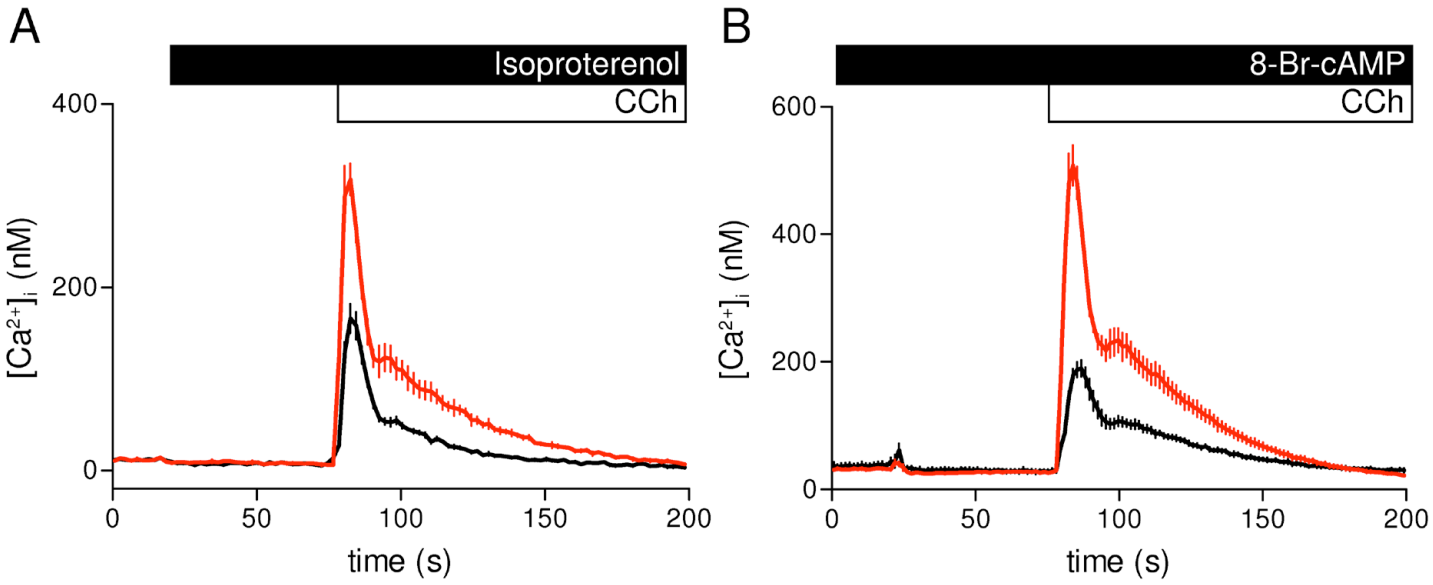

Fig. S1. Potentiation of CCh-evoked Ca^{2+} signals by endogenous β2-adrenoceptors in HEK cells or 8-Br-cAMP in HEK-PR1 cells. Experiments, similar to those shown in Fig. 1B, show the Ca^{2+} signals evoked by CCh alone (black) or after addition of isoproterenol ((**A**), 1 μM, red) or pre-incubation with 8-Br-cAMP ((**B**) 10 mM, 20 min, red). Results show means±s.e.m. for 3–4 wells from a single experiment, each typical of at least 3 similar experiments.

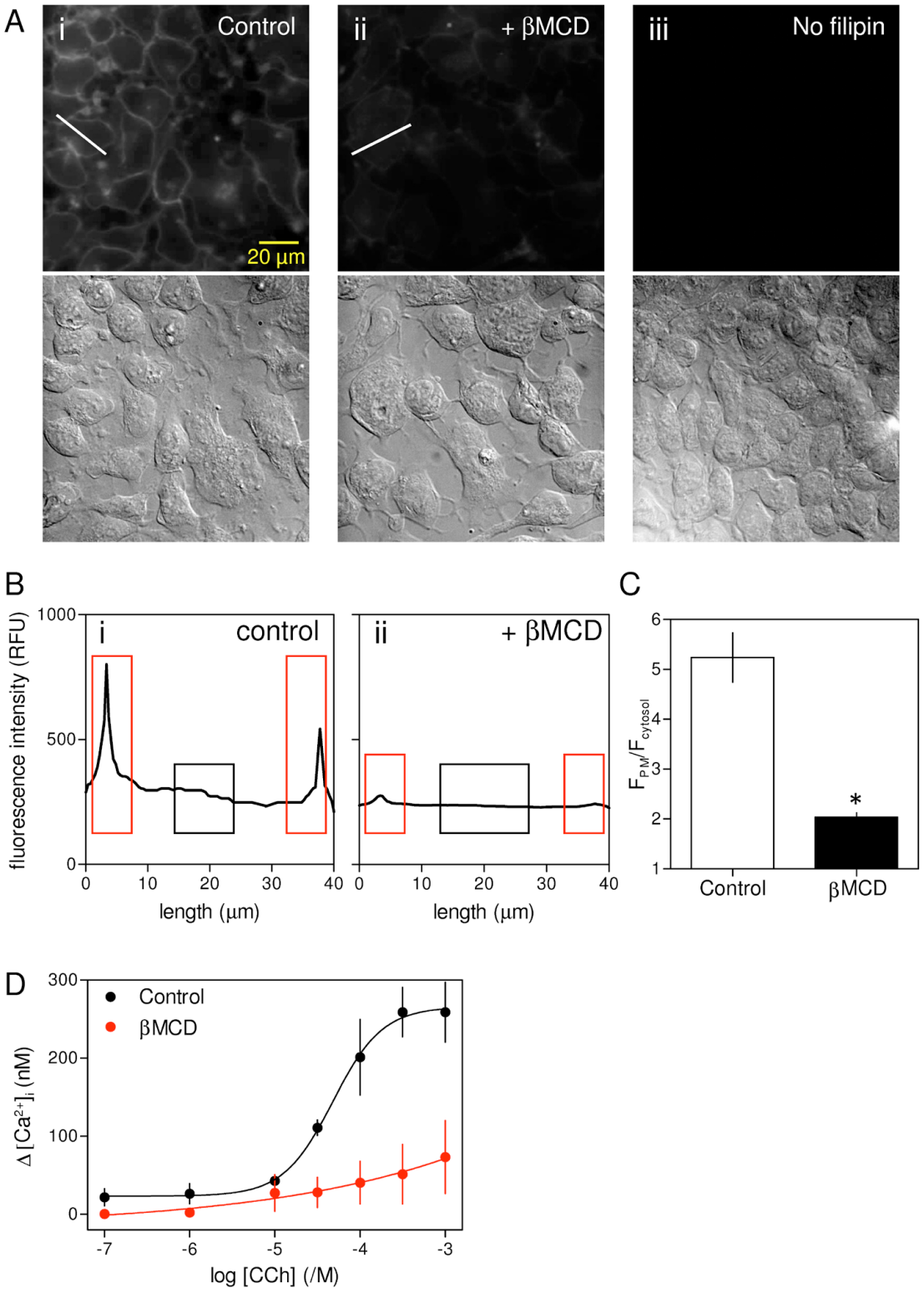

Fig. S2. Brief treatment with βMCD at 37°C depletes the plasma membrane of cholesterol and attenuates CCh-evoked Ca^{2+} signals. (**A–C**) The analysis is similar to that shown in Fig. 1C–G, but with a briefer treatment (10 min) with βMCD (2%, w/v) at 37°C. Typical fields of cells show filipin staining (upper panels) or differential interference contrast images (lower panels) for control cells (i), cells treated with βMCD (ii), or unstained control cells (iii) (A). Images are typical of at least 3 experiments. The scale bar (20 µm) applies to all images. Free cholesterol levels in the plasma membrane were quantified by measuring filipin fluorescence intensities along lines drawn across cells. (B) Typical fluorescence profiles, corresponding to the lines shown in Ai (i) and Aii (ii). (C) From the fluorescence transect, ROI corresponding to the plasma membrane (red boxes in B) or cytosol (black in B) were identified and the average fluorescence intensities within each were calculated (FPM and Fcytosol, respectively). The histogram shows the FPM/Fcytosol ratios from control cells and after treatment with βMCD (2%, 10 min, 37°C). Results are means±s.e.m. from 3 experiments with 10 cells analysed in each field. *P<0.05. (**D**) Effects of βMCD (2%, 10 min, 37°C) on CCh-evoked Ca^{2+} signals. Results are means±s.e.m. from 3 experiments, each with matched control and treated cells.

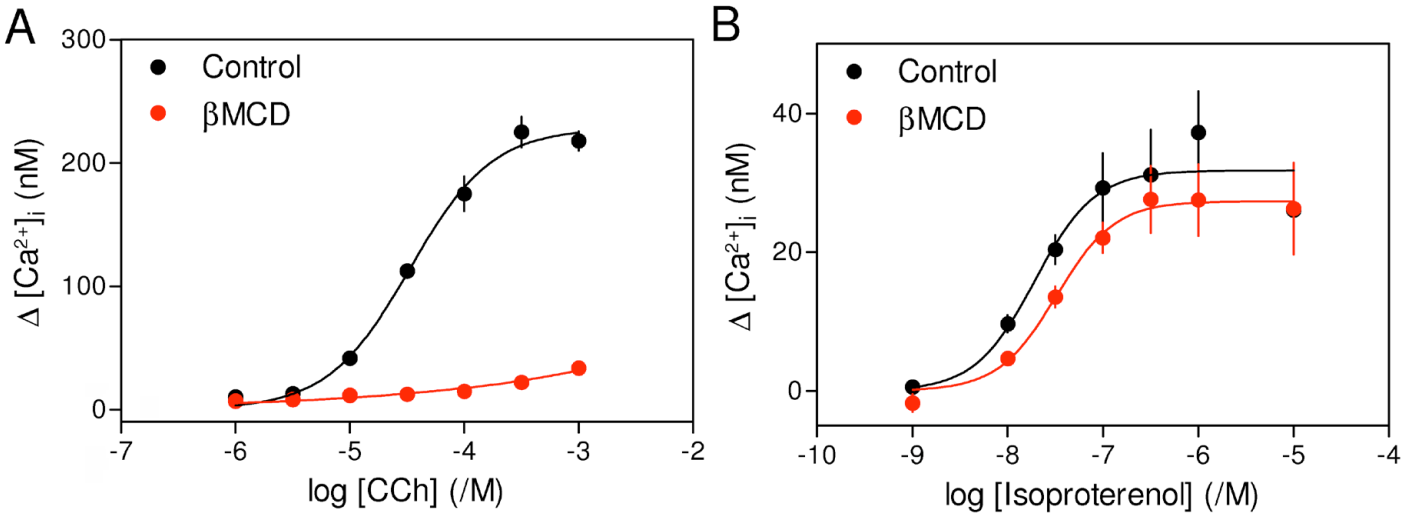

Fig. S3. Loss of cholesterol attenuates Ca^{2+} signals evoked by CCh alone, but not those evoked by isoproterenol with CCh. Ca^{2+} signals were recorded during sequential stimulation with CCh and then isoproterenol from populations of control HEK cells (black) or after treatment with βMCD (at 20°C, red). Results (means±s.e.m. from 3 experiments) show concentration-dependent effects of CCh (**A**) and concentration-dependent effects of isoproterenol for cells stimulated with 1 mM CCh (**B**).

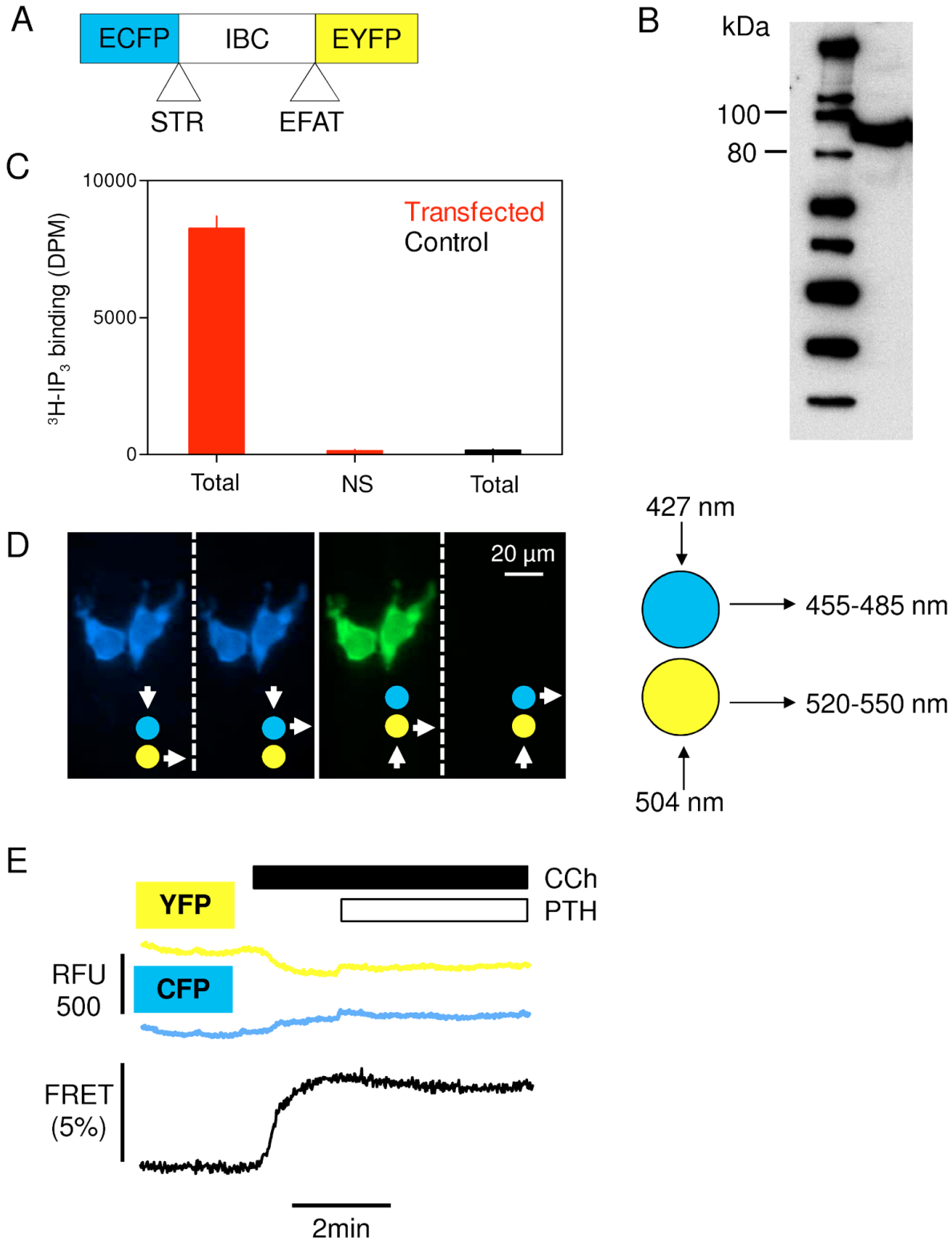

Fig. S4. FRET sensor for IP3. (**A**) Structure of the IP3-sensor used. The fluorescent proteins (ECFP and EYFP) are linked by the short sequences shown to the IP3-binding core (IBC). (**B**) Supernatants from HEK cell lyzates transiently expressing the IP3-sensor were immunoblotted (50 µg protein/lane) with an anti-GFP antibody. The only band detected has the size expected of the full-length IP3-sensor (~96 kDa). Molecular weight makers are shown on the left. (**C**) Total and non-specific (NS) 3H-IP3 binding to supernatants (150 µg total protein) from HEK cells transiently expressing the IP3-sensor (red) or untransfected HEK cells (black). Results show means±s.e.m. of three replicates. (**D**) Images show the uniform cytosolic distribution of the IP3-sensor in two typical HEK-PR1 cells. The excitation and emission wavelengths used are shown alongside the images. Insets illustrate the wavelengths used to collect each image. The scale bar (20 µm) applies to all images. (**E**) Traces, each typical of at least 4 experiments, show CFP and YFP emission and the resultant change in FRET in a HEK-PR1 cell stimulated with CCh (1 mM) and then PTH (100 nM). RFU, relative fluorescent units. The fluorescence of YFP is pH-sensitive, but we have confirmed that CCh does not affect cytosolic pH in HEK-PR1 cells (López Sanjurjo et al., 2013).

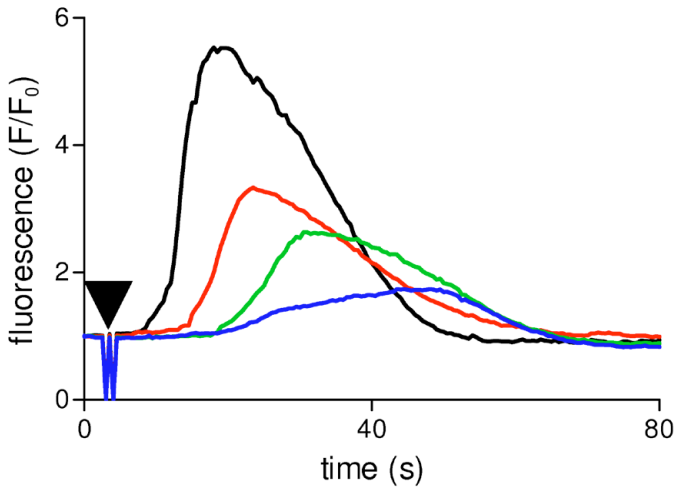

Fig. S5. Responses to photolysis of ciIP3. Typical traces from single HEK-PR1 cells showing responses to photorelease of ciIP3 (at arrow). Each trace shows the average change in fluorescence (ΔF/Fo) for a single HEK-PR1 cell.

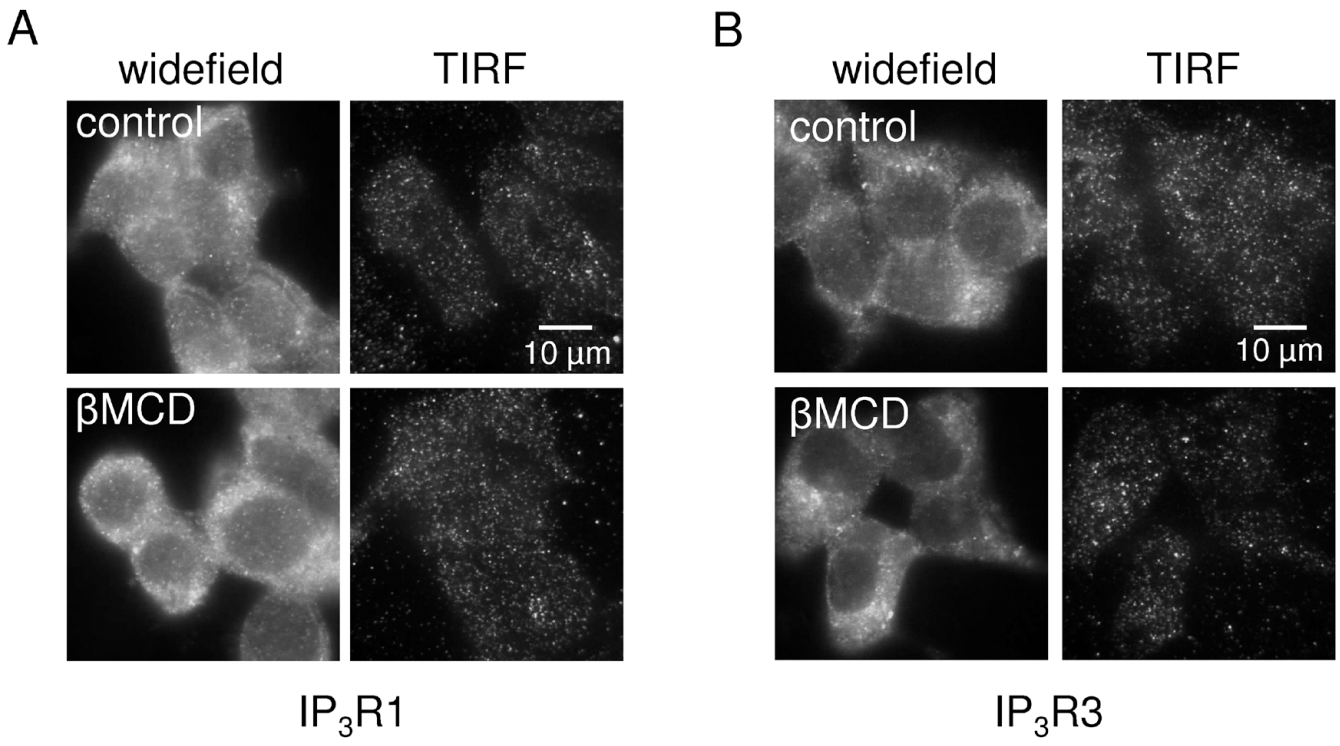

Fig. S6. Cholesterol depletion does not affect the subcellular distribution of IP3R. Immunostaining for IP3R1 (**A**) or IP3R3 (**B**) in control and βMCD-treated HEK-PR1 cells. Images show fluorescence detected in widefield and in the region immediately beneath the plasma membrane using TIRFM. The scale bar (10 µm) applies to all images. Images are representative of at least 3 experiments. Similar analyses of D1ER and IP3R2 are shown in Fig. 4F,G.

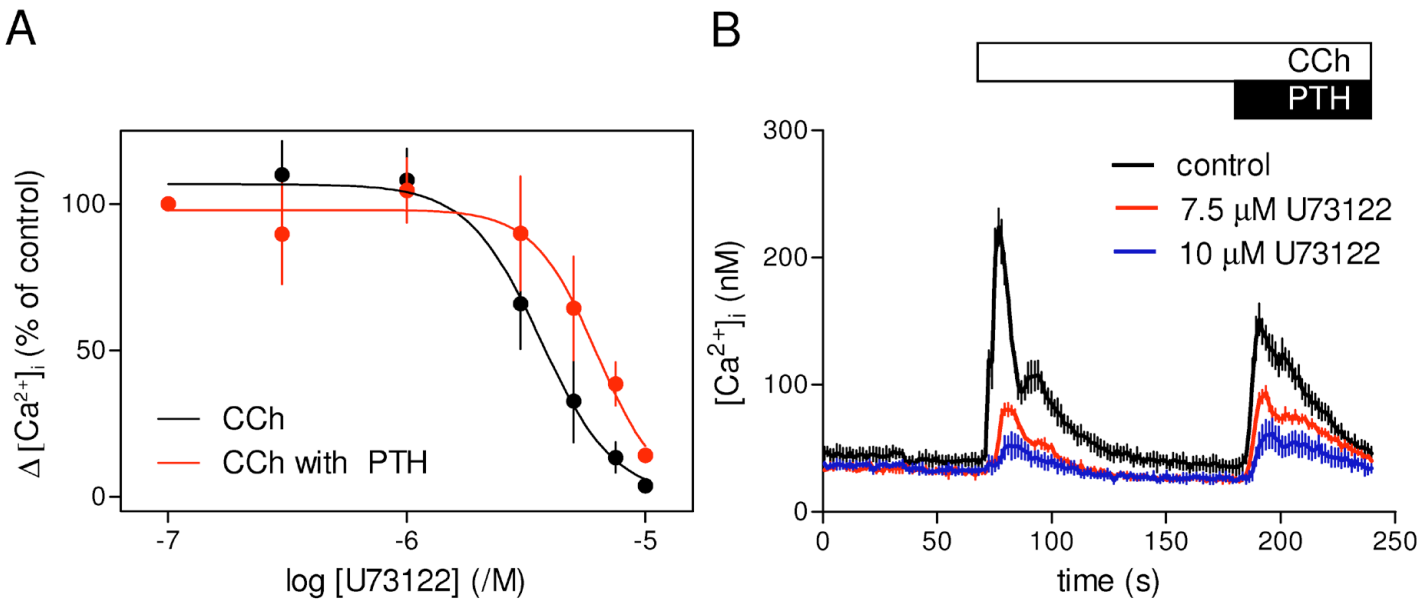

Fig. S7. Inhibition of PLC with U73122 does not mimic cholesterol depletion. (**A**) Concentration-dependent effects of U73122 (added 20 min before stimulation) on Ca^{2+} release evoked by CCh alone (1 mM) or CCh with PTH (100 nM). Results are means±s.e.m. from 3 experiments. (**B**) Typical traces from cells stimulated with CCh and then CCh with PTH after treatment with the indicated concentrations of U73122 show that no effective concentration of U73122 mimics the ability of βMCD to abolish responses to CCh alone without affecting responses to PTH with CCh. Traces show means±s.e.m. for 3–4 wells from a single experiment, each typical of at least 3 similar experiments.

Table S1. Sequence of primers used, highlighting the sites inserted.

Primer		Sequence	Insertions
1	F	TAA**GGATCC**GCCACCATGGCCTGCTGTCAAGGATGTTGC**ACGCGT**ATGAAATGGAGTGATAACAAA	*BamHI* and *MluI*
2	R	ATT**CTCGAG**TTAGGCGCAACATCCTCCACAGCA**GAATTC**CTTTCGGTTGTTGTGGAGCAG	*XhoI* and *EcoRI*
3	F	ACT**GGATCCGAATTC**GCCACCATGGTGAGCAAGGGCGAGGAG	*BamHI* and *EcoRI*
4	R	ATT**ACGCGT**GGACTTGTACAGCTCGTCCAT	*MluI*
5	R	ATT**CTCGAGTTA**CTTGTACAGCTCGTCCAT	*XhoI* and stop codon

F and R denote forward and reverse primers.