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Obligatory Role of Src Kinase in the Signaling Mechanism for TRPC3 Cation Channels*

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Members of the canonical transient receptor potential (TRPC) subfamily of cation channels are candidates for capacitative and non-capacitative Ca^{2+} entry channels. When ectopically expressed in cell lines, TRPC3 can be activated by phospholipase C-mediated generation of diacylglycerol or by addition of synthetic diacylglycerols, independently of Ca^{2+} store depletion. Apart from this mode of regulation, little is known about other receptor-dependent signaling events that modulate TRPC3 activity. In the present study the role of tyrosine kinases in receptor- and diacylglycerol-dependent activation of TRPC3 was investigated. In HEK293 cells stably expressing TRPC3, pharmacological inhibition of tyrosine kinases, and specifically of Src kinases, abolished activation of TRPC3 by muscarinic receptor stimulation and by diacylglycerol. Channel regulation was lost following expression of a dominant-negative mutant of Src, or when TRPC3 was expressed in an Src-deficient cell line. In both instances, wild-type Src restored TRPC3 regulation. We conclude that Src plays an obligatory role in the mechanism for receptor and diacylglycerol activation of TRPC3.

In many types of cells, membrane receptors coupled to phospholipase C (PLC)[1] promote inositol 1,4,5-trisphosphate (IP_3)-mediated release of Ca^{2+} from endoplasmic reticulum and Ca^{2+} entry across the plasma membrane through both capacitative or store-operated and non-capacitative calcium entry pathways (1–3). Although the molecular identity of capacitative and non-capacitative calcium entry channels, as well as the signaling mechanisms involved, remain uncertain, mammalian homologues of the *Drosophila melanogaster* transient receptor potential (TRP) channel have been considered as potential candidates (4, 5), particularly members of the canonical TRP (TRPC) subfamily (designated TRPC1 through TRPC7 (6)). The human isoform of TRPC3, originally cloned by Zhu *et al.* (7), has been shown in many heterologous expression systems to behave as a receptor-activated channel that can be activated also by exogenous application of diacylglycerols (DAGs) independently of store depletion. In fact, DAG-induced activation of TRPC3 and its structural relatives TRPC6 and TRPC7 (8, 9) provides the likely mechanism of activation of these channels by phosphoinositide-specific PLC-linked receptors, independently of IP_3 and store depletion. Apart from its regulation by DAG generated from PLC stimulation through either G-protein-coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs), little is known about additional signaling pathways downstream of receptor-stimulation involved in activation of TRPC3. Stimulation of either of these receptor pathways results in rapid tyrosine phosphorylation of cellular proteins (10, 11), and so in the present study we investigated whether tyrosine kinases might play a role in the signaling mechanism underlying receptor- and DAG-dependent activation of TRPC3. We found that inhibition of tyrosine kinase activity completely abrogated the ability of either GPCR stimulation or exogenous application of diacylglycerol to induce TRPC3 activation. The results indicate an obligatory requirement for functional Src kinase for TRPC3 activation, likely involving Src-dependent phosphorylation and/or recruitment of an as yet unidentified accessory protein. In addition, the results show that activation of the channels by diacylglycerol is unlikely to involve a direct action of diacylglycerol on the channels.

MATERIALS AND METHODS

Cell Culture, Transfection, and Measurement of Intracellular Calcium—Human embryonic kidney cells (HEK293) stably expressing the human TRPC3 (TRPC3-HEK293) with either the hemagglutinin (HA)-epitope (TRPC3-HA) or the green fluorescent protein (TRPC3-GFP) fused to its C terminus, were cultured as previously described (12, 13). For transient transfections, either TRPC3-HA or TRPC3-GFP cells were grown in 6-well plates and transfected as indicated with 1 μg of either wild-type Src (WT-Src, kindly provided by Dr. Thomas E. Smithgall, Department of Molecular Genetics and Biochemistry, School of Medicine, University of Pittsburgh, Pittsburgh, PA), a dominant-negative mutant of Src (DN-Src, kindly provided by Dr. Sara Courtneidge, Van Andel Research Institute, Grand Rapids, MI), a constitutively active form of avian Src (CA-Src, courtesy of Dr. John O'Bryan, NIEHS, National Institutes of Health (NIH), Research Triangle Park, NC) or the corresponding vector alone (pcDNA3) and EYFP-C1 (EYFP, 0.5 μg; Clontech) as a transfection marker by using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. 24 h after transfection, cells were plated onto glass coverslips and an additional 24 h later used for calcium measurements. Real-time fluorescence measurements with coverslip-plated cells were performed as in Trebak *et al.* (14). When only Ca^{2+} was measured in a particular experiment, calibrated $[Ca^{2+}]_i$ values are reported (*i.e.* see Fig. 1). In most instances, when experiments involved measurement of Ba^{2+} entry, fluorescence

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[1] The abbreviations used are: PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; TRP, transient receptor potential; TRPC, canonical transient receptor potential; TRPM, melastatin-related transient potential; TRPV, vanilloid receptor-related transient receptor potential; GPCR, G-protein-coupled receptor; RTK, receptor tyrosine kinase; HBSS, Hepes-buffered saline solution; PKC, protein kinase C; HA, hemagglutinin; GFP, green fluorescence protein; WT, wild-type; DN, dominant-negative; EYFP, enhanced yellow fluorescent protein; EGF, epidermal growth factor; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]-pyrimidine; bpV(phen), bisperoxo(1,10-phenantroline)oxovanadate; BCR, B cell receptor.

ratios (fluorescence at 510 nm with excitation at 340 nm divided by that with excitation at 380 nm) are reported. The DT40 chicken B lymphocyte cell line and the mutant variant deficient in PLCγ-2 (PLCγ-KO) were kindly provided by Dr. Tomohiro Kurosaki (Department of Molecular Genetics, Kansai Medical University). DT40 cells culture, transfection, loading with the Ca^{2+} indicator Fura-2, and ratiometric measurement of intracellular Ca^{2+} were accomplished as described previously (15). Transient transfections of the wild-type and PLCγ-KO DT40 cells were performed by electroporation, with either the human isoform of TRPC3 (100 μg/ml TRPC3 into pcDNA3 vector, provided by Dr. Lutz Birnbaumer, NIEHS, NIH), the human M5 muscarinic receptor (50 μg/ml, in pcDNA3), vector alone (pcDNA3, mock transfected cells), or a combination of them, along with EYFP (5 μg) as marker for transfection. Cells were used for real-time fluorescence measurements 17–25 h post-transfection.

SYF ($Src^{-/-}$, $Yes^{-/-}$, and $Fyn^{-/-}$) and YF ($Yes^{-/-}$, $Fyn^{-/-}$) cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C under humidified air (5% CO_2). For transient transfections, SYF and YF cells grown in 6-well plates were transfected by using Lipofectamine 2000 following the manufacturer's instructions, with either human TRPC3 (1 μg), human M5 muscarinic receptor (1 μg), vector alone (pcDNA3, mock transfected cells), or a combination of them, along with EYFP (0.5 μg) as marker for transfection. In some experiments, either WT-Src (1 μg) or CA-Src (1 μg) was co-transfected with TRPC3, M5 receptor, and EYFP into SYF cells. 24 h after transfection, cells were plated onto glass coverslips, and an additional 24 h later used for calcium measurements as described above for HEK293 cells. In all experiments involving transiently transfected cells, real-time fluorescence measurements were performed under the conditions indicated with single EYFP-positive cells, selected by their yellow/green fluorescence (excitation: 485 nm; emission: 520 nm).

In figures involving transient transfections, average traces from 8–12 EYFP-positive cells are shown for a single experiment, whereas for stably transfected cells (*i.e.* for the pharmacological experiments) averages of 30–50 are shown. These are representative of at least three independent experiments. Because the cells are not confluent and behave independently, statistical analyses treat each cell as an individual observation. The use of at least three different coverslips with experiments carried out independently assures that spurious errors do not confound the statistical conclusion. In some instances, statistical significance is assumed on the basis of non-parametric statistical principles when large numbers of cells either do or do not show a qualitative response; *i.e.* when for example each of 100 different cells shows a strong Ca^{2+} signal under control conditions, whereas not one of 100 different cells responds in the presence of an inhibitor. Comparisons of quantitative differences between mean levels of $[Ca^{2+}]$ or rates of Ca^{2+} or Ba^{2+} entry were made using a two-tailed t test for two means, or analysis of variance for three or more means. When the analysis of variance was found to be significant at $p < 0.05$, it was followed by Tukey-Kramer multicomparison tests carried out using GraphPad InStat software. Summarized data are given as means ± S.E.

Cell Lysis, Immunoprecipitation, and Immunoblotting—Wild-type and HA-TRPC3-expressing HEK293 cells were plated onto polylysine-coated 60-mm tissue culture dishes. After 24 h, cells were washed two times in HEPES-buffered physiological saline solution (HBSS, composition in mM: 140 NaCl, 4.7 KCl, 1 $MgCl_2$, 2.0 $CaCl_2$, 10 glucose, 10 HEPES, pH 7.4) and equilibrated in the same solution for 20 min at room temperature. Following treatment with either vehicle, carbachol (300 μM), EGF (150 ng/ml), or OAG (1-oleoyl-2-acetyl-*sn*-glycerol, 100 μM), the cells were washed twice with ice-cold phosphate-buffered saline (PBS) solution and lysed in lysis buffer containing: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.2% sodium deoxycholate, 1 mM Na_3VO_4, 1 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 4 μg/ml leupeptin, 10 μg/ml aprotinin. After 30-min incubation on ice with shaking, samples were centrifuged for 20 min at maximal speed in an Eppendorf microcentrifuge, and supernatants were used for immunoprecipitation with the desired antibody. The supernatants (800 μg) were then incubated with 2 μg of anti-HA mouse monoclonal antibody (12CA5 clone, Roche Applied Science) or anti-phosphotyrosine antibody (clone 4G10, Upstate Biotechnology, Charlottesville, VA) and 50 μl of protein A-agarose (Invitrogen) for 2 h at 4 °C. Immunoprecipitates were washed four times with PBS (containing inhibitors for proteases and phosphatases) and then heated at 95 °C in 2× SDS sample buffer and resolved on SDS-polyacrylamide gels (10% polyacrylamide). Proteins from gels were electrotransferred onto nitrocellulose membranes (0.45-μm pore size, Invitrogen) and subjected to immunoblotting with the indicated antibody. After incubation with the secondary antibody (HRP-conjugated anti-mouse, Amersham Biosciences) immunoreactive bands were visualized by ECL chemiluminescence (Amersham Biosciences), following the manufacturer's instructions.

For evaluation of tyrosine phosphorylation of total cellular proteins, cells were treated and processed as above. Where indicated, cells were incubated for 30 min with 100 μM genistein before carbachol or OAG treatment. Aliquots (20 μg) from each cell lysate were subjected to SDS-PAGE (10% polyacrylamide) electrophoresis and immunoblotted with anti-phosphotyrosine antibody 4G10.

Evaluation of Src Activation—TRPC3-HEK293 cells plated onto polylysine-coated 60-mm tissue culture dishes were washed two times in HBSS, equilibrated in the same solution for 20 min at room temperature, and then treated with either vehicle, carbachol (300 μM), or OAG (100 μM), either in the presence (2 mM) or absence of extracellular calcium (nominally Ca^{2+} free) and in the presence of 5 μM Gd^{3+}. Where indicated, the protein kinase C inhibitor Gö6976 (1 μM) was added to the incubation medium 15 min prior to OAG stimulation. The cells were then washed twice with ice-cold PBS and lysed as detailed above. The supernatants (800 μg) were then incubated with 4 μg of anti-Src mouse monoclonal antibody (clone GD11, Upstate Biotechnology) and 50 μl of protein G-agarose (Calbiochem) for 2 h at 4 °C. Immunoprecipitates were washed four times with PBS (containing inhibitors for proteases and phosphatases) and then processed as above for SDS-PAGE (10% polyacrylamide). Immunoblotting was performed using a specific anti-phosphoSrc antibody (Calbiochem), which detects Src phosphorylated at Tyr-418 (16). After incubation with the secondary antibody, immunoreactive phosphoSrc was detected by ECL chemiluminescence.

Materials—Fura-2-acetoxymethylester was from Molecular Probes Inc. Carbachol, OAG, Gö6976 (12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-*a*)pyrrolo(3,4-*c*)carbazole), genistein, daidzein, erbstatin analogue, herbimycin, lavendustin A, bpV(phen) (bisperoxo(1,10-phenantroline)oxovanadate), AG1478, PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine), salicylate, piceatannol (3,4,3′,5′-tetrahydroxy-*trans*-stilbene), and sodium orthovanadate were purchased from Calbiochem. Recombinant human epidermal growth factor was from Upstate Biotechnology (Charlottesville, VA). The anti-chicken IgM antibody (clone M4) was from Southern Biotechnology Associates Inc. (Birmingham, AL). Nonidet P-40, sodium deoxycholate, sodium pyrophosphate, β-glycerophosphate, sodium fluoride, phenylmethylsulfonyl fluoride, $CaCl_2$, $BaCl_2$, and $GdCl_2$ were from Sigma. Complete® protease inhibitor tablets were from Roche Applied Science. All other reagents used were of analytical grade. Sources of antibodies and reagents for immunoprecipitation, electrophoresis, and immunoblotting are indicated in the corresponding methods sections.

RESULTS

We first evaluated the impact of inhibition of cellular tyrosine kinases on receptor-dependent TRPC3 activation by Fura-2 imaging of multiple single cells as indicated under "Materials and Methods" (and see Ref. 14). As in previous work, 5 μM Gd^{3+} in the extracellular medium was used throughout these experiments to fully inhibit the endogenous capacitative calcium entry pathway (2, 17). As shown in Fig. 1*A*, muscarinic stimulation of cells in Ca^{2+}-free medium resulted in a transient release of Ca^{2+} as a consequence of IP_3-mediated mobilization from endogenous stores. Re-addition of Ca^{2+} (2 mM) to the extracellular medium resulted in a robust Gd^{3+}-insensitive Ca^{2+} entry, indicating activation of TRPC3. Genistein caused a dose-dependent reduction in TRPC3 activation, with complete inhibition at 100 μM genistein. The inactive genistein analogue, daidzein (100 μM), did not inhibit (not shown). Erbstatin analogue, a tyrosine kinase inhibitor not chemically related to genistein (18), also completely suppressed receptor- activation of TRPC3 (Fig. 1*A*). At the concentrations used in this study, the tyrosine kinase inhibitors herbimycin (1 μM) and lavendustin A (5 μM) partially inhibited TRPC3-mediated Ca^{2+} entry (not shown).

When TRPC3 is ectopically expressed in most cell lines, OAG-induced Gd^{3+}-insensitive Ca^{2+} entry constitutes a hallmark of TRPC3 activation (8). We next examined the effect of tyrosine kinase inhibitors on OAG-induced activation of

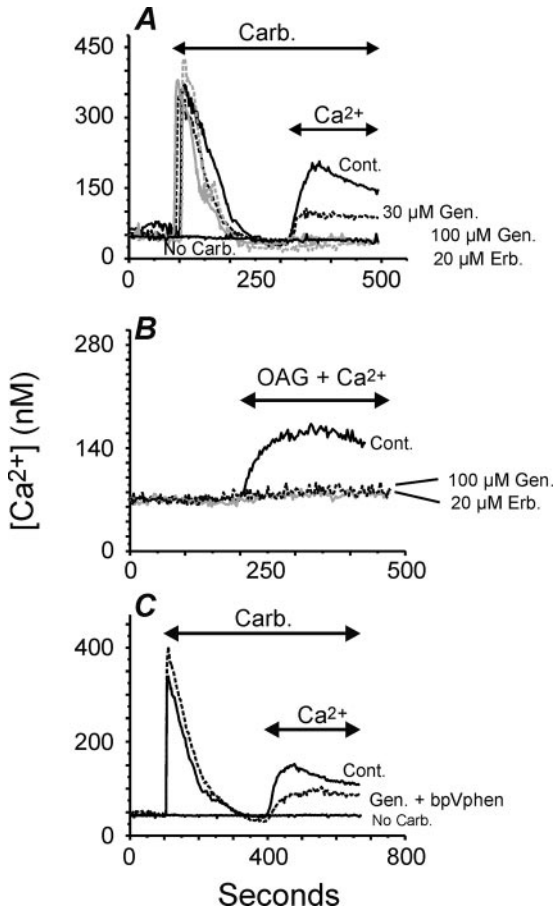

FIG. 1. **Inhibition of tyrosine kinases suppresses receptor- and OAG-induced activation of TRPC3.** Fura-2-loaded HEK293 cells stably expressing TRPC3 were incubated in a nominally Ca^{2+}-free medium containing 5 μM Gd^{3+} and then treated with 300 μM carbachol (*A*) or 100 μM OAG (*B*). In *A*, after the transient cytosolic Ca^{2+} rise returned to basal levels, Ca^{2+} (2 mM) was added to the medium. The conditions were: control (*solid black trace*), 30 μM genistein (*dotted black trace*), 100 μM genistein (*solid gray trace*), 20 μM erbstatin analogue (*dotted gray trace*), non-stimulated control (*lower flat solid black trace*). In *B*, Ca^{2+} (2 mM) was simultaneously added with OAG. The conditions were: control (*solid black trace*), 100 μM genistein (*dotted black trace*), 20 μM erbstatin analogue (*solid gray trace*). *C*, as in *A*, but where indicated cells were incubated for 30 min simultaneously with 200 μM bpV(phen) and 100 μM genistein (*dotted trace*; control is shown by the *solid trace*, and a non-stimulated control by the *flat solid trace*). Shown are average traces from 38–50 cells on a single coverslip, representative of three separate experiments. Statistical analysis of all cells revealed that for *A* the differences among the two concentrations and control were significant at $p < 0.0001$, $n = >100$ for each group. For *B*, all of the 90 cells responded to OAG plus Ca^{2+} in the control condition, whereas none of the 138 responded in the presence of genistein, and none of 110 responded in the presence of erbstatin. For *C*, all of 105 cells responded to the re-addition of Ca^{2+} in cells treated with genistein and bpV(phen), and the response was less than the control (126 cells, $p < 0.0001$).

TRPC3. Both genistein (100 μM) and erbstatin analogue (20 μM) completely abrogated OAG-induced activation of TRPC3 (Fig. 1*B*). Importantly, TRPC5, a member of the TRPC family, which has been shown to behave as a receptor-activated non-selective cation channel insensitive to OAG and store depletion (19), was not affected by the tyrosine kinase inhibitors used here (not shown), thus ruling out potential nonspecific effects of the tyrosine kinase inhibitors such as plasma membrane depolarization. Finally, because further evidence that the effects of genistein result from inhibition of tyrosine kinases, treatment of TRPC3-expressing HEK293 cells with 200 μM of the tyrosine phosphatase inhibitor bisperoxo(1,10-phenantroline)oxovanadate (bpV(phen)) (20) resulted in a partial recovery (~30–40%) from genistein inhibition of TRPC3 activation (Fig. 1*C*).[2] Together, these observations strongly suggest that receptor- and OAG-dependent activation of TRPC3 requires tyrosine kinase activity.

To obtain preliminary insight into the putative identity of the tyrosine kinase/s involved in receptor-dependent activation of TRPC3, a panel of inhibitors specific for different families of tyrosine kinases was utilized. The potential roles of epidermal growth factor receptor-associated kinase activity, Syk and Src-family kinases, and the proline-rich tyrosine kinase Pyk2, all known to be activated downstream of stimulation of the G_q-coupled muscarinic receptor (21–26), were evaluated.

Pre-treatment of HEK293 cells with the potent antagonist of the epidermal growth factor receptor, AG1478 (2 μM (27)), did not affect the ability of muscarinic receptor stimulation to activate TRPC3-mediated calcium entry (not shown). Neither salicylate (20 mM), a compound reported as a specific inhibitor of Pyk2 (28), nor piceatannol (3,4,3′,5′-tetrahydroxy-*trans*-stilbene), a specific inhibitor for Syk (29, 30), a cytoplasmic protein-tyrosine kinase not belonging to the Src family, affected either receptor- or OAG-dependent activation of TRPC3, indicating that neither of these tyrosine kinases was required for signaling to TRPC3 (not shown). However, preincubation of HEK293 cells with the Src kinase-specific inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2 (31)) resulted in a dose-dependent reduction in both muscarinic receptor stimulated- and OAG-induced TRPC3-mediated cation entry, with almost complete inhibition at 5 μM (Fig. 2). This result suggests that an Src family kinase may be involved in both receptor and OAG activation of TRPC3.

To investigate more directly the involvement of Src, we determined the effect of transient expression of a kinase-dead dominant-negative mutant of human Src (DN-Src) into TRPC3-expressing HEK293 cells. This mutant carries a kinase inactivating mutation (Lys-295 → Met) that results in complete suppression of Src kinase activity without altering its ability to interact with SH2 and/or SH3 binding partners (32). As shown in Fig. 3, both muscarinic receptor- and OAG-induced TRPC3-mediated cation entry were completely abrogated by DN-Src, whereas overexpression of the wild-type form of Src (WT-Src) had no such inhibitory effect. In parallel experiments, the endogenous store-operated pathway was assessed by observing thapsigargin-activated Ca^{2+} entry (in the absence of Gd^{3+}). Significantly, the endogenous store-operated Ca^{2+} entry pathway was not affected by DN-Src (not shown), ruling out alterations in membrane potential that might reduce the driving force for Ca^{2+} entry. Also, DN-Src expression in HEK293 cells stably expressing TRPC3 tagged to GFP (TRPC3-GFP) showed that overexpression of the mutant did not affect either expression or the membrane targeting of the channel (not shown). Importantly, co-expression of WT-Src in TRPC3-HEK293 cells together with DN-Src partially rescued both agonist- and OAG-dependent activation of TRPC3 (Fig. 3). Expression of DN-Src and WT-Src was confirmed by Western analysis (not shown). Quantitation of the Western blots indicated that similar amounts of both forms were expressed and that the amount of protein expressed with the combination was approximately twice that for expression of each alone.

As an additional test to the hypothesis that Src activity is obligatory for signaling to TRPC3, we transiently expressed

[2] Examination of gels from whole cell extracts immunoblotted for tyrosine phosphates shows that, although the block of tyrosine phosphorylation by genistein is nearly complete, some faint bands remain, indicating a small degree of kinase persists. Thus, we would suppose that a small degree of Src activity persists in the presence of genistein, which can by amplified by the tyrosine phosphatase inhibitor.

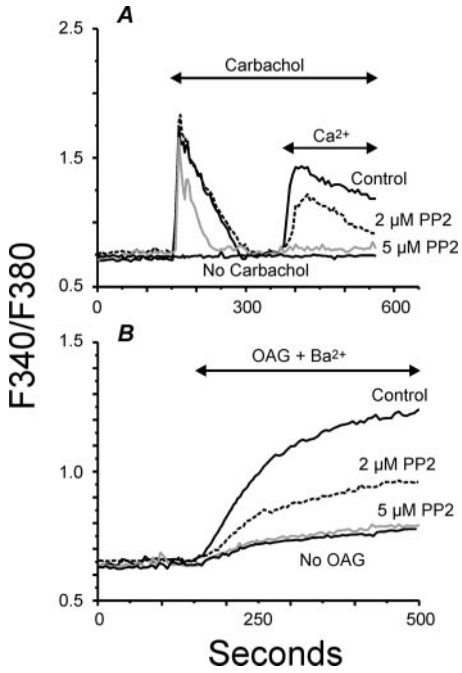

FIG. 2. **Pharmacological inhibition of Src tyrosine kinase suppresses receptor- and OAG-induced activation of TRPC3.** Fura-2-loaded HEK293 cells stably expressing TRPC3 were incubated in a nominally Ca^{2+}-free medium containing 5 μM Gd^{3+} and then exposed to 300 μM carbachol (*A*) or 100 μM OAG (*B*). In *A*, after the transient cytosolic Ca^{2+} rise returned to basal levels, Ca^{2+} (2 mM) was re-added to the medium. In *B*, Ba^{2+} (2 mM) was simultaneously added with OAG. For both panels, conditions were: control (*solid black trace*), 2 μM PP2 (*dotted black trace*), 5 μM PP2 (*solid gray trace*). Control responses in TRPC3-expressing cells in the absence of agonist are shown by the *lower solid black trace*. Shown are average traces from 30–46 cells representative of three separate experiments. Statistical analysis of all experiments revealed no significant inhibitory effect of PP2 on Ca^{2+} release (in *A*), but the rates of entry in both agonist- and OAG-treated cells were significantly reduced by 2 μM PP2 ($p < 0.0001$ for both), and completely blocked by 5 μM (for agonist, 105 cells, for OAG, 141 cells).

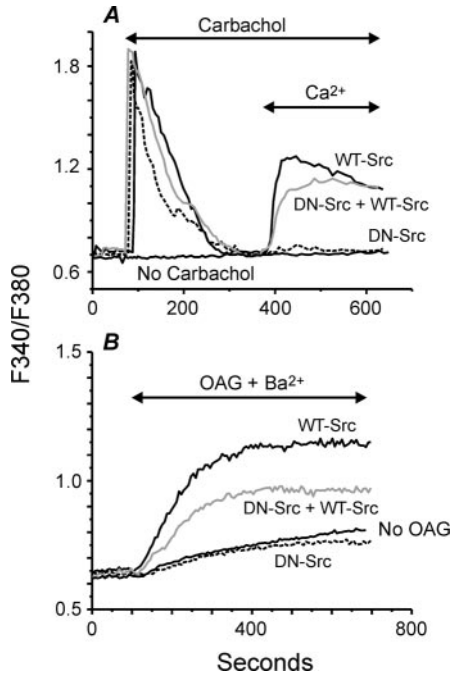

FIG. 3. **TRPC3 regulation is abrogated by a dominant-negative form of Src.** HEK293 cells stably expressing TRPC3 were transiently transfected with either DN-Src (*dotted traces*), WT-Src (*upper solid black traces*), or both (*solid gray traces*), using EYFP as a marker for transfection, as indicated under "Materials and Methods." Control responses in TRPC3-expressing cells in the absence of agonist are shown by the *lower solid black trace*. Cells were loaded with Fura-2, incubated in a nominally Ca^{2+}-free medium containing 5 μM Gd^{3+}, and then exposed to 300 μM carbachol (*A*) or 100 μM OAG (*B*). In *A*, after the transient cytosolic Ca^{2+} rise returned to basal levels, Ca^{2+} (2 mM) was re-added to the medium. In *B*, Ba^{2+} (2 mM) was simultaneously added with OAG. Ba^{2+} entry in cells expressing DN-Src was not significantly different from TRPC3 constitutive activity. Shown are *traces* representative of at least three separate experiments. Statistical analysis of all experiments revealed a small but significant reduction in the peak of Ca^{2+} release in the DN-Src transfected cells: control, 1.08 ± 0.014, n = 95; DN-Src, 0.98 ± 0.007, n = 154; $p < 0.001$. DN-Src produced a complete block of entry in all of 154 cells for the experiments with carbachol, and in all of the 143 cells with OAG. The combination of DN-Src and WT-SRC resulted in significant entry in all of 125 cells for carbachol stimulation and all of 98 cells for OAG stimulation. However, this entry was significantly less than that seen in the presence of WT-Src ($p < 0.0001$ for both carbachol and OAG).

TRPC3 (together with the M5 muscarinic receptor) into SYF ($Src^{-/-}$, $Yes^{-/-}$, and $Fyn^{-/-}$) cells. SYF is a mouse embryo fibroblast cell line harboring functional null mutations in both alleles of the Src family protein-tyrosine kinases (33). As shown in Fig. 4*A*, addition of 2 mM Ba^{2+} to the extracellular bath resulted in the appearance of a significant Gd^{3+}-insensitive cation entry in many transfected cells due to TRPC3 constitutive activity, because it was absent in cells transfected only with the M5 receptor (not shown). Subsequent activation of the muscarinic pathway resulted in a transient increase in intracellular Ca^{2+} due to release from endogenous stores, but the rate of constitutive Ba^{2+} entry, an indicator of TRPC3 activity, was not further increased. To minimize the cation entry due to TRPC3 constitutive activity, the Ba^{2+} concentration was reduced to 0.5 mM. Under these conditions, constitutive Ba^{2+} entry was minimized, and there was no evidence of carbachol-stimulated TRPC3 activity (Fig. 4*A*). Similarly, OAG failed to increase TRPC3-mediated Ba^{2+} entry under these conditions (Fig. 4*B*). That the lack of TRPC3 regulation in these cells was in fact due to the absence of Src expression was confirmed by two separate strategies. First, TRPC3 was transiently co-expressed with the muscarinic M5 receptor into YF ($Yes^{-/-}$ and $Fyn^{-/-}$) cells, which were obtained by stably reintroducing the wild-type form of Src into SYF cells (33). As shown in Fig. 5 (*A* and *B*), challenging these cells with either carbachol or OAG significantly stimulated a Gd^{3+}-insensitive cation entry that was absent in YF cells transfected with either the M5 receptor (Fig. 5*A*) or vector (pcDNA3, mock cells in Fig. 5*B*) alone. Second, transiently co-expressing WT-Src into SYF cells together with TRPC3 and M5 receptor resulted in restitution of both receptor and OAG activation of the channel (Fig. 5, *C* and *D*).

The findings to this point indicate a clear obligatory role for active Src kinase in the mechanism for activation of TRPC3 channels. Tyrosine phosphorylation events are known to occur downstream of both GPCR- or RTK-activated pathways, and we confirmed that treatment of TRPC3-expressing HEK293 cells with either carbachol or OAG increased total tyrosine phosphorylation of cellular proteins, and this was blocked by genistein (not shown). We next sought to determine whether this tyrosine kinase activation resulted in tyrosine phosphorylation of TRPC3 and/or activation of Src. HEK293 cells stably expressing HA-epitope-tagged TRPC3 (13) were exposed for 10 min to either vehicle, the muscarinic agonist carbachol (300 μM), OAG (100 μM), or epidermal growth factor (EGF, 150 ng/ml). After treatment, cells were lysed and tyrosine phosphorylation of TRPC3 was evaluated by anti-phosphotyrosine immunoblotting the corresponding anti-HA immunoprecipitates (Fig. 6*A*). We found no detectable phosphotyrosine signal under these experimental conditions. The reverse protocol, *i.e.* by anti-HA immunoblotting of anti-phosphotyrosine immunoprecipitates, also resulted in a negative result (not shown).

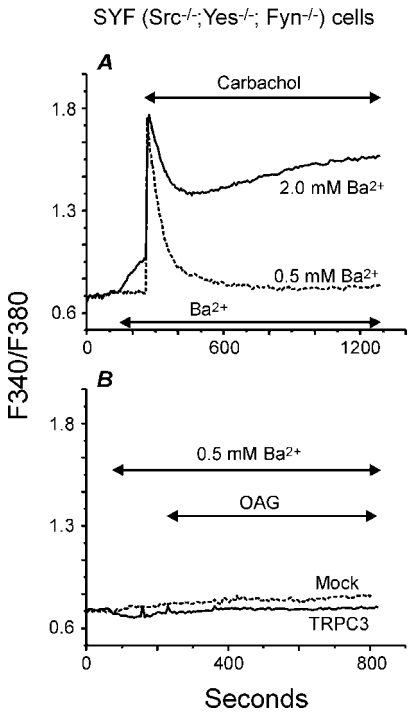

FIG. 4. **Loss of receptor- and OAG-dependent regulation of TRPC3 when expressed in a Src deficient cell line.** Ba^{2+} influx was measured in Fura-2-loaded SYF ($Src^{-/-}$, $Yes^{-/-}$, and $Fyn^{-/-}$) cells transfected with the M5 muscarinic receptor plus TRPC3 (*A*), or either TRPC3 (*solid trace*) or its vector alone (pcDNA3, *Mock*, *dotted trace*) (*B*). EYFP was used as a transfection marker. The cells were maintained in a nominally Ca^{2+}-free medium containing 5 μM Gd^{3+} and then exposed to 300 μM carbachol (*A*) or 100 μM OAG (*B*). Ba^{2+} (0.5 or 2 mM for *A*, as indicated; see text) was added to the medium 2 min before stimulation to monitor TRPC3 constitutive activity and maintained throughout the experiment. Shown are *traces* representative of at least three separate experiments. Statistical analysis of all experiments showed that with 2 mM Ba^{2+}, all of 70 cells showed constitutive Ba^{2+} entry (0.89 ± 0.01), whereas at 0.5 mM Ba^{2+}, none of 87 cells showed either Ba^{2+}- or carbachol-stimulated Ba^{2+} entry. Likewise, with 0.5 mM Ba^{2+}, none of 90 cells showed OAG-stimulated entry.

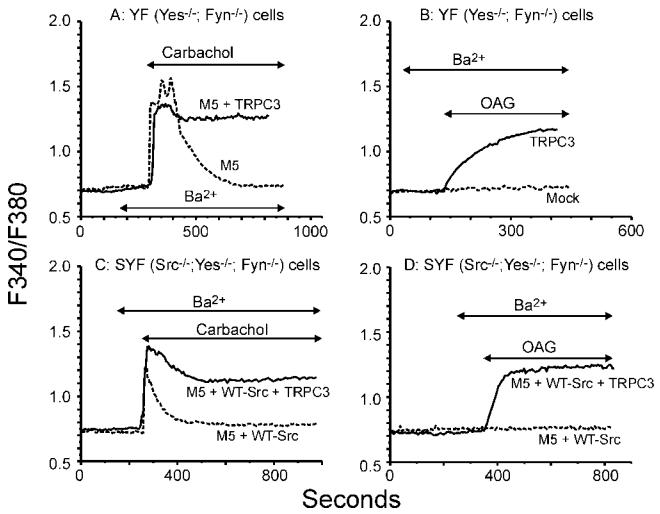

FIG. 5. **TRPC3 regulation is restored when Src is expressed.** *A* and *B*, Ba^{2+} influx was measured in Fura-2-loaded YF ($Yes^{-/-}$ and $Fyn^{-/-}$) cells transfected with: *A*, either the M5 muscarinic receptor alone (*dotted trace*) or the M5 receptor plus TRPC3 (*solid trace*), or *B*, either TRPC3 (*solid trace*) or its vector alone (pcDNA3, *Mock*, *dotted trace*). *C* and *D*, Ba^{2+} influx was measured in Fura-2-loaded SYF ($Src^{-/-}$, $Yes^{-/-}$, and $Fyn^{-/-}$) cells co-transfected with either the M5 muscarinic receptor and WT-Src (*dotted traces*), or the M5 receptor, WT-Src, and TRPC3 (*solid traces*). EYFP was used as a transfection marker. The cells were maintained in a nominally Ca^{2+}-free medium containing 5 μM Gd^{3+} and then exposed to 300 μM carbachol (*A* and *C*) or 100 μM OAG (*B* and *D*). Ba^{2+} (0.5 mM) was added to the medium 2 min before agonist or OAG stimulation and maintained throughout the experiment. Shown are *traces* representative of at least three separate experiments. For *A*, the mean increase in ratio due to Ba^{2+} entry in cells transfected with TRPC3 was 0.53 ± 0.01 (n = 96). In non-TRPC3-transfected cells, there was no net increase in steady-state ratio in any of 115 cells. For *B*, in TRPC3-transfected cells, addition of OAG increased the fluorescence ratio by 0.40 ± 0.005 (n = 101). In non-TRPC3-transfected cells, there was no net increase in steady-state ratio in any of 92 cells. For *C*, in TRPC3-transfected cells, carbachol increased the steady-state ratio by 0.44 ± 0.006 (n = 91). In non-TRPC3-transfected cells, there was no net increase in steady-state ratio in any of 95 cells. For *D*, in TRPC3-transfected cells, addition of OAG increased the fluorescence ratio by 0.50 ± 0.008 (n = 100). In non-TRPC3-transfected cells, there was no net increase in steady-state ratio in any of 95 cells.

We next measured Src activation by immunoblotting using a specific anti-phosphoSrc antibody, which specifically detects Src autophosphorylation at Tyr-418, a process indicative of Src activation (16). We confirmed that Src was significantly activated following stimulation of the muscarinic pathway (Fig. 6*B*, *lane 2*). Furthermore, consistent with the pharmacological data indicating a role for Src in OAG-dependent activation of TRPC3, OAG also caused activation of Src (Fig. 6*B*, *lane 3*). However, neither carbachol nor OAG were able to induce activation of Src when extracellular calcium was omitted (Fig. 6*B*, *lanes 5–8*). Also, preincubation with the PKC inhibitor Gö6976 (1 μM) almost completely reduced the ability of the DAG analogue to induce activation of Src (Fig. 6*B*, *lane 4*), consistent with a PKC-mediated mechanism (34). These results indicated that carbachol- and OAG-dependent activation of Src is unlikely to be causally related to their ability to activate TRPC3, a process known to be independent of both extracellular calcium and PKC activity. Because Src family kinases may associate with and regulate effector proteins through tyrosine phosphorylation-independent interactions (35, 36), we sought evidence for association of TRPC3 with Src by co-immunoprecipitation. However, the results from co-immunoprecipitation experiments with both anti-HA and anti-Src antibodies were essentially negative, providing no evidence for physical association of the channel with Src (not shown).

We next addressed the question as to whether activation of Src could activate TRPC3 in the absence of agonist stimulation of DAG formation. Two approaches were used. First, TRPC3-expressing HEK293 cells were transfected with a constitutively active form of avian Src (CA-Src), and the constitutive activity of TRPC3 (12, 37) was evaluated and compared with that from both mock- and DN-Src-transfected cells. Ba^{2+} (10 mM) was used as a surrogate for Ca^{2+}, and the initial rate (first 2 min following Ba^{2+} addition) of Ba^{2+} entry was calculated (17). Neither DN-Src (n = 53) nor CA-Src (n = 70) significantly altered TRPC3 constitutive activity (0.065 ± 0.006 and 0.060 ± 0.007 ratio units per min, for DN-Src and CA-Src, respectively) compared with that of mock (n = 41) or non-transfected (n = 46) cells (0.050 ± 0.007 and 0.056 ± 0.008 ratio units per min, respectively), indicating that active Src by itself was not able to activate the channel. The failure of DN-Src to diminish constitutive activity is consistent with the observation that DN-Src does not influence the expression or targeting of GFP-tagged TRPC3. Thus, because TRPC3 is clearly expressed and divalent cation-permeable channels are clearly present in the plasma membrane, this indicates that inhibition of Src activity prevents the *activation* mechanism of TRPC3, rather than altering the expression or basic permeation properties of the channels. Expression of the different Src mutants under our experimental conditions was confirmed by immunoblot (not shown).

A second approach to investigating the ability of Src to act on the channels independently involved use of a PLCγ2-deficient DT40 B lymphocyte cell line (PLCγ-KO DT40). These cells

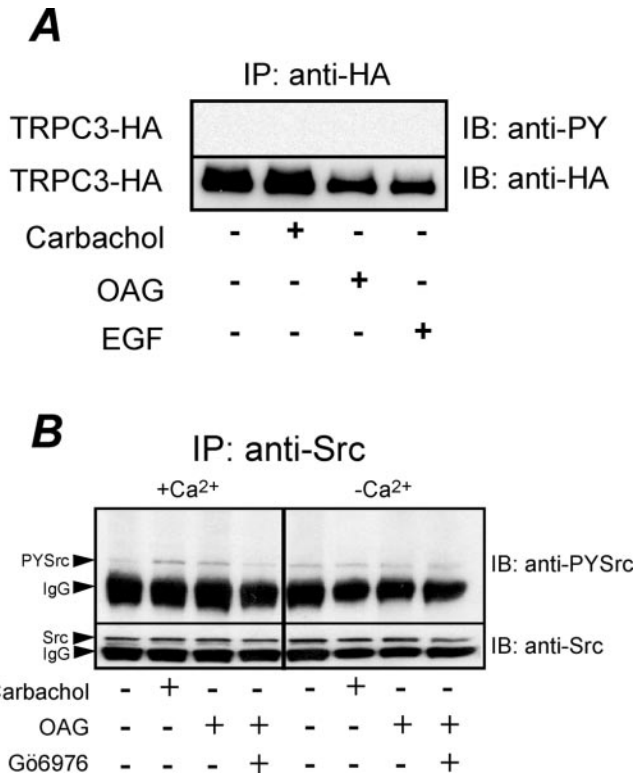

FIG. 6. **Receptor stimulation and OAG induce tyrosine phosphorylation of Src, but not TRPC3.** *A*, HEK293 cells stably expressing TRPC3-HA were challenged with either vehicle, carbachol (300 μM), epidermal growth factor (EGF, 150 ng/ml), or OAG (100 μM) for 10 min and then lysed as described under "Materials and Methods." TRPC3-HA was immunoprecipitated (*IP*) from each cell lysate by incubating the corresponding aliquots (800 μg) with 2 μg of anti-HA mouse monoclonal antibody (*anti-HA*) as detailed under "Materials and Methods." Immunoprecipitates were resolved by SDS-PAGE (10% polyacrylamide), immunoblotted (*IB*) with anti-phosphotyrosine antibody 4G10 (*anti-PY*), and following incubation with secondary antibody (HRP-conjugated anti-mouse), immunoreactive bands were visualized by ECL chemiluminescence. The accompanying anti-HA immunoblot was obtained by reprobing the same membrane with anti-HA antibody to confirm immunoprecipitation of TRPC3-HA (~100 kDa). Shown are immunoblots representative from five separate experiments. *B*, HEK293 cells stably expressing TRPC3 were challenged with either vehicle, carbachol (300 μM), or OAG (100 μM) for 10 min, in the presence (2 mM, $+Ca^{2+}$) or absence ($-Ca^{2+}$) of Ca^{2+}, and in the presence of 5 μM Gd^{3+}, and then lysed as described under "Materials and Methods." Where indicated, cells were incubated for 15 min with the PKC inhibitor Gö6976 (1 μM) before OAG treatment. Src was immunoprecipitated from the corresponding supernatants (800 μg) with 4 μg of anti-Src mouse monoclonal antibody (clone GD11, anti-Src) as detailed under "Materials and Methods." Immunoprecipitates were resolved by SDS-PAGE (10% polyacrylamide), immunoblotted (*IB*) with anti-phosphoSrc antibody (anti-PYSrc), and following incubation with secondary antibody (HRP-conjugated anti-rabbit) immunoreactive bands were visualized by ECL chemiluminescence. Comparable levels of total Src in all conditions were confirmed by reprobing the same membrane with anti-Src antibody (*bottom immunoblot*). Shown are immunoblots representative from three separate experiments. *PYSrc*, Tyr-418-phosphorylated Src; *IgG*, immunoglobulin heavy chain.

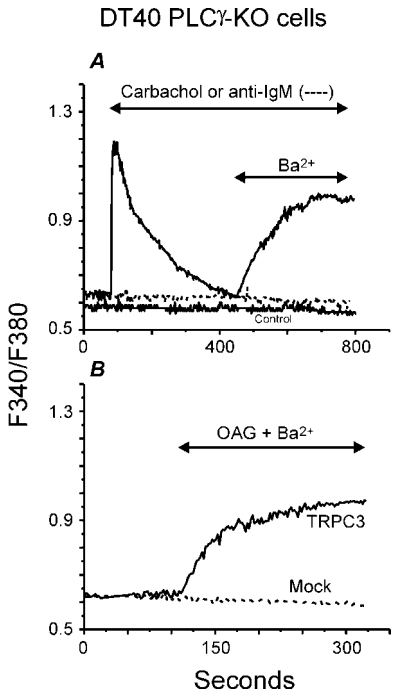

FIG. 7. **TRPC3 regulation in PLCγ-deficient DT40 cells: stimulation of the muscarinic pathway but not the B cell receptor, results in channel activation.** *A*, Ba^{2+} influx was measured in Fura-2-loaded PLCγ-deficient (PLCγ-KO) DT40 cells transfected with either TRPC3 alone or TRPC3 plus the M5 muscarinic receptor. In *B*, PLCγ-KO cells were transfected with either TRPC3 or its vector (pcDNA3, *Mock*). EYFP was used as a transfection marker. The cells were maintained in a nominally Ca^{2+}-free medium containing 5 μM Gd^{3+} and then exposed to: *A*, 5 μg/ml anti-IgM (*dotted trace*, cells transfected with TRPC3) or 300 μM carbachol (*upper solid trace*, cells transfected with TRPC3 and M5 receptor) or no agonist (*flat solid trace*), or *B*, 100 μM OAG (*solid trace*, TRPC3-transfected cells; *dotted trace*, mock-transfected cells). Where indicated, Ba^{2+} (2 mM) was added to the extracellular medium. Shown are *traces* representative of at least three separate experiments. In *A*, none of the control cells (no agonist, 38 cells) nor anti-IgM-treated cells (46 cells) showed an increase on Ba^{2+} addition, whereas the addition of carbachol caused an increase in all of 42 cells (average increase at 2 min was 0.32 ± 0.01).

respond to B cell receptor (BCR) stimulation with activation of several tyrosine kinases, including Src protein-tyrosine kinases, but they are unable to generate IP_3 and DAG because they lack the phosphoinositide-specific PLCγ2 normally coupled to BCR activation (38, 39). Transient expression of human TRPC3 in the wild-type DT40 cell line under appropriate conditions produces a channel that can be activated through either BCR stimulation or muscarinic receptor stimulation (15, 40). In the experiments shown in Fig. 7, TRPC3 was transiently expressed in PLCγ-KO DT40 cells together with EYFP, and TRPC3-dependent Ba^{2+} entry was evaluated. As expected, BCR stimulation of PLCγ-KO DT40 cells did not lead to Ca^{2+} release from endogenous stores. BCR stimulation did not result in activation of TRPC3 (Fig. 7*A*, *dotted trace*), in contrast with the efficient receptor-dependent activation of the channel when expressed in wild-type DT40 cells (data not shown and Ref. 15). These results indicated an absolute requirement of PLCγ expression for BCR-dependent activation of TRPC3, most likely due to the requirement for PLC-dependent DAG generation for channel activity. More importantly, because Src activation is known to be normal in these cells, this result clearly shows that Src activation *per se* is not sufficient to activate TRPC3 channels. In support of this interpretation, carbachol stimulation of PLCγ-KO DT40 cells transiently co-transfected with the G_q/PLCβ-coupled M5 muscarinic receptor and TRPC3, resulted in rapid IP_3-induced release of Ca^{2+} from stores followed by activation of TRPC3-mediated Ba^{2+} entry (Fig. 7*A*, *solid trace*), which was not observed in cells transfected with the M5 receptor alone (not shown). Additionally, as in TRPC3-expressing WT-DT40 cells (not shown, but see Fig. 5*A* in Ref. 15), 100 μM OAG significantly stimulated TRPC3-mediated Ca^{2+} influx in PLCγ-KO DT40 cells (Fig. 7*B*), whereas there was no effect of the DAG analogue on cation entry in mock-transfected cells. As in TRPC3-expressing HEK293 cells, both muscarinic and OAG activation of the channel in PLCγ-KO DT40 cells were completely blocked by pre-treatment with genistein (100 μM) and erbstatin analogue (20 μM; not shown). These results, together

with those derived from TRPC3-HEK293 cells transiently expressing DN-Src and CA-Src, indicate that, although Src kinase activity appears to be absolutely *necessary* for channel activation, it is not *sufficient* in the absence of a DAG stimulus to cause channel activation.

DISCUSSION

Despite being one of the most extensively studied members of the canonical TRP subfamily, the precise signaling mechanism underlying TRPC3 activation and regulation remains uncertain. In many cell types, ectopic expression of TRPC3 produces a receptor-activated channel that can also be activated by exogenous application of membrane-permeant diacylglycerols (*i.e.* OAG). Activation by diacylglycerols is also a characteristic of the other members of the subfamily, TRPC6 and TRPC7 (8, 9), and provides a potential mechanism for activation of these channels by PLC-linked receptors, independently of IP_3 and Ca^{2+} store depletion. Although DAG generation through stimulation of either GPCRs or RTKs is currently widely accepted as a mechanism for TRPC3 activation, little is known about additional signaling pathways downstream of receptor stimulation involved in regulation of TRPC3 activity. Despite the existence of multiple potential phosphorylation sites in the TRPC3 primary sequence, there are very few studies specifically addressing the role of kinases in TRPC3 activity. Recent studies provided evidence that PKC (14, 41) and protein kinase G negatively regulate TRPC3 (42). By using HEK293 cells stably expressing TRPC3 we found that the non-receptor tyrosine kinase Src is absolutely required for signaling to TRPC3, whether through receptor activation, or through application of OAG. Several lines of evidence support this concept. First, pretreatment of the cells with a specific inhibitor of Src, but not inhibitors of other tyrosine kinases, abrogated both receptor- and OAG-dependent activation of TRPC3. Second, transient expression of a dominant-negative form of Src (DN-Src) into TRPC3-HEK293 cells completely suppressed both receptor- and OAG-dependent TRPC3 activation. The endogenous store-operated pathway was not affected by DN-Src, indicating that the requirement for Src activity was specific for signaling to TRPC3. Note that this finding is inconsistent with the conclusion drawn by other investigators that Src plays a role in the store-operated pathway (43–45). That DN-Src was in fact exerting a genuine dominant-negative action by competing out endogenous Src was confirmed by rescuing the response by concomitantly overexpressing the wild-type form of Src. Third, expression of TRPC3 into SYF cells ($Src^{-/-}$, $Yes^{-/-}$, and $Fyn^{-/-}$) resulted in a channel with the expected constitutive activity for TRPC3 but devoid of regulation by either receptor activation or OAG. However, when TRPC3 was transiently expressed into YF ($Yes^{-/-}$ and $Fyn^{-/-}$) cells, which do express Src (33), or when WT-Src and TRPC3 were co-expressed into SYF cells, receptor and OAG activation of TRPC3 was in both instances restored. These findings strongly support the concept of an obligatory role for Src activity for signaling to TRPC3.

Then, a more fundamental question arises: having established that Src kinase activity is absolutely required for TRPC3 activation, is it sufficient to activate the channel in the absence of DAG production? The evidence indicates that it is not. First, a constitutively active form of Src caused no significant activation of TRPC3. Second, expression of TRPC3 in a PLCγ2-deficient DT40 cell line resulted in a channel unresponsive to BCR stimulation. BCR stimulation of these cells results in normal activation of Src family tyrosine kinases, but there is no receptor-dependent generation of either IP_3 or DAG due to the lack of PLCγ2 (38, 39). Together, these findings indicate that, although Src clearly has an obligatory role in TRPC3 regulation, it is not sufficient to regulate the channel in the absence of a DAG stimulus. On the other hand, the data also clearly show that DAG cannot be acting by simply interacting, alone, with TRPC3 channels. Rather, a concerted role of both diacylglycerol and Src seems to be necessary for TRPC3 activation, perhaps through a mechanism involving Src-dependent phosphorylation and/or recruitment of an as yet unknown accessory/regulatory protein within the vicinity of TRPC3. The action of Src does not appear to involve direct or indirect tyrosine phosphorylation of TRPC3 protein. However, we cannot absolutely rule out that some direct phosphorylation of TRPC3 might occur that might be transient and of modest stoichiometry and that might be undetectable by the anti-phosphotyrosine immunoblotting technique used here. Also, immunoprecipitation of TRPC3 failed to bring down detectable Src, and immunoprecipitation of Src failed to bring down detectable TRPC3, indicating that, if the two proteins physically interact *in situ*, the interaction is very labile. Additional studies, including *in vitro* analysis of channel protein phosphorylation and protein interactions, might be needed to specifically address this issue.

Patterson *et al.* (46) reported the failure of B-cell receptor or muscarinic receptor stimulation to activate entry in DT40 PLCγ2-deficient cells. This they interpreted as being indicative of a coupling, lipase-independent role of PLCγ in agonist-dependent cation entry regulation. However, our experiments showing the inability of BCR stimulation to activate TRPC3 when expressed in the PLCγ2-deficient DT40 cell line indicate a requirement for PLC-dependent DAG generation for channel activity (8, 14, 40). This is supported by the finding that stimulation of the Gq/PLCβ-coupled muscarinic receptor pathway or challenging the cells with OAG, efficiently activated TRPC3-mediated cation entry in PLCγ-KO DT40 cells, *i.e.* irrespectively of the status of PLCγ expression.

A recent report described evidence for the Src family kinase Fyn having a role in EGF-dependent regulation of TRPC6, a close structural relative of TRPC3 (47). By overexpressing both Fyn and TRPC6, these authors showed association of Fyn with TRPC6 and that Fyn phosphorylated the channel in an *in vitro* kinase assay. In contrast, in a previous study by Hassock *et al.* (48) endogenous TRPC6 in human platelets challenged with thrombin was found to be a substrate for cyclic AMP-dependent protein kinase but not for tyrosine kinases. Differences in channel protein level when both the channel and the kinase are overexpressed in a heterologous system (47) compared with those found under native conditions (48), may in part account for this discrepancy. An increasing body of evidence implicates different kinases in regulation of ion channel function in the greater TRP superfamily. For example, PKC and protein kinase A modulate TRPV1 activity by direct channel phosphorylation (49), whereas Src appears to be involved in regulation of both TRPM7 (50) and TRPV4 (51). Thus, protein phosphorylation in general, and Src family tyrosine kinases in particular, are emerging as critical regulatory elements in the signaling mechanisms to TRP superfamily channels.

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