Regulation of a Transient Receptor Potential (TRP) Channel by Tyrosine Phosphorylation

SRC FAMILY KINASE-DEPENDENT TYROSINE PHOSPHORYLATION OF TRPV4 ON TYR-253 MEDIATES ITS RESPONSE TO HYPOTONIC STRESS*

Received for publication, October 29, 2002, and in revised form, January 17, 2003
Published, JBC Papers in Press, January 21, 2003, DOI 10.1074/jbc.M211061200

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The recently identified transient receptor potential (TRP) channel family member, TRPV4 (formerly known as OTRPC4, VR-OAC, TRP12, and VRL-2) is activated by hypotonicity. It is highly expressed in the kidney as well as blood-brain barrier-deficient hypothalamic nuclei responsible for systemic osmosensing. Apart from its gating by hypotonicity, little is known about TRPV4 regulation. We observed that hypotonic stress resulted in rapid tyrosine phosphorylation of TRPV4 in a heterologous expression model and in native murine distal convoluted tubule cells in culture. This tyrosine phosphorylation was sensitive to the inhibitor of Src family tyrosine kinases, PP1, in a dose-dependent fashion. TRPV4 associated with Src family kinases by co-immunoprecipitation studies and confocal immunofluorescence microscopy, and this interaction required an intact Src family kinase SH2 domain. One of these kinases, Lyn, was activated by hypotonic stress and phosphorylated TRPV4 in an immune complex kinase assay and an in vitro kinase assay using recombinant Lyn and TRPV4. Transfection of wild-type Lyn dramatically potentiated hypotonicity-dependent TRPV4 tyrosine phosphorylation whereas dominant negative-acting Lyn modestly inhibited it. Through mutagenesis studies, the site of toxicity-dependent tyrosine phosphorylation was mapped to Tyr-253, which is conserved across all species from which TRPV4 has been cloned. Importantly, point mutation of Tyr-253 abolished hypotonicity-dependent channel activity. In aggregate, these data indicate that hypotonic stress results in Src family tyrosine kinase-dependent tyrosine phosphorylation of the toxicity sensor TRPV4 at residue Tyr-253 and that this residue is essential for channel function in this context. This is the first example of direct regulation of TRP channel function through tyrosine phosphorylation.

Signaling by hypotonic stress and cell swelling has been well studied in diverse models (reviewed in Ref. 1). Nonetheless, the sensor for hypotonic stress, both at the single cell and organismal levels, remains elusive. A member of the transient receptor potential (TRP) family of cation channels has recently been described that fulfills multiple criteria for an osmosensing protein. Based upon homology with OSM-9, an osmosensing TRP-like channel expressed in Caenorhabditis elegans (2), the mammalian candidate osmosensing TRP channel family member, was cloned independently as OTRPC4 (3) and VR-OAC (4). This protein, also identified as TRP12 and vanilloid receptor-related protein-2 in other contexts, was recently renamed TRPV4 (5).

TRPV4, as a member of the TRPV subfamily of TRP channels, shares close homology with the following channels: 1) the capsaicin (capsaicin) receptor and sensor of noxious heat, TRPV1; 2) the heat-sensitive vanilloid receptor-related channel, TRPV2; 3) the calcium channels, TRPV5 (formerly known as CaCaC1 and CaTa2) and TRPV6 (formerly known as CaTa1, CaCaC2, and CaTa-L) (all reviewed in Ref. 5 and references therein); and 4) the very recently described temperature-sensitive receptor, TRPV3 (6–8). TRPV4 forms nonselective cation channels that are activated by changes in extracellular osmolality; hypertonicity, in contrast, inactivates the channel (3, 4).

Although TRPV4 is expressed at the mRNA level in multiple tissues including liver, heart, lung, spleen, and adipose tissue, it is by far most abundant in the kidney (3, 4). By in situ hybridization, renal expression was localized principally to the distal convoluted tubule (3). Interestingly, TRPV4 expression was also detected in the specific blood-brain barrier-deficient hypothalamic nuclei responsible for systemic osmosensing and in the mechanosensing hair cells of the inner ear (4). Functional and expression data were both consistent with a role for TRPV4 in osmosensing at both the single cell and organismal levels.

Apart from its gating by toxicity, little is known about TRPV4 regulation. We and others have shown that signaling events in response to hypotonic stress are reminiscent of activation of a receptor-tyrosine kinase (9–12). We observed that hypotonic stress resulted in rapid tyrosine phosphorylation of TRPV4 in a heterologous expression model and in native murine distal convoluted tubule cells in culture. This tyrosine phosphorylation was sensitive to the inhibitor of Src family cytoplasmic tyrosine kinases, PP1, in a dose-dependent fashion. TRPV4 associated with Src family tyrosine kinases by co-immunoprecipitation studies and confocal immunofluorescence microscopy, and this interaction required an intact SH2 domain. Lyn, a Src family kinase, was activated by hypotonic stress and phosphorylated TRPV4 in an immune complex ki-

* These studies were supported by the National Institutes of Health (Grant DK52494 to D. M. C.), by the American Heart Association, and by the Department of Veterans Affairs. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: TRP, transient receptor potential; SH, Src homology domain; HEK, human embryonic kidney cell.
nase assay and kinase assay with recombinant proteins. Transfection of wild-type Lyn dramatically potentiated hypotonicity-dependent TRPV4 tyrosine phosphorylation whereas dominant negative-acting Lyn modestly inhibited it. Phosphorylation occurred principally at residue Tyr-253, and point mutation of this residue abolished tonicity-dependent calcium entry. In aggregate, these data indicate that Src family kinase-dependent tyrosine phosphorylation of Tyr-253 regulates TRPV4 activation in response to hypotonicity.

**Materials and Methods**

**Cells and Cell Culture**—mMCD3 cells were maintained and passaged as previously described (13). The murine distal convoluted tubule dMCT cell line (14), kindly provided by Peter Friedman, was maintained in Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal bovine serum.

**Marine TRPV4 Cloning, Transient and Stable Transfection, and Mutagenesis—**TRPV4 was cloned from murine kidney poly(A) RNA via RT-PCR using the SuperScript Pre amplification System (Invitrogen) for first strand synthesis, followed by amplification with TRPV4-specific primers, 5'-AGT AGC GCA GAT CCT GGT GA-3' and 5'-GTA CAG TGG GCC ATC GTC C-3'. Amplified fragment was TA-cloned into pGEM3.Zf(+)-Topo (Invitrogen) to create an expression plasmid encoding a carboxyl-terminal V5-epitope-tagged murine TRPV4; identity was confirmed by sequencing (Core Facility, Vollum Institute for Advanced Biomedical Research). Following the demonstration of tyrosine phosphorylation of TRPV4, high probability tyrosine phosphorylation sites were sought using ProSite (us.expasy.org/prosite, Ref. 15) and NetPhos (www.cbs.dtu.dk/services/NetPhos, Ref. 16; see "Results"). The deduced amino acid sequence of the TRPV4 clone in the present study was identical to that of the canonical sequence, NP_071300, with the following exceptions: Leu instead of Arg at position 90, Thr instead of Ile at position 389, and His instead of Tyr at position 860. Leu-90 was previously reported in the context of murine TRP12 and murine VRAC. Of note, the extreme carboxy-terminal Tyr-860, present in NM_022017 and absent from our clone, was predicted to encode an extremely low-probability tyrosine phosphorylation site (probability 0.075, NetPhos). HEK293 cells were transfected with LipofectAMINE Plus in accordance with the manufacturer's directions (Invitrogen). Stable cell lines were derived from pooled clones propagated under selection pressure with Gly-418. Lyn-WT and Lyn-DN were originally selected with Gly-418. Lyn-WT and Lyn-DN were originally selected with Gly-418. Lyn-WT and Lyn-DN were originally selected with Gly-418.

**Antibodies, Immunoblotting, Immunoprecipitation, and Kinase Assay**—Antibodies—Anti-peptide polyclonal antibody was raised in two rabbits against a synthetic peptide representing the carboxyl-terminal sequence of TRPV4 (CDG HQQ GYA PKW RTD DAP L) and then subjected to affinity purification with the peptide antigen (Alpha Diagnostic International, Inc., San Antonio, TX). Sequence analysis (BLAST search with peptide) indicated that this sequence was not conserved across many other TRP family members (TRP1, TRP2, TRP5, TRPV1, TRPV3, TRPV4). It was conserved across murine VRL-2, TRP12, OTRPC4, and TRPV4, all of which are nearly identical at the amino acid level and have been collectively designated TRPV4 (5). Other antibodies were commercially available including anti-PY99 (Upstate Biotechnology), anti-V5, anti-Lyn, anti-Src, anti-Fyn, anti-Lck, anti-Hck, anti-Yes (Santa Cruz Biotechnologies). Immunoblot analysis was performed as described below (18). Cell monolayers were lysed in Lysis Buffer (125 mM NaCl, 50 mM Tris (pH 7.5), 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 µM leupeptin, 1 µM meprin, 1 µM pepstatin A, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate) for 30 min at 4°C and immunoprecipitated with appropriate antibody and protein A/G bound to Sepharose beads. Lyn immune complex kinase assay was performed essentially as described by Yoshida et al. (17). Briefly, monolayers were lysed in Lysis Buffer, immunoprecipitated with antibody, washed, and subjected to kinase assay in Complete Kinase Buffer (50 mM HEPES, pH 7.4, 2 mM dithiothreitol, 10 mM MgCl2, 10 mM MnCl2, 0.1 mM sodium orthovanadate, 20 µM ATP, 1% (v/v) 32P[ATP] (250 µCi/ml), 75 ng/µl avidin-activated enolase) for 30 min at 30°C prior to SDS/PAGE. Adult mouse kidney tissue blot was obtained from Alpha Diagnostic International. For Lyn co-immunoprecipitation kinase assay, monolayers of control or hypotonicity-stressed HEK293 cells were lysed in Lysis Buffer, immunoprecipitated with anti-Lyn, washed extensively, and then subjected to in vitro kinase assay in Complete Kinase Buffer for 30 min at 30°C in the absence of acid-activated enolase. In vitro TRPV4-directed Lyn kinase assay was performed essentially as described by Chin et al. (18). Briefly, in vitro transcribed and translated TRPV4 or ΔTRPV4 (see below), synthesized using the TNT kit (Promega) in accordance with the manufacturer's instructions, was immunoprecipitated with anti-V5 antibody. After washing, bead-bound TRPV4 or ΔTRPV4 was incubated in In Vitro Kinase Buffer (10 mM HEPES, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 5 mM MnCl2, 0.1 mM ATP, 5% (v/v) [γ-32P]ATP, 32P (50 Ci/ml), 20 mM NaOH, 0.36 µg/µl LNA (Calbiochem)) for 30 min at 30°C. ΔTRPV4 (murine TRPV4 absent membrane-spanning domains 2 through 6) was prepared through BsaI restriction digestion of TRPV4-pcDNA3.I/V5-His-TOPO and self-ligation following gel purification of the larger fragment. In some experiments (e.g., anti-V5 immunoprecipitation followed by anti-Src family protein-tyrosine kinase immunoblotting), anti-V5 was covalently cross-linked to protein A/G-Sepharose (Amersham Biosciences) using dimethyl pimelimidate (20) to eliminate the secondary antibody detection of immunoglobulin heavy chain, which co-migrates with many Src family protein-tyrosine kinases.

**Intracellular Calcium Measurement—**Intracellular [Ca2+]i measurement were conducted on cell suspensions obtained by trypan blue staining of confluent cell monolayers of wild-type and TRPV4 stably transfected HEK293 cells. The suspended cells were loaded with Fura-2-AM, washed twice with HEPES-buffered saline solution (HBSS: 130 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM NaHPO4, 2 mM CaCl2, 10 mM HEPES, pH 7.4), and assayed for intracellular calcium concentration ([Ca2+]i) in a cuvette under constant, gentle stirring (2-ml final volume). Fluorescent emission was monitored at 510 nm with alternate excitation at 340 and 380 nm using a Hitachi F2000 fluorescence spectrophotometer (Hitachi Instruments, Inc., Naperville, IL). Calibration of the Fura-2 signal was performed as previously described (21) using Fura-2Ca^2+ dissociation constant of 224 nm (22).

**Immunofluorescence**—Monolayers were grown on LabTek Chamber slides (Nalgene Nunc) and labeled with primary antibody (anti-Lyn (Santa Cruz Biotechnology sc-15), anti-Src (Santa Cruz Biotechnology sc-14), or anti-V5 (In vitro 46-0705)) and secondary antibody (AlexaFluor 594 goat anti-rabbit IgG (Molecular Probes A-11037) or AlexaFluor 488 goat anti-mouse IgG (Molecular Probes A-11091) prior to confocal epifluorescence imaging. Studies were performed on wild-type HEK293 cells and on HEK293 cells stably transfected with TRPV4. The affinity-purified anti-TRPV4 antibody was insufficiently sensitive in the immunofluorescence assay to permit examination of the native (mDCT) system. For confocal imaging, consecutive sections 500 nm apart, bracketing the center of the cell, were acquired in alternating between sets of 488 and 568 nm units as a Zeiss TCS SP confocal laser scanning microscope. System settings were held constant for all imaging. Images were digitally captured and then deconvolved using Power HazeBuster™ imaging software (VayTek, Inc., Fairfield, VA). All imaging analysis determining colocalization was carried out using software by IPLab™ (Senealitics Inc., Fairfield, VA).

**Results**

Because tyrosine phosphorylation events may accompany hypotonic stress, we sought to determine whether hypotonicity resulted in tyrosine phosphorylation of the tonicity-responsive TRPV4. V5-epitope-tagged TRPV4 was stably overexpressed in HEK293 cells, which were reported to lack this channel (3). By anti-phosphotyrosine immunoblotting of anti-V5 immunoprecipitates, hypotonic stress (150 mosmol/kg H2O) resulted in pronounced and transient up-regulation of TRPV4 tyrosine phosphorylation (Fig. 1A). This phenomenon was specific to hypotonic stress as it was not observed in the presence of treatment with the peptide growth factor, epidermal growth factor, or the activator of classical protein kinase C, 12-O-tetradecanoylphorbol-13-acetate (TPA) (Fig. 1B). There was no effect upon total TRPV4 abundance in these experiments (data not shown). Two other solute stressors, hypertonic stress and urea stress, both failed to increase anti-phosphotyrosine immunoreactivity in this model (data not shown). Of note, additional less intense bands migrating more slowly than the principal
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**Fig. 1.** TRPV4 is tyrosine-phosphorylated in response to hypotonic stress. Anti-phosphotyrosine immunoblots of anti-V5 immunoprecipitates prepared from TRPV4 stable transfectants of HEK293 and subjected to the indicated duration of hypotonic stress (150 mosmol/kg H2O) (A) or exposure to other cell activators (EGF, 100 ng/ml) (B). C, effect of incubation with the indicated concentration of endoglycosidase-F upon electrophoretic mobility of TRPV4. NS, non-specific.

A band corresponding to newly tyrosine-phosphorylated TRPV4 were frequently observed in this heterologous expression model. Treatment of lysates with endoglycosidase-F resulted in the complete disappearance of the higher molecular mass bands and enhancement of the principal lower molecular mass band (Fig. 1C). These data were consistent with the presence of forms of TRPV4 exhibiting varying degrees of glycosylation.

We next sought to confirm that: 1) stable heterologous transfection of epitope-tagged TRPV4 resulted in expression of functional TRPV4 and thereby mimicked endogenous expression, and 2) addition of the epitope tag did not interfere with channel function. Calcium transients in response to hypotonicity were examined in untransfected and TRPV4-transfected HEK293 cells. In both untransfected and TRPV4-transfected cells, there was negligible calcium entry following sham (isotonic) treatment (Fig. 2A). In response to hypotonic stress (150 mosmol/kg H2O), there was a robust response in the TRPV4-transfected cells but virtually no response in the wild-type cell line. Next, to confirm that the calcium transients required calcium entry, similar studies were performed with only the TRPV4-transfected cell line in the presence and absence of extracellular calcium (Fig. 2B). The response to hypotonic stress was noted again, in the presence of extracellular calcium ([Ca2+]o = 1.25 mM) but was completely absent when calcium was omitted from the assay buffer ([Ca2+]o ~0 mM). However, the hypotonicity-dependent, intracellular calcium signal was restored upon addition of calcium (CaCl2, 1.25 mM) to the cuvette. In aggregate, these data indicated that the heterologous expression model functionally mimicked the native system.

To demonstrate that tyrosine phosphorylation of TRPV4 in response to hypotonic stress occurred in a native system, in a fashion similar to the HEK293-based heterologous expression system, a rabbit polyclonal, affinity-purified anti-peptide antibody was generated against the carboxyl terminus of murine TRPV4 (see “Materials and Methods”). The specific peptide was chosen to avoid cross-reactivity with closely related members of the TRP channel family (Fig. 3A); VRL-2, OTRPC4, VR-OAC, and TRP12 are names independently assigned to the protein now designated TRPV4 (5). Murine renal tissue was screened for anti-TRPV4 immunoreactivity. Expression was detected in whole kidney lysate (Fig. 3B), most of which appeared to be confirmed by expression in the outer cortex. The distal convoluted tubule, where TRPV4 mRNA had previously been detected (3), is confined to the outer cortex. Several cell lines were also screened for the presence of TRPV4 by anti-TRPV4 immunoblotting (Fig. 3C). Consistent with the reports of others, anti-TRPV4 immunoreactivity was absent in 3T3 and HEK293 cells. It was, however, present, in the renal distal convoluted tubule cell line (14), mDCT, and in the renal inner medullary cell line, mIMCD3. As expected, abundant TRPV4 expression was detected in a HEK293 cell line stably transfected with TRPV4. To assess utility of the anti-TRPV4 antiserum in immunohistochemistry, and to confirm that the TRPV4 overexpression system exhibited membrane-associated immunoreactivity, anti-TRPV4 immunofluorescence microscopy was performed with wild-type HEK293 cells and with the TRPV4/HEK stable cell line studied in Fig. 2. Anti-TRPV4 confocal immunofluorescence microscopy demonstrated modest nonspecific staining in wild-type HEK293 cells (Fig. 3D) and robust, primarily cell membrane-associated, expression in TRPV4/HEK stable transfectants (Fig. 3D). The effect of hypotonicity upon TRPV4 tyrosine phosphorylation was next examined in the native model of the mDCT cell line. Hypotonic stress (~150 mosmol/kg H2O) resulted in robust time-dependent tyrosine phosphorylation of TRPV4 in this model (Fig. 3E), confirming that neither heterologous expression nor, potentially, overexpression, was required for this phenomenon.

To ascertain in preliminary fashion the nature of this tyrosine phosphorylation event, a panel of tyrosine kinase inhibitors was applied prior to hypotonic stress. The general tyrosine kinase inhibitor, genistein, only modestly inhibited the effect of hypotonicity whereas the Src family kinase-specific inhibitor, PP1, was more effective. In addition, this effect was dose-de-
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Fig. 3. Tonicity-dependent tyrosine phosphorylation of TRPV4 is evident in a native system. A, alignment of carboxyl termini of TRPV family members (c, C. elegans; h, human; m, murine; r, rat) with TRPV4 (formerly known as VRL-2, OTRPC4, VR-OAC, or TRP12) depicting location of synthetic peptide to which anti-TRPV4 polyclonal antibody was raised. B, immunodetection of TRPV4 in murine kidney tissue lysates prepared from whole kidney (K), inner cortex (IC), outer cortex (OC), inner medulla (IM), and papilla (P). C, anti-TRPV4 immunoblot of whole cell lysates prepared from the indicated untransfected (wild-type) cell types, as well from TRPV4-HEK293 stable transfectants. D, confocal microscopy of subconfluent monolayers of wild-type HEK293 cells (left) and TRPV4/HEK293 stable transfectants (right) labeled with anti-TRPV4 and AlexaFluor-594-conjugated anti-rabbit secondary antibody (see “Materials and Methods”). E, anti-phosphotyrosine immunoblot of anti-TRPV4 immunoprecipitates prepared from a murine distal convoluted tubule cell line (mDCT) treated for the indicated interval with hypotonic stress.

To confirm our inference from the inhibitor data, we first sought to confirm the interaction in the native system (Fig. 4). Of note, piceatannol, which is reportedly specific for Syk (23), a related cytoplasmic protein-tyrosine kinase not belonging to the Src family (reviewed in Ref. 24), failed to influence tonicity-dependent tyrosine phosphorylation. These data suggested in a preliminary fashion that tonicity-dependent activation of a Src family kinase may be responsible for TRPV4 tyrosine phosphorylation.

To further support a physical interaction between these proteins, confocal immunofluorescence microscopy was performed. In HEK293 cells stably transfected with TRPV4, near total co-localization of TRPV4 with Lyn was observed (Fig. 6). This co-localization, however, was unaffected by up to 30 min of exposure to hypotonicity. In contrast, only a small fraction of immunodetectable TRPV4 exhibited co-localization with Src (data not shown).

We next sought to confirm the interaction in the native mDCT system and to determine in preliminary fashion the Src family of tyrosine kinases.

Fig. 4. Effect of hypotonicity is inhibited by PP1. Anti-phosphotyrosine immunoblot of anti-V5 immunoprecipitates prepared from TRPV4-HEK293 stable transfectants subjected to control treatment or hypotonic stress for 30 min, in the presence or absence of the tyrosine kinase inhibitors, genistein (100 μM), PP1 (100 nM/1 μM), or piceatannol (30 μM).

Fig. 5. TRPV4 co-immunoprecipitates with Src family tyrosine kinases. A, anti-V5 immunoprecipitates prepared from wild-type HEK293 cells and TRPV4-HEK293 stable transfectants subjected to hypotonic stress for the indicated interval, and then immunoblotted for the indicated Src family tyrosine kinase. B, anti-V5 immunoblot of immunoprecipitates prepared from wild-type HEK293 cells and TRPV4-HEK293 stable transfectants; cells were subjected to hypotonic stress for the indicated duration and then immunoprecipitated with antibodies specific for the indicated Src family protein-tyrosine kinase.
TRPV4 and Lyn co-localize by confocal microscopy. TRPV4-transfected HEK293 cells were subjected to confocal imaging analysis after immunofluorescence staining with anti-Lyn and anti-V5. Panel A depicts Lyn immunolocalization, panel B is TRPV4 localization, and panel C is a merging of the Lyn and V5-TRPV4 images showing co-localization. A, B, and C are corresponding individual layers (1 µm); D represents the sum of all merged layers.

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Fig. 7. TRPV4 associates with Src family kinases via SH2 domain. A, lysates prepared from control-treated TRPV4-HEK293 stable transfectants, affinity-precipitated with agarose bead-bound Fyn-(85–247) or control protein A/G-bound beads, and then immunoblotted with anti-V5 to detect the presence of V5-TRPV4. (Fyn-(85–247) encodes the SH3-SH2 domains of Fyn.) B, lysates prepared from wild-type mDCT cells treated for the indicated interval with hypotonic stress, affinity-precipitated with agarose bead-bound Fyn-(85–247) or control protein A/G-bound beads, and then immunoblotted with anti-TRPV4. C, lysates prepared from wild-type mDCT cells (upper panel) and TRPV4-HEK293 stable transfectants (lower panel) treated for the indicated interval with hypotonic stress, affinity-precipitated with agarose bead-bound Fyn-(85–139) or Fyn-(145–247), and then immunoblotted with anti-TRPV4. Fyn-(85–139) encodes the SH3 domain and Fyn-(145–247) encodes the SH2 domain of Fyn.

Fig. 8. Transfection of Lyn enhances and dominant negative-acting Lyn inhibits tonicity-dependent TRPV4 tyrosine phosphorylation. TRPV4-HEK293 stable transfectants were transiently transfected with the indicated plasmid and subjected to control treatment or hypotonic stress (150 mosmol/kgH₂O × 30 min) prior to anti-V5 immunoprecipitation and anti-phosphotyrosine immunoblotting. The discontinuity between lanes 2 and 3 reflects the removal of several intervening lanes, all depicted lanes are taken from the same exposure of the same autoradiograph.
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were present in this domain of TRPV4. Recombinant Lyn phosphorylated the in vitro transcribed and translated TRPV4 and ΔTRPV4 producing bands precisely co-migrating with immunodetectable TRPV4 and ΔTRPV4 (Fig. 9C). Importantly, no substrate phosphorylation was evident in the absence of exogenous Lyn (data not shown). These data suggested that in addition to associating with Lyn, TRPV4 serves as a bona fide Lyn substrate.

We next sought to identify the site of the tyrosine phosphorylation in TRPV4 through site-directed mutagenesis studies. Preliminary sequence analysis of mTRPV4 using a standard approach, the single “classical” tyrosine phosphorylation motif in this protein (upon which we based our initial mutagenesis approach, Tyr-411) was actually a suboptimal candidate for phosphorylation in vivo because of an adjacent bulky tryptophan residue rendering the tyrosine sterically inaccessible. Eight other sites were identified with a high likelihood of undergoing regulated tyrosine phosphorylation. Based upon conservation across species in which TRPV4 has been cloned and upon predicted intracellular localization, we selected five sites for mutagenesis. The 5×-mutant TRPV4 construct included the following mutations (numbered according to accession number NP_071300, the murine consensus TRPV4): Y253F, Y281F, Y415F, Y508F, and Y805F. Hypotonicity-inducible tyrosine phosphorylation was evident in anti-V5 immunoprecipitates of mDCT cells transiently transfected with wild-type TRPV4 but not in cells transfected with the 5×-mutant (Fig. 10A, upper panel). Background nonspecific immunoreactivity co-migrating with TRPV4 (Fig. 10A) precluded detailed analysis of the tyrosine residues undergoing phosphorylation so an additional model system was sought. The COS7 cell line, in which essentially no co-migrating background was detectable, was selected for further study. Consistent with the mDCT model, mutagenesis of the 5 key tyrosine residues of TRPV4 prevented hypotonicity-dependent phosphorylation in the COS7 cells (Fig. 10A, lower panel).

A series of stable COS7 cell lines expressing single Tyr → Phe mutants of TRPV4 was generated and compared with cells expressing wild-type TRPV4 (Fig. 10B). Although there was some variability in the level of expression of each of the mutants, it was clear that Tyr-253 accounted for the vast majority of regulated (and constitutive) tyrosine phosphorylation of TRPV4. Interestingly, Tyr-253 is conserved across all species from which TRPV4 has been cloned (mouse, rat, and human), yet is not shared by any of the other TRPV family members or the prototypical C. elegans channel, OSM-9 (Fig. 10C). In addition, it is not shared by any members of the TRPC or TRPM families (data not shown).

Further studies were carried out to determine the importance of the putative tyrosine phosphorylation sites in TRPV4 function in response to hypotonicity. Wild-type HEK293 cells were compared with cells stably transfected with TRPV4 or with TRPV4 harboring mutations (Tyr → Phe) in the five key tyrosine residues (Fig. 11A). Again, a robust increase in intracellular calcium was observed in TRPV4-transfected cells but not in untransfected HEK cells. Importantly, the 5×-mutant transfected essentially lacked hypotonicity responsiveness, with a response indistinguishable from that of untransfected HEK. This effect was not a consequence of lack of expression of the mutant protein or aberrant targeting of the mutant protein because immunodetectable TRPV4 was evident in both whole cell lysates and membrane preparations from the 5×-mutant cell line (Fig. 11A). Because Tyr-253 was the principal target of tyrosine phosphorylation in response to hypotonic stress based upon our mutagenesis data (Fig. 10), we next assessed the effect of the single Tyr-253 point mutant upon TRPV4 channel function (Fig. 11B). The Y253F mutant, similar to the 5×-mutant, exhibited negligible hypotonicity-dependent calcium response. Again, TRPV4 expression and membrane targeting were comparable to the overexpressed wild-type TRPV4 as demonstrated through immunoblotting and preparation of membrane and cytosolic proteins (Fig. 11B). In addition, the subcellular distribution of the heterologously expressed Tyr-253 was indistinguishable from that of the heterologously ex-

Fig. 9. Lyn is activated by hypotonic stress and phosphorylates TRPV4. A, autoradiogram of immune complex kinase assay of anti-Lyn immunoprecipitates prepared from control and hypotonicity treated (150 mosmol/kgH2O × 15 min) HEK293; arrowhead denotes in vitro phosphorylated Lyn substrate (enolase). B, co-immunoprecipitation kinase assay of anti-Lyn immunoprecipitates prepared from TRPV4-HEK293 stable transfectants; immunoprecipitates were subjected to kinase assay in absence of exogenous substrate. Phosphorylation of band co-migrating with TRPV4 is detected following hypotonic treatment. C, in vitro kinase assay with recombinant LynA and in vitro transcribed/translated TRPV4 or ΔTRPV4 (a TRPV4 deletion mutant lacking five of six membrane-spanning domains to facilitate expression). A specifically phosphorylated band, co-migrating with either immunodetectable TRPV4 or ΔTRPV4 was evident. No phosphorylation, specific or nonspecific, was noted in the absence of LynA (data not shown).
pressed wild-type TRPV4 (data not shown). These data indicate that Tyr-253 is essential not only for tyrosine phosphorylation of TRPV4 but also for function of TRPV4 in response to hypotonic stress; they are also consistent with the observation of Liedtke et al. (4) and Watanabe et al. (28) who noted a decrease in the hypotonicity responsiveness of mutant TRPV4 proteins lacking much of the amino terminus. Of note, mutation of a different single tyrosine among the five principal candidates (Tyr-281), upon stable transfection of the resultant plasmid, virtually abolished tonicity-dependent calcium mobilization.

In contrast to the present model of direct regulation, several TRP family members are indirectly regulated by upstream signaling in anisotonicity has been extensively studied in diverse models (reviewed in Ref. 1), comparatively fewer data describe the role of tyrosine kinase activity in this process. In cell culture models, tyrosine kinase activity is up-regulated by hypotonic stress (9, 10) and is required for immediate-early gene transcription and expression (10, 32). Pharmacological inhibition of tyrosine kinase activity blocks, and potentiation of

observed the physical and functional interaction between TRPV4 and Src family kinases in both the heterologous over-expression model and in the native expression model afforded by the murine distal convoluted tubule cell line. We show that TRPV4 becomes newly tyrosine-phosphorylated in response to hypotonic stress, and that point mutation of the tyrosine residue conferring essentially all of the de novo phosphorylation virtually abolishes toxicity-dependent calcium mobilization.

Although kinase activation and the role of kinase-dependent signaling in anisotonicity has been extensively studied in diverse models (reviewed in Ref. 1), comparatively fewer data describe the role of tyrosine kinase activity in this process. In cell culture models, tyrosine kinase activity is up-regulated by hypotonic stress (9, 10) and is required for immediate-early gene transcription and expression (10, 32). Pharmacological inhibition of tyrosine kinase activity blocks, and potentiation of

To the best of our knowledge, these are the first data demonstrating regulated tyrosine phosphorylation of a TRP channel or its association with a Src family protein-tyrosine kinase. Moreover, they are the first to describe regulation of function of a TRP channel through direct tyrosine phosphorylation. We

FIG. 10. Hypotonicity results in phosphorylation of Tyr-253 of TRPV4. A, anti-PY99 immunoreactivity (tyrosine phosphorylation) of anti-V5 immunoprecipitates prepared from untransfected mDCT cells (None), or mDCT cells transiently transfected with either wild-type (+TRPV4) or 5× tyrosine mutant (5×-mut) TRPV4 (upper panel); the lower panel depicts the identical experiment performed using stable transfectants in COS7 cells. B, anti-PY99 immunoreactivity (tyrosine phosphorylation; upper panel) and anti-V5 immunoreactivity (lower panel) of anti-V5 immunoprecipitates prepared from COS7 cells stably transfected with an expression vector encoding wild-type TRPV4 (WT) or the indicated tyrosine-directed Tyr-as the point mutant of TRPV4, and then subjected to the presence (+) or absence (−) of hypotonicity. The smear in the left-most lane of the upper panel indicates molecular mass markers. C, alignment of murine (m), rat (r), and human (h) TRPV4 with TRPV1-TRPV6 (murine clones, where available), and C. elegans OSM-9 showing restriction of Tyr-253 to TRPV4.

FIG. 11. Mutation of Tyr-253 abolishes hypotonicity-dependent calcium transients. A, HEK293 cells (HEK) or HEK cells stably transfected with TRPV4, or TRPV4 in which the five principal putative tyrosine phosphorylation sites have been mutated (5×-mut-TRPV4), were subjected to isotonicity or hypotonic stress and the effect upon intracellular calcium concentration was determined ratiometrically. The filled arrowhead indicates the time of addition of the cells to the cuvette; the transient upward and downward deflections noted at this time are artifacts of chamber opening. Expression of TRPV4 in whole-cell extracts and in cell membrane preparations was assessed via anti-TRPV4 immunoblotting. B, studies similar to those performed in A were performed with HEK cells stably transfected with either TRPV4 or the point mutant Y253F-TRPV4. The effect of hypotonicity was virtually abolished (i.e. indistinguishable from untransfected HEK cells) by the point mutation. Whole-cell and membrane-associated expression were again confirmed through immunoblotting.

DISCUSSION

To the best of our knowledge, these are the first data demonstrating regulated tyrosine phosphorylation of a TRP channel or its association with a Src family protein-tyrosine kinase. Moreover, they are the first to describe regulation of function of a TRP channel through direct tyrosine phosphorylation. We
Tyrosine kinase activity inhibits, regulatory volume decrease- associated efflux of cations (9), chloride (33–39), and the amino acid osmolyte, taurine (39, 40).

Several cytoplasmic protein-tyrosine kinases have previously been implicated in the hypotonic stress response. In a marine elasmobranch model of hypotonic stress, Syk- and Lyn-phosphorylated band 3, an efflux pathway for the osmolyte, taurine (41). In lymphocytes, lck may mediate activation of swelling-induced chloride efflux, based in part upon experiments using p56
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Acknowledgment—We thank Peter Friedman for the generous gift of the mDCT cell line.

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