Homer 1 Mediates Store- and Inositol 1,4,5-Trisphosphate Receptor-dependent Translocation and Retrieval of TRPC3 to the Plasma Membrane

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Store-operated Ca\textsuperscript{2+} channels (SOCs) mediate receptor-stimulated Ca\textsuperscript{2+} influx. Accumulating evidence indicates that members of the transient receptor potential (TRP) channel family are components of SOCs in mammalian cells. Agonist stimulation activates SOCs and TRP channels directly and by inducing translocation of channels in intracellular vesicles to the plasma membrane (PM). The mechanism of TRP channel translocation in response to store depletion and agonist stimulation is not known. Here we use TRPC3 as a model to show that IP\textsubscript{3} and the scaffold Homer 1 (H1) regulate the rate of translocation and retrieval of TRPC3 from the PM. In resting cells, TRPC3 exists in TRPC3-H1b/c-IP3Rs complexes that are located in part at the PM and in part in intracellular vesicles. Binding of IP\textsubscript{3} to the IP3Rs dissociates the interaction between IP3Rs and H1 but not between H1 and TRPC3 to form IP3Rs-TRPC3-H1b/c. TIRFM and biotinylation assays show robust receptor- and store-dependent translocation of the TRPC3 to the PM and their retrieval upon termination of cell stimulation. The translocation requires depletion of stored Ca\textsuperscript{2+} and is prevented by inhibition of the IP3Rs. In HEK293, dissociating the H1b/c-IP3R complex with H1a results in TRPC3 translocation to the PM, where it is spontaneously active. The TRPC3-H1b/c-IP3Rs complex is reconstituted by infusing H1c into these cells. Reconstitution is inhibited by IP3, deletion of H1 in mice markedly reduces the rates of translocation and retrieval of TRPC3. Conversely, infusion of H1c into H1\textsuperscript{−/−} cells eliminates spontaneous channel activity and increases the rate of channel activation by agonist stimulation. The effects of H1c are inhibited by IP3. These findings together with our earlier studies demonstrating gating of TRPC3 by IP3Rs were used to develop a model in which assembly of the TRPC3-H1b/c-IP3Rs complexes by H1b/c mediates both the translocation of TRPC3-containing vesicles to the PM and gating of TRPC3 by IP3Rs.

Ca\textsuperscript{2+} influx is a critical component of the receptor-evoked Ca\textsuperscript{2+} signal and plays a role in many physiological functions (1). The best described form of Ca\textsuperscript{2+} influx is mediated by the store-operated Ca\textsuperscript{2+} channels (SOCs), which are activated by agonist-dependent or agonist-independent depletion of Ca\textsuperscript{2+} stored in the ER (1). The molecular identity of the SOCs and \textit{I}_{\text{crac}} is still not known with certainty, although recent work points to ORAI1/CRACM1/olf186-F as a potential \textit{I}_{\text{crac}} (2–5). However, accumulating evidence indicates that members of the transient receptor potential (TRP) family of ion channels are associated with SOCs in mammalian cells. Thus, deletion of TRPC4 in mice (6, 7) or of TRPC1, TRPC3, TRPC6, and TRPC7 by antisense or siRNA (8–10) and dominant negative TRPC1, TRPC3, or TRPC5 (11–14) partially inhibit SOCs and/or receptor-stimulated Ca\textsuperscript{2+} influx.

The mechanism by which agonist stimulation activates Ca\textsuperscript{2+} influx by TRPC channels is not well understood. TRPC1, -4, and -5 can be activated by store depletion, whereas TRPC3, -6, and -7 can be activated by the lipid diacylglycerol (11, 15, 16). However, depending on cell type and expression levels, TRPC3 can also be activated by store depletion (17–19). Several mechanisms have been proposed to explain how store depletion leads to activation of SOCs and TRPC channels; conformational coupling between TRPC channels and IP3 receptors (IP3Rs) (18, 20–22), exocytotic insertion of the channels in the plasma membrane (PM) (23–25), and activation by a diffusible messenger (26, 27). Biochemical and functional evidence showed regulatory interaction between IP3Rs and several TRPC channels, including TRPC1 and TRPC3 (18, 28–32). The interaction between TRPC1 and IP3Rs and gating of TRPC1 by IP3Rs is mediated by Homers (33). Homers are scaffolding proteins that bind many Ca\textsuperscript{2+}-signaling proteins, including all TRPC channels and IP3Rs (33–37). In addition, a C-terminal domain...
of several TRPC channels directly interacts with an N-terminal sequence in the IP₃Rs (28, 32) that participates in gating of TRPC channels by IP₃Rs (30, 32).

A newly discovered and apparently a general regulatory mechanism of TRPC channel activity is agonist-stimulated translocation of the channels to the PM. In HEK293 cells, receptor stimulation but not passive store depletion was reported to stimulate the translocation of TRPC3 to the PM in a mechanism that was inhibited by cleavage of VAMP2 (vesicle-associated membrane protein 2) with tetanus toxin (38). Another form of regulation of TRPC3 is by interaction with phospholipase Cγ (22, 39). However, unlike the role of VAMP2, phospholipase Cγ does not affect the acute expression or translocation of TRPC3 but rather the steady-state level of TRPC3 in the PM (22). Stimulation of the epidermal growth factor receptor resulted in translocation of TRPC5 to the PM in a mechanism that was dependent on phosphoinositide 3-kinase, the Rho GTPase Rac1, and phosphatidylinositol-4-5-phosphate 5-kinase (PIP5Kα) (24). Finally, stimulation of the muscarinic M3 receptor resulted in translocation of TRPC6 to the PM in a time course that coincides with activation of Ca²⁺ influx (25).

For the most part, TRPC channel translocation has been studied in cell lines. Whether such a mechanism also operates in native cells is not known. Furthermore, TRPC3, -5, and -6 bind Homers and IP₃Rs (33) (present work). The potential role of Homer and IP₃Rs in this form of regulation of TRPC channels activity is not known. Here, we used cells transfected with TRPC3 and Homer 1 (H1) isoforms and cells prepared from WT and H1⁻/⁻ mice to report that H1 regulates the agonist- and store-dependent translocation and antagonist-mediated retrieval of TRPC3 from the PM. This process requires the dissociation of the IP₃Rs-H1-TRPC3 complex by binding of IP₃ to the IP₃Rs. These findings suggest a novel mechanism by which store depletion leads to activation of TRPC channels and Ca²⁺ influx.

**EXPERIMENTAL PROCEDURES**

*Materials and Solution—* Anti-TRPC3 antibodies were a generous gift from Dr. Craig Montell (Johns Hopkins University) or batch 2 from Alomone (Jerusalem, Israel). Anti-IP₃R3 antibodies were from BD Transduction Laboratories, and anti-HA antibodies were from Covance (Princeton, NJ). pRK5-HA-TRPC3, pRK5-HA-Homer 1a, and pRK5-HA-Homer 1c were prepared as detailed elsewhere (33), and PCMV-TRPC3-YFP was generously provided by Dr. Thomas Gudermann (Philips-Universitat-Marburg, Germany). All biotinylation-related products were purchased from Pierce.

*Cell Culture, Transfection, Co-immunoprecipitation, Pull-down Assay, and IP₃ Competition Assay—* M3-HEK cell lysates and pancreatic microsomal lysates were incubated with or without 100 μM IP₃ for 30 min at 0 °C (ice water bath) to bind IP₃ to the IP₃Rs. GST, GST-H1α, or GST-H1c coupled to beads was then added to the extracts, and the incubation at 0 °C continued for 4–12 h. The beads were washed with lyss buffer, and proteins were released by incubation in SDS sample buffer. Pulled proteins were analyzed by SDS-PAGE and Western blotting. Due to the long incubation required for the pull-down and co-IP assays (≥4.5 h), 100 μM IP₃ was used to ensure the presence of IP₃ during the entire incubation periods.

*Isolation of Pancreatic Acinar Cells—* The pancreases of 1–2 mice were removed and digested with collagenase and trypsin to isolate single cells to improve access to biotin. Single pancreatic acinar cells were prepared as described before (40). Briefly, the pancreases were finely minced and digested with 4 mg/15 ml collagenase P for 5 min at 37 °C in a solution named PSA that contained 10 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES (pH 7.4 with NaOH), 10 mM glucose, 0.1% bovine serum albumin, and 0.02% soybean trypsin inhibitor. The cells were then washed with Ca²⁺⁺-, and Mg²⁺⁻-free PBS and treated for 2 min at 37 °C with 0.05% trypsin-EDTA solution (Sigma), washed with PSA, and digested again with collagenase for 3–4 min at 37 °C. Finally, the cells were washed with PSA and stored on ice until use.

*Protein Extraction—* Mice were scarified, and the tissues of interest were collected. To prepare microsomes, each tissue was washed with a buffer containing 250 mM sucrose, 10 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride and homogenized in 5 ml of buffer by 20 strokes with a Dawn’s homogenizer. After centrifugation for 3 min at 1000 rpm, the supernatants were centrifuged for 20 min at 18,000 rpm. The microsomes were dissolved in lysis buffer containing 20 mM Tris, pH 8.0, 137 mM NaCl, 5 mM NaEDTA, 5 mM NaEGTA, 10% glycerol, 0.5% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 20 mM benzamidine and kept on ice for 30 min. Insoluble material was removed by 15-min centrifugation at 14,000 rpm, 4 °C, and the supernatants were collected. Protein concentration in the extracts was measured by the Bradford method.

*Surface Biotinylation Assay—* Sulfo-NHS-ss-Biotin (Pierce) in PBS supplemented with 1 mM MgCl₂ and 0.5 mM CaCl₂ was added to control cells or cells treated with agonist, antagonist, or CPA, and the mixtures were incubated for 30 min at 0 °C. Free biotin was quenched by the addition of 1% bovine serum albumin in PBS supplemented with Ca²⁺⁺ and Mg²⁺⁻, and then the cells were washed once with PBS. Lysates were prepared in lysis buffer by passing 7–10 times through a 27-gauge needle. The lysates were centrifuged at 14,000 rpm for 10 min at 4 °C, and protein concentration in the supernatants was determined. Volume and protein content were adjusted to be the same in all samples, and to each sample was added 10% streptavidin beads (Pierce) in 300 μl. The mixtures were incubated overnight at 4 °C, the beads were washed five times with lysis buffer, and the proteins were extracted by suspending the beads in sample loading buffer. Precipitated proteins were analyzed by Western blot.


**Immunocytochemistry**—Immunostaining was performed as detailed previously (41). Pancreatic, parotid, or submandibular cells were immobilized on a poly-l-lysine-coated coverslip and were permeabilized with 0.5 ml of cold methanol for 10 min at –20 °C. The samples were washed with PBS, and the non-specific sites were blocked by a 1-h incubation with a solution containing 5% goat serum, 1% bovine serum albumin, and 0.1% gelatin in PBS (blocking medium). The medium was aspirated and replaced with 50 μl of blocking medium containing control serum or a 1:50 dilution of anti-TRPC3, 1:100 dilution of anti-

**RESULTS AND DISCUSSION**

**H1a Translocates TRPC3 to the Plasma Membrane**—Homer 1a (H1a) has an EVH domain but lacks the multimerizing coiled-coil and leucine zipper domains and dissociates complexes formed by dimerizing H1b/c. H1a is thus a useful tool to study the role of Homer proteins in Ca2+ signaling (33). The role of Homer in agonist-mediated translocation of TRPC3 to the plasma membrane (PM) was evaluated by TIRF in HEK293 cells expressing the muscarinic M3 receptors and transfected with TRPC3-YFP or TRPC3-YFP and H1a. The images in Fig. 1A and the time lapse movies in the supplement show that a significant portion of TRPC3 was found in intracellular vesicles. Stimulation with carbachol resulted in a rapid disappearance of the vesicles as they fused with the PM. The rapid fusion precluded observing accumulation of the vesicles at the PM, but this was confirmed by surface biotinylation assays in Fig. 1B. Removal of carbachol and treatment of the cells with atropine resulted in partial recovery of the vesicles within 40 s of the treatment with atropine. Expression of H1a inhibited translocation of TRPC3-YFP that remained in intracellular vesicles. However, the frequency of finding intracellular vesicles was low, and when found, the vesicles appeared smaller (average size of vesicles in the presence of H1a was 61 ± 4% (n = 32) smaller than in the absence of H1a). These vesicles were not translocated to the PM upon agonist stimulation.

The effect of H1a was quantitated by a biotinylation assay to measure the surface expression of TRPC3. Fig. 1B shows that cell stimulation resulted in rapid translocation of TRPC3 to the PM. Expression of H1a increased the level of TRPC3 present at the PM of resting cells to a level caused by cell stimulation. Subsequent stimulation of cells expressing H1a resulted in only a small further increase in PM expression of TRPC3. The dimerizing H1b increased the total cellular level of TRPC3, perhaps by decreasing degradation of the channel, but did not change its surface expression at the resting state or its translocation to the PM by cell stimulation. Similar results were observed with TRPC3-YFP (not shown).

The functional consequences of the effects of H1a and H1b on TRPC3 translocation are depicted in Fig. 1, C–E. Fig. 1C shows examples of current traces, Fig. 1D shows the typical TRPC3 I/V in TRPC3-transfected cells, and Fig. 1E is the summary. HEK293 cells transfected with empty vector, H1a alone, or H1b alone did not show a typical TRPC3 current (Fig. 1, C and D). Resting HEK293 cells expressing TRPC3 showed very small spontaneous TRPC3-specific current. Stimulation of the M3R resulted in activation of TRPC3. In the presence of H1a, mostly spontaneous TRPC3 current was detected, consistent with PM localization of the channel and indicating that TRPC3 was fully active in the presence of H1a. H1b only increased the stimulated activity of TRPC3 (Fig. 1E, red trace), as expected
from increased expression of the channel and receptor-mediated translocation to the PM.

**IP₃ Dissociates IP₃Rs-H₁ Interaction**—Co-IP experiments showed that expression of H₁b/c increases, whereas expression of H₁a decreases, the co-IP of TRPC3 and IP₃ receptors (not shown), suggesting that H₁b/c assembles and H₁a dissociates TRPC3-H₁b/c-IP₃Rs complexes. The question that arises is how disassembly of the complexes occurs under physiological conditions and its role in regulating TRPC3 activity. A clue was provided by a recent crystal structure of the ligand-binding suppressor domain of the IP₃R1, which showed that the Homer binding motif of the IP₃Rs is adjacent to the IP₃ binding core (43). We reasoned that binding of IP₃ to activate the IP₃Rs and evoke Ca²⁺ release from the stores may also allosterically inhibit binding of H₁ to the IP₃Rs and dissociate the TRPC3-H₁b/c-IP₃Rs complexes. The pull-down (PD) assays in Fig. 2A indicate that this is the case. GST-H₁a and GST-H₁c specifically pulled down the native IP₃R3 and transfected TRPC3. Remarkably, the addition of IP₃ inhibited the binding of both H₁a and H₁c to the IP₃ receptors but did not affect the binding of the Homers to TRPC3.

To determine the functional significance of H₁b/c-IP₃R dissociation by IP₃, we measured the effect of IP₃ on modulation of the TRPC3 current by the Homers. Maximal activation of TRPC3 by H₁a was verified by showing that infusion of 100 μM IP₃ into cells expressing H₁a and TRPC3 did not further activate the channel (Fig. 2C). Importantly, infusing the TRPC3-H₁a-expressing cells with recombinant multimerizing H₁c recoupled TRPC3 to reduce the spontaneous activity and increase the receptor-activated portion of the current to that found in control cells (Fig. 2D). Most notably, IP₃ completely inhibited the effect of H₁c (Fig. 2E), as expected from inhibition of binding of H₁c to the IP₃Rs by IP₃ (Fig. 2A). The recoupling indicates that after dissociation of the complex by cell stimulation and binding of IP₃ to the IP₃Rs, the channels remain in

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**FIGURE 1. H₁a translocates TRPC3 to the plasma membrane.** A, HEK293 cells transfected with TRPC3-YFP (left images) of TRPC3-YFP and H₁a (right images) were stimulated with 100 μM carbachol for 60 s and then inhibited with 10 μM atropine for 40 s, and TIRF fluorescence was recorded. B, biotinylation assay. HEK293 cells transfected with HA-TRPC3 (all blots), H₁a (middle blot), or H₁b (lower blot) were stimulated with 100 μM carbachol for the indicated times, and surface TRPC3 was assayed by biotinylation as detailed under “Experimental Procedures.” The columns are the summary of three experiments showing the -fold increase in TRPC3 at the plasma membrane relative to the level in control, unstimulated cells. The results were corrected for input of TRPC3 and show that most TRPC3 was in the plasma membrane of resting cells expressing TRPC3 and H₁a. C–E, channel function. The whole cell current was measured at a holding potential of −100 mV in cells transfected with TRPC3 alone (C), H₁a alone (solid blue symbols), TRPC3 and H₁a (open blue symbols), TRPC3 and H₁b (open red symbols), as indicated. Spontaneous current was measured by incubating resting cells with media containing Na⁺ of NMDG⁺, and then the cells were stimulated with 100 μM carbachol to record the stimulated TRPC3 current, as evident from the I/V plots in D. The summary in E is of spontaneous (Spon; green) or agonist-stimulated current (Carb; blue) at −100 mV from 4–6 cells, which were normalized for cell capacitance (pA/picofarads) and averaged to obtain the mean ± S.E.
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FIGURE 2. Functional consequences of inhibition by IP$_3$ of Homers binding to the IP$_3$R. A, the blots show inhibition by IP$_3$ of H1a and H1c binding to the IP$_3$R. Extracts prepared from HEK293 cells transfected with TRPC3 were incubated at 0 °C with and without 100 μM IP$_3$, and were used to pull down TRPC3-H1b/c-IP$_3$Rs complexes by beads bound with GST alone (control), GST-H1a, or GST-H1b. Binding of IP$_3$ to the IP$_3$Rs inhibited the binding and pulldown (PD) of IP$_3$Rs by H1a and H1b. B–E, whole cell current was measured in HEK293 cells transfected with TRPC3 and H1a (B–E). The cells were also infused with 100 μM IP$_3$, to show that, once activated by H1a, the current is no longer sensitive to store depletion (C). Cells expressing TRPC3 + H1a were infused with 50 μg of recombinant H1c (D) or H1c and 100 μM IP$_3$ to inhibit binding of H1c to the IP$_3$Rs. Inward current was measured at a holding potential of −100 mV, and monovalent cation current was evaluated by incubating the cells in medium containing 150 mM NMDG$^+$ or Na$^+$ and resting or stimulated with 100 μM carbachol. F, summary of spontaneous or agonist-stimulated current from 8–13 cells under each condition.

close proximity to allow the recoupling. This point will be further addressed below.

TRPC3 Is Expressed at the Lateral Pole of Polarized Cells—The results in Figs. 1 and 2 suggest that Homers play a role in translocation of TRPC3 to the PM and regulation of its activity. To extend these findings to the in vivo situation, we used pancreatic and salivary gland cells, which express high levels of TRPC3. Fig. 3 shows that TRPC3 is expressed mainly at the lateral membrane and next to the apical pole of all cells examined. Previous work used immunohistochemistry to suggest that TRPC3 may be expressed at the luminal membrane of the submandibular gland (44). The more sensitive immunofluorescence localization in Fig. 3B shows concentration of TRPC3 and IP$_3$Rs at the junctional site at the apical pole (yellow arrowheads), expression at the lateral and basal poles (white arrowheads), and weak expression at the luminal membrane. Transfection in Madin-Darby canine kidney cells grown on permeable support confirmed targeting of TRPC3 mainly to the basolateral membrane, proximal to ZO1 and the same as β-catenin (Fig. 3C). The same localization was found with four different anti-TRPC3 antibodies (Montell, Schilling, Alomone, and Sigma).

The specificity of the antibodies used for the staining, the co-IP, and the biotinylation experiments is shown in Fig. 3D. The anti-TRPC3 antibodies detected a single band of about 78 kDa in 37 μg of brain and pancreatic extracts. Moreover, the antibodies detected a single band in 6 μg of extract prepared from HEK293 cells transfected with TRPC3-YFP. The anti-IP$_3$R3 antibodies detected a single band of about 270 kDa in pancreatic and HEK293 cell extracts and two bands in HEK293 cells transfected with green fluorescent protein-IP$_3$R3 (Fig. 3D). Hence, the antibodies used in the present work had the required specificity. The Co-IP experiments in Fig. 3E show that in secretory cells and the brain, TRPC3 exists in complexes with IP$_3$Rs and the plasma membrane Ca$^{2+}$ ATPase pump (PMCA).

Receptor- and Store-dependent Translocation of TRPC3 to the PM—A biotinylation assay was used to follow TRPC3 translocation in native cells. Fig. 4A shows that some TRPC3 is present at the PM of resting cells and that stimulation of the G$_q$-coupled M3 receptors resulted in a robust translocation of TRPC3 to the PM. Significantly, termination of cell stimulation with atropine resulted in retrieval of TRPC3 from the PM. The state of the interaction of IP$_3$Rs with TRPC3 at the PM was assayed by biotinylation. Fig. 4B shows that in unstimulated cells, some IP$_3$Rs were pulled down by the avidin beads, probably by binding to surface proteins, such as TRPC3. The specificity of the biotinylation is shown by the lack of pull-down of the cytoplasmic protein aldolase. Cell stimulation reduced the amount or the strength of interaction of IP$_3$Rs with surface proteins. Again, the reduced interaction was reversed by subsequent inhibition of cell stimulation with atropine, indicating that after the dissociation by cell stimulation, the IP$_3$Rs remain in close proximity to TRPC3 at the PM. That at least one of the PM proteins interacting with IP$_3$Rs is TRPC3 is shown in Fig. 4C, which shows that IP of TRPC3 co-immunoprecipitated IP$_3$R3, the co-IP was reduced by cell stimulation, and the IP$_3$R3-TRPC3 complex was reformed by inhibition of carbachol-stimulated cells with atropine.

Although several studies (17, 19, 28, 45, 46), including ours (18, 30), suggested that TRPC3 can function as SOCs, others concluded that this is not the case (15, 47, 48). However, the behavior of TRPC3 in native cells was examined before only in one study using pontine neurons from P3–P8 rats. In these cells, activation of TRPC3 required IP$_3$-mediated Ca$^{2+}$ release,
although the channel could not be activated by treating the cells
with thapsigargin (49). Here we examined the role of Ca^{2+}/H{\text{11001}}
stores in the translocation of TRPC3. Fig. 4, A and B, shows that
store depletion with the SERCA pump inhibitor CPA was as
effective as agonist stimulation in causing TRPC3 translocation
to the PM and in altering its interaction with IP3Rs. TRPC3 translocation was dependent on both IP3Rs and
depletion of stored Ca^{2+}/H{\text{11001}}. Store depletion with CPA reduced the
co-IP of IP3Rs and TRPC3 in native cells (Fig. 4C). Further-
more, Fig. 4D shows that inhibition of the IP3Rs with xesto-
spongion C (Xest C) inhibited the agonist- and CPA-mediated
translocation of TRPC3 to the PM. Since inhibition of SERCA
pumps with CPA depletes the Ca^{2+}/H{\text{11001}} stores also in the presence
of Xest C, inhibition of TRPC3 translocation by CPA indicates
that the translocation required functional IP3Rs. The protocol
of Fig. 4E was designed to show that TRPC3 translocation and
retrieval were dependent on Ca^{2+} content of the stores. Acinar
cells were incubated in Ca^{2+}/H{\text{11001}}-free medium and stimulated with
carbachol to deplete stored Ca^{2+}, which resulted in the usual
translocation of TRPC3 to the PM. Inhibition by atropine while
the cells were kept in Ca^{2+}/H{\text{11001}}-free medium to prevent store
reloading inhibited retrieval of TRPC3. The subsequent addi-
tion of Ca^{2+} to the medium to reload the stores resulted in
retrieval of TRPC3 from the PM. We have previously used the
same protocol to show that SOCs remained fully active as
long as the stores were not allowed to reload with Ca^{2+} and
were inhibited only when the stores were reloaded with Ca^{2+}
(50, 51).

The implication of the results in Figs. 3 is that translocation
of TRPC3 to the PM is dependent on IP3Rs and on store Ca^{2+}
content. This indicates that (a) in vivo TRPC3 behaves as SOCs,
(b) TRPC3 is a subunit of SOCs, and (c) in vivo the TRPC3-

FIGURE 3. Basolateral localization of TRPC3. Large clusters of pancreatic (A) and parotid (B, left) acini or submandibular duct (B, right) were co-stained for TRPC3 (green) and ZO1 (A) or IP3R3 (A and B, red). The yellow arrowheads in the duct mark expression of TRPC3 at the apical pole junctions, and the white arrowheads mark the basolateral membrane. In C, HA-TRPC3 was expressed in polarized Madin-Darby canine kidney cells, and the cells were co-stained for TRPC3 (green) and ZO1 (upper images) or β-catenin (lower images) (red). In each panel, the upper image is the x/y scan at the apical region, and the lower image is the z scan. D shows the high specificity of the antibodies used in the present work as revealed by blotting for TRPC3 (left) and IP3R3 (right) of extracts prepared from the mouse brain and pancreas (panc) from untransfected HEK293 cells of HEK293 cells transfected with TRPC3-YFP or green fluorescent protein (GFP)-IP3R3, as indicated. In E, native TRPC3 (left) or plasma membrane Ca^{2+} ATPase pump (PMCA) (right) was immunoprecipitated, and the precipitates were probed for TRPC3 and IP3R3 (left) or TRPC3 and PMCA (right).
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FIGURE 4. IP₃R₃- and store-dependent translocation and retrieval of TRPC3 from the plasma membrane. A, pancreatic acinar cells from WT mice were stimulated with 1 mM carbachol for 5 min or stimulated with carbachol for 2 min and inhibited with 10 μM atropine for 3 min. The cell surface proteins were biotinylated, and extracts were prepared to pull down and analyze plasma membrane levels of TRPC3. The columns show a summary of 4–5 experiments. *, p < 0.01 relative to unstimulated WT cells. B, the same protocol as in A was used, except that the blots were analyzed for IP₃R₃. For a control, the blots were analyzed for the cytosolic protein aldolase. C, extracts were prepared from acini treated as in A and were used to immunoprecipitate TRPC3 and blot for IP₃R₃ to assay for co-IP of IP₃Rs and TRPC3. D, the same protocol as in A was used, except that a portion of the cells was treated with 5 μM xestospongin C (Xest C) for 10 min before treatment with carbachol or CPA. The columns summarize the results of three similar experiments. E, effect of store Ca²⁺ content on TRPC3 retrieval. Cells in Ca²⁺-free medium were stimulated with carbachol for 5 min (Car/0Ca) or with carbachol for 2 min and inhibited with atropine for 3 min (Car/Atro/0Ca). Ca²⁺ to a final concentration of 2 mM was added to cells treated with carbachol and atropine in Ca²⁺-free medium, and the incubation continued for an additional 3 min (Car/Atro/0Ca(+Ca)). The cells were then used to determine surface expression of TRPC3. The columns show the summary of three similar experiments. *, p < 0.01 relative to unstimulated cells; #, p < 0.05 relative to Car/Atro/0Ca cells. Con, control.

H1 facilitates IP₃R₃- and store-dependent translocation and retrieval of TRPC3 from the plasma membrane. A, pancreatic acinar cells from WT (○) and H1⁻/⁻ cells (△) were stimulated with 1 mM carbachol (○ and △) or treated with 30 μM CPA (△) for the indicated times, and surface TRPC3 was determined by biotinylation. B, a typical blot and the summary of 4–5 similar experiments. C, effect of H1 on time course of TRPC3 retrieval. WT (○) and H1⁻/⁻ (△) acinar cells were stimulated with 1 mM carbachol for 5 min, and samples were removed to determine surface level of TRPC3 before termination of cell stimulation with 10 μM atropine. Samples were removed at the indicated times to measure the rate of TRPC3 retrieval. Con, control.

Homer 1 Regulates the Rate of TRPC3 Translocation and Retrieval—The role of Homers in agonist- and store-dependent TRPC3 translocation to the PM was examined using pancreatic acinar cells from WT and H1⁻/⁻, H2⁻/⁻, and H3⁻/⁻ mice. Deletion of Homer 2 and Homer 3 had no apparent effect on TRPC3 translocation (not shown), indicating that the effects described below are specific to Homer 1 (H1). Deletion of H1 had no effect on overall expression of TRPC3 but reduced the steady-state level of TRPC3 at the PM by about 50% (Fig. 5A). This is probably an adaptation to reduce the spontaneous activity of TRPC3 at the PM (see Ref. 33) (see below). Stimulation of H1⁻/⁻ cells resulted in translocation of TRPC3 to increase its level at the PM to that found in stimulated WT cells. However, store depletion by CPA was less effective in stimulating TRPC3 translocation in H1⁻/⁻ cells. Furthermore, inhibition of cell stimulation by atropine for 3 min resulted in only partial retrieval of TRPC3 from the PM.

To determine the reason for the poor TRPC3 translocation stimulated by store depletion and its poor retrieval on termination of cell stimulation in H1⁻/⁻ cells, we compared the time course of the translocation and retrieval of TRPC3 in WT and H1⁻/⁻ cells. Fig. 5B shows that most TRPC3 translocation was completed within 30 s of stimulation with agonist, whereas little translocation was measured after 30 s of treatment with CPA. Comparable translocation was measured after 5 min of treatment with agonist or CPA, consistent with the fast and slow components of store depletion of the Ca²⁺ stores by agonist stimulation and inhibition of SERCA pump, respectively. Fig. 4B shows that deletion associated SOCs are regulated in part by translocation to the PM.
of H1 markedly reduced the rate of TRPC3 translocation in response to agonist stimulation. Furthermore, Fig. 5C shows that deletion of H1 also reduced the rate of TRPC3 retrieval initiated by atropine inhibition of stimulated cells. Thus, the combined results in Fig. 5 show that H1 regulates the rate of stores and IP$_3$Rs-mediated translocation and retrieval of TRPC3.

Functional Correlate—To determine the relationship between IP$_3$Rs, H1 and TRPC3 in native cells, we measured the effect of IP$_3$ on the interaction between the proteins. As was found in HEK293 cells (Fig. 2A), GST-H1a and GST-H1c specifically pulled down TRPC3 and IP$_3$R3 from pancreatic acinar cell extracts, and binding of IP$_3$ to the IP$_3$Rs dissociated the Homers-IP$_3$Rs interaction without dissociating the Homers from TRPC3 (Fig. 6A). Similar results were also obtained by pull-down (not shown) and co-IP from brain extract (Fig. 6B). Brain extract was used for the co-IP experiments, since the level of H1 in the pancreas was low and could not be reliably detected in the immunoprecipitate. Fig. 6B shows that IP of IP$_3$Rs co-immunoprecipitated TRPC3 and H1, and binding of IP$_3$ to the IP$_3$Rs reduced the co-IP of TRPC3 and H1 with the IP$_3$Rs.

TRPC3 functions as a nonselective monovalent cation channel (18, 52). Therefore, we measured the properties of the monovalent cation current in WT and H1$^{-/-}$ acinar cells. The cells were dialyzed with 10 mM EGTA to inhibit all Ca$^{2+}$-activated currents, prevent inhibition of TRPC3 current by [Ca$^{2+}$]$_o$, and facilitate store depletion. Fig. 5C shows that resting WT cells have a small spontaneous cation current, and cell stimulation prominently and rapidly activated the current. Interestingly, infusion of IP$_3$ (Fig. 6D) or recombinant H1a (Fig. 6E) maximally activated the same cation current, as evident from the minimal effect of agonist stimulation after activation of the current by IP$_3$ or H1a.

Parallel measurement in H1$^{-/-}$ cells showed that about half of the cation current was spontaneously active in these cells. Stimulation of H1$^{-/-}$ cells resulted in further activation of the current. However, the current was only slowly activated, consistent with the slow translocation of TRPC3 to the PM shown in Fig. 5B. The most significant findings for the purpose of the present discussion are shown in Fig. 6, G and H, and are summaries in Fig. 6, I and J. Fig. 6G shows that infusion of recombinant dimerizing H1c recoupled the current to reduce the spontaneous and increase the receptor-stimulated current. In addition, H1c increased the rate of current activation by agonist (Fig. 6f), as expected from the effect of H1 on the rate of TRPC3 translocation. Fig. 6H shows that IP$_3$ completely inhibited the effect of H1c, as predicted from inhibition of binding of H1c to the IP$_3$Rs by IP$_3$, shown in Fig. 6A.

Pancreatic acinar cells express TRPC3, TRPC6, and probably other TRP channels. Preliminary studies showed that TRPC3 and TRPC6 exist in Ca$^{2+}$-signaling complexes with other Ca$^{2+}$-signaling proteins, including IP$_3$Rs and Homers (not shown). The excellent agreement between regulation of recombinant TRPC3 by Homers and IP$_3$Rs in HEK293 cells...
and native TRPC3 in pancreatic and salivary gland cells, including properties of the nonselective monovalent cation current, suggest that TRPC3 is a major component of SOCs in native cells and that other TRP channels that participate in SOCs are likely to be regulated by H1. Previous work with TRPC3 and TRPC6 expressed individually in HEK293 cells concluded that both channels do not behave as SOCs (15). Furthermore, TRPC3 and TRPC6 did not translocate to the PM in response to store depletion (25, 38). The present findings emphasize the need to study the TRPC channels in vivo to understand their physiological behavior and contribution to Ca\(^{2+}\) influx. In vivo TRPC3 behaves as SOCs and appears to contribute to agonist-stimulated and store-dependent Ca\(^{2+}\) influx.

The key findings of the present work are that binding of IP\(_3\) to the IP\(_3\)Rs inhibited binding of H1 to the IP\(_3\)Rs to disassemble the TRPC3-H1b/c-IP\(_3\)Rs complexes and that H1 regulates the rates of translocation and retrieval of TRPC3 from the PM. At the same time, in a previous work we showed that IP\(_3\) (18) and its N-terminal domain (30) bound with IP\(_3\) activate TRPC3. TRPC3- and IP\(_3\)R-interacting domains were subsequently identified (28), and the IP\(_3\)R domain that binds to TRPC3 was sufficient to activate TRPC3 (32). Since cell stimulation and binding of IP\(_3\) to IP\(_3\)Rs result in dissociation of H1 from IP\(_3\)Rs and reduced interaction of IP\(_3\)Rs with TRPC3, we propose that H1 may have two roles in controlling TRPC3 activity: facilitation of TRPC3 translocation and retrieval from the PM and strengthening of the interaction and gating of TRPC3 by IP\(_3\)Rs. The model in Fig. 7 attempts to account for both effects of H1. In resting cells, TRPC3 exists in a complex with H1 and IP\(_3\) receptors. IP\(_3\)Rs and TRPC3 also interact directly, and this interaction is stabilized by H1. While in the complex, TRPC3 is not active, whether the TRPC3-H1b/c-IP\(_3\)Rs complex is at the PM or in intracellular vesicles. IP\(_3\) generated by receptor stimulation binds to the IP\(_3\)Rs to release Ca\(^{2+}\) from the stores and dissociate between IP\(_3\)Rs and H1 to form the IP\(_3\)Rs-TRPC3-H1b/c complex, in which interaction between IP\(_3\)Rs-TRPC3 is flexible and is not maintained in extracts of stimulated cells or extracts incubated with IP\(_3\). The IP\(_3\)Rs-TRPC3-H1 complex rapidly translocates to the PM. The IP\(_3\)Rs-TRPC3-H1b/c complex is active and mediates Ca\(^{2+}\) influx. Maintained binding of IP\(_3\) to the IP\(_3\)Rs and interaction of IP\(_3\)Rs with TRPC3 is required for activation of TRPC3 (18, 28, 30, 32). H1a can also dissociate the IP\(_3\)Rs-H1-TRPC3 complexes to form IP\(_3\)Rs-TRPC3-H1a complexes that translocate and fuse with the PM. As long as the IP\(_3\)Rs is bound with IP\(_3\), it does not bind to H1, and TRPC3 remains active at the PM. Termination of cell stimulation results in hydrolysis of IP\(_3\), reloading of the stores with Ca\(^{2+}\), restoration of binding of H1 to IP\(_3\)Rs, and stabilization of the TRPC3-H1b/c-IP\(_3\)Rs complexes and retrieval of TRPC3 from the PM.

Recent studies identified STIM1 (stromal interaction molecule-1) as a sensor of ER Ca\(^{2+}\) stores that is essential for agonist- and store-dependent activation of L\(_{\text{crac}}\) (53) and SOCs (54, 55). Cell stimulation translocates STIM1 from the ER to the PM (53). Hence, regulation of SOCs by STIM1 and H1 are probably two different modes of channel regulation.

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