TRPC4 forms store-operated Ca\(^{2+}\) channels in mouse mesangial cells

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In both excitable and nonexcitable cells, depletion of intracellular stores of Ca\(^{2+}\) causes a flux of Ca\(^{2+}\) into the cell until the sarcoendoplasmic reticulum refills to its original Ca\(^{2+}\) concentration. This process of refilling Ca\(^{2+}\) is designated Ca\(^{2+}\) release-activated Ca\(^{2+}\) (SOC) channel. Using TRPC4-specific primers and RT-PCR, we found that cultured MMC contained mRNA for TRPC1 and TRPC4 but not for TRPC2, TRPC3, TRPC5, TRPC6, and TRPC7. Immunocytochemical staining of MMC revealed predominantly cytoplasmic expression of TRPC1 and plasmalemmal expression of TRPC4. The role of TRPC4 in SOC was determined with TRPC4 antisense and fura 2 ratiometric measurement of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). SOC was measured as the increase in [Ca\(^{2+}\)]\(_i\) after extracellular Ca\(^{2+}\) was increased from <10 nM to 1 mM in the continued presence of thapsigargin. We found that TRPC4 antisense, which reduced plasmalemmal expression of TRPC4, inhibited SOC by 83%. Incubation with scrambled TRPC4 oligonucleotides did not affect SOC. Immunohistochemical staining identified expressed TRPC4 in the glomeruli of mouse renal sections. The results of RT-PCR performed to distinguish between TRPC4-α and TRPC4-β were consistent with expression of both isoforms in brain but with only TRPC4-α expression in MMC. These studies show that TRPC4-α may form the homotetrameric SOC in mouse mesangial cells.

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94°C and the extension temperature was 72°C, and 35–40 PCR cycles were performed. The PCR products were visualized on 1.5% agarose gel containing ethidium bromide under UV light.

To distinguish splice variants TRPC4-α and TRPC4-β, isolated mRNA was amplified by PCR with the use of a forward primer (5’-CATTTCAGTCCGTCTTC-3’) paired with a reverse primer (5’-TCTTCAACCACACTCTC-3’). Annealing was performed at 60°C for 1 min. All other cycle conditions were as described above.

Western blotting. For the detection of proteins by Western blot analysis, MMC proteins were collected and isolated according to standard protocols by sonication and centrifugation. Protein concentration was assayed with the use of Bio-Rad protein assay dye (Bio-Rad, Hercules, CA), and proteins were loaded onto a 12.5% polyacrylamide gel after being boiled in Laemmli sample buffer. After electrophoresis, proteins were transferred to a polyvinylidene difluoride nitrocellulose membrane blocked in 5% milk in Tris-buffered saline containing Tween 20 (TBST: 0.1% Tween 20) before exposure to the primary antibody (TRPC1, 1:200, or TRPC4, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA). After being washed in TBST, the membranes were exposed to a horseradish peroxidase (HRP)-conjugated secondary antibody and washed once again, and then the signal was visualized with the use of SuperSignal West Femto substrate (Pierce, Rockford, IL). Images were captured with a UVP bioimaging system (Upland, CA) and saved as digital image files.

Immunocytochemical staining. For immunocytochemical staining, MMC were plated on glass coverslips, fixed with 4% paraformaldehyde for 10 min at 23°C, and then washed briefly in PBS followed by methanol (10 min at 23°C). Next, the cells were incubated with PBS containing 1% BSA for 1 h at 23°C, incubated with primary antibodies overnight at 4°C, and then washed with PBS three times for 5 min each. After this first incubation, the MMC were incubated for 1 h at 23°C with the secondary antibodies. For TRPC1, the primary antibody used was rabbit anti-human TRPC1 (1:50), commercially available from Santa Cruz Biotechnology. The secondary antibody was goat anti-rabbit IgG (1:400) conjugated with Alexa Fluor 594 (Molecular Probes, Eugene, OR). For TRPC4, the primary antibody used was rabbit polyclonal anti-TRPC4 (1:100; Sigma) or goat polyclonal anti-TRPC4 (1:50; Santa Cruz Biotechnology). The secondary antibody for TRPC4 was Alexa 594-conjugated donkey anti-rabbit IgG or Alexa 488-conjugated donkey anti-goat (1:400; Molecular Probes). The negative control was IgG incubated in the absence of the primary antibody but at the same concentration. Samples were viewed with an Olympus BX50 microscope. Images were captured with a digital camera (Princeton Scientific Instruments, Monmouth Junction, NJ), and the final layout of images was created with Adobe Photoshop software.

Fura 2 ratiometry. For fura 2 ratiometric measurement of Ca2+, cells were grown to confluence, passed onto 22 × 22-mm glass coverslips (Fisher Scientific, Pittsburgh, PA), and studied within 6 h at 25°C. Measurements of intracellular Ca2+ concentration ([Ca2+]i) in MMC were obtained by fura 2 and dual excitation wavelength fluorescence microscopy as previously described (4, 7). In brief, after loading with fura 2, the glass coverslips with MMC were placed into a perfusion chamber (Warner RC-20H; Warner Instruments, Hamden, CT) and then mounted on the stage of a Nikon Diaphot 300 inverted microscope. With light provided by a DeltaScan dual monochromator system (Photon Technology International, Lawrenceville, NJ), the cells were illuminated alternately at 340- and 380-nm wavelengths (3-nm bandwidths). An adjustable optical sampling window was positioned within the light path before detection with a photon-counting photomultiplier to monitor fluorescence emission (510 nm, 20-nm bandpass) from a single cell. Background-corrected data were collected at 50 ms from points and then stored and processed with the use of the FeliX software package (Photon Technology International). Calibration of the fura 2 signal was performed according to established methods (4, 7). Cells were loaded with fura 2 by incubation for 60 min (23°C) in physiological salt solution (135 mM NaCl, 5 mM KCl, 2 mM MgCl2, 1 mM CaCl2, and 10 mM HEPES) containing 7 μM fura 2-acetoxymethyl ester, 0.09 g/dl DMSO, and 0.018 g/dl Pluronic F-127 ( Molecular Probes). For all experiments, the initial bathing solution contained (in mM) 135 NaCl, 5 KCl, 10 HEPES, and 1 CaCl2. Free [Ca2+] was reduced to <10 nM by the addition of EGTA.

SOC activity was measured as previously described by investigators at our laboratory (15) and by others (1). In brief, after stable baseline [Ca2+]i was obtained, 1 μM thapsigargin was applied to the bathing solution containing 1 mM Ca2+. After an initial rapid rise and a subsequent plateau phase, the bath [Ca2+]i was reduced to <10 nM by the addition of EGTA. When [Ca2+]i declined to the lowest level, 1 mM Ca2+ was returned to the bath. Once added, thapsigargin was present throughout the experiment. The difference in [Ca2+]i ([ΔCa2+]i) in response to the return of 1 mM Ca2+ to the external solution was used as the measurement of SOC.

TRPC4 antisense. To determine the function of TRPC4 in MMC, the cells were pretreated for 48 h with TRPC4 antisense or scrambled oligonucleotides (4 nM). The antisense and scrambled sequences were TTTGATAATAGCTTGCCT and CATTAGGTCCGTAACCTATA, respectively (corresponding to GenBank accession no. NM_016984). Δ[Ca2+]i values were determined by following the same procedures as described above. The differences in Δ[Ca2+]i among the three groups (control, antisense, and scrambled) were compared by performing ANOVA plus the Student-Newman-Keuls test. Significance was established as P < 0.05.

Immunohistochemistry. For histological analyses, kidneys were removed from 8-wk-old mice (C57BL/6). Coronal cross sections containing the hilus were removed from kidneys, fixed in neutral buffered formalin, embedded in paraffin, and mounted on glass slides. For immunohistochemical analysis, 5-μm sections of paraffin-embedded tissue were deparaffinized in two changes of xylene for 10 min each. The sections were rehydrated in a series of graded alcohol solutions (100, 95, 85, 50, and 30%) for 2 min each. Antigen was retrieved by continuous boiling in 10 mM citrate buffer (pH 6.0) for 20 min. The sections were blocked with 3% H2O2 for 15 min followed by 1% BSA for 15 min at 23°C. After blocking solution was removed, the specimens were covered and incubated in a mixture of goat anti-human TRPC1 antibody (1:50, 1% BSA/PBS; Santa Cruz Biotechnology) overnight at 4°C. The slides were then washed in PBS three times for 5 min each. Each section was then covered and incubated for 1 h at 23°C with sufficient HRP-conjugated donkey anti-goat IgG (1:200; Santa Cruz Biotechnology). The slides were washed again in PBS three times for 5 min each. Each section was then covered with sufficient solution (liquid DAB substrate kit; Zymed Laboratories, South San Francisco, CA) at 23°C for 2 min in the dark and then counterstained with hematoxylin. Samples were viewed with an Olympus BX50 microscope. Images were captured with a Princeton Scientific Instruments digital camera and manipulated with the use of Adobe Photoshop software.

RESULTS

RT-PCR for TRPC RNA. Figure 1 shows the results of experiments in which RT-PCR was performed and specific primers were used to probe for TRPC1–TRPC7 RNA in MMC. Figure 1A demonstrates the presence of RNA for TRPC1–TRPC7 in mouse brain (positive controls). The sizes of the bands correctly matched the expected lengths of the amplified fragments of each TRPC (365, 297, 309, 304, 365, 309, 260, and 469 bp for TRPC1, TRPC2, TRPC3, TRPC4, TRPC5, TRPC6, TRPC7, and GAPDH, respectively).

As shown in Fig. 1B, only bands corresponding to TRPC1 and TRPC4 were amplified by RT-PCR and mRNA derived from MMC. Bands representing TRPC2, TRPC3, TRPC5,
TRPC6, and TRPC7 were not detected. We conclude that MMC contain mRNA for TRPC1 and TRPC4 but not for TRPC2, TRPC3, TRPC5, TRPC6, and TRPC7.

**Immunocytochemical localization of TRPC1 and TRPC4.** The localization of TRPC1 and TRPC4 in MMC was determined by immunocytochemistry with the use of anti-TRPC1 and anti-TRPC4, both of which were purchased from Santa Cruz Biotechnology. The specificities of TRPC1 and TRPC4 antibodies were established by Western blot analysis of MMC proteins. As shown in Fig. 2A, two distinct bands, at 90 and 50 kDa, were detected with anti-TRPC1. The molecular mass of 90 kDa is in the range (80–95 kDa) previously reported for TRPC1 protein (2, 9, 24, 32, 44, 47, 51). The 50-kDa band, demonstrated previously with the use of a variety of TRPC1 antibodies, has been identified as the heavy chain of IgG (24). Figure 2B shows a Western blot for TRPC4 using MMC proteins in which a single band of ~115 kDa was detected. This size is in agreement with previous reports regarding TRPC4 (29, 39, 43).

As shown in Fig. 3, immunofluorescence staining of single mesangial cells with anti-TRPC1 (Fig. 3B) and anti-TRPC4 (Fig. 3D) revealed a diffuse cytoplasmic pattern for TRPC1 and a distinct plasmalemmal pattern for TRPC4. Cytosolic localization of TRPC1 was confirmed with the use of two other antibodies (Alomone Laboratories, Jerusalem, Israel; a kind gift from Dr. L. Tsiokas, Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK). Minimal nonspecific staining was observed for TRPC1 and TRPC4 (Fig. 3, A and C).

**Distinguishing TRPC4-α and TRPC4-β.** RT-PCR performed with primers to amplify a region encompassing the 84-amino acid deletion for TRPC4-β amplified 329- and 581-bp products in the positive control (mouse brain) (Fig. 4A). These products were the expected sizes for TRPC4-β and TRPC4-α, respectively. However, the 581-bp product, but not the 329-bp product, was amplified from MMC RNA (Fig. 4B). An additional product of ~620 bp was amplified (Fig. 4B). It is not known whether this 620-bp band is a new splice variant or a nonspecific product of the RT-PCR. For a future project, we are obtaining the full-length sequence of the mesangial TRPC4. In this study, we concluded that MMC contain transcripts consistent with the expression of TRPC-α but not TRPC-β.

**Antisense determination of SOC role of TRPC4.** Immunocytochemical staining was used to establish the effectiveness of TRPC4 antisense. As shown in Fig. 5, antisense reduced the expression of TRPC4 in the plasma membrane. However, the intensity of cytoplasmic staining of TRPC1 was unaffected by TRPC4 antisense.

Fura 2 ratiometric analysis and antisense experiments were conducted to determine whether TRPC4 is involved in the function of SOC in MMC. Figure 6A shows a typical experiment in which SOC was determined by fura 2 ratiometric measurement of [Ca²⁺]. In control experiments, 1 μM thapsigargin caused an initial elevation of cytosolic [Ca²⁺] due to inhibition of Ca²⁺-ATPase in the sarcoplasmic reticulum. After an initial thapsigargin-induced peak elevation in [Ca²⁺], the [Ca²⁺], relaxed to a lower level. After removal of external Ca²⁺, [Ca²⁺] decreased to 50 nM. The return of 1 mM Ca²⁺ resulted in a 160-nM increase in [Ca²⁺]. This increase in [Ca²⁺] after the bath [Ca²⁺] was increased from <10 nM to 1 mM in the presence of thapsigargin was previously shown to be a measure of SOC (15). When cells were pretreated with TRPC4 antisense for 48 h, the first (thapsigargin-induced) peak was not affected; however, SOC (the second peak) was reduced to 30 nM (Fig. 6B). The scrambled oligonucleotides did not affect SOC (Fig. 5C).

As shown in Fig. 7, the first peak was not affected by TRPC4 antisense or by scrambled oligonucleotides. However, SOC was reduced by ~83% with TRPC4 antisense treatment. Treating MMC with TRPC4 scrambled oligonucleotides did not affect SOC. These results show that TRPC4 is a contributor to SOC in MMC.

**Immunohistochemical analysis of in vivo TRPC4 expression.** Immunohistochemical techniques were used to determine whether TRPC4 was expressed in glomerular sections from C57BL/6 mice. Figure 8A shows that the glomeruli were stained with anti-TRPC4 antibody (brown areas), consistent with mesangial expression. In contrast to the negative control, the anti-TRPC4-stained sections showed that mesangial cells express an abundance of TRPC4 in vivo. Note that TRPC4 also

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**Fig. 1. RT-PCR amplification of canonical transient receptor potential (TRPC) isozymes with the use of specific primers designed for TRPC1–TRPC7.** A: bands in 1.5% agarose gel correspond to sizes for amplified products of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TRPC1–TRPC7 in mouse brain. B: amplification of RNA corresponding to TRPC1 and TRPC4 but not TRPC2, TRPC3, TRPC5, TRPC6, and TRPC7 in mouse mesangial cells (MMC).

**Fig. 2. Immunoblot analysis of TRPC1 (A) in MMC with rabbit anti-TRPC1 polyclonal IgG (1:200).** A: 90- and 50-kDa bands were consistent with sizes for TRPC1 and heavy chain of IgG (IgG H ), respectively. B: lane ~115-kDa band is consistent with previous reports in which TRPC4 was detected.
is abundantly expressed in some extraglomerular structures. These experiments show that TRPC4 SOC is expressed both in vivo and in vitro and is due not merely to a biochemical change in the cultured environment.

**DISCUSSION**

In this study, RT-PCR revealed the presence of TRPC1 and TRPC4 mRNA in MMC; however, immunocytochemistry showed that only TRPC4 resided in the plasma membrane. In functional experiments, TRPC4 antisense reduced the SOC response measured by fura 2 ratiometry. TRPC4 was identified in the glomeruli of renal sections obtained from mice with the use of immunohistochemical techniques.

Expression of TRPC isoforms in MMC. Because mesangial cells have a smooth muscle-like phenotype, it is not surprising that TRPC1, previously identified in smooth muscle (24, 37, 38, 51), was also found in MMC. However, TRPC1 was predominately expressed in the cytoplasm of MMC and therefore would not serve as a component of SOC, which occurs in membranes. Because the cytoplasmic localization of TRPC1 was a surprising result, we used three different TRPC1 antibodies (sc-20110, Santa Cruz Biotechnology; ACC-010, Alomone Laboratories; a gift from Dr. L. Tsiokas) and seven fixation methods to ensure the accuracy of this result. In each experiment, we found the localization of TRPC1 to be intracellular. The localization of TRPC1 in the cytoplasm is not understood and is somewhat contradictory to studies showing that TRPC1 is endogenously localized in the plasma membranes of some cells. For example, TRPC1 associates with caveolin-1 in the plasma membrane region of human submandibular glands (13, 41) and sperm cells (42). In the MMC used in this study, we did not find clear evidence of plasma membrane localization of TRPC1; however, we are unable to conclude that TRPC1 is not in the plasma membrane. It remains possible that TRPC1 is localized to the plasma membrane but is not resolvable by our methods. Interestingly, Philipp et al. (29) also found transcripts of both TRPC1 and TRPC4 in adrenal cells. That TRPC4 antisense reduced \( I_{\text{CRAC}} \) in adrenal cells led to the conclusion that TRPC4 was at least one of the proteins forming SOC. However, it was not determined whether TRPC1 was also a component of \( I_{\text{CRAC}} \) in the Philipp et al. study.

Function of TRPC4 in native cells. In the absence of pharmacological inhibitors of specific isoforms of TRPC, antisense knockdown technology has been used extensively to study the function of TRPC channels in native environments. Antisense methods provided the first compelling evidence that TRP
homologs were involved in SOC activity (52). After the Zhu et al. (52) study, Wu et al. (50) used antisense to show that human TRPC1 is a functional component of SOC in human embryonic kidney (HEK)-293 cells. TRPC1 antisense oligonucleotides successfully inhibited SOC in endothelial cells (3) and in salivary glands (12). Patch-clamp experiments showed that TRPC4 antisense silenced SOC in bovine adrenal cortical cells (29). Thus antisense is a proven method of establishing the SOC function of specific isoforms of TRPC in their native environment.

By performing fura 2 ratiometry in the present study, we demonstrated that TRPC4 antisense reduced SOC by 83% (from 213 to 36 nM), a value close to the 20 μM La3+ reduction that inhibited SOC by 93% [also determined with fura 2 techniques (15)]. Thus TRPC4-antisense appears to be effective in silencing the SOC response to releasing Ca2+ stores with thapsigargin. That TRPC4 antisense did not affect expression of TRPC1, which has 35% amino acid identity with TRPC4, demonstrates the specificity of this particular knockdown strategy.

RNA transcripts of TRPC4 were previously described in a variety of tissues, including brain, kidney, lung, heart, testis, ovary, and adrenal glands (6, 20, 33). Consistent with our results, transcripts of TRPC4 have been discovered in rat (33) and human (19) kidney. However, a study of bovine adrenal cells may have been the first to describe a functional role for TRPC4 in its native environment (29). More recent studies with TRPC4 knockout mice showed that TRPC4, although not necessary for survival, has an important role in regulating blood flow and vascular permeability (5, 40). Freichel et al. (5) demonstrated that TRPC4−/− expressed with impaired agonist-dependent vasorelaxation and concluded that TRPC4 forms the
SOC channels necessary for Ca\(^{2+}\) stimulation of nitric oxide synthase in endothelial cells. More recently, Tiruppathi et al. (40) showed that lung vascular endothelial cells of TRPC4\(^{-/-}\) mice lack the thrombin-induced endothelial cell retraction response. These studies show that in some cells, TRPC4 is a crucial component not only of maintaining intracellular Ca\(^{2+}\) stores but also of Ca\(^{2+}\)-regulated physiological responses.

**Properties of mesangial TRPC4 channels.** The membrane-spanning structure of TRPC channels is similar to the molecules that form tetrameric ion-selective channels in the plasma membranes of cells. Therefore, it has been concluded that TRPC isoforms can form either heterotetrameric or homotetrameric channels. Because only TRPC4 was localized in the plasma membrane of MMC, our study results imply that the mesangial SOC is a homotetramer formed by only TRPC4. However, we cannot rule out the possibility that other membrane-associated TRPCs exist that were not detected with our methods. Clearly, further studies are necessary to clarify the role of TRPC4 in store-operated Ca\(^{2+}\) entry. Although several studies have reported SOC currents at the whole-cell level, few studies have reported single-channel currents. Our (15) previous results showed that thapsigargin-induced SOC was expressed in cultured human mesangial cells as a small, La\(^{3+}\)-sensitive, Ca\(^{2+}\)-selective, 2-pS channel with Ba\(^{2+}\) as the current carrier. The present study shows that the same channels, when examined with fura 2 methods, are silenced by TRPC4 antisense.

In agreement with our findings, previous studies showed that bovine TRPC4 (formerly CCE1), which is closely homologous to mTRPC4-\(\alpha\), was Ca\(^{2+}\) selective and activated by store depletion when heterologously expressed in HEK-293 or Chinese hamster ovary cells (27, 48). However, our findings are in disagreement with the described properties in many studies which heterologously overexpress TRPC4 (34, 35). It was shown that TRPC4 channels, presumably expressed as homotetramers, formed receptor-operated, nonselective cation channels activated independently of IP\(_3\) or store depletion (17, 30, 34). The single-channel conductance of these nonselective TRPC4 channels was much higher (41 pS) (34) than we found in our previous study (15). It was therefore suggested that TRPC4 did not form I\(_{CRAC}\), which was originally described as Ca\(^{2+}\)-selective, inwardly rectifying, and having a small single-channel conductance (8, 16). However, Philipp et al. (29) showed that the endogenous TRPC4 in adrenal cells is a Ca\(^{2+}\)-selective SOC. These data are consistent with studies showing that TRPC4\(^{-/-}\) mice lack a highly Ca\(^{2+}\)-selective, store-dependent current in endothelial cells (5, 40). This large discrepancy between the overexpressed and the endogenous TRPC4 is not surprising in light of a recent study by Vazquez et al. (45), who found that TRPC3 functioned as a SOC when expressed at low levels in chicken DT40 B lymphocytes. However, when expressed at higher levels, TRPC3 no longer formed a SOC but could be activated by receptor-coupled phospholipase C. Thus the native TRPC4 may be interacting with another endogenous protein that influences its properties. It is now known that several types of channels have accessory subunits that influence the biophysical, pharmacological, and regulatory properties of the pore-forming subunit (11, 22). It will be interesting to determine whether TRPC channels are similarly influenced by accessory subunits.

**Splice variants of TRPC4.** Although there are multiple splice variants of TRPC4 (30), isoforms TRPC4-\(\alpha\) and TRPC4-\(\beta\) have been shown to be the most abundantly expressed in tissues (19, 34, 35). Northern blot analysis revealed an abundance of both TRPC4-\(\alpha\) and TRPC4-\(\beta\) RNA in human renal tissue (19). Satoh et al. (33) also showed that TRPC4-\(\alpha\) transcripts were specifically expressed in rat kidney. Because Satoh et al. found both TRPC4-\(\alpha\) and TRPC4-\(\beta\) in rat brain, we used mouse brain as a positive control. Our results revealed amplified transcripts consistent with TRPC-\(\alpha\) but not TRPC-\(\beta\) expression in MMC. That TRPC4-\(\alpha\) forms the SOC channel in...
MMC is consistent with a study by Mery et al. (19), who showed, using the yeast two-hybrid method, a strong interaction between human TRPC4-α (but not TRPC4-β) and the intracellular Ca2+ release channel (IP3R). An association between TRP and IP3R is the sine qua non of the conformational coupling hypothesis regarding activation of SOC (10).

In summary, we have found that both TRPC1 and TRPC4 are expressed in MMC. TRPC4 was clearly localized in the plasma membrane of glomerular MMC, where it may form a homotetrameric SOC. Recent studies with TRPC4−/− mice revealed important roles for TRPC4 SOC in the normal function of cardiovascular and pulmonary systems. It therefore will be interesting to examine the renal function of TRPC4−/− mice at the cellular and integrative levels.

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