Supplementary Information

Synergistic FRET Assays for Drug Discovery Platform Targeting RyR2 Channels

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Supplementary Methods

Myocyte indicator loading, $[Ca^{2+}]_i$ calibration and SR Ca^{2+} content calculations

To help ensure consistency and limit possible Ca^{2+} buffering by the indicator when acquiring our wide-field fluorescent $[Ca^{2+}]_i$ data, obtained as described in the main text, we loaded myocytes with Fluo8-AM only sufficiently to ensure acceptable signal-to-noise ratio and interpretability. To achieve this, we controlled loading of esterified dye according to incubation duration and temperature (room temperature), as well as the total myocyte mass (based on the mass or size of the myocyte pellet) relative to the total number of dye molecules (for which bath concentration and volume together form a surrogate). Fluorescence emission detected by photomultipliers in our photometer was integrated for 5 ms to establish each data point, the longest sampling period we consider consistent with representing Ca^{2+} transient kinetics faithfully.

Fluorescence was converted to $[Ca^{2+}]$ using the pseudoratio method introduced by Cheng et al., 1993 (in their footnote 18)[1]. The pseudoratio equation is:

$$[Ca^{2+}] = K_d \times (F/F_0) / [(K_d/[Ca^{2+}]_{rest} + 1) - F/F_0],$$

where F_0 is the cell fluorescence at rest, when $[Ca^{2+}]_{rest}$ reaches steady state, which we assumed to be 100 nM under control conditions. We used $K_d = 1100$ nM. While the assumption of a specific value for $[Ca^{2+}]_{rest}$ is an unavoidable limitation with nonratiometric indicators, this value was used during all control, drug and wash periods of every experiment, facilitating our ability to represent any changes in fluorescence as actual changes in cytosolic [Ca].

The pseudoratio equation is readily derived assuming that fluorescence reports Ca^{2+} binding to a single site on the fluorophore, $F = F_{max} \times [Ca^{2+}] / (K_d + [Ca^{2+}])$ and simple algebraic rearrangement. F_{max} is not known in an experiment, but is an implicit function of, and can be determined from, observed rest fluorescence F_0, using only the assumption that F_0 represents $[Ca^{2+}]_{rest}$. Minimum fluorescence F_{min} with $[Ca^{2+}] = 0$ was taken to be negligible (0) and as noted in text, non-specific background recorded from a cell-free region of the same subtense as each cell studied was first subtracted from all raw fluorescence data before conversion.

For quantification of SR Ca^{2+} content, we converted the caffeine-induced Ca^{2+} transient amplitude ($\Delta[Ca^{2+}]_i$) to the change in cytosolic total $[Ca^{2+}]$ ($[Ca^{2+}]_{Tot}$) by accounting for cytosolic Ca^{2+} buffering above 100 nM $[Ca^{2+}]_i$ as described by Shannon *et al.*, 2000[2], and detailed in Bers, 2001[3], simplified to:

$$\Delta[Ca]_{Bound} = B_{max}/\{(1 + K_{Ca}/[Ca^{2+}]_i)\}2+ + B_{min},$$

where B_{max} is 244 µmol/l cytosol, K_{Ca} is 673 nM $[Ca^{2+}]_i$, and B_{min} is -28 µmol/l cytosol (the theoretical value at $[Ca^{2+}]_i = 0$, i.e., below 100 nM)[2, 3]. This provides SR Ca^{2+} content in units normalized to cytosolic volume. Since SR volume in rabbit ventricular myocytes is ~5.2% of cytosolic volume (or 3.5% of cell volume -including mitochondria), one would multiply this inferred $\Delta[Ca^{2+}]_{Tot}$ by 19.1 to estimate the

concentration of total intra-SR Ca^{2+} (e.g., 60 μmol/l cytosol, typical of bars in Fig 5F), which would correspond to 1.15 μM total intra- SR Ca^{2+}, roughly half of which would be bound by calsequestrin (K_{dCa} ~500 μM).

References

[1] H. Cheng, W.J. Lederer, M.B. Cannell, Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle, Science 262(5134) (1993) 740-4.

[2] T.R. Shannon, K.S. Ginsburg, D.M. Bers, Reverse mode of the sarcoplasmic reticulum calcium pump and load-dependent cytosolic calcium decline in voltage-clamped cardiac ventricular myocytes, Biophys J 78(1) (2000) 322-33.

[3] D.M. Bers, Excitation-Contraction Coupling and Cardiac Contractile Force., 2nd ed., Kluwer Academic Press, Dordrecht, Netherlands, 2001.

Supplementary Tables

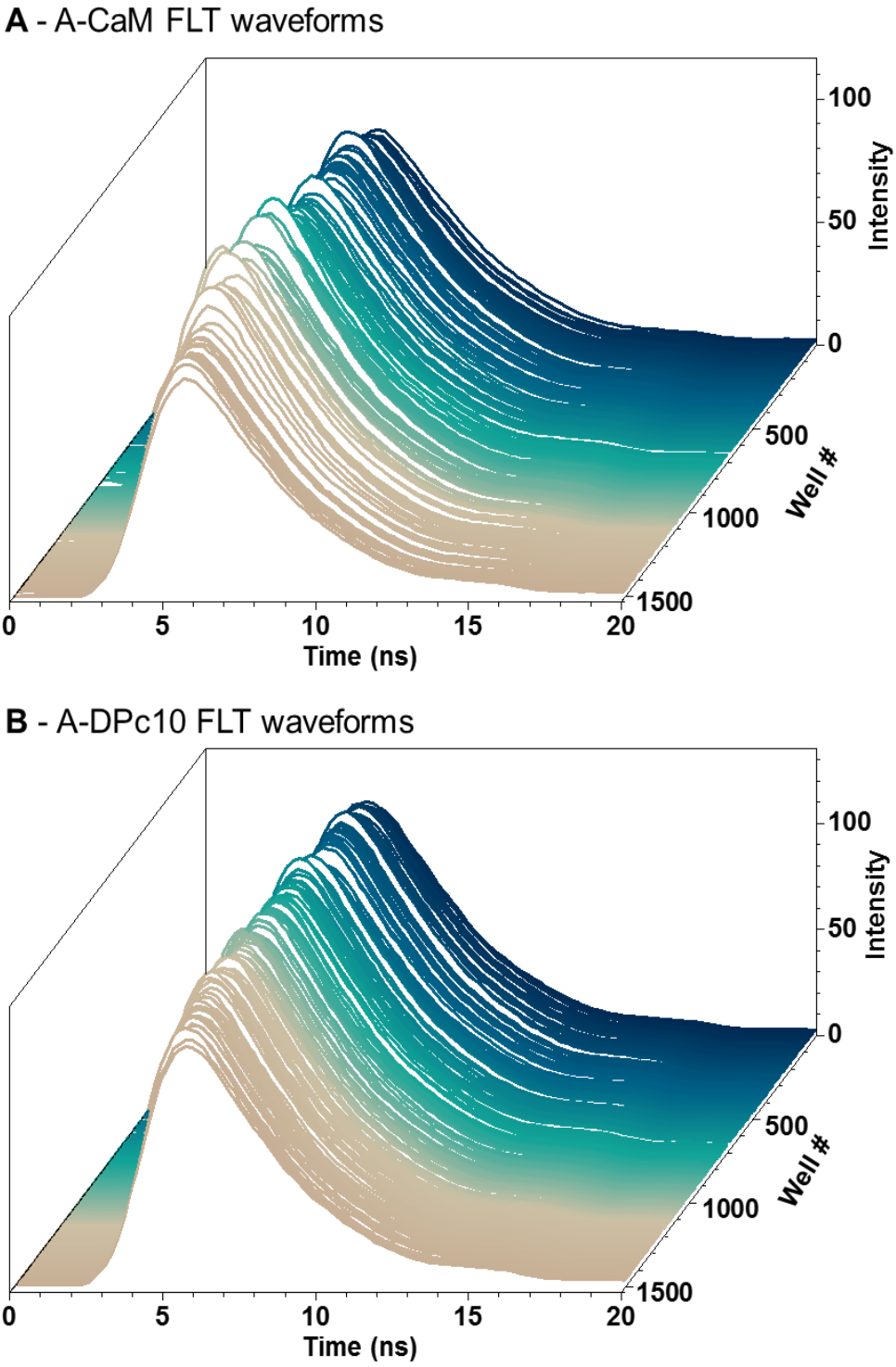
Supplementary Table 1 Number (#) of Hits and Hit reproducibility for 3SD and 5SD threshold.

	A-CaM FRET	**No H_2O_2**			**H_2O_2 treatment**		
		Plate 1	**Plate 2**	**Plate 3**	**Plate 1**	**Plate 2**	**Plate 3**
3SD	# of Hits 20 min	122	30	56	147	69	59
	# of Hits 120 min	114	74	82	179	29	84
	% of Repeated Hits in 2 plates[1]	43.0	62.2	59.8	26.8	82.8	56.0
	% of Repeated Hits in 3 plates[1]	33.3	51.4	46.3	12.8	79.3	27.4
5SD	# of Hits 20 min	52	18	37	49	34	36
	# of Hits 120 min	52	41	45	72	22	49
	% of Repeated Hits in 2 plates[1]	63.5	75.6	71.1	44.4	77.3	65.3
	% of Repeated Hits in 3 plates[1]	53.8	68.3	62.2	23.6	77.3	35.0

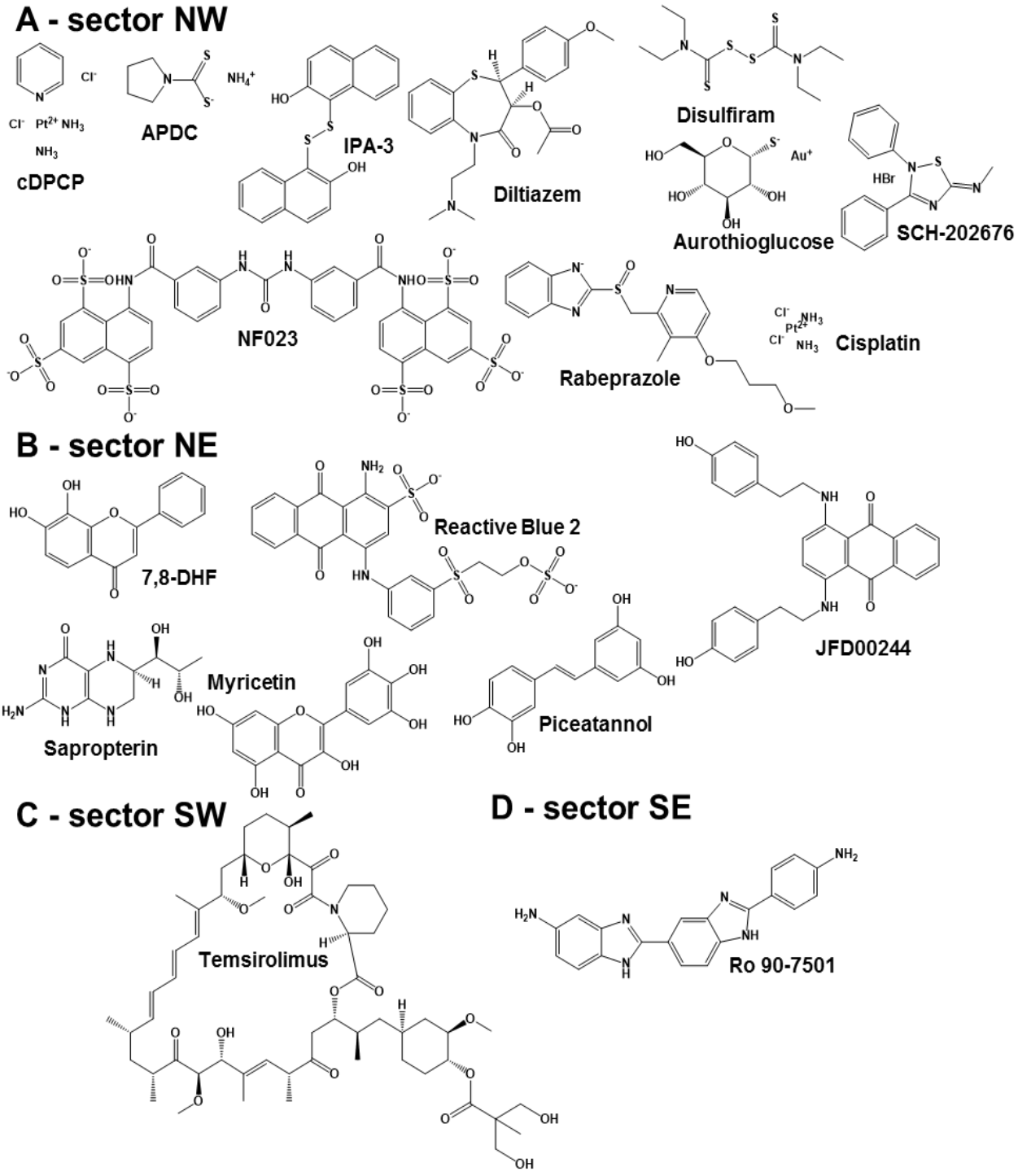
	A-DPc10 FRET	**No H_2O_2**			**H_2O_2 treatment**		
		Plate 1	**Plate 2**	**Plate 3**	**Plate 1**	**Plate 2**	**Plate 3**
3SD	# of Hits 20 min	62	81	39	62	16	48
	# of Hits 120 min	64	114	40	65	43	62
	% of Repeated Hits in 2 plates[1]	35.9	31.6	75	43.1	65.1	45.2
	% of Repeated Hits in 3 plates[1]	26.6	14.9	42.5	36.9	55.8	38.7
5SD	# of Hits 20 min	26	38	18	26	18	25
	# of Hits 120 min	16	41	18	15	20	25
	% of Repeated Hits in 2 plates[1]	56.3	39.0	77.8	93.3	70.0	52.0
	% of Repeated Hits in 3 plates[1]	43.8	17.1	38.9	73.3	55.0	44.0

[1] Data for 120 min incubation.

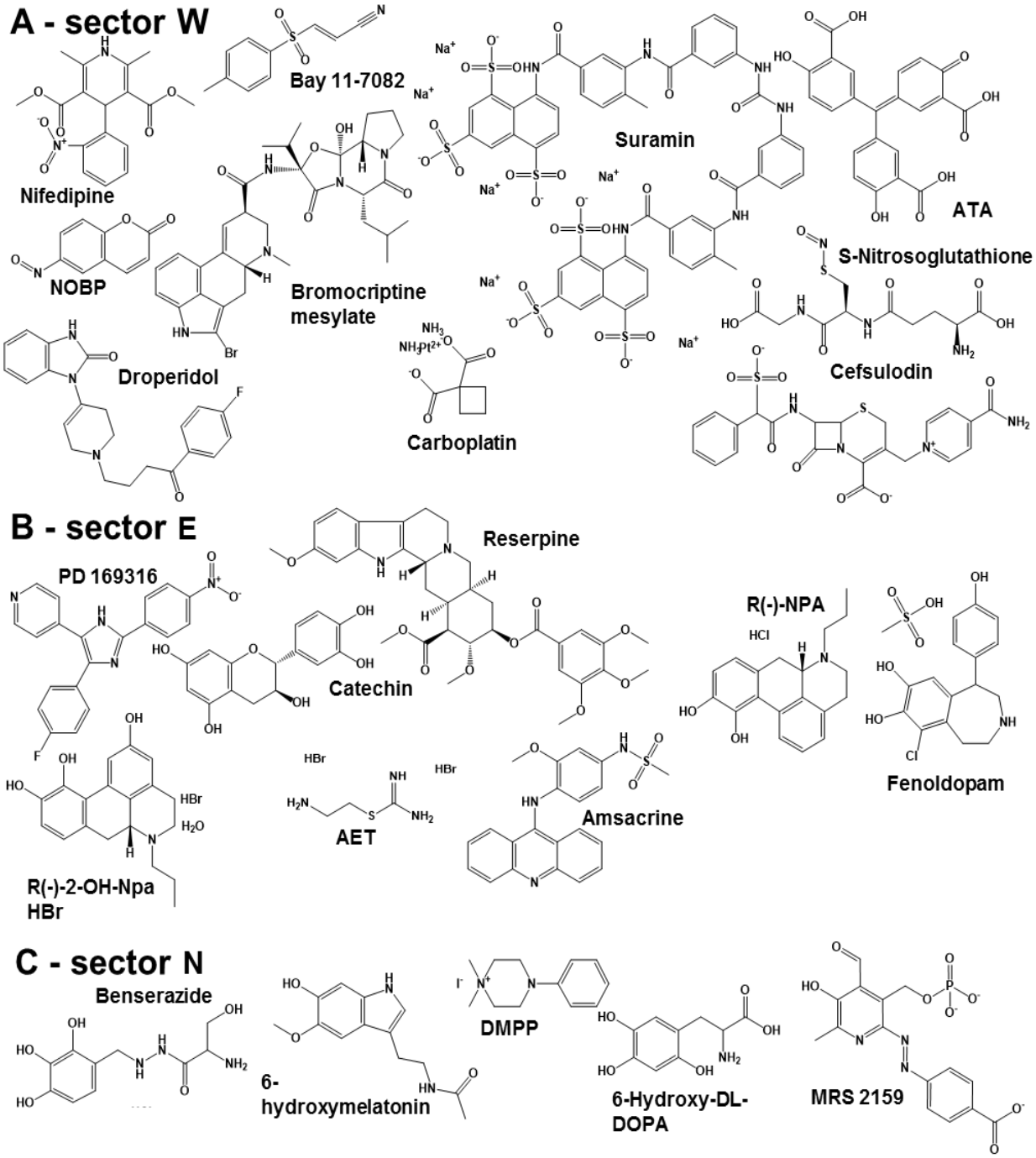
Supplementary Figures



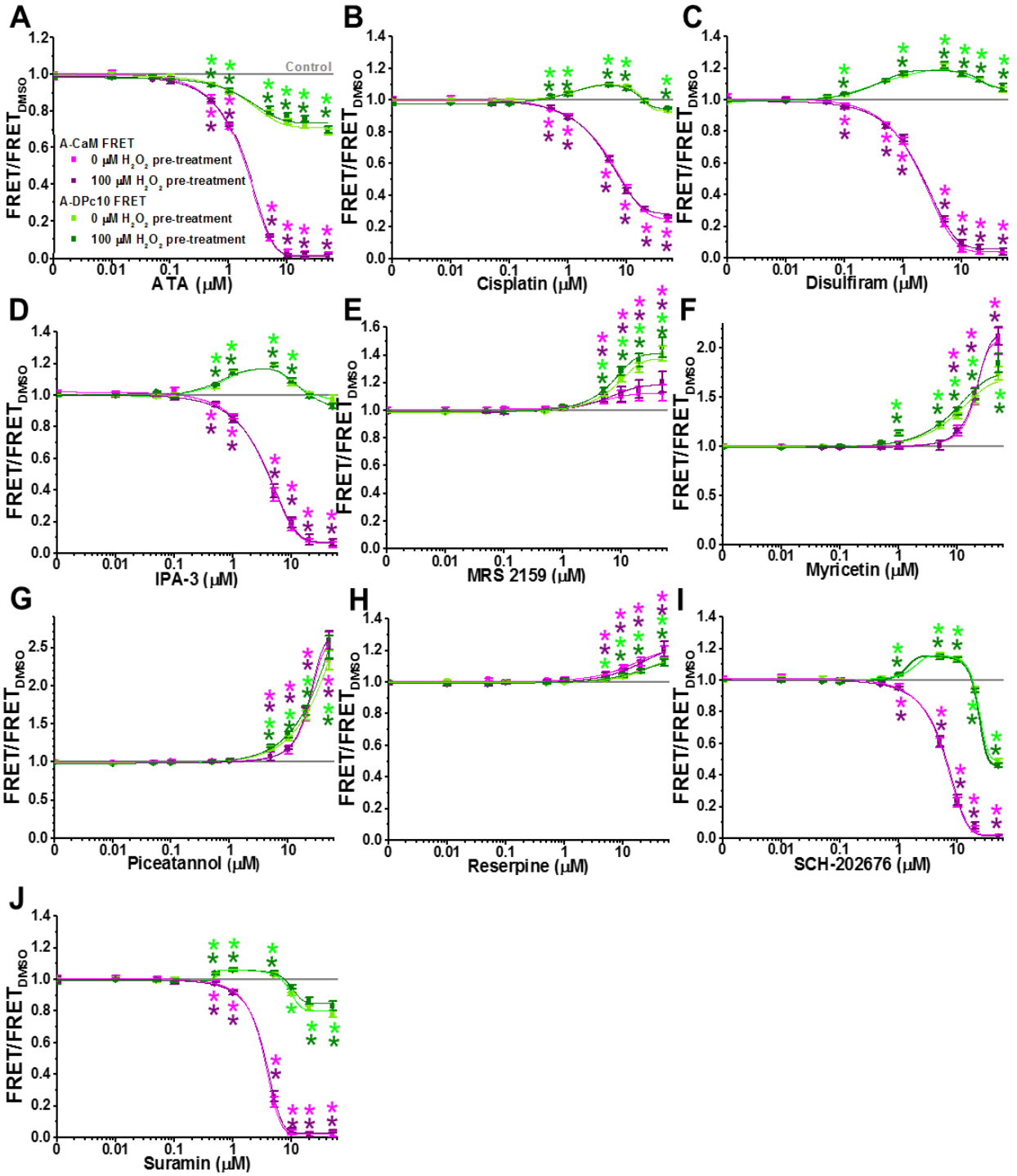
Supplementary Fig. 1 Fluorescence lifetime measurements of FRET. Fluorescence waveforms acquired from 1536-well plates loaded with the 1280 compounds of LOPAC and samples corresponding to the A) A-CaM or B) A-DPc10 FRET assays. These measurements were performed using a high-throughput fluorescence lifetime plate-reader (Fluorescence Innovations, Inc. MN, USA) provided by Photonic Pharma LLC (MN, USA), as described in Materials and Methods.



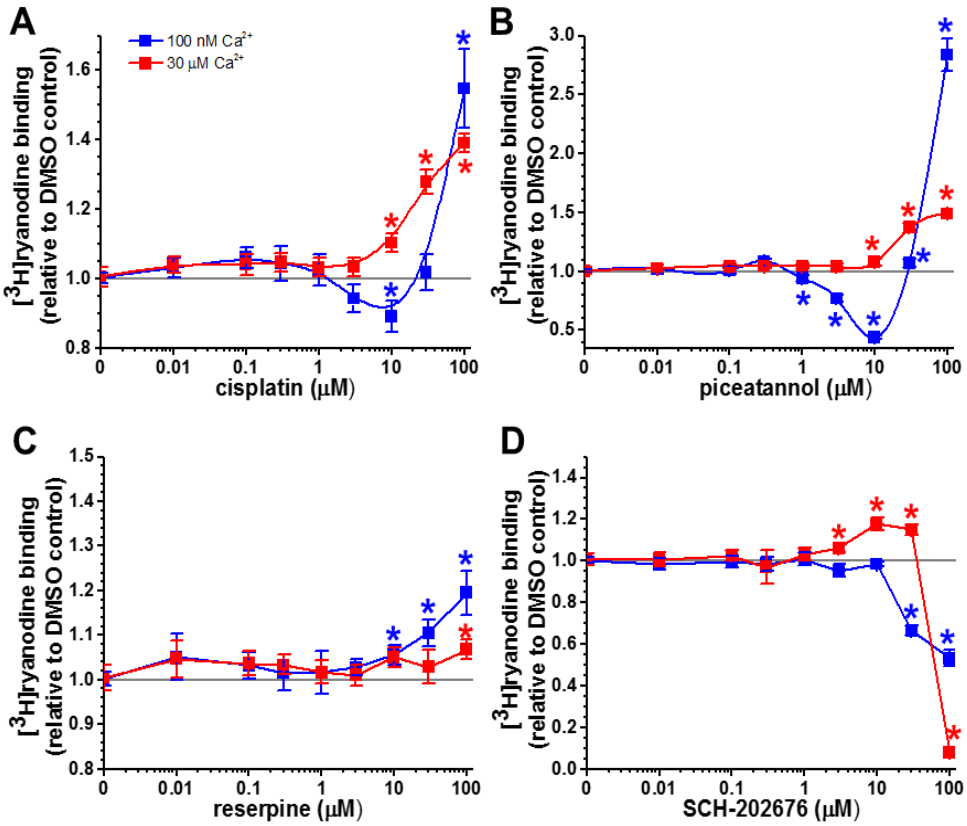
Supplementary Fig. 2 Chemical structures of Hit compounds that reproducibly altered both A-CaM and A-DPc10 FRET in LOPAC screens. Sector assignment (Fig. 3) attributed to effect on A-CaM and A-DPc10 FRET. A) Sector NW indicates that these compounds decreased A-CaM FRET and increased A-DPc10 FRET. B) Sector NE indicates that these compounds increased both A-CaM and A-DPc10 FRET. C) Sector SW indicates that this compound decreased both A-CaM and A-DPc10. D) Sector SE indicates that this compound increased A-CaM and decreased A-DPc10.



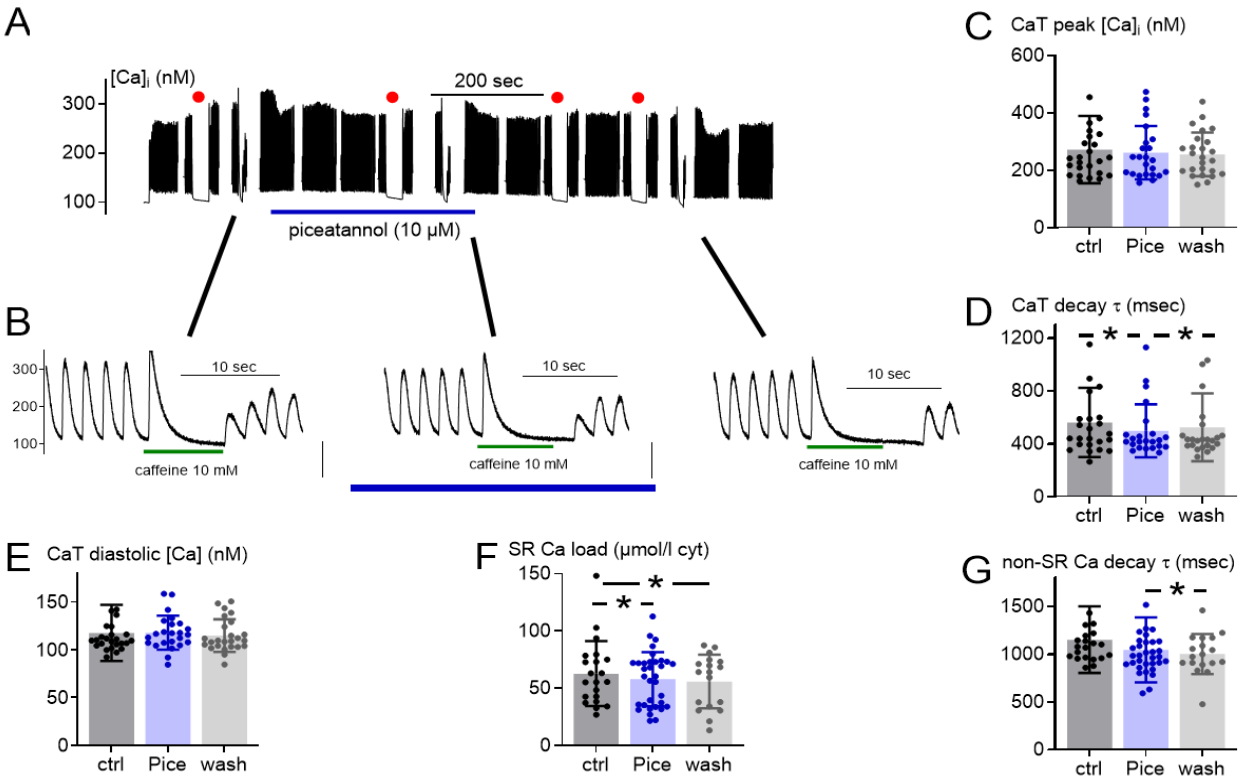
Supplementary Fig. 3 Chemical structures of compounds that reproducibly altered only A-CaM or A-DPc10 FRET in LOPAC screens. Sector assignment (Fig. 3) attributed to effect on A-CaM and A-DPc10 FRET. A) Sector W indicates that these compounds increased A-CaM FRET. B) Sector E indicates that these compounds decreased A-CaM FRET. C) Sector N indicates that these compounds increased A-DPc10 FRET.



Supplementary Fig. 4 A-CaM or A-DPc10 FLT-FRET profiles for RyR2 over a range of Hit concentrations. Normalized FLT-FRET dose response to A) ATA, B) cisplatin, C) disulfiram, D) IPA-3, E) MRS 2159, F) myricetin, G) piceatannol, H) reserpine, I) SCH0202676, and J) suramin, using cardiac D-FKBP-decorated RyR2 (in CSR) and A-CaM (purple) or A-DPc10 (green), at 30 nM Ca^{2+} following pre-treatment with no H_2O_2 (light color), or 100 μM H_2O_2 (dark color). Data are normalized relative to the values for no-drug DMSO control (grey line). A-CaM $FRET_{control}$ values of SR exposed to no H_2O_2 and plus H_2O_2 treatment were 0.11±0.02 and 0.1±0.02, respectively. A-DPc10 $FRET_{control}$ values of SR exposed to no H_2O_2 and plus H_2O_2 treatment were 0.13±0.03 and 0.13±0.03, respectively. Means ± SEM, n=3. *P < 0.05 vs. DMSO by 2-sided Student's unpaired T-test.



Supplementary Fig. 5 [^{3}H]ryanodine binding to RyR2 over a range of Hit concentrations. A) cisplatin, B) piceatannol, C) reserpine, and D) SCH-202676, at 100 nM (blue) or 30 µM (red) free Ca^{2+}. Data are normalized relative to the values for no-drug DMSO control (grey line). Data are shown as means ± SEM, n=3. *P < 0.05 vs. DMSO by 2-sided Student's unpaired T-test.



Supplementary Fig. 6 Piceatannol did not affect Ca^{2+} handling in normal rabbit myocytes. A) Time series of a representative experiment with piceatannol (blue underbar), decimated from >1x10^6 data points to a more practical 1x 10^4 points. Red dots (30s rests) indicate that the resting fluorescence was stable and justifies free $[Ca^{2+}]_i$ pseudo-ratio measurements with resting $[Ca^{2+}]_i$ at baseline assumed to be 100 nM. B) Detail of same experiment before, during and after drug contact, each showing final 10 sec of steady state Ca^{2+} transients, followed by 10 mM caffeine (duration 8 s, green underbars) to assay SR Ca^{2+} loading. C) Peak transient $[Ca^{2+}]_i$, D) transient $[Ca^{2+}]_i$ decay time constant and (E) residual (diastolic) $[Ca^{2+}]_i$ at the end of each transient, predicted by exponential fitting. (C-E) #cells/#animal (n/N) = 24/8, 23/8 and 24/8 for control, piceatannol and wash, respectively. F) SR Ca^{2+} loading, represented as total $[Ca^{2+}]$ released to cytosol by caffeine (calculated as in text). G) Ca^{2+} transient decay time constant measured with caffeine present, representing non-SR Ca^{2+} removal, mainly via Na/Ca exchange. (F and G) n/N = 21/8, 33/8 and 18/8 for control, piceatannol and wash, respectively. $[Ca^{2+}]$ in (C), (E) and (F) shown with one dot per cell, measured via pseudo-ratio relative to the same cell's rest value (red dots in A). *P < 0.05 vs. control (ctrl) by 2-sided Student's paired T-test.