Supplemental Data

CRACM1 Multimers Form the Ion-Selective Pore of the CRAC Channel

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Supplemental Experimental Procedures

Electrophysiology

Patch-clamp experiments were performed in the tight-seal whole-cell configuration at 21–25°C. High-resolution current recordings were acquired with the EPC-9 (HEKA, Lambrecht, Germany). Voltage ramps of 50 ms duration spanning a range of −100 to +150 mV were delivered from a holding potential of 0 mV at a rate of 0.5 Hz over a period of 100–240 s. All voltages were corrected for a liquid-junction potential of 10 mV. Currents were filtered at 2.9 kHz and digitized at 100 μs intervals. Capacitive currents were determined and corrected before each voltage ramp. Low-resolution temporal development of inward and outward currents was obtained by extracting the current amplitude at −80 mV and +130 mV from individual ramp-current records. Where applicable, statistical errors of averaged data are given as means ± SEM with n determinations. Standard external solutions were as follows (in mM): 120 NaCl, 10 TEACl, 2 $MgCl_2$, 10 $CaCl_2$, 10 glucose, 10 HEPES-NaOH (pH 7.2 with NaOH), and 300 mOsm. Standard internal solutions were as follows (in mM): 120 Cs-glutamate, 20 Cs·BAPTA, 3 $MgCl_2$, 10 HEPES-CsOH, 0.02 IP_3 (pH 7.2 with CsOH), and 300 mOsm. For 0 Ca solution, $CaCl_2$ was omitted from the standard external solution. Monovalent replacement solutions were based on the external 0 Ca solution but with 120 mM NaCl and 10 mM HEPES-NaOH (pH 7.2 with NaOH), replaced by either 120 mM CsCl and 10 mM HEPES-CsOH (pH 7.2 with CsOH) or 120 mM KCl and 10 mM HEPES-KOH (pH 7.2 with KOH). For 10 Ca^{2+}/0 Na^+ and 0 Ca^{2+}/0 Na^+ solution, NaCl was replaced by TEACl in standard external and 0 Ca external solution, respectively. For divalent replacement solutions, 10 mM $BaCl_2$ or $SrCl_2$ was added to the 0 Ca^{2+}/0 Na^+ solution. The nominally divalent-free external solution used in Figure 2F was based on the standard Na^+-based external solution and in the absence of $CaCl_2$ and $MgCl_2$. For absolute divalent-free (DVF) external solutions used in all other experiments, 10 mM EDTA was added. For the anomalous mole-fraction experiments, extracellular Ca^{2+} was adjusted by adding 10, 100, and 1000 μM $CaCl_2$ to nominally divalent-free solution, which was assumed to contain 1 μM Ca^{2+}. If necessary, osmolarity was adjusted by adding glucose. All chemicals were purchased from Sigma-Aldrich.

Subcloning and Overexpression of CRACM1 and Mutant CRACM1

Wild-type STIM1 was subcloned into pcDNA/4TO/myc-His. Wild-type CRACM1 was subcloned into pcDNA/4TO/myc-His, pcDNA/4TO/Flag (Invitrogen), and pIRES2-EGFP (Clontech) plasmids as described [S1, S2]. Point mutants of CRACM1 were generated with QuikChange site-directed mutagenesis kit (Stratagene) and subcloned into pcDNA/4TO/Flag (Invitrogen) and pIRES2-EGFP (Clontech) plasmids. For electrophysiological analysis, CRACM1 and its mutants were overexpressed in HEK293 cells stably expressing STIM1 by using lipofectamine 2000 (Invitrogen). These cells were generated by electroporation of human STIM1 subcloned into pIRESneo (Clontech) and selection with G418 (250 μg/ml) [S3]. Green cells were used for analysis 36–48 hr after transfection.

Immunoprecipitation and Western Blotting

HEK293 cells were cotransfected with STIM1 and wild-type CRACM1, and its mutants were cloned in pcDNA/4TO/myc-His or pcDNA/4TO/Flag. Twenty-four hours after transfection, cell extracts were prepared with lysis buffer: 50 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1% NP-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF. Tagged CRACM1 and its mutants were immunoprecipitated with 2.5 μg/ml of anti-myc (Invitrogen), anti-His (Invitrogen), or mouse monoclonal antibodies directed against the Flag epitope (Sigma). Proteins were resolved with SDS-PAGE and analyzed with the relevant antibodies in western blots. For immunoblotting, His (Invitrogen) and myc (Invitrogen) antibodies were used at 200 ng/ml, and flag antibody was used at 100 ng/ml.

Confocal Imaging

HEK293 cells were transfected with wild-type CRACM1 or its mutants. Twenty-four hours after transfection, cells were fixed with 2% paraformaldehyde, permeabilized, and blocked with BSA. Immunostaining was done with corresponding tag primary antibodies (2.5 μg/ml) and the detection with Cy3-conjugated mouse secondary antibody (Jackson Immuno) at 1:300 dilution. Images were captured on a Zeiss LSM 510 confocal microscope.

Supplemental References

S1. Vig, M., Peinelt, C., Beck, A., Koomoa, D.L., Rabah, D., Koblan-Huberson, M., Kraft, S., Turner, H., Fleig, A., Penner, R., et al. (2006). CRACM1 is a plasma membrane protein essential for store-operated Ca^{2+} entry. Science *312*, 1220–1223.

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