The Third Transmembrane Segment of Orai1 Protein Modulates Ca$^{2+}$ Release-activated Ca$^{2+}$ (CRAC) Channel Gating and Permeation Properties

Received for publication, May 27, 2011, and in revised form, July 19, 2011. Published, JBC Papers in Press, August 24, 2011, DOI 10.1074/jbc.M111.265884

Sonal Srikanth, Ma-Khin Win Yee, You sang Gwack¹, and Bernard Ribbalet²

From the Department of Physiology, University of California, Los Angeles, California 90095

Orai1, the pore subunit of Ca$^{2+}$ release-activated Ca$^{2+}$ channels, has four transmembrane segments (TMs). The first segment, TM1, lines the pore and plays an important role in channel activation and ion permeation. TMIII, on the other hand, does not line the pore but still regulates channel gating and permeation properties. To understand the role of TMIII, we have mutated and characterized several residues in this domain. Mutation of Trp-176 to Cys (W176C) and Gly-183 to Ala (G183A) had dramatic effects. Unlike wild-type channels, which exhibit little outward current and are activated by STIM1, W176C mutant channels exhibited a large outward current at positive potentials and were constitutively active in the absence of STIM1. G183A mutant channels also exhibited substantial outward currents but were active only in the presence of 2-aminoethyldiphenyl borate (2-APB), irrespective of STIM1. With outward currents but were active only in the presence of 2-aminophenylborate (2-APB), irrespective of STIM1. With G183A mutant channels also exhibited substantial outward currents but were active only in the presence of 2-aminophenylborate (2-APB), irrespective of STIM1. With W176C mutant channels inward, monovalent currents were blocked by Ca$^{2+}$ with a high affinity similar to the wild type, but the Ca$^{2+}$-dependent blocking of outward currents differed in the two cases. Although a 50% block of the WT outward current required 250 μM Ca$^{2+}$, more than 6 mM was necessary to have the same effect on W176C mutant channels. In the presence of extracellular Ca$^{2+}$, W176C and G183A outward currents developed slowly in a voltage-dependent manner, whereas they developed almost instantaneously in the absence of Ca$^{2+}$. These changes in permeation and gating properties mimic the changes induced by mutations of Glu-190 in TMIII and Asp-110/Asp-112 in the TMI/TMII loop. On the basis of these data, we propose that TMIII maintains negatively charged residues at or near the selectivity filter in a conformation that facilitates Ca$^{2+}$ inward currents and prevents outward currents of monovalent cations. In addition, to controlling selectivity, TMIII may also stabilize channel gating in a closed state in the absence of STIM1 in a Trp-176-dependent manner.

* This work was supported, in whole or in part, by National Institute of Health Grants AI-083432 and AI-088393 (to Y. G.). This work was also supported by a fellowship from the American Heart Association (to S. S.).

¹ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–5.

1 To whom correspondence may be addressed: Dept. of Physiology, David Geffen School of Medicine, 53-266 CHS, 10833 Le Conte Ave., Los Angeles, CA 90095. Tel.: 310-794-2003; Fax: 310-206-5661; E-mail: ygwang@mednet.ucla.edu.

2 To whom correspondence may be addressed: Dept. of Physiology, David Geffen School of Medicine, 53-364 CHS, 10833 Le Conte Ave., Los Angeles, CA 90095. Tel.: 310-825-9743; Fax: 310-206-5661; E-mail: bribalet@mednet.ucla.edu.

3 The abbreviations used are: SOCE, store-operated Ca$^{2+}$ entry; CRAC, Ca$^{2+}$ release-activated Ca$^{2+}$; ER, endoplasmic reticulum; PM, plasma membrane; I/V, current-to-voltage relationship; 2-APB, 2-aminoethyldiphenylborate; TIRF, total internal reflection fluorescence; MEF, murine embryonic fibroblast; HEDTA, N-(2-hydroxyethyl)ethylenediaminetriacetic acid; DVF, divalent-free.
opened in a STIM1-independent manner by extracellular application of 2-APB (> 30 μM) (12–14). In this case, the I/V exhibits inward currents at hyperpolarized membrane potentials and large outward currents at potentials more positive than +50 mV (11, 12, 14, 15). The mechanisms responsible for the differences in gating and permeability between Orai1 and Orai3 remain largely unknown. Recent studies using chimeras have shown that an Orai1 chimera containing the intracellular loop between TMII and TMIII and the TMIII derived from Orai3 behaves similar to Orai3 and shows large outward currents upon external application of 50 μM 2-APB (14).

In a previous study, we systematically mutated residues in the intracellular loop of Orai1 and found all the mutants to behave like the wild type in terms of inward rectification and STIM-dependent activation (16). In this study, we performed mutagenesis of residues in Orai1 TMIII and identified two amino acids, Trp-176 and Gly-183, the mutation of which confer STIM1 independence and affect channel gating and permeation properties. Although the W176C mutant channel was constitutively active, the G183A mutant channel carried currents only in the presence of 2-APB. We found that both mutations induce large outward currents. Although the binding affinity for Ca2+ at hyperpolarized membrane potentials remained similar to the wild type, a dramatic decrease in Ca2+ blocking efficacy of the outward current was observed in these mutant channels. It has been suggested that the high-affinity Ca2+-binding site, which is responsible for ion selectivity, comprises a single site formed by Glu-106 in TMII (8). On the basis of these data, we propose a model whereby the negatively charged residues Asp-110 and Asp-112 in the TMI-TMII extracellular loop, which are known to evoke a large outward current at depolarized potentials (13), facilitate local Ca2+ accumulation within the outer vestibule of the pore and prevent Ca2+ release from the Glu-106 Ca2+-binding site at depolarized potentials. Such a mechanism would account for the inwardly rectifying I/V of WT CRAC currents. Accordingly, we propose that the W176C and G183A mutations in TMIII allosterically prevent Ca2+ accumulation near Asp-110/D112, resulting in the expulsion of Ca2+ from the Glu-106 site at depolarized potentials, the generation of large outward currents, and the loss of ion selectivity.

EXPERIMENTAL PROCEDURES

Reagents—Thapsigargin and 2-APB were purchased from EMD Biochemicals. Polyclonal rabbit antibody for detection of Orai1 was generated, affinity-purified (Open Biosystems, Huntsville, AL), and used at 1:1000 dilution for immunocytochemistry. Alexa Fluor 568-labeled secondary antibodies were purchased from Invitrogen and used at 1:1000 dilutions for immunocytochemistry.

Plasmids—Full-length cDNA of human Orai1 subcloned into the bicistronic retroviral expression vector pMSCV-CITE-eGFP-PGK-Puro, which allows simultaneous expression of Orai1, GFP, and a puromycin resistance gene, has been described previously (17). Single-point mutants were generated using the QuikChange XL site-directed mutagenesis kit (Stratagene) following the manufacturer’s instructions. All the clones were verified by sequencing. For total internal reflection fluorescence (TIRF) analysis, WT, G183A, and W176C mutant Orai1 cDNAs were fused in frame with pEGFP to have a C-terminal GFP tag. The STIM1-mCherry plasmid has been described previously (18).

Cell Lines and Transductions—HEK293 cells were obtained from the ATCC and cultured in DMEM (Mediatech, Hargrave, VA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 10 mM HEPES, 10 mM Glutamine and 1% penicillin/streptomycin (Mediatech, Hargrave, VA). Cells were transfected at 80–90% confluency using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For retroviral transductions, phoenix cells stably expressing gag-pol and ecotropic env (purchased from the ATCC) were transfected with plasmids encoding Orai proteins to produce an ecotropic, replication-incompetent retrovirus using the calcium phosphate transfection method. Virus-containing supernatant was collected 2 and 3 days after transfection, and immortalized Orai1−/− mouse embryonic fibroblasts (MEFs) (16, 19) were transduced twice on day 2 and day 3 in the presence of 8 μg/ml polybrene. Transduction efficiencies were evaluated visually by GFP expression and Orai1 expression using immunoblotting.

Single-cell Ca2+ Imaging—Orai1−/− MEFs or HEK293T cells were grown directly on UV-sterilized coverslips and loaded with 2 μM fura-2/acetoxyl methyl ester for 45 min at 22–25 °C. For [Ca2+]i measurements, cells were mounted in a RC-20 closed-bath flow chamber (Warner Instrument Corp., Hamden, CT) and analyzed on an Olympus IX51 epifluorescence microscope with Slidebook 5.0 (Intelligent Imaging Innovations, Inc.) imaging software. Cells were perfused with Ca2+-free Ringer’s solution, and Ca2+ stores were passively depleted with 1 μM thapsigargin. Fura-2 emission was detected at 510 nm with excitation at 340 and 380 nm, and the Fura-2 emission ratio (340/380) was acquired at every 5-s interval after subtraction of the background. For each experiment, 35–50 individual cells were analyzed using OriginPro (Originlab) analysis software. [Ca2+]i was estimated from the relation [Ca2+]i = K*(Ri/(Rmax−Ri)), K, Ri (minimum ratio value), and Rmax (maximum ratio value) were measured independently in control cells.

Immunocytochemistry—Immunocytochemistry for Orai1 staining was performed as described (16). Briefly, transfected HEK293T cells were fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with wash buffer containing 0.5% Nonidet P-40, and incubated with anti-Orai1 antibodies (16) and Alexa Fluor-labeled secondary antibodies. Immunofluorescence was analyzed by epifluorescence microscopy using an Olympus IX51 epifluorescence microscope (Olympus, Inc.) and a Plan Aposphotomat ×40 oil differential interference contrast objective with numerical aperture of 1.3.

TIRF Microscopy Analysis—HEK293 cells were transfected with plasmids encoding STIM1-mCherry along with either WT or mutant Orai1-GFP fusion protein encoding cDNAs at a molar ratio of 1:1. TIRFM was performed using an Olympus IX2 illumination system mounted on an Olympus IX51 inverted microscope. Lasers beams from a 488-nm argon ion laser (Melles Griot) and a 594-nm diode laser (Coherent Instruments) were combined and controlled using an Olympus OMAC TIRF dual port condenser and controller system. The angle of the incident light at the interface between the glass...
coverslip and the aqueous medium was controlled by independently adjusting the position of each laser beam before passing through a ×60 oil immersion objective (NA 1.49). The emission was filtered either at D525/50 nm or 660/50 nm filter (Chroma) and captured by a Hamamatsu ORCA cooled CCD (Roper Scientific) camera. Acquisition and image analysis were performed using Slidebook (Intelligent Imaging Innovations, Inc.) and OriginPro 8.5 software.

**Measurement of CRAC Currents by Whole Cell Recording**—For recording of CRAC currents, HEK293T cells were cotransfected with plasmids encoding Orai1 WT or mutant cDNAs in the presence or absence of a STIM1-encoding plasmid at a molar ratio of 1:1 using Lipofectamine 2000 (Invitrogen). Cells were used for experiments 24–48 h post-transfection. Patch-clamp recordings were performed using an Axopatch 200B amplifier (Molecular Devices, CA) interfaced to a Digidata 1320A (Axon Instruments, CA) for stimulation and data acquisition. Currents were recorded at 1 kHz with a four-pole Bessel filter and sampled at 5 kHz. Recording electrodes were pulled from borosilicate glass capillaries (WPI, Sarasota, FL) using a Flaming Brown pipette puller (Sutter Instrument, CA) to a final resistance of 2–7 MΩ. Stimulation, data acquisition, and analysis were performed using pCLAMP8 and SigmaPlot or Origin software. The standard extracellular Ringer solution contained 145 mM Cs-glutamate, 8 mM MgCl₂, 2 mM CaCl₂, 10 mM Cs-glucose, and 10 mM Na-Hepes (pH 7.35). The standard internal solution contained 145 mM Cs-aspartate, 4.5 mM KCl, 6 mM CaCl₂, 10 mM D-glucose, and 10 mM Na-Hepes (pH 7.35). The standard internal solution contained 145 mM Cs-glutamate, 8 mM MgCl₂, 12 mM EGTA, and 10 mM Cs-Hepes (pH 7.3). Experiments described in Figs. 2 and 3 were performed using symmetrical Na⁺-containing solutions with extracellular Na-aspartate and intracellular Na-glutamate. Unless stated otherwise, the cell membrane was held at 0 mV, and pulses were applied between −110 mV to +115 mV at 15-mV intervals for 250 ms. We used HEDTA/Ca²⁺ for the extracellular solutions to obtain varying amounts of free Ca²⁺, depicted in Figs. 3 and 6. For each solution, the concentration of HEDTA and Ca²⁺ was calculated using the WEBMAXC program and validated using a Ca²⁺ electrode.

**Analysis of Patch Clamp Data**—Ionic currents from cells expressing WT and mutant channels were recorded using only the analog compensation of the membrane linear components. In most cases (mutant channels), 2-APB had no inhibitory effects, and the current traces could not be corrected for leak currents. The time course of outward current activation was fitted by a single exponential function. The first 1 ms of recorded data following the onset of the voltage pulses was not included in the fitting to minimize the effect of uncompensated membrane capacitance on the estimated time course of the current. For the I/V, we used steady-state currents measured at the end of the pulse unless specified otherwise.

**RESULTS**

**Analysis of TMIII Mutants of Orai1 for Store-operated Ca²⁺ Entry**—To determine the role of TMIII in the permeation properties of CRAC channels, we mutated a series of residues in TMIII and examined their properties (Fig. 1, A and B). All the mutants examined in TMIII are highlighted in red in Fig. 1A. Among these mutants, E190Q has been characterized previously and shows altered ion selectivity (15, 20). To measure the activity of individual mutants without interference from endogenous Orai1, mutants were retrovirally expressed in Orai1-deficient (Orai1⁻/⁻) MEFs (19). SOCE was strongly reduced in Orai1-null MEFs but was restored to WT levels by expression of WT Orai1 (Fig. 1B and Ref. 19). The W176C mutant showed very high levels of basal intracellular Ca²⁺ concentration ([Ca²⁺]i), whereas mutants S179C and G183A did not reconstitute SOCE in Orai1-null MEFs. All the other mutants (E173C, A175C, A177C, F178C, C195G, and W196C) showed normal Ca²⁺ entry as measured by ratiometric Ca²⁺-imaging and current recordings (Fig. 1B and data not shown). All the mutants showed comparable expression levels as judged by immunoblotting (Fig. 1C) and showed plasma membrane localization similar to wild-type Orai1 (Fig. 1D and supplemental Fig. 1). These studies suggested that the Ser-179 and Gly-183 residues play an important role in channel gating and that the W176C mutant may be constitutively active and independent from store-depletion.

The **W176 Residue Is Important for Gating and Permeation Properties of CRAC Channels**—To examine the biophysical profile of currents generated by W176C mutant channels, we expressed W176C mutant channels with and without STIM1 in HEK293 cells. As reported previously (21–23), coexpression of wild-type Orai1 and STIM1 results in macroscopic CRAC currents exhibiting large inward currents with negligible outward currents at potentials more positive than +60 mV (Fig. 2A). In agreement with SOCE measurements, we observed currents in HEK293 cells expressing W176C-Orai1 even in the absence of STIM1, demonstrating the constitutive activity of these channels. Additionally, W176C mutant channels showed large outward currents in addition to inward currents, yielding an I/V with an S shape (Fig. 2B). Thus, in W176C mutant channels, currents are strongly suppressed up to +40 mV, but in contrast to WT channels, large outward currents of monovalent Na⁺ or Cs⁺ ions develop at potentials more positive than +50 mV (Fig. 2B and supplemental Fig. 2A). These data suggest an important role of W176 in regulating the permeation properties of CRAC channels. Little is known about the molecular mechanism of CRAC current rectification, but it has been shown that removal of extracellular divalent ions is accompanied by loss of ion selectivity and permeation of Na⁺ and Ca²⁺ both in the inward and outward direction (8). In this case, the I/V relationship of WT CRAC currents becomes more linear with symmetrical Na⁺ (see also Fig. 2C). Thus, extracellular Ca²⁺ may play a role in limiting the outward current of monovalent cations at physiological Ca²⁺ concentrations. Interestingly, the I/V relationship of W176C mutant Orai1 channels was completely linear in divalent free (DVF) solutions (Fig. 2D). Together these data suggest that large outward currents observed with W176C mutant channels may result from a change in CRAC channel pore affinity for extracellular Ca²⁺.

**W176C Channels Show the Same Kᵢ for Ca²⁺ Block of Monovalent Current as WT Channels**—To further characterize the Ca²⁺-dependent blocking of inward and outward currents for WT and W176C mutant channels, we generated I/V relationships in the presence of various external Ca²⁺ concentrations (Fig. 3, A and B). In the presence of increasing extracellular
Ca²⁺, the inward monovalent currents decreased for both WT and W176C mutant channels. In some instances, the inward current amplitude for WT channels recovered at Ca²⁺ concentrations greater than 6 mM. To quantify Ca²⁺ blocking efficacy, we measured current amplitudes at fixed voltages (-110 mV and 115 mV) for varying Ca²⁺ concentrations. For inward currents, the plot of the current amplitude normalized to the maximum current amplitude in the absence of Ca²⁺ as a function of extracellular Ca²⁺ concentration is well fitted by a Hill equation with a coefficient approaching 1, suggesting a single binding site, and an IC₅₀ between 20–40 μM for both WT (Fig. 3A, center panel) and W176C (B, center panel) channels. This Ca²⁺ affinity for channel blocking is similar to a previous report (8).

Regarding the Ca²⁺ blocking efficacy of the Na+/H¹⁺ inward current in W176C, it should be noted that at intermediary concentrations (100 μM to 1 mM), the effect of Ca²⁺ is time- and voltage-dependent (supplemental Fig. 2B). Thus, in contrast to WT channels where Ca²⁺ blocking of the Na⁺ inward current is almost instantaneous, it takes tens of milliseconds to block the current in W176C. As a result, the affinity for Ca²⁺ blocking measured at a steady state is similar to that observed in WT channels, but the value measured at the beginning during the peak current shifted 5- to 10-fold. Although extracellular Ca²⁺ blocked WT and W176C inward currents with similar efficacy, blocking of the outward current was greatly different. In fact, WT outward currents were almost completely blocked by 1 mM Ca²⁺ (IC₅₀ = 255 μM), but 100 mM Ca²⁺ (IC₅₀ = 6.2 mM) was required to achieve similar results with W176C mutant channels (Fig. 3, A and B, right panels).

We also examined how these outward currents developed and found further evidence in favor of Ca²⁺-dependent blocking of outward currents. In the absence of extracellular Ca²⁺, the outward current from W176C channels developed quasi-instantaneously (Fig. 3C, left panel) but developed slowly in the presence of external Ca²⁺, with a time constant of 2 to 5 ms depending on the voltage (Fig. 3C, right panel). These data sug-
suggest that the slow activation phase in the presence of Ca$^{2+}$ involves Ca$^{2+}$ unblocking. Because the rate of rise of the outward current is a function of voltage at the membrane potentials greater than the Ca$^{2+}$ reversal potential (Fig. 3D, left panel), it follows that unblocking may be due to Ca$^{2+}$ dissociation from a site within the pore. As expected, the rate of unblocking was independent of the concentration of extracellular Ca$^{2+}$ (Fig. 3D, right panel).

So far, the W176C data uncover a blocking effect of Ca$^{2+}$ binding to a site within the membrane electrical field. In WT CRAC channels, extracellular Ca$^{2+}$ also blocks Na$^{+}$ inward currents by binding at a site with an IC$_{50}$ close to 20 μM (8). Because this IC$_{50}$ increases to 500 μM in E106D mutant channels, which also show loss of ion selectivity, it has been suggested that this site was located at the selectivity filter (10). Interestingly, W176C mutant channels showed a very sim-
ilar IC$_{50}$ (20 μM, Fig. 3A) for blockage of Na$^+$ inward currents at hyperpolarized membrane potentials, suggesting that the binding site encompassing Glu-106 is not affected by the W176C mutation. However, our data also show that Ca$^{2+}$ efficacy at blocking Na$^+$ outward currents is much lower in W176C mutant channels as compared with WT. As shown by our data, this loss in ion selectivity at the depolarized membrane potential is very likely due to Ca$^{2+}$ leaving its high-affinity binding
The Mutation of Gly-183 Causes STIM1-independent Channel Opening by Application of 2-APB—2-APB is widely used as a modulator of CRAC channel activity, which either activates or inhibits CRAC currents at concentrations of 10 μM and 50 μM, respectively (24). To examine whether currents observed with W176C and other mutants of Orai1 were modulated by 2-APB, we measured SOCE in the presence of 2-APB. Although W176C-mediated SOCE was unaffected by 2-APB (data not shown), one of the other mutants, G183A, which initially did not show significant Ca²⁺ entry upon store depletion (Fig. 1B), showed a robust increase in cytoplasmic Ca²⁺ in the presence of 2-APB (Fig. 4A). To examine whether this effect was dependent on store depletion, we exposed Orai1-deficient cells expressing W176C or G183A mutants to 2-APB, even in the absence of store depletion (Fig. 4B). We then examined the permeation and gating properties of this mutant. Whole-cell patch clamp recordings showed that G183A mutant channels conducted Ca²⁺ only in the presence of 2-APB. In this case, addition of 2-APB caused a rapid and simultaneous increase of inward and outward currents (Fig. 4C). It is unlikely that the development of outward and inward currents in G183A is due to separate processes because both effects occur.

FIGURE 4. Gly-183 in TMIII modulates CRAC channel gating and permeation. A, SOCE measurements in Orai1−/− MEFs expressing WT and G183A mutant Orai1 proteins. Intracellular Ca²⁺ stores were first depleted by thapsigargin (1 μM) and then SOCE was measured by addition of 10 mM external Ca²⁺ prior to addition of 2-APB. Each trace represents an average from 30–40 cells. B, measurement of the effect of 2-APB on Ca²⁺ entry in Orai1−/− MEFs. Orai1−/− MEFs expressing WT (black trace), W176C (blue trace), or G183A (green trace) Orai1 proteins were exposed to 10 mM Ca²⁺-containing external solution in the absence and presence of 100 μM 2-APB. Each trace represents an average from 30–40 cells. C, measurements of currents from HEK293 cells expressing G183A mutant Orai1. Currents elicited by +90- and −90-mV pulses were recorded in the presence of 2-APB (50 μM) applied externally. The center panel shows representative current traces obtained with voltage steps of 0.2 s from −110 mV to +115 mV (15-mV intervals) from a holding potential of 0 mV (only alternate traces are shown for clarity). The right panel shows the I/V taken between −110 mV and +115 mV from the same cells. D, average steady-state currents from WT, W176C, and G183A Orai1 channels with and without STIM1 coexpression in HEK293 cells. The numbers at the top represent the number of cells used for the experiments, and the bars represent mean ± S.D. from these cells.
concomitantly. In WT channels, 2-APB activates outward currents, but this effect is delayed compared with the early activation and block of inward currents (supplemental Fig. 3, A and B). Thus, in this respect, the channel permeation properties of G183A and W176C appear to be similar, both exhibiting large outward currents. The lack of G183A mutant channel activation by STIM1 suggests that this mutant may cause a collapse of the channel gate, which can be opened by 2-APB.

To further investigate the interaction of STIM1 with these Orai1 mutants, we measured W176C and G183A mutant channel currents in the presence and absence of STIM1. Both inward and outward currents were enhanced in W176C mutant channels by coexpression of STIM1. In contrast, currents remained unaltered by STIM1 in G183A mutant channels (Fig. 4D). In this case, the lack of effect of STIM1 is very likely due to the requirement of 2-APB to open the channel, which maximally activates CRAC channels, thereby masking the effect of STIM1.

Because Gly residues are considered helix breakers, we surmised that mutation in Gly-183 may uncouple the relay of the STIM1 signal for opening the gate at the selectivity filter region. To examine whether any other Gly residues within TMs of Orai1 function similarly, we systematically mutated all the Gly residues within TMs of Orai1 (supplemental Fig. 4). Orai1 encodes three glycine residues, one in TM1, a second in TMIII, and the third one in TMIV. All the Gly mutants showed a similar expression as judged by immunoblotting and immunocytochemistry (Fig. 1C and supplemental Fig. 4B). Mutation of Gly in TM1 (G98A) showed a very low SOCE that was blocked instantaneously and remained unaffected by external application of 2-APB (supplemental Fig. 4C). Accordingly, we could not detect any CRAC currents by expression of this mutant in HEK293 cells in the presence or absence of STIM1 (data not shown). On the other hand, Gly in TMIV (G247A) showed increased SOCE (supplemental Fig. 4C) but other than that behaved exactly like WT Orai1 in terms of permeation and gating characteristics (supplemental Fig. 4D and data not shown). In summary, we observed STIM1-dependent Ca$^{2+}$ entry in the presence of 2-APB only with G183A mutant, indicating a specific role of TMIII.

The W176C Mutant Localizes and Translocates Normally under Resting and Store-depleted Conditions—It has been shown that interaction of STIM1 with Orai regulates at least two separate processes: one responsible for clustering of Orai1 into the junctional regions between the PM and ER membrane and the other required for channel activation. Because both W176C and G183A mutants show STIM1-independent currents, we examined whether they exhibited proper clustering with STIM1. To this end, we measured their translocation to PM/ER junctional regions upon stimulation with thapsigargin using TIRF microscopy. As reported previously (22), WT Orai1 channels are homogenously distributed in the PM, whereas STIM1 is distributed in the ER membrane and hence shows a very low signal under resting conditions (Fig. 5A, top panels). Upon store depletion, however, STIM1 clusters and translocates to near PM junctional areas, and Orai1 accumulates into those clusters (Fig. 5A, lower panels). Under the same condition, both W176C and G183A mutant channels clustered into junctional areas with kinetics similar to that of WT channels (Fig. 5, B and C, and data not shown). These results suggest that the W176C and G183A mutants retain at least the interaction with STIM1 required for their clustering in junctional areas. Thus, despite their clustering into junctional areas upon coexpression with STIM1 (Fig. 5), the G183A mutant channels remained closed upon store depletion and were activated only by extracellular 2-APB. This observation supports the hypothesis that TMIII plays a role in transmitting a STIM1-dependent
Orai1TMIII Modulates CRAC Channel Gating and Permeation

activation signal from the cell interior to the upper channel gate, and G183A alters this process.

The W176C Mutant and D110A/D112A Have Similar Effects on CRAC Channel Permeation Properties—It has been reported that the Asp-110A/Asp-112A mutant Orai1 channels showed a Ca\(^{2+}\) affinity similar to WT despite the presence of outward currents at depolarized membrane potentials (13). On the basis of the similarity between the effects of W176C and D110A/D112A on CRAC channel permeation properties, we surmised that the W176C mutation may affect the positioning of Asp-110A/Asp-112A on the outside of the channel pore. To test this hypothesis, we measured CRAC currents generated by the double mutant channel D110A/D112A coexpressed with STIM1 in HEK293T cells. With this double mutant, we observed large outward currents that develop with a slow time course (Fig. 6, A and B), suggesting Ca\(^{2+}\) unblocking at depolarized membrane potentials. Also, as with W176C mutant channels, high concentrations of extracellular Ca\(^{2+}\) (100 mM) were necessary to block these outward currents (Fig. 6, B–D), and this block occurs very slowly in minutes, supporting the hypothesis that the double D110A/D112A mutation prevents Ca\(^{2+}\) accumulation in the outer vestibule. These data, together with that of others showing that Na\(^{+}\) inward currents generated by the double mutation are blocked by Ca\(^{2+}\) with high affinity (13), demonstrate that the W176C and D110A/D112A mutations similarly affect CRAC channel permeation properties.

**DISCUSSION**

The key properties of CRAC currents include a very high selectivity for Ca\(^{2+}\) over monovalent ions and inward rectification. Our data show that mutation of the Trp-176 and Gly-183 residues (W176C and G183A) in Orai1 TMIII exhibited strong outward currents. In addition, W176C was constitutively active in the absence of STIM1. On the other hand, G183A mutant channels were closed even when coexpressed with STIM1, but addition of 2-APB activated the channel irrespective of STIM1. These data suggest an important role of TMIII in CRAC channel gating, although it has been shown to not line the pore (25, 26).

In cation-selective channels, inward rectification may result from two processes: concentration of ions at the outer mouth of the channel by pore-associated negative charges, which facilitates inward currents (27), or blocking of the outward current as seen in inward-rectifying K\(^{+}\) channels. The latter may result from Mg\(^{2+}\) and polyamines entering the channel (28, 29), asymmetry of the ion conduction pathway, or positive charges located at the inner mouth of the pore that repel cations (30). Inward rectification in CRAC channels may involve both, facilitation of inward currents and blocking of outward currents. Our data obtained with W176C and G183A indicate that blocking of the outward current by Ca\(^{2+}\) plays a role in the rectification process. The I/V of W176C and G183A is characterized by strong current suppression up to +40 mV and large outward currents above +50 mV. This pattern is abolished in the absence of extracellular Ca\(^{2+}\), giving rise to a linear I/V. We suggest that current inhibition up to +40 mV is due to Ca\(^{2+}\) blocking on the basis of a number of observations. The outward currents in W176C and G183A mutant channels develop slowly in the presence of Ca\(^{2+}\), with a time constant of 2–5 ms, whereas activation is instantaneous in the absence of Ca\(^{2+}\) (31) (Fig. 3 and data not shown). The rate of rise is strongly voltage-dependent, increasing with positive membrane potentials, and independent of extracellular Ca\(^{2+}\) concentration. This suggests that the Ca\(^{2+}\) blocking site lies within the membrane electrical field. Furthermore, the K\(_{\text{off}}\) (rate of Ca\(^{2+}\) from its blocking site) derived from the rate of activation of outward currents are approximately 650 s\(^{-1}\) at 115 mV and 300 s\(^{-1}\) at 60 mV, which is consistent with a K\(_{\text{off}}\) of 125 s\(^{-1}\) estimated for the blocking effect of Ca\(^{2+}\) on inward currents at −100 mV (10). Finally, the IC\(_{50}\) for Ca\(^{2+}\), close to 20 μM, is almost identical to the value estimated for Ca\(^{2+}\)-dependent blocking of the Na\(^{+}\) inward current (8). On the basis of the observation that Ca\(^{2+}\) blocking of Na\(^{+}\) inward currents in WT CRAC channels decreased with the mutation of Glu-106 (E106D), the IC\(_{50}\) shifting from 20 μM to 500 μM, it has been suggested that the Ca\(^{2+}\) blocking binding site was at the selectivity filter. In addition, a Hill coefficient of 1 deduced from the blocking effect of Ca\(^{2+}\) in WT and W176C mutant channels indicates that Ca\(^{2+}\) binds to a single site (Glu-106) that remains unaltered in the W176C mutant. Because this site is functional in W176C mutant channels as judged by a Ca\(^{2+}\) affinity identical to WT channels for inward currents, the observation of outward currents at depolarized potentials in W176C channels is puzzling. In fact, these observations parallel those with D110A/D112A mutant Orai1 channels, showing a Ca\(^{2+}\) affinity similar to WT and the presence of outward currents at depolarized membrane potentials (Fig. 6 and Ref. 13). On the basis of these observations, we propose a model for Orai1 (Fig. 7) derived from a BK\(_{Ca}\) channel model (31). In Orai1, the Glu-106 residue forms the sole Ca\(^{2+}\) binding site that controls...
Orai1TMIII Modulates CRAC Channel Gating and Permeation

ion selectivity and the Asp-110 and Asp-112 residues, which also line the pore but above the selectivity filter allow for local Ca\(^{2+}\) concentration in the outer vestibule. Such Ca\(^{2+}\) accumulation has two major effects: increase Ca\(^{2+}\) inward current via a mechanism similar to that proposed for BK\(_{Ca}\) channels (31) and retention of Ca\(^{2+}\) at the Glu-106 binding site at depolarized membrane potentials. The presence of Ca\(^{2+}\) at this site prevents the loss of ion selectivity and generation of outward currents at depolarized monovalent cations at depolarized membrane potentials. Mutation of Asp-110 and Asp-112, on the other hand, dissipated the high Ca\(^{2+}\) ion density in the outer vestibule. Under this condition, the affinity of the Glu-106 binding site for Ca\(^{2+}\) is not affected, but association of Ca\(^{2+}\) with the site may be slower at low concentrations. The major effect of this mutation would be to promote the displacement of Ca\(^{2+}\) from the Glu-106 binding site at depolarized membrane potential and account for the loss of ion selectivity and generation of outward currents. Our data indicate that the mutations in TMIII do not affect Ca\(^{2+}\) binding to the Glu-106 site. However, it may affect the mechanism of Ca\(^{2+}\) accumulation in the outer vestibule. Although mutation of Glu-190 in TMIII causes a similar activation of the Na\(^{+}\) outward current (supplemental Fig. 5), it is unlikely to directly affect the negative charge distribution in the outer mouth of the channel because TMIII does not line the pore (25, 26). It follows, therefore, that the effects of TMIII on the ion selectivity at depolarized membrane potentials is likely mediated via allosteric rearrangement of Asp-110 and Asp-112 in the outer vestibule (Fig. 7, lower panel). Whether Glu-190 or the TMIII/TMIV extracellular loop play a role in this process remains to be demonstrated.

As reported for many Ca\(^{2+}\) and K\(^{+}\) channels (32–34) CRAC channel gating may occur at the selectivity filter (10, 11). Accordingly, our W176C and G183A data may be explained assuming a gate located at the outer mouth of the channel near the selectivity filter. In WT channels, a Trp-176-dependent process maintains the channel gate in a closed state in the absence of STIM1. TMIII may transmit this Trp-176-dependent closing signal to the gate. STIM1, but also the mutations W176C and G183A, release the closing mechanism, allowing the channel to open. Thus, the W176C and G183A mutants channel open in a STIM-independent fashion. Whether the Orai channel gate involves the selectivity filter itself or a structure near it is unclear, but it is also possible that the gate is above the selectivity filter and comprises the negatively charged residues Asp-110 and Asp-112. Such a mechanism has been postulated to account for Kv4.3 gating (34). Finally, the CRAC channel gate may be located at the intracellular face of the pore, and the large side chain of Trp-176 may regulate this mechanism. There is no direct evidence in favor of this hypothesis, but it should be noted that the mutations R91W and K85A/K87A (Ref. 35 and data not shown) at the base of TMI block channel permeation, suggesting an important role of this region in channel gating.

In summary, our data obtained with the W176C and G183A mutant channels indicate that TMIII regulates both permeation and gating of CRAC channels. Allosteric interaction of TMIII with the outer mouth of the pore may prevent activation of monovalent cation outward currents. This process does not directly involve the high-affinity binding site for Ca\(^{2+}\) in the selectivity filter at Glu-106 but rather some negative charges that cause local concentration of Ca\(^{2+}\) in the outer vestibule. This local accumulation of Ca\(^{2+}\) maintains Ca\(^{2+}\) binding to Glu-106 and thus prevents monovalent cation outward currents at depolarized membrane potentials. Finally, our data indicate that Glu-176 is part of a mechanism that maintains Orai in a closed state. STIM frees this mechanism, and the opening signal is transmitted to the gate via TMIII.

Acknowledgments—We thank Dr. Riccardo Olcese for useful discussions and comments on the manuscript and Marcus Jew for technical help.

REFERENCES
1. Gwack, Y., Feske, S., Srikanth, S., Hogan, P. G., and Rao, A. (2007) Cell Calcium 42, 145–156
2. Hogan, P. G., Lewis, R. S., and Rao, A. (2010) Annu. Rev. Immunol. 28, 491–533
3. Putney, J. W. (2011) Neurochem Res. 36, 1157–1165
4. Kawasaki, T., Lange, I., and Feske, S. (2009) Biochem. Biophys. Res. Commun. 385, 49–54
5. Muik, M., Fahrner, M., Derler, I., Schindl, R., Bergsmann, J., Frischauf, I., Groschner, K., and Romanin, C. (2009) J. Biol. Chem. 284, 8421–8426
6. Park, C. Y., Hoover, P. J., Mullins, F. M., Bachhawat, P., Covington, E. D.,
Orai1TM III Modulates CRAC Channel Gating and Permeation

Raunser, S., Walz, T., Garcia, K. C., Dolmetsch, R. E., and Lewis, R. S. (2009) Cell 136, 876–890
7. Yuan, J. P., Zeng, W., Dorwart, M. R., Choi, Y. J., Worley, P. F., and Muallem, S. (2009) Nat. Cell Biol. 11, 337–343
8. Prakriya, M., and Lewis, R. S. (2006) J. Gen. Physiol. 128, 373–386
9. Prakriya, M. (2009) Immunol. Rev. 231, 88–98
10. Yamashita, M., Navarro-Borelly, L., McNally, B. A., and Prakriya, M. (2007) J. Gen. Physiol. 130, 525–540
11. Peinelt, C., Lis, A., Beck, A., Fleig, A., and Penner, R. (2008) J. Physiol. 586, 3061–3073
12. DeHaven, W. I., Smyth, J. T., Boyles, R. R., Bird, G. S., and Putney, J. W., Jr. (2008) J. Biol. Chem. 283, 19265–19273
13. 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
14. Zhang, S. L., Kozak, J. A., Jiang, W., Yeromin, A. V., Chen, J., Yu, Y., Penna, A., Shen, W., Chi, V., and Cahalan, M. D. (2008) J. Biol. Chem. 283, 17662–17671
15. Lis, A., Peinelt, C., Beck, A., Parvez, S., Monteilh-Zoller, M., Fleig, A., and Penner, R. (2007) Curr. Biol. 17, 794–800
16. Srikanth, S., Jung, H. J., Ribalet, B., and Gwack, Y. (2010) J. Biol. Chem. 285, 5066–5075
17. 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
18. Srikanth, S., Jung, H. J., Kim, K. D., Souda, P., Whitelegge, J., and Gwack, Y. (2010) Nat. Cell Biol. 12, 436–446
19. Gwack, Y., Srikanth, S., Oh-Hora, M., Hogan, P. G., Lamperti, E. D., Yamashita, M., Gelinas, C., Neems, D. S., Sasaki, Y., Feske, S., Prakriya, M., Rajewsky, K., and Rao, A. (2008) Mol. Cell. Biol. 19, 2802–2817
20. Prakriya, M., Feske, S., Gwack, Y., Srikanth, S., Rao, A., and Hogan, P. G. (2006) Nature 443, 230–233
21. Peinelt, C., Vig, M., Koomoa, D. L., Beck, A., Nadler, M. J., Koblan–Huberson, M., Lis, A., Fleig, A., Penner, R., and Kinet, J. P. (2006) Nat. Cell Biol. 8, 771–773
22. Mercer, J. C., Dehaven, W. I., Smyth, J. T., Wedel, B., Boyles, R. R., Bird, G. S., and Putney, J. W., Jr. (2006) J. Biol. Chem. 281, 24979–24990
23. Soboloff, J., Spassova, M. A., Tang, X. D., Hewavitharana, T., Xu, W., and Gill, D. L. (2006) J. Biol. Chem. 281, 20661–20665
24. Prakriya, M., and Lewis, R. S. (2001) J. Physiol. 536, 3–19
25. McNally, B. A., Yamashita, M., Engh, A., and Prakriya, M. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 22516–22521
26. Zhou, Y., Ramachandran, S., Oh-Hora, M., Rao, A., and Hogan, P. G. (2010) Proc. Natl. Acad. Sci. U.S.A. 107, 4896–4901
27. Babich, O., Matveev, V., Harris, A. L., and Shirokov, R. (2007) J. Gen. Physiol. 129, 477–483
28. Vandenberg, C. A. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 2560–2564
29. Lopatin, A. N., Makhina, E. N., and Nichols, C. G. (1995) J. Gen. Physiol. 106, 923–955
30. Lear, J. D., Schneider, J. P., Kienker, P. K., and DeGrado, W. F. (1997) J. Am. Chem. Soc. 119, 3212–3217
31. Haug, T., Sigg, D., Ciani, S., Toro, L., Stefani, E., and Olcese, R. (2004) J. Gen. Physiol. 124, 173–184
32. Liu, Y., Jurrman, M. E., and Yellen, G. (1996) Neuron 16, 859–867
33. Kiss, L., LoTurco, J., and Korn, S. J. (1999) Biophys. J. 76, 253–263
34. Eghbali, M., Olcese, R., Zarei, M. M., Toro, L., and Stefani, E. (2002) J. Membr. Biol. 188, 73–86
35. Derler, I., Fahrner, M., Carugo, O., Mui, M., Bergmann, J., Schindl, R., Frischau, I., Eshaghi, S., and Romanin, C. (2009) J. Biol. Chem. 284, 15903–15915