Supporting Information

Molecular characterization of flubendiamide sensitivity in lepidopterous ryanodine receptor Ca^{2+} release channel

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Supplemental Experimental Procedures

Materials. W7 and cyclopiazonic acid were obtained from Sigma, FK506 was gifted from Fujisawa.

cDNA cloning and construction of expression vectors. Genomic DNA sequence encoding sSERCA was identified by computer search of homologues of the cDNA sequence encoding *Drosophila* SERCA (GenBank accession number AE013599) through the silkworm genomic DNA shotgun sequence using KAIKOBLAST (*29*). RT-PCR and cDNA library screening were used to amplify the entire coding sequence of the sSERCA cDNA from silkworm RNA isolated from muscle, using forward and reverse primers designed on the basis of the genomic DNA sequence of sSERCA. The cDNA encoding sSERCA (GenBank accession number FJ586237) was subcloned into pCI-neo vector (Promega).

RT-PCR. Expression levels of RyR1 (GenBank Accession Number NM_000540), RyR2 (GenBank Accession Number NM_001035), and RyR3 (GenBank Accession Number NM_001036) mRNA in the HEK293 cells were determined by RT-PCR using specific primers 5'-CGGATTGCTGAGCTACTGGGCATGG-3' (sense) and 5'-AAGCTGGTCCTCATACTGCTTACGG-3' (anti-sense) for RyR1, 5'-AGAATCAGTGAATTACTTGGCATGG-3' (sense) and 5'-TAGCTGGTCTTCATACTGTTTCCGG-3' (anti-sense) for RyR2, and 5'-TCTGGTGTCATGGCTAAGTTCC-3' (sense) and 5'- CACCTTTCTTGGTACATCTTCCAG-3' (anti-

sense) for RyR3 as described previously (*41*). Temperature cycles were as follows: 94 °C for 60 s followed by 30 cycles at 94 °C for 45 s, 56 °C for 45 s, 72 °C for 1 min 10 s for RyR1, RyR2, and RyR3.

*3-(3-Benzoic acid)-3-trifluoromethyl-3H-diazirine (**2**).* $KMnO_4$ (9.50 g, 60.1 mmol) was added to a solution of **1** (ref *36*) (9.25 g, 46.2 mmol) in pyridine (30 ml) and H_2O (30 ml). The reaction mixture was stirred at 50 °C for 16 h, and then CH_2Cl_2 and H_2O were added. After washing with H_2O, the organic layer was dried over $MgSO_4$ and the solvent was removed *in vacuo*. The remaining oil was purified by column chromatography (silica, CH_3Cl:MeOH = 1:0 → 20:1 → 50:1) to give a white solid **2** (10.1 g, 43.9 mmol, 95%). ^{1}H NMR (250 MHz, $CDCl_3$): δ/ppm 7.26–7.64 (m, 4H).

*1,6-Diazidohexane (**3**).* NaN_3 (2.60 g, 40.0 mmol) was added to a solution of 1,6-dibromohexane (4.88 g, 20.0 mmol) in DMF (50 ml). The reaction mixture was refluxed for 20 h. The solvent was removed *in vacuo*, and Et_2O and H_2O were added. The organic layer was washed with H_2O (100 ml × 3). The organic layer was dried over $MgSO_4$, and the solvent was removed *in vacuo* to give a colorless oil **3** (3.02 g, 18.0 mmol, 90%). ^{1}H NMR (250 MHz, $CDCl_3$): δ/ppm 1.41 (tt, J=5.4 Hz and 3.0 Hz, 4H), 1.62 (t, J=3.0 Hz, 4H), 3.28 (t, J=5.4 Hz, 4H).

*6-Azidohexylamine (**4**).* Triphenylphosphine (4.72 g, 18.0 mmol) was added to a solution of 3 (3.02 g, 18.0 mmol) in Et_2O (30 ml), EtOAc (30 ml), and 5% HCl (60 ml). The reaction mixture was refluxed

for 1 h, and then the mixture was stirred at room temperature for 24 h. The organic layer was discarded, and the aqueous layer was washed with CH_2Cl_2 (50 ml × 2). The resulting aqueous phase was carefully basified with NaOH, and then extracted with CH_2Cl_2 (80 ml × 3). The combined organic extracts were dried over $MgSO_4$, and the solvent was removed *in vacuo* to give a colorless solid **4** (1.36 g, 9.56 mmol, 53 %). ^{1}H NMR (300 MHz, DMSO): δ/ppm 1.22–1.41 (m, 4H), 1.45-1.75 (m, 4H), 3.20–3.40 (m, 4H).

*Biotin-hexylazido (**5**)*. A solution of biotin N-succinimidyl ester (bitoin-NHS) (500 mg, 1.46 mmol) in MeOH (10 ml) was slowly added to a solution of **4** (284 mg, 1.31 mmol) and triethylamine (0.5 ml) in MeOH (50 ml). The reaction mixture was stirred at room temperature for 20 h. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography ($CHCl_3$:MeOH = 1:0 → 20:1) to give a pale yellow solid **5** (449 mg, 1.22 mmol, 93%). ^{1}H NMR (300 MHz, DMSO): δ/ppm 1.25–1.54 (m, 14H), 2.04 (t, J=7.4 Hz, 2H), 2.58 (d, J=12.5, 1H), 2.82 (dd, J=12.5 Hz and 5.1 Hz, 1H), 3.01 (dt, J=12.5 Hz and 6.7 Hz, 2H), 3.06–3.12 (m, 1H), 3.31 (t, J=6.9 Hz, 2H),4.11-4.15 (m, 1H), 4.29–4.32 (m, 1H), 6.38 (br-s, 1H), 6.44 (br-s, 1H), 7.76 (t, J=5.5 Hz, 1H).

*N-(1,1-Dimethyl-prop-2-ynyl)-3-iodo-phthalamic acid (**6**)*. A solution of 1,1-dimethylprop-2-ynylamine (0.17 g, 2.0 mmol) in acetonitrile (2 ml) was slowly added to a suspension of 3-iodophthalic anhydride (Supporting Information of ref *1, 2*) (0.55 g, 2.0 mmol) in acetonitrile (5 ml). The reaction mixture was stirred at room temperature for 1 h. The precipitate was collected by filtration and washed

with a small amount of cold acetonitrile. Yield: 0.61g (85%), mp 162-164 °C. ^{1}H NMR (DMSO): δ/ppm 1.75 (s, 6H), 2.44 (s, 1H), 7.11 (t, 1H), 7.17 (br s, 1H), 7.97 (d, 2H).

*3-(1,1-Dimethyl-prop-2-ynylimino)-4-iodo-3H-isobenzofuran-1-one (**7**)*. Trifluoroacetic anhydride (0.53 g, 2.5 mmol) was added to a suspension of **6** (0.61 g, 1.7 mmol) in toluene (10 ml) and the reaction mixture was stirred at room temperature for 30 min. The solvent was removed under reduced pressure to obtain a product (0.58 g) in a crude form quantitatively, which was used in the subsequent reaction without purification. ^{1}H NMR ($CDCl_3$): δ/ppm 1.74 (s, 6H), 2.39 (s, 1H), 7.38 (t, 1H), 7.91 (d, 1H), 8.24 (d, 1H).

*N^2-[(1,1-Dimethyl-prop-2-ynyl)-3-iodo-N^1-(2-methyl-4-nitro-phenyl)-1,2-benzene-dicarboxamide (**8**).* 2-methyl-4-nitroaniline (0.26 g, 1.7 mmol) and trifluoroacetic acid (0.01 g) was added to a solution of **7** (0.58 g, 1.7 mmol) in acetonitrile (10 ml). The reaction mixture was then stirred for 3 h. The precipitate was collected by filtration and washed with a small amount of cold acetonitrile. This material was used in the subsequent reaction without further purification. Yield: 0.65g (78%). ^{1}H NMR ($CDCl_3$): δ/ppm 1.62 (s, 6H), 1.85 (s, 1H), 2.47 (s, 3H), 7.24 (t, 1H), 7.74 (br s, 1H), 7.83 (d, 1H), 8.00 (d, 1H), 8.06 (s, 1H), 8.09 (d, 1H), 8.52 (d, 1H), 8.94 (br s, 1H).

*N^1-(4-amino-2-methyl-phenyl)-N^2-(1,1-Dimethyl-prop-2-ynyl)-3-iodo-1,2-benzene-dicarboxamide (**9**).* Iron powder (0.28 g, 5.0 mmol) and ammonium chloride (0.16 g, 3.0 mmol) was added to a suspension of **8** (0.49 g, 1.0 mmol) in EtOH (50 ml) and H_2O (10 ml) and then the reaction mixture was stirred at room temperature for 8 h. The reaction mixture was filtered, and the filtrate was evaporated under reduced pressure. The residue obtained was dissolved in a mixture of EtOAc and H_2O, and the organic layer was separated, dried over $MgSO_4$, and evaporated. The crude product was further purified by chromatography (silica, hexane:ethyl acetate = 1:2) as eluent to afford **9**. Yield: 0.38 g (82%), mp 222-223 °C. ^{1}H NMR ($CDCl_3$): δ/ppm 1.67 (s, 6H), 2.08 (s, 1H), 2.22 (s, 3H), 3.57 (br s, 2H), 6.13 (br s, 1H), 6.51 (s, 1H), 6.52 (d, 1H), 7.18 (t, 1H), 7.72 (d, 1H), 7.79 (d, 1H), 7.94 (d, 1H), 8.08 (br s, 1H).

*Flubendiamide photoaffinity probe (**flubendiamide-PP**).* **9** (107 mg, 0.290 mmol) was added to a solution of **5** (40.0 mg, 0.109 mmol) in DMF (2 ml), followed by copper (II) sulfate pentahydrate (6.5 mg, 0.026 mmol) in H_2O (1 ml) and sodium ascorbic acid (15.3 mg, 86.9 μmol) in H_2O (1 ml). The reaction mixture was stirred at room temperature for 21 h. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography ($CHCl_3$:MeOH = 19:1 → 10:1) to give **10** as a white solid. m/z (FAB+) 830 ([M + H]+). WSC-HCl (50.0 mg, 0.261 mmol) and DIPEA (1 ml) were added to a solution of **10** and **2** (60.0 mg, 0.261 mmol) in DMF (10 ml). The reaction mixture was stirred at room temperature for 13 h. The solution was concentrated *in vacuo*, and the residue was purified by

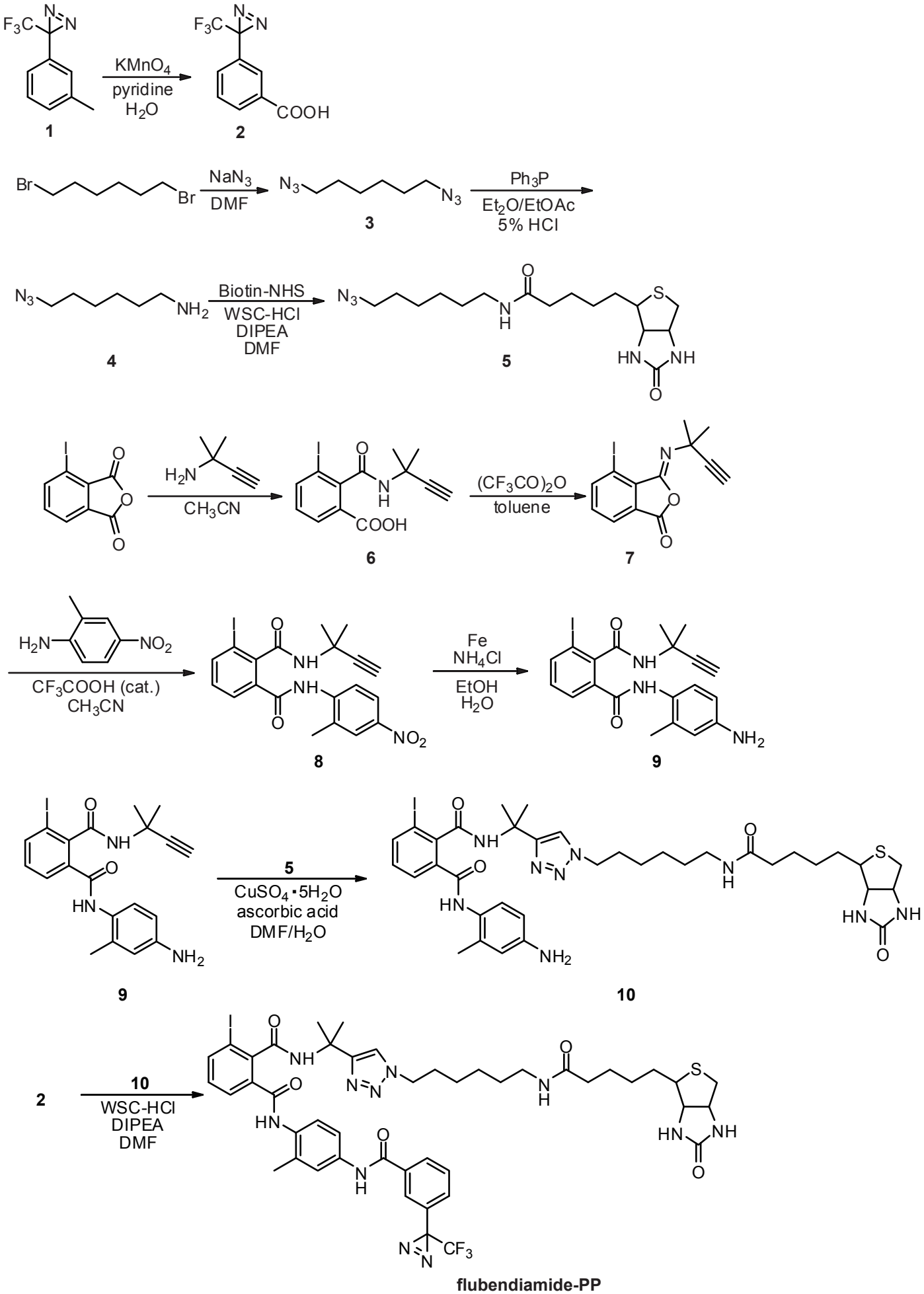
chromatography (silica, $CHCl_3$:MeOH = 1:0 → 4:1) to give a yellow solid (**flubendiamide-PP**) (51.8 mg, 49.7 μmol, 46%). LRMS (FAB+) m/z calcd for 1042 [M+], found 1042.

Supplemental References

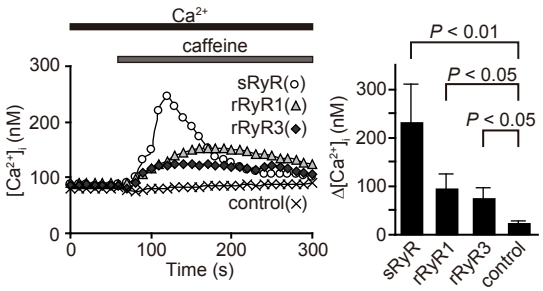
1. Machiya, K., and Seo, A. (2000) Production of iodophthalic acid compounds, *Jpn. Kokai Tokkyo Koho* JP2000-080058, Japan.

2. Abe, N., Takagi, K., and Tsubata, K. (2001) Method for producing phthalic anhydride, *Jpn. Kokai Tokkyo Koho* JP2001-335571, Japan.

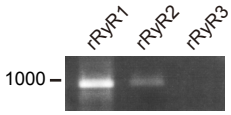
3. Seidler, N. W., Jona, I., Vegh, M., and Martonosi, A. (1989) Cyclopiazonic acid is a specific inhibitor of the Ca^{2+}-ATPase of sarcoplasmic reticulum, *J. Biol. Chem. 264*, 17816–17823.



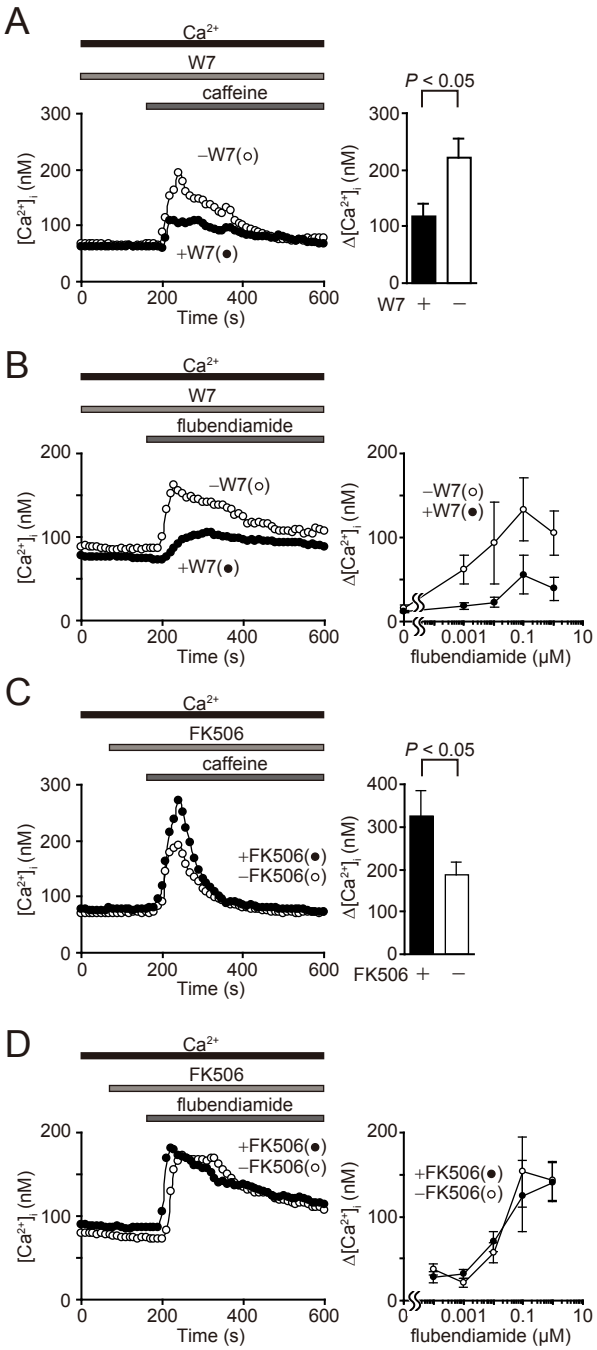
Supplemental Scheme 1. Synthetic schemes of flubendiamide-PP.



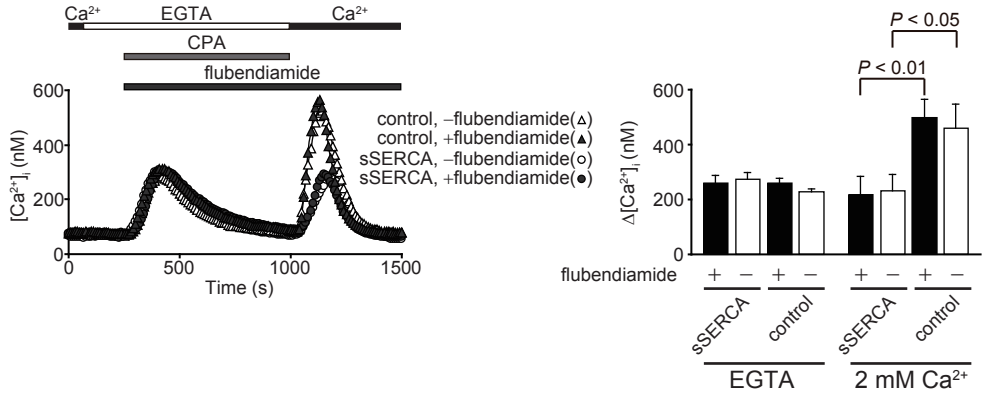
Supplemental Figure 1. Caffeine-induced $[Ca^{2+}]_i$ rises in sRyR (○)-, rRyR1 (▲)-, rRyR3 (♦)-, or control vector (×)-transfected HEK293 cells. Average time courses of Ca^{2+} responses (left panel) and maximum $[Ca^{2+}]_i$ rises (right panel) induced by 10 mM caffeine (n = 24–37).



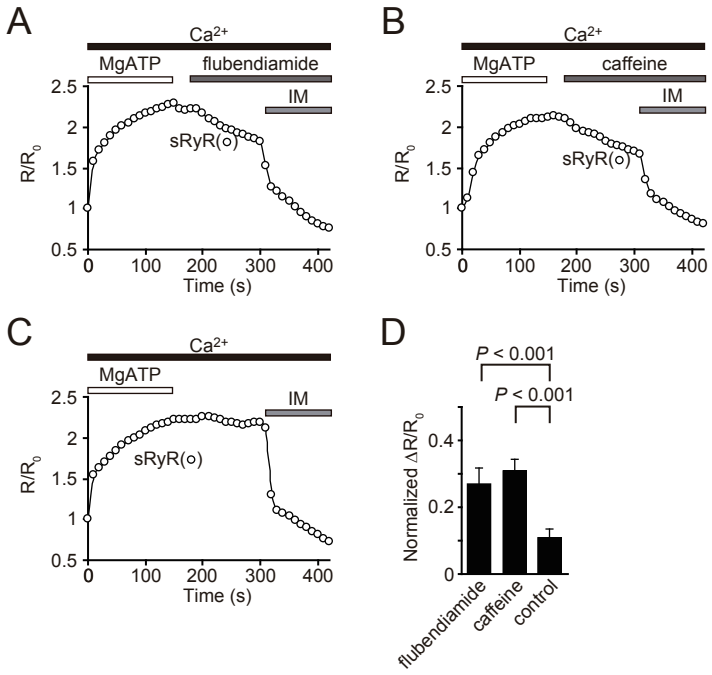
Supplemental Figure 2. RT-PCR analysis of endogenous RNA expression of RyR1, RyR2, and RyR3 in HEK293 cells.



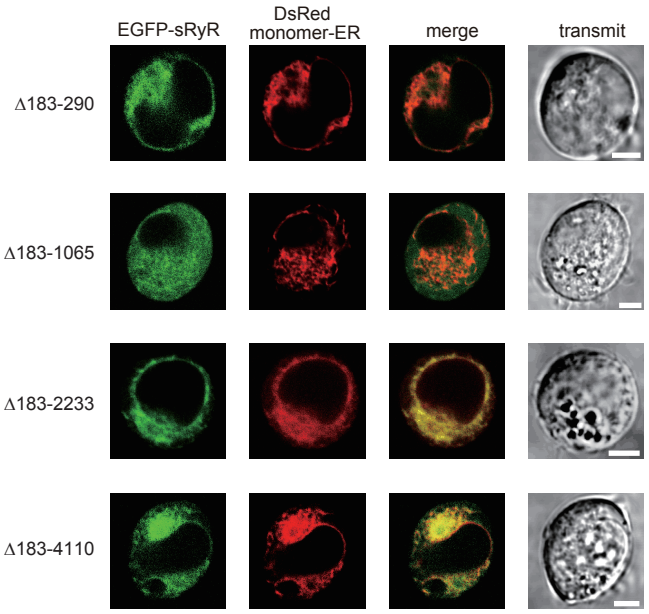
Supplemental Figure 3. Effects of 10 μM W7 and 10 μM FK506 on $[Ca^{2+}]_i$ rises in sRyR-transfected HEK293 cells. (A and B) W7 inhibits caffeine- or flubendiamide-induced $[Ca^{2+}]_i$ rises. W7 is applied 20 min before and during caffeine or flubendiamide administration. (A) Average time courses of Ca^{2+} responses (left panel) and maximum $[Ca^{2+}]_i$ rises (right panel) induced by 10 mM caffeine in the presence (●) or absence (○) of W7 (n = 43–78). (B) Average time courses of Ca^{2+} responses induced by 1 μM flubendiamide in the presence (●) or absence (○) of W7 (left panel) (n = 16–21). Concentration dependence of maximum flubendiamide-induced $[Ca^{2+}]_i$ rises (right panel) (n = 12−28). (C and D) FK506 enhances caffeine-induced $[Ca^{2+}]_i$ rises, but fails to affect flubendiamide-induced $[Ca^{2+}]_i$ rises. FK506 is applied 2 min before and during caffeine or flubendiamide administration. (C) Average time courses of Ca^{2+} responses (left panel) and maximum $[Ca^{2+}]_i$ rises (right panel) induced by 10 mM caffeine in the presence (●) or absence (○) of FK506 (n = 62–100). (D) Average time courses of Ca^{2+} responses induced by 1 μM flubendiamide in the presence (●) or absence (○) of FK506 (left panel) (n = 43–79). Concentration dependence of maximum flubendiamide-induced $[Ca^{2+}]_i$ rises (right panel) (n = 25−82).



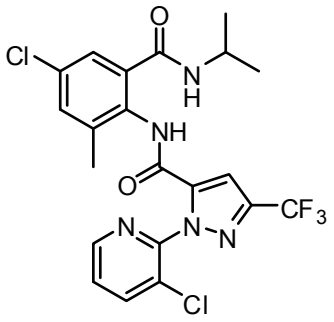
Supplemental Figure 4. Flubendiamide fails to affect Ca^{2+} uptake by SERCA. $[Ca^{2+}]_i$ rises induced by cyclopiazonic acid (CPA), a reversible inhibitor of SERCA (Supporting Information of ref *3*), in sSERCA- or control vector-transfected HEK293 cells. The perfusion solution is first changed to Ca^{2+}-free, 0.5 mM EGTA-containing external solution, and 10 μM CPA with or without 3 μM flubendiamide are applied to the cells in the absence of extracellular Ca^{2+}. Eleven minutes after the application of CPA, 2 mM Ca^{2+} is further added to the extracellular solution (left panel). Maximum $[Ca^{2+}]_i$ rises induced by CPA in the presence or absence of extracellular Ca^{2+} (right panel) (n = 13–22).



Supplemental Figure 5. Flubendiamide mobilizes Ca^{2+} from a fraction of Ca^{2+} store. (A–C) ER luminal Ca^{2+} concentration is increased with activation of the Ca^{2+} pump in the presence of MgATP, and is declined upon application of 100 nM flubendiamide (A) or 10 mM caffeine (B) in HEK293 cells transfected with sRyR (○). Two min after the application of flubendiamide or caffeine, 10 μM ionomycin (IM) is further added to the extracellular solution. (C) For the control experiment, flubendiamide or caffeine is omitted from the external solution (n = 26–47). The ratio (R) between intensities of the fluorescence at 340 nm and 380 nm excitation is divided by the initial ratio (R_0) to yield R/R_0. (D) Maximum decreases of R/R_0 induced by flubendiamide (A) or caffeine (B) alone or in the absence of these agents (C) are normalized to those induced by IM.



Supplemental Figure 6. Confocal imaging of recombinant EGFP-sRyR (Δ183−290), EGFP-sRyR (Δ183−1065), EGFP-sRyR (Δ183−2233), or EGFP-sRyR (Δ183−4110) and DsRed monomer-ER in HEK293 cells.



Supplemental Figure 7. Chemical structure of DP-23.