Gated regulation of CRAC channel ion selectivity by STIM1

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Two defining functional features of ion channels are ion selectivity and channel gating. Ion selectivity is generally considered an immutable property of the open channel structure, whereas gating involves transitions between open and closed channel states, typically without changes in ion selectivity1. In store-operated Ca2+ release-activated Ca2+ (CRAC) channels, the molecular mechanism of channel gating by the CRAC channel activator, stromal interaction molecule 1 (STIM1), remains unknown. CRAC channels are distinguished by a very high Ca2+ selectivity and are instrumental in generating sustained intracellular calcium concentration elevations that are necessary for gene expression and effector function in many eukaryotic cells2. Here we probe the central features of the STIM1 gating mechanism in the human CRAC channel protein, ORAI1, and identify V102, a residue located in the extracellular region of the pore, as a candidate for the channel gate. Mutations at V102 produce constitutively active CRAC channels that are open even in the absence of STIM1. Unexpectedly, although STIM1-free V102 mutant channels are not Ca2+-selective, their Ca2+ selectivity is dose-dependently boosted by interactions with STIM1. Similar enhancement of Ca2+ selectivity is also seen in wild-type ORAI1 channels by increasing the number of STIM1 activation domains that are directly tethered to ORAI1 channels, or by increasing the relative expression of full-length STIM1. Thus, exquisite Ca2+ selectivity is not an intrinsic property of CRAC channels but rather a tunable feature that is bestowed on otherwise non-selective ORAI1 channels by STIM1. Our results demonstrate that STIM1-mediated gating of CRAC channels occurs through an unusual mechanism in which permeation and gating are closely coupled.

Functional CRAC channels are tetramers of ORAI1 subunits3–5, with the pore flanked by residues of the first transmembrane domain (TM1) of each subunit6–7 (Fig. 1a). To localize the gate region that governs STIM1-dependent activation, we mutated individual pore-lining residues to Cys and analysed state-dependent differences in the sensitivity of mutant channels to methanethiosulphonate (MTS) Cys-reactive reagents6. Because the unusually narrow CRAC channel lining residues to Cys and analysed state-dependent differences in potential candidates for the gate, E106 controls Ca2+ selectivity13,14 and is not thought to regulate store-operated gating15, leaving V102 as the most promising residue for further study.

Previous reports suggest that V102 is located very close to the central symmetry axis of the channel10, that is, in a narrow constriction of the pore. If V102 is a component of the gating mechanism, mutations at this locus would be predicted to destabilize channel gating. Consistent with this possibility, a Cys mutation of V102 eliminated store-dependent gating. Cells expressing V102C ORAI1 and STIM1 displayed a large standing ICRAC after whole-cell break-in (Fig. 2a). Moreover, resting cells exhibited constitutive Ca2+ entry and activation of the Ca2+ -dependent nuclear factor of activated T cell (NFAT) transcription factor (Supplementary Fig. 2), indicating that V102C ORAI1 channels are constitutively active.

Several lines of evidence indicated that the constitutive activation of V102C ORAI1 is STIM1-independent. Large La3+-sensitive standing currents were seen in cells expressing V102C ORAI1 alone (Fig. 2b). Furthermore, Ca2+ imaging and NFAT activation experiments revealed constitutive Ca2+ entry in these cells (Fig. 2c and Supplementary Fig. 2c). Recent evidence indicates that STIM1 drives the redistribution of ORAI1 into discrete puncta after endoplasmic reticulum (ER) store depletion16. However, when expressed alone, V102C ORAI1 remained diffusely distributed in the plasma membrane (Fig. 2d).

Moreover, ICRAC in these cells did not show Ca2+-dependent fast inactivation (CDI) (Supplementary Fig. 3). Because puncta formation and CDI require STIM1 (ref. 14–16), these results indicate that when over-expressed alone, the mutant channels are functionally free of STIM1. Consistent with this interpretation, knockdown of endogenous STIM1 in HEK293 cells did not affect the constitutive V102C current (Supplementary Table 1). However, when V102C ORAI1 was co-expressed with STIM1, puncta formation, interaction with STIM1, and CDI were indistinguishable from the behaviour of wild-type ORAI1 (Fig. 2d and Supplementary Figs 3 and 4). Additional analyses indicated that introducing the mutations E106A or R91W, which abrogate store-operated ORAI1 activity17,18, strongly diminished V102C ORAI1 currents (Supplementary Fig. 5a), indicating that these residues are essential for both store-operated and constitutive activation modes of ORAI1.

Mutation of the equivalent residue in Orai3 (V77C) also resulted in a STIM1-independent activation phenotype similar to that seen in V102C ORAI1 (Supplementary Fig. 6). Together, these results indicate that the V102C mutation destabilizes the channel gate, resulting in STIM1-independent constitutive ORAI1 activation.

Many ion channels including nicotinic acetylcholine receptors and the mechanosensitive channel MscL are reported to use hydrophobic...
Figure 1 | State-dependent accessibility of pore-lining residues localizes the activation gate to the extracellular TM1 region. 

a, Schematic representation of the key pore-lining residues in ORAI1 (refs 6, 7). b, MTSEA modification of G98C is protected by La3+. A HEK293 cell co-expressing G98C/E106D ORAI1 (O1) and STIM1 was exposed to two applications of MTSEA (100 μM), the first in the presence of La3+ (100 μM) and the second after washout of La3+. Periodic applications of a divalent free (DVF, red rectangles) solution facilitated washout of La3+. MTSEA inhibition was quantified by the relief of block induced by BMS (5 mM) (arrows). c, State-dependent modification of G98C. MTSEA (200 μM) was applied for 120 s to resting cells and then washed off. After whole-cell break-in, ICRAC was activated by passive store depletion by dialysing in BAPTA. BMS was applied to examine relief from MTSEA blockade (arrows). A second application of MTSEA and BMS provides a measure of blockade in open channels. A DVF solution was periodically applied to monitor Na+–ICRAC.

Figure 2 | Mutations at V102 cause STIM1-independent constitutive ORAI1 activation.

a, Time course of the development of ICRAC in cells expressing wild-type (WT) or V102C ORAI1 and STIM1 after whole-cell break-in. Intracellular Ca2+ stores were depleted by dialysing cells with 8 mM BAPTA. b, V102C ORAI1 currents are constitutively active in the absence of STIM1 co-expression. c, Intracellular calcium concentration [Ca2+]I measurements in HEK293 cells expressing the indicated ORAI1 constructs in the absence of STIM1. UT, untransfected. d, Localization of V102C ORAI1–cyan fluorescent protein (CFP) before, and after, ER Ca2+ store depletion in the absence (left) or presence (right) of STIM1–yellow fluorescent protein (YFP). e, Mutational analysis of V102. Normalized current densities of V102 substitutions plotted against the solvation energies of the substituted amino acids in the presence or absence of STIM1 co-expression. Currents were normalized to the mutant that yielded the maximal current density for each condition (Ala for STIM1-free cells and Ile in STIM1-co-expressing cells). Green, mutants that yield large constitutively active currents in the absence of STIM1; red, mutants that are not constitutively active but that require STIM1 for activation. TG, thapsigargin.
residues (Leu, Val and Ile) as gates to inhibit the flux of ions\textsuperscript{18-20}. To explore the possibility that V102 comprises a hydrophobic gate in ORAI1, we investigated the side-chain dependence at this position for constitutive activation. We observed constitutive activity with several mildly hydrophobic and polar substitutions, including Cys, Gly, Ala, Ser and Thr (Fig. 2e). Conversely, substitutions to the highly hydrophobic amino acids Leu, Ile and Met resulted in only STIM1-dependent activation, as seen in wild-type ORAI1. Large hydrophobic residues such as Trp, Tyr and Phe attenuated both the constitutive and STIM1-induced currents, probably because of pore occlusion, as expected for a position that is nestled in a narrow region of the pore\textsuperscript{6,7} (Supplementary Fig. 5b). Substitutions to extremely polar residues such as Glu, Asp, Lys and Arg resulted in non-functional channels with or without STIM1, probably owing to secondary effects of these mutations on the nearby selectivity filter at E106. Despite these deviations, however, the overall pattern is consistent with the hypothesis that V102 comprises a hydrophobic gate, with less hydrophobic substitutions producing a leaky gate.

CRAC channels are extraordinarily Ca\textsuperscript{2+} selective and poorly permeable to the large monovalent cation Cs\textsuperscript{+}. However, the ion selectivity of STIM1-free V102C ORAI1 channels differed from wild-type ORAI1 channels in both respects. STIM1-free V102C ORAI1 channels showed significantly lower Ca\textsuperscript{2+} permeability (P<0.0001), as demonstrated by the left-shifted reversal potentials of mutant Ca\textsuperscript{2+} currents (Fig. 3a). Consistent with this interpretation, replacement of extracellular Na\textsuperscript{+} with NMDG\textsuperscript{+}, an impermeant ion, revealed significant Na\textsuperscript{+} conduction in these channels (P<0.0001) (Fig. 3b). Direct estimates of fractional Ca\textsuperscript{2+} currents using fluo-4 indicated that V102C ORAI1 conducts only 36% of the Ca\textsuperscript{2+} carried by wild-type ORAI1 in 20 mM Ca\textsuperscript{2+} (Supplementary Fig. 7a). In addition, unlike wild-type channels, STIM1-free V102C channels were highly permeable to the large monovalent cation Cs\textsuperscript{+} (Fig. 3a–c and Supplementary Table 1).

Unexpectedly, co-expressing exogenous STIM1 together with V102C ORAI1 increased the Ca\textsuperscript{2+} permeability and lowered the Cs\textsuperscript{+} permeability of V102C ORAI1, effectively correcting its aberrant ion selectivity (Fig. 3a–c). STIM1 also modified permeation of V102C ORAI1 for Ba\textsuperscript{2+} and Sr\textsuperscript{2+} (Supplementary Fig. 7b). Modification of ion selectivity by STIM1 was not unique to V102C ORAI1 but occurred in all constitutively active V102X mutants (Supplementary Table 1). These changes in ion selectivity required direct STIM1–ORAI1 interactions, as modification of V102C ORAI1 ion selectivity was nullified in the V102C/L276D ORAI1 double mutant (Fig. 3d), in which STIM1–ORAI1 binding was impaired (Supplementary Fig. 4b, c).\textsuperscript{21} STIM1–ORAI1 binding was impaired (Supplementary Fig. 4b, c).\textsuperscript{21} STIM1–ORAI1 binding was impaired (Supplementary Fig. 4b, c).\textsuperscript{21} STIM1–ORAI1 binding was impaired (Supplementary Fig. 4b, c).\textsuperscript{21} STIM1–ORAI1 binding was impaired (Supplementary Fig. 4b, c).\textsuperscript{21} STIM1–ORAI1 binding was impaired (Supplementary Fig. 4b, c).\textsuperscript{21} STIM1–ORAI1 binding was impaired (Supplementary Fig. 4b, c).\textsuperscript{21} STIM1–ORAI1 binding was impaired (Supplementary Fig. 4b, c).\textsuperscript{21} STIM1–ORAI1 binding was impaired (Supplementary Fig. 4b, c).\textsuperscript{21} STIM1–ORAI1 binding was impaired (Supplementary Fig. 4b, c).

Using concatenated ORAI1 dimers (see Supplementary Methods) indicated that the subunit stoichiometry of STIM1-free and STIM1-bound mutant channels are identical, arguing that their distinct permeation properties are due to different pore structures of fully assembled, tetrameric channels rather than different subunit stoichiometries (Supplementary Fig. 8). Collectively, these results indicate that STIM1 binding modifies the structural features of the mutant channel.

Figure 3 | STIM1 regulates ion selectivity of constitutively active V102C ORAI1 channels. a. Current–voltage (I–V) relationships of V102C ORAI1 currents in 20 mM Ca\textsuperscript{2+} and DVF Ringer’s solutions. Arrows emphasize the reversal potential (V\textsubscript{rev}) in each case. The bar graphs summarize the V\textsubscript{rev} in the presence or absence of STIM1. Values are mean ± s.e.m. b. Effects of substituting extracellular Na\textsuperscript{+} with NMDG\textsuperscript{+} on V102C ORAI1 currents in the absence or presence of STIM1. Values are mean ± s.e.m. c. Effects of replacing the standard extracellular Ringer’s solution with Na\textsuperscript{+}- or Ca\textsuperscript{2+}-based DVF solutions. In the absence of STIM1, large Ca\textsuperscript{2+} currents are seen in V102C ORAI1 channels. By contrast, no Ca\textsuperscript{2+} conduction is observed in the presence of STIM1. d. I–V relationship of currents in the V102C/L276D ORAI1 double mutant in the presence or absence of STIM1. The bar graphs summarize the V\textsubscript{rev} values of this mutant in the presence or absence of STIM1. Values are mean ± s.e.m. e. Relative permeabilities of V102C ORAI1 channels to different organic monovalent cations plotted against the size of each cation (test ion P\textsubscript{x} and Na\textsuperscript{+} P\textsubscript{Na}). Dotted lines are fits to the hydrodynamic relationship. Values of d\textsubscript{pore} (the apparent width of the pore) estimated from the fits are 4.9 Å for V102C ORAI1 + STIM1 channels and 6.9 Å for V102C ORAI1.
pore, bestowing permeation properties that are associated with CRAC channels.

The normalization of ion selectivity of mutant channels by STIM1 suggests that their altered ion selectivity in the absence of STIM1 is not merely a byproduct of the mutations, but that it is indicative of a native intermediate activation state that is not readily seen in wild-type channels owing to a closed gate. Recent studies indicate that ORAI1 channels are activated in a nonlinear and cooperative manner by STIM1, with maximal channel activation requiring binding of eight STIM1 molecules per channel.15,22,23, We reasoned that if modification of ion selectivity is coupled to the stoichiometry of STIM1 binding, then partial activation of wild-type ORAI1 by a sub-saturating concentration of STIM1 may lead to incomplete normalization of ion selectivity, revealing an intermediate activation state. To test this hypothesis, we used constructs in which wild-type ORAI1 was tethered to either one or two functional STIM1 (S or SS) domains (resulting in four or eight S domains per channel, respectively), as recently described.22 We found that wild-type ORAI1 channels tethered to one S domain per subunit produced currents that were smaller and displayed diminished CDI that wild-type ORAI1 channels tethered to one S domain per subunit.24,25,26

Collectively, our results show that mutations at V102 cause constitutive activation of ORAI1 channels through a mechanism that probably involves destabilization of the channel gate at V102. This disposition of the STIM1 activation gate, in the extracellular region of the pore close to the selectivity filter, is markedly different from the familiar structural designs in K+ channels and by extension, voltage-gated Ca2+- (CaV) channels, which are constructed with the gate located at the cytoplasmic end of the pore.24 We exploited the constitutive channel activity that resulted from mutations in the putative gate to identify an unusual ion channel gating mode in which STIM1 regulates ion selectivity and the pore architecture of CRAC channels. Activation by STIM1 bestows several key distinctive characteristics that are associated with CRAC channels including high Ca2+- selectivity, low Cs+ permeability and a narrow pore to otherwise non-selective ORAI1 channels. Although the underlying mechanism remains to be established, the close proximity of the putative STIM1 activation gate (V102) to the selectivity filter (E106) probably contributes to the tight coupling of permeation and gating during channel activation. The altered ion selectivity of ORAI1 channels when STIM1 is limiting is reminiscent of the ion selectivity of ORAI1 and ORAI3 channels directly activated by the compound 2-APB, which exhibit lower Ca2+- selectivity and higher Cs+ selectivity than STIM1-activated ORAI1 channels.19,20,21,22 These findings indicate that the exquisite Ca2+- selectivity of CRAC channels is not an intrinsic and immutable property of ORAI1 but is instead uniquely manifested only in response to STIM1 gating. Given the emerging evidence that indicates that ORAI1 can be activated in a STIM1-independent manner by other cellular activators,23 these results raise the possibility that activation of

Figure 4 | STIM1 dose-dependently modulates the ion selectivity of ORAI1 channels. a, The addition of S domains to wild-type ORAI1 produces a rightward shift in the Vrev of ICRAC vs. V relationships of wild-type ORAI1 channels that are tagged to either one or two S domains in the 20 mM Ca2+ and DVF Ringer’s solutions. The bar graphs summarize the Vrev in each solution. Values are mean ± s.e.m. The top and bottom traces for each condition are from the same cell. b, Effects of substituting extracellular Na+ with an impermeant ion, NMDG+. Removal of Na+ diminishes the inward current in ORAI1–S channels, but not ORAI1–SS channels. c, I-V relationships and reversal potentials of V102C, V102C–S and V102C–SS channels. Values are mean ± s.e.m. Increasing the number of S domains to the V102C monomer causes a progressive rightward shift in the Vrev of ICRAC. d, A plot of V102C ORAI1 activation with two increasing current density of wild-type ORAI1 channels tagged to zero, one or two S domains.
highly Ca\(^{2+}\) selective or non-selective ORAI1 currents may be a general mechanism for cells to tune Ca\(^{2+}\) and Na\(^+\) entry through ORAI1 channels depending on the nature of the upstream activation signal.

**METHODS SUMMARY**

\(I_{\text{CRAC}}\) was recorded in the standard whole-cell patch-clamp configuration in HEK293 cells transfected with the indicated ORAI1 mutants, which were cloned into a bicistronic expression vector that co-expressed GFP. For recording \(I_{\text{CRAC}}\), the membrane potential was hyperpolarized from +30 mV (holding) to −100 mV (100 ms) and then ramped from −100 mV to +100 mV (100 ms). MTS reagents were added to a 20 mM Ringer’s or divalent-free Ringer’s solution at the indicated concentrations. Second-order rate constants of MTSEA blockade were determined at a constant holding potential of −80 mV, as previously described.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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METHODS

Cells. HEK293 cells were maintained in suspension in a medium containing CD293 supplemented with 4 mM GlutaMAX (Invitrogen) at 37°C, 5% CO₂. For imaging and electrophysiology, cells were plated and adhered to poly-L-lysine-coated coverslips at the time of passage, and grown in a medium containing 44% DMEM (Mediatech), 44% Ham’s F12 (Mediatech), 10% fetal calf serum (HyClone), 2 mM glutamine, 50 U ml⁻¹ penicillin and 50 µg ml⁻¹ streptomycin.

Plasmids and transfections. Cys mutations that were used for characterization using electrophysiology were engineered into the previously described C-terminal Myc-tagged ORAI1 construct (M070 ORAI1) in the bicistronic expression vector pMSCV-CITE-eGFP-PGK-Puro11. For generating the tandem dimers, the basic ‘building blocks’ (monomeric ORAI1 (M070) and the Bluescript SK⁺ vector with ORAI1 attached to a linker) were obtained from T. Shuttlerworth and constructed as previously described1. The orientation and number of subunits in the final constructs were confirmed by restriction enzyme analysis and western blot analysis. The ORAI1–S–eGFP and ORAI1–SS–eGFP constructs were kind gifts of T. Xu. The ORAI1–CFP, STIM1–YFP, and CFP–ORAI1 plasmids have been previously described21–28. Site-directed mutagenesis to generate ORAI1 mutations was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer’s instructions and the results were confirmed by DNA sequencing. For electrophysiology, the indicated ORAI1 constructs were transfected into HEK293 cells either alone or together with a construct expressing unlabelled STIM1 (pCMV6-XL5, Origene Technologies). Cells were transfected with the indicated STIM1 and/or ORAI1 complementary DNA (ratio of 10:1 by mass for electrophysiology) using TransPass D2 (NEB Labs) and studied 24 h later. Cells that were transfected with sSTIM1 (Ambion) were studied 72 h following transfection.

Electrophysiology. Currents were recorded in the standard whole-cell configuration at room temperature on an Axopatch 200B amplifier (Molecular Devices) interfaced to an ITC-18 input–output board (Instrutech). Routines developed by R. S. Lewis and M. Prakriya on the Igor Pro software (Wavemetrics) were used for stimulation, data acquisition and data analysis. Data are corrected for the liquid junction potential of the pipette solution relative to Ringer’s in the bath (−10 mV). The holding potential was +30 mV. The standard voltage stimulus consisted of a 100-ms step hyperpolarization to −100 mV followed by a 100-ms ramp depolarization from −100 to +100 mV, applied at 1-s intervals. Unless noted otherwise, the peak currents during the −100 mV pulse were measured for data analysis. For examining Ca²⁺−dependent fast inactivation, the voltage protocol consisted of a 100-ms step decrease to −100 mV, applied at 2-s intervals. Unless otherwise indicated, ICa,K was activated by passive depletion of intracellular Ca²⁺ stores by internal dialysis of 8 mM BAPTA through the pipette solution. To prevent complications arising from the changing membrane potential in the standard step–ramp voltage protocol, rate constants of blockade by MTS reagents were determined at a constant potential of −80 mV by acquiring 200-ms sweeps of current at 4 Hz. All currents were acquired at 5 kHz and low-pass filtered with a 1-kHz Bessel filter built into the amplifier. All data were corrected for liquid junction potential of the pipette solution and for leak currents collected in 50–150 µM LaCl₃.

MTS reagent protocol. The protocol for analysis of state-dependent modification of ORAI1 bearing Cys mutants is described in Fig. 1c. For the D110C mutant, this protocol was slightly modified to adjust for the formation of spontaneous disulphide bonds in this mutant29. The protocol included an additional application of the reducing agent BMS 90–120 s before the first MTSEA application to remove pre-existing disulphide bonds, as described previously4.

Solutions. The standard extracellular Ringer’s solution contained 130 mM NaCl, 4.5 mM KCl, 20 mM CaCl₂, 10 mM tetrathylammonium chloride (TEA-Cl), 10 mM D-glucose, and 5 mM Na-HEPES (pH 7.4). The DVF Ringer’s solution contained 150 mM NaCl, 10 mM HEDTA, 1 mM EDTA, 10 mM TEA-Cl and 5 mM HEPES (pH 7.4). The 110-mM Ca²⁺ solution contained 110 mM CaCl₂, 10 mM D-glucose and 5 mM HEPES (pH 7.4). The standard internal (pipette) solution contained 125 mM Cs-aspartate, 8 mM MgCl₂, 8 mM BAPTA and 10 mM Cs HEPES (pH 7.2). In experiments examining CDI, BAPTA was replaced with EGTA to accentuate CDI. In these experiments, the internal solution contained 125 mM Cs-aspartate, 10 mM EGTA, 3 mM MgCl₂, 8 mM NaCl and 10 Ca HEPES (pH 7.2). Stock solutions of MTS reagents (Toronto Research Chemicals) were prepared as previously described4.

Data analysis. Reversal potentials were measured from the average of several leak-subtracted sweeps (4–6 sweeps) in each cell. Measurements were taken from 6–15 cells per mutant per condition. In cases in which the I−V curve asymptotically approached the x axis at very positive membrane potentials with no clear reversal (for example, in wild-type ORAI1-expressing cells), the reversal potential was assigned as +80 mV. The MTSEA reaction rate constant was estimated from single exponential fits to the current decline after MTSEA application. The apparent second-order modification rate constant kon was calculated from the relationship:

\[
kon = \frac{1}{t_{1/2}/[MTS]}
\]

where [MTS] is the concentration of the MTS reagent.

Relative permeabilities were calculated from changes in the reversal potential using the Goldman–Hodgkin–Katz voltage equation:

\[
\frac{P_X}{P_{Na}} = \frac{[Na]}{[X]} e^{\Delta E_{rev}/RT}
\]

where R, T and F have their usual meanings, PX and PNa are the permeabilities of the test ion and Na⁺, respectively, [X] and [Na] are the ionic concentrations, and ΔErev is the shift in reversal potential when the test cation is exchanged for Na⁺. To estimate the minimal width of ORAI1 channels, the relative channel permeabilities for a series of organic monovalent cations of increasing size were examined as described before30. The cations used were ammonium (3.2Å), methylammonium (3.78Å), dimethylammonium (4.6Å) and trimethylammonium (5.34Å). These experiments were carried out in buffered Ca²⁺-free solutions to avoid the potent blocking effects of Ca²⁺ ions on monovalent ICa,K. The data were fitted to the hydrodynamic relationship31:

\[
P_X/P_{Na} = k(\frac{d_{ion}}{d_{pore}})^2
\]

where \(d_{ion}\) is the diameter of the tested ion and \(d_{pore}\) is the apparent width of the pore.

All data were corrected for leak currents collected in 20 mM Ca²⁺ + 50–150 µM La³⁺. All curve fitting was done by least-squares methods using built-in functions in Igor Pro 5.0.