Supplemental Data

CRACM1, CRACM2, and CRACM3 Are Store-Operated Ca^{2+} Channels with Distinct Functional Properties

Annette Lis, Christine Peinelt, Andreas Beck, Suhel Parvez, Mahealani Monteilh-Zoller, Andrea Fleig, and Reinhold Penner

Supplemental Experimental Procedures

Subcloning and Overexpression

Full-length human CRACM1 and CRACM1-E106Q were subcloned as described [S1]. Full-length human CRACM2 (accession no. NM_032831) and CRACM3 (accession no. NM_152288) were amplified from cDNAs (purchased from OriGene) with Pfu Ultra High Fidelity polymerase (Stratagene) and subcloned into a pCAGGS-IRES-GFP vector [S2]. We introduced the ribosome-binding site ACC GCC ACC and a HA-tag in frame immediately 5′ to the start codon of CRACM2 and CRACM3 cDNAs, which were subsequently cloned into pCAGGS-IRES-GFP for transient dicistronic expression of CRACM2 and CRACM3 together with the green fluorescent protein (GFP). For electrophysiological analysis, CRACM proteins were overexpressed in HEK293 cells stably expressing STIM1 [S3] with lipofectamine 2000 (Invitrogen), and the GFP expressing cells were selected by fluorescence. Experiments were performed 24–48 hr after transfection.

Immunoprecipitation

HEK293 cells were transiently cotransfected with CRACM1-Myc [S4] and HA-CRACM1, HA-CRACM2, and HA-CRACM3 (described above). Forty-eight hours after transfection, cells were harvested in PBS and lysed in 1 ml lysis buffer with the following: 75 mM NaCl, 40 mM NaF, 20 mM Iodocetamide, 50 mM HEPES, 1% IGEPAL, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.25 mM sodium orthovanadate, and protease inhibitor cocktail (Sigma). The cell lysates were precipitated with anti-HA rat monoclonal antibody (2.5 μg, Roche) or anti-c-Myc mouse monoclonal antibody (2.5 μg, Calbiochem) for 2 hr at 4°C. Samples were resolved by SDS-PAGE and analyzed with anti-HA rat monoclonal antibody at a dilution 1:1000. Anti-Rat IgG (whole molecule) peroxidase conjugate (Sigma) were used as secondary antibody in accordance with the manufacturer's instructions. Proteins were detected by development with the ECL Plus Western Blotting Detection System (Amersham).

Electrophysiology

Patch-clamp experiments were performed in the tight-seal whole-cell configuration at 21°C–25°C. High-resolution current recordings were acquired with the EPC-9 (HEKA). Voltage ramps of 50 ms duration spanning a range of −150 to +150 mV were delivered from a holding potential of 0 mV at a rate of 0.5 Hz over a period of 100–300 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. Extracting the current amplitude at −80 mV from individual ramp current records assessed the low-resolution temporal development of currents. Where applicable, statistical errors of averaged data are given as means ± SEM with n determinations. Standard external solutions were as follows: 120 mM NaCl, 2 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM TEA-Cl, 10 mM HEPES, 10 mM glucose, pH 7.2 with NaOH, 300 mOsm. In some experiments, we applied Na^+-free solutions, where NaCl was replaced equimolarly by tetraethylammonium-chloride (TEA-Cl). For Ca^{2+}-free external solutions $CaCl_2$ was omitted, but Mg^{2+} was retained. The divalent-free external solution (DVF) was based on the standard external solution but in the absence of $CaCl_2$ and $MgCl_2$ and was additionally supplemented with 10 mM EDTA. Divalent replacement solutions were based on the standard external solution but with 10 mM $CaCl_2$ replaced by 10 mM $BaCl_2$. In some experiments, 2-aminoethyldiphenyl borate (2-APB) was added to the standard external solution at a final concentration of 50 μM. Standard internal solutions were as follows: 120 mM Cs-glutamate, 20 mM Cs·BAPTA, 3 mM $MgCl_2$, 10 mM HEPES, 0.02 mM IP_3, pH 7.2 with CsOH, 300 mOsm. In the experiments of Figure 2, 10 mM EGTA was used, and in Figure 3, $[Ca^{2+}]_i$ was buffered to defined levels with 20 mM Cs·BAPTA, and appropriate concentrations of $CaCl_2$ as calculated with WebMaxC (http://www.stanford.edu/~cpatton/webmaxcS.htm). For passive-depletion experiments, IP_3 was omitted from the internal solution. All chemicals were purchased from Sigma-Aldrich.

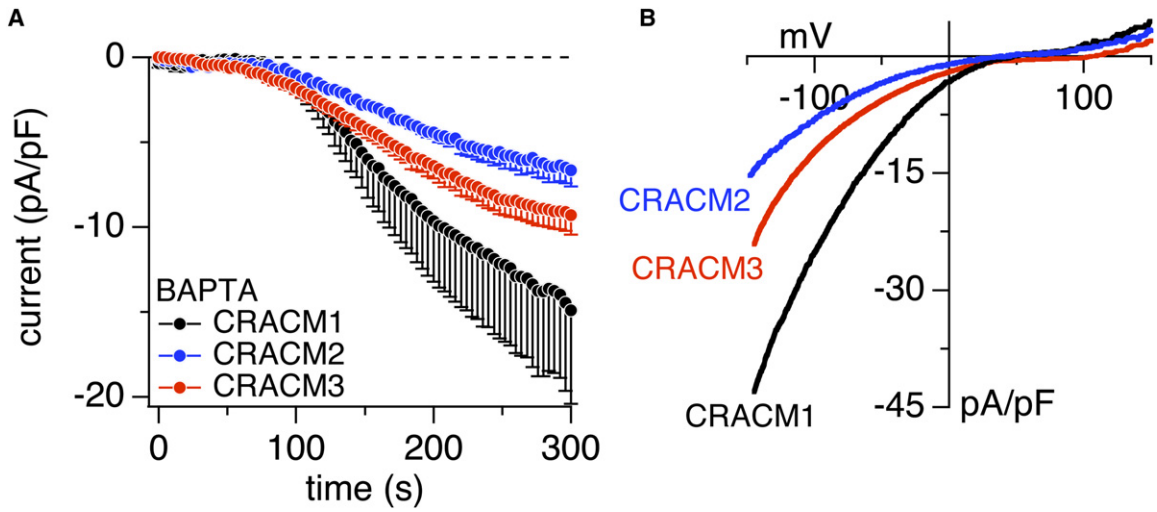

Figure S1. Store-Operated Currents Induced by BAPTA

(A) Average CRAC current densities after store depletion with 20 mM BAPTA and omitting IP_3 in cells expressing CRACM1 (black, n = 12), CRACM2 (blue, n = 7), and CRACM3 (red, n = 7). Currents were analyzed as shown in (A).

(B) Average I/V traces extracted from representative HEK293 cells shown in (A) at 300 s into the experiment. Traces correspond to CRACM1 (black, n = 9), CRACM2 (blue, n = 7), and CRACM3 (red, n = 7).

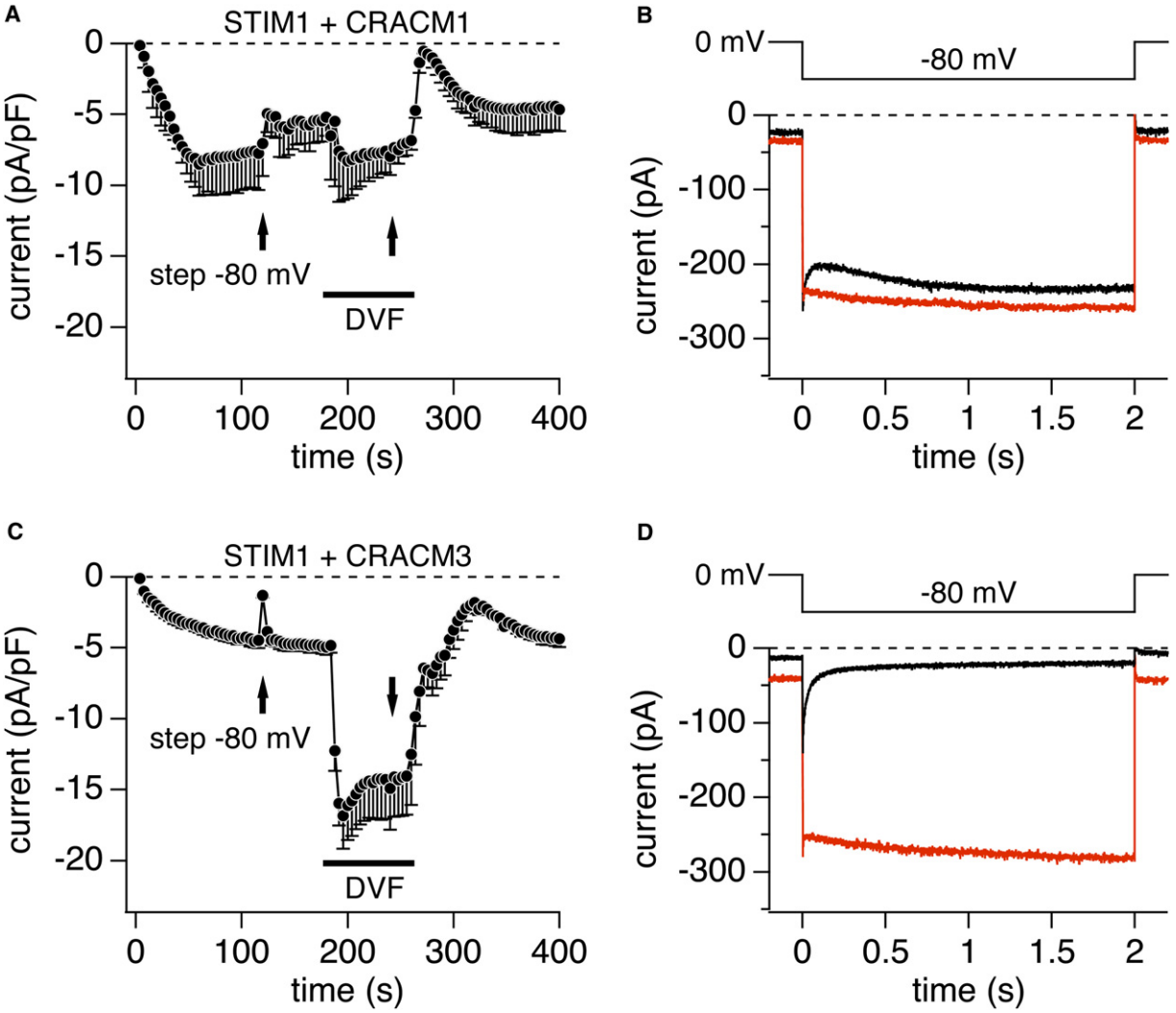

Figure S2. Ca^{2+}-Dependent Inactivation of CRACM3 Currents

(A) Average CRAC current densities at −80 mV induced by IP_3 (20 μM) with 10 mM EGTA in stable STIM1-expressing HEK293 cells transiently overexpressing CRACM1 (n = 3). CRAC currents were monitored continuously by voltage ramps spanning −100 mV to +100 mV over 50 ms delivered at a rate of 0.5 Hz. After CRAC currents were fully activated (120 s), a rectangular voltage pulses of 2 s duration was delivered to −80 mV (see [B]). Then the cell was exposed to divalent-free (DVF) extracellular solution and another voltage pulse was applied.

(B) Average CRAC currents evoked by step pulses (2 s duration) to −80 mV in the presence of 10 mM Ca^{2+} (black) and in DVF solution (red, n = 3, same cells as shown in [A]). Note the loss of initial fast inactivation and subsequent reactivation in DVF solution. The remaining slow increase in inward currents is probably voltage-dependent facilitation.

(C) Same experimental conditions and protocol as in (A), but in stable STIM1-expressing HEK293 cells transiently overexpressing CRACM3 (n = 3).

(D) Average CRAC currents evoked by step pulses (2 s duration) to −80 mV in the presence of 10 mM Ca^{2+} (black) and in DVF solution (red, n = 3, same cells as shown in [C]). Note the loss of inactivation in DVF solution, revealing the same slow facilitation as CRACM1 that is presumably voltage dependent.

Fluorescence Measurements

For Ca^{2+} measurements, fura-2 AM (Molecular Probes)-loaded cells (1 μM/60 min/37°C) were kept in extracellular saline containing the following: 140 mM NaCl, 2.8 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES·NaOH (pH 7.2). Store depletion was induced by addition of 2 μM thapsigargin to the bath, and for assessing store-operated Ca^{2+} entry, 2 mM Ca^{2+} was added. Experiments were performed with a Zeiss Axiovert 100 fluorescence microscope equipped with a dual excitation fluorometric imaging system (TILL-Photonics), with a 40× Plan NeoFluar objective. Data acquisition and computation was controlled by TILLvisION software. Dye-loaded cells were excited by wavelengths of 340 and 380 nm, produced by a monochromator (Polychrome IV). The fluorescence emission of several single cell bodies was simultaneously recorded with a video camera (TILL-Photonics Imago) with an optical 440 nm long-pass filter. The signals were sampled at 0.5 Hz and computed into relative ratio units of the fluorescence intensity at the different wavelengths (340/380 nm). Results are given as the approximate $[Ca^{2+}]_i$, calculated from the 340/380 nm fluorescence values, with an in vivo Ca^{2+} calibration performed in patch-clamp experiments with defined Ca^{2+} concentrations combined with fura-2 in the patch pipette.

Supplemental References

S1. Peinelt, C., Vig, M., Koomoa, D.L., Beck, A., Nadler, M.J., Koblan-Huberson, M., Lis, A., Fleig, A., Penner, R., and Kinet,

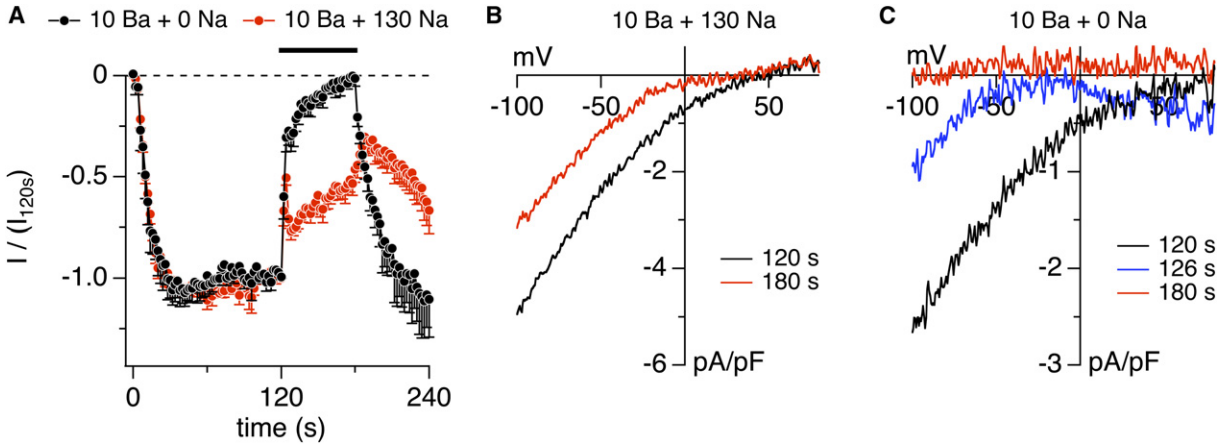

Figure S3. Ba^{2+} Conductivity in Jurkat T Cells

(A) Average normalized CRAC currents (I/I_{120s}) at −80 mV induced by IP_3 (20 μM) in Jurkat T cells. The bar indicates application of an external solution containing 10 mM Ba^{2+} in the presence of Na^+ (red, n = 8) and when Na^+ was replaced by TEA^+ (black, n = 5)

(B) Average I/V relationships of CRAC currents extracted from cells shown in (A), obtained at 120 s (black) and 180 s (red) during Ba^{2+} application in the presence of Na^+ (n = 8). Data represent leak-subtracted current densities (pA/pF) evoked by 50 ms voltage ramps from −150 to +150 mV (voltage range shown is from −100 to +80 mV).

(C) Average I/V relationships of CRAC currents from cells shown in (A) obtained at 120 s (black), 126 s (blue), and 180 s (red) during Ba^{2+} application when Na^+ was replaced by TEA^+ (n = 5).

J.P. (2006). Amplification of CRAC current by STIM1 and CRACM1 (Orai1). Nat. Cell Biol. *8*, 771–773.

S2. Warnat, J., Philipp, S., Zimmer, S., Flockerzi, V., and Cavalie, A. (1999). Phenotype of a recombinant store-operated channel: Highly selective permeation of Ca2+. J. Physiol. *518*, 631–638.

S3. Soboloff, J., Spassova, M.A., Hewavitharana, T., He, L.P., Xu, W., Johnstone, L.S., Dziadek, M.A., and Gill, D.L. (2006). STIM2 is an inhibitor of STIM1-mediated store-operated Ca2+ entry. Curr. Biol. *16*, 1465–1470.

S4. 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 Ca2+ entry. Science *312*, 1220–1223.