Voltage Gating at the Selectivity Filter of the Ca\(^{2+}\) Release-activated Ca\(^{2+}\) Channel Induced by Mutation of the Orai1 Protein*

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The Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channel is a plasma membrane (PM) channel that is uniquely activated when free Ca\(^{2+}\) level in the endoplasmic reticulum (ER) is substantially reduced. Several small interfering RNA screens identified two membrane proteins, Orai1 and STIM1, to be essential for the CRAC channel function. STIM1 appears to function in the PM membrane proteins, Orai1 and STIM1, to be essential for the depletion with Ca\(^{2+}\) recently, the molecular identity of the machinery linking store release-activated Ca\(^{2+}\) (CRAC) channel function. STIM1 appears to function in the PM and as the Ca\(^{2+}\) sensor in the ER. Orai1 is forming the pore of the CRAC channel. Despite the recent breakthroughs, a mechanistic understanding of the CRAC channel gating is still lacking. Here we reveal new insights on the structure-function relationship of STIM1 and Orai1. Our data suggest that the cytoplasmic coiled-coil region of STIM1 provides structural means for coupling of the ER membrane to the PM to activate the CRAC channel. We mutated two hydrophobic residues in this region to proline (L286P/L292P) to introduce a kink in the first \(\alpha\)-helix of the coiled-coil domain. This STIM1 mutant caused a dramatic inhibition of the CRAC channel gating compared with the wild type. Structure-function analysis of the Orai1 protein revealed the presence of intrinsic voltage gating of the CRAC channel. A mutation of Orai1 (V102I) close to the selectivity filter modified CRAC channel voltage sensitivity. Expression of the Orai1 V102I mutant resulted in slow voltage gating of the CRAC channel by negative potentials. The results revealed that the alteration of Val102 develops voltage gating in the CRAC channel. Our data strongly suggest the presence of a novel voltage gating mechanism at the selectivity filter of the CRAC channel.

The CRAC\(^{2+}\) channel is activated by depletion of the intracellular Ca\(^{2+}\) stores and mediates sustained Ca\(^{2+}\) entry in hematopoietic cells (1–3) and some other cell types (1, 3). Until recently, the molecular identity of the machinery linking store depletion with Ca\(^{2+}\) entry remained elusive. However, recent studies identified two proteins, STIM1 and Orai1, as being essential for CRAC function (4–11). Although STIM1 resides predominantly in the ER and the plasma membrane (PM) (11), Orai1 is almost exclusively expressed in the PM (12). STIM1 functions as the Ca\(^{2+}\) sensor in the ER that triggers the CRAC channel activation (5, 6, 11). However, its translocation to the PM upon activation (11, 13) and functional role in the PM have also been demonstrated (6, 14). Even though a report by Mignen et al. (14) revealed that PM-STIM1 regulates another Ca\(^{2+}\) channel (arachidonate-regulated Ca\(^{2+}\) channels), the same group later revealed that arachidonate-regulated Ca\(^{2+}\) channels have the same molecular identity as CRAC channels (Ref. 41; see also Ref. 15). STIM1 aggregates into puncta and translocates toward the PM, when the stores are depleted (5, 16). Physical incorporation of STIM1 molecules into the PM after Ca\(^{2+}\) store depletion has been clearly demonstrated (11, 13). The time course of the ER Ca\(^{2+}\) depletion followed by CRAC activation correlates with the time course of STIM1 translocation that is ~52 s (5, 17).

How STIM1 relays signals about ER Ca\(^{2+}\) levels from the ER to the PM has not been determined. STIM1 contains one transmembrane domain and a number of potential protein-interacting domains on its N and C termini. The luminal/extracellular N terminus contains a helix-rich sterile \(\alpha\)-motif in addition to an EF-hand Ca\(^{2+}\) sensor (6, 18). The cytoplasmic C terminus contains two prominent coiled-coil regions followed by a flexible proline-rich domain (6, 18). Based on the protein structure and several studies on STIM1, we hypothesize that two distinct types of interactions are likely important in the function of STIM1. First, homotypic interactions likely mediate the aggregation of the molecule into puncta after Ca\(^{2+}\) store depletion (19). Recent studies indicate that an N-terminal fragment of STIM1 that contains the sterile \(\alpha\)-motif and the EF-hand domains is able to undergo self-association upon removal of Ca\(^{2+}\) (19). These results suggest that the ER luminal N terminus of STIM1 mediates the punctal self-assembly of STIM1 when the ER Ca\(^{2+}\) is depleted. Second, the “punctal” STIM1, which lies within ~25 nm of the PM (5, 17), likely interacts directly with components in the PM, through its cytosolic domain. The latter can be achieved through homotypic interactions between the ER-bound STIM1 (STIM1-ER) and the PM-bound STIM1 (STIM1-PM). Earlier studies revealed that the coiled-coil region of STIM1 is indeed involved in homotypic interactions between STIM1 molecules (18). Alternatively STIM1-ER might
be interacting with other PM protein or directly with the pore-forming protein Orai1. We consider the later less likely, based on a recent study that showed a lack of detectable interaction between STIM1 and Orai1 (20). Furthermore, biochemical cross-linking of the ER with the PM revealed that the distance between the ER and the PM at the sites of STIM1 puncta is too large (11–14 nm) for direct interactions of STIM1-ER with the PM Orai1 (21). That, however, is not the case for putative STIM1-ER and STIM1-PM interactions; hence the first coiled-coil region of STIM1 is an approximately 8-nm-long α-helix containing 15 turns (6) and can provide the structural means for ER-PM interactions during CRAC activation. The possibility that this cytosolic segment of STIM1 is essential for bridging the ER with the PM to activate CRAC channel was explored in the present study. Our data reveal that the intact structure of the first coiled-coil domain of STIM1 protein is crucial for the CRAC channel activation.

Orai1 is an integral membrane protein that works in synergy with STIM1 to reconstitute CRAC channel function (8, 9). In resting cells, Orai1 is diffusely distributed in the PM, but following store depletion it migrates to areas in close proximity to the near-PM STIM1 puncta, where local Ca\(^{2+}\) entry occurs (22). Orai1 has four predicted transmembrane domains (TM), a topology similar to the KCNK two pore domains K\(^{+}\) channels (23). The putative TM1-TM2 extracellular loop of human Orai1 contains a number of acidic residues, and the first one, Glu\(^{106}\), is the most conserved across species. Several groups simultaneously identified Glu\(^{106}\) (Glu\(^{180}\) in *Drosophila*) as a residue that coordinates the Ca\(^{2+}\) binding to the selectivity filter of the CRAC channel (24, 25). These experiments were repeated by another group using our Orai1\(^{106D}\) construct (26). All these results provided unequivocal evidence that Orai1 is a pore-forming subunit of the CRAC channel. In the present study we further examined the function of this residue and adjacent residues by structure-function analysis. Uniquely, our study was further consistent with an intrinsic mechanism.

### Experimental Procedures

**Cells, Cell Culture, and Reagents**—HEK293 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1% penicillin/streptomycin. RBL cells were grown in the same medium but with heat-inactivated serum. ATP and G418 were from Sigma. Thapsigargin was from EMD Biosciences (San Diego, CA). Stealth siRNA duplexes for rat STIM1 and rat Orai1 were from Invitrogen. Enhanced yellow fluorescent protein (YFP) vector was from BD Biosciences (Mountain View, Ca).

**RNA Interference Design and RNA Interference/DNA Transfection**—All siRNA sequences were designed using Invitrogen Block-It software. A mixture of two siRNA duplex sequences exclusively targeting rat and not human STIM1 were used with start nucleotides 935 and 970. Two rat-specific siRNA targeting different regions of rat Orai1 and not human Orai1 were used for Orai1 knockdown with start nucleotides 488 and 837. Scrambled double-stranded RNA sequences were used as negative control. Transfections were performed by electroporation using the Gene Pulser II Electroporation system (Bio-Rad) at 350 V, 960 microfarads, and infinite resistance. Transfection with siRNA was followed by co-transfection with the indicated DNA expression constructs and YFP after 24 h. After 48 h the cells were selected by YFP fluorescence for recordings as previously described (6). In some cases the cells were transfected with one of two transfection reagents (FuGENE 6, Roche Applied Science; or Lipofectamine, Invitrogen) with 2–3 μg of DNA, ~24 h after plating in 35-mm dishes of 250,000 cells.

**DNA Expression Constructs and Mutagenesis**—Wild type human STIM1 (STIM1\(^{WT}\)) was subcloned into pIREsNeo (Clontech, Palo Alto, CA) as previously described (18, 32). Orai1, expressed in pCMV6-XL4, was obtained from Origene (Rockville, MD). YFP conjugated to the N terminus of STIM1 and STIM1\(^{D76A}\) constructs were gifts from Dr. Tobias Meyer. The human STIM1 and Orai1 mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by sequencing.

**Immunoblot Analysis of Protein Expression**—The cells were grown in 35-mm dishes and harvested 48 h after transfection as previously described (33). Protein concentrations were determined using a Bradford assay. The samples were retained on ice, mixed with prewarmed 2X loading buffer and immediately loaded onto the gel. Standard SDS-PAGE and transfer buffers were used for running the gel and for transferring the gel to a 0.45-μm pore nitrocellulose membrane (Schleicher & Schuell) at ~70 mAh. The membrane was blocked in 5% dry milk in washing buffer containing Tween (0.05%) and Tris-buffered saline and incubated overnight with primary anti-green fluorescent protein antibody at 1:3000 dilution (Sigma-Aldrich). The membrane was washed and incubated with a horseradish peroxidase-labeled secondary antibody and visualized with the

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3 M. Spassova, unpublished observations.
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enhanced chemiluminescence kit (LumiGlo, Upstate Cell Signaling Solutions, Lake Placid, NY).

Fluorescence Localization and Immunocytochemistry—To characterize the fluorescence distribution of the YFP-STIM1, YFP-STIM1D76A, and YFP-STIM1L286P/L292P proteins, the cells grown on coverslips were fixed 48 h after transfection, with or without 20 min stimulation by thapsigargin, using 4% paraformaldehyde in phosphate-buffered saline. Cell imaging was performed using a confocal microscope (Leica TCS-NT).

Electrophysiology—We used conventional whole cell recordings as described previously (6). The cells were transferred into the recording chamber on poly-L-lysine-coated coverslips prior to experiments and allowed to settle. Immediately after establishing the whole cell configuration, linear voltage ramps of 50-ms duration spanning the voltage range of −100 to +100 mV were delivered from a holding potential of 0 mV at a rate of 0.5 Hz, in some cases preceded by a prestep to various voltages as described in the text. The currents were filtered at 6 kHz and sampled at 10–50-μs intervals. We used automatic capacitive and series resistance compensation of the EPC-10 amplifier (HEKA). PatchMaster, Pulsetools, FitMaster, and Origin software were used for acquisition and analysis. The temporal development of inward (at −80 mV) and outward (at +80 mV) currents were measured from the individual ramps. The intra- 

RESULTS AND DISCUSSION

Introduction of a Kink in the First STIM1 Coiled-coil α-Helix Prevents the CRAC Channel Activation—The STIM1 protein is a Ca2+ sensor in the ER that transmits information to the PM to activate the CRAC channels when ER Ca2+ is depleted (5, 6, 11). Following store depletion, STIM1 aggregates into “punctal” regions of ER that lie within ~25 nm of the PM (17, 22). Interaction with the PM proteins is likely through the large C-terminal cytoplasmic domain of STIM1, which includes two extended coiled-coil regions and a less structured proline-rich region (6). We previously demonstrated that deletion of the proline-rich region of STIM1 still permits CRAC channel activation (6). Here we address the role of the coiled-coil region of STIM1, in coupling of the ER with the PM to activate the CRAC channel. We expressed human STIM1WT and mutants thereof on a rat STIM1 knockdown background in RBL cells, as we undertook previously (6). We introduced a kink in the first, unusually long coiled-coil region of STIM1 by substituting two close leucine residues with prolines to perturb the α-helical structure. Expression of the single STIM1L292P mutation did not result in significant change in CRAC channel amplitudes compared with the STIM1WT-expressing cells. However, expression of the double mutant STIM1L286P/L292P dramatically reduced the amplitude of CRAC, measured at its maximum activation in DVF (Fig. 1A). On average the current was decreased by 85% in cells expressing the mutated STIM1 compared with the STIM1WT (Fig. 1A, right panel). We also compared the ability of STIM1WT and STIM1L286P/L292P to increase CRAC-mediated Ca2+ entry in HEK293 cells using intracellular Ca2+ imaging (Fig. 1B). Co-overexpression of STIM1 and Orai1 resulted in a large increase of CRAC-mediated Ca2+ entry (Fig. 1B). However, when STIM1L286P/L292P was co-expressed with Orai1, the resulting CRAC-mediated Ca2+ entry was not higher than the background level (Fig. 1B). In fact, on average, the resulting Ca2+ elevation with co-expression of STIM1D76A/E87A and Orai1X mutants on a combined knockdown background of rOrai1 and rSTIM1, we refer to it as “Orai1X-CRAC channel” activity. The cells with transient expression of STIM1D76A/E87A when co-expressed with Orai1 or its mutants were healthy for recordings within 48 h after the transfection.

Ca2+ Measurement—HEK cells grown on coverslips were placed in “cation safe” medium free of sulfate and phosphate anions (107 mM NaCl, 7.2 mM KCl, 1.2 mM MgCl2, 11.5 mM glucose, 20 mM HEPES-NaOH, pH 7.2) and loaded with fura-2/acetoxymethylester (2 μM) for 30 min at 20 °C. The cells were washed, and dye was allowed to de-esterify for a minimum of 30 min at 20 °C. The cells on coverslips were placed in cation safe medium in the absence or presence of 1 mM CaCl2. Ca2+ measurements were performed using an InCyt dual wavelength fluorescence imaging system (Intracellular Imaging Inc.). Fluorescence emission at 505 nm was monitored with excitation at 340 and 380 nm; intracellular Ca2+ measurements are shown as 340/380-nm ratios. Ca2+ entry through CRAC channel was induced by incubation with 2 μM thapsigargin. All traces are averages from multiple cells and are representative of at least three separate experiments.
Constitutively active YFP-STIM1 D76A was distributed in RBL cells (measured in RBL cells expressing STIM1WT or STIM1L286P/L292P on a rSTIM1 knockdown background. The_cur-expressing mutated STIM1L286P/L292P had protein levels similar to the wild type as detected by Western blot (Fig. 1). These results indicated that the STIM1L286P/L292P mutant is able to aggregate into puncta but is likely defective in the conformational coupling that allows successful gating of the CRAC channel (Fig. 2).

Analysis of the CRAC Channel Selectivity by Co-expression of Orai1 Mutants with a Constitutively Active STIM1 Mutant—The recently discovered Orai1 protein is a pore-forming protein of the CRAC channel (24, 25, 36). These results were later confirmed by Vig et al. (26) using our Orai1/E106D construct. Orai1 has predicted topology similar to the KCNK class of K+ channel proteins (23), with four TMs, one extracellular loop between the TM1 and TM2, and a second one between TM3 and TM4. We examined the function of Orai1 mutants that affect the CRAC channel pore properties using RBL cells. In these cells the endogenous rat STIM1 was knocked down by rat-specific RNA interference, and the constitutively active sterile α-motif domain region (19), whereas the interactions of ER-STIM1 with the PM proteins are structurally supported by the coiled-coil region of STIM1. Such interactions likely involve homomultimerization of ER-STIM1 with the PM-STIM1 (Fig. 2). That is consistent with an earlier study showing that the coiled-coil region of STIM1 plays a role in STIM1 homomultimerization (18) and with the lack of direct physical interactions between STIM1 and Orai1 (20, 21). The study by Varnai et al. (21) hypothesized the presence of intermediate players that connect the ER-STIM1 with the PM-Orai1 to open CRAC channel. That hypothesis is based on the finding that the distance between the ER and the PM at the sites of STIM1 puncta is ~11–14 nm, too large for direct interaction between ER-STIM1 and PM-Orai1. However, interactions between the ER-STIM1 and PM-STIM1 can span distance larger than 11 nm, based on the ~8-nm length of the first coiled-coil domain (based on its predicted α-helical structure with 15 turns (6)). Overall our results reveal that the long coiled-coil region of STIM1 is necessary for successful gating of the CRAC channel.

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FIGURE 1. The STIM1 coiled-coil domain is essential for CRAC channel activation. A, CRAC channels were measured in RBL cells expressing STIM1WT or STIM1L286P/L292P on a rSTIM1 knockdown background. The current-voltage relationship of CRAC channel was recorded at the time of maximal activation in DVF (left panel). Average currents at +100 mV reveal significant reduction of the CRAC currents in STIM1L286P/L292P expressing RBL cells (n = 5) compared with STIM1WT expressing cells (n = 4) (right panel). HEK cells co-expressing STIM1 or STIM1L286P/L292P together with Orai1 were pretreated with thapsigargin (Tg, 2 μM) in the absence of extracellular Ca2+ (10 min) to deplete Ca2+ stores. Control cells were transfected with empty vector. 1 mM Ca2+ was added at the arrow to assess the store operated channel. A summary of the experiments is presented on the right with a Western blot of empty vector, STIM1, and STIM1L286P/L292P overexpressed in HEK cells (right panel, bottom). The STIM1-Orai1 co-expressing HEK cells had significantly elevated store operated channel, whereas STIM1L286P/L292P-Orai1 co-expressing cells had the store operated channel at the background level. C, top panel, Western blot of YFP-STIM1, YFP-STIM1L286P/L292P, and YFP-STIM1D76A overexpressed in HEK cells; bottom panel, distribution of the YFP-STIM1, YFP-STIM1L286P/L292P, and YFP-STIM1D76A proteins in HEK cells before (left column) and after (right column) store depletion by 2 μM thapsigargin.

FIGURE 2. Model of STIM1 mediated ER-PM interactions via its coiled-coil domain. ER-STIM1 forms oligomers after store depletion that relocalize close to the PM. A, ER-STIM1 oligomers relocalized close to the PM interact with PM-STIM1 and/or Orai1 protein to activate CRAC channel. B, the interaction between the ER and the PM is abolished by the kink introduced in the coiled-coil domain of STIM1 by L286P/L292P mutation.
examined the reconstitution of CRAC channel function by depletion. We used cells expressing STIM1D76A/E87A on rSTIM1 knockdown background (6). Ca\textsuperscript{2+} specific problems associated with the harsh conditions of cells ethoxydiphenyl borate (2-APB) (14942 JOURNAL OF BIOLOGICAL CHEMISTRY) expressed alone in the rSTIM1 knockdown background cells is expressed. The mutant hSTIM1D76A/E87A EF-hand mutant was expressed. The mutant and numbered. FIGURE 3. Voltage Gating of the CRAC Channel. hSTIM1D76A/E87A EF-hand mutant was expressed. The mutant causes constitutive CRAC channel activity obviating the non-specific problems associated with the harsh conditions of cells Ca\textsuperscript{2+} store-emptying. When co-expressed, STIM1 and Orai1 are known to work in synergy to massively amplify CRAC channel function (8–10); thus we used this system to examine structure-function parameters of the Orai1 protein with precision. We initially focused on the domains within the Orai1 molecule that appear to form the functional CRAC channel pore. The first extracellular loop of Orai1 has a number of acidic residues, reminiscent of the pore-forming loop of Ca\textsuperscript{2+} channels known to have carboxylate-rich selectivity filters. Alignment of the first extracellular loop of Orai1 with Drosophila Orai and with Orai1 orthologs in other species is shown in Fig. 3A. The most conserved acidic residue, Glu\textsuperscript{106}, coordinates Ca\textsuperscript{2+} binding at the selectivity filter. We introduced conserved mutations at and close to this position that were likely to influence the permeability properties of the channel. To knockdown endogenous rat Orai1 we used rat-specific siRNA, as previously described (6), and examined human Orai1 and mutants thereof on this knockdown background. Application of a mixture of two siRNAs targeted to different regions of Orai1 resulted in 98% reduction of the endogenous CRAC current (Fig. 3B). Thus, we examined the reconstitution of CRAC channel function by human STIM1\textsuperscript{D76A/E87A} and Orai1 or its mutants. The cells with combined knockdown of STIM1 and Orai1 did not have measurable CRAC current. Characterization of STIM1\textsuperscript{D76A/E87A} expressed alone in the rSTIM1 knockdown background cells is shown in Fig. 3 (C–E). The resulting CRAC channel was constitutively active with highly positive reversal potentials in 20 mM Ca\textsuperscript{2+}. The most conserved residues are labeled with stars. The residues mutated in this study are labeled with a black bar and numbered. B, transfection with mixture of two siRNAs targeting rat Orai1 resulted in 98% reduction of the CRAC channel activity measured at \(-100\) mV in 20 mM Ca\textsuperscript{2+} (\(n = 3\)) compared with the cells transfected with control scrambled siRNA and YFP (\(n = 3\)). C–E, characterization of the CRAC channels in cells expressing the constitutively active STIM1\textsuperscript{D76A/E87A} mutant used with dif-

DVF (Fig. 3D) and DVF (Fig. 3E), demonstrating preserved Ca\textsuperscript{2+} and monovalent ions selectivity. The current was rapidly inhibited by 5 \(\mu\text{M}\) 2-aminoethoxydiphenyl borate (Fig. 3C), similar to our previous report on STIM1\textsuperscript{E87A} expression-induced CRAC channel activity (6). The selectivity for Cs\textsuperscript{+} versus Na\textsuperscript{+} was \(-0.1\) calculated from the reversal potential (Fig. 3E).

With STIM1 and Orai1 co-expression on a knockdown background, the CRAC current levels were increased up to 60 pA/pF in 10 mM Ca\textsuperscript{2+} (Fig. 4, A–C), 900-fold higher than the knockdown background (Fig. 3B). The initial level of the constitutively active current was further increased by perfusing the cell cytosol with 10 mM BAPTA (Fig. 4, A and B). This partial initial inactivation of the current at the time of break-in, later recovered by BAPTA perfusion, is likely because of saturation of the endogenous Ca\textsuperscript{2+} buffers and CRAC inactivation by elevated cytosolic Ca\textsuperscript{2+} (37). To measure the fully activated CRAC current, we perfused the cell cytosol after break-in for at least 2 min with 10 mM BAPTA to allow diffusion of BAPTA into the cell before taking any statistical measurements. The current through Orai\textsuperscript{WT}-CRAC was decreased in proportion to the Ca\textsuperscript{2+} concentration, when extracellular Ca\textsuperscript{2+} was decreased from 10 to 1 mM (Fig. 4B). The current was highly Ca\textsuperscript{2+}-selective, with a reversal potential above 50 mV, and was not affected by replacement of extracellular Na\textsuperscript{+} with nonpermeant NMDG\textsuperscript{+} (38) (Fig. 4B, see also Fig. 6C). The I/V curves in 10 mM Ca\textsuperscript{2+} and DVF (Fig. 4C) were identical to endogenous CRAC current, with a reversal potential in DVF of \(-50\) mV.

Using the knockdown background of Orai1 and STIM1, we further re-examined the properties of the E106D mutant under different ionic conditions (Fig. 4, D–F). The Orai1\textsuperscript{E106D}-CRAC current was outwardly rectifying and constitutively active in 10 mM external Ca\textsuperscript{2+} (Fig. 4, D–F). However, the inward current was reduced by more than 50% when Na\textsuperscript{+} solution was replaced by NMDG\textsuperscript{+} (Fig. 4, D and E), indicating that it was carried largely by Na\textsuperscript{+}. Similar substitution did not have any effect on the Orai\textsuperscript{WT}-CRAC activity (Fig. 4C, see also Fig. 6C), demonstrating that similar to the endogenous CRAC, the constitutively active Orai\textsuperscript{WT}-CRAC current is highly selective for Ca\textsuperscript{2+}, the major charge carrier in high extracellular Ca\textsuperscript{2+}. The negative reversal potential of approximately \(-50\) mV measured from the I/V relationship in 10 mM Ca\textsuperscript{2+}-NMDG\textsuperscript{+} further revealed that the Orai\textsuperscript{E106D}-CRAC channel is not selective for Ca\textsuperscript{2+} versus Cs\textsuperscript{+} (Fig. 4E). Furthermore, when the external solution was switched to DVF (Fig. 4, D–F), the Orai1\textsuperscript{E106D}-CRAC current exhibited a reversal potential of 5 \(\pm\) 1 mV on

FIGURE 3. Functional characterization of the constitutively active STIM1\textsuperscript{D76A/E87A} mutant used with different Orai1 mutants. A, alignment of the first extracellular loop (gray) of Orai1 generated using Clustal W. The
affected by replacement of Na

mutants of Orai1 were co-expressed with the STIM1D76A/E87A mutant in RBL

activation at

cells and the CRAC current amplitude was measured at the time of maximal

activation. The observed activation is likely due to perfusion with 10 mM BAPTA (see text). A, time course of the constitutively active CRAC current after

break-in. The observed activation was slightly inhibited after a hyperpolarizing prestep to

−100 mV, demonstrating inhibition of the Na

current by reduction of Ca2+

versus Na+

and selectivity ratio increased from ~0.1 for Orai1WT CRAC to ~0.8 for the Orai1E106D, CRAC channel. The selectivity of Ca2+

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was also drastically reduced, suggesting that the E106D residue coordinates the binding of both monovalent and divalent ions to the selectivity filter. These results agree well with those published after completion of this work (24,

25) and further establish that the Orai1E106D-CRAC channel is non-

selective for Ca2+ versus Cs+. (25, 26). We present these results here to
demonstrate that the nonselective mutant Orai1E106D-CRAC has pre-

erved inward rectification in DVF, suggesting that the channel has an intrinsic voltage gating mechanism. This conclusion is supported by the data on another nonselective mutant, Orai1E190Q-CRAC (26).

E190Q-CRAC has similar to

Orai1E106D, CRAC reversal potential and outward current amplitude in DVF but much smaller inward current amplitude and thus lacks the inward rectifying properties (26).

We characterized the Orai1 protein function, when residues close to the E106D residue were mutated to further examine the voltage gating properties of the CRAC channel. CRAC currents in 10 mM Ca2+ in cells expressing the V105I, V102I, E106D, and E106A mutants of Orai1 co-expressed with the STIM1D76A/E87A mutant were measured at −100 mV and summarized in Fig. 5A. Expression of E106A resulted in a complete absence of current above the background level (dead pore mutant) and expression of the E106D, V105I, and V102I mutants revealed drastic reduction of CRAC current in 10 mM Ca2+.

Single Point Mutation of Orai1, V102I, Close to the Selectivity Filter Induces Slow Voltage Gating of the CRAC Channel—We examined in more detail the Orai1 function using the mutated V102I at the end of the first putative transmembrane domain (TM1) of Orai1 (Fig. 6). On average Orai1V102I-CRAC was constitutively active, with severely reduced whole cell Ca2+ conductance after equilibration in high Ca2+ (Fig. 5). However, atypical potentiation of the Orai1V102I-CRAC current by negative voltages was present, and we studied this phenomenon more thoroughly. The control Orai1WT-CRAC current was slightly inhibited after a hyperpolarizing prestep to −100 mV, likely because of fast Ca2+ inactivation. Fig. 6B compares the

average (n = 3), revealing low selectivity of Na+ versus Cs+ compared with Orai1WT-CRAC. Further characterization of the Orai1E106D-CRAC channel revealed that the inward current

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FIGURE 4. Mutation E106D in the first extracellular loop of Orai1 changes the CRAC channel selectivity. A–C, characterization of the CRAC current in cells co-expressing Orai1WT and STIM1D76A/E87A on combined rSTIM1 and rOrai1 knockdown background. A, time course of the constitutively active CRAC current after

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Orai1E106D, CRAC reversal potential and outward current amplitude in DVF but much smaller inward current amplitude and thus lacks the inward rectifying properties (26).

We characterized the Orai1 protein function, when residues close to the E106D residue were mutated to further examine the voltage gating properties of the CRAC channel. CRAC currents in 10 mM Ca2+ in cells expressing the V105I, V102I, E106D, and E106A mutants of Orai1 co-expressed with the STIM1D76A/E87A mutant were measured at −100 mV and summarized in Fig. 5A. Expression of E106A resulted in a complete absence of current above the background level (dead pore mutant) and expression of the E106D, V105I, and V102I mutants revealed drastic reduction of CRAC current in 10 mM Ca2+.

Single Point Mutation of Orai1, V102I, Close to the Selectivity Filter Induces Slow Voltage Gating of the CRAC Channel—We examined in more detail the Orai1 function using the mutated V102I at the end of the first putative transmembrane domain (TM1) of Orai1 (Fig. 6). On average Orai1V102I-CRAC was constitutively active, with severely reduced whole cell Ca2+ conductance after equilibration in high Ca2+ (Fig. 5). However, atypical potentiation of the Orai1V102I-CRAC current by negative voltages was present, and we studied this phenomenon more thoroughly. The control Orai1WT-CRAC current was slightly inhibited after a hyperpolarizing prestep to −100 mV, likely because of fast Ca2+ inactivation. Fig. 6B compares the

average (n = 3), revealing low selectivity of Na+ versus Cs+ compared with Orai1WT-CRAC. Further characterization of the Orai1E106D-CRAC channel revealed that the inward current
CRAC I/V after a prestep to $-100$ mV to the control CRAC I/V, where the cell was held at 0 mV before the voltage ramp. All of the recordings were done after equilibration in 10 mM extracellular Ca$^{2+}$ to avoid effects of Ca$^{2+}$-dependent potentiation. The control Orai1 WT-CRAC I/V curves in Fig. 6B were recorded immediately after the voltage presteps shown in Fig. 6A. Replacement of Na$^+$ with NMDG$^-$ did not have any effect on the current (Fig. 6C). However, with Orai1 V102I expression, a prestep to $-100$ mV increased CRAC current $\sim3$-fold with a time constant of $\approx200$ ms (Fig. 6D). The amount of the inward rectification of Orai1 WT-CRAC correlated with the amplitude of the negative voltage prestep (Fig. 6E). Similar voltage potentiation of Orai1 V102I-CRAC was observed in DVF (Fig. 6F), revealing that it is a phenomenon independent from the extracellular ion composition. Consistently, when extracellular Ca$^{2+}$ was elevated to $30$ mM, the voltage potentiation was preserved (not shown). Furthermore, the voltage dependence of the Orai1 V102I-CRAC current was steeper compared with the control Orai1 WT-CRAC current-voltage dependence after holding at 0 mV (Fig. 6E, inset). That further confirmed increased voltage dependence of the Orai1 V102I-CRAC channel. The potentiated portion of the current in 10 mM Ca$^{2+}$ (Fig. 6G) had inwardly rectifying properties, consistent with the notion that the potentiated current has similar properties to CRAC channel current. The current amplitude of Orai1 V102I-CRAC in 10 mM Ca$^{2+}$ was not affected by replacement of Na$^+$ with NMDG$^-$ (Fig. 6H), revealing that the Ca$^{2+}$ selectivity of the Orai1 V102I-CRAC channel is preserved and is similar to the Orai1 WT-CRAC current. It is important to note that the reduced conductance of Orai1 V102I-CRAC was not a result of lower expression or reduced plasma membrane localization of the Orai1 V102I proteins, because the potentiated current revealed maximal whole cell currents similar to Orai1 WT-CRAC (Fig. 6I). We hypothesize the presence of a gating charge at the selectivity filter of the CRAC channel. A similar phenomenon is observed in the KcsA K$^+$ channel, where the reorientation of the Glu71 residue in the electric field induces destabilization of the inactivated conformation of the channel pore (39, 40). Our results, however, suggest that the gating charge assumes a position that stabilizes the open channel conformation (destabilizes the closed conformation) at negative voltages. We further propose that in the Orai1 V102I-CRAC channel, the gating charge movement has a much slower component, compared with the WT-CRAC. The data reveal that the Val102 residue likely interacts with the gating charge, and the V102I mutation of this residue alters the gating charge movement.

Overall, we present strong evidence that the STIM1 coiled-coil domain plays a structural role in CRAC channel activation, probably by bridging important protein-protein interactions between the ER and the PM. The unusually long coiled-coil domain of STIM1 likely provides the structural features needed for connection between the two membranes to transfer the signal from the ER to the PM that activates CRAC channel. A likely candidate for such interactions is PM-STIM1 (6, 11, 13, 14). However, direct interactions with Orai1 are also possible. Furthermore, our results suggest that the Glu106 residue of Orai1 coordinates both Ca$^{2+}$ and monovalent ions binding to the selectivity filter of the CRAC channel pore and is important for the channel selectivity. The nonselective Orai1 E106D-CRAC channel revealed preserved inward rectification in DVF, suggesting intrinsic voltage gating in CRAC channels. Another mutation of Orai1, V102I, close to the selectivity site, modified the CRAC channel voltage dependence. The Orai1 V102I-CRAC channel whole cell current was potentiated by negative voltages in a time-dependent manner. Overall the results demonstrate...
intrinsic voltage gating of CRAC channel with a voltage gating sensor at the selectivity filter. The CRAC channel voltage gating switch is coordinated by the Val102 residue in the first transmembrane domain of the protein. This last result reveals a mechanism of voltage gating in channels that lack the classical voltage sensor and is fundamentally important for understanding the phenomenon of voltage gating.

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REFERENCES

1. Parekh, A. B., and Penner, R. (1997) Physiol. Rev. 77, 901–930
2. Prakriya, M., and Lewis, R. S. (2003) Cell Calcium 33, 311–321
3. Parekh, A. B., and Putney, J. W., Jr. (2005) Physiol. Rev. 85, 757–810
4. Roos, J., DiGregorio, P. I., Yeromin, A. V., Ohlsen, K., Lioudyno, M., Zhang, S., Safrina, O., Kozak, J. A., Wagner, S. L., Cahalan, M. D., Velicelebi, G., and Stauderman, K. A. (2005) J. Cell Biol. 169, 435–445
5. Liou, J., Kim, M. L., Heo, W. D., Jones, J. T., Myers, J. W., Ferrell, J. E., and Meyer, T. (2007) J. Biol. Chem. 282, 20661–20665
6. Spassova, M. A., Soboloff, J., He, L. P., Xu, W., Dziadek, M. A., and Gill, D. L. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 4040–4045
7. Pesce, S., Gwack, Y., Prakriya, M., Srikanth, S., Puppel, S. H., Tanasa, B., Hogan, P. G., Lewis, R. S., Daly, M., and Rao, A. (2006) Nature 441, 179–185
8. Peinelt, C., Vig, M., Koomoa, D. L., Beck, A., Nadler, M. J., Kohlan-Huberson, M., Lis, A., Fleig, A., Penner, R., and Kinet, J. P. (2006) Nat. Cell Biol. 8, 771–773
9. Soboloff, J., Spassova, M. A., Tang, X. D., Hewavitharana, T., Xu, W., and Gill, D. L. (2006) J. Biol. Chem. 281, 20661–20665
10. Zhang, S. L., Yeromin, A. V., Zhang, Y. H., Yu, Y., Safrina, O., Penna, A., Roos, J., Stauderman, K. A., and Cahalan, M. D. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 9357–9362
11. Zhang, S. L., Yu, Y., Roos, J., Kozak, J. A., Deerinck, T. I., Ellisman, M. H., Stauderman, K. A., and Cahalan, M. D. (2005) Nature 437, 902–905
12. Vig, M., Peinelt, C., Beck, A., Koomoa, D. L., Rabah, D., Kohlan-Huberson, M., Kraft, S., Turner, H., Fleig, A., Penner, R., and Kinet, J. P. (2006) Science 312, 1220–1223
13. Hauser, C. T., and Tsien, R. Y. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 3693–3697
14. Mignen, O., Thompson, J. L., and Shuttleworth, T. J. (2007) J. Physiol. 579, 703–715
15. Lis, A., Peinelt, C., Beck, A., Parvez, S., Monteilh-Zoller, M., Fleig, A., and Penner, R. (2007) Curr. Biol. 17, 794–800
16. Liou, J., Fivaz, M., Inoue, T., and Meyer, T. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 9301–9306
17. Wu, M. M., Buchanan, J., Luik, R. M., and Lewis, R. S. (2006) J. Cell Biol. 174, 803–813
18. Williams, R. T., Senior, P. V., Van Stekelenburg, L., Layton, J. E., Smith, P. J., and Dziadek, M. A. (2002) Biochim. Biophys. Acta 1596, 131–137
19. Stathopulos, P. B., Li, G. Y., Plevin, M. J., Ames, J. B., and Ikura, M. (2006) J. Biol. Chem. 281, 35855–35862
20. Gwack, Y., Srikanth, S., Feske, S., Cruz-Guilloty, F., Oh-Hora, M., Neems, D. S., Hogan, P. G., and Rao, A. (2007) J. Biol. Chem. 282, 16232–16243
21. Varnai, P., Toth, B., Toth, D. J., Hunyady, L., and Balla, T. (2007) J. Biol. Chem. 282, 29678–29690
22. Luik, R. M., Wu, M. M., Buchanan, J., and Lewis, R. S. (2006) J. Cell Biol. 174, 815–825
23. Talley, E. M., Sirois, J. E., Lei, Q., and Bayliss, D. A. (2003) Neuron 39, 46–56
24. Yeromin, A. V., Zhang, S. L., Jiang, W., Yu, Y., Safrina, O., and Cahalan, M. D. (2006) Nature 443, 226–229
25. Prakriya, M., Feske, S., Gwack, Y., Srikanth, S., Rao, A., and Hogan, P. G. (2006) Nature 443, 230–233
26. Vig, M., Beck, A., Billingsley, J. M., Lis, A., Parvez, S., Peinelt, C., Koomoa, D. L., Soboloff, J., Gill, D. L., Fleig, A., Kinet, J. P., and Penner, R. (2006) Curr. Biol. 16, 2073–2079
27. Guo, D., and Lu, Z. (2000) J. Gen. Physiol. 116, 561–568
28. Spassova, M., and Lu, Z. (1998) J. Gen. Physiol. 112, 211–221
29. Lu, Z., and MakiNisson, R. (1994) Nature 371, 243–246
30. Bakowski, D., and Parekh, A. B. (2000) J. Physiol. 529, 295–306
31. Hermosura, M. C., Monteilh-Zoller, M. K., Scharenberg, A. M., Penner, R., and Fleig, A. (2002) J. Physiol. 539, 445–458
32. Williams, R. T., Manji, S. S., Hancock, M. S., Van Stekelenburg, L., Eid, J. P., Senior, P. V., Kazenwadel, J. S., Shandala, T., Saint, R., Smith, P. I., and Dziadek, M. A. (2001) Biochem. J. 357, 673–685
33. Patel, K. A., Bartoli, K. M., Fandino, R. A., Ngatchou, A. N., Woch, G., Carey, J., and Tanaka, J. C. (2005) Investig. Ophthalmol. Vis. Sci. 46, 2282–2290
34. Prakriya, M., and Lewis, R. S. (2002) J. Gen. Physiol. 119, 487–508
35. Neher, E. (1992) Methods Enzymol. 207, 123–131
36. Yeromin, A. V., Roos, J., Stauderman, K. A., and Cahalan, M. D. (2004) J. Gen. Physiol. 123, 167–182
37. Zweifach, A., and Lewis, R. S. (1995) J. Gen. Physiol. 105, 209–226
38. Bakowski, D., and Parekh, A. B. (2002) Pflugers Arch. Eur. J. Physiol. 443, 892–902
39. Cordero-Morales, J. F., Cuello, L. G., and Perozo, E. (2006) Nat. Struct. Mol. Biol. 13, 319–322
40. Cordero-Morales, J. F., Cuello, L. G., Zhao, Y., Jorgina, V., Cortes, D. M., Roux, B., and Perozo, E. (2006) Nat. Struct. Mol. Biol. 13, 311–318
41. Mignen, O., Thompson, J. L., and Shuttleworth, T. J. (2008) J. Physiol. (Lond.) 586, 185–195