Tyrosine Phosphorylation Modulates the Activity of TRPV4 in Response to Defined Stimuli*§

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Src family tyrosine kinases (SFKs) regulate the function of several transient receptor potential (TRP) family members, yet their role in the regulation of the vanilloid subfamily member 4 protein (TRPV4) remains controversial. TRPV4 is a calcium-permeable channel activated by numerous physical and chemical stimuli. Here we show that SFKs mediate tyrosine phosphorylation of TRPV4 in different cell lines. Using mass spectrometric analysis, we identified two novel phosphorylation sites in the cytosolic N- and C-terminal tails of TRPV4. Substitution of either tyrosine with phenylalanine led to a substantial reduction in the overall tyrosine phosphorylation level of TRPV4, suggesting that these two tyrosines constitute major phosphorylation sites. Both mutants efficiently localized to the plasma membrane, indicating that neither tyrosine is required for trafficking of TRPV4 in the secretory pathway. Analysis of the channel function demonstrated a crucial role of the N-terminal tyrosine residue in the activation of TRPV4 by heat, mechanical (shear) stress, hypotonic cell swelling, and phorbol 12-myristate 13-acetate, but not in the activation by synthetic ligand 4α-phorbol 12,13-didecanoate. Furthermore, the response of TRPV4 to phorbol 12-myristate 13-acetate was SFK-dependent. Because the SFK-mediated phosphorylation of the N-terminal tyrosine occurred before TRPV4 activation, tyrosine phosphorylation appears to sensitize rather than activate this channel. Reactive oxygen species, known to mediate inflammatory pain, strongly up-regulated TRPV4 phosphorylation in the presence of SFKs. Our findings indicate that tyrosine phosphorylation of TRPV4 represents an important modulatory mechanism, which may underlie the recently described function of TRPV4 in inflammatory hyperalgesia.

The transient receptor potential (TRP) superfamily consists of Ca2+-permeable cation channels with a remarkable diversity of activation mechanisms (1). They perform a wide range of physiological functions and are involved in the pathogenesis of several diseases (2). All TRP proteins share the same topology: six transmembrane (TM) segments, a pore-loop situated between TM5 and TM6, and intracellular N- and C-terminal tails (3). Based on sequence similarity, the TRP superfamily can be divided into as many as eight subfamilies, including the vanilloid subfamily (TRPV) (1). The TRPV subfamily contains six mammalian members named TRPV1−6, as well as several invertebrate proteins such as osm-9 from Caenorhabditis elegans.

TRPV4 was initially identified as an osm-9-related channel that is activated by hypotonic cell swelling (4–6). Subsequently, experiments with cultured cells indicated that TRPV4 can also be activated by moderate heat with a threshold of 25–34 °C (7, 8), mechanical (shear) stress (9), synthetic ligand 4α-phorbol 12,13-didecanoate (4αPDD) (10), and endogenous compounds such as anandamide, arachidonic acid, and 5,6′-epoxyeicosatrienoic acid (11). Although viable, mice with a genetically disrupted TRPV4 gene are impaired in osmoregulation (12, 13), avoidance of high temperatures (14), responses to noxious mechanical stimuli (12, 15), inflammation-induced thermal and mechanical hyperalgesia (16−18), as well as in shear stress-induced vasodilation (19). TRPV4 protein is strongly expressed in epithelial cells of kidneys (20) and airways (21), endothelial cells (22), keratinocytes (7), and in sensory neurons (12, 15). This distribution indicates that TRPV4 is well positioned to sense systemic and local environmental changes.

The polymodal nature of the TRPV4 channel raises the question whether the activating stimuli converge on the same pathway or act independently. Recent studies demonstrate that opening of TRPV4 in response to hypotonic cell swelling involves phospholipase A2 (PLA2),-mediated release of arachidonic acid, which is further metabolized by cytochrome P450 epoxygenase to 5,6′-epoxyeicosatrienoic acid (23). Although direct gating of TRPV4 by mechanical force has not been excluded (24), the TRPV4-mediated responses to shear stress and high viscous load appear to depend on PLA2 activity as well (19, 25). In contrast, 4αPDD stimulates TRPV4 directly by

12,13-didecanoate; PP2, 4-aminophosphonate-5-(4-chlorophenyl)-7-(3-butyl)pyrazolo[3,4-d]pyrimidine; BIM I, bisindolylmaleimide I hydrochloride; HTS, hypotonic solution; TM, transmembrane; HEK, human embryonic kidney; MDCK, Madin-Darby canine kidney; MOPS, 4-morpholinepropanesulfonic acid; MRM, multiple reaction monitoring; WT, wild type; BisTris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propan-1,3-diol; IRES, internal ribosome entry site.
binding to its TM3 and TM4 segments (26). Finally, activation by heat appears to share mechanistic requirements both with 4αPDD and cell swelling pathways (23, 27). Thus, TRPV4 can be gated by at least two independent mechanisms.

In addition to the stimuli described above, activity of TRPV4 can also be regulated by phosphorylation. Treatment with phorbol 12-myristate 13-acetate (PMA) activates TRPV4 in a protein kinase C (PKC)-dependent way (9, 28), which is reminiscent of the well studied PKC-mediated phosphorylation and regulation of TRPV1 channels (29). Furthermore, Xu et al. (30) have shown that phosphorylation of TRPV4 on tyrosine 253 by Src family kinases (SFKs) is required for channel activation upon treatment with hypotonic solution. In agreement with this finding, SFKs positively regulate several other TRP channels (31–37). However, both the involvement of SFKs and the role of tyrosine 253 in the activation of TRPV4 were subsequently contradicted by others (23). Prompted by this controversy, we further explored the function of the SFK-mediated tyrosine phosphorylation of TRPV4. Using mass spectrometry, we unequivocally identified the phosphorylation sites of Src kinase in TRPV4 as tyrosines 110 and 805, and characterized the TRPV4 variants with point mutations at these sites. Our results demonstrate a major role of the Tyr110 residue in the stimulus-specific modulation of TRPV4 channel function, and contribute to the understanding of the polymodal nature of TRPV4 activation.

EXPERIMENTAL PROCEDURES

**Plasmids**—TRPV4 with a C-terminal FLAG tag was generated from a pcDNA3-based plasmid containing the mouse Trpv4 cDNA (6). We verified that the addition of the FLAG tag did not inhibit TRPV4 channel function (data not shown). The Trpv4 cDNA was re-cloned into pcDNA6-V5/His vector (Invitrogen) to obtain C-terminal V5/His-tagged constructs. For retrovirus production, Trpv4 cDNA was cloned into pLXSN vector (Clontech). For Ca²⁺ imaging with HeLa cells, TRPV4 was expressed from the pCAGGS/IRE-S-GFP vector (6). Y110F and Y805F substitutions were generated by PCR, and TRPV4 was expressed from the pCAGGS/IRE-S-GFP vector (6). Y110F and Y805F substitutions were generated by PCR, and for short DNA fragments containing these mutations were used to replace corresponding wild-type fragments in target plasmids, to eliminate the possibility of introducing undesired nucleotide changes. The resulting plasmids were verified by sequencing. The T7-tagged fragment of the N-terminal tail of TRPV4 (amino acids 1–321) containing Tyr110 as the only tyrosine (T7-TRPV4 110Y) was prepared by DNA synthesis and cloned into pcDNA3 vector (Invitrogen). V-src was expressed from a plasmid containing Rous sarcoma virus DNA fragment (Schmidt-Ruppin A strain). Human Src cDNA was cloned into EcoRI and Sall sites of the pIRES-hrGFP-1a vector (Stratagene). Src kinase-dead (KD) mutant was constructed by introducing K297R substitution into the above construct.

**Cell Cultures, Transfections, and Retrovirus Production**—Human embryonic kidney (HEK) 293T, HeLa, and Madin-Darby canine kidney (MDCK) cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Transient transfections were carried out using the calcium phosphate method or FuGENE 6 reagent (Roche Applied Science). Retroviruses were produced in HEK 293T cells co-transfected with appropriate pLXSN-based plasmid and helper plasmids. Transduction of MDCK cells was carried out in the presence of 8 μg/ml Polybrene (Sigma). TRPV4-expressing cells were selected with 0.25 mg/ml geneticin (Invitrogen), and analyzed by Western blotting and immunofluorescence.

**Reagents**—PMA, 4αPDD, bisindolylmaleimide I hydrochloride (BIM I) (Sigma), and 4-amino-5-(4-chlorophenyl)-7-(t-buty)pyrazolo[3,4-d]pyrimidine (PP2) (Calbiochem) were dissolved in dimethyl sulfoxide at 2–5 mM. Ruthenium red (Latoxan) was dissolved in water at 10 mM.

**Immunoprecipitations and Western Blotting**—Cells were washed with ice-cold phosphate-buffered saline and lysed in the immunoprecipitation buffer (20 mM Tris, pH 7.5, 1% Triton X-100, 50 mM NaCl, 50 mM sodium fluoride, 15 mM Na₃P₂O₇, 0.1 mM EDTA) supplemented with 1 mM sodium orthovanadate and protease inhibitor mixture (Roche). The lysates were cleared by centrifugation at 125,000 × g for 30 min at 4 °C, and incubated with the anti-FLAG M2 resin (Sigma). The bound proteins were washed five times with the immunoprecipitation buffer and analyzed by Western blotting with the following antibodies: mouse anti-FLAG, mouse anti-actin (clone AC-15), rabbit anti-phosphotyrosine (all Sigma), mouse anti-v-src (clone 327) (Calbiochem), mouse anti-V5 (Serotec), mouse anti-phosphotyrosine (clone 4G10) (Upstate), and rabbit anti-phospho-Src (Tyr416) (Cell Signaling).

**Enzyme-linked Immunosorbsent Assay**—The assay was performed as previously described (36). In short, HEK 293T cells transfected by the calcium phosphate method were split into poly-L-lysine-coated 48-well dishes. The cells were incubated with α-V5 antibody (1.33 μg/ml) in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 20 mM HEPES at 4 °C for 45 min, washed three times, and fixed with 3.7% paraformaldehyde in phosphate-buffered saline. Following incubation with alkaline phosphatase-coupled α-mouse antibodies (Sigma), the enzymatic reaction was performed using 1 mg/ml p-nitrophenyl phosphate (Sigma), and read at 405 nm in a microplate spectrophotometer. Cells split in parallel were lysed and analyzed by Western blotting. The blots were scanned and the bands were quantified using ImageJ software (National Institutes of Health).

**Mass Spectrometry**—Purified TRPV4-FLAG proteins were separated by one-dimensional SDS-PAGE using a 6 × 6-cm precast 4–12% Bis-Tris gel (Invitrogen) under MOPS buffer conditions. After colloidal Coomassie G-250 staining (39) bands corresponding to TRPV4 were excised and processed for mass spectrometric detection as previously described (40). An in-gel proteolytic digest was performed with 12.5 ng/ml trypsin (Promega) overnight at 37 °C. Peptides were extracted from the gel slices with 15 μl of 0.1% trifluoroacetic acid for 30 min.

Mass spectrometric analyses were performed on a linear ion trap coupled online to a nanoLC system (Famos, Switchos, Ultimate, Dionex, Idstein, Germany) comprising a common precolumn concentration setup. For trapping and desalting of peptides from in-gel digests a custom-made 100-μm inner diameter × 2-cm length precolumn (Ace C₅, 5 μm particle size, 100 Å pore size, HiChrom Ltd., Berkshire, UK) with 0.1% trifluoroacetic acid as loading buffer was used. Reversed-phase separation was performed on custom-made 75-μm inner diam-
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Tyrosine phosphorylation modulates TRPV4 activity.

\[ \tau = 6 \times \eta \times Q/(b \times h^2) \]  
(Eq. 1)

where \( \tau \) is shear stress (dyne/cm²); \( \eta \) is fluid viscosity (0.69 cP); \( Q \), flow rate (ml/s); \( b \), chamber width (0.2 cm); \( h \), chamber height (0.02 cm).

Cells were exposed to the light from a xenon lamp (Hamamatsu) passed through 340- and 380-nm filters. The emitted fluorescence was recorded and analyzed using Metafluor software (Molecular Devices). Background fluorescence was subtracted before calculation of 340/380 ratios. Typically several GFP-positive HeLa or HEK 293T cells and 20–30 MDCK cells were recorded in one measurement.

Calibration of 340/380 ratios to intracellular resting calcium concentration [Ca\(^{2+}\)] was performed as described in Ref. 41, using the following equation,

\[ [\text{Ca}^{2+}] = \beta \times K_d \times [(R - R_{\text{min}})/(R_{\text{max}} - R)] \]  
(Eq. 2)

where \( \beta \) is the ratio of fluorescence emission intensity at 380 nm excitation in Ca\(^{2+}\)-depleting and Ca\(^{2+}\)-saturating conditions, \( R \) is 340/380 ratio at any time, \( R_{\text{min}} \) is the minimum ratio in Ca\(^{2+}\)-depleting conditions (5 mM EGTA, 2 \( \mu \)M ionomycin), \( R_{\text{max}} \) is the maximum ratio in Ca\(^{2+}\)-saturating conditions (5 mM Ca\(^{2+}\), 2 \( \mu \)M ionomycin), and \( K_d \) is the Ca\(^{2+}\) dissociation constant of fura-2 (220 nM).

Immunofluorescence—Cells grown on glass coverslips were fixed in 3.7% paraformaldehyde, permeabilized with 0.5% Triton X-100, blocked in 2% horse serum, and incubated either with rabbit anti-TRPV4 antibody (38) followed by anti-rabbit Cy3-coupled antibody (Jackson Immunoresearch) or with anti-FLAG antibody followed by anti-mouse Cy3-coupled antibody. Nuclei were stained with 1 \( \mu \)g/ml Hoechst 33342 (Molecular Probes). Images were taken with Zeiss LSM 510 confocal microscope using C-Apochromat \( \times 63/1.2 \) W (for Fig. 3B) or Plan-Neofluar \( \times 100/1.3 \) oil (for Fig. 5B) objectives.

Chemical Cross-linking—Cross-linking of TRPV4 with bis(sulfosuccinimidyl)suberate (BS3) (Pierce) was performed as described previously (42).

Statistical Analysis—Statistical significance was calculated using one-sample t test (for Fig. 2) or unpaired t test (for Figs. 4, 6, supplemental S2, and S4). Unless otherwise stated, \( n \) refers to one independent measurement. Significance in figures was depicted as follows: N.S., not significant; *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \).

RESULTS

Src Phosphorylates TRPV4 on Tyrosines 110 and 805—To investigate Src-mediated phosphorylation of TRPV4, we generated MDCK cell lines stably expressing FLAG-tagged mouse TRPV4 by retroviral gene transfer. TRPV4-FLAG protein purified from these cells contained phosphorylated tyrosine residues, as evidenced by the immunostaining with anti-phosphotyrosine antibodies (Fig. 1A). The phosphotyrosine signal in TRPV4 purified from cells pre-treated with PP2, a specific inhibitor of SFKs, was reduced in a dose-dependent manner, suggesting that Src or related kinases are largely responsible for the basal level of tyrosine phosphorylation of this protein (Fig. 1A). To confirm the involvement of SFKs in the phosphorylation of TRPV4, we transfected HEK 293T cells with plasmids

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encoding v-src from Rous sarcoma virus together with either FLAG-tagged TRPV4 or CD2-associated protein (FLAG-CD2AP). Co-expression of v-src led to a clear increase in the phosphotyrosine level of TRPV4 but not of CD2AP (Fig. 1B).

Subsequently, TRPV4 purified from HEK 293T cells expressing v-src was subjected to mass spectrometry analysis to identify tyrosine phosphorylation sites in this protein. The employed MRM-scanning technique was sufficient to reduce sample complexity and to detect phosphorylation sites. Therefore, no further enrichment technique was required for phosphopeptide detection. This approach identified two phosphorylation sites within the peptide sequences KAPMDSLFDY*GTYR and SEIY*QYYGF8HTVGR, corresponding to Tyr110 and Tyr805 in the protein sequence of TRPV4 (Fig. 1C, upper spectra). The spectra allowed an unambiguous assignment of the respective phosphorylation sites by directly annotating sequence ions. Importantly, mass spectrometry analysis of TRPV4-FLAG purified from MDCK cells indicated the same phosphorylation sites (Fig. 1C, lower spectra). Thus, tyrosines 110 and 805 are phosphorylated by endogenous SFKs in TRPV4 at normal cell culture conditions. Applying the outlined experimental conditions, we did not detect phosphorylation at Tyr253, a previously reported target site of SFKs during hypotonic cell swelling (30).

We next extended our analysis of TRPV4 phosphorylation in HEK 293T cells with V5/His-tagged TRPV4 variants after replacing either tyrosine by phenylalanine. Tyrosine phosphorylation of wild-type (WT) TRPV4 was induced in the presence of active Src kinase, but not in the presence of kinase-dead (KD) mutant of Src (Fig. 1D).

**Figure 1.** Src family kinases phosphorylate TRPV4 on tyrosines 110 and 805. A, tyrosine phosphorylation of TRPV4 is sensitive to the Src kinase inhibitor PP2. MDCK cells were pre-treated with the indicated dose of PP2 for 30 min prior to lysis. TRPV4-FLAG protein, immunoprecipitated (IP) from cell lysates, was analyzed by Western blotting (WB) with anti-FLAG and anti-phosphotyrosine (pY) antibodies. MDCK pLXSN cells containing empty vector pLXSN were used as a control. B, HEK 293T cells were transfected with plasmids encoding v-src, TRPV4-FLAG, or FLAG-CD2AP, as indicated. Purified FLAG-tagged proteins were analyzed by Western blotting. Note that TRPV4-FLAG and FLAG-CD2AP have very similar apparent molecular weight. C, TRPV4-FLAG proteins, purified from HEK 293T cells co-expressing v-src (upper spectra) or from MDCK cells (lower spectra) were analyzed by mass spectrometry using targeted MRM scans in conjunction with tandem mass spectrometric sequencing. Phosphorylation of Tyr110 and Tyr805 was identified by significant Mascot scores. Asterisks and hashes within the peptide sequences mark the sites of phosphorylation and methionine oxidation, respectively. D, TRPV4 proteins with Y110F and/or Y805F substitutions exhibit reduced levels of Src-induced tyrosine phosphorylation. HEK 293T cells were transfected with plasmids encoding various V5/His-tagged TRPV4 variants (WT, Y110F, Y805F, or Y110F Y805F) along with WT Src or kinase-dead (KD) Src, as indicated. TRPV4 proteins were immunoprecipitated with anti-V5 antibodies and analyzed by Western blotting with antibodies, as indicated. Expression of Src kinase was detected in the lysates with anti-Src antibodies.
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FIGURE 2. TRPV4 containing Y110F or Y805F substitutions localizes at the cell surface. A. HEK 293T cells were transfected with plasmids encoding variants of TRPV4 (WT, Y110F, or Y805F) with a V5-tag engineered into the first extracellular loop, or with the empty vector as a control. The amount of the V5 epitope at the cell surface was quantified by enzyme-linked immunosorbent assay, using an alkaline phosphatase enzymatic reaction. Total levels of V5-tagged TRPV4 proteins were controlled by Western blotting. Shown is one representative experiment. B, ratios of surface levels to total levels for Y110F and Y805F mutants as compared with wild-type TRPV4. Shown are mean ± S.E. from n independent experiments (n = 5 for Y110F, n = 4 for Y805F). p value was calculated using the one-sample t test.

The results described above suggested that the Tyr<sup>110</sup> residue may be important for the channel function of TRPV4. To investigate this possibility in more detail, we decided to compare responses of the TRPV4 channel and its Y110F variant to known stimuli by measuring Ca<sup>2+</sup> transients with fura-2 imaging. For this analysis we used HeLa cells, because these cells respond more robustly to TRPV4 activation than HEK 293T cells. We used TRPV4 wild-type and Y110F cDNAs fused with IRES-GFP sequences to directly identify transfected (GFP-positive) cells. Western blot analysis demonstrated that both TRPV4 variants were expressed at comparable levels with respect to the co-transcribed GFP, justifying the use of GFP signal both as the qualitative marker of transfected cells and the quantitative marker of TRPV4 expression (Fig. 3A). In addition, both TRPV4 variants displayed similar localization in HeLa cells (Fig. 3B).

TRPV4 can be activated by an increase in the temperature of bath solution with a threshold of 25–34 °C (7, 8). In agreement with this, GFP-positive HeLa cells expressing wild-type TRPV4 exhibited a rise in [Ca<sup>2+</sup>], upon rapid warming of bath solution to 35 °C (Fig. 4A). The response to the flow of heated solution was significantly and substantially diminished in GFP-positive HeLa cells expressing TRPV4 Y110F. Because Ca<sup>2+</sup> influx in response to the flow of the heated solution may reflect either heat-evoked activation of TRPV4 or, alternatively, temperature-stimulated activation of TRPV4 by mechanical stress imposed by the flow (9, 43), we analyzed Ca<sup>2+</sup> responses of TRPV4-transfected HeLa cells to defined fluid shear stress. HeLa
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A

![Western blot (WB) analysis of TRPV4 WT and Y110F proteins.](image)

B

![Confocal fluorescence microscopy of immunostained HeLa cells expressing TRPV4 WT and Y110F.](image)

FIGURE 3. TRPV4 WT and Y110F proteins exhibit the same expression levels and localization in transiently transfected cells. A, Western blot (WB) analysis of TRPV4 WT and Y110F proteins. B, Confocal fluorescence microscopy of immunostained HeLa cells expressing TRPV4 WT (upper panels) and Y110F (lower panels). TRPV4 and GFP proteins were transiently expressed from TRPV4-IRES-GFP constructs. GFP is shown in green and nuclei in blue. Scale bars denote 10 μm.

cells expressing wild-type TRPV4, subjected to low level of shear stress (0.3 dyne/cm² for 10 min at 37 °C), exhibited a clear elevation in [Ca²⁺], upon increasing fluid shear stress to 9 dyne/cm² (Fig. 4B). In contrast, such response was absent in HeLa cells expressing the TRPV4 Y110F mutant. These results suggest that the Tyr¹¹⁰ residue may be essential for mediating responses of TRPV4 to mechanical stimuli at physiological temperatures. To avoid the influence of mechanical stress and elevated temperatures on TRPV4 activation triggered by other stimuli, we performed the subsequent Ca²⁺ imaging experiments with HeLa cells at room temperature.

We analyzed responses of Trpv4-transfected HeLa cells to two well established stimuli, HTS and the PKC-non-activating phorbol ester 4aPDD, which activate TRPV4 through different mechanisms (23). Although HeLa cells expressing TRPV4 Y110F generally responded to HTS, the mean amplitude of Ca²⁺ transients was significantly smaller and amounted to 35% of that in HeLa cells expressing wild-type TRPV4 (Fig. 4C). In contrast, TRPV4 Y110F mediated responses to 1 μM 4aPDD that were only moderately and not significantly smaller from those mediated by wild-type TRPV4 (Fig. 4D). Finally, we investigated TRPV4 channel function following the treatment with 1 μM PMA, a PKC-activating phorbol ester that causes TRPV4 channel opening in a PKC-dependent manner (9, 28). In response to PMA, HeLa cells expressing wild-type TRPV4 exhibited a rise in [Ca²⁺], with a kinetics slower than treatments with HTS, 4aPDD, or increasing shear stress, whereas HeLa cells expressing TRPV4 Y110F virtually showed no Ca²⁺ transients (Fig. 4E). Cytosolic Ca²⁺ levels in untransfected (GFP-negative) cells or in cells transfected with empty IRES-GFP vector were not clearly affected by any of the described stimuli, indicating absence of endogenous TRPV4 channel in HeLa cells (supplemental Fig. S3, and data not shown). In aggregate, replacement of tyrosine 110 with phenylalanine disrupts normal TRPV4 channel function in response to TRPV4 activating stimuli such as mechanical stress, HTS, and PMA, but not to 4aPDD.

Subsequently we asked whether induction of TRPV4 tyrosine phosphorylation would facilitate its activation. To this end, we co-expressed TRPV4 and v-src in HeLa cells. We found that the cells transfected with both proteins exhibited a higher rise in [Ca²⁺], in response to PMA than the cells transfected with TRPV4 alone (supplemental Fig. S4). Thus, up-regulation of the SFK activity and associated TRPV4 phosphorylation appears to lead to an increased Ca²⁺ influx through TRPV4 channel.

TRPV4-mediated Response to PMA in MDCK Cells Requires the Activity of PKC and SFK—To investigate whether SFK-mediated TRPV4 phosphorylation, occurring in unstimulated MDCK cells (see Fig. 1A), is required for TRPV4 activation, we characterized Ca²⁺ transients following PMA treatment in MDCK cell lines stably expressing TRPV4-FLAG wild-type (WT), Y110F mutant, or empty pLXSN vector. We confirmed that TRPV4-FLAG WT and Y110F proteins were expressed at comparable levels in these cells (Fig. 5A) and localized at the cell border (Fig. 5B). Furthermore, both TRPV4 proteins were detected in chemically stabilized oligomeric structures, suggesting that the TRPV4 Y110F protein is not defective in the assembly of mature tetrameric channels (supplemental Fig. S5). Because MDCK cells, in contrast to Trpv4-transfected HeLa cells, did not strongly react to an abrupt increase in the temperature of bath solution (data not shown), all experiments were performed at 35 °C. Control, wild-type TRPV4 and TRPV4 Y110F cells exhibited an initial rise in [Ca²⁺], in response to 1 μM PMA followed by a further rise in [Ca²⁺], upon subsequent exposure to HTS (Fig. 6A). Amplitudes of Ca²⁺ transients upon treatment with PMA were most pronounced in MDCK cells expressing FLAG-tagged wild-type TRPV4, and significantly higher compared with cells expressing TRPV4 Y110F (Fig. 6A). The responses observed in control cells expressing empty vector pLXSN can be explained by the presence of endogenous TRPV4 in MDCK cells (44). The amplitudes of Ca²⁺ signals following treatments with PMA and HTS in individual cells expressing TRPV4 WT showed a positive correlation (r = 0.46, p ≤ 7.86e-14 for n = 236 individual cells taken from 8 independent measurements presented in Fig. 6A), suggesting that both signals are mediated by TRPV4. The responses to PMA and HTS were not evident when the cells were perfused with Ca²⁺-free solution (Fig. 6B), and were almost entirely abolished when the cells were pre-treated with ruthenium red (Fig. 6C), an inhibitor of TRPV channels. Together, these results strongly suggest that Ca²⁺ transients in MDCK cells upon treatments with PMA and HTS are mediated by the plasma membrane-localized TRPV4 channel, a conclusion supported by a recent
finding that responses to either stimulus in renal epithelial M-1 cells are dependent on endogenous TRPV4 (45). In addition, these experiments confirmed that the TRPV4 Y110F mutant is defective in response to activation by 1 μM PMA.

Phorbol esters can activate TRPV4 in PKC-dependent and independent manners (28). Pretreatment of MDCK cells expressing TRPV4-FLAG WT with BIM I, a broad-spectrum PKC inhibitor, significantly reduced the mean amplitude of Ca²⁺ transients to 51% in response to 1 μM PMA (Fig. 6D). Thus, TRPV4 activation by PMA depends, at least partially, on PKC activity, as shown previously for TRPV4-expressing HEK 293 cells (9, 28). Furthermore, we found that responses to PMA in these cells were severely reduced upon pretreatment with the SFK inhibitor PP2 (Fig. 6E). The latter finding indicates that SFKs positively regulate TRPV4 activation by PKC-mediated pathways and is consistent with the severely attenuated responses of the TRPV4 Y110F mutant to PMA.

The Increase in Tyrosine Phosphorylation of TRPV4 upon PMA Treatment Does Not Depend on the Tyr¹¹⁰ Residue—The phosphotyrosine level of TRPV4 increases upon hypotonic cell swelling (30). This increase was sensitive to pharmacological inhibition of the SFK activity, indicating an involvement of Src or related kinases. Because PKCs may activate Src leading to tyrosine phosphorylation and sensitization of TRPV1 (37), we investigated whether stimulation of MDCK cells with PMA promotes tyrosine phosphorylation of TRPV4. For this purpose, TRPV4-FLAG WT and Y110F mutant proteins were purified from MDCK cells and their phosphorylation status was examined with an anti-phosphotyrosine antibody. The basal level of tyrosine phosphorylation in the Y110F mutant was decreased, although not completely abolished, as compared with the wild-type.
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FIGURE 5. Steady state levels and localization of TRPV4-FLAG WT and Y110F proteins stably expressed in MDCK cells. A, the levels of TRPV4-FLAG proteins in lysates from MDCK cells were analyzed by Western blotting (WB) and compared with actin levels. The lysates from MDCK cells expressing pLXSN empty vector were analyzed as control. B, confocal fluorescence microscopy of immunostained MDCK cells stably expressing TRPV4-FLAG WT (left) and Y110F (right) proteins. TRPV4 was detected with anti-FLAG antibodies. Scale bars denote 10 μm.

observed increased autophosphorylation of Src within its activation loop, indicating enhanced Src kinase activity (Fig. 8A). Both tyrosine phosphorylation of TRPV4 and autophosphorylation of Src could be blocked by pre-treating the cells with the PP2 inhibitor prior to the application of hydrogen peroxide (Fig. 8A). Our results confirm that Src is activated by hydrogen peroxide in renal epithelial cells (47). Hydrogen peroxide-induced phosphorylation of the TRPV4 Y110F mutant was substantially weaker than the wild-type TRPV4 (Fig. 8B). Thus, hydrogen peroxide, and presumably oxidative stress, leads to a strong up-regulation of Src-dependent phosphorylation of TRPV4 channel, mostly at the Tyr\(^{110}\) residue.

**DISCUSSION**

Although the involvement of SFKs in the function of TRP channels is well documented, the functional consequences of SFK-mediated TRPV4 phosphorylation remain controversial. Therefore, we took a novel approach to explore the role of SFK-mediated TRPV4 regulation. Using mass spectrometry, we identified Tyr\(^{110}\) and Tyr\(^{805}\) as v-src-induced phosphorylation sites in the N- and C-terminal cytosolic tails of TRPV4, respectively. The same TRPV4 sites were phosphorylated in HEK 293T and in renal epithelial MDCK cells, which natively express this channel, suggesting that phosphorylation at these sites may be physiologically important. Although homologous sites are absent in other mammalian TRPV channels, the tyrosine residue corresponding to Tyr\(^{110}\) is present in osm-9, a functional homolog of TRPV4 in *C. elegans*. Substitution of tyrosine 110 with phenylalanine in TRPV4 severely altered its Ca\(^{2+}\) channel function. In comparison to wild-type channel, the TRPV4 Y110F mutant exhibited a diminished spontaneous activity, and mediated strongly reduced Ca\(^{2+}\) transients in response to most known stimuli, including the phorbol ester PMA. Importantly, we excluded that the Y110F mutation decreases the abundance of the TRPV4 channel at the plasma membrane by two different immunological methods in various cell types. Thus, replacement of tyrosine 110 with phenylalanine impairs TRPV4 channel function, without affecting its secretory trafficking. These changes were specific for the Y110F mutant because the spontaneous activity of the TRPV4 Y805F variant was comparable with that of the wild-type, although we did not extensively investigate the channel activity of TRPV4 Y805F.

Our data revealed that TRPV4-dependent Ca\(^{2+}\) transients in MDCK cells triggered by PMA were significantly reduced by prior inhibition of SFKs with PP2. Together with the findings discussed above, this indicates that phosphorylation at Tyr\(^{110}\) regulates the activity of TRPV4, at least in response to PMA. This conclusion is in agreement with several reports showing that SFKs increase phosphorylation and activation potential of various TRP channels (31, 33–35, 37). Moreover, Xu et al. (30) have shown that cell swelling induces SFK-mediated phosphorylation of TRPV4 at tyrosine 253, and proposed that Tyr\(^{253}\) phosphorylation regulates the channel permeability of TRPV4. However, our data indicate that TRPV4 phosphorylation at Tyr\(^{110}\) does not occur concomitantly with TRPV4 activation. Thus, we suggest that Tyr\(^{110}\) phosphorylation modulates rather than activates TRPV4, and provide the following rationale. First, phosphorylation of TRPV4 at Tyr\(^{110}\) occurs in unstimu-
lated cells; second, a modest increase in PMA-induced TRPV4 tyrosine phosphorylation does not depend on Tyr\(^{110}\); third, the reduction of PMA-stimulated Ca\(^{2+}\) transients in MDCK cells requires a prolonged incubation with the SFK-inhibitor PP2 (≈45 min in total) as well as prior serum starvation of cells, suggesting that inhibition of SFKs per se is not sufficient to block responses of TRPV4, but entails TRPV4 dephosphorylation. The requirement of Tyr\(^{253}\) phosphorylation for the activation of TRPV4 by cell swelling has remained controversial. Vriens et al. (23) presented evidence for the involvement of PLA\(_2\) and its metabolites in the cell swelling-induced TRPV4 response. Small Ca\(^{2+}\) transients in cells expressing TRPV4 Y110F upon hypotonic swelling (Fig. 4C) indicate that phosphorylation at this site is not absolutely required for this response but appears to lower the threshold of TRPV4 activation by PLA\(_2\) metabolites.

Phosphorylation of TRPC4 and TRPV1 by SFKs increases their presence at the cell surface, suggesting that SFKs regulate TRP channels by inducing their exocytosis (35, 37). Although we cannot completely exclude such a role for Tyr\(^{110}\) phosphorylation of TRPV4, the following two observations argue against it: first, under steady-state conditions, TRPV4 Y110F was more abundant than wild-type TRPV4 at cell surface of HEK 293T cells; second, if Tyr\(^{110}\) phosphorylation increases TRPV4 plasma membrane localization, we would expect to see equally impaired responses of the TRPV Y110F mutant to all stimuli. However, the TRPV4 Y110F mutant was strongly defective in responses to shear stress and PMA, and partially defective in response to hypotonic cell swelling, whereas the response to 4αPDD was not significantly different from that of wild-type TRPV4. These activation properties of TRPV4 Y110F are consistent with recent findings that TRPV4 can be activated through at least two distinct mechanisms, one involving PLA\(_2\) metabolites and the other involving direct binding of 4αPDD to the TM3 and TM4 of the channel (23, 26). We favor the hypothesis that phosphorylation of Tyr\(^{110}\) triggers a conformational change in TRPV4 and/or affects its interaction with

FIGURE 6. Murine TRPV4 stably expressed in MDCK cells mediates responses to PMA in PKC- and SFK-dependent manner. A, Fura-2 calcium imaging of MDCK cells stably expressing mouse TRPV4-FLAG WT, Y110F, or empty pLXSN vector. Cells were stimulated with 1 μM PMA and subsequently with HTS at 35 °C, as indicated with bars above the graphs. Shown are traces of representative measurements (the black line denotes mean 340/380 ratio for all cells recorded in the measurement, the gray area indicates 1 S.D.), and mean amplitudes of Ca\(^{2+}\) transients with S.E. of n independent measurements (n indicated in brackets above the error bars) in response to PMA treatment. B, MDCK cells expressing TRPV4-FLAG WT were treated with 1 μM PMA, HTS, and 50 μM ATP in Ca\(^{2+}\)-free solution (Ca\(^{2+}\) replaced by 5 mM EGTA). Shown is a representative trace (repeated 4 times). C–E, Representative traces and statistical analysis of mean amplitudes (depicted as above) of Ca\(^{2+}\) responses to 1 μM PMA in MDCK cells expressing TRPV4-FLAG WT with or without the following chemical inhibitors in the bath solution: C, 1 μM ruthenium red (RR); D, 100 nM BIM I; and E, 1 μM PP2. The inhibitors were also present during the incubation of cells with Fura-2 AM (RR was used at 100 nM during incubation).
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auxiliary proteins, thereby modulating TRPV4 gating in response to certain stimuli. Strikingly, the phenotypes of the TRPV4 Y110F mutant appear to recapitulate the effects of a recently discovered binding partner of TRPV4, PACSIN3 (48). The interaction with PACSIN3 increases TRPV4 apparent levels at the cell surface, inhibits its spontaneous activity, and impairs its responses to heat and hypotonic swelling, but not to 4μM PDD (27, 48). PACSIN3 binds to a proline-rich region in TRPV4 that is separated by only 21 amino acids from the Tyr110 residue. Thus, it is conceivable that binding to PACSIN3 competes with TRPV4 Tyr110 phosphorylation.

Nociception, the perception of pain, differs from other senses in that its sensitivity increases with time in the continued presence of painful stimulus, a process called hyperalgesia. This sensitization is in part achieved through up-regulation of TRPV1 via diverse mechanisms, including Src-mediated phosphorylation (37). Although TRPV1 appears to be the major player among TRP channels in mediating nociception, other family members including TRPV4 were shown to participate in this process as well (2). Trpv4 knock-out mice are defective in sensing noxious but not low-threshold mechanical stimuli (12, 15). In addition, these mice fail to develop mechanical and thermal hyperalgesia in inflamed tissues (16–18). Finally, TRPV4 is required for taxol-induced neuropathic pain in rats, a process that also depends on the integrin/Src pathway (49). These findings indicate that TRPV4 is involved in the sensitization of pain detection under pathological conditions, yet the underlying mechanisms remain unclear. In this study, we found that hydrogen peroxide strongly up-regulates Src-dependent tyrosine phosphorylation of TRPV4, including the Tyr110 residue. Supported by the established function of reactive oxygen species and SFKs in mediating inflammation and pain (46, 50, 51), our results suggest that SFK-mediated phosphorylation of Tyr110 may contribute to the sensitization mechanism of TRPV4 channel in hyperalgesia.

In summary, we identified two sites of Src-mediated phosphorylation in TRPV4. We present evidence that phosphorylation of Tyr110 is an important mechanism for the modulation of TRPV4 function. Finally, our results demonstrate the importance of the cytosolic N-terminal tail in mediating stimulus-specific responses of TRPV4, reinforcing recent findings by D’Hoedt et al. (27).
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