Regulation of TRPC6 Channel Activity by Tyrosine Phosphorylation*

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Various growth factors and hormones activate the mammalian canonical transient receptor potential (TRPC) channel through phospholipase C (PLC) activation. However, the precise mechanism of the regulation of TRPC channel activity remains unknown. Here, we provide the first evidence that direct tyrosine phosphorylation by Src family protein-tyrosine kinases (PTKs) is a novel mechanism for modulating TRPC6 channel activity. We found that TRPC6 is tyrosine-phosphorylated in COS-7 cells when coexpressed with Fyn, a member of the Src family PTKs. We also found that Fyn interacts with TRPC6 and that the interaction is mediated by the SH2 domain of Fyn and the N-terminal region of TRPC6 in a phosphorylation-independent manner. In addition, we demonstrated the physical association of TRPC6 with Fyn in the mammalian brain. Moreover, we showed that stimulation of the epidermal growth factor receptor induced rapid tyrosine phosphorylation of TRPC6 in COS-7 cells. This epidermal growth factor-induced tyrosine phosphorylation of TRPC6 was significantly blocked by PP2, a specific inhibitor of Src family PTKs, and by a dominant negative form of Fyn, suggesting that the direct phosphorylation of TRPC6 by Src family PTKs could be caused by physiological stimulation. Furthermore, using single channel recording, we showed that Fyn modulates TRPC6 channel activity via tyrosine phosphorylation. Thus, our findings demonstrated that tyrosine phosphorylation by Src family PTKs is a novel regulatory mechanism of TRPC6 channel activity.

Ca²⁺ channels, because modulation of the channel activities can profoundly affect these various physiological processes. The transient receptor potential (TRP) channel superfamily has emerged as candidates responsible for such a PLC-dependent Ca²⁺ influx. The TRP channel superfamily can be divided into at least three subfamilies of Ca²⁺-permeable nonselective cation channels (TRPC, TRPV, and TRPM families), having closely related structures comprised of six transmembrane domains, a large NH₂-terminal cytoplasmic domain, and a COOH-terminal cytoplasmic domain (4). Among the three subfamilies, TRPC channels are one of the molecules that have been extensively characterized.

The TRPC channel family is composed of seven non-selective ion channels that can be divided into four subgroups (TRPC1; TRPC4 and -5; TRPC3, -6, and -7; and TRPC2) based on their amino acid sequences and functional similarities (4–6). Recent investigations have extensively studied the regulation of TRPC channel activity. TRPC1, -4, and -5 appear to be activated by a store-operated mechanism (7–9). TRPC3 and TRPC1 are physically associated with IP₃ receptors (10–13) and ryanodine receptors (14) and are activated when a gated conforma- tional change in the IP₃ receptors or ryanodine receptors is sensed. Calmodulin (CaM) directly binds to TRPC3 and TRPC6 in a Ca²⁺-dependent manner, thereby reducing channel activity (15, 16). DAG directly activates TRPC3, -6, and -7 channel activities (17, 18). More recently, it is reported that Homer, a scaffolding protein, binds TRPC1 and regulates gating of TRPC1 by IP₃ receptors (19). However, considering that the TRPC channels appear to be organized into macromolecular assemblies (5), several unidentified mechanisms that regulate TRPC channel activities are thought to exist.

The activation of various growth factors and hormonal recep- tors that stimulate the TRPC channel through PLC activation may also induce tyrosine phosphorylation of multiple pro- teins. Here, we provide evidence that direct tyrosine phosphorylation by Fyn is a novel mechanism for modulating TRPC6 channel properties. We found that Fyn physically inter- acts with and directly phosphorylates TRPC6 in COS-7 cells. Src, another type of Src family protein-tyrosine kinases (PTKs) also phosphorylates TRPC6 in vitro and in COS-7 cells. We also found that tyrosine phosphorylation on TRPC6 was caused by the stimulation of the epidermal growth factor (EGF) receptor and that this tyrosine phosphorylation event was absolutely diminished by inhibition of Src family PTK activity. Moreover, we showed that tyrosine phosphorylation increases TRPC6 channel activity. Our results suggest that Fyn-mediated tyrosine phosphorylation enhances DAG-induced TRPC6 channel activity, resulting in a more efficient calcium influx through the TRPC6 channel upon EGF receptor stimulation.

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§The abbreviations used are: PLC, phospholipase C; IP₃, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; TRP, transient receptor potential; PTK, protein-tyrosine kinase; EGF, epidermal growth factor; HA, hemagglutinin; GST, glutathione S-transferase; PBS, phosphate-buffered saline; OAG, oleoyl-2-acyetyl-sn-glycerol; SH2, Src homology domain 2; EGFR, epidermal growth factor receptor.
**EXPERIMENTAL PROCEDURES**

**Culture—** COS-7 cells, CHO-K1, and HEK 293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 units/ml of penicillin, and 50 μg/ml of streptomycin. HEK 293 cells stably expressing TRPC6 were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 400 μg/ml G418, 50 μg/ml penicillin, and 50 μg/ml streptomycin (20).

**DNA Constructs and Mutagenesis—** Mouse TRPC6 expression vector (pcDNA3-TRPC6) was constructed as follows. The EcoRI-BamHI fragment encoding mouse TRPC6 cDNA was excised from TRPC6-HA/pVL139 (a kind gift from Dr. M. Zhu). The fragment was inserted into the EcoRI-BamHI sites of pcDNA3 expression vector (Clontech). The NH2-terminal deletion mutant of TRPC6 (Δ1–388 amino acids) was constructed as follows. The TRPC6 cDNA in the EcoRI-BamHI site of pBlueScriptII SK− was cut with BamHI and NdeI and a double strand DNA encoding the HA tag (sense: 5′-GATCGGATGATATCGGCGTCCCCAGACTATGCTAGCTCCA-3′, antisense: 5′-TATGGAGCTGATAGCTGTGGAGGCAGCTAAAGTATGATG-3′) was inserted between the sites. The truncated TRPC6 fragment in pBlueScriptII was excised with BamHI and EcoRI, and transferred into the BamHI-EcoRI restriction site of pcDNA3. The COOH-terminal truncated mutant of TRPC6 (Δ634–930 amino acids) was constructed as follows. The truncated TRPC6 expression vector (pcDNA3-TRPC6) was cut with EcoRV and Apal, and a double strand oligo DNA encoding HA tag (sense: 5′-ATCTATCCCTATGACGTCCACACTAGTCTAGCCTCCA-3′, antisense: 5′-TATGGAGCTGATAGCTGTGGAGGCAGCTAAAGTATGATG-3′) was inserted into the sites. For the construction of the TRPC6-EGFP expression vector, the DNA fragment of mouse TRPC6 was amplified using two primers (sense primer: 5′-CCGATATGTCAGGAGCCAGCCGAG-3′, antisense primer: 5′-CCGATATGTCAGGAGCCAGCCGAG-3′) using Megazyme polymerase (Invitrogen), and pCMV-TRPC6 as a template. PCR cycles used were: 30 s at 94°C, 30 s at 55°C, and 3 min at 72°C for 25 cycles. After digested with EcoRI and Sall, the amplified fragment was inserted into the restriction site between EcoRI and Sall of pEGFP-N3 vector (Clontech). The nucleotide sequences of these constructs were confirmed by DNA sequencing. Expression vectors encoding wild-type and various mutants of Fyn kinase (pME-FynYF and pME-FynKM) and plasmids encoding glutathione S-transferase (GST) fusion with the SH2 or SH3 regions of Fyn were kind gifts from Dr. T. Yamamoto (21).

**Transfection and Immunoprecipitation—** The cells (5 × 105) in 6-cm dishes were transfected with total of 3 μg of various plasmids encoding HA-tagged TRPC6, TRPC6-GFP, Fyn YF, or Fyn KM by the lipid method using TransIT-LT1 (Mirus) according to the manufacturer’s recommendations. After 36 h, the cells were lysed by 1.0 ml of TNE buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.0% Nonidet P-40, 1 mM EDTA, and 1 mM Na3VO4) for 30 min at 4°C. The lysates were centrifuged at 15,000 × g for 10 min. Then, 5 μg of appropriate antibodies (rabbit polyclonal anti-HA antibodies (Zymed Laboratories Inc.) mouse monoclonal anti-GFP antibodies (MBL), mouse monoclonal anti-Fyn antibodies (Wako Biochemicals), or rabbit polyclonal anti-TRPC6 antibody (Chemicon)) and 20 μl of protein G-Sepharose or protein A-Sepharose were added to the supernatants. After incubation for 2 h at 4°C, the resultant immunocomplexes were washed 5 times with the same buffer. For immunoblotting, the precipitated proteins were separated by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was treated with the blocking solution (0.05% Tween/PBS) containing 5.0% skim milk, and then, the membrane was incubated with the indicated antibodies. The antibodies were used at the following concentrations: rabbit polyclonal anti-HA antibody (1.0 μg/ml, Zymed Laboratories Inc.), rabbit polyclonal anti-Fyn antibody (1.0 μg/ml, Santa Cruz Biochemicals), and mouse monoclonal anti-GFP antibody (1.0 μg/ml, MBL). After incubation with horseradish peroxidase-labeled anti-rabbit IgG (from donkey) or anti-mouse IgG (from sheep) antibodies (Amersham Biosciences), the immobilized specific antigen was visualized with ECL plus detection kit (Amersham Biosciences). For the detection of tyrosine phosphorylation, the membrane filter was treated with blocking reagents containing 5.0% bovine serum albumin and probed with an anti-PY antibody (RC-20, Chemicon International, Inc.).

**Preparation of Rat Brain Microsomal Fractions—** Rat brains were dissected and homogenized in 10 volumes of homogenization buffer (0.32 M sucrose, 5 mM Hepes-NaOH (pH 7.5)). The homogenate was centrifuged at 800 × g for 10 min and the supernatant at 100,000 × g centrifuged at 800 × g for 10 min and the supernatant at 100,000 × g. The supernatant was centrifuged again at 100,000 × g for 1 h. The resulting pellet was resuspended in 10 volumes of homogenization buffer and used as rat brain microsomal fraction.
for 30 min at 4 °C. The pellet was resuspended in homogenization buffer. Three hundred micrograms of the soluble proteins in TNE buffer were used for immunoprecipitation with 2 μg of the appropriate antibodies (rabbit polyclonal anti-TRPC6 antibody (Chemicon), mouse monoclonal anti-synaptophysin antibody (Sigma), rabbit polyclonal anti-Src antibody (Sigma), rabbit normal IgG, rabbit polyclonal anti-Fyn antibody (Sigma)) and 20 μl of protein G-Sepharose. Immune complexes were washed five times with TNE buffer. Immunoblotting was performed with rabbit polyclonal anti-TRPC6 antibody (Chemicon) as described above.

EGF Stimulation—The COS-7 cells transiently expressing HA-tagged TRPC6 were serum starved for 1.5 h in PBS containing 1.0 mM Ca²⁺ and 0.8 mM Mg²⁺, to reduce the basal tyrosine phosphorylation level of TRPC6. For the inhibition of Src family PTKs or EGFR, the cells were treated with 4 μM PP2, a chemically synthesized specific Src family PTKs inhibitor (Calbiochem) for the last 15 min or 10 μM AG1478 (Calbiochem) for the last 10 min. Then, the cells were stimulated with 200 ng/mL EGF (Sigma) for 1, 3, or 5 min. The cells were quickly washed with ice-cold PBS, and lysed with 1 ml of TNE buffer for 30 min at 4 °C. Then, the cell lysates were subjected to immunoprecipitation and Western blot analysis.

In Vitro Kinase Assay—COS-7 cells (5 × 10⁶) were transfected with HA-tagged TRPC6 expression vector, and lysed with TNE buffer containing 0.5% SDS and then diluted with 4 volumes of TNE buffer. HA-TRPC6 was immunoprecipitated as described above. Immunoprecipitated HA-tagged TRPC6 was incubated with 3 units/ml Fyn (UBI) in the kinase buffer (40 mM Hepes (pH 7.4), 10 mM MgCl₂, 3 mM MnCl₂, 10 μM ATP) for 30 min at 37 °C. After washing 3 times with TNE buffer, the immunoprecipitates were subjected to immunoblot analysis.

Immunofluorescence and Confocal Microscopy—Transfected CHO-K1 cells with HA-TRPC6 and wild-type Fyn grown on glass coverslips were washed once with PBS, fixed in 4% paraformaldehyde/PBS for 10 min, permeabilized in 0.5% Triton X-100/PBS for 5 min, and blocked in 5% skim milk/PBS for 1 h at room temperature. The cells were stained with rabbit polyclonal anti-HA antibody (Zymed Laboratories Inc.) and mouse monoclonal anti-Fyn antibody (Clone 1872574). After washing 3 times with PBS, the coverslips were washed with Alexa 488-conjugated goat anti-rabbit IgG and Alexa 594-conjugated goat anti-mouse IgG (Molecular Probes) for 1 h at room temperature. After washing with PBS for 15 min, the coverslips were mounted with Vectashield (Vector Laboratories) and observed under IX-70 confocal fluorescence microscopy (Olympus).

In Vitro Binding Assay—HEK 293T cells were transfected with 3 μg of plasmids encoding HA-tagged TRPC6. After 24 h, the cells were lysed with 1.0 ml of TNE buffer, and the cell lysates were incubated with 10 μg of the GST fusion proteins in TNE buffer containing 0.5% SDS and then diluted with 4 volumes of TNE buffer. HA-tagged TRPC6 expression vector, and lysed with TNE buffer containing 0.5% SDS and then diluted with 4 volumes of TNE buffer. Then, the cell lysates were subjected to immunoblot analysis.

Single Channel Recording—Ionic currents through single TRPC6 channels were recorded from inside-out patches excised from HEK 293 cells stably expressing TRPC6 using a method as reported previously (17). Borosilicate glass pipettes (4–5 megohms) filled with an extracellular solution (120 mM sodium isothionate, 5.87 mM calcium gluconate, 1.0 mM magnesium gluconate, 10 mM EGTA, 10 mM glucose, 10 mM Hepes, pH 7.4) containing 100 μM 1-oleoyl-2-acetyl-sn-glycerol (OAG) (Calbiochem). For the analysis of the effect of Fyn on the channels, the intracellular solution was changed with the solution containing 100 μM OAG, 3 unit/ml recombinant wild-type Fyn (UBI), and 100 μM ATP. For boiled Fyn, Fyn boiled for 10 min in a water bath was used. The membrane patches were voltage-clamped at −60 mV with an Axopatch 200A amplifier (Axon Instruments, Inc.). Single channel currents were low pass-filtered at 2 kHz by a filter with Bessel characteristics (48 dB/octave) (model 362E, NF Instruments), sampled at 10 kHz, displayed, and stored in a Macintosh computer with custom-made software, TI work bench (developed by T. Inoue). Data were analyzed by Patch Analyst Pro software (version 1.16, MT Corp) after digital filtering at 500 Hz and baseline adjustments.
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RESULTS

Fyn Interacts with and Directly Phosphorylates the TRPC6 Channel—As various ion channel activities are regulated by direct tyrosine phosphorylation by Src family PTKs (N-methyl-d-aspartate receptors, K+ channels, etc.) (23, 24), we set out to examine whether TRPC channels are tyrosine phosphorylated by Src family PTKs. For this, we transiently expressed HA-tagged TRPC6 along with constitutively active form of Fyn (Fyn YF), one of the Src family PTKs, in COS-7 cells and examined tyrosine phosphorylation of TRPC6. When we expressed HA-tagged TRPC6 in COS-7 cells, we detected TRPC6 as a glycosylated broad band migrating between 100 and 150 kDa that was absent in control COS-7 cells, as reported previously (20). Immunoprecipitation of TRPC6 from the cell lysates followed by immunoblotting with antibody to phosphotyrosine revealed that TRPC6 is efficiently tyrosine-phosphorylated by Fyn (Fig. 1A, a). To test the possibility that the observed tyrosine-phosphorylated band was a TRPC6-associated protein, we constructed a fusion protein of TRPC6 with EGFP (TRPC6-EGFP) that had a higher molecular mass (−150 kDa) than HA-tagged TRPC6 (−100 kDa). We next transiently expressed TRPC6-EGFP along with Fyn YF in COS-7 cells and examined the tyrosine phosphorylation of TRPC6-GFP. If TRPC6 is really phosphorylated by Fyn, the tyrosine-phosphorylated band should be detected at a molecular mass of TRPC6-GFP (~150 kDa). As shown in Fig. 1A, b, we observed the decreased motility of both the tyrosine-phosphorylated protein and TRPC6-EGFP (lane 2) compared with HA-tagged TRPC6 (lane 1), strongly supporting the idea that TRPC6 was tyrosine phosphorylated by Fyn. Moreover, an in vitro kinase assay showed that recombinant Fyn directly tyrosine phosphorylates TRPC6 (Fig. 1B). Taken together, these results demonstrated that Fyn directly tyrosine phosphorylates the TRPC6 channel. We also observed tyrosine phosphorylation of TRPC6 by Src, another member of the Src family PTKs, in vitro and in COS-7 cells (data not shown).

To examine the physical interaction between Fyn and TRPC6, we transiently expressed HA-tagged TRPC6 along with either Fyn KM or Fyn YF in COS-7 cells, and tested for their phosphorylation-dependent interactions by immunoprecipitation followed by immunoblotting with specific antibodies to HA and Fyn. Fyn coimmunoprecipitated with TRPC6 at similar levels irrespective of the phosphorylation state of TRPC6 (Fig. 1C, lanes 7 and 8). Conversely, the result of co-immunoprecipitation using anti-Fyn antibodies showed that TRPC6 is precipitated with Fyn, confirming the physical interaction between TRPC6 and Fyn (Fig. 1D). As a control experiment, no TRPC6 was observed in the immunoprecipitates with normal control IgG, indicating the specific interaction of TRPC6 with Fyn (Fig. 1D). Thus, these results indicate that Fyn constitutively interacts with TRPC6 regardless of the tyrosine phosphorylation of TRPC6.

Co-localization of TRPC6 with Fyn in Heterologously Expressed Cells.—To further confirm the physical interaction of TRPC6 with Fyn, we examined the subcellular localization of TRPC6 and wild-type Fyn in heterologously expressed cells using confocal immunofluorescence microscopy. As shown in Fig. 2, A and B, we found that the immunoreactivities of both TRPC6 and Fyn were observed at or near the plasma membrane in the transfected cells and extensively overlapped each other. These data strongly support the physical interaction of TRPC6 with Fyn.

Identification of the Sites That Are Responsible for TRPC6-Fyn Interaction.—To determine the region responsible for TRPC6-Fyn interaction, we performed an in vitro binding assay using GST fusion proteins with the Src homology 2 (SH2) and Src homology 3 (SH3) domains of Fyn (GST-Fyn-SH2 and GST-Fyn-SH3). Pull-down experiments performed on lysates of HEK 293T cells expressing TRPC6 using these GST fusion proteins showed that TRPC6 is efficiently precipitated with GST-Fyn-SH2, but not with GST-Fyn-SH3 and GST alone (Fig. 3A). To determine the region of TRPC6 necessary for Fyn binding, we constructed deletion mutants of TRPC6 lacking either the NH2- or COOH-terminal cytoplasmic regions as shown in Fig. 3B, and performed in vitro binding experiments using GST-Fyn-SH2. As shown in Fig. 3C, whereas the COOH-terminal deletion mutant of TRPC6 still bound to GST-Fyn-SH2, the NH2-terminal deletion mutant of TRPC6 no longer did so. Taken together, these results indicate the TRPC6-Fyn association to be dependent on the NH2-terminal cytoplasmic region of TRPC6 and the SH2 domain of Fyn.

Interaction between TRPC6 and Fyn in the Mammalian Brain.—To demonstrate that TRPC6 associates with Src family PTKs in vivo, we prepared microsome fractions from rat brain and examined their interaction by coimmunoprecipitation ex-
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Experiments. We used TRPC6 antibody that efficiently recognized both HA-tagged TRPC6 exogenously expressed in COS cells and endogeneous TRPC6 in the brain lysate (Fig. 4A). As shown in Fig. 4B, TRPC6 was coimmunoprecipitated with Fyn from brain lysates (lane 5). We also found that TRPC6 interacted with Src, another member of the Src family PTKs, in rat brain (lane 4). As a control, no TRPC6 was detected in the immunoprecipitates either with control IgG (lane 1) or with anti-synaptophysin antibody, a presynaptic vesicle protein (lane 2). Thus, TRPC6 interacts with Fyn in the mammalian brain.

Stimulation of the EGF Receptor Induces Rapid Tyrosine Phosphorylation of TRPC6 through the Activation of Src Family PTKs Activation—To examine whether tyrosine phosphorylation of the TRPC6 channel was occurring in response to physiological stimuli, we examined tyrosine phosphorylation of TRPC6 following EGF stimulation in COS-7 cells. We found that EGF application rapidly increased tyrosine phosphorylation of TRPC6 within 1 min (Fig. 5A, lanes 6 and 7). As a control experiment, no tyrosine-phosphorylated protein was detected in immunoprecipitates from non-transfected control COS-7 cells stimulated with EGF for 3 min (Fig. 5A, lane 8). Pretreatment of cells with an inhibitor of EGF receptor (EGFR), AG1478, significantly inhibited the TRPC6 tyrosine phosphorylation (Fig. 5B). These results indicate that TRPC6 is tyrosine phosphorylated following EGF stimulation in COS-7 cells.

Because EGFR itself has intrinsic tyrosine kinase activity and can also activate Src family PTKs via its downstream signaling pathways (25), there are at least two possible mechanisms for the EGF-induced TRPC6 tyrosine phosphorylation: (i) direct tyrosine phosphorylation of TRPC6 by the EGFR; or (ii) tyrosine phosphorylation of TRPC6 by Src family PTKs activated by the EGFR. To assess the involvement of Src family PTKs in the EGF-induced TRPC6 tyrosine phosphorylation, we treated TRPC6 expressing COS-7 cells with PP2, a specific inhibitor of Src family PTKs, before EGF application. PP2 treatment dramatically reduced EGF-induced tyrosine phosphorylation of TRPC6 (Fig. 5C, lane 8). Moreover, EGF-induced tyrosine phosphorylation of TRPC6 was completely abolished by co-expression of a kinase negative form of Fyn (Fyn KM), one of the Src family PTKs (Fig. 5D, lane 8). EGF-induced tyrosine phosphorylation level of the cell lysates from PP2-treated and Fyn KM-expressed COS cells was similarly increased as compared with the control cell lysate, suggesting that neither PP2 treatment nor expression of Fyn KM interfered with EGFR activation (Fig. 5C, lanes 2 and 4, and D, lanes 2 and 4). Indeed, this was confirmed by the evidence that tyrosine phosphorylation of EGFR after EGF stimulation was similarly detected, irrespective of the presence of PP2 or Fyn KM expression (Fig. 5E).

Fyn Modulates TRPC6 Channel Activity via Tyrosine Phosphorylation—To explore the functional effect of tyrosine phosphorylation on the TRPC6 channel, we next examined the effect of Fyn on the channel properties of TRPC6 using an
inside-out patch clamp recording technique. As previously reported (17), the TRPC6 channel opening events occurred, when OAG (100 μM) was applied to the cytoplasmic face of the membrane patch excised from stably TRPC6 expressing HEK 293 cells (Fig. 6B). No similar channel events were detected from control HEK 293 cells (n = 7). Generally, the opening events of TRPC6 channels increased gradually after 100 μM OAG application, reached the maximum within 40 s, and declined gradually thereafter as shown in Fig. 6C, a (n = 7). Therefore, we applied Fyn to the TRPC6 channel at 45 s after the start of recording, while examining the effect of Fyn on TRPC6 channel activity, as illustrated in Fig. 6A. Application of Fyn (3 units/ml) and 100 μM ATP to the cytoplasmic face of the patch from TRPC6 expressing cells substantially increased channel activity (n = 6) (Fig. 6, B and C, b). Because application of Fyn, OAG, and ATP to the cytoplasmic face of the patch excised from control HEK 293 cells did not result in similar events (data not shown, n = 7), the increase in events presumably resulted from the modification of TRPC6 channel events altered by Fyn. Application of 100 μM ATP alone (n = 3) or boiled Fyn and ATP (n = 8) caused no significant change in TRPC6 channel activity, which excluded the possible involvement of any other kinases or a direct effect by ATP (Fig. 6C, c and e). Moreover, application of Fyn without ATP did not change TRPC6 channel activity (n = 6) (Fig. 6C, d), strongly suggesting the increased TRPC6 channel activity to be because of tyrosine phosphorylation of TRPC6 by Fyn rather than the physical association with Fyn. Fig. 6D shows the fold increases in TRPC6 channel activity expressed as a relative increase of mean amplitude (M.A.2/M.A.1); OAG, 0.93 ± 0.34 (n = 7); OAG + Fyn + ATP, 2.91 ± 0.79 (n = 6); OAG + ATP, 0.93 ± 0.31 (n = 3); OAG + Fyn - ATP, 1.32 ± 0.13 (n = 6); OAG + boiled Fyn + ATP, 1.31 ± 0.29 (n = 8). Thus, our findings clearly demonstrate that Fyn increases TRPC6 channel activity via tyrosine phosphorylation.

**DISCUSSION**

Here, we describe the first evidence that Fyn tyrosine kinase physically interacts with TRPC6 and increases TRPC6 channel activity via tyrosine phosphorylation. In addition, we also show that this tyrosine phosphorylation event occurs rapidly in response to EGF receptor stimulation in COS-7 cells. Thus, two simultaneous events, opening by DAG and modulation by Fyn-induced tyrosine phosphorylation, are thought to contribute to

**Fig. 6.** Fyn increases the TRPC6 channel activity via tyrosine phosphorylation. A, the experimental procedure for the TRPC6 single channel recording. Ion currents through TRPC6 channels were recorded from inside-out patches excised from HEK 293 cells stably expressing TRPC6. Following recording of the resting state for 15 s, the intracellular solution containing OAG (100 μM) was perfused onto the cytoplasmic face of the excised membrane patch, and then at 45 s the solution was changed to solutions containing both OAG and X (X: Fyn or boiled Fyn + ATP). The mean amplitudes (M.A.) of 20-s recordings before (M.A.1) and after change of the solution (M.A.2) were calculated in analysis. B, representative continuous 3-s recordings of TRPC6 channel currents before OAG application (control) and in the presence of OAG (OAG) or OAG + Fyn + ATP (Fyn). C, representative TRPC6 channel activity (expressed as mean amplitudes). The first arrow indicates OAG application onto the excised patches and the second arrow indicates OAG + X application. n, OAG, n = 7; b, X = OAG + Fyn + ATP, n = 6; c, X = OAG + ATP, n = 3; d, X = OAG + Fyn-ATP, n = 6; e, X = OAG + boiled Fyn + ATP, n = 8. D, fold increase (M.A.2/M.A.1) of TRPC6 channel activity expressed as mean ± S.E. Asterisk indicates a statistically significant difference between the result obtained from control patches (OAG) and those from treated patches; *, p < 0.05 (t test).
the efficient influx of calcium through the TRPC6 channel upon EGF stimulation. Recent studies have shown that several protein kinases are involved in the regulation of Drosophila TRP channels and some non-classical and classical TRP-related cation channels. TRPV1 activity is modulated by protein kinase C (26). Cyclic AMP-dependent protein kinase A potentiates TRPV1 by direct phosphorylation (27). TRPC6 is a substrate for protein kinase A, although phosphorylation appears to not affect cation permeation (28). The TRPM7 activity seems to be dependent on the Src family PTKs, although whether TRPM7 is directly phosphorylated by tyrosine kinases or not is unclear (29). In particular, TRPV4 is regulated by Src family kinase-dependent tyrosine phosphorylation (30). Thus, protein phosphorylation appears to be a common important regulatory mechanism for TRP superfamily channels.

The Drosophila TRP channel is assembled as a result of macromolecular signaling complexes that include INAD (inactive afterpotential D), TRPL, PLCβ, rhodopsin, protein kinase C, calmodulin, and myosin III (5). These macromolecular complexes contribute to the localization and stability of component proteins, which is necessary for proper phototransduction (5, 31). Like Drosophila TRP channels, mammalian TRPC channels also appear to be organized into macromolecular assemblies of cellular signaling proteins. TRPC3 physically associates with the brain-derived nerve growth factor receptor and is activated in response to brain-derived nerve growth factor stimulation in neurons (32). TRPC4 and TRPC5 associate with the Na+/H+ exchange regulatory factor (NhERF), a protein that binds to PLCβ through its PDZ (PSD-95, Dlg, ZO-1) domains (33). TRPC1 appears to be assembled in a signaling complex containing IP₃R3, caveolin-1, and Go₁q₁₁ (34). Such assemblies of signaling proteins in mammalian cells may contribute to proper signal transduction, as in Drosophila. Indeed, recent study indicates that caveolin-1 regulates the localization of TRPC1 on the plasma membrane and contributes to the proper regulation of the TRPC1 channel activity in human submandibular gland cells (35). In this study, we have shown that Fyn is involved in the establishment of TRPC6 macromolecular complexes and that EGF stimulation induces the rapid tyrosine phosphorylation of TRPC6. Because physical interactions between Src PTKs and their target proteins are critical for efficient target phosphorylation (36, 37), TRPC6-Fyn interaction may also contribute to the rapid and efficient tyrosine phosphorylation of the TRPC6 channel and the regulation of TRPC6 channel properties.

The Fyn-TRPC6 interaction is mediated by the SH2 domain of Fyn and the NH₂-terminal cytoplasmic domain of TRPC6; this interaction is not dependent on phosphorylation. Although associations involving the SH2 domain are usually mediated by an SH2-phosphotyrosine-based interaction, a phosphorylation-independent interaction between the SH2 domain and its target proteins has been reported in several SH2-dependent protein interactions (36, 38–40). TRPC6 might also recruit other SH2-containing signaling molecules in the macromolecular signaling complex to maintain proper TRPC6 channel regulation via the NH₂-terminal cytoplasmic domain.

TRPC6 is reported to be highly expressed in vascular smooth muscles (41, 42). Inoue et al. (42) reported that heterologous expression of TRPC6 in HEK 293 cells reproduces almost all the essential properties of α₁-adrenoreceptor-activated nonselective cation channels in portal vein smooth muscle. In addition, they reported that the treatment of portal smooth muscle cells with TRPC6 antisense oligonucleotides results in marked inhibition of the adrenoreceptor-activated nonselective cation channel (42). Thus, TRPC6 is likely to be an essential component of the α₁-adrenoreceptor-activated non-selective cation channel in portal vein smooth muscles. Worthy of mention is the fact that a peptide of Src PTK activator and a phosphatase inhibitor, Na₂VO₄, can produce non-selective cation currents similar to noradrenaline-induced currents in portal vein smooth muscle (43). These findings lead us to speculate that the non-selective cation channel induced by Src PTK activation might be a TRPC6-mediated current and that TRPC6 might be regulated by tyrosine phosphorylation in portal vein smooth muscle. It is an interesting future issue to determine whether the TRPC6 channel can be tyrosine phosphorylated in portal vein smooth muscle cells.

In summary, we have shown that Fyn tyrosine kinase interacts with and phosphorylates tyrosine residues in the TRPC6 channel. This tyrosine phosphorylation event is caused by EGF receptor stimulation. In addition, we have shown, using single channel recording, that tyrosine phosphorylation increases TRPC6 channel activity. Thus, tyrosine phosphorylation is a novel regulatory mechanism of TRPC6 channel activity. Because DAG stimulation and Fyn-mediated stimulation are additive (Fig. 6), tyrosine phosphorylation seems to modulate the TRPC6 channel properties activated by DAG. However, the possibility cannot be completely excluded that tyrosine phosphorylation directly affects TRPC6 gating, because tyrosine phosphorylation of TRPV4 by Src PTKs is known to be crucial for channel opening (30). Because the basal TRPC6 channel activity was observed before OAG application in our experiments (Fig. 6C, a and d), we could not clearly distinguish direct opening of the TRPC6 channel by tyrosine phosphorylation from modification of the TRPC6 channel activity by tyrosine phosphorylation, at present. Identification of the tyrosine residue(s) of TRPC6 phosphorylated by Src family kinases would help in understanding the mechanical insight into how tyrosine phosphorylation regulates TRPC6 channel properties, and this project is underway.

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REFERENCES
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