

Role of *Src* in C3 transient receptor potential channel function and evidence for a heterogeneous makeup of receptor- and store-operated Ca^{2+} entry channels

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Receptor-operated Ca^{2+} entry (ROCE) and store-operated Ca^{2+} entry (SOCE) are known to be inhibited by tyrosine kinase inhibitors and activation of C-type transient receptor potential channel (TRPC) isoform 3 (TRPC3), a cation channel thought to be involved in SOCE and/or ROCE, was recently shown to depend on *src* tyrosine kinase activity. What is not known is the step at which *src* acts on TRPC3 and whether the role for tyrosine kinases in ROCE or SOCE is a general phenomenon. Using *in vitro* and in cell protein-protein interaction assays we now report that *src* phosphorylates TRPC3 at Y226 and that formation of phospho-Y226 is essential for TRPC3 activation. This requirement is unique for TRPC3 because (i) mutation of the cognate tyrosines of the closely related TRPC6 and TRPC7 had no effect; (ii) TRPC6 and TRPC7 were activated in *src*-, *yes*-, and *fyn*-deficient cells; and (iii) *src*, but not *yes* or *fyn*, rescued TRPC3 activation in *src*-, *yes*-, and *fyn*-deficient cells. The *Src* homology 2 domain of *src* was found to interact with either the N or the C termini of all TRPCs, suggesting that other tyrosine kinases may play a role in ion fluxes mediated by TRPCs other than TRPC3. A side-by-side comparison of the effects of genistein (a general tyrosine kinase inhibitor) on endogenous ROCE and SOCE in mouse fibroblasts, HEK and COS-7 cells, and ROCE in HEK cells mediated by TRPC3, TRPC6, TRPC7, and TRPC5 showed differences that argue for ROCE and SOCE channels to be heterogeneous.

G protein-coupled receptors | signal transduction | store depletion

Changes in cytosolic Ca^{2+} in nonexcitable cells in response to hormones and growth factors that activate C-type phospholipases (PLCs) are biphasic: An initial transient rise is followed by a less pronounced but sustained elevation (reviewed in refs. 1–3). This sustained phase of elevated Ca^{2+} persists until the hormones and growth factors that activate PLCs cease to act (4). The initial phase originates from the inositol trisphosphate-induced release of Ca^{2+} from cellular stores and is transient because of the action of membrane ATPases that pump Ca^{2+} back into the stores and out of the cells. The sustained phase of the increase in cytosolic Ca^{2+} requires the continued activation of a Ca^{2+} influx pathway to maintain sustained responses.

Although agonist-induced Ca^{2+} influx has been the object of many studies over the last 20 years, neither its molecular basis nor the mechanism of its activation are well understood. This type of Ca^{2+} influx is referred to as receptor-operated Ca^{2+} entry (ROCE). Store-operated Ca^{2+} entry (SOCE) is a related form of Ca^{2+} entry that may or may not involve the same molecular entities that are responsible for ROCE. SOCE can be triggered independently of PLC activation by mere depletion of the same stores that occurs upon inactivation of endogenous sarcoplasmic reticulum–endoplasmic reticulum Ca^{2+} ATPases or by simple chelation of Ca^{2+} that leaks from the stores into the cytoplasm. SOCE is also referred to as capacitative Ca^{2+} entry. Thus, capacitative Ca^{2+} entry and SOCE are operational terms used to indicate Ca^{2+} entry triggered by the depletion of the stores in the absence of PLC activation. As is the case for ROCE, the molecular basis for and mechanism(s) of activation of SOCE channels are poorly understood.

The C-type transient receptor potential ion channels (TRPCs) have been postulated as candidates for forming ROCE and/or capacitative Ca^{2+} entry channels (5–7). There are seven TRPC channels (TRPC1–TRPC7) (8, 9), which, when expressed in model cells, form Ca^{2+} -permeable, largely nonselective cation channels. Expression and assembly studies have shown that TRPCs can form heteromeric channels (10), and all but TRPC6 can be activated by store depletion (reviewed in ref. 11). The main attribute not yet recapitulated by expressing TRPCs alone or in combination in model cells is the high Ca^{2+} selectivity seen for CCE channels in normal cells (ref. 12; reviewed in ref. 13).

A likely scenario by which SOCE occurs is one in which SOCE channels coexist with Ca^{2+} -permeable nonselective ROCE channels (14). As is the case with SOCE channels, the activation mechanism(s) operating on ROCE channels also is largely a matter for speculation. Investigations into the factors and variables that affect activation of ROCE and SOCE may therefore be fruitful avenues to improving our molecular knowledge of how ROCE or SOCE come about.

One such variable is involvement of tyrosine phosphorylation in the activation of ROCE and SOCE. The original observation that tyrosine phosphorylation may be an important regulator participating in activation of ROCE and SOCE in nonexcitable cells came from studies showing an inhibitory effect of tyrosine kinase inhibitors on these forms of Ca^{2+} entry in human foreskin fibroblasts (15). This finding was followed by studies that showed total loss of bradykinin-stimulated ROCE and partial absence of SOCE in embryonic fibroblasts derived from mice lacking the *src* tyrosine kinase (16). Furthermore, evidence has accumulated in the literature showing that tyrosine phosphorylation is a common consequence associated with stimulation of cells that signal through the heterotrimeric G protein with $\alpha\text{q}\beta\gamma$ subunit composition (Gq)–PLC– Ca^{2+} pathway (17–19). We therefore became interested in the possibility that tyrosine phosphorylation may be an activating signal for one of the events that leads to ROCE and SOCE. Indeed, experiments parallel to ours showed that in HEK cells the activation of TRPC3 by a PLC-stimulating G protein-coupled receptor (GPCR) is inhibited by inhibitors of tyrosine kinases and that, when expressed in *src* kinase negative cells, TRPC3 is not activated by Gq-coupled receptor (20). These data recapitulated the earlier findings with an endogenous (bradykinin-activated) GPCR acting via Gq activation on the endogenous complement of the ROCE pathway (16).

The present work extends these observations in three ways. First, we sought to establish whether the site of action of the *src* kinase

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Abbreviations: AVP, arginine vasopressin; CCh, carbachol; GPCR, G protein-coupled receptor; Gq, heterotrimeric G protein with $\alpha\text{q}\beta\gamma$ subunit composition; HA, hemagglutinin; M5R, M5 muscarinic acetylcholine receptor; PLC, phospholipase C; ROCE, receptor-operated Ca^{2+} entry; SOCE, store-operated Ca^{2+} entry; SYF, *src*-, *yes*-, and *fyn*-deficient; TRPC, C-type transient receptor potential; V1aR, type 1a vasopressin receptor; YF, SYF expressing transfected *src*.

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was at the level of TRPC3 or some other rate-limiting step in the pathway leading from a Gq-coupled GPCR to TRPC3, including other molecules that may form part of the ROCE channel to which TRPC3 belongs. Second, we tested whether the requisite for a tyrosine phosphorylation step was unique to TRPC3 or whether other TRPCs tested in the same cellular context shared with TRPC3 the dependence of their activation on the *src* tyrosine kinase. Third, we sought to learn whether tyrosine kinase activity is a universal feature of ROCE and/or SOCE. We report that TRPC3 is a direct target of the *src* tyrosine kinase, and, although all TRPCs can be shown to interact physically with the *src* tyrosine kinase *in vitro*, only TRPC3 depends on phosphorylation by *src*. Furthermore, although inhibition of tyrosine phosphorylation can be shown to abolish SOCE in one cell type, tyrosine phosphorylation appears to play much less of a role in another cell type. We propose that these results reflect molecular complexities in the regulation of ROCE and SOCE, which, in turn, reflect the complexity and diversity of the molecular components that make up the ROCE and SOCE pathways.

Experimental Procedures

Materials, reagents, cDNAs, transfection methods, and ratio-metric imaging protocols were as described in refs. 8 and 11. $[Ba^{2+}]_i$ was calculated assuming that the affinity of Fura-2 for Ba^{2+} and the spectroscopic properties of the Fura-2: Ba^{2+} complex are equal to those of the Fura-2: Ca^{2+} complex. The GST pull-down assays, coimmunoprecipitation assays, and Western blotting were as described in Boulay *et al.* (21) using the antibodies mentioned in the legend for Fig. 1.

Interacting cellular proteins were crosslinked by overlaying transfected COS-7 cells in 100-mm dishes with 5 ml of 2 mM dithiolbis(succinimidylpropionate) (Pierce) in PBS (GIBCO) solution for 30 min at room temperature, followed by addition of an equal volume of 20 mM Tris-HCl, pH 7.4, for 15 min. The cells were lysed with 500 μ l of ice-cold *src* lysis buffer (1% Triton X-100/150 mM NaCl/5 mM EDTA/5 μ g/ml aprotinin/5 μ g/ml leupeptin/1 μ g/ml trypsin inhibitor). The lysates were cleared by incubation for 20 min on ice with 25 μ l of a 1:1 slurry of protein-A Sepharose (Amersham Pharmacia) followed by centrifugation at 10,000 $\times g$ for 5 min. Cleared lysates were then used as sources for immunoprecipitation with the antibodies indicated in Fig. 1 and subjected to Western blot analysis.

GST fusion proteins were phosphorylated *in vitro* by immunoprecipitated *src* as follows. *Src* was expressed transiently in COS-7 cells and isolated from 500 μ l of lysates prepared as described above (without the crosslinking step) by immunoprecipitation with mAb 327 bound to protein A Sepharose. The immunocomplex was washed once and resuspended in 150 μ l of kinase buffer (10 mM Tris, pH 7.5/5 mM $MnCl_2$ /100 μ M $NaVO_4$ /1 mM NaF/1 mM sodium pyrophosphate). GST fusion proteins (≈ 1 μ g in 5 μ l of kinase buffer) were mixed with 5 μ l of *src* immunocomplex in kinase buffer and brought to a final volume of 35 μ l. The kinase reaction was started with the addition of 10 Ci/pmol (1 Ci = 37 GBq) $[\gamma\text{-}^{32}P]ATP$ at 1 μ M and incubated for 60 min at room temperature. The reactions were terminated by addition of 40 μ l of 2 \times Laemmli's sample buffer with 20% 2-mercaptoethanol and subjected to SDS/PAGE and autoradiography. For positive control, the GST fusion proteins were replaced with enolase (Sigma).

For confocal microscopy, HEK cells expressing hemagglutinin (HA)-tagged TRPC3 and its variants were fixed with 4% paraformaldehyde and immunostained with HA-Alexa Fluor 433 antibody. Images were acquired with a Carl Zeiss LSM 51M laser scanning microscope at a z resolution of 0.6 μ m.

Results

TRPCs Interact with *src* and TRPC3 Is Phosphorylated at 4 of 11 N-Terminal Tyrosines. We first investigated whether TRPCs (including TRPC3) interacted with *src* in *in vitro* binding assays. The

N and C termini of the TRPC channels were labeled with ^{35}S by *in vitro* translation with a reticulocyte lysate-based transcription-translation system (TnT, Promega) and incubated with a GST-*src* fusion protein bound to glutathione agarose beads. The retention of the labeled TRPC fragment was visualized by autoradiography after electrophoretic separation. The GST-*src* fusion protein but not GST alone interacted in a stable manner with each of the tested TRPCs either by interacting with the N terminus (Fig. 1*A Left*, TRPC1, TRPC3, and TRPC6), the C terminus (Fig. 1*A Right*, TRPC2 and TRPC4), or both termini (Fig. 1*A*, TRPC5). Given the previous report that TRPC3 depends on *src* for its activation, we focused next on TRPC3-*src* interaction. Fig. 1*B* shows that TRPC3-*src* interaction occurs through the src homology 2 domain of *src*. A weak interaction could also be seen between the C terminus and GST-*src*. This interaction was highly variable and, thus, was not explored further.

To test the relevance of the TRPC3 interaction with *src* seen in the GST pull-down experiments in the context of a living cell, we coexpressed TRPC3 and *src* in COS-7 cells and asked whether expressed proteins could be coimmunoprecipitated from cell lysates. We found that TRPC:*src* complexes can be immunoprecipitated by using antibodies directed against TRPC3 (tagged with the HA epitope) or the *src* protein (Fig. 1*C*). Thus, immunoprecipitating HA-tagged protein brought down not only protein corresponding to the relative molecular weight of TRPC3-HA (Fig. 1*Ci*) but also protein immunoreactive for *src* (Fig. 1*Cii*). In addition, immunoprecipitating *src* brought down HA-tagged TRPC3 (Fig. 1*Ciii*), and the TRPC3-HA immunoprecipitated from cells expressing *src* reacted positive for phosphotyrosine (Fig. 1*Civ*). These experiments argue strongly for a direct interaction between TRPC3 and *src* in a cellular context and for TRPC3 being a substrate for the *src* tyrosine kinase.

The susceptibility of the interacting TRPC3 N terminus to be phosphorylated by *src* was directly tested by incubating the N terminus fused to GST with *src* in the presence of $[\gamma\text{-}^{32}P]ATP$ and Mn^{2+} using as a source of active *src* lysates from COS-7 cells in which *src* had been transiently expressed. As illustrated in Fig. 2*B*, lane 4, the ≈ 63 -kDa GST-C3 N terminus [TRPC3(1-370)] fusion protein was intensely phosphorylated by the kinase activity immunopurified from COS-7 cell lysates. Lanes 1 and 2 of Fig. 2*B* show the results from negative (GST only) and positive (enolase) control reactions. We concluded that the TRPC3 N terminus is a target of the tyrosine kinase activity of *src*.

The N terminus of TRPC3 (848-aa splice variant, GenBank accession no. U47050) has 11 tyrosines (Fig. 2*A*). Through systematic site-directed mutation of these 11 tyrosines singly and in groups (Fig. 2*B*), we identified Y49, Y148, Y150, and Y226 as *src* substrates. Thus, a tyrosine-free N terminus in which all 11 tyrosines had been mutated to phenylalanine was no longer a *src* substrate (Fig. 2*B*, lanes 5 and 13), and reintroduction of tyrosines only at positions 49, 148, 150, and 226 [Fig. 2*B*, lane 14: rescue F(49,148,150,226)Y] yielded a substrate equally as phosphorylated as the wild-type N terminus shown in lane 11. Removal of only Y226 (lane 10) resulted in partial loss of phosphorylation; Y226 contributed partially to the phosphorylation of the N-terminal tyrosines (lane 15).

Functional Correlate to Lack of Phosphorylation of TRPC3 Y226.

Expression of TRPC3 with mutated N-terminal tyrosines followed by the standard test for activation by a cotransfected GPCR, either the muscarinic M5 acetylcholine receptor (M5R) or the type 1a vasopressin receptor (V1aR), showed that TRPC3 activation was lost whenever Y226 was mutated to phenylalanine either singly (Fig. 2*C Left*) or as part of the removal of all N-terminal tyrosines (Fig. 2*C Right*). Moreover, the activation of an N-terminally tyrosine-free TRPC3 was restored by reintroducing only Y226 (Fig. 2*C Right*, \odot).

These experiments showed that TRPC3 critically depends on

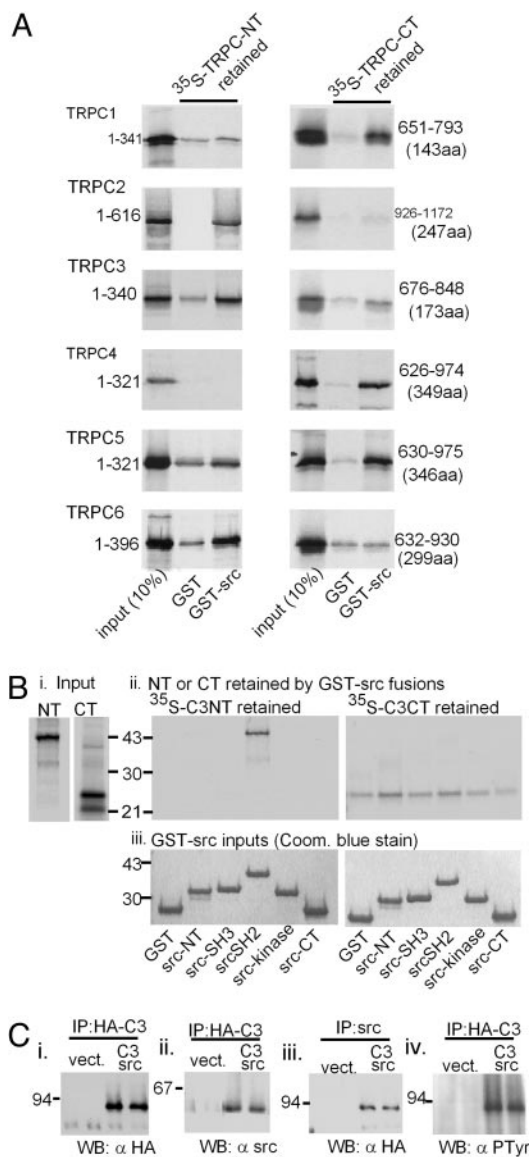


Fig. 1. Interactions of *src* with TRPC channel proteins. (**A**) *Src* interacts with all members of the TRPC family of TRP-related channels. N termini (NT) and C termini (CT) of TRPC1–TRPC6 were translated *in vitro* in a reticulocyte lysate in the presence of ^{35}S -labeled methionine and cysteine and incubated with a GST fusion protein of *src* bound to glutathione Sepharose. After washing, the radioactive N and C termini retained by the GST–*src* fusion protein were separated by SDS/PAGE and quantified by autoradiography. Samples equivalent to 10% of the N and C termini offered to the GST–*src* fusion protein were electrophoresed alongside. (**B**) The N terminus of TRPC3 interacts preferentially with the *src* homology 2 (SH2) domain of *src*. (**B** and **Bii**) Input components. (**Bii**) Retention of the ^{35}S -labeled N terminus (*Left*) or C terminus (*Right*) of TRPC3 by the different domains of *src* (fused to GST), coom., Coomassie. (**C**) TRPC3 and *src* interact upon coexpression in COS-7 cells, and the TRPC3 associated with *src* reacts with anti-phosphotyrosine antibodies. COS-7 cells were transfected with empty vectors (vect) or vectors carrying insert coding for HA-tagged TRPC3 and *src* (C3 *src*). Lysates from the transfected cells were then immunoprecipitated with either anti-HA 12CA5 or anti-*src* mAb327 monoclonal antibodies. The precipitates were washed and subjected to 10% SDS/PAGE in duplicate, followed by electrophoretic transfer onto nitrocellulose membranes and immunostaining with 12CA5 α -HA, mAb327 α -*src*, or 4G10 (α -phosphotyrosine, Upstate Biotechnology, Lake Placid, NY) antibodies. IP, immunoprecipitation; WB, Western blot.

the presence of Y226, a substrate of the *src* tyrosine kinase. However, the failure of agonists to activate Ca^{2+} influx in cells expressing the mutant TRPC3s could also be attributed to

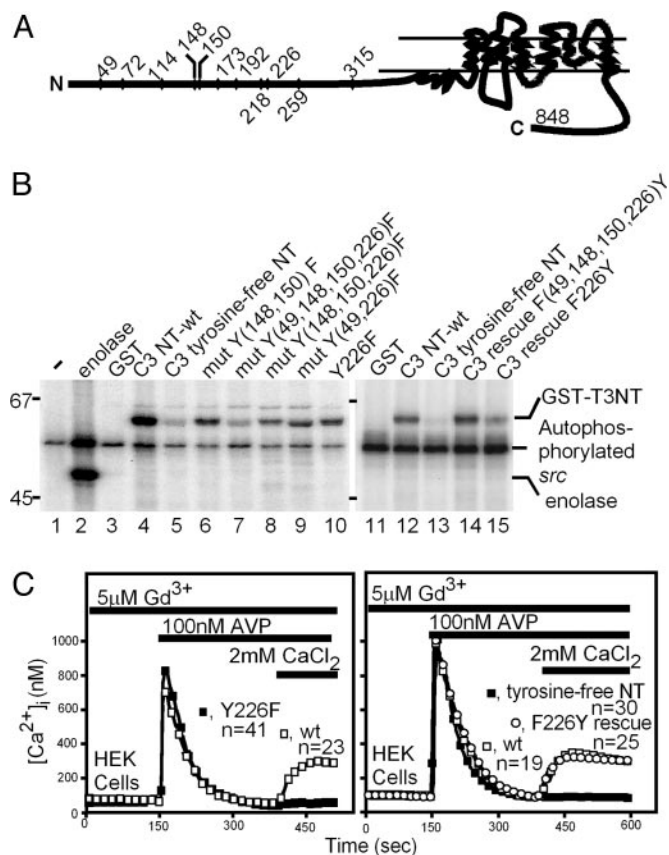


Fig. 2. *In vitro* phosphorylation of TRPC3 with unmodified N terminus (NT) or with N termini in which the indicated tyrosines had been mutated to phenylalanine. (**A**) Diagram of the location of the 11 N-terminal tyrosines in the 848-aa TRPC3 protein. (**B**) Autoradiogram of the N terminus–GST fusion proteins subjected to an *in vitro* tyrosine kinase reaction using [γ - ^{32}P]ATP and *src* isolated by immunoprecipitation from lysates of COS-7 cells transfected with the pcDNA3 expression vector carrying the chicken *src* cDNA. The products from the tyrosine kinase reaction were separated by SDS/PAGE and analyzed by autoradiography. Lanes 1 and 2 show negative (GST only) and positive (GST fusion protein replaced with enolase) controls, respectively. (**C**) Y226 is required for activation of TRPC3 by a Gq-coupled receptor. Fura-2-loaded HEK cells expressing TRPC3 or the indicated mutants and cotransfected V1aR were stimulated with AVP in the absence of extracellular Ca^{2+} or Ba^{2+} to activate the Gq–PLC β signaling system and deplete internal Ca^{2+} stores. At the time indicated by the bar, the Ca^{2+} -free medium was exchanged with the same medium plus 2 mM CaCl_2 to record ROCE. Changes in $[\text{Ca}^{2+}]_i$ were monitored in individual cells by ratiometric fluorescence spectroscopy and video microscopy as described in ref. 11. GdCl_3 at $5 \mu\text{M}$ was present throughout to suppress Ba^{2+} entry through endogenous ROCE and SOCE channels. (*Left*) A single mutation, Y226F, abrogates activation of TRPC3. (*Right*) Mutating all 11 N-terminal tyrosines to phenylalanine (tyrosine-free NT) abolishes receptor-operated cation entry; reintroduction of only Y226 into the tyrosine-free NT of TRPC3 (F226Y rescue), restores receptor-operated cation entry.

structural interference. Two types of experiments showed that TRPC3 with mutated Y226 reaches the plasma membrane and is capable of forming divalent cation-permeable ion channels. In the first experiment, we tagged the C termini of the nonresponsive N-terminally tyrosine-free TRPC3 molecules, Y226F mutant TRPC3 molecules, and wild-type TRPC3 molecules with the HA epitope and visualized their subcellular distribution by confocal microscopy. We saw no significant difference in their localization within the cells, including their periphery (Fig. 3*A*). The second experiment took advantage of the unique property of TRPC3 to exhibit a low level of constitutive activity, which is readily apparent if influx is tested with 10 mM BaCl_2 instead of

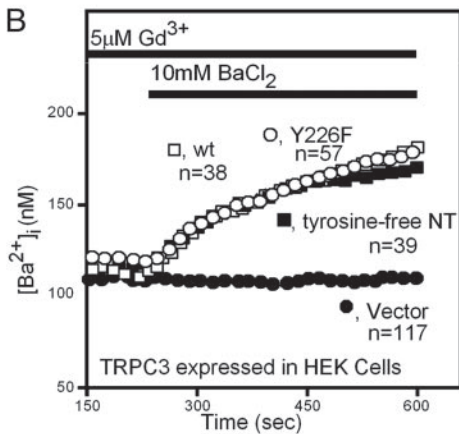
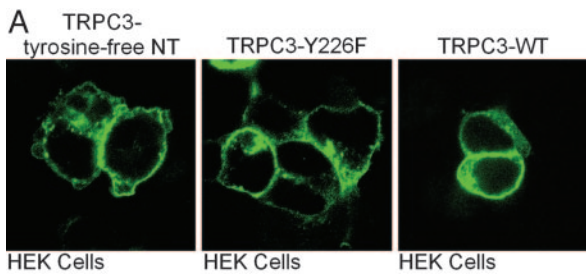


Fig. 3. Mutating N-terminal (NT) tyrosines in TRPC3 has no effect on its subcellular localization or its basal divalent cation channeling activity. (A) HA-tagged wild-type or mutated TRPC3 were transiently expressed in HEK cells, and their subcellular localization was analyzed 24 h after transfection by confocal microscopy. (B) Divalent cation entry into HEK cells mediated by wild-type TRPC3 or TRPC3 with either the Y266F mutation or a tyrosine-free N terminus was recorded as described for in Fig. 2C, except that the cells were not stimulated with agonist and 10 mM BaCl₂ was substituted for 2 mM CaCl₂.

2 mM CaCl₂. Indeed, basal Ba²⁺ entry into HEK cells expressing TRPC3 did not differ among wild-type TRPC3, the TRPC3 with an N terminus free of tyrosines, and TRPC3 with a single Y226F mutation (Fig. 3B).

The cognate of TRPC3 Y226 is present in the closely related TRPC6 and TRPC7 but absent in other TRPCs. We therefore tested whether mutation of the TRPC6 and TRPC7 “Y226” tyrosines (TRPC6 Y284 and TRPC7 Y230) would affect the behavior of these channels. Contrary to our expectation, neither mutation affected the behavior of TRPC6 and TRPC7 (Fig. 4A and B). Moreover, expression of TRPC6 and TRPC7 in embryonic fibroblasts lacking the *src*, *yes*, and *fyn* tyrosine kinases (SYF cells) led to normal TRPC6- and TRPC7-mediated ROCE, indicating that these channels do not depend on these kinases for their activity. In contrast and in agreement with published observations (20), TRPC3 could not be activated by a GPCR coupled by Gq in SYF cells (Fig. 5) unless they were supplemented with *src*. Furthermore, neither *yes* nor *fyn* could substitute for *src* (Fig. 5). In these experiments, TRPC3 activation was evaluated at 1 mM BaCl₂ to minimize interference from its basal activity (Fig. 5 Inset).

Heterogeneity of Channels Mediating ROCE and SOCE as Seen in Different Cells: Comparison of Endogenous ROCE and SOCE to ROCE Mediated by TRPC3, TRPC6, TRPC7, and TRPC5. TRPC3, TRPC6, and TRPC7 are closely related not only from a structural point of view (being >70% identical in the amino acid sequence that spans 150 aa on either side of Y226) but also from biochemical and functional points of view, because all three interact through their N termini with *src* in GST pull-down tests (Fig. 1), and all

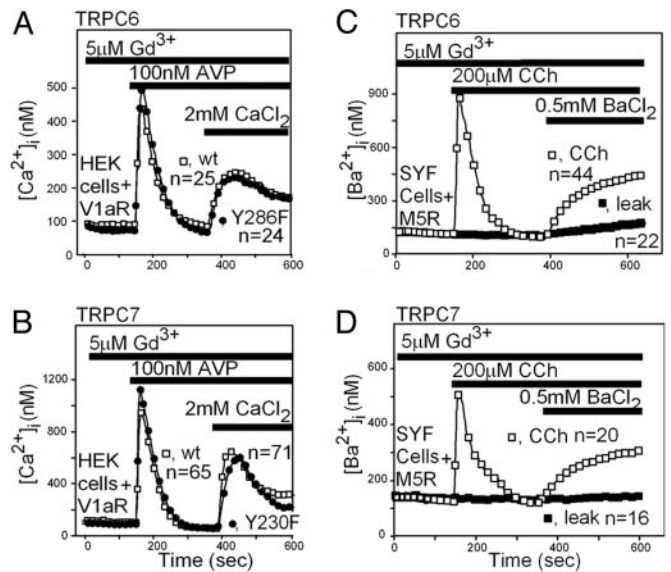


Fig. 4. TRPC6 and TRPC7 do not share with TRPC3 a similar dependence on tyrosine phosphorylation. (A and B) Mutating the tyrosines of TRPC6 (A) and TRPC7 (B) that are cognates of TRPC3 Y226 has no effect on their ability to respond to the activation of a Gq-coupled receptor. ROCE mediated by TRPC6, TRPC7, and the indicated mutant forms of these TRPCs was recorded as described for Fig. 2C. (C and D) TRPC6 (C) and TRPC7 (D) do not require *src*, *yes*, or *fyn* for activation by the Gq-PLC β signaling pathway. TRPC cDNAs were cotransfected with M5R into SYF embryonic fibroblasts and tested for activation by CCh. For these experiments, ROCE was assessed by measuring Ba²⁺ instead of Ca²⁺ influx.

three are directly TRPCs activated by diacylglycerol (22). The failure to observe an effect upon mutating the cognates of TRPC3 Y226 in TRPC6 and TRPC7 (Fig. 4A and B) and the lack of impairment of their activation by a Gq-coupled receptor in SYF cells (Fig. 4C and D) was therefore surprising.

We next tested whether genistein would inhibit TRPC6- and TRPC7-mediated ROCE as seen with TRPC3. We again found that the activation of the TRPC3-related TRPC6 and TRPC7 differed from TRPC3 and were unaffected by genistein (Fig. 6 Top). The insensitivity of TRPC6 and TRPC7 activation to genistein is in sharp contrast to that seen with TRPC3 in the

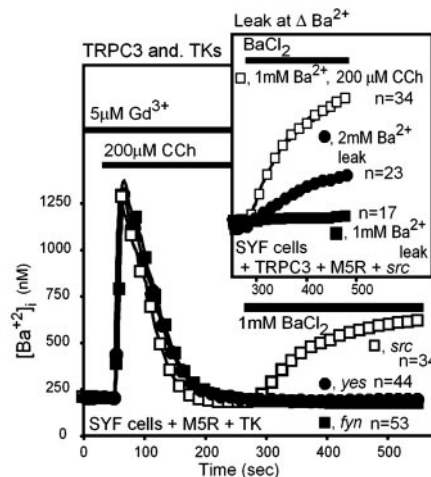


Fig. 5. Expression of *src* but not of *yes* or *fyn* in SYF cells rescues activation of TRPC3 by the Gq-PLC β signaling pathway. (Inset) Lack of interference by the basal activity of TRPC3 in ROCE assessment by using 1 mM BaCl₂ to monitor ROCE. TK, tyrosine kinase.

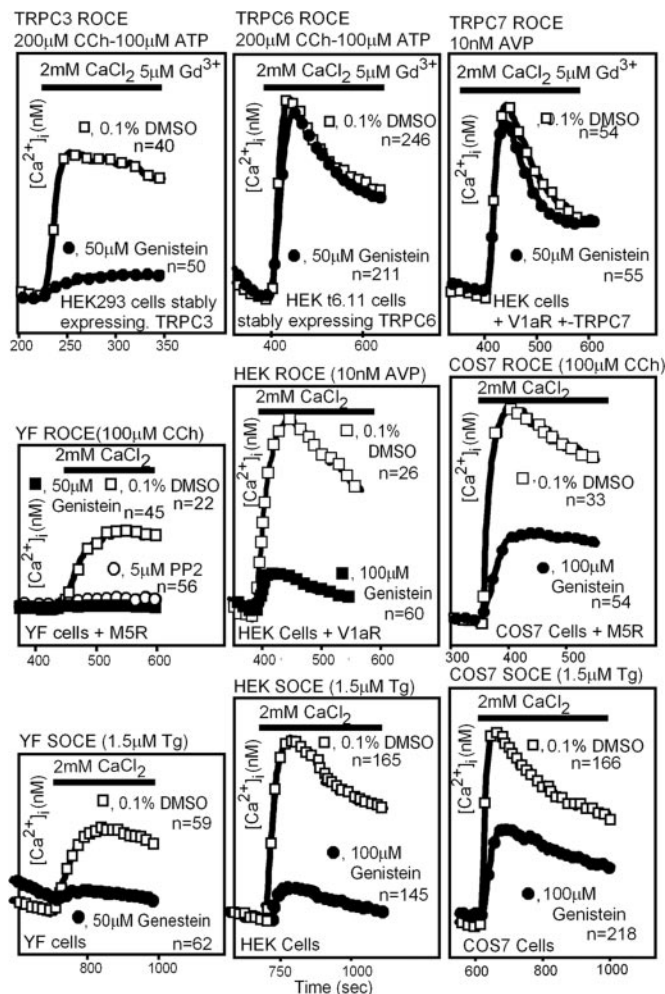


Fig. 6. Endogenous ROCE, endogenous SOCE, and TRPC-mediated ROCE show different sensitivities to tyrosine kinase inhibition. (*Top*) ROCE mediated by TRPC3 but not by TRPC6 or TRPC7 is sensitive to inhibition by the nonselective tyrosine kinase inhibitor genistein in HEK cells. TRPC cDNAs were cotransfected with V1aR into HEK cells, and TRPC-mediated ROCE was recorded. (*Middle*) Variable effectiveness of genistein to inhibit the Ca^{2+} entry phase mediated by endogenous ROCE channels of YF, HEK, and COS-7 cells. YF mouse fibroblasts were the most sensitive to genistein, and COS-7 cells were the least sensitive. (*Bottom*) Variable effectiveness of genistein as an inhibitor of endogenous SOCE activated by thapsigargin-mediated store depletion in YF, HEK, and COS-7 cells. For all experiments, only the Ca^{2+} entry phase is shown. Tg, thapsigargin.

same cells (Fig. 6 *Top Left*). In other experiments, TRPC5-mediated ROCE in HEK cells was also found to be insensitive to genistein, thus resembling TRPC6 and TRPC7 in this respect (data not shown).

These differences prompted us to test whether natural ROCE and SOCE channels would show cell-to-cell differences in terms of their susceptibility to inhibition by genistein. Such differences were indeed found (Fig. 6 *Middle and Bottom*). Note that the $5 \mu\text{M}$ Gd^{3+} used to inhibit endogenous ROCE and SOCE when studying Ca^{2+} entry mediated by transfected TRPCs (11, 22) was omitted from the external media when endogenous ROCE and SOCE were tested. Thus, YF cells (cells lacking *yes* and *fyn*), HEK cells, and COS-7 cells were plated without prior manipulations, loaded with Fura-2, and tested for SOCE activation by thapsigargin (Fig. 6 *Bottom*) or plated after transfection of a suitable Gq-coupled receptor [V1aR activated by arginine vasopressin

(AVP) or M5R activated by carbachol (CCh)] (Fig. 6 *Middle*) in the absence and the presence of genistein. The degree to which genistein inhibited SOCE and ROCE was similar in each cell but differed when comparing one cell to another, from total inhibition in YF embryonic fibroblasts to $\approx 80\text{--}90\%$ inhibition in HEK cells and $\approx 40\text{--}50\%$ inhibition in COS-7 cells. SYF cells behaved like YF cells (data not shown).

Discussion

Two methodologies are currently used to study ROCE and SOCE into cells. One method is based on changes in $[\text{Ca}^{2+}]_i$ (intracellular Ca^{2+} concentration) (or $[\text{Ba}^{2+}]_i$) monitored by using a Ca^{2+} indicator such as Fura-2. The other method involves recording by electrophysiological means the activities of the channels that are activated when ROCE and SOCE are induced. However, it is not clear whether both techniques look at the same event. For example, endogenous ROCE and SOCE channels are studied, are often of very low amplitude and are slow in becoming apparent. ROCE and SOCE measured by means of Fura-2 fluorescence changes do not predict such differences. ROCE channels are often rapidly inactivated by Ca^{2+} (in the millisecond range), whereas feedback inhibition by entering Ca^{2+} is slow in ROCE and SOCE measured with Fura-2. In fact, Fura-2-based measurements of ROCE and SOCE do not lend themselves to observations in the millisecond range and reflect $[\text{Ca}^{2+}]_i$ changes over tens of seconds. The gradual decreases in $[\text{Ca}^{2+}]_i$ seen in Fura-2 experiments are very likely the sum of not only influx rates but also plasma membrane Ca^{2+} pump-mediated extrusion rates.

The present studies did not address these issues and focused on ROCE and SOCE as originally defined in studies that measured macroscopic ion fluxes with the Fura-2 Ca^{2+} indicator dye. In this context we probed the ROCE and SOCE phenomena with the nonselective tyrosine kinase inhibitor genistein and compared effects on endogenous ROCE and SOCE to its effect on TRPC-mediated Ca^{2+} entry. Our hope was that we might be able to establish correlations that would be informative not only about the mechanism of action by which genistein inhibits TRPC function but also about the nature of the channels that mediate native ROCE and SOCE.

We were able to elucidate the mechanism by which genistein inhibits ROCE mediated by TRPC3 by pinpointing the tyrosine phosphorylated by *src* that is required for its stimulation by the GPCR-Gq-PLC β signaling pathway. Removal of this tyrosine, TRPC3 Y226, causes loss of the ability of TRPC3 to respond to the Gq-PLC β signal (presumably diacylglycerol), because genistein inhibits activation not only by a receptor agonist but also by oleyl-acetyl-glycerol, a mimic of PLC β activation (22). *Src* is a candidate kinase for enabling activation of TRPC3 (Fig. 1). PP2, another tyrosine kinase inhibitor that is more selective toward *src* than genistein, also inhibits activation of TRPC3 in HEK cells (20), and restitution of Y226 in a TRPC3 channel lacking all N-terminal tyrosines shown to be *src* substrates in *in vitro* kinase assays rescues activation by the Gq-PLC β pathway (Fig. 2). Transfection of *yes* or *fyn* into SYF cells did not rescue stimulation of TRPC3 by the Gq-PLC β pathway (Fig. 5). Thus, the closely related *yes* and *fyn* tyrosine kinases cannot substitute for *src*. Although functionally important, the interaction of *src* with TRPC3 was not very stable and, to be reliably observed, required chemical stabilization with a crosslinker (see *Experimental Procedures*) before cell solubilization and immunoprecipitation. Furthermore, phosphorylation of transfected TRPC3 was not observable unless exogenous *src* was cotransfected. Yet we conclude that activation of TRPC3 depends on *src*-mediated phosphorylation on Y226. Phosphorylation on Y226 is not required for channel assembly because Y226I TRPC3 shows a subcellular distribution indistinguishable from that of wild-type

TRPC3 (Fig. 3A) and exhibits the same degree of basal activity as wild-type TRPC3 (Fig. 3B).

The finding that TRPC6 is active in SYF and YF cells was unexpected, because Hisatsune *et al.* (24) reported that TRPC6 is a substrate of *fyn* and that it behaves essentially the same as TRPC3 in *in vitro* and cell expression assays. Thus, TRPC6 is phosphorylated by coexpression with *fyn* in COS-7 cells and associates with *fyn* in GST pull-down assays by interaction of its N terminus with the src homology 2 domain of *fyn* (24). Furthermore, TRPC6 activation by a receptor-tyrosine kinase (RTK)-PLC γ pathway (triggered by EGF) is inhibited by PP2, a tyrosine kinase inhibitor selective for the *src* family of tyrosine kinases to which *fyn* belongs. Moreover, addition of *fyn* to inside-out membrane patches from cells expressing TRPC6 increased basal and oleyl-acetyl-glycerol-stimulated TRPC6 activity (24). Yet, we find that TRPC6 ROCE is activated in cells lacking not only *fyn* but also *yes* and *src* (Fig. 4C). We do not know whether this discrepancy is due to our use of the GPCR-Gq-PLC β activation pathway instead of a RTK-PLC γ pathway or to the method used to assess TRPC6 ROCE, Fura-2 in our case and electrophysiological inside-out membrane patches in the case of Hisatsune *et al.* (24). If, however, the lack of sensitivity of TRPC6 to genistein in our experiments and TRPC6 being active in SYF cells are not due to the use of different activation pathways or the method used to assess TRPC6 activation, then the data may also indicate that, in addition to *src*, *fyn*, and *yes*, functionally there is at least one more "PP2-sensitive *src*-family tyrosine kinase" able to regulate TRPC6. The fact that TRPC1-TRPC7 all interacted with *src* in GST pull-down assays (Fig. 1A) also raises the possibility that all TRPC channels depend on tyrosine phosphorylation for their functioning as an effector system for the activation of the GPCR-Gq-PLC β /RTK-PLC γ pathways.

The interaction of *src* with N and C termini of TRPCs fell into three types (Fig. 1A). Given the unexpected differences in the kinase(s) that may confer regulation to TRPC3 vs. those acting on TRPC6 and TRPC7, it is clear that the essential tyrosines involved and the specific phosphorylating kinase or kinases need to be determined in separate TRPC-specific studies. Indeed, although our studies have conclusively shown that *src* phosphorylates and enables TRPC3 to respond to the activating signal generated by the activation of the Gq-PLC β pathway, they have not ruled out the possibility that another tyrosine kinase not expressed in SYF and YF cells may not also have the same enabling properties as *src*. In this sense, our studies support the postulate of Hisatsune *et al.* (24) that tyrosine phosphorylation may be an obligatory step for the activation of TRPC channels. Moreover, results presented in Fig. 6 showing various degrees of inhibition of ROCE and SOCE by genistein strongly argue not only that the type of kinase may vary but also that the makeup of endogenous ROCE and SOCE channels is likely to vary from cell to cell. Results from the studies presented above may aid not only in understanding the mechanisms and molecular makeup of channels responsible for ROCE and/or SOCE but also in establishing a bridge between phenomena measured on a time scale of tens of seconds using fluorescent indicator dyes and phenomena measured in time scales of milliseconds by electrophysiological means.

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