Cannabinoids are known to have multiple sites of action on 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. Since phosphorylation of transient receptor potential potential subtype V1 (TRPV1) 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 ganglia cultures with WIN leads to dephosphorylation of TRPV1, specifically on threonine amino acid residues. Utilizing CHO cell lines, we demonstrate that threonine residues at positions 144 and 370 are dephosphorylated, leading to desensitization of the TRPV1 receptor. This post-translational modification occurs through activation of the phosphatase calcineurin (PP2B) following WIN treatment. Furthermore, knock-down of transient receptor potential subtype A1 (TRPA1) expression in sensory neurons by specific siRNA 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 CHO 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 mediated by cannabinoid type 1 and/or type 2 receptor (CB1/CB2) 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 less than a 5-10% co-localization of CB1/CB2 metabotropic receptors with nociceptive neuronal markers such as TRPV1 and calcitonin gene-related peptide (CGRP) in trigeminal and dorsal root ganglia neurons, suggesting that cannabinoids could act on nociceptors through non-CB1/CB2 mechanism(s) (9-11). Certain cannabinoids have been shown to activate channels such as TRPV1, including arachidonyl-2-chloroethylamide (ACEA, (12)), N-arachidonoyldopamine (NADA, ((13), and anandamide (AEA, (14)), as well as TRPA1, by Δ9-THC (15). In addition, the synthetic cannabinoid R(+)-WIN 55,212-2 (WIN) has demonstrated non-CB1/CB2 receptor activities in the trigeminal ganglia (11). Results from these studies suggest that cannabinoids may activate calcium channel function similar to non-
cannabinoid TRP 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 (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). Coincidently, calcium-dependent sensitization of the receptor can also occur, through activation of Ca^{42+}-calmodulin-dependent kinase II (CaMKII, (28)) and PKC (29). The balance between calcium-stimulated kinase and phosphatase activities results in a tightly regulated system responsible for modulating TRPV1 activity.

In the present study we examined whether certain cannabinoids could 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 T144 and T370, but does so in a manner dependent upon TRPA1 co-expression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection of cDNA** - All procedures utilizing animals were approved by the Institutional Animal Care and Use Committee of The University of Texas Health Science Center at San Antonio and were conducted in accordance with policies for the ethical treatment of animals established by the National Institutes of Health. Trigeminal ganglia were removed bilaterally from male Sprague-Dawley rats (200-250g, Charles River, Wilmington, MA), and dissociated by collagenase treatment (30 min, Worthington, Lakewood, NJ), followed by trypsin treatment (15 min, Sigma, St.Louis, MO), and DNAse-1 (Roche, Indianapolis, IN) treatment (5 min). Cells were centrifuged and re-suspended between each treatment with Pasteur pipettes. Cells were centrifuged, aspirated, and re-suspended in DMEM (Gibco, Grand Island, NY) with 10% FBS (Gibco), 250 ng/ml NGF (Harlan, Indianapolis, IN), 1% 5-fluoro deoxyuridine (Sigma), 1% penicillin/streptomycin (Gibco), and 1% L-glutamine (Sigma), and then plated onto plates coated with poly-D lysine. Cultures were maintained at 37°C, 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, 5% CO₂ and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following manufacturer’s instructions. TG neurons were transfected using a PDS-1000/He Biolistic system (BioRad, Hercules, CA) according to manufacturer’s instructions.

**cDNA Constructs and Site-Directed Mutagenesis** - Rat TRPV1 cDNA was kindly provided by Dr. David Julius (UCSF, San Francisco, CA) and mouse TRPA1 cDNA was kindly provided by Dr. Ardem Patapoutian (The Scripps Research Institute, San Diego, CA). The entire coding sequence of mouse TRPA1 (30), apart from the starting codon, was used to generate a myc-TRPA construct in pCMV-Myc (Clontech, Palo Alto, CA). pEGFP-N1 cDNA was purchased from Clontech, and bradykinin receptor type 2 and muscarine receptor type 1 cDNA were purchased from UMR cDNA Resource Center (Rolla, MO). Site-directed mutagenesis was performed using the Quickchange XL Site-Directed Mutagenesis
Kit (Stratagene, La Jolla, CA) following manufacturer’s instructions. rTRPV1 T144A was kindly provided by Dr. Carla Nau (Friedrich-Alexander-University, Erlangen, Germany). To create rTRPV1 T370A, the utilized forward primer was 5’- CCAGGAAGTTCCGGAATGGGCCTATGGG. To create rTRPV1 T704A, the utilized forward primer was 5’- GCAGAGAGCATCGCACCACCTGGATAACAG. All mutations were confirmed by sequencing (UTHSCSA Advanced Nucleic Acids Core Facility, San Antonio, TX).

**Immunoprecipitation and Western Blot Analysis** - For each experimental condition, cells were treated with appropriate compounds and harvested as previously described (31). Protein determination was completed following instructions from the manufacturer of the Bradford Assay (BioRad, Hercules, CA). For radioactivity experiments, 10 cm plates of TG were incubated with 1 mCi of 32P orthophosphate (Perkin Elmer, Boston, MA) and 6 cm plates of CHO were incubated with 125 μCi for 4 h at 37°C in phosphate-free DMEM (Gibco). Following harvesting, cleared lysates were immunoprecipitated with 1 μg of anti-TRPV1 antisera (Ab-2, Calbiochem, La Jolla, CA), resolved on 15% SDS-PAGE, and transferred to polyvinyl difluoride (PVDF, Millipore, Bedford, MA). Western blots were either exposed to film at –80°C overnight for autoradiography, or blocked in 5% BSA in TBS-Tw and visualized using antibodies to TRPV1 (anti-TRPV1, Ab-1, Calbiochem), phospho-serine (Calbiochem), or phospho-threonine (Calbiochem), followed by appropriate secondary antisera linked to HRP and enhanced chemiluminescence (GE Healthcare, Piscataway, NJ) following manufacturer’s instructions.

Other antibodies used in these studies include rabbit polyclonal anti-TRPA1 that recognizes an N-terminal epitope (amino acid sequence C-KRSLRRVLRPEERKE), and anti-FKBP12 (Affinity BioReagents, Golden, CO). Figure 1A-G illustrates the specificities of the TRPV1 and TRPA1 antibodies used.

Autoradiography and Western blot results were scanned and quantified using NIH Image 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 3-5 independent experiments, and statistical significance was determined using two-way ANOVA or paired t-test as appropriate.

**Electrophysiology** - All recordings were made in a perforated-patch voltage clamp configuration at a holding potential (Vh) 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 pCLAMP9.0 software (Axon Instruments, Union City, CA). Cells were transfected with indicated cDNAs, along with pEGFP-N1 vector for identification of channel-expressing cells. Data were filtered at 0.5 kHz and samples at 2 kHz. Borosilicate pipettes (Sutter, Novato, CA) were polished to resistances of 4-7 MΩ in perforated patch pipette solution. If necessary, access resistance (Ra) was compensated by 40-80% to 10-15 MΩ.

All recordings are made in the presence of 2 mM Ca2+ in external solution. Standard external solution (SES) contained (in mM): 140 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 D-glucose and 10 HEPES, pH 7.4. The pipette solution for the perforated-patch consisted of (in mM): 110 K-methanesulfonate, 30 KCl, 1 MgCl2, 10 HEPES pH 7.3, and 250 μg/ml amphotericin B (Sigma, St. Louis, MO). Drugs were applied using a computer controlled pressure-driven 8-channel system (ValveLink8; AutoMate Scientific, San Francisco, CA).

**Ca2+ and Fluorescent Imaging in TG Neurons** - To measure intracellular [Ca2+] levels, the dye Fura-2 AM (2 μM; Molecular Probes, Carlsbad, CA) was loaded for 30 min at 37°C into cells in the presence of 0.05% Pluronic (Calbiochem). Fluorescence was detected with a Nikon Eclipse TE 2000-U microscope fitted with a 40x/1.30 NA Fluor objective. Fluorescence images from excitation wavelengths were collected and analyzed with the MethaFluor Software (MethaMorph, Web Universal Imaging Corporation, Downingtown, PA). The net change
in Ca\(^{2+}\) was calculated by subtracting the basal Ca\(^{2+}\) level (mean value collected for 60 s prior to agonist addition) from the peak Ca\(^{2+}\) level achieved after exposure to the agonists. Ratiometric data were converted to \([Ca^{2+}]_i\) (in \(\mu\)M) by using the equation \([Ca^{2+}]_i = K^* (R – R_{min})/(R_{max} – R)\), where R is the 340/380 nm fluorescence ratio. \(R_{min}\), \(R_{max}\) and \(K^*\) (0.1, 1.6 and 0.65 \(\mu\)M, respectively) were measured according to a previously described method (32).

Calcineurin Activity Assay - Cultured TG neurons were grown for 5 days, and harvested following manufacturer’s instructions (BIOMOL, Plymouth Meeting, PA). Cells were rinsed twice with 4°C 1X PBS and harvested in lysis buffer (50mM Tris, pH 7.5, 0.1mM EDTA, 0.1 mM EGTA, 1mM DTT, 0.2% NP-40) with protease inhibitor cocktail. Cells were gently triturated via 10 passes through a 20g needle, and lysed via 3 freeze/thaw cycles (45 sec liquid N2, 45 sec 30°C water bath). Cell lysates were centrifuged at 1000 x g to remove nuclei and unlysed cells. The ensuing supernatant was retained and protein determination was completed following instructions from the manufacturer of the Bradford Assay (BioRad). Lysates were purged of free phosphates by gel filtration, and calcineurin dephosphorylation of RII phosphopeptide substrate (BIOMOL) was monitored colorimetrically (OD620nm) on a Versa Max microplate reader (Molecular Devices, Sunnydale, CA).

siRNA Transfection - siRNA directed against rat TRPA1 (A-1, GGAACUGCAUACCAACUU-dTdT, sense) and drosophila TRPA1 (Adr-1, GCAUGUCATCGAUUUCA-dTdT, sense) were custom synthesized by Dharmacon RNA Technologies (Chicago, IL). Silencer Negative Control #1 siRNA was used as the scrambled negative control (Ambion, Austin, TX). For transfection, 20 \(\mu\)l of HiPerFect transfection reagent (Qiagen, Valencia, CA) and 625 ng of siRNA were combined in F12 media (Gibco, Carlsbad, CA), and incubated at 25°C 15 minutes. TG neurons were cleared of all cellular debris, and incubated overnight with siRNA/HiPerFect/F12 at 37°C. Post-transfection, TG neurons were moved to normal media (see above), and incubated as described here. This procedure was conducted twice (on day 2 and day 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 media. Coverslips were rinsed with PBS, and fixed with 4% paraformaldehyde for 10 min at 25°C. Following fixation, coverslips were rinsed twice with PBS, and incubated with 5% normal goat serum, 0.5% Triton X-100 in PBS for 30 min at 25°C. Coverslips were then incubated with antisera directed specifically towards FKBP12 (1:500, Affinity Bioreagents, Golden, CO) and TRPV1 (Guinea pig 1:500, Neuromics, Edina, MN) overnight at 4°C. Coverslips were then rinsed three times and incubated with appropriate secondary antibodies (TRITC, 1:250; FITC, 1:250) for one hour at 25°C. Following rinsing three times with PBS, coverslips were attached to microscope slides and dried overnight. For double-label immunofluorescence, cover-slipped images were acquired using a 40X objective lens mated to a Nikon E600 microscope (Melville, NY, USA) equipped with a Photometrics SenSys digital CCD camera (Roper Scientific, Tucson, AZ, USA) connected to a computer equipped with Metamorph V4.1 image analysis software (Universal Image Corporation, Downingtown, PA, USA).

Mass Spectrometry (MS) – Immunoprecipitated TRPV1 was resolved via 15% SDS-PAGE and protein bands were stained with Coomassie Blue. The band of interest was excised, digested \textit{in situ} with trypsin (modified; Promega, Madison, WI). The resulting digests were subjected to capillary HPLC-electrospray ionization tandem mass spectrometry (HPLC-ESI/MS/MS) 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 as follows: column, PicoFrit™ (New Objective; 75 \(\mu\)m i.d.) packed to 10 cm with C18 adsorbent (Vydac; 218MSB5; 5 \(\mu\m, 300 \AA\); mobile phase A, 0.5% acetic acid/0.005% TFA; mobile phase B, 90% acetonitrile/0.5% acetic acid/0.005% trifluoroacetic acid; linear gradient of 2 to 72% B in 30 min; flow rate, 0.4 \(\mu\)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 MS/MS spectra were analyzed by Mascot (Matrix Science; London, UK). Further assessment of probabilities of protein identification was obtained through use of Scaffold (Proteome Software, Inc.; Portland, OR). The probability of identification of TRPV1 (rat, 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 Figure 1H. MS analyses were carried out in the UTHSCSA Institutional Mass Spectrometry Laboratory, San Antonio, TX.

RESULTS

Cannabinoids Dephosphorylate Threonine Residues of TRPV1 in TG Neurons - The cannabinoids ACEA, AEA, and WIN were evaluated in this individual study since all have been shown to result in significant peripheral antihyperalgesia 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 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. Figure 2A illustrates a significant reduction in 32p-phospho-labeling of immunoprecipitated TRPV1 receptor following treatment with ACEA (25μM), AEA (25μM), or WIN (25μM), in comparison to vehicle treated cells. ACEA, AEA, and WIN treatment led to 20.1 ± 3.8%, 30.1 ± 7.6%, and 27.2 ± 6.5% reductions in TRPV1 phosphorylation, respectively, when compared to vehicle (*p<0.05, n=3). It is important to note that the concentrations of cannabinoids used in these studies are between 1000- and 10,000-fold higher than those concentrations initially characterized to activate 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 antibodies specific for phospho-serine or phospho-threonine. Cannabinoid treatment produced a significant reduction in the phospho-threonine immunoreactivity of immunoprecipitated TRPV1 from TG neurons compared with vehicle-treated neurons (Figure 2B). In contrast, cannabinoid treatment produced no significant change in phospho-serine immunoreactivity (1.7 ± 6.5% reduction for ACEA [p=0.75], 20.3 ±9.9% increase for AEA [p=0.16], and 18.3 ± 6.2% increase for WIN [p=0.09], all with n=4). ACEA, AEA, and WIN treatment led to 35.2 ± 4.7%, 57.8 ± 8.7%, and 63.9 ± 9.5% reduction in TRPV1 threonine-phosphorylation, respectively, when compared to vehicle (*p<0.05, n=4). These results suggest that cannabinoid treatment produces a significant dephosphorylation of threonine residues on 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 FK-506 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 it to express at the correct size (~12kDa, Figure 3A) and with TRPV1-positive neurons (Figure 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 FK-506 pretreatment (Figure 3C). Furthermore, dephosphorylation of threonine residues on TRPV1 from TG neurons following WIN treatment was reversed following FK-506 pre-treatment (Figure 3D, p<0.05, n=4), suggesting a role for calcineurin in TRPV1 modulation by WIN. Dose-response experiments conducted previously were utilized to determine the optimal concentration of WIN used in these and following studies (41).

**TRPA1 is Necessary for WIN-Induced TRPV1 Dephosphorylation** – CB1/CB2 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 mechanisms of peripheral cannabinoid actions on nociceptive neurons could exist (42-44). Recent reports (and this study) also indicate that inhibition of TRPV1 by WIN is G-protein independent, occurs at low micromolar concentrations of WIN, and is Ca^{2+}-dependent (41). Moreover, TRPA1 was revealed as a candidate receptor activated by WIN (N. Ruparel et al., 2005, Soc. Neurosci., abstract).

Therefore, we investigated the role of TRPA1 in WIN-induced dephosphorylation of TRPV1 in TG neurons. Firstly, 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)). Figure 4 illustrates that application of 25 μM WIN, unlike 100 nM capsaicin, did not gate a current in CHO cells transfected with TRPV1. In contrast, TRPA1-transfected CHO cells robustly responded to WIN as well as mustard oil (20 μM) applications. Control CHO cells expressing GFP did not generate currents upon WIN application. Thus, WIN selectively gates TRPA1 but not TRPV1. To verify the dependency 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 transfected with rat TRPA1 siRNA (A-1), mustard oil responses, accessed by Ca^{2+} imaging, were reduced dramatically (~80%) 2 days post-transfection in comparison to cells transfected with a negative control siRNA (Adr-1, Figure 5F), 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± 12 % reduction in TRPA1 expression (Figure 5) without significant changes in TRPV1 expression. In addition, the dephosphorylation of TRPV1 threonine residues from TG neurons treated with WIN (25μM) was abolished in neurons transfected with A-1 siRNA (Figure 5). Negative control siRNA transfections had no significant effect (Adr-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 Gα 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 PLC via CB1 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 TRP channels (46), WIN could indirectly activate TRPA1 through the liberation of diacylglycerol (DAG) following PLC activation.

To address this possible cellular pathway of WIN activity, we employed the green fluorescent protein-tagged pleckstrin homology domain (GFP-PHD) PLC activity sensor. Specifically, translocation of membrane-localized GFP-PHD to the cytosol serves as a measure for PIP_2 hydrolysis by receptor-stimulated activation of PLCβ (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 muscarinic type 1 receptor (m1R), or bradykinin type 2 receptor (B2R), together with GFP-PHD, revealed neither GFP-PHD translocation (Figures 6A and C), nor Ca^{2+} accumulation (Figures 6B and C). However, the positive control experiments evoked Gα–coupled receptor activation by subsequent stimulation of the same CHO cells with oxotremorine (10μM, n=19/29) or bradykinin (200nM, n=9/12), and resulted in robust GFP-PHD translocation (Figure 6A and C). These cells also displayed an increase in Ca^{2+}...
influx (Figure 6B), likely due to the release of intracellular stores of Ca\textsuperscript{2+} following PIP\textsubscript{2} degradation to IP\textsubscript{3} (49). In cells transfected additionally with TRPA1, WIN treatment led to a dramatic increase in Ca\textsuperscript{2+} influx over cells transfected with GFP-PHD alone (n=8/18; Figures 6A, B and D). TRPA1 transfected cells also displayed a modest increase in GFP-PHD translocation, likely due to activation of Ca\textsuperscript{2+}-sensitive PLC isoforms, as has been reported previously (50).

It has been reported that the enzymatic activities of Ca\textsuperscript{2+}-sensitive PLC isoforms are dependent on the amount of Ca\textsuperscript{2+} influx following receptor activation (51,52). Indeed, TRPA1-expressing CHO cells that accumulated less than 600 nM Ca\textsuperscript{2+} after WIN stimulation failed to display GFP-PHD translocation. Since WIN rarely triggers accumulation greater than 600 nM [Ca\textsuperscript{2+}]i in sensory neurons (data not shown), we transfected sensory neurons with the GFP-PHD construct to monitor PIP\textsubscript{2} depletion following WIN treatment. Figure 6E demonstrates that WIN (25 \u03bcM) was unable to activate PLC\(\beta\) (n=10) in TG neurons containing GFP-PHD, whereas application of a positive control, bradykinin (1 \u03bcM), 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\textalpha\textsubscript{s} signaling pathways in either CHO cells or TG neurons.

**TRPV1 Residues T144 and T370 are Dephosphorylated Following WIN Treatment** - As previously mentioned, both threonine and serine residues of the TRPV1 channel have been confirmed as phosphorylation sites for various kinases that serve to sensitize channel activity. Data from Figure 2B suggests that threonine residues are specifically dephosphorylated by WIN treatment, so site-directed mutants of TRPV1 were created, mutating three individual threonine residues to alanine residues: T144 and T370 (53), and T704 (28). CHO cells were transiently transfected with mouse TRPA1 cDNA and either WT TRPV1, TRPV1 T144A, TRPV1 T370A, or TRPV1 T704A. Perforated-patch electrophysiology was utilized to measure inward capsaicin (CAP) current (\(I_{\text{CAP}}\)) following vehicle or WIN (25 \u03bcM) pre-treatment for each transfection set (Figure 7). Figure 7A shows that the magnitude of \(I_{\text{CAP}}\) was affected by certain threonine mutations. Since \(I_{\text{CAP}}\) tachyphylaxis is proposed to follow a similar mechanism as WIN-induced inhibition of \(I_{\text{CAP}}\) (i.e. Ca\textsuperscript{2+}-, calcineurin- and dephosphorylation-dependency), we employed \(I_{\text{CAP}}\) tachyphylaxis as a positive control. Pretreatment with WIN, like CAP, led to a significant reduction in \(I_{\text{CAP}}\) in WT TRPV1 transfected CHO cells compared to \(I_{\text{CAP}}\) of vehicle treated cells (Figure 7A, B, and D). In contrast to WT and T704A TRPV1, the significant \(I_{\text{CAP}}\) reduction by WIN as well as CAP pre-treatments was not apparent in CHO cells transfected with TRPV1 T144A, TRPV1 T370A (Figure 7E), or the double-mutant TRPV1 T144A/T370A (Figure 7G). In agreement with previous reports (25), T144 and T370 were found to be essential for \(I_{\text{CAP}}\) tachyphylaxis (Figure 7B). Results presented here suggest that T144 and T370 are essential for WIN-induced TRPV1 desensitization (Figure 7C).

Studies on acute and pharmacological (i.e. tachyphylaxis) desensitization of \(I_{\text{CAP}}\) demonstrate 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. Figure 8A illustrates representative traces recorded for various TRPV1 phosphorylation site mutants, including TRPV1 T704A, T144A, T370A, and T144A/T370A, after vehicle treatment. In agreement with results reported by others (25), analysis of these traces indicates that acute desensitization of TRPV1 is significantly reduced in the absence of phosphorylation on T144 and T370 of the receptor (Figure 8B). In summary, T144A and T370A mutations of TRPV1 affect not only acute and pharmacological desensitization of \(I_{\text{CAP}}\), as previously reported (25), but also WIN-induced reduction in \(I_{\text{CAP}}\).

In the next set of the experiments, we evaluated whether the essential mutation T144A/T370A in TRPV1 alters WIN induced dephosphorylation of TRPV1. CHO cells were transiently transfected with TRPA1 and WT TRPV1, TRPA1 and TRPV1 T144A/T370A, or pcDNA3 empty vector and WT TRPV1, and then monitored for changes in \(^{32}\text{P}\)-orthophosphate
incorporation by TRPV1 following application of WIN (25μM). As anticipated, WIN application led to significant reduction by 26.7 ± 7.7% in TRPV1 phosphorylation compared to vehicle-treated cells (p<0.05, n=3, Figure 9). In cells transfected with TRPA1 and TRPV1 T144A/T370A, TRPV1 phosphorylation was unaffected by WIN (Figure 9), in agreement with data presented in Figure 7. TRPV1 phosphorylation had similar results 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

The studies presented herein detail a mechanism by which cannabinoids act to dephosphorylate, and as a result, desensitize TRPV1 activity. A major finding of this study is that WIN exerts 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, WIN was found to act directly on TRPA1 in both CHO cells and TG neurons. In addition, the co-expression of TRPA1 with TRPV1 was discovered to be pertinent to the dephosphorylation of TRPV1 on threonine residues 144 and 370 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 that contrasts with the more classical, metabotropic cannabinoid receptors.

The identification of amino acid residues T144 and T370 as important determinants of TRPV1 sensitivity to CAP activation is an interesting discovery. Despite the activation of calcineurin by WIN, total TRPV1 phosphorylation is 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 phospho-residues on TRPV1. Indeed, numerous other functionally important phosphorylated residues have been identified on TRPV1, including, but not limited to, S800 (36,54), S116 (39), S502 (53,54), and T704 (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 would suggest that TRPV1 is chronically phosphorylated under these conditions, and that the 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.

Data reported here on the ability of WIN to desensitize I_{CAP} are in agreement with the tachyphylaxis reported with repeated applications of CAP (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 CAP (25), (41), it is believed that WIN-induced TRPV1 desensitization is also dependent on Ca^{2+}. It is interesting to note that desensitization of I_{CAP} by WIN in this study was approximately half as efficacious as that by CAP (Figure 7B-C). This difference may be due to the different sites of action of WIN and CAP. CAP, acting on TRPV1, is known to induce a robust inward conductance of Ca^{2+} ions with a current amplitude of about 6-8 nA (by 10 μM CAP) in sensory neurons (55), while WIN, acting on TRPA1, gates a much smaller inward current of approximately 250 pA (by 50 μM WIN) in TG neurons (N. Ruparel et al., 2005, Soc. Neurosci., abstract). Reduced current amplitude with WIN activation of TRPA1 in comparison with that for CAP on TRPV1 could partly explain the differences in the ability of each to desensitize I_{CAP}.

Selective knock-down by directed siRNA, coupled with the transient transfection of CHO cells, jointly support 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 has been shown here to require TRPA1. Recent findings by Lauckner et al. have suggested that WIN acts through a pertussis-insensitive, G_{αq}-coupled CB1 receptor in transfected HEK 293 cells (47). 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 HEK cells (50). Nevertheless, data presented in this study support a non Gq-coupled mechanism in this particular action of the cannabinoid in TG neurons or naïve CHO cells. Furthermore, the increase in [Ca2+]i following WIN treatment in CHO cells in this study was greater than that demonstrated by Lauckner et al. in HEK 293 cells, suggesting a direct gating effect by TRPA1, and not the release of Ca2+ from intracellular stores as previously reported (47).

The concentration of WIN required to activate TRPA1 and lead to dephosphorylation of TRPV1 in this study was relatively high (25 μM) compared to 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 found 100nM WIN to reversibly inhibit neuroblastoma and glioma cell voltage-gated calcium currents by 40% in a PTX-sensitive fashion (56). Moreover, Khosabova and colleagues recently reported that voltage-gated calcium channels of only large sensory neurons (i.e. non-nociceptors) are affected by nanomolar concentrations of WIN (43). In contrast, micromolar concentrations of WIN attenuate depolarization-evoked CGRP release from TG neurons (11). Similarly, the present study focused on activation of the channel by a cannabinoid, and it is unlikely that the 25 μM concentration of WIN exerted non-specific effects, given that the effects reported in this study were completely dependent on TRPA1 expression (see also Figure 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 of OAG (a membrane permeable analog of DAG) is required to gate TRPA1, whereas PIP2 depletion by bradykinin, for example, leads to a smaller amount of intracellular DAG that is still able to gate the channel (46). Therefore, despite the high WIN concentrations used, reported results support the specificity of the cannabinoid for TRPA1.

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 the subsequent dephosphorylation of TRPV1 on amino acid residues T144 and T370. The implications of these results lend support to the use of cannabinoids as analgesics in the clinical setting, potentially attenuating peripheral inflammatory hyperalgesia caused tissue damage and inflammation.

ACKNOWLEDGEMENTS

We would like to thank Gabriela Helesic, Dr. Sue Weintraub, and the UTHSCSA Mass Spectrometry Core for expert technical assistance, Dr. David Julius for providing rTRPV1 cDNA, Dr. Carla Nau for providing rTRPV1 T144A cDNA, and Dr. Ardem Patapoutian for providing mTRPA1 cDNA. Research was supported by NIH grants F32 DE016500 (N.A.J.), R21 DE014928 (A.N.A.), and R01 DA19585 (K.M.H.).

REFERENCES


The abbreviations used are: ACEA, arachidonyl-2-chloroethylamide; AEA, anandamide; B2R, bradykinin type 2 receptor; BSA, bovine serum albumin; CAMKII, calmodulin kinase II; CAP, capsaicin; CB1 1/2, cannabinoid receptor type 1/2; CGRP, calcitonin-related gene peptide; CHO, Chinese hamster ovary; DAG, diacylglycerol; DMEM, Dulbecco’s modification of Eagle’s medium; FKBP12, FK binding protein 12; GFP-PHD, green fluorescent protein-pleckstrin homology domain; HEK 293, human embryonic kidney 293; HRP, horse radish peroxidase; m1R, muscarinic type 1 receptor; MO, mustard oil; NGF, nerve growth factor; PKC, protein kinase C; PLC, phospholipase C; PP2B, calcineurin, protein phosphatase 2B; PTX, pertussis toxin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS-Tw, Tris-buffered saline-Tween; TG, trigeminal ganglia; TRPA1, transient receptor potential A type 1; TRPV1, transient receptor potential V type 1; WB, Western blot; WIN, WIN 55, 212-2;
FIGURE LEGENDS

Figure 1. Western blot and Mass Spectrometry (MS) data illustrating the specificity of the antibodies used to identify TRPV1 and TRPA1. Western blot data were generated utilizing several antibodies and sources, including (A) anti-TRPV1 Ab-2 from Calbiochem on 50 μg TG cell lysate, (B) anti-TRPV1 Ab-1 from Calbiochem on 50 μg TG cell lysate, (C) anti-TRPV1 Ab-1 pre-incubated with 25 μg antigenic peptide from Calbiochem on 50 μg TG cell lysate, (D) anti-TRPV1 Ab-1 from Calbiochem on 25 μg cell lysate of rat-TRPV1 transiently transfected CHO cells, (E) anti-TRPA1 N-terminal antibody on 50 μg TG cell lysate, (F) anti-TRPA1 N-terminal antibody pre-incubated with 100 μg antigenic peptide on 50 μg TG cell lysate, (G) anti-TRPA1 N-terminal antibody on 25 μg cell lysate of mouse-TRPA1 transiently transfected CHO cells. Black arrows to the left of each blot correspond to size markers (in kDa), grey arrows on the right of each blot correspond to 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 via 15% SDS-PAGE stained with Coomassie Blue, excised from the gel, and analyzed for trypsin digestion by Mass Spectrometry. 13 unique peptides were identified (highlighted in yellow), providing 15% coverage of the amino acid sequence for rat TRPV1 (accession number AF029310). Mascot analytical software made a 100% match between the cumulative peptides to rat TRPV1.

Figure 2. Cannabinoids reduce TRPV1 phosphorylation. A) TG neurons were treated with ACEA (25μM), AEA (25μM), and WIN (25μM), and analyzed by SDS-PAGE and WB for 32P phosphate incorporation by TRPV1. Autoradiographic results normalized to total immunoprecipitated TRPV1 (B), with band densities quantified and expressed as a percentage of vehicle treated cells (C), *p<0.05, ANOVA, n=3. TG neurons treated with ACEA (25μM), AEA (25μM), and WIN (25μM) and immunoprecipitated TRPV1 was analyzed for phospho-serine (D) and –threonine (E) immunoreactivities. Phospho- results normalized to total immunoprecipitated TRPV1, with band optical densities quantified and expressed as a percentage of vehicle treated cells (F), *p<0.05 (significant from vehicle), ANOVA, n=4. Black arrows to the left of each blot correspond to size markers (in kDa), grey arrows on the right of each blot correspond to the immunoreactive band of interest. WB results are representative of 3-4 independent trials.

Figure 3. WIN dephosphorylates TRPV1 through calcineurin. A) FKBP12 expression in TG neurons analyzed by WB in the absence/presence of antibody blocking peptide (BP). Results are representative of 3 independent trials. B) FKBP12 expression analyzed by immunofluorescence in a TG neuron, co-expressing TRPV1. Yellow bar = 25μm, results representative of two independent trials. C) Calcineurin activity from TG neurons treated with DMSO vehicle, CAP (100 nM), WIN (25 μM), and FK-506 (100μM) + WIN, analyzed by RII phosphopeptide assay, *p<0.05, ANOVA and 1 tailed- t-test, n=3. D) TG neurons treated with WIN (25μM) or FK-506 (100μM) + WIN, and immunoprecipitated TRPV1 analyzed for phospho-threonine immunoreactivity. Phospho-threonine results normalized to total immunoprecipitated TRPV1, with band optical densities quantified and expressed as a percentage of vehicle treated cells, *p<0.05, ANOVA, n=4. Results are representative of 4 independent trials.

Figure 4. Current traces from transfected CHO cells. CHO cells were transfected with GFP, TRPV1, or TRPA1 vectors, and treated with WIN (25 μM), CAP (100 nM) and/or mustard oil (MO, 20 μM). Recordings have been made in perforated patch voltage clamp configuration. Cells were analyzed for currents, results are representative of 5-9 trials. Drug application durations are marked by horizontal bars.
Figure 5. TRPA1 mediates WIN-induced dephosphorylation of TRPV1 in TG neurons. TG neurons transfected with siRNA directed against TRPA1 (A-1), negative silencer siRNA ((-)), siRNA directed against drosophila TRPA1 (Adr-1), or no siRNA (mock) are analyzed for TRPV1 phospho-threonine by WB following WIN (25μM) treatment (1: mock transfection, DMSO vehicle, 2: mock transfection, WIN, 3: A-1 transfection, WIN, 4: (-) silencer siRNA control, WIN, 5: Adr-1 control, WIN). Phospho-results (A) normalized to total immunoprecipitated TRPV1 (B), or TRPA1 expression (C) normalized to TRPV1 expression (D), with band optical densities quantified and expressed as a percentage of vehicle treated cells (E). *p<0.05 (significant from vehicle/mock), *p<0.05, ANOVA, n=4. Gray bars indicate threonine phosphorylation (T), white bars indicate TRPA1 protein expression (A) by WB. Black arrow to the left of each blot correspond to size markers (in kDa), grey arrows on the right of each blot correspond to the immunoreactive band of interest. Results are representative of 4 independent trials. F) Mustard oil (20 μM) evoked calcium imaging of mock, Adr-1 (FITC-labeled), or A-1 transfected TG cultures over a 4-day period of post-transfection.

Figure 6. WIN directly activates TRPA1 to increase [Ca^{2+}] through a Gc_{αs}-independent mechanism. TG neurons were transfected with the GFP-PHD PLC sensor, while CHO cells were transiently transfected with GFP-PHD PLC sensor and different combinations of TRPA1, m1R, or B2R. Translocation of GFP-PHD from the plasma membrane to the cytosol and accumulation of intracellular Ca^{2+}(Δ[Ca^{2+}]) were concurrently imaged by a set of filters. Data for analysis were collected every 5 sec after exposure to WIN (25μM, 3 min), oxotremorine (Oxo, 10μM, 5 min), or bradykinin (BK, 200nM, 5 min), with 5 min wash out period. A) Translocation of GFP-PHD (F/F₀) were calculated from the measurement of F₄₇₀ within a pre-determined area of the cytosol by MetaFluor software, *p<0.05, ANOVA, n values are indicated. B) Δ[Ca^{2+}], was calculated from measurements of changes in F₃₄₀/₃₈₀ ratio by MetaFluor software, **p<0.005, ANOVA, n values are indicated. C-D) Typical traces of GFP-PHD translocation and calcium influx on the same temporal scale for m1R/GFP-PHD transfected CHO cells and TRPA1/m1R/GFP-PHD transfected CHO cells. GFP images of a cell within indicated time points are presented. E) Time course of GFP-PHD translocation in a TG neuron stimulated by WIN (25μM) or BK (1μM) plotted against GFP fluorescent density (F₄₇₀) in the cytosol.

Figure 7. TRPV1 desensitization of I_{CAP} by WIN is dependent on T144 and T370 phosphorylation. CHO cells were transiently transfected with TRPA1 and either TRPV1 wt, or T144A, T370A, T704A, and T44A/T370A mutants of TRPV1 and treated with vehicle 0.1% DMSO or WIN (25μM) for 10 min, washed for 5 min, patched, and I_{CAP} recorded during 30 sec application of 300nM CAP. Data normalized to the mean peak of I_{CAP} measured from vehicle-treated cells, n=8-15/treatment. A) Inward I_{CAP} measured from CHOs expressing TRPV1 wt, T144A, T370A, T704A, and T144A/T370A, *p<0.05, ***p<0.001, paired t-test. B) Tachyphylaxis after re-application of CAP to cells expressing phospho-site mutants, **p<0.005, ANOVA. C) Normalized data on desensitization of I_{CAP} by WIN in TRPV1 phospho-site mutants, *p<0.05, **p<0.005, ANOVA. D-G) Typical I_{CAP} traces of CAP- and WIN-induced desensitization in wt and the phospho-site mutants of TRPV1. Durations of drug application are indicated by horizontal bars. Number of cells in trials is noted inside bar graphs.

Figure 8. Acute TRPV1 desensitization is modulated by phosphorylation at T144 and T370. A) Current traces of five separate transiently transfected CHO cells to a 30 sec application of 300 nM CAP expressing the indicated TRPV1 mutants (WT, T704A, T370A, T144A, T144A/T370A). 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 sec after peak of I_{CAP} is reached. The number of neurons in each trial is indicated within each bar, **p<0.005, ***p<0.001, ANOVA.
Figure 9. TRPA1 co-expression is necessary for WIN-induced dephosphorylation of TRPV1. CHO cells transiently transfected with indicated cDNAs, and analyzed for $^{32}$p phosphate incorporation by TRPV1 following WIN (25μM) or vehicle. Autoradiographic results (A) normalized to total immunoprecipitated 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 to the left of each blot correspond to size markers (in kDa), grey arrows on the right of each blot correspond to the immunoreactive band of interest. Results are representative of three independent trials.
Figure 1

A.  

B.  

C.  

D.  

E.  

F.  

G.  

H.  

| MEORASLDSE | ESESPPOENS | CLDPDPDRDPN | CKPVPVPHI |
| FTTRSRTRLF | GKDSEEASP | LCDPYEEGGL | ASCPIITVSS |
| VLTIPPGDG | PASYRPSSQD | SVSAGEKPRP | LYDRRSIFDA |
| VAQSNCOELE | SLPLPFQLRSK | KRLTDSEFKD | PETGKTCLLK |
| AMLNQLHGNQ | DTVIALLDVA | RKTDSLKNQV | NASYTDSSYYK |
| GQTAHIAIE | RINMTLYTL | VENFGADVQAA | ANGDFFKKTK |
| GRPGFYFGEL | PLSLAACTNQ | LAVKFFLLQN | SWQPADISAR |
| DSVGNTVLHA | LAVEVADNTVD | NTKFVTSMYN | ELILIAGK |
| PTOLKLEEITN | RCLKTPLALA | ASSGKIGVLA | YILQREIHEP |
| ECRHLRSRKFT | EWVAYQPHSS | LVDLSCIDTC | EKNSVLEVIA |
| YCSSETPNRH | DMMLLVEPLNR | LLQDKWDRFV | KRIFYFNFFV |
| YCLMIFITA | AAYRPVEG | PPKKLKNTVG | DYZEURGTE |
| SVSGGYVFFF | RGIQYFLQR | PSKSLLFYDS | YSELFFYOS |
| LFMVLSVLY | FSQREYRVAS | MVFSLAMGWT | NMLYTRGQ |
| QOMGIAYVMI | KMLRDLCRF | MFVLYVLF | FSTAVTLIE |
| DGKNSSLPM | STHKCRGSA | CKPGNSYNLS | YSTCLELFK |
| TIGMGDLFT | ENYDFAKVFI | ILLLAYVILT | YILLNLMLIA |
| LMGGETVNKIA | QEKSNICKWLQ | RAITILDTEK | SFLKCMRKAF |
| RSGKLLQVGF | TPDGKDDYRW | CFRVEDVNTNT | TWTNVGIIIN |
| EDLPNGCEGVK | RFLSFSLRS | RGVSRRWKNF | ALVPLLDRAS |
| TRDRHATAQGE | EVOULKHTGS | LKPEDAEVF | DSMVPGEK |
Figure 2

A. IP: TRPV1, 32P AutoRad

B. IP: TRPV1, WB: TRPV1

C. Optical Density (% of vehicle)

D. IP: TRPV1, WB: P-Ser

E. IP: TRPV1, WB: P-Thr

F. Optical Density (% of vehicle)
Figure 3

A.  

B.  

C.  

D.  

[Description of Figure 3A, 3B, 3C, 3D]
Figure 5

A. IP:TRPV1, WB: P-Thr

B. IP:TRPV1, WB:TRPV1

C. WB:TRPA1

D. WB:TRPV1

E. Optical Density (% of Veh/mock)

F. M0-evoked ACO-1
Figure 6
Figure 7

A. Trapped Tset. Quat. (pF)

B. Normalization of the mean of "CAP" (pF)

C. Normalization of the mean of "WIN" (pF)

D. WT TrpV1 + TrpA1

E. TrpV1 T370A + TrpA1

F. TrpV1 T704A + TrpA1

G. TrpV1 T144A/T370A + TrpA1
Figure 8

A. 

B. 

TRPV1 Mutants

% Acute Desensitization

WT T144A T370A T144A/T370A T704A

15 9 15 9 10
Figure 9

A. IP: TRPV1, 32P AutoRad
   pcDNA + TRPV1 + TRPA1 + TRPV1 M + TRPV1
   WIN - + - + - +

B. IP: TRPV1, WB: TRPV1
   pcDNA + TRPV1 + TRPA1 + TRPV1 M + TRPV1
   WIN - + - + - +

C. Optical Density (% of vehicle)
   WIN - - + + - +
Cannabinoid WIN 55,212-2 regulates TRPV1 phosphorylation in sensory neurons

J. Biol. Chem. published online September 5, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M603220200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts