Direct Phosphorylation of Capsaicin Receptor VR1 by Protein Kinase Cε and Identification of Two Target Serine Residues*

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Mitsuko Numazaki‡§, Tomoko Tominaga‡¶,
Hidenori Toyooka‡, and Makoto Tominaga‡

From the ‡Department of Physiology, Mie University School of Medicine, Edobashi 2-174, Tsu, Mie 514-8507, Japan, the §Department of Anesthesiology, University of Tsukuba School of Medicine, Tsukuba 305-0006, Japan, and the ¶Foundation for Advancement of International Science, Tsukuba 305-0062, Japan

The capsaicin receptor, VR1, is a sensory neuron-specific ion channel that serves as a polymodal detector of pain-producing chemical and physical stimuli. It has been reported that ATP, one of the inflammatory mediators, potentiates the VR1 currents evoked by capsaicin or protons and reduces the temperature threshold for activation of VR1 through metabotropic P2Y2 receptors in a protein Kinase C (PKC)-dependent pathway, suggesting the phosphorylation of VR1 by PKC. In this study, direct phosphorylation of VR1 upon application of phorbol 12-myristate 13-acetate (PMA) was proven biochemically in cells expressing VR1. An in vitro kinase assay using glutathione S-transferase fusion proteins with cytoplasmic segments of VR1 showed that both the first intracellular loop and carboxyl terminus of VR1 were phosphorylated by PKCε. Patch clamp analysis of the point mutants where Ser or Thr residues were replaced with Ala in the total 16 putative phosphorylation sites showed that two Ser residues, Ser502 and Ser800, were involved in the phosphorylation of both PMA or ATP. In the cells expressing S502A/S800A double mutant, the temperature threshold for activation was not reduced upon PMA treatment. The two sites would be promising targets for the development of substance modulating VR1 function, thereby reducing pain.

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† To whom correspondence should be addressed: Dept. of Physiology, Mie University School of Medicine, Edobashi 2-174, Tsu, Mie 514-8507, Japan. Tel./Fax: 81-59-231-5004; E-mail: tominaga@doc.medic.mie-u.ac.jp.

EXPERIMENTAL PROCEDURES

In Vivo Phosphorylation—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (supplemented with 10% fetal bovine serum, penicillin, streptomycin, and l-glutamine) and plated at 60–70% confluence in 100-mm dishes, then transfected with 1 μg of rat VR1 plasmid DNA using LipofectAMINE plus reagent (Invitrogen) as described previously (6). In vivo phosphorylation was confirmed as described previously (15). In brief, after transfection the cells were serum-deprived for 36 h in serum-free medium and then labeled with [32P]orthophosphate (300 μCi/ml) for 3 h at 37 °C. Following PMA (Sigma) stimulation (50 ng/ml) for 10 min at 37 °C, the cells were washed with ice-cold phosphate-buffered saline and resuspended in TNE buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, complete EDTA-free protease inhibitor mixture (Roche Molecular Biochemicals),
phosphatase inhibitor mixture (Sigma). Samples were centrifuged for 15 min at 100,000 × g. The pellets were resuspended in TNE buffer with 1% Nonidet P-40 and sonicated for 30 s. Following centrifugation at 100,000 × g for 30 min, the supernatants were pre-cleared with protein A and then incubated at 4 °C for 3 h with 1 μg of rabbit anti-rat VR1 antibodies. Anti-rabbit IgG was added and incubated at 4 °C for 1 h. Immunoprecipitated proteins were boiled in SDS sample buffer and separated by SDS-PAGE (8% polyacrylamide). The gel was exposed for autoradiography.

Anti-rat VR1 antibody was made as follows. A peptide encoding the predicted carboxyl terminus of VR1 (EDAVEFKDSMVPMGERK) was coupled to keyhole limpet hemocyanin via an amino-terminal cysteine and used to immunize rabbits.

Bacterial Expression of Glutathione S-Transferase (GST)-VR1 Fusion Proteins—Fusion proteins consisting of GST at the amino terminus in-frame with amino (NH2)-terminal, the first intracellular loop and carboxyl (COOH)-terminal were generated by PCR and standard cloning techniques as described previously (16). The PCR products were subcloned into the pcEX vector (Amersham Biosciences). The final constructs were verified by sequencing. GST-VR1 protein was purified according to the manufacturer’s manuscript (Amersham Biosciences).

In Vitro Kinase Assays—For the in vitro kinase assays, each purified fusion protein was incubated with the following reagents: 20 mM Tris-HCl, 0.1% Triton-X, 100 mM MgCl2, 200 μg/ml phosphatidylinerine, 10 μM PMA, 5 mM KCl, 2 mM MgCl2, 5 mM EGTA, 10 mM EDTA, 10 mM glucose, pH 7.4 (adjusted with NaOH). Pipette solution contained 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, 5 mM EGTA, 10 mM HEPES, 10 mM glucose, pH 7.4 (adjusted with KOH). Whole-cell patch clamp recordings were carried out at one or 2 days after transfection of VR1 cDNA to HEK293 cells as described previously (6). Standard bath solution contained 140 mM NaCl, 2 mM MgCl2, 5 mM EGTA, 10 mM HEPES, 10 mM glucose, pH 7.4 (adjusted with KOH). All patch clamp experiments were performed at room temperature (22 °C) unless otherwise noted. When examining the heat-evoked current responses, bath temperature was increased using a preheated solution with the rate of 1–1.5 °C/s. When the heat-activated currents started to inactivate, the heat solution was changed to a 22 °C one. Chamber temperature was monitored (accuracy ±0.1 °C) with a thermocouple placed within 4 mm of the patch-clamped cell. The solutions containing drugs were applied to the chamber (180 μl) by a gravity at a flow rate of 5 ml/min.

RESULTS AND DISCUSSION

To confirm the in vivo phosphorylation of VR1 by PKC, we used HEK293 cells expressing VR1 heterologously. Activation of PKC was achieved by incubating the transfected cells for 10 min with 50 ng/ml PMA, a potent and specific PKC activator. Following the treatment with [γ-32P]ATP, the cells were stimulated with PMA. VR1 protein immunoprecipitated with anti-rat VR1 antibody showed more 32P incorporation into VR1 upon PMA stimulation compared with the VR1 without PMA stimulation (Fig. 1A), indicating the direct phosphorylation of VR1 by PKC. There are 16 putative Ser or Thr residues that are candidate substrates for PKC-dependent phosphorylation in the VR1 NH2 terminus, first intracellular loop, and COOH terminus (Fig. 1B). To distinguish among these possibilities, recombinant proteins carrying GST fused to the three segments of the cytoplasmic domains of VR1 were generated for use in an in vitro kinase assay. This assay demonstrated that the first intracellular loop and the COOH-terminal contained the substrates for PKC (Fig. 1C, panel b).

To identify the specific VR1 amino acids involved, eight Ser or Thr residues in the first intracellular loop and the COOH-terminal were individually replaced with Ala, and the resulting mutant proteins were subjected to functional analysis using a whole-cell patch clamp technique. In voltage clamp experiments, a low dose of capsaicin (20 nM) evoked small inward currents in the HEK293 cells expressing VR1. In the absence of extracellular calcium, no change was observed in the magnitude of responses evoked by repetitive capsaicin applications. In contrast, after a 1-min pretreatment with 100 nM PMA, the same dose of capsaicin produced a much larger current response (7.95 ± 2.72 (means ± S.E.)-fold, n = 8) (Fig. 2A).

Other electrophysiological properties of these capsaicin-evoked responses were unchanged by the presence of PMA (data not shown). Among the mutants tested, S502A and S800A showed significantly smaller potentiation of capsaicin-evoked current responses by PMA, although normalized currents after treatment of PMA varied in the eight mutants (2.13 ± 0.52-fold, n = 7; 4.58-fold, n = 5 for S502A; 2.76 ± 0.52-fold, n = 11 for S800A) (p < 0.05) (Fig. 2, B, C, and E). In wild type VR1, PKC activation works by increasing the potency of capsaicin but not its efficacy (12). Therefore, we ruled out the possibility that the two mutants, S502A and S800A, already have a high affinity for capsaicin by examining that higher doses of capsaicin produced bigger currents in the two mutants like wild type (data not shown). Patch clamp recordings in mutants bearing Ala substitutions of eight other Ser and Thr residues in the NH2 terminus of VR1 provided no evidence for the involvement of these residues in PMA-mediated potentiation (9.18 ± 4.05-fold, n = 7; 14.7 ± 4.56-fold, n = 9; 10.4 ± 4.26-fold, n = 5; 7.20 ± 1.50-fold, n = 6; 9.85 ± 2.37-fold, n = 5; 7.32 ± 3.77-fold, n = 5; 14.6 ± 5.39-fold, n = 6 and 20.4 ± 7.46-fold, n = 9 for T42A, S93A, S139A, S153A, S185A, T322A, T329A, and S366A, respectively). Furthermore, double mutant S502A/S800A exhibited almost no PMA potentiation effect (0.95 ± 0.04-fold, n = 7) (p < 0.05) (Fig. 2, D and E), suggesting that these two Ser residues were the major substrates for PKC-dependent phosphorylation. Because ATP is a more physiological stimulus.
leading to PKC activation and because PMA has been reported to have some direct effects on VR1 (17), ATP was applied to HEK293 cells expressing wild type and point mutants of VR1. Currents were normalized to the currents evoked by capsaicin (20 nM) before application of ATP, and the normalized values represent the means ± S.E. Normalized currents were 2.61 ± 0.19 (n = 7), 1.05 ± 0.24 (n = 7), 0.63 ± 0.10 (n = 6) and 0.79 ± 0.12 (n = 7) for wild type, S502A, S800A and S502/S800A, respectively. *, p < 0.05 versus wild type; two-tailed unpaired t test.

FIG. 2. Two Ser residues are involved in phosphorylation of VR1 by PMA. A–D, representative traces of the increase of capsaicin (CAP)-activated currents in transfected HEK293 cells expressing wild type (A) and mutants (B–D) of VR1. PMA (100 nM) increased the currents a little in S502A and S800A mutants (B and C), whereas PMA greatly increased the current in wild type VR1 (A). PMA did not increase the current in a S502A/S800A double mutant (D). Currents initially activated by capsaicin (20 nm) were 330 ± 52 pA (means ± S.E.) (30–1600 pA) without any significant change in the wild type and the mutants. Positions Ser502 and Ser800 were shown in Fig. 1B. Cells were perfused for 1 min with solution containing PMA before exposure to capsaicin. Holding potential was −60 mV. E, effects of PMA on the capsaicin-activated currents in HEK293 cells expressing wild type and point mutants of VR1. Currents were normalized to the currents evoked initially by capsaicin (20 nm) before application of PMA, and the normalized values represent the means ± S.E. Normalized currents were 7.95 ± 2.72 (n = 8), 6.36 ± 2.92 (n = 5), 12.8 ± 4.94 (n = 8), 5.58 ± 0.74 (n = 5), 7.1 ± 3.47 (n = 5), 14.6 ± 8.27 (n = 5), 12.6 ± 2.91 (n = 8), 2.13 ± 0.41 (n = 9), 2.76 ± 0.52 (n = 11), and 0.95 ± 0.04 (n = 7) for wild type, T708A, S722A, S776A, S778A, S783A, S800A, S502A, S800A and S502/S800A, respectively. *, p < 0.05 versus wild type; one-way analysis of variance and two-tailed unpaired t test.

FIG. 3. Two Ser residues are involved in phosphorylation of VR1 by ATPs. A and B, representative traces of increase of capsaicin (CAP)-activated currents in transfected HEK293 cells expressing wild type (A) and S502A/S800A mutant (B) of VR1. ATP (100 μM) did not increase the currents in S502A/S800A mutant (B), whereas ATP increased the current in wild type VR1 (A). Cells were perfused for 1 min with solution containing ATP before exposure to capsaicin. Holding potential was −60 mV. C, effects of ATP on the capsaicin-activated currents in HEK293 cells expressing wild type and point mutants of VR1. Currents were normalized to the currents evoked initially by capsaicin (20 nm) before application of ATP, and the normalized values represent the means ± S.E. Normalized currents were 2.61 ± 0.19 (n = 7), 1.05 ± 0.24 (n = 7), 0.63 ± 0.10 (n = 6) and 0.79 ± 0.12 (n = 7) for wild type, S502A, S800A and S502/S800A, respectively. *, p < 0.05 versus wild type; two-tailed unpaired t test.

applied to HEK293 cells expressing wild type VR1, heat-evoked currents developed at about 42 °C with an extremely steep temperature dependence (Fig. 4A). PMA (100 nM) treatment lowered the temperature threshold for wild type VR1 activation significantly (41.9 ± 0.9 °C, n = 3 and 31.8 ± 1.6 °C, n = 4, without and with PMA treatment, respectively, p < 0.01) (Fig. 4, A and C). On the other hand, HEK293 cells expressing S502A/S800A mutant showed a little lower temperature threshold for activation without PMA treatment, although there was no significant difference between wild type and S502A/S800A mutant. However, no reduction of the threshold was observed in the mutant upon PMA treatment (38.0 ± 1.4 °C, n = 7 and 37.4 ± 0.7 °C, n = 9 without and with PMA treatment, respectively, p = 0.7). These data further indicate the involvement of these two Ser residues in VR1 sensitization.

To further confirm that Ser502 and Ser800 function as substrates for PKCe-dependent phosphorylation, an in vitro kinase assay was carried out in those mutants. Phosphorylation was significantly reduced in both S502A and S800A mutants upon PKCe application when the same amount of proteins were loaded (Fig. 1C, panels a and b). Some residual signals in both S502A and S800A mutants might suggest phosphorylation of other amino acids in the fusion proteins by PKCe. However, it is not likely that the phosphorylation in the mutants has significant meaning in terms of potentiation of VR1 currents by PKC, since the double mutant, S502A/S800A, showed no potentiation of VR1 currents evoked by capsaicin upon both PMA and ATP stimuli (Figs. 2 and 3) and no reduction of temperature threshold for activation upon PMA stimulus (Fig. 4).

One of the mechanisms underlying inflammatory pain is sensitization of ion channels expressed in nociceptor terminals such as VR1 (1–3, 5). Sensitization is triggered by extracellular inflammatory mediators, including ATP and bradykinin, re-
leashed from surrounding damaged, inflamed, or ischemic tissues and from nociceptors themselves. Our data and those of others suggest that a system consisting of VR1 and certain metabotropic receptors exists that causes nociceptor sensitization by increasing VR1 sensitivity to noxious stimuli (12, 13, 17, 19). In addition, a series of observations indicate that PKC plays an important role in this system (12–14, 20). PKCs, among many PKC isoforms, has been reported to be predominantly and specifically involved in nociceptor sensitization (21–23). In the present study, direct in vivo phosphorylation of VR1 by PKC was proven for the first time, and PKC was found to phosphorylate two Ser residues. The replacement of these residues with Ala results in blunting of PKC-mediated VR1 phosphorylation and a complete loss of VR1 potentiation or sensitization by ATP or PMA.

Another isoform of PKC, PKCγ, has been shown to be pivotal for enhancing the sensation of pain in the spinal cord dorsal horn neurons (24, 25). Therefore, two different isoforms of PKC, PKCe and PKCγ, exhibit distinct roles at two levels of the pain pathway: the primary afferent neuron and the spinal cord dorsal horn, respectively. Our findings suggest that inhibitors of PKC (especially PKCe) as well as compounds acting at Ser502 or Ser800 of VR1 could prove useful in the treatment of pain by interfering with phosphorylation-mediated sensitization events.

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FIG. 4. Thermal sensitivity is increased by PMA in wild type VR1, but not in S502A/S800A mutant. A, representative temperature-response profiles of heat-activated currents obtained by a temperature ramp in wild type VR1 without (left) and with (right) PMA treatment. Temperature-response profiles were made with only current responses during the heat stimulation. Dashed lines show the threshold temperature for heat activation of VR1. Holding potential was −60 mV. B, representative temperature-response profiles of heat-activated currents in a S502A/S800A mutant without (left) and with (right) PMA treatment. C, temperature threshold for activation of wild type VR1 in the presence of PMA (31.8 ± 1.6 °C, n = 4) was significantly lower than that in the absence of PMA (41.9 ± 0.9 °C, n = 3), *p < 0.05 versus wild type without PMA; #, p < 0.05 versus S502A/S800A mutant without or with PMA; two-tailed unpaired t test. There was no significant difference in temperature threshold for activation of S502A/S800A mutant (38.0 ± 1.4 °C, n = 7, and 37.4 ± 0.7 °C, n = 9, with and without PMA treatment, respectively, p = 0.7; two-tailed unpaired t test). Threshold was defined as a temperature at which clear current increase was observed in the temperature-response profile.
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