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Supplementary Materials for

TRPM2 Functions as a Lysosomal Ca^{2+}-Release Channel in β Cells

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Materials and Methods

Cells: HEK293-TRex non-transfected (wild type) and Tetracycline-inducible HEK-293 Flag-TRPM2-expressing cells (*2*) were cultured at 37°C with 5% CO2 in DMEM supplemented with 10% fetal bovine serum. The medium was supplemented with blasticidin (5 µg/ml; Invitrogen) and zeocin (0.4 mg/ml; Invitrogen). TRPM2 expression was induced by adding 1 µg/ml tetracycline to the medium 16 to 22 hours before experiments. The insulinoma rat cell line INS-1 was kept at 37°C with 5% CO_2 in RPMI containing 10% fetal bovine serum. Pancreatic β-cells were isolated from C57BL/6 wild type or CD38 knockout mice. Adult experimental mice (10 to 40 g) were anesthesized by enflurane inhalation and subsequently euthanized by cervical dislocation. An incision was made in the abdomen to expose the pancreas. The pancreatic duct was clamped at the duodenal insertion with a hemostat before inserting a cannula into the duct. The pancreas was perfused with 1.5 mg/mL collagenase, then isolated, placed in a conical tube, and incubated at 37∞C for 20 minutes. The pancreatic tissue was rinsed three times with ice-cold RPMI 1640 medium, and digested tissue was filtered through a 400 µM metal sieve to separate the pancreatic islets. The islets were further purified in Histopaque overlaid with RPMI 1640 medium by centrifuging for 20 minutes at 4∞C. The islets were handpicked, then digested in 0.1% trypsin-EDTA in RPMI 1640, washed three times in RPMI 1640, plated on cover slips, and incubated in RPMI 1640 supplemented with 10% FBS overnight at 37∞C. The experimental protocol was performed in accordance with institutional and national regulations and was approved by the Institutional Animal Care and Use Committee (IACUC), University of Hawaii and the Animal Care Committee (ACC), The Queen's Medical Center.

Solutions and Chemicals: Cells were kept in standard external Ringer's solution (in mM): 140 NaCl, 2.8 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 glucose, 10 HEPES-NaOH (pH 7.2 adjusted with NaOH). Standard internal pipette-filling solutions contained (in mM): 140 Cs-glutamate for INS-1 and primary mouse β-cells, 140 K-glutamate for HEK293 WT/TRPM2, 8 NaCl, 1 $MgCl_2$, 10 HEPES-Cs/KOH (pH 7.2 adjusted with CsOH/KOH). Ca^{2+} was left unbuffered by leaving out Ca^{2+} chelators. ADPR was added to its final concentrations as appropriate. All reagents were purchased from Sigma-Aldrich except for suramin (Fluka). 8-Br-ADPR was synthesized as described previously (*22*). Relative purity of 8-Br-ADPR was assessed by HPLC analyses and found grater than 95%. No traceable 8-Br-NAD^+ or 8-Br-cADPR contaminants were detected (*45*). Note that AMP-monophosphate-monohydrate (Sigma-Aldrich) was used.

Electrophysiology: Patch-clamp experiments were performed in the whole-cell configuration at 21 to 25 ˚C. All data were acquired with "Pulse" software controlling an EPC-9 amplifier (HEKA, Lambrecht, Germany) and analyzed using FitMaster (HEKA) and Igor Pro (Wavemetrics). Voltage ramps of 50 ms spanning the voltage range from -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 s to 150 s. Voltages were corrected for liquid junction potentials. Currents were filtered at 2.9 kHz and digitized at 100 µs intervals. Capacitive currents and series resistance were determined and corrected before each voltage ramp. For analysis, current amplitudes were extracted at –80 mV and normalized to cell capacitance. External solution changes were performed using a wide-mouthed glass pipette controlled by a pneumatic pressure devise (Lorenz Messgeraetebau, Katlenburg-Lindau, Germany).

INS-1 siRNA experiments: Three siRNA 25-mers (20 nM) matching the rat TRPM2 sequences were obtained from Invitrogen using their BLOCK-iT™ RNAi Designer for custom Stealth

siRNA duplex oligoribonucleotides and diluted in diethylpyrocarbonate (DEPC) treated water (1 ml) to a concentration of 20 pmol/µl. Uptake of RNA was confirmed using AM4620 Silencer® FAM™ labeled Negative Control #1 siRNA from Ambion (20 pmol/µl) with Olympus IX70 Inverted Microscope fluorescence microscopy. Stealth RNAi Negative Control Medium GC Duplex (Cat. No. 12935-300, Invitrogen) (20 pmol/µl) was used as a control for sequence-independent effects. INS-1 cells were seeded in six well tissue culture plates at $2x10^5$ cells per well in 2 ml RPMI containing 10% FBS. Cells were cultured in a CO_2 incubator at 37°C until 70-80% confluence. Cells were transfected according to manufacturer's protocol using Invitrogen Lipofectamine 2000. Cells were cultured after transfection for 18 to 24 hours, trypsinized, washed, and plated on glass coverslips. Patch clamp and imaging experiments were performed 40 to 60 hours post-transfection. Screened sequence of efficient knockdown of rat-TRPM2 was (RNA) - 5' UAA GCG UUC AUG CUC UUC UGC CAG C 3'. Rat GAPDH [Ambion; (*29*)] siRNA was used as control siRNA for flow cytometric experiments.

Cell death assay: INS-1 cells were cultured for 24 hours after transfection with siRNA directed against GAPDH or TRPM2 siRNA, trypsinized, washed, and seeded at 1 x 10^5 cells/well in 24-well culture plates. H_2O_2-induced cell death was assayed 40 to 50 hours post-transfection. After H_2O_2 stimulation for 10 min in RPMI 1640 containing either 0.42 mM Ca^{2+} or left Ca^{2+} free, cells were trypsinized, pelleted, washed with cold PBS, and stained with 6.25 mg/ml propidium iodide (PI; Invitrogen). PI-positive cells were analyzed by EPICS Altra flow cytometer (Beckman Coulter).

Polyclonal anti-TRPM2 Antibody Generation: Rabbits were immunized with a synthetic peptide CNHKTILQKVASLFGA, located in the C-terminal portion of mouse TRPM2, conjugated to KLH, and sera from three bleeds as well as preimmune serum were collected.

TRPM2 and ER Fluorescence labeling in INS-1 cells: INS-1 cells were grown on coverslips. The cells were washed with PBS, fixed with 2% paraformaldehyde (Sigma) and permeabilized with 0.2% Triton X-100 (0.05%) for 5 min at room temperature. Slides were blocked with 10% of goat serum and then incubated with anti-mTRPM2 specific serum or preimmune serum (1:500) and anti-PDI (Invitrogen 1:1000), mouse antibody directed against the ER-associated protein disulfide isomerase, for 2 hours at 37°C. Goat anti-rabbit Alexa Fluor 488 and Goat anti-mouse Alexa Fluor 594 (Molecular Probes 1:1000) were used as a secondary antibodies. Nucleic acids were stained with 4'-6'-diamine-2 phenylindole dihydrochloride (DAPI)-containing mounting medium ProLong gold (Invitrogen) Slides were mounted and cells visualized with a Zeiss ApoTome Axiovert 200 imaging microscope at 63x using an Axiocam MRM CCD camera and the Zeiss AxioVision software.

TRPM2 and Lysosome Fluorescence labeling in INS-1 cells: INS-1 cells were grown on coverslips, washed with PBS, fixed with ice cold methanol and permeabilized with 0.2% Triton X-100 (0.2%) for 5 min at room temperature. Slides were blocked with 1% of fish gelatin and then incubated with anti-mTRPM2 specific serum or preimmune serum (1:400) and anti-mLAMP1 (LY1C6, 1:100, Abcam) for 1.5 hours at 37° C. Goat anti-rabbit Alexa Fluor 568 and Goat anti-mouse Alexa Fluor 488 (Molecular Probes 1:1250) were used as a secondary antibodies. Slides were mounted in ProLong gold medium (Invitrogen) and cells were visualized on a Zeiss 5 Pascal confocal laser microscope.

Multipeak analysis of Ca^{2+} release signals: The number of Ca^{2+} transients was assessed by a fitting routine convoluting a Gaussian fit and an exponential decay. Only the first oscillation was

selected for detailed analysis, including time-to-peak and Ca^{2+} peak amplitude according to $y(x) = ae^{-bx} + a\exp(-((x-b)/c)^2)$, where *a* is the peak amplitude, *b* is the peak centroid, and *c* is related to the peak width. The resulting values were averaged and their mean and S.E.M. plotted in Table S1 and selectively displayed in Fig. S4.

Supplemental Figures

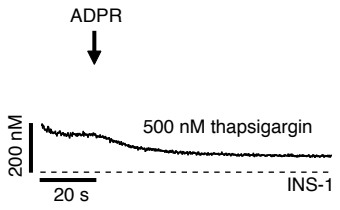

Fig. S1. Thapsigargin prevents Ca^{2+} release induced by extracellular ADPR. Average Ca^{2+} signals measured in INS-1 cells in response to application of 100 µM ADPR in the absence of extracellular Ca^{2+} and presence of 1 µM CGS-15943, 100 µM suramin, and 500 nM thapsigargin (n = 11). Cells were loaded with 5 µM Fura-2-AM at 37° C for 30 min.

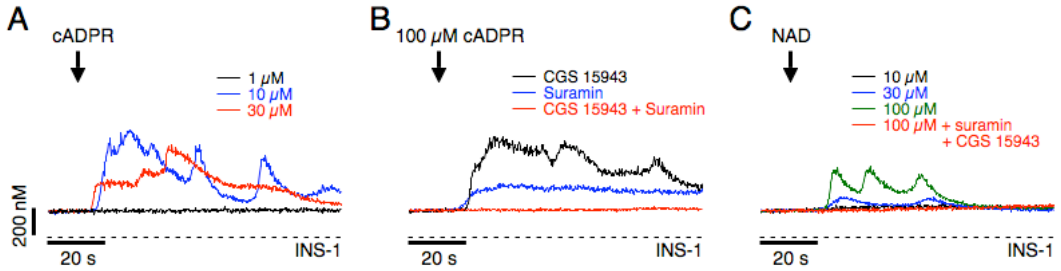

Fig. S2. Extracellular cADPR and NAD^+ evoke Ca^{2+} signals in INS-1 β-cells. (A) Average Ca^{2+} signal measured in intact Fura-2 AM-loaded INS-1 cells in response to increasing concentrations of extracellular cADPR [1 µM (black trace, n = 5), 10 µM (blue trace, n = 8), 30 µM (red trace, n = 5)] and in the absence of extracellular Ca^{2+}. Application start as indicated by the arrow. (**B**) Average Ca^{2+} signal measured in intact Fura-2 AM-loaded INS-1 cells in response to application of 100 µM cADPR in the absence of extracellular Ca^{2+} and in the presence of either 100 µM suramin (blue trace, n = 6) or 1 µM CGS-15943 (black trace, n = 5) or both suramin and CGS-15943 (red trace, n = 6) in the external solution. (**C**) Average Ca^{2+} signal measured in intact Fura-2 AM-loaded INS-1 cells in response to application of 10 µM (black trace, n = 5), 30 µM (blue trace, n = 7), or 100 µM (green trace, n = 10) NAD^+ in the absence of extracellular Ca^{2+}. The red trace indicates application of 100 µM NAD^+ in the presence of 100 µM suramin and 1 µM CGS-15943 (n = 7) in the external solution.

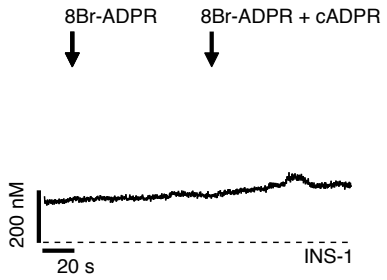

Fig. S3. 8-Br-ADPR application inhibits Ca^{2+} release by cADPR. Average Ca^{2+} signals measured in INS-1 cells in response to application of extracellular 8-Bromo-ADPR (100 µM) and, shortly thereafter, 8-Bromo-ADPR and 100 µM cADPR without extracellular Ca^{2+} (n = 12) in the standard external solution. Application started as indicated by arrows and was maintained throughout the experiment. Cells were loaded with 5 µM Fura-2 AM at 37° C for 30 min.

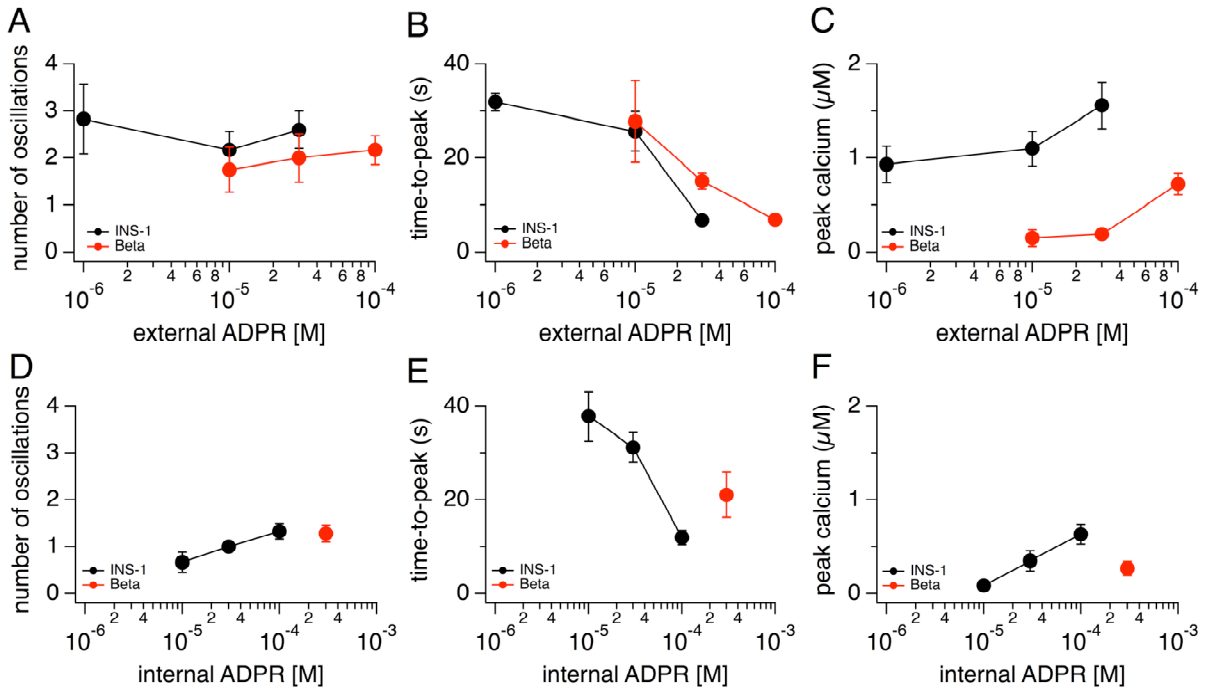

Fig. S4. Statistical analysis of Ca^{2+} signals induced by external or internal ADPR in β-cells. Ca^{2+} signals were analyzed for number of oscillations using a Gaussian fitting routine (panels A,D). The first oscillation (if any) was assessed for time-to-peak (panels B,E) and Ca^{2+}-peak amplitude (Ca^{2+} peak; panels C,F). Black circles are data for INS-1 cells, red circles are data for primary mouse β-cells. Error bar indicates S.E.M. Data plotted from Table S1.

Table S1. Statistical analysis of agonist-induced Ca^{2+} signals in TRPM2 HEK and β-cells. Where feasible, Ca^{2+} signals were analyzed for number of oscillations (no. of peaks). The first oscillation was analyzed for time-to-peak (TTP) and Ca^{2+}-peak amplitude (Ca^{2+} peak) ± S.E.M. with n determinations. The first column indicates figures, panels and conditions. The last column details the cell type used.

	TTP (s)	SEM	Ca^{2+} Peak (M)	SEM	Area (M)	SEM	no. of peaks	SEM	n	Cell Type
Fig. 1 HEK Cells										
A										
1 mM Ca	26.98	1.42	7.44×10^{-7}	2.63×10^{-7}	7.18×10^{-6}	3.17×10^{-6}	1	–	8	TRPM2 HEK
0 mM Ca	30.19	2.002	9.64×10^{-7}	1.58×10^{-7}	8.92×10^{-6}	1.36×10^{-6}	1	–	7	
B										
1 mM ADPR	29.52	1.1	7.17×10^{-7}	1.82×10^{-7}	1.09×10^{-5}	1.07×10^{-6}	1	–	7	WT HEK
C										
ATP/ADPR	28.95	0.98	1.04×10^{-6}	2.58×10^{-7}	1.15×10^{-5}	1.88×10^{-6}	1.3	0.21	6	TRPM2 HEK
D										
ADPR	14.34	3.07	6.06×10^{-7}	2.39×10^{-7}	9.46×10^{-6}	2.18×10^{-6}	1	–	4	TRPM2 HEK
E										
1 mM ADPR	19.65	4.40	6.77×10^{-7}	1.48×10^{-7}	1.39×10^{-5}	4.47×10^{-6}	1.17	0.16	6	TRPM2 HEK
0.1 mM ADPR	17.25	1.10	3.87×10^{-7}	5.10E-08	1.14×10^{-5}	2.80×10^{-6}	1.6	0.2	7	
AMP	–	–	–	–	–	–	–	–	8	
Fig. 2 INS-1 Cells	**$[ADPR]_{exter-1}$**									
D										
100 nM ADPR	–	–	–	–	–	–	–	–	6	INS-1 Cells
1 µM ADPR	31.92	1.90	9.31×10^{-7}	1.94×10^{-7}	1.13×10^{-5}	3.15×10^{-6}	2.8	0.74	6	
10 µM ADPR	25.67	4.20	1.10×10^{-6}	1.86×10^{-7}	2.22×10^{-5}	8.84×10^{-6}	2.17	0.4	6	
30 µM ADPR	6.744	0.67	1.56×10^{-6}	2.48×10^{-7}	1.67×10^{-5}	2.86×10^{-6}	2.6	0.4	6	
E										
100 µM ADPR	18.16	1.78	1.26×10^{-6}	2.01×10^{-7}	2.36×10^{-5}	5.47×10^{-6}	2.55	0.34	11	INS-1 Cells
suramin	15.75	1.81	5.62×10^{-7}	1.42×10^{-7}	1.67×10^{-5}	6.23×10^{-6}	3.1	0.78	8	
CGS	16.1	5.42	5.26×10^{-7}	1.97×10^{-7}	6.87×10^{-6}	2.39×10^{-6}	2.5	0.52	11	
F										
ADPR CGS	16.64	1.51	8.31×10^{-7}	2.06×10^{-7}	1.41×10^{-5}	2.48×10^{-6}	4.17	1.01	6	INS-1 Cells

	TTP (s)	SEM		Ca^{2+} Peak (M)	SEM		Area (M)	SEM		no. of peaks	SEM	n	Cell Type
Fig. 3 INS-1 Cells	**$[ADPR]_{inter-1}$**												
A													
10 µM ADPR	37.81	5.25		8.69E-08	3.06E-08		4.97×10^{-6}	2.97×10^{-6}		0.67	0.21	6	INS-1 Cells
30 µM ADPR	31.17	3.17		3.50×10^{-7}	1.05×10^{-7}		1.40×10^{-5}	4.77×10^{-6}		1	–	8	
100 µM ADPR	11.99	1.57		6.35×10^{-7}	1.08×10^{-7}		1.38×10^{-5}	2.94×10^{-6}		1.3	0.16	9	
B													
H2O2	16.51	2.16		5.43×10^{-7}	1.15×10^{-7}		2.21×10^{-5}	6.35×10^{-6}		1.3	0.15	10	INS-1 Cells
C													
scramble	12.05	1.34		6.32×10^{-7}	1.11×10^{-7}		1.60×10^{-5}	3.01×10^{-6}		1.7	0.39	10	INS-1 Cells
M2 KO	–	–		–	–		–	–		–	–	10	
Fig. 5 Mouse beta													
D													
1 µM ADPR	–	–		–	–		–	–		–	–	4	Mouse Beta
10 µM ADPR	27.82	8.77		1.47×10^{-7}	8.79E-08		1.62×10^{-6}	5.77×10^{-7}		1.75	0.47	5	
30 µM ADPR	15.08	1.70		1.88×10^{-7}	4.84E-08		1.65×10^{-6}	3.89×10^{-7}		2	0.51	6	
100µM ADPR	6.87	0.81		7.23×10^{-7}	1.15×10^{-7}		8.52×10^{-6}	2.29×10^{-6}		2.17	0.30	6	
E													
ADPR CGS	5.32	0.82		9.48×10^{-7}	2.13×10^{-7}		2.73×10^{-6}	4.95×10^{-7}		1	–	8	Mouse Beta
F													
$[ADPR]_{inter-1}$	21.06	4.80329		2.71×10^{-7}	7.13E-08		2.54×10^{-5}	1.15×10^{-5}		1.29	0.18	7	Mouse Beta
Fig. 6	**$[X]_{exter-1}$**												
A													
10 mM cADPR	113.43	21.88		1.60×10^{-7}	6.73E-08		1.14×10^{-5}	3.32×10^{-6}		1	–	8	WT HEK
10 mM –D	39.49			1.88×10^{-7}			3.86×10^{-6}			1	–	1/6	
B													
30 µM cADPR	17.62	3.38		6.85×10^{-7}	1.62×10^{-7}		7.78×10^{-6}	2.37×10^{-6}		2	0.44	5	INS-1 Cells
100 µM –D	37.15	10.25		6.46×10^{-7}	2.29×10^{-7}		8.96×10^{-6}	3.24×10^{-6}		1.2	0.29	10	
C													
300µM cADPR	15.73	2.42		3.83×10^{-7}	1.10×10^{-7}		4.45×10^{-6}	1.34×10^{-6}		1.17	0.16	6	Mouse Beta
1 mM –D	–	–		–	–		–	–		–	–	8	
D													
ADPR	8.96	0.93		3.86×10^{-7}	9.05E-08		3.65×10^{-6}	7.03×10^{-7}		1.5	0.5	4	CD38 KO
ATP	5.51	0.61		4.16×10^{-7}	1.01×10^{-7}		4.13×10^{-6}	1.16×10^{-6}		1.25	0.16	8	
cADPR	–	–		–	–		–	–		–	–	20	
Fig. S2 INS-1 Cells	**$[cADPR]_{exter-1}$**												
A													
1 µM cADPR	–	–		–	–		–	–		–	–	5	INS-1 Cells
10 µM cADPR	14.27	1.82		6.58×10^{-7}	2.58×10^{-7}		7.89×10^{-6}	3.70×10^{-6}		2	0.88	8	
B													
cADPR SUR	22.76	3.50		1.67×10^{-7}	4.59E-08		5.38×10^{-6}	1.87×10^{-6}		1.8	0.47	6	INS-1 Cells
cADPR CGS	19.93	2.52		7.04×10^{-7}	2.11×10^{-7}		1.18×10^{-5}	4.92×10^{-6}		3	0.44	5	