SUPPLEMENTAL DATA

Two novel 2-Aminoethyl diphenylborinate (2-APB) analogues differentially activate and inhibit store-operated Ca^{2+} entry via STIM proteins

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Materials and Methods

DNA constructs

Full length of human CRACM1 (Orai1), CRACM2 (Orai2) and CRACM3 (Orai3) were subcloned as described previously (Lis A, Peinelt C, Beck A, Parvez S, Monteilh-Zoller M, *et al*. (2007) *Curr Biol* 17:794-800).

Human STIM1 stable HEK cells and human STIM2 stable HEK cells were kindly provided by D.L. Gill's lab.

Mouse CRACM1 was purchased from DNAFORM (Kanagawa, Japan) and HA tag was added by inserting the PCR product amplified with 5'-AAGGTACCTACTCCATGCATCCGGAGCC-3' and 5'-GAGAATTCTTAGGCATAGTGGGTGCCCG-3' primers into pHM6 vector.

Human STIM1 was cloned into pcDNA3.1 myc-His vector (Invitrogen) after the EcoRI/XhoI digestion of the PCR product from cDNA of HeLa cells amplified with 5'-GGGAATTCGAGTCATGGATGTATGCGTC-3' and 5'-GGCTCGAGCTTCTTAAGAGGCTTCTTAA-3' primers. EGFP-STIM1 was prepared by inserting EGFP into human STIM1. EGFP fragment was amplified from pEGFP-C1 (Clontech) with PCR primers of 5'-GGGATATCATGGTGAGCAAGGGCGAGGAG-3' and 5'-GGGATATCCTTGTACAGCTCGTCCATGCC-3' to introduce the EcoRV restriction site. Human STIM1 was introduced mutations from 29Glu/30Lys to

29Asp/30Ile (from GAGAAG to GATATC) to obtain EcoRV restriction site and EGFP fragment was inserted.

STIM1 deletion constructs were produced from EGFP-STIM1 by PCR amplification, enzymatic digestion and ligation. For Δ28–209, PCR primers of 5'-GGGAATTCGAGTCATGGATGTATGCGTC-3' and 5'-GTCCTTGAGGTGATTCTTGTACAGCTCGTCCATGCC-3'; and 5'-GACGAGCTGTACAAGAATCACCTCAAGGACTTCATG-3' and 5'-GGCTCGAGCTACTTCTTAAGAGGCTTCT-3' were used and the products of both reactions were mixed, used for subsequent PCR reaction with 5'-GGGAATTCGAGTCATGGATGTATGCGTC-3' and 5'-GGCTCGAGCTACTTCTTAAGAGGCTTCT-3' primers, then cloned into pcDNA3.1-mycHis after EcoRI/XhoI digestions. Δ208C was obtained with 5'-GGGAATTCGAGTCATGGATGTATGCGTC -3' and 5'-ACCTCGAGCTAATGGCGAGTCAAGAGAGGAGG-3' primers and EcoRI/XhoI digestions, cloned into pcDNA3.1-mycHis. Δ246C was obtained with 5'-GGGAATTCGAGTCATGGATGTATGCGTC -3' and 5'-ACCTCGAGCTACATCTTCTTCATGTGCTCCTT-3' primers and EcoRI/XhoI digestions, cloned into pcDNA3.1-mycHis. ΔN251 was obtained with 5'-ACGAATTCTTTACACCGAGCTGAGCAGAG-3' and 5'-TGGGATCCCTACTTCTTAAGAGGCTTCT-3' primers and EcoRI/BamHI digestions, cloned into pEGFP-C1.

Ca^{2+} imaging of DT40 cells

IP_3R-deficient DT40 cells in log-phase were transferred to a Ca^{2+}-containing Hepes-buffered salt solution (HBSS(+)), consisting of (in mM): 107 NaCl, 6 KCl, 1.2 $MgSO_4$, 2 $CaCl_2$, 1.2 KH_2PO_4, 11.5 glucose, and 20 Hepes, with the pH adjusted to 7.4, loaded with 3 μM fura-2 acetoxy-methyl ester (fura-2/AM; Dojindo, Kumamoto, Japan) at 30 °C in the dark for 30 min, washed three times with

Ca^{2+}-free HBSS (HBSS(–)) (identical to HBSS(+) except that the Ca^{2+} was omitted and 0.5 mM EGTA was included). The washed cells were resuspended in 500 μl HBSS(–) in a stirred cuvette at a density of 1 x 10^6 cells/ml, and fluorescence measurements were carried out at 30 °C using a dual-wavelength spectrofluorometer (CAF-110, Jasco, Tokyo, Japan). Fura-2 fluorescence intensities were monitored at the emission wavelength of 510 nm using alternate excitation wavelengths of 340 and 380 nm (F_{340nm} and F_{380nm}), and the ratio of the emissions (F_{340nm}/F_{380nm}) was calculated. The maximum and minimum fluorescence ratios (R_{max} and R_{min}) were obtained in the presence of 0.2% Triton X-100 or 5 mM EGTA, respectively. The Ca^{2+} concentration was calculated according to Grynkiewicz's equation (Grynkiewicz G, Poenie M & Tsien RY (1985) *J Biol Chem* 260:3440-3450).

Animal experiments

All the animal experiments were done under the guidelines of Institute of Medical Science, The University of Tokyo.

Overexpression of STIM and/or CRACM in HEK293 and HeLa cells

For electrophysiological analysis, CRACM proteins were overexpressed in HEK293 cells (wild type, stably expressing STIM1 or STIM2; Soboloff J, Spassova MA, Hewavitharana T, He LP, Xu W, *et al*. (2006) *Curr Biol* 16:1465-1470) using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Experiments were performed 24–48 h post-transfection. CRACM proteins expressing cells were identified by the fluorescence of GFP, which is expressed in transfected cells under the control of IRES sequence.

For STIM1 clustering experiment, HeLa cells were transfected with N-terminal EGFP-tagged human STIM1. Experiments were performed 24–48 h post-transfection.

Data analysis and fitting

In the experiments of SOCE in DT40 cells, data points of DPB162-AE were fitted with single exponential dose-response function ($f(x) = Ymax / (1 + (k / x)^n)$): where Ymax is maximum value of ratio (% of control), k indicates IC_{50}, and n indicates Hill coefficient) and 2-APB and DPB163-AE data were fitted with double exponential dose-response function ($f(x) = –Y1 / (1 + (x / k1)^{n1}) + Y2 / (1 + (x / k2)^{n2})$): where k1 indicates IC_{50} for inhibition, k2 indicates EC_{50} for activation and n1 and n2 indicate Hill coefficients).

For the experiments of IICR and SOCE in CHO-K1 cells, data points were fitted with single exponential dose-response function.

For the experiments of SOCE in HeLa cells, data points of DPB162-AE were fitted with single exponential dose-response function ($f(x) = Ymin + (Ymax – Ymin) / (1 + (k / x)^n)$): where Ymin is minimum value of ratio (% of control), Ymax is maximum value of ratio (% of control), k indicates IC_{50}, and n indicates Hill coefficient). Data points of DPB163-AE were fitted with double exponential dose-response function ($f(x) = Y1 / (1 + (x / k1)^{n1}) + Y2 / (1 + (k2 / x)^{n2})$): where k1 indicates IC_{50} for inhibition, k2 indicates EC_{50} for activation and n1 and n2 indicate Hill coefficients).

NMR analysis of compounds

NMR spectra were recorded on JEOL JNM-GSX 270 FT NMR SYSTEM or JNM-Alpha400 (JEOL Inc.). High-resolution mass spectra (HRMS) were recorded on JMS-700 (JEOL Inc.) using a mass resolution of 5000.

DPB162-AE

^{1}H NMR (270 MHz, DMSO-d_6) δ 7.40-7.23 (m, 8H), 7.14-7.00 (m, 10H), 6.06 (br, 4H, NH_2), 4.40 (s, 4H), 3.75 (t, 4H, J = 6.3 Hz), 2.81 (tt, 4H, J = 6.3 Hz, 5.9 Hz) ^{13}C NMR (67.5 MHz, DMSO-d_6) δ 136.0, 131.3, 130.9, 130.6, 126.4, 126.3, 124.7, 124.4, 72.2, 62.4, 41.4 HRMS(FAB)(m/z): calculated for $C_{30}H_{35}O_3N_2B_2^+$ [M+H$^+$] 493.2834, found 493.2849

DPB163-AE

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.39-7.35 (m, 8H), 7.14-7.00 (m, 10H), 6.04 (br, 4H, NH_2), 4.37 (s, 4H), 3.75 (t, 4H, J = 6.0 Hz), 2.81 (t, 4H, J = 6.0 Hz) ^{13}C NMR (100 MHz, DMSO-d_6) δ 134.9, 131.5, 131.4, 126.6, 126.2, 124.9, 71.6, 62.4, 41.3 HRMS(FAB)(m/z): calculated for $C_{30}H_{35}O_3N_2B_2^+$ [M+H$^+$] 493.2834, found 493.2838

Figure legend

Figure S1: DPB compounds do not activate individual CRACM/Orai channels without STIM

(*A–F*) Average CRAC current densities at –80 mV in HEK293 cells expressing each subtype of CRACM/Orai proteins. I_{CRAC} was activated by IP_3 mediated store depletion and the effect of each compound at 1 μM was tested. DPB162-AE was applied to CRACM1/Orai1 (*A*, n = 5), CRACM2/Orai2 (*C*, n = 7) or CRACM3/Orai3 (*E*, n = 8) expressing HEK cells. DPB163-AE was applied to CRACM1/Orai1 (*B*, n = 8), CRACM2/Orai2 (*D*, n = 5) or CRACM3/Orai3 (*F*, n = 5) expressing HEK cells. Data are shown as mean ± SEM.

Figure S1 Goto et al.