Endothelin-1 Potentiates Capsaicin-Induced TRPV1 Currents *Via* the Endothelin A Receptor

TIM D. PLANT,* CHRISTIAN ZÖLLNER,† SHAABAN A. MOUSA,† AND ALEXANDER OKSCHE‡,[1]

*Institut für Pharmakologie und Toxikologie, Philipps-Universität Marburg, Karl-von-Frisch Str. 1, 35033 Marburg, Germany; †Klinik für Anästhesiologie und operative Intensivmedizin, Charité–Universitätsmedizin Berlin, Campus Benjamin-Franklin, Hindenburgdamm 30, 12200 Berlin, Germany; and the ‡Institut für Pharmakologie, Charité–Universitätsmedizin Berlin, Campus Benjamin-Franklin, Thielallee 67-73, 14195 Berlin-Dahlem, Germany

Endothelin-1 (ET-1) both stimulates nociceptors and sensitizes them to painful stimuli. The cellular mechanisms of the ET-1–mediated effects are only poorly understood. TRPV1, the heat-, proton-, and capsaicin-sensitive cation channel already known to be modulated by a number of cellular mediators released by painful stimuli and during inflammation, is a potential target for the action of ET-1. In immunocytochemistry of rat lumbar dorsal root ganglion using TRPV1- and ET_A receptor–specific antibodies, both proteins were found to be co-expressed in small sensory neurons. To provide evidence that ET-1 can modulate TRPV1 activity *via* the ET_A receptor, we used HEK 293 cells transiently co-expressing a fusion protein of TRPV1 and the yellow fluorescent protein (TRPV1-YFP) and the ET_A receptor. In whole-cell patch clamp recordings of HEK293 cells co-expressing TRPV1-YFP and the ET_A receptor, capsaicin (10 n*M*) elicited small currents, which were markedly potentiated when capsaicin (10 n*M*) and ET-1 (100 n*M*) were applied simultaneously. The data indicate that ET-1 potentiates TRPV1 activity *via* the ET_A receptor and that this process is likely to play a crucial role in the pain-producing and pain-potentiating effects of ET-1. Thus, ET_A receptor antagonists may be of importance in painful states with increased circulating ET-1 levels, as found in cancer and in chronic inflammation. Exp Biol Med 231:1161–1164, 2006

Key words: G protein–coupled receptor; TRP channel; capsaicin; immunohistochemistry

Introduction

Endothelin-1 (ET-1), a peptide of 21 amino acids, is one of the most potent vasoconstrictors known (1). Besides its cardiovascular effects, it can also elicit pain (2). However, the effector molecules involved in ET-1–induced pain and the signaling pathways underlying endothelin receptor–mediated nociception are poorly understood. ET-1 is released upon tissue injury, from tumor cells, or from macrophages and monocytes in inflammation (3, 4). It then acts on ET_A receptors expressed on Aδ and C fibers, causing the release of calcitonin gene-related peptide, substance P, and other factors leading to pain (5).

Little is known about the cellular effects of ET-1 that lead to the nociceptive responses in primary sensory neurons. It has recently been shown that activation of ET_A receptors results in an increase in intracellular Ca^{2+} concentration in model sensory neurons and that ET_A receptor activation lowers the threshold for activation of tetrodotoxin (TTX)-insensitive Na^+ channels in dorsal root ganglion (DRG) neurons (6, 7). However, it is not known whether ET_A receptor stimulation also results in a modulation of other channels involved in transduction of noxious stimuli, like the nonselective cation channel TRPV1. TRPV1, which is expressed in a subpopulation of sensory neurons, is an integrator of a number of noxious stimuli and is essential for thermal hyperalgesia (8, 9). TRPV1 is activated by noxious heat (>42°C), capsaicin, endocannabinoids, and protons. Its activation results in membrane depolarization and excitation of sensory neurons. For a number of different mediators and hormones that act on G protein–coupled receptors such as prostaglandins (10), bradykinin (11), and ATP (12), as well as for nerve growth factor acting *via* TrkA (13), sensitization of TRPV1 was observed. The mechanisms involved in the potentiation of TRPV1 responses involve phosphorylation of serine and threonine residues by protein kinases (PKs), such as PKA and/or PKC (14, 15). In addition, hydrolysis of phospha-

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[1] To whom correspondence should be addressed at Institut für Pharmakologie, Charité–Universitätsmedizin Berlin, Campus Benjamin-Franklin, Thielallee 67-73, 14195 Berlin-Dahlem. E-mail: alexander.oksche@charite.de

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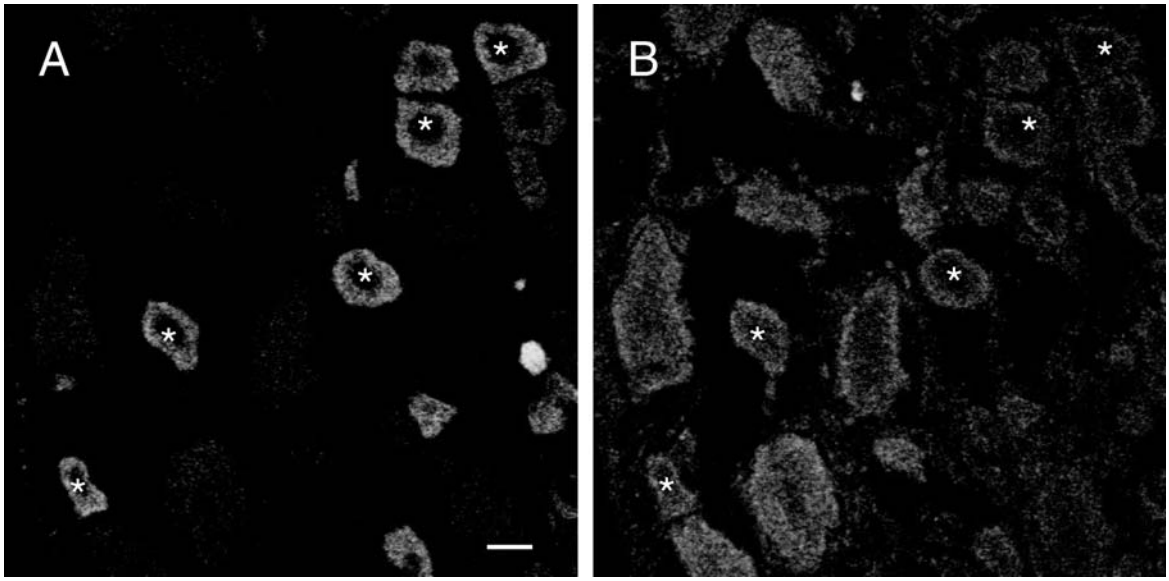

Figure 1. Demonstration of TRPV1 and ET_A receptor expression in rat lumbar DRG by immunohistochemistry. Shown is a representative image of a double-labeled section of rat lumbar DRG, using specific antibodies for TRPV1 and ET_A receptors. (A) TRPV1 immunoreactivity in rat lumbar DRG. TRPV1 is found in the plasma membrane of small-diameter sensory neurons but is also observed in the cell's interior. (B) ET_A receptor immunoreactivity in rat lumbar DRG. ET_A receptors are predominantly found in the plasma membrane of small-, medium-, and large-diameter neurons. *, sensory neurons that show clear co-expression of TRPV1 and the ET_A receptor. Bar, 20 μm.

tidylinositolbisphosphate was shown to disinhibit TRPV1 directly (11).

In this study, we investigated the expression of TRPV1 and ET_A receptors in dorsal root ganglion neurons and analyzed the effects of ET-1 on TRPV1-mediated responses to capsaicin in HEK293 cells co-expressing the ET_A receptor and TRPV1 using the patch clamp technique.

Materials and Methods

Isolation of DRG and Immunohistochemistry. Isolation of DRG and immunohistochemistry was performed as described recently (15). Tissue of isolated DRG was embedded in Tissue-Tek compound (OCT; Miles, Inc., Elkhart, IN) and frozen. Consecutive sections (9 μm) were prepared with a cryostat and mounted onto gelatin-coated slides.

To prevent nonspecific binding, the sections were incubated for 60 mins in phosphate-buffered saline (PBS) containing 0.3% Triton X-100, 1% bovine serum albumin, 4% goat serum, and 4% horse serum (block solution). The sections were then incubated overnight at 4°C with a guinea pig polyclonal antibody against TRPV1 (1:1000; Chemicon, Temecula, CA) in combination with a rabbit polyclonal antibody against the ET_A receptor (1:100). The ET_A receptor antibody was generated against a synthetic peptide corresponding to amino acids 402–427 of the ET_A receptor's C terminus (Swiss-Prot entry P25101; TSIQWKNHDQNNHNTDRSSHKDSMN). The specificity of the ET_A receptor peptide antibody was verified by Western blot analysis of HEK cells transiently expressing ET_A·GFP fusion proteins with and without peptide competition employing the peptide used for immunization. The tissue sections were washed with PBS and then incubated with Texas red-conjugated goat anti-rabbit antibody and fluorescein isothiocyanate (FITC)-conjugated donkey anti-guinea pig antibody. Thereafter, sections were washed with PBS, mounted in vectashield (Vector Laboratories, Burlingame, CA), and viewed with a Zeiss 510 laser scanning microscope (Zeiss, Oberkochen, Germany).

Cell Culture and Transient Transfection. HEK293 cells were cultured in MEM-Earle medium (Biochrom, Berlin, Germany), supplemented with 10% (v/v) fetal calf serum (Biochrom) and 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cells were plated onto glass cover slips 24–48 hrs before transfection. The cells were transiently transfected with 1 μg of the plasmid pTRPV1-YFP (kindly provided by Dr. Michael Schaefer, Berlin, Germany) (16) and 2 μg of the plasmid pET_Amyc (17) using 6 μl of FuGENE 6 Transfection Reagent (Roche Diagnostics, Mannheim, Germany) in 94 μl of OptiMEM medium (Invitrogen, Karlsruhe, Germany) per 85-mm dish. Electrophysiological experiments were performed 24–48 hrs after transfection.

Patch Clamp Recordings. Recordings of whole-cell currents from single cells were made with an EPC-7 amplifier using Pulse software (HEKA, Lambrecht, Germany), as described previously (18). Experiments were performed using the standard whole-cell mode of the patch clamp technique. Cells were held at a potential of –60 mV and the current recorded continuously using XChart (HEKA). Ramps from –100 to +100 mV, with a duration of 400 msecs, were applied at a frequency of 0.2 Hz.

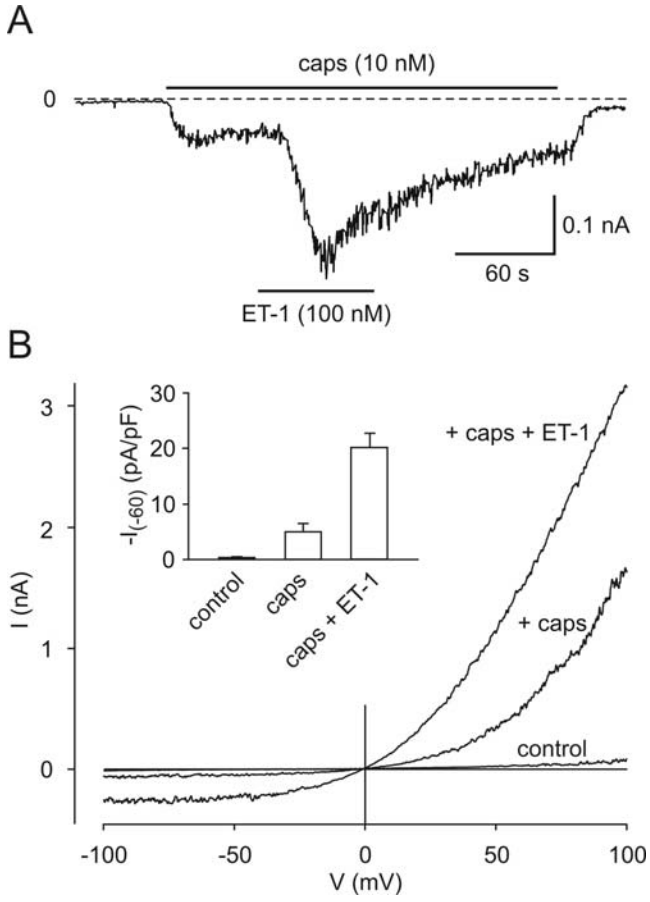

Figure 2. Potentiation of capsaicin-activated TRPV1 currents by ET-1. (A) Current at –60 mV recorded from a HEK293 cell transfected with TRPV1-YFP and the myc-tagged ET_A receptor. Application of capsaicin (caps, 10 n*M*) activated a current through TRPV1 that was strongly potentiated by the addition of ET-1 (100 n*M*). (B) Current voltage relations recorded during potential ramps from –100 to +100 mV before the addition of capsaicin (control), during the application of capsaicin (caps), and at the peak of the response to application of capsaicin and ET-1 (caps + ET-1). The inset shows the mean peak current densities (±SEM, $n = 4$) at –60 mV in the control, in capsaicin, and in capsaicin + ET-1. Currents were recorded in a standard extracellular solution containing 2 m*M* Ca^{2+}.

Currents during ramps were acquired at a frequency of 4 kHz after filtering at 1 kHz.

The standard pipette solution contained 100 m*M* CH_3O_3SCs (cesium methane sulfonate), 25 m*M* CsCl, 3 m*M* $MgCl_2$, 2 m*M* Na_2ATP, 3.62 m*M* $CaCl_2$, 10 m*M* EGTA, and 30 m*M* HEPES (pH 7.2 with CsOH). Pipette tips were filled with the same solution without ATP to avoid the activation of purinergic receptors before seal formation. The standard extracellular solution contained 140 m*M* NaCl, 5 m*M* CsCl, 2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 10 m*M* glucose, and 10 m*M* HEPES (pH 7.4 with NaOH). Experiments were performed at room temperature, 20°–25°C.

Results and Discussion

Using immunohistochemistry to look at rat lumbar DRG with TRPV1- and ET_A receptor–specific antibodies in a double-staining analysis, immunoreactivity of the TRPV1-specific antibody was exclusively found in small- to medium-diameter neurons (Fig. 1A). TRPV1 immunoreactivity was found in the plasma membrane and also in the cell interior. For the ET_A receptor antibody, immunoreactivity was observed in small- to medium-diameter neurons, but also in larger neurons (Fig. 1B). It is of note that a significant proportion of small- to medium-diameter neurons revealed both immunoreactivity for ET_A receptors and for TRPV1 (asterisks, Fig. 1), indicating co-expression of the two proteins.

After demonstrating that a subset of sensory neurons in the DRG co-expresses TRPV1 and ET_A receptors, we studied the regulation of TRPV1 by ET-1. To investigate whether activation of ET_A receptors by ET-1 modulates TRPV1 activity, we used HEK293 cells transiently expressing a fusion protein of TRPV1 and the yellow fluorescent protein (TRPV1-YFP) and the ET_A receptor. The YFP moiety at the very C terminus of TRPV1 did not alter the functional properties of the nonselective cation channel, but it allowed for easy identification of HEK293 cells expressing TRPV1 by fluorescence microscopy. In a whole-cell patch clamp analysis of single cells, 10 n*M* capsaicin activated currents that displayed the characteristic outwardly rectifying IV-relation of TRPV1 (Fig. 2). Addition of ET-1 in the presence of capsaicin for 1 min resulted in a marked potentiation of currents through TRPV1 (Fig. 2). Currents recorded during combined capsaicin (10 n*M*) and ET-1 (100 n*M*) application were, on average, 6-fold larger (6.1 ± 1.8, $n = 4$) than those activated during the initial capsaicin response.

Previous studies have postulated that part of the effects of ET-1 on nociception occur *via* modulation of TTX-resistant Na^+ channels (7). ET-1 shifts the potential dependence of Na^+ channel activation to more negative membrane potentials, and this could result in enhanced excitability of the sensory neurons. We show here for the first time at the cellular level that ET-1 is also a potent modulator of TRPV1 in HEK293 cells transiently co-expressing the ET_A receptor and TRPV1. Furthermore, since TRPV1 and ET_A receptor are co-expressed in DRG neurons, modulation of TRPV1 activity by ET-1 is likely to be involved in ET-1–elicited painful states. Our data are in line with those of a previous poster presentation on slice preparations from newborn rat DRG, demonstrating an ET-1–mediated potentiation of capsaicin-evoked currents (19). Thus, ET-1 contributes to nociception by both sensitization of TTX-resistant Na^+ channels and potentiation of TRPV1 currents.

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