0026-895X/07/7101-12–18$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2007 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 71:12–18, 2007

Vol. 71, No. 1
26740/3159178
Printed in U.S.A.

μ-Opioid Receptor Activation Modulates Transient Receptor Potential Vanilloid 1 (TRPV1) Currents in Sensory Neurons in A Model of Inflammatory Pain

Jeannette Endres-Becker, Paul A. Heppenstall, Shaaban A. Mousa, Dominika Labuz, Alexander Oksche, Michael Schäfer, Christoph Stein, and Christian Zöllner

Klinik für Anaesthesiologie und operative Intensivmedizin (J.E.-B., P.A.H., S.A.M., D.L., M.S., C.S., C.Z.) and Institut für Pharmakologie (A.O.), Charité Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany

Received May 29, 2006; accepted September 27, 2006

ABSTRACT

Current therapy for inflammatory pain includes the peripheral application of opioid receptor agonists. Activation of opioid receptors modulates voltage-gated ion channels, but it is unclear whether opioids can also influence ligand-gated ion channels [e.g., the transient receptor potential vanilloid type 1 (TRPV1)]. TRPV1 channels are involved in the development of thermal hypersensitivity associated with tissue inflammation. In this study, we investigated μ-opioid receptor and TRPV1 expression in primary afferent neurons in the dorsal root ganglion (DRG) in complete Freund's adjuvant (CFA)-induced paw inflammation. In addition, the present study examined whether the activity of TRPV1 in DRG neurons can be inhibited by μ-opioid receptor (μ-receptor) ligands and whether this inhibition is increased after CFA inflammation. Immunohistochemistry demonstrated colocalization of TRPV1 and μ-receptors in DRG neurons. CFA-induced inflammation increased significantly the number of TRPV1- and μ-receptor-positive DRG neurons, as well as TRPV1 binding sites. In whole-cell patch clamp studies, opioids significantly decreased capsaicin-induced TRPV1 currents in a naloxone- and pertussis toxin-sensitive manner. The inhibitory effect of morphine on TRPV1 was abolished by forskolin and 8-bromo-cAMP. During inflammation, an increase in TRPV1 is apparently rivaled by an increase of μ-receptors. However, in single dissociated DRG neurons, the inhibitory effects of morphine are not different between animals with and without CFA inflammation. In in vivo experiments, we found that locally applied morphine reduced capsaicin-induced thermal allodynia. In summary, our results indicate that μ-receptor activation can inhibit the activity of TRPV1 via $G_{i/o}$ proteins and the cAMP pathway. These observations demonstrate an important new mechanism underlying the analgesic efficacy of peripherally acting μ-receptor ligands in inflammatory pain.

The treatment of patients with inflammatory pain includes opioids acting outside the central nervous system (Stein et al., 2003). All three types of opioid receptors are synthesized and expressed in the cell bodies of dorsal root ganglion (DRG) neurons. These opioid receptors are intra-axonally transported into the neuronal processes and are detectable on peripheral C- and A-δ -nerve terminals. High-voltage activated calcium currents can be reduced by opioid receptor-activated inhibitory G-proteins (G_i), as suggested by experiments in cultured DRG neurons (Schroeder and McCleskey, 1993). In addition, opioids suppress tetrodotoxin-resistant sodium-selective and nonselective cation currents, which are mainly expressed in nociceptors (Ingram and Williams, 1994; Gold and Levine, 1996). The nonselective ion channel TRPV1 is also predominantly expressed in nociceptive sensory neurons (Caterina et al., 1997) and can be sensitized and/or up-regulated during conditions associated with tissue damage (Tominaga et al., 1998). TRPV1 seems to be essential for the development of thermal hypersensitivity associated with tissue inflammation but not that associated with nerve injury (Caterina and Julius, 2001). Inflammation-induced ther-

This work was supported by Deutsche Forschungsgemeinschaft (DFG) grant KFO 100/2 and a European Society of Anaesthesiology research grant.

Parts of this work have been published previously in abstract form [Endres-Becker J, Heppenstall PA, Mousa S, Labuz D, Schäfer M, Stein C, Zöllner C. Opioids modulate the transient receptor potential vanilloid 1 (TRPV1) ion channel. International Narcotic Research Conference; July 10–15, 2005; Annapolis, MD].

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.106.026740.

ABBREVIATIONS: DRG, dorsal root ganglion; TRPV1, transient receptor potential vanilloid type 1; μ-receptor, μ-opioid receptor; PKA, protein kinase A; PCR, polymerase chain reaction; CFA, complete Freund's adjuvant; RTX, resiniferatoxin ; PBS, phosphate-buffered saline; MEM, minimal essential medium; PTX, pertussis toxin; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; FSK, forskolin; 8-Br-cAMP, 8-bromo-cAMP; PWL, paw withdrawal latency; ANOVA, analysis of variance.

mal hypersensitivity may result from the actions of heat, low pH, and other inflammatory mediators on TRPV1 (Caterina and Julius, 2001). However, other mechanisms, such as up-regulation of TRPV1 expression, may also come into play. Because peripheral μ-receptor agonists are particularly effective in inflammatory hyperalgesia, we hypothesized that opioids can modulate the activity of TRPV1. To elucidate possible TRPV1–μ-receptor interactions, we investigated G-protein and second messenger pathways. After opioids bind to their receptors, dissociation of the heterotrimeric G protein complex into G_{α} and $G_{\beta\gamma}$ subunits can subsequently lead to the inhibition of adenylyl cyclase isoforms. As a result, formation of cAMP is inhibited, and protein kinase A (PKA) cannot be activated (Law et al., 2000). TRPV1 can be phosphorylated and subsequently regulated by several kinases, including PKA (De Petrocellis et al., 2001; Bhave et al., 2002; Mohapatra and Nau, 2003), and protein kinase C (Bhave et al., 2003). In this study, we investigated the activity of TRPV1 after μ-receptor activation and its modulation by the cAMP/PKA pathway in single DRG neurons using patch clamp technique. We examined expression and colocalization of μ-receptors and TRPV1 in DRG using immunohistochemistry, real-time PCR, and radioligand binding in animals with and without painful hindpaw inflammation, a model that resembles postoperative pain, arthritis and other types of inflammatory pain (Machelska et al., 2003).

Materials and Methods

Animals. Experiments were performed in individually housed male Wistar rats (180–200 g). Control animals were treated with intraplantar saline (0.15 ml) injections. Inflammation was induced by intraplantar CFA (0.15 ml; Calbiochem, San Diego, CA) administered into the right hindpaw. All intraplantar injections were performed under brief isoflurane (Willy Rüsch GmbH, Böblingen, Germany) anesthesia. The animal protocol was approved by the state animal care and use committee ("Landesamt für Arbeitsschutz, Gesundheit und Technische Sicherheit Berlin") and the guidelines on ethical standards for investigations of experimental pain in animals were followed (Zimmermann, 1983).

TRPV1 Light Cycler PCR Experiments. Real time-PCR was performed as described previously (Puehler et al., 2004). The following primers for TRPV1 were used (Ji et al., 2002): 5′-AAA CTC CAC CCC ACG CTG AA-3′ and 5′-GTC GGT TCA AGG GTT CCA CG-3′, corresponding to bases 1031 and 1321 (GenBank accession number NM_031982). A mathematical model was used to determine the relative quantification of a target gene compared with a reference gene (Pfaffl, 2001). Ribosomal protein L19 (RPL-19) was chosen as a reference gene and the following primers were used: 5′-AAT CGC CAA TGC CAA CTC TCG-3′ and 5′-TGC TCC ATG AGA ATC CGC TTG-3′ corresponding to bases 1521 and 3274 (GenBank accession number X82202). Target and reference genes were quantified using triplicate samples. Light cycler PCR was performed with a DNA Sybr Green kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Amplification was carried out for 45 cycles, each consisting of 10 s at 95°C, 5 s at 57°C, and 16 s at 72°C. A temperature was determined just below the product-specific melting temperature (T_m), and this temperature step was incorporated into the PCR for detection of fluorescence (TRPV1, T_m 89°C; RPL-19, T_m 88°C). As a control for the PCR reaction, reverse transcriptase (RT)-negative samples were used. PCR products and a size marker (DNA Marker XIV; Roche Diagnostics, Mannheim, Germany) were separated by electrophoresis on a 2% agarose gel containing ethidium bromide (Sigma, Taufkirchen, Germany) and were visualized under UV light. PCR products were sequenced in forward and backward direction (AGOWA, Berlin, Germany) and were compared with the published sequences of TRPV1 (homologies ≥99%). TRPV1 mRNA was determined at 2, 6, 12, 24, 48, 72, and 96 h after CFA inoculation.

Membrane Preparations. All chemicals and drugs were purchased from Sigma (Taufkirchen, Germany) unless indicated otherwise. Rats were killed by isoflurane anesthesia 96 h after intraplantar saline or CFA application. Ipsilateral lumbar (L4/5) DRG neurons were removed from saline-injected animals. In CFA animals, ipsilateral and contralateral (internal controls) lumbar (L4/5) DRG neurons were removed. The tissue was placed immediately on ice in ice-cold assay buffer (50 mM Tris-HCl and 1 mM EGTA, pH 7.4). Tissue was pooled from 10 rats, homogenized, centrifuged twice at 42,000*g* and 4°C for 20 min, and resuspended in assay buffer.

TRPV1 Binding. Binding studies with the labeled TRPV1 agonist resiniferatoxin ([^{3}H]RTX) were carried out according to a modified protocol (Szallasi et al., 1999). In brief, appropriate concentrations of cell membranes (40 μg) were prepared and incubated in assay buffer (5 mM KCl, 5.8 mM NaCl, 0.75 mM $CaCl_2$, 2 mM $MgCl_2$, 320 mM sucrose, and 10 mM HEPES) with increasing doses of [^{3}H]RTX (15–2000 pM, 43 Ci/mmol; GE Healthcare, Little Chalfont, Buckinghamshire, UK) in the absence or presence of 10 μM unlabeled RTX. Because adenosine (released during sample preparation) can directly interact with TRPV1 (Puntambekar et al., 2004), we pretreated DRG membranes with adenosine deaminase (1 U/ml) for 15 min at 37°C to degrade adenosine. Membranes were incubated in a final volume of 750 μl for 1 h at 30°C in assay buffer. Filters were soaked in 0.1% (w/v) polyethylenimine solution for 30 min before use. Bound and free ligand were separated by rapid filtration under vacuum through Whatman GF/B glass fiber filters, followed by four washes with ice-cold buffer (50 mM Tris-HCl, pH 7.4). Bound radioactivity was determined by liquid scintillation spectrophotometry at 70% counting efficiency for [^{3}H] after overnight extraction of the filters in 3 ml of scintillation fluid (PerkinElmer Wallac, Turku, Finland).

Immunohistochemistry. Immunofluorescence staining for μ-receptors and TRPV1 was performed as described previously (Mousa et al., 2001). Rats were deeply anesthetized with isoflurane and perfused transcardially with 100 ml of 0.1 M PBS, pH 7.4, and 300 ml of ice-cold PBS containing 4% paraformaldehyde and 0.2% picric acid (pH 6.9; fixative solution). Lumbar DRG (L4–L5) were removed, postfixed for 90 min at 4°C in the fixative solution, and cryoprotected overnight at 4°C in PBS containing 10% sucrose. The tissues were embedded in Tissue-Tek compound (Bayer Healthcare, Pittsburgh, PA) and consecutive sections (9 μm) mounted onto gelatin-coated slides were prepared on a cryostat. To prevent nonspecific binding, the sections were incubated in PBS containing 0.3% Triton X-100, 1% bovine serum albumin, 4% goat serum, and 4% donkey serum (block solution). The sections were incubated overnight at 4°C with rabbit anti-μ-receptor (Drs S. Schulz and V. Höllt, Magdeburg, Germany) in combination with guinea pig anti-TRPV1 antibody (1:1000 dilution; Neuromics, Minneapolis, MN) and then incubated with goat anti-rabbit conjugated with Texas Red (1:250 dilution; Vector Laboratories, Burlingame, CA) and donkey anti-guinea pig conjugated fluorescein isothiocyanate (1:250 dilution; Vector Laboratories). Thereafter, sections were washed with PBS, mounted in Vectashield, and viewed under a confocal laser scanning microscope (Zeiss, Jena, Germany) by an experimenter blinded to the treatment regime. Settings for excitation of fluorescein isothiocyanate (488 nm) and Texas Red (543 nm) and of photodetectors (pinhole, amplifier gain) were identical throughout the analysis. Controls included (1) preabsorption of diluted antibodies with their respective immunizing peptides and (2) omission of either the primary antisera or the secondary antibodies. These control experiments did not show staining.

Quantification of Immunostaining. The method of quantification for DRG staining has been described previously (Ji et al., 1995). In brief, we stained every fourth section of DRG that was serially cut at 9 μm for each animal ($n = 5$). Square grids avoid double count of

neurons. The cell body diameter was measured with the nucleus in the focal plane and was estimated from the average length and width determined with a calibrated micrometer. A total number of 60 immunoreactive neurons with nucleus were measured for each animal. The μ-receptor-ir and TRPV1-ir neurons were counted, and the proportion was calculated as percentage of total number of DRG cells. In addition, the μ-receptor-ir neurons, which were also immunoreactive for TRPV1, were counted, and the proportion was calculated as percentage of TRPV1-ir neurons. For neuron counting, only those immunostained neurons containing a distinct nucleus were counted for a total of 332 neurons. Data were obtained from four sections of each DRG and five rats per group.

Cultures of DRG Neurons. DRG were prepared as described previously (Bolyard et al., 2000). In brief, L4/L5 DRG were removed and placed in sterile MEM at 4°C. DRG were digested with collagenase type 2 in MEM at 37°C for 50 min and 0.025% trypsin for 10 min at 37°C. After digestion, DRG were carefully dissociated by mechanical agitation and centrifuged at 500*g* for 5 min and at 300*g* for 5 min. The cells were maintained in MEM (Biochrom AG, Berlin, Germany) growth media supplemented with 10% horse serum/50 μg/ml penicillin and streptomycin and plated in six-well polylysine-coated culture plates at 37°C for 2 to 4 h in a 5% CO_2 atmosphere.

Drug and Heat Stimulation. For drug application and heat stimulation of single neurons, a fast channel system with common outlet was used (Warner Instruments, Hamden, CT). Magnetic valves to open and close the syringes were controlled manually from a switchboard. Heat was applied using a Peltier device (ESF Electronic, Goettingen, Germany) that heated the extracellular buffer at approximately 1°C per second from 24°C to 46°C. Temperature was monitored with a thermocouple placed within the flow of buffer and close to the cells.

Electrophysiology in Dissociated DRG Cells. Whole-cell measurements in the voltage-clamp configuration of the patch-clamp technique were performed 2 to 4 h after dissociation at −60 mV holding potential with an EPC-10 patch clamp amplifier and PULSE software (HEKA Elektronik, Lambrecht, Germany). For pertussis toxin (PTX) experiments, dissociated DRG cells were kept in culture for 2 h. Afterward, DRG cells were pretreated with PTX for 6 h. Borosilicate glass electrodes (Hilgenberg, Malsfeld, Germany) pulled on a horizontal puller (Sutter Instrument Company, Novato, CA) had resistances of 2 to 7 MΩ after filling with 145 mM KCl, 1 mM $MgCl_2$, 2 mM Na-ATP, 10 mM HEPES, 10 mM glucose, and 0.2 mM Na-GTP, pH adjusted to 7.3 with KOH. The external solution consisted of 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, at pH adjusted to 7.3 with NaOH. Only small sensory neurons (cell diameter ≤26 μm) that were sensitive to the nociceptor excitant capsaicin (1 μM) or heat (46°C) were included in the study, and these cells were considered responsive if the inward current magnitude was at least 100 pA. Because in preliminary experiments repeated applications of capsaicin to the same neuron produced currents of progressively smaller magnitude, Ca^{2+} was removed from the extracellular buffer. We compared responses in dissociated DRG cells from animals with and without CFA inflammation. After the first capsaicin application, dissociated DRG cells were incubated with buffer or the μ-receptor agonists morphine (10 μM) or DAMGO (10 μM), and capsaicin (1 μM) was applied again 60 and 360 s later. To examine whether the morphine-evoked inhibitory effect was reversible, DRG cells were treated with morphine for 60 s. Thereafter, cells were treated with buffer or morphine and capsaicin was applied again 360 and 720 s later. Temperature ramps were used to heat the extracellular buffer from room temperature to 46°C. After the first increase in temperature, DRG cells were incubated with morphine (10 μM) or buffer and heat was applied again 60 and 360 s later. To investigate the involvement of μ-receptors and $G_{i/o}$-proteins, dissociated DRG neurons were treated with naloxone (10 μM) concomitantly with morphine or pretreated for 6 h with PTX (100 ng), respectively. To study the role of cAMP pathways, dissociated DRG neurons were treated with morphine (10 μM) and forskolin (FSK; 10 μM), a potent stimulator of adenylyl cyclase activity. In addition, the reversibility of the morphine effect was investigated by concomitant application of the cAMP analog 8-bromo-cAMP (8-Br-cAMP) (10 μM) with morphine (10 μM) for 5 min.

Measurement of Thermal Hyperalgesia. Nociceptive thresholds were determined by measuring the paw withdrawal latency (PWL) upon application of acute thermal stimulation (Hargreaves et al., 1988). Rats were placed in clear plastic chambers positioned on a glass surface (model 336 Analgesia Meter; IITC Life Science, Woodland Hills, CA). Radiant heat was applied to the plantar surface of a hindpaw from underneath the glass floor with a high-intensity projector lamp bulb and PWL was measured using an electronic timer. Nociceptive testing was performed twice per paw, separated by at least 30 s, and the mean value was calculated for each animal. The heat intensity was adjusted to obtain a basal PWL of approximately 9 to 10 s. A 20-s cut-off was used to prevent tissue damage. Animals were tested before and 15, 30, 45, and 60 min after intraplantar injection of capsaicin (30 μg/10 μl). In a separate experiment, morphine (intraplantar; 100 μg/20 μl) was injected 10 min after capsaicin, and PWL was measured 5 min later. For control (Ctrl), this same volume of vehicle was injected into the paw.

Statistics. Statistical differences are represented as means ± S.E.M. Light cycler PCR experiments were made using one-way ANOVA followed by Student-Newman-Keuls test in the case of normally distributed data and ANOVA on ranks followed by Kruskal-Wallis analysis in the case of not normally distributed data. Statistical differences in electrophysiology were determined with two-way ANOVA and Bonferroni post hoc test. Unpaired, two-tailed Student's *t* test was used to assess statistical significance in immunohistochemistry, ligand binding and behavioral experiments. All ligand binding data are reported as means ± S.E.M. of at least four experiments, performed in duplicate. Differences were considered significant at $p < 0.05$. All tests were performed using Prism 4 (GraphPad, San Diego, CA) or Sigma Stat 2.03 (SyStt Software, Point Richmond, CA) statistical software.

Results

TRPV1 mRNA in DRG Cells. TRPV1 mRNA levels did not differ significantly in DRG cells from animals with and without 96 h CFA inflammation. Our previous experiments have shown a biphasic transcriptional regulation of μ-receptor mRNA in response to inflammation (Puehler et al., 2004). Therefore, TRPV1 mRNA was determined at additional time points (2, 6, 12, 24, 48, and 72h). No significant differences were detected (one-way ANOVA; $p > 0.05$) (Fig. 1A). Reverse transcriptase-negative samples ruled out the idea that

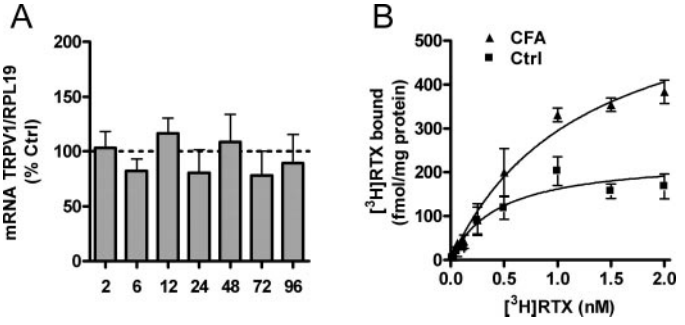

Fig. 1. A, quantification of TRPV1 mRNA after hindpaw inflammation. Changes of TRPV1-mRNA were not significantly different from animals without inflammation over the entire time course ($n = 6$ animals per time point; ANOVA, $p > 0.05$). Data are shown as percentage of change in relation to the housekeeping gene RPL-19. B, number of [^{3}H]RTX binding sites increased on DRG cells from animals treated with CFA (B_{max} = 655 ± 114 fmol/mg of protein) compared with animals without CFA treatment (B_{max} = 233 ± 38 fmol/mg of protein) (*t* test, $p < 0.05$).

genomic DNA was amplified. Sequencing of the real-time PCR product confirmed that primers were specific for TRPV1 (data not shown).

TRPV1 Binding in DRG Neurons. Saturation binding analysis with [^{3}H]RTX showed no significant difference in binding affinities (K_d) in DRG membranes of animals treated with saline (K_d = 0.44 ± 0.2 nM) or CFA (K_d = 1.22 ± 0.4 nM). The number of TRPV1 binding sites (B_{max}) increased significantly in CFA-treated animals compared with saline-treated animals (Fig. 1B) (t test, $p < 0.05$). This increase was not detectable in DRG cells on the contralateral noninflamed site (data not shown).

Coexpression of TRPV1 and μ-Receptors in DRG. The mean cell body diameter of μ-receptor and TRPV1-positive neurons was 31 ± 3 μm; the majority lay between small- and medium-diameter neurons (Fig. 2, C, F, and I). Four days after induction of paw inflammation, a significant increase in DRG neurons positive for μ-receptor (21 ± 2 to 29 ± 3%) (Fig. 2, E and H) and TRPV1 (20 ± 3 to 32 ± 4%) (Fig. 2, D and G) was detectable compared with saline-treated animals (Fig. 2, A and B) (t test, $p < 0.05$). Coexpression of μ-receptors and TRPV1 was seen in 14 ± 2% of all DRG neurons from untreated animals (Fig. 2C) and in 22 ± 4% from CFA-treated animals (Fig. 2F). (percentage expression relates to total number of DRG cells). The number of μ-receptor-TRPV1-ir divided by TRPV1-ir was 67 ± 6% in saline treated and 71 ± 8% in CFA-treated animals. The difference between both groups did not reach statistical significance (t test, $p > 0.05$).

Opioids Inhibit Capsaicin-Induced TRPV1 Activity. In Ca^{2+}-free buffer, capsaicin, applied repeatedly at three different time points (0, 60, and 360 s) produced similar currents in dissociated DRG neurons from animals treated with saline (0, 60, and 360 s, −2.1 ± 0.3, −2.0 ± 0.1, and −1.8 ± 0.1 nA, respectively) (Fig. 3B) or CFA (0, 60, and 360 s, −2.1 ± 0.2, −1.9 ± 0.1, and −1.6 ± 0.1 nA, respectively) (Fig. 3D). Only small-sized sensory neurons (cell diameter, ≤26 μm) were used for patch-clamp experiments. Morphine (10 μM), applied after the first capsaicin stimulus, induced a significant decrease in TRPV1 activity in 79% of dissociated DRG cells from animals without CFA inflammation (Fig. 3B). Another μ-receptor agonist (DAMGO; 10 μM) induced a similar decrease in TRPV1 activity compared with morphine (0 s, −2.0 ± 0.3 nA [= 100%]; ANOVA, **, $p < 0.001$) (data not shown). Washout experiments revealed that additional capsaicin applications 360 and 720 s later progressively increased TRPV1 activity in the absence of morphine but not in the presence of morphine (Fig. 3C). In addition, morphine reduced capsaicin-activated currents in 70% of dissociated DRG cells with CFA inflammation (Fig. 3D). Morphine (10 μM) also inhibited heat-evoked currents in 86% of dissociated DRG cells from naive animals (Fig. 3E). The inhibitory effect of morphine was not significantly different

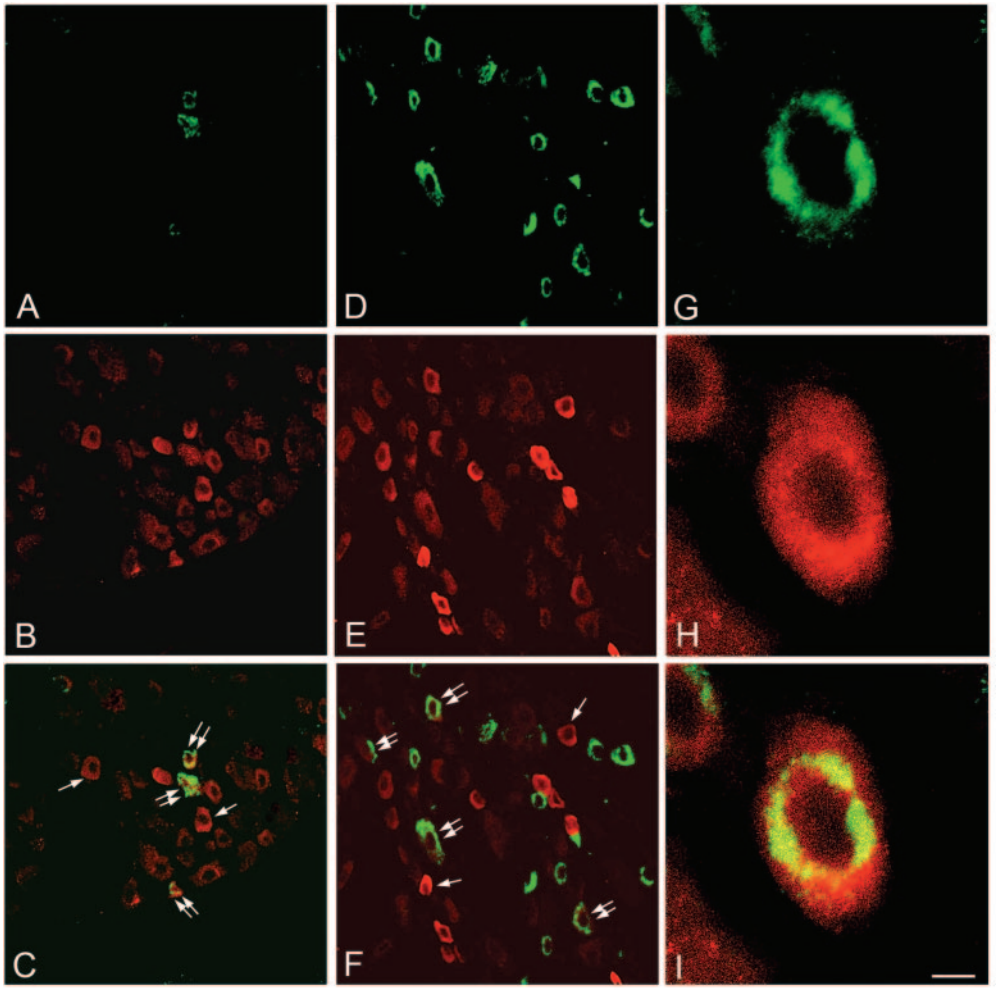

Fig. 2. Effects of CFA-induced paw inflammation on TRPV1 and μ-receptor expression in DRG. In control animals, 20 ± 3% of total DRG neurons expressed TRPV1 (green) (A) and 21 ± 2% expressed μ-receptors (red) (B). Coexpression of μ-receptors and TRPV1 was seen in 14 ± 2% of all DRG neurons (C). Double arrow, representative cell coexpressing μ-receptors and TRPV1; arrow, representative cell expressing μ-receptors only. In CFA-treated animals 32 ± 4% of total DRG neurons expressed TRPV1 (green) (D) and 29 ± 3% expressed μ-receptors (red) (E). Coexpression of μ-receptors and TRPV1 was seen in 22 ± 4% of all DRG neurons (F). Double arrow, representative cell coexpressing μ-receptors and TRPV1; arrow, representative cell expressing μ-receptors only. TRPV1 (green) (G), μ-receptor (red) (H), and coexpression of μ-receptor and TRPV1 (I) on a representative single DRG neuron from an animal with CFA inflammation. Scale bars, 50 μm (A–F); 10 μm (G–I).

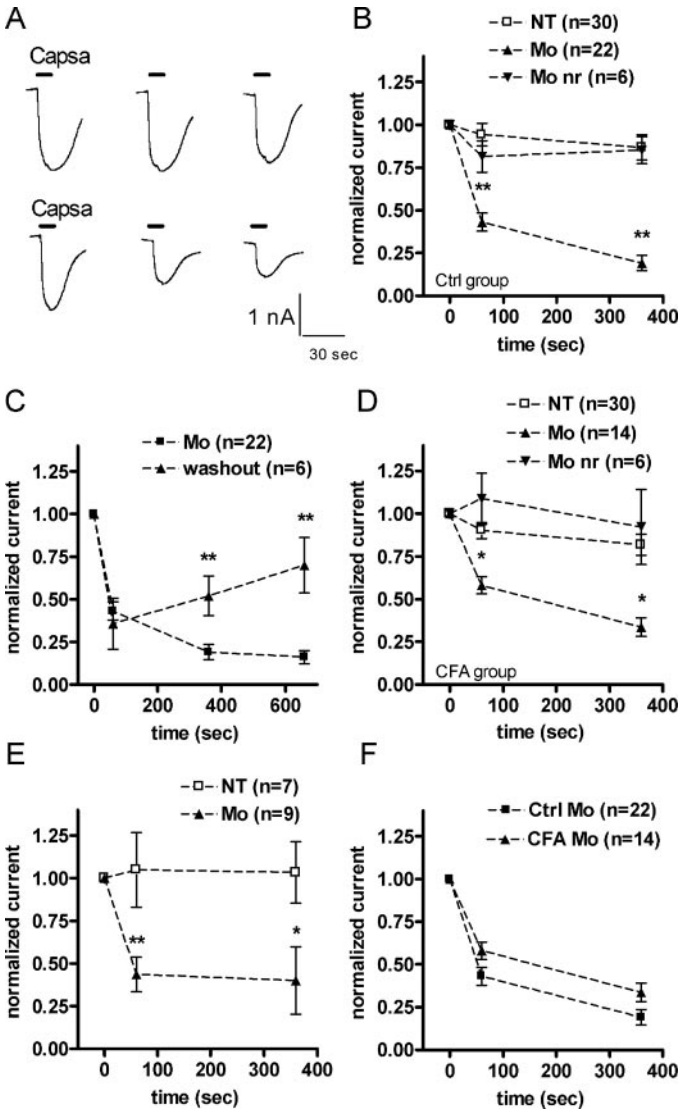

Fig. 3. Inhibitory effects of opioids on TRPV1 currents. A, examples of whole-cell current responses to 5-s applications of 1 μM capsaicin in the absence (top) and presence (bottom) of morphine in DRG neurons from control animals. B, current responses to repeated applications of 1 μM capsaicin (nontreated; NT) were normalized to the amplitude obtained with the first capsaicin application in DRG neurons from control animals (Ctrl) (0 s, -2.1 ± 0.3 nA [= 100%]). Morphine significantly decreased capsaicin-activated currents in Ctrl DRG neurons (0 s, $-1{,}9 \pm 0.3$ nA [= 100%]; ANOVA, **, $p < 0.01$). Cells that did not respond to morphine (Mo nr) showed no significant differences in capsaicin activated currents compared with current responses in the absence of morphine (0 s, -1.8 ± 0.4 nA [= 100%], ANOVA, $p > 0.05$). C, morphine application for 60 s decreased capsaicin-activated currents in Ctrl DRG neurons (0 s, -1.8 ± 0.2 nA [= 100%]). Washout of morphine for 360 and 720 s restored capsaicin-mediated TRPV1 activity. D, current responses to repeated applications of 1 μM capsaicin (nontreated; NT) were normalized to the amplitude obtained with the first capsaicin application in DRG neurons from animals with CFA inflammation (CFA) (0 s, -2.1 ± 0.2 nA [= 100%]). Morphine significantly decreased capsaicin-activated currents (0 s, 2.3 ± 0.3 nA [= 100%]; ANOVA, *, $p < 0.05$). Cells that did not respond to morphine (Mo nr) showed no significant differences in capsaicin-activated currents compared with responses in the absence of morphine (0 s, 2.5 ± 0.4 nA [= 100%], ANOVA, $p > 0.05$). E, morphine significantly decreased heat-activated currents in Ctrl DRG neurons (0 s, -2.7 ± 0.8 nA [= 100%], ANOVA, **, $p < 0.01$; *, $p < 0.05$). F, current responses to repeated applications of 1 μM capsaicin in the presence of morphine did not differ between animals with and without CFA inflammation.

between animals with and without CFA inflammation (Fig. 3F). These effects were reversed by naloxone (10 μM; Fig. 4A), PTX (100 ng, 6 h; Fig. 4B), FSK (10 μM; Fig. 4C), and 8-Br-cAMP (10 μM, 5 min; Fig. 4D). FSK and 8-Br-cAMP treatment in the absence of morphine did not alter TRPV1 activity significantly compared with untreated DRG neurons (Fig. 4, C and D).

Opioids Inhibit Capsaicin-Induced Hyperalgesia. The mean baseline PWL was 10.4 ± 0.3 s. Intraplantar injection of capsaicin (30 μg/10 μl) induced a time-dependent decrease in PWL with a maximum effect at 15 min (Fig. 5A). Capsaicin-induced thermal hyperalgesia was significantly reduced 5 min after local application of morphine (100 μg/20 μl) compared with control group (Fig. 5B) (t test, $p < 0.05$). No PWL changes were observed in the contralateral paws after capsaicin or morphine application (data not shown).

Discussion

This study showed that:

1. TRPV1 and μ-receptors are coexpressed in DRG neurons. Paw inflammation induced an increase in TRPV1/μ-receptor-expression and TRPV1 binding sites but not in TRPV1 mRNA.
2. μ-Receptor agonists inhibit capsaicin-elicited TRPV1 activity in dissociated DRG neurons via opioid receptors and inhibitory G-proteins in a cAMP-dependent manner;
3. Morphine-mediated inhibition at TRPV1 was not aug-

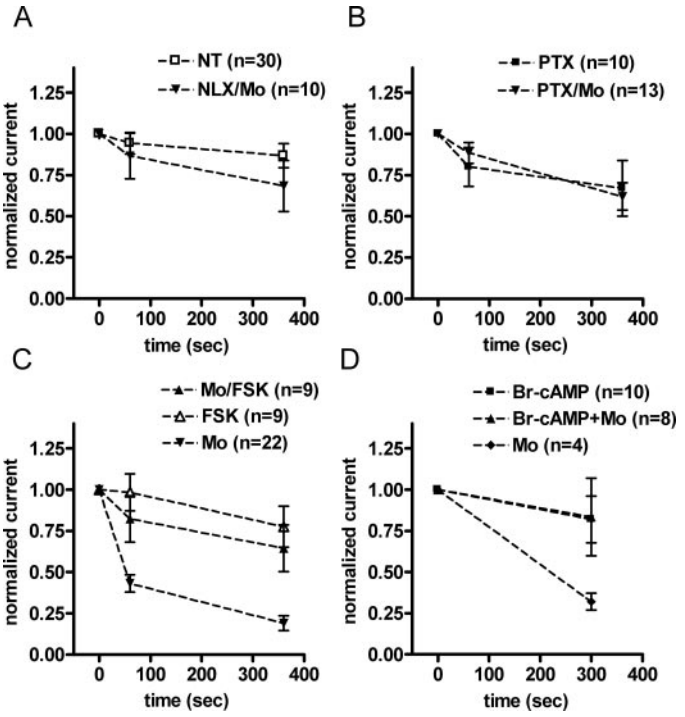

Fig. 4. Inhibitory effects of opioids on TRPV1 currents. Naloxone (NLX/Mo) (0 s, -2.5 ± 0.4 nA [= 100%]; ANOVA, $p > 0.05$ compared with NT) (A) and pertussis toxin (PTX/Mo) (0 s, -2.7 ± 0.3 nA [= 100%]; ANOVA, $p > 0.05$ compared with PTX alone) (B) blocked morphine (Mo)-induced inhibition of TRPV1. C, forskolin (Mo/FSK) (0 s, -2.4 ± 0.5 nA [= 100%]; ANOVA, $p > 0.05$ compared with FSK alone) and (D) 8-Br-cAMP (Mo/8-Br-cAMP) (0 s, -2.6 ± 0.4 nA [= 100%]; ANOVA, $p > 0.05$ compared with 8-Br-cAMP alone) pretreatment reversed morphine-induced inhibition of TRPV1. *, statistically significant difference compared with the respective control.

mented in DRG neurons from animals with CFA inflammation.

4. Locally applied morphine abolishes capsaicin-induced hyperalgesia in vivo.

The nonselective ion channel TRPV1 is predominantly expressed in nociceptive sensory neurons (Caterina et al., 1997) and can be sensitized and/or up-regulated during inflammation (Tominaga et al., 1998). Because peripheral opioid agonists are particularly effective in inflammatory hyperalgesia, we hypothesized that μ-receptor ligands can modulate in addition the activity of TRPV1. In agreement with the putative role of TRPV1 in nociception, the majority of TRPV1-expressing cells belong to small DRG neurons (Nagy et al., 2004). Paw inflammation induced a substantial increase in TRPV1 and μ-receptor positive DRG neurons. This confirms previous immunohistochemical studies showing an increase in TRPV1-like immunoreactivity in DRG during inflammation from 27 to 44% (Amaya et al., 2004), 23 to 36% (Ji et al., 2002), and 11 to 25% (Breese et al., 2005). In addition, we found that μ-receptors and TRPV1 are colocalized to a substantial amount on the same DRG neurons.

[^{3}H]RTX binding to TRPV1 was performed previously and showed specific binding to DRG membranes with a K_d of 270 pM and a B_{max} of 160 fmol/mg of protein (Szallasi and Blumberg, 1990). Our experiments revealed comparable results for K_d and B_{max} in DRG cells from saline-treated animals. In addition, specific TRPV1 binding to DRG membranes increases 2.8-fold during inflammation, without changing the affinity of RTX to TRPV1. This confirms our immunohistochemical experiments and supports the hypothesis that inflammation-induced thermal hypersensitivity may result, at least in part, from an up-regulation of TRPV1 expression. In addition, we could confirm previous studies showing that TRPV1 mRNA remained unchanged during inflammation (Ji et al., 2002). This might indicate that inflammation can increase translation of TRPV1 without changing its transcription or that insertion of preformed TRPV1 into the plasma membrane is promoted, as shown for TRPC5 after growth factor stimulation (Bezzerides et al., 2004). For the μ-receptor, previous studies from our group have shown that CFA inflammation can induce an up-regulation of μ-receptor binding sites and G-protein coupling in DRG cells (Zöllner et al., 2003). mRNA content for μ-receptors showed a biphasic up-regulation with an early peak at 1 to 2 h and a late increase at 96 h after CFA inflammation (Puehler et al., 2004). Together, these data indicate that TRPV1 and μ-receptor binding sites increase during peripheral hindpaw inflammation.

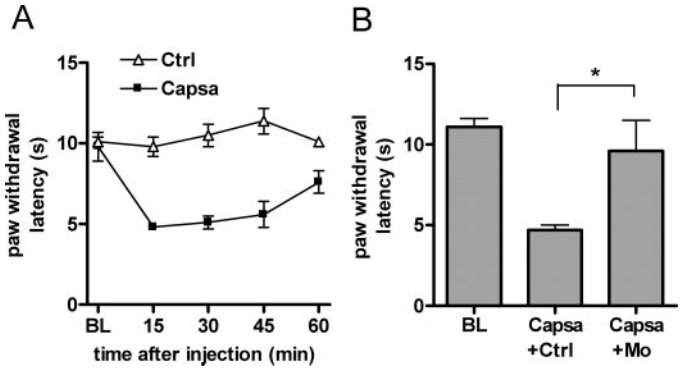

Fig. 5. Hyperalgesia to heat after plantar capsaicin injection in animals pretreated with morphine. The mean baseline PWL (BL, 10.4 ± 0.3 s) showed a time-dependent decrease after local capsaicin injection (Capsa) compared with control (Ctrl) animals (A). This hyperalgesia (Capsa+Crtl) was significantly reduced by local application of morphine (Capsa+Mo) (t test, $*p < 0.05$) (B).

A molecular interaction between TRPV1 and μ-receptors in DRG neurons has not been shown so far. Consistent with the colocalization of both proteins, our electrophysiological experiments revealed that morphine and DAMGO can attenuate capsaicin-induced TRPV1 activity. The morphine evoked inhibitory effect was reversible in washout experiments. We could show in addition that this attenuation was opioid receptor-mediated, because it was blocked by naloxone, a specific opioid receptor antagonist. Furthermore, the opioid-induced inhibition of TRPV1 was G protein dependent, because it was blocked by PTX, which catalyzes the ADP-ribosylation of α subunits of the G_i family. We cannot rule out the possibility that $G_{\beta\gamma}$ -subunits might interfere with TRPV1 directly. However, our observations that both FSK and 8-Br-cAMP can reverse morphine-induced inhibition of TRPV1 activity suggest that this effect is mediated mainly via a cAMP/PKA-dependent pathway. PKA-dependent modifications of TRPV1 currents have been shown in various cell systems (Bhave et al., 2002; Hu et al., 2002), including DRG neurons (Rathee et al., 2002). Activators of PKA increased TRPV1 phosphorylation and the sensitivity of the channel (Lee et al., 2005). In addition, it was shown that PKA-mediated phosphorylation not only potentiates heat-activated TRPV1 currents (Rathee et al., 2002), but also counteracts Ca^{2+}-dependent desensitization (Mohapatra and Nau, 2003). This was confirmed in experiments showing that TRPV1 is strongly phosphorylated in the resting state, that phosphorylation of the channel is reduced by stimulation with capsaicin, and that PKA can rephosphorylate and subsequently resensitize TRPV1 (Bhave et al., 2002). Our data suggest that an opioid receptor-mediated decrease in intracellular cAMP levels (Law et al., 2000), which controls the activity of PKA, results in reduced TRPV1 channel activity. Consistent with this notion, pretreatment of DRG neurons with 8-Br-cAMP, a potent cell-permeable cAMP analog that is more resistant to phosphodiesterases than is cAMP, reversed opioid mediated inhibition at TRPV1. This suggests that the inhibition of the cAMP/PKA-pathway by opioids rather than a direct Gβγ effect at TRPV1 is involved, in that it was suggested before for TRPV1-transfected HEK293 cells (Vetter et al., 2006).

Because analgesic effects of peripheral opioids are particularly prominent during inflammatory pain (for review, see Stein et al., 2003), we tested whether the observed interaction between μ-receptors and TRPV1 is augmented after CFA treatment. We found a significant morphine-induced decrease in capsaicin-mediated TRPV1 activity in DRG from CFA-treated animals. However, the individual TRPV1 channel after capsaicin stimulation was not more susceptible to opioid modulation during inflammatory pain compared with naive animals. Heat plays an important role in the development of hyperalgesia during inflammation. Therefore, we tested the role of morphine on heat-evoked responses at TRPV1. Similar to capsaicin, TRPV1 activity stimulated by heat was blocked by morphine, suggesting that opioids can inhibit TRPV1 activity after its activation by different ligands.

Finally, our behavioral experiments show that the local injection of morphine results in a significant attenuation of capsaicin-induced thermal hyperalgesia similar to a previous study in primates (Butelman et al., 2004). The applied dose of morphine was not systemically effective, which excludes centrally mediated analgesic effects. However, this experiment does not exclude the possibility that opioids influence other ion channels such as calcium and sodium channels. Because morphine is known to act upon these targets, the analgesic effects might occur without a direct effect on TRPV1. Nonetheless, together with our in vitro studies, our behavioral data suggest that the ligand-gated ion channel TRPV1 as a molecular target for opioids to inhibit inflammatory pain.

In conclusion, this work shows that effects of μ-receptor agonists on dissociated DRG neurons are not only restricted to voltage-activated calcium and tetrodotoxin-resistant sodium channels. Opioids can also inhibit the activity of the ligand-gated ion channel TRPV1. This inhibition is μ-receptor-specific, mediated via $G_{i/o}$ proteins and the cAMP/PKA pathway. These observations demonstrate an important mechanism underlying the analgesic efficacy of peripherally acting μ-receptor ligands in inflammatory pain.

Acknowledgments

We thank N. Siegemund for technical assistance.

References

Amaya F, Shimosato G, Nagano M, Ueda M, Hashimoto S, Tanaka Y, Suzuki H, and Tanaka M (2004) NGF and GDNF differentially regulate TRPV1 expression that contributes to development of inflammatory thermal hyperalgesia. *Eur J Neurosci* **20:**2303–2310.

Bezzerides VJ, Ramsey IS, Kotecha S, Greka A, and Clapham DE (2004) Rapid vesicular translocation and insertion of TRP channels. *Nat Cell Biol* **6:**709–720.

Bhave G, Hu HJ, Glauner KS, Zhu W, Wang H, Brasier DJ, Oxford GS, and Gereau RW (2003) Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). *Proc Natl Acad Sci USA* **100:**12480–12485.

Bhave G, Zhu W, Wang H, Brasier DJ, Oxford GS, and Gereau RW (2002) cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. *Neuron* **35:**721–731.

Bolyard LA, Van Looy JW, and Vasko MR (2000) Sensitization of rat sensory neurons by chronic exposure to forskolin or 'inflammatory cocktail' does not downregulate and requires continuous exposure. *Pain* **88:**277–285.

Breese NM, George AC, Pauers LE, and Stucky CL (2005) Peripheral inflammation selectively increases TRPV1 function in IB4-positive sensory neurons from adult mouse. *Pain* **115:**37–49.

Butelman ER, Harris TJ, and Kreek MJ (2004) Antiallodynic effects of loperamide and fentanyl against topical capsaicin-induced allodynia in unanesthetized primates. *J Pharmacol Exp Ther* **311:**155–163.

Caterina MJ and Julius D (2001) The vanilloid receptor: a molecular gateway to the pain pathway. *Annu Rev Neurosci* **24:**487–517.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, and Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature (Lond)* **389:**816–824.

De Petrocellis L, Harrison S, Bisogno T, Tognetto M, Brandi I, Smith GD, Creminon C, Davis JB, Geppetti P, and Di Marzo V (2001) The vanilloid receptor (VR1)-mediated effects of anandamide are potently enhanced by the cAMP-dependent protein kinase. *J Neurochem* **77:**1660–1663.

Gold MS and Levine JD (1996) DAMGO inhibits prostaglandin E_2-induced potentiation of a TTX-resistant Na^+ current in rat sensory neurons in vitro. *Neurosci Letters* **212:**83–86.

Hargreaves K, Dubner R, Brown F, Flores C, and Joris J (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32:**77–88.

Hu HJ, Bhave G, and Gereau RWt (2002) Prostaglandin and protein kinase A-dependent modulation of vanilloid receptor function by metabotropic glutamate receptor 5: potential mechanism for thermal hyperalgesia. *J Neurosci* **22:**7444–7452.

Ingram SL and Williams JT (1994) Opioid inhibition of Ih via adenylyl cyclase. *Neuron* **13:**179–186.

Ji RR, Samad TA, Jin SX, Schmoll R, and Woolf CJ (2002) p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron* **36:**57–68.

Ji RR, Zhang Q, Law PY, Low HH, Elde R, and Hokfelt T (1995) Expression of mu-, delta-, and kappa-opioid receptor-like immunoreactivities in rat dorsal root ganglia after carrageenan-induced inflammation. *J Neurosci* **15:**8156–8166.

Law PY, Wong YH, and Loh HH (2000) Molecular mechanisms and regulation of opioid receptor signaling. *Annu Rev Pharmacol Toxicol* **40:**389–430.

Lee SY, Lee JH, Kang KK, Hwang SY, Choi K_d, and Oh U (2005) Sensitization of vanilloid receptor involves an increase in the phosphorylated form of the channel. *Arch Pharm Res (NY)* **28:**405–412.

Machelska H, Schopohl JK, Mousa S, Labuz D, Schäfer M, and Stein C (2003) Different mechanisms of intrinsic pain inhibition in early and late inflammation. *J Neuroimmunol* **141:**30–39.

Mohapatra DP and Nau C (2003) Desensitization of capsaicin-activated currents in the vanilloid receptor TRPV1 is decreased by the cyclic AMP-dependent protein kinase pathway. *J Biol Chem* **278:**50080–50090.

Mousa SA, Zhang Q, Sitte N, Ji R, and Stein C (2001) beta-Endorphin-containing memory-cells and mu-opioid receptors undergo transport to peripheral inflamed tissue. *J Neuroimmunol* **115:**71–78.

Nagy I, Santha P, Jancso G, and Urban L (2004) The role of the vanilloid (capsaicin) receptor (TRPV1) in physiology and pathology. *Eur J Pharmacol* **500:**351–369.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29:**e45.

Puehler W, Zöllner C, Brack A, Shaqura MA, Krause H, Schäfer M, and Stein C (2004) Rapid upregulation of mu opioid receptor mRNA in dorsal root ganglia in response to peripheral inflammation depends on neuronal conduction. *Neuroscience* **129:**473–479.

Puntambekar P, Van Buren J, Raisinghani M, Premkumar LS, and Ramkumar V (2004) Direct interaction of adenosine with the TRPV1 channel protein. *J Neurosci* **24:**3663–3671.

Rathee PK, Distler C, Obreja O, Neuhuber W, Wang GK, Wang SY, Nau C, and Kress M (2002) PKA/AKAP/VR-1 module: A common link of G_s-mediated signaling to thermal hyperalgesia. *J Neurosci* **22:**4740–4745.

Schroeder JE and McCleskey EW (1993) Inhibition of Ca^{2+} currents by a μ-opioid in a defined subset of rat sensory neurons. *J Neurosci* **13:**867–873.

Stein C, Schäfer M, and Machelska H (2003) Attacking pain at its source: new perspectives on opioids. *Nat Med* **9:**1003–1008.

Szallasi A and Blumberg PM (1990) Specific binding of resiniferatoxin, an ultrapotent capsaicin analog, by dorsal root ganglion membranes. *Brain Res* **524:**106–111.

Szallasi A, Blumberg PM, Annicelli LL, Krause JE, and Cortright DN (1999) The cloned rat vanilloid receptor VR1 mediates both R-type binding and C-type calcium response in dorsal root ganglion neurons. *Mol Pharmacol* **56:**581–587.

Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI, and Julius D (1998) The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* **21:**531–543.

Vetter I, Wyse BD, Monteith GR, Roberts-Thomson SJ, and Cabot PJ (2006) The mu opioid agonist morphine modulates potentiation of capsaicin-evoked TRPV1 responses through a cyclic AMP-dependent protein kinase A pathway. *Mol Pain* **2:**22–38.

Zimmermann M (1983) Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* **16:**109–110.

Zöllner C, Shaqura MA, Bopaiah CP, Mousa S, Stein C, and Schäfer M (2003) Painful inflammation-induced increase in mu-opioid receptor binding and G-protein coupling in primary afferent neurons. *Mol Pharmacol* **64:**202–210.

Address correspondence to: Christian Zöllner, MD, Klinik für Anaesthesiologie und operative Intensivmedizin, Charité – Universitätsmedizin Berlin, Campus Benjamin Franklin, Hindenburgdamm 30, 12200 Berlin, Germany. E-mail: christian.zoellner@charite.de