Animal Cells and Systems
Vol. 14, No. 2, June 2010, 99–114

Taylor & Francis
Taylor & Francis Group

The modulation of TRPV4 channel activity through its Ser 824 residue phosphorylation by SGK1

Eun Jeoung Lee[a,c], Sung Hwa Shin[a], Jaesun Chun[b], Sunghee Hyun[c], Yangmi Kim[d] and Sang Sun Kang[a,e]*

[a]*Department of Biology Education, Chungbuk National University, 410 Seongbong Road, Heungdok-gu, Cheongju, Chungbuk 361-763, Republic of Korea;* [b]*Department of Biology Education, Korea National University of Education, Cheongwon, Chungbuk 363-791, Republic of Korea;* [c]*Department of Pre-medicine, Eulji University School of Medicine, Daejeon 301-832, Republic of Korea;* [d]*Department of Physiology, College of Medicine, Chungbuk National University, 410 Seongbong Road, Heungdok-gu, Cheongju, Chungbuk 361-763, Republic of Korea;* [e]*Biotechnology Research Institute, Chungbuk National University, 410 Seongbong Road, Heungdok-gu, Cheongju, Chungbuk 361-763, Republic of Korea*

(*Received 17 February 2010; final version received 31 March 2010*)

With the consensus sequence information of the serum glucocorticoid-induced protein kinase-1 (SGK1) phosphorylation site {R-X-R-X-X-(S/T)Φ; where Φ is any hydrophobic amino acid}, we noticed that the transient receptor potential vanilloid 4 (TRPV4) cation channel, a member of the TRP vanilloid subfamily, harbors the putative SGK1 phosphorylation site (on its Ser 824). We have demonstrated that TRPV4 is an SGK1 authentic substrate protein, with the phosphorylation on the Ser 824 of TRPV4 by SGK1. Further, using TRPV4 mutants (S824A and S824D), we noted that the modification of the Ser 824 activates its Ca^{2+} entry, and sensitizes the TRPV4 channel to 4-α-phorbol 12,13-didecanoate (4-αPDD) or heat, simultaneously enhancing its active state. Additionally, we determined that the modification of the Ser 824 controls both its plasma membrane localization and its protein interactions with calmodulin. Thus, we have proposed herein that phosphorylation on the Ser 824 of TRPV4 is one of the control points for the regulation of its functions.

Keywords: TRPV4; SGK1; phosphorylation; calmodulin

Introduction

The transient receptor potential vanilloid 4 (TRPV4) cation channel, a member of the TRP vanilloid subfamily, is expressed in a broad range of tissues, in which it contributes to the generation of a Ca^{2+} signal and/or the depolarization of membrane potential (Nilius et al. 2003; Cohen 2006; Vennekens et al. 2008; Everaerts 2009). The participation of TRPV4 in osmo- and mechanotransduction is relevant to several important functions, including cellular and systemic volume homeostasis, arterial dilatation, nociception, bladder voiding, and the regulation of ciliary beat frequency (Suzuki et al. 2003a, b; Andrade et al. 2005; Becker et al. 2005; Earley et al. 2005; Birder et al. 2007). TRPV4 channel activity can be sensitized via the co-application of a variety of stimuli and by the participation of a number of cell signaling pathways, which is suggestive of the presence of different regulatory sites (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), with no lysine protein kinase, and pacsin3 (Strotmann et al. 2003; Suzuki et al. 2003a, b; Cuajungco et al. 2006; Fu et al. 2006). Fernandes et al. have demonstrated a functional and physical interaction between inositol trisphosphate receptor 3 and TRPV4, which sensitizes the latter to the mechano- and osmotransducing messenger, 5′,6′-epoxieicosatrienoic acid (Nilius et al. 2003; Cohen 2006; Birder et al. 2007; Everaerts 2009; Vennekens et al. 2008). TRPV4 responds to temperature, endogenous arachidonic acid metabolites, and phorbol esters, including 4-α-phorbol 12,13-didecanoate (4-αPDD), and participates in receptor-operated Ca^{2+} entry, thus evidencing multiple modes of activation (Watanabe et al. 2002, 2003; Fernandes et al. 2008). However, the precise manner in which TRPV4 is regulated in the cell by these protein interactions, chemicals, and stimuli remains to be thoroughly elucidated.

Serum glucocorticoid induced protein kinase1 (SGK1) is a Ser/Thr protein kinase which is transcriptionally regulated by serum and/or glucocorticoids in mammary epithelial cells and by Rat-2 fibroblasts (Webster et al. 1993; Maiyar et al. 1996). SGK1 is also transcriptionally regulated by corticosteroids in several types of cells (Chen et al. 1999). The SGK1 protein has previously been reported to promote cell survival; in this regard, it serves essentially

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

ISSN 1976-8354 print/ISSN 2151-2485 online
© 2010 Korean Society for Integrative Biology
DOI: 10.1080/19768354.2010.486939
http://www.informaworld.com

the same function as Akt kinase (Casamayor et al. 1999). A few SGK1 substrates have been characterized thus far, and it appears that they, in concert with SGK1, play a role in propagating the effects of PI3K, which include the promotion of cell-cycle progression and, consequently, cell survival (Tessier and Woodgett 2006). We also determined that SGK1 performs a function in the phosphorylation of MEKK3, FE65 and Tau, thus, regulates their activities (Chun et al. 2003, 2004; Lee et al. 2008). However, many physiological SGK1 substrates remain to be identified, and the manner in which they are modulated in the presence of SGK1 remains to be clearly elucidated. The consensus SGK1 substrate site is described by the structural formula {R-X-R-X-X-(S/T)Φ}, similar to the substrate specificity of Akt kinase. The arginine residues are conserved at positions -5 and -3 relative to the positions of the Ser/Thr residues that are phosphorylated in the presence of SGK1. Proteins harboring such amino acid sequences appear to function as potent SGK1 substrates (Kobayashi et al. 1999; Murray et al. 2005).

Because the Ser824 residue of TRPV4 has been detected in the consensus SGK1 substrate sequences {R-X-R-X-X-(S/T)Φ} (Liedtke et al. 2000), we attempted to ascertain whether or not SGK1 phosphorylates the Ser 824 residue of TRPV4, as one of its specific substrate proteins. In this study, we demonstrated that the SGK1-mediated modification of the TRPV4 Ser 824 residue exerts a synergistic effect on its functional Ca^{2+} entry, as well as its reactivity to 4-αPDD, interactions with CaM, subcellular localization, and cell survival.

Materials and methods

Site-directed mutagenesis

In order to obtain the mutants, amino acid changes were introduced using mutated oligonucleotides for S/A (up 5′-agg gat cgt tgg Gcc Gcg gtg gtg ccc cgc gta-3′, down 5′ -gcg ggg cac cac cgC ggC cca acg atc cct acg-3′) or S/D (up 5′-agg gat cgt tgg GAc GAC gtg gtg ccc cgc gta -3′, down 5′-gcg ggg cac cac GTC gTC cca acg atc cct acg-3′) and wild-type TRPV4 as a template. The TRPV4 mutant constructs were conducted using the QuikChange®XL Site-Directed Mutagenesis Kit (Stratagene). To obtain truncated TRPV4 (aa718–871), we used primers (up 5′-ata gga tcc atg ggt gag acc gtg ggc cag-3′, down 5′–gg ttg gat cca cag tgg ggc atc gtc cgt-3′). All TRPV4 mutants were confirmed by DNA sequencing. Human embryonic kidney (HEK 293) cells were transfected with TRPV4 and with mutant constructs as previously described.

Electrophysiology

Patch-clamp recordings on single transfected HEK 293 cells were conducted 1 day after transfection, as described previously (Strotmann et al. 2003; Suzuki et al. 2003a, b; Cuajungco et al. 2006; Fu et al. 2006). Voltage clamping was performed using an Axopatch-200B (Axon Instruments, Union City, CA). For whole cell recording, the pipette solution contained (in mM): 100 aspartic acid, 20 $CsCl_2$, 10 HEPES, 1 $MgCl_2$, 4 Na-ATP, 10 BAPTA (pH 7.4 with NaOH). The bath solution contained (in mM): 150 NaCl, 6 CsCl, 1 $MgCl_2$, 5 $CaCl_2$, 10 glucose, 10 HEPES (pH 7.4 with NaOH). Whole-cell configuration was formed by rupturing the membrane by applying suction. Whole cell currents were recorded every 10 s via the application of a 300-ms ramp pulse from −100 mV to +100 mV from a holding potential of 0 mV. For single-channel recordings, the pipette solution contained (in mM): 150 NaCl, 1 $MgCl_2$, 10 HEPES, pH adjusted to 7.4 with NaOH. The bath solution contained (in mM) 150 KCl, 5 $MgCl_2$, 5 $CaCl_2$, 10 HEPES, 10 glucose for cell-attached patches. The holding potential was −60 mV for single-channel recording. The recorded signal was filtered at 2 kHz and transferred to a computer using the digidata 1322A interface (Axon Instruments, Union City, CA) at a sampling rate of 10 kHz. Single-channel currents were analyzed with the pClamp program (Version 9.02, Axon Instruments, Union City, CA). Data are expressed as the means ± SEM. All experiments were conducted at room temperature (22–24°C). Perfusion rate was 1 ml/min. The SGK1 inhibitors bisindolylmaleimide and the 4-αPDD were acquired from Calbiochem Co.

Glutathione S-transferase (GST)-TRPV4 fusion proteins and pull-down assays

TRPV4 sequences were PCR-amplified, subcloned into pGEX-5X-1, sequenced, and expressed in *Escherichia coli* BL21. S824A-agarose or GST-TRPV4 fusion proteins bound to glutathione-Sepharose were equilibrated in TBS buffer containing 0.1% Triton X-100 and 1 mM $CaCl_2$ or 2 mM EGTA. Incubation with the total cell lysates or S824A was followed by three washes with the appropriate buffers, and bound proteins were eluted with sample buffer, subjected to SDS gel-electrophoresis, and exposed to X-ray film (Fuji Las 3000 mini, Japan).

Phosphorylation assay with SGK1

The GST C-terminal domain (aa718-871) of TRPV4 WT or S824A was expressed in *E. coli* and purified with the glutathione affinity bead. The fusion protein was phosphorylated with SGK1 (Santa Cruz

Biotechnology) in a buffer containing 20 mM Tris HCl, pH 7.5, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 5 μM ATP, 100 μg/ml l-α-phosphatidyl-L-serine in a final volume of 20 μl. The reaction was initiated via the addition of 2 nM SGK1. After 3 min of incubation at 30°C, the reaction was halted via the addition of sample buffer. After SDS-PAGE electrophoresis, the phosphorylation of protein was detected with an anti-phosphor Ser-specific antibody. To assess the phosphorylation of TRPV4 WT and S824A including the His epitope was expressed in HEK 293cells, harvested 24 h after transfection, and membrane proteins were solubilized in 50 mM TrisHCl, pH 7.5, 150 mM NaCl, and 2.5% polyoxyethylene lauryl ether (Lubrol) with a mixture of protease inhibitors, and the tagged proteins were purified with nickel-agarose (Invitrogen, USA). TRPV4 phosphorylation was conducted as described above using a 1/50 volume of the nickel-agarose eluate and 4 nM SGK1 for 20 min at 37°C in 80 μl. The reaction was halted via the addition of sample buffer, and Western blotting was conducted with an anti-phosphor Ser residue-specific antibody which was purchased from AbChem Co.

Fluorescence measurements of $[Ca^{2+}]_I$

We measured $[Ca^{2+}]_i$ using the fluorescent Ca^{2+} indicator Fluo4-acetoxymethyl ester (Fluo4-AM) as described previously. In brief, cells growing on coverslips were incubated for 40 min in DMSO solution containing 1 μM 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) at wavelengths of 495 nm (excitation) and 519 nm (emission). The absorption (as an arbitrary unit) at 488 nm by argon-ion laser was measured as a relative intracellular Ca^{2+} ion concentration $[Ca^{2+}]_i$. All experiments were conducted at 24°C. After stimulating with mild heat (from 24 to 42°C within 45 s for 2 min), $[Ca^{2+}]_i$ was measured in single cells in a 24°C solution (Birder et al. 2007).

Solutions and drugs

Cells were superfused normally with a solution containing (mM): 88 NaCl, 5 KCl, 5.5 glucose, 1 $CaCl_2$, 10 HEPES and 100 mannitol, adjusted to pH 7.4 with NaOH (300 mosm kg^{-1} H_2O). The HTS was adjusted to 200 mosm kg^{-1} H_2O by omitting mannitol. Fluo4-AM, wortmannin, insulin, and 4-αPDD were acquired from Sigma (St Louis, MO). Stock solutions of phorbol esters were initially prepared in dimethyl sulphoxide (DMSO) at a concentration of 1 mM, and then stored at −20°C. The final DMSO concentration in the experimental bath solution containing phorbol esters never exceeded 0.5%.

Statistics

Data are expressed as the means ± standard error of the mean (s.e.m.). Statistical significance was determined via Student's *t*-test or Welch's *t*-test. We utilized the Mann–Whitney test to compare the effects of 4-αPDD to normal conditions. Values of $P < 0.05$ were considered statistically significant.

FACS

His TRPV4 (wt), TRPV4 S824A, S824D mutant, His vector was transfected into cells and the rates of apoptosis were measured with an Annexin V-PE apoptosis detection kit I (BD Biosciences, USA). Transfected cells were washed twice in cold PBS and resuspended in binding buffer (0.01 M Hepes/NaOH (pH 7.4) 0.14 M NaCl, 2.5 mM $CaCl_2$). Cells (1×10^5) in 100 ml were transferred to 5-ml culture tubes and 5 ml of Annexin V-PE and 5 ml of 7-aminoactinomycin D were added. The cells were vortexed gently and incubated for 15 min at 25°C in the dark. Binding buffer (400 ml) was added to each tube. Within 1 h, FACS was performed on a Coulter Epics Elite equipped with a gated amplifier and upgraded to give enhanced system performance in the Core Facility of Chungbuk National University (Lee et al. 2008).

Results

The phosphorylation on Ser 824 residue in (^{819}RdRwsSV^{825}) of TRPV4 by SGK1

The participation of the transient receptor potential vanilloid 4 (TRPV4) in osmo- and mechanotransduction contributes to a variety of 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). The regulation of TRPV4 has been shown to occur via posttranslational modifications (phosphorylation, glycosylation, and ubiquitinization) and protein–protein interactions, including ankyrin, pacsin3, calmodulin (Figure 1A) (Strotmann et al. 2003; Suzuki et al. 2003a, b; Cuajungco et al. 2006; Fu et al. 2006; Fernandes et al. 2008). After recognizing the putative phosphorylation site (Ser 824 residue) of TRPV4 with the consensus sequence information {R-X-R-X-X-(S/T)Φ} (Figure 1A,B), we evaluated both its phosphorylation and its biological significance. We subsequently

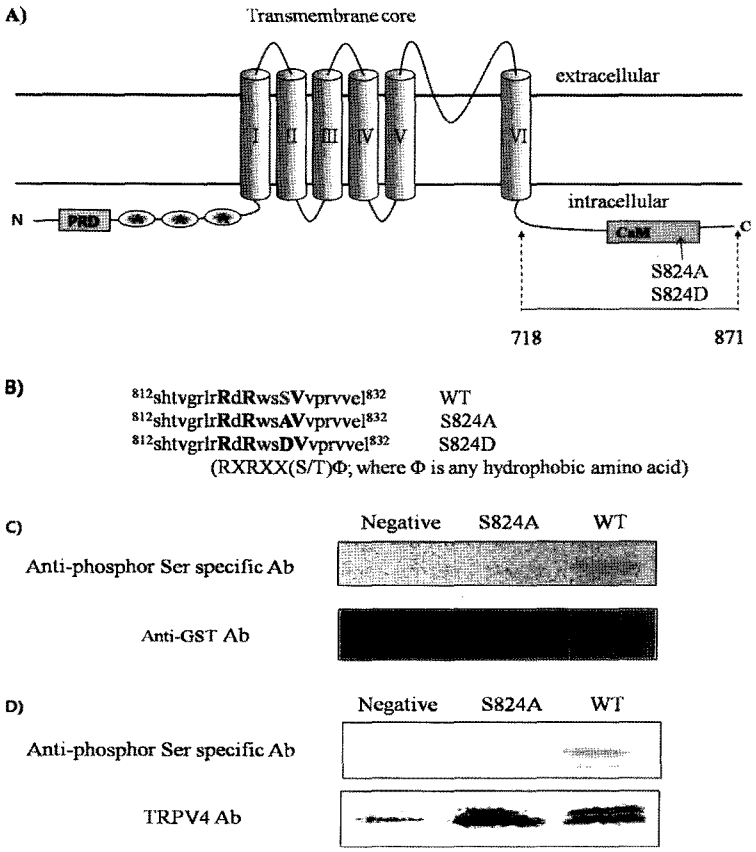

Figure 1. The phosphorylation of TRPV4 in response to activation of SGK1. (A) Transmembrane topology of the mouse TRPV4 (871aa length). Indicated are the three ankyrin binding repeats (ANK; as gray circles), the six trans-membrane regions (TM1–TM6), the Ca^{2+}/CaM binding site (CaM), and the putative SGK1 phosphorylation site (S814). The putative cytoplasmic region of TRPV4 (718–871aa) is also indicated. The putative SGK1 phosphorylation site (Ser 824) is found in CaM (812–832aa) of the TRPV4 channel. The line indicates the GST C-terminal cytoplasmic region of TRPV4 (718–871aa) or mutant (S824A) fusion protein, TRPV4 mutant site (S824A or S824D) is compared with its wild-type (WT; Gene Bank no. BC127052). The arrow points to phosphorylation protein by SGK1. TRPV4 S824A or S824D mutant was used as the unphosphorylated or phosphorylated TRPV4 analog form, respectively. (B) The alignment of TRPV4 WT, S824A, and S824D with the consensus SGK1 substrate motif. The putative SGK1 phosphorylation site (Ser 824) of TRPV4 is located in the specific conserved SGK1 substrate {R-X-R-X-X-(S/T)Φ}. With site-directed mutagenesis, S824A and S824D were constructed and aligned with WT and SGK1 substrate-specific motif. (C) SGK1 kinase assay with GST-TRPV4 fusion proteins. With GST-TRPV4 WT or mutant (S824A or S824D) fusion protein purified from *E. coli*, SGK1 assay was performed. After electrophoresis, Western blotting was conducted with an anti-phosphor Ser-specific antibody. The arrow points to the phosphorylation protein by SGK1. The picture below represents the Western blot results, using a TRPV4 antibody in the same samples. (D) The phosphorylation of the Ser 824 residue in (RdRwsSV) of TRPV4 in HEK 293 cells. The host cells were transiently transfected with His-TRPV4 WT or S824A plasmid. After 48 h, the cells were lysed, total proteins were re-collected, and immunoprecipitation was conducted with His monoclonal mouse antibody. Western blot assays were then conducted with a rabbit TRPV4 Ab or an anti-phosphor Ser-specific antibody in order to confirm whether the Ser 824 residue in (RdRwsSV) of TRPV4 could be phosphorylated by SGK1 in HEK 293 cells. After electrophoresis of the immunoprecipitants with TRPV4 Ab, Western blotting was performed with an anti-phosphor Ser specific antibody. The negative control was the vector (pcDNA 3.0) alone.

attempted to pinpoint the target Ser residue for the phosphorylation on Ser 824 {R-X-R-X-X-(S/T)Φ} or Ser 824 of TRPV4. Because Ser residues that are subject to phosphorylation have been demonstrated in a number of studies to reside within the consensus motif, we initially placed our focus on Ser 824. After determining the SGK1 consensus sequences in TRPV4 (Figure 1A,B), we attempted to determine whether or not TRPV4 is phosphorylated by SGK1 with its protein kinase assay in vitro (Figure 1C,D).

GST-tagged mouse C-terminal (aa 718–871) TRPV4 WT fusion protein and its S824A mutant fusion protein were expressed in *E. coli*, purified with glutathione beads, and utilized as substrate proteins in

the SGK1 phosphorylation assay to determine whether SGK1 phosphorylation occurred on the S^{824} residue in ^{819}RdRwsSV^{825} of TRPV4. As is shown in Figure 1C top lane, we detected phosphorylation on the C-terminal (aa 718–871) fusion protein of TRPV4 WT, but not on its TRPV4 S824A mutant with anti-phosphor Ser-specific antibody in the Western blot experiment. To monitor the amount TRPV4 fusion, a Western blot was also performed with an anti-GST antibody (Figure 1C bottom). Thus, these results indicated that the S^{824} residue in ^{819}RdRwsSV^{825} of TRPV4 is the SGK1 phosphorylation site.

In order to further confirm the SGK1 phosphorylation of TRPV4 in a HEK 293 cell, we conducted Western blotting on the immunopurified TRPV4 from the HEK 293 cell using anti-phosphor Ser-specific antibody, as described in the Materials and methods section. As is shown in Figure 1D, the SGK1 phosphorylation of TRPV4 was detected as high molecular weight protein bands (top lane), similar to the results shown in Figure 1C. For the negative control, an unrelated mouse antibody was employed (left lane). Together, these results suggested that the S^{824} residue of TRPV4 is one of SGK1 phosphorylation site.

The effect of IGF1 or wortmannin on TRPV4 activity

Next, we determined the effect of phosphorylation on Ser 824 residue of TRPV4 with IGF1 or wortmannin treatment (Figure 2). Because SGK1 is an effecter of the downstream of PI3 kinase, we used IGF1 or wortmannin as a modulator of SGK1 activity to delineate the TRPV4 regulation mechanism (Casamayor et al. 1999; Chen et al. 1999; Tessier and Woodgett 2006). The single-channel current elicited in the TRPV4 WT was recorded. We observed that the application of IGF1 (at 100 μM) enhanced the relative channel activity of TRPV4 approximately 5-fold from 4.12 ± 0.23 to 24.8 ± 2.51 pA (Figure 2A, $n = 6$), whereas wortmannin (at 100 nM) reduced the TRPV4 WT channel activity approximately 0.5-fold from 17.4 ± 4.3 to 8.12 ± 2.4 (Figure 2B, $n = 6$). After IGF1 or wortmannin treatment, the cell was washed with a normal buffer (wash) to monitor the cell state. After washing, the single-channel activity of TRPV4 with the IGF1 treatment remained active (Figure 2A), while that of TRPV4 with wortmannin treatment was returned to the control state (Figure 2B). Thus, considering that the difference of channel activity was significant after IGF1 or wortmannin treatment, it seems to be that the modulation of TRPV4 channel activity with these treatments is due to the SGK1 phosphorylation on its S^{824} residue (Figure 1).

The enhancement of TRPV4 activity and its sensitivity to 4-αPDD through S824D or S824A mutation

In order to determine the effects of Ser824 phosphorylation on its channel activity in HEK 293 cells, we conducted a patch clamp experiment to assess the functional consequences of TRPV4 WT, S824A (as analogous of the TRPV4 Ser 824 unphosphorylation form) or S824D (as analogous of the TRPV4 Ser 824 phosphorylation form) channel. We used HEK 293 cells as the host, because it is known that this cell line does not express its endogenous TRPV4 (Strotmann et al. 2003; Suzuki et al. 2003a, b; Andrade et al. 2005). As shown in Figure 3A, the channel open probability (P_O) elicited in the TRPV4 S824D mutant (bottom lane, P_O; 0.95 ± 0.31) was more than 5-times the P_O of TRPV4 WT (top lane, P_O; 0.18 ± 0.02), and the relative channel activity of the TRPV4 was enhanced approximately 2-fold (from 11.2 ± 0.2 to 23.8 ± 2.5 pA, $n = 6$), with the application of 4-αPDD which is known as a PKC independent TRPV4 activator (Birder et al. 2007; Fernandes et al. 2008; Watanabe et al. 2002; Watanabe

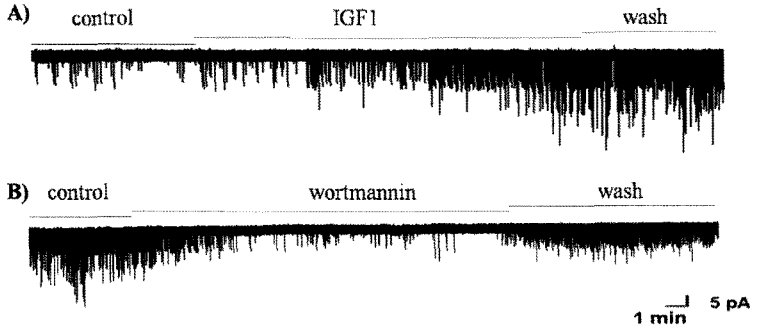

Figure 2. TRPV4 WT channel activity change in response to IGF1 or wortmannin. The single-channel activity change of TRPV4 by IGF1 (A) or wortmannin (B) treatment. Cells were exposed to IGF1or wortmannin by the perfusion of extracellular solution containing 1 μM IGF or 300 nM wortmannin. Expressed TRPV4 currents were recorded with a cell-attached patch, using an Axopatch 200B amplifier (Axon Instruments). The holding potential was − mV and the perfusion rate (1ml/min).

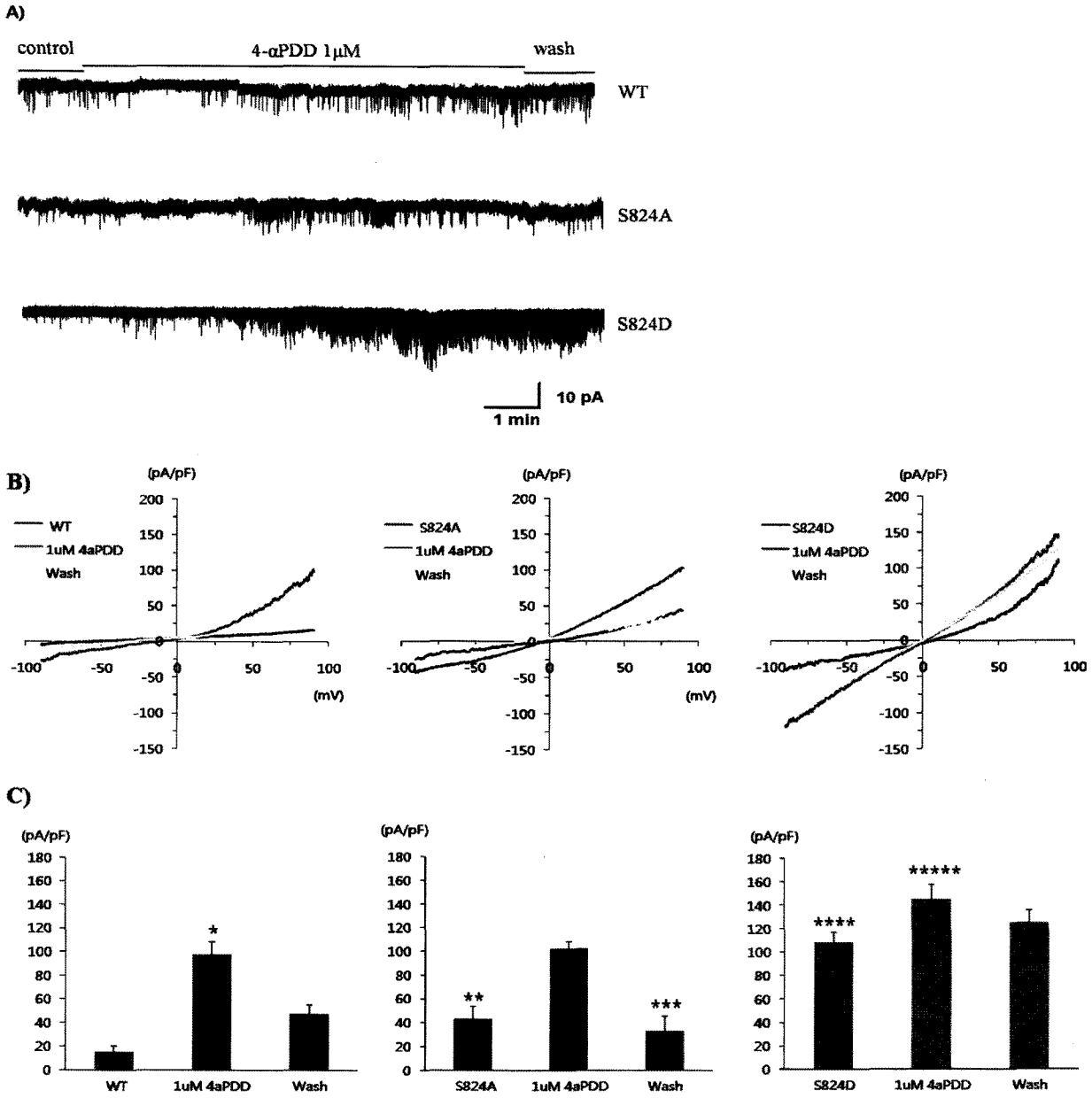

Figure 3. The comparison of TRPV4 wt, TRPV4 S824A and TRPV4 S824D single-channel properties in HEK 293 cells. HEK 293 cells were transfected with either TRPV4WT/pcDNA3.1, TRPV4 (S824A)/pcDNA3.1, or TRPV4 (S824D)/pcDNA3.1, using ExGen500 (Fermentas) in accordance with the manufacturer's instructions.(A) Expressed TRPV4 currents were recorded with a cell-attached patch using an Axopatch 200B amplifier (Axon Instruments). Single TRPV4 currents were recorded a holding potential (Vh) of −60 mV. (B) Current-voltage relations of peak whole-cell cationic currents recorded from different HEK 293 cells transfected with TRPV4 WT, TRPV4 S824A or TRPV4 S824D and exposed to 1 μM 4-α PDD, dialyzed with normal buffer again. Normalized (pA/pF) current traces in response to a voltage step to 80 mV for TRPV4 WT, S824A, and S824D constructs. (C) The bar graph shows the difference in relative channel activity of TRPV4 WT, TRPV4 S824A or TRPV4 S824D with/without 4-α PDD treatment. Mean current densities (±S.E.) at +80 mV in HEK 293 cells expressing TRPV4-WT, and TRPV4 S824A and dialyzed with 1 nM 4-αPDD or washed with normal buffer. Mean current density obtained from HEK 293 cells overexpressing TRPV4-WT and dialyzed with 1 nM 4-α PDD ($n = 6$) is also shown, with the perfusion rate (1 ml/min). *$P < 0.05$ versus the control. **$P < 0.05$ versus TRPV4 WT or SD. ***$P < 0.05$ versus TRPV4 SA with 1μM 4-α PDD. ****$P < 0.05$ versus TRPV4 WT or SA. *****$P < 0.05$ versus TRPV4 SD.

et al. 2003). However, 4-α PDD at 1 μM only enhanced P_O of TRPV4 S824A channel (middle lane, 0.37±0.09) twice than that of TRPV4 WT (0.18±0.02), without great change of amplitude (Figure 3A). Thus, the single-channel activity of TRPV4 S824D mutant was increased, as compared with the WT and S824A

variant, thereby supporting the notion that the Ser 824 residue is a major regulation site in TRPV4, and the phosphorylation of TRPV4 Ser 824 is likely to directly gate TRPV4 and contributes to a prolongation of its active stage as a Ca^{2+} channel.

Next, we compared the whole-cell current property with TRPV4 WT, TRPV4 S824A, or S824D mutant (Figure 3B) to obtain more information on the role of Ser 824 phosphorylation. As expected from the single-channel current in Figure 3A, the basal current elicited in the TRPV4 S824D mutant (right, 115 $\pm$12 pA/pF, $n=6$) was more than 6-times the amplitude of TRPV4 WT (left lane, 17$\pm$4 pA/pF, $n=6$) without any treatment (Figure 3B black line). However, the current induced by 1 μM of 4-αPDD showed a dramatic reduction of difference between TRPV4 WT and S824D (Figure 3B, red line, TRPV4 WT; 106$\pm$8 pA/pF, $n=6$, S824D: 150$\pm$14 pA/pF, $n=6$). After washing, however, the current of TRPV4 S824D was not reduced more than that of WT (green line), suggesting that TRPV4 S824D has more affinity to 4-αPDD than WT.

Consistent with the single-channel current result (Figure 3A), the basal whole cell current amplitude elicited in the TRPV4 S824A mutant (46$\pm$6 pA/pF, $n=6$) was 2.7-times higher than that of TRPV4 WT (17$\pm$4 pA/pF, $n=6$), (Figure 3B bottom; black line). This result, interestingly, suggested that the activation of TRPV4 channel does not solely depend on the phosphorylation of the Ser 824 residue, but that other regulation (inactivation) mechanisms, such as the protein–protein interaction with the C-terminal domain of TRPV4 (see the next discussion section), are required. After stimulation with 4-αPDD, the difference in extent of channel activation between he TRPV4 S824A mutant and the WT was not observed (red line), similar to the single-channel result (Figure 3A,B). However, the current of TRPV4 S824A (green line) was reduced to the basal level (black line) in the wash stage, suggesting that TRPV4 S824A has less affinity to 4-αPDD than WT. Thus, these findings (Figure 3B) with the previouslynoted single-channel properties (Figure 3A) indicated that phosphorylation on the Ser 824 residue enhances TRPV4 activity and prolongs its period of activity, after the stimulation with 4-αPDD.

Ca^{2+} image analysis of TRPV4 WT, S824A or S824D after 4-αPDD or heat stimulation

It has been suggested that TRPV4 forms constitutively open ion channels which show an inwardly rectifying current–voltage relationship with a high selectivity of calcium over monovalent cations. In addition, their activity has been correlated with the electrochemical driving force for Ca^{2+} and the level of intracellular Ca^{2+} ($[Ca^{2+}]_i$), which is suggestive of the existence of calcium-dependent regulatory mechanisms (Strotmann et al. 2003; Suzuki et al. 2003a, b; Cuajungco et al. 2006; Fernandes et al. 2008). To determine the consequences of the phosphorylation on the Ser 824 residue of TRPV4, we employed a Ca^{2+} image experiment (Figure 4 and Supplementary Figures 1–3, available on the Supplementary content tab on the article's online page at http://dx.doi.org/10.1080/19768354.2010.486939). The application of 4-αPDD also increased $[Ca^{2+}]_i$ of TRPV4 WT, S824A, and S824D, similar to the electric current result in Figure 3. Although all three recombinant TRPV4 showed a robust elevation of $[Ca^{2+}]_i$ in response to 4-αPDD treatment of HEK 293 cells, the difference in its responding pattern was as dramatic in TRPV4 (Figure 4). Comparing the optical response change (as arbitrary $[Ca^{2+}]_i$ concentration;% unit to the maximum OD) of TRPV4 S824D with that of WT at a given time period (30 min) of 1 μM 4-αPDD treatment, it contributes 4.3-times higher $[Ca^{2+}]_i$ than TRPV4 WT does (Figure 4B). Interestingly, TRPV4 S824D did not decrease (or was not inactivated), after rising (Figure 4A right, and Supplementary Figure 3), suggesting that TRPV4 S824D is a constitutively active stage (because it is defective in the inactivation step). However, the representative trace of TRPV4 WT showed a regular rise and fall immediately in the response signal to 4-αPDD (Figure 4A left, Supplementary Figure 1), whereas both TRPV4 D and A mutants did not respond to it with a similar pattern (Figure 4A middle and right, Supplementary Figures 2 and 3).

Consistent with the electrophysiology results in Figure 3, we also observed that $[Ca^{2+}]_i$ of TRPV4 S824A was not reduced more than that of TRPV4 WT for the given time period (30 min) with the treatment of 1 μM 4-αPDD (Figure 4A,B middle). Comparing the representative trace of the optical density of TRPV4 WT (Figure 4A left), the decline side of graph (Figure 4A, middle) was not smooth, suggesting that its Ser 824 mutation to Ala also contributes to the inactivation step of this channel protein, a similar phenomenon to the case of its S824D mutant (Figure 4A, right). This result also supported the notion that the phosphorylation on Ser 824 residue is not absolutely required for its activation. However, we do not know why the difference in $[Ca^{2+}]_i$ between TRPV4 WT and S824A is greater than the whole-cell current amplitude between them (Figure 3B) on treatment with 4-αPDD. One of the possible explanations seems to be that TRPV4 is also involved in the intracellular membrane Ca^{2+} transport (more than the plasma membrane Ca^{2+} channel), similarly to TRPV2 (Saito et al. 2007). We observed that TRPV4 S824A mutant was predominantly localized in the intracellular membrane (see the next section; Figure 6).

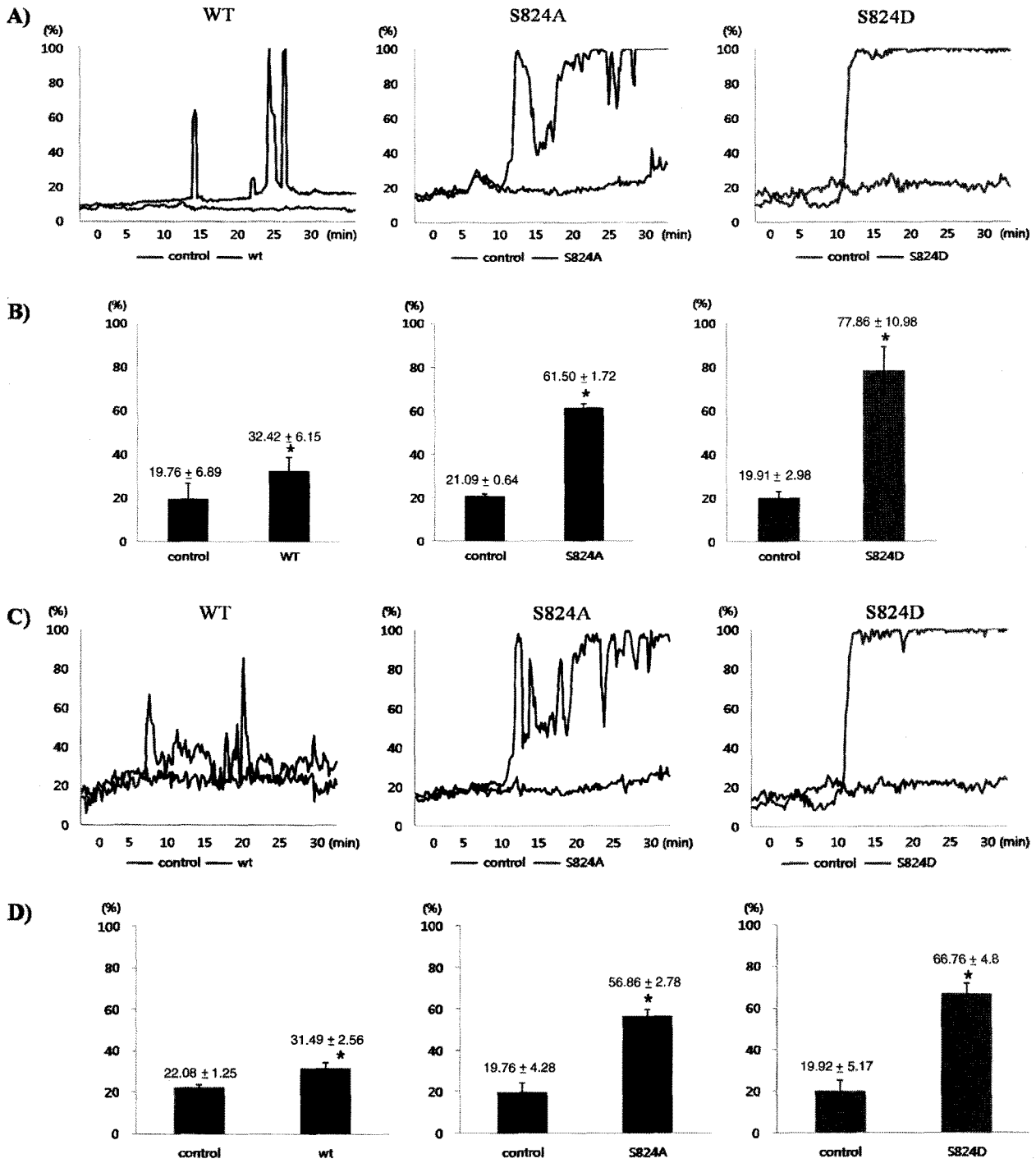

Figure 4. Effects of 4-αPDD or heat on TRPV4 WT, S824A or S824D intracellular calcium concentration change $[Ca^{2+}]_i$; expressed by the absorption at 488 nm of argon-ion laser in HEK 293 cells (as an arbitrary% unit). (A) Representative trace of the optical density showing the effect of 1 nM 4-αPDD in TRPV4-transfected or His vector-transfected (mock) HEK 293 cells. (B) Summary of $[Ca^{2+}]_i$ fluctuation caused by 4-αPDD. Each column represents the mean ± s.e.m.; numbers of cells examined are indicated above each column. Responses (with the arbitrary units;% of total area) were measured as the total area of each representative trace from 10 min to 35 of (A) minus each basal total area of the same period. (C) Representative trace of the optical density of TRPV4-transfected or His vector-transfected (mock) HEK 293 cells by heat stimulation (from 24 to 42°C for 2 min). (D) Summary of $[Ca^{2+}]_i$ fluctuation caused by mild heat stimulation. Each column represents the mean ± s.e.m.; 6 numbers of cells examined are indicated above each column. Responses (with arbitrary units;% of total area) were measured as the total area of each representative trace from 10 min to 35 min of (C) minus each basal total area of the same period. $^*P < 0.05$ versus the control.

To elucidate the TRPV4 activation/inactivation pattern further, we measured $[Ca^{2+}]_i$ with Ca^{2+} image analysis with heat (42°C for 1 min) stimulation (Figure 4C and D). The mild heat treatment also increased TRPV4 WT, S824A, and S824D current amplitudes, similar to the results after 4-αPDD treatment (Figure 4A). In comparison with the WT channel (Figure 4C left), the TRPV4 S824D (Figure 4C right) and S824A (middle) mutants exhibited 9- and 3-times increased spontaneous activity, respectively, and mediated a strong induction of Ca^{2+} entry in response to the heat (Figure 4D). In the given time period (30 min) after heat treatment, TRPV4 S824D shows a 4.3-times higher $[Ca^{2+}]_i$ than TRPV4 WT (Figure 4D). The $[Ca^{2+}]_i$ of TRPV4 S824D did not decrease for 30 min after rising (Figure 4C right), corroborating that TRPV4 S824D is in the prolonged active stage by delaying its inactivation. The representative trace patterns of TRPV4 WT, S824A or D mutants with heat treatment are similar to the results of heat stimulation (Figure 4A,C), and the replacement of the 824 Ser residue of TRPV4 with Ala or Asp enhanced its channel activity (Figure 4C and D). These changes were specific for the S824D mutant, because the spontaneous activity of the TRPV4 S824D variant (right) was comparable to that of the WT (left) or S824A mutant (middle). Taken together, these data confirmed again that the S824A/D mutant seems to be more active than WT, because of delaying (or lacking) its inactivation mechanism. However, we were unable to dismiss the possibility that the S824A/D mutation may have increased the abundance of the TRPV4 channel at the plasma (or intracellular) membrane (see the next section; Figure 6).

The comparison of TRPV4 WT, S824A or S824D interaction with CaM

To find out why TRPV4 S824A/D mutant is more active than WT, we pursued the protein which interacts with the C-terminal of TRPV4 (Figure 1). One candidate protein for our consideration is calmodulin (CaM), because it is known to be involved in the feedback dual (positive or negative) regulation of a variety of ion channels (Nilius et al. 2003; Strotmann et al. 2003; Earley et al. 2005). Further, Strotmann et al. reported that the CaM binding site (aa 811–850) is located within the C-terminal domain (aa 718–871) of TRPV4 (Strotmann et al. 2003). Thus, we tried to identify whether the Ser 824 residue affects the binding between TRPV4 and CaM. GST-fusion proteins encompassing C-terminal TRPV4 domains were constructed and expressed in *E. coli*. Approximately 2 μg of WT, S824A or S824D fusion protein (C-terminal domain (aa 718–871) of TRPV4) bound to the glutathione-Sepharose bead was incubated with HEK 293 cell lysate in the presence (or absence) of 2 mM EGTA.

As shown in Figure 5A, we observed that the GST fusion C-terminal domain of TRPV4 WT protein interacts with CaM (Figure 5A left). However, the TRPV4 C-terminal domain S824A or S824D did not pull down CaM from HEK 293 cell lysates (Figure 5A middle and right). Further, the GST fusion C-terminal domain of TRPV4 did not pull down CaM in the presence of 2 mM EGTA, suggesting that the interaction between the C-terminal domain of TRPV4 WT and CaM is dependent on the presence of Ca^{2+} (Figure 5A second left). However, the TRPV4 C-terminal domain S824A or S824D did not pull down CaM from HEK 293 cell lysates (Figure 5A middle and right).

To confirm this observation again, we performed the co-immunoprecipitation of CaM with His TRPV4 WT, S824A, or S824D in HEK 293. Similarly to the pull down experiment result in Figure 5 A, we also observed that TRPV4 S824A or S824D did not co-immunoprecipitate CaM in HEK 293 cells, while TRPV4 WT interacts with CaM, depending on 2 mM EGTA treatment (Figure 5B). Considering that TRPV4 can be phosphorylated at its CaM binding site (Figure 1) and is then unable to bind to Ca^{2+}-CaM, we wondered whether a fraction of the expressed wild-type channels had already been phosphorylated, resulting in as S824D. These channels would then be unable to bind to Ca^{2+}-CaM and would consequently reduce the effect of Ca^{2+}-CaM binding disruption. TRPV4 S824D (the phosphorylation analogous form of TRPV4 by SGK1) and Ca^{2+}-CaM binding in the activation state seem to be mutually exclusive. If indeed this is the case, all of the expressed S/A mutant TRPV4 channels, unable to become phosphorylated at their CaM-binding site, should be capable of binding to Ca^{2+}-CaM and should thus show more rapid inactivation than the wild-type channels. However, as shown in Figure 4-5, TRPV4 S824A, which does not bind with Ca^{2+}-CaM, is also an activation state, even though its activity is less than that of TRPV4 S824D. Thus, these results suggested that the interaction between the C-terminal domain of TRPV4 and CaM is dependent on the presence of Ser 824 residue and Ca^{2+} ion, not solely dependent on Ser 824 phosphorylation.

The subcellular localization of TRPV4 WT, S824A, or S824D

Other researchers reported that the phosphorylation of the TRPV4 Y110 residue is important to regulate its abundance at the plasma membrane (Wegierski et al. 2009). In an effort to determine whether or not the Ser

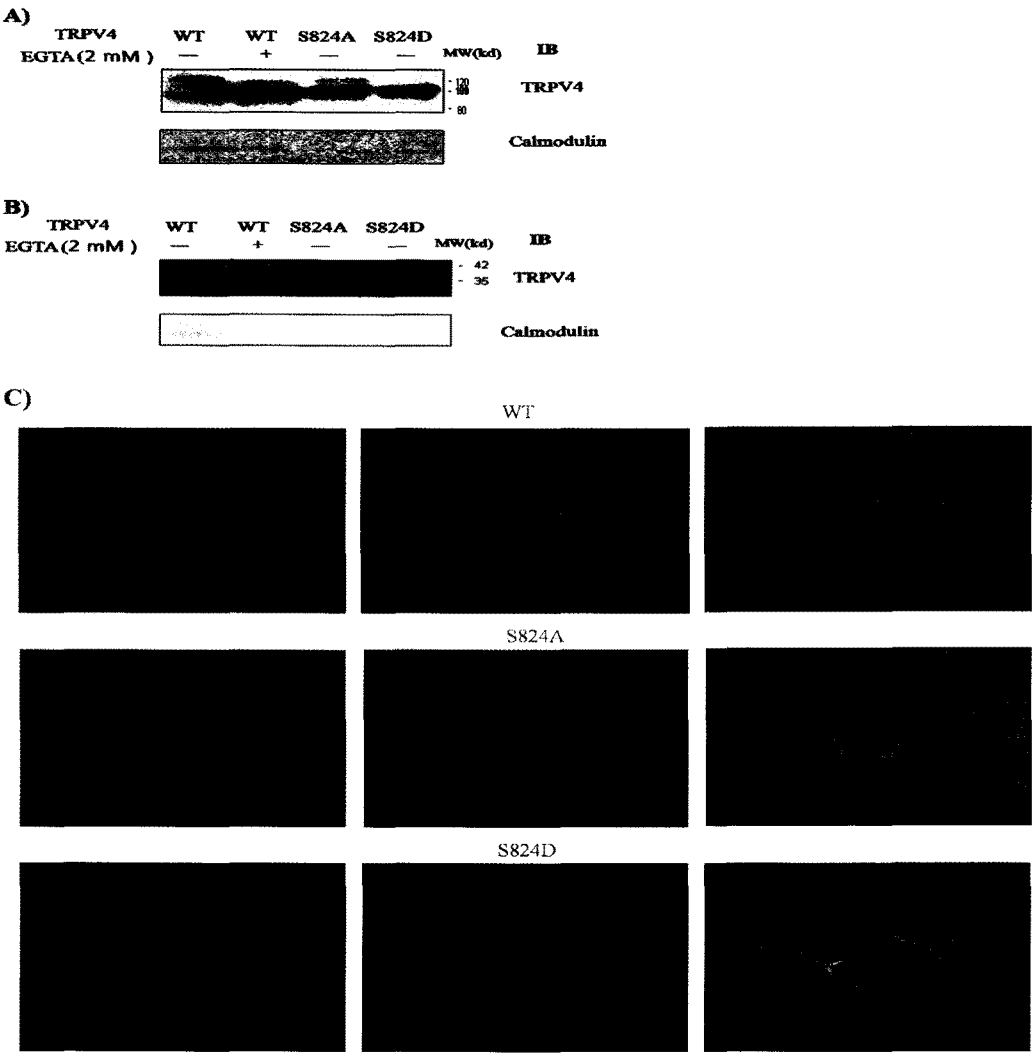

Figure 5. TRPV4 WT, S824A or S824D interaction with CaM. (A) Co-immunoprecipitation of TRPV4 WT, S824A or S824D with CaM. HEK 293 cells were transiently transfected with His-TRPV4 WT or S824A plasmid. After 48 h, the cells were lysed, total proteins were recollected, and immunoprecipitation was conducted with nickel beads. Western blot assays were then conducted with a rabbit TRPV4 Ab or an anti- CaM antibody in HEK 293 cells. (B) Pull-down analysis of CaM with TRPV4 WT, S824A or S824D. To identify in vitro whether the Ser 824 residue affects the binding between TRPV4 and CaM, GST-fusion proteins encompassing C-terminal TRPV4 domains were constructed and expressed in *E. coli*. Approximately 2 μg of WT, S824A or S824D fusion protein bound to the glutathione-Sepharose bead was incubated with HEK 293 cell lysates in the presence of 1 mM Ca^{2+} or 2 mM EGTA. (C) Confocal microscopic analysis of transfected His-TRPV4 WT or mutant (S824A or S824D) (all constructs are shown in green) with CaM (red). The transfected His-TRPV4 WT was merged as yellow. However, the transfected His-TRPV4 S824A or S824D was not merged with CaM (shown in red).

824 residue change of TRPV4 S824A and S824D is also attributable to its subcellular localization or to an expression-level deviation, we conducted the the confocal microscopic analysis of transfected His-TRPV4 WT, S824A, or S824D (all red) with direct immunofluorescence microscopy (Figure 6A). Compared with TRPV4 WT (Figure 6A left), S824A was predominantly shown in the cytoplasm (Figure 6A middle), whereas S824D was noticed at the plasma membrane (Figure 6A right).

To confirm further whether or not the Ser 824 residue change of TRPV4 S824A, S824D is also attributable to its subcellular localization or to an expression-level deviation, we conducted a confocal microscopic examination of the S824A TRPV4 WT, S824A, and S824D with Rab11b (a cytoplasmic Golgi network protein). We noticed the presence of a putative motif for Rab11b binding in the C-terminal domain of TRPV4 which is demonstrated in TRPV5 and 6 (van de Graaf et al. 2006; Fan et al. 2009), and also detected

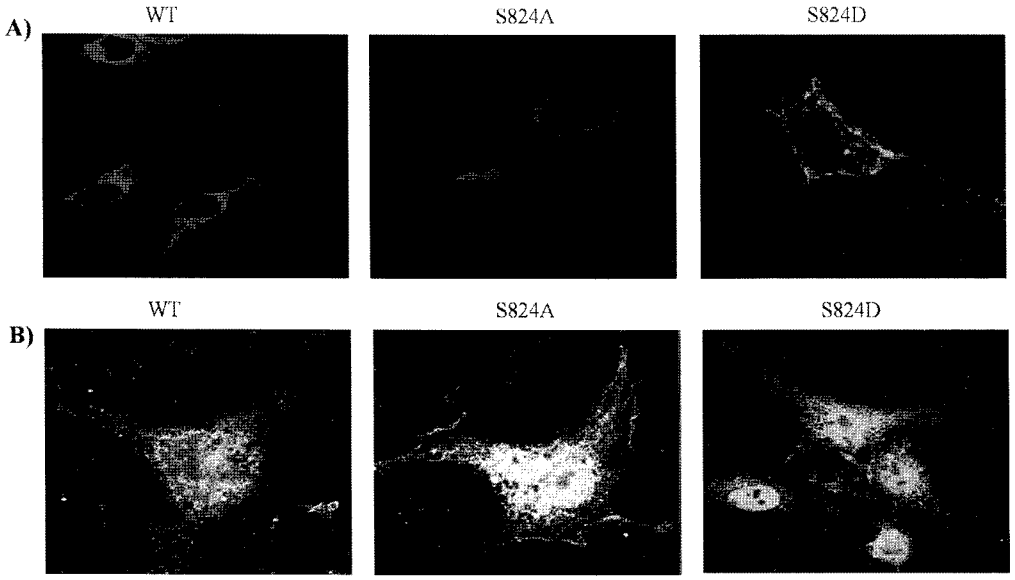

Figure 6. The subcellular localization of TRPV4 WT, TRPV4 S824A or S824D in HEK 293 cells. (A) The confocal microscopic image of transfected His-TRPV4 WT, S824A, or S824D (all red). The image was taken with the direct immunofluorescence microscopy (× 640). (B) The analysis of the merged confocal microscopic image of His-TRPV4 WT or mutant (S824A or S824D) (all constructs are shown in green) with Rab11b (shown in red). The transfected His-TRPV4 WT or S824D was less merged (yellow) with CaM at the Golgi apparatus (B left and right). However, the transfected HIS-TRPV4 (S824A) was merged with CaM at the Golgi apparatus (B middle lane), whereas His-TRPV4 S824D was principally detected in the plasma membrane (right).

the protein–protein interaction between TRPV4 and Rab11b in vitro (data not shown). Thus, we compared the subcellular localization of TRPV4 WT, S824A, and S824D with Rab11b. As is shown in Figure 6B (only merged image), we noted that the significant difference in TRPV4 WT, S824A, or S824D localization is distinguishable. Even though these TRPV4 variants (red) are found both in the plasma membrane and in the cytoplasm where Rab11b (green) is also co-localized with them (yellow), the plasma membrane localization of TRPV4 S824D (Figure 6 right) is more prominent than that of TRPV4 WT or S824A (Figure 6 right). Meanwhile, the co-localization of TRPV4 S824A and Rab11b was mainly observed in the cytoplasm (Figure 6A,B middle). Thus, as with the effect of TRPV4 tyrosine phosphorylation on its plasma membrane localization (Wegierski et al. 2009), our results also indicated that the Ser 824 residue of TRPV4 seems to be a control point for its plasma membrane localization through the regulation of its exocytosis.

The reduction of cell apoptosis rate by TRPV4 S824D mutant

His TRPV4 WT or its S824A or S824D mutant or the vector was transfected into the cells, and the apoptosis rate was determined by FACS. His-TRPV4 WT or His TRPV4 S824A or S824D or His vector was also transfected, and the apoptosis rate was determined by FACS. As shown in Figure 7A bottom, TRPV4 S824D mutant significantly promoted cell survival approximately 2-fold compared to the TRPV4? WT or S824A construct. TRPV4? S824A, which showed improved Ca^{2+} entry compared with TRPV4 WT (Figure 3 and 4) also reduced cell apoptisis (Figure 7A middle) compared with TRPV4 WT. The order of channel activity in Figure 3 and the level of $[Ca^{2+}]_i$ with TRPV4 constructs in Figure 4 were observed as S824D > S824A > WT. This order is inversely correlated with the HEK 293 cell apoptosis rate (S824D $15 \pm 2\%$ < S824A $26 \pm 2\%$ < WT $27 \pm 2\%$, $n = 6$), compared with that of control ($7 \pm 1\%$, $n = 6$) in Figure 7B. Thus, contrasting with the reports that the elevation of Ca^{2+} in pancreas or neuronal cell causes ER stress and apoptosis (Tessier and Woodgett 2006), the enhancement of TRPV4 activity (or the property of Ca^{2+} entry) and S824 phosphorylation may contribute to cell survival in HEK 293 cells synergistically, as an effect of SGK1 signal transduction (Casamayor et al. 1999; Chun et al. 2003).

In summary, these results demonstrated that TRPV4 is modulated through the phosphorylation on its 824 serine residue by SGK1, one of its authentic substrate proteins. As a consequence of SGK1 phosphorylation on Ser824 of TRPV4, Ca^{2+} channel activity was increased through its delayed inactivation, coupled with a prolongation of the active state, an

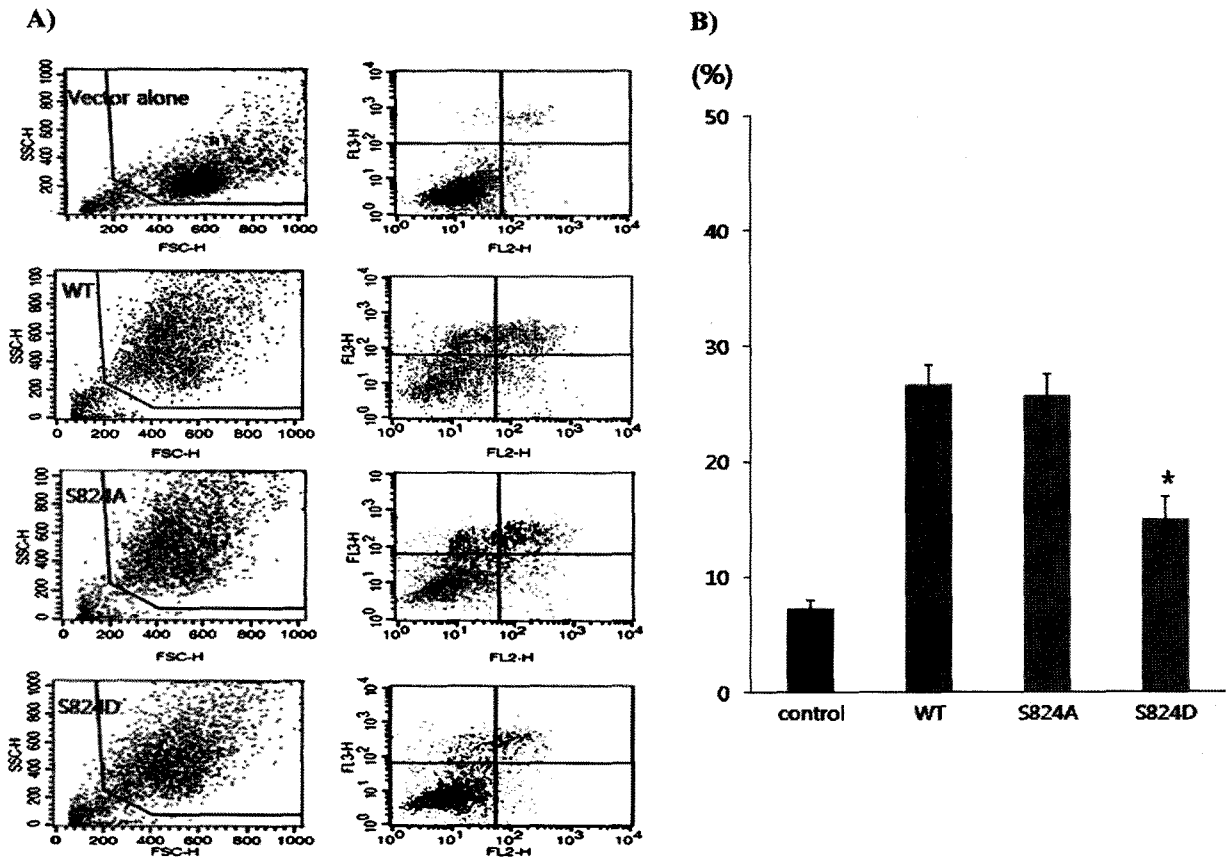

Figure 7. Comparison of TRPV4 cell viability with its WT, S824A or S824D mutant. His TRPV4 WT or its S824A or S824D mutant or His vector was transfected and the rate of apoptosis was measured by FACS. (A) One of six repeat of FACS results of FACS with the vector alone, its WT, S824A, or S824D mutant. (B) Summary of the cell apoptosis rates. His-TRPV4 S824D mutant showed cell survival rates significantly better than the TRPV4 wt or TRPV4 S824A constructs. Each column represents mean ± s.e.m. ($n = 6$); $^*P < 0.05$ versus TRPV4 WT or S824A.

increase in sensitivity to PDD and heat, a promotion of its plasma membrane localization, and enhanced cell survival.

Discussion

In our experiments, we revealed some intriguing features of the mechanism relevant to TRPV4 regulation by SGK1 phosphorylation on the Ser 824 residue, one of the important regulatory sites for this Ca^{2+} channel. In this study we have demonstrated that, in addition to a feedback inhibitory effect of the modulation on 824 Ser of TRPV4, the potentiatory effect of Ca^{2+} occurs via its action at an intracellular site in the C-terminus of the channel protein, and is mediated by CaM binding (Nilius et al. 2003; Strotmann et al. 2003; Cohen 2006; Vennekens et al. 2008; Everaerts et al. 2009;).

It has been previously reported that TRPV4 channel activation and serine phosphorylation were enhanced by exposure to the non-PKC activator 4-αPDD, or by the application of bradykinin, which activates PKC via a G-protein-coupled mechanism or PKA (Watanabe et al. 2003; Birder et al. 2007; Fernandes et al. 2008; Fan et al. 2009). This enhancement was inhibited by the PKC inhibitors staurosporine, BIM and rottlerin, and by mutation of the serine/threonine residues S162, T175 and S189 (Xu et al. 2003; Cao et al. 2009). In line with these reports, we also expected that TRPV4 activity enhancement by S824 phosphorylation to be agonized or antagonized by the selective SGK1 activity modulators (IGF1 and wortmannin) (Figure 2). With treatment with wortmannin or IGF1, we observed TRPV4 WT single-channel property changes, as expected (Figure 2). However, we observed that the basal activity and sensitivity to 4-α PDD of TRPV4 S824A (an analog of unphosphorylated TRPV4 by SGK1) was higher than those of TRPV4 WT (Figure 5–7). This observation suggested that the C-terminal domain of TRPV4 near serine residue 824 seems to be assigned to regulate its function by an unknown controlling mechanism beyond phosphorylation modification, such as

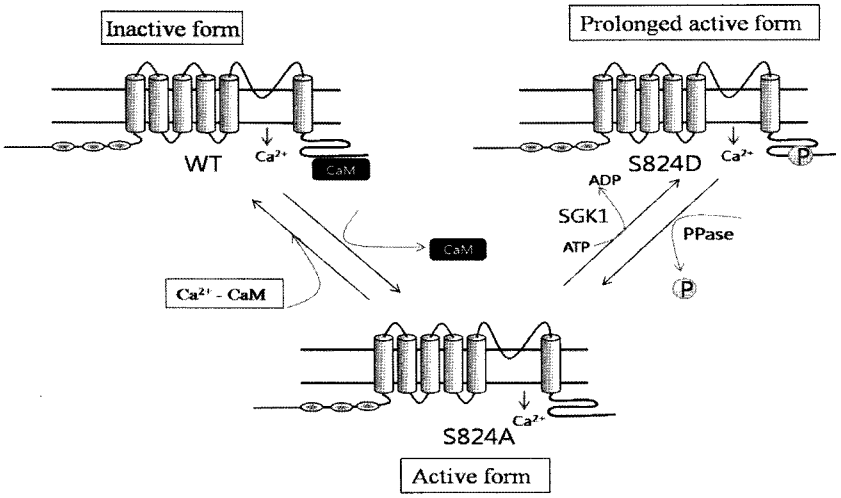

Figure 8. Schematic representation of TRPV4 activity regulation through S824phosphorylation by SGK1. TRPV4 WT can be activated by the putative dual (activator/inhibitor) function protein (such as CaM) association/dissociation from its C-terminal cytoplasmic domain (a short-term activation/inactivation loop on the left side) and the C-terminal phosphorylation (a long-term activation/inactivation loop on the right side). In contrast with TRPV4 WT, TRPV4 S824A and S824D mutants cannot be readily associate with an inhibitory protein, and thus return back to inactive form (for more detail, see the Discussion section).

protein–protein interaction with CaM (Strotmann et al. 2003; Fernandes et al. 2008; Vennekens et al. 2008).

We evaluated our hypothesis as described in Figure 8, and our observations indicated that TRPV4 is modulated by a short-term (dependent on a Ca^{2+} ion) and a long-term (dependent on the phosphorylation of Ser 824 residue) negative/positive feedback regulation loop. In a short-term regulation loop, TRPV4 WT appears to be dually modulated by the association of regulatory proteins (such as Ca^{2+}-bound CaM) on its C-terminal cytoplasmic domain. At first, the naive TRPV4 begins to open as a 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 Ca^{2+} ion concentration. However, at high Ca^{2+} ion levels, the fully active TRPV4 is negatively feedback-inhibited and returned to the inactive form: these mechanisms constitute the short-term negative/positive feedback regulation loop (Figure 8). This phenomenon also explains our observation that, after activation with 4-αPDD or heat, TRPV4 WT showed oscillations of Ca^{2+} ion concentration in the cytoplasm (Figure 4). On the other hand, after TRPV4 WT is phosphorylated on its Ser 824 residue by SGK1, the prolonged active (phosphorylated) form (an analogue of TRPV4 S824D) remains active until being dephosphorylated on its Ser 824 residue by a protein phosphatase (PPase) and the association of a putative regulatory protein (such as Ca^{2+}-CaM). Consistent with this assumption, TRPV4 S824D (an analogue with a prolonged active form) showed high Ca^{2+} channel activity with/without 4-αPDD treatment (see Figure 3 and 4), abolishing the protein interaction with the inhibitory factor. This model is also able to explain why TRPV4 E797 mutant is constitutively active (Nilius et al. 2003).

Following 4-αPDD or heat treatment, TRPV4 S824A appears to be analogous with an active form. TRPV4 S824A, which is not associated with an inhibitory protein Ca^{2+}-CaM (as an inhibition complex), returns to an inactive form. This may explain why the channel activity and Ca^{2+} entry of TRPV4 S824A appear to be higher than those of TRPV4 WT after 4-αPDD treatment (see Figure 3, 4 and 8). It seems to be valuable to elucidate the protein which interacts with the C-terminal region of TRPV4, in this respect.

CaM is a ubiquitous dual-function protein, which is known to regulate the activity of different ion channels, Ca^{2+} pumps and other proteins in a Ca^{2+}-dependent manner (Strotmann et al. 2003; Lambers et al. 2004; van de Graaf 2006; Fernandes et al. 2008). Previously, it has been reported that mutant channels, containing a single mutation in the C-terminus of TRPV4 (E797), are constantly open, and increase $[Ca^{2+}]_i$ in non-stimulated cells (Nilius et al. 2003). The inactivation of TRPV4 via binding with an inhibitory protein (such as Ca^{2+}-CaM) was blockaded and TRPV4 S824D activity was prolonged to a somewhat greater degree than that of TRPV4 WT (Figure 5 and 7).

It may be that unidentified proteins also regulate TRPV4 function through protein–protein interaction with its C-terminal domain. In this regard, the CaM binding site in its TRPV4 C-terminal seems to be involved in Ca^{2+}-induced conformational changes.

It seems likely that the Ser 824 of the C-terminal domain induces a conformational change in the TRPV4 channel protein that results in increased channel activity, as both TRPV4 S824D and S824A (and such as TRPV4 E797 mutant which is regardless of the phosphorylation) are more active than TRPV4 WT (Nilius et al. 2003). Interestingly, TRPV4's closest relatives, the Ca^{2+} transporter TRPV6, seem to be regulated at the corresponding region by CaM through SGK1 phosphorylation (Lambers et al. 2004; van de Graaf et al. 2006). A putative SGK1 phosphorylation site (Thr 702 residue) in the C-terminal domain of TRPV6 is present (unpublished data). Thus, even though other researchers reported that CaM-binding domains in similar regions of related proteins exert opposite effects on channel function among TRPV4, 5, and 6, its C-terminal CaM-binding domain function involved in the Ca^{2+}-dependent potentiation of TRPV4 and 6 channels seems to be regulated similarly (van de Graaf et al. 2006; Hardie 2007).

Consistent with this notion, our data demonstrated a relationship between the increase in Ca^{2+} permeability by TRPV4 and cell survival (van de Graaf et al. 2006). However, the deleterious increase in intracellular Ca^{2+} resulting from Ca^{2+}-activated Ca^{2+} entry appears to be prevented by Ca^{2+}-dependent negative feedback (Strotmann et al. 2003). The results of this study provide evidence suggesting the importance of the TRPV4 C-terminal Ser 824 residue in the regulation of channel gating in response to 4-αPDD, CaM binding, and its subcellular localization to the plasma membrane via SGK1 phosphorylation. It has been reported that Ca^{2+} also inactivates TRPV4 channels in several different ways. The first of these is a slow inactivation of TRPV4 channels by other posttranslational modifications (such as *S*-nitrosylation), which is clearly independent of CaM binding and may be the result of a local effect of Ca^{2+} on the intracellular pore-forming region; this mechanism has yet to be investigated in detail (Yoshida et al. 2006). The second is a rapid inactivation, which was determined to be Ca^{2+}-CaM dependent (Nilius et al. 2003; Strotmann et al. 2003; Cohen 2006; Vennekens et al. 2008; Everaerts et al. 2009). The removal of Ca^{2+}-CaM binding does affect the initial rapid phase of inactivation, or it abolishes all of the slow inactivation, as in the case of TRPV4 S824A mutant (Figure 8). However, it remains to be identified how the modification or protein–protein interaction of C-terminal of TRPV4 regulates its channel opening/closing (Nilius et al. 2003; Cohen 2006; Vennekens et al. 2008; Everaerts 2009).

The biphasic current responses observed after Ca^{2+} entry after 4-αPDD or heat stimulation (Figure 4 and 8) suggest that smaller increases in $[Ca^{2+}]_i$ potentiate TRPV4, but larger increases exert an inhibitory effect (Xu et al. 2003). It has been suggested that the dual regulation of other TRPV channels by Ca^{2+} shape the cellular Ca^{2+} response to channel stimuli, and the response will be oscillatory in nature (Xu et al. 2003). The positive/negative feedback effect of Ca^{2+} entry on channel activation could result in the delayed regenerative Ca^{2+} responses. However, the combination of Ca^{2+}-dependent potentiation and inactivation will result in transient responses with a rapid rising phase. Thus, this dual regulation is consistent with an oscillatory response of TRPV4 WT to 4-αPDD, while the inactivation of TRPV4 S824D and S824A was prolonged with no regular oscillation. The reason seems to be that a negative regulatory protein (such as CaM) does not bind with TRPV4 properly due to its mutation, or because TRPV4 S824D shows its prolonging 4-αPDD effect by preventing the diffusion/release of 4-αPDD out from the central part of TM4 (Leu 584 and Trp 586) (Watanabe et al. 2002, 2003). Indeed, oscillatory Ca^{2+} responses have been noted in cells expressing high levels of TRPV4 (Xu et al. 2003). Without knowing the physiological role of the channel, any further speculation regarding the significance of Ca^{2+}-dependent potentiation is premature.

TRPV4 expression has been suggested as a marker in human hepatoblastoma; thus, it is tempting to speculate that a finely tuned feedback mechanism must exist in TRPV4-expressing cells (Vriens et al. 2006). Given TRPV4's selective expression in malignant cancer cells of hepatic origin, an understanding of the feedback mechanism may prove critical to our understanding of the functions and differentiation behaviors of these cells. Our result (Figure 8), namely that an increase of TRPV4 activity (such as S824D) enhanced the survival of HEK 293 cells, appears consistent with their observation.

It is also worth noting that both TRPV4 and SGK1 are involved in the regulation of cell volume, interestingly (Becker et al. 2005; Tessier and Woodgett 2006). The C-terminal tail motif of TRPV4 is also similar to that of SGK1, suggesting that these two proteins interact in the cell together by intermediation of PDZ scaffolding protein, such as NHERF2 (Garcia-Elias et al. 2008). Moreover, our results suggested that TRPV4 Ser 824 residue phosphorylation by SGK1 also contributes to the cell survival-related effect or its volume regulation through the interaction between CaM and TRPV4. Therefore, in order to control both cell volume and survival, these two proteins appear to work together in a synergistic fashion (Figure 7). During the preparation of this manuscript, others reported that Ser 824 of TRPV4 is also phosphorylated by PKA and its Ca^{2+} entry is enhanced through the phosphorylation (Fan et al. 2009).

Regardless of the mechanisms (Figure 8), however, the gating diversity of the TRPV4 channel demonstrated in the current study is reflective of the presence of multiple physical and chemical signaling pathways that converge on the channel. As a consequence, the TRPV4 channel would appear to function as a molecular integrator of a complex array of diverse signals. Because multiple environmental signals and transduction pathways converge on TRPV4, the channel may function as a molecular integrator of microenvironmental chemical and physical signals. Thus, further study for the elucidation of the TRPV4 regulation mechanism will be required in order to clearly determine the physiological role and activation mechanism of this channel.

Acknowledgements

We thank Dr. David M. Cohen (Oregon Heath and Science University) for kindly providing the TRPV4 gene. This work was supported by National Research Foundation of Korea (NRF) grants (2009-0076024 and 2009-0069007) funded by the Korea government (MEST) to S.S. Kang. We also appreciate the help of The Core Facility of Chungbuk National University.

References

Andrade YN, Fernandes J, Vázquez E, Fernández-Fernández JM, Arniges M, Sánchez TM, Villalón 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.

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, de Groat W, Kanai A, Caterina M. 2007. Activation of urothelial transient receptor potential vanilloid 4 by 4alpha-phorbol 12,13-didecanoate contributes to altered bladder reflexes in the rat. J Pharmacol Exp Ther. 323:227–235.

Cao DS, Yu SQ, Premkumar LS. 2009. Modulation of transient receptor potential Vanilloid 4-mediated membrane currents and synaptic transmission by protein kinase C. Mol Pain. 10:5:1–15.

Casamayor A, Torrance PD, Kobayashi T, Thorner J, Alessi DR. 1999. Functional counterparts of mammalian protein kinases PDK1 and SGK in budding yeast. Curr Biol. 9:186–197.

Chen SY, Bhargava A, Mastroberardino L, Meijer OC, Wang J, Buse P, Firestone GL, Verrey F, Pearce D. 1999. Epithelial sodium channel regulated by aldosterone-induced protein sgk. Proc Natl Acad Sci USA. 96:2514–2519.

Chun J, Kwon T, Kim DJ, Park I, Chung G, Lee EJ, Hong SK, Chang SI, Kim HY, Kang SS. 2003. Inhibition of mitogen-activated kinase kinase kinase 3 activity through phosphorylation by the serum- and glucocorticoid-induced kinase 1. J Biochem. 133:103–108.

Chun J, Kwon T, Lee EJ, Kim CH, Han YS, Hong SK, Hyun S, Kang SS. 2004. 14–3-3 Protein mediates phosphorylation of microtubule-associated protein tau by serum- and glucocorticoid-induced kinase 1. Mol Cells. 18:360–368.

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 Ca^{2+} signaling complex with ryanodine receptors and BKCa channels. Circ Res. 97:1270–1279.

Everaerts W, Nilius B, Owsianik G. 2009. The vallinoid 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.

Garcia-Elias A, Lorenzo IM, Vicente R, Valverde MA. 2008. IP_3 receptor binds to and sensitizes TRPV4 channel to osmotic stimuli via a calmodulin-binding site. J Biol Chem. 283:31284–31288.

Hardie RC. 2007. TRP channels and lipids: from Drosophila to mammalian physiology. J Physiol. 578:9–24.

Kobayashi T, Deak M, Morrice N, Cohen P. 1999. Characterization of the structure and regulation of two novel isoforms of serum- and glucocorticoid-induced protein kinase. Biochem J. 1:189–197.

Lambers TT, Weidema AF, Nilius B, Hoenderop JG, Bindels RJ. 2004. Regulation of the mouse epithelial $Ca^{2(+)}$-sensor calmodulin. J Biol Chem. 279:28855–28861.

Lee EJ, Chun J, Hyun S, Ahn HR, Jeong JM, Hong SK, Hong JT, Chang IK, Jeon HY, Han YS, Auh CK, Park JI, Kang SS. 2008. Regulation Fe65 localization to the nucleus by SGK1 phosphorylation of its Ser566 residue. BMB Rep. 41:41–47.

Liedtke W, Choe Y, Marti-Renom MA, Bell AM, Denis CS, Sali A, Hudspeth AJ, Friedman JM, Heller S. 2000. Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. Cell. 103:525–535.

Maiyar AC, Huang AJ, Phu PT, Cha HH, Firestone GL. 1996. p53 stimulates promoter activity of the sgk. Serum/glucocorticoid-inucible serin/threonine protein kinase gene in rodent mammary epithelial cells. J Biol Chem. 271:12414–12422.

Murray JT, Cummings LA, Bloomberg GB, Cohen P. 2005. Identification of different specificity requirements between SGK1 and PKBalpha. FEBS Lett. 579:991–994.

Nilius B, Watanabe H, Vriens J. 2003. The TRPV4 channel: structure-function relationship and promiscuous gating behaviour. Pflugers Arch. 446:298–803.

Saito M, Hanson PI, Schlesinger P. 2007. Luminal chloride-dependent activation of endosome calcium channels: patch clamp study of enlarged endosomes. J Biol Chem. 282:27327–27333.

Strotmann R, Schultz G, Plant TD. 2003. Ca-dependent potentiation of thenonselective cation channel TRPV4 is

mediated by a C-terminal calmodulin binding site. J Biol Chem. 278:26541–26549.

Suzuki M, Hirao A, Mizuno A. 2003a. Microtubule-associated [corrected] protein 7 increase the membrane expression of transient receptor potential vanilloid 4 (TRPV4). J Biol Chem. 278:51448–51453.

Suzuki M, Mizuno A, Kodaira K, Imai M. 2003b. Impaired pressure sensation in mice lacking TRPV4. J Biol Chem. 278:22664–22668.

Tessier M, Woodgett JR. 2006. Serum and Glucocorticoid-regulated protein kinases: variations on a theme. J Cell Biochem. 98:1391–1407.

van de Graaf SF, Hoenderop JG, Bindels RJ. 2006. Regulation TRPV5 and TRPV6 by associated proteins. Am J Physiol Renal Physiol. 290:1295–1302.

Vennekens R, Owsianik G, Nilius B. 2008. Vanilloid transient receptor potential cation channels: an overview. Curr Pharm Des. 14:18–31.

Vriens J, Janssens A, Prenen J, Nilius B, Wondergem R. 2006. TRPV channels and modulation by hepatocyte growth factor/scatter factor in human hepatoblastoma (HepG2) cells. Cell Calcium. 36:19–28.

Vriens J, Owsianik G, Janssens A, Voets T, Nilius B. ?2007. Determinants of 4 alpha-phorbol sensitivity in transmembrane domains 3 and 4 of the cation channel TRPV4. J Biol Chem. 282:12796–12803.

Watanabe H, Davis JB, Smart D, Jerman JC, Smith GD, Hayes P, Vriens J, Cairns W, Wissenbach U, Prenen J, Flockerzi V, Droogmans G, Benham CD, Nilius B. 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.

Webster MK, Goya L, Ge Y, Maiyar AC, Firestone GL. 1993. Characterization of sgk, a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum. Mol Cell Biol. 13:2031–2040.

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.

Xu F, Satoh E, Iijima T. 2003. Protein kinase C-mediated Ca^{2+} entry in HEK 293cells transiently expressing human TRPV4. Br J Pharmacol. 140:413–421.

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-nitryosylation. Nat Chem Biol. 2:596–607.