Expression of Truncated Transient Receptor Potential Protein 1α (Trp1α)

EVIDENCE THAT THE TRP1 C TERMINUS MODULATES STORE-OPERATED Ca2+ ENTRY

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Transient receptor potential protein 1 (Trp1) has been proposed as a component of the store-operated Ca2+ entry (SOCE) channel. However, the exact mechanism by which Trp1 is regulated by store depletion is not known. Here, we examined the role of the Trp1 C-terminal domain in SOCE by expressing hTrp1α lacking amino acids 664–793 (ΔTrp1α) or full-length hTrp1α in the HSG (human submandibular gland) cell line. Both carbachol (CCh) and thapsigargin (Tg) activated sustained Ca2+ influx in control (nontransfected), ΔTrp1α-, and Trp1α-expressing cells. Sustained [Ca2+]i, following stimulation with either Tg or CCh in ΔTrp1α-expressing cells, was about 1.5–2-fold higher than in Trp1α-expressing cells and 4-fold higher than in control cells. Importantly, (i) basal Ca2+ influx and (ii) Tg- or CCh-stimulated internal Ca2+ release were similar in all the cells. A similar increase in Tg-stimulated Ca2+ influx was seen in cells expressing Δ2Trp1α, lacking the C-terminal domain amino acid 649–793, which includes the EWFKAR sequence. Further, both inositol 1,4,5-trisphosphate receptor-3 and caveolin-1 were immunoprecipitated with ΔTrp1α and Trp1α. In aggregate, these data suggest that (i) the EWFKAR sequence does not contribute significantly to the Trp1-associated increase in SOCE, and (ii) the Trp1 C-terminal region, amino acids 664–793, is involved in the modulation of SOCE.

Ca2+ influx in non-excitatory cells occurs via the store-operated Ca2+ entry (SOCE)1 mechanism, which is activated by the depletion of Ca2+ from the internal Ca2+ store (1–4). However, the nature of the signal that is transmitted from the internal Ca2+ store to the plasma membrane to trigger activation or inactivation of SOCE is not yet known. Three main models have been proposed for the activation of SOCE: (i) activation by second messengers such as cGMP or inositol 1,4,5-trisphosphate (IP3) or mediators such as the calcium influx factor, which are either generated with, or in response to, the release of Ca2+ from the internal Ca2+ store; (ii) recruitment of channels into the plasma membrane by a process involving vesicle fusion; (iii) a physical interaction between the SOCE channel in the plasma membrane and the IP3 receptor (IP3R) in the internal Ca2+ store membrane, i.e. the conformational-coupling hypothesis (1, 2, 5, 6). A major hurdle in establishing the mechanism of activation for SOCE has been the lack of information regarding the identity of the SOCE channel protein(s).

Recently, mammalian homologues of the Drosophila trp gene have been suggested to encode the SOCE channel protein (2, 3, 7). Seven different trps have been cloned. Expression of trp1 and trp4 was associated with increased SOCE, whereas expression of their antisense cDNAs resulted in a loss of SOCE (7–10). Expression of trp3 and trp6 induced increases in agonist- but not thapsigargin-stimulated Ca2+ influx (8, 11–14). Thus, although it is possible that some Trp proteins might be involved in SOCE, it remains to be established whether the Trps in fact form the SOCE channel. More importantly, studies with Trp proteins have provided data consistent with the conformational coupling hypothesis proposed for the regulation of SOCE. Two Trp proteins that have been shown to be localized in the plasma membrane, Trp3 and Trp1, appear to interact with the IP3 receptor(s) (15–18).

We have recently reported that Trp1 is a strong candidate for the SOCE mechanism in the human submandibular gland cell line (HSG) and that it is associated with a caveolar lipid domain where it is assembled in a signaling complex with proteins such as IP3R, Goq,, and caveolin (8, 17). We proposed that protein-protein interactions coordinated within this domain are involved in the activation or inactivation of SOCE. To determine which region of the Trp1 molecule is involved in the activation of SOCE, we have now expressed truncated forms of Trp1α that lack (i) the C-terminal domain aa 664–793 (ΔTrp1α) or (ii) the C-terminal domain aa 649–793, which includes the highly conserved EWFKAR sequence (Δ2Trp1α), in HSG cells and measured SOCE. The data demonstrate that activation of SOCE was not altered by expression of truncated Trp1. Importantly, SOCE was increased in these cells compared with those expressing full-length Trp1α, and IP3R3 was immunoprecipitated with ΔTrp1α. These data are novel and suggest that the C-terminal domain of Trp1 is involved in the modulation of Ca2+ influx via the SOCE pathway.

MATERIALS AND METHODS

DNA Manipulation, HSG Cell Culture, and Transfection—The 3’-untranslated 1.5-kilobase pair region of htrp1α cDNA used previously (8, 10) was deleted and full-length htrp1α was cloned into the pcDNA3 vector at the KpnI site. For the truncation, htrp1α was cleaved at the Xhol site (Δtrp1a, Fig. 1A) and religated. The plasmid was used to transform DH5α competent cells (Life Technologies, Inc.), and individual clones were selected. The truncated Trp1α protein (ΔTrp1α) has 9 amino acids (GGALFYSVT) carried over from the vector at the C-terminal domain (649–793), which includes the highly conserved EWKFAR sequence (Δ2Trp1α), in HSG cells and measured SOCE. The data demonstrate that activation of SOCE was not altered by expression of truncated Trp1. Importantly, SOCE was increased in these cells compared with those expressing full-length Trp1α, and IP3R3 was immunoprecipitated with ΔTrp1α. These data are novel and suggest that the C-terminal domain of Trp1 is involved in the modulation of Ca2+ influx via the SOCE pathway.
was different from that in ΔTrp1α. All of the constructs were analyzed using restriction analysis and DNA sequencing.

HSG cells were cultured and stably transfected as described earlier (8, 19, 20). Cells were lysed and crude membranes were prepared as described previously (8). Protein concentration was determined by the Bio-Rad protein assay.

Immunoprecipitation—Crude membranes were treated with 0.5% Nonidet P-40 or with 1.5 mM octylglucoside + 0.5 M KI, and centrifuged at 45,000 × g for 60 min. 200 μg of the pre-cleared supernatant was incubated with 10 μg of anti-HA antibody (Roche Molecular Biochemicals (17, 21). Immunocomplexes were pulled down with protein A, washed, and treated with SDS solubilization buffer. Proteins were detected by Western blotting as described previously (8, 21). Anti-caveolin-1 and anti-IP3-R3 (Roche Molecular Biochemicals) were used at 1:1000 dilution.

Confocal Microscopy—Cells were fixed, permeabilized, and treated with anti-HA antibody (1:50) and rhodamine-linked secondary antibody as described previously (8). Images were collected for confocal microscopy (8). The entire series of images was then collected into a single focused image using Confoal Assistant software supplied by the manufacturer.

(Ca²⁺) Measurements—Fura2 fluorescence in single cells was measured as described earlier (8, 17). Analog plots of the fluorescence ratio (340/380) in single cells are shown.

RESULTS

Expression of Truncated and Full-length Trp1α in HSG Cells—The C terminus of hTrp1α was deleted between aa 664 and 793 (see Fig. 1A). Truncated hTrp1α (ΔTrp1α) and full-length (Trp1α; both with N-terminal HA tag) were stably expressed in HSG cells. Molecular sizes of the inserts (Fig. 1B) were consistent with the predicted sizes, i.e. ΔTrp1α was 387 base pairs less than the full-length gene. These inserts were further characterized by digestion with restriction enzymes and sequencing (data not shown).

Fig. 1C shows Trp1α and ΔTrp1α in crude membranes isolated from stably transfected cells. The lower band (78 kDa) corresponds to the expected molecular weight of Trp1α. The proteins were also detected by the anti-Trp1 antibody. Notably, the relative level of endogenous Trp1 in ΔTrp1α-expressing cells was not higher than in Trp1α-expressing cells (data not shown).

Fig. 1D shows the immunolocalization of ΔTrp1α in HSG cells. Fluorescence was detected in the plasma membrane region of transfected cells, similar to the localization of stably expressed HA-tagged Trp1α in HSG cells (data not shown; see Ref. 8). Thus, deletion of the C terminus does not interfere with the localization of the Trp1α protein in HSG cells. Further, these data demonstrate that relatively similar levels of ΔTrp1α and Trp1α are expressed in HSG cells stably transfected with the respective cDNAs.

Thapsigargin- and Carbachol-stimulated Ca²⁺ Influx in ΔTrp1α- and Trp1α-expressing HSG Cells—Fig. 2 shows thapsigargin (Tg)-stimulated [Ca²⁺] changes in control cells (non-transfected, labeled HSG) in Trp1α- and ΔTrp1α-expressing HSG cells. Measurements were made in Ca²⁺-containing medium (Fig. 2, A and B) and in nominally Ca²⁺-free medium (Fig. 2, C and D). Average data are shown in the bar graphs (Fig. 2, B and D). In the presence of external Ca²⁺ (1.0 mM), Tg induced an initial, relatively fast increase in [Ca²⁺] in control cells, which returned to resting values in about 10 min. Part of the initial increase in [Ca²⁺], and the relatively sustained increase in [Ca²⁺], are caused by SOCE (8, 17). However, in cells stimulated with Tg in a Ca²⁺-free medium, peak [Ca²⁺], increases were lower, and the resting levels were reached within 4 min (Fig. 1C). The transient [Ca²⁺] increase is the result of internal Ca²⁺ release. With external Ca²⁺, Tg-stimulated initial peak increase was significantly higher (about 1.5-fold) in HSG cells expressing Trp1α. Further, the sustained elevation in [Ca²⁺], measured at 7.5 min after stimulation was also increased in these cells (by about 3-fold). These results are consistent with our previous studies (8). Importantly, cells expressing ΔTrp1α, also demonstrated an initial peak increase in [Ca²⁺], in response to Tg stimulation, which was similar to Trp1α cells but significantly higher than in control cells. More interestingly, the sustained elevation of [Ca²⁺] (at 7.5 min after stimulation) seen in these cells was about 1.5- and 4-fold higher than in Trp1α-expressing and control cells, respectively. Fig. 2C shows that Tg-stimulated similar [Ca²⁺], changes when cells were stimulated in a Ca²⁺-free medium. Thus, the internal Ca²⁺ store status and Ca²⁺ release are similar in control, Trp1α-, and ΔTrp1α-expressing cells and do not account for the increase in Ca²⁺ influx. Additionally, the resting Ca²⁺ permeabilities of the three sets of cells were similar. These were determined by the re-addition of 1.0 mM Ca²⁺ to cells in a Ca²⁺-free medium or by adding 5 or 10 mM Ca²⁺ to cells in a Ca²⁺-containing medium (data not shown).

To exclude effects due to the vector sequence carried over in ΔTrp1α, another truncated Trpl (ΔTrp1αv) was expressed in HSG cells, which lacked the vector sequence. Δ2Trp1α was also expressed, which lacked the C-terminal domain aa 649–793. Note that a vector sequence different from that in ΔTrp1α was carried over in Δ2Trp1α. Significantly, SOCE in cells stably transfected with these cDNAs, was similar to that in cells transfected with ΔTrp1α (Fig. 2, D and E). In all three cases, SOCE was higher than in control cells and those expressing Trp1α. Thus, the increased SOCE in cells expressing the truncated Trpl(s) is not due to contributions from the vector sequence that was carried over. Interestingly, the EWFKAR sequence does not appear to significantly affect Trp1 function (Fig. 2E). In aggregate, the data from Fig. 2 show that the activation of SOCE by Tg is not affected in HSG cells expressed-
ing Trp1α lacking the C-terminal domain. Importantly, these cells have a higher level of SOCE than cells expressing the full-length protein.

Carbachol (CCh)-stimulated Ca\(^{2+}\) mobilization was also measured in control and Trp1α and ΔTrp1α cells in Ca\(^{2+}\)-containing (Fig. 3, A and B) and Ca\(^{2+}\)-free (Fig. 3, C and D) medium. As seen with Tg, sustained [Ca\(^{2+}\)]\(_i\) increase (measured 5 min after stimulation) was significantly higher in ΔTrp1α cells than in Trp1α cells and control cells, by 1.8 and 4.4-fold, respectively. However, the initial peak increase was not significantly different in the three sets of cells. Fig. 3C shows that CCh-stimulated internal Ca\(^{2+}\) release is similar in all the cells. Thus, these data demonstrate that: (i) initial Ca\(^{2+}\) signaling events related to CCh stimulation of HSG cells are not altered by expression of ΔTrp1α or Trp1α; and (ii) the sustained Ca\(^{2+}\) increase seen in cells in Ca\(^{2+}\)-containing medium, i.e. Ca\(^{2+}\) influx, is increased in cells expressing the ΔTrp1α proteins. The Ca\(^{2+}\) entry component was also assessed by adding Ca\(^{2+}\) to cells treated with either CCh or Tg in Ca\(^{2+}\)-free medium. These results also demonstrated significantly (2-fold) higher [Ca\(^{2+}\)]\(_i\) elevation in ΔTrp1α cells compared with Trp1α cells (data not shown).

In aggregate, the data from Figs. 2 and 3 clearly demonstrate that SOCE is increased in HSG cells expressing the Trp1α proteins. More significantly, the data show that higher levels of Ca\(^{2+}\) influx are achieved when the truncated Trp1 proteins are expressed. Our data also rule out the possibility that this increase in SOCE is due to changes in the status of internal Ca\(^{2+}\) stores, an increase in basal Ca\(^{2+}\) influx, or differences in the level of protein expression.

Interaction of ΔTrp1α with IP\(_3\)R3 and Caveolin 1—To examine whether the C-terminal deletion alters the interaction of Trp1α with IP\(_3\)R, an anti-HA antibody was used to immunoprecipitate either ΔTrp1α (Fig. 4, lane 2) or Trp1α (Fig. 4, lane 1). The immunoprecipitates were then analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting using either anti-IP\(_3\)R3 or anti-caveolin1. The reactions shown in Fig. 4 clearly demonstrate that the relative level of IP\(_3\)R3 or caveolin1 co-immunoprecipitated with ΔTrp1α is similar to that seen with Trp1α.

**DISCUSSION**

We have previously reported that (i) expression of hTrp1 in HSG cells induces an increase in SOCE, and (ii) Trp1α in HSG cells interacts with IP\(_3\)R and caveolin 1 (8, 17). The main findings of this study are that deletions in the C-terminal region of Trp1α do not appear to alter its ability to increase SOCE in HSG cells. Further, the ability of Trp1α to interact with IP\(_3\)R3 and caveolin 1 is not altered, as shown by the coimmunoprecipitation of these proteins with ΔTrp1α. Importantly, the relative amounts of IP\(_3\)R3 coimmunoprecipitated with ΔTrp1α or Trp1α were similar. Previous studies have shown that the IP\(_3\)R N-terminal peptide (aa 1–787) induced spontaneous Trp3 channel activity, although when the IP\(_3\)R N terminus was linked to the IP\(_3\)R-transmembrane regions, IP\(_3\) was required for channel activation (15). Thus, it was proposed that Trp3 interactions with the IP\(_3\)R under “resting” conditions inhibits the channel. When Ca\(^{2+}\) is released from the store in the presence of IP\(_3\), there was an alteration in the Trp3-IP\(_3\)R interaction resulting in opening of the channel. Another study (16) demonstrated that a C-terminal domain of Trp3 (aa 777–797) is conserved among various Trps and is the putative site for interaction with IP\(_3\)R. Expression of the Trp3 domain aa 742–795 induced an inhibition of agonist- and thapsigargin-stimulated Ca\(^{2+}\) influx in cells expressing Trp3. An N-terminal domain in the IP\(_3\)R3 (aa 638–926) was identified as the region...
interacting with the C terminus of hTrp3. Two subdomains within this IP$_3$R region, aa 751–821 and aa 669–698, were identified as having stimulatory and inhibitory effects, respectively, on Trp3 activity. Also, some homology was identified in these domains among the various IP$_3$Rs.

Based on these previous reports and our studies showing that Trp1 expression increases SOCE in HSG cells and Trp1 interacts with IP$_3$R3 (8, 17), we predicted the following possible outcomes of $\Delta$Trp1$\alpha$ expression in HSG cells. (i) There would be no effect on SOCE if the Trp1 C terminus interaction with IP$_3$R was required for opening the channel. In this case, $\Delta$Trp1$\alpha$ would not interact with IP$_3$R because of lack of the C terminus. (ii) There would be no effect on SOCE if Trp1 interacted with IP$_3$R via another domain but gating was mediated via the C terminus. In this case, $\Delta$Trp1$\alpha$ and IP$_3$R would interact, but the channel would be silent. (iii) SOCE would be constitutively activated if IP$_3$R interaction with the Trp1 C terminus during gating relieved an inhibition of the gate by the Trp1 C terminus. In this case, removing the inhibitory domain would allow the gate to be opened and $\Delta$Trp1$\alpha$ would function as an open channel. (iv) Inhibition of SOCE would occur if the truncated channel could not be gated but it interacted with IP$_3$R and competed with the endogenous SOCE channel, i.e., a dominant negative effect. All four of these possibilities were excluded by the data discussed above. We have shown that expression of $\Delta$Trp1$\alpha$ induced no change in the ability of either carbacephal or thapsigargin to induce SOCE in HSG cells, ruling out predictions i, ii, and iv. Further, no increase in basal Ca$^{2+}$ permeability was noted in $\Delta$Trp1$\alpha$-expressing cells, ruling out possibility iii. Importantly, the level of SOCE seen in $\Delta$Trp1$\alpha$ cells was greater than that seen in Trp1$\alpha$-expressing cells. The data suggest that the difference in the SOC between $\Delta$Trp1$\alpha$ and Trp1$\alpha$ cells is primarily in the sustained level of [Ca$^{2+}$], indicating that there is relatively more Ca$^{2+}$ entering the $\Delta$Trp1$\alpha$-expressing cells.

The data described above suggest that the C terminus of Trp1 likely acts as an inhibitory domain of the SOCE channel and restricts the amount of Ca$^{2+}$ entering the cells. This suggestion is based on the observation that there is an increase in SOCE when this domain is removed. As proposed previously, Trp1 might form the SOCE channel either by itself or in association with other subunits. Thus, the marked effects of the C-terminal truncation on SOCE are consistent with this proposal. Interestingly, a similar increase in Ca$^{2+}$ influx activity was reported following deletion of the C terminus of the $\alpha$-subunit of the cardiac t-type voltage-gated Ca$^{2+}$ channels (22). However, electrophysiological measurements of single-channel events will be required to understand exactly how C-terminal deletion of Trp1 results in increased SOCE. It is possible that putative phosphorylation sites, or direct binding of lipids or proteins such as calmodulin, might be involved in the Trp1 C terminus-mediated feedback regulation of SOCE. Alternatively, as suggested by previous reports and analogous to Trp3 (15, 16, 18), direct interactions of Trp1 with the IP$_3$R could modulate Ca$^{2+}$ influx. Because IP$_3$R communoprecipitates with $\Delta$Trp1$\alpha$, which lacks the C-terminal domain, our data suggest that IP$_3$R might bind to other sites on Trp1. However, presently, we cannot rule out the possibility that $\Delta$Trp1$\alpha$ indirectly associates with IP$_3$R via interactions with endogenous Trp or other protein(s), which in turn is associated with IP$_3$R. Despite how $\Delta$Trp1$\alpha$ interacts with IP$_3$R, the present data strongly suggest that the C-terminal region of hTrp1 exerts an inhibitory effect on SOCE, which likely provides a feedback regulation of Ca$^{2+}$ influx. Further, we report here for the first time that the EWKFAR region in Trp1 does not contribute significantly to its functional effects on SOCE.

In conclusion, we have shown that deletion of the C terminus of hTrp1 does not affect its ability to be activated via store-depletion and increase SOCE in HSG cells. Further, the levels of Ca$^{2+}$ influx associated with the expression of the truncated Trp1 proteins are higher than that associated with the expression of the full-length Trp1. Thus, our data support the suggestion that the C-terminal of hTrp1 is involved in modulating SOCE, likely by exerting a feedback inhibitory effect.

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![Figure 4](image-url)