Role in Apical Ca\textsuperscript{2+} Influx

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Receptor-coupled [Ca\textsuperscript{2+}] increase is initiated in the apical region of epithelial cells and has been associated with apically localized Ca\textsuperscript{2+}-signaling proteins. However, localization of Ca\textsuperscript{2+} channels that are regulated by such Ca\textsuperscript{2+}-signaling events has not yet been established. This study examines the localization of TRPC channels in polarized epithelial cells and demonstrates a role for TRPC3 in apical Ca\textsuperscript{2+} uptake. Endogenously and exogenously expressed TRPC3 was localized apically in polarized Madin-Darby canine kidney cells (MDCK) and salivary gland epithelial cells. In contrast, TRPC1 was localized basolaterally, whereas TRPC6 was detected in both locations. Localization of Ga\textsubscript{q/11}, inositol 1,4,5-trisphosphate receptor-3, and phospholipase C\(\beta\)1 and -\(\beta\)2 was also predominantly apical. TRPC3 co-immunoprecipitated with endogenous TRPC6, phospholipase C\(\beta\)3 and syntaxin 3 but not with TRPC1. Furthermore, 1-oleoyl-2-acetyl-glycerol (OAG)-stimulated apical \(\text{Ca}^{2+}\) uptake was higher in TRPC3-MDCK cells compared with control (MDCK) cells. Bradykinin-stimulated apical \(\text{Ca}^{2+}\) uptake and transepithelial \(\text{Ca}^{2+}\) flux were also higher in TRPC3-expressing cells. Consistent with this, OAG induced [Ca\textsuperscript{2+}] increase in the apical, but not basolateral, region of TRPC3-MDCK cells that was blocked by EGTA addition to the apical medium. Most importantly, (i) TRPC3 was detected in the apical region of rat submandibular gland ducts, whereas TRPC6 was present in apical as well as basolateral regions of ducts and acini; and (ii) OAG stimulated apical \(\text{Ca}^{2+}\) influx into dispersed ductal cells. These data demonstrate functional localization of TRPC3/TRPC6 channels in the apical region of polarized epithelial cells. In salivary gland ducts this could contribute to the regulation of salivary [Ca\textsuperscript{2+}] and secretion.

Stimulation of plasma membrane receptors that are coupled to the Ga\textsubscript{q/11} family of G proteins leads to activation of PLC\(\beta\), hydrolysis of PIP\(_2\), and generation of IP\(_3\) and DAG (1–3). Stimulation of tyrosine kinase-coupled receptors also activates PIP\(_2\) hydrolysis via stimulation of PLC\(\gamma\). In either case, the signaling cascade results in an increased intracellular [Ca\textsuperscript{2+}](\([\text{Ca}^{2+}]\)), due to IP\(_3\)-induced release of Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+} stores and influx of extracellular Ca\textsuperscript{2+}. Such increases in [Ca\textsuperscript{2+}] have a critical role in regulating a wide variety of cellular functions, such as secretion and gene expression. In polarized cells, e.g. neuronal and epithelial cells, Ca\textsuperscript{2+}-regulated cellular functions are often restricted to specific microdomains (4–9). It has been proposed that local changes in [Ca\textsuperscript{2+}], are determined by the targeted localization of specific Ca\textsuperscript{2+}-signaling proteins within that domain.

In polarized exocrine gland epithelial cells, there are convincing functional and biochemical data to demonstrate that Ca\textsuperscript{2+}-signaling proteins as well as initial [Ca\textsuperscript{2+}], changes occur in the apical region. More importantly, cellular functions such as protein and fluid secretion as well as ion re-absorption occur via the apical membrane of acinar and ducal cells, respectively, in these glands and are regulated by agonist-stimulated [Ca\textsuperscript{2+}], increases. Thus, it has been proposed that Ca\textsuperscript{2+}-signaling events are strategically localized in order to facilitate the regulation of these cellular functions (4, 6, 8). For example, Ca\textsuperscript{2+}-mobilizing agonists activate apical chloride channels in salivary gland and pancreatic acinar cells, and it has been demonstrated that this is mediated via an increase in [Ca\textsuperscript{2+}], at the apical pole of these cells. This has been further confirmed by studies showing that agonist-stimulated release of Ca\textsuperscript{2+} initially occurs from internal stores localized in the apical region of cells (6–8). Most importantly, this internal Ca\textsuperscript{2+} release has been correlated with the relative abundance of IP\(_3\)Rs in this region. However, relatively less is known about the site of Ca\textsuperscript{2+} influx that is stimulated in response to receptor-activated PIP\(_2\) hydrolysis. Based on recent studies, it can be suggested that two types of Ca\textsuperscript{2+} influx pathways can be activated following stimulation of plasma membrane receptors that are coupled to PIP\(_2\) hydrolysis. The first type of influx depends on PIP\(_2\) hydrolysis (but not on store depletion), whereas the second influx mechanism is associated with store depletion per se (10). It was reported previously that store depletion-regulated Ca\textsuperscript{2+} influx occurs via the basolateral
membrane of exocrine gland acinar cells (11). However, whether additional receptor-stimulated Ca\(^{2+}\) influx occurs in the apical region of cells where the Ca\(^{2+}\)-signaling proteins and initial Ca\(^{2+}\) increases occur and are localized is not yet known.

The TRPC subfamily of ion channel proteins form Ca\(^{2+}\)-permeable cation channels that are activated by agonist stimulation of PIP\(_2\) hydrolysis (12, 13). TRPC3, -6, and -7 form agonist-regulated channels that can also be directly activated by DAG (14), whereas TRPC1 and TRPC4 appear to form store-operated channels in some cell types (15–17). Furthermore, a number of the TRPC proteins, e.g. TRPC1, TRPC3, and TRPC4, form heteromeric channels and are assembled in a complex with key Ca\(^{2+}\)-signaling proteins (18–20). Thus, it can be proposed that interaction of TRPCs with Ca\(^{2+}\)-signaling proteins localizes the channels to the same microdomain where Ca\(^{2+}\)-signaling events are initiated. This localization likely facilitates not only activation of the TRPC channels in response to stimulation of Ca\(^{2+}\)-signaling events but also regulation of the downstream cellular functions that are dependent on Ca\(^{2+}\) influx. A number of TRPC proteins are expressed in every cell type (12, 13, 21, 22). However, with the exception of murine TRPC2 (23), the physiological function of TRPC channels is not yet known. Furthermore, cellular factors that determine the heteromeric interactions between TRPCs have also not been defined. As noted above, (i) Ca\(^{2+}\)-signaling proteins are present in the apical region of salivary and pancreatic epithelial cells, and (ii) TRPC proteins have been reported to associate with each other and with Ca\(^{2+}\)-signaling proteins. Thus, we have examined the localization of TRPC channels (TRPC1, TRPC3, and TRPC6) in polarized epithelial cells and have assessed the site of Ca\(^{2+}\) entry.

**Experimental Procedures**

**Cell Culture and Transfection**—Madin-Darby canine kidney (MDCK) cells and submandibular immortalized epithelial cells (SMIE) were maintained in DMEM, supplemented with 10% fetal calf serum, 2 mm L-glutamine, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin (Biowhittaker, Rockville, MD). For stably transfected MDCK cells, media were supplemented with 600 \(\mu\)g/ml geneticin (G418; Invitrogen). MDCK cells (at 70–80% confluency) were treated with a mixture containing 1 \(\mu\)g of the required plasmid and 40 \(\mu\)g of Lipofectamine (Invitrogen) in serum-free DMEM. After 5 h, the mixture was replaced with fresh DMEM containing serum, penicillin, streptomycin, and 600 \(\mu\)g/ml G418 (Invitrogen). 48–72 h after transfection, cells were harvested at low density and placed in G418 media for 2–3 weeks to select for stably transfected cells. Clones of G418-resistant cells were screened for protein expression, and those with the highest expression were chosen for use in this study. For each protein, the results were verified at least two independently derived stable cell clones. Transient transfection of SMIE cells was performed using 1 \(\mu\)g of the required plasmid combined with 2.4 \(\mu\)g of Lipofectamine 2000 (Invitrogen) in serum-free DMEM, incubated at room temperature for 20 min, and added to cells grown overnight in a 24-mm Transwell filter. After 5 h, serum-free medium was replaced with normal growth medium. The positions of the TAGs in the TRPC proteins were as follows: exo-HA-TRPC3, between TM-3 and 4; FLAG-TRPC3, C-terminal; HA-TRPC1, N-terminal; HA-TRPC4, C-terminal.

**Measurement of Transepithelial Electrical Resistance** (TER)—TER across cell monolayers was measured as described previously (24). Briefly, cells were seeded at a density of 1 \(\times\) 10\(^5\) per well and grown to confluence in 24-mm Transwell culture chambers (Corning Costar). TER measurements were made with a Millicell ERS epithelial volt-ohmmeter (Millipore, Billerica, MA) as described by the manufacturer. Experiments were performed in triplicate with three separate cell preparations. Data are presented as the mean \pm S.E.M. Student's t test was used for statistical evaluation of the values; *p < 0.05 was considered to be statistically significant.

**Crude Membrane Preparation**—Cells were grown in tissue culture plates and harvested by scraping into ice-cold PBS containing 1% (v/v) aprotonin (Sigma) and centrifuged for 5 min at 2000 \(\times\) g. The cell pellet was resuspended in a lysis buffer containing (in mM) the following: 10 Tris-HCl (pH 8.0), 1 Mgly, 0.5 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (INC Biomedical Inc., Aurora, OH, 0.1 phenylmethylsulfonyl fluoride (Calbiochem) and frozen at −80 °C for at least 2 h, before use. Crude membranes were prepared from cell lysates by homogenization and centrifugation as described previously (18–19). Protein concentration was determined by using the Bio-Rad protein assay (microassay procedure).

**Generation of Anti-TRPC3 Antibody**—Rabbit polyclonal antisera against TRPC3 was generated by injecting New Zealand White male rabbits with the peptide MREKGRRQAVRGPAFMNDRC (corresponding to a C-terminal sequence) coupled to keyhole limpet hemocyanin (Pierce). Initial injection was with Complete Freund’s Adjuvant, and boost injections at days 14, 21, and 49 were with Incomplete Freund’s Adjuvant. Rabbit injections, bleeds, and housing were performed by Cocalico Biologicals (Reamstown, PA). Antibodies were affinity-purified by using the antibody peptide coupled to the Pierce min. ELISA-Ultralink solid phase. Immunoblots were performed by Dr. Craig Montell, and anti-FLAG antibody to confirm the specific- ity of the antibody. Furthermore, incubation with the peptide decreased detection of the TRPC3 protein.

**Confocal Fluorescence and Confocal Imaging**—All steps were performed at room temperature unless otherwise mentioned. The cells were rinsed with PBS, fixed with 3% paraformaldehyde in PBS (pH 7.4) for 30 min, rinsed with PBS, and treated with 100 \(\mu\)m glycine in PBS for 20 min. Cells were then washed and permeabilized with methanol at −20 °C for 5 min. Following incubation with a blocking solution containing 5% donkey serum and 0.5% bovine serum albumin in PBS for 20 minutes, cells were incubated with primary antibodies for 1 h at room temperature, washed with PBS/bovine serum albumin, and probed with the required FITC- or rhodamine-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA). The filter was excised from its support and mounted onto a slide with an anti-fade reagent (Ted Pella, Inc.). Fluorescence images were taken by using the confocal laser-scanning microscope Leica TCS-SL2 attached to an upright Leica DM-RE7 microscope. Details of the images are in the figure and text.

**Immunoprecipitation and Immunoblotting**—Polarized MDCK cells were lysed by addition of octyl glucoside and KI (18, 19) directly to the filter. Immunoprecipitation from the cell lysate was done as described previously (19). For TRPC3-FLAG, 200 \(\mu\)g of Sepharose beads crosslinked to anti-FLAG antibody (Sigma) was used. For TRPC6, anti-TRPC6 antibody (Sigma) was used followed by addition of Sepharose beads coupled to the anti-FLAG- or anti-TRPC6-bound beads. The supernatant was saved (unbound fraction). The beads were washed, and bound proteins (IP fraction) were eluted by boiling in SDS-PAGE sample buffer for 5 min and were separated by SDS-PAGE. Proteins were detected by Western blotting as described previously (19) using anti-FLAG (1:1000), anti-\(\beta\)-actin (1:1000, Roche Applied Science), anti-syntaxin 3 (1:500, Santa Cruz Biotechnology), anti-syntaxin 3 (1:250), anti-PLC-\(\gamma\) (1:500), anti-PLC-\(\beta\)2 and -3 (1:500, Santa Cruz Biotechnology), anti-PLC-\(\gamma\)2 (1:500), -3 (1:500), and -2 (1:500, Santa Cruz Biotechnology), the required secondary antibody, and treatment with ECL reagent.

**\(4^{45}\)Ca\(^{2+}\) Uptake Measurements**—Cells were grown until confluent (with High TER) on Transwell filters. The cell culture medium was removed, and the upper (apical) and lower (basal) chambers were washed with SES medium (details are given in the figure legend). As indicated, medium in the required chamber (0.7 ml per chamber) was supplemented with \(4^{45}\)Ca\(^{2+}\). Other reagents were added as described in the text and figures. Cells were incubated at 37 °C for the required time periods, after which the filters were removed, rinsed in ice-cold medium containing 500 \(\mu\)M La\(^{3+}\) cut out from the manifold, and placed in scintillation vials with scintillation fluid, and radioactivity was determined. For transepithelial flux, an aliquot of the basal medium was sampled. Values are represented relative to the respective controls in which uptake was measured from the same chamber without stimulation of cells.

**Immunocytochemistry**—Adult male Wistar rats (weighing 200–250 g) were killed by decapitation under controlled light/dark cycle (light/ dark cycle, 20–22 °C) with food and water available ad libitum until used. All animals were treated according to guidelines approved by the NICDR, National Institutes of Health Animal Care and Use Committee. Submandibular glands were excised from the animals and fixed in 10% formalin solution for 24 h for immunocytochemistry, dehydrated in graded concentrations of ethanol, embedded in paraffin, and used to prepare 5–10-μm-thick sections (American Histolabs, Gaithersburg,
MD). Immunocytochemistry was performed on paraffin sections of rat submandibular gland. Sections were dewaxed in xylene, rehydrated in ethanol solution (70–100%), and incubated for 10 min in 0.5% Triton X-100 in PBS (pH 7.5) for permeabilization. Sections were then treated with 3% hydrogen peroxide in PBS for 10 min and preincubated for 1 h in PBS containing 5% goat serum, followed by avidin-biotin blocking. Sections were incubated overnight at 4 °C in PBS containing rabbit polyclonal anti-TRPC3 (1:100) and TRPC6 (1:150) antibodies, rinsed, and incubated for 30 min at room temperature with a biotinylated goat polyclonal anti-rabbit IgG antibody, followed by streptavidin-peroxidase complex, and finally developed with 3,3′-diaminobenzidine using the Histostain kit (Zymed Laboratories Inc.). In control sections, rabbit IgG was used instead of the primary antibody.

**Crude Membrane Preparation**—For crude membrane preparation, the brain and submandibular gland were collected immediately after rats were euthanized and were immersed in chilled sucrose buffer containing the protease inhibitors. Membranes were prepared as described above after mincing and dispersion of the tissue by using a Polytron tissue homogenizer.

**Dispersed Cell Preparation from Rat SMG and [Ca^{2+}] Measurements**—Submandibular glands were removed and placed in ice-cold external solution with 0.02% soybean trypsin inhibitor and 0.1% bovine serum albumin. Each gland was cleaned and finely minced. The minced tissue was transferred to 8 ml of external solution containing 4 mg of collagenase P. The tube was gassed and capped. After 15–20 min of incubation at 37 °C, the digest was washed twice with the normal external solution followed by a 2–4-min centrifugation at 100 × g and resuspended in about 4 ml of external solution + 0.1% BSA + 0.02% soybean trypsin inhibitor. For microfluorimetry, cells were loaded with fura 2 for 45–60 min at 30 °C and placed in a poly-L-lysine-coated glass bottom dish (Matek Corp.) and allowed to attach. Ducts and acinar cells from the same glands were morphologically identified by their distinctive appearance under microscopic examination (26). Fluorescence measurements were made using a Till Photonics-Polychrome IV spectrophotometer and Metafluor Imaging System, Universal Imaging Corp.

**Measurements of Fluo-4 Fluorescence by Confocal Microscopy in Polarized MDCK Cells**—MDCK cells stably expressing TRPC3 were grown in Transwell filters to establish polarization, and TER was monitored to confirm this. Cells were incubated with 5 μM fluo-4-acetoxyethyl ester (Molecular Probes, Eugene, OR) at 37 °C for 1 h in 0.8% SES medium. Samples were scanned on a TCS SP2 Leica confocal microscope system using a 40 × 0.8 NA dipping objective. Single Transwell inserts were washed; 2 ml of SES medium was added (apical chamber), and the inserts were placed in 35 mm chambers containing 2.5 ml of SES medium (basal chamber). All additions were made to the apical chamber. Samples were scanned in X2 time mode for 5 min total time with 12-s increments between sampling. Calculations of the mean intensity in a defined region of interest were made using the histogram function in the Leica LCS Lite software and analyzing whole cell, basal, and apical regions throughout the time series.

**RESULTS**

**Localization of TRPC Proteins in Polarized Epithelial Cells**—TRPC3-FLAG, TRPC1-HA, and TRPC6-HA were stably expressed in MDCK cells. To determine their localization in polarized cells, TRPC-expressing MDCK cells were cultured on Transwell filters, and TER was monitored to assess the formation of a tight monolayer of polarized cells. Control MDCK cells developed maximum TER in about 3 days, and this was not altered by TRPC expression (Fig. 1A). Note that in all experiments described below, TER measurements were first made to ensure polarization of cells. Fig. 1B shows the expression of TRPC1, TRPC3, and TRPC6 in two clones that were used, in each case, in the experiments described below. After 3 days in culture, immunofluorescence was used to detect syntaxin 3, a marker protein for apical membranes in MDCK cells; Na+/K+-ATPase, a marker protein for basolateral membrane; and ZO1, a marker protein for the tight junction. The localization of these proteins in control polarized MDCK cells is shown in Fig. 1C (upper panels show stacked images, left; and a section with the x-z and y-z views, right; lower panels show enlarged images of the latter). As expected for MDCK cells (27, 28), syntaxin 3 was apically localized; Na+/K+-ATPase was basolateral, and ZO1 was detected in the tight junction region, between the cells, toward the apical pole. Note that when MDCK cells were grown on plastic, the proteins were detected all over the cell membrane (images are not shown). Furthermore, the localization of these marker proteins was similar in polarized MDCK cells stably expressing TRPC3 (Fig. 1D).

Localization of exogenously expressed TRPC3-FLAG was detected using the anti-FLAG antibody and FITC-conjugated secondary antibody (green denotes localization of TRPC3-FLAG, and red shows the location of the nucleus, which was stained
with propidium iodide). In a stacked image of the cells grown on the filter (Fig. 2A), TRPC3 appears to be in the apical region. This is more clearly seen in the x-z and z-y sections, where TRPC3 signal is predominantly detected in the apical membrane region of the cells (Fig. 2, B–D). Fig. 2E shows co-localization of syntaxin 3 and TRPC3-FLAG, further confirming the apical localization of TRPC3-FLAG in polarized MDCK cells. TRPC3 did not show polarized localization when grown on plastic (data not shown). We further examined localization of TRPC3 in a salivary gland epithelial cell line (SMIE) (24), which also forms polarized, high resistance monolayers on Transwell filters (TER measurements are not shown). As in MDCK cells, TRPC3-FLAG was seen in the apical membrane of polarized SMIE cells (SMIE, Fig. 2F). Notably, this localization was not an artifact due to the FLAG epitope tag because TRPC3-HA was similarly localized in the apical region of SMIE cells (Fig. 2G) and in MDCK cells (image not shown). Most importantly, the HA tag in this TRPC3 was inserted in the external domain (between TM-3 and -4), and apical TRPC3-HA was detected in nonpermeabilized cells. These data demonstrate surface expression of TRPC3 in the apical membrane.

The localization of exogenously expressed TRPC1-HA and TRPC6-HA was also examined in MDCK cells. Consistent with our previous studies with salivary gland acinar cells (15, 29), TRPC1-HA was localized in the basolateral region of polarized MDCK cells (Fig. 2H). TRPC6-HA was localized in both basolateral and apical regions of the cells (Fig. 2I). Thus, both TRPC3 and TRPC6 are localized in the apical region of the cells as would be predicted by previous reports demonstrating that these two proteins interact to form heteromeric channels.

TRPC1, TRPC3, and TRPC6 are endogenously present in MDCK cells (Fig. 2J). Consistent with the localization of TRPC6-HA, endogenous TRPC6 was detected in both basolateral and apical regions (Fig. 2K). Most importantly, endogenous TRPC3, like TRPC3-FLAG, was detected (using a rabbit polyclonal anti-TRPC3 antibody, 1:50 dilution, see “Experimental Procedures” for details).

**Association of TRPC3 and TRPC6 with Apical Ca\(^{2+}\)-signaling Proteins**—Among the various TRP channels, TRPV5 and TRPV6 are apically localized in distal regions of renal tubules and gastric epithelium (30). These proteins are suggested to be involved in apical Ca\(^{2+}\) reabsorption in distal tubules and collecting tubules of the kidney as well as from the gut lumen. However, these channels have been reported to be spontaneously active and not regulated by PIP\(_2\)-PLC activation. TRPC3 and TRPC6, on the other hand, are activated in response to receptor-activated PIP\(_2\) hydrolysis, possibly by the DAG that is
cells. Endogenous Gq/11, PLCβ1, and -β2, as well as IP3R-3 and syntaxin 3 all appear to be relatively enriched in the IP fraction (compared with the unbound fraction). We also detected endogenous TRPC6 and IP3R-1 in the TRPC3-IP, although these did not appear to be enriched in this fraction. This is most likely due to the fact that a significant fraction of TRPC6 and IP3R-1 are not apically localized and therefore are not associated with TRPC3. Most importantly, and consistent with its localization in the basolateral membrane, TRPC1 was not immunoprecipitated with the apically localized TRPC3. These findings are very significant because they show that TRPC3 is assembled in an apically localized complex with TRPC6 and key Ca2+-signaling proteins.

An important finding of this study is that endogenously expressed TRPC6 and TRPC3 also demonstrate similar association. Because endogenous TRPC3 and TRPC6 were localized in the apical region of MDCK cells, we examined possible association between them by immunoprecipitating endogenous TRPC6. Endogenous TRPC3, syntaxin 3, an apical membrane marker, and Gq/11 were detected in the immunoprecipitated fraction (Fig. 4B, other proteins were not determined). These data show that like exogenously expressed TRPC3, endogenous TRPC3 also forms an apical Ca2+-signaling complex with TRPC6. Based on the regulation of several ion channels by syntaxin 3, it will be important in future studies to determine whether syntaxin 3 has any direct effects on the function or trafficking of TRPC3. These data are also consistent with our recent report (33) showing that TRPC3 interacts with SNARE proteins.

**TRPC3 Increases Apical Ca2+ Entry and Apico-basal Trans-epithelial Ca2+ Flux in Polarized MDCK Cells**—The function of TRPC3 in the apical membrane of MDCK cells was assessed by measuring 45Ca2+ uptake in TRPC3-FLAG-MDCK cells and control MDCK cells grown on Transwell filters. OAG (50 μM) was added to the apical side of the filter, whereas 45Ca2+ was added to the apical side or basal side (incubation was done for 10–15 min). In control assays, Me2SO was added apically (note that Me2SO did not change the 45Ca2+ permeability of the cells). The function of TRPC3 in the apical membrane of MDCK cells was assessed by measuring 45Ca2+ uptake in TRPC3-FLAG-MDCK cells and control MDCK cells grown on Transwell filters. OAG (50 μM) was added to the apical side of the filter, whereas 45Ca2+ was added to the apical side or basal side (incubation was done for 10–15 min). In control assays, Me2SO was added apically (note that Me2SO did not change the 45Ca2+ permeability of the cells). The function of TRPC3 in the apical membrane of MDCK cells was assessed by measuring 45Ca2+ uptake in TRPC3-FLAG-MDCK cells and control MDCK cells grown on Transwell filters. OAG (50 μM) was added to the apical side of the filter, whereas 45Ca2+ was added to the apical side or basal side (incubation was done for 10–15 min). In control assays, Me2SO was added apically (note that Me2SO did not change the 45Ca2+ permeability of the cells). The function of TRPC3 in the apical membrane of MDCK cells was assessed by measuring 45Ca2+ uptake in TRPC3-FLAG-MDCK cells and control MDCK cells grown on Transwell filters. OAG (50 μM) was added to the apical side of the filter, whereas 45Ca2+ was added to the apical side or basal side (incubation was done for 10–15 min). In control assays, Me2SO was added apically (note that Me2SO did not change the 45Ca2+ permeability of the cells). The function of TRPC3 in the apical membrane of MDCK cells was assessed by measuring 45Ca2+ uptake in TRPC3-FLAG-MDCK cells and control MDCK cells grown on Transwell filters. OAG (50 μM) was added to the apical side of the filter, whereas 45Ca2+ was added to the apical side or basal side (incubation was done for 10–15 min). In control assays, Me2SO was added apically (note that Me2SO did not change the 45Ca2+ permeability of the cells). The function of TRPC3 in the apical membrane of MDCK cells was assessed by measuring 45Ca2+ uptake in TRPC3-FLAG-MDCK cells and control MDCK cells grown on Transwell filters. OAG (50 μM) was added to the apical side of the filter, whereas 45Ca2+ was added to the apical side or basal side (incubation was done for 10–15 min). In control assays, Me2SO was added apically (note that Me2SO did not change the 45Ca2+ permeability of the cells). The function of TRPC3 in the apical membrane of MDCK cells was assessed by measuring 45Ca2+ uptake in TRPC3-FLAG-MDCK cells and control MDCK cells grown on Transwell filters. OAG (50 μM) was added to the apical side of the filter, whereas 45Ca2+ was added to the apical side or basal side (incubation was done for 10–15 min). In control assays, Me2SO was added apically (note that Me2SO did not change the 45Ca2+ permeability of the cells). The function of TRPC3 in the apical membrane of MDCK cells was assessed by measuring 45Ca2+ uptake in TRPC3-FLAG-MDCK cells and control MDCK cells grown on Transwell filters. OAG (50 μM) was added to the apical side of the filter, whereas 45Ca2+ was added to the apical side or basal side (incubation was done for 10–15 min). In control assays, Me2SO was added apically (note that Me2SO did not change the 45Ca2+ permeability of the cells).
Ca\(^{2+}\) uptake from the apical medium suggests the involvement of the apically localized TRPC3/TRPC6. This was further confirmed by using polarized TRPC3-FLAG-MDCK cells in which the expressed TRPC3 protein is apically localized (see Fig. 2). In these cells, OAG-stimulated apical Ca\(^{2+}\) uptake was 3–4-fold higher than that in control MDCK cells. Control 45Ca\(^{2+}\) uptake from either apical or basal medium was similar in the two groups, suggesting that the expressed TRPC3 does not form spontaneously active channels. In aggregate, these data demonstrate that TRPC3 forms functional OAG-sensitive Ca\(^{2+}\)-permeable channels in the apical membrane of MDCK cells.

Possible regulation of TRPC3 by receptor activation was examined by measuring apical 45Ca\(^{2+}\) uptake as well as apico-basal transepithelial 45Ca\(^{2+}\) flux in TRPC3-FLAG-expressing MDCK and control MDCK and in cells stimulated with bradykinin, which has been shown previously to induce Ca\(^{2+}\) mobilization in these cells. 1 \(\mu\)M bradykinin was added either apically or basolaterally, and 45Ca\(^{2+}\) was added apically. After 30 min of incubation, 45Ca\(^{2+}\) was counted in the filters and in aliquots from the medium on the basal side of the filter. In control MDCK cells stimulated with bradykinin (Fig. 5B), values marked by * are significantly different from similarly filled columns in the control MDCK cell group (\(n\) in each case was greater than 4, and duplicate samples were run in each experiment). OAG-stimulated increase in the apical (AP) region was significantly higher (\(p < 0.01\)) than in basal. I, model summarizing localization of TRPC proteins in polarized MDCK cells. Arrows indicate proposed TRPC3/TRPC6-mediated apical Ca\(^{2+}\) uptake and direction of transepithelial Ca\(^{2+}\) flux.
Apical Localization and Function of TRPC3/TRPC6 Channels in Rat Submandibular Gland—The data presented above demonstrate that TRPC3/TRPC6 channels are localized in the apical membrane of polarized MDCK cells and SMIE cells that are derived from rat submandibular glands (SMG). To confirm this, in situ localization of TRPC3 and TRPC6 was determined in sections of excised SMG by immunocytochemistry. Consistent with the data shown above, TRPC3 was detected primarily in the apical region of salivary gland ducts (Fig. 6, B and C). There was no significant signal in acini (Fig. 6, small arrows), compare signal in B and C with A, which shows control staining in a serial section using rabbit IgG. TRPC3 was present in the apical region of all types of salivary gland ducts (striated, shown by asterisks, and excretory ducts, shown by boldface arrow, can be seen in the sections shown in Fig. 6B; an enlarged excretory duct is shown in Fig. 6C). The weakest signal was detected in the intercalated ducts and progressively increased in striated and excretory ducts. Furthermore, consistent with its localization in MDCK and SMIE cells, a more diffused localization of TRPC6 was seen in both SMG ducts as well as acini (Fig. 6, F and G; Fig. 6E shows control staining of a serial section using rabbit IgG). Most importantly, TRPC6 was clearly detected in the apical region where TRPC3 was also localized (see enlarged images of the same excretory duct in Fig. 6, C and G). TRPC6 is detected in both basal and luminal areas, Fig. 6G). The presence of TRPC3 and TRPC6 in SMG was further confirmed by Western blots (Fig. 6, D and H), crude membrane from brain was used as a control.

The function of TRPC3/TRPC6 was examined by measuring OAG-stimulated changes in [Ca\(^{2+}\)] in fura 2-loaded dispersed SMG ducts. OAG stimulated a rapid increase in [Ca\(^{2+}\)], in ductal cells bathed in a Ca\(^{2+}\)-containing medium (Fig. 6I, an
image of the duct is shown in the inset). Addition of carbachol after OAG induced a further smaller increase in [Ca$$^{2+}$$], (carbachol alone induced [Ca$$^{2+}$$], increase that was similar to that seen with OAG, data not shown). In a nominally Ca$$^{2+}$$-free medium, OAG did not induce any detectable increase in [Ca$$^{2+}$$]. However, re-addition of Ca$$^{2+}$$ induced a rapid increase in [Ca$$^{2+}$$], which was similar to that seen in the presence of 1 mM external Ca$$^{2+}$$. These data suggest that OAG stimulates Ca$$^{2+}$$ entry into SMG ducts, although further studies will be required to confirm the exact location of this Ca$$^{2+}$$ entry.

**DISCUSSION**

The data presented above demonstrate a role for TRPC3/TRPC6 channels in Ca$$^{2+}$$ flux across the apical membrane of epithelial cells. In polarized MDCK and SMIE cells, kidney and rat submandibular gland ductal cell lines, respectively, TRPC3 was localized primarily in the apical membrane, whereas TRPC6 was detected both in the basal and apical regions. Furthermore, this localization was displayed by both endogenous and exogenously expressed TRPC3 and TRPC6 in both these cell types. Most importantly, we have demonstrated that the localization of TRPC3 and TRPC6 in situ in rat SMG was similar to that in the polarized ductal epithelial cell lines. TRPC3 in rat SMG was detected primarily in the apical region of all types of ducts. In contrast, TRPC6 was present in acinar and ductal cells where it was detected in both basal and apical regions. It has been reported previously (5, 6) that key Ca$$^{2+}$$-signaling proteins are localized in the apical region of salivary and pancreatic gland cells. Consistent with this, the present data demonstrate that most of the same Ca$$^{2+}$$-signaling proteins display polarized localization in MDCK cells. Most importantly, we show that TRPC3 forms a heteromeric complex with TRPC6 and the Ca$$^{2+}$$-signaling proteins, Goq11, PLC$$\beta$$1 and $$\beta$$3, and IP$$_3$$R-1 and -3. In aggregate, these data suggest that apically localized TRPC channels can be regulated by Ca$$^{2+}$$-signaling mechanisms, as proposed previously for these channels (10, 14, 21).

Apically localized Ca$$^{2+}$$ channels in ductal epithelia, such as those of kidney and salivary glands, most likely mediate influx Ca$$^{2+}$$ because [Ca$$^{2+}$$]$$_i$$ in the urine or saliva is relatively high. TRPV5 and TRPV6 are spontaneously activated channels that are localized in the apical membrane of the distal convoluted ducts of kidney and have been proposed to mediate Ca$$^{2+}$$ re-absorption. To our knowledge, Ca$$^{2+}$$ channels that are regulated by receptor-mediated PIP2 hydrolysis have not yet been identified in the apical membrane of epithelial cells. By examining Ca$$^{2+}$$ uptake in polarized MDCK cells, we report here that TRPC3/TRPC6 forms functional channels in the apical membrane of MDCK cells. Furthermore, both OAG, which directly activates TRPC3/TRPC6/TrPC7 channels (14), and bradykinin, a Ca$$^{2+}$$-mobilizing agonist acting on bradykinin receptors, increased Ca$$^{2+}$$ uptake via the apical membrane. This increase was greater in cells overexpressing TRPC3 than in control MDCK cells. Most importantly, our data show that (i) apical Ca$$^{2+}$$ was delivered to the basal medium via transepithelial flux, and (ii) this transepithelial Ca$$^{2+}$$ flux was higher in TRPC3-expressing cells. Furthermore, we have confirmed the spatial localization of the apical influx by confocal imaging of fluo-4. Thus, we suggest that the apically localized heteromeric TRPC3/TRPC6 channels can potentially mediate Ca$$^{2+}$$ re-absorption and transepithelial Ca$$^{2+}$$ flux in absorptive epithelial cells in response to stimulation of receptors that lead to PIP2 hydrolysis (see the model in Fig. 5f that summarizes our data).

It has been well established that in pancreatic and salivary gland acinar as well as ductal cells, receptor-activated cellular Ca$$^{2+}$$ signals are initiated in the apical region (36–38). However, relatively less is known regarding the site(s) of Ca$$^{2+}$$ influx that are initiated in response to receptor-mediated PIP2 hydrolysis. It has been suggested that store-operated Ca$$^{2+}$$ uptake occurs across the basolateral membrane of exocrine gland acinar cells (8, 11). In an earlier study we had reported that TRPC1 is localized in the basolateral region of rat SMG acini, and overexpression of TRPC1 in this region of the cell increased store-operated calcium entry in rat SMG acini (29). The data described above provide evidence for the presence of Ca$$^{2+}$$ influx channels in the apical membrane of polarized epithelial cells that can mediate Ca$$^{2+}$$ influx in response to stimuli that induce PIP2 hydrolysis. We have shown that TRPC3/TRPC6 channels are localized in the apical membrane of rat submandibular gland ducts and that OAG stimulates Ca$$^{2+}$$ influx in dispersed SMG ducts. Whether these channels are also regulated store-operated mechanisms has not been determined in this study. Although we have not presently confirmed whether Ca$$^{2+}$$ uptake occurs via the apical membrane of rat SMG ducts, the apical Ca$$^{2+}$$ entry we have demonstrated in MDCK cells, together with the similar apical localization of TRPC3/TRPC6 in rat SMG, strongly suggests that the TRPC3/TRPC6 channel complex has the potential to do so. Further studies will be required to directly demonstrate this and to establish which Ca$$^{2+}$$-signaling receptor(s) regulate these apically localized channels in rat SMG ducts. We have recently reported that surface expression of TRPC3 channels is regulated by a PIP2 hydrolysis-dependent exocytosis mechanism (33). It is interesting to note that several apically active channels, e.g. cystic fibrosis transmembrane regulator, AQP5, are present in recycling vesicles and are recruited to the surface membrane upon activation.

The apical membrane of rat SMG ducts is involved in protein as well as ion (K$$^+$$ and HCO$$^3$$) secretion and ion re-absorption (Na$$^+$$ and Cl$$^-$$). Several of these ion channels are regulated by changes in [Ca$$^{2+}$$]. Most interestingly, G protein-coupled receptors such as purinergic and bradykinin receptors have been identified in apical membranes of salivary gland and kidney ductal cells, and stimulation of these receptors regulates secretion as well as re-absorption mechanisms (37–39). We suggest that Ca$$^{2+}$$ entry into the apical region of SMG ducts can regulate local [Ca$$^{2+}$$]i, in this region of the cell and thus contribute to the mechanisms involved in protein and fluid secretion as well as re-absorption of ions. Furthermore, re-absorption of Ca$$^{2+}$$ from the primary saliva can also regulate the [Ca$$^{2+}$$]i of saliva that is critical for maintaining oral health. Re-absorption of Ca$$^{2+}$$ can also have an impact on pathological conditions such as sialoliths that are formed when salivary [Ca$$^{2+}$$]i is increased (40). Most interestingly, it has been suggested that Ca$$^{2+}$$ is reabsorbed from the lumen of pancreatic ducts and that failure of this mechanism could lead to formation of pancreatic stones or pancreatitis (41). Thus, determining the exact localization of TRPC channels in different epithelial tissues and identifying the Ca$$^{2+}$$-signaling system they are coupled to will provide a better understanding of the physiological function and regulation of these channels.

**Acknowledgments**—We thank Drs. Robert Wellner, Brij Singh, and Timothy Lockwich for their help. We also thank Burton Rankie for technical assistance; Dr. Mike Zhu for the HA-TRPC3, exo-HA-TRPC3, and HA-TRPC1 plasmids; Dr. Lutz Birnbaumer for HA-TRPC6 plasmids; Dr. Sue Goo Rhee for the anti-PLC$$\gamma$$1 antibody; and Dr. Craig Montell for the anti-TRPC3 antibody. We especially thank Dr. Mark Knepper for providing us with the anti-syntaxin 3 antibody and for helpful discussions and input in the preparation of this manuscript.

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