Trp1, a Candidate Protein for the Store-operated $\text{Ca}^{2+}$ Influx Mechanism in Salivary Gland Cells*

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The trp gene family has been proposed to encode the store-operated $\text{Ca}^{2+}$ influx (SOC) channel(s). This study examines the role of Trp1 in the SOC mechanism of salivary gland cells. htrp1, htrp3, and Trp1 were detected in the human submandibular gland cell line (HSG). HSG cells stably transfected with htrp1α cDNA displayed (i) a higher level of Trp1, (ii) a 3–5-fold increase in SOC (thapsigargin-stimulated $\text{Ca}^{2+}$ influx), determined by [Ca$^{2+}$]i, and Ca$^{2+}$-activated K$^+$ channel current measurements, and (iii) similar basal Ca$^{2+}$ permeability, and inhibition of SOC by Gd$^{3+}$ but not by Zn$^{2+}$, as compared with control cells. Importantly, (i) transfection of HSG cells with antisense trp1α cDNA decreased endogenous Trp1 level and significantly attenuated SOC, and (ii) transfection of HSG cells with htrp3 cDNA did not increase SOC. These data demonstrate an association between Trp1 and SOC and strongly suggest that Trp1 is involved in this mechanism in HSG cells. Consistent with this suggestion, Trp1 was detected in the plasma membrane region, the proposed site of SOC, of acinar and ductal cells in intact rat submandibular glands. Based on these aggregate data, we propose Trp1 as a candidate protein for the SOC mechanism in salivary gland cells.

[Ca$^{2+}$], regulates the physiological function of a variety of nonexcitable cell types, including salivary gland cells (1–3). In salivary gland cells, a sustained elevation of [Ca$^{2+}$], is required for the activation of the ion channels, such as the Ca$^{2+}$-activated K$^+$ channel and the Ca$^{2+}$-activated Cl$^-$ channel, which critically regulate the secretion of fluid and electrolytes. [Ca$^{2+}$], increase in these cells results from intracellular Ca$^{2+}$ release and from extracellular Ca$^{2+}$ influx. While release of Ca$^{2+}$ from the intracellular store(s) triggers a transient increase in [Ca$^{2+}$], and fluid secretion, sustained fluid secretion is dependent on the influx of Ca$^{2+}$ from the extracellular medium (3, 4). Intracellular Ca$^{2+}$ release is induced by inositol 1,4,5-trisphosphate (IP$_3$), that is generated in response to agonist-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) (1, 5). Ca$^{2+}$ influx across the plasma membrane is mediated via an as yet unknown mechanism referred to as store-operated Ca$^{2+}$ influx (SOC), or capacitative calcium entry, that is activated by the depletion of Ca$^{2+}$ from the internal Ca$^{2+}$ store (3, 6–8).

The molecular basis for the activation and regulation of SOC has not yet been determined in any nonexcitable cell type. The two critical questions that remain are regarding (i) the signal that is transmitted from the internal Ca$^{2+}$ store to the plasma membrane to trigger activation or inactivation of SOC and (ii) the molecule that mediates Ca$^{2+}$ influx into the cells. Recent studies with salivary gland and other nonexcitable cells indicate that store-operated Ca$^{2+}$ influx is mediated via a channel-like mechanism (8, 9). Electrophysiological measurements demonstrate that Ca$^{2+}$ influx is associated with the activation of an inward Ca$^{2+}$ current, which appears to be selective for Ca$^{2+}$ in some cell types and relatively nonspecific for cations in others (2, 8). However, the mechanism involved in gating the putative Ca$^{2+}$ influx channel is not known. Studies toward identifying the molecule(s) mediating Ca$^{2+}$ influx have led to the cloning of mammalian homologues of the Drosophila transient receptor potential gene, trp (10, 11). Presently, full-length or partial sequences have been reported for seven trp genes (trp1–trp7) in various mammalian species, including human, mouse, rat, rabbit, and bovine. It has been proposed that the trp gene(s) encode the store-operated Ca$^{2+}$ channel in nonexcitable cells. However, studies examining the functional effects induced by expression of trp genes have demonstrated that the characteristics of the expressed activity are distinct from those of the endogenous SOC activity, suggesting that the encoded Trp protein is functionally different from the endogenous SOC mechanism present in the cells used for the expression. Additionally, several studies also show an increase in receptor-operated, or basal, Ca$^{2+}$ influx in cells transfected with trp cDNAs. Thus, only a few trp gene products meet the functional criteria for SOC, typically defined as an increase in Ca$^{2+}$ influx stimulated in cells following internal Ca$^{2+}$ store-depletion by thapsigargin.

Expression of the Drosophila trp cDNA, dtrp (12); human trp1β, the alternatively spliced variant, short form of htrp1, (13), but not htrp1α (14, 15); bovine trp4, btrp4 (16); rat trp4, rtrp4 (17); and rabbit trp5 (18) but not mouse trp5, mtrp5 (19), was associated with increased Ca$^{2+}$ influx in response to treatment with thapsigargin. mtrp2 expression in HEK-293 cells induced a relatively small increase in SOC and also increased receptor-stimulated Ca$^{2+}$ influx (20). On the other hand, rtrp2 has been suggested to be associated with the sensory signaling mechanism in brain, apparently independent of store depletion (21). The reported data strongly suggest that Trp3, Trp6, and Trp7 proteins are associated with receptor- or second messenger-regulated Ca$^{2+}$ influx (22–25). Consistent with this, it was

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1 The abbreviations used are: IP$_3$, inositol 1,4,5-trisphosphate; SOC, store-operated Ca$^{2+}$ influx; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Trp, human Trp; HA, hemagglutinin; K$_{1e}$, Ca$^{2+}$-activated K$^+$ channel current; Tg, thapsigargin; Trp, transient receptor potential; IP$_{3b}$, phosphatidylinositol 4,5-bisphosphate.

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recently suggested that Trp3 might be regulated by IP₃ via interaction with the IP₃ receptor (26). However, it remains to be established whether the Trp3-IP₃ receptor interaction is involved in the mechanism regulating SOC.

More convincing data regarding a role for Trp in SOC has been obtained by expression of antisense sequences of trp cDNA. Expression of either a mixture of antisense cDNA fragments of mtrp1–mtrp6 or of mtrp4 alone attenuated carbachol-stimulated Ca²⁺ influx (10, 14). However, it is difficult to assess the role of the trp gene products based on these data, since thapsigargin-stimulated influx was not tested in these studies and, as mentioned above, expression of several trp isoforms induce increases in second messenger- or receptor-operated type of Ca²⁺ influx. In another study, expression of an antisense oligodeoxynucleotide against the region of the start codon of mtrp1 in Xenopus oocytes attenuated thapsigargin stimulation of a 1,3,4-inositol trisphosphate (17). This sequence is present in all four variants of mtrp1 genes, although only trp1a and -b genes have been suggested to encode a polypeptide (27).

The present study uses the human submandibular gland ductal cell line, HSG, to examine the role of the trp1 gene product in the SOC mechanism in salivary gland cells. Similar internal Ca²⁺ release and store-operated Ca²⁺ influx mechanisms are triggered upon activation of the muscarinic receptor in salivary acinar and ductal cells (28). Consistent with this, HSG cells exhibit a robust activation of Ca²⁺ influx when the internal Ca²⁺ store(s) is depleted by either muscarinic receptor stimulation (via an IP₃-dependent mechanism) or by treatment with thapsigargin or tert-butylhydroxyquinone (9, 29). Furthermore, SOC appears to be the primary mechanism for Ca²⁺ influx in these cells following muscarinic receptor stimulation or treatment with thapsigargin. Thus, HSG cells provide an excellent, well characterized model system to study the mechanism of SOC. The results presented below demonstrate that transfection of HSG cells with htrp1a, but not htrp3, cDNA induced dramatic increases in thapsigargin-stimulated SOC, while expression of the htrp1a cDNA in the antisense direction significantly attenuated the endogenous SOC activity. Importantly, changes in SOC were associated with corresponding changes in the levels of Trp1 in HSG cell plasma membranes. Further, and consistent with its proposed function, we show that Trp1 is localized in the plasma membrane region of acinar and ductal cells in intact rat submandibular glands. In aggregate, these data strongly suggest that Trp1 is a candidate protein for the SOC mechanism in salivary gland cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HSG cells were a kind gift from Dr. Mitsunabo Sato (Takushima University, Japan). The conditions for cell culture were similar to those described previously (7, 29). Briefly, cells were grown in Eagle’s minimum essential medium under 5% CO₂ at 37 °C. The culture medium was supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Biofluids, Rockville, MD). Cells were passaged when confluent by detaching from the tissue culture dish with 0.25% trypsin, 1.0 mM EDTA.

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Sections were then washed for 5 min in PBS containing 0.2% BSA and incubated for 20 min with a FITC-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch) diluted 1:150 in PBS containing 0.2% BSA. The secondary antibody was then removed by washing the sections twice in PBS containing 0.2% BSA (5 min/wash) followed by two washes with PBS (5 min each). Each immunostained section received a drop of Citifluor (Ted Pella, Redding, CA), was mounted on a coverslip, and sealed. Samples were stored in the dark at 4 °C until examined.

Confocal images were collected using an MRC 1024 krypton/argon laser-scanning confocal system (Bio-Rad) equipped with a Nikon Optiphot II photomicroscope (Melville, NY). Images of control and experimental samples were obtained using identical conditions (laser intensities, gain, iris aperture, black level, scan speed). Images were collected in the x-y plane using a 60 × 60 oil immersion PlanApo objective (Nikon, Japan) with a confocal aperture of 2 mm. The entire series of images was then collected into a single focused image using the Confocal Assistant software supplied by the manufacturer. A Codonics NP-1600 Photographic Network Printer (Middleton Heights, OH) was used to print the final single focused image.

Patch Clamp Measurements—A piece of coverslip (0.5 × 0.5 mm) with cells was placed in the perfusion chamber (Warner Instrument Corp., Hamden, CT). Perfusion, at approximately 5 ml/min, was achieved by gravity-fed plastic tubes. A vacuum line continuously removed the bath solution. Complete solution changes were achieved within 25 s. The standard extracellular solution for measuring Ca$^{2+}$-activated K$^+$ channel current (Ihk) contained 145 mM NaCl, 5 mM KCl, 20 mM HEPES, pH 7.4. The pipette was filled with 150 mM KCl, 2 mM MgCl$_2$, 1 mM ATP, 10 mM HEPES, pH 7.2. Any changes in the composition of the pipette and external solutions are given under "Results.

Patch clamp in a whole cell configuration was performed on single HSG cells attached to coverslips using the standard patch clamp technique described previously (9, 29). Membrane currents were measured with an Axopatch 200A amplifier in conjunction with pClamp 6.1 software and a Digidata 1200 A/D converter (Axon Instruments, Foster City, CA). The currents were filtered at 2 kHz (low pass bessel filter) and sampled with an interval of 10 ms. The currents were digitized and recorded directly onto the hard drive of a Dell Pentium computer.

(Ca$^{2+}$)-Measurements—The culture medium was removed, and the cells were washed and incubated in medium containing 1 μM Fura-2/AM (Molecular Probes, Inc., Eugene, OR) for 45 min at 37 °C. Fura-2 fluorescence in single cells was measured at excitation wavelengths of 340 and 380, with emission at 510 nm, using a SLM 8000/DMX 100 spectrophotometer attached to an inverted Nikon Diaphot inverted 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., PA). Analog plots of the fluorescence in single cells are shown. The 340/380 nm ratio of Fura-2 fluorescence have been used to represent [Ca$^{2+}$]. Other details of the experiments are given under "Results" and in the figure legends. All experiments were performed at room temperature.

RESULTS

Expression of Trp1 in HSG Cells—Fig. 1A shows RT-PCR products amplified from HSG cell RNA by using htrp1-specific primers for the conserved (400-base pair region) of trp genes (30, 33) (lane 1). RT-PCR products from rat submandibular gland (lane 2) and rat brain (lane 3) are also shown (rat-specific primer sequences used were described previously; see Ref. 30). Lane 4 shows DNA size markers. Sequencing the product from HSG cells confirmed the presence of htrp1 (data not shown). Using htrp3-specific primers and RT-PCR, a similar region in htrp3 was also amplified from HSG cell mRNA and sequenced (data not shown). Fig. 1B shows the RT-PCR products representing the 2.3-kilobase pair 3'—end of trp1 (lane 1, HSG cells; lane 2, rat submandibular glands; lane 3, size markers). Similar 2.3-kilobase pair products were amplified from rat parotid gland (data not shown) and brain mRNA (30).

Endogenous Trp1 protein expression in HSG cells was determined using the Trp1-specific antibody we have previously described (30). Trp1 protein (shown by an arrow) was detected in HSG cell plasma membranes by Western blotting (Fig. 1C, lane 1). Since antibody to Trp3 is presently unavailable, the presence of endogenous Trp3 in HSG cells could not be determined.

Stable Transfection of HSG Cells with htrp1α cDNA—To examine the role of Trp1 protein in salivary gland cell store-operated Ca$^{2+}$ influx, HSG cells were stably transfected with the HA-tagged hTrp1α cDNA. The transfected cells were then used for detection of the Trp1 protein and measurement of store-operated Ca$^{2+}$ influx. Fig. 1C, lane 2, shows that the plasma membrane fraction of transfected cells contains a higher (about 2-fold) level of Trp1 protein than nontransfected cells (lane 1; detected using anti-Trp1 antibody). The localization of the expressed HA-tagged hTrp1α protein was further examined by confocal microscopy. Fig. 1D shows an extended focus (stacked) image of FITC fluorescence using an anti-HA antibody. Consistent with the data obtained with the immunoblotting technique, considerable reactivity was detected in the transfected cells, and fluorescence was mainly associated with the plasma membrane and sub-plasma membrane region of the cell. A similar pattern was observed when the anti-Trp1 anti-
body was used (data not shown). The fluorescence intensity was dramatically diminished when the anti-HA antibody was preincubated with the HA peptide (Fig. 1E).

**Fig. 2.** Thapsigargin-stimulated Ca\(^{2+}\) influx in HSG cells. Fluorescence was measured in Fura-2-loaded HSG cells as described under "Experimental Procedures." A, Tg test. Tg was added to control (HSG) and htrp1a-transfected (hTrp1) HSG cells in a Ca\(^{2+}\)-free medium to measure internal Ca\(^{2+}\) release (first increase in fluorescence). 10 mM Ca\(^{2+}\) was then added to measure the influx component (second increase in fluorescence). B, basal control. Fluorescence was measured by the addition of Ca\(^{2+}\) to untreated (i.e. basal control) hTrp1 and HSG cells. C, Ca\(^{2+}\) influx. Average data of peak [Ca\(^{2+}\)]\(_{i}\) increase due to Ca\(^{2+}\) influx in Tg-treated and basal control hTrp1 and control HSG cells are shown. An asterisk indicates values significantly different (\(p < 0.05\)) from each other and from their respective basal control values.

Thapsigargin-stimulated Ca\(^{2+}\) Influx in htrp1a-transfected HSG Cells—Fig. 2A shows Fura-2 fluorescence measurements in control (HSG cell) and htrp1a-transfected (hTrp1 cell) cells. The 340/380 nm fluorescence ratio has been used to represent changes in cytosolic [Ca\(^{2+}\)]\(_{i}\) ([Ca\(^{2+}\)]\(_{i}\)). The experimental protocol used involved stimulating cells with thapsigargin (Tg test) in a Ca\(^{2+}\)-free medium to detect internal Ca\(^{2+}\) release, following which 10 mM Ca\(^{2+}\) was added to the cell medium to detect Ca\(^{2+}\) influx. This experimental protocol was employed, since we have previously shown that SOC in HSG cells is strongly subject to feedback inhibition by [Ca\(^{2+}\)]\(_{i}\) when treated with thapsigargin in a Ca\(^{2+}\)-containing medium (9).

Thapsigargin induced a rapid release of Ca\(^{2+}\) from intracellular stores in both control and transfected cells, demonstrated by the first transient increase in [Ca\(^{2+}\)]\(_{i}\). There was no significant difference in this initial increase in [Ca\(^{2+}\)]\(_{i}\), in the two sets of cells (\(p > 0.05\); number of cells tested was 65 and 90, respectively, for control and transfected cells). When Ca\(^{2+}\) was readded to the cells, a second increase in [Ca\(^{2+}\)]\(_{i}\), was obtained, representing the Ca\(^{2+}\) influx component. The peak of this increase was about 3-fold higher in transfected cells compared with control cells (\(p < 0.01\); number of cells was 65 and 90 for control and transfected cells, respectively). This increase was transient in both types of cells and decreased to a relatively sustained level that was also consistently higher in the transfected cells. Importantly, Fig. 2B shows that the addition of 10 mM Ca\(^{2+}\) to cells incubated in a Ca\(^{2+}\)-free medium without thapsigargin treatment induced a similar increase in fluorescence in both sets of cells (\(p > 0.05\), \(n = 43\) for either set). These data demonstrate that the transfected HSG cells, which express higher levels of the Trp1 protein, display (i) a significantly higher level of SOC (see average data in Fig. 2C), (ii) similar thapsigargin-stimulated internal Ca\(^{2+}\) release, and (iii) similar basal Ca\(^{2+}\) influx as compared with control cells (see Fig. 2C). The SOC component (i.e. the difference between the peak [Ca\(^{2+}\)]\(_{i}\), increase obtained upon readdition of 10 mM Ca\(^{2+}\) in untreated cells and that obtained in thapsigargin-treated cells) measured in transfected cells was about 3–5-fold higher than in control HSG cells (Fig. 2C).

Thapsigargin-stimulated K\(_{Ca}\) Current in htrp1a-transfected HSG Cells—Since Ca\(^{2+}\) influx is strongly affected by changes in the membrane potential, measurement of K\(_{Ca}\) current, performed under voltage-clamped conditions, was also used to assess the increase in SOC seen in htrp1a-transfected HSG cells. We have previously reported that (i) the K\(_{Ca}\) current in...
HSG cells represents underlying changes in [Ca^{2+}]_i, and (ii) its sustained activation is determined by Ca^{2+} influx (29). Thus, K_{Ca} activity in these cells is a physiological readout for the Ca^{2+} entering the cell. The experimental protocol used was similar to that used for [Ca^{2+}]_i measurements, and the data are shown in Fig. 3. Following the addition of thapsigargin to the cell maintained at 0 mV in a Ca^{2+}-free medium, a transient increase in the outward current (due to internal Ca^{2+} release) was detected, which was similar in control (A) and transfected (B) cells. Upon the readdition of 10 mM Ca^{2+}, a second increase in the current, due to Ca^{2+} influx, was detected that was significantly higher in the transfected cells. Importantly, and consistent with the [Ca^{2+}]_i measurements, the addition of Ca^{2+} to cells incubated for the same length of time in Ca^{2+}-free medium without thapsigargin treatment induced K_{Ca} currents of similar magnitudes in control and transfected HSG cells (data not shown). Based on the present data and our previous studies, we suggest that the higher level of K_{Ca} current obtained upon the readdition of Ca^{2+} to transfected cells is due to a higher level of Ca^{2+} influx in these cells and not due to an increase in the activity of K_{Ca} per se in cells overexpressing hTrp1. Note that two different clones of htrp1 cDNA-transfected HSG cells were tested for thapsigargin-stimulated Ca^{2+} influx using the experimental protocol described above. Fig. 5A shows a representative trace of Fura-2 fluorescence. As seen in htrp1a-transfected cells, thapsigargin-stimulated internal Ca^{2+} release and Ca^{2+} influx using the experimental protocol described above. Fig. 5A shows a representative trace of Fura-2 fluorescence. As seen in htrp1a-transfected cells, thapsigargin-stimulated internal Ca^{2+} release and Ca^{2+} influx using the experimental protocol described above. Fig. 5A shows a representative trace of Fura-2 fluorescence. As seen in htrp1a-transfected cells, thapsigargin-stimulated internal Ca^{2+} release and Ca^{2+} influx using the experimental protocol described above. Fig. 5A shows a representative trace of Fura-2 fluorescence. As seen in htrp1a-transfected cells, thapsigargin-stimulated internal Ca^{2+} release and Ca^{2+} influx using the experimental protocol described above. Fig. 5A shows a representative trace of Fura-2 fluorescence. As seen in htrp1a-transfected cells, thapsigargin-stimulated internal Ca^{2+} release and Ca^{2+} influx using the experimental protocol described above. Fig. 5A shows a representative trace of Fura-2 fluorescence. As seen in htrp1a-transfected cells, thapsigargin-stimulated internal Ca^{2+} release and Ca^{2+} influx using the experimental protocol described above. Fig. 5A shows a representative trace of Fura-2 fluorescence. As seen in htrp1a-transfected cells, thapsigargin-stimulated internal Ca^{2+} release and Ca^{2+} influx using the experimental protocol described above.
TABLE I
Characteristics of SOC in control and htrp1a-transfected HSG cells

Experimental conditions were as described for Fig. 4. The values given below are the peak increases in Fura-2 fluorescence (340/380 ratios) obtained upon the addition of 10 μM Ca2+ to the external medium after internal Ca2+ store depletion by treatment with thapsigargin in control (HSG) and htrp1a-transfected cells (Trp1-HSG). The other ions were added prior to the addition of Ca2+ (see Fig. 4). The number of cells tested in each case (n) is given in parentheses. Values marked with an asterisk are significantly different (p < 0.01) from the unmarked values. The values obtained with Zn2+ in either group are not significantly different (p > 0.05) from those obtained without any additions.

| Additions      | Peak fluorescence (340/380) |
|----------------|----------------------------|
|                | HSG                        | Trp1-HSG                   |
| None           | 6.1 ± 1.1 (65)              | 12.9 ± 2.1 (90)            |
| 1 mM Zn2+      | 5.8 ± 1.0 (48)              | 12.5 ± 1.6 (51)            |
| 1 mM La3+      | 0.6 ± 0.1 (56)*             | 0.8 ± 0.1 (54)*            |
| 1 mM Gd3+      | 0.5 ± 0.1 (51)*             | 0.9 ± 0.1 (50)*            |

(lane 1). These data clearly show that increasing the levels of hTrp3 in HSG cells does not induce any changes in thapsigargin-stimulated Ca2+ influx. These results are similar to those previously reported showing that htrp3 expression does not increase thapsigargin-stimulated Ca2+ influx in HEK-293, COS, and Chinese hamster ovary cells (22, 24).

Expression of Antisense trp1a cDNA in HSG Cells—The data presented above provide evidence for an increase in the SOC activity in HSG cells transfected with the htrp1a, but not htrp3, cDNA. The data also rule out secondary effects due to changes in (i) the membrane potential or (ii) the basal Ca2+ permeability of the cells. To demonstrate more directly that the Trp1 protein is involved in the endogenous SOC mechanism, HSG cells were stably transfected with htrp1a cDNA in the antisense direction. Two clones were tested for activity, and similar results were obtained.

Fig. 5C is a representative trace showing thapsigargin-stimulated [Ca2+]i changes in HSG cells transfected with antisense htrp1a cDNA. Thapsigargin-stimulated internal Ca2+ release was not altered in these cells (compare traces in Figs. 5A and B, and 2A). However, the thapsigargin-stimulated Ca2+ influx (second peak of [Ca2+]i increase) was significantly reduced compared with that in control cells (compare with trace in Fig. 2A). The magnitude of [Ca2+]i increase upon the readaptation of Ca2+ to these cells is similar to that in the basal, unstimulated condition (see Fig. 2B). Average values for the SOC component measured in the HSG cells transfected with antisense htrp1a cDNA is shown in Fig. 5E. The SOC component in these cells is significantly lower (p < 0.05, n = 80) than in the other three groups of cells (i.e. control, htrp1a-transfected, and htrp3-transfected).

The effect of transfection of HSG cells with antisense htrp1a on the levels of endogenous Trp1 was determined. A dramatic decrease in the level of Trp1 protein was detected in plasma membrane fraction isolated from these cells. Fig. 5D shows a representative Western blot using anti-Trp1 antibody (similar results were obtained in three experiments using cells from different passages). Lane 1 shows endogenous hTrp1 in plasma membranes isolated from HSG cells transfected with antisense htrp1a cDNA, and lane 2 shows endogenous hTrp1 in control cells. In agreement with these data, strongly demonstrates that the expression of the htrp1a cDNA in HSG cells in the antisense direction decreases the level of Trp protein and compromises activation of SOC by thapsigargin. Thus, the data demonstrate for the first time a direct association between SOC and the level of Trp1 in the plasma membrane.

Immunolocalization of the Endogenous Trp1 Protein in Rat Submandibular Gland Cells—The data presented above strongly suggest that Trp1 is involved in the SOC mechanism of HSG cells. Consistent with this suggestion and with the detection of trp1 in rat submandibular glands, Fig. 6 shows that endogenous Trp1 protein is present in this salivary gland. Two methods were used to localize Trp1 in intact rat submandibular gland: immunocytochemistry, using a horseradish peroxidase-linked secondary antibody (Fig. 6, A and B), and confocal microscopy, using an FITC-linked secondary antibody (Fig. 6, C-E). In both cases, anti-Trp1 antibody (30) was used as the primary antibody. A reddish color was detected in the basolateral region of the acinar cells (shown by the arrow marked a) but not in the luminal regions (A). The intensity of the reaction seen in the ductal cells (shown by the arrow marked d) was higher than that of the acinar cells, although the localization was not as distinct. Since this reactivity was blocked either by preincubating the antibody with the Trp1- peptide (B) or in the absence of the secondary antibody (data not shown), the reactivity in the ductal cells does not appear to be nonspecific. Fig. 6, C–E, shows the images obtained by confocal microscopy. Consistent with the immunocytochemistry results, Trp1 protein was detected in the basolateral plasma membrane region of acinar cells (see the arrow marked a in Fig. 6C), while ductal cells (see the arrow marked d in Fig. 6D) showed a relatively stronger reactivity that did not appear to be localized to any specific region. The reactivity detected in the absence of the primary antibody is shown in Fig. 6E. Notably, the store-operated Ca2+ influx activity has been suggested to occur via the basolateral membrane of salivary gland and other exocrine gland acinar cells (3, 34). Thus, the localization of the Trp1 protein in intact submandibular gland cells is consistent with its proposed physiological function.

DISCUSSION

A number of previous studies have suggested that trp gene products are involved in SOC. However, the data presently available do not provide convincing evidence linking the localization and expression of any Trp protein with the SOC function in any nonexcitable cell type or tissue. The present study demonstrates the localization of the Trp1 protein in an intact noneexcitable tissue and functional effects of trp1a expression in the same cell type. Importantly, the data show for the first time an association between the level of Trp1 protein and SOC and suggest that Trp1 is a candidate protein for the SOC mechanism in salivary gland cells. HSG cells stably transfected with the htrp1a cDNA expressed higher levels of Trp1 and displayed a 3–5-fold increase in the thapsigargin-stimulated Ca2+ influx component. This was unequivocally demonstrated by measuring (i) [Ca2+]i changes, by microfluorimetry and imaging of Fura-2 fluorescence in single transfected HSG cells and (ii) Ca2+-activated K+ channel current. We have shown that more Ca2+ enters thapsigargin-treated htrp1a cDNA-transfected cells than control cells, thus accounting for the higher level of Kca current measured in these cells. Consistent with this, the sustained level of [Ca2+]i, in these cells was about 2-fold higher than in nontransfected cells. Further, the data rule out the possibility that the increase in SOC is indirectly induced due to a change in the membrane potential or an increase in the basal Ca2+ permeability of the cell. An important and novel finding of this study is that the characteristics of Ca2+ influx in htrp1a-transfected HSG cells are similar to those of SOC in the nontransfected HSG cells. This is in contrast to previous reports, where the Ca2+ influx activity associated with the expressed trp was shown to be distinct from the endogenous SOC in the cell used for expression (11). We have reported earlier that SOC in HSG cells can be inhibited by Gd3+ but not by Zn2+ (9, 29). We now show that SOC activity in transfected cells displays the same differential sensitivity to these cations. Notably, the ac-
Fig. 5. Thapsigargin-stimulated Ca\(^{2+}\) influx in HSG cells transfected with htrp3 cDNA or htrp1a cDNA in the antisense direction. Experimental conditions were as described for Fig. 2. Peak [Ca\(^{2+}\)]\(_i\) (340/380 ratio) was measured upon the addition of Tg in a Ca\(^{2+}\)-free medium and after the readdition of 10 mM Ca\(^{2+}\) to cells stably transfected with htrp3 cDNA (A) or htrp1a cDNA in the antisense direction (C). B, HA-tagged Trp3 was detected (shown by an arrow) using anti-HA antibody in control (lane 1) and hTrp3-expressing cells (lane 2). D, Trp1 (shown by an arrow) was detected using anti-Trp1 antibody in the plasma membrane fraction of cells transfected with antisense htrp1a (lane 1) and control HSG cells (lane 2). E, SOC component (difference in peak [Ca\(^{2+}\)]\(_i\), increase upon the readdition of 10 mM Ca\(^{2+}\) to Tg-treated and -untreated cells) in control (HSG), htrp1a cDNA-transfected (hTrp1), antisense htrp1a cDNA-transfected (Antisense htrp1), and htrp3-cDNA transfected (hTrp3) cells was measured. **, values (HSG and hTrp3) are not significantly different from each other but are significantly different from the value marked with one asterisk (hTrp1) and the unmarked value (Antisense htrp1). *, value (hTrp1) significantly different from all other values.

Fig. 6. Localization of Trp1 protein in rat submandibular glands. Immunocytochemistry using HistoStain (A and B, \(\times 100\) objective lens) or confocal microscopy using an FITC-linked anti-rabbit IgG (C–E, extended focus images) was used to detect the localization of Trp1. Reactivity to anti-Trp1 was detected in acinar and ductal cells (arrows labeled a and d, respectively). Reactivity in the absence of primary antibody is shown in E, and reactivity after incubation of anti-Trp1 antibody with the peptide is shown in B.

Activity associated with htrp1a (13) was also inhibited by Gd\(^{3+}\). In contrast, the store-operated \(_{\text{CRAC}}^I\) channel found in mast cells was highly sensitive to Zn\(^{2+}\) (8). We suggest that the sensitivity of SOC to various cations might be a useful tool for fingerprinting the type of Ca\(^{2+}\) influx channel present.

Further evidence for an association between hTrp1 and SOC was provided by our demonstration that transfection of HSG cells with antisense htrp1a cDNA resulted in a decrease in endogenous hTrp1 and a significant attenuation of SOC in these cells. In aggregate, these data strongly suggest that Trp1 is involved in the SOC mechanism of HSG cells. Notably, transfection of HSG cells with htrp3 cDNA and increase in hTrp3 did not induce an increase in thapsigargin-stimulated Ca\(^{2+}\) influx. This is consistent with a number of previous reports that suggest that Trp3 activity is associated with agonist-stimulation of cells and probably regulated by IP\(_3\) (11, 22, 24) or diacylglycerol (35). An interaction between Trp3 and the IP\(_3\) receptor has been proposed as a possible mechanism for Trp3 activation (26). Thus, Trp3 is more likely to be involved in mediating Ca\(^{2+}\) influx following stimulation of cells by agonists that induce PIP\(_2\) hydrolysis and internal Ca\(^{2+}\) store depletion. The Trp1-associated SOC activity we have described above in HSG cells is activated by internal Ca\(^{2+}\) store depletion in the absence of PIP\(_2\) turnover or an increase in the level of IP\(_3\). Although a role for IP\(_3\) and the IP\(_3\) receptor in Trp1 activation cannot be presently excluded, it is possible that cells might have an IP\(_3\)-independent SOC mechanism that (i) serves a more housekeeping role to ensure maintenance of the internal Ca\(^{2+}\) stores or (ii) achieves refill when store depletion involves an IP\(_3\)-independent process. However, more detailed studies are required to determine the specific physiological function of these two Trp proteins in HSG cells.

Localization of trp transcripts has been previously shown by...
in situ hybridization in the brain (36, 37). More recently, rat Trp2 protein was shown to be localized exclusively in rat brain VNO neurons and sensory villi (21). The present data describe for the first time the localization of a Trp protein in an intact nonexcitable tissue. By using a Trp1-specific antibody that we have previously characterized (30), we have shown here that endogenous Trp1 protein is localized in the basolateral plasma membrane region of submandibular gland acinar cells. In ductal cells, the localization appears to be less distinct, since reactivity against the antibody is seen on both the apical and basolateral membrane regions. The present findings are consistent with our previous findings of trp1 homologous sequences in rat submandibular gland RNA (30). Notably, the proposed route for Ca2+ entry in salivary gland acinar cells is via the basolateral plasma membrane (3). Although, presently, there are no data to demonstrate the route of Ca2+ entry in ductal cells, these cells appear to have a more sustained level of Ca2+ influx than the acinar cells (28). Thus, the localization of Trp1 protein in submandibular gland cells described above is consistent with its proposed function (i.e. a Ca2+ influx pathway in the plasma membrane). However, further studies are required to determine the role of Trp1 in salivary gland function.

As mentioned above, the function of the trp gene product(s) has not yet been clearly established. With regard to studies involving trp1 genes, the trp1a (long form) has been expressed in COS-M6 cells and SF9 insect cells (14, 15). In COS-M6 cells, trp1a expression induced a relatively small increase in the Ca2+ influx activity associated with carbobalach stimulation. However, this study did not assess Tg-stimulated Ca2+ influx, a primary criterion used to define SOC. Transient expression of trp1a in SF9 cells induced nonselective constitutively activated cation channel activity, which did not appear to be sensitive to internal Ca2+ store depletion. In contrast, transient expression of htrp1b (the alternatively spliced, short form of the gene) in Chinese hamster ovary and SF9 cells induced a nonselective cation channel activity and an increase in the Ca2+ influx component in response to Tg treatment. However, since the Chinese hamster ovary cells used in this study did not display an endogenous SOC activity (13), it is difficult to ascribe a function to trp1b in this cell type. Overall, these previous studies were more consistent with a role for trp1b, rather than trp1a, in SOC. However, here we have demonstrated that expression of trp1a increased the SOC mechanism of salivary gland cells. Thus, both trp1a and trp1b appear to be capable of regulating SOC.

The exact reasons for the conflicting data and different effects seen following expression of the various trp genes are not yet understood. It is possible that distinct and tissue-specific regulatory factors are involved in regulating the function of various Trp proteins. Thus, the activity of the expressed protein will probably be determined by the endogenous SOC and associated regulatory mechanism(s) present in the cells used for the functional expression. It has been previously proposed that Trp proteins might form multimeric complexes, either homomers or heteromers with other Trp proteins, or with other as yet unidentified proteins (10). For example, interactions have been suggested between hTrp1 and hTrp3 (38). It has also been recently suggested that hTrp3 activity is regulated via an interaction with the IP3 receptor (26). Thus, such interactions and other possible regulatory mechanisms could also determine the characteristics and regulation of expressed trp activity in various cells. Clearly, further studies are required for a more complete understanding of the function of Trp proteins. An important aspect of these studies will be to determine the presence and localization of various Trp proteins in different tissues and cell types.

Based on the rather ubiquitous distribution of the SOC mechanism in various cell types, it is reasonable to hypothesize that the molecules involved in this mechanism would also be similarly widespread in distribution. Interestingly, data with htrp1 and rtrp1 demonstrate that trp1 is more widely expressed than the other trp genes. In addition to brain and heart, both rtrp1 and htrp1 transcripts are present in a number of nonexcitable tissues, such as kidney, lung, and colon (30, 33). trp1 homologous sequences have also been identified in cells such as rat and human pulmonary endothelial cells, Xenopus oocytes, and megakaryocytes. On the other hand, htrp3, trp5, and rtrp2 transcripts have been predominantly found in the brain, mtrp2 was abundant in the testsis, btrp4 was abundant in the adrenal gland and testis, and mtrp6 was abundant in the lung. Thus, the functional differences between various trp gene products might be related to their tissue-specific distribution. Whether these molecules fulfill a specific physiological role in these tissues remains to be established.

In summary, this study demonstrates that Trp1 protein is endogenously present in the plasma membrane region of HSG cells and of acinar and ductal cells in the intact salivary gland. Further, stable expression of trp1a cDNA in HSG cells induced an approximately 2-fold increase in the levels of the Trp1 protein and a 3–5-fold increase in SOC. Importantly, the increased Ca2+ influx activity associated with trp1a expression displayed characteristics similar to the endogenous SOC in HSG cells. Thus, the data presented demonstrate that expression of htrp1a, like trp1b, also results in an increase in SOC activity. In addition, we have shown that (i) expression of the trp1a cDNA in the antisense direction significantly reduced the endogenous SOC activity in HSG cells and (ii) expression of htrp3 cDNA did not induce any change in SOC. In aggregate, these findings provide evidence for the involvement of the Trp1 protein in the SOC mechanism in HSG cells, thus suggesting a molecular basis for this process in this salivary cell line. Notably, these are the first data to directly correlate changes in the level of the Trp1 protein with changes in SOC. Further studies will be required to address the important question of whether the Trp1a protein forms the SOC channel and how it is regulated by internal Ca2+ store depletion.

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Trp1, a Candidate Protein for the Store-operated Ca$^{2+}$ Influx Mechanism in Salivary Gland Cells

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