

Epidermal Growth Factor Induces Tyrosine Phosphorylation, Membrane Insertion, and Activation of Transient Receptor Potential Channel 4*

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Various members of the canonical family of transient receptor potential channels (TRPCs) exhibit increased cation influx following receptor stimulation or Ca^{2+} store depletion. Tyrosine phosphorylation of TRP family members also results in increased channel activity; however, the link between the two events is unclear. We

nel, which led the authors to suggest that the interaction between hTRPC4 and NHERF is required for the retention and stabilization of hTRPC4 channels at the cell membrane (22). NHERF is also involved in regulating the cell surface expression levels of numerous other plasma membrane proteins, including the β -adrenergic receptor and platelet-derived growth factor receptor β , in a phosphorylation-dependent manner (24, 25). The activity of TRPC5 channels has also been shown to be influenced by interactions with NHERF (26). Dynamic regulation of the interaction between TRPC4 and NHERF may provide a mechanism for controlling TRPC4 surface expression and channel activation.

The present study investigates the basis for EGFR-mediated Ca^{2+} entry through hTRPC4 channels. Evidence is presented that this key physiological activity results through Src family tyrosine kinase-mediated phosphorylation of hTRPC4 causing an increase in channel association with NHERF and a resultant increase in the plasma membrane expression of hTRPC4.

MATERIALS AND METHODS

Antibodies—Anti-M2 FLAG antibody was obtained from Sigma-Aldrich, anti-PY20 antibody from BD Transduction Laboratories, and anti-TRPC4 and -TRPC5 antibodies from Alomone Laboratories. Anti-TRPC6 antibody was from Chemicon Laboratories. Rabbit polyclonal and mouse monoclonal anti-NHERF antibodies were from Calbiochem and Abcam Ltd., respectively. Anti-FYN (FYN3), anti-Lyn, anti-EGFR, anti-Src, anti- β 4 integrin, and anti-TRPC4 (sc-20) antibodies were purchased from Santa Cruz Biotechnology. Streptavidin horseradish peroxidase and alkaline phosphatase conjugates were from Silenus Laboratories. Horse anti-mouse biotin was from Vector; Rabbit anti-GFP antibody and Streptavidin Alexa-594 were from Molecular Probes. All other reagents were from Amersham Biosciences.

Immunoprecipitation and Biotinylation—Cell lines were maintained in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 10% fetal bovine serum at 37 °C in 5% CO_2 . Cell culture reagents were obtained from Invitrogen. HEK293 cells stably expressing FLAG-hTRPC4 were maintained in the above media containing 400 $\mu\text{g}/\text{ml}$ Zeocin (Invitrogen). COS-7 and HEK293 cells were transfected with expression plasmids with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and routinely achieved between 35 and 60% transfection efficiency.

For immunoprecipitation studies, cells were washed in ice-cold PBS and lysed in 1.2 ml of RIPA buffer (1% Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.05% (w/v) SDS, 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 0.2 mM Na_3VO_4 , 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride) supplemented with Complete Protease Inhibitors (Roche Applied Science). Protein content was determined by BCA analysis (Pierce Biotechnology) and equal amounts of protein subjected to immunoprecipitation with protein G-Sepharose (Amersham Biosciences) and 4 μg of antibody for 2 h at 4 °C. For immunoprecipitation from rat brain membranes, lysates were pre-cleared with protein G-Sepharose (Amersham Biosciences) followed by centrifugation. Immune complexes were washed four times with lysis buffer and resolved, along with lysates, by 8% SDS-PAGE followed by Western blotting onto Hybond ECL nitrocellulose membrane (Amersham Biosciences). Membranes were blocked and probed with indicated antibodies, visualized with ECL Plus reagent on Typhoon Imaging System (Amersham Biosciences), and quantified with ImageQuaNT software.

For biotinylation studies A431 or COS-7 cells treated with human EGF (Sigma-Aldrich) either in the presence or absence of PP2, PP3, or AG1478 (Calbiochem, Merck Biosciences) were washed in PBS and either lysed in RIPA buffer or biotinylated with 0.2 mg/ml EZ-Link Sulfol-NHS-Biotin

(Pierce Biotechnology) in PBS for 30 min at 4 °C. Cells were then washed three times with 10 mM glycine in PBS prior to lysis in RIPA buffer. Cell lysates were subjected to immunoprecipitation and Western blot analysis with streptavidin-HRP or streptavidin-AP. Total biotinylated membrane proteins were isolated by incubating lysates with 50 μl of NeutrAvidin-agarose (Pierce Biotechnology) for 2 h at 4 °C.

DNA Constructs and Mutagenesis—N-terminal FLAG-tagged hTRPC4 was generated by PCR from pCMVSPORT-hTRPC4 (gift of Dr. J. Putney, NIEHS, National Institutes of Health) (27) using Expand High Fidelity Taq (Roche Diagnostics) and cloned into BamHI-NotI sites of pcDNA3.1/Zeo(+) with an inserted internal ribosome entry site-GFP sequence (Invitrogen). All clones were verified by DNA sequencing (Biomolecular Research Facility, Newcastle, Australia). The Y959F, Y972F, and Y959F/Y972F hTRPC4 mutants were generated using QuikChange II (Stratagene). Primer pairs for all PCR steps were supplied upon request. YFP-hTRPC4 was generated by BamHI-NotI digestion of FLAG-hTRPC4 from pcDNA3.1/Zeo(+) and insertion after the C terminus of YFP in pYFP-NT-3.1/Zeo(+) (Dr. F. Ross, University of Newcastle). peGFP-hTRPC1 was amplified by PCR from pTRPC1-N-FLAG (gift of Dr. G. Barritt, Flinders University and Dr. C. Montell, John Hopkins University) with Expand High Fidelity Taq and inserted into BamHI-XbaI sites of peGFP-C3 (Clontech). Truncated C-terminal GFP-hTRPC4 was generated by PCR amplification of pCMVSPORT-hTRPC4 and ligated in-frame with the C terminus of GFP in peGFP-C2 (Clontech). GST fusions were generated by PCR amplification from pCMVSPORT-hTRPC4 and insertion into the BamHI-NotI sites of pGEX-4T-1 (Amersham Biosciences). GST-NT-T was generated by EcoRI-SalI digestion of GST-NT and sub-cloning liberated DNA into pGEX-4T-1. The hTRPC5 construct was obtained from Dr. D. Beech (University of Leeds). Fyn constructs were provided by Dr. T. Yamamoto (University of Tokyo, Japan) (28), Lyn constructs by Dr. M. Hibbs (29), and the wild-type-EGFR construct by Dr. F. Walker (Ludwig Institute, Melbourne) (30).

In Vitro Kinase Assay—HEK293 cells were transfected with pMET-Fyn-YF expression vector and lysed with RIPA buffer. Fyn-YF was precipitated with FYN3 antibody, washed two times with kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl_2 , 3 mM MnCl_2 , 10 μM ATP, with 0.25% Triton X-100), and immune complexes were incubated with equal quantities of purified GST fusion proteins in kinase buffer for 30 min at 37 °C. Complexes were cleared by centrifugation and analyzed by SDS-PAGE and immunoblotting.

Immunofluorescence and Confocal Microscopy—COS-7 cells transfected with YFP-hTRPC4 and HA-NHERF, were grown on glass coverslips, washed twice with PBS, then fixed in 3.7% formaldehyde/PBS/5% sucrose and permeabilized with 50 $\mu\text{g}/\text{ml}$ digitonin. Cells were blocked in 5% normal goat serum/5% normal horse serum for 45 min, then stained with anti-NHERF antibodies followed by anti-mouse biotin prior to development with streptavidin-conjugated Alexa 594. Cells were mounted with Anti-Fade (Dako Cytomation) and observed under 63 \times oil-immersion objective on a Zeiss Axiovert 100M confocal microscope and analyzed with Zeiss LSM510.

Intracellular Ca^{2+} Imaging—Cells were cultured on glass coverslips and loaded with 2–4 μM fura-2-acetoxymethyl ester in Dulbecco's modified Eagle's medium at 37 °C for 30 min and incubated in HEPES-buffered saline (HBS, 140 mM NaCl, 5 mM KCl, 0.5 mM MgCl_2 , 5.5 mM HEPES, 10 mM glucose, 2 mM CaCl_2 , pH 7.4) for 30 min prior to stimulation. In experiments performed in Ca^{2+} -free medium, cells were placed in HEPES-buffered saline without CaCl_2 containing 200 μM EGTA. Fluorescence was recorded from coverslips mounted in 4.5-ml chambers, alternatively excited at 340 and 380 nm wavelengths and emission measured at 510 nm.

hTRPC4 Tyrosine Phosphorylation

Measurements were made using a PerkinElmer Life Sciences LS50B luminescence spectrophotometer sampled every 1.4 s at 30 °C and analyzed with an FL Data Manager (PerkinElmer Life Sciences). Changes in $[Ca^{2+}]_i$ were monitored as 340/380 fluorescence ratio and converted to relative Ca^{2+} concentration using the formula of Grynkiewicz *et al.* (31), after establishment of daily R_{min} and R_{max} . Peak Ca^{2+} change was measured as the integral under the rise in $[Ca^{2+}]_i$ above basal for the period of stimulation or Ca^{2+} re-addition. Single cell imaging was performed on transfected HEK293 cells illuminated with Lambda DG-4 lamp, captured with an Orca ER camera, and analyzed with the MetaFluor program (Universal Imaging Devices) using an objective with 40 \times magnification. All statistics were performed using Prism, one-way analysis of variance, and Tukey's post test.

RESULTS

EGF Receptor Stimulation Induces Tyrosine Phosphorylation of hTRPC4—Activation of EGFR is known to induce both Ca^{2+} entry and store release with a number of TRPC channels, including TRPC4, -5, and -6 activated following EGF application. We sought to examine whether tyrosine phosphorylation of the TRPC member, hTRPC4, is involved in its activation. A recent report has questioned the specificity of commercially available TRPC4 antibodies (32); therefore, we first sought confirmation of the ability of the Alomone antibody to detect hTRPC4. An immunoblot was performed on whole cell lysates from control, FLAG-hTRPC4, and YFP-tagged FLAG-hTRPC4 transfected COS-7 cells to demonstrate the ability of the anti-TRPC4 antibody to recognize transfected hTRPC4 and endogenous TRPC4. As shown in Fig. 1A, the anti-TRPC4 antibody recognizes both endogenous TRPC4 and transfected hTRPC4. The endogenous product has a lower apparent molecular weight, possibly due to a species difference between the proteins or differential processing of the products in COS-7 cells. Interestingly, a variation in size between the endogenous and transfected proteins has also been reported for TRPC1 (33). Significantly, neither the endogenous nor transfected protein were recognized by the antibody in the presence of immunizing peptide (data not shown) suggesting that the antibody is specific for the TRPC4 epitope.

To address the role of phosphorylation in the activation of TRPC4 by EGF, serum-starved COS-7 cells transfected with FLAG-hTRPC4 were stimulated with EGF and assessed for tyrosine phosphorylation of hTRPC4 by immunoblotting with anti-phosphotyrosine antibody (PY20). At 20 min post stimulation, a 5-fold increase in tyrosine phosphorylation of hTRPC4 was observed (Fig. 1B); an effect also shown to occur with heterologously expressed hTRPC4 tagged with YFP (Fig. 1C). This shift in apparent molecular weight of the band recognized by the PY20 antibody to match the corresponding TRPC4 band upon addition of YFP suggests the species recognized by the PY20 antibody is the exogenously expressed immunoprecipitated TRPC4 protein in both FLAG and GFP precipitates and not a TRPC4-associated protein. Further, anti-FLAG immunoprecipitates failed to isolate any significant PY20 or TRPC4 reactive bands in the absence of expressed FLAG-TRPC4.

To determine if endogenous hTRPC4 is phosphorylated, lysates from EGF stimulated A431 cells, which express both TRPC4 and large amounts of EGFR, were reacted with PY20 and precipitated proteins probed for the presence of TRPC4. In agreement with the results obtained with transfected hTRPC4, endogenous TRPC4 was phosphorylated upon EGFR stimulation (Fig. 1D), an effect that was prevented upon application of the specific EGFR inhibitor, AG1478 (10 nM) (Fig. 1D). Tyrosine phosphorylation in response to EGF stimulation was not common to all TRPC members, because GFP-hTRPC1, a closely related TRPC member, was not phosphorylated under similar conditions (Fig.

1E). Taken together, these data show that hTRPC4 is tyrosine phosphorylated following EGFR stimulation.

Numerous proteins are regulated by tyrosine phosphorylation following activation of both G-protein-coupled receptors and receptor tyrosine kinases, including members of the mitogen-activated protein kinase (MAPK) and STK-signaling cascades. Therefore, we sought to determine whether tyrosine phosphorylation of hTRPC4 was unique to EGFR activation. Transiently transfected COS-7 cells were treated with a range of stimuli known to activate TRPC channels and initiate Ca^{2+} entry. G-protein receptor pathways were activated with the muscarinic acetylcholine receptor agonist, carbachol, the PKC agonist oleoylacyl-*sn*-glycerol, and histamine. The effect of store depletion on hTRPC4 tyrosine phosphorylation was investigated using the sarco-endoplasmic reticulum Ca^{2+} -ATPase inhibitor, thapsigargin. Cells were examined for activation of the classic G-protein and tyrosine kinase receptor signaling pathway, Ras/Raf/MAPK by immunoblotting lysates for phosphorylated ERK1/2. The effect on global tyrosine phosphorylation was also studied by probing lysates with PY20. All stimuli used were shown to induce ERK activation and global tyrosine phosphorylation (Fig. 1F), however only EGF induced significant tyrosine phosphorylation of hTRPC4 (Fig. 1F). These data indicate that tyrosine phosphorylation of hTRPC4 is unique to receptor tyrosine kinase activation.

Tyrosine Phosphorylation of hTRPC4 Is Mediated by Src Family Non-receptor Tyrosine Kinases—Src family tyrosine kinases, including Fyn and Lyn, are important downstream signaling intermediates of the EGF receptor and are implicated in the regulation of ion channels (34, 35). Therefore, their role in EGFR-induced hTRPC4 phosphorylation was investigated. COS-7 cells transfected with FLAG-hTRPC4 were stimulated with EGF in the presence of the specific STK-inhibitor, PP2 (5 μ M), or an equivalent concentration of inactive analogue, PP3, and cell lysates examined for hTRPC4 tyrosine phosphorylation. When STK activity was inhibited, EGF failed to induce tyrosine phosphorylation of hTRPC4 (Fig. 2A), an effect not observed upon treatment with PP3 (Fig. 2A). Because the application of PP2 did not directly affect EGFR activation, as determined by phosphotyrosine levels of immunoprecipitated receptor (Fig. 2A), it was concluded that tyrosine phosphorylation of hTRPC4 following EGF receptor stimulation is mediated by Src family tyrosine kinases.

Recent findings have implicated the STKs, Fyn and Lyn, in the regulation of TRPC channels. Therefore, their role in EGF-stimulated TRPC4 tyrosine phosphorylation was investigated. Either kinase-inactive Fyn (K299M, Fyn-KM), wild-type Fyn (Fyn-WT), or constitutively active Fyn (Y531F, Fyn-YF) was co-expressed with FLAG-hTRPC4 in COS-7 cells, and lysates were examined for hTRPC4 tyrosine phosphorylation. Co-expression of Fyn-WT or Fyn-YF with hTRPC4 enhanced the phosphorylation of the channel by ~7- and 17-fold, respectively, in the absence of EGF (Fig. 2B). Fyn-KM had no effect on hTRPC4 tyrosine phosphorylation (Fig. 2B). Similar results were obtained when the experiments were repeated in HEK293 cells (data not shown), which express lower levels of EGFR than COS-7 cells. Further evidence for a role of Fyn in EGFR signaling and hTRPC4 tyrosine phosphorylation was gained by expressing either Fyn-KM or empty vector together with FLAG-hTRPC4 in EGF-stimulated COS-7 cells. In the presence Fyn-KM, EGF failed to increase hTRPC4 tyrosine phosphorylation (Fig. 2C). Importantly, Fyn-KM expression did not affect EGFR activation as determined by tyrosine phosphorylation levels of the receptor (Fig. 2C). These results indicate that Fyn is a signaling intermediate between EGFR activation and hTRPC4 phosphorylation.

The ability of Lyn co-expression to induce the phosphorylation of hTRPC4 was also assessed. Co-expression of constitutively active p53 and p56 Lyn (Lyn-YF) isoforms with FLAG-hTRPC4 in COS-7 cells

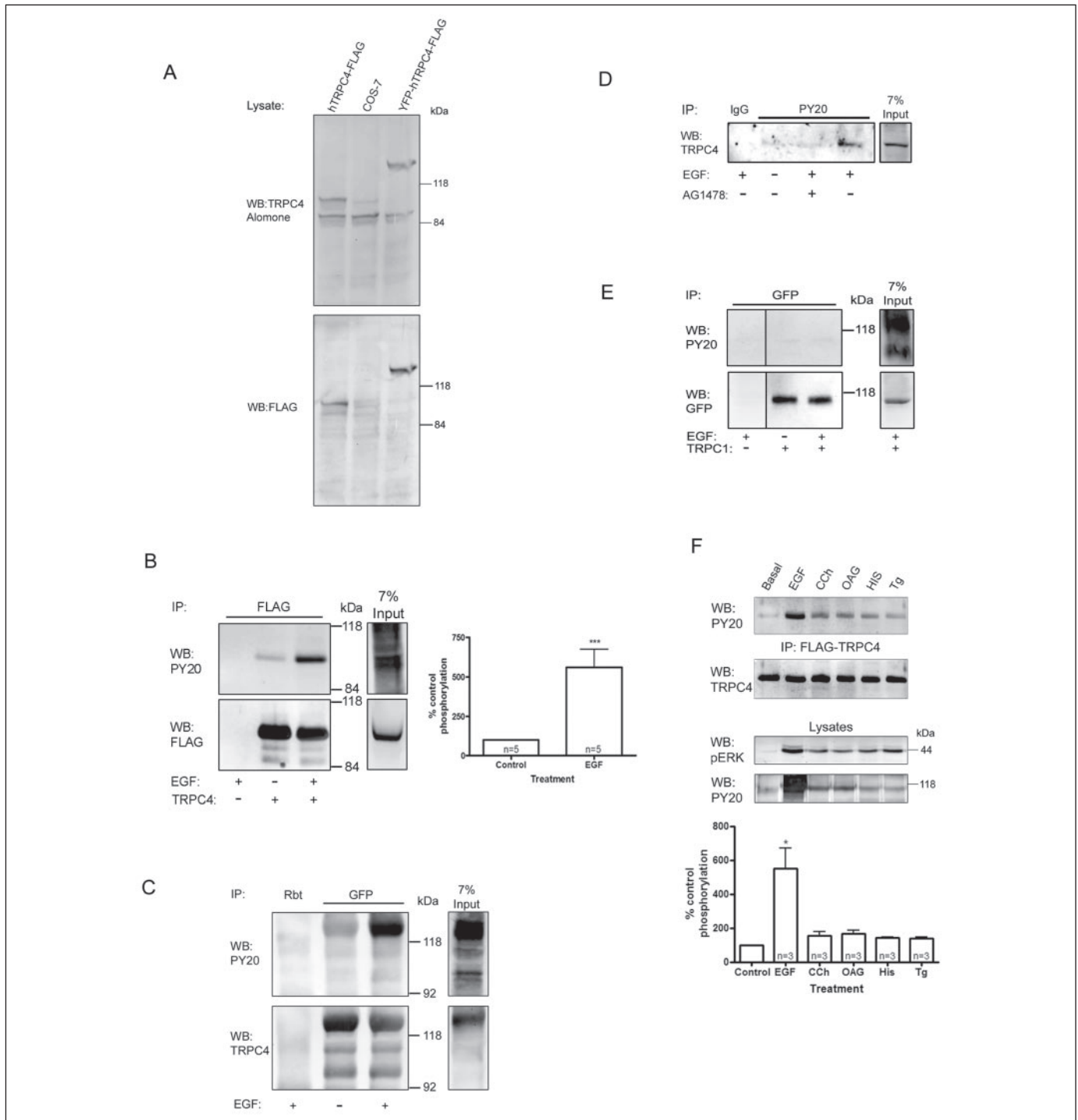


FIGURE 1. hTRPC4 is tyrosine-phosphorylated following EGF stimulation. *A*, Alomone anti-TRPC4 antibody recognizes transfected hTRPC4 and an endogenous species of TRPC4. COS-7 cells were transfected with FLAG-hTRPC4 or YFP-FLAG-hTRPC4, and 20 μ g of total protein was immunoblotted with anti-TRPC4 antibody, then stripped and re-probed with anti-FLAG antibody. *B*, EGF receptor stimulation promotes rapid tyrosine phosphorylation of hTRPC4. COS-7 cells transiently transfected with FLAG-hTRPC4 were stimulated with EGF (100 ng/ml, 20 min), reacted with anti-FLAG antibody, and immunoprecipitated hTRPC4 was blotted for phosphorylated tyrosine residues with anti-PY20 (*top*) or anti-FLAG M2 (*bottom*) antibodies. Precipitation from untransfected COS-7 cells was used as a negative control. Shown also is whole cell lysate (7% of immunoprecipitation input). *Lower panel*, phosphorylated tyrosine levels were determined using ImageQuant software and expressed as a percentage of unstimulated levels and normalized for total protein content (***, $p < 0.001$ compared with control). *C*, YFP-hTRPC4 is tyrosine-phosphorylated in response to EGF. COS-7 cells transfected with YFP-hTRPC4 stimulated with EGF (100 ng/ml, 20 min). YFP-hTRPC4 precipitated with anti-GFP antibody was blotted with anti-PY20 (*top*) and anti-TRPC4 (*bottom*) antibodies. *D*, endogenous hTRPC4 is tyrosine-phosphorylated following EGF treatment. A431 cells were stimulated with EGF (50 ng/ml, 20 min) in the presence or absence of AG1478 (10 nM) followed by precipitation with anti-PY20 (PY20) antibody. Bound proteins were analyzed by blotting with anti-TRPC4 antibody. *E*, TRPC1 does not undergo tyrosine phosphorylation following EGF stimulation. COS-7 cells transfected with GFP-hTRPC1 were stimulated with EGF (100 ng/ml, 20 min) and reacted with anti-GFP antibody, and bound proteins were analyzed by blotting with anti-PY20 (*top*) or anti-GFP (*bottom*) antibodies. *F*, only EGFR stimulation induced significant tyrosine phosphorylation of hTRPC4. FLAG-TRPC4 transfected COS-7 cells were treated with EGF (100 ng/ml, 15 min), carbachol (CCh, 10 μ M, 10 min), oleoylacyl-*sn*-glycerol (OAG, 50 μ M, 10 min), histamine (His, 100 μ M, 10 min), or thapsigargin (Tg, 1 μ M, 10 min), and immunoprecipitated hTRPC4 was assessed for tyrosine phosphorylation with anti-PY20 antibody (*top*). Total protein was determined with anti-TRPC4 antibody (*middle*). Whole cell lysates separated by SDS-PAGE and analyzed by blotting with anti-phosphorylated ERK1/2 or anti-PY20 (*bottom*) antibodies are shown for comparison of global phosphorylation levels. Phosphorylated tyrosine levels were determined using ImageQuant software and expressed as a percentage of unstimulated levels and normalized for total protein content (*, $p < 0.05$ compared with control).

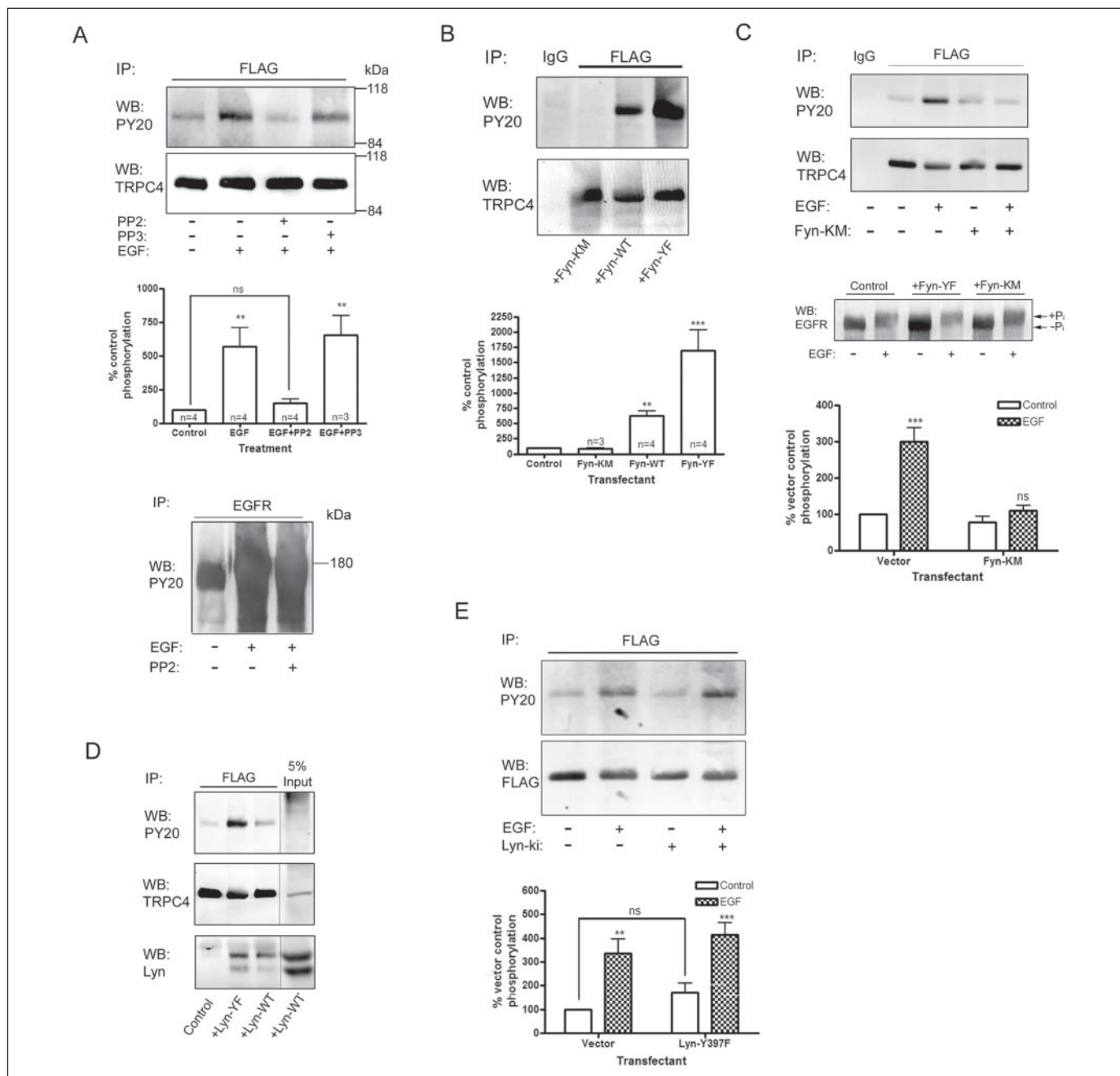
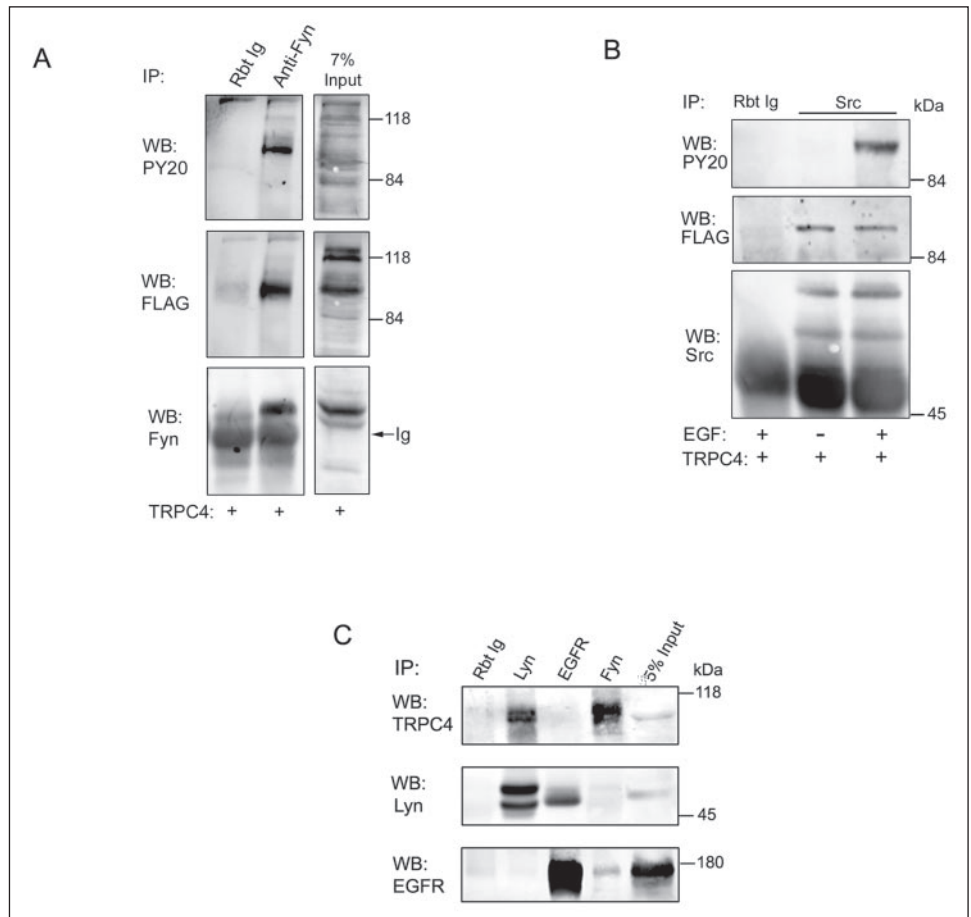


FIGURE 2. Src family tyrosine kinases (STKs) mediate hTRPC4 tyrosine phosphorylation. A, PP2 inhibits EGF-mediated tyrosine phosphorylation of hTRPC4. COS-7 cells transfected with FLAG-hTRPC4 were preincubated with either PP2 (5 μ M) or PP3 (5 μ M) for 10 min prior to EGF (100 ng/ml, 20 min) application. FLAG-hTRPC4 precipitated with anti-FLAG antibody was blotted with anti-PY20 antibody (top). Total protein levels were determined by blotting with anti-TRPC4 antibody (bottom). Phosphorylated tyrosine levels were determined using ImageQuant software and expressed as a percentage of unstimulated control levels and normalized for total protein content (**, $p < 0.01$ compared with control). EGFR was also precipitated from COS-7 cells stimulated with EGF in the presence of PP2 and analyzed for phosphorylated tyrosine levels (middle panel). The bottom panel shows precipitated EGFR immunoblotted for anti-PY20. B, Fyn promotes hTRPC4 tyrosine phosphorylation. COS-7 cells were transfected with FLAG-hTRPC4 and either Fyn-KM, Fyn-WT, or Fyn-YF, and at 48 h post-transfection hTRPC4 was precipitated with anti-FLAG antibody and blotted with anti-PY20 (top) or anti-TRPC4 (bottom) antibodies. Phosphorylated tyrosine levels were determined using ImageQuant software and expressed as a percentage of unstimulated control levels and normalized for total protein content (**, $p < 0.01$; ***, $p < 0.001$ compared with control). C, expression of Fyn-KM prevents EGF-induced phosphorylation of hTRPC4. COS-7 cells transfected with FLAG-hTRPC4 and either control vector or Fyn-KM were stimulated at 48 h post-transfection with EGF (100 ng/ml, 20 min), cell lysates reacted with anti-FLAG antibody and precipitates analyzed by blotting with anti-PY20 (top) or anti-TRPC4 (middle) antibodies. Phosphotyrosine immunoblotting of immunoprecipitated EGFR from COS-7 cells transfected with Fyn constructs is shown below ($n = 7$). D, Lyn also interacts with and promotes tyrosine phosphorylation of hTRPC4. COS-7 cells transfected with Lyn-WT or p53 and p56 Lyn-YF together with FLAG-hTRPC4 were lysed, reacted with anti-FLAG antibody, and blotted with anti-PY20 (top), anti-TRPC4 (middle), and anti-Lyn (bottom) antibodies. E, kinase-inactive Lyn (Lyn-Y397F/ki) does not prevent hTRPC4 phosphorylation. COS-7 cells co-transfected with Lyn-ki and hTRPC4 were stimulated with EGF, precipitated with anti-FLAG, and blotted for phosphotyrosine and TRPC4. The bottom panel shows collated data analyzed from seven individual experiments with ImageQuant.

elevated the tyrosine phosphorylation of hTRPC4 in the absence of EGF (Fig. 2D). However, in contrast to the results obtained with Fyn, wild-type isoforms of Lyn did not significantly increase basal hTRPC4 tyrosine phosphorylation, possibly due to lower transfection levels of Lyn

(data not shown). Significantly, Lyn was found to co-precipitate with hTRPC4 from transfected cells, providing further evidence that Lyn also closely associates with hTRPC4 and is involved in the phosphorylation event (Fig. 2D). Co-expression of a kinase inactive form of Lyn (Lyn-ki,

FIGURE 3. Fyn, Src, and Lyn associate with TRPC4. *A*, Fyn co-immunoprecipitates hTRPC4 from COS-7 cells. Cells were co-transfected with Fyn-WT and hTRPC4, immunoprecipitated with anti-Fyn antibody, and blotted with PY20, anti-FLAG, and anti-Fyn antibodies. *B*, Src associates with hTRPC4 in COS-7 cells. EGF-stimulated FLAG-hTRPC4-transfected COS-7 cells were immunoprecipitated with anti-Src antibody, and precipitates were probed for the presence of phosphotyrosine, TRPC4, and Src. *C*, Lyn and Fyn physically associate with native TRPC4 in COS-7 cells. COS-7 cell lysates were immunoprecipitated with indicated antibodies and immunoblotted for TRPC4 (*top*), Lyn (*middle*), and EGFR (*bottom*).



Y397F) did not significantly alter the EGF-induced phosphorylation of TRPC4 (Fig. 2E). This may indicate a preference for Fyn in the EGFR signaling pathway to hTRPC4.

To further assess the interaction of STKs with TRPC4, a series of co-immunoprecipitations were performed from transfected COS-7 cells. Lysates from cells co-transfected with Fyn-WT and hTRPC4 were immunoprecipitated with anti-Fyn antibody or control rabbit immunoglobulin. Bound proteins were probed for the presence of phosphotyrosine (PY20), TRPC4 (FLAG), and Fyn. Only Fyn precipitates contained TRPC4 and a corresponding PY20 band (Fig. 3A). To assess the potential involvement of Src in hTRPC4 phosphorylation, EGF-stimulated FLAG-hTRPC4-transfected COS-7 cells were immunoprecipitated with anti-Src antibody and lysates analyzed by immunoblotting for phosphotyrosine and hTRPC4 (Fig. 3B). Similar to Fyn and Lyn, Src was found to associate with hTRPC4 and may be involved in mediating the EGF response, however, its role was not explored further due to the strong functional evidence for Fyn and Lyn.

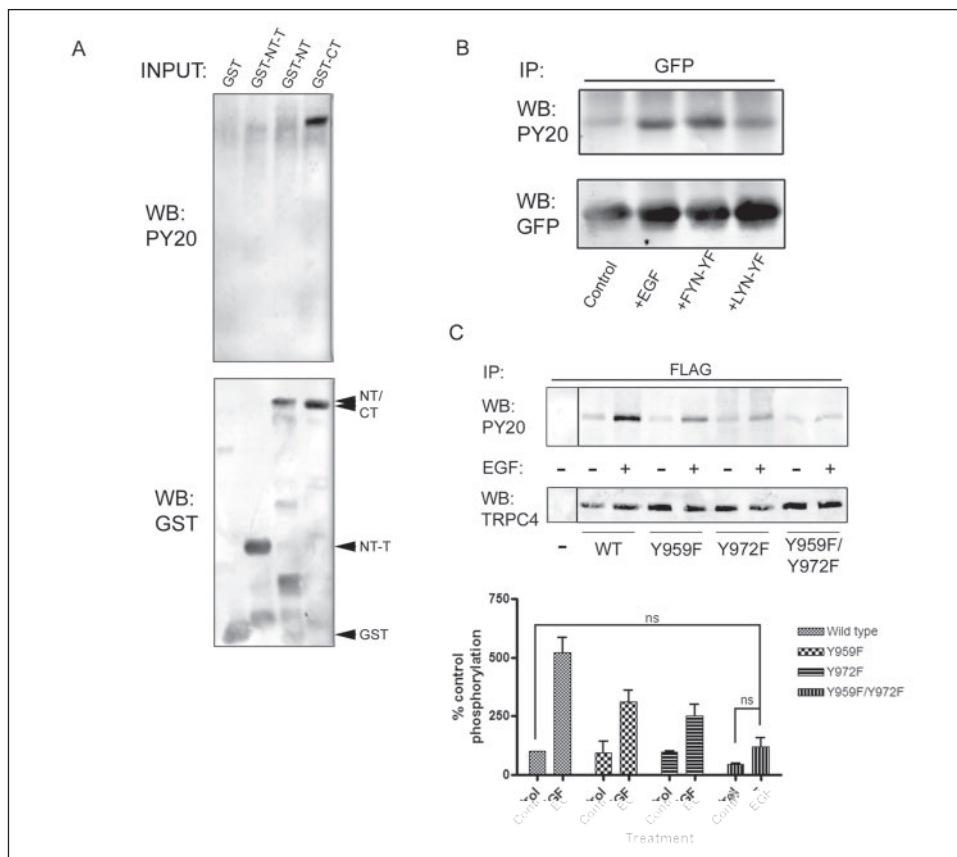
Associations between endogenous TRPC4 and Fyn and Lyn were also investigated. Fyn, Lyn, or EGFR were immunoprecipitated from COS-7 cell lysates, and bound proteins were analyzed by Western blotting. Both Fyn and Lyn immunoprecipitates contained a strong TRPC4-immunoreactive band at the molecular weight predicted for TRPC4 protein (Fig. 3C), indicating a close association between endogenous TRPC4 and the respective STKs. This band was not present in immunoprecipitates performed with control rabbit immunoglobulin. Fyn immunoprecipitates were also shown to contain low levels of EGFR immunoreactivity, suggesting these two molecules complex in COS-7 cells. In contrast, TRPC4 was not present in EGFR immunoprecipita-

tions indicating that the proteins do not interact directly in COS-7 cells under the conditions examined. Reverse precipitations with anti-TRPC4 antibody were not performed due to the inability of the commercially available antibodies to precipitate significant amounts of endogenous TRPC4 (data not shown). Taken together, these results demonstrate a physical association between TRPC4 and STKs. This association appears responsible for the tyrosine phosphorylation of TRPC4 in activated cells.

Specific Tyrosine Residues in the C Terminus of hTRPC4 Are Phosphorylated in Response to EGF Stimulation—To define the region of hTRPC4 that undergoes Fyn-mediated tyrosine phosphorylation, truncated GST-hTRPC4 fusion proteins were generated; GST-NT (amino acids 0–350), GST-NT-T (amino acids 236–350), and GST-CT (amino acids 686–977) of hTRPC4. The truncated fusion proteins were purified from BL21 *Escherichia coli* and used as substrates for *in vitro* kinase assays in the presence of immunopurified constitutively active Fyn (Fyn-YF). Under these conditions, only the C terminus of hTRPC4 was phosphorylated (Fig. 4A). A series of hTRPC4 C-terminal truncations were generated, expressed as GFP fusion proteins in COS-7 cells, and assessed for Fyn and EGF-induced tyrosine phosphorylation. Phosphorylation was shown to occur in the most distal 131 amino acids (G846–L977) of hTRPC4 (Fig. 4B). Site-directed mutagenesis of C-terminal amino acids in FLAG-hTRPC4 was used to analyze potential tyrosine phosphorylation sites in this region. Mutated constructs were expressed in COS-7 cells and assessed for their ability to undergo tyrosine phosphorylation in response to EGF stimulation. Mutation of either tyrosine 959 or tyrosine 972 to phenylalanine (Y959F and Y972F) resulted in reduced EGFR-mediated hTRPC4 tyrosine phosphorylation (Fig. 4C).

hTRPC4 Tyrosine Phosphorylation

FIGURE 4. The C terminus of hTRPC4 is dually phosphorylated on tyrosine residues. A, Fyn phosphorylates tyrosine residues in the C terminus of TRPC4 *in vitro*. GST fusion proteins of the N and C termini of hTRPC4 were purified and subjected to an *in vitro* kinase assay with immunoprecipitated Fyn-YF. Reactions were analyzed by blotting with anti-PY20 antibody. Anti-GST antibody was used to indicate total protein concentration (bottom panel). B, the distal 131 amino acids of hTRPC4 are critical for Fyn-mediated tyrosine phosphorylation of hTRPC4. COS-7 cells were transfected with either truncated C-terminal GFP-hTRPC4 (GFP-CT-T, amino acids 846–977) alone or together with the constructs indicated, reacted with anti-GFP antibody, and bound protein analyzed for phosphotyrosine activity using anti-PY20 antibody (top). The second lane is from COS-7 cells treated with EGF (100 ng/ml, 20 min). Anti-GFP antibody was used to confirm equal fusion protein levels (bottom). C, Tyr-959 and Tyr-972 are tyrosine-phosphorylated in response to EGF stimulation. Point mutations were introduced into FLAG-hTRPC4 (Y959F, Y972F, and Y959F/Y972F) and FLAG-hTRPC4 and mutated constructs transfected into COS-7 cells. Cells were stimulated with EGF (100 ng/ml, 20 min), and precipitated hTRPC4 was analyzed by blotting with anti-PY20 (top) and anti-TRPC4 (bottom) antibodies. Phosphotyrosine levels were quantified with ImageQuant software and corrected for total hTRPC4 content in immunoprecipitates. Collated data are expressed as a percentage of control unstimulated wild-type (WT) hTRPC4 levels.



Mutation of both residues ablated hTRPC4 phosphorylation following EGF stimulation, indicating that these residues may be phosphorylated additively in response to EGF (Fig. 4C). Interestingly, these residues are absent from all other TRPC members, including TRPC5, and may represent a mechanism for selectively controlling TRPC4 activation.

EGF Stimulation Promotes the Insertion of hTRPC4 into the Plasma Membrane—Recent data suggest that TRPC channels may be reversibly trafficked between the cell surface and an intracellular compartment in numerous cell types (36–38). Therefore, it was of interest to investigate whether, in addition to inducing tyrosine phosphorylation of hTRPC4, EGF stimulation also promotes the translocation of hTRPC4 to the plasma membrane. To assess this, COS-7 cells transfected with FLAG-hTRPC4 were treated with EGF and the surface expression of FLAG-hTRPC4 assessed by biotinylation. Under these conditions, a 2-fold increase in surface expression of hTRPC4 was observed (Fig. 5A). The translocation of hTRPC4 was time-dependent, becoming significant after 5-min stimulation and peaking at 20 min (Fig. 5B). Elevated plasma membrane levels persisted for at least 60 min in the continual presence of EGF. The time course of the membrane insertion of hTRPC4 closely mimicked that of EGF-induced hTRPC4 tyrosine phosphorylation in COS-7 cells with both events peaking at 20-min post stimulation (Fig. 5B), indicating that the two events may be closely linked. The surface expression of endogenous hTRPC4 in response to EGF stimulation was also examined in A431 cells, which overexpress human EGFR. Surface biotinylated proteins isolated by NeutrAvidin agarose binding were assessed for the presence of hTRPC4 by immunoblotting with anti-TRPC4 antibody. Similar to the results obtained with transfected hTRPC4, EGF stimulation produced a significant increase in surface expression of endogenous TRPC4 (Fig. 5C). Surface expression of β 4-integrin, a control integral membrane protein, was not altered under these conditions (Fig. 5C).

Actin polymerization and store depletion have been shown to influence the plasma membrane expression of TRPC channels, with membrane levels of TRPC6 increased by store depletion and carbachol treatment in HEK293 cells and TRPC1, -3, and -4 proteins internalized after cytoskeletal reorganization in neutrophils (21, 37). Therefore, the effect of store depletion and actin polymerization on the insertion of hTRPC4 into the plasma membrane was investigated. COS-7 cells transfected with FLAG-hTRPC4 were pretreated with either cytochalasin D to disrupt actin polymerization, or the store-depleting agent, thapsigargin, prior to EGF stimulation and immunoprecipitation of surface biotinylated hTRPC4. Neither agent had any effect on the translocation of FLAG-hTRPC4 to the surface of non-treated nor EGF-stimulated cells (Fig. 5D). The effect of these agents on tyrosine phosphorylation of hTRPC4 was also investigated. Similar to the findings on membrane insertion, neither agent alone altered the tyrosine phosphorylation of hTRPC4 (Fig. 5D). Further, treatment with cytochalasin D or thapsigargin also had no effect on the level of EGF-induced phosphorylation of transfected hTRPC4. Taken together, these results demonstrate that a proportion of hTRPC4 resides in an intracellular compartment and undergoes plasma membrane insertion following EGF stimulation. Furthermore, the time course of membrane insertion closely mimics that of hTRPC4 tyrosine phosphorylation indicating that the two events may be related.

The role of STK-mediated tyrosine phosphorylation of hTRPC4 in promoting membrane insertion of the channel was investigated further by stimulating COS-7 cells transiently expressing hTRPC4 with EGF in the presence of the STK inhibitor, PP2, and assessing for surface biotinylation of transfected hTRPC4. In the presence of PP2, the EGF-induced increase in hTRPC4 surface expression was reduced to non-stimulated control levels (Fig. 6A), indicating that STKs and tyrosine phosphorylation of hTRPC4 regulate the membrane insertion of

β 4 integrin). hTRPC4 protein levels were determined by Western blotting. The amount of hTRPC4 protein was normalized to the amount of β 4 integrin protein. The amount of hTRPC4 protein was determined by Western blotting. The amount of hTRPC4 protein was normalized to the amount of β 4 integrin protein. The amount of hTRPC4 protein was determined by Western blotting. The amount of hTRPC4 protein was normalized to the amount of β 4 integrin protein.

μ M

hTRPC4 channel protein. To confirm a role for Fyn in this process, hTRPC4 together with either Fyn-YF or Fyn-KM were expressed in COS-7 cells and surface expression of hTRPC4 assessed by biotinylation. Overexpression of active Fyn-YF was shown to enhance the cell surface localization of hTRPC4 2-fold, whereas expression of dominant-negative Fyn-KM had no effect on the level of hTRPC4 at the plasma membrane (Fig. 6B). The enhanced level of hTRPC4 surface expression observed in the presence of Fyn-YF was similar to that shown in transfected cells following EGF stimulation. These results demonstrate an important role for STK-mediated tyrosine phosphorylation in regulating the level of TRPC4 at the cell surface.

The role of Fyn and tyrosine phosphorylation in hTRPC4 insertion was explored further using dominant negative Fyn-KM to suppress the EGF response. Co-expression of Fyn-KM with FLAG-hTRPC4 prevented the membrane insertion of hTRPC4 consistent with the blockade of hTRPC4 tyrosine phosphorylation (Fig. 6C).

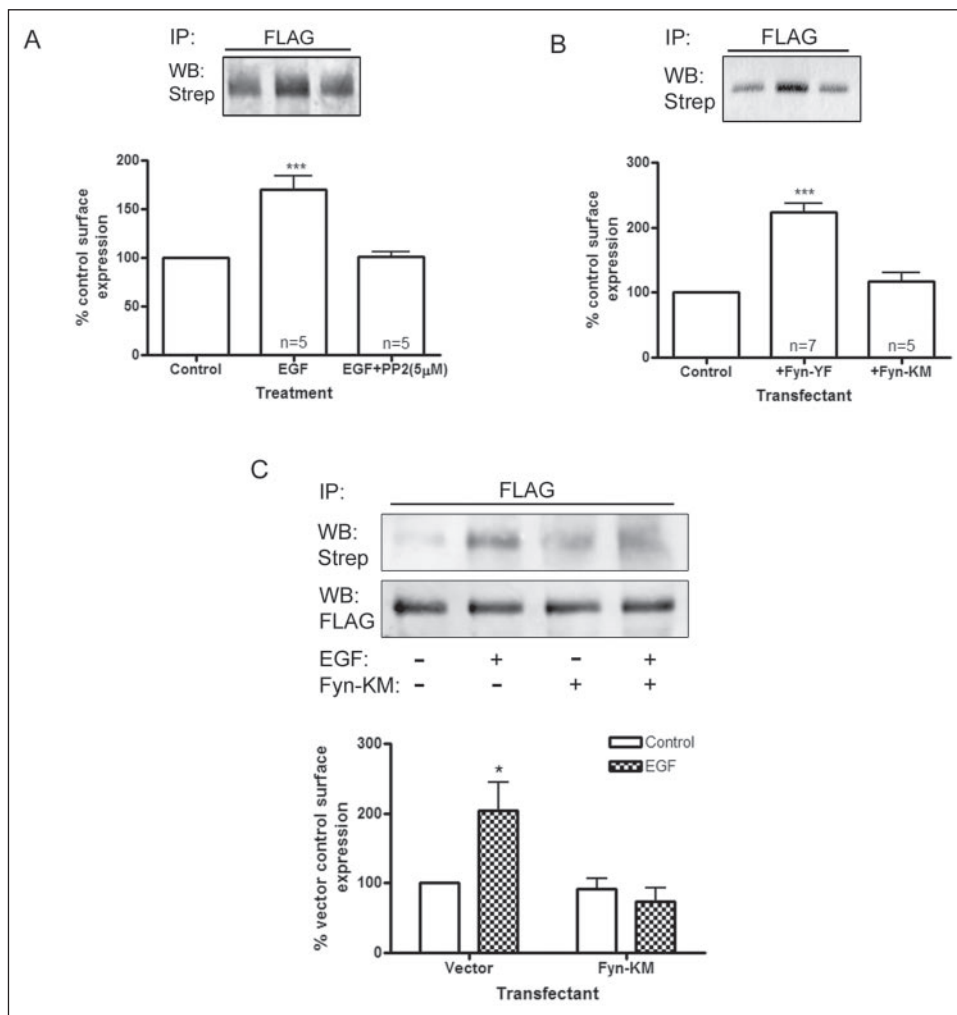
EGF-stimulated Tyrosine Phosphorylation of hTRPC4 Enhances the Association between hTRPC4 and NHERF—Previous studies have shown that the interaction between hTRPC4 and the Na^+/H^+ exchanger regulatory factor, NHERF, is critical in controlling the cell surface expression and localization of hTRPC4 channels (22). Recently, the interaction between NHERF and platelet-derived growth factor β and β -adrenergic receptor was found to be disrupted by phosphorylation of serine residues near the PDZ interaction motif on the receptors (24). These findings coupled with the proximity of the tyrosine phosphorylation sites to the C terminus PDZ motif (V_TTRL) of hTRPC4 implies that phosphorylation may regulate the interaction of hTRPC4

with NHERF. An alteration in this interaction may be responsible for increasing the surface expression of hTRPC4. These results suggest that tyrosine phosphorylation of hTRPC4 may be important for its surface expression.

To assess the levels of NHERF1 associated with hTRPC4 in the presence of EGF, COS-7 cells were transfected with FLAG-hTRPC4 and NHERF1. The amount of NHERF1 associated with hTRPC4 was determined by Western blotting. The amount of NHERF1 associated with hTRPC4 was normalized to the amount of FLAG-hTRPC4. The amount of NHERF1 associated with hTRPC4 was determined by Western blotting. The amount of NHERF1 associated with hTRPC4 was normalized to the amount of FLAG-hTRPC4.

lower panel,

FIGURE 6. Tyrosine phosphorylation promotes plasma membrane insertion of hTRPC4. *A*, preincubation of COS-7 cells with PP2 prevents EGF-induced hTRPC4 translocation. FLAG-hTRPC4-transfected COS-7 cells stimulated with EGF (100 ng/ml, 20 min) either in the presence or absence of 5 μ M PP2 were biotinylated, cell lysates were reacted with anti-FLAG antibody, and precipitates were blotted with streptavidin-AP followed by anti-TRPC4 antibody. Biotinylated proteins were analyzed with ImageQuant and compiled in Prism. *B*, co-expression of Fyn-YF increases the plasma membrane levels of hTRPC4 in the absence of EGF stimulation. COS-7 cells were transfected with FLAG-hTRPC4 together with either control plasmid, Fyn-YF, or Fyn-KM and biotinylated, and lysates were incubated with anti-FLAG antibody. Biotinylated hTRPC4 was visualized with streptavidin-AP followed by anti-TRPC4 antibody. Protein levels were analyzed with ImageQuant, normalized for total hTRPC4 protein, and compiled in Prism. *C*, Fyn-KM prevents EGF-stimulated hTRPC4 membrane insertion. COS-7 cells transfected with indicated constructs were stimulated with EGF, and lysates were precipitated with anti-FLAG antibody. Bound proteins were analyzed by Western blotting for biotin incorporation (*upper*) and total TRPC4 content (*lower*). Below are compiled data from five experiments.



phosphorylation. Indirect confocal immunofluorescence analysis of HA-tagged-NHERF (Fig. 7D, *left panel*) and YFP-hTRPC4 (Fig. 7D, *middle panel*) transfected COS-7 cells also confirmed the enhanced association between the molecules and membrane translocation of hTRPC4 evident upon EGF stimulation. YFP-hTRPC4 adopted a more classic membrane staining pattern and exhibited greater co-localization with NHERF at the cell membranes in the presence of EGF (Fig. 7D). Taken together, these results implicate STK-mediated tyrosine phosphorylation of hTRPC4 in the enhanced association of NHERF with hTRPC4.

This was investigated further using mutated hTRPC4 constructs devoid of the C-terminal tyrosine phosphorylation sites (Y959F, Y972F, and Y959F/Y972F) expressed in COS-7 cells and assessed for their ability to bind NHERF. Mutation of either or both tyrosine residues of hTRPC4 decreased the amount of NHERF bound to hTRPC4 by 40% in untreated cells and in the presence of EGF (Fig. 7E). The previously observed increase in NHERF binding to hTRPC4 upon EGF stimulation was also ablated (Fig. 7E). Mutation of both C-terminal tyrosine phosphorylation sites reduced NHERF interaction by over 55% in comparison to wild-type hTRPC4 (Fig. 7E). These results confirm a role for Tyr-959 and Tyr-972 in regulating the interaction of hTRPC4 with NHERF. Phosphorylation of these residues appears responsible for the enhanced NHERF-hTRPC4 interaction evident upon EGF stimulation and potentially for promoting the insertion or stabilization of hTRPC4 channels in the plasma membrane. Indeed, mutation of both C-terminal

residues completely prevented the increase in membrane expression normally induced by EGF (Fig. 7F). The failure of the dual mutant (Y959F/Y972F) to translocate in response to EGF highlights the importance of the phosphorylation event in regulating the membrane insertion of hTRPC4.

STKs Are Required for the Activation of hTRPC4-mediated Ca^{2+} Entry Induced by EGF Stimulation—Both mouse TRPC4 and TRPC5 are known to form cation channels activated by carbachol and EGF receptor stimulation (12), however the mechanisms of activation are unknown. Stimulation of serum-starved COS-7 cells with EGF (50 ng/ml) was shown to induce a transient rise in intracellular Ca^{2+} followed by a sustained influx of extracellular Ca^{2+} (Fig. 8A). A peak increase in $[Ca^{2+}]_i$ of 600 nM was a typical response following EGF stimulation and was largely dependent on the presence of extracellular Ca^{2+} . Ca^{2+} entry was abolished upon transfer to Ca^{2+} free buffer (Fig. 8B). To address whether or not a tyrosine phosphorylation event was responsible for mediating the sustained influx of extracellular Ca^{2+} following EGF receptor stimulation, COS-7 cells were preincubated with the STK inhibitor, PP2 (5 μ M), for 10 min prior to EGF application. The addition of PP2 reduced both peak Ca^{2+} influx and the sustained phase of Ca^{2+} entry by over 50% (Fig. 8, A and B). Cells were also incubated with PP2 prior to stimulation with thapsigargin. In contrast to reports in other cell lines (39), the thapsigargin response was largely unaffected by STK inhibition (Fig. 8C). Neither Ca^{2+} release from the passively depleted stores or Ca^{2+} entry upon re-addition of Ca^{2+} were affected by

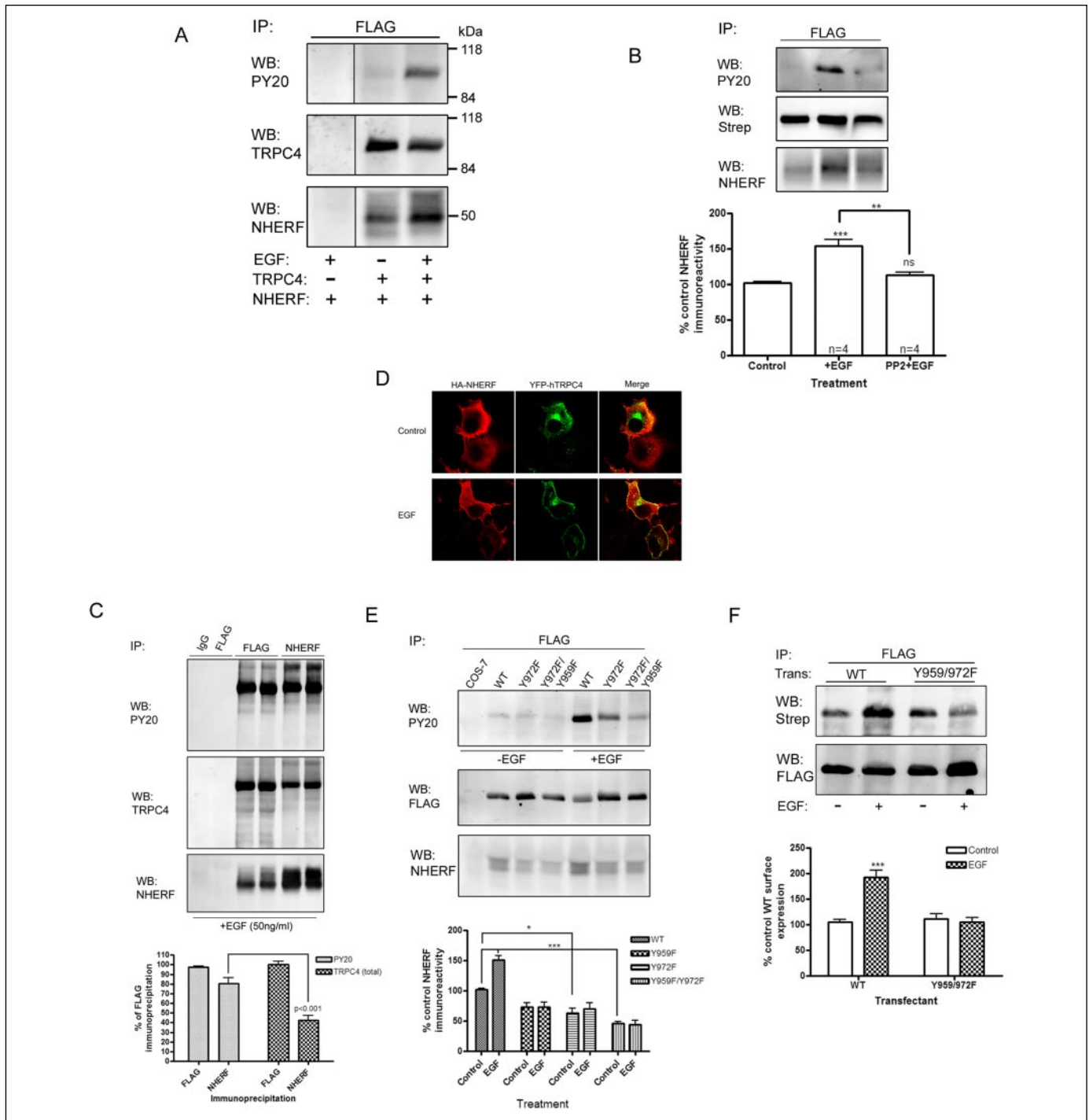


FIGURE 7. Tyrosine phosphorylation enhances the association of hTRPC4 with NHERF. A, EGF stimulation enhances the association of hTRPC4 with NHERF. COS-7 cells transiently expressing HA-NHERF and FLAG-hTRPC4 were stimulated with EGF (100 ng/ml, 20 min), and cell lysates were reacted with anti-FLAG antibody. Bound proteins were analyzed for phosphorylated tyrosine levels (top), total hTRPC4 (middle), and NHERF (lower) content. B, EGF-stimulated NHERF association is inhibited by PP2. COS-7 cells transfected with FLAG-hTRPC4 and HA-NHERF were stimulated with EGF (100 ng/ml) in either the presence or absence of PP2 (5 μ M) and biotinylated, and lysates were reacted with anti-FLAG antibody. Western blots were performed as indicated. C, NHERF preferentially associates with tyrosine-phosphorylated hTRPC4. COS-7 cells transfected with HA-NHERF and FLAG-hTRPC4 were stimulated with EGF (100 ng/ml for 15 min), and lysates were immunoprecipitated with either anti-NHERF or anti-FLAG antibodies. Bound proteins were immunoblotted with anti-phosphotyrosine antibody (PY20, upper panel), then stripped and re-probed with anti-TRPC4 antibody (middle panel). Also shown is an immunoblot of precipitated NHERF. Data were collated and expressed as a percentage of either phosphotyrosine or anti-TRPC4 immunoreactivity relative to FLAG immunoprecipitates. D, EGF induces the movement of hTRPC4 to the cell membrane. HA-NHERF- and YFP-hTRPC4-transfected COS-7 cells were treated with or without EGF (100 ng/ml, 20 min), fixed, stained with anti-HA antibody, and visualized with streptavidin-Alexa594 tertiary. E, C-terminal tyrosine residues influence the binding of hTRPC4 to NHERF. Wild-type and mutated hTRPC4 constructs (WT, Y959F, Y972F, or Y959F/Y972F) transfected into COS-7 cells were precipitated with anti-FLAG antibody and assessed for NHERF (bottom) and tyrosyl (top) immunoreactivity following EGF stimulation (100 ng/ml, 20 min). NHERF levels were quantified and expressed as a percentage of binding to unstimulated WT-hTRPC4. F, Y959F/Y972F mutant fails to translocate in response to EGF stimulation. COS-7 cells transfected with either wild-type hTRPC4 or dual mutant (Y959F/Y972F) were treated with EGF, biotinylated, and assessed for membrane insertion. Below are collated data from 10 experiments.

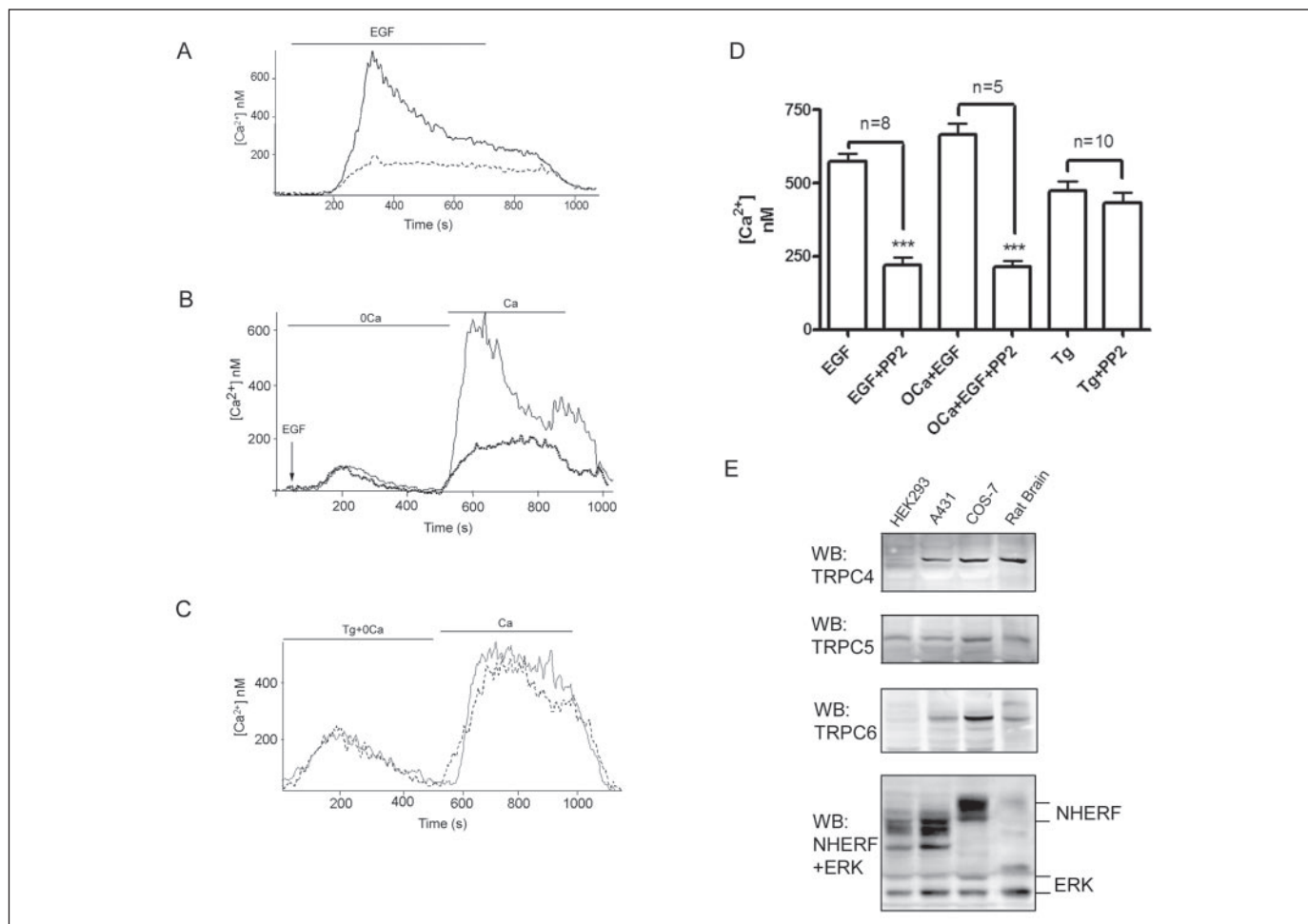


FIGURE 8. EGF stimulation initiates Ca^{2+} entry in native COS-7 cells. *A*, EGF application induces sustained Ca^{2+} entry in COS-7 cells. Fura-2-loaded COS-7 cells were stimulated with 50 ng/ml EGF in the presence (*broken trace*) or absence (*continuous trace*) of PP2 (5 μM) with 2 mM external Ca^{2+} . Fluorescence was recorded from a cell population at excitation wavelengths of 340 and 380 nm and ratio values converted to absolute $[\text{Ca}^{2+}]_i$, according to Grynkiewicz. Shown are individual traces from single representative experiments expressed as summary data of peak Ca^{2+} levels in Fig. 8*D*. *B*, Representative traces of COS-7 cells stimulated with EGF (50 ng/ml) in the presence (*broken trace*) or absence (*continuous trace*) of PP2 (5 μM). External Ca^{2+} was removed prior to EGF application and cells returned to 2 mM Ca^{2+} at the indicated time. Collated data from five individual experiments is shown in *D*. *C*, thapsigargin-stimulated Ca^{2+} release and entry are unaffected by STK inhibition. Trace represents Fura-2-loaded cells passively depleted with thapsigargin (100 nM) in the absence of external Ca^{2+} prior to 2 mM Ca^{2+} re-addition at the indicated time with (*broken trace*) or without 5 μM PP2 (*continuous trace*). *D*, collated data from Fura-2 experiments analyzed for peak intracellular Ca^{2+} concentration (nanomolar). For thapsigargin and EGF in 0- Ca^{2+} media, peaks occurred upon re-addition of Ca^{2+} . *E*, TRPC protein expression in COS-7 cells. 25 μg of total protein from indicated cell lines was analyzed by immunoblotting with the indicated antibodies.

the presence of PP2 (Fig. 8*C*). Fig. 8*D* represents collated peak Ca^{2+} levels in the presence of extracellular Ca^{2+} . This suggests that STKs are involved in mediating the Ca^{2+} changes initiated by EGF application, but are not involved in classic store-operated Ca^{2+} entry in COS-7 cells.

Previously, TRPC4, -5, and -6 have been reported to be activated by receptor tyrosine kinase stimulation (11, 12). All three subtypes, along with NHERF, are expressed in COS-7 and A431 cells, with only TRPC5 apparent in HEK293 cells (Fig. 8*E*). Of those three TRPC members only mTRPC6 and now hTRPC4, are known to be phosphorylated by STKs. All three TRPC proteins are candidates for mediating the sustained Ca^{2+} entry phase of EGF stimulation. However, the large endogenous EGF response in COS-7 cells impeded analysis of the contribution of overexpressed hTRPC4 to EGF-induced Ca^{2+} entry in these cells. To investigate the possible involvement of hTRPC4 in the EGF response we generated stable HEK293 cell lines expressing FLAG-tagged hTRPC4. As a consequence of the lower EGFR background we were able to demonstrate a 2-fold increase in $[\text{Ca}^{2+}]_i$ in response to EGF application in Fura-2-AM-loaded cells (Fig. 9*A*, *unbroken trace*). Although the response was small, in part because of the low EGFR levels in these cells, it was still significantly greater than the control cell response (Fig. 9*A*,

broken middle trace). When cells were pre-treated with PP2 (5 μM for 10 min) the hTRPC4-mediated EGFR response was reduced to near control levels (Fig. 9*A*, *dashed lower trace*), demonstrating a requirement for STKs in the activation of Ca^{2+} entry by EGF. Also shown as Fig. 9*B* is the collated data of peak Ca^{2+} levels evident upon EGF application, and the *inset* is a representative immunoblot of FLAG-hTRPC4 immunopurified from EGF-stimulated HEK293 cells stably expressing hTRPC4. Taken together with previous data, this observation indicates that EGF-stimulated Ca^{2+} entry may in part be mediated by tyrosine phosphorylation of hTRPC4.

To confirm the role of hTRPC4 tyrosine phosphorylation in the activation of the channel, single cell Fura-2 Ca^{2+} imaging was performed on HEK293 cells transiently expressing both the wild-type human EGFR and either control GFP vector (GFP, $n = 37$), wild-type hTRPC4 (WT-hTRPC4, $n = 34$), or dual mutant hTRPC4 (Y959F/Y972F, $n = 35$) (Fig. 9*C*). Cells expressing WT-hTRPC4 showed a greater response to EGF than control cells, consistent with results in Fig. 9*A*. Importantly, mutant hTRPC4 exhibited a reduced EGF response compared with the wild-type hTRPC4 control; however, it failed to reach control levels (Fig. 9*C*). This may indicate that other factors are involved in regulating

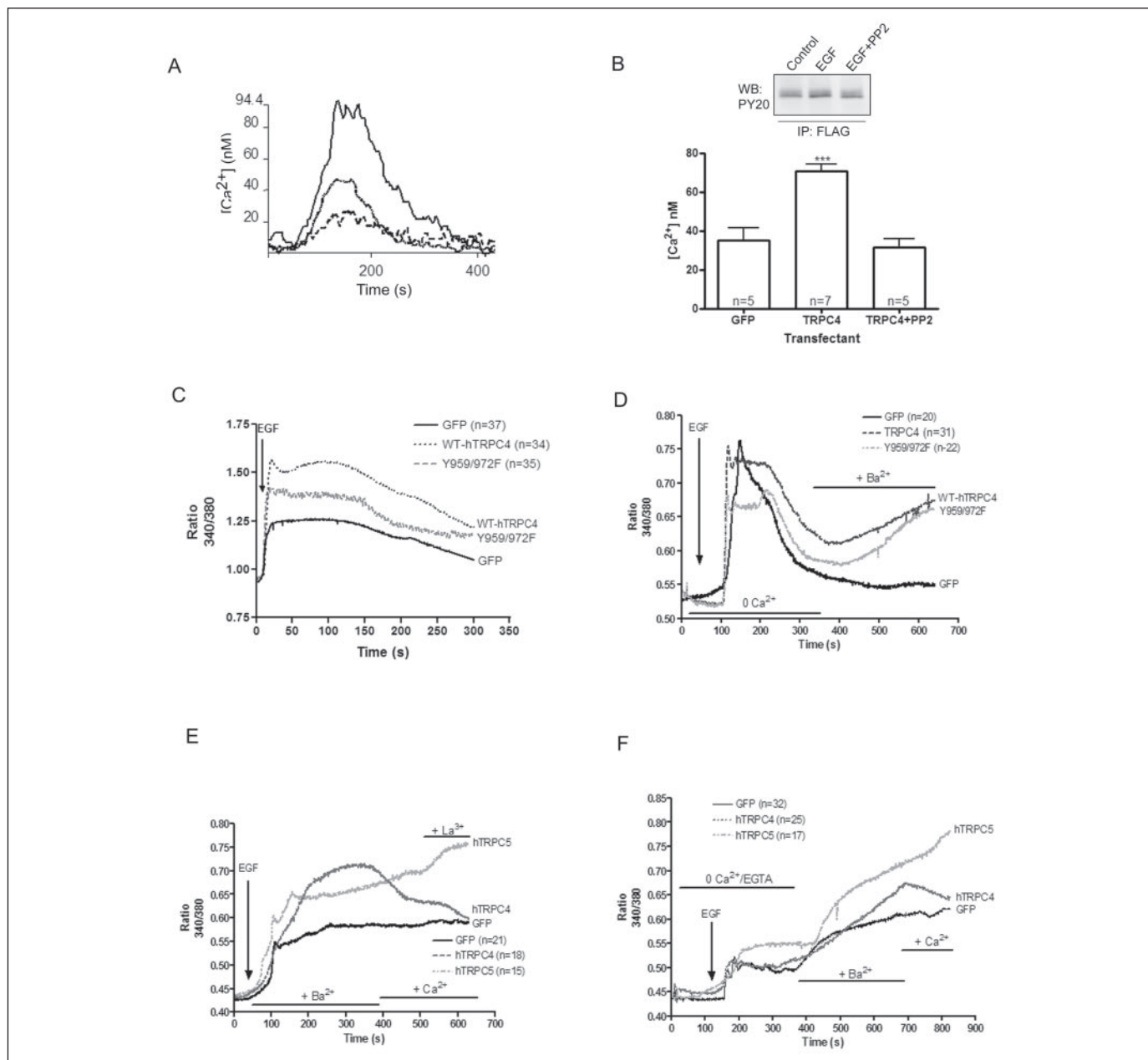


FIGURE 9. PP2 inhibits Ca^{2+} entry in response to EGF application in FLAG-hTRPC4-expressing HEK293 cells. *A*, FLAG-hTRPC4 expression enhances Ca^{2+} entry by EGF. Fura-2-loaded control hTRPC4-transfected cells were stimulated with EGF (50 ng/ml) in the presence (*broken, lower trace*) or absence of 5 μM PP2 (*unbroken trace*). EGF response in control GFP-expressing cells is also shown (*dashed, middle trace*). *B*, representative traces from 5–7 experiments collated as peak Ca^{2+} levels. *Inset*, immunoblot of tyrosine phosphorylation of immunoprecipitated FLAG-hTRPC4 induced by EGF. *C*, HEK293 cells transiently expressing wild-type EGFR and either GFP control, wild-type hTRPC4 (WT-hTRPC4) or dual Y959F/Y972F (Y959F/Y972F) were loaded with Fura-2, and a field of 5–7 cells was analyzed for ratiometric Ca^{2+} imaging following EGF application (50 ng/ml). Shown are the average traces from the indicated number of cells. *D*, EGFR and either GFP, WT-hTRPC4, or Y959F/Y972F were transfected in HEK293 cells and loaded with Fura-2, and a field of 5–7 cells was analyzed for single-cell ratiometric Ca^{2+} imaging following EGF application (50 ng/ml) in the absence of external Ca^{2+} , followed by re-addition of 2 mM Ba^{2+} . Shown are averaged traces of the indicated number of cells. *E*, HEK293 cells transfected with the EGFR and either GFP, hTRPC4, or hTRPC5 were stimulated with EGF (50 $\mu\text{g}/\text{ml}$) in bath solution containing 2 mM Ba^{2+} supplemented for Ca^{2+} . Buffer was switched as indicated to 2 mM Ca^{2+} followed by supplementation with 50 μM La^{3+} . Shown are averaged traces from the indicated number of cells. *F*, HEK293 cells transfected with the EGFR and either GFP, hTRPC4 or hTRPC5 were loaded with Fura-2 stimulated with EGF (50 ng/ml) in 0 mM external Ca^{2+} (supplemented with 250 μM EGTA) followed by re-addition of 2 mM Ba^{2+} , which was replaced by 2 mM Ca^{2+} as indicated. Shown are averaged traces from the indicated number of cells.

hTRPC4 channel activation in addition to tyrosine phosphorylation. Similarly, when transfected cells were stimulated with EGF (50 ng/ml) in nominally Ca^{2+} free buffer (0 Ca^{2+}) followed by the re-addition of 2 mM barium (Ba^{2+}), WT-hTRPC4 transfectants ($n = 31$) exhibited greater cation entry than both control ($n = 20$) and mutant Y959F/Y972F-expressing ($n = 22$) HEK293 cells (Fig. 9D). Taken together, these results demonstrate a role for the tyrosine phosphorylation of C-terminal residues in the activation of hTRPC4 by EGF.

To further define the hTRPC4-mediated Ca^{2+} responses elicited by EGF, additional experiments were performed on EGFR- and either GFP-, hTRPC4-, or hTRPC5-expressing HEK293 cells. When cells were stimulated with EGF (50 ng/ml) in the presence of Ba^{2+} , both hTRPC4- and hTRPC5-expressing HEK293 cells demonstrated enhanced cation influx compared with control cells (GFP, *lower trace*). However, upon switching to buffer containing 2 mM Ca^{2+} , the hTRPC4 cation influx was reduced and further impaired by the addition of 50 μM La^{3+} (Fig.

hTRPC4 Tyrosine Phosphorylation

9E). In contrast the presence of hTRPC5 resulted in a cation influx that appeared unaffected by the Ca^{2+} switch and was stimulated by La^{3+} application (Fig. 9E). This is consistent with previous findings regarding activation of hTRPC5 channels by lanthanides (40, 41). To verify that transfected hTRPC4 participates in the formation of a cation permeable channel, the effect of EGF (50 ng/ml) stimulation on transfected cells in the absence of Ca^{2+} (supplemented with 250 μM EGTA), followed by the sequential addition of 2 mM Ba^{2+} and 2 mM Ca^{2+} was also examined (Fig. 9F). Cells expressing hTRPC4 (*broken, middle trace*) demonstrated a sustained Ba^{2+} influx compared with control cells (*unbroken, lower trace*), which was reduced upon transfer to Ca^{2+} -containing buffer. This result is consistent with the Ca^{2+} influx data in Fig. 9E and indicates that hTRPC4 forms cation channels responsive to EGF stimulation. In contrast, the expression of hTRPC5 resulted in the formation of cation channels similarly selective for Ba^{2+} and Ca^{2+} (Fig. 9F), demonstrating that the channels formed by hTRPC5 possess different properties to hTRPC4, despite both being activated by EGF stimulation.

DISCUSSION

The findings presented here provide evidence that hTRPC4 undergoes rapid tyrosine phosphorylation mediated by Src family tyrosine kinases (STKs) following activation of the EGF receptor. Although several reports have previously implicated tyrosine phosphorylation by STKs in TRP channel regulation (13, 38), our finding that phosphorylation of hTRPC4 by STKs enhances the interaction of hTRPC4 with the scaffold protein NHERF is the first report of TRPC protein interactions undergoing modulation by phosphorylation. Indeed, it represents the first report of PDZ-mediated interactions undergoing modulation by tyrosine phosphorylation.

The interaction of hTRPC4 with NHERF was modulated by the phosphorylation of two C-terminal tyrosine residues of hTRPC4. The proximity of these tyrosyl residues to the C-terminal PDZ (PSD-95, Dlg A, ZO-1) motif of hTRPC4 allows their phosphorylation to regulate interactions with PDZ domain containing proteins and potentially influence channel activity. Consistent with this, mutation of Tyr-959 and Tyr-972 to phenylalanine reduced the interaction of hTRPC4 with NHERF in the absence of stimulation, implying that phosphorylation of these tyrosine residues induces a conformational change in the C terminus of hTRPC4 to facilitate NHERF binding. Mutation of these residues also prevented the membrane insertion of hTRPC4 consistent with reduced NHERF binding, strengthening the argument of enhanced NHERF binding as responsible for the membrane trafficking upon EGF stimulation. The candidate tyrosine residues are confined to TRPC4, being absent from both TRPC5 and TRPC6. Thus, by manipulating NHERF binding through phosphorylation of hTRPC4, channel surface expression and resultant activity of hTRPC4 may be closely and dynamically regulated independent of other TRPC members. Furthermore, together with the recent findings by Gordon Gill and coworkers (42) regarding the NHERF-dependent stabilization of EGFRs on the plasma membrane, our data hint at a role for NHERF in the assembly and maintenance of signal transduction events linking EGFR stimulation to hTRPC4 channel activation and Ca^{2+} entry.

For members of the TRP family of non-selective cation channels, reversible membrane trafficking is becoming a widely reported occurrence. For example, the activation of TRPC6 by store depletion and carbachol application has been shown to involve the exocytotic insertion of channels into the plasma membrane (21). This insertion was not mediated by the classic secretory pathway involving an elevation of intracellular Ca^{2+} and was prevented by condensation of the actin cytoskeleton. In contrast, the insertion of hTRPC4 by EGF application is not affected by inhibition of actin polymerization nor store depletion or

carbachol application, but is instead reliant on the activity of STKs. This demonstrates a clear difference in the regulation of hTRPC4 externalization from that proposed for TRPC6.

EGF has been found to induce the activation of TRPC5 in HEK293 and PC12 cells, a process mimicked by NGF application to hippocampal neurons (38). This activation was accompanied by increased plasma membrane expression of TRPC5. We have found that hTRPC4 is similarly regulated by EGF receptor stimulation in COS-7 and A431 cells and undergoes rapid membrane insertion following agonist application. However, the membrane translocation of hTRPC4 is accompanied by tyrosine phosphorylation of the channel, with both exhibiting similar kinetic properties. A role for phosphorylation in regulating plasma membrane expression of hTRPC4 is consistent with previous reports where the surface levels of both K^{+} and Na^{+} channels are altered by channel phosphorylation (43, 44). Phosphorylation may therefore represent a common mechanism for controlling ion channel localization.

TRPV2 and TRPC5 translocation in response to receptor tyrosine kinase activation is dependent on phosphatidylinositol 3-kinase, with TRPC5 incorporation enhanced by constitutively active Rac1 through a phosphatidylinositol-4-phosphate (5) $\text{K}\alpha$ -dependent mechanism (38). We found hTRPC4 translocation to be dependent on STK activity with Fyn able to promote the phosphorylation of hTRPC4 in a time-dependent manner consistent with membrane insertion. The over-expression of a constitutively active form of Fyn not only induced tyrosine phosphorylation of hTRPC4 in the absence of EGFR stimulation, it also promoted its membrane insertion. In contrast, a dominant-negative Fyn isoform blocked both EGF-induced tyrosine phosphorylation and membrane insertion (Fig. 6). This effect appears limited to Fyn, with a dominant-negative Lyn failing to prevent both hTRPC4 tyrosine phosphorylation and membrane insertion in response to EGF. These results suggest that translocation of hTRPC4 is influenced by its tyrosine phosphorylation state as regulated by Fyn kinase rather than membrane phosphatidylinositol 4,5,-bisphosphate levels. However, whether STK activity is influenced by phosphatidylinositol 3-kinase-Rac1-phosphatidylinositol-4-phosphate (5) kinase or vice versa remains to be determined.

A previous report by Schaeffer and colleagues (12) described the activation of TRPC4 channels by EGF application. Here we provide evidence that the tyrosine phosphorylation of hTRPC4 by STKs is involved in this activation. The time course of hTRPC4 tyrosine phosphorylation corresponds with the sustained phase of Ca^{2+} entry in COS-7 cells and hTRPC4-expressing HEK293 cells following EGF application. The reduction in Ca^{2+} entry by STK inhibition suggests an important role for tyrosine phosphorylation in the maintenance of receptor-operated Ca^{2+} influx. This was confirmed by the reduced Ca^{2+} entry profile of HEK293 cells expressing the dual C-terminal tyrosine-deficient hTRPC4 mutant and was consistent with the defective phosphorylation and membrane insertion displayed by this mutant upon EGF stimulation (Figs. 7 and 9). The failure of the Y959F/Y972F mutant to exhibit a Ca^{2+} entry profile consistent with control GFP-expressing cells indicates that other regulatory factors are involved in controlling channel activation apart from phosphorylation. This may involve activation by other signaling intermediates, possibly phospholipid derivatives, generated by EGFR stimulation. Indeed, it may involve interactions with phospholipase C γ 1 through regeneration of partial pleckstrin homology domains as recently suggested by Solomon Snyder's group (45). Regardless, STKs have previously been implicated in the control of Ca^{2+} entry following store-depletion, possibly by influencing the polymerization of the actin cytoskeleton (39, 46, 47), and a role for STKs in the activation and maintenance of store-operated Ca^{2+} entry in mouse pancreatic acinar cells has also been reported with STK inhibition reducing thapsigargin-

stimulated calcium entry (48). In contrast, our findings show that only receptor-operated Ca^{2+} entry and not store-operated Ca^{2+} entry is reduced by STK inhibition suggesting a fundamental difference in the regulation of Ca^{2+} entry between pancreatic acinar cells and COS-7 cells. These results coupled with the recent report of the involvement of TRPC4 in mediating store-operated Ca^{2+} entry in human corneal epithelial cells in response to EGF (13), suggest an important role for TRPC4 channels in regulating Ca^{2+} entry following EGFR stimulation, either by a receptor-operated or a store-operated mechanism.

Characterization of the EGF-induced cation entry profile in HEK293 cells expressing both hTRPC4 and hTRPC5 demonstrated that both channels are capable of mediating cation entry, with hTRPC4 channels exhibiting an enhanced permeability to Ba^{2+} over Ca^{2+} (Fig. 9, E and F). The ability of hTRPC5 to increase EGF-stimulated cation influx in the absence of corresponding C-terminal phosphorylatable tyrosine residues indicates the involvement of alternative mechanisms for regulating Ca^{2+} influx through these channels. This provides a mechanism to differentially modulate the activity of these two closely related channels. Contrary to a previous report (41), the addition of micromolar La^{3+} did not stimulate further cation entry through hTRPC4 channels (Fig. 9E). Our results are, however, consistent with another report investigating the effects of lanthanides on currents mediated by both native and transfected TRPC4 (49). In contrast, and as others have reported, hTRPC5 channels are stimulated by micromolar La^{3+} application (Fig. 9E) (40, 41). These data further highlight the functional differences between channels formed by hTRPC4 and hTRPC5.

In summary, we have shown that tyrosine phosphorylation of C-terminal residues in hTRPC4 induces channel translocation from inside the cell to the plasma membrane in response to EGF application. Channel phosphorylation is dependent on STKs, is directly mediated by Fyn, and promotes the association of hTRPC4 with the scaffolding protein NHERF. This association appears responsible for the enhanced membrane insertion of hTRPC4 through the recruitment and stabilization of channels at the plasma membrane. This cascade contributes to the resultant Ca^{2+} influx initiated by EGFR activation and is unique to growth factor-induced activation of hTRPC4 with other stimuli failing to alter channel tyrosine phosphorylation and membrane expression levels. Tyrosine phosphorylation of hTRPC4 represents a novel mechanism for regulating plasma membrane channel levels and activation through modulation of PDZ-mediated interactions. This activation may be important in providing Ca^{2+} entry to drive the growth factor-stimulated events of cell proliferation, differentiation, and motility.

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