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Multiple Types of Calcium Channels Arising from Alternative Translation Initiation of the *Orai1* Message

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Abstract

In mammals exclusively, the pore-forming Ca^{2+} release-activating Ca^{2+} (CRAC) channel subunit, Orai1, occurs in two forms due to alternative translation initiation. The longer, mammal-specific Orai1 α contains an additional 63 amino acids upstream of the conserved start site for Orai1 β , which occurs at methionine 64 in Orai1 α . Orai1 participates in the generation of three distinct Ca^{2+} currents, including two store-operated currents, I_{crac} , which involves the Ca^{2+} -sensing protein STIM1 activation of Orai1 channels, and I_{soc} , which involves a tertiary interaction among Orai1, the transient receptor potential (TRP) family member TRPC1, and STIM1. Orai1 is also a pore-forming subunit of an arachidonic acid (leukotriene C_4)-regulated current I_{arc} that involves interactions of Orai1, Orai3, and STIM1. Here, we evaluated the roles of the two Orai1 forms in the Ca^{2+} currents I_{crac} , I_{soc} , and I_{arc} . We found that Orai1 α and Orai1 β are largely interchangeable for I_{crac} and I_{soc} , although Orai1 α exhibits stronger inhibition by Ca^{2+} . Only the mammalian-specific Orai1 α functions in the arachidonic acid-regulated current, I_{arc} . Thus, alternative translation initiation of *Orai1* message produces at least three types of Ca^{2+} channels with distinct signaling and regulatory properties.

Introduction

Store-operated Ca^{2+} entry, mediated by store-operated Ca^{2+} channels, is among the most widely encountered signaling mechanism in the animal kingdom, and the most extensively characterized store-operated channel is the Ca^{2+} -release activated Ca^{2+} (CRAC) channel. CRAC channels are comprised of the pore-forming subunits Orai1, Orai2, or Orai3 and are

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gated by an interaction with the endoplasmic reticulum-localized Ca^{2+} sensor STIM1 or STIM2 (1). These components are also necessary for a Ca^{2+} entry pathway involving a complex interaction between Orai1, STIM1, and channels of the canonical transient receptor potential family (TRPC), and this complex produces a current that is less selective than I_{crac} , termed I_{soc} . There are two views regarding the molecular underpinnings of I_{soc} , one holding that the current represents the sum of inwardly rectifying I_{crac} and a less selective current through TRPC channels (2), whereas another view suggests that the current involves distinct channels comprised of both TRPC and Orai pore-forming subunits (3). In addition, Orai1 is a constituent of a non-store-operated current, I_{arc} , involving Orai1, Orai3 and STIM1, which is gated by arachidonic acid (4) or a metabolite of arachidonic acid, leukotriene C4 (5). Recent studies indicate that currents previously shown to be mediated by arachidonic acid, I_{arc} (6), as well as currents mediated by leukotriene C4 (5, 7), arise from the same channel. In a recent study, their activation was inhibited when metabolism of exogenous arachidonic acid to leukotriene C₄ is prevented (8), whereas in earlier studies that was not the case (9, 10). For the sake of simplicity, we will adhere to the original term for this current, I_{arc} , although we believe it may be regulated by a metabolite of arachidonic acid. Not surprisingly, I_{arc} is similar to I_{crac} in many ways, because its pore is also comprised of Orai subunits. However, the ARC channels appear to be heteromers of Orai1 and Orai3 (11), whereas the CRAC channels are thought to be homomers of Orai1, Orai2, or Orai3 (12, 13). Like I_{crac} , I_{arc} is a small current, and strongly inwardly rectifying. Whereas I_{crac} is activated by Ca^{2+} store depletion detected by STIM1 (14) and is inhibited by 2-aminoethyldiphenylborate (2-APB); I_{arc} is activated by a ligand, has a different pH sensitivity (15), exhibits reduced or lacks fast Ca^{2+} -dependent inactivation (15), does not rapidly de-potentiate (5, 16), and is not inhibited by 2-aminoethyldiphenylborate (2-APB) (5, 10).

Orai1 is expressed in two forms, Orai1 α (long) and Orai1 β (short), due to alternative translation initiation (17). Here, we have examined the role of these distinct forms of Orai1 in the three Orai1 currents, I_{crac} , I_{soc} , and I_{arc} . We show that both Orai forms can support I_{crac} and I_{soc} . However, the longer form, Orai1 α , undergoes much stronger calcium-dependent inactivation than Orai1 β . Additionally, only the longer Orai1 α formed channels underlying I_{arc} . Thus, alternative forms of an ion channel encoded by a single gene can give rise to three distinctly different channels. This finding adds considerable breadth to the roles of Orai1 subunits in signaling and changes the way experimental work with Orai1 mouse models can be interpreted. It also provides a molecular framework for separating and analyzing the distinct functions of Orai1-containing channels in mammalian cell signaling.

Results

Generation of Orai1 α -optimized and Orai1 β -optimized constructs

Here, we used a construct in which the Kozak sequence for the first methionine is optimized, such that the ribosomal complex does not skip the first methionine, resulting in efficient formation of only the Orai1 α form because the ribosomal complex now starts essentially every time at the first strengthened Kozak sequence (Orai1 α). In a second construct, the ribosomal complex continues past a nonconsensus start site at the first methionine and starts

essentially every time at the second Kozak sequence at methionine 64 (Orai1 β). Thus, introduction of the Orai1 α -optimized construct or the Orai1 β -optimized construct results in the production of approximately equal amounts of either Orai1 α or Orai1 β (17).

Rescue of SOCE in Orai1-KO MEF Cells

We investigated the efficacy of Orai1 α and Orai1 β in supporting store-operated Ca²⁺ entry (SOCE) with a line of mouse embryonic fibroblasts (MEFs) from an Orai1-knockout mouse (18) (Orai1-KO MEFs). To minimize overexpression of Orai1, we subcloned wild-type Orai1, Orai1 α , and Orai1 β into expression plasmids carrying a *thymidine kinase* (TK) promoter, which gives considerably less expression than in previous studies utilizing the stronger CMV promoter (19). All constructs were tagged at the C-terminus with enhanced green fluorescent protein (EGFP). With the strong CMV promoter, it has not generally been possible to study Orai1 expressed on its own because these constructs actually had a partial inhibitory effect, presumably due to inappropriate stoichiometry between Orai1 and endogenous STIM1 (20). We determined from the fluorescence of the EGFP-tagged proteins that the three constructs were expressed at similar amounts, although there was a small but significantly greater abundance of the wild-type and Orai1 β constructs compared to that of Orai1 α (Fig. 1A). We then measured SOCE from wild-type MEFs and Orai1-KO MEFs stably expressing wild-type Orai1, Orai1 α , or Orai1 β . SOCE in response to the passive Ca²⁺-depleting agent, thapsigargin, was undetectable in Orai1-KO MEFs, but was restored by transfection with wild-type Orai1, Orai1 α or Orai1 β (Fig. 1B). There were no significant differences in the thapsigargin-induced release of Ca²⁺ in the various cell lines (Fig. 1C). All three constructs rescued SOCE, whereas stable transfection with GFP did not. However, the Ca²⁺ entry in the wild-type Orai1-expressing and Orai1 β -expressing MEFs was significantly greater than in the Orai1 α -expressing cells (Fig. 1D).

Rescue of I_{crac} in Orai1-KO MEF Cells

We assessed I_{crac} in these same MEF cell lines. Because I_{crac} is extremely small in MEFs, we utilized the strategy of observing store-operated currents that were amplified in solutions lacking divalent cations (divalent-free, DVF) (21, 22). In wild-type MEFs dialyzed with 20 mM BAPTA, a small inward current developed that was amplified by a transient switch to a DVF external solution (Fig. 2A). Shortly after breaking in with the patch pipet, a switch to the DVF solution resulted in a symmetrical (equal inward and outward) increase in current that we presume reflects an increase in leak at the cell membrane or pipet seal due to the absence of divalent cations. At the second addition, when the DVF inward current was transiently increased, this outward current was unchanged such that the leak-subtracted DVF current showed the typical inward rectification expected of I_{crac} (Figure 2A). This current was completely blocked by subsequent addition of 5 μ M Gd³⁺, again as expected for I_{crac} (20). In Orai1-KO MEFs, no such current was observed (Figure 2B).

Stable expression of any of the three constructs wild-type Orai1 (Figure 2C), Orai1 α (Figure 2D), or Orai1 β (Figure 2E) in the Orai1-KO MEF cells rescued the inwardly rectifying inward current and produced similar leak-subtracted peak inward Na⁺ currents to those in wild-type MEFs (Figure 2F).

Wild-type Orai1, expected to yield a mixture of the two forms, as well as the Orai1 α -specific and Orai1 β -specific constructs, all restored I_{crac} to levels similar to the wild type cells. This was somewhat unexpected because, in the Ca^{2+} experiments, Orai1 α resulted in a significantly lower Ca^{2+} entry signal. Because in the I_{crac} measurements, Ca^{2+} feedback on the channels is abrogated by the use of the fast buffer, BAPTA, we considered that the smaller Ca^{2+} entry in the Orai1 α cells, as measured in the fluorescence assays (Fig. 1), might reflect a greater degree of Ca^{2+} feedback, also known as Ca^{2+} -dependent inactivation (CDI) (23). We thus carried out experiments specifically designed to reveal the presence and extent of CDI by comparing the kinetics of inactivation of currents with the fast calcium chelator BAPTA or the slower chelator EGTA in the pipette. CDI is revealed by holding membrane potential at a positive value, where CDI is prevented, then rapidly jumping to a negative value, allowing Ca^{2+} to enter and then rapidly inactivate I_{crac} . For Orai1 α , we observed rapid and considerable inactivation with the slower Ca^{2+} buffer EGTA, and this inactivation was diminished with the faster buffer BAPTA (Fig. 3A); whereas for Orai1 β this difference was absent (Fig. 3B). Quantification of the extent of inactivation data for different final membrane potentials showed that Orai1 α and Orai1 β behaved similarly in the presence of BAPTA (Fig. 3C) and Orai1 α produced a smaller current at all potentials tested in the presence of EGTA (Fig. 3D). Thus, we concluded that, although both Orai1 α and Orai1 β can support I_{crac} , the two forms differ in that Orai1 α exhibits stronger CDI.

Function of Orai1 Forms in I_{soc}

We considered the possibility that one of the forms of Orai1 might specifically function in the less selective store-operated current, I_{soc} (24). These currents are believed to result from a complex mechanism whereby Ca^{2+} entering through classical Orai-containing CRAC channels recruits TRPC channels to the plasma membrane where they are activated by STIM1 (2).

We transfected HEK293 cells with either STIM1 and wild-type Orai1, which produces large I_{crac} , or STIM1, wild-type Orai1, and TRPC1, which produces a larger current with a more significant outward component, resembling I_{soc} (Fig. 4A). We performed similar transfections utilizing either the Orai1 α -optimized construct (Fig. 4B) or the Orai1 β -optimized construct (Fig. 4C). The I_{crac} that we observed was somewhat smaller than in earlier publications (20), possibly due to variations in lines of HEK293 cells. We routinely observed small outward currents associated with the I_{crac} protocol in the cells not transfected with TRPC1, which could result from endogenous TRPC1 in HEK293 cells (25); this was not further investigated. Both Orai1 α and Orai1 β supported I_{soc} equally well, but unlike the findings in MEFs, the single forms of Orai gave greater I_{crac} currents than the wild-type Orai1 (Fig. 4D). Inward I_{soc} was also greater with the specific constructs than with wild-type Orai1, perhaps in part because the amount of TRPC1 recruited should depend on the amount of Ca^{2+} entering through CRAC channels (2). However, for the outward I_{soc} , this difference was substantially less and was statistically significant only for Orai1 β . This might support the model in which I_{soc} is comprised of separate CRAC and TRPC1 channels (2, 26). In confirmation of a previous report (3) utilizing the same TRPC1 construct and recording conditions, we failed to see currents in cells transfected with only STIM1 and TRPC1 (Fig. 4A and D).

We noted that the larger I_{crac} seen in HEK293 cells with the Orai1 α and Orai1 β constructs, compared to cells with wild-type Orai1 (Figure 4D), was not seen in I_{crac} measurements in MEFs (Figure 2F). One difference in the two protocols (aside from the different cell types) is that in the HEK293 experiments, exogenous STIM1 was supplied, whereas it was not in the MEFs, raising the possibility that STIM1 was limiting in the MEFs. Indeed, when we transiently transfected the stable MEF lines with ##STIM1, compared with the I_{crac} in the previous experiment (Fig. 2F), I_{crac} was significantly amplified in all three lines based on two-way ANOVA (Fig. 5B) but to a significantly greater extent in the cell lines expressing either Orai1 α or Orai1 β alone rather than in the cells expressing wild-type Orai1 cells (Fig. 5B). The basis for this small but apparently reproducible difference is currently under investigation.

The data to this point indicated that the two forms support I_{crac} equally well. In our previous study, we found that in unstimulated cells, the mobility of Orai1 α was significantly less than that for Orai1 β (17). Thus, we examined the rates at which Orai1 α and Orai1 β associate with STIM1 in cells following Ca^{2+} store depletion (see for example (17)). By monitoring the area of puncta containing fluorescently tagged STIM1 and fluorescently tagged Orai1 α or Orai1 β , we determined that the rate of association of the two forms with STIM1 was similar for the two Orai1 forms (Fig. 6).

Orai1 Forms and I_{arc}

Finally, we investigated the possible role of a specific form of Orai1 in the non-store-operated Ca^{2+} entry pathway activated by arachidonic acid or its metabolite leukotriene C_4 (5–7, 27). We utilized HEK293 cells for these studies, because they exhibit a small endogenous I_{arc} (8, 16) and because the function of Orai1 in I_{crac} can be compared in the same cells. We used exogenous arachidonic acid to activate I_{arc} currents. Due to the small size of the currents, we examined I_{crac} - and I_{arc} -associated Na^+ currents. In wild-type HEK293 cells, dialysis with an intracellular solution containing 20 mM BAPTA resulted in the development of an inwardly rectifying Na^+ current that rapidly diminished by a process known as de-potentialization (28) and was inhibited by 2-APB (Figure 7A), which are characteristics of I_{crac} . This current was absent or substantially diminished following siRNA-mediated knockdown of Orai1 (Figure 7B). In cells in which Orai1 had been knocked down and subsequently transfected with plasmids for either the Orai1 α (Figure 7C) or Orai1 β (Figure 7D) constructs, both forms of Orai1 were equally efficacious in rescuing I_{crac} Na^+ current (Fig. 7E). Note that both the Na^+ and Ca^{2+} currents for I_{crac} and I_{arc} were substantially smaller in the current study than in earlier published studies (for example (16, 22, 29)). The reason for this difference is not known, but as pointed out previously, may be due to differences in lines of HEK293 cells.

With an intracellular patch pipet solution containing Ca^{2+} buffered to 150 nM to prevent development of I_{crac} , HEK293 cells developed inwardly rectifying Na^+ currents in response to extracellular addition of 8 μM arachidonic acid (Figure 8A). Unlike I_{crac} (Fig. 7), this Na^+ current did not de-potentiate and was not blocked by 2-APB, consistent with the properties of I_{arc} (5, 7, 8, 16). Knockdown of Orai1 substantially reduced the Na^+ current (Figure 8B). Subsequent transfection with a plasmid carrying a construct for Orai1 α fully rescued Na^+ I_{arc}

(Figure 8C), whereas Orai1 β was completely ineffective (Figure 8D; summarized in Figure 8E). To insure that this striking distinction in the function of the two Orai1 forms also applied to the channels when the physiological ion was carrying the current, we examined Ca²⁺ currents as well. These currents are extremely small, and so we show the results as a scatter plot of individual leak-subtracted currents (Figure 8F). Despite the very small current values, only Orai1 α rescued the Ca²⁺ I_{arc}

Discussion

In a previous study reporting I_{crac} measurements (17), a construct that produced only Orai1 α was made by mutating the start site at methionine 64, and a construct that produced only Orai1 β was made by mutating the first methionine start site. Alternative translation initiation usually occurs through a process of ribosomal scanning. The two forms of Orai1 arise because the Kozak sequence at the first methionine is weak, thus will be skipped about half of the time. Therefore, with the Orai1 α construct used in the previous study (17), the ribosomal complex would still fail to initiate much of the time at the first methionine, resulting in poorer expression than for the Orai1 β construct. This likely explains why the Orai1 α construct gave considerably less I_{crac} than did the Orai1 β construct [Figure 4 in (17)].

In this same study (17), we provided evidence that Orai1 α and β could not form heteromeric channels. Nonetheless, on the basis of the current findings, we concluded that channels comprised of either form are equally capable of forming CRAC channels or participating in the generation of I_{soc} . In addition, despite the previously documented difference in plasma membrane mobility under unstimulated conditions (17), we found that following Ca²⁺-store depletion, the two forms associated with STIM1 in near plasma membrane puncta at similar rates. We did observe a difference in Ca²⁺ regulation of the two forms, because only Orai1 α exhibited CDI. However, more strikingly it appeared that Orai1 α is specifically required for the channels underlying I_{arc} . Interestingly, only mammals express the N-terminal extended Orai1 α , and only mammals express Orai3, which associates with Orai1 to form ARC channels (5, 11). Thus, mammalian evolution has exploited the process of alternative translation initiation to produce from the same gene calcium channels with distinct modes of regulation and activation. Our previous work demonstrated that the two forms of Orai1 do not assemble to form channels that are heteromultimeric with respect to Orai1 form (17). Thus, cells may utilize Orai1 in three functionally distinct channel structures: homomeric Orai1 α and homomeric Orai1 β CRAC channels, with differing sensitivities to Ca²⁺-dependent inactivation, as well as heteromeric Orai1 α -Orai3 ARC channels. We expect the presence of at least some Orai1 α CRAC channels because the N-terminal extension contains the site at which the Ca²⁺-activated adenylyl cyclase 8 is tethered permitting specific linkage between Ca²⁺-activation of cAMP (adenosine 3'-5'-monophosphate) formation and store-operated CRAC channels (30).

A number of mouse models for studying the function of Orai1 are now in use, and the general presumption is that a deficit in Orai1 means a loss of I_{crac} . However, it is clear that Orai1 functions in other Ca²⁺ channels and the loss of I_{soc} or I_{arc} or both may underlie or contribute to the various phenotypes that these knockout mice exhibit. Understanding

distinctions at the molecular level between these different Ca^{2+} signaling mechanisms will permit the generation of other mouse models to shed light on the specific roles of Orai1 in different physiological and pathological contexts.

Methods

Reagents

Arachidonic acid, Cs-methanesulfonate and Na-methanesulfonate were purchased from *Sigma*. Fura-5F-AM was purchased from *Setareh Biotech, LLC*. 2-aminoethoxydiphenyl borate (2-APB) was purchased from *Calbiochem*. Cs-BAPTA was purchased from *Invitrogen*. GdCl_3 was from *Acros Organics*. All dicer-substrate siRNAs (dsiRNAs) were purchased from *IDT*. The transfection kit (VCA-1003) for HEK293 cells was from *Lonza*. All other chemicals were from *Fisher*.

Cell Culture

MEFs were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 100 units/ml penicillin and 100 units/ml streptomycin and maintained in a humidified 95% air, 5% CO_2 incubator at 37°C . In preparation for Ca^{2+} measurements, cells were cultured to about 70% confluence and then transferred onto 30-mm round glass coverslips (#1.5 thickness). The cells were allowed to attach for a period of 6 h, after which additional DMEM was added to the coverslip, and the cells were maintained in culture for an additional 24 h before use in Ca^{2+} measurements.

cDNA constructs

Human Orai1 cDNA fused to EGFP under the control of a *TK* promoter was provided by Tamas Balla, NICHD-NIH. This wild type Orai1-EGFP cDNA with the *TK* promoter was subcloned into CD510B-1 Lentivector (System Biosciences) for transfections and making Lentivirus. The *CMV* promoter in CD510B-1 Lentivector was deleted during subcloning. Site-direct mutagenesis of the first methionine to alanine or to a strong Kozak sequence [as in (17)] was performed with Quikchange II XL (Stratagene) according to the manufacturer's instructions. These mutations produce constructs that give essentially 100% Orai1 β and 100% Orai1 α , respectively (17). For transient transfections, STIM1, EYFP-STIM1, mCherry-STIM1, wild-type Orai1-EGFP, Orai1 α -EGFP, and Orai1 β -EGFP constructs were as described previously (17). The EYFP (enhanced yellow fluorescent protein)-STIM1 plasmid was obtained from Tobias Meyer, Stanford University. TRPC1 construct was obtained from Leonidas Tsiokas, University of Oklahoma (3).

Production of lentivirus and stable Orai1-expressing MEF cells

All lentivirus were packaged in HEK293T/17 cells (ATCC # CRL-11268) (31). Briefly, 293T cells were transiently transfected with pMD2G, psPAX2, and transfer vector containing wild-type Orai1-EGFP, Orai1 α -EGFP, or Orai1 β -EGFP using Lipofectamine 2000. Supernatant was collected 48 hours post transfection and concentrated by centrifugation at 50,000 g for 2 hours. Pellets were resuspended in PBS (pH 7.4) and used for infection. All titers were determined by performing quantitative PCR to measure the number of lentiviral particles that integrated into the host genome. In addition to quantitative

PCR, biological titration of viruses that coexpressed fluorescent moieties was determined by flow cytometry. MOI ranging from 3 to 1 were used for infection of MEF cells. The stable Orai1-expressing MEF cells were established by selecting with 4 $\mu\text{g/ml}$ puromycin in the medium.

Transient Transfections

For experiments examining Orai1 α and Orai1 β in I_{crac} and I_{soc} , the HEK293 cells were transfected with the Amaxa electroporation system (Amaxa Inc., a Lonza Cologne company) following the guidelines set forth by the company for the cell line using the HEK293 (ATCC) cell line setting and the nucleofector kit V buffer. HEK293 cells were transfected with following amounts of cDNA: EYFP-STIM1 cDNA (0.1 μg), and wild-type Orai1-EGFP cDNA (0.1 μg) with or without TRPC1 cDNA (0.1 μg). To compare the α and β forms of Orai1, HEK293 cells were transfected with 0.1 μg cDNA encoding either of these forms with or without 0.1 μg TRPC1 cDNA. After a 6–8 h incubation period, the medium bathing the cells was replaced with complete DMEM and the cells were maintained in culture. The following day, cDNA-transfected cells were transferred to 30-mm glass coverslips in preparation for electrophysiological studies. For experiments examining the roles of Orai1 α and Orai1 β in I_{crac} and I_{arc} , and for examining calcium-dependent inactivation of Orai1 α -mediated and Orai1 β -mediated I_{crac} , transfections were done using the Nucleofector device II (*Amaxa Biosystems*) with program Q-001 according to the manufacturer's instructions. For the calcium-dependent inactivation studies, cells were also transfected with EYFP-STIM1 as in (17). After transfection, HEK293 cells were seeded on glass coverslips and maintained in DMEM supplemented with 10% fetal bovine serum (*HyClone Laboratories*), 1% L-glutamine, and 1% Antibiotic-Antimycotic (*Invitrogen*) in a humidified, 5% CO_2 incubator at 37 $^{\circ}\text{C}$.

For knockdown experiments, we used dsRNA at 1 nmol dsRNA per 1 million cells. Sequences are listed below:

siOrai1 sequence:

5'- GGGAGGUUGAGACGGACAGGACAGGAG -3'
3'- CCCUCCAACUCUGCCUGUCCUGUCC -5'

Negative control sequence:

5'- CGUUAUUCGCGUAUAAUACGCGUAT -3'
3'- AUACGCGUAUUAUACGCGAUUAACGAC -5'

Calcium Measurements on Single MEF Cells

Fluorescence measurements were made with MEFs loaded with the ratiometric calcium sensitive dye, fura-5F. Briefly, coverslips with attached MEFs were mounted in a Teflon chamber and incubated in DMEM with 4 μM fura-5F/AM at 37 $^{\circ}\text{C}$ in the dark for 25 min. Before $[\text{Ca}^{2+}]_i$ measurements were made, cells were washed 3 times and incubated for 10–15 min at room temperature (25 $^{\circ}\text{C}$) in a HEPES-buffered salt solution (HBSS, in mM: NaCl 120; KCl 5.4; MgCl_2 0.8; HEPES 20; CaCl_2 1.8 and glucose 10 mM, with pH 7.4 adjusted

by NaOH). In these experiments, nominally Ca^{2+} -free solutions were HBSS with no added CaCl_2 .

Coverslips with attached MEFs were mounted onto a Nikon TS-100 inverted microscope equipped with a 20 $\times$ fluor objective (0.75 NA) and fluorescence images recorded and analyzed with a digital fluorescence imaging system (InCyt Im2, Intracellular Imaging Inc., Cincinnati, OH) equipped with a light-sensitive CCD camera (Cooke PixelFly, ASI, Eugene, OR). Fura-5F fluorescence was monitored by alternately exciting the dye at 340 and 380 nm, and collecting the emission at 510 nm. Changes in intracellular calcium are represented as the ratio of fura-5F fluorescence due to excitation at 340 nm to that due to excitation at 380 nm (Ratio = F340/F380). We utilized the lower affinity indicator fura-5F (K_d = 400 nM) to assure that the data are well below dye saturation; most of the ratios were <1.0, well below the average observed R_{max} of 5.6. The ratio changes in fields of fura-5F-loaded cells were collected from multiple regions of interest (ROI), with each ROI representing an individual cell. Typically, 40 to 50 ROIs were monitored per experiment. In all cases, ratio values were corrected for contributions of autofluorescence, which was measured after treating cells with 10 μM ionomycin and 20 mM MnCl_2 .

Assessment of expression of EGFP-tagged Orai1 forms in MEF cells

Orai1-EGFP expression in MEF cells was quantitated by recording cell fluorescence on a LSM 780 confocal microscope (Carl Zeiss, Jena, Germany) using an Plan-Apochromat 40 $\times$ /1.4 oilDIC M27 objective at a zoom of 1 $\times$. EGFP was excited with the 488-nm laser line. Fluorescence emission was detected using spectral imaging between 491 nm – 695 nm with a 9-nm bandwidth. Digital photon counting mode was used for detection, with pinhole set at 180 μm and 4 line averaging. Linear unmixing was used to deconvolute the fluorescence spectral overlap of Orai1-EGFP and autofluorescence. After elimination of autofluorescence, total GFP signal intensity was calculated in individual cells using the ImageJ “Particle Analysis” method.

Confocal imaging of puncta formation of Orai1 forms

Fluorescence time-lapse images were taken with a Zeiss (LSM710) confocal laser-scanning microscope using the 488-nm line of an argon laser for excitation and a 493 nm to 558 nm emission for EGFP, 561-nm line of a HeNe laser for excitation and 578 nm to 700 nm for emission for mCherry. The specimens were viewed at a zoom of 2.4 $\times$ using plan oil objectives (Plan-Apochromat 63 $\times$, numerical aperture 1.4, Oil DIC). Images were taken every 30 seconds for 20 minutes. Focal plane was maintained by using Perfect Focus System sampling before each time point. The puncta area of colocalization of Orai1 and STIM1 were calculated using Zen2012 (Zeiss Inc) and ImageJ (NIH) software. All data were analyzed using a laboratory-written macro (available upon request) of SigmaPlot (Systat Software, CA).

Patch clamp electrophysiology

Patch clamp electrophysiological recordings were carried out using an Axopatch 200B and Digidata 1440A (Molecular Devices) as previously published (7, 8). Pipettes were pulled from borosilicate glass capillaries (World Precision Instruments) with a P-1000 flaming/

brown micropipette puller (*Sutter Instrument Company*) and polished using DMF1000 (*World Precision Instruments*). Resistances of filled pipettes were 2–4 M Ω . The liquid-junction potential offset due to different internal and external saline composition was around –4.5 mV and was corrected. Series resistances were between 5 ~ 10 M Ω . Under whole-cell configuration, only cells with tight seals (>13 G Ω) were selected to perform recordings. Cells were maintained at a +30 mV holding potential during experiments. Clampfit 10.1 software was used for data analysis.

Store depletion-activated CRAC currents (MEFs)

Bath solution—115 mM Na-methanesulfonate, 10 mM CsCl, 1.2 mM MgSO₄, 10 mM HEPES, 20 mM CaCl₂, and 10 mM glucose (pH=7.4 with NaOH).

Pipette solution—115 mM Cs-methanesulfonate, 20 mM Cs-BAPTA, 8 mM MgCl₂, and 10 mM HEPES (pH=7.2 with CsOH).

DVF solution—155 mM Na-methanesulfonate, 10 mM HEDTA, 1 mM EDTA, and 10 mM HEPES. (pH=7.4 with NaOH).

Protocol—Reverse voltage ramps from +100 to –140 mV lasting 250 ms every 2 s.

I_{crac} and I_{soc} in HEK293 Cells

The solutions and recording protocol were those described by (3).

Bath solution—140 mM NaCl, 1.2 mM MgCl₂, 10 mM CaCl₂, 5 mM CsCl, 30 mM d-glucose, 10 mM HEPES (pH 7.4).

Pipette solution—145 mM cesium methanesulfonate, 20 mM BAPTA, 10 mM HEPES, 8 mM MgCl₂, IP₃ 25 μ M, pH to 7.2 with CsOH.

Protocol—Reverse voltage ramps from (–100 to +100 mV) of 250 ms were recorded every 2 s.

I_{ARC} currents in HEK293 cells

Bath solution—115 mM Na-methanesulfonate, 10 mM CsCl, 1.2 mM MgSO₄, 10 mM HEPES, 20 mM CaCl₂, and 10 mM glucose (pH=7.4 with NaOH). 8 μ M AA was added to the bath where indicated in figures.

Pipette solution—115 mM Cs-methanesulfonate, 10 mM Cs-BAPTA, 5 mM CaCl₂, 8 mM MgCl₂, and 10 mM HEPES (pH=7.2 with CsOH). Calculated free Ca²⁺ was 150 nM using Maxchelator software (<http://maxchelator.stanford.edu/>).

DVF solution—155 mM Na-methanesulfonate, 10 mM HEDTA, 1 mM EDTA, and 10 mM HEPES. (pH=7.4 with NaOH).

Protocol—Reverse voltage ramps from +100 to –140 mV lasting 250 ms every 2 s.

CDI experiments

Bath solution—115 mM Na-methanesulfonate, 10 mM CsCl, 1.2 mM MgSO₄, 10 mM HEPES, 20 mM CaCl₂, and 10 mM glucose (pH=7.4 with NaOH).

Pipette solution 1—115 mM Cs-methanesulfonate, 20 mM Cs-BAPTA, 8 mM MgCl₂, and 10 mM HEPES (pH=7.2 with CsOH).

Pipette solution 2—135 mM Cs-methanesulfonate, 10 mM EGTA, 8 mM MgCl₂, and 10 mM HEPES (pH=7.2 with CsOH).

Recording method and protocols of voltage stimulation: After break-in, we ran a stimulus protocol consisting of families of 150-ms voltage steps (from +30 mV holding potential to −120 mV, −100 mV, −80 mV, and −60 mV). There is a 5-second interval between steps. The first recording was used for leak subtraction. Next, a 250-ms voltage ramp from +100 mV to −140 mV was administered every 2 seconds until CRAC currents reached a steady-state level (usually 150 s to 300 s). Once steady state for CRAC current activation was achieved, we performed a second voltage step protocol similar to the first one.

Statistics

All values are expressed as mean ± SEM. Statistical analyses comparing two or more experimental groups were performed using ANOVA followed by Tukey's test for differences between rows, columns, or cells as appropriate, or two-tail t-test, with Origin 8.1 software (*OriginLab*) or Graph Pad Prism 6.0. Throughout the figures *, ** and *** indicates P values < 0.05, 0.01 and 0.001, respectively. Differences were considered significant when P < 0.05.

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Fig. 1. Expression and rescue of Ca²⁺ entry in Orai1-KO MEFs by Orai1 constructs

A: Fluorescence intensity (arbitrary units) as an indicator of expression of Orai1-EGFP forms was measured and calculated as described in the Methods. Data are means $\pm$ SEM. ($n=421$ for KO + WT, 490 for KO + Orai1 α and 838 for KO + Orai1 β). WT and KO cells are negative controls, and give threshold fluorescence levels in absence of Orai1-EGFP. ($n=540$ for KO, 456 for WT). Fluorescence unit is an arbitrary value in 8bit scale. *** $P<0.001$, one-way ANOVA followed by Tukey's test. B: Traces showing realtime measurements of intracellular Ca²⁺ in MEFs. Thapsigargin was added as indicated initially in the absence of extracellular Ca²⁺, and then as indicated Ca²⁺ (3 mM) followed by Gd³⁺ (5 μ M) was added. C: Summary of the extent of thapsigargin-induced Ca²⁺ release, taken as the

peak Ca^{2+} (ratio) rise after thapsigargin addition minus baseline. D: Summary of the magnitude of thapsigargin-induced Ca^{2+} entry, taken as the peak Ca^{2+} (ratio) rise after restoration of extracellular Ca^{2+} minus baseline. * $p < 0.05$ vs WT; # $p < 0.05$ vs KO + WT and KO + Orai1 β .

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Fig. 2. Na⁺-carried I_{crac} in wild-type and Orai1-KO MEFs

A pipette solution containing 20 mM BAPTA was dialyzed through the patch pipette to activate CRAC currents. Representative time course of CRAC current recordings are shown.

A: CRAC currents amplified by DVF bath solution were measured in wild-type MEF cells.

B: Absence of CRAC currents in Orai1-KO MEF cells. C, D, E: CRAC currents in Orai1-KO MEF cells stably expressing the indicated Orai1 constructs. Below each time course

trace are CRAC sodium current I-V relationships, which are taken from traces in A-E where indicated by asterisks. F: Summary of Na⁺-carried CRAC currents from the five

different conditions. * $p < 0.05$ vs WT, XX test. N values represent the number of independently determined currents.

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Fig. 3. Calcium-dependent inactivation of I_{crac} in HEK293 cells expressing STIM1 and Orai1 α or Orai1 β

CRAC currents were recorded in HEK293 cells cotransfected with 1.0 μ g of plasmid coding for EYFP-STIM1 and plasmids coding for either 2.0 μ g of Orai1 α (A) or Orai1 β (B) (under TK promoter). As indicated, either 20 mM BAPTA or 10 mM EGTA was used in patch pipette. C and D: For quantification of CDI, extent of inactivation at 146 ms (R₁₄₆ ms), the residual current remaining at the end of the test pulse at 146 ms relative to the peak current at 3 ms, was plotted against test potential (as in (23)). N values represent the number of independent current measurements.

Fig. 4. Orai1 α and Orai1 β in I_{crac} and I_{soc} in HEK293 cells

HEK293 cells were transiently transfected with the indicated combinations of constructs. Successfully transfected cells were identified by the fluorescence from EYFP-STIM1. A–C: Whole cell recordings of CRAC (solid line from cells expressing STIM1 + Orai1 constructs) or SOC (dotted line from cells expressing STIM1 + Orai1 constructs + TRPC1) currents by dialysis of BAPTA (10 mM) and inositol trisphosphate (IP₃) (25 μ M) through the patch pipette. Note in A that the current measured in the STIM1 + TRPC1 cells is barely detectable. D. Summary data for current density at +100 mV and –100 mV in cells transfected with STIM1 and the indicated Orai1 constructs with (I_{soc}) or without (I_{crac}) TRPC1. * p < 0.05, two-way ANOVA followed by Tukey's test. N values are the number of independently determined currents.

Fig. 5. Effect of expression of STIM1 on I_{crac} in MEFs transfected with Orai1 α or Orai1 β constructs

A: CRAC currents in Orai1-KO MEF cells stably expressing the indicated Orai1 constructs and transiently expressing EYFP-STIM1. Below each time course trace are CRAC sodium current I–V relationships, which are taken from traces where indicated by asterisks. **B:** A summary of the Na⁺-carried inward currents, including currents from Figure 2 (without STIM1). Two-way ANOVA indicated highly significant increase with all three constructs in the presence of overexpressed STIM1 ($P < 0.001$). Post-hoc Tukey's test indicated that in

the presence of STIM1, both Orai1 α and Orai1 β have significantly larger currents than Orai1-WT ($P < 0.05$). N represents the number of independent current measurements.

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Fig. 6. Orai1 puncta formation in HEK293 cells in response to store depletion by activation of muscarinic acetylcholine receptors

Orai1-positive puncta over time were measured in HEK293 cells expressing either Orai1 α -EGFP and mCherry-STIM1 or Orai1 β -EGFP and mCherry-STIM1 and that were exposed to 0.5 mM carbachol. Puncta size was calculated by averaging the area of each individual puncta in the single cells at each time point of 30 sec. Puncta size was defined as the area of colocalization of Orai1 and STIM1. Maximal puncta size was set at 100%. 6 cells were analyzed in 6 independent experiments, means $\pm$ SEM.

Fig. 7. Rescue of I_{crac} by Orai1 α or Orai1 β following knockdown of Orai1 in HEK293 cells
 CRAC currents amplified in DVF bath solution were measured in HEK293 cells. In each case, Na⁺ current was obtained by brief substitution with the divalent-free (DVF) solution. The first substitution of DVF occurs before substantial store depletion and provides a measure of leak current which was subtracted by the current developing later. A: CRAC currents in HEK293 cells transfected with nontargeting control siRNA. B: The effect of Orai1 knockdown with siRNA on CRAC currents. C and D: Rescue of CRAC currents by Orai1 α or Orai1 β expression in Orai1-silenced cells. For each trace, sodium CRAC current

I–V relationships are shown below, which are taken from traces in A–D where indicated by asterisks. E: Summary of leak-subtracted Na⁺-carried CRAC currents. * $p < 0.05$ vs. siControl, ANOVA followed by Tukey's test, N values represent the number of independently determined current measurements.

Fig. 8. Rescue of I_{arc} by Orai1 α , but not Orai1 β , following knockdown of Orai1 in HEK293 cells
 Arachidonic acid (8 μM) was added to bath solution to activate ARC currents (15) in HEK293 cells. In each case, Na^+ current was obtained by brief substitution with the divalent-free (DVF) solution. The first substitution of DVF occurs before addition of arachidonic acid and provides a measure of leak current which was subtracted by the current developing later. A: ARC currents in HEK293 cells transfected with nontargeting control siRNA. B: The effect of Orai1 knockdown on ARC currents. C and D: Rescue of ARC currents by Orai1 α or Orai1 β expression in Orai1-silenced cells. Below each trace are Na^+ -

carried ARC current I–V relationships taken from traces in A – D where indicated by asterisks. E: Summary of Na⁺-carried ARC current from the four experimental conditions in A–D. * p < 0.05 vs. siControl; # NS compared to siOrai, ANOVA followed by Tukey's test, N values represent the number of independently determined current measurements. F: Summary of Ca²⁺-carried ARC currents in HEK293 cells from the four experimental conditions in Figure 8.

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