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**ABT-102 ((*R*)-(5-*tert*-butyl-2,3-dihydro-1*H*-inden-1-yl)-3-(1*H*-indazol-4-yl)-urea)
Blocks Polymodal Activation of TRPV1 Receptors *In Vitro* and Heat-Evoked Firing
of Spinal Dorsal Horn Neurons *In Vivo***

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Abbreviations:

TRPV1, transient receptor potential vanilloid-1; CNS, central nervous system; DRG, dorsal root ganglion; ABT-102, (*R*)-(5-*tert*-butyl-2,3-dihydro-1*H*-inden-1-yl)-3-(1*H*-indazol-4-yl)-urea; D-PBS, Dulbecco's phosphate-buffered saline; D-MEM, Dulbecco's modified Eagle's medium; TRPV3, transient receptor potential vanilloid-3; TRPV4, transient receptor potential vanilloid-4; TRPM8, transient receptor potential-melastatin-8; TRPA1,

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transient receptor potential A-1; PDBu, phorbol-12,13-dibutyrate; CGRP, calcitonin gene-related peptide; PKC, protein kinase C; FBS, fetal bovine serum; MEM, minimal essential medium; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; MES, 2-morpholinoethanesulfonic acid; ATP·Mg, adenosine 5'-triphosphate magnesium salt; NGF, nerve growth factor; NS, nociceptive-specific; WDR, wide dynamic range; A-425619, 1-isoquinolin-5-yl-3-(4-trifluoromethyl-benzyl)-urea; AMG 6880, (*E*)-3-(2-(piperidin-1-yl)-6-(trifluoromethyl)pyridin-3-yl)-*N*-(quinolin-7-yl)acrylamide; AMG 517, (*N*)-{4-[6-(4-trifluoromethyl-phenyl)-pyrimidin-4-yloxy]-benzothiazol-2-yl}-acetamide; BCTC, *N*-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2*H*)-carbox-amide; SB-452533, 1-(2-bromo-phenyl)-3-[2-ethyl-*m*-tolyl-amino)-ethyl]-urea; CAP, capsaicin; CPZ, capsazepine; NADA, N-arachidonoyl-dopamine; FLIPR, fluorometric imaging plate reader; DMSO, dimethylsulfoxide; RFU, relative fluorescence units; agonist 6, 1-(benzo[*b*]thiophen-3-yl)-2-(cyclohexyl(ethyl)amino)ethanone; 2-APB, 2-aminoethoxydiphenyl borate; ELISA, enzyme-linked immunosorbent assay; CFA, complete Freund's adjuvant.

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Abstract

The TRPV1 receptor, a non-selective cation channel expressed on peripheral sensory neurons and in the CNS, plays a key role in pain. TRPV1 receptor antagonism is a promising approach for pain management. In this report, we describe the pharmacological and functional characteristics of a structurally novel TRPV1 antagonist, ABT-102, which has entered clinical trials. At the recombinant human TRPV1 receptor ABT-102 potently ($IC_{50} = 5 - 7$ nM) inhibits agonist (capsaicin, NADA, anandamide and proton)-evoked increases in intracellular Ca^{2+} levels. ABT-102 also potently ($IC_{50} = 1 - 16$ nM) inhibits capsaicin-evoked currents in rat DRG neurons and currents evoked through activation of recombinant rat TRPV1 currents by capsaicin, protons or heat. ABT-102 is a competitive antagonist ($pA_2 = 8.344$) of capsaicin-evoked increased intracellular Ca^{2+} , and shows high selectivity for blocking TRPV1 receptors over other TRP receptors and a range of other receptors, ion channels, and transporters. In functional studies ABT-102 blocks capsaicin-evoked CGRP release from rat DRG neurons. Intraplantar administration of ABT-102 blocks heat-evoked firing of wide dynamic range and nociceptive-specific neurons in the spinal cord dorsal horn of the rat. This effect is enhanced in a rat model of inflammatory pain induced by administration of complete Freund's adjuvant. Therefore, ABT-102 potently blocks multiple modes of TRPV1 receptor activation and effectively attenuates downstream consequences of receptor activity. ABT-102 is a novel and selective TRPV1 antagonist with pharmacological and functional properties that support its advancement into clinical studies.

Introduction

The transient receptor potential vanilloid-1 (TRPV1) receptor is a non-selective cation channel which is expressed on peripheral and central terminals of small and medium-sized primary sensory neurons and plays a key role in the detection and modulation of nociceptive stimuli (Caterina and Julius, 2001). In the central nervous system (CNS), the channel is expressed presynaptically in lamina I and postsynaptically in lamina II of the spinal cord dorsal horn as well as at supraspinal sites in pain pathways (Valtschanoff et al., 2001; McGaraughty et al., 2003). Recently, both peripheral and central TRPV1 receptors have been implicated in contributing to the broad-spectrum analgesia demonstrated by TRPV1 antagonists (Cui et al., 2006).

The TRPV1 receptor can be activated by the exogenous agonists capsaicin and resiniferatoxin, by noxious heat ($>42^{\circ}\text{C}$), and by extracellular protons ($\text{pH} < 6.0$) (Caterina et al., 1997; Tominaga et al., 1998). The receptor is also activated by several endogenous substances, including the endocannabinoid anandamide and N-arachidonyl dopamine (NADA) (Smart et al., 2000; Huang et al., 2002), as well as by metabolites from the lipoxygenase pathway, such as 12-hydroperoxyeicosatetraenoic acid (Hwang et al., 2000). These diverse stimuli not only directly activate the TRPV1 receptor but also sensitize the receptor, thereby reducing the threshold for activation by other stimuli. Inflammatory mediators, such as prostaglandins, bradykinin and nerve growth factor (NGF), also modulate TRPV1 receptors. Acting via downstream signaling pathways that include protein kinase C (PKC), these inflammatory agents act to sensitize the receptor (Bonnington and McNaughton, 2003; Moriyama et al., 2005; Sikand and Premkumar, 2007). For example, phosphorylation of the TRPV1 receptor by PKC lowers the threshold for activation such that it can be activated at normal body temperature (Sikand and Premkumar, 2007). PKC activation has been reported to contribute to chronic pain (Hua et al., 1999). In addition to activation and sensitization of TRPV1 receptors, inflammatory stimuli also affect TRPV1 receptor expression. Up-regulation of TRPV1

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receptor expression in axons of unmyelinated neurons and an increased transport of TRPV1 mRNA from the cell bodies to central (superficial laminae of the spinal cord dorsal horn) and peripheral terminals (Carlton and Coggeshall, 2001; Tohda et al., 2001) are both associated with inflammatory pain.

TRPV1 receptor activation results in activation of nociceptive-specific (NS) and wide dynamic range (WDR) neurons in the spinal cord (Eckert et al., 2006; McGaraughty et al., 2006). These two classes of neurons play a significant role in pain transmission. At the molecular level, TRPV1 receptor activation results in the release of neuropeptides such as calcitonin gene-related peptide (CGRP) from the peripheral and central terminals of sensory neurons (Rigoni et al., 2003). CGRP plays a role in pain through sensitization of the CNS, as well as in neurogenic inflammation and associated pain in the peripheral nervous system (Kilo et al., 1997; Bird et al., 2006).

The search for potent and selective TRPV1 antagonists that are efficacious in animal models of chronic pain and exhibit pharmacokinetic properties suitable for clinical development has been the subject of intense research in the pharmaceutical industry in recent years (Szallasi et al., 2007). However, many of the reported TRPV1 antagonists, although potent, have poor pharmaceutical properties and only a fraction of them have advanced beyond preclinical studies (Szallasi et al., 2007). In addition, an efficacious TRPV1 antagonist may also need to demonstrate the ability to potently inhibit TRPV1 receptor activation by the multiple stimuli that could directly or indirectly enhance the activity of this polymodal receptor. In the present study we describe a novel TRPV1 antagonist, (*R*)-(5-*tert*-butyl-2,3-dihydro-1*H*-inden-1-yl)-3-(1*H*-indazol-4-yl)-urea (ABT-102), which blocks TRPV1 channel opening by multiple stimuli, CGRP release from DRG neurons and neuronal firing in the rat spinal cord. Collectively these properties support the advancement of ABT-102 into clinical trials.

Methods

Materials

Cell culture media and fetal bovine serum (FBS) were obtained from Sigma-Aldrich (St. Louis, MO). G418 sulfate was obtained from Calbiochem (San Diego, CA). Dulbecco's phosphate-buffered saline, pH 7.4 (D-PBS) (with calcium, magnesium, and 1 mg/ml D-glucose) was obtained from Invitrogen (Carlsbad, CA). Fluo-4 AM was purchased from Tef Labs (Austin, TX). NADA was purchased from Tocris Cookson, Inc. (Ellisville, MO). ((*R*)-(5-*tert*-butyl-2,3-dihydro-1*H*-inden-1-yl)-3-(1*H*-indazol-4-yl)-urea) (ABT-102), 1-isoquinolin-5-yl-3-(4-trifluoromethyl-benzyl)-urea (A-425619) (El Kouhen et al., 2005), (*E*)-3-(2-(piperidin-1-yl)-6-(trifluoromethyl)pyridin-3-yl)-*N*-(quinolin-7-yl)acrylamide (AMG 6880) (Gavva et al., 2005a), (*M*)-{4-[6-(4-trifluoromethyl-phenyl)-pyrimidin-4-yloxy]-benzothiazol-2-yl}-acetamide (AMG 517) (Doherty et al., 2007), *N*-(4-*tert*butylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2*H*)-carbox-amide (BCTC) (Valenzano et al., 2003), 1-(2-bromo-phenyl)-3-[2-ethyl-*m*-tolyl-amino)-ethyl]-urea (SB-452533) (Rami et al., 2004) and 1-(benzo[*b*]thiophen-3-yl)-2-(cyclohexyl(ethyl)amino)ethanone (agonist 6) (Casillas et al., 2006) were synthesized at Abbott Laboratories (Abbott Park, IL). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Animals

Male Sprague-Dawley rats (200-400g) (Charles River Laboratories, Inc., Wilmington, MA) were used in experiments (specific size ranges are described below) and were housed in a temperature-controlled room with a 12/12hr day/night cycle. Food and water were available ad libitum. All animal handling and experimental protocols were approved by Abbott's Institutional Animal Care and Use Committee (IACUC) and conducted in

accordance with the ethical principles for pain-related animal research of the American Pain Society.

Assay of TRPV1 Receptor-Mediated Changes in Intracellular Ca²⁺ Levels

Cloning and stable expression of the human TRPV1 receptor in HEK 293 cells and measurement of TRPV1-mediated elevation of intracellular calcium levels using the fluorescent calcium chelating dye Fluo-4, was as previously described (El Kouhen et al., 2005). The TRPV1 receptor cDNA sequence expressed in these cells corresponds to accession number AL136801. Assay of the effect of compounds on TRPV1 receptor activity stimulated by capsaicin, anandamide, NADA, or protons, or under conditions of phorbol ester-mediated sensitization, was also as previously described (El Kouhen et al., 2005). In washout studies, cells were washed in 250 μ l D-PBS multiple times prior to treatment with capsaicin. In all of these studies, changes in fluorescence were recorded over time in a fluorometric imaging plate reader (FLIPR) (Molecular Devices Corp., Sunnyvale, CA) ($\lambda_{EX} = 488$ nm, $\lambda_{EM} = 540$ nm). Antagonists were tested at 11 concentrations (indicated on each graph). The peak increase in fluorescence over baseline [relative fluorescence units (RFU)] was calculated and expressed as a percentage of the maximal agonist response (in absence of antagonist). EC₅₀ and IC₅₀ values were calculated from curve-fits of the concentration-effect data using a four-parameter logistic Hill equation (GraphPad Prism[®], GraphPad Software, Inc., San Diego, CA). Significant differences were calculated by unpaired, two-tailed Student's *t* tests. IC₅₀ values at the human TRPV1 receptor obtained in this study, using TRPV1 stably expressed in HEK 293 cells (see Results), are very similar to those we have observed using human TRPV1 stably expressed in 1321N cells (Gomtsyan et al., 2008).

Transient Expression of Other TRP Receptors and Assay of TRP Receptor-Mediated Increases in Intracellular Ca²⁺ Levels

Human recombinant TRP receptors with cDNA sequences corresponding to the following accession numbers were used in assays to assess selectivity: transient receptor potential vanilloid-3 (TRPV3) (NM_145068), transient receptor potential vanilloid-4 (TRPV4) (NM_021625), transient receptor potential melastatin-8 (TRPM8) (NM_024080), and transient receptor potential A-1 (TRPA1) (NM_007332). TRP channels were transiently expressed and assayed for responses to agonist-induced Ca^{2+} flux in the absence or presence of ABT-102 using FLIPR technology. Transient expression of TRPV3, TRPV4, TRPM8 and TRPA1 channels was performed in FreeStyle™ HEK 293-F cells using the FreeStyle™ 293 expression system (Invitrogen, Grand Island, NY), as previously described (Bianchi et al., 2007).

For assay, cells were thawed and prepared as previously described (Bianchi et al., 2007). Cells (25,000 cells/well for TRPV3 and TRPV4, 100,000 cells per well for TRPA1 and TRPM8) were then seeded into black-walled clear-bottom 96-well Biocoat™ poly-D-lysine assay plates (BD Biosciences, Bedford, MA) and incubated 2 to 24hr at 37°C under a humidified 5% CO_2 atmosphere in preparation for assay.

Ca^{2+} influx was measured using Fluo-4 AM loading dye (Invitrogen, Carlsbad, CA), as described above for TRPV1. Activators for these other TRP receptors were as follows: 62.5 μM 2-aminoethoxydiphenyl borate (2-APB) (Tocris, Ellisville, MO) for TRPV3; 2 μM 1-(benzo[*b*]thiophen-3-yl)-2-(cyclohexyl(ethyl)amino)ethanone (agonist 6 from WO2006029210 (Casillas et al., 2006) for TRPV4; 10 μM menthol for TRPM8; 30 μM mustard oil for TRPA1.

Dorsal Root Ganglion (DRG) Neuronal Cultures for Electrophysiological and CGRP Release Studies

All experiments were carried out in accordance with the guidelines and the approval of the Institutional Animal Care and Use Committee (IACUC). All media, antibiotics, and enzymes were obtained from Invitrogen (Carlsbad, CA.), unless indicated.

For electrophysiological studies, DRG neurons (L4-L6) were dissected from 200-300 g male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) and placed in D-MEM containing 0.3% collagenase B (Roche Diagnostics Corporation, Indianapolis, IN) for 60 min at 37°C. The collagenase was replaced with 0.25% trypsin (Invitrogen) in Ca²⁺/Mg²⁺-free D-PBS, followed by further incubation for 30 min. at 37°C. After washing in fresh D-MEM, neurons were dissociated by trituration using a sterile fire-polished Pasteur pipette and plated on polyethylenimine-treated 12 mm glass coverslips in 1 ml D-MEM supplemented with 10% FBS, NGF (50 ng/ml) and 100 U/ml Penicillin and 100 µg/ml streptomycin. Electrophysiological recordings were performed 15-30 hrs later on individual neurons.

For CGRP release studies, DRG neurons were prepared similarly to previous studies (El Kouhen et al., 2005), with minor modifications. Seven-to-eight week old Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were deeply anesthetized with CO₂ and euthanized by decapitation. DRG were rapidly removed and collected in ice-cold dissection solution (D-MEM containing 10% heat-inactivated horse serum plus 100 U/ml penicillin and 100 µg/ml streptomycin). Isolated DRG were transferred to a tube containing 0.1% Collagenase B (Roche Diagnostics Corporation, Indianapolis, IN.) in minimal essential medium (MEM):Ham's F12 (1:1) medium and allowed to incubate for 90 min at 37°C. Following the incubation, the tissue was centrifuged at 1000 rpm (170xg) for 5 minutes, the supernatant removed, and the pellet resuspended in 20 U/ml papain (Worthington Biochemical Corp., Lakewood, NJ) plus 50 µg/ml DNase IV in phosphate buffered saline, pH 7.4, and allowed to incubate for an additional 60 min at 37°C. The tissue was then dissociated by trituration with the use of a plastic Pasteur pipette. Undisrupted tissue fragments were allowed to settle and the supernatant was layered over Earle's balanced salt solution containing 15% FBS and centrifuged at 1000 rpm (170xg) for 5 min. The resulting pellet was resuspended in MEM:Ham's F12 (1:1) medium containing 10% heat-inactivated horse serum, 4 mM L-glutamine, 100 U/ml

penicillin and 100 µg/ml streptomycin. Cells were plated in a 96-well Poly-L-Ornithine/Laminin BioCoat plate (BD Bioscience, Bedford, MA.) at 10,000 cells/well.

In Vitro Electrophysiology

Patch-clamp recordings using rat DRG neurons and HEK 293 cells stably expressing recombinant rat TRPV1 were performed in an extracellular recording solution (pH 7.4, 325 mOsm) consisting of (mM): 155 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 12 glucose. The recombinant rat TRPV1 cDNA sequence used in this study corresponds to accession number NM_031982. For experiments using pH 5.5, 2-morpholinoethanesulfonic acid (MES) was used in place of HEPES in the external solution. Patch-pipettes composed of borosilicate glass (1B150F-3; World Precision Instruments, Inc., Sarasota, FL) were pulled and fire-polished using a DMZ-Universal micropipette puller (Zeitz Instrumente GmbH, Munich, Germany). Pipettes (2-6 MΩ) were filled with an internal solution (pH 7.3, 295 mOsm) consisting of (mM): 122.5 K-aspartate, 20 KCl, 1 MgCl₂, 10 ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 5 HEPES, 2 adenosine 5'-triphosphate, magnesium salt (ATP•Mg).

Coverslips plated with cells were placed in a perfusion chamber and bath perfusion (~2 ml/min) was initiated following establishment of whole-cell recording conditions. Standard whole-cell recording techniques were employed using an Axopatch 200B amplifier (Molecular Devices Corp., Sunnyvale, CA). Unless otherwise noted, membrane potentials of the cells were voltage-clamped at -60 mV. Application of control bath solution through a multi-barrel application device with a common 360 µm polyimide tip (Cell Microcontrols, Norfolk, VA), positioned ~100 µm from the cell, was continued throughout the recording except during drug application. Each drug reservoir was connected to solenoid teflon or pinch valves that were controlled by a ValveLink16 system (AutoMate Scientific, San Francisco, CA). Drug application protocols were established using pCLAMP (Axon instruments) software that controlled rapid valve

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switching through the ValveLink system. Drugs were applied once the amplitude of the agonist response stabilized, typically after an initial (5 minute) current run down. Drugs were applied by gravity feed through the drug application device for durations described in the Results section. Each drug application sequence was followed by a washout period of 90 to 120 s. Capsaicin, protons (acidic pH), or heat were applied for 5 s at 2 min intervals to individual cells. For antagonist studies, ABT-102 was applied 1 minute prior to application of agonist, except when the rate of block of capsaicin- or proton-mediated TRPV1 currents was assessed; in these studies antagonist was added after pre-incubation in capsaicin or acidic solution. For all studies, a temperature calibrated MPRE8 multitube pre-heater was connected to the polyimide tip and controlled by a TC2PKG temperature control system (Cell MicroControls, Norfolk, VA, U.S.A). Unless otherwise indicated, the temperature of the solutions applied to the cells was maintained at room temperature (~22°C). Any baseline currents were observed to be stable. Activation of heat-activated currents using this system was shown to exhibit biophysical (outward rectification), pharmacological (blocked by capsazepine) and kinetic properties consistent with TRPV1 channels.

Data acquisition and analysis were performed using pCLAMP 9.0. The effect of ABT-102 on TRPV1 receptor currents was assessed by measurement of the average current amplitude (compared to the agonist control) during the last second of recording, which accommodates for any desensitization that might have occurred during the recording period. Graphs were plotted and statistical analysis performed using GraphPad Prism (Graphpad Software, San Diego, CA, U.S.A). Agonist and antagonist concentration-response curves were fitted by a least-squares regression to the logistic equation provided in the GraphPad software:

$$Y = \min + [(\max - \min) / (1 + 10^{(\log EC_{50} - X)nH})]$$

CGRP Release

CGRP release was measured using the protocol described by (Gavva et al., 2005b) with minor modifications. Rat DRG were dissociated and plated onto pre-coated poly-L-ornithine/Laminin Biocoat 96 well plates at a density of 10,000 cells per well and neurons cultured for 72 hr before initiation of the CGRP release assay. After removing media, the neuronal cultures were rinsed once with CGRP release buffer containing 135 mM NaCl, 3.5 mM KCl, 2.5 mM CaCl₂, 3.3 mM glucose, 25 mM HEPES, 2 μM phosphoramidon, 0.1% bovine serum albumin, pH7.4. The cultures were stimulated with 300 nM of capsaicin for 20 min at room temperature to evoke CGRP release. To observe the effect of antagonist, neurons were pre-incubated with 90 μl of different concentrations of antagonist at room temperature for 15 min followed by addition of 10 μl of 3μM capsaicin for another 20 min. At the end of the incubation, 100 μl of supernatant solution was analyzed using a CGRP enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical, Ann Arbor, MI) and CGRP levels measured according to the manufacturer's instructions.

In Vivo Electrophysiology

In vivo electrophysiology experiments were conducted as described previously (McGaraughty et al., 2006). To induce chronic inflammatory hyperalgesia, a 150 μl solution of 1:1 Complete Freund's adjuvant (CFA) and PBS was injected subcutaneously into the plantar region of the rat's right hind paw 48 hr before the experiment. In these studies, CFA-inflamed and uninjured rats (350-400g) were initially anesthetized with pentobarbital (50 mg/kg, i.p.). A catheter was placed into an external jugular vein, and a laminectomy was performed to remove vertebral segments T12-L3. Animals were then secured in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) supported by clamps attached to the vertebral processes on either side of the exposure site. A stable plane of anesthesia was maintained throughout the experiment by a continuous infusion of propofol at a rate of 8-12 mg/kg/hr (i.v.). Body temperature was kept at approximately 37°C by placing the animals on a circulating water blanket. Platinum-iridium

microelectrodes (Frederick Haer, Brunswick, ME) were used to record extracellular activity of WDR or NS neurons. Spike waveforms were monitored on an oscilloscope throughout the experiment, digitized (32 points), and then stored for off-line analysis (SciWorks, Datawave Technologies, Longmont, CO) to ensure that the unit under study was unambiguously discriminated throughout the experiment. Except for 5 experiments in which easily distinguished neurons were simultaneously recorded on one electrode, only one cell was studied in each experiment. Thermal evoked responses of WDR and NS neurons were recorded before and after administration of ABT-102 (30-300 nmol in 50 μ l) into the neuronal receptive field on the hind paw in CFA-inflamed and uninjured rats. In order to equate baseline levels of evoked firing between the two groups of rats (McGaraughty et al., 2006), uninjured and CFA-treated animals received different intensities of thermal stimulation. Thus, the ipsilateral hind paw of uninjured rats was immersed in 52°C water for 10 s while the hindpaw of CFA-injected rats was immersed in 47°C water for 10 s. Statistical significance was established by using a Wilcoxon's matched-pairs test for comparisons to baseline firing levels, and by a Kruskal-Wallis analysis of variance followed by a Mann-Whitney U test was used for comparison across groups ($P < 0.05$).

Results

ABT-102 is a potent antagonist at recombinant human and rat TRPV1 receptors

The ability of ABT-102 (Figure 1A) to inhibit TRPV1 receptor activation was investigated in HEK 293 cells stably expressing the human recombinant receptor in a FLIPR-based assay of intracellular calcium levels, as previously described (El-Kouhen et al, 2005). In this assay, we have previously demonstrated that activation of human TRPV1 receptors by capsaicin, anandamide, NADA (shown in supplemental Figure 1A) and protons (pH 5.5) (shown in supplemental Figure 1B), leads to concentration-dependent increases in intracellular calcium levels (El Kouhen et al., 2005). A

concentration of each agonist that corresponds to the approximate EC_{50} value obtained in those studies (capsaicin $EC_{50} = 36$ nM, NADA $EC_{50} = 2870$ nM, anandamide $EC_{50} = 8650$ nM, and protons $EC_{50} = pH5.5$) was used to determine the potency of ABT-102 in this study (Figure 1B). ABT-102 blocked recombinant human TRPV1 activation by 50 nM capsaicin in a concentration-dependent manner, with a pIC_{50} value of 8.13 ± 0.04 ($IC_{50} = 7$ nM; $n = 11$). ABT-102 also blocked recombinant human TRPV1 receptor activation by NADA (3 μ M), anandamide (10 μ M) and protons (pH 5.5) with similar potency, with IC_{50} values of 4 nM, 5 nM and 6nM, respectively (Figure 1B). The ability of ABT-102 to block the effects of NADA or anandamide was not complete and likely reflects non-TRPV1 mediated effects of these agonists (El Kouhen et al., 2005). The potency of ABT-102 against TRPV1 receptor activation by 50 nM capsaicin compared with the potency of other TRPV1 receptor antagonists: 1-isoquinolin-5-yl-3-(4-trifluoromethyl-benzyl)-urea (A-425619), (*E*)-3-(2-(piperidin-1-yl)-6-(trifluoromethyl)pyridin-3-yl)-*N*-(quinolin-7-yl)acrylamide (AMG-6880), 1-(2-bromo-phenyl)-3-[2-ethyl-*m*-tolyl-amino)-ethyl]-urea (SB-452533), *N*-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2*H*)-carbox-amide (BCTC) and capsazepine (CPZ), is shown in Figure 1C. pIC_{50} values obtained were 8.13 ± 0.04 ($IC_{50} = 7$ nM; $n = 11$) for ABT-102 versus 8.45 ± 0.04 ($IC_{50} = 4$ nM; $n = 10$) for A-425619, 6.90 ± 0.06 ($IC_{50} = 127$ nM; $n = 6$) for AMG-6880, 6.71 ± 0.03 ($IC_{50} = 195$ nM; $n = 3$) for SB-452533, 7.67 ± 0.05 ($IC_{50} = 21$ nM; $n = 9$) for BCTC and 6.83 ± 0.02 ($IC_{50} = 148$ nM; $n = 16$) for capsazepine. The ability of ABT-102 to block TRPV1 receptor activation was reversible as indicated by the restoration of the ability of capsaicin to stimulate calcium influx following washout of the antagonist (Figure 1D). In addition, ABT-102, at concentrations up to 100 μ M, did not show any effects on human TRPV1 receptors in the absence of agonist (data not shown).

ABT-102 potently blocked recombinant rat TRPV1 receptor activation in whole-cell voltage clamp electrophysiology assays. ABT-102 inhibited capsaicin-induced currents (Figure 2A), with a pIC_{50} value of 9.01 ± 0.10 ($IC_{50} = 1$ nM; $n = 3$) (Figure 2B). ABT-102 also blocked proton (pH 5.5)-mediated currents at rat TRPV1 receptors (Figure 2C), with

a pIC₅₀ value of 7.78 ± 0.12 (IC₅₀ = 16 nM; n = 5) (Figure 2D). At 100 nM ABT-102 completely blocked heat (50°C)-activated rat TRPV1 currents to background levels (n=4) (Figure 3A). In these cells, a small background non-TRPV1-mediated heat-evoked current is apparent (Figure 3A). As shown in Figure 3B, ABT-102 clearly reduced heat-evoked TRPV1-mediated currents in a concentration-dependent manner, and at 100 nM reduced the TRPV1 receptor mediated current to background levels. Thus, ABT-102 potently blocks recombinant human and rat TRPV1 receptors activated by different stimuli.

ABT-102 is a potent antagonist at the native rat TRPV1 receptor

ABT-102 produced a concentration-dependent inhibition of currents elicited by 1 μM capsaicin in small diameter (20-30 μm) DRG neurons (Figure 4A), with a pIC₅₀ value of 8.24 ± 0.08 (IC₅₀ = 6 nM; n = 8) (Figure 4B). This value agrees well with that obtained at the recombinant rat TRPV1 receptor, using both electrophysiology and calcium influx assays, and confirms the high potency of ABT-102 at endogenously expressed TRPV1 in a native system.

ABT-102 is a highly selective TRPV1 antagonist

The selectivity of ABT-102 was evaluated versus other TRP receptors. EC₅₀ values were determined for the agonist for each TRP receptor (Figure 5A). The concentration of each agonist that corresponded to its EC₅₀ value at its respective receptor was then used to assess potency of ABT-102 at these receptors. As shown in Figure 5B, ABT-102 was highly selective as an antagonist of TRPV1 versus other TRP receptors, with IC₅₀ values of > 100 μM for TRPV3, >100 μM for TRPV4, >30 μM for TRPM8, and >30 μM for TRPA1. Thus, ABT-102 shows greater than 3000-fold selectivity for the TRPV1 receptor over the other TRP receptors evaluated.

To further determine the specificity of ABT-102 for the TRPV1 receptor the compound was profiled in a large panel of in vitro binding assays (CEREP, Poitiers, France). The

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assays included ion channels, G-protein coupled receptors, other receptors and transporters (listed in supplemental Table 1). ABT-102 showed little or no activity at all receptors, ion channels, and transporters evaluated (supplemental Table 1). In additional, in-house, assays ABT-102 produced only 0 -18% inhibition (at 3 –100 μ M) of ligand binding to adrenergic, cannabinoid, dopamine, histamine or nicotinic receptors or to the $\alpha 2\delta 1$ subunit of voltage-gated calcium channels. In Ca^{2+} influx assays, ABT-102 (up to 100 μ M) did not block activation of P2X₇ receptors or NMDA NR1/NR2B receptors. ABT-102 was also inactive (at 1-3 μ M) at human fatty acid amide hydrolase (FAAH) and at tetrodotoxin-resistant voltage-gated sodium channel currents in rat DRG neurons (data not shown). These data support the highly selective nature of ABT-102 for TRPV1.

ABT-102 is a competitive antagonist at the capsaicin-binding site of the TRPV1 receptor

To determine the nature of ABT-102 antagonism at the TRPV1 receptor, capsaicin concentration-effect curves were generated in the presence of increasing concentrations of ABT-102 using the HEK 293-based stable cell line expressing recombinant human TRPV1. As shown in Figure 6A, capsaicin concentration-effect curves were shifted to the right with increasing concentrations of ABT-102, without affecting the maximal response for capsaicin. These results indicate that ABT-102 acts as a competitive antagonist at the TRPV1 receptor capsaicin-binding site. A Schild plot analysis yielded a pA₂ of 8.344 and a slope factor of 1.07 ± 0.18 (Figure 6B).

ABT-102 potently blocks activation of the sensitized TRPV1 receptor by various agonists

To determine the potency of ABT-102 under conditions where the TRPV1 receptor is sensitized prior to the addition of agonist, the receptor was sensitized through activation of PKC, since this appears to be a common mechanism through which various agents sensitize TRPV1 (Bonnington and McNaughton, 2003; Moriyama et al., 2005; Sikand and

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Premkumar, 2007). The recombinant human TRPV1 receptor was sensitized with a concentration (100 nM) of the PKC activator PDBu that does not by itself activate TRPV1, as previously demonstrated (El Kouhen et al., 2005). The pEC₅₀ values for capsaicin and NADA were 7.99 ± 0.04 (EC₅₀ = 10 nM) and 6.08 ± 0.03 (EC₅₀ = 0.83 μM), respectively (Figure 7A). Compared to basal conditions (supplemental Figure 1A), PKC-mediated sensitization of recombinant human TRPV1 enhanced the potency of capsaicin and NADA by approximately 4-fold. A concentration of each agonist that corresponded to approximately its EC₅₀ value at the PKC-sensitized receptor, 10 nM for capsaicin and 0.8 μM for NADA, was used to assess the potency of ABT-102. ABT-102 blocked activation of the PKC-sensitized receptor with similar potency to that at the naïve receptor, with average pIC₅₀ values for capsaicin (Figure 7B) of 8.40 ± 0.09 (IC₅₀ = 4 nM; n = 6) and for NADA (Figure 7C) of 8.41 ± 0.08 (4 nM; n = 6) at the sensitized receptor. ABT-102 also blocked heat activation of the PKC-sensitized receptor (Figure 7D), with a similar average pIC₅₀ value, of 8.32 ± 0.03 (IC₅₀ = 5 nM; n = 9).

ABT-102 potently blocks TRPV1 activation-induced CGRP release from rat DRG neurons

The effect of ABT-102 on CGRP release by cultured rat DRG neurons was evaluated to determine whether ABT-102 blocked TRPV1-mediated downstream events. CGRP release in response to TRPV1 activation has previously been demonstrated (Tognetto et al., 2001). Capsaicin induced a dose-dependent increase in release of CGRP from rat DRG neurons (supplemental Figure 2A), with a pEC₅₀ value of 7.28 ± 0.10 (EC₅₀ = 52 nM). ABT-102 potently inhibited capsaicin-induced CGRP release with a pIC₅₀ value of 7.24 ± 0.11 (IC₅₀ = 57 nM; n = 8) (supplemental Figure 2B).

ABT-102 potently blocks heat-evoked activity of spinal WDR and NS neurons through an inhibition of peripheral TRPV1 receptors

The effects of ABT-102 on neuronal excitability *in vivo* were evaluated in anesthetized rats. Discharge activity was recorded from 56 WDR and 22 NS neurons ($891.8 \pm 144.3 \mu\text{m}$ from the surface of the spinal cord) in CFA-inflamed and uninjured rats ($n = 5 - 9$ per treatment group). In CFA rats, administration of ABT-102 (30-300 nmol) into the neuronal receptive field dose-dependently reduced heat-evoked firing of WDR neurons compared to baseline levels ($p < 0.05$; Figure 8A, C). The greatest reduction ($69.5 \pm 6.7 \%$) in thermal-evoked WDR firing occurred 35 min after the injection of 300 nmol ABT-102 (Figure 8C). Similarly, 300 nmol ABT-102 also reduced the responses of NS neurons to noxious thermal stimulation in CFA-inflamed rats ($p < 0.05$, Figure 8B) with an observed maximal effect of $71.1 \pm 8.4 \%$ 35 min after injection. Injections of ABT-102 (300 nmol) into the contralateral non-inflamed hind paw did not alter heat-evoked neuronal firing (data not shown).

In uninjured rats, injections of ABT-102 (300 nmol) into the ipsilateral hind paw also significantly reduced ($p < 0.05$) the thermal-evoked discharges of both WDR ($38.6 \pm 7.8 \%$) and NS neurons ($33.2 \pm 9.1 \%$). However, the effects of ABT-102 in uninjured rats were significantly less than those observed in CFA-inflamed rats (Figure 8D).

Discussion

The present study characterizes functional and pharmacological properties of ABT-102, a structurally novel TRPV1 receptor antagonist. ABT-102 is a potent antagonist of multiple modes of TRPV1 activation. ABT-102 blocks capsaicin-induced activation of recombinant human and rat TRPV1 receptors, as well as native rat TRPV1 receptors, with similar potency. ABT-102 also shows equivalent potency at human TRPV1 receptors activated by protons or by the putative endogenous ligands NADA and anandamide, and at the PKC-sensitized and activated human TRPV1 receptor. In addition, ABT-102 potently blocks heat-activated TRPV1 receptor currents. ABT-102 is a competitive and reversible antagonist of capsaicin-activated TRPV1 receptors, and

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shows greater potency versus capsaicin-induced activation than several other reported TRPV1 receptor antagonists. ABT-102 is highly selective for TRPV1 over other TRP receptors, and is also highly selective versus other receptors, ion channels, and transporters. ABT-102 potently blocks functional consequences of TRPV1 activation, including capsaicin-mediated CGRP release from DRG neurons *in vitro*, and heat-evoked neuronal firing of WDR and NS spinal cord neurons after intraplantar administration *in vivo*.

The ability of ABT-102 to antagonize capsaicin- and proton-mediated TRPV1 receptor activation with equal potency places ABT-102 into a group with a limited number of other TRPV1 receptor antagonists, A-425619 (El Kouhen et al., 2005), (N)-{4-[6-(4-trifluoromethyl-phenyl)-pyrimidin-4-yloxy]-benzothiazol-2-yl}-acetamide (AMG 517) (Doherty et al., 2007), and AMG 6880 (Gavva et al., 2005a). In contrast, other reported TRPV1 antagonists effectively block capsaicin-induced TRPV1 receptor activation, but either have no effect on proton-induced activation (Gavva et al., 2005a) or show reduced potency for proton- versus capsaicin-induced TRPV1 activation (Gavva et al., 2005b; Gunthorpe et al., 2007). Capsaicin binding and proton binding sites have been mapped to different regions of the TRPV1 receptor (Jordt et al., 2000; Gavva et al., 2004; Gavva et al., 2005a). Thus, the equal potency of ABT-102 to block capsaicin- and proton-induced TRPV1 receptor activation suggests an ability to bind TRPV1 in a manner that prevents activation, irrespective of the distinct binding sites for capsaicin and protons. (Gavva et al., 2005a) proposed that TRPV antagonists with similar potency to block activation by capsaicin and protons might lock the channel in a closed state, thus preventing activation by either agent. ABT-102 showed a somewhat slower rate of block of proton-activated currents compared with block of capsaicin-activated currents (Figure 2). It is possible that the different kinetics may be due to indirect block of proton-activated currents caused by binding of ABT-102 to the capsaicin-binding site. In this study our aim was to determine potency of ABT-102 under conditions where the TRPV1 receptor was activated by several different stimuli. In future studies it may be of interest

to investigate, using more detailed kinetic analyses, the mechanism(s) involved in receptor antagonism.

The ability of TRPV1 receptors to be activated by diverse stimuli (Caterina et al., 1997; Smart et al., 2000; Huang et al., 2002; Cortright and Szallasi, 2004) highlights not only a key role for TRPV1 as a pain sensor, but also the challenge of identifying potent antagonists that can effectively block many or all of the mechanisms of TRPV1 activation relevant to chronic pain. Moreover, TRPV1 sensitization and/or up-regulation in response to inflammatory mediators, PKC and growth factors (Sikand and Premkumar, 2007) adds a further layer of complexity to targeting this receptor. Several selective TRPV1 receptor antagonists that block polymodal receptor activation show good efficacy in preclinical animal models of pain (Szallasi et al., 2007). Similarly, ABT-102 is a potent blocker of these stimulus modalities both *in vitro* (this study) and *in vivo* (Honore et al (submitted)). A hallmark of inflammation and associated pain is a decreased pH (to ~ pH 6.1 - 5.4) at the site of inflammation (Steen et al., 1992) and TRPV1 receptors expressed on nociceptors have been implicated in acid-mediated pain (Leffler et al., 2006). Moreover, anandamide and NADA may play an enhanced role in evoked and spontaneous pain under sensitized pathological conditions (Premkumar et al., 2004; Singh Tahim et al., 2005; Huang and Walker, 2006). Thus, the ability of ABT-102 to inhibit multiple modes of TRPV1 activation, including activation of the sensitized receptor, with the same high potency when assayed under equivalent conditions/levels of receptor activation, suggests a highly favorable pharmacological profile for this antagonist.

It is well known that CGRP plays a role in neurogenic inflammation and in central sensitization in pain (Kilo et al., 1997; Bird et al., 2006), and that TRPV1 receptor activation induces CGRP release from specific subsets of DRG neurons at central and peripheral terminals (Price and Flores, 2007). ABT-102 shows potent and concentration-dependent block of TRPV1-mediated CGRP release from rat DRG neurons clearly demonstrates the ability of ABT-102 to block a relevant downstream effect of TRPV1 activation that is strongly implicated in both central and peripheral aspects of pain.

Injection of ABT-102 into a rat hind paw decreased spinal cord WDR and NS neuronal responses to noxious thermal stimulation. These results confirm previous work from our laboratory and others that in vivo antagonism of TRPV1 receptors on peripheral terminals is sufficient to attenuate the transmission of noxious thermal signals to the spinal cord (Jhaveri et al., 2005; McGaraughty et al., 2006). The effects of peripheral ABT-102 in both inflamed and uninjured rats suggests that TRPV1 has a thermal transduction role in both “normal” and pathological states. Nonetheless, ABT-102 had a greater effect in the CFA-inflamed rats than in the uninjured rats. This outcome is consistent with an injury-related upregulation of TRPV1 receptors on peripheral terminals (Carlton and Coggeshall, 2001), and with the increased efficacy of intraplantar injected iodo-resiniferatoxin in the carrageenan model of acute inflammation compared to uninjured rats (Jhaveri et al., 2005). In contrast, we had previously shown that intraplantar injection of another TRPV1 receptor antagonist, A-425619, was equally effective in inflamed and uninjured rats, despite a greater effect in inflamed rats following systemic and intra-DRG administration (McGaraughty et al., 2006). Physicochemical properties of A-425619 may have contributed to the lack of differential action between inflamed and uninjured rats following intraplantar injection of this compound. The increased efficacy of ABT-102 (and iodo-resiniferatoxin) in models of inflammation is consistent with the amplified role of TRPV1 receptors following injury. The ability of intraplantar ABT-102 to block heat-evoked WDR and NS neuronal firing in the dorsal horn of the spinal cord also underscores the important contribution made by the TRPV1 receptor in nociceptive pathways, its contributions to the summation of afferent inputs in the spinal cord (in addition to its expected sensitization of afferent nociceptive fibers), and its impact on central aspects of pain.

In summary, ABT-102 is a structurally novel TRPV1 receptor antagonist with overall superior potency and selectivity for TRPV1 receptors activated by multiple pain-related stimuli. Importantly, ABT-102 also attenuates functional consequences of TRPV1 activation in pain pathways. The compound shows a favorable pharmacokinetic profile,

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oral bioavailability, and predicted good absorption in humans, as well as low *in vitro* metabolism (Gomtsyan et al., 2008). ABT-102 shows analgesic efficacy in several rodent pain models (including chronic inflammatory, bone cancer and post-operative pain) and an acceptable therapeutic index in preclinical studies (Gomtsyan et al., 2008) (Honore et al (submitted)). ABT-102, like other TRPV1 antagonists, also produced a transient increase in core body temperature in rodents, but this effect attenuates following repeated dosing (Honore et al (submitted)). Interestingly, the analgesic activity of ABT-102 increases with repeated dosing (Honore et al (submitted)). ABT-102 recently advanced to clinical trials. TRPV1 antagonism represents a novel approach for pain management since it targets a key pain sensor that integrates multiple stimuli. The potential of this approach will be more fully revealed from clinical trials that have been initiated with several TRPV1 antagonists.

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Legends for Figures

Fig. 1. ABT-102 potently blocks recombinant human TRPV1 activation by a variety of stimuli. A, Chemical structure of ABT-102: (*R*)-(5-*tert*-butyl-2,3-dihydro-1*H*-inden-1-yl)-3-(1*H*-indazol-4-yl)-urea. B, Concentration-effect curves of ABT-102 on TRPV1 receptor activation induced by different stimuli. ABT-102 was added prior to activation of the human TRPV1 receptor and subsequent measurement of Ca²⁺ flux, as described in *Methods*. Data are presented as percentage of the maximal response observed in the absence of antagonist (control) minus basal fluorescence. pIC₅₀ values obtained at the human TRPV1 receptor are presented in the text. C, Comparison of concentration effect curves for ABT-102, A-425619, AMG-6880, SB-452533, BCTC and capsazepine on TRPV1 receptor activation induced by 50 nM capsaicin. pIC₅₀ values obtained are presented in the text. D, Washout of ABT-102 shows reversal of inhibition of capsaicin-induced activation of human TRPV1. Representative FLIPR traces after 1 μM capsaicin application (control), 200 nM ABT-102 block of 1 μM capsaicin stimulated calcium influx, and 1 μM capsaicin stimulated calcium influx following washout of 200 nM ABT-102.

Fig. 2. ABT-102 potently blocks capsaicin and proton evoked current at recombinant rat TRPV1 receptors stably expressed in HEK 293 cells. A, Representative current traces illustrating the inhibition of 1 μM (~EC₅₀) capsaicin-activated rat TRPV1 currents by 100 nM ABT-102 added subsequent to the activation of TRPV1. B, Concentration-effect curve of ABT-102 added subsequent to activation of the recombinant rat TRPV1 receptor by capsaicin. C, Representative current traces illustrating the inhibition of proton (pH 5.5) - activated rat TRPV1 currents by 100 nM ABT-102 added subsequent to the activation of TRPV1. D, Concentration-effect curve of ABT-102 added subsequent to proton-evoked activation of the TRPV1 receptor. Data are presented as percentage of the maximal

response observed in the absence of antagonist (control) minus basal fluorescence.
 pIC_{50} values obtained at rat TRPV1 receptor are presented in the text.

Fig. 3. ABT-102 potently blocks heat-evoked currents of recombinant rat TRPV1 receptors. A, Representative current traces illustrate the inhibition of heat-activated rat TRPV1 currents by 100 nM ABT-102. B, Currents evoked by 50°C in rat TRPV1 expressing cells are inhibited, in a concentration-dependent manner, by ABT-102 to background levels. Data are the mean of $n = 3 - 6 \pm$ S.E.M.

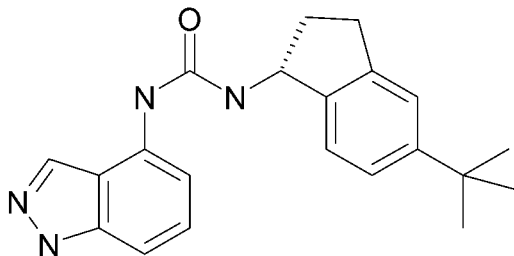
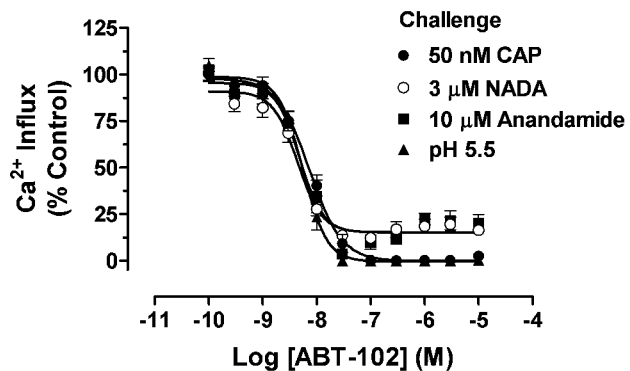
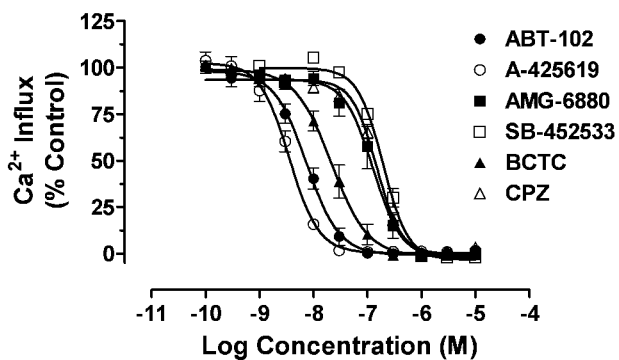
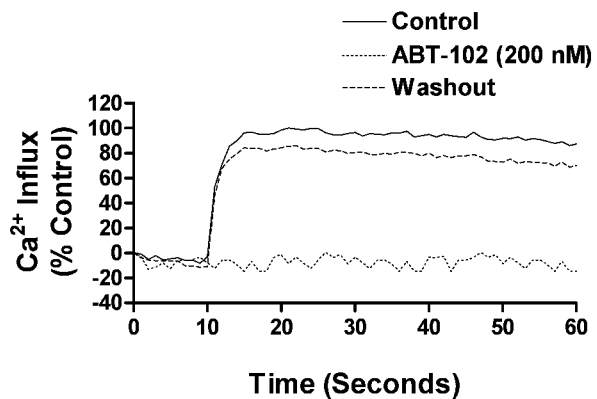
Fig. 4. Concentration-dependent inhibition of native rat TRPV1 channels by ABT-102. A, Representative current traces illustrating the concentration-dependent inhibition of 1 μ M capsaicin-activated currents by 1, 10 and 100 nM ABT-102 in cultured rat DRG neurons. B, Average normalized data plotted as a function of ABT-102 concentration and fitted to a logistic equation. Data are the mean of $n = 3 - 8 \pm$ S.E.M.

Fig. 5. ABT-102 is highly selective for TRPV1 over other TRP receptors. A, Concentration-effect curves for activation of TRPV1-, TRPV3-, TRPV4-, TRPM8- and TRPA1-induced increase in intracellular Ca^{2+} by capsaicin, 2-APB, agonist 6 (1-(benzo[*b*]thiophen-3-yl)-2-(cyclohexyl(ethyl)amino)ethanone) from WO2006029210 (Casillas et al., 2006), menthol and mustard oil, respectively. Data are expressed as percentage of maximal response (minus basal fluorescence). B, Concentration-effect curves of the antagonism by ABT-102 on TRP receptor activation. TRPV1, TRPV3, TRPV4, TRPM8 and TRPA1 were activated by 50 nM capsaicin, 62.5 μ M 2-APB, 2 μ M agonist 6 (1-(benzo[*b*]thiophen-3-yl)-2-(cyclohexyl(ethyl)amino)ethanone), 10 μ M menthol and 30 μ M mustard oil, respectively. ABT-102 was added prior to activation of the TRP receptors and subsequent measurement of Ca^{2+} flux, as described in *Methods*. Data are presented as percentage of the maximal response observed in the absence of antagonist (control) minus basal fluorescence.

Fig. 6. ABT-102 is a competitive antagonist at the capsaicin-binding site of TRPV1. A, Concentration-effect curves for capsaicin-induced increase in intracellular Ca^{2+} in the presence of increasing concentrations of ABT-102. B, Schild plot analysis of the antagonism produced by ABT-102. Slope = 1.07 ± 0.18 , $\text{pA}_2 = 8.344$.

Fig. 7. ABT-102 potently blocks responses at the sensitized human TRPV1 receptor. A, Capsaicin and NADA concentration-effect curves at the PDBu-sensitized human TRPV1 receptor. Percentage maximum effect is expressed relative to the capsaicin response. B, Concentration-effect curve for ABT-102 to block 10 nM capsaicin at PDBu-sensitized TRPV1 receptors. C, Concentration-effect curve for ABT-102 to block 0.8 μM NADA at PDBu-sensitized TRPV1 receptors. D, Concentration-effect curve for ABT-102 to block heat (38°C)-evoked responses at PDBu-sensitized TRPV1 receptors.

Fig. 8. ABT-102 potently blocks heat-evoked firing activity of WDR and NS neurons in spinal cord dorsal horn and with greater efficacy in CFA-treated vs. naïve rats. A and B, Injection of ABT-102 (in 50 μl) into the neuronal receptive field of CFA-inflamed rats significantly reduced WDR (A, 30-300 nmol) and NS (B, 300 nmol) responses to noxious thermal stimulation. C, Representative ratemeter showing the responses of a single WDR neuron to noxious thermal stimulation (47°C for 10 s) both before (base) and after the administration of ABT-102 (300 nmol). Shaded region on the paw represents the neuronal receptive field; $n = 5-9$ per group, $*p < 0.05$ vs. baseline evoked activity; $^+p < 0.05$, $^{++}p < 0.01$ vs. vehicle-treated group. D, Injection of ABT-102 (300 nmol in 50 μl) also decreased the thermal-evoked responses of WDR and NS neurons in uninjured rats, but these effects were significantly less than those observed in CFA-inflamed rats; $n = 5-6$ per group, $*p < 0.05$ vs. baseline evoked activity; $^+p < 0.05$, vs. uninjured group.

A.**B.****C.****D.****Figure 1**

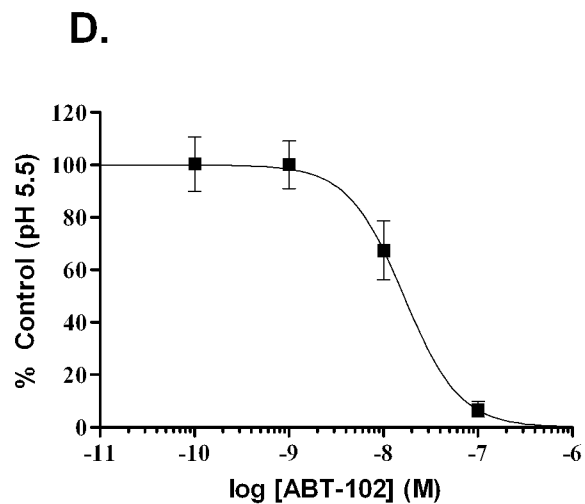
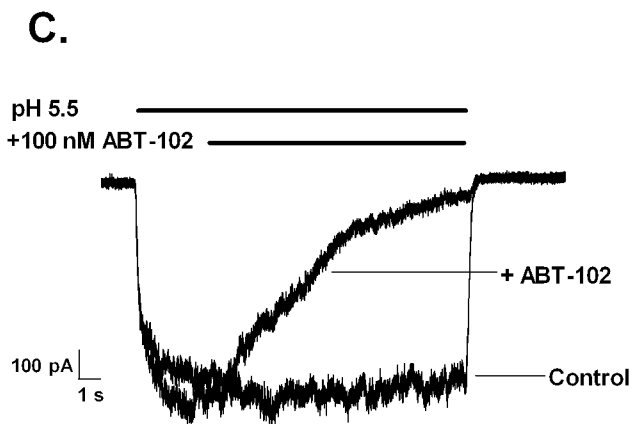
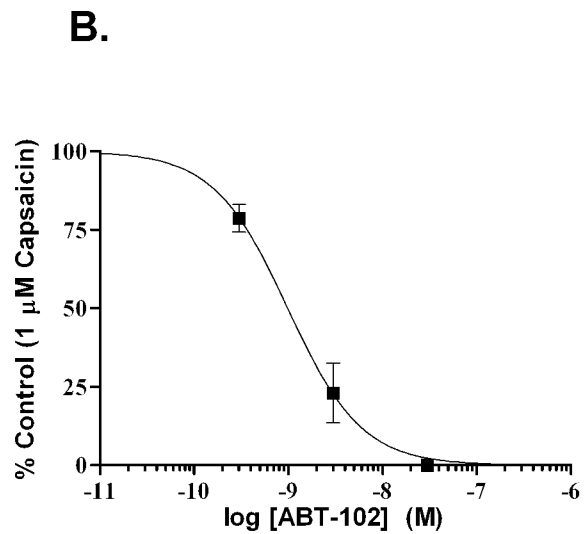
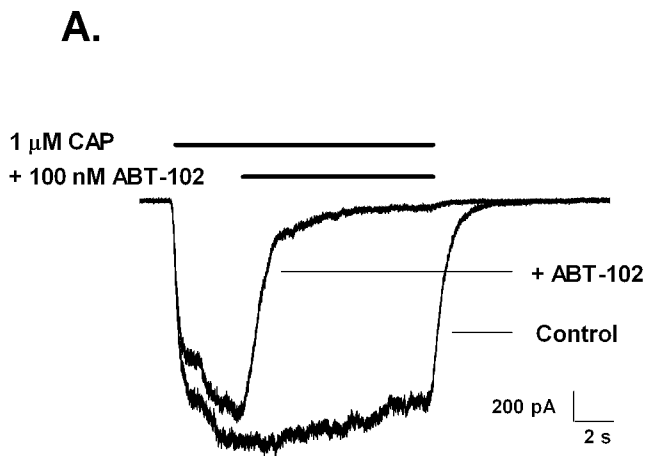
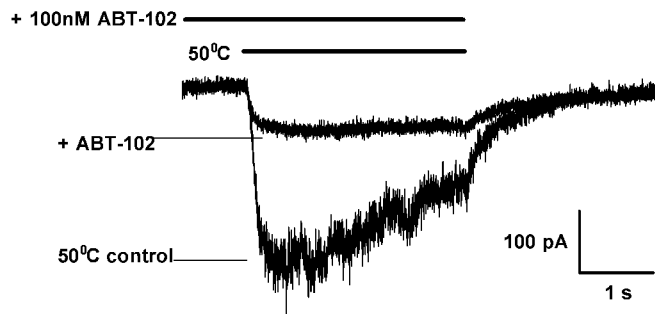
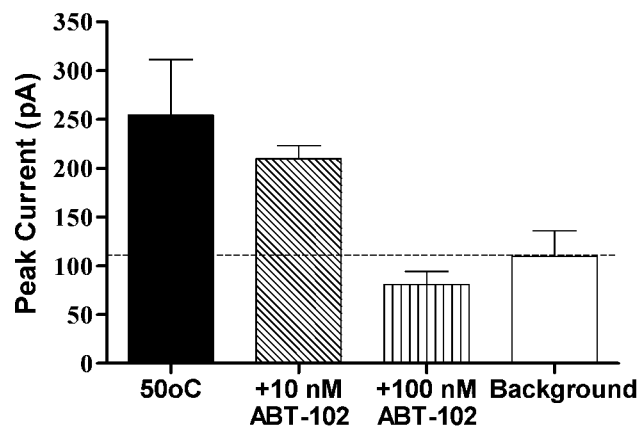
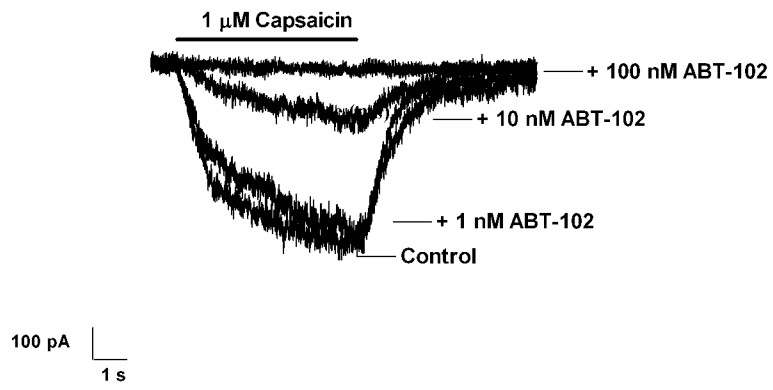
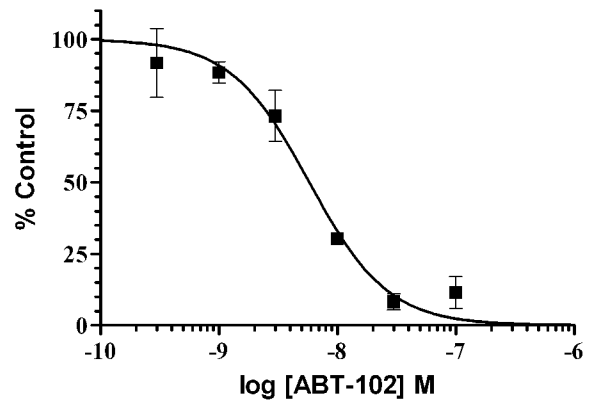
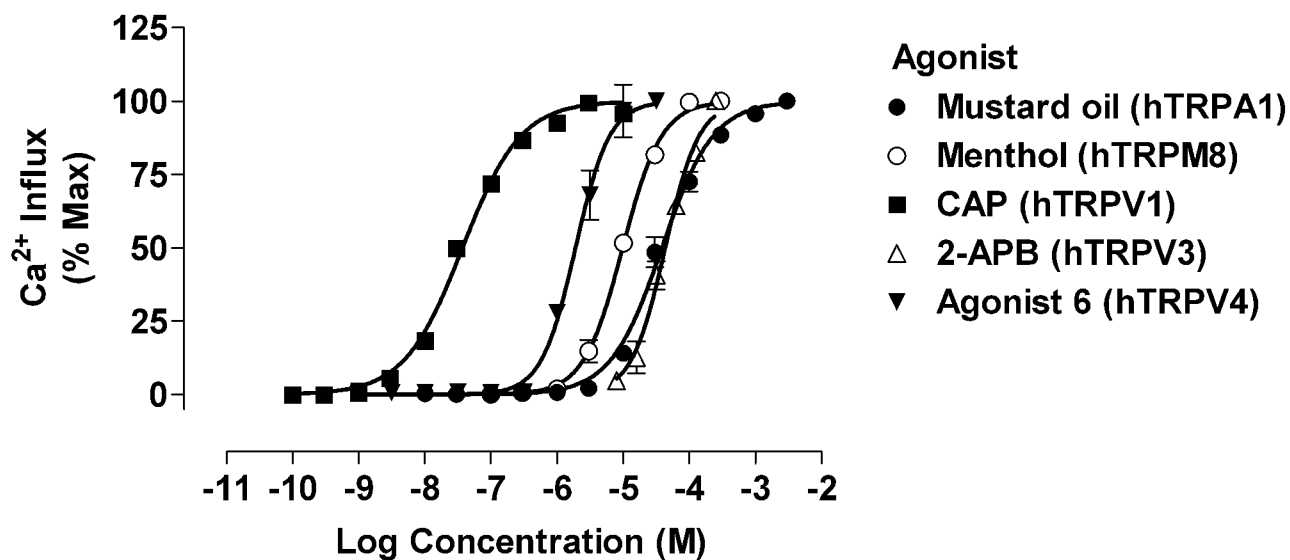
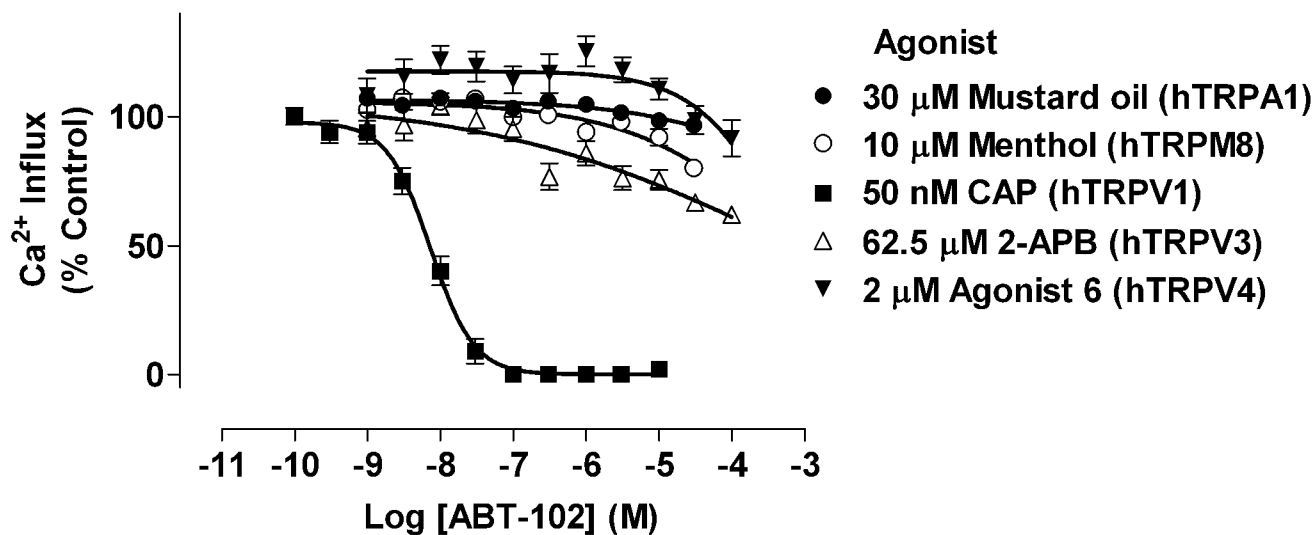
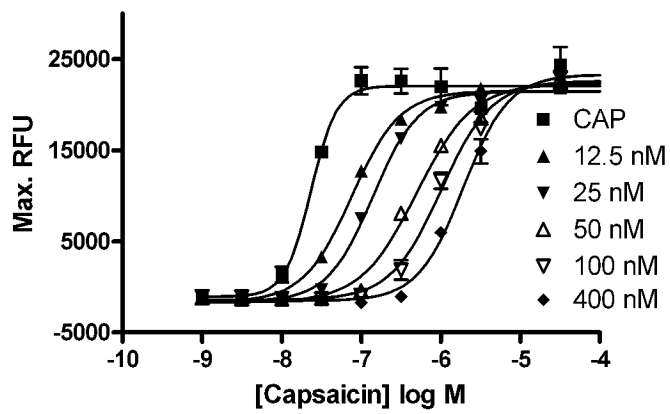
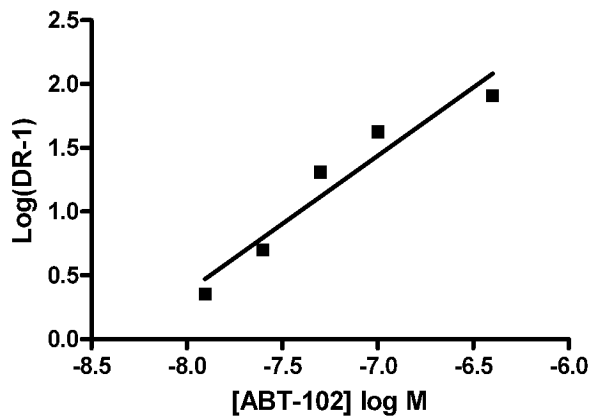


Figure 2

A.**B.****Figure 3**

A.**B.****Figure 4**

A.**B.****Figure 5**

A.**B.****Figure 6**

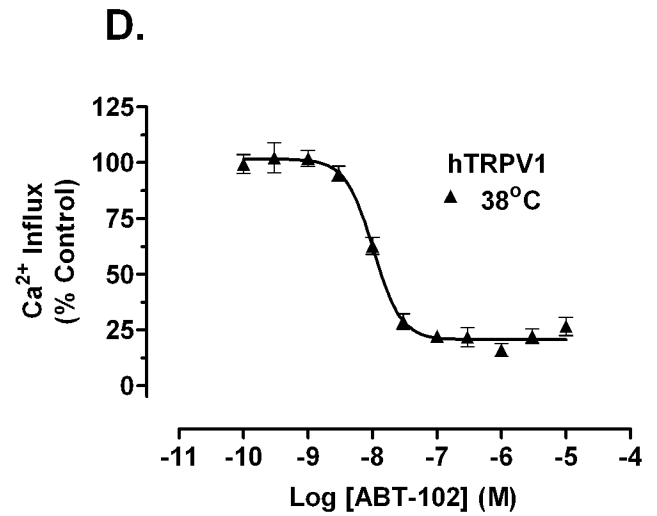
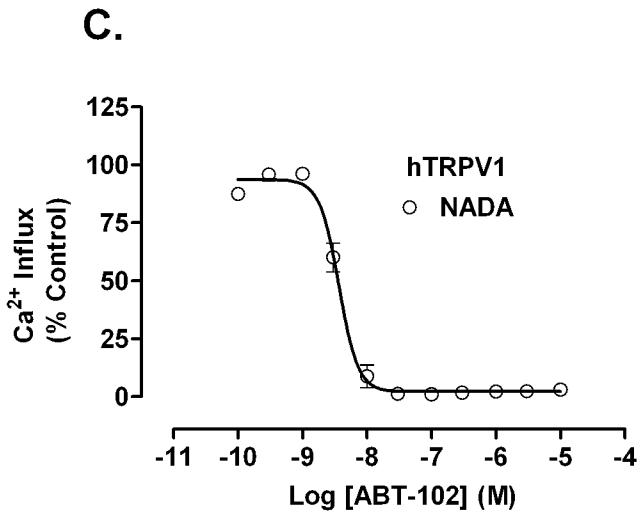
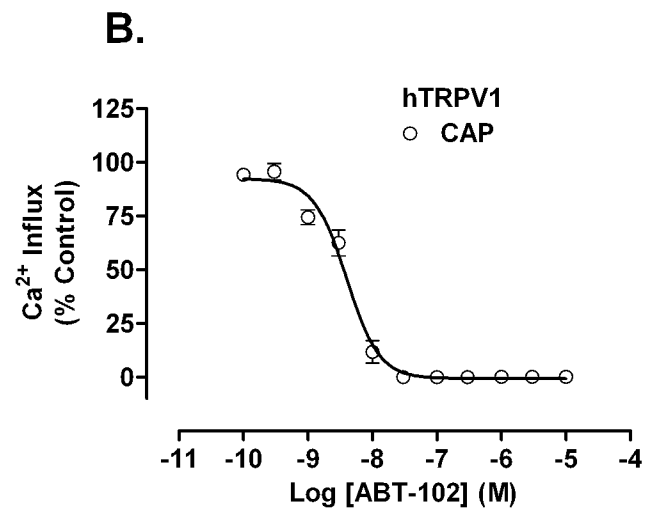
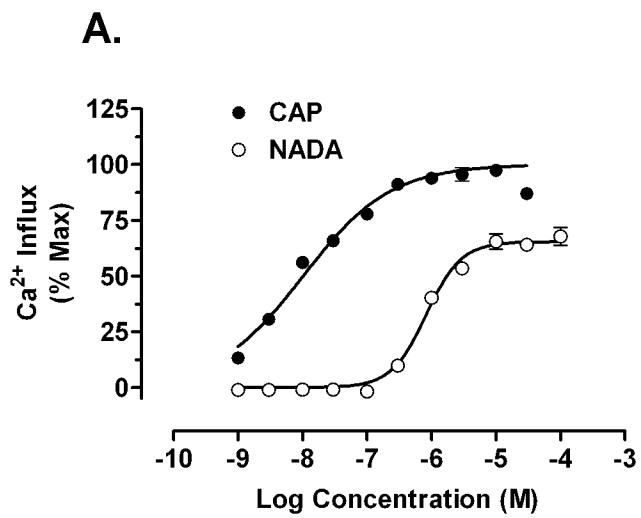


Figure 7

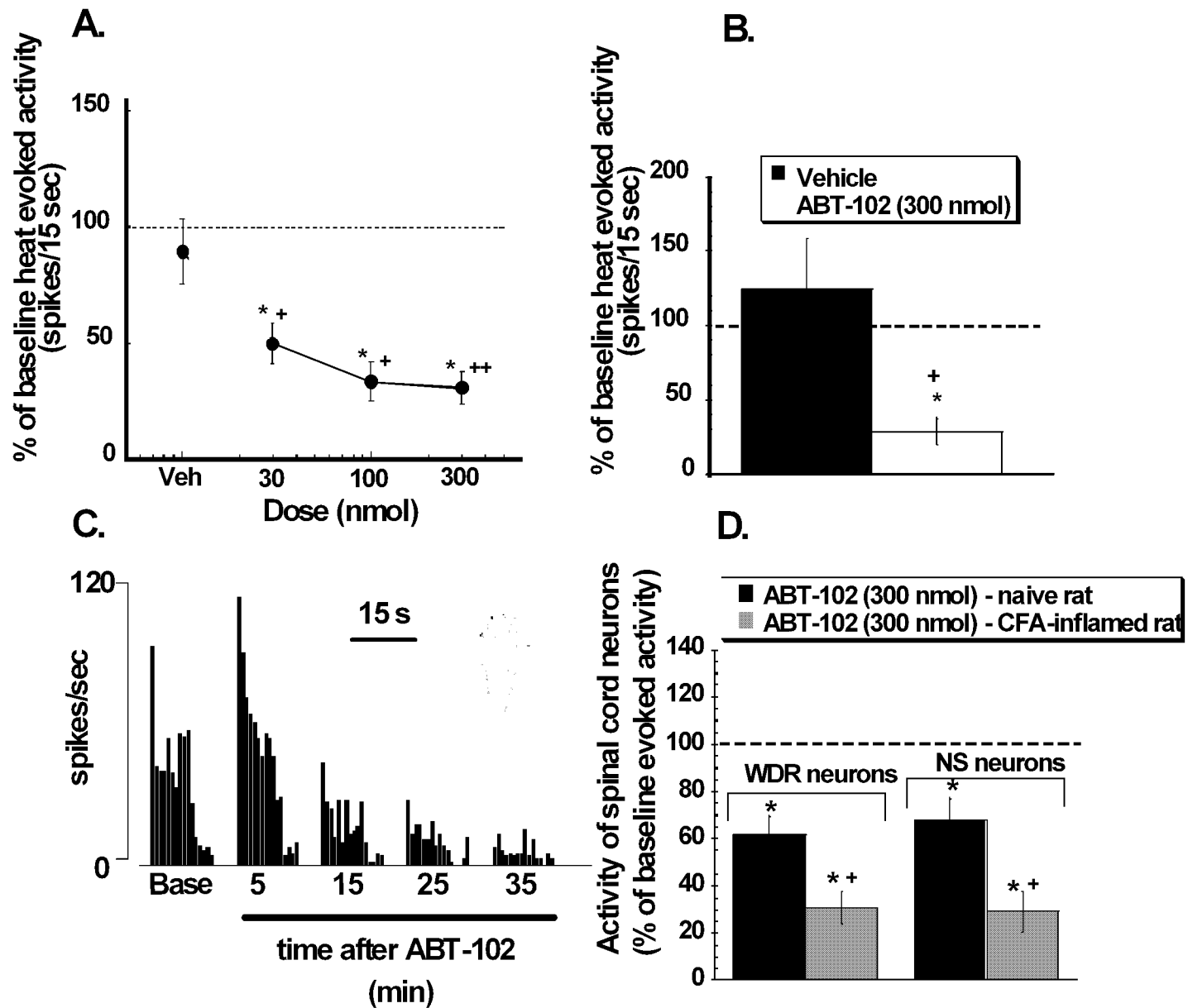


Figure 8