Assembly of Trp1 in a Signaling Complex Associated with Caveolin-Scaffolding Lipid Raft Domains

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Trp1 has been proposed as a component of the store-operated Ca\(^{2+}\) entry (SOC) channel. However, neither the molecular mechanism of SOC nor the role of Trp in this process is yet understood. We have examined possible molecular interactions involved in the regulation of SOC and Trp1 and report here for the first time that Trp1 is assembled in signaling complex associated with caveolin-scaffolding lipid raft domains. Endogenous hTrp1 and caveolin-1 were present in low density fractions of Triton X-100-extracted human submandibular gland cell membranes. Depletion of plasma membrane cholesterol increased Triton X-100 solubility of Trp1 and inhibited carbachol-stimulated Ca\(^{2+}\) influx. Importantly, we have shown that Trp1 is assembled in signaling complex associated with caveolin and inhibited carbachol-stimulated Ca\(^{2+}\) signaling. Importantly, thapsigargin stimulated Ca\(^{2+}\) influx, but not internal Ca\(^{2+}\) release, and inositol 1,4,5-trisphosphate (IP3)-stimulated \(I_{\text{soc}}\) were also attenuated. Furthermore, both anti-Trp1 and anti-caveolin-1 antibodies co-immunoprecipitated hTrp1, caveolin-1, \(G_{\alpha q/11}\), and IP3 receptor-type 3 (IP3R3). These results demonstrate that caveolar microdomains provide a scaffold for (i) assembly of key Ca\(^{2+}\) signaling proteins into a complex and (ii) coordination of the molecular interactions leading to the activation of SOC. Importantly, we have shown that Trp1 is also localized in this microdomain where it interacts with one or more components of this complex, including IP3R3. This finding is potentially important in elucidating the physiological function of Trp.

Store-operated Ca\(^{2+}\) entry (SOC), present in all non-excitable cells, is activated via an as yet unknown mechanism when internal Ca\(^{2+}\) stores are depleted. Following neurotransmitter stimulation of cells, SOC is activated by inositol 1,4,5-trisphosphate (IP3)-dependent release of Ca\(^{2+}\) from internal Ca\(^{2+}\) stores via the IP3 receptor (IP3R). However, SOC is also activated by store depletion in the absence of any increase in IP3. The nature of the signal which links the internal Ca\(^{2+}\) store to the plasma membrane, and the molecule that mediates Ca\(^{2+}\) influx across the plasma membrane, is not yet known in any non-excitable cell type (1, 2).

Several, mammalian homologues of the Drosophila trp gene have been proposed as candidate genes encoding the SOC channel protein (1, 3), although the exact physiological role of trp gene products remains to be clarified. Important questions that need to be addressed are (i) whether Trp proteins form the SOC channel and if they do, is the channel a homomer or multimer of one or more Trp protein(s), and (ii) whether SOC regulation is more complex, involving Trp interactions with other scaffolding or regulatory proteins. The Drosophila Trp (dTrp) has been shown to be assembled in a signaling complex, binding directly to the scaffolding protein (INAD) and calmodulin and indirectly to phospholipase C and rhodopsin via INAD. Protein kinase C, Trp-like channel, and G-protein subunits (\(G_{\alpha q, \beta, \gamma}\)) are also associated with this complex. It has been suggested that protein-protein interactions within the complex, localized in the photoreceptor membrane, facilitate key reactions in the signaling cascade and lead to Trp activation (4–6). A mammalian homologue of the Drosophila inaD has been recently cloned (7), although presently there are no data to suggest that the mammalian Trp protein(s), like dTrp, is assembled in a larger signaling complex where it is associated with regulatory or scaffolding proteins.

We have recently proposed that Trp1 is a strong candidate for the SOC mechanism in salivary epithelial cells (8). Our studies demonstrated that the endogenous Trp1 was localized in the plasma membrane of these cells and could be correlated with SOC. We showed that a decrease in endogenous Trp1 was associated with a decrease in SOC while an increase in Trp1, following stable transfection of the cells with htrp1 cDNA, induced an increase in SOC. However, the molecular mechanism(s) involved in the Trp1-SOC association is not yet clear. For example, it remains to be established whether Trp1, either by itself or in association with other proteins, constitutes the SOC channel. Recent studies reported by Muallem and co-workers (9) have shown that hTrp3 is a Ca\(^{2+}\) channel that is activated by internal Ca\(^{2+}\) store depletion (9). Furthermore, these investigators have shown that Trp3 is activated via binding to the N-terminal domain of the IP3R (10). These results are consistent with the conformational coupling hypothesis (1) according to which SOC is regulated via an interaction with IP3R which acts as the “sensor” of the status of the internal Ca\(^{2+}\) store(s). In addition to this Trp-IP3R interaction, Trp proteins have also been proposed to be regulated via association either as homomers, or heteromers with other Trps, to form the SOC channel (3). However, little is known regarding the molecular mechanisms that govern the interactions between the various Trp proteins or between Trp and other proteins. Also unclear is...
how such interactions result in the activation, or inactivation, of the putative Trp channels or SOC.

Functionally distinct microdomains, formed by the lateral packing of glycosphingolipids and cholesterol within the membrane bilayer, have been identified in plasma membranes (11). These domains, called lipid raft domains (LRD), are characterized by their insolubility in nonionic detergents under certain conditions, e.g. 1% Triton X-100 at 4 °C. LRD have been proposed to be involved in the sorting of proteins in the Golgi and their delivery to the plasma membrane (12). LRD have also been implicated in cellular signaling mechanisms, where they have been proposed to serve as scaffolds to facilitate the association of signaling complexes, increase the rate of interactions, and enhance crosstalk networks (13). Among the various signaling proteins known to be associated with raft domains are G-protein α subunits (14), Src family tyrosine kinases (15), and eNOS (16). Furthermore, key protein and non-protein molecules involved in the Ca²⁺ signaling cascade, such as phosphatidylinositol 4,5-biphosphate (PIP₂) (17), Go-µ1 (18), muscarinic receptor (19), and Ca²⁺ signaling events such as the turnover of PIP₂ have been localized to such microdomains in the plasma membrane (20). Notably, Ca²⁺ signaling mediated via bradykinin receptor stimulation (18) was altered when LRD were disrupted by cholesterol depletion. In aggregate, these previous reports suggest that the integrity of the LRD is a critical element in the regulation of Ca²⁺ signaling.

Plasma membrane domains, called caveolae, have also been shown to play a crucial role in signal transduction mechanisms (13). It has now been shown that caveolae are formed through a coalescence of LRDs. Caveolin-1, a cholesterol-binding protein which is enriched in LRD, has been proposed to be a scaffolding protein involved in the recruitment/association of specific proteins into LRD (13). Notably, a number of proteins, that are known to be present in LRD, have one or more binding sites for caveolin. Thus, arrangement of the lipids and scaffolding proteins within LRD forms a platform for the coordination of cellular signaling events.

To elucidate the role of Trp1 in SOC, we have investigated possible molecular interactions involved in the regulation of endogenous hTrp1 and SOC in human submandibular gland cells, HSG. These cells provide a well characterized experimental system to study the regulation of SOC (8, 21, 22). The data presented below demonstrate for the first time that hTrp1 is localized in a caveolin-scaffolding LRD in the plasma membrane where it interacts, either directly or indirectly, with a complex of Ca²⁺ signaling proteins, such as IP₃R and Go-µ1. Furthermore, the integrity of the LRD appeared to be critical in the regulation of SOC and for the association of Trp1 with LRD. Thus, this study reveals that the molecular architecture of the LRD is likely to be important in determining the localization and regulation of Trp1 and the SOC mechanism.

MATERIALS AND METHODS

Preparation of HSG Cell Lysates—HSG cells were cultured as described earlier (8, 21, 22). HSG cells were stably transfected with hTrp1 cDNA as described earlier (8). Cells were detached, suspended in ice-cold phosphate-buffered saline containing 1% (w/v) aprotinin (Sigma), centrifuged for 5 min at 900 x g, and resuspended in a lysis buffer containing (in mM): 100 Tris-HCl (pH 8.0), 1 M MgCl₂, 0.5 4-(2-aminoethyl)-benzenesulfonfluoride hydrochloride (ICN Biomedicals Inc., Aurora, OH), 0.1 phenylmethylsulfonfluoride (Calbiochem, La Jolla, CA), and 0.1% (v/v) Triton X-100 for at least 2 h before use.

Preparation of Crude Membranes—HSG cell lysates (3-5 ml) were thawed on ice, homogenized in a Dounce homogenizer, and diluted into sucrose buffer consisting of (final concentration): 0.25 M sucrose, 10 mM Tris-HEPES (pH 7.4), 1% aprotinin (w/v), 1 mM diithiothreitol (Calbiochem, Ultrlgrade). The homogenate was centrifuged at 3000 x g for 15 min at 4 °C and the resultant supernatant centrifuged at 50,000 x g for 30 min at 4 °C. The pellet was resuspended in a minimal volume of the sucrose buffer and stored at ~80 °C until use. Protein concentration was determined by using the Bio-Rad protein assay (microassay procedure).

Detergent Extraction of HSG Cell Membranes—Crude membranes were solubilized essentially using a modified method described by Skibbens et al. (23). 1 mg of crude membranes (2 mg/ml) was sonicated for 20 min at 4 or 37 °C, in a medium containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA with either 1.5% n-octyl-β-D-glucopyranoside (w/v) or 1% Triton X-100 (w/v). 1 mM KI was added where indicated to disrupt possible interactions with cytoskeletal proteins (24). Samples were centrifuged at 4 °C for 1 h at 145,000 x g. The supernatant was stored at ~80 °C until use.

SDS-PAGE and Western Blotting—Samples were diluted in a buffer containing 50 mM Tris-HCl (pH 8.8), 5 mM EDTA, and 1% SDS. Protein concentrations were determined according to the method of Schaffner and Weissmann (25). Conditions for SDS-PAGE and Western blotting were as described previously (26). Polyvinylidene difluoride membranes were blocked and incubated for 1 h with 1:1000 dilution of the primary antibodies (as indicated in the figure legends). The membrane was washed with TTBS, incubated as required with either anti-rabbit IgG or anti-mouse IgG, washed, treated with ECL reagents (Amersham Pharmacia Biotech), and exposed to X-Omat™ films (Kodak).

Depletion of Cholesterol with Methyl-β-cyclooctatrin—Confluent HSG cells were treated with 10 mM methyl-β-cyclooctatrin (CD) for 30 min at 37 °C as described (27, 28). Cells were then washed with 20 ml of ice-cold phosphate-buffered saline with 1% (v/v) aprotinin and harvested in ice-cold extraction buffer containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% (w/v) Triton X-100, and 1% (v/v) aprotinin. Cells were homogenized in a Dounce homogenizer, incubated on ice for 20 min, and centrifuged at 10,000 x g for 2 min. The supernatant was then centrifuged at 10,000 x g for 30 s and the combined pellets were resuspended at room temperature in a minimal volume of a solubilization buffer consisting of 50 mM Tris-Cl (pH 8.8), 5 mM EDTA, and 1% SDS. The supernatants were concentrated using Centriprep (Amicon Corp.) concentrators. Protein concentration was determined according to Schaffner and Weissmann (25).

Optiprep Flotation Gradients—Flotation gradients were performed as described in Scheiffele et al. (29) with the following modifications. 250 μg of HSG crude membranes were pelleted by centrifugation for 15 min at 60,000 x g at 4 °C and resuspended in 250 μl of extraction medium containing 1% Triton X-100 and 1 mM KI, and incubated for 20 min at either 4 or 37 °C as indicated in figure legends. In some experiments, 1.5% n-octyl-β-D-glucopyranoside (w/v) was included in the buffer instead of Triton X-100.

Immunoprecipitation—Cells were lysed and crude membrane was prepared as described above. Membranes were treated with 0.5% Nonidet P-40 and the solubilized fraction was collected by centrifugation at 20,000 x g for 60 min and precleared by incubation with an excess of the protein A beads and centrifugation. 200 μl of the cleared sample was incubated either with 5 μg of monoclonal anti-caveolin-1 (Santa Cruz Biotechnology, Inc.) or 10 μg of polyclonal anti-Trp1 (26) for 3 h at 4 °C. Immunocomplexes were pulled down with 30 μl of 50% protein A beads for 1 h at 4 °C. Beads were washed with buffer containing 500 mM NaCl, 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonfluoride, 0.5% Nonidet P-40, 10% sucrose, 1 μg/ml aprotinin, leupetin, and pepstatin and treated with SDS solubilization buffer. Proteins were detected by SDS-PAGE and Western blotting. The following primary antibodies were used at 1:1000 dilution: anti-Trp1, anti-caveolin-1, anti-IP₃R, and anti-calmodulin (Transduction Laboratories), and anti-Go-µ1 (Upstate Biologicals). The secondary antibodies and conditions for the ECL reaction were as described above.

Ca²⁺ Measurement—Fura2 fluorescence in single cells was measured as described earlier (8, 21) using an SLM 8000/DMX 100 spectrophotometer attached to an inverted Nikon Diaphot microscope with a Fluor ×40 oil-immersion objective. Images were acquired using an enhanced CCD camera (CCD-72, MTI) and the Image-1 software (Universal Imaging Corp., West Chester, PA). Analog plots of the fluorescent ratio (340/380) in single cells are shown.

Measurement of Ca²⁺ Current—The store-operated inward Ca²⁺ current (iSC) was measured using the whole cell patch clamp technique as described before (22). The external solution contained 135 mM K-glutamate, 1 mM MgCl₂, 10 mM CaCl₂, 10 mM gluconate, 10 mM HEPES (pH 7.4) (NaOH). The pipette solution contained 135 mM N-methyl-D-glutamate, 10 mM CsCl, 1 mM MgCl₂, 1 mM ATP, 10 mM HEPES (pH 7.2) (CsOH), and 10 mM BAPTA. When required, 100 μM IP₃ was included in the pipette solution. Membrane potential was maintained at 0 mV. Within the time frame of these experiments, no inward currents.
FIG. 1. Detergent solubility of hTrp1. HSG cell membranes were treated with buffer containing either 1% Triton X-100 (A) or 1.5% octylglucoside (OG, B) at 4 °C (A, left panel, and B) or 37 °C (A, right panel). Soluble (S) and insoluble (P) were separated by SDS-PAGE, and endogenous hTrp1 was detected (shown by arrow) by Western blotting using an anti-Trp1 antibody. Similar results were obtained in at least three experiments for each condition.

were activated in the absence of IP₃. Other details are mentioned in the figure legends and text.

RESULTS

Association of hTrp1 with the Triton X-100 Insoluble Fraction of HSG Cell Membranes—Fig. 1A shows that when HSG cell membranes were extracted with 1% (w/v) Triton X-100 at 4 °C, the endogenous Trp1 (hTrp1) remained associated with the insoluble pellet (P, lane 2) and was not detected in the solubilizate (S, lane 1). Endogenous Trp1 was detected using the Trp1-specific polyclonal antibody we have described previously (8, 26). Since cytoskeletal components of the cell are also resistant to Triton X-100, cell membranes were treated with Triton X-100 in the presence of 1 M KI, which is known to disrupt protein-cytoskeletal interactions (24). Lane 3 shows that in the presence of KI, relatively more hTrp1 was extracted into the solubilizate (S) and less was left in the insoluble pellet (P, lane 4). When membranes were treated with Triton X-100 alone at 37 °C (right panel, Fig. 1A), again hTrp1 was not solubilized (see lanes 1 and 2). However, when KI was included in the solubilization medium the protein was almost completely solubilized (see lanes 3 and 4). These results are consistent with the association of Trp1 in a detergent-resistant membrane fraction. Furthermore, the increased solubility of hTrp1 when KI was included during solubilization suggests that it interacts with cytoskeletal proteins. However, the insolubility in Triton X-100 + KI at 4 °C suggests that part of the total Trp1 protein is associated with LRD. LRD have been shown to be more effectively solubilized by octylglucoside (30). Thus, to further demonstrate the association of hTrp1 with LRD, HSG cell membranes were treated with 1.5% (w/v) octylglucoside at 4 °C. hTrp1 was not solubilized (Fig. 1B, lanes 1 and 3) unless KI was included during solubilization, which resulted in complete solubilization of hTrp1 by the detergent, even at 4 °C (Fig. 1B, lanes 2 and 4). In aggregate, these data show that the fraction of Trp1 that is insoluble in Triton X-100 + KI at 4 °C can be solubilized by octylglucoside and thus is associated with LRD.

Association of hTrp1 with Low Density Fractions of Triton X-100-treated HSG Cell Membrane—Proteins interacting with lipid raft domains have been shown to associate with low density fractions of Triton X-100-extracted cell membranes, while solubilized proteins and those linked to the cytoskeleton are recovered in the high density fractions (30). The differential density of proteins following treatment with 1% Triton X-100 at 4 °C versus 37 °C, is another criterion used to determine their association with lipid raft domains. When HSG cell membranes were treated with Triton X-100 + KI at 4 °C (Fig. 2A), hTrp1 was detected in both the low and high density fractions. This result is consistent with the data in Fig. 1 showing that Trp1 is only partially solubilized by treatment with Triton X-100 + KI at 4 °C. Importantly, following treatment with Triton X-100 at 37 °C, hTrp1 was enriched in the heaviest fraction (number 6, Fig. 2B). This again is consistent with the data in Fig. 1 showing that almost all of the Trp1 was solubilized under these conditions. A similar pattern was obtained when membranes were treated with Triton X-100 at 37 °C in the absence of KI (data not shown). Together with the insolubility of hTrp1 in Triton X-100 at 37 °C (Fig. 1A), these data suggest that (i) a fraction of Trp1 is associated with LRD, and (ii) Trp1 also interacts with the cytoskeleton. Notably, when membranes were treated with octylglucoside at 4 °C in the presence of KI, hTrp1 is completely recovered in the high density fractions (Fig. 2C). These results are consistent with the data in Fig. 1B and suggest that lipid raft-associated hTrp1 is solubilized by octylglucoside. Thus, in aggregate the data in Figs. 1 and 2 suggest that the detergent insolubility of hTrp1 is due to its interactions with both LRD and with cytoskeletal or other protein components in the plasma membrane.

As mentioned above caveolin-1 is a cholesterol-binding scaffolding protein known to be present in LDR. Thus, the presence of caveolin-1 is used to identify LRD membrane fractions. We examined the presence of caveolin-1 in the fractions described above for hTrp1. In the data shown in Fig. 2D, the lower portion of the same membrane used for hTrp1 was used to probe for caveolin-1. Caveolin-1 was also detected in the low density fractions following treatment of membranes with Triton X-100 at 4 °C and high density fractions after treatment with Triton X-100 at 37 °C (data not shown). These data provide further evidence for the association of hTrp1 with caveolin-enriched lipid raft domains.

Effect of Plasma Membrane Cholesterol Depletion on the Solubility of hTrp1—It is well established that cholesterol is required for the formation and structural integrity of LRD (11, 12). Furthermore, depletion of membrane cholesterol has been shown to alter the association and function of proteins in this domain. HSG cells were treated with methyl-β-cyclodextrin, CD, an agent reported to deplete plasma membrane cholesterol (31). Fig. 3 shows that more Trp1 protein was obtained in the Triton X-100 extract of CD-treated cells (lane 1) than control, untreated, cells (lane 2). These data convincingly demonstrate that hTrp1 is associated with cholesterol-sphingolipid raft domains in the plasma membrane of HSG cells.

Effect of Disruption of Lipid Raft Domains on SOC—The involvement of lipid raft domains in HSG cell Ca²⁺ signaling was assessed by examining carbachol-stimulated [Ca²⁺], mobilization in control and CD-treated HSG cells.
tion was determined by stimulating the cells with carbachol (100 μM) in the presence or absence of external Ca\(^{2+}\). In the presence of 1 mM Ca\(^{2+}\) in the external medium, control HSG cells display a typical response (21, 32, 33); an initial rapid increase in [Ca\(^{2+}\)]\(_i\) (represented as the ratio of 340/380 nm fura 2 fluorescence) which was followed by a relatively sustained elevation of [Ca\(^{2+}\)]\(_i\), (Fig. 4A). The initial rise in [Ca\(^{2+}\)]\(_i\) is a result of IP\(_3\)-dependent internal Ca\(^{2+}\) release, as it is also detected in cells in the absence of external Ca\(^{2+}\), i.e. in a nominally Ca\(^{2+}\)-free medium (Fig. 4D). The sustained increase in [Ca\(^{2+}\)]\(_i\), which is due to the influx of extracellular Ca\(^{2+}\), is not detected in the absence of external Ca\(^{2+}\) (Fig. 4D) or in the presence of inhibitors of Ca\(^{2+}\) influx (data not shown). Carbachol-stimulated [Ca\(^{2+}\)]\(_i\) elevation was completely blocked in CD-treated HSG cells in a Ca\(^{2+}\)-containing medium (compare Fig. 4, A and B, average data shown in 4C). [Ca\(^{2+}\)]\(_i\) in CD-treated cells after stimulation with carbachol was not different from resting [Ca\(^{2+}\)]\(_i\). Additionally, resting [Ca\(^{2+}\)]\(_i\) was not affected by the CD treatment. More importantly, the loss of [Ca\(^{2+}\)]\(_i\) increase was also seen when CD-treated cells were stimulated with carbachol in a Ca\(^{2+}\)-free medium (Fig. 4D and E, average data shown in F). These data demonstrate that disruption of LRD in HSG cells results in the loss of carbachol-stimulated internal Ca\(^{2+}\) release. This could be due to alterations in the muscarinic receptor-mediated signaling mechanisms which result in the inhibition of IP\(_3\) generation. The lack of internal Ca\(^{2+}\) release accounts for the inhibition of carbachol-stimulated Ca\(^{2+}\) influx.

To directly assess the role of LRD in SOC and to examine the effects of CD treatment on the internal Ca\(^{2+}\) store, thapsigargin-stimulated [Ca\(^{2+}\)] mobilization was measured as described above in control and CD-treated HSG cells. When thapsigargin was added to control HSG cells in a Ca\(^{2+}\)-containing medium (Fig. 5A), there was an initial fast increase in [Ca\(^{2+}\)]\(_i\), which was followed by a relatively sustained elevation of [Ca\(^{2+}\)]\(_i\). Although the [Ca\(^{2+}\)]\(_i\) gradually decreased, it remained above resting for more than 10 min. Stimulation of HSG cells with thapsigargin in a Ca\(^{2+}\)-free medium induced a lower and transient increase in [Ca\(^{2+}\)]\(_i\), (compare Fig. 5, A and D). The lower peak [Ca\(^{2+}\)]\(_i\), is consistent with previous observations and indicates that both release of Ca\(^{2+}\) from internal stores and Ca\(^{2+}\) influx contribute to the maximum increase in [Ca\(^{2+}\)]\(_i\), induced by thapsigargin (21, 32, 33). The transient [Ca\(^{2+}\)]\(_i\) increase (which reaches resting [Ca\(^{2+}\)]\(_i\), within 3–5 min) represents thapsigargin-stimulated emptying of internal Ca\(^{2+}\) stores. Thapsigargin stimulation of CD-treated cells in a Ca\(^{2+}\)-containing medium resulted in [Ca\(^{2+}\)]\(_i\) elevation that was significantly lower than that in control cells. This was true for both the peak (i.e. maximum [Ca\(^{2+}\)]\(_i\)), elevation as well as sustained [Ca\(^{2+}\)]\(_i\), elevation (compare Fig. 5, A and B). Average data are presented in Fig. 5C, sustained [Ca\(^{2+}\)]\(_i\), is represented by the [Ca\(^{2+}\)]\(_i\), at 7.5 min. At 7.5 min, [Ca\(^{2+}\)]\(_i\), in CD-treated cells was similar to resting [Ca\(^{2+}\)]\(_i\). Stimulation of CD-treated HSG cells with thapsigargin in a Ca\(^{2+}\)-free medium induced a transient increase in [Ca\(^{2+}\)]\(_i\), that was not significantly different from that in control cells (compare Fig. 5, D and E, average data are presented in Fig. 5F, peak [Ca\(^{2+}\)]\(_i\), and that at 3 min are shown). These data demonstrate that thapsigargin-stimulated Ca\(^{2+}\) influx, i.e. SOC, is directly affected by disruption of cholesterol-sphingolipid raft regions in the plasma membrane of HSG cells. These data also demonstrate that CD treatment does not affect the internal Ca\(^{2+}\) store.

We have previously shown that stable transfection of HSG cells with htrp1a cDNA induced an increase in: (i) the Trp1 protein in the plasma membrane, and (ii) thapsigargin-stimulated SOC (8). To further establish a link between Trp1 and SOC, hTrp1a-expressing cells were treated with CD and thapsigargin-stimulated internal Ca\(^{2+}\) release and sustained}

Fig. 3. Plasma membrane cholesterol depletion increases solubility of hTrp1. Conditions for β-cyclodextrin (CD) treatment are described under “Experimental Procedures.” Western blot shows hTrp1 (indicated by arrow) in Triton X-100 extracts from CD-treated (lane 1) and control (lane 2) HSG cells.

Fig. 4. Effect of β-cyclodextrin treatment on carbachol-stimulated [Ca\(^{2+}\)] mobilization in HSG cells. Cells grown on coverslips were treated with CD as described for Fig. 3. Fura 2 fluorescence was measured microfluorimetrically in control (A and D) or CD-treated (B and E) HSG cells in either Ca\(^{2+}\)-containing or Ca\(^{2+}\)-free medium as indicated. Carbachol (100 μM) was added where shown by the arrow. Figures show representative traces and average data are shown in C and F; number of cells are indicated (n). Statistical analysis was performed using the Student’s t test. ** indicates values that are significantly different (p < 0.05) from that in CD-treated cells. Resting fura 2 fluorescence ratio in control cells was 0.56 ± 0.02 and this was not significantly different from that in CD-treated cells.
[Ca\textsuperscript{2+}]i elevation were measured as described above (Fig. 6).

Consistent with our previous observation, thapsigargin-stimulated [Ca\textsuperscript{2+}]i mobilization was measured as described for carbachol in the legend to Fig. 4 in non-transfected HSG cells (control cell, A and C) and CD-treated cells (B and E). Representative traces are shown and average data are presented in C and F. [Ca\textsuperscript{2+}], (340/380 nm ratio) obtained for peak and at 7.5 min (i.e. sustained level) in cells in Ca\textsuperscript{2+}-containing medium and for peak and at 3 min in cells in Ca\textsuperscript{2+}-free medium are given. Statistical analysis was performed using the Student's t test. **, denotes values significantly different (p < 0.05) from that in CD-treated cells.

FIG. 5. Effect of \(\beta\)-cyclodextrin treatment on thapsigargin-stimulated [Ca\textsuperscript{2+}]i mobilization in HSG cells. Thapsigargin (Tg)-stimulated internal [Ca\textsuperscript{2+}]i mobilization was measured as described for carbachol in the legend to Fig. 4 in non-transfected HSG cells (control cell, A and C) and CD-treated cells (B and E). Representative traces are shown and average data are presented in C and F. [Ca\textsuperscript{2+}], (340/380 nm ratio) obtained for peak and at 7.5 min (i.e. sustained level) in cells in Ca\textsuperscript{2+}-containing medium and for peak and at 3 min in cells in Ca\textsuperscript{2+}-free medium are given. Statistical analysis was performed using the Student's t test. **, denotes values significantly different (p < 0.05) from that in CD-treated cells.

FIG. 6. Effect of \(\beta\)-cyclodextrin treatment on thapsigargin-stimulated [Ca\textsuperscript{2+}]i mobilization in htrp1a-transfected HSG cells. Thapsigargin (Tg)-stimulated internal [Ca\textsuperscript{2+}]i mobilization was measured as described in the legend to Fig. 5 in HSG cells stably transfected htrp1a-CDNA (Trp1 cell); control cells (A and D) and CD-treated cells (B and E). Figure shows representative traces and average data are presented in C and F, number of cells in each case is indicated (n). [Ca\textsuperscript{2+}], values obtained for peak and at 7.5 min (sustained) are given. Statistical analysis was performed using the Student's t test. **, denotes values significantly different (p < 0.05) from that in the CD-treated cells. Note that peak and sustained [Ca\textsuperscript{2+}], values in thapsigargin-stimulated, htrp1a-transfected cells in complete medium were significantly higher than in the non-transfected cells (p < 0.05, comparing values from Fig. 6A with those from Fig. 5A, see Figs. 5C and 6C). Peak and sustained increase in [Ca\textsuperscript{2+}], measured under these conditions were similar to that in CD-treated non-transfected HSG cells (see Fig. 5) or in htrp1a-transfected cells in a Ca\textsuperscript{2+}-free medium (Fig. 6, D and E). Importantly, in the Ca\textsuperscript{2+}-free medium, thapsigargin-stimulated [Ca\textsuperscript{2+}], increase was similar in control and CD-treated htrp1a-transfected cells (compare Fig. 6, D and E, average data shown in F). Thus, CD treatment inhibited SOC in HSG cells expressing only the endogenous Trp1 protein, with normal levels of Ca\textsuperscript{2+} influx, and those expressing exogenous hTrp1a protein, with increased Ca\textsuperscript{2+} influx. These data suggest that...
the SOC activity associated with the expressed Trp1α, like the endogenous SOC, is also functionally associated with LRD. These results are consistent with our finding that Trp1 is localized in LRD.

Effect of CD Treatment on \(I_{\text{SOC}}\) in HSG Cells—Depletion of internal Ca\(^{2+}\) stores in HSG cells, by either IP\(_3\) or thapsigargin, generates an inward Ca\(^{2+}\) current \(I_{\text{SOC}}\) (see Ref. 22). The data described above demonstrate that carbachol-mediated Ca\(^{2+}\) signaling was blocked by CD treatment. As mentioned above, this could be due to inhibition of IP\(_3\) generation. Alternatively, it could also be due to an effect of CD at a step distal to IP\(_3\) generation. To examine the latter possibility, \(I_{\text{SOC}}\) was measured using the whole cell patch clamp technique with IP\(_3\) stores. Consistent with the data shown in Figs. 5 and 6, there was no increase in SOC activity in CD-treated cells from resting current.

Association of Trp1 with Caveolin-1 and Ca\(^{2+}\)-Signaling Proteins in HSG Cell Plasma Membranes—The data presented above suggest that caveolin-1 and Trp1 are localized in LRD in HSG cell plasma membranes. As discussed above, caveolin-1 is known to be involved in the recruitment of proteins into LRD by interacting with caveolin-binding domains that have been identified in various proteins (34). Thus, we examined the amino acid sequence of hTrp1 for potential caveolin-1-binding domains. We identified two such domains in hTrp1 between amino acid residues 281–307 and 626–635, respectively. To assess possible interactions between hTrp1 and caveolin-1, we used anti-Trp1 (Fig. 8, lane 1) or anti-caveolin-1 (Fig. 8, lane 2) for immunoprecipitation of hTrp1 and caveolin-1, respectively, from Nonidet P-40-solubilized extracts of HSG cell membranes. Both hTrp1 (upper panel) and caveolin-1 (lower panel) were detected in the immunoprecipitates obtained with either antibody, suggesting an interaction between these two proteins. Notably, more Trp1 was detected in the immunoprecipitate with anti-Trp1 than with anti-caveolin-1 (compare the Trp1 band in lanes 1 and 2, upper panel). In the case of caveolin-1 (lower panel), the pattern was reversed. More caveolin-1 was detected in the immunoprecipitate with anti-caveolin-1 than with anti-Trp1. These data demonstrate that part of the Trp1 in HSG cell membranes is associated with caveolin-1, a finding consistent with the data in Figs. 1 and 2 that show that part of the Trp1 is localized in LRD.

Since β-cycloexetrin treatment disrupted carbachol-stimulated signaling, we examined the possible association of the G-protein involved in this signaling (Goq/11) with Trp1 and caveolin-1. Notably, Goq/11 has previously been localized to LRD (13). As shown in the middle panel, Goq/11 was also detected in both immunoprecipitated fractions. Thus, the same component of proteins was detected with either antibody. The detection of Goq/11 in the immunoprecipitates suggests that hTrp1 is either directly or indirectly associated with this G-protein. PKCa, a protein reported to associate with caveolin-1, is not immunoprecipitated with anti-Trp1 under the same experimental conditions (data not shown).

Mammalian Trp proteins have been proposed to be regulated via an interaction with IP\(_3\)R (1–3). hTrp3 has been shown to immunoprecipitate with IP\(_3\)R1 (10). We therefore examined the presence of IP\(_3\)R subunits in the caveolin-scaffolding LRD which contain Trp1 and Goq/11. The data in Fig. 9 show that both anti-caveolin-1 and anti-Trp1 can immunoprecipitate IP\(_3\)R3. These data are highly significant since they demonstrate that IP\(_3\)R, a key molecule in the activation of SOC, is assembled in a signaling complex along with Trp1, caveolin-1, and Goq/11. We have not yet determined whether the other two IP\(_3\)R subtypes are also present in this signaling complex.

**DISCUSSION**

The data presented above demonstrate for the first time that endogenous Trp1 is assembled in a multimeric complex of Ca\(^{2+}\) signaling proteins. We have shown that this complex is associated with caveolin-scaffolding LRD in the plasma membrane and includes the proteins IP\(_3\)R, caveolin-1, and Goq/11. Trp1 fulfilled several criteria that have been used to describe the...
association of proteins with such domains: (i) insolubility following treatment of HSG cell membranes with Triton X-100 at 4 °C, even after inclusion of KI to disrupt cytoskeletal interactions; (ii) presence in low density fractions of membranes treated with Triton X-100 + KI at 4 °C; (iii) appearance in the high density fraction of membranes treated with Triton X-100 + KI at 37 °C; (iv) increased solubilization with Triton X-100 following treatment of cells with CD to deplete plasma membrane cholesterol; (v) co-migration with caveolin-1 on density gradients and co-immunoprecipitation with caveolin-1. In addition, these data reveal that a part of the Trp1 (the fraction solubilized with Triton X-100 + KI at 4 °C, which is recovered in the high density fraction on the density gradient) is associated with non-raft regions in HSG cell. The requirement of KI for Trp1 solubilization by either Triton X-100 or octylglucoside at 4 °C suggests that it is also associated with cytoskeletal proteins in both the raft and non-raft regions. Trp1 has dystrophin and ankyrin domains which can be affected by KI. Thus, the increased solubilization of Trp1 by KI might involve alterations in the interactions of Trp1 with other proteins that involve these domains. Notably, cytoskeletal proteins like actin have been shown to be concentrated in caveolin-rich membrane raft regions of the plasma membrane (35, 36). Furthermore, it is interesting to note that actin polymerization was reported to induce complete inhibition of SOC in DDT1MF-2 smooth muscle cells, while cytoskeletal disruption with cytochalasin D did not affect SOC (32). Thus, Trp-cytoskeletal interactions might be potentially important in the regulation of its function.

The role of LRD in the activation or inactivation of proteins is not similar for all proteins associating with these domains. There are several examples of signaling proteins that appear to partition into membrane raft regions under specific conditions. For example, Ras is known to associate with LRD only in its inactive GDP-bound state (14). In contrast, the muscarinic receptor is reported to move into these domains upon binding to an agonist but not to an antagonist (19). It has been suggested that caveolae can act as scaffolds for pre-assembled signaling complexes which can be readily activated following stimulation of cells. The available data demonstrate that the function of Ca^{2+} signaling proteins depends on the integrity of the cholesterol-rich LRD (13). Furthermore, as discussed above, several components involved in Ca^{2+} signaling have been localized to LRD. Consistent with this, we have shown here that muscarinic agonist (carbachol)-stimulated Ca^{2+} mobilization and IP_{3}-dependent activation of I_{SOC} in HSG cells are blocked by disruption of LRD. However, while our data demonstrate that a step distal to IP_{3} generation is affected by CD treatment, earlier steps in the signaling cascade leading to IP_{3} generation might also be blocked. In addition, G_{a_{q/11}} and IP_{3},R were co-immunoprecipitated with caveolin-1. These data suggest that the muscarinic receptor-associated Ca^{2+} signaling system is functionally associated with LRD in HSG cells. More importantly, we have also shown that thapsigargin-stimulated Ca^{2+} influx is attenuated when these domains are disrupted. Thus, the SOC channel itself, or its associated regulatory proteins, also appears to be associated with these specialized microdomains. Notably, we have also established a link between SOC, Trp1, and LRD by demonstrating that disruption of LRD also blocks the increased Ca^{2+} influx associated with hTrp1 expression in HSG cells. Thus, these data functionally localize Trp1 to caveolar LRD in the plasma membrane. However, the present data do not clarify whether LRD disruption affects SOC channel activity per se or interferes with the mechanism involved in its activation.

An important finding of this study is that the IP_{3}R3 can be immunoprecipitated by using either anti-caveolin-1 or anti-Trp1. Thus, hTrp1 behaves like Trp3 (10) in that it is apparently associated with the IP_{3},R. In addition, these data are also consistent with previous observations that the IP_{3},R is localized in caveolar domains (37, 38). It will be important to determine whether all three IP_{3},R subtypes are localized in these domains and whether there is any specificity to the IP_{3}R-Trp interaction(s). A key question that arises out of these findings is how IP_{3},R, which is localized in the endoplasmic reticulum interacts with caveolin-1, Trp1, or LRD, that are present in the plasma membrane. Recent studies (39) have suggested that PIP_{2} is an inhibitor of the IP_{3},R in resting cells and that agonist-stimulated hydrolysis of PIP_{2} induces activation of IP_{3},R. This study proposes that the N-terminal domain of the IP_{3}, receptor would span the region between the endoplasmic reticulum and the plasma membrane and interact with PIP_{2} via a PIP_{2}-binding domain. PIP_{2} has been reported to be a major component of caveolar LRD and bulk of the PIP_{2} hydrolysis has been reported to occur in these domains (17). Thus, the present data are consistent with these previous studies and suggest that the PIP_{2}-IP_{3},R interactions are facilitated by the molecular architecture of the LRD. Another significant conclusion from our findings is that IP_{3},R likely interacts with both Trp1 and PIP_{2}
in the same spatially restricted membrane domain. Thus, we suggest that caveolar LRD provide a scaffold to coordinate and increase the rate of interactions between these three key Ca\(^{2+}\) signaling molecules, which are likely to be critical in the activation of internal Ca\(^{2+}\) release and SOC.

Several post-translational modifications such as glycosylation, palmitoylation, and myristoylation have been shown to direct proteins to LRD (11, 12, 40). In addition, certain amino acid alignments within transmembrane domains can also interact with the lipid bilayer and determine the partitioning of proteins into the LRD (27). Recently, protein-protein interactions have been implicated in the recruitment of proteins into LRD. The scaffolding domain in caveolin-1, a major protein component of LDR, has been suggested to interact with caveolin-binding domains in proteins (13), thus facilitating the association or recruitment of such proteins into LDR. We have identified two putative caveolin-binding domains (34) in hTrp1 in the N- and C-terminal regions, amino acid residues 281–307 and 626–635, respectively. Consistent with the presence of these domains, our data demonstrate that Trp1 and caveolin-1 coimmunoprecipitate when either anti-Trp1 or anti-caveolin-1 antibody is used. This result strongly suggests that there is a physical interaction between hTrp1 and caveolin-1. It is interesting to note that the second putative caveolin-binding domain includes the conserved amino acid motif which is present in all currently identified Trps (EWKFKAR). In addition, as mentioned above Trp1 also has dystrophin, ankyrin, and coiled-coiled domains in its C-terminal and N-terminal regions. Thus, Trp1 might associate with other proteins via these domains or might associate more directly with cytoskeletal or other scaffolding proteins. Trp1 might also interact with the lipid components of LRD via other domains such as pleckstrin homology domains. The Drosophila Trp has been shown to be assembled in a signaling complex through interactions mediated by the PDZ domain protein, INAD. Protein interactions with INAD have been proposed to have a role both in the activation and inactivation of phototransduction. As mentioned earlier, a mammalian homolog of INAD has been cloned (7). We have also detected INAD-like transcripts in HSG cells. Further studies will be required to determine whether an INAD-like protein is also involved in the localization and regulation of the signaling complex associated with mammalian Trp protein(s).

In summary, the data presented here demonstrate for the first time that Trp1, a candidate protein for the SOC channel mechanism, is associated with a caveolar LRD in the HSG cell plasma membrane, where it is assembled into a signaling protein complex that includes caveolin-1, IP\(_3\)R3, and G\(_{\alpha_{11}}\). Fig. 10 shows our proposed model for the localization of Trp1 and associated signaling molecules in a LRD. It will be important in future studies to determine what other proteins are present in this complex and which proteins interact directly with Trp1. We have shown here that disruption of LRD: (i) alters the Trp1-membrane interactions, as demonstrated by the change in the buoyancy of the protein, and (ii) attenuates thapsigargin-activation of endogenous SOC and the increased SOC associated with exogenous hTrp1\(_{10}\) expression. Thus, the present data localize Trp1 and the SOC mechanism to this specialized, spatially restricted plasma membrane microenvironment and suggest that the integrity of the LRD is critical for the regulation of SOC. Importantly, we have also shown that the IP\(_3\)R, which has been proposed as a key molecule in the regulation of SOC, also interacts with hTrp1 and is associated with the LRD. We hypothesize that protein-protein interactions facilitated by the molecular architecture of this membrane domain determine the activation and inactivation of Trp and thus SOC. Our observation that only a fraction of Trp is associated with caveolar LRD, and that Trp cannot support Ca\(^{2+}\) influx when LRD is disrupted, indicates that Trp1 in non-raft regions is not associated with activation of SOC. Thus, activation of SOC might involve recruitment of Trp1 into the lipid raft domains. Alternatively, protein-protein interactions could activate Trp1 which is preassembled in a protein complex within the LRD. This is consistent with the suggestion that caveolae contain pre-assembled complexes of signaling proteins that can be activated upon stimulation of the cells (11–13). In aggregate, the present data suggest a novel structural and molecular basis for the localization and regulation of SOC, i.e., via interactions between Ca\(^{2+}\) signaling proteins that are facilitated by a caveolin-raft LRD. Importantly, these studies also show for the first time that Trp1 is assembled in a signaling complex where it interacts with one or more key Ca\(^{2+}\) signaling proteins, including IP\(_3\)R3 and G\(_{\alpha_{11}}\). Identification of the mechanisms that determine these interactions will be critical in elucidating the physiological role of Trp1.

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Assembly of Trp1 in a Signaling Complex Associated with Caveolin-Scaffolding Lipid Raft Domains
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