



Mutation of a putative *S*-nitrosylation site of TRPV4 protein facilitates the channel activation

Eun Jeoung Lee^b, Sung Hwa Shin^a, Sunghee Hyun^b, Jaesun Chun^c and Sang Sun Kang^{a,d*}

^aDepartment of Biology Education, Chungbuk National University, 410 Seongbong Road, Heungdok-gu, Cheongju, Chungbuk 361-763, Republic of Korea; ^bDepartment of Pre-medicine, Eulji University School of Medicine, Daejeon 301-832, Republic of Korea;

^cDepartment of Biology Education, Korea National University of Education, Chongwon, Chungbuk 363-791, Republic of Korea;

^dBiotechnology Research Institute, Chungbuk National University, 410 Seongbong Road, Heungdok-gu, Cheongju, Chungbuk 361-763, Republic of Korea

(Received 8 September 2010; received in revised form 28 October 2010; accepted 1 November 2010)

The transient receptor potential vanilloid 4 (TRPV4) cation channel, a member of the TRP vanilloid subfamily, is expressed in a broad range of tissues. Nitric oxide (NO) as a gaseous signal mediator shows a variety of important biological effects. In many instances, NO has been shown to exhibit its activities via a protein *S*-nitrosylation mechanism in order to regulate its protein functions. With functional assays via site-directed mutagenesis, we demonstrate herein that NO induces the *S*-nitrosylation of TRPV4 Ca²⁺ channel on the Cys⁸⁵³ residue, and the *S*-nitrosylation of Cys⁸⁵³ reduced its channel sensitivity to 4- α phorbol 12,13-didecanoate and the interaction between TRPV4 and calmodulin. A patch clamp experiment and Ca²⁺ image analysis show that the *S*-nitrosylation of Cys⁸⁵³ modulates the TRPV4 channel as an inhibitor. Thus, our data suggest a novel regulatory mechanism of TRPV4 via NO-mediated *S*-nitrosylation on its Cys⁸⁵³ residue.

Keywords: TRPV4; NO; *S*-nitrosylation; 4- α phorbol 12; 13-didecanoate; Ca²⁺ channel; protein-protein interaction

Introduction

The participation of transient receptor potential vanilloid 4 (TRPV4) in osmo- and mechanotransduction contributes to important functions including cellular and systemic volume homeostasis, arterial dilatation, nociception, epithelial hydroelectrolyte transport, bladder voiding and the regulation of ciliary beat frequency (Nilius et al. 2003; Cohen 2006; Vennekens et al. 2008; Everaerts et al. 2009). TRPV4 also responds to temperature, endogenous arachidonic acid (AA) metabolites, and phorbol esters including the inactive 4- α phorbol 12,13-didecanoate (4- α PDD), and also participates in receptor-operated Ca²⁺ entry, evidencing multiple activation modes (Suzuki et al. 2003a; Andrade et al. 2005; Becker et al. 2005; Earley et al. 2005; Birder et al. 2007). Therefore, the elucidation of important molecular pathways that modulate TRPV4 transport sensitivity has promoted our understanding of its biological functions in the cell (Nilius et al. 2003; Cohen 2006; Vennekens et al. 2008; Everaerts et al. 2009). In this regard, several proteins have been proposed to modulate TRPV4 subcellular localization and/or function: microtubule-associated protein 7, calmodulin (CaM), OS-9, actin, and pacsin3 (Strotmann et al. 2003; Suzuki et al. 2003b; Cuajungco et al. 2006; Fu et al. 2006). It has been reported that fairly intimate functional and physical interactions occur between the inositol trisphosphate

receptor 3 (IP3R3) and TRPV4, which sensitize the latter to the mechano- and osmotransducing messenger 5',6'-epoxyicosatrienoic acid (Nilius et al. 2003; Cohen 2006; Birder et al. 2007; Vennekens et al. 2008; Everaerts et al. 2009). Muramatsu et al. also demonstrated that TRPV4 regulates the SOX9 [SRY (sex-related Y)-type high mobility group box] pathway and contributes to the process of chondrogenesis (Muramatsu et al. 2007)

Until now, only a little information has been available concerning the molecular players responsible for the processes regulating the sensitivity of TRPV4 for Ca²⁺ flux (Watanabe et al. 2002, 2003; Fernandes et al. 2008). In this regard, a number of regulatory posttranslational modifications, including phosphorylation, ubiquitinylation and glycosylation, have recently been implicated in the control of the biophysical, pharmacological and expression properties of ion channels and transporters via direct interactions (Fan et al. 2009; Wegierski et al. 2009).

Nitric oxide (NO) is a pleiotropic cell signaling molecule which controls a variety of biological processes (Olson and Garbán 2008; Robertson and Sillar 2009). The importance of a protein *S*-nitrosylation (a posttranslational modification in which a cysteinyl thiol is converted to a nitrosothiol by NO) is being increasingly recognized in the field of NO signal transduction (Miyamoto et al. 2009). Even though

*Corresponding author. Email: jin95324@cbu.ac.kr

the direct *S*-nitrosylation appears to be an important NO-mediated regulatory mechanism of various classes of proteins, the link between protein *S*-nitrosylation and functional relevance has been proven only in limited examples, and should be experimentally determined (Kimura et al. 2000; Gavathiotis et al. 2008; Asada et al. 2009). The characterization of *S*-nitrosylation protein may provide a clue to further understand the mechanism and the functional significance underlying the preferential *S*-nitrosylation of the target Cys. This reversible posttranslational modification may function as analogous to a protein phosphorylation in the broad spectrum of cellular signal regulatory mechanism of various classes of proteins (Haendeler et al. 2004; Lipton 2007; Teixeira et al. 2008), including ion channels such as the transient receptor potential canonical 5 (TRPC5), the skeletal muscle type ryanodine receptor (ryanodine receptor type 1) channel, the *N*-methyl-D-aspartate receptor channel, the cardiac L-type Ca^{2+} channel, and the cardiac Na^+ channel (Choi et al. 2000; Sun et al. 2001, 2007; Yoshida et al. 2006). In this paper, we have provided, for the first time, evidence that the Cys⁸⁵³ residue of TRPV4 is one of the *S*-nitrosylation target sites by NO, as a preferential target for its inactivation, which is presumably mediated for the regulation of negative feedback in HEK293 cells. Additionally, as compared with TRPV4 WT, the C853A mutant evidenced more sensitivity to 4- α PDD, with a higher level of cellular Ca^{2+} influx.

Materials and methods

Site-directed mutagenesis and plasmid construction

In order to generate the mutants, amino acid changes Cys 853 Ala or Cys 645 Ala were introduced using mutated oligonucleotides for C645A (up 5'-aac atg aag gtc GCt gac gag gac cag agc aac-3', down 5'-gct ctg gtc ctc gtc aGC gac ctt cat gtt ggt-3') or C853A (up 5'-ggg aac ccc aac GCt gac ggc cac cag cag-3', down 5'-ctg gtg gcc gtc aGC gtt ggg gtt ccc tag-3') and wild-type TRPV4 as a template. The TRPV4 mutant constructs were prepared using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). In order to acquire truncated TRPV4 (aa718-871), we used primers (up 5'-ata gga tcc atg ggt gag acc gtg ggc cag-3', down 5'-ata ctc gag cta cag tgg ggc atc gtc cgt-3'). All TRPV4 mutants were confirmed via DNA sequencing. To create the GST fusion protein (aa718-871) TRPV4 WT cDNA or its C853 mutant were PCR amplified, and were subcloned into the pGEX-5X-1 vector (Amersham). Human embryonic kidney (HEK 293) cells were transfected with TRPV4 and with mutant constructs as described previously.

Preparation of GST fusion protein and biotin-switch assay

GST fusion proteins were purified as previously described. In brief, the expression of GST fusion proteins in *Escherichia coli* strain BL21 (DE3) (RBC) was induced with 0.1 mM isopropyl 1-thio- β -D-galactopyranoside overnight at 18–20°C. Cells were harvested via centrifugation and were re-suspended in lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM EDTA). Cell lysis was achieved by treating the cells with lysozyme (1 mg/ml) for 1 h on ice, sonicating them with 1.5% Sarkosyl, and then subjecting them to a freeze-thaw cycle. Soluble fractions from a 10-min centrifugation at 24,000 $\times g$ (4°C) were rotated for 1 h in the presence of GST-Sepharose beads (Amersham Biosciences) at 4°C. Beads containing bound fusion proteins in HEN buffer (100 mM HEPES, pH 7.7, 400 μM EDTA, 40 μM neocuproine) were incubated with 1 mM *S*-nitroso-*N*-acetyl penicillamine (SNAP) for 1 h at a room temperature, and SNAP was removed by five washings with HEN buffer.

The biotin-switch assay was conducted as previously described by Jaffrey et al. In brief, GST fusion proteins immobilized to GST-Sepharose beads were washed with HEN buffer containing 0.05% Triton X-100, and free thiol (R-SH) residues were blocked (R-S-S-CH₃) for 30 min with 20 mM methylmethanethiol sulfonate (MMTS, Sigma-Aldrich) in HEN buffer containing 2.5% SDS at 53°C. After 1 min of centrifugation at 2500 rpm (at room temperature), the supernatant was incubated for 30 min at -20°C with two volumes of pre-chilled acetone to remove MMTS. Only *S*-nitrosylated thiol (R-S-NO) residues were then reduced to free thiol (R-SH) with 1 mM sodium ascorbate in HEN buffer and were biotinylated (R-S-S-biotin) with 4 mM biotin-HPDP (Pierce). The biotinylated samples were subsequently electrophoresed in SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane (Amersham Biosciences), and immunoblotted with a 1:4000-diluted monoclonal anti-biotin antibody (BN-34, Sigma-Aldrich). The proteins were detected with an advanced enhanced chemiluminescence system (Amersham Biosciences) using a LAS-3000 mini lumino-image analyzer (Fujifilm, Japan).

Fluorescence measurements of $[\text{Ca}^{2+}]_i$

We measured $[\text{Ca}^{2+}]_i$ using the fluorescent Ca^{2+} indicator Fluo4-acetoxymethyl ester (Fluo4-AM) as previously described. In brief, cells growing on coverslips were incubated for 40 min in a DMSO solution containing 1 M Fluo4-AM at 24°C in darkness, then washed and incubated for 15 min to hydrolyze

internalized Fluo4-AM. We measured $[Ca^{2+}]_i$ in single cells that emitted fluorescence, using confocal microscopy (LSM710 Zeiss, Germany). The absorption (as arbitrary unit) at 488 nm by argon-ion laser was measured as the relative intracellular Ca^{2+} ion concentration $[Ca^{2+}]_i$. All data are expressed as the means \pm SEM. All experiments were conducted at 24°C.

Biotin-switch assay for culture cell lysates

For the *S*-nitrosylation assay in living cells, human embryonic kidney (HEK) 293 cells (ATCC) were transfected with one of TRPV4WT/pcDNA3.1, TRPV4(C853A)/pcDNA3.1, or pcDNA3.1 alone or with either of CaM/pcDNA3.1 or CaM1234/pcDNA3.1 using ExGen500 (Fermentas) in accordance with the manufacturer's instructions. After 24–36 h after transfection, the cells were collected and lysed in HEN buffer containing 0.5% Triton X-100. The cells were treated for 15 min with 0.5 mM NOBF₄, washed twice in phosphate-buffered saline, collected, and lysed in HEK 293 cells with 0.5% Triton X-100, in accordance with the manufacturer's recommendations (S-Nitrosylated Protein Detection Kit, Cayman Chemical Co.)

Patch clamp experiments

HEK 293 cells were transfected either with TRPV4WT/pcDNA3.1, TRPV4 (C853A)/pcDNA3.1, or pcDNA3.1 using ExGen500 (Fermentas) according to the manufacturer's instructions. The expressed TRPV4 currents were recorded via a whole cell patch clamp technique using an Axopatch 200B amplifier (Axon Instruments) as previously described. The pCLAMP software (version 8.0, Axon) was used to generate voltage-pulse protocols and to acquire and analyze the data. TRPV4 currents were elicited by 2-s test pulses (Vt) to +60 mV followed by 2-s repolarization pulses to -40 mV from a holding potential (Vh) of -65 mV at 0.1 Hz. Peaks of tail current amplitude were measured during the repolarization pulse. All experiments were conducted at a room temperature of $22 \pm 2^\circ\text{C}$. Pipette solution contained (in mM), 110 aspartic acid, 1 CaCl₂, 1 MgCl₂, 5 ATP potassium salt, 10 HEPES, 11 EGTA (pH 7.3 adjusted with KOH): in some experiments, 11 mM EGTA was replaced with equimolar BAPTA to maintain low concentrations of intracellular Ca^{2+} . The external (bath) solution was Tyrode's solution containing (in mM), 135 NaCl, 4.8 KCl, 2 CaCl₂, 1.2 MgCl₂, 5 glucose, and 10 HEPES (pH 7.4 adjusted with NaOH).

Statistics

All numerical values are presented as means \pm SE. Statistical significance was evaluated via an analysis of variance followed by Bonferroni's multiple comparison test. $P < 0.05$ was established as the significance level.

Results

Specific S-nitrosylation on the TRPV4 Cys⁸⁵³ residue

Several ion-channel regulations have been shown to occur as the result of *S*-nitrosylation, including the cyclic nucleotide-gated channel and the delayed rectifier potassium channel (Choi et al. 2000; Sun et al. 2001, 2007; Yoshida et al. 2006; Asada et al. 2009). TRPC5 (including other TRP channels) is also activated by NO via cysteine *S*-nitrosylation, as a general mechanism for the regulation of stimulus-coupled cellular Ca^{2+} flux (Teixeira et al. 2008; Yoshida et al. 2006; Olson and Garbán 2008).

After recognizing the putative *S*-nitrosylation site (Cys⁸⁵³ residue) of TRPV4 with the consensus sequence information (Lys/Arg/His/Asp/Glu)Cys(Asp/Glu) (sites -1, 0, and +1, respectively), we investigated its *S*-nitrosylation and its biological significance (Figure 1A). We attempted to pinpoint the target Cys residue for *S*-nitrosylation on Cys⁶⁴⁵ of Val/Cys/Asp or Cys⁸⁵³ of Lys/Cys/Asp in human TRPV4. We focused first on Cys⁸⁵³, because of its greater similarity with the consensus sequence than Cys⁶⁴⁵. We determined whether TRPV4 is modified by NO via *S*-nitrosylation using a Biotin-Switch Assay (Figure 1B and C). To corroborate Cys⁸⁵³ as the principal target of *S*-nitrosylation, we expressed His-tagged full-length TRPV4 in which Cys⁸⁵³ had been replaced with Ala, leaving the remaining Cys⁸⁵³ intact (TRPV4 C853A) in HEK293 cells (Figure 1B). The cell lysates (left panel) were treated with 0.5 mM NOBF₄ for 15 min, and subjected to biotin-switch assay (right panel). As shown in Figure 1B, the application of an NO donor induced *S*-nitrosylation of TRPV4 WT (lane 3) but not C853A (lane 2). Thus, these results confirmed that TRPV4 is modified by NO as *S*-nitrosylation, and its Cys⁸⁵³ is the principal target of *S*-nitrosylation. However, the *S*-nitrosylation of TRPV4 mutant (C645A, which is located at the extracellular side near its pore region) was unchanged (data not shown), even though Cys⁶⁴⁵ was also proposed by other researchers as a putative *S*-nitrosylation site. As a consequence, *S*-nitrosylation detected TRPV4 WT (lane 3 Figure 1B), but was completely eliminated for the C853A mutant (lane 2, Figure 1B).

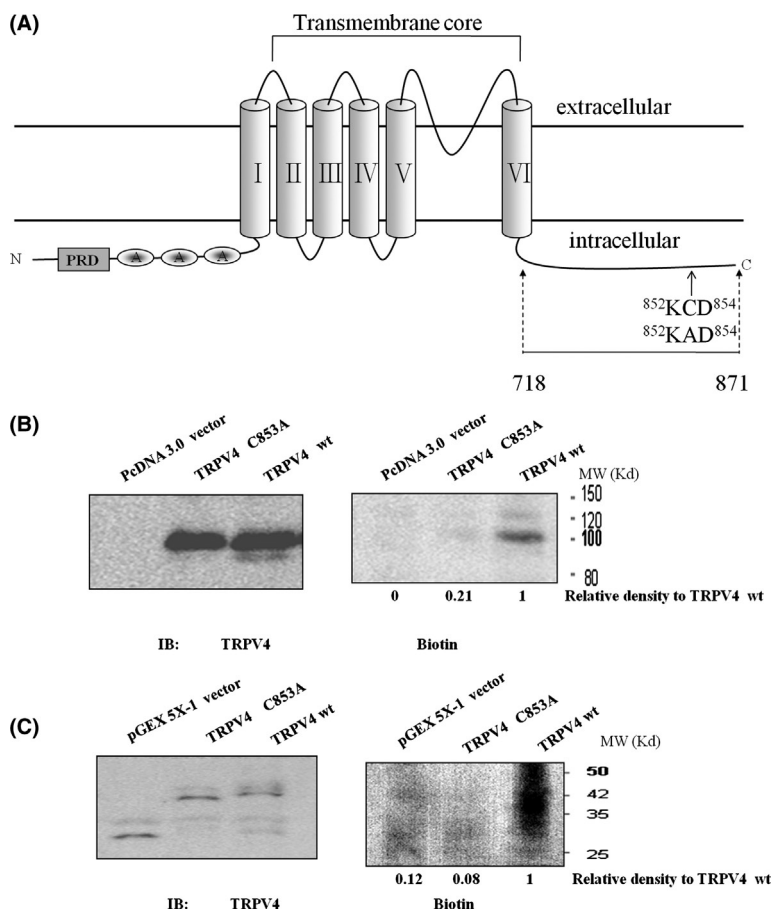


Figure 1. The S-nitrosylation of TRPV4. (A) The putative S-nitrosylation site was noted in human TRPV4 (Cys⁸⁵³). Cys⁸⁵³ (⁸⁵²KCD⁸⁵⁵) was replaced with alanine (C853A). Among 14 Cys residues in TRPV4, the Cys⁸⁵³ residue is followed immediately by the acidic amino residue (Lys/Arg/His/Asp/Glu)Cys(Asp/Glu) (sites -1, 0, and +1, respectively) which is required for S-nitrosylation by nitric acid (NO). Shaded blocks denote the N-terminal ankyrin repeats and the transmembrane helices. Underlines indicate the C-terminal cytoplasmic region (aa718–871) which is expressed in *E. coli* as GST fusion protein. The TRPV4 Cys⁸⁵³ which is replaced with alanine is marked with an arrow. (B) HEK293 cells expressing His-tagged TRPV4 WT, TRPV4 (C853A) with 0.5 mM NOBF₄. The cell lysates were prepared and subjected to biotin-switch assay. The left panel is the representative data for immunoblotting with an anti-His antibody and biotin antibody. The number at the bottom represents the density relative to TRPV4 WT. (C) GST fusion proteins with TRPV4 fragments containing Cys⁸⁵³ (as shown in A) were prepared from *E. coli*, and subjected to biotin-switch assay. GST fusion proteins with TRPV4 fragments harboring Cys⁸⁵³ were prepared from *E. coli*, and subjected to the biotin-switch assay. The density relative to TRPV4 WT is shown at the bottom.

To confirm again that the Cys⁸⁵³ residue of TRPV4 is the specific site for the S-nitrosylation, we used the GST-TRPV4 (aa 718-871) fusion protein expressed in *E. coli* (Figure 1C). The purified proteins, 2 g/ml of WT or C853A fusion protein in HEN buffer (left panel Figure 1C), were treated with 0.5 mM NOBF₄ for 1 h and subjected to biotin-switch assay (right panel in Figure 1C). As shown in Figure 1C, the application of an NO donor induced S-nitrosylation of TRPV4 WT (lane 3 in right panel) but not C853A (lane 2 in right panel). Together these results in Figure 1 B and C also confirmed that TRPV4 is modified by NO as S-nitrosylation on its Cys⁸⁵³ residue, consistent with the expectation from Figure 1A.

TRPV4 C853A inhibits the protein-protein interaction with calmodulin, and affects its subcellular localization

One protein known to be involved in the feedback regulation of a variety of ion channels is calmodulin (CaM) (Nilius et al. 2003; Strotmann et al. 2003; Earley et al. 2005). The CaM binding site is known to be located within the C-terminal domain of TRPV4 (aa 718–871). In order to determine whether the S-nitrosylation on the Cys⁸⁵³ residue affects the binding between TRPV4 and CaM, GST-fusion proteins encompassing the C-terminal TRPV4 domains were constructed and expressed in *E. coli*. Approximately 2 g/ml of WT or C853A fusion protein bound to glutathione-Sepharose was incubated with HEK293

cell lysates with/without treatment with 0.5 mM NOBF₄ for 1 h. As is shown in Figure 2A, the pull-down of CaM by TRPV4 WT from the HEK293 cell lysates was affected by the treatment of 0.5 mM NOBF₄, but C853A did not affect it.

Considering that TRPV4 can be *S*-nitrosylated at its CaM binding site and is then unable to bind to Ca²⁺-CaM, we wondered whether a fraction of the expressed wild-type channels had already been *S*-nitrosylated. These channels would then prove unable to bind to Ca²⁺-CaM and would consequently reduce the effect of Ca²⁺-CaM binding disruption. If this is indeed the case, all of the expressed C853A mutant TRPV4 channels that cannot be *S*-nitrosylated at their CaM-binding site should be capable of binding to Ca²⁺-CaM and should therefore show more activation than the wild-type channels. Thus, these results suggested that the *S*-nitrosylation of TRPV4 and Ca²⁺-CaM binding in the activation state are mutually exclusive.

In an effort to determine whether the Cys⁸⁵³ residue change of TRPV4 C853A is attributable to its subcellular localization or to a deviation in CaM binding at the expression level, we conducted a confocal microscopic examination of TRPV4 WT, C853A, or with CaM. TRPV4 WT was principally detected within the plasma membrane, whereas TRPV4 C853A was primarily detected in the cytoplasm (Figure 2B). Furthermore, we noted that a significant difference in TRPV4 WT subcellular localization could be distinguished with/without the treatment of 0.5 mM NOBF₄ for 30 min. The co-localization of TRPV4 WT and CaM was disrupted principally in the cytoplasm by the treatment of 0.5 mM NOBF₄ for 30 min (compare the top two lanes), but that of C853A and CaM was not changed with/without NOBF₄ treatment (compare the bottom two lanes).

Taken together, these results showed that the Cys⁸⁵³ residue of TRPV4 (by NO through *S*-nitrosylation) appears to be the control point which binds with CaM and contributes its plasma membrane localization.

Mutation of the S-nitrosylation site (C853A) of TRPV4 enhances its activity and the sensitization to 4- α PDD

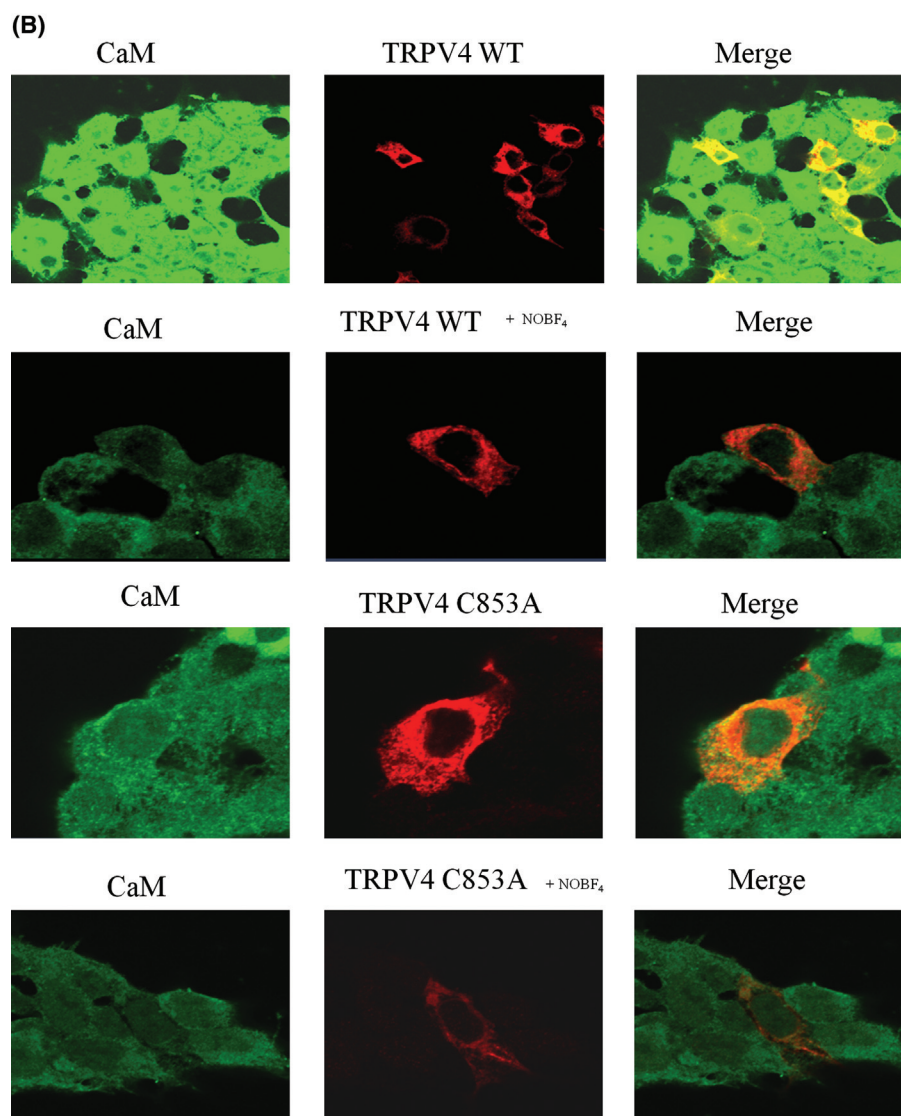
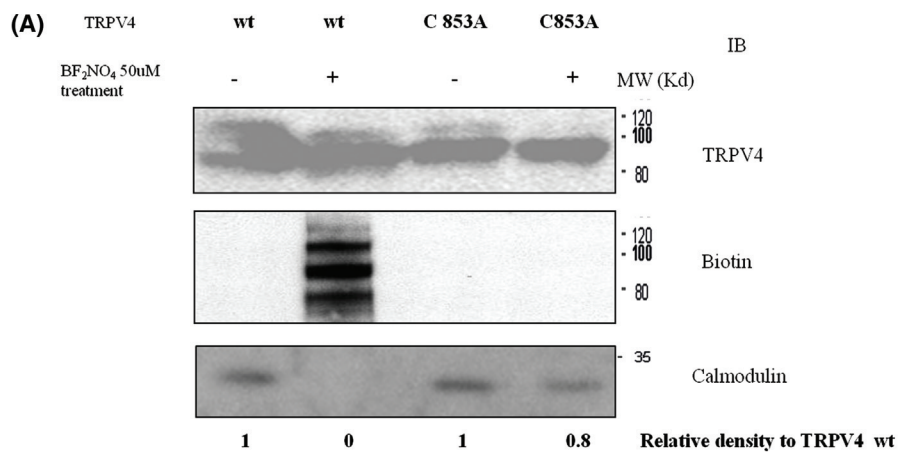
We employed a patch clamp experiment to evaluate the functional consequences of the *S*-nitrosylation of Cys⁸⁵³ of the TRPV4 channel. HEK 293 cells express TRPV4 and conduct slowly activating outward currents. As is shown in Figure 3A, the single channel current elicited in the TRPV4 WT was recorded. We observed that the application of NOBF₄ (at 50 μ M) decreased the relative channel activity of the TRPV4 by approximately 1/4-fold from 24.8 ± 2.51 to 4.12 ± 0.23 pA (Figure 3A upper lane, $n = 6$), whereas NOBF₄ did

not change the TRPV4 C853A channel activity (Figure 3A bottom lane, $n = 6$). Further, the channel open probability (P_O) declined in the TRPV4 WT (Figure 3A upper lane, $P_O 0.18 \pm 0.02$) to less than 1/5 the P_O of TRPV4 C853A (Figure 3A bottom lane, $P_O 0.95 \pm 0.31$), after treatment with NOBF₄. Comparing with the properties of TRPV4 WT, the amplitude and P_O of TRPV4 C853A were unchanged with the NOBF₄ treatment (Figure 3A bottom). However, with the infusion of 1 M 4- α PDD (a known PKC-independent TRPV4 activator; Watanabe et al. 2002, 2003; Birder et al. 2007; Fernandes et al. 2008;), the channel open probability (P_O) elicited in the TRPV4 C853A (Figure 3A bottom lane, $P_O 0.95 \pm 0.31$) was more than 5 times that of TRPV4 WT (Figure 3A upper lane, $P_O 0.18 \pm 0.02$), and the relative channel activity of the TRPV4 C853A was enhanced approximately 3-fold (from 11.2 ± 0.2 to 23.8 ± 2.5 pA, $n = 6$), with the application of 4- α PDD (Figure 3A). Thus, the single channel activity of the TRPV4 C853A mutant was increased as compared with the WT, thereby supporting the notion that the *S*-nitrosylation on its Cys⁸⁵³ residue is a major regulation for the direct TRPV4 channel inactivation.

Next, to obtain more information regarding the role of Cys⁸⁵³ *S*-nitrosylation, we compared the whole cell current property with TRPV4 WT and the TRPV4 C853A mutant (Figure 3B). As expected from the single channel current in Figure 3A, the current elicited in the TRPV4 WT was decreased from 25 ± 2 pA/pF ($n = 6$) to 17 ± 3 pA/pF with NOBF₄ treatment (Figure 3B left), even though the current elicited in the TRPV4 C853A was almost unchanged with NOBF₄ treatment (16 ± 2 pA/pF, $n = 6$, Figure 3B right). Thus, those results also supported the idea that the activity of the TRPV4 channel is regulated by the *S*-nitrosylation on its Cys⁸⁵³ residue as a negative effect. Unexpectedly, however, the current induced by 1 μ M of 4- α PDD showed a dramatic difference between TRPV4 WT and C853A (Figure 3B upper lane, TRPV4 WT, 106 ± 8 pA/pF, $n = 6$, C853A: 150 ± 14 pA/pF, $n = 6$), suggesting that TRPV4 C853A was more sensitive than TRPV4 WT to 4- α PDD. Therefore, other regulation mechanisms, including protein-protein interaction with the C-terminal domain of TRPV4 (see the next discussion section), seem to be required.

Ca²⁺ image analysis of TRPV4 WT and C853A with 4- α PDD stimulation

It has been proposed that TRPV4 may form constitutively open ion channels that show an inwardly rectifying current-voltage relationship with a high selectivity of calcium over monovalent cations (Strotmann et al. 2003; Suzuki et al. 2003a; Cuajungco et al. 2006;



Fernandes et al. 2008). Additionally, their activity has been correlated with the electrochemical driving force for Ca^{2+} and the level of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$), which is suggestive of the existence of calcium-dependent regulatory mechanisms. In order to determine the consequences of *S*-nitrosylation on the Cys⁸⁵³ residue of TRPV4, we employed a Ca^{2+} image experiment (Figure 4). With the transfection of TRPV4 WT, or C823A, the application of 4- α PDD increased the current amplitudes of TRPV4 (Figure 3). Although the recombinant TRPV4 expression elicited a robust elevation of $[\text{Ca}^{2+}]_i$ in response to 4- α PDD treatment of HEK 293 cells, the difference was as dramatic in TRPV4 (Figure 4). Comparing the optical response change (as arbitrary $[\text{Ca}^{2+}]_i$ concentration) of TRPV4 C823A with that of WT at a given time period (30 min) of 1 μM 4- α PDD treatment, it shows a 3.3 times higher $[\text{Ca}^{2+}]_i$ than does TRPV4 WT (Figure 4). Interestingly, $[\text{Ca}^{2+}]_i$ of TRPV4 C823A fluctuated without declining to the base level (Figure 4), thereby suggesting that TRPV4 C823A is in the active stage without the inactivation step. However, the representative trace of TRPV4 WT showed a regular response signal to 4- α PDD (Figure 4 left). The response of TRPV4 WT to 4- α PDD showed a regular up-and-down change, whereas TRPV4 C853A did not respond in this fashion (Figure 4).

Consistent with the electrophysiology results shown in Figure 3, we also noted that TRPV4 C853A contributed 3 times the Ca^{2+} entry as compared to TRPV4 WT within a given time period (30 min) in 1 μM 4- α PDD treatment (Figure 4). Comparing the inactivation pattern of TRPV4 WT (Figure 4 left) with that of C853A (Figure 4 right), TRPV4 C853A appears to be activated, thereby suggesting that the mutation of Cys⁸⁵³ to Ala is relevant to the activation step of this channel protein.

In summary, these results showed that TRPV4 is modulated by *S*-nitrosylation on its Cys⁸⁵³, one of its authentic *S*-nitrosylation proteins. As a consequence of the *S*-nitrosylation on the Cys⁸⁵³ of TRPV4, the reduction in Ca^{2+} channel activity was coupled to a prolongation of the inactive state (Figure 5). Meanwhile,

TRPV4 C853A mutant seems to be an active form, with increased sensitivity to 4- α PDD.

DISCUSSION

An abundant variety of stimuli activate members of the various TRPV subfamilies. Vanilloid compounds such as capsaicin, the compound responsible for the spiciness of hot chili peppers, but also moderate heat and protons can activate TRPV4 channels, which function as an integrator of a variety of painful stimuli. Further, TRPV4 is sensitive to noxious heat and can be constitutively activated by growth factors (Nilius et al. 2003; Strotmann et al. 2003; Cohen 2006; Vennekens et al. 2008; Everaerts et al. 2009).

In this study, we demonstrate that an NO donor induces *S*-nitrosylation at Cys⁸⁵³ in the C terminus of TRPV4 (Figures 1 and 5). Our results provide convincing evidence to suggest that the Cys⁸⁵³ residue of TRPV4 is required for site-specific *S*-nitrosylation (Figure 1). Further, *S*-nitrosylation at the Cys⁸⁵³ residue in the TRPV4 channel functionally and directly regulates its channel sensitivity. Here, we proposed that NO conversely inhibits the Ca^{2+} response and direct *S*-nitrosylation on the Cys⁸⁵³ of TRPV4 as a negative feedback regulation (Figure 5). These findings reveal the structural motif for the NO-sensitive activation gate in TRP channels, and indicate that NO sensors are a new functional category of cellular receptors extending over different TRP families. Among 14 Cys residues in human TRPV4, the Cys⁸⁵³ residue is immediately followed by the acidic amino residue, and the *in vitro* biotin-switch assay indicates that the presence of acidic amino acid at the (Lys/Arg/His/Asp/Glu)Cys(Asp/Glu) (sites -1, 0, and +1, respectively) residue is required for the *S*-nitrosylation of Cys⁸⁵³ (Figure 1).

It was demonstrated previously that endothelial NO synthase and TRPV4 co-localize in the caveolar fraction of guinea pig hearts, thereby indicating the close proximity of the NO donor and the target protein. Thus, multiple factors are responsible for making the Cys⁸⁵³ of TRPV4 a target of molecule-specific and site-specific *S*-nitrosylation. This may

Figure 2. Effects of *S*-nitrosylation on the interaction between TRPV4 with calmodulin. (A) Representative superimposed TRPV4 currents without (upper panel) and with (lower panel) NO infusion. HEK293 cells were transiently transfected with His-TRPV4 WT or C853A plasmid. After 48 h, the cells were lysed, total proteins were re-collected, and immunoprecipitation was conducted with nickel beads. Western blot assays were then conducted with a rabbit TRPV4 Ab or an anti-CaM antibody in HEK293 cell. The number at the bottom represents the density to relative TRPV4 WT. (B) Effects of *S*-nitrosylation on the subcellular localization of TRPV4 and calmodulin. The subcellular localization of TRPV4 WT, TRPV4 C853A or C823A in HEK 293 cells with/without NO infusion. Confocal microscopic analyses of transfected His-TRPV4 WT, or mutant (C853A, all constructs are shown in green) were conducted in order to determine whether it merged with CaM (shown in red). The transfected HIS-TRPV4 WT was merged (yellow) without NO infusion (top two columns). However, the transfected HIS-TRPV4 C853A was not merged with CaM (bottom two column) with/without NO infusion. His-TRPV4 WT was principally detected within the plasma membrane, whereas HIS-TRPV4 C853A was primarily detected in the cytoplasm.

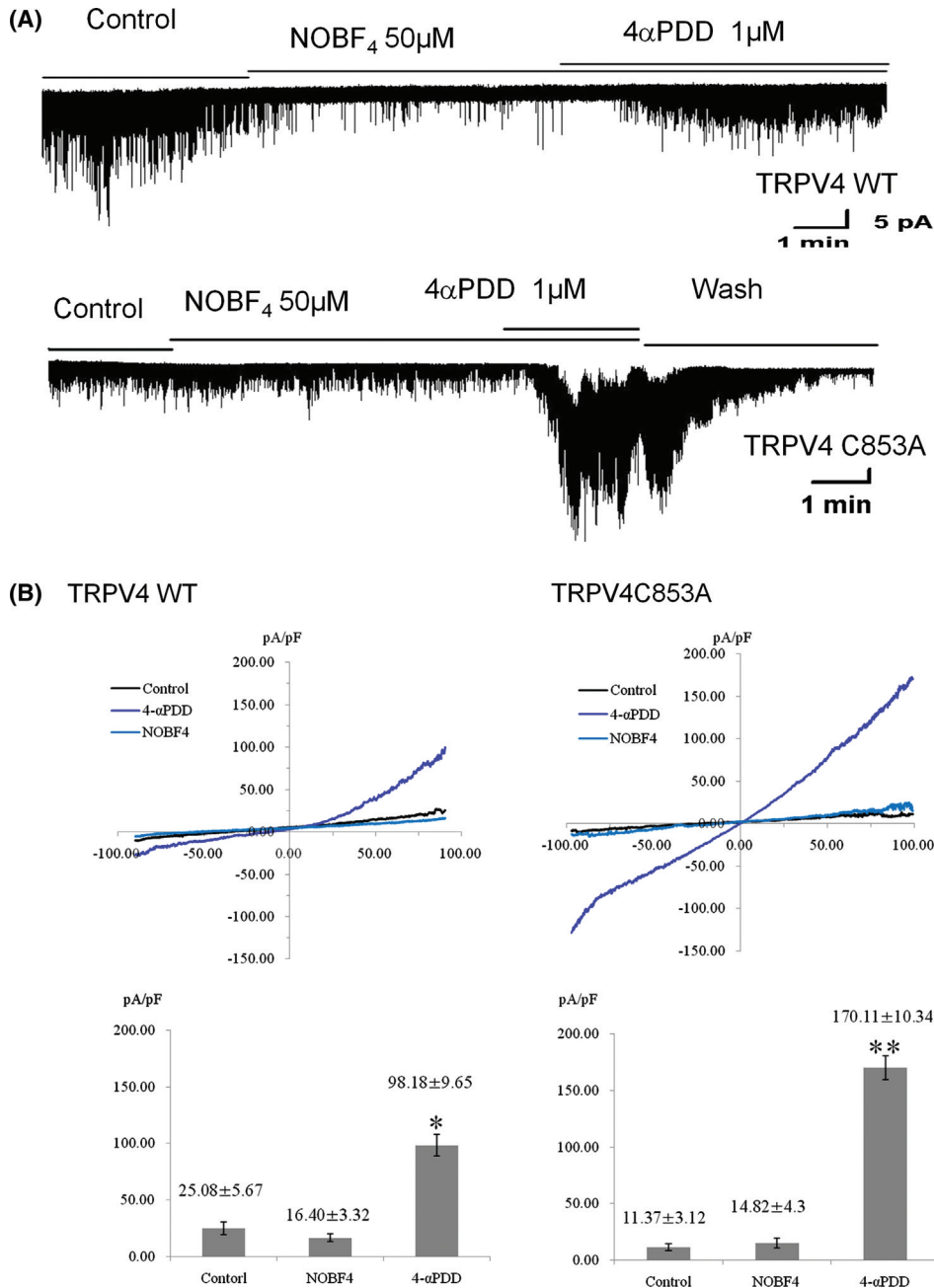


Figure 3. Effects of S-nitrosylation of TRPV4 on its channel current. (A) Representative superimposed TRPV4 WT currents without (upper panel) and with (lower panel) infusion of NO. The black trace represents the basal current, the green trace represents the presence of NOBF₄, 100 nM, and the blue trace represents post-washout of NOBF₄. HEK 293 cells were transfected with either TRPV4 WT/pcDNA3.1 or TRPV4 (C853A)/pcDNA3.1, using ExGen500 (Fermentas) in accordance with the manufacturer's instructions. Expressed TRPV4 currents were recorded with a patch clamp technique using an Axopatch 200B amplifier (Axon Instruments). pCLAMP software (version 8.0, Axon) was used to generate voltage-pulse protocols and for data acquisition and analysis. TRPV4 currents were elicited by 2-s test pulses (V_t) to +60 mV followed by 2-s repolarization pulses to -40 mV from a holding potential (V_h) of -65 mV at 0.1 Hz. (B) (Upper panel) Current-voltage relations of peak whole-cell cationic currents recorded from different HEK293 cells transfected with TRPV4-WT, or TRPV4-C853A and exposed to 1 μM α 4PDD, then again dialyzed with normal buffer again. Normalized (pA/pF) current traces in response to a voltage step to -100 mV for the wild-type (WT) and C853A constructs. All experiments were conducted at 24°C. (Lower panel) Mean current densities (± S.E.) at +60 mV in HEK293 cells expressing TRPV4-WT (n=6), and TRPV4 C853A (n=6) and dialyzed with 1 μM 4-α PDD or NOBF₄. * P < 0.05 versus to the control. ** P < 0.05 versus to TRPV4 WT.

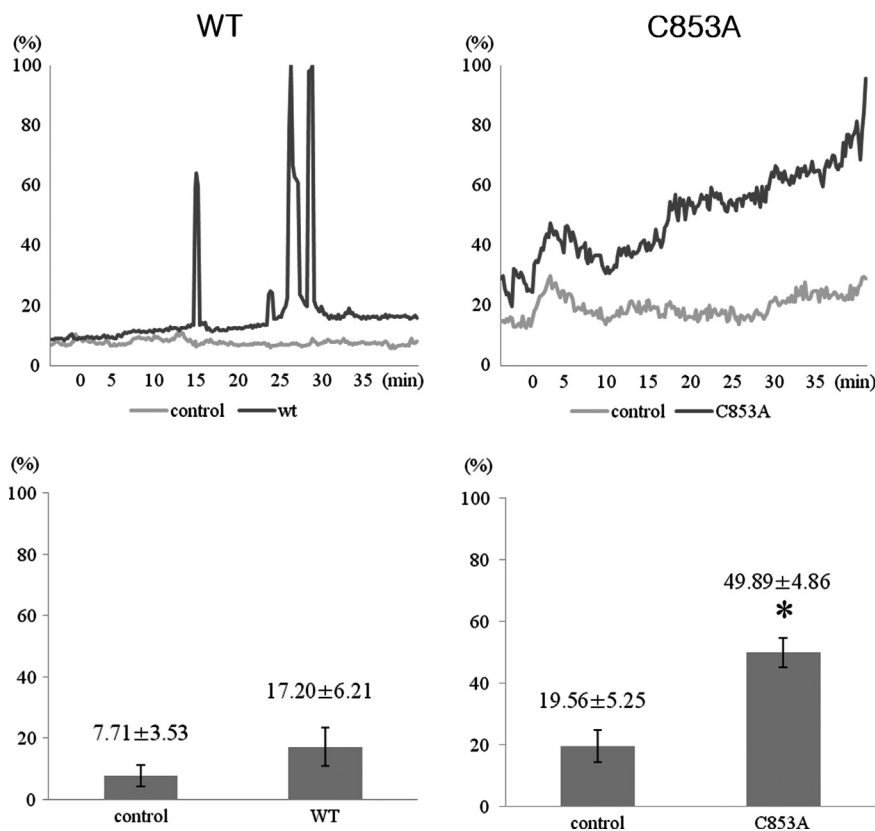


Figure 4. Effects of WT or C853A on TRPV4 intracellular calcium concentration change ($[Ca^{2+}]_i$). Representative trace showing the effect of $1 \mu M$ 4- α PDD in TRPV4 WT or C853A transfected or His vector-transfected (mock) HEK 293 cells. Summary of $[Ca^{2+}]_i$, expressed as the absorption (in arbitrary units) at 488 nm (argon-ion laser), after 4- α PDD in HEK 293 cells. The fluctuation caused by 4- α PDD is expressed as means \pm SE: numbers of cells examined are indicated above each column. Responses (with the arbitrary units) were measured as the total area of each representative trace from 10 min to 35 min minus each basal total area of the same period. All experiments were conducted at $24^\circ C$. * $P < 0.05$ versus TRPV4 WT.

explain why, to induce *S*-nitrosylation in the reducing intracellular milieu, the presence of NO is required. The *S*-nitrosylation of the TRPV4 channel is a novel regulatory mechanism of this channel, which may have a profound impact on the physiological regulation of cardiac electrical sensitivity, as the Ca^{2+} channel composed of TRPV4 has been identified to perform a crucial regulatory function in cardiac electrophysiology, including responses to autonomic nervous stimulation and adaptation to changes in heart rate. Even though it has been reported that TRPV5 and TRPV6 are tightly controlled by the membrane potential, including a voltage-dependent open-pore block by a Ca^{2+} -dependent feedback mechanism, it is not known that TRPV4 is also regulated as the same manner. The *S*-nitrosylation of the TRPV4 channel seems to be one of the Ca^{2+} -dependent feedback mechanisms (Figure 5).

Interestingly, we noted that the sensitivity of the C853A mutant to 4- α PDD is higher than that of WT (Figures 3 and 4). However, we do not at present know

the reason for this phenomenon; rather, it appears that the *S*-nitrosylation of TRPV4 Cys⁸⁵³ residue prevents the access of 4- α PDD to the channel. The mechanism underlying the *S*-nitrosylation-induced inactivation of the TRPV4 channel also remains to be addressed. Biochemical, spectroscopic and crystallographic analyses have provided the structural model for the C-terminal region of TRPV4 binding sites for NO. Although the functional assembly of TRPV4 channels has been demonstrated to require its interaction with NO, similar TRPV4 channel currents were recorded in the absence and presence of NO co-expression. Endogenous levels of NO in HEK293 cells were approximately one-eighth of those in NO-transfected cells, which may prove sufficient for the functional assembly of TRPV4, but not for TRPV4 *S*-nitrosylation. Certainly, more work will be necessary to decipher the role of NO in *S*-nitrosylation and the mechanism linking the *S*-nitrosylation of the TRPV4 channel to its regulation. The mechanism of TRPV inactivation by Ca^{2+} in general has yet to be clearly elucidated.

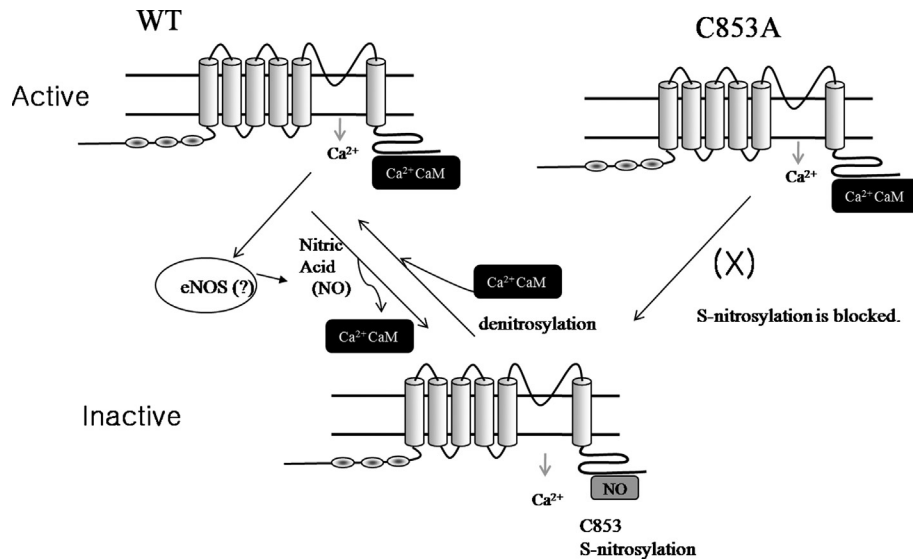


Figure 5. Schematic representation for the regulation of TRPV4 by S-nitrosylation on its Cys⁸⁵³ residue. When the normal cell receives the proper stimuli, such as mechanical, chemical, heat or nociception stimuli, the outside Ca²⁺ enters the cell through TRPV4. The entered Ca²⁺ stimulates eNOS, which produces NO. The increased NO then S-nitrosylates the Cys⁸⁵³ residue of TRPV4, and dissociates the activator proteins that bind to its C-terminal region (such as Ca²⁺-CaM), resulting in the inactivation of TRPV4 (as a negative feedback regulation). The TRPV4 desensitized (inactivated) by S-nitrosylation returns to the active state via denitrosylation, the mechanisms for which currently remain unknown. The cells that contain mutant C853A do not properly respond to NO, even though the outside Ca²⁺ enters the cell through the TRPV4 channel upon stimulus, and increases the concentration of NO via the activation of eNOS. However, the S-nitrosylation (inactivation) of the TRPV4 C853A mutant is blocked, as it cannot receive nitric oxide on its Cys⁸⁵³ residue. Thus, the TRPV4 C853A mutant appears to remain in an active state for a longer period than TRPV4 WT.

Mutant channels, harboring a single mutation in the C-terminus of TRPV4 (E797A), are constitutively open (Nilius et al. 2003). Such mutants increase [Ca²⁺]_i in non-stimulated cells. It is likely that this constitutive activation reflects a defect in the inactivation of Ca²⁺. We do not at present know whether (E797A) also affects the S-nitrosylation of TRPV4, or whether its C853A mutant is also defective in inactivating its channel activity, like E797A. However, even though it remains unclear as to how the C853A mutant affects its activation, it is probably modulated by NO and may prove essential for the regulation of the inward driving force for Ca²⁺ entry (Figure 5).

Recombinant TRPC1, TRPC4, TRPV4, TRPV1, TRPV3 and TRPV4 of the TRPC and TRPV families, which are commonly classified as receptor-activated channels and thermosensor channels, induce Ca²⁺ entry into cells in response to nitric oxide (NO) (Yoshida et al. 2006; Miyamoto et al. 2009). Even though our data demonstrate that the cytoplasmically accessible Cys⁸⁵³ is an S-nitrosylation site that mediates NO sensitivity in TRPV4, the other responsive TRP proteins have conserved cysteines on the same N-terminal side of the pore region. In living cells, an NO donor failed to S-nitrosylate TRPV4 in the presence of NO (Miyamoto et al. 2009), implying

that both the redox motif flanking Cys⁸⁵³ and the absence of NO are required. Infusions of NO are required for the S-nitrosylation of TRPV4 in living cells, but not in the cell lysates.

It has been reported that the nitrosylation of native TRPV4 upon G protein-coupled ATP receptor stimulation elicits Ca²⁺ entry into endothelial cells. In the case of TRPV4, however, even though the increase of NO in the cells also appears to enhance the nitrosylation of native TRPV4, the nitrosylation site (Cys⁸⁵³) of TRPV4 is located in its C-terminal tail, and the Ca²⁺ transport by the channel appears to be inactivated by this modification, as a negative feedback Ca²⁺ transport regulation mechanism (Figures 1 and 5).

Yoshida et al. predicted that the NO responsiveness of TRPV4 channels is localized to its Cys⁶⁴⁵ residue in (⁶⁴⁴VCD⁶⁴⁶) by the alignment of amino acid sequences surrounding the Cys⁵⁵³ and Cys⁵⁵⁸ of TRPC5 with counterpart sequences, including TRPC1 and TRPC4, as well as the thermosensor channels TRPV1, TRPV3 and TRPV4, which harbor conserved cysteines on the N-terminal side of the H7 putative pore-forming region in the linker region located between the fifth and sixth transmembrane domains S5 and S6 (Yoshida et al. 2006). However, the actual S-nitrosylation site of human TRPV4 is not the Cys⁶⁴⁵ residue (⁶⁴⁴VCD⁶⁴⁶)

conserved on the extracellular side of the putative pore-forming region found between the fifth and sixth transmembrane domains, but rather the Cys⁸⁵³ residue (⁸⁵²KCD⁸⁵⁵) in its intracellular C-terminal domain (Figure 1). Therefore, the *S*-nitrosylation site among TRP family proteins appears to show protein specificity.

We evaluated our hypothesis as described in Figure 5, and our observations indicated that TRPV4 is modulated by *S*-nitrosylation (dependent on a Ca²⁺ ion) as a negative feedback regulation loop. In a short-term regulation loop, TRPV4 WT appears to be modulated by the association of regulatory proteins (such as Ca²⁺-bound CaM) on its C-terminal cytoplasmic domain. At first, the naive TRPV4 begins to open in response to several over-threshold environmental signals (e.g. mechanical, chemical temperature, osmolality). The activated TRPV4 is positively activated by bound regulatory proteins such as CaM, at low levels of Ca²⁺ ion concentration. However, at high Ca²⁺ ion levels, the fully active TRPV4 is inhibited by negative feedback and returned to the inactive form, as negative feedback regulatory loop by NO (*S*-nitrosylation) (Figure 5). The NO is putatively generated by eNOS under high Ca²⁺ ion concentration conditions. This phenomenon also explains our observation that, after activation with 4- α PDD, TRPV4 WT showed oscillations in the concentration of the Ca²⁺ ion in the cytoplasm [Ca²⁺]_i (Figure 4 left). On the other hand, TRPV4 WT is *S*-nitrosylated on its Cys⁸⁵³ residue by NO, which remains inactive until being de-nitrosylated on its Cys⁸⁵³ residue and the association of a putative regulatory protein (such as CaM). Consistent with this assumption, we noted high Ca²⁺ channel activity with/without 4- α PDD treatment (see Figures 3 and 4), abrogating the interaction of the protein with the activation factor. This model also explains, to some degree, why the TRPV4 E797 mutant is constitutively active (Nilius et al. 2003).

In conclusion, we demonstrated herein that NO can control the selective and direct activation of the TRPV4 channel. This novel negative regulatory mechanism of the Ca²⁺ channel by NO (Figure 5) may perform a role in the TRPV4-mediated cell signal phenomenon in which homeostasis is maintained to prevent the apoptosis induced by Ca²⁺ toxicity during the response to intracellular Ca²⁺.

Acknowledgements

We thank Dr. David M. Cohen (Oregon Health and Science University) for kindly providing the TRPV4 gene. This work was supported by a National Research Foundation of Korea grant funded by the Korea government (KRF-2009-0076024 and 2009-0069007) to S.S. Kang and a National Research Foundation of Korea grant funded by the Korea government

(KRF-2009-351-C00139) to E.J. Lee. We also appreciated The Core Facility of Chungbuk National University.

References

- Andrade YN, Fernandes J, VJ, an E, Fernández-Fernández JM, Arnanges M, Singe TM, Villales M, Valverde MA. 2005. TRPV4 channel is involved in the coupling of fluid viscosity changes to epithelial ciliary activity. *J Cell Biol.* 168: 869–874.
- Asada K, Kurokawa J, Furukawa T. 2009. Redox- and calmodulin-dependent *S*-nitrosylation of the KCNQ1 channel. *J Biol Chem* 27:6014–6020.
- Becker D, Blasé C, Bereiter-Hahn J, Jendrach M. 2005. TRPV4 exhibits a functional role in cell-volume regulation. *J Cell Sci* 118:2435–2440.
- Birder L, Kullmann FA, Lee H, Barrick S, Groat W de, Kanai A, Caterina M. 2007. Activation of urothelial transient receptor potential vanilloid 4 by 4 α -phorbol 12,13-didecanoate contributes to altered bladder reflexes in the rat. *J Pharmacol Exp Ther.* 323: 227–235.
- Choi YB, Tenneti L, Le DA, Ortiz J, Bai G, Chen HS, Lipton SA. 2000. Molecular basis of NMDA receptor-coupled ion channel modulation by *S*-nitrosylation. *Nat Neurosci* 3:15–21.
- Cohen DM. 2006. Regulation of TRP channels by N-linked glycosylation. *Semin Cell Dev Biol* 17:630–637.
- Cuajungco MP, Grimm C, Oshima K, D'hoedt D, Nilius B, Mensenkamp AR, Bindels RJ, Plomann M, Heller S. 2006. PACSINs bind to the TRPV4 cation channel. PACSIN 3 modulates the subcellular localization of TRPV4. *J Biol Chem* 281:18753–18762.
- Earley S, Heppner TJ, Nelson MT, Brayden JE. 2005. TRPV4 forms a novel Ca²⁺ signaling complex with ryanodine receptors and BKCa channels. *Circ Res* 97:1270–1279.
- Everaerts W, Nilius B, Owsianik G. 2009. The vanilloid transient receptor potential channel Trpv4: From structure to disease. *Prog Biophys Mol Biol.* 1:1–25.
- Fan HC, Zhang X, McNaughton PA. 2009. Activation of the TRPV4 ion channel is enhanced by phosphorylation. *J Biol Chem* 284:27884–27891.
- Fernandes J, Lorenzo IM, Andrade YN, Garcia-Elias A, Serra SA, Fernández-Fernández JM, Valverde MA. 2008. IP3 sensitizes TRPV4 channel to the mechano- and osmotransducing messenger 5'-6'-epoxyeicosatrienoic acid. *J Cell Biol* 181:143–155.
- Fu Y, Subramanya A, Rozansky D, Cohen DM. 2006. WNK kinases influence TRPV4 channel function and localization. *Am J Physiol Renal Physiol.* 290:1305–1314.
- Gavathiotis E, Suzuki M, Davis ML, Pitter K, Bird GH, Katz SG, Tu HC, Kim H, Cheng EH, Tjandra N, Walensky LD. 2008. BAX activation is initiated at a novel interaction site. *Nature* 455:1076–1081.
- Haendeler J, Hoffmann J, Zeiher AM, Dimmeler S. 2004. Antioxidant effects of statins via *S*-nitrosylation and activation of thioredoxin in endothelial cells: a novel vasculoprotective function of statins. *Circulation* 110:856–61.
- Kimura H, Weisz A, Kurashima Y, Hashimoto K, Ogura T, D'Acquisto F, Addeo R, Makuuchi M, Esumi H. 2000. Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia-inducible factor-1 activity by nitric oxide. *Blood.* 95:189–197.

- Lipton SA. 2007. Pathologically-activated therapeutics for neuroprotection: mechanism of NMDA receptor block by memantine and S-nitrosylation. *Curr Drug Targets*. 8:621–632.
- Miyamoto T, Dubin AE, Petrus MJ, Patapoutian A. 2009. TRPV1 and TRPA1 mediate peripheral nitric oxide-induced nociception in mice. *PLoS One*. 4: e7596.
- Muramatsu S, Wakabayashi M, Ohno T, Amano K, Ooishi R, Sugahara T, Shiojiri S, Tashiro K, Suzuki Y, Nishimura R, et al. 2007. Functional gene screening system identified TRPV4 as a regulator of chondrogenic differentiation. *J Biol Chem*. 282:32158–32167.
- Nilius B, Watanabe H, Vriens J. 2003. The TRPV4 channel: structure-function relationship and promiscuous gating behavior. *Pflugers Arch*. 446:298–303.
- Olson SY, Garbán HJ. 2008. Regulation of apoptosis-related genes by nitric oxide in cancer. [Review]. *Nitric Oxide*. 19:170–176.
- Robertson RM, Sillar KT. 2009. The nitric Oxide/cGMP pathway tunes the thermosensitivity of swimming motor patterns in *Xenopus laevis* tadpoles. *J Neurosci*. 29:13945–13951.
- Strotmann R, Schultz G, Plant TD. 2003. Ca²⁺-dependent potentiation of the nonselective cation channel TRPV4 is mediated by a C-terminal calmodulin binding site. *J Biol Chem*. 278:26541–26549.
- Sun J, Xin C, Eu JP, Stamler JS, Meissner G. 2001. Cysteine-3635 is responsible for skeletal muscle ryanodine receptor modulation by NO. *Proc Natl Acad Sci U S A*. 98:11158–11162.
- Sun J, Morgan M, Shen RF, Steenbergen C, Murphy E. 2007. Preconditioning results in S-nitrosylation of proteins involved in regulation of mitochondrial energetics and calcium transport. *Circ Res*. 101:1155–1163.
- Suzuki M, Mizuno A, Kodaira K, Imai M. 2003a. Impaired pressure sensation in mice lacking TRPV4. *J Biol Chem*. 278:22664–22668.
- Suzuki M, Hirao A, Mizuno A. 2003b. Microtubule-associated [corrected] protein 7 increases the membrane expression of transient receptor potential vanilloid 4 (TRPV4). *J Biol Chem*. 278:51448–51453.
- Teixeira CC, Agoston H, Beier F. 2008. Nitric oxide, C-type natriuretic peptide and cGMP as regulators of endochondral ossification. [Review] *Dev Biol*. 319:171–178.
- Vennekens R, Owsianik G, Nilius B. 2008. Vanilloid transient receptor potential cation channels: an overview. *Curr Pharm Des*. 14:18–31.
- Watanabe H, Davis JB, Smart D, Jerman JC, Smith GD, Hayes P, Vriens J, Cairns W, Wissenbach U, Prenen J, et al. 2002. Activation of TRPV4 channels (hVRL-2/mTRP12) by phorbol derivatives. *J Biol Chem*. 277:13569–13577.
- Watanabe H, Vriens J, Prenen J, Droogmans G, Voets T, Nilius B. 2003. Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. *Nature*. 424:434–438.
- Wegierski T, Lewandrowski U, Müller B, Sickmann A, Walz G. 2009. Tyrosin phosphorylation modulates the activity of TRPV4 in response to defined stimuli. *J Biol Chem*. 284:2923–2933.
- Yoshida T, Inoue R, Morii T, Takahashi N, Yamamoto S, Hara Y, Tominaga M, Shimizu S, Sato Y, Mori Y. 2006. Nitric oxide activates TRP channels by cysteine S-nitrosylation. *Nat Chem Biol* 2:596–607.