Supporting Information

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SI Materials and Methods

Reagents. Chemicals, unless otherwise indicated, were obtained from Sigma or Merck. Ryanodine, Ruthenium Red, and CALP-3 were purchased from Tocris Biosciences. All fluorescent dyes and rhodamine 110 bis-(CBZ-L-isoleucyl-L-prolyl-L-arginine amide) dihydrochloride (BZiPAR) were purchased from Invitrogen, and FFP-18 (K^+) salt was from TEF Labs. Antibodies against IP_3Rs and control antibodies were from Brain Science Institute, RIKEN, Saitama, Japan (KM1112, KM1083, and KM1082), Millipore (AB3000, AB9076, and CBL600B), and Santa Cruz Biotechnology (sc-28614).

Isolation of Pancreatic Acinar Cells. Single pancreatic acinar cells and clusters of two or three acinar cells were isolated from the pancreas of adult CD 1 male mice as described previously (1, 2). Briefly, animals were killed according to United Kingdom Schedule 1 regulations and the pancreas was rapidly dissected, transferred into collagenase (220 units/mL; Worthington), and incubated at 36.5 °C for 14–15 min. After digestion, the tissue was mechanically disrupted in Na-Hepes–based extracellular media, containing 140 mM NaCl, 4.7 mM KCl, 10 mM Hepes KOH, 1 mM $MgCl_2$, 10 mM glucose, 1 mM $CaCl_2$ (pH 7.2). Cells were then incubated with fluorescent dye following the manufacturer's description. All experiments were carried out with freshly isolated pancreatic acinar cells, attached to the coverslip of the perfusion chamber at room temperature (23 °C).

$[Ca^{2+}]$ Measurements in Intact Cells. Intact cells were loaded with either 2.5 μM Fluo-4 acetoxymethyl ester (AM) or 5 μM Fura Red AM or Fura-2 AM for 30 min at room temperature. Cells were transferred into a flow chamber and perfused with the Na-Hepes–based extracellular solution described above. Cells were visualized using a Leica SP2 MP dual or SP5 MPII two-photon confocal microscope, with a 63× 1.2-NA objective lens. Fluo-4 and Fura Red were excited with a 488-nm Argon laser, at 2.5–3% power, and emitted light was collected at 510–640 nm. Generally, a series of images was recorded at 512 × 512 pixels resolution (at the speed of 0.61 frames/s) and analyzed using Leica Confocal Software. Fluorescence signals were plotted as F/F_0 (F_0 is the initial level of fluorescence). Fura-2 measurements were performed with a TILL Photonics monochromator-based imaging system using 340 ± 5- and 380 ± 5-nm band excitation and collecting emission at 510–550 nm. Statistical analysis was performed using Origin 7.5 Pro software (Origin Labs), and statistical significance and P values were calculated using an ANOVA test.

$[Ca^{2+}]$ Measurements in Permeabilized Cells. Cells to be permeabilized were loaded with 5–7.5 μM Fluo-5N AM, for 45 min at 36.5 °C, and then transferred to poly-L-lysine–coated coverslips in a flow perfusion chamber. Cells were first washed with an intracellular solution based on K-Hepes, containing 127 mM KCl, 20 mM NaCl, 10 mM Hepes KOH, 2 mM ATP, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.075 mM $CaCl_2$ (pH 7.2), 291 mOsM. Then cells were permeabilized using a two-photon microscope, as described previously (1, 2). Briefly, FFP-18 K^+ salt (1 μM) was added to the solution in the flow chamber containing the cells, and usually a single cell was selected to be permeabilized at the apical membrane using a short pulse of a high-intensity two-photon laser beam, with a typical wavelength of 745 nm (Spectraphysics 8W Millenia or Mai-Tai femto second laser). We used the K-Hepes–based intracellular solution described above except in the $[Ca^{2+}]$ clamp experiments when 10 mM BAPTA and 2 mM $CaCl_2$ were included. Cells were observed using a Leica confocal microscope (described previously). Measurements were performed using the area of interest placed either over the whole cell (mainly the ER calcium store) or (where indicated, acidic store after thapsigargin) over the granular area. A total of 10 μM thapsigargin for 10 min was routinely used to empty the ER calcium store. Fluo-5N AM was excited at 476 nm, and emission was collected at a 500- to 600-nm wavelength.

Measurements of Trypsin Activation in Permeabilized Cells. BZiPAR (10 μM) was used to continuously measure the activity of trypsin by adding the substrate to the perfusion chamber after cell permeabilization. BZiPAR was excited at 488 nm, and emission collected at 510–600 nm.

Transgenic Mice. IP_3R2 knockout mice, IP_3R2/IP_3R3 double-knockout mice, and wild-type control mice were generated in the Laboratory of Developmental Neurobiology (Brain Science Institute, RIKEN, Saitama, Japan) (3).

Calmodulin Experiments. Cell-permeable calmodulin activator CALP-3 (Tocris Biosciences) was used in both intact and permeabilized cell preparations in a concentration of 50 or 100 μM (4, 5). Calmodulin inhibitory peptide (Merk) was added in a concentration of 20 μM to a permeabilized cell preparation for Ca^{2+} store measurements (6).

1. Gerasimenko JV, Sherwood M, Tepikin AV, Petersen OH, Gerasimenko OV (2006) NAADP, cADPR and IP_3 all release Ca^{2+} from the endoplasmic reticulum and an acidic store in the secretory granule area. *J Cell Sci* 119:226–238.
2. Gerasimenko JV, et al. (2006) Bile acids induce Ca^{2+} release from both the endoplasmic reticulum and acidic intracellular calcium stores through activation of inositol trisphosphate receptors and ryanodine receptors. *J Biol Chem* 281:40154–40163.
3. Futatsugi A, et al. (2005) IP_3 receptor types 2 and 3 mediate exocrine secretion underlying energy metabolism. *Science* 309:2232–2234.
4. Villain M, et al. (2000) De novo design of peptides targeted to the EF hands of calmodulin. *J Biol Chem* 275:2676–2685.
5. Manion MK, Su Z, Villain M, Blalock JE (2000) A new type of $Ca^{(2+)}$ channel blocker that targets $Ca^{(2+)}$ sensors and prevents $Ca^{(2+)}$-mediated apoptosis. *FASEB J* 14:1297–1306.
6. Török K, et al. (1998) Inhibition of calmodulin-activated smooth-muscle myosin light-chain kinase by calmodulin-binding peptides and fluorescent (phosphodiesterase-activating) calmodulin derivatives. *Biochemistry* 37:6188–6198.

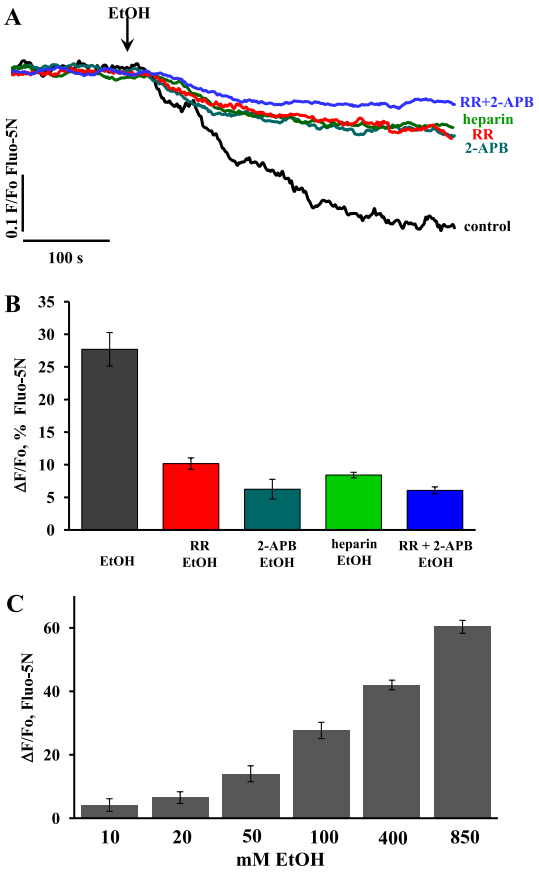

Fig. S1. In permeabilized cells, ethanol evokes a marked reduction in $[Ca^{2+}]$ in the intracellular stores ($[Ca^{2+}]_{store}$). (*A*) Ethanol (EtOH) (100 mM) elicits a marked reduction in $[Ca^{2+}]_{store}$ (n = 12). The ethanol response is markedly reduced in the presence of 2-APB (100 μM) (n = 9), heparin (250 μg/mL) (n = 7), RR (10 μM) (n = 6), or a mixture of 2-APB and RR (n = 5). (*B*) Comparison of averaged amplitudes of ethanol-induced reduction in $[Ca^{2+}]_{store}$ measured 300 s after ethanol application. Error bars indicate SEM. (*C*) Concentration dependence of ethanol-induced reduction in $[Ca^{2+}]_{store}$ (n > 6). Error bars indicate SEM.

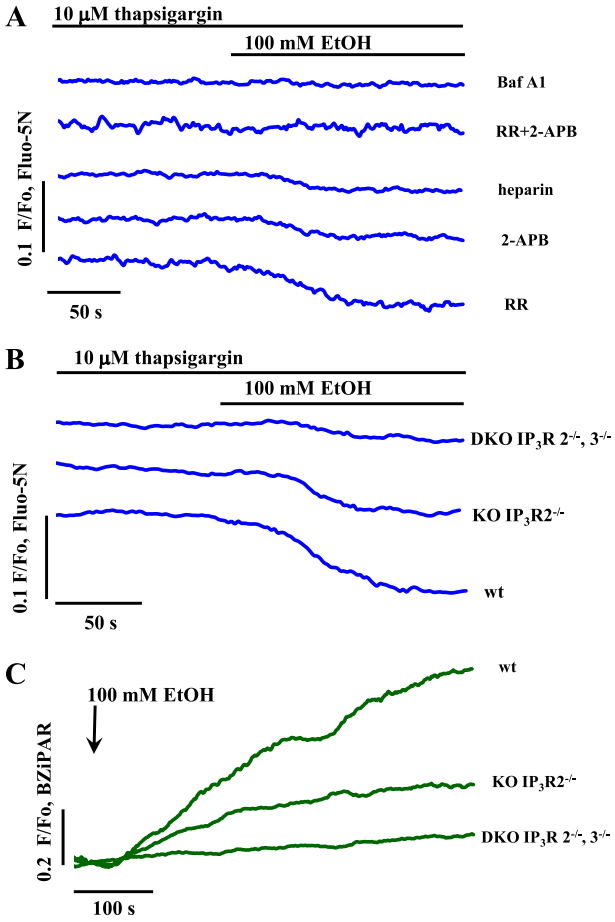

Fig. S2. Ethanol elicits Ca^{2+} release from thapsigargin-insensitive calcium stores. Ethanol-induced Ca^{2+} release from the acidic stores as well as ethanol-elicited trypsinogen activation is substantially reduced in permeabilized cells isolated from $IP_3R2^{-/-}$ knockout and even more in those from $IP_3R2^{-/-}$, $3^{-/-}$ double-knockout mice. (*A*) Representative $[Ca^{2+}]_{acid\ store}$ traces showing examples of the effects of ethanol (100 mM) in the granular region, after depletion of the ER store with thapsigargin (10 μM) and in the absence or presence of various inhibitors. Preincubation with bafilomycin A1 (100 nM Baf A1, 30 min), heparin (250 μg/mL), 2-APB (100 μM), or Ruthenium Red (RR) (10 μM) or with a mixture of 2-APB and RR (100 μM and 10 μM, respectively) reduced to varying extents the ethanol-induced release of Ca^{2+} from intracellular stores. (*B*) Representative fluorescence traces of ethanol-induced reduction in $[Ca^{2+}]_{acid\ store}$ after thapsigargin treatment in permeabilized cells from wild-type control mice and from $IP_3R2^{-/-}$ as well as $IP_3R2^{-/-}$, $3^{-/-}$ mice. (*C*) Ethanol elicits reduced trypsin activity in permeabilized cells isolated from $IP_3R2^{-/-}$ mice compared with wild-type control mice and the response is even more severely reduced in cells isolated from $IP_3R2^{-/-}$, $IP_3R3^{-/-}$ mice.

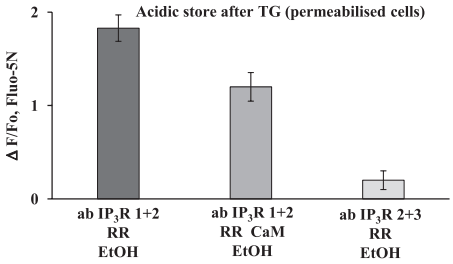

Fig. S3. Inhibition of ethanol-induced Ca^{2+} release from thapsigargin-insensitive calcium stores using antibodies against IP_3Rs and in the presence of the RyR blocker RR. CaM (2.5 μM) partially inhibited ethanol-induced Ca^{2+} release in the presence of antibodies against IP_3Rs 1 and 2 (center bar, 1.2 ± 0.2, n = 8) compared with experiments without CaM (left bar, 1.8 ± 0.1, n = 8). The right bar shows almost complete inhibition of ethanol-induced Ca^{2+} release in the presence of antibodies against IP_3Rs 2 and 3 and RR (0.2 ± 0.1, n = 6), which was not significantly different from data shown in Fig. 3*B* (100 μM 2-APB + 10 μM RR, $P > 0.8$). Comparison of averaged amplitudes of ethanol-induced reduction in $[Ca^{2+}]_{acid\ store}$ is shown. Cells were loaded with Fluo-5N in AM form. Error bars indicate SEM.

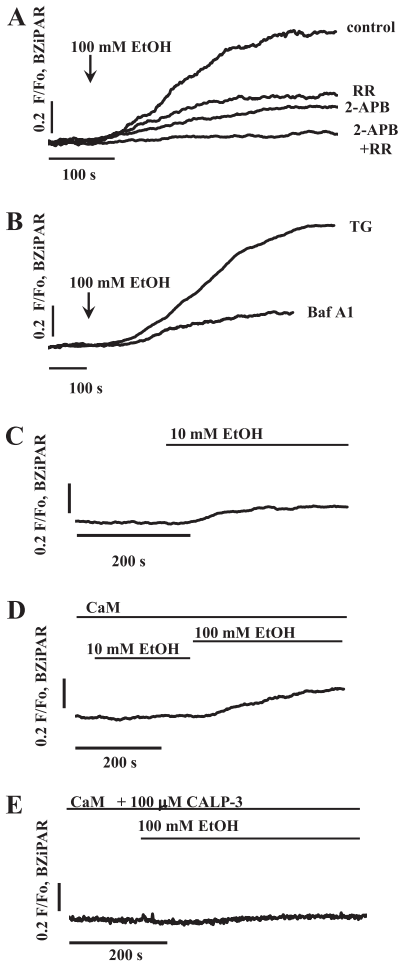

Fig. S4. Ethanol-induced trypsin activity in permeabilized cells. Inhibitors of IP_3Rs and RyRs as well as bafilomycin dramatically reduced ethanol-induced responses. Bafilomycin A1 blocks activation, but thapsigargin does not inhibit. (*A*) Representative fluorescence traces showing the time course of trypsin activity induced by ethanol (100 mM, control). Ruthenium Red (RR) as well as 2-APB inhibited trypsin activation. Both inhibitors together practically abolished trypsin activity. (*B*) Representative fluorescence traces showing that preincubation with thapsigargin (TG) (initial concentration 1 nM, thereafter 10 μM) did not change 100 mM ethanol-induced trypsin activation (compare with control trace in *A*). Preincubation with bafilomycin A1 (Baf A1) (100 nM) substantially inhibited ethanol-induced trypsin activation. (*C*) Control experiment illustrating trypsin activation by 10 mM ethanol. (*D*) In the presence of CaM (2.5 μM) ethanol at the same concentration (10 mM) fails to induce trypsin activity. A higher concentration of ethanol (100 mM) does induce small trypsin activation. (*E*) Trypsin activation by 100 mM ethanol in the presence of CaM (2.5 μM) is practically blocked by the calmodulin activator CALP-3 (100 μM).