Depletion of intracellular Ca\(^{2+}\) stores leads to the activation of Ca\(^{2+}\) influx through store-operated Ca\(^{2+}\) channels. Although the identity of these channels is unknown, there is considerable evidence that the transient receptor potential channel 1 (TRPC1) participates in the formation of these channels. We show that TRPC1 physically interacts with the α-isof orm of the inhibitor of the myogenic family (I-mfa), a known inhibitor of basic helix-loop-helix transcription factors, in vitro and in vivo. The interaction is mediated by the C-terminal cytoplasmic tail of TRPC1 and the C-terminal cysteine-rich domain of I-mfa. Using the whole cell configuration of the patch clamp technique, we show that ectopic expression of I-mfa in CHO-K1 cells reduces native store-activated Ca\(^{2+}\) currents, whereas knock-down of endogenous I-mfa in A431 cells by RNA interference enhances these currents. Pipette perfusion of purified recombinant I-mfa rescues the effect of I-mfa knock-down on store-operated conductance. Finally, cell dialysis with a monoclonal antibody specific to TRPC1 results in the suppression of store-activated conductance in cells lacking I-mfa, but not in I-mfa expressing cells. We propose that I-mfa functions as a molecular switch to suppress the store dependence of TRPC1.

G protein-coupled receptors and receptor tyrosine kinases comprise a very large group of cell surface receptors that elicit their physiological responses through the production of inositol (1,4,5)-trisphosphate (IP\(_3\)) (1). Upon receptor activation, newly synthesized IP\(_3\) acts on IP\(_3\) receptors (IP\(_3\)Rs) to trigger a rapid increase in the intracellular Ca\(^{2+}\) concentration by releasing free Ca\(^{2+}\) from intracellular stores (2). Intracellular Ca\(^{2+}\) concentration returns to normal levels by extrusion of cytoplasmic Ca\(^{2+}\) into the extracellular space by plasma membrane Ca\(^{2+}\)-ATPase and Na\(^{+}\)-Ca\(^{2+}\) exchangers, re-admission of Ca\(^{2+}\) into the endoplasmic reticulum by the SERCA pump, and Ca\(^{2+}\) entry via the store-operated Ca\(^{2+}\) channels (3, 4). Whereas all of these pathways contribute to the maintenance of normal Ca\(^{2+}\) homeostasis, store-operated Ca\(^{2+}\) entry is of particular interest because it is responsible for the regulation of diverse cellular functions (5) ranging from cell proliferation and gene expression in T lymphocytes (6) to endothelial cell function (7) and regulation of the acrosome reaction in germ cells (8).

Despite intensive investigation, the identity of the store-operated channels and the cellular mechanisms underlying the coupling of store depletion and Ca\(^{2+}\) entry remain elusive (9, 10). Mammalian TRPCs have been proposed to function as store-operated channels (11, 12). However, it is not clear whether they are store-, receptor-operated (13, 14), and/or channels serving other functions.

TRPC1 was the first mammalian TRP channel cloned (15, 16) and there has been considerable evidence to support its role in store-operated Ca\(^{2+}\) entry. First, genetic elimination of TRPC1 by homologous recombination significantly reduces Ca\(^{2+}\) release-activated Ca\(^{2+}\) currents in DT40 cells (17). Second, TRPC1 knock-down by RNAi reduces store-operated currents in CHO-K1 cells (18). Third, heterologous expression of TRPC1 augments store-operated Ca\(^{2+}\) entry in some cell types including CHO-K1 cells (19, 20). Fourth, TRPC1 is responsible for the Ca\(^{2+}\)/calmodulin-dependent feedback inhibition of store-operated Ca\(^{2+}\) entry, a hallmark of native store-activated Ca\(^{2+}\) conductance (21). Fifth, TRPC1 can physically interact with various isoforms of the IP\(_3\)R (22–24) and polycystin-2 (25), both functioning as intracellular Ca\(^{2+}\) release channels activated in response to IP\(_3\) (26, 27). This is consistent with the conformational coupling model of the store-operated Ca\(^{2+}\) entry by which store-operated channels are gated by Ca\(^{2+}\) release channels via protein-protein interactions (28).

In contrast, there are independent functional and biochemical studies showing that TRPC1 does not have channel function by itself, but it is required for the formation of receptor- but not store-activated channel complexes with TRPC4 and TRPC5 (29–31). In addition, when TRPC1 is successfully expressed in CHO-K1 cells it does not reconstitute the properties of the prototypical Ca\(^{2+}\) release-activated Ca\(^{2+}\) current extensively studied in hematopoietic cells (19, 32). Although these findings are difficult to reconcile with each other, most of the controversy appears to be because of results obtained from gain-versus loss-of-function experiments. Thus, it has been suggested that as yet unidentified proteins may be required to successfully reconstitute a true store-operated channel containing one or more of the currently known TRPCs (6, 33, 34). Another possibility is that proteins with inhibitory activity may prevent activation of mammalian TRPCs by store-depletion, similar to the Calliphora vicina TRP homolog (35). It was recently shown that the PDZ containing domain protein, INAD (inactivation no afterpotential D) inhibited the activation of C. vicina TRP by store depletion (36).

I-mf is the founding member of a group of related proteins...
I-mfa Modulates TRPC1 Activity

with an unusual cysteine-rich domain (36), first identified as an interacting protein with MyoD in a yeast two-hybrid screen. I-mfa exists in three isoforms (a, b, and c) produced by alternative splicing (37), with each isoform containing a common N-terminal and a variable C-terminal region (a, b, or c) (37). All three transcripts encode small cytoplasmic proteins lacking any predicted transmembrane segments or nuclear localization signals (37). I-mfa, the a-isoform, inhibits the transcriptional activity of MyoD and other basic helix-loop-helix (bHLH) transcription factors such as myogenin, Myf5, Mash2, and Hand1 by preventing their nuclear localization (37, 38). I-mfa has also been shown to function within the Wnt/β-catenin pathway (39, 40). Specifically, it binds and inhibits the transcriptional activity of the Xenopus HMG box transcription factor XTCd3 (39) and also participates in a multiprotein complex containing Axin (40). Consistent with its molecular role as an inhibitor of both the bHLH proteins and canonical Wnt/β-catenin pathway, activation of the I-mfa gene by homologous recombination in mice resulted in skeletal patterning and placental defects (38). I-mfa mutant mice on a C57Bl/6 background died around E10.5 because of a placental defect associated with a reduced number of trophoblast giant cells, whereas mutant mice on a 129/Sv background survived to adulthood and showed skeletal patterning defects associated with deregulated osteogenic differentiation (38). However, the exact mechanisms by which I-mfa mediates its effects during development still remain unknown.

In the present study, we have identified and characterized a physical interaction between I-mfa and TRPC1 in vitro and in vivo. Furthermore, gain- and loss-of-expression experiments show that I-mfa functions as an inhibitor of store-operated currents by negatively modulating TRPC1 activity.

EXPERIMENTAL PROCEDURES

Cell Culture—CHO-K1 and A431 cells were obtained from ATCC and maintained in F-12 (Ham’s) supplemented with 0.05% sodium bicarbonate and 10% fetal bovine serum or Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum, respectively. I-mfa mutant mice on a C57Bl/6 background died around E10.5 because of a placental defect associated with a reduced number of trophoblast giant cells, whereas mutant mice on a 129/Sv background survived to adulthood and showed skeletal patterning defects associated with deregulated osteogenic differentiation (38). However, the exact mechanisms by which I-mfa mediates its effects during development still remain unknown.

In the present study, we have identified and characterized a physical interaction between I-mfa and TRPC1 in vitro and in vivo. Furthermore, gain- and loss-of-expression experiments show that I-mfa functions as an inhibitor of store-operated currents by negatively modulating TRPC1 activity.

EXPERIMENTAL PROCEDURES

Cell Culture—CHO-K1 and A431 cells were obtained from ATCC and maintained in F-12 (Ham’s) supplemented with 0.05% sodium bicarbonate and 10% fetal bovine serum or Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum, respectively. I-mfa mutant mice on a C57Bl/6 background died around E10.5 because of a placental defect associated with a reduced number of trophoblast giant cells, whereas mutant mice on a 129/Sv background survived to adulthood and showed skeletal patterning defects associated with deregulated osteogenic differentiation (38). However, the exact mechanisms by which I-mfa mediates its effects during development still remain unknown.

In the present study, we have identified and characterized a physical interaction between I-mfa and TRPC1 in vitro and in vivo. Furthermore, gain- and loss-of-expression experiments show that I-mfa functions as an inhibitor of store-operated currents by negatively modulating TRPC1 activity.

Experimental Procedures

Cell Culture—CHO-K1 and A431 cells were obtained from ATCC and maintained in F-12 (Ham’s) supplemented with 0.05% sodium bicarbonate and 10% fetal bovine serum or Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum, respectively.

Plasmids—F-I-mfa was made by inserting the coding region of human I-mfa downstream of the FLAG epitope in the pCMV5-FLAG vector (Kodak). Mouse myogenin in pCMV-SPORT6 was obtained from Incyte Genetics. The “a-region” of human I-mfa was fused to pMAL-c2 (New England Biolabs) to generate maltose-binding protein (MBP)-I-mfa(a). Portions of the C-terminal (Asp639-Ser750, accession number Z3903) and the N-terminal cytoplasmic region of human TRPC1 (Ser20-Ser390) were cloned in pGEX-3X (Pharmacia) to generate glutathione S-transferase (GST)-TRPC1-C or GST-TRPC1-N, respectively. F-I-mfa15 and TRPC1N were generated by the introduction of termination codons at positions Ser20 and Arg20 of human I-mfa (accession number NM_005586) and human TRPC1, respectively, by site-directed mutagenesis using QuickChange (Stratagene). All other constructs were described previously (25).

Production of Polyclonal Antibodies—Mouse α-TRPC1-N, rabbit α-TRPC1-C, or rabbit α-TRPC1-N/W was mouse polyclonal against the N-terminal cytoplasmic region, rabbit polyclonal against the C-terminal cytoplasmic tail, or rabbit polyclonal antibody made against the N-terminal region of human TRPC1, respectively. Mouse or chicken α-I-mfa was raised against the entire I-mfa molecule. All antibodies were raised against fusions of TRPC1 or I-mfa with GST. Antibody titers were initially determined by enzyme-linked immunosorbent assay using purified TRPC1 or I-mfa fusions with MBP. Rabbit α-TRPC1-C and chicken α-I-mfa were further affinity purified using the AminoLink method (Pierce). All polyclonal antibodies recognized human, mouse, and rat TRPC1 or I-mfa protein.

Production of Monoclonal Antibody (1F1)—Monoclonal antibody was produced by standard methods (41). Briefly, female Balb/c mice were immunized with purified GST-TRPC1-N by intraperitoneal and subcutaneous injections (intraperitoneally and subcutaneously) of 15 μg each. Serum levels of specific antibody were determined by direct enzyme-linked immunosorbent assay. Four days following the final injection, the spleen of the animal determined to have the highest specific titer was removed and then fused to Sp2/0 myeloma cells in 50% polyethylene glycol-500. Following hyoxanthine-aminopterin-thymidine selection, resulting clones were screened for specific antibody production by direct enzyme-linked immunosorbent assay using a purified fusion of the N-terminal of human TRPC1 with MBP (MBP-TRPC1-N). Positive clones were subcloned twice by limiting dilution. 1F1 showed the highest titers and was further used for ascites production. 1F1 was isotype mouse IgG1, and its specificity to TRPC1 was additionally characterized by immunoblotting and immunofluorescence staining in transfected cells. 1F1 recognized mouse, human, and rat TRPC1.

Yeast Two-hybrid—The yeast strain containing both His and LacZ reporters under the control of LexA binding sites was sequentially transformed with a bait plasmid in pLEXA containing a portion of the C-terminal cytoplasmic region of human TRPC1 (Asp639-Ser750, accession number Z3903) and a human fetal kidney library (Clontech) in pGD10 or a whole mouse embryonic library in pVP16. Four million independent clones from each library were screened with pLexA-TRPC1. Two overlapping clones of human I-mfa were obtained from the human fetal kidney library and a single mouse clone was obtained from the mouse embryonic library.

In Vitro Binding Using Recombinant Proteins—MBP or GST fusions were expressed and purified from bacteria using amylose resin or glutathione-Sepharose 4B column, respectively. GST fusions were retained on the column, whereas MBP fusions were eluted by 10 mM maltose. The concentration and integrity of purified soluble or immobilized MBP or GST fusions, respectively, were determined by Coomassie Brilliant Blue staining of 12% SDS-PAGE gel containing known amounts of purified bovine serum albumin or MBP. In a typical in vitro binding assay, 2 μg of immobilized GST fusions were incubated with 5 μg of soluble MBP or MBP fusion in a total volume of 1 ml of immunoprecipitation solution (1% Triton X-100 buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Nonident P-40, aprotinin (1 μg/ml), and pepstatin). Binding was done overnight at 4 °C. The beads were washed five times in immunoprecipitation buffer, suspended in 40 μl of gel loading buffer, separated by 12% SDS-PAGE, and followed by Western blotting using α-MBP (1:10,000) (New England Biolabs).

Transient Transfections and Immunoprecipitations—Transient transfections and immunoprecipitations were done in HEK293T cells as described previously (42).

Stable Transfections in CHO-K1 Cells—CHO-K1 cells were transfected with pSV2-neo (CHOneo cells) or F-I-mfa, pSV2-neo, and CD4 (CHOneo) using LipofectAMINE PLUS (Invitrogen) and stable transfecants were selected in 500 μg/ml G418 for 2 weeks. CD4 did not affect the interaction between TRPC1 and I-mfa, as determined by transient transfections followed by co-immunoprecipitations (data not shown). Individual clones were isolated, expanded, and sorted twice for CD4− cells using a phycoerythrin-conjugated monoclonal antibody against CD4 to obtain pure populations of F-I-mfa expressing cells. Individual clones were first tested for F-I-mfa expression by immunoprecipitation followed by immunoblotting using α-FLAG. Clone CHOneo demonstrated the highest levels of F-I-mfa.

Electrophysiology—The conventional whole cell voltage-clamp configuration was used to measure transmembrane currents in single cells. Patch clamp recordings were obtained from single cells at room temperature using a Warner PC-505B amplifier (Warner Instrument Corp., Hamden, CT) and pClamp 8 software (Axon Instrument, Foster City, CA). Glass pipettes (plain, Fisher Scientific) with resistances of 5–10 MΩ were prepared with a pipette puller and polisher (PP-830 and MP-830, respectively, Narishige, Tokyo, Japan). After the whole cell configuration was achieved, cell capacitance and series resistance were compensated before each recording. From a holding potential of −80 mV, voltage steps were applied from −100 to 80 mV in 20-mV increments with 100 ms duration at 5-s intervals. I-V relationships were measured by voltage ramping from −80 to 90 mV within 100 ms. Current traces were filtered at 1 kHz and analyzed off-line with pClamp 8. Statistical analysis was employed with the SigmaStat (Chicago, IL) software. Data were tested as mean ± S.E. One-way repeated measures analysis of variance followed by Student-Newman-Keuls’s tests were used for comparisons among sequential treatments in the same group. Student’s t test was used for comparisons between groups. Differences were considered significant at p < 0.05. The pipette solution contained (in mM): 140 NaCl, 1 MgCl2, 0.3 Mg-ATP, 0.03 GTP, 10 HEPS, and 10 EGTA (pH 7.4). The extracellular solution contained (in mM): 110 NaCl, 5 CaCl2, 1 MgCl2, 20 CaCl2, 10 HEPS, and 10 glucose (pH 7.4). Permeability ratio of Ca2+ to Na+ was calculated from the modified GHK equation as described in Ref. 43, assuming a permeability ratio of Ca2+ to Na+ equals to 1.
**Results**

**I-mfa Is an Interacting Partner of TRPC1**—A yeast two-hybrid screen using two independent libraries, a human fetal kidney or whole mouse embryonic (E9.5) library, resulted in the identification of I-mfa as an interacting partner with TRPC1. Two overlapping clones containing the entire I-mfa cDNA including a portion of the 5'-untranslated region or just the a-region were obtained from the human fetal kidney library, and a partial clone containing the a-region and a portion of the common region was obtained from the whole mouse embryonic library. DNA sequence analysis revealed that all interacting clones were in-frame with the transactivation domain of GAL4. Because the smaller interacting clone contained just the a-region of I-mfa, we inferred that TRPC1 might specifically interact with the a- but not the b- or c- isoforms of I-mfa.

**In Vitro Interaction and Co-localization of I-mfa and TRPC1 in Transfected Cells**—To test whether the a-region of I-mfa specifically interacted with the C-terminal cytoplasmic region of TRPC1 (TRPC1-C), we employed purified recombinant proteins to examine these interactions. 2 μg of immobilized GST, a fusion protein with GST and the C-terminal tail of human TRPC1 (GST-TRPC1-C), or the N-terminal region of human TRPC1 (GST-TRPC1-N), were incubated with 5 μg of soluble, purified MBP or MBP fused to the a-region of human I-mfa (MBP-I-mfa(a)). Fig. 1A shows that MBP-I-mfa(a), but not MBP (lanes 3, 5, and 7), bound to GST-TRPC1-C (lane 6) but not GST (lane 4) or GST-TRPC1-N (lane 8). These results show that the C-terminal cytoplasmic tail of TRPC1 mediates a direct, physical interaction with the a-region of I-mfa.

To determine whether full-length TRPC1 and I-mfa could interact in mammalian cells, we transfected FLAG-tagged I-mfa (F-I-mfa), myc-tagged TRPC1 (M-TRPC1), or myc-tagged TRPC3 (M-TRPC3) in HEK293T cells and tested their interactions by co-immunoprecipitation experiments. F-I-mfa co-immunoprecipitated with M-TRPC1 (Fig. 1B, lane 3) but failed to interact with M-TRPC3 (Fig. 1B, lane 2) showing specificity of I-mfa for TRPC1 but not for a homologous channel protein. A TRPC1-truncation mutant lacking the domain used in the yeast two-hybrid screen to identify I-mfa (TRPC1a) failed to interact with I-mfa (Fig. 1C, lane 2). Conversely, a truncation mutant of I-mfa lacking almost the entire a-region (F-I-mfaa5) could not associate with TRPC1 (Fig. 1C, lane 4). HA-tagged I-mfb (HA-I-mfb) was also unable to associate with TRPC1 (Fig. 1D, lane 2) supporting the finding that the interaction between TRPC1 and F-I-mfa is mediated by the a-region of I-mfa. Altogether, these results demonstrate not only the specificity of the interaction but also the lack of additional interaction domains in the two proteins.

**Myogenin Competes with TRPC1 for Binding to I-mfa**—Because the a-region of I-mfa was independently found to mediate an interaction with bHLH proteins (37), we tested whether the bHLH protein myogenin could compete with TRPC1 for binding to I-mfa. Fig. 1E shows that increasing amounts of transfected myogenin did inhibit the TRPC1-I-mfa association in a concentration-dependent fashion. These results suggest that I-mfa may not bind to TRPC1 and myogenin at the same time, or to other bHLH-binding partners, thereby ruling out the possibility of a tripartite formation.

**Endogenous I-mfa and TRPC1 Interact in Native Tissues**—To determine whether endogenous TRPC1 associated with I-mfa, we examined whether they co-existed in a native complex in adult rat tissues. Fig. 2A shows that a mouse polyclonal antibody made against TRPC1 or I-mfa (mouse polyclonal α-TRPC1-N or α-I-mfa, respectively) immunoprecipitated native TRPC1 from rat liver (lanes 7 and 8), Nalm-6 cells (a human pre-B cell line) (lanes 11 and 12), and rat kidney (lanes 14 and 15) lysates. Endogenous TRPC1 was detected by a rabbit polyclonal antibody raised against the C-terminal cytoplasmic tail of human TRPC1 (α-TRPC1-C) (Fig. 2A, lanes 5–15) but not by rabbit preimmune serum (Fig. 2A, lanes 1–4). The possibility that proteins recognized in lanes 7, 8, 11, 12, 14, and 15 represented mouse antibodies non-specifically recognized by rabbit α-TRPC1-C was ruled out by probing the immunocomplexes captured by preimmune, α-TRPC1-N, or α-I-mfa antiserum from rat liver lysates with sheep anti-mouse IgG coupled to horseradish peroxidase. Fig. 2B shows that anti-mouse IgG identified just the heavy and light chains of the mouse antibodies used in immunoprecipitations but not any other protein with a molecular weight similar to transfected TRPC1. The specificity of the α-TRPC1-C was further confirmed by recognizing a single protein in native rat tissue lysates having a molecular size similar to transfected TRPC1 (Fig. 2C, upper panel). Transfected F-TRPC1 was used as a positive size control in Fig. 2A (lanes 5 and 9) and Fig. 2C (lane 1) and was detected by α-TRPC1-C in both experiments. The additional bands detected by α-TRPC1-C in lysates of transfected F-TRPC1 in Fig. 2C (lane 1) compared with Fig. 2A (lanes 5 and 9) are likely to represent nonspecific binding because of long exposure to identify endogenous TRPC1 in various tissues (Fig. 2C, lanes 2–5). It should be noted that endogenous TRPC1 is expressed at very low levels in the tissues tested and therefore long exposures were required for detection, in our hands. The relative expression of endogenous I-mfa in various rat tissues is shown in Fig. 2C (lower panel). The highest I-mfa expression was seen in lung followed by kidney, liver, and heart. This pattern of expression partially mirrored the expression of TRPC1, which showed the highest levels in liver and lung followed by heart and kidney. Overall, these results provided evidence for a physical association between TRPC1 and I-mfa in adult rat tissues. The interaction was also supported by the co-expression pattern of these proteins.

**Ectopic Expression of I-mfa Inhibits Native Store-operated Currents in CHO-K1 Cells**—To determine whether I-mfa was
sufficient to regulate the function of TRPC1, we stably transfected I-mfa into CHO-K1 cells. We chose these cells because they express TRPC1 (Fig. 3A, lane 2) and lack endogenous I-mfa (Fig. 3B, lane 1). More importantly in CHO-K1 cells, TRPC1 knock-down by siRNA resulted in the elimination of store-operated currents suggesting that TRPC1 is an essential component of the native store-operated conductance in these cells (18). Mock-transfected (CHOmock) or cells stably expressing I-mfa (CHOI-mfa) were challenged with 1 μM TG, an agent traditionally used to irreversibly deplete intracellular Ca\(^{2+}\) stores, resulting in the activation of store-operated conductance without the interference of post-receptor activated events.

Whole cell currents were sequentially measured in CHOmock or CHOI-mfa cells using voltage step and ramp protocols before and after the addition of TG. Under basal conditions (Pre-TG), small currents were recorded in both cell types (Fig. 3, C, D, G, and H). Depletion of the internal stores with 1 μM TG enhanced the whole cell current density in CHOmock cells from -4.66 ± 0.61 pA/pF to -12.69 ± 2.1 pA/pF (mean ± S.E.) at -100 mV (Fig. 3I). Similar results were obtained in a green fluorescent protein-expressing clone of CHO-K1 cells (data not shown) and in the parental CHO-K1 cells diazylated with control mouse IgG, (see below).

Examination of the I-V relation curve derived from the ramp protocol showed that TG induced a slightly inward and outward rectifying current, which reversed at positive potentials (Fig. 3G). A large, characteristic of higher Ca\(^{2+}\) over Na\(^{+}\) selectivity (PCa:PNa = 12.6:1). In contrast to CHOmock cells, CHOI-mfa cells did not show a significant response to TG (from -5.4 ± 1.09 to -6.7 ± 1.15 pA/pF at -100 mV) (Fig. 3I). Because La\(^{3+}\) has been shown to inhibit store-operated channels at nanomolar- to micromolar concentrations (44–46), depending on the cell type, we examined the TG-evoked responses in the presence of 200 nM or 20 μM La\(^{3+}\).
TG-induced, but not background currents were completely blocked by 20 μM La^{3+} in CHOmock cells (Fig. 3I), whereas they were only partially inhibited by 200 nM La^{3+} (data not shown). In summary, these data showed that TG was able to induce native currents in CHO-K1 cells but not in cells expressing I-mfa, suggesting that I-mfa has a suppressive effect on native store-operated currents.

To determine whether the effect of I-mfa was direct and did not result from changes in transcription affecting the expression of proteins that may regulate TRPC1 activity, and to also determine the specificity of I-mfa on TG-induced currents, we tested whether pipette-perfused recombinant MBP-I-mfa(a), MBP-I-mfa(b), or MBP-I-mfc at 200 ng/ml could affect TG-induced currents in CHO-K1 cells. To allow adequate time for the recombinant proteins to diffuse into the cytoplasm, we initiated recording 5 min following break-in. Consistent with previous results, only MBP-I-mfa(a) suppressed these currents (Fig. 3J).

To determine whether the I-mfa-mediated reduction in store-operated currents could also result in a reduction in store-operated Ca^{2+} entry, we measured TG-induced Ca^{2+} influx in CHOmock and CHO-I-mfa cells by ratiometric measurements of intracellular Ca^{2+} concentration in cell populations using indo-1/AM. Ca^{2+} entry in response to store depletion by 1 μM TG was determined in the presence of a nominally Ca^{2+}-free extracellular solution (0.5 mM EGTA) using a Ca^{2+} re-addition protocol (Fig. 3K). Ca^{2+} entry was significantly reduced in CHO-I-mfa cells compared with CHOmock cells (Fig. 3K, n = 4). Ca^{2+} imaging experiments also verified the inhibitory effect of I-mfa on TG-induced Ca^{2+} entry in individual cells (data not shown).

Thus, ratiometric results supported previous current measurements providing evidence for an inhibitory role of transfected I-mfa in native store-operated Ca^{2+} entry in CHO-K1 cells.

**Endogenous TRPC1 Functionally Contributes to Store-operated Currents in CHO-K1 Cells**—TRPC1 was shown to be an
essential component of store-operated currents in CHO-K1 cells (18). To confirm these findings using an independent approach, we measured store-operated currents in the presence of 10 or 100 ng/ml 1F1, a mouse TRPC1-specific monoclonal antibody (47), or mouse IgG1 in the intrapipette solution. The specificity of 1F1 for TRPC1 was confirmed by immunoblotting or flow cytometry in transfected HEK293T or permeabilized CHO-K1 cells, respectively (data not shown).

Fig. 4 shows that TG failed to induce store-operated currents in 4 of 4 CHO-K1 cells dialyzed with 1F1 at 100 ng/ml (B, D, and E). The inclusion of 100 ng/ml mouse IgG1 or 10 ng/ml 1F1 to the intrapipette solution did not affect store-operated currents ($n = 4$) (Fig. 4, A, C, and E). These data showed that TRPC1 contributes greatly to native store-operated currents in these cells, thus confirming previous data (18) and validating the use of 1F1 as a TRPC1-specific neutralizing antibody.

Fig. 3. F-I-mfa suppresses native TG-induced currents in CHO-K1 cells. A, expression of endogenous TRPC1 in CHO-K1 cells. 3 μl of transfected F-TRPC1 HEK293T cell lysates (positive control, lane 1) or 50 μg of crude CHO-K1 lysates (lane 2) were probed with affinity purified rabbit α-TRPC1-C. Arrow in the right indicates the position of transfected and endogenous TRPC1. B, levels of expression of F-I-mfa and endogenous I-mfa in CHOmock (lane 1) and CHO-I-mfa cells (lane 2). C, expression of I-mfa in CHO mock and CHO-I-mfa cells was determined by immunoprecipitation using mouse α-I-mfa and subsequent immunoblotting with the same antibody. The positions of the heavy (IgG HC) and light chains (IgG LC) of the mouse IgG used in the immunoprecipitation are shown in the right. C–F, representative traces of single CHOmock (C and E) or CHO-I-mfa (D and F) cells showing background currents (Pre-TG, C and D) or currents recorded 1 min following the addition of 1 μM TG to the extracellular solution (Post-TG, E and F) using a voltage step protocol. Current is expressed as current density. Dashed lines indicate 0 current. G and H, I-V relation curves before (Pre-TG) and 2 min after (Post-TG) the addition of 1 μM TG to the extracellular solution in CHOmock (G) and CHO-I-mfa cells (H). Curves were generated by a ramp protocol. I, summary data showing current density (pA/pF) of background currents (Pre-TG), TG-induced currents (Post-TG), and TG-induced currents in the presence of 20 μM La3⁺ (TG + La3⁺) in CHOmock and CHO-I-mfa cells. Step currents recorded 50 ms after the initiation of the −100 mV voltage pulse were used in the analysis. *, $p < 0.05$, comparison among pre-, post-TG, and TG + La3⁺ treatments in the same group. n indicates the number of cells analyzed in each group. J, summary data showing the effect of pipette-perfused (at 200 ng/ml) purified recombinant MBP, MBP-I-mfa(a), MBP-I-mfa(b), or MBP-I-mic on TG-induced current density in CHO cells obtained by a voltage step protocol, as described in F. n indicates the number of cells analyzed in each group. *, $p < 0.05$, comparison between pre- and post-TG current density. K, suppression of store-operated Ca²⁺ entry in CHO-K1 cells by F-I-mfa in CHO-K1 cells. Effect of Ca²⁺ re-addition (CaCl₂, 6.25 mM) on intracellular Ca²⁺ concentration (shown as 405/485 fluorescence ratio) in CHOmock (black) and CHO-I-mfa cells (red) incubated in a nominally Ca²⁺-free solution with (left) or without (right) TG stimulation (1 μM for 1 h). I-mfa Modulates TRPC1 Activity

Inactivation of Endogenous I-mfa Increases Store-operated Currents in A431 Cells—Whereas transfected I-mfa suppressed native currents (Fig. 3), we wished to determine whether endogenous I-mfa had a similar effect on native store-activated conductance. Therefore, we knocked down I-mfa expression by RNAi using a short hairpin I-mfa-specific RNA (48) in A431 cells, a human epidermoid carcinoma cell line. The interfering sequence was designed to the common region of human I-mf. We chose the human A431 cell line because they showed the highest levels of I-mfa expression compared with all other cell lines tested, including Nalm-6 (data not shown). They also express detectable levels of TRPC1 (Fig. 5A, lanes 3 and 4). Immunoblotting of total cell lysates from A431 and cells stably transfected with our I-mfa-specific RNAi vector (A431-I-mfa-RNAi) revealed that endogenous I-mfa was reduced by ~90% in the transfected cells (A431-I-mfa-RNAi) (Fig. 5B). To confirm these results with two I-mfa-specific antibodies, we

Fig. 4. I-mfa Modulates TRPC1 Activity

I-mfa Modulates TRPC1 Activity

Inactivation of Endogenous I-mfa Increases Store-operated Currents in A431 Cells—Whereas transfected I-mfa suppressed native currents (Fig. 3), we wished to determine whether endogenous I-mfa had a similar effect on native store-activated conductance. Therefore, we knocked down I-mfa expression by RNAi using a short hairpin I-mfa-specific RNA (48) in A431 cells, a human epidermoid carcinoma cell line. The interfering sequence was designed to the common region of human I-mf. We chose the human A431 cell line because they showed the highest levels of I-mfa expression compared with all other cell lines tested, including Nalm-6 (data not shown). They also express detectable levels of TRPC1 (Fig. 5A, lanes 3 and 4). Immunoblotting of total cell lysates from A431 and cells stably transfected with our I-mfa-specific RNAi vector (A431-I-mfa-RNAi) revealed that endogenous I-mfa was reduced by ~90% in the transfected cells (A431-I-mfa-RNAi) (Fig. 5B). To confirm these results with two I-mfa-specific antibodies, we

essential component of store-operated currents in CHO-K1 cells (18). To confirm these findings using an independent approach, we measured store-operated currents in the presence of 10 or 100 ng/ml 1F1, a mouse TRPC1-specific monoclonal antibody (47), or mouse IgG1 in the intrapipette solution. The specificity of 1F1 for TRPC1 was confirmed by immunoblotting or flow cytometry in transfected HEK293T or permeabilized CHO-K1 cells, respectively (data not shown).

Fig. 4 shows that TG failed to induce store-operated currents in 4 of 4 CHO-K1 cells dialyzed with 1F1 at 100 ng/ml (B, D, and E). The inclusion of 100 ng/ml mouse IgG1 or 10 ng/ml 1F1 to the intrapipette solution did not affect store-operated currents ($n = 4$) (Fig. 4, A, C, and E). These data showed that TRPC1 contributes greatly to native store-operated currents in these cells, thus confirming previous data (18) and validating the use of 1F1 as a TRPC1-specific neutralizing antibody.

Fig. 3. F-I-mfa suppresses native TG-induced currents in CHO-K1 cells. A, expression of endogenous TRPC1 in CHO-K1 cells. 3 μl of transfected F-TRPC1 HEK293T cell lysates (positive control, lane 1) or 50 μg of crude CHO-K1 lysates (lane 2) were probed with affinity purified rabbit α-TRPC1-C. Arrow in the right indicates the position of transfected and endogenous TRPC1. B, levels of expression of F-I-mfa and endogenous I-mfa in CHOmock (lane 1) and CHO-I-mfa cells (lane 2). Expression of I-mfa in CHOmock and CHO-I-mfa cells was determined by immunoprecipitation using mouse α-I-mfa and subsequent immunoblotting with the same antibody. The positions of the heavy (IgG HC) and light chains (IgG LC) of the mouse IgG used in the immunoprecipitation are shown in the right. C–F, representative traces of single CHOmock (C and E) or CHO-I-mfa (D and F) cells showing background currents (Pre-TG, C and D) or currents recorded 1 min following the addition of 1 μM TG to the extracellular solution (Post-TG, E and F) using a voltage step protocol. Current is expressed as current density. Dashed lines indicate 0 current. G and H, I-V relation curves before (Pre-TG) and 2 min after (Post-TG) the addition of 1 μM TG to the extracellular solution in CHOmock (G) and CHO-I-mfa cells (H). Curves were generated by a ramp protocol. I, summary data showing current density (pA/pF) of background currents (Pre-TG), TG-induced currents (Post-TG), and TG-induced currents in the presence of 20 μM La3⁺ (TG + La3⁺) in CHOmock and CHO-I-mfa cells. Step currents recorded 50 ms after the initiation of the −100 mV voltage pulse were used in the analysis. *, $p < 0.05$, comparison among pre-, post-TG, and TG + La3⁺ treatments in the same group. n indicates the number of cells analyzed in each group. J, summary data showing the effect of pipette-perfused (at 200 ng/ml) purified recombinant MBP, MBP-I-mfa(a), MBP-I-mfa(b), or MBP-I-mic on TG-induced current density in CHO cells obtained by a voltage step protocol, as described in F. n indicates the number of cells analyzed in each group. *, $p < 0.05$, comparison between pre- and post-TG current density. K, suppression of store-operated Ca²⁺ entry in CHO-K1 cells by F-I-mfa in CHO-K1 cells. Effect of Ca²⁺ re-addition (CaCl₂, 6.25 mM) on intracellular Ca²⁺ concentration (shown as 405/485 fluorescence ratio) in CHOmock (black) and CHO-I-mfa cells (red) incubated in a nominally Ca²⁺-free solution with (left) or without (right) TG stimulation (1 μM for 1 h).
I-mfa Modulates TRPC1 Activity

Conductance in A431 I-mfa-RNAi but Not in A431 Cells—Although down-regulation of I-mfa enhanced store-activated currents in A431 cells, the contribution of TRPC1 to this enhancement was unknown. Fig. 6 shows that dialysis of the cytoplasm of A431 cells with 1F1 did not result in a significant change in the whole cell store-operated currents compared with non-dialyzed A431 cells (Figs. 5J and 6C) suggesting that TRPC1 did not significantly contribute to TG-induced currents in these cells. In contrast, TG-induced currents were reduced by 85% in A431 I-mfa-RNAi cells dialyzed with 1F1 as compared with mouse IgG1 (Fig. 6, E). The increase in current density at −100 mV in response to TG was reduced from −5.9 ± 0.9 pA/pF in cells dialyzed with 100 ng/ml mouse IgG1 to −0.9 ± 0.4 pA/pF in cells dialyzed with 100 ng/ml 1F1 (Fig. 6H). These results indicated that TRPC1 was required for the function of native store-operated channels in A431 I-mfa-RNAi cells supporting the idea that removal of I-mfa from A431 cells enhanced the function of TRPC1 as a store-operated channel.

Recombinant MBP-I-mfa(a) Rescues the Effect of I-mfa Knock-down in A431 Cells—To confirm that the effect of I-mfa inactivation on store-operated conductance in A431 cells was specific to I-mfa knock-down, we pipette-perfused recombinant MBP-I-mfa(a) in A431 I-mfa-RNAi cells and tested whether it could reverse the increase in store-operated currents induced by I-mfa knock-down. Fig. 6H shows that MBP-I-mfa(a) significantly reduced the increase in current density in response to TG from −5.9 ± 0.9 pA/pF to −1.9 ± 0.38 pA/pF in A431 I-mfa-RNAi cells.

DISCUSSION

We provide three lines of evidence supporting a physical and functional interaction between I-mfa and TRPC1. First, TRPC1 and I-mfa associate in vitro and in vivo. Second, overexpression of I-mfa suppresses store-operated conductance, whereas down-regulation of I-mfa increases store-operated currents. Third, TRPC1 functionally contributes to store-operated conductance in cells lacking I-mfa but not in cells expressing I-mfa. These data provide evidence for a new function of I-mfa as a negative regulator of store-operated Ca²⁺ entry.
I-mfa Modulates TRPC1 Activity

Fig. 5. Knock-down of I-mfa by RNAi augments TG-induced currents in A431 cells. A, expression levels of endogenous TRPC1 in A431 and A431\textsuperscript{mfa-RNAi} cells. Lysates of mock-transfected (lane 1), M-TRPC1-transfected HEK293T (lane 2), A431 (lane 3), or A431\textsuperscript{mfa-RNAi} (lane 4) cells were immunoblotted with mouse α-TRPC1-N at 1:5,000 dilution. B and C, expression levels of I-mfa in A431 and A431\textsuperscript{mfa-RNAi} cells. Lysates of A431 (lane 1) or A431\textsuperscript{mfa-RNAi} (lane 2) cells were immunoblotted with a chicken affinity purified α-I-mfa (B) or immunoprecipitated with mouse α-I-mfa and then immunoblotted with chicken α-I-mfa (C). D–G, representative traces of single A431 (D and F) or A431\textsuperscript{mfa-RNAi} (E and G) cells showing background currents (Pre-TG, D and E) or currents recorded 1 min following the addition of 1 μM TG to the extracellular solution (Post-TG, F and G) using a voltage step protocol. Currents are expressed as current densities. H and I, I-V relation curves generated from a ramp protocol before (Pre-TG) and 2 min after (Post-TG) the addition of 1 μM TG to the extracellular solution in A431 (H) and A431\textsuperscript{mfa-RNAi} cells (I). J, summary data showing current density (pA/pF) of background (Pre-TG), TG-induced currents (Post-TG), or TG-induced currents in the presence of 20 μM La\textsuperscript{3+} (TG + La\textsuperscript{3+}) in A431 and A431\textsuperscript{mfa-RNAi} cells. Step currents recorded 50 ms after establishing the initiation of the -100 mV voltage pulse were used in the analysis. *p < 0.05, comparison among pre-, post-TG, and TG + La\textsuperscript{3+} treatments in the same group; #, p < 0.05, comparison of post-TG treatments between groups. n indicates the number of cells analyzed in each group.

Using the yeast two-hybrid assay as an unbiased screen, we identified I-mfa as an interacting protein with TRPC1. The interaction was further verified in \textit{in vitro} and \textit{in vivo} co-immunoprecipitation experiments and \textit{in vitro} binding assays using recombinant proteins. Furthermore, the interaction sites were mapped to the C terminus of both peptides. The a-region of I-mfa has been shown to mediate interactions with a divergent group of proteins such as a subset of bHLH (37, 38), XTcf3 (39), and axin (40). \textit{In vitro} data showed that the I-mfa-TRPC1 interaction could be competed away by I-mfa interacting proteins, such as myogenin. We speculate that I-mfa exists in two separate but dynamically regulated pools; one pool of I-mfa associated with TRPC1 and another pool of I-mfa associated with bHLH/LEF1/TCF3 transcription factors. Although the functional and physiological significance of this competition is unknown at present, it provides an \textit{in vitro} example of the dynamic nature of the interaction between TRPC1 and I-mfa and raises the intriguing possibility that TRPC1 function could be regulated by proteins that interact with I-mfa. Thus, the I-mfa-mediated inhibition of TRPC1 could be reversed under conditions limiting the amount of I-mfa available for interaction with TRPC1. Conversely, TRPC1-interacting proteins may displace inhibitory I-mfa from TRPC1. It is tempting to speculate that the latter may underlie a gating mechanism for store-operated channels via TRPC1.

Several independent studies, including our own, show that endogenous TRPC1 functionally contributes to native store-operated conductance in a variety of cells including hamster fibroblast CHO-K1, human submandibular gland HSG cells, and DT40 B lymphocytes (17, 18, 49). However, it cannot be concluded from these studies that store-operated currents are carried through TRPC1 and evidence from heterologous expression studies argue against this idea (19, 32). Our data show that removal of I-mfa from A431 cells resulted in the activation of slightly Ca\textsuperscript{2+} selective store-operated currents. In addition, TG activated a conductance with similar selectivity in CHO-K1 cells. The TG-induced currents are unlikely to be carried by TRPC1 itself, as TRPC1 forms a non-selective cation channel when successfully expressed alone in CHO-K1 cells (19). Furthermore, knock-down of either TRPC1 (18) or TRPC2 (50) in CHO-K1 cells resulted in more than 90% reduction in Ca\textsuperscript{2+} entry in response to store depletion implying a functional interaction between TRPC1 and TRPC2. Thus, we favor the idea that native TRPC1 is an essential component of store-
operated conductance, but that it may require other channel proteins to form a store-operated channel. Identification of these interacting proteins will be instrumental in the determination of the molecular entity of the elusive store-operated Ca\(^{2+}\) channel complex.

In a previous study (18), TG-induced current densities were 4–5 pA/pF in CHO-K1 cells, whereas in our case, TG-induced current densities were 8 pA/pF in the same cells. There are several explanations for these disparities. First, in the previous study, 200 nM TG was used to activate store depletion-sensitive currents instead of 1 μM used in our experiments. It is unknown whether 200 nM TG had completely depleted internal Ca\(^{2+}\) stores in these experiments. It has been reported that the magnitude of Ca\(^{2+}\) entry through store-operated Ca\(^{2+}\) channels is closely correlated with the extent of store depletion (51). The Ca\(^{2+}\) concentration in the bathing solution was 2 mM in the experiments of Vaca and Sampieri (18), whereas 20 mM in ours. Because store-activated channel is permeable to Ca\(^{2+}\), the extracellular Ca\(^{2+}\) concentration may have an impact on the amplitude of the whole cell currents. In addition, the Mg\(^{2+}\) concentration in their pipette solution was dramatically different from ours. Specifically, the total intrapipette Mg\(^{2+}\) concentration was 5 mM (2 mM MgCl\(_2\), 2 mM Mg-ATP, and 1 mM Mg-GTP) in the previous study, whereas it was 1.33 mM (1 mM MgCl\(_2\), 0.3 Mg-ATP, and 0.03 mM GTP) in our case. The effect of intracellular Mg\(^{2+}\) on currents carried by CRAC, MIC, or TRP channels is summarized in a recent review (9). Despite differences in current amplitudes, two independent approaches, RNAi (18) and the use of a neutralizing TRPC1-specific antibody, have demonstrated a necessary role of TRPC1 in native store-operated conductance in CHO-K1 cells. Because I-mfa expression suppressed store-activated currents as completely as 1F1 or the double stranded RNA used to inactivate TRPC1 in another study (18), we propose that I-mfa inhibited store-activated currents in CHO-K1 cells by a TRPC1-dependent mechanism. This was further supported in A431 cells in which 1F1 reversed the increase in store-operated currents following I-mfa inactivation.

We found that knock-down of I-mfa by ~90% resulted in a 3-fold increase in the TG-induced currents in A431 cells. This increase was reduced by 85% using a TRPC1 neutralizing antibody or by 68% with recombinant I-mfa, both delivered via pipette-perfusion. These results support a direct functional interaction between I-mfa and TRPC1 and prompt us to conclude that native I-mfa negatively regulates the activity of TRPC1 as a store-activated channel in these cells. Store-operated channels in A431 have been studied extensively (52–57). In a recent detailed study (58), it was shown that store-operated conductance in A431 cells is composed of two major currents, the Ca\(^{2+}\) release-activated current (I\(_{CRAC}\)) and store-
I-mfa Modulates TRPC1 Activity

operated current (I_{SOC}). Some of the differences between these two currents in relation to our studies were that I_{CRAC} showed high Ca$^{2+}$-selectivity and lack of significant outward currents at positive potentials, whereas I_{SOC} was less selective to Ca$^{2+}$ and showed outward currents. Under our experimental conditions, it is likely that both of these currents contribute to the TG-induced response. However, based on the shape of our I-V curve, we propose that I_{ISOC} might be the predominant contributor. Because I_{F1} had a minimal effect on the TG-induced current in native cells, we speculate that TRPC1 may not significantly contribute to I_{SOC} in these cells. In contrast, it may contribute up to 85% (Fig. 6H) of the same current in cells lacking I-mfa. A detailed analysis to determine the specific suppressive effect of I-mfa on I_{CRAC} and I_{SOC} will provide insight into the mechanism by which TRPC1 contributes to I_{CRAC} and/or I_{SOC}.

The interaction between TRPC1 and I-mfa was demonstrated in adult rat kidney and liver lysates, whereas significant co-expression of TRPC1 and I-mfa was seen in lung and heart. A widespread distribution of TRPC1 (for review see, Ref. 14) and I-mfa mRNAs (38) in adult tissues has also been reported. Based on our biochemical and functional studies, it is tempting to suppose that the expression of TRPC1 by I-mfa may have physiological consequences. However, it is also equally possible that one of the physiological roles of I-mfa may be the modulation of the activity of TRPC1 to function as a receptor-operated channel through the association with TRPC4 or TRPC5. Because TRPC1 is likely to be co-expressed with TRPC4 and/or TRPC5 in native tissues, a more intriguing possibility is that I-mfa may function as a mode switch between store-operated and receptor-operated modes of TRPC1 activation. Experiments are underway to determine whether I-mfa can modulate TRPC1/TRPC4 function in transfected cells.

Overall, our study provides both biochemical and functional data supporting a role for I-mfa on Ca$^{2+}$ signaling via a protein-protein interaction with TRPC1. Given the role of I-mfa in the regulation of the activity of a diverse group of transcription factors (37–39), our results provide evidence for a novel mechanism by which a transcriptional regulator is involved in Ca$^{2+}$ signaling by direct interaction with a channel. Because of the latter, the interaction between TRPC1 and I-mfa could function as a ubiquitous switch influencing many developmental and physiological processes.

Acknowledgments—We thank Drs. R. E. Anderson, B. Ceresa, P. A. McKee, K. Lau, G. Dale, and H. Akbarali for critical reading of the manuscript, C. Montell for helpful discussions, S. Altman and C. Guerrier-Takada for H1 R-gene promoter, S. Hollenberg for the pLexA vector and mouse embryonic library, S. Tapscott and L. Snider for mouse HA-I-mfa, MBP-I-mfa, and MBP-I-mfa constructs, and A. Kern for F-1mfa plasmid.

REFERENCES

1. Berridge, M. J., and Irvine, R. F. (1984) Nature 312, 315–321
2. Burgess, G. M., Godfrey, P. P., McKinney, J. S., Berridge, M. J., Irvine, R. F., and Rosado, J. A., and Sagi, S. O. (2000) Biochem. J. 350, 631–635
3. Rosado, J. A., and Sagi, S. O. (2000) Biochem. J. 350, 191–199
4. Josiak, I., Arnold, T., Zhu, C., Kim, E., Walz, G., and Schultze, G. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3934–3939
5. Thromer, C., Jaeger, R., and Ehrlich, B. E. (2001) Trends Pharmacol. Sci. 22, 580–586
6. Kiyosu, T., Cai, Y., Geng, L., Maeda, Y., Nishimura, S., Witzgall, R., Ehrlich, B. E., and Somlo, S. (2002) Nat. Cell Biol. 4, 191–197
7. Patterson, R. L., van Rossum, D. B., and Gill, D. L. (1999) Cell 98, 487–499
8. Strubing, C., Krapivinsky, G., Krapivinsky, L., and Clapham, D. E. (2001) J. Biol. Chem. 276, 6436–6969
9. Ma, R., Smith, S., Child, A., Carmines, P. K., and Sansom, S. C. (2000) Am. J. Physiol. 278, F954–F961
10. Venkatachalam, K., van Rossum, D. B., Patterson, R. L., Ma, H. T., and Gill, D. L. (2002) Nat. Cell Biol. 4, E263–E272
11. Minke, B., and Selinger, Z. (1996) Mol. Neurobiol. 12, 163–180
12. Birnbaumer, L., Zhu, X., Jiang, M., Boulay, G., Peyton, M., and Vannier, B., Brown, D., Platano, D., Sadeghi, H., Stiefani, E., and Birnbaumer, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11518–11522
13. Clapham, D. E., Runnels, L. W., and Strubing, C. (2001) Nat. Rev. Neurosci. 2, 387–396
14. Montell, C., Birnbaumer, L., and Flockerzi, V. (2002) Cell 108, 595–598
15. Zhu, X., Chu, P. B., Peyton, M., and Birnbaumer, L. (1995) FEBS Lett. 373, 193–198
16. Wes, P. D., Chevesich, J., Jeromin, A., Rosenberg, C., Stetten, G., and Montell, C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9652–9656
17. Mori, Y., Wakamori, M., Miyakawa, T., Hermosa, M., Hara, Y., Nishida, M., Hirose, K., Mizushima, A., Kuroski, M., Mori, R., Gotob, K., Okada, T., Fleig, A., Penner, R., and Kuryshev, Y. A. (2002) J. Exp. Med. 195, 673–681