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Supporting Online Material for

The Calcium Store Sensor, STIM1, Reciprocally Controls Orai and $Ca_V1.2$ Channels

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The calcium store-sensor, STIM1, reciprocally controls Orai and CaV1.2 channels

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Experimental Procedures

DNA constructs, RNAi, cell culture and transfection — YFP-STIM1 D76A, YFP-STIM2, YFP-STIM1, YFP-STIM1-Ct (235-685), and Orai1 E106A-CFP were all inserted in the pIRESneo plasmid (Clontech, Palo Alto, CA). YFP-STIM1Δ441-448 was inserted in pDS plasmid. YFP-SOAR (344-442) was inserted in pEYFP-C1 plasmid. YFP-STIM1ΔK(1-666), GFP-SOAR (344-442), GFP-SOAR-LQ347/348AA and, mCherry-Orai1 were kindly provided by Dr. Shmuel Muallem (UT Southwestern) . GFP-α_1C plasmid was a kind gift from Dr. Kurt Beam (Univ. Colorado). mCherry-STIM1 was provided by Dr. Madesh Muniswamy (Temple Univ.). Rabbit α_{1C}, β_{2a}, and $\alpha_2\delta_1$ in vector were gifts from Dr. Michael Davis (Univ. Missouri). For knockdown studies, cells were mixed with 200 nM control (CAUGGUUUCCUUACUUUACA-CCUUU) or corresponding rat RNAi (STIM1: AUCAUCAUCCAUCAGCUUGUGGA-UG; Orai1: UGAACGGCAAAGACGAUAAACACCA) and then transfected using the following methods. For smooth muscle cells, transfection was achieved with the AMAXA nucleofector system. The transfection of DNA in HEK293 cells was done with electroporation using the BioRad Xcel electroporation system as previously described (*2*).

Intracellular Ca^{2+} measurements — Intracellular Ca^{2+} concentration was measured with a ratiometric dye fura-2, as previously described (*3*). Coverslips with cells grown on

them were placed in ion-safe solution (mM) 107 NaCl, 7.2 KCl, 1.2 $MgCl_2$, 11.5 glucose and 20 Hepes-NaOH, pH=7.2 containing 2 μM fura-2/acetoxymethylester for 30 min at 20°C. Cells were then washed with ion-safe solution and fura-2 trapped inside cells was allowed to de-esterify for 30 min at 20°C. With this dye loading protocol, approximately 95% of the dye was confined in the cytosol (*4*). Ca^{2+} measurements were performed on an InCyt dual-wavelength fluorescence imaging system (Intracellular Imaging Inc.). Briefly, Fura-2 was excited at 340 and 380 nm (OMEGA Optical, XF1093, 1094 respectively) alternatively at rate of 1 HZ, the fluorescence emission was first passed through a 415 nm long pass dichroic filter (OMEGA Optical, XF2002) and then monitored at 515 nm (OMEGA Optical, XF3043). Intracellular Ca^{2+} concentrations are represented by the ratio of the fluorescence intensities excited at 340 nm and 380 nm from groups of single cells. Data are shown as means ± SEM of traces from groups of individual cells (15 – 40 cells), and are representative of three or more independent experiments.

Fluorescence imaging: Fluorescence was examined using a Leica DMI 6000B fluorescence microscope equipped with CFP ($438_{Ex}/483_{Em}$), YFP ($500_{Ex}/542_{Em}$), GFP($472_{Ex}/520_{Em}$) and mCherry ($562_{Ex}/641_{Em}$) filters controlled by Slidebook Software (Intelligent Imaging Innovations; Denver, CO). All images were obtained at room temperature with 40 X oil len (N.A. 1.35, Leica). For studies on co-localization of GFP-α_{1C} with mCherry-STIM1 and mCherry Orai1 in HEK293 cells, high resolution images of ER-PM junctions were obtained by focusing on the PM at the cell-coverslip interface.

Electrophysiological measurements — Conventional whole cell recordings were undertaken in A7r5 or various HEK cell lines as described earlier (*1*). Right after establishment of the whole-cell configuration, a 50 ms voltage step (-100mV) from a holding potential of 0 mV, followed by a voltage ramp spanning from -100 to +100 mV in 50 ms was delivered every 2 seconds. For CRAC current measurements, the intracellular solution contained (mM): 145 CsGlu, 10 HEPES, 8 NaCl, 6 $MgCl_2$, 2 Mg-ATP (total 8 mM Mg^{2+}), 0.03 IP_3, 10 EGTA (or BAPTA where specified), pH 7.2. TRPM7 activity was suppressed by the presence of 8 mM Mg^{2+} and ATP (*3*). The

extracellular solutions contained (mM): 145 NaCl, 10 $CaCl_2$, 10 CsCl, 2 $MgCl_2$, 2.8 KCl, 10 HEPES, 10 glucose, pH 7.4. A 10 mV junction potential compensation was applied. For TRPC current experiments, IP_3 was omitted from the pipette solution and $CaCl_2$ was added to clamp the cytosolic Ca^{2+} at resting level (calculated via WEBMAXCLITE). To get a bigger TRPC current, Mg^{2+} concentration in internal solution was reduced to 2mM, Ca^{2+} concentration in external solution was chelated to 50 μM by 350 μM $CaCl_2$, and 300 μM EGTA (WEBMAXCLITE). For Ca_V current measurement, same pipette solution as that used for TRPC current were used. To minimize TRPC or CRAC current, Na was replaced by NMDG, and 10 mM $CaCl_2$ was substituted by 10 mM $BaCl_2$. Changes in whole cell Ba^{2+} currents were evaluated using 100-ms step pulses to different voltages. In A7r5 cells, linear leakage current was subtracted using scaled currents elicited by 10 mV hyperpolarizing pulses (*5*). With overexpressing HEK pIRES cells, current was obtained by subtracting nimodipine resistant current. All whole cell currents were filtered at 3.3 kHz and sampled at 10 kHz.

Real Time PCR — It was performed using ABI 7300 Fast Real Time PCR system (PerkinElmer Life Sciences). Total RNA was extracted under sterile conditions from A7r5 and primary rat aortic vascular smooth muscle cells with TriZOL reagent (Molecular Research Center Inc.). Primers (Table S1) were designed against the CDS sequence for each protein (NCBI protein database) using Primer premier software (5^{th} version) (Premier Biosoft) and purchased from Invitrogen. To reduce pipetting errors, 63μl master mixes for triplicates were made first. Each mix contained 33μl SYBR/Reverse Transcriptase Master Mix (Invitrogen EXPRESS One-Step SYBR Green ER Kit), 20μl primer master mix (0.1μg/μl) and 1μg total RNA. The mix was subsequently divided to 3 separate wells, 20μl each, on a 96 well plate. Standard amplification comprised 40 cycles of two stages (95º for 15secs followed by 60º for 1min). Fluorescent product was detected at the end of the second stage of each cycle. Data were analyzed using ABI system software and Relative Quantization of gene expression was calculated by the comparative threshold cycle method (ΔC_t) (*3*). C_t is the threshold cycle at which PCR product is detected. To reduce variations due to RNA quality and quantity, mRNA levels of GAPDH, a housekeeping gene in each cell type

were used as an internal control. Subtracting the C_t of the housekeeping gene from the C_t of the gene of interest yields the ΔC_t.

Western analyses and Immunoprecipitation — Cells were lysed in chilled Nonidet P-40 buffer (1% (v/v) Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, containing 100 μM phenylmethylsulfonyl fluoride and Sigma protease inhibitor mixture I followed by incubation for 30 min at 4°C and subsequent centrifugation (14,000 x g, 10 min at 4°C). The protein content of the supernatants was quantified with Bio-Rad DC protein assay kits. For immunoprecipitation, lysates (200 μg) were mixed with 50μl of a 50% protein G slurry (Calbiochem) that was precoated for 1 h with the indicated antibodies (5-10μl/sample). The samples were incubated for 2 h at 4 °C with rotation and were then washed three times with lysis buffer. After the final wash, beads were resuspended in gel-loading buffer and boiled for 10 min. Anti-GFP antibody is from Abcam; anti-GOK antibody is from BD Sciences; anti-actin antibody is from Millipore; anti-STIM1 antibody is purified in our lab from a poly-clonal anti-STIM1 serum. For western blot, protein extracts (25 μg per lane) were resolved on 6% SDS-polyacrylamide gels and electroblotted onto Bio-Rad Immun-Blot PVDF membranes. The resulting PVDF membranes were blocked (1h, room temperature) in Tris-buffered saline-Tween 20 (TBST; 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) containing membrane blocking agent (5%) (Amersham Biosciences) and subsequently incubated with corresponding primary antibodies (1h, 22°C). Membranes were washed two times (7 min) in TBST and incubated with secondary antibody (30 min, IgG conjugated to horseradish peroxidase). Subsequently, membranes were washed three times (5 min) in TBST followed by a single wash (5 min) in Tris-buffered saline (TBS; 150 mM NaCl, 10 mM Tris-HCl, pH 8.0). Peroxidase activity was visualized using the ECL kit as according to the manufacturer's instructions (Amersham Biosciences) with FluorChem®HD2 imaging system from Alpha Innotech.

Supplemental Literature Cited

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2. T. Hewavitharana *et al.*, *J. Biol. Chem.* **283**, 26252 (2008).
3. J. Soboloff *et al.*, *J. Biol. Chem.* **280**, 39786 (2005).
4. H. T. Ma *et al.*, *Science* **287**, 1647 (2000).
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Table S1. Primer sequences used in experiments

Target (Rat)	*GenBank™ accession number*	*Orientation*	*Sequence (5'-3')*	*Primer Location*
Orai1	*NM_001013982.1*	*Forward*	*CGTCTTTGCCGTTCACTTC*	*847-865*
		Reverse	*CTGTCGGTCCGTCTTATGG*	*901-883*

Orai2	XM_222288.4	Forward	GGGTCTCATCTTCGTGGTCTTT	798-819
		Reverse	CGCTCTGTCTTATGTCGCACC	863-843
Orai3	NM_001014024.1	Forward	ACACCAACGACTCCACCGATAT	570-591
		Reverse	TGACCCAGCCCACCAAAA	670-653
STIM1	NM_001108496.1	Forward	ATGATGCCAATGGTGATGT	606-624
		Reverse	TAGGTCCTCCACGCTGATA	736-711

	2	e		8
STIM1(2)	NM_001108496.2	Forward	TTGACCCATTCCGATTC	1922-1938
		Reverse	CATTGGAAGACGTGGC	2039-2024
STIM2	NM_001105750.1	Forward	CATAGTAATTGGCGTTGG	882-899
		Reverse	TGCTCTGCGGTTTGTAGG	992-975
GAPD	NM_01	Forwar	GCAAGTTCAACGGCACAG	231-24

H	7008.3	d Reverse	GCCAGTAGACTCCACGACAT	8 3701-3511

Supplemental Figure Legends

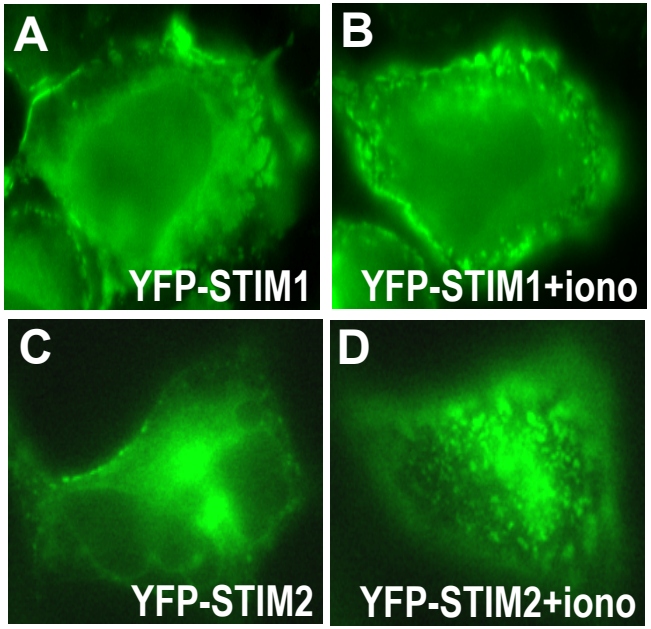
Fig. S1. STIM1 and STIM2 translocation in HEK293 cells is unaffected by co-expression of $Ca_V1.2$ channels (α1C+ β2a+ α2δ1). (**A,B**) Imaging of YFP-STIM1 in a single cell before and 5 min after 2 μM ionomycin to empty stores. (**C,D**) Imaging of YFP-STIM2 in a single cell before and 5 min after 2 μM ionomycin.

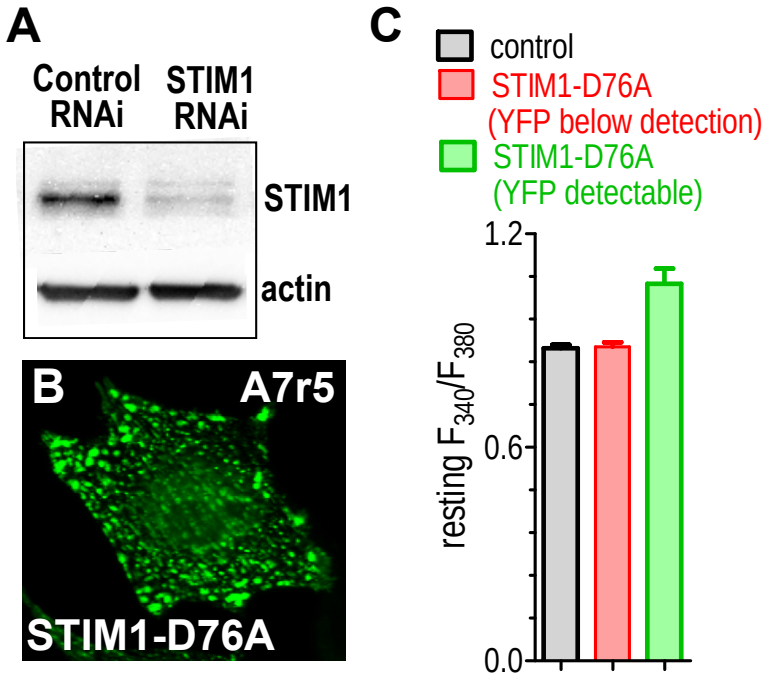
Fig. S2. (**A**) Effect of STIM1 RNAi knock down on STIM1 protein expression in A7r5 cells visualized with anti-STIM1 antibody. (**B**) Overexpressed YFP-STIM1D76A constitutively exists in localized puncta in A7r5 cells even without store depletion. (**C**) Effect of expression of YFP-STIM1 D76A on resting cytosolic Ca^{2+} levels in A7r5 cells. Compared to untransfected cells (grey), transfected cells with no detectable YFP fluorescence (red) have similar resting cytosolic Ca^{2+} level (p=0.8, n=7, paired T-test). Cells transfected with detectable YFP fluorescence (green) have only slightly higher resting cytosolic Ca^{2+} level (p=0.005, n=7, paired T-test).

Fig. S3. (**A-C**) HEK293 cells expressing the α_{1C} subunit; fura-2 responses to K^+/Sr^{2+} pulses (bars) before (**A**) and after (**B**) store-emptying with 2 μM ionomycin and subsequent 2 μM nimodipine addition (arrow). (**C**) Statistics (n=6, p=0.006 for STIM1, p=0.0001 for STIM2; paired T-test). (**D**) Western analysis of YFP-STIM1-D76A and GFP-α_{1C} in HEK293 cells. (**E**) Overexpressed YFP-STIM1D76A constitutively exists in localized puncta in HEK293 cells and is unaltered by co-expression of α_{1C}.

Fig. S4. (**A**) Statistics for data shown in Fig. 4B-D (n=7, paired T-test, p=0.04). (**B**) Relative YFP-SOAR expression in the two cell populations (Orai-coupled and Orai-uncoupled) shown in Fig. 4C (n=10, paired T-test, p=0.0005). (**C**) Statistics for data shown in Fig. 4K (n=7, paired T-test, STIM1 VS STIM1Δ441-448, p=0.7; STIM1ΔK VS STIM1, p=0.0008; STIM1ΔK VS STIM1Δ441-448, p=0.002).

SUPPL. FIG. 1




A
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STIM1
actin
B
A7r5
STIM1-D76A
C
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STIM1-D76A
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STIM1-D76A
(YFP detectable)
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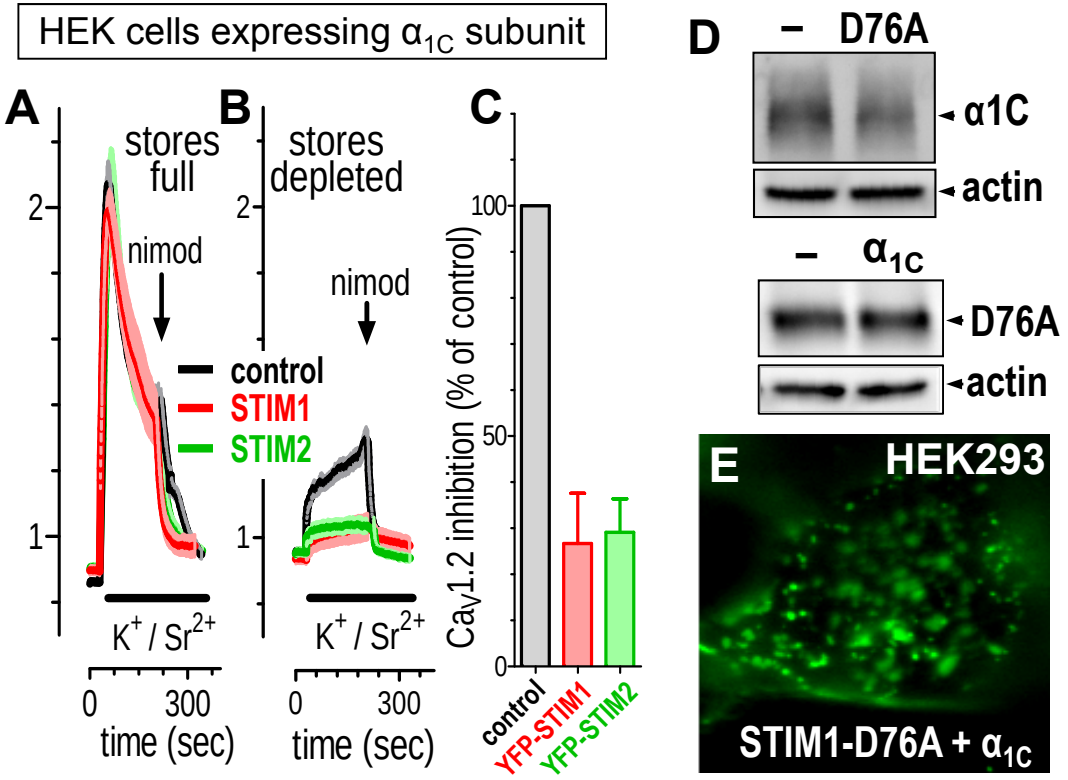
0.6
0.0
resting F_{340}/F_{380}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nimod
B
stores depleted
nimod
control
STIM1
STIM2
K^+ / Sr^{2+}
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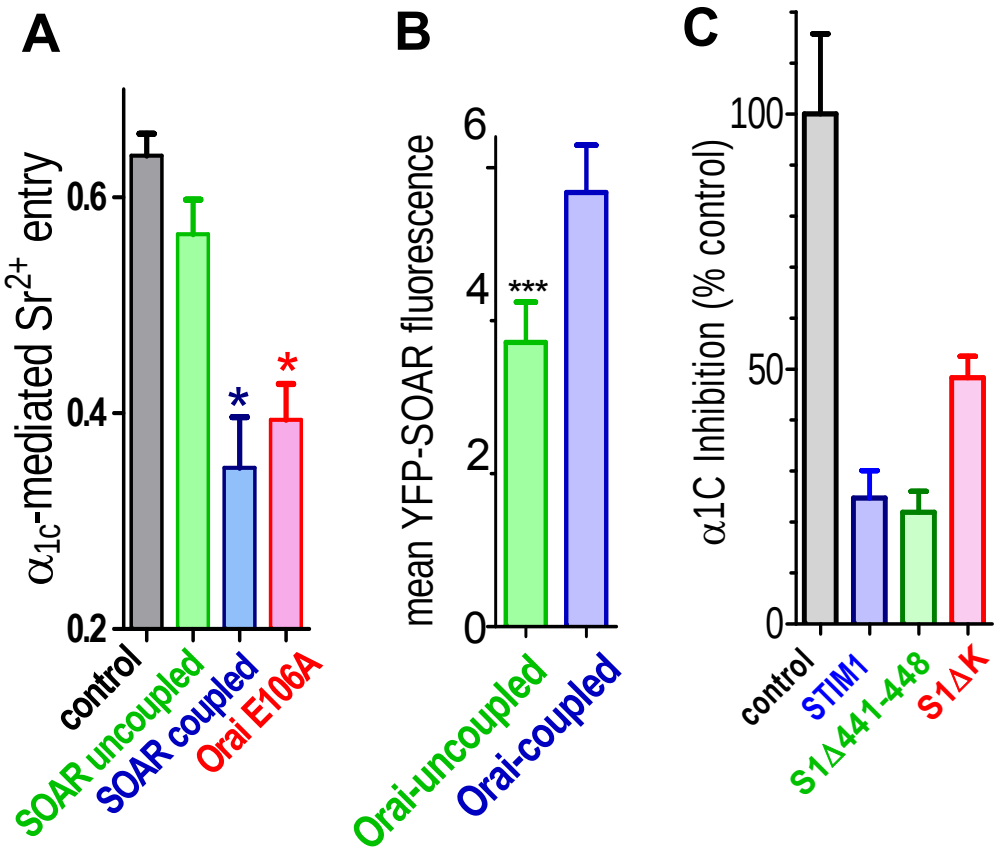
time (sec)
C
$Ca_V1.2$ inhibition (% of control)
100
50
0
control
YFP-STIM1
YFP-STIM2
D
– D76A
α1C
actin
– α_{1C}
D76A
actin
E
HEK293
STIM1-D76A + α_{1C}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0.4
0.2
*
*
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SOAR coupled
Orai E106A
B
mean YFP-SOAR fluorescence
6
4
2
0
***
Orai-uncoupled
Orai-coupled
C
α1C Inhibition (% control)
100
50
0
control
STIM1
S1Δ441-448
S1ΔK