

Amplification of CRAC current by STIM1 and CRACM1 (Orai1)

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Depletion of intracellular calcium stores activates store-operated calcium entry across the plasma membrane in many cells. STIM1, the putative calcium sensor in the endoplasmic reticulum, and the calcium release-activated calcium (CRAC) modulator CRACM1 (also known as Orai1) in the plasma membrane have recently been shown to be essential for controlling the store-operated CRAC current (I_{CRAC})^{1–4}. However, individual overexpression of either protein fails to significantly amplify I_{CRAC} . Here, we show that STIM1 and CRACM1 interact functionally. Overexpression of both proteins greatly potentiates I_{CRAC} , suggesting that STIM1 and CRACM1 mutually limit store-operated currents and that CRACM1 may be the long-sought CRAC channel.

Receptor-mediated release of Ca^{2+} from intracellular stores induces Ca^{2+} entry through calcium release-activated calcium (CRAC) channels^{5–7}. Previous studies have identified STIM1 as the potential sensor for endoplasmic reticulum luminal Ca^{2+} concentration^{1,8,9}. When Ca^{2+} is depleted from intracellular stores, STIM1 translocates to vesicular structures (punctae) underneath the plasma membrane, where it is hypothesized to activate CRAC channels residing in the plasma membrane. A second protein, CRACM1, has recently been identified as essential for activating CRAC channels^{3,4}. This protein contains four transmembrane domains, is located in the plasma membrane and, therefore, may represent the CRAC channel itself, a subunit of the channel, or a regulatory molecule that couples to the channel. When overexpressed individually, neither STIM1 nor CRACM1 can significantly potentiate I_{CRAC} ^{1–4}.

To address the potential interaction of STIM1 and CRACM1, both proteins were overexpressed individually, or in combination, in HEK293 and Jurkat T cells and the CRAC currents were measured in response to Ca^{2+} store depletion by 20 μM inositol 1,4,5-trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$). Both cell types normally exhibit native CRAC currents of approximately 0.5 pA pF^{-1} and approximately 3 pA pF^{-1} , respectively⁴ (Fig. 1a, c), with typical inwardly rectifying current-voltage (I/V) relationships^{5,10,11} (Fig. 1b, d). Consistent with previous work^{1,9}, overexpression of STIM1 alone caused a small-to-modest increase in I_{CRAC} in HEK293 and Jurkat cells (Fig. 1a, c).

CRACM1 overexpression alone did not affect the CRAC currents induced by store depletion in HEK293 cells (Fig. 1a, b) and caused a small reduction in I_{CRAC} in Jurkat cells (Fig. 1c, d). Unless simply due to a general effect of transfection or variability of I_{CRAC} across preparations, this reduction may be due to some kind of dominant-negative effect. Taken together, the available data on CRACM1 and STIM1 suggest that the individually expressed proteins, although essential for I_{CRAC} manifestation, cannot significantly amplify the current. This would indicate that these proteins are either not sufficient to generate large CRAC currents or that they are stoichiometrically linked and limit each others' ability to generate CRAC currents above normal. Therefore, we co-overexpressed both proteins in HEK293 cells (see Supplementary Information, Fig. S1) and assessed store-operated currents by patch clamp.

The co-overexpression of STIM1 and CRACM1, in both HEK293 and Jurkat cells, is sufficient to generate enormous membrane currents of approximately 30 pA pF^{-1} on store depletion by $\text{Ins}(1,4,5)\text{P}_3$ (Fig. 1e). These currents are significantly larger than the corresponding native currents evoked by the same experimental protocol and amount to an approximately 60-fold increase in HEK293 cells and approximately tenfold in Jurkat cells. The currents exhibit a similar time course of activation (Fig. 1e) and the same inwardly rectifying I/V relationship (Fig. 1f), as the well-characterized CRAC current^{5,10}. It should be noted that the average current presented in the graph represents an underestimate, as it excludes cells in which I_{CRAC} increased to well above 50–100 pA pF^{-1} . In these cells, the massive Ca^{2+} influx presumably saturated the intracellular Ca^{2+} chelator BAPTA (1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid), causing the current to inactivate^{5,10} (see Supplementary Information, Fig. S2).

To further characterize these currents, some specific properties that are considered the hallmarks of native CRAC currents were assessed. We established that the current could be activated by other stimuli that cause store depletion. Passive store depletion by 10 mM BAPTA, in the absence of $\text{Ins}(1,4,5)\text{P}_3$, also recruited CRAC-like currents (Fig. 2a). These developed after a characteristic delay that is likely to represent the time required for Ca^{2+} to leak out of the stores⁵. The cells were next perfused with a solution in which the Ca^{2+} concentration was buffered to

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BRIEF COMMUNICATION

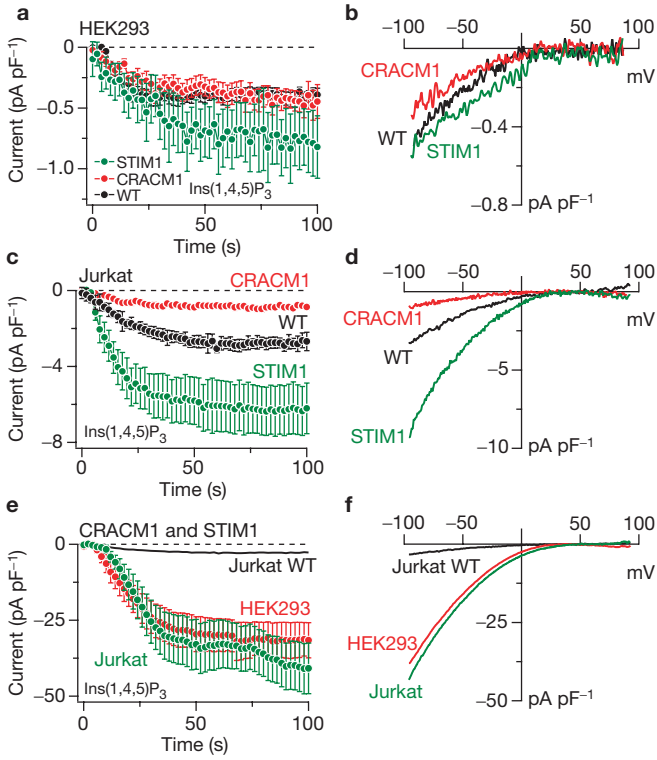


Figure 1 Individual and combined overexpression of STIM1 and CRACM1. (a) Normalized average time course of $\text{Ins}(1,4,5)\text{P}_3$ -induced ($20\ \mu\text{M}$) I_{CRAC} in HEK293 cells. Currents of individual cells were measured at $-80\ \text{mV}$, normalized by their respective cell size, averaged and plotted versus time ($\pm\text{s.e.m.}$). Cytosolic calcium was clamped to near zero with $10\ \text{mM}$ BAPTA. Traces represent native I_{CRAC} in wild-type cells (WT, black circles; $n=10$), cells transfected with CRACM1+GFP (red circles; $n=28$) or STIM1+GFP expressing cells (green circles; $n=13$). (b) Average current–voltage (I/V) relationships of I_{CRAC} extracted from representative HEK293 cells at 60s, representing leak-subtracted currents evoked by 50ms voltage ramps from -100 to $+100\ \text{mV}$, normalized to cell size (pF). Traces represent native I_{CRAC} in wild-type cells ($n=6$), cells transfected with CRACM1+GFP ($n=13$) and STIM1+GFP expressing cells ($n=5$). (c) Normalized average time course of $\text{Ins}(1,4,5)\text{P}_3$ -induced ($20\ \mu\text{M}$) I_{CRAC} in Jurkat cells. Currents were analysed as in (a) ($n=21$ for control; $n=11$ for CRACM1; $n=12$ for STIM1). (d) Averaged I/V traces of I_{CRAC} extracted from representative Jurkat cells at 60s. Analysis as in (b) ($n=19$ for wild type; $n=7$ for CRACM1; $n=12$ for STIM1). (e) Normalized average time course of I_{CRAC} in HEK293 or Jurkat cells expressing STIM1+CRACM1. Analysis as in (a) ($n=14$ for HEK293; $n=17$ for Jurkat cells). The time course of I_{CRAC} in wild-type Jurkat cells is included for comparison (same data as in c). (f) Average current–voltage (I/V) data traces of I_{CRAC} extracted from representative HEK293 (red) or Jurkat (green) cells expressing STIM1+CRACM1. Analysis as in (b) ($n=14$ for HEK293; $n=17$ for Jurkat cells). The Jurkat wild-type data trace is plotted for comparison (same data as in d).

approximately $150\ \text{nM}$ to avoid store depletion and this did prevent current activation (Fig. 2b). Subsequent application of ionomycin to release Ca^{2+} from intracellular stores, however, rapidly activated large currents that exhibited the same rectifying I/V as I_{CRAC} (Fig. 2c).

The current produced by STIM1–CRACM1 overexpression was as specific for Ca^{2+} ions as I_{CRAC} ^{5,10}. Removal of extracellular Ca^{2+} (nominally Ca^{2+} -free), while retaining normal levels of Mg^{2+} , inhibited the inward currents evoked by store depletion (Fig. 2d). Another specific characteristic of I_{CRAC} is that it can carry Ba^{2+} and Sr^{2+} currents, albeit at smaller levels than Ca^{2+} (refs 5,11,12). Ion substitution experiments in which $10\ \text{mM}$ extracellular Ca^{2+} was replaced by equimolar amounts of Ba^{2+}

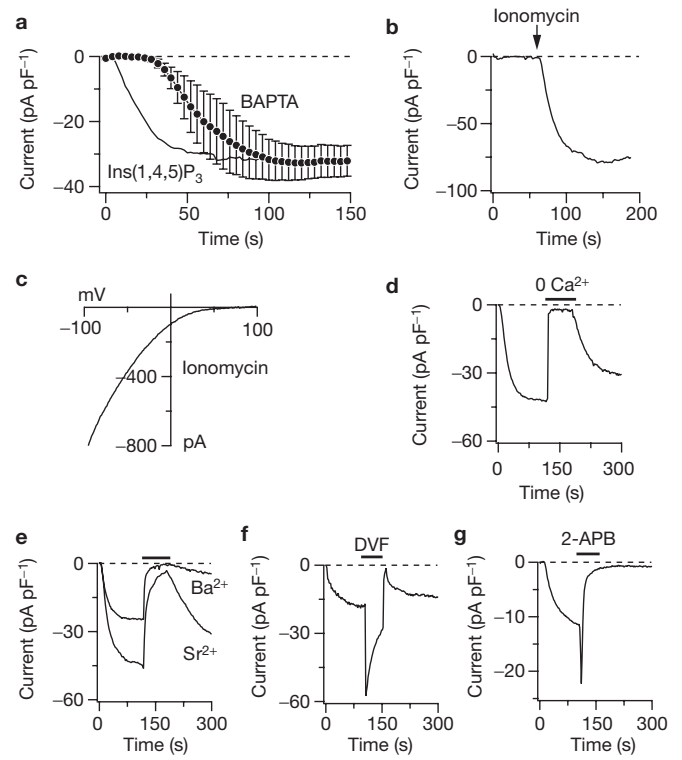


Figure 2 Co-expression of CRACM1 and STIM1 produces a current with the characteristic features of I_{CRAC} . (a) Normalized average time course of I_{CRAC} in STIM1+CRACM1 expressing HEK293 cells (black circles; $n=3$). Currents of individual cells were measured at $-80\ \text{mV}$, normalized by cell size (pF), averaged and plotted versus time ($\pm\text{s.e.m.}$). Passive store-depletion was induced by clamping cytosolic calcium to near zero using $10\ \text{mM}$ BAPTA. For comparison, the solid line reproduces the $\text{Ins}(1,4,5)\text{P}_3$ -induced data shown in Fig. 1e. (b) Time course of a representative HEK293 cell expressing STIM1+CRACM1 ($n=3$), where I_{CRAC} was induced by application of $2\ \mu\text{M}$ ionomycin for 3s, as indicated by the arrow. Intracellular calcium was clamped to $150\ \text{nM}$ with $10\ \text{mM}$ BAPTA and $4\ \text{mM}$ CaCl_2 to prevent passive store-depletion before treatment with ionomycin. (c) I/V data trace of I_{CRAC} extracted from the same cell as in (b) at 110s. The voltage protocol was as in Fig. 1b. (d) Representative time course of I_{CRAC} evoked in a HEK293 cell expressing STIM1+CRACM1 by $20\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$. Extracellular calcium was removed as indicated by the black bar (average inhibition at the end of application = $94\% \pm 1\%$, $n=15$). (e) Representative HEK293 cell expressing STIM1+CRACM1 where $10\ \text{mM}$ extracellular Ca^{2+} was replaced with equimolar Ba^{2+} or Sr^{2+} during the time indicated by the bar (average inhibition by Ba^{2+} = $95\% \pm 1\%$, $n=14$; and by Sr^{2+} = $81\% \pm 2\%$, $n=16$). (f) Representative HEK293 cell expressing STIM1+CRACM1 superfused with a divalent-free (DVF) external solution ($n=3$). (g) Application of $50\ \mu\text{M}$ 2-APB initially facilitates and then inhibits $\text{Ins}(1,4,5)\text{P}_3$ -induced I_{CRAC} in a representative HEK293 cell expressing STIM1+CRACM1 ($n=5$).

and Sr^{2+} resulted in a strong inhibition of the current and very limited steady-state permeation of these ions compared with Ca^{2+} (Fig. 2e). I_{CRAC} can also carry monovalent cations, such as Na^+ , when removing all divalent ions from the extracellular solution and this transiently increases inward currents¹⁰. The large currents in STIM1–CRACM1 overexpressing cells showed the same behaviour (Fig. 2f). Whether or not the current exhibited the pharmacological profile of I_{CRAC} , which is known to be enhanced by low concentrations of 2-aminoethoxydiphenylborate (2-APB) and inhibited at higher doses^{13,14}, was also assessed. Treatment with $50\ \mu\text{M}$ 2-APB, after an initial increase as 2-APB concentration built up, completely blocked the current. Although these properties of the

large CRAC currents are generally in excellent agreement with those of native I_{CRAC} , it is noteworthy that Sr^{2+} and Ba^{2+} currents seem smaller than those described for native I_{CRAC} (refs 5,11,12). This may reflect a genuine, small Ba^{2+} - and Sr^{2+} -permeability of the CRAC channels in STIM1–CRACM1 overexpressing cells, and/or compromised channel function, as a consequence of the removal of extracellular Ca^{2+} (ref. 15). This difference in divalent permeation may also hint at the possibility of species differences, or that additional proteins — possibly other members of the STIM or CRACM families — may participate in shaping native CRAC currents.

In summary, our data establish that the co-overexpression of STIM1 and CRACM1 greatly amplifies store-operated currents and that these currents possess most of the defining characteristics of I_{CRAC} . This suggests that STIM1 and CRACM1 are entirely sufficient to control the magnitude of the CRAC current. As individual overexpression of either protein fails to augment I_{CRAC} , we conclude that they mutually represent limiting factors for CRAC current manifestation, although in Jurkat cells there may be a surplus of CRACM1 compared with STIM1, as STIM1 overexpression can enhance I_{CRAC} approximately twofold (Fig. 1c). Although more complex interpretations are conceivable, the most parsimonious interpretation of the fact that additional CRACM1 overexpression can amplify I_{CRAC} 10–60-fold (and in some cells well above 100-fold) would be that CRACM1 itself constitutes the CRAC channel. □

Note: Supplementary Information (including Methods) is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

J.P.K., R.P. A.F. and M.N. are consultants to Synta Pharmaceuticals Corp. (Lexington, MA). J.P.K. and R.P. are members of the scientific advisory board of Synta Pharmaceuticals Corp.

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Supplementary Information

Amplification of Ca^{2+} Release-Activated Ca^{2+} (CRAC) current by STIM1 and CRACM1 (ORAI1)

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Supplementary Figures

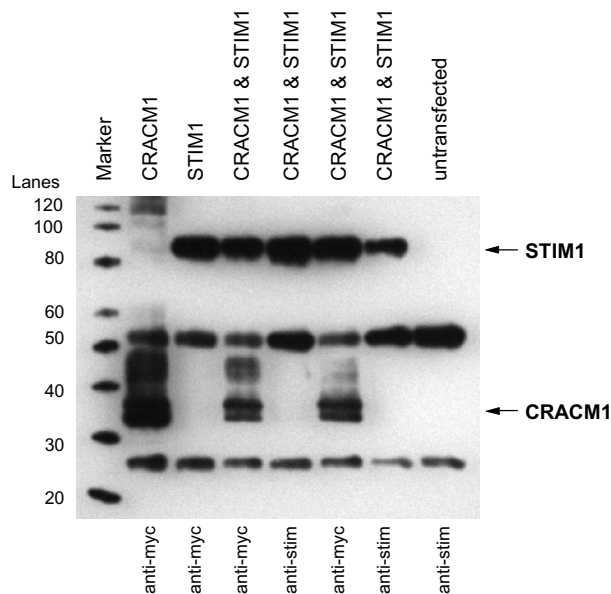


Figure S1: Western blot of combined overexpression of STIM1 and CRACM1. Over-expression of both STIM1 and CRACM1 proteins was confirmed by making whole cell lysates of the co-transfected HEK cells. Since both the proteins are tagged on the C-terminal end with myc and His tags, overexpressed proteins were immunoprecipitated using anti-myc monoclonal antibody, resolved on SDS-PAGE and immunoblotted using anti-His antibody. The co-transfected cells showed the expected 84 KDa band corresponding to STIM1 and a nearly 37 KDa double band corresponding to CRACM1. The STIM1 alone and CRACM1 alone transfected cells only showed the bands corresponding to STIM1 and CRACM1 respectively. Untransfected control cells did not show any bands corresponding to these two proteins, demonstrating that the anti-His antibody is specifically binding to the overexpressed, tagged proteins. All the lanes had bands corresponding to the heavy and the light chain of the antibodies used for immunoprecipitations at around 25 and 50 KDa. The co-expression strategy is described in the methods section.

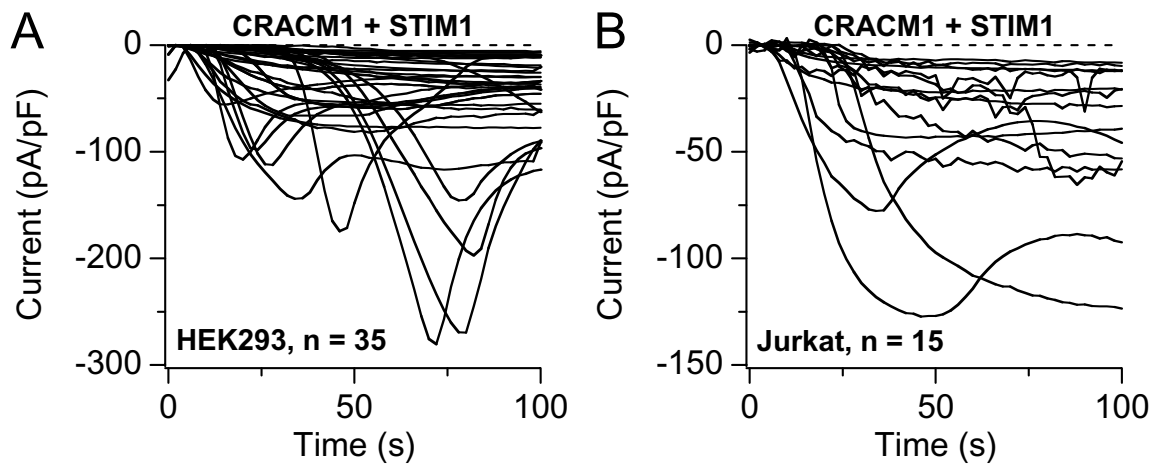


Figure S2: Combined overexpression of STIM1 and CRACM1. (A) Normalized time course of I_{CRAC} in individual HEK293 cells expressing STIM1+CRACM1. Currents of individual cells were measured at -80 mV, normalized by their respective cell size (pF) and plotted versus time. Cytosolic calcium was clamped to near zero with 10 mM BAPTA. Note the delay of current onset in some cells and the inactivation in cells with I_{CRAC} larger than ~ 50 pA/pF, which is likely due to high $[Ca^{2+}]_i$ as the Ca^{2+} buffers are saturated. (B) Normalized time course of I_{CRAC} in individual Jurkat T cells expressing STIM1+CRACM1. Currents were analyzed and plotted as in panel A. Cytosolic calcium was clamped to near zero with 10 mM BAPTA. Note the delay of current onset in some cells and the inactivation in cells with I_{CRAC} larger than ~ 50 pA/pF.

METHODS

Electrophysiology. Patch-clamp experiments were performed in the tight-seal whole-cell configuration at 21–25 °C. High-resolution current recordings were acquired using the EPC-9 (HEKA, Lambrecht, Germany). Voltage ramps of 50 ms duration spanning a range of –100 to +100 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 μ s 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 both currents. Where applicable, statistical errors of averaged data are given as mean \pm s.e.m. with *n* determinations. Standard external solutions were as follows: 120 mM NaCl, 2.8 mM KCl, 10 mM CsCl, 2 mM MgCl₂, 10 mM CaCl₂, 10 mM HEPES, 10 mM glucose, at pH 7.2 with 300 mOsm NaOH. In HEK293 cells, 10 mM tetraethylammonium (TEA) was added to suppress delayed rectifier K⁺ currents. Standard internal solutions were as follows: 120 mM Cs-glutamate, 8 mM NaCl, 10 mM Cs-BAPTA, 3 mM MgCl₂, 10 mM HEPES, 0.02 mM Ins(1,4,5)P₃, at pH 7.2 with 300 mOsm CsOH. As indicated in the figure legends, for some experiments the Ca²⁺ concentration was buffered to 150 nM by 10 mM Cs-BAPTA and 4 mM CaCl₂. For passive-depletion experiments, the internal solution was supplemented with Cs-BAPTA in the absence of Ins(1,4,5)P₃ and calcium. In some cells, 2 μ M ionomycin was applied for 3 s using a wide-mouth glass pipette. The divalent-free external solution (DVF) was based on the standard external solution but in the absence of CaCl₂ and MgCl₂ and also contained 10 mM EDTA. Divalent replacement solutions were based on the standard external solution but with 10 mM CaCl₂ replaced by either 10 mM BaCl₂ or 10 mM SrCl₂. 2-aminoethoxydiphenyl borate (2-APB) was added to the standard external solution at a final concentration of 50 μ M. All chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO).

Subcloning and overexpression of CRACM1 and STIM1. Full-length human *CRACM1* and *STIM1* were amplified from human cDNA (PfuTurbo; Stratagene, La Jolla, CA) and subcloned in frame with the carboxy-terminal Myc–His tag in a pcDNA-4TO–Myc–His plasmid (Invitrogen, Carlsbad, CA). The full-length *CRACM1* was re-amplified along with the C-terminal Myc–His tag and subcloned into a pIRES2–EGFP plasmid (Clontech, Mountain View, CA). For overexpression of *CRACM1*, HEK293 or Jurkat cell lines were transfected with *CRACM1*–IRES–EGFP using lipofectamine 2000 (Invitrogen). Green cells were analysed for effects of *CRACM1* overexpression. For *STIM1* overexpression, HEK293 and Jurkat cells were cotransfected with C-terminal Myc–His tagged *STIM1* and GFP at a 10:1 ratio, respectively. GFP-expressing cells were analysed by single cell patch-clamp experiments to analyse the effects of *STIM1* overexpression. For co-overexpression experiments, *STIM1* was cotransfected with *CRACM1*–IRES–EGFP plasmid at a ratio of 10:1, respectively and green cells were analysed for the effects of co-expression of the two molecules. In approximately 80% of the green fluorescent HEK293 and Jurkat cells, large currents well in excess of 10 pA pF^{–1} were observed. These cells were presumed to co-overexpress *STIM1* and *CRACM1*–IRES–EGFP.