Modulation of TRPV1 by nonreceptor tyrosine kinase, c-Src kinase

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Jin, Xiaochun, Nemat Morsy, John Winston, Pankaj J. Pasricha, Kennon Garrett, and Hamid I. Akbarali. Modulation of TRPV1 by nonreceptor tyrosine kinase, c-Src kinase. Am J Physiol Cell Physiol 287: C558–C563, 2004. First published April 14, 2004; 10.1152/ajpcell.00113.2004.—The capsaicin receptor TRPV1 is a nonselective cation channel that is expressed in sensory neurons. In this study, we examined the role of the nonreceptor cellular tyrosine kinase c-Src kinase in the modulation of the rat TRPV1. Capsaicin-induced currents in identified colonic dorsal root ganglion neurons were blocked by the c-Src kinase inhibitor PP2 and enhanced by the tyrosine phosphatase inhibitor sodium orthovanadate. PP2 also abolished currents in human embryonic kidney-293 cells transfected with rat TRPV1, whereas cotransfection of TRPV1 with v-Src resulted in fivefold increase in capsaicin-induced currents. In cells transfected with dominant-negative c-Src and TRPV1, capsaicin-induced currents were decreased by approximately fourfold. TRPV1 co-immunoprecipitated with Src kinase and was tyrosine phosphorylated. These studies demonstrate that TRPV1 is a potential target for cellular tyrosine kinase-dependent phosphorylation.

capsaicin; pp60, cation channel; immunoprecipitation; inflammatory bowel disease; pain

THE VANILLOID RECEPTOR, VR1 or TRPV1, is a nonselective cation channel with homology to the transient receptor potential (TRP) family of ion channels. Expression of the TRPV1 channel in nociceptors, and its polymodal nature of activation by heat and protons, define the physiological role of this channel in pain sensation. In addition to the direct activation by capsaicin, which was originally used to characterize and clone the TRPV1 channel, both G-protein-coupled receptors (e.g., bradykinin receptor) and tyrosine kinase receptors (e.g., receptor for nerve growth factor) indirectly modulate the kinetics of the channel through pathways involving release of phosphatidylinositol 4,5-bisphosphate (PIP2)-mediated inhibition (5).

Phosphorylation/dephosphorylation of ion channels is a major mechanism by which channel function is regulated, under both basal conditions, as well as in response to various ligands. Both protein kinases C and A have been demonstrated to regulate TRPV1 currents (1, 4, 8, 10, 14, 16, 18, 22, 31). The potential phosphorylation sites for protein kinase Cε were recently identified as Ser602 and Ser800 on TRPV1 (14). Protein kinase A phosphorylates Ser116 of TRPV1 under basal conditions, and dephosphorylation appears to be a primary mechanism that initiates desensitization of the channel (4). There is now accumulating evidence that cellular tyrosine kinases may also play a potential role in the regulation of ion channel function. For instance, our laboratory (9, 11) and others (3, 13, 20, 25, 26, 28) have previously defined a significant role for the cellular tyrosine kinase c-Src kinase in regulation of the L-type calcium channel, both under basal conditions and in response to G-protein-coupled receptors and tyrosine kinase receptors. Src-kinase-mediated regulation of store-operated channels, presumably due to TRP channels, has also been demonstrated in fibroblasts (2, 27). A direct role for Src-mediated regulation of TRPV4, a close homolog of TRPV1, was recently identified by Xu et al. (29). Site-directed mutagenesis defined Tyr253 as the phosphorylation site. However, Vriens et al. (23) were unable to demonstrate a similar regulation by tyrosine kinase in TRPV4. There are several potential tyrosine phosphorylation sites in TRPV1, as determined from the NetPhos server (www.cbs.dtu.dk/services/NetPhos). In the present study, we examined the role of c-Src kinase in the regulation of TRPV1 currents.

MATERIALS AND METHODS

All animal protocols were approved by the Institutional Animal Use and Care Committee of the University of Oklahoma Health Science Center.

Labeling of colonic dorsal root ganglion nociceptors. Adult male Sprague-Dawley rats (250–300 g) were anesthetized with pentobarbital sodium (60 mg/kg). The descending colon was exposed by a midline laparotomy, and dicarbocyanine dye, 1,1'-dioleyl-3,3',3'-tetramethylindocarbocyanine methanesulfonate (Di; 20 mg in 1 ml methanol; Molecular Probes, Eugene, OR), was injected into the colon wall with the use of a Hamilton syringe. The injections were performed in ~10 sites. Any leak of the Di was immediately wiped away with cotton swabs. The surgical wound was sutured in layers, and the rats were allowed to recover for 1–2 wk to permit Di to be transported to the cell soma of the sensory neurons. Di-labeled neurons were easily identified under epifluorescence illumination with a Texas-red/rhodamine filter set.

Cell dissociation and culture. Rats were killed by euthanasia with pentobarbital sodium. Dorsal root ganglia (DRG) were dissected bilaterally from T11 to S2 and transferred into ice-cold F-12 culture medium (Invitrogen, Carlsbad, CA). Connective tissue was dissected away under a dissecting microscope, and the ganglia were placed in F-12 medium containing 2.4 mg/ml collagenase type II (Worthington) for 1.5 h at 37°C. After the initial enzyme treatment, the tissue was rinsed twice in PBS and then incubated for 15 min with trypsin (1 mg/ml). The enzymatic reactions were terminated by replacing the media with DMEM (containing 10% fetal bovine serum) and trypsin inhibitor (1 mg/ml). Single cells were obtained by gently triturating the tissues in the presence of DNase (0.05 mg/ml). The tissue fragments were centrifuged at 700 rpm for 3 min, and the pellet was resuspended in DMEM culture media (Invitrogen). Cells were plated onto poly-l-lysine-coated culture plates and maintained at 37°C in 5%
CO₂ incubator. Electrophysiological recordings were carried out within 24 h of isolation.

**Electrical recordings.** Membrane currents were measured in a standard whole cell voltage-clamp configuration by using the Axopatch 200A amplifier. The data were low-pass filtered at 1 kHz and sampled at a 10- to 100-μs rate. The pipettes were prepared on a Flaming-Brown horizontal puller (P-87; Sutter Instruments) and fire polished. The resistance of the pipettes was ~5 MΩ when filled with pipette solution. The pipette solution contained (in mM) 135 NaCl, 5.4 KCl, 0.33 GTP, 1 MgCl₂, 2 CaCl₂, and 5.5 glucose. The pH was adjusted to 7.4 with NaOH. The solutions were equilibrated with NaH₂PO₄, 5 HEPES, 1 MgCl₂, 2 CaCl₂, and 5.5 glucose. The pH was adjusted to 7.4 with NaOH. The solutions were equilibrated with 100% O₂ during the perfusion. All patch-clamp experiments were performed at room temperature (22°C).

**Transient transfection.** Human embryonic kidney (HEK-293) cells were cultured in DMEM with 10% FBS and transiently transfected by using Lipofectamine (Invitrogen) according to the manufacturer’s protocols. Cells were either transfected with cDNA for TRPV1 alone or cotransfected in a 4:1 ratio of TRPV1 and v-Src plasmid DNA or TRPV1 and dominant-negative mutant c-Src plasmid DNA (K296R/Y528F) (rat TRPV1 cDNA subcloned into pcDNA3) was kindly provided by Dr. David Julius, University of California; plasmid cDNA for v-Src by Dr. Leo Tsikas, University of Oklahoma Health Science Center; dominant-negative Src cDNA was obtained from Upstate Biotechnology, Lake Placid, NY). Transfections were performed in media without serum. After 6–12 h, cells were detached and replated on a new culture dish with medium-containing serum. To identify transfected cells, an enhanced green fluorescence protein receptor plasmid was also transfected at 1/12 the concentration of total cDNA. Transfected cells were used for either electrophysiological recordings or immunoprecipitation and Western blot within 72 h after transfection.

**Immunoprecipitation and Western blotting.** Protein extracts were prepared by treating cells in 1–1.5 ml of ice-cold lysis buffer containing a cocktail of proteinase and phosphatase inhibitors with the following composition: 20 mM Tris (pH 7.5), 1% (vol/vol) Triton X-100, 1 mM EGTA, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 5 μM each of leupeptin, pepstatin A, and aprotinin. Cell lysates were mixed and vortexed every 15 min for 1 h in ice and centrifuged at 14,000 rpm for 30 min at 4°C, and the supernatants were collected for either immunoprecipitation or Western blot. Protein concentrations were determined by means of the bicinchoninic acid assay. For immunoprecipitation, 4–5 μg of anti-Src antibody GD11 (Upstate Biotechnology, Lake Placid, NY), anti-phospho-Src antibody Tyr416 (Upstate Biotechnology), or anti-phosphotyrosine antibody PY20 (BD Transduction Laboratories) were first incubated with washed protein G agarose beads for 1 h at 4°C. After three washes, beads were incubated with cell lysate (200–500 μg) for 1 h at 4°C. The beads were collected, washed, and resuspended in 2× Laemml sample buffer containing 0.5% β-mercaptoethanol and boiled for 5 min. Protein samples of supernatant fraction were used in Western blot. Proteins were separated on a SDS-PAGE 8% gel and transferred to polyvinylidene difluoride filters. The blots were blocked with 5% milk for 1 h and incubated with anti-Src GD11 (1:1,000), anti-pTyr416 (1:1,000), or anti-TRPV1 (1:2,000; Santa Cruz Biotechnology) for 1 h at room temperature. The blots were then incubated with secondary antibody (1:3,000) for 1 h at RT, developed in enhanced chemiluminescence solution (NEN) for 1 min, and exposed onto X-ray films for 2–30 min.

**RESULTS**

The average capacitance of Dil-labeled colonic DRG neurons was 28.4 ± 2.1 pF (n = 16). Capsaicin (10 μM) induced inward currents from a holding potential of ~50 mV, with the peak currents averaging ~41.5 ± 6.0 pA/pF (n = 18) (Fig. 1A). When labeled colonic neurons were pretreated with the c-Src kinase inhibitor PP2 (10 μM) (7) for 3 min, capsaicin-induced currents were completely abolished in 13 of 20 cells each of control and cell dialyzed with the tyrosine phosphatase inhibitor sodium orthovanadate (bottom). Cells were held at ~50 mV. B: current traces in presence of c-Src kinase inhibitor PP2 (10 μM). Cells were perfused with PP2 for 3 min before addition of capsaicin. PP2 completely abolished capsaicin-induced currents in some DRG cells (a), but not in others (b). C: normalized peak amplitude of capsaicin-induced currents in control, in the presence of sodium orthovanadate, or pretreated with PP2. Values are means ± SE; n, no. of cells. **P ≤ 0.01. D: Western blot of TRPV1 and Src protein from native DRG. IB, immunoblot; VR1, vanilloid receptor 1.
Fig. 2. Capsaicin response in transfected human embryonic kidney (HEK-293) cells from a holding potential of ~70 mV. A: raw traces obtained in cells either transfected with TRPV1 alone (top) or cotransfected with v-Src (bottom). B: bar graph showing peak amplitude normalized to cell capacitance for capsaicin-induced currents in cells transfected with TRPV1, TRPV1 pretreated with PP2 (1 μM), cotransfected with TRPV1 plus v-Src, and TRPV1 plus dominant-negative c-Src. Values are means ± SE; n, no. of cells. **p ≤ 0.01. C: Western blot of TRPV1 protein from TRPV1 alone or TRPV1 + v-Src-transfected cells, demonstrating equal protein expression in both groups. Three different protein amounts (10, 20, 30 μg) were examined. UT, untransfected cells.

(Fig. 1B, a), whereas in 7 other cells capsaicin-induced currents averaged ~41.96 ± 6.0 pA/pF, similar to control (Fig. 1B, b). The mean current in all PP2-treated cells was ~7.98 ± 7.3 pA/pF (n = 20) (Fig. 1C). To further elucidate whether the effects of PP2 were due to inhibition of basal tyrosine kinase activity, we examined the effect of a tyrosine phosphatase inhibitor, sodium orthovanadate, on capsaicin-induced currents. When cells were dialyzed with sodium orthovanadate (1 μM) through the patch pipette, mean capsaicin-induced current increased to ~64.4 ± 6.9 pA/pF in all cells examined (n = 8) (Fig. 1, A and C). These data indicate that capsaicin-induced currents in DRG neurons may be regulated by Src kinase. We further confirmed the presence of endogenous Src kinase and TRPV1 in DRG neurons by Western blots. Fig. 1D shows expression of both TRPV1 and c-Src kinase in DRG neurons.

We next examined the ability of c-Src kinase to modulate TRPV1 currents in HEK-293 cells transfected with cDNA from rat TRPV1. In TRPV1 transfected cells, capsaicin-induced currents measured ~122.2 ± 15.5 pA/pF from a holding potential of ~70 mV (n = 12). Prior exposure to PP2 (1 μM) for 3 min inhibited capsaicin response by almost 90% (Fig. 2B), suggesting that an endogenous kinase likely regulates TRPV1 currents. In cells cotransfected with activated Src (v-Src) cDNA and TRPV1, the response to capsaicin was significantly enhanced with peak amplitudes measuring ~537.4 ± 75.6 pA/pF (n = 9, holding potential = ~70 mV) (Fig. 2A). This was not due to increased expression of TRPV1, because Western blots demonstrated equal expression of the channel in both TRPV1 and TRPV1 plus v-Src-transfected cells (Fig. 2C). When cells were cotransfected with the dominant-negative Src cDNA and TRPV1, the response to capsaicin was significantly decreased to ~30.1 ± 15.1 pA/pF (n = 16, holding potential = ~70 mV) (Fig. 2B).

To determine whether the Src-enhanced responses to capsaicin are PKC dependent, TRPV1 plus v-Src-transfected cells were pretreated with 1 μM BIM I, a selective PKC inhibitor, for 20 min. In the presence of BIM I, capsaicin-induced currents measured ~402.03 ± 53.92 pA/pF (n = 5), which was not significantly different from that of untreated TRPV1/v-Src-transfected HEK-293 cells (~58.57 pA/pF; n = 4) (Fig. 3).

Immunoblot with anti-TRPV1 antibody showed a ~95-kDa band in cell lysates from transfected, but not untransfected, cells (Fig. 4A). Immunoblotting with the anti-c-Src antibody (GD11) detected a 60-kDa band in untransfected cells, suggesting the presence of endogenous c-Src kinase in HEK-293 cells. The intensity of this band was significantly enhanced in cells cotransfected with v-Src (Fig. 4A).

The association of Src kinase with the TRPV1 channel in native DRG and TRPV1/v-Src-cotransfected HEK-293 cells was examined by immunoprecipitating with anti-Src antibo-
ies followed by immunoblot with anti-TRPV1 antibody. Two different Src antibodies were used for immunoprecipitation. Because Src is known to be phosphorylated in the 416Y position when activated, we also determined the association of TRPV1 with an anti-phospho-416Y Src antibody. Figure 4B shows that immunoprecipitation with either Src antibodies followed by immunoblot with anti-TRPV1 resulting in a distinct band corresponding to the TRPV1 protein. To further confirm that TRPV1 is tyrosine phosphorylated, TRPV1 and TRPV1 plus v-Src-transfected cells were subject to immunoprecipitation with an anti-phospho-tyrosine antibody. Immunoblot with anti-VR1 revealed enhanced tyrosine phosphorylation in TRPV1 plus v-Src-transfected cells (Fig. 4C).

**DISCUSSION**

In this study, we show that 1) the c-Src kinase inhibitor PP2 specifically abolishes capsaicin-induced currents both in identified colonic DRG neurons and in cells transfected with TRPV1 channel; 2) in colonic DRG neurons, the tyrosine phosphatase inhibitor sodium orthovanadate enhances capsaicin-induced currents; 3) in HEK-293 cells, cotransfection of TRPV1 with an activated Src kinase, v-Src, results in an almost fivefold increase in the peak amplitude of capsaicin-induced currents, whereas cotransfection with the dominant-negative c-Src kinase resulted in an almost fourfold decrease; and 4) biochemical evidence demonstrated the association of active Src kinase with the TRPV1 channel in rat DRG neurons and in transfected cells. We further show that TRPV1 is tyrosine phosphorylated. Collectively, these data strongly suggest a significant role for Src kinase(s) in the regulation of TRPV1 currents.

It has been previously shown that not all labeled colonic DRG neurons respond to capsaicin. Su et al. (21) found that almost 50% of labeled colonic DRG from the S1 segment were unresponsive to capsaicin. It could, therefore, be argued that the inhibition of the capsaicin response in PP2-treated cells may represent unresponsive neurons. However, our findings that the tyrosine phosphatase inhibitor enhances TRPV1 currents and PP2 induces marked inhibition of TRPV1 transfected cells strongly suggest a direct role of Src kinase(s) in the regulation of TRPV1. Interestingly, not all DRG neurons were inhibited by PP2. Almost one-third of the cells pretreated with PP2 produced currents in response to capsaicin to the same extent as controls. This may reflect possible subgroups of DRG neurons, as demonstrated by Petruska et al. (17), or PP2 concentration may have been insufficient to block the capsaicin response in these cells. In contrast to the DRG, in all transfected HEK cells, PP2 almost completely abolished capsaicin-induced responses.

TRPV1 belongs to the family of TRP channels and shares homology to the osmosensing TRPV4 receptor. Although Xu et al. (29) found that TRPV4 is directly regulated by the family of Src kinases, others have been unable to demonstrate that mutation of Tyr253 of TRPV4 results in inhibition of the channel (23). Whereas Tyr253 is conserved across TRPV4 in all species, it is distinctly absent in the rat TRPV1. Evaluation of possible tyrosine phosphorylation sites by using the NetPhos phosphorylation prediction server shows several other tyrosine residues that are potential phosphorylation sites. Of particular significance is the role of Y511 at the cytoplasmic loop, linking TM2 and TM3 of the TRPV1. This tyrosine residue appears to be required for the binding of capsaicin, and mutation leads to inhibition of capsaicin-induced currents (12, 15). Our data indicate that phosphorylation of the tyrosine residue may be an important component in the activation of the current by capsaicin. A role for tyrosine kinase(s) in the activation of TRPV1 has been previously suggested by Obreja et al. (15), who showed that genistein, a nonspecific tyrosine kinase inhibitor, blocked interleukin 1β-induced enhancement of heat-activated currents in DRG. This effect was, however, identified as upstream of protein kinase C. We have found that, in TRPV1/v-Src-cotransfected cells, the PKC inhibitor BIM I did not block capsaicin-induced currents, suggesting a direct tyrosine kinase modulation of TRPV1. Consistent with this were the
findings that TRPV1 was tyrosine phosphorylated, as shown by direct immunoprecipitation with anti-phospho-tyrosine antibody. Interestingly, mutation of Y511 does not affect the heat-induced responses (12, 15), suggesting that heat-induced gating differs from that of capsaicin.

Receptor-mediated regulation of the TRPV1 is dependent on PIP$_2$ hydrolysis after phospholipase C activation. PIP$_2$ binding is associated with the COOH terminus (19). Several G-protein-coupled receptors and tyrosine kinase receptors also activate c-Src. Although our data demonstrate potential regulation of capsaicin-induced responses by c-Src, further studies are required to determine whether this pathway is utilized by G-protein or tyrosine kinase receptors.

Regulation of ion channels by tyrosine kinases has been demonstrated for both voltage-gated and ligand-gated channels, including Ca$^{2+}$ channels, K$^+$ channels, and Na$^+$ channels. The fact that capsaicin-induced currents were enhanced by a tyrosine phosphatase inhibitor and abolished by the c-Src kinase-specific inhibitor points toward a basal regulation of the TRPV1 channel by cellular tyrosine kinases. This resembles the regulation of voltage-dependent L-type Ca$^{2+}$ channels, which are blocked by the tyrosine kinase inhibitors, enhanced by tyrosine phosphatase inhibitor, and directly associated with c-Src kinase (6, 9).

Abdominal pain is a common phenomenon in inflammatory bowel diseases such as ulcerative colitis and Crohn’s disease. Su et al. (21) have previously identified capsaicin-sensitive currents in the sensory afferents of the colon, and Yiangou et al. (30) recently demonstrated an increase in the TRPV1 immunoreactivity within the submucosal plexus of patients with ulcerative colitis. Changes in ion channel activity due to transcriptional regulation (24) or those involving altered signaling are known to occur in response to various insults. Our findings that c-Src kinase potentiates TRPV1 currents in colonic DRG neurons provides a novel therapeutic target in the treatment of visceral hypersensitivity.

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GRANTS

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