Molecular determinants of fast Ca^{2+}-dependent inactivation and gating of the Orai channels

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Ca^{2+} influx by store-operated Ca^{2+} influx channels (SOCs) mediates many cellular functions regulated by Ca^{2+}, and excessive SOC-mediated Ca^{2+} influx is cytotoxic and associated with disease. One form of SOC is the CRAC current that is mediated by Orai channels activated by STIM1. A fundamental property of the native CRAC and of the Orais is fast Ca^{2+}-dependent inactivation, which limits Ca^{2+} influx to guard against cellular damage. The molecular mechanism of this essential regulatory mechanism is unknown. We report here the fast Ca^{2+}-dependent inactivation is mediated by three conserved glutamates in the C termini (CT) of Orai2 and Orai3, which show prominent fast Ca^{2+}-dependent inactivation compared with Orai1. Transfer of the CT between the Orais transfers both the extent of channel opening and the mode of fast Ca^{2+}-dependent inactivation. Fast Ca^{2+}-dependent inactivation of the Orais also requires a domain of STIM1; fragments of STIM1 that efficiently open Orai channels do not evoke fast inactivation unless they include an anionic sequence that is C-terminal to the STIM1-Orai activating region (SOAR). Our studies suggest that Orai CT are necessary and sufficient to control pore opening and uncover the molecular mechanism of fast Ca^{2+}-dependent inactivation that has implications for Ca^{2+} influx by SOC in physiological and pathological states.

calcium | fast inactivation | STIM1

The receptor-evoked Ca^{2+} signal entails Ca^{2+} release from the endoplasmic reticulum (ER) that is followed by activation of plasma membrane store-operated Ca^{2+} influx channels (SOCs) (1, 2). Ca^{2+} entry through SOCs sustains the Ca^{2+} response to mediate cellular functions, and when aberrant leads to cell toxicity and cell death (2, 3). The molecular nature of the SOCs and their gating by Ca^{2+} stored in the ER have been elucidated recently with the discovery of STIM1 (4, 5) and the Orai channels (6–8). STIM1 is a single transmembrane spanning, multidomain Ca^{2+}-binding protein. The STIM1 N-terminal EF hand and SAM domains reside in the ER lumen whereas the C-terminal ERM (ezrin/radixin/moesin), S/P-rich and polybasic lysine (K)-rich domains reside in the cytoplasmic face of the ER (9). Ca^{2+} binding to the EF hand maintains STIM1 in a monomeric state in the ER (10). Upon Ca^{2+} release from the ER, Ca^{2+} dissociates from the EF hand and STIM1 clusters next to the plasma membrane to activate SOCs (4, 5).

The two-channel families that are activated by STIM1 and function as SOCs are the TRPC (TRPCs) (11–13) and Orai channels (Orais) (7, 14). We have shown recently that the STIM1 ERM domain binds the TRPCs (11) to heteromultimerize them (12) and allow gating of the TRPCs by electrostatic interaction between STIM1(K684,K685) and TRPC1(D639,D640) (13). However, the Orai channels are gated by the STIM1 SOAR domain, which resides within the ERM domain, and interacts with the Orai1 C terminus to open the channels (15–17). The polybasic K-domain of STIM1 also regulates Orai1 by interaction with the Orai1 N-terminal proline-rich domain (16).

The Orai channels function as highly selective Ca^{2+} channels and mediate the Ca^{2+}-release activated Ca^{2+} (CRAC) current (7, 14). A defining and important regulatory feature on the CRAC current is fast inactivation by Ca^{2+} with millisecond time constants (2, 18, 19) that likely limits Ca^{2+} influx to prevent cell toxicity (2). Previous work reported distinct fast Ca^{2+}-dependent inactivation by the Orais, with Orai1, Orai2, and Orai3 showing minimal, moderate and very prominent fast Ca^{2+}-dependent inactivation, respectively (20). Recently, prominent fast Ca^{2+}-dependent inactivation of Orai1 was observed when Orai1 was expressed with excess STIM1 (21). However, the molecular mechanism of the fast Ca^{2+}-dependent inactivation and the role of STIM1 and Orai domains in the inactivation are not known. In the present work, we examined fast Ca^{2+}-dependent inactivation and found that although both STIM1 and SOAR are capable of activating Orai channels, SOAR does not evoke Ca^{2+}-dependent inactivation. A strategy of swapping the C termini (CT) between the Orai channels and mutation analysis was used to study the role of the CT in fast Ca^{2+}-dependent inactivation and gating of Orai channels. Remarkably, the Orai1 C terminus opens Orai2 and Orai3 much better than native CT. Because STIM1 activates Orai via interactions with the C terminus, these findings provide conclusive evidence that interaction of SOAR with the Orai CT is necessary and sufficient to open the channels. The C-terminal chimeras further showed that fast Ca^{2+}-dependent inactivation is a property that is conferred by the Orai CT. We identified three glutamates that are conserved in the CT of Orai3 and Orai2, but not in Orai1, which are required for fast Ca^{2+}-dependent inactivation. Surprisingly, structure-function studies identified a string of negative charges in STIM1 (478–484) that are downstream of SOAR that are also required for fast Ca^{2+}-dependent inactivation. These findings define the role of the Orai CT in channels opening, and identify the molecular mechanism of fast Ca^{2+}-dependent inactivation of the Orai channels relevant for understand its role in regulation of Ca^{2+} influx in physiological and pathological conditions.

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Results and Discussion

The Orai Channel CT Mediate Channel Gating. To study the role of the Orai CT in channel gating and fast Ca^{2+}-dependent inactivation we swapped their CT and asked whether gating and fast Ca^{2+}-dependent inactivation are transferred with the CT. We focused on the CT because SOAR (16) and similar STIM1 domains (15, 17) interact with Orai1 C terminus to activate the channel and because SOAR fully activates (Δ1–73)Orai1 (16). All of the Orai chimeras used in the present studies, except

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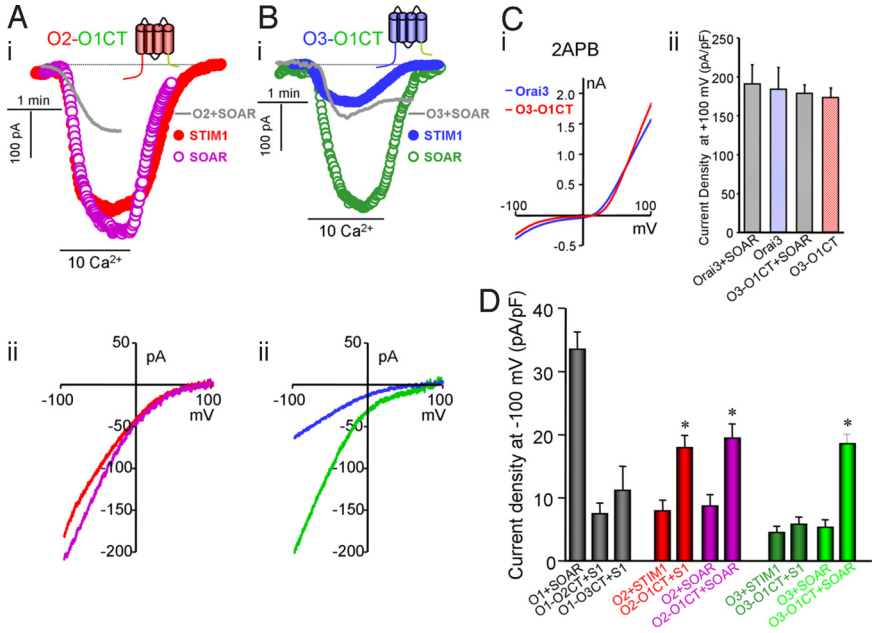

Fig. 1. Orai1 C terminus potently opens Orai2 and Orai3. The CRAC current was measured in HEK cells transfected with the indicated chimera. (*Ai*) Current mediated by Orai2-Orai1CT and STIM1 (●) or SOAR (○). The gray trace shows example of typical current mediated by Orai2+STIM1. (*Aii*) Examples I/V for Orai2-Orai1CT with STIM1 (red) and SOAR (purple). (*Bi*) Current mediated by Orai3–Orai1 CT and STIM1 (●) or SOAR (○). The gray trace shows example of typical current mediated by Orai3+STIM1. (*Bii*) Examples I/V for Orai3–Orai1 CT with STIM1 (blue) and SOAR (green). (*Ci*) I/V of the current recorded in cells transfected with STIM1 and Orai3 (blue) or Orai3–Orai1 CT (red) activated by 50 μM 2-ABP. (*Cii*) Mean ± SEM of four experiments at each condition. (*D*) Mean ± SEM of 4–6 experiments of the current density recorded under the indicated conditions. *, $P < 0.01$ relative to the respective control.

Orai3–Orai1 CT, expressed normally (Fig. S1*a*), similarly coimmunoprecipitated with STIM1 (Fig. S1*a*) and were targeted to the cell surface (Fig. S1*b*). All chimeras showed coclustering with STIM1 in response to store depletion (Fig. S2). Hence, effects of swapping the CT are not due to altered expression, targeting, or interaction with STIM1.

Orai1–Orai2 CT and Orai1–Orai3 CT showed low channel activity (Fig. 1*D*), more typical of the current density of Orai2 and Orai3, respectively. More important, strikingly, the Orai1 C terminus opened Orai2 and Orai3 much better than their CT (Fig. 1). Thus, the current density mediated by the Orai2–Orai1 CT chimera was 2-fold higher than that of Orai2 when activated by STIM1 or by SOAR (Fig. 1 *A* and *D*). The current density of the Orai3–Orai1 CT chimera was similar to that mediated by Orai3 when activated by STIM1. By contrast, SOAR markedly activated Orai3–Orai1 CT with current ≈3-fold larger than the current mediated by Orai3 activated by SOAR. Replacing the Orai3 C terminus with the Orai1 C terminus had no apparent effect on the properties of the channel (Fig. 1*Bii*). Moreover, the Orai1 C terminus does not act on the Orai3 pore, but rather functions to open the Orai3 pore better than the Orai3 C terminus. This is concluded from the findings in Fig. 1*C* in which 2APB, which directly activates Orai3 by acting on the Orai3 pore (22–24), similarly activates Orai3 and Orai3-Orai1 CT without affecting the channel current/voltage relationship.

The findings in Fig. 1, showing that the extent of channel opening is determined by the C terminus, have several implications: i) the C terminus of Orais function as the gate that opens the channel; ii) interactions of STIM1 and SOAR with the C terminus are necessary and sufficient to open the channels and interactions of other STIM1 domains with Orai1 serve only to modulate channel opening by the CT; and iii) the Orai2 and Orai3 pores can conduct large CRAC currents. The conductance of these channels pore is determined by their CT rather than intrinsic properties of the pores.

Fast Ca^{2+}-Dependent Inactivation Transfers with the CT. The chimeras were then used to determine their mode of fast Ca^{2+}-dependent inactivation. Fast Ca^{2+}-dependent inactivation was measured after maximal activation of the channels by store depletion with the slow Ca^{2+} buffer EGTA (2, 19, 25). The protocol used throughout to record fast Ca^{2+}-dependent inactivation is stepping the membrane potential from a holding potential of 0 mV to negative potentials between −100 to −20 mV for 200 ms in 20-mV increments. Fig. 2*A* shows that transferring the C terminus of Orai2 to Orai1 (Orai1–Orai2 CT) resulted in a channel with an Orai2 mode of fast Ca^{2+}-dependent inactivation (20). Moreover, transferring the Orai1 C terminus to Orai2 (Orai2–Orai1 CT, Fig. 2*B*) or Orai3 (Orai3–Orai1 CT, Fig. 2*C*) resulted in channels with no fast Ca^{2+}-dependent inactivation, typical of the Orai1 mode.

Truncation of the Orai1 C terminus (15, 16) and replacing the entire Orai1 C terminus with that of Orai3 resulted in inactive channel. Therefore, we first determined the extent to which Orai1 can be truncated without losing channel activity. Fig. 2*D* shows that Orai1 (1–283) (Orai1×283) is a fully active channel when activated by STIM1 (or SOAR). Unexpectedly, Orai1×283 gained modest fast Ca^{2+}-dependent inactivation that is similar to the inactivation observed with Orai2 (Fig. 2*F*) and with the native CRAC current (2, 19, 25). This raises the possibility that the last 18 residues of Orai1 mask fast Ca^{2+}-dependent inactivation when measured at comparable Orai1-STIM1 expression levels. Further studies are required to clarify this point. Orai1×283 was used to generate chimeras with the Orai3 C terminus (Orai1×283-Orai3CT, Fig. 2*E*). Orai1-Orai3CT showed prominent fast Ca^{2+}-dependent inactivation typical of Orai3 (Fig. 2 *E* and *F*). Hence, fast Ca^{2+}-dependent inactivation resides at the CT of the Orais.

Fast Ca^{2+}-Dependent Inactivation Is Mediated by Three Conserved CT Glutamates. We reasoned that because Orai2 and Orai3, but not Orai1, show fast Ca^{2+}-dependent inactivation, inactivation might

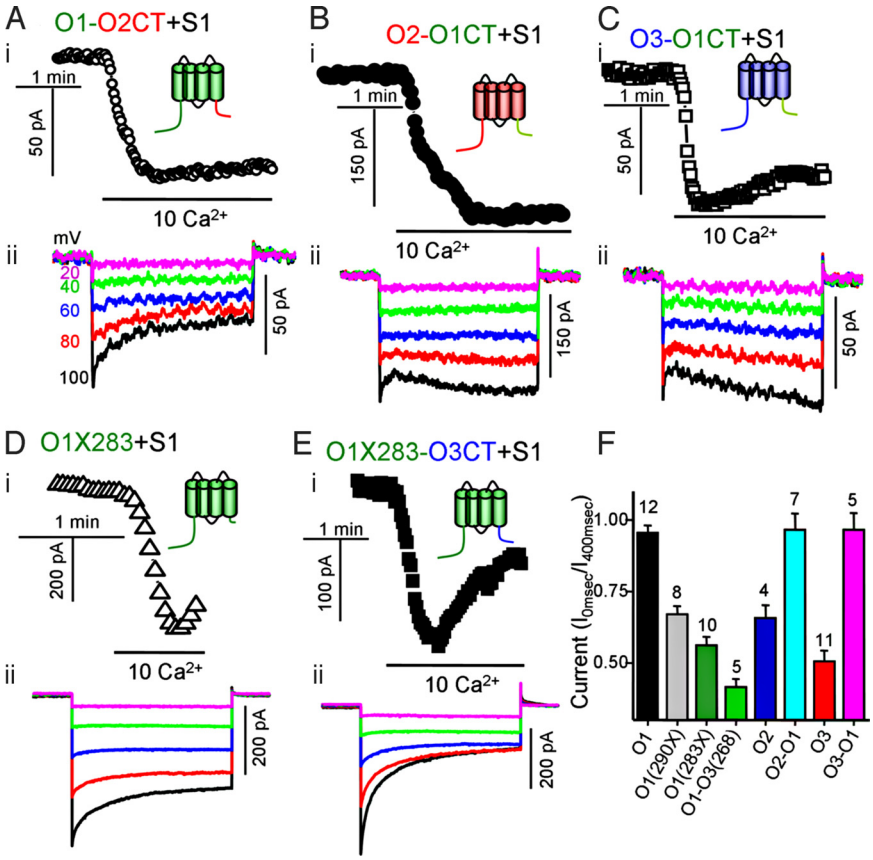

Fig. 2. Fast Ca^{2+}-dependent inactivation transfers with the Orais CT. CRAC current was measured in HEK cells transfected with STIM1 and Orai1-Orai2CT (*Ai* and *Aii*), Orai2-Orai1CT (*Bi* and *Bii*), Orai3-Orai1CT (*Ci* and *Cii*), Orai1×283 (*Di* and *Dii*) or Orai1×283-Orai3CT (*Ei* and *Eii*). All *i* images show the time course of current activation. At the peak current, fast Ca^{2+}-dependent inactivation was measured as detailed in *Methods* (*ii*). (*F*) Mean ± SEM of the indicated number of experiments. Extent of inactivation was calculated from the current measured at −100 mV as detailed in methods. All Orai1 mutants are different from Orai1 at $P < 0.001$, Orai2–Orai1 CT is different from Orai2 at $P = 0.04$ and Orai3–Orai1 CT is different from Orai3 at $P < 0.001$.

be mediated by negative charges that are conserved in the CT of Orai2 and Orai3. Glutamates E233, E235, and E236 of Orai2 are conserved and correspond to E281, E283, and E284 of Orai3. Orai3, which displays the most prominent fast Ca^{2+}-dependent inactivation, has additional negative charges at D273 and E291. We systematically mutated these sites to alanine and examined the effect of neutralizing the charges on fast Ca^{2+}-dependent inactivation. Figs. 3 *A* and *I* show that Orai2(E235,236A) displays nearly normal fast Ca^{2+}-dependent inactivation. However, the extent and rate of fast Ca^{2+}-dependent inactivation of Orai2(E233,235,236A) was reduced (Fig. 3 *B* and *I*). Inactivation of Orai2 proceeded along 2 exponentials with $\tau_1 = 9.7 \pm 1.3$ and $\tau_2 = 65.3 \pm 5.4$ whereas inactivation of Orai2(E233,235,236A) is described by a short fast and a long slow exponentials with $\tau_1 = 7.6 \pm 1.4$ and $\tau_2 = 62.6 \pm 7.4$ that are significantly different from those of Orai2.

Fig. 3*C* shows the typical Orai3 fast Ca^{2+}-dependent inactivation whereas Fig. 3 *D–G* and the summary in Fig. 3*I* show that Orai3(D273A), Orai3(E291A), Orai3(E281A), and Orai3(E283,284A) have normal fast Ca^{2+}-dependent inactivation. However, mutating all glutamates to alanines [Orai3(E281,283,284A)] largely eliminated the fast Ca^{2+}-dependent inactivation. Together, these observations suggest that the glutamates conserved in Orai2 and Orai3 form a motif that mediates fast Ca^{2+}-dependent inactivation. One of the glutamates is not conserved in Orai1, which may account in part for the lack of fast Ca^{2+}-dependent inactivation with this Orai isoform.

STIM1 (475–483) Is Required for Fast Ca^{2+}-Dependent Inactivation of the Orai Channels. The isoform specific fast Ca^{2+}-dependent inactivation of the Orais when activated by STIM1 and expressed at a 1:1 STIM1:Orai1 ratio (20) is confirmed in Figs. 4 *A*, *C*, and *E*. Hence, Orai1 (Fig. 4*Aii*) shows no inactivation, Orai2 (Fig. 4*Cii*) is partially inactivated whereas Orai3 (Fig. 4*Eii*) strongly inactivates, particularly at negative membrane potentials.

Recently, we showed that the STIM1 domain SOAR [STIM1 (344–442)] fully activates all Orai channels and recapitulates all kinetic properties of Orai1 (16). Here, we asked whether Orai channels activated by SOAR also undergo fast Ca^{2+}-dependent inactivation. Surprisingly, Orai1 (Fig. 4*Bii*), Orai2 (Fig. 4*Dii*), and Orai3 (Fig. 4*Fii*) activated by SOAR show no or minimal fast Ca^{2+}-dependent inactivation. This suggests that a sequence in STIM1 may participate in fast Ca^{2+}-dependent inactivation. To identify this sequence we tested the effect of STIM1 fragments longer than SOAR searching for the minimal STIM1 fragment that retains fast Ca^{2+}-dependent inactivation of Orai3. The search revealed that extending SOAR up to STIM1 (344–470) does not restore the inactivation (Fig. 4*Gii*) whereas full fast Ca^{2+}-dependent inactivation is observed with STIM1 (344–485) (Fig. 4*Hii*). Fast Ca^{2+}-dependent inactivation of Orai3 observed with STIM1 (344–485) is reduced by the fast Ca^{2+} buffer BAPTA (Fig. S3), indicating that the fast inactivation is mediated by Ca^{2+}. The STIM1 region 470HFIMT**DDV**D**D**M**DEE**IV485 has high density of negative charges (highlighted in bold) that may participate with fast Ca^{2+}-dependent inactivation.

The role of the charges in STIM1 (475–483) was studied by

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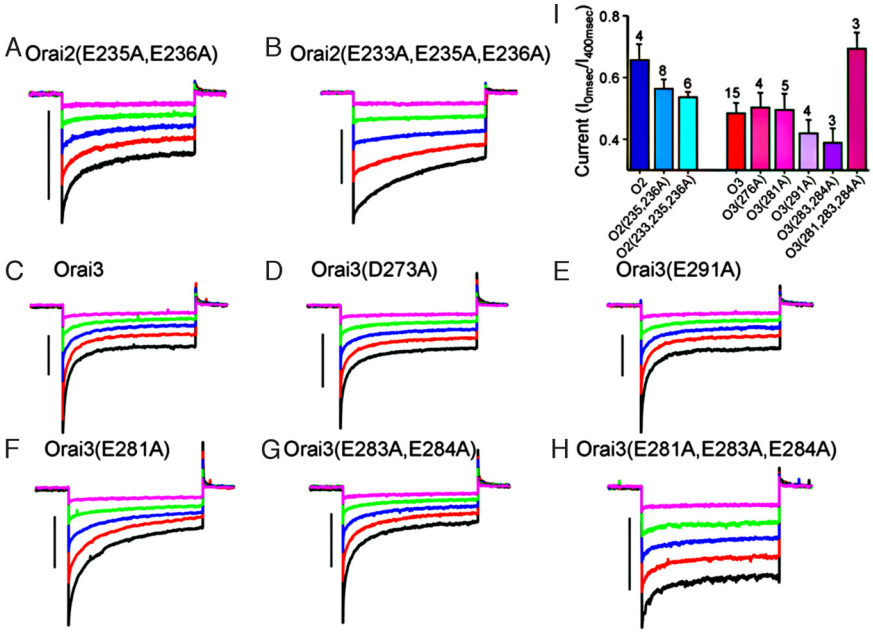

Fig. 3. Fast Ca^{2+}-dependent inactivation is mediated by conserved C-terminal glutamates. CRAC current was measured in HEK cells transfected with STIM1 and the indicated Orai2 (*A* and *B*) and Orai3 mutants (*C–H*). Vertical bars next to the current traces mark 100 pA. The mean ± SEM of current inactivation is shown in *I*. *, $P < 0.05$ for Orai2(E233A,E235A,E236A) relative to Orai2 and $P < 0.01$ for Orai3(E281,E283A,E284A) relative to Orai3.

their neutralizing in full-length STIM1 (Fig. 5). Mutating (D475,476A) (Fig. 5*A*) and (D478,479A) (Fig. 5*B*) alone had minimal effect whereas mutating all 4 aspartates (Fig. 5*C*) significantly reduced fast Ca^{2+}-dependent inactivation of Orai3. Similar reduced fast Ca^{2+}-dependent inactivation of Orai3 was observed with the mutants STIM1(D481A,E482A,E483A) and STIM1(D475,476,478,479A) (Fig. 5*D*). Although neutralizing all 7 charges in STIM1 (475–483) resulted in STIM1 mutant that poorly activated Orai3 (≈30% of WT), this mutant also showed minimal fast Ca^{2+}-dependent inactivation (Fig. 5*E*). The residual fast Ca^{2+}-dependent inactivation observed with Orai2(E233,235,236A) (Fig. 3 *B* and *I*) was eliminated when Orai2(E233,235,236A) was activated by either STIM1(D481A,E482A,E483A) (Fig. 5*F*) or STIM1(D475,476,478,479A) (Fig. 5*G*). These findings suggest that the STIM1 475**DDVDDMDEE**483 sequence participates in formation or stabilizes an interaction of STIM1 with Orais that mediates fast Ca^{2+}-dependent inactivation.

Conclusions

SOC channels mediate many physiological and pathological effects of Ca^{2+}. As such, their activity must be regulated by

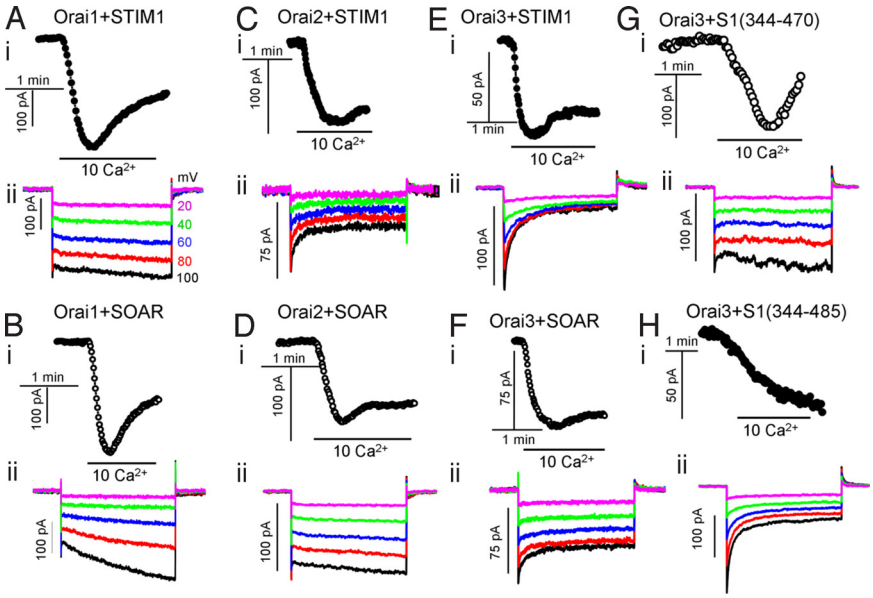

Fig. 4. Fast Ca^{2+}-dependent inactivation is observed when the Orais are activated by STIM1 but not by SOAR. CRAC current was measured in HEK cells transfected with STIM1 (*A*, *C*, and *E*) or SOAR (*B*, *D*, and *F*) and Orai1 (*A* and *B*), Orai2 (*C* and *D*) or Orai3 (*E* and *F*). CRAC current was also measured in cells transfected with Orai3 and STIM1 (344–470) (*G*) or STIM1 (344–485) (*H*). The time course of the currents is shown in *i* images and the instantaneous current at the indicated voltages is show in images *ii*. Similar results were obtained in 4–15 experiments under each condition.

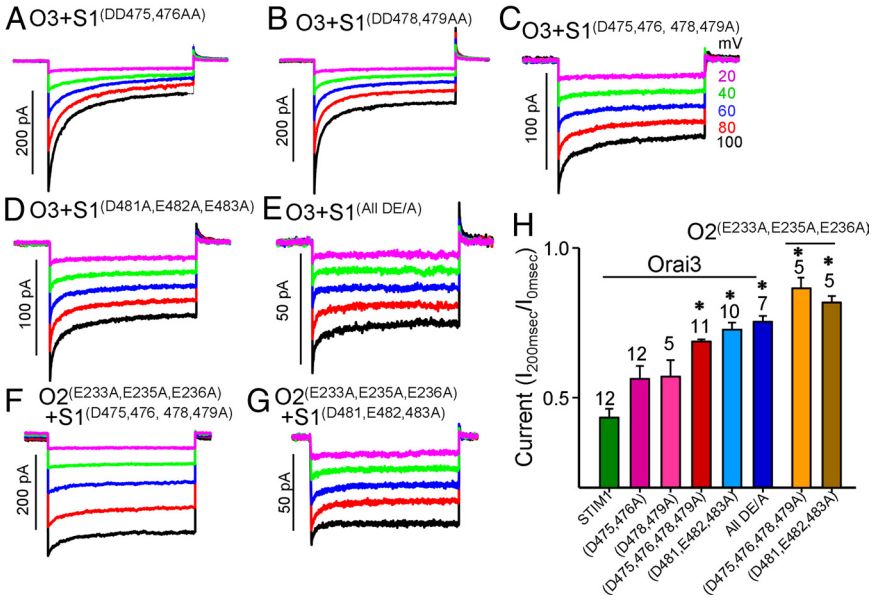

Fig. 5. STIM1 (475–483) is required for fast Ca^{2+}-dependent inactivation. CRAC current was measured in HEK cells transfected with Orai3 (*A–E*) or Orai2(E233A,E235A,E236A) (*F* and *G*) and the indicated STIM1 mutants. The mean ± SEM of current inactivation is shown in *H*. *, $P < 0.01$ relative to STIM1.

limiting the amount of Ca^{2+} entering the cells upon their opening. An important regulatory mechanism is inhibition of the channels by Ca^{2+}, a phenomenon known as fast Ca^{2+}-dependent inactivation (2, 19, 25). The present study identified 3 conserved glutamates in the C terminus of Orai2 and Orai3 that mediates channels inactivation. It was quite unexpected that negative charges in STIM1 are also required for the fast Ca^{2+}-dependent inactivation. At present it is not clear how the charges in Orais and STIM1 coordinate fast Ca^{2+}-dependent inactivation because neither sequence is predicted to fold into an established Ca^{2+}-binding site. Presumably, the Orais and STIM1 sequences function as part of a sensor of intracellular Ca^{2+}. The similar reduction of fast Ca^{2+}-dependent inactivation by neutralizing STIM1(D475,476,478,479) or STIM1(D481,E482,E483) without affecting extent of channel activation by these mutants suggests that all charges in 475**DD**V**DD**M**DEE**483 contribute to the mechanism. One intriguing possibility is that aspartates in Orai and STIM1 play a direct role in chelation of cytosolic Ca^{2+} at a site close to the Orai channel.

The native CRAC current shows prominent fast Ca^{2+}-dependent inactivation (2, 19, 25), and deletion of Orai1 eliminates the CRAC current (6–8). Yet, when expressed at comparable levels with STIM1, Orai1 shows minimal fast Ca^{2+}-dependent inactivation [(21) and the present work]. A possible explanation for this conundrum is that the native CRAC comprises a combination of Orai1–Orai2 or Orai1–Orai3. However, an alternative explanation is that Orai1 does undergo fast Ca^{2+}-dependent inactivation (21), but an Orai1 domain within its last 18 residues functions to inhibit the fast Ca^{2+}-dependent inactivation. Truncation of the 18 residues resulted in fast Ca^{2+}-dependent inactivation of Orai1 (Fig. 2*D*) that is similar to that observed with the native CRAC. Reconstitution of Orai1 fast Ca^{2+}-dependent inactivation may require interaction of another protein or factor with the 18 residues inhibitory domain. This may not be observed with the over-expressed channels, perhaps by a limited level of the regulatory factor.

The spatial localization of the Orais-STIM1 dependent Ca^{2+} sensor is not known with certainty. However, it must be close to the channel pore, because the Ca^{2+} inhibiting the channel passes through the channel and its access to the inhibitory site can be controlled by the Ca^{2+} buffer (19, 25). Thus, fast Ca^{2+} buffers like BAPTA markedly reduce fast Ca^{2+}-dependent inactivation compared with slow Ca^{2+} buffers like EGTA (see Fig. S3). An important implication of this model is that the entire C terminus of the Orais that gates the channels is in close spatial proximity and may interact with the pore. Thus, the C-terminal sequence upstream of the conserved Orai glutamates binds SOAR whereas the domain containing the glutamates interact with STIM1 (475–483) that are downstream of SOAR. When the stores are filled, the Orai CT keep the channel pores in a closed state. Store depletion facilitates interaction of SOAR with Orais C terminus to open the channels. At the same time STIM1 (475–483) and the conserved Orai glutamates act as a Ca^{2+} sensor adjacent to the SOAR-Orais interacting site to rapidly reduce channel activity.

Materials and Methods

Cell Culture, Reagents, Clones, and Mutations. The human Orai1, Orai2, Orai3, and STIM1 clones and the SOAR and various STIM1 fragments were described (11–13, 16). All point mutations on STIM1 and Orai channels were generated using the site-directed mutagenesis kit from Stratagene. The antibodies used were monoclonal HRP-conjugated anti-GFP (Santa Cruz Biotechnology) and monoclonal anti-FLAG and HRP-conjugated anti-FLAG (Sigma–Aldrich). Anti-FLAG antibodies were used for co-IP whereas HRP-conjugated anti-GFP and anti-FLAG antibodies were used for western blot analysis. HEK293 cells were transfected with plasmid for 6 h. The total amount of cDNA used per 35-mm dish was 0.5 μg/mL. Localization assays using confocal microscopy were done, current was measured, or cells were harvested and extracted for coimmunoprecipitation/biotinylation analysis the following day.

Immunoprecipitation, Biotinylation, and Western Blot Analysis. Transfected cells were washed once with 1× PBS on ice. EZ-Link Sulfo-NHS-SS-Biotin (0.5 mg/mL, Pierce) was added to the cells for 30 min on ice. Afterward, the biotin was quenched with 50 mM glycine on ice for 10–15 min. The cells were then harvested and lysed using 500 μL binding buffer: 1× PBS buffer containing 1 mM $NaVO_3$, 10 mM sodium pyrophosphate, 50 mM NaF (pH 7.4), and 1% Triton X-100. The cell extracts were sonicated, and insoluble material was spun down at 30,000 × *g* for 20 min. For the co-IP experiments, 1 μg of FLAG antibody was added to 100 μL cell extract and incubated for 1 h at 4 °C. Then, 50 μL of 1:1 slurry of protein G Sepharose 4B beads were added to the antibody-extract mix and incubated for an additional h at 4 °C. For the biotinylation assay, 50 μL of 1:1 slurry of immobilized neutravidin beads (Pierce) were added to 100 μL cell extract and incubated for 2 h at 4 °C. Beads were washed 3 × 10 min with binding buffer, proteins were released from the beads with 50 μL SDS-loading buffer. Five microliters were loaded onto 8%

Tris-glycine SDS/PAGE gels and blotted for Orai (FLAG) whereas 20 μL were loaded onto a separate gel and blotted for STIM1 (GFP). Gels were transferred onto PVDF membrane, and western blot analysis was done.

Confocal Imaging. After HEK cells grown on glass coverslips were transfected with the eGFP-STIM1 or fragments and mCherry-Orai WT or chimeras, the cells were treated with 25 μM CPA in Ca^{2+}-free medium for 10 min to deplete the stores, fixed with 4% paraformaldehyde, and mounted onto microscope slides. Images were collected under 400× magnification using the LaserSharp 2000 (Bio-Rad) software and a Bio-Rad confocal microscope.

Current Measurements. The whole-cell configuration was used to measure the Orai CRAC current in HEK cells cotransfected with Orais and with STIM1, SOAR, or STIM1 fragments, as detailed (13, 16). The standard pipette solution contained (in mM): 140 Cs aspartate, 6 $MgCl_2$, 10 EGTA, and 10 Hepes (pH 7.2 with CsOH), expect in Fig. 1 in which EGTA was replaced with 10 BAPTA. The standard bath solution contained (in mM): 130 NaCl, 5 KCl, 10 $CaCl_2$, 1 $MgCl_2$, and 10 Hepes (pH 7.4 with NaOH). To measure current-voltage relationship (I/V), 200 ms RAMP plus from −100 mV to + 100 mV were applied every 2 s and Ca^{2+} current were compared at −100 mV after normalization by capacitance (pA/pF). The current recorded at −100 mV was used to plot the time course of current development. Inactivation studies were performed by 200-ms voltage steps ranging from −100 mV to −20 mV in 20 mV increments from a holding potential of 0 mV. After establishing the whole cell configuration, current was gradually developed as a result of chealation of intracellular Ca^{2+} by EGTA and intracellular Ca^{2+} store depletion. Step voltage pulses were applied when current was fully developed. Peak currents were taken from the current recorded 3 ms after the start of the pulse to minimize contributions from capacitative current. Steady state currents were measured at the end of the pulse. The current traces evoked by the −100 mV pulse were fitted to a double exponential function to analyze the kinetic parameters as follows: $y = y_0 + A_1e^{-t/\tau 1} + A_2e^{-t/\tau 2}$.

Where y_0 is the steady state current, A1 and A2 are the relative amplitude of the fast and slow exponentials, respectively, and τ1 and τ2 are the time constants of the corresponding exponentials. The averages of multiple experiments are given as mean ± SEM of the number of experiments performed. Differences between groups were analyzed by unpaired Student *t* test and significance are considered at *P* values <0.05.

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1. Kiselyov K, Wang X, Shin DM, Zang W, Muallem S (2006) Calcium signaling complexes in microdomains of polarized secretory cells. *Cell Calcium* 40:451–459.
2. Parekh AB, Putney JW, Jr (2005) Store-operated calcium channels. *Physiol Rev* 85:757–810.
3. Berridge MJ, Bootman MD, Roderick HL (2003) Calcium signalling: Dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* 4:517–529.
4. Liou J, et al. (2005) STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx. *Curr Biol* 15:1235–1241.
5. Roos J, et al. (2005) STIM1, an essential and conserved component of store-operated Ca2+ channel function. *J Cell Biol* 169:435–445.
6. Feske S, et al. (2006) A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature* 441:179–185.
7. Zhang SL, et al. (2006) Genome-wide RNAi screen of Ca(2+) influx identifies genes that regulate Ca(2+) release-activated Ca(2+) channel activity. *Proc Natl Acad Sci USA* 103:9357–9362.
8. Vig M, et al. (2006) CRACM1 is a plasma membrane protein essential for store-operated Ca2+ entry. *Science* 312:1220–1223.
9. Dziadek MA, Johnstone LS (2007) Biochemical properties and cellular localisation of STIM proteins. *Cell Calcium* 42:123–132
10. Stathopulos PB, Zheng L, Li GY, Plevin MJ, Ikura M (2008) Structural and mechanistic insights into STIM1-mediated initiation of store-operated calcium entry. *Cell* 135:110–122.
11. Huang GN, et al. (2006) STIM1 carboxyl-terminus activates native SOC, I(crac) and TRPC1 channels. *Nat Cell Biol* 8:1003–1010.
12. Yuan JP, Zeng W, Huang GN, Worley PF, Muallem S (2007) STIM1 heteromultimerizes TRPC channels to determine their function as store-operated channels. *Nat Cell Biol* 9:636–645.
13. Zeng W, et al. (2008) STIM1 gates TRPC channels, but not Orai1, by electrostatic interaction. *Mol Cell* 32:439–448.
14. Peinelt C, et al. (2006) Amplification of CRAC current by STIM1 and CRACM1 (Orai1). *Nat Cell Biol* 8:771–773.
15. Park CY, et al. (2009) STIM1 clusters and activates CRAC channels via direct binding of a cytosolic domain to Orai1. *Cell* 136:876–890.
16. Yuan JP, et al. (2009) SOAR and the polybasic STIM1 domains gate and regulate Orai channels. *Nat Cell Biol* 11:337–343.
17. Muik M, et al. (2009) A cytosolic homomerization and a modulatory domain within STIM1 C terminus determine coupling to ORAI1 channels. *J Biol Chem* 284:8421–8426.
18. Parekh AB (2003) Mitochondrial regulation of intracellular Ca2+ signaling: More than just simple Ca2+ buffers. *News Physiol Sci* 18:252–256.
19. Zweifach A, Lewis RS (1995) Rapid inactivation of depletion-activated calcium current (ICRAC) due to local calcium feedback. *J Gen Physiol* 105:209–226.
20. Lis A, et al. (2007) CRACM1, CRACM2, and CRACM3 are store-operated Ca(2+) channels with distinct functional properties. *Curr Biol* 17:794–800.
21. Scrimgeour N, Litjens T, Ma L, Barritt GJ, Rychkov GY (2009) Properties of Orai1 mediated store-operated current depend on the expression levels of STIM1 and Orai1 proteins. *J Physiol* 587:2903–2918.
22. Peinelt C, Lis A, Beck A, Fleig A, Penner R (2008) 2-Aminoethoxydiphenyl borate directly facilitates and indirectly inhibits STIM1-dependent gating of CRAC channels. *J Physiol* 586:3061–3073.
23. Schindl R, et al. (2008) 2-aminoethoxydiphenyl borate alters selectivity of Orai3 channels by increasing their pore size. *J Biol Chem* 283:20261–20267.
24. Zhang SL, et al. (2008) Store-dependent and -independent modes regulating Ca2+ release-activated Ca2+ channel activity of human Orai1 and Orai3. *J Biol Chem* 283:17662–17671.
25. Bakowski D, Parekh AB (2007) Regulation of store-operated calcium channels by the intermediary metabolite pyruvic acid. *Curr Biol* 17:1076–1081.