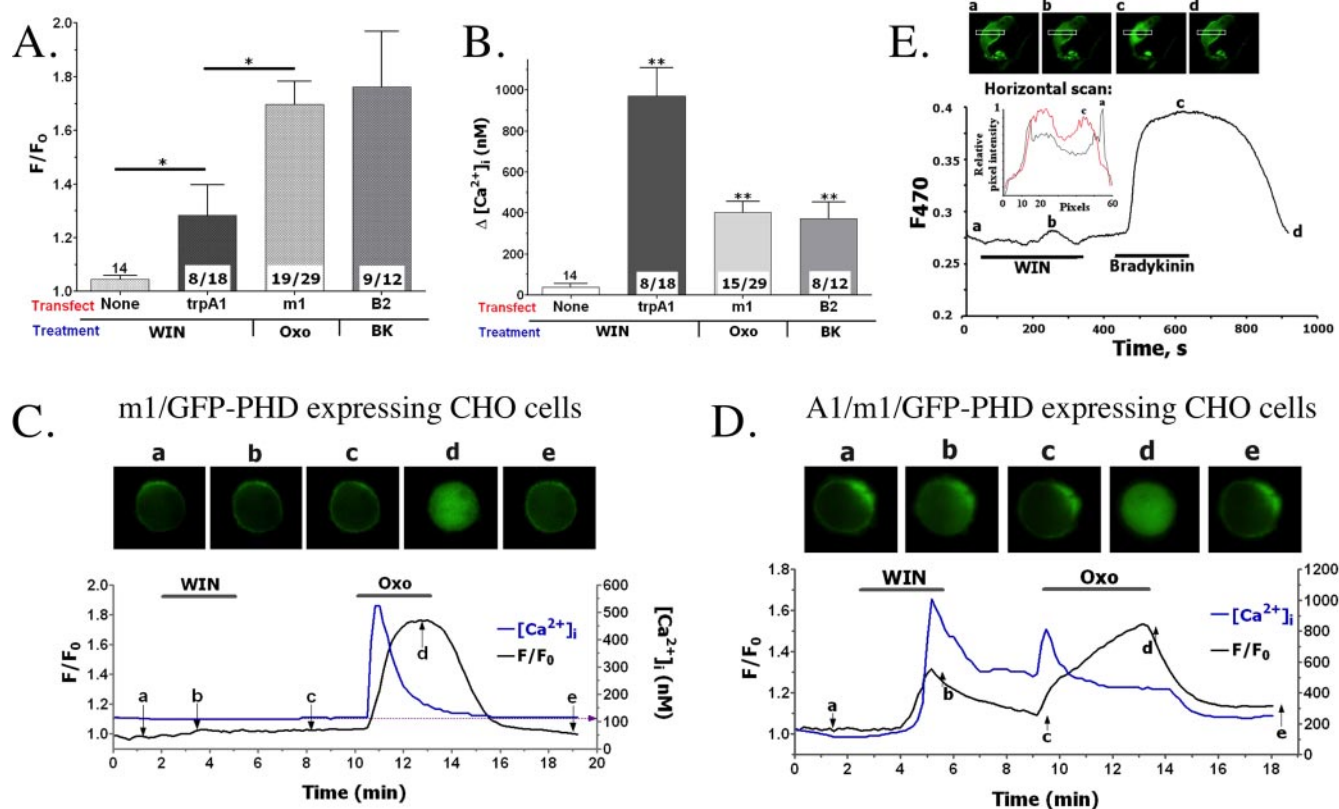


FIGURE 6. WIN directly activates TRPA1 to increase $[Ca^{2+}]_i$ through a $G\alpha_q$ -independent mechanism. TG neurons were transfected with the GFP-PHD PLC sensor, whereas CHO cells were transiently transfected with the GFP-PHD PLC sensor and different combinations of TRPA1, the muscarinic type 1 receptor (*m1*), or the bradykinin type 2 receptor (*B2*). Translocation of GFP-PHD from the plasma membrane to the cytosol and accumulation of intracellular Ca^{2+} ($\Delta[Ca^{2+}]_i$) were concurrently imaged by a set of filters. Data for analysis were collected every 5 s after exposure to WIN (25 μM , 3 min), oxotremorine (*Oxo*; 10 μM , 5 min), or bradykinin (*BK*; 200 nM, 5 min) with 5-min washout period. *A*, translocation of GFP-PHD (F/F_0) was calculated from the measurement of $F_{340/380\text{ nm}}$ within a predetermined area of the cytosol using MetaFluor software. *, $p < 0.05$ (ANOVA, with *n* values indicated). *B*, $\Delta[Ca^{2+}]_i$ was calculated from measurements of changes in the $F_{340/380\text{ nm}}$ ratio using MetaFluor software. **, $p < 0.005$ (ANOVA, with *n* values indicated). *C* and *D*, shown are typical traces of GFP-PHD translocation and calcium influx on the same temporal scale for muscarinic type 1 receptor/GFP-PHD-transfected CHO cells and TRPA1 (*A1*)/muscarinic type 1 receptor/GFP-PHD-transfected CHO cells, respectively. GFP images of a cell within the indicated time points are presented. *E*, shown is the time course of GFP-PHD translocation in a TG neuron stimulated by WIN (25 μM) or bradykinin (1 μM) plotted against GFP fluorescence density ($F_{470\text{ nm}}$) in the cytosol.

transfected with rat TRPA1 siRNA (A-1), mustard oil responses (accessed by Ca^{2+} imaging) were reduced dramatically ($\approx 80\%$) 2 days post-transfection in comparison with cells transfected with a negative control siRNA (A-1) (Fig. 5*F*), suggesting that the A-1 siRNA specifically knocked down rat TRPA1 function. Moreover, A-1 siRNA transfection of TG neurons resulted in a significant and specific $58.1 \pm 12\%$ reduction in TRPA1 expression (Fig. 5) without significant changes in TRPV1 expression. In addition, dephosphorylation of TRPV1 threonine residues from TG neurons treated with WIN (25 μM) was abolished in neurons transfected with A-1 siRNA (Fig. 5). Negative control siRNA transfections had no significant effect (A-1 transfection, $p = 0.13$), suggesting that TRPA1 expression is necessary for dephosphorylation of TRPV1 by WIN in TG neurons.

WIN Actions Are Independent of $G\alpha_q$ Mechanisms—Recent reports have indicated that second messenger signaling pathways could form a tenable link between WIN activity and increases in intracellular Ca^{2+} (46, 47). Specifically, it was demonstrated that low micromolar concentrations of WIN can activate phospholipase *C* β (PLC β) via the CB1 receptor and, as a result, deplete intracellular Ca^{2+} stores in hippocampal neurons. As the TRPA1 channel is known to belong to the family of second messenger-operated transient receptor potential chan-

nels (46), WIN could indirectly activate TRPA1 through the liberation of diacylglycerol following PLC β activation.

To address this possible cellular pathway of WIN activity, we employed the GFP-tagged pleckstrin homology domain (PHD) PLC activity sensor. Specifically, translocation of membrane-localized GFP-PHD to the cytosol serves as a measure for phosphatidylinositol 4,5-bisphosphate hydrolysis by receptor-stimulated activation of PLC β (48). Throughout the experiment, both GFP-PHD translocation and Ca^{2+} influxes were simultaneously monitored and analyzed. WIN (25 μM) treatment of CHO cells transfected with the muscarinic type 1 or bradykinin type 2 receptor together with GFP-PHD revealed neither GFP-PHD translocation (Fig. 6, *A* and *C*) nor Ca^{2+} accumulation (*B* and *C*). However, the positive control experiments evoked $G\alpha_q$ -coupled receptor activation by subsequent stimulation of the same CHO cells with oxotremorine (10 μM ; $n = 19/29$) or bradykinin (200 nM; $n = 9/12$) and resulted in robust GFP-PHD translocation (Fig. 6, *A* and *C*). These cells also displayed an increase in Ca^{2+} influx (Fig. 6*B*), likely because of the release of intracellular stores of Ca^{2+} following phosphatidylinositol 4,5-bisphosphate degradation to inositol 1,4,5-trisphosphate (49). In cells transfected additionally with TRPA1, WIN treatment led to a dramatic increase in Ca^{2+} influx compared with cells

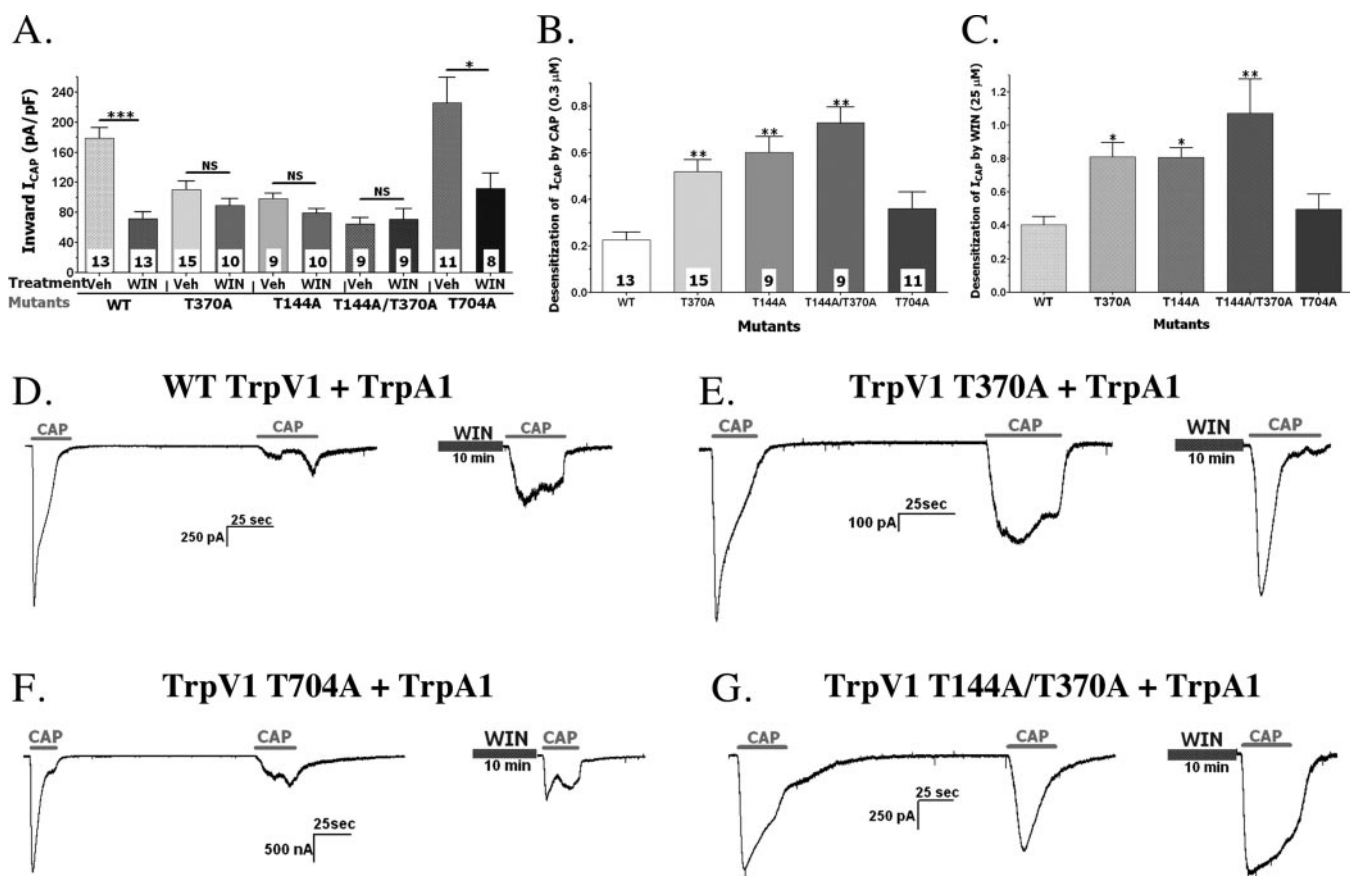


FIGURE 7. TRPV1 desensitization of I_{CAP} by WIN is dependent on Thr¹⁴⁴ and Thr³⁷⁰ phosphorylation. CHO cells were transiently transfected with TRPA1 and wild-type TRPV1 (WT), TRPV1(T144A), TRPV1(T370A), TRPV1(T704A), or TRPV1(T144A/T370A); treated with vehicle (Veh; 0.1% Me₂SO) or WIN (25 μ M) for 10 min; washed for 5 min; and patched. I_{CAP} was recorded during a 30-s application of 300 nM capsaicin (CAP). Data were normalized to the mean peak of I_{CAP} measured from vehicle-treated cells ($n = 8-15$ /treatment, noted inside each bar). *A*, I_{CAP} measured from CHO cells expressing wild-type TRPV1, TRPV1(T144A), TRPV1(T370A), TRPV1(T704A), or TRPV1(T144A/T370A). *pF*, picofarads. *, $p < 0.05$; ***, $p < 0.001$ (paired *t* test); NS, not significant. *B*, tachyphylaxis after re-application of capsaicin to cells expressing phosphorylation site mutants. **, $p < 0.005$ (ANOVA). *C*, normalized data on desensitization of I_{CAP} by WIN in TRPV1 phosphorylation site mutants. *, $p < 0.05$; **, $p < 0.005$ (ANOVA). *D-G*, typical I_{CAP} traces of capsaicin- and WIN-induced desensitization in wild-type TRPV1 and the phosphorylation site mutants. Durations of drug application are indicated by horizontal bars.

transfected with GFP-PHD alone ($n = 8/18$) (Fig. 6, *A*, *B*, and *D*). TRPA1-transfected cells also displayed a modest increase in GFP-PHD translocation, likely because of activation of Ca²⁺-sensitive PLC isoforms, as has been reported previously (50).

It has been reported that the enzymatic activities of Ca²⁺-sensitive PLC isoforms are dependent on the amount of Ca²⁺ influx following receptor activation (51, 52). Indeed, TRPA1-expressing CHO cells that accumulated <600 nM Ca²⁺ after WIN stimulation failed to display GFP-PHD translocation. Because WIN rarely triggered accumulation of >600 nM Ca²⁺ in sensory neurons (data not shown), we transfected sensory neurons with the GFP-PHD construct to monitor phosphatidylinositol 4,5-bisphosphate depletion following WIN treatment. Fig. 6*E* demonstrates that WIN (25 μ M) was unable to activate PLC β ($n = 10$) in TG neurons containing GFP-PHD, whereas application of a positive control, bradykinin (1 μ M), triggered a real-time translocation of GFP-PHD in a subset of sensory neurons ($n = 6/10$). Taken together, these results imply that WIN directly activates TRPA1 without mediation of G α_q signaling pathways in either CHO cells or TG neurons.

TRPV1 Thr¹⁴⁴ and Thr³⁷⁰ Are Dephosphorylated following WIN Treatment—As mentioned above, both threonine and serine residues of the TRPV1 channel have been confirmed as

phosphorylation sites for various kinases that serve to sensitize channel activity. The data from Fig. 2*B* suggest that threonine residues are specifically dephosphorylated by WIN treatment, so site-directed mutants of TRPV1 were created by mutating three individual threonine residues to alanine residues: Thr¹⁴⁴ and Thr³⁷⁰ (53) and Thr⁷⁰⁴ (28). CHO cells were transiently transfected with mouse TRPA1 cDNA and wild-type TRPV1, TRPV1(T144A), TRPV1(T370A), or TRPV1(T704A). Perforated patch electrophysiology was utilized to measure inward capsaicin current (I_{CAP}) following vehicle or WIN (25 μ M) pretreatment for each transfection set (Fig. 7). Fig. 7*A* shows that the magnitude of I_{CAP} was affected by certain threonine mutations. Because I_{CAP} tachyphylaxis is proposed to follow a mechanism similar WIN-induced inhibition of I_{CAP} (*i.e.* Ca²⁺, calcineurin, and dephosphorylation dependence), we employed I_{CAP} tachyphylaxis as a positive control. Pretreatment with WIN, like that with capsaicin, led to a significant reduction in I_{CAP} in wild-type TRPV1-transfected CHO cells compared with that in vehicle-treated cells (Fig. 7, *A*, *B*, and *D*). In contrast to wild-type TRPV1 and TRPV1(T704A), the significant I_{CAP} reduction by WIN as well as capsaicin pretreatment was not apparent in CHO cells transfected with TRPV1(T144A) or TRPV1(T370A) (Fig. 7*E*) or the with double mutant

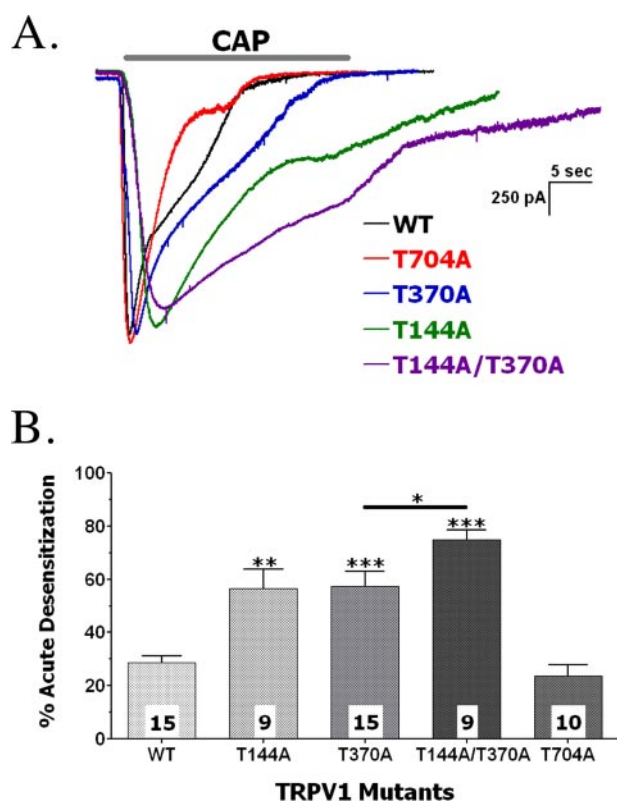


FIGURE 8. Acute TRPV1 desensitization is modulated by phosphorylation at Thr¹⁴⁴ and Thr³⁷⁰. *A*, current traces of five separate transiently transfected CHO cells expressing wild-type TRPV1 (WT), TRPV1(T704A), TRPV1(T370A), TRPV1(T144A), or TRPV1(T144A/T370A) upon a 30-s application of 300 nM capsaicin (CAP). Current amplitudes were normalized to that of the largest response to demonstrate differences in desensitization kinetics. *B*, summary graph of the percentage of acute desensitization measured 10 s after the peak of I_{CAP} was reached. The number of neurons in each trial is indicated within each bar. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ (ANOVA).

TRPV1(T144A/T370A) (Fig. 7G). In agreement with a previous report (25), Thr¹⁴⁴ and Thr³⁷⁰ were found to be essential for I_{CAP} tachyphylaxis (Fig. 7B). The results suggested that Thr¹⁴⁴ and Thr³⁷⁰ are essential for WIN-induced TRPV1 desensitization (Fig. 7C).

Studies on acute and pharmacological (*i.e.* tachyphylaxis) desensitization of I_{CAP} have demonstrated that the two types of desensitization could share similar, if not identical, calcineurin-dependent mechanisms (24–26, 28). Following this hypothesis, we analyzed data from previous experiments to identify possible changes in acute desensitization of TRPV1 mutants. Fig. 8A illustrates representative traces recorded for various TRPV1 phosphorylation site mutants, including TRPV1(T704A), TRPV1(T144A), TRPV1(T370A), and TRPV1(T144A/T370A), after vehicle treatment. In agreement with results reported by others (25), analysis of these traces indicated that acute desensitization of TRPV1 was significantly reduced in the absence of phosphorylation of the receptor at Thr¹⁴⁴ and Thr³⁷⁰ (Fig. 8B). In summary, the T144A and T370A mutations of TRPV1 affect not only acute and pharmacological desensitization of I_{CAP} , as reported previously (25), but also WIN-induced reduction in I_{CAP} .

In the next set of experiments, we evaluated whether the essential T144A/T370A mutation of TRPV1 alters WIN-induced dephosphorylation of TRPV1. CHO cells were tran-

siently transfected with TRPA1 and wild-type TRPV1, with TRPA1 and TRPV1(T144A/T370A), or with pcDNA3 empty vector and wild-type TRPV1 and then monitored for changes in [³²P]orthophosphate incorporation by TRPV1 following application of WIN (25 μ M). As anticipated, WIN application led to a significant $26.7 \pm 7.7\%$ reduction in TRPV1 phosphorylation compared with vehicle-treated cells ($p < 0.05$, $n = 3$) (Fig. 9). In cells transfected with TRPA1 and TRPV1(T144A/T370A), TRPV1 phosphorylation was unaffected by WIN (Fig. 9), in agreement with the data presented in Fig. 7. Similar results were obtained for TRPV1 phosphorylation following WIN treatment in cells transfected with pcDNA3 and TRPV1, suggesting that TRPA1 expression is required for WIN-induced dephosphorylation of TRPV1 in CHO cells.

DISCUSSION

In this study, we have described a mechanism by which cannabinoids act to dephosphorylate and, as a result, desensitize TRPV1 activity. A major finding of this study is that WIN induces dephosphorylation of TRPV1 in sensory neurons via activation of TRPA1. Moreover, by comparing an indirect measure of PLC activity (*i.e.* GFP-PHD translocation) with Ca^{2+} influx, we found that WIN acts directly on TRPA1 in both CHO cells and TG neurons. In addition, the coexpression of TRPA1 with TRPV1 was discovered to be pertinent to dephosphorylation of TRPV1 at Thr¹⁴⁴ and Thr³⁷⁰ by calcineurin following WIN treatment. These results confirm the importance of an ionotropic target for WIN (*i.e.* TRPA1) in the modulation of nociceptor sensitivity, which contrasts with the more classical metabotropic cannabinoid receptor targets.

The identification of Thr¹⁴⁴ and Thr³⁷⁰ as important determinants of TRPV1 sensitivity to capsaicin activation is an interesting discovery. Despite the activation of calcineurin by WIN, total TRPV1 phosphorylation was reduced by only 20–30% following cannabinoid treatment. Although these data agree with previous reports (25, 28, 39), it is difficult to determine whether these residues serve as the only phosphorylated targets of calcineurin among other phosphorylated residues in TRPV1. Indeed, numerous other functionally important phosphorylated residues have been identified in TRPV1, including, but not limited to, Ser⁸⁰⁰ (36, 54), Ser¹¹⁶ (39), Ser⁵⁰² (53, 54), and Thr⁷⁰⁴ (28). These residues could, in principle, be sensitive to phosphatases other than calcineurin or may exist in a persistent phosphorylated state. This hypothesis is supported by our data, as TRPV1 exhibited basal levels of phosphorylation before calcineurin activation by WIN. This suggests that TRPV1 is chronically phosphorylated under these conditions and that dephosphorylation of only a select few residues can have far greater effects on channel basal conductance. However, WIN modulation of TRPV1 residues that may undergo phosphorylation following inflammatory insult remains to be determined.

The data reported here on the ability of WIN to desensitize I_{CAP} are in agreement with the tachyphylaxis reported upon repeated applications of capsaicin (16, 24–26, 28), leading to Ca^{2+} -dependent TRPV1 desensitization (24). Given the similar activation profiles of the Ca^{2+} -dependent phosphatase calcineurin by WIN and capsaicin (25, 41), it is believed that WIN-induced TRPV1 desensitization is also dependent on Ca^{2+} . It is

WIN Modulates TRPV1 through TRPA1

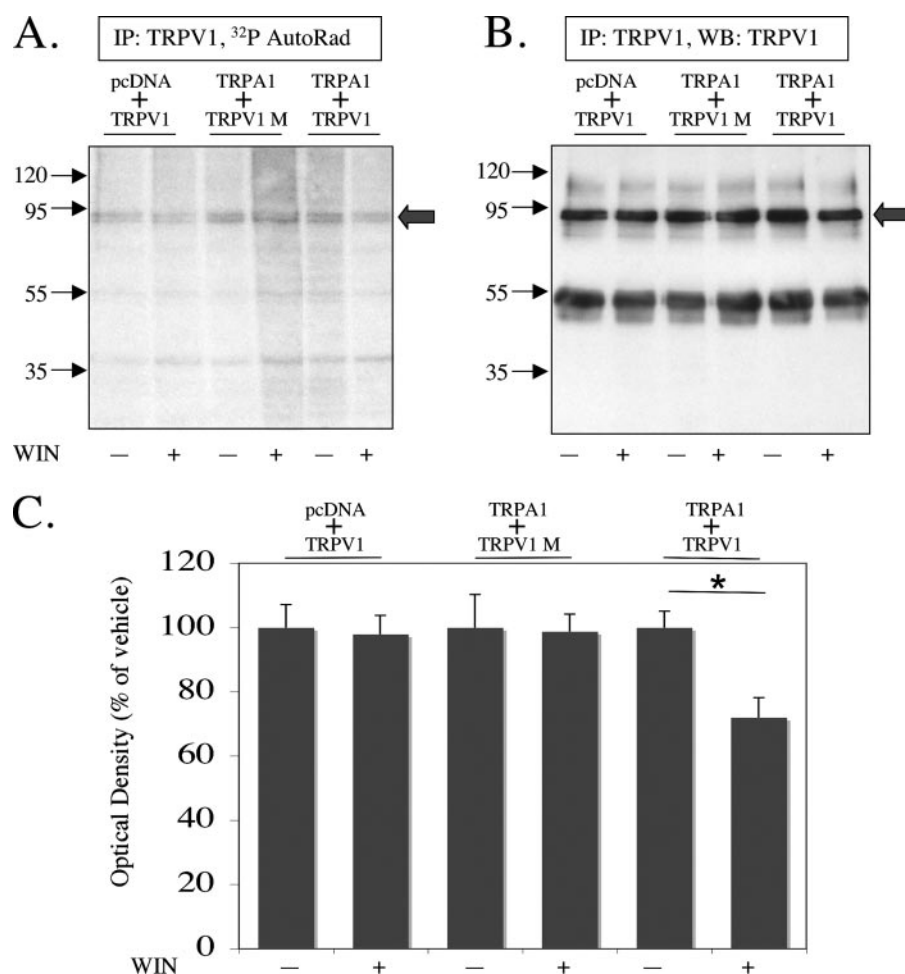


FIGURE 9. TRPA1 coexpression is necessary for WIN-induced dephosphorylation of TRPV1. CHO cells were transiently transfected with the indicated cDNAs and analyzed for [³²P]phosphate incorporation by TRPV1 following WIN (25 μM) or vehicle treatment. Autoradiographic (AutoRad) results (A) were normalized to total immunoprecipitated (IP) TRPV1 (B), with band optical densities quantified and expressed as a percentage of vehicle-treated cells (C). *, *p* < 0.05 (paired *t* test; *n* = 3). Black arrows indicate size markers (in kilodaltons), and gray arrows indicate the immunoreactive band of interest. The results are representative of three independent trials. TRPV1 M, TRPV1(T144A/T370A) mutant; WB, Western blot.

interesting to note that desensitization of I_{CAP} by WIN in this study was approximately half as efficacious as that by capsaicin (Fig. 7, B and C). This difference may be due to the different sites of action of WIN and capsaicin. Capsaicin, acting on TRPV1, is known to induce a robust inward conductance of Ca^{2+} ions with a current amplitude of ~6–8 nA (by 10 μM capsaicin) in sensory neurons (55), whereas WIN, acting on TRPA1, gates a much smaller inward current of ~250 pA (by 50 μM WIN) in TG neurons.⁵ The reduced current amplitude with WIN activation of TRPA1 in comparison with that with capsaicin activation of TRPV1 could partly explain the differences in the ability of each to desensitize I_{CAP} .

Selective knockdown by directed siRNA coupled with the transient transfection of CHO cells jointly supported the hypothesis that WIN-induced dephosphorylation of TRPV1 is mediated by TRPA1 in sensory neurons. The nature of the association between the two receptor channels has yet to be fully characterized, yet TRPV1 dephosphorylation and desensitization by WIN have been shown here to require TRPA1. Recent findings by Lauckner *et al.* (47) have suggested that WIN acts through a pertussis-insensitive, $G\alpha_q$ -coupled CB1 receptor in

transfected human embryonic kidney 293 cells. It is apparent that WIN-evoked Ca^{2+} influx (>600 nM) in TRPA1-expressing CHO cells triggers slight translocation of GFP-PHD, supporting recent reports on activation of PLC by menthol-gated TRPM8 in human embryonic kidney cells (50). Nevertheless, the data presented in this study support a non- $G\alpha_q$ -coupled mechanism in this particular action of the cannabinoid in TG neurons or naïve CHO cells. Furthermore, the increase in $[Ca^{2+}]_i$ following WIN treatment in CHO cells in this study was greater than that demonstrated by Lauckner *et al.* (47) in human embryonic kidney 293 cells, suggesting a direct gating effect by TRPA1, and not the release of Ca^{2+} from intracellular stores as reported previously.

The concentration of WIN required to activate TRPA1 and to lead to dephosphorylation of TRPV1 in this study was relatively high (25 μM) compared with that in studies evaluating activation of metabotropic cannabinoid receptors. Initial studies on the characterization of WIN pharmacology found that the aminoalkylindole preferentially binds the CB2 receptor with an affinity of 0.28 nM (38) over the CB1 receptor with an affinity of 1.89 nM (37). Indeed, Mackie

and Hille (56) found 100 nM WIN to reversibly inhibit neuroblastoma and glioma cell voltage-gated calcium currents by 40% in a pertussis toxin-sensitive fashion. Moreover, Khasabova *et al.* (43) reported recently that voltage-gated calcium channels of only large sensory neurons (*i.e.* non-nociceptors) are affected by nanomolar concentrations of WIN. In contrast, micromolar concentrations of WIN attenuate depolarization-evoked calcitonin gene-related peptide release from TG neurons (11). Similarly, in this study, we focused on activation of the channel by a cannabinoid, and it is unlikely that the 25 μM concentration of WIN exerted nonspecific effects, given that the effects reported in this study were completely dependent on TRPA1 expression (see also Fig. 4). The need for higher concentrations of WIN to activate the TRPA1 channel are believed to be due to ligand-receptor binding kinetics. Whereas the WIN-binding region of CB1/2 receptors, including the third transmembrane helix (57), is located extracellularly and is more accessible to the agonist, it is possible that the TRPA1-binding site is intracellular. For example, the extracellular application of 100 μM 1-oleoyl-2-acetyl-*sn*-glycerol (a membrane-permeable analog of diacylglycerol) is required to gate TRPA1, whereas

phosphatidylinositol 4,5-bisphosphate depletion by bradykinin leads to a smaller amount of intracellular diacylglycerol that is still able to gate the channel (46). Therefore, despite the high WIN concentrations used, the reported results support the specificity of the cannabinoid for TRPA1.

The results from this study indicate that WIN desensitizes TRPV1 in trigeminal neurons employing mechanisms similar to TRPV1 pharmacological desensitization. WIN-induced activation of TRPA1 leads to an influx of calcium, activation of calcineurin, and subsequent dephosphorylation of TRPV1 at Thr¹⁴⁴ and Thr³⁷⁰. The implications of these results lend support to the use of cannabinoids as analgesics in the clinical setting, potentially attenuating tissue damage and inflammation caused by peripheral inflammatory hyperalgesia.

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