

Supplemental Material

Bromination Pattern of Hydroxylated Metabolites of BDE-47 Affects their Potency to Release Calcium from Intracellular Stores in PC12 Cells

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Abbreviations:

AB	Alamar Blue
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
ER	endoplasmic reticulum
F_{340}	fluorescence intensity evoked by 340 nm excitation wavelength
F_{380}	fluorescence intensity evoked by 380 nm excitation wavelength
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
MeO-PBDE	methoxylated polybrominated diphenyl ether
NR	Neutral Red
OH-PBDE	hydroxylated polybrominated diphenyl ether
PBDE	polybrominated diphenyl ether
R_{max}	maximum F_{340}/F_{380} ratio
R_{min}	minimum F_{340}/F_{380} ratio
SERCA	sarcoplasmic/endoplasmic reticulum (ER) Ca^{2+} ATPase
TG	thapsigargin

Materials and Methods supplemental

Cell culture. Rat pheochromocytoma (PC12) cells (Greene and Tischler 1976), obtained from ATCC (American Type Culture Collection, Manassas, VA, USA), were cultured as described previously (Dingemans et al. 2008). Briefly, PC12 cells were cultured for up to 15 passages in RPMI 1640 (Invitrogen, Breda, The Netherlands) supplemented with 5% fetal calf serum and 10% horse serum (ICN Biomedicals, Zoetermeer, The Netherlands). For Ca^{2+} imaging experiments, PC12 cells were subcultured in poly-L-lysine coated glass-bottom dishes (MatTek, Ashland MA, USA).

Cell viability assay. To investigate possible acute effects of the PBDEs on cell viability, the Alamar Blue (AB) and neutral red (NR) uptake assays were used with minor modifications (Magnani and Bettini 2000; Repetto et al. 2008). Briefly, PC12 cells were plated at a density of 2×10^5 cells/well 24 h before exposure in 24-wells plates. Cells were exposed for 20 min to 20 μM polybrominated diphenyl ether (PBDE) or 0.2-20 μM hydroxylated PBDE (OH-PBDE) in saline (containing 1.8 mM CaCl_2 , 24 mM glucose, 10 mM HEPES, 5.5 mM KCl, 0.8 mM MgCl_2 , 125 mM NaCl, and 36.5 mM sucrose, adjusted to pH 7.3 with NaOH). Cells were subsequently incubated with 400 μl AB (6.25 μM) in saline for 30 min at 37°C in the dark. Fluorescence was measured, with excitation at 530 nm and emission at 590 nm using a FLUOstar Galaxy V4.30-0 platereader (BMG Labtechnologies, Offenburg, Germany). The same plate was then incubated with 800 μl NR (33 $\mu\text{g/ml}$) in saline for 60 min at 37°C in the dark. The cells were lysed using 400 μl

extraction solution (50% ethanol absolute, 49% MilliQ water, 1% acetic acid). After 10 min shaking, fluorescence was measured, with excitation at 530 nm and emission at 645 nm using a FLUOstar Galaxy V4.30-0.

Intracellular Ca²⁺ imaging. Changes in intracellular Ca²⁺ concentration ([Ca²⁺]_i) were measured using the Ca²⁺-sensitive fluorescent ratio dye Fura-2 as described previously (Dingemans et al. 2008; Dingemans et al. 2007). Briefly, cells were loaded with 5 μM Fura-2 AM (Molecular Probes; Invitrogen, Breda, the Netherlands) in saline for 20 min at room temperature; this was followed by 15 min de-esterification in saline. The cells were then placed on the stage of an Axiovert 35M inverted microscope (Zeiss, Göttingen, Germany) equipped with a TILL Photonics Polychrome IV (TILL Photonics GmbH, Gräfelfing, Germany). Fluorescence, evoked by 340 and 380 nm excitation wavelengths (F₃₄₀ and F₃₈₀), was recorded every 12 sec at 510 nm with an Image SensiCam digital camera (TILL Photonics GmbH). The digital camera and polychromator were controlled by imaging software (TILLvisION, version 4.01), which was also used for data collection and processing. After 5 min baseline recording, cells were exposed to 0.1-20 μM of the (MeO/OH-)PBDEs. Maximum and minimum ratios (R_{max}/R_{min}) were determined after 25 min recording (20 min exposure) by addition of ionomycin (5 μM) and EDTA (17 mM), respectively.

Where applicable, cells were washed with Ca²⁺-free saline (containing 10 μM EDTA to remove residual extracellular Ca²⁺) just before the imaging experiments. In specific experiments, thapsigargin (TG)-responsive endoplasmic and mitochondrial Ca²⁺ stores were emptied by incubation with

respectively 1 μM TG and 1 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) in Ca^{2+} -free saline for 10 min (Dingemans et al. 2008). TG is a high-affinity inhibitor of sarcoplasmic/endoplasmic reticulum (ER) Ca^{2+} ATPase (SERCA; Toyoshima and Inesi 2004). FCCP depolarizes the mitochondrial membranes, resulting in the uncoupling of oxidative phosphorylation and subsequent Ca^{2+} release from mitochondria (Taylor et al. 2000). These compounds are commonly used under experimental conditions to empty intracellular Ca^{2+} stores.

Free cytosolic $[\text{Ca}^{2+}]_i$ was calculated using Grynkiewicz's equation $[\text{Ca}^{2+}]_i = K_d \times (R - R_{\min}) / (R_{\max} - R)$ (as described in Deitmer and Schild, 2000), where K_d is the dissociation constant of Fura-2 determined in the experimental set-up used for the fluorescence measurements using Fura-2 Ca^{2+} imaging calibration buffers (Molecular Probes; Invitrogen). For Ca^{2+} -free experiments, in which baseline $[\text{Ca}^{2+}]_i$ is lower, a correction factor is applied to allow for comparison with experiments in normal (1.8 mM) Ca^{2+} -conditions.

Reference list supplemental

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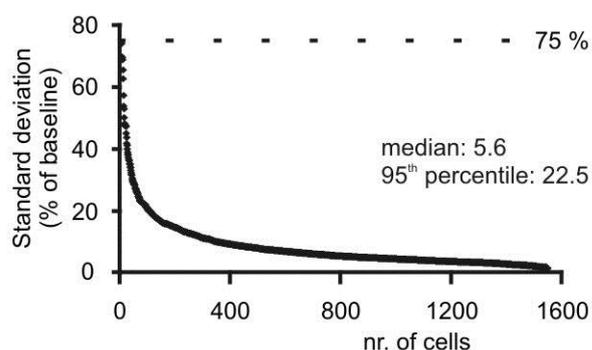
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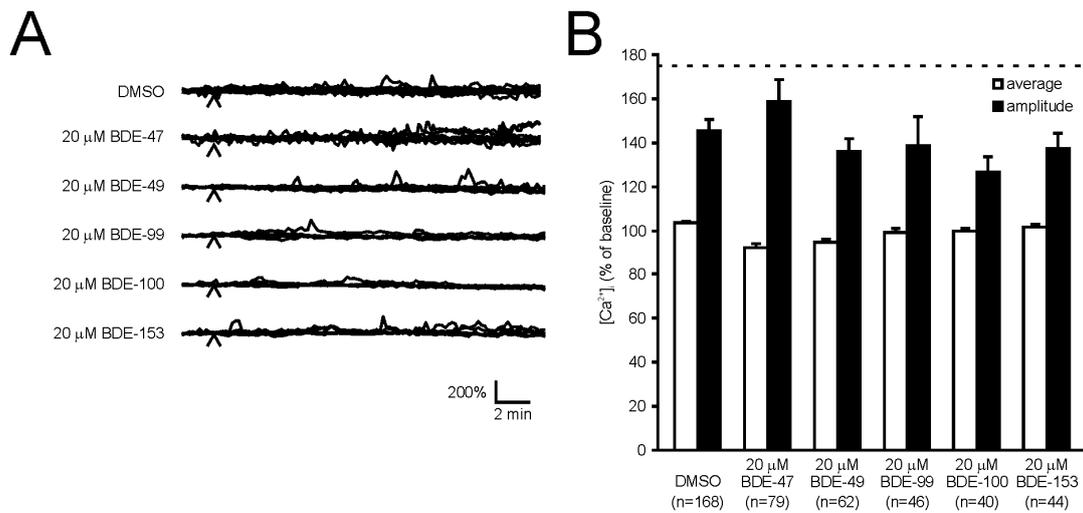
Figures and tables supplemental

3-OH-BDE-47	3-hydroxy-2,2',4,4'-tetrabromodiphenyl ether
4'-OH-BDE-49	4'-hydroxy-2,2',4,5'-tetrabromodiphenyl ether
5-OH-BDE-47	5-hydroxy-2,2',4,4'-tetrabromodiphenyl ether
6-MeO-BDE-47	6-methoxy-2,2',4,4'-tetrabromodiphenyl ether
6-OH-BDE-47	6-hydroxy-2,2',4,4'-tetrabromodiphenyl ether
6'-OH-BDE-49	6'-hydroxy-2,2',4,5'-tetrabromodiphenyl ether
BDE-47	2,2',4,4'-tetrabromodiphenyl ether
BDE-49	2,2',4,5'-tetrabromodiphenyl ether
BDE-99	2,2',4,4',5-pentabromodiphenyl ether
BDE-100	2,2',4,4',6-pentabromodiphenyl ether
BDE-153	2,2',4,4',5,5'-hexabromodiphenyl ether
BDE-209	2,2',3,3',4,4',5,5',6,6'-decabromodiphenyl ether

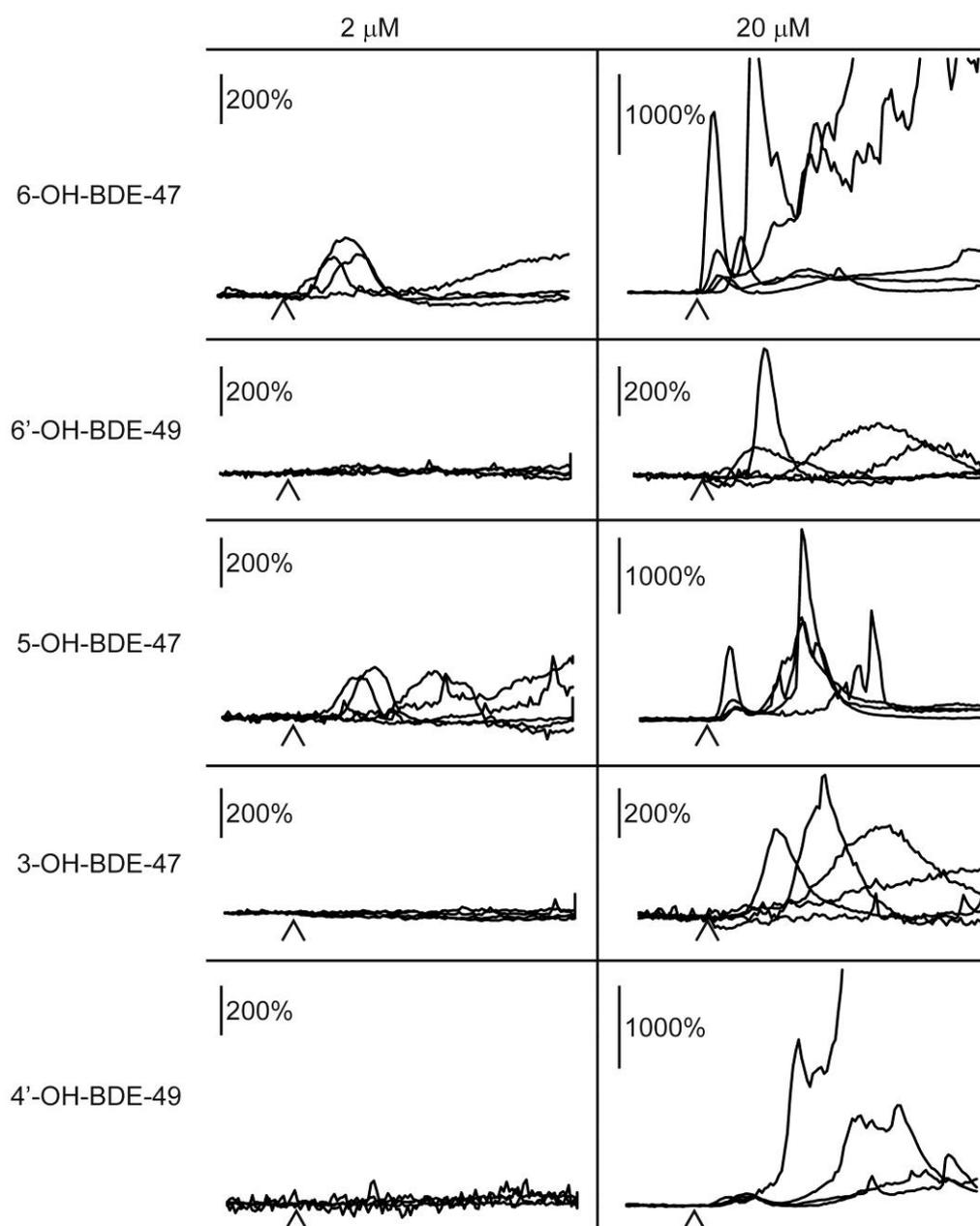
Supplemental Material, Table 1. Full names of the PBDEs and hydroxylated PBDEs discussed in this paper.



Supplemental Material, Figure 1. Distribution of standard deviations (sorted descending) calculated from 5 min baseline Ca^{2+} recordings ($n = 1538$, 160 experiments) in normal Ca^{2+} conditions (1.8 mM Ca^{2+}), to determine the minimum change in $[\text{Ca}^{2+}]_i$. To prevent false positive results, increases of $[\text{Ca}^{2+}]_i$ to $>175\%$ of baseline are considered relevant.



Supplemental Material, Figure 2. Lack of increase in $[Ca^{2+}]_i$ in PC12 cells during exposure to PBDEs. Results are shown as representative traces (A) of normalized $[Ca^{2+}]_i$ from individual PC12 cells exposed to DMSO, 20 μ M BDE-47, BDE-49, BDE-99, BDE-100 and BDE-153 for 20 min (applied at $t=0$ min as indicated by the arrowheads) and as average (B, open bars) and amplitude (closed bars) of $[Ca^{2+}]_i$ during exposure to the PBDEs. None of the PBDEs increases $[Ca^{2+}]_i > 175\%$ of baseline (as indicated with dashed line). Data are shown from 5 to 19 experiments per concentration; numbers below each bar indicate the number of cells used for data analysis.



Supplemental Material, Figure 3. Increase in $[Ca^{2+}]_i$ in PC12 cells during exposure to hydroxylated metabolites of BDE-47. Results are shown as representative traces of normalized $[Ca^{2+}]_i$ from individual PC12 cells exposed to 2 or 20 μ M 6-OH-BDE-47, 6'-OH-BDE-49, 5-OH-BDE-47, 3-OH-BDE-47 or 4'-OH-BDE-49 for 20 min, applied as indicated by arrowheads. Note the difference in scaling for 20 μ M 6-OH-BDE-47, 5-OH-BDE-47 and 4'-OH-BDE-49 compared to the other traces.

	concentration (μM)	$[\text{Ca}^{2+}]_i$ amplitude of initial increase (% of baseline)	$[\text{Ca}^{2+}]_i$ amplitude of late increase (% of baseline)
6-OH-BDE-47	0.2	n.a.	n.a.
	1	249 ± 9 ($n = 40$)	n.a.
	2	280 ± 15 ($n = 43$)	247 ± 15 ($n = 18$)
	5	316 ± 14 ($n = 71$)	301 ± 17 ($n = 38$)
	20	481 ± 40 ($n = 89$)	2485 ± 408 ($n = 83$)
6'-OH-BDE-49	2	n.a.	n.a.
	20	566 ± 95 ($n = 32$)	235 ± 9 ($n = 24$)
5-OH-BDE-47	0.2	n.a.	n.a.
	1	248 ± 22 ($n = 11$)	n.a.
	2	273 ± 13 ($n = 40$)	306 ± 24 ($n = 18$)
	5	443 ± 56 ($n = 31$)	302 ± 32 ($n = 22$)
	20	553 ± 70 ($n = 51$)	1803 ± 155 ($n = 52$)
3-OH-BDE-47	2	n.a.	n.a.
	20	868 ± 127 ($n = 42$)	236 ± 15 ($n = 8$)
4'-OH-BDE-49	1	n.a.	n.a.
	2	n.a.	231 ± 27 ($n = 6$)
	20	224 ± 5 ($n = 33$)	2518 ± 486 ($n = 40$)

Supplemental Material, Table 2. Effects of OH-PBDEs on the amplitude of initial and late increases in $[\text{Ca}^{2+}]_i$ in PC12 cells during 20 min exposure. No initial or late increases were observed during exposure to DMSO, the parent PBDE congeners (BDE-47, BDE-49, BDE-99, BDE-100 and BDE-153) or 6- CH_3O -BDE-47. Data are expressed as mean \pm SE from the number of cells (n) indicated.

	concentration (μM)	% of cells showing fluctuations	fluctuation frequency (fluctuations/h)	fluctuation duration (min)	fluctuation amplitude (% of baseline)
DMSO	n.a.	14 ($n = 168$)	1.2 ± 0.3 ($n = 168$)	0.4 ± 0.0 ($N = 65$)	205 ± 4 ($N = 65$)
BDE-47	1	23 ($n = 26$) ^{n.s.}	2.4 ± 1.1 ($n = 26$) ^{n.s.}	0.4 ± 0.1 ($N = 18$) ^{n.s.}	194 ± 4 ($N = 18$) ^{n.s.}
	2	31 ($n = 48$) [#]	2.8 ± 0.9 ($n = 48$) [*]	0.7 ± 0.1 ($N = 140$) [*]	212 ± 50 ($N = 140$) ^{n.s.}
	20	23 ($n = 79$) ^{n.s.}	2.3 ± 0.6 ($n = 79$) [*]	0.8 ± 0.2 ($N = 60$)	202 ± 8 ($N = 60$) ^{n.s.}
BDE-49	20	16 ($n = 62$) ^{n.s.}	1.0 ± 0.4 ($n = 62$) ^{n.s.}	0.5 ± 0.2 ($N = 21$) ^{n.s.}	203 ± 6 ($N = 21$) ^{n.s.}
BDE-99	20	7 ($n = 46$) ^{n.s.}	0.5 ± 0.4 ($n = 46$) ^{n.s.}	- ^a	- ^a
BDE-100	20	5 ($n = 40$) ^{n.s.}	0.2 ± 0.1 ($n = 40$) ^{n.s.}	- ^a	- ^a
BDE-153	20	11 ($n = 44$) ^{n.s.}	0.6 ± 0.2 ($n = 44$) ^{n.s.}	- ^a	- ^a
6-MeO-BDE-47	20	24 ($n = 42$) ^{n.s.}	1.6 ± 0.5 ($n = 42$) ^{n.s.}	0.3 ± 0.0 ($N = 23$) ^{n.s.}	193 ± 4 ($N = 23$) ^{n.s.}
6-OH-BDE-47	0.1	20 ($n = 35$) ^{n.s.}	1.5 ± 0.7 ($n = 35$) ^{n.s.}	1.1 ± 0.4 ($N = 18$) ^{n.s.}	209 ± 11 ($N = 18$) ^{n.s.}
	0.2	31 ($n = 58$) ^{**}	1.7 ± 0.4 ($n = 58$) ^{n.s.}	1.3 ± 0.4 ($N = 33$) ^{**}	223 ± 19 ($N = 33$) ^{n.s.}
6'-OH-BDE-49	2	10 ($n = 49$) ^{n.s.}	0.3 ± 0.2 ($n = 49$) ^{n.s.}	no data ($N = 5$)	no data ($N = 5$)
5-OH-BDE-47	0.2	14 ($n = 50$) ^{n.s.}	0.5 ± 0.2 ($n = 50$) ^{n.s.}	no data ($N = 7$)	no data ($N = 7$)
	1	33 ($n = 52$) ^{**}	1.6 ± 0.4 ($n = 52$) ^{n.s.}	2.4 ± 0.5 ($N = 28$) [#]	203 ± 4 ($N = 28$) ^{n.s.}
3-OH-BDE-47	2	10 ($n = 49$) ^{n.s.}	2.0 ± 0.8 ($n = 49$) ^{n.s.}	0.4 ± 0.0 ($N = 50$) ^{n.s.}	238 ± 24 ($N = 50$) ^{n.s.}
4'-OH-BDE-49	1	14 ($n = 22$) ^{n.s.}	1.2 ± 0.8 ($n = 22$) ^{n.s.}	no data ($N = 9$)	no data ($N = 9$)
	2	35 ($n = 43$) ^{**}	4.3 ± 1.3 ($n = 43$) [#]	0.8 ± 0.1 ($N = 88$) [*]	288 ± 20 ($N = 88$) [#]

a. The number of data points to investigate duration and amplitude of the fluctuations in $[\text{Ca}^{2+}]_i$ is insufficient.

Supplemental Material, Table 3. Effects of (OH-)PBDEs, at NOECs based on the amplitude of increase of $[\text{Ca}^{2+}]_i$, on the percentage of cells showing fluctuations and the frequency, duration and amplitude of these fluctuations. Data are expressed as mean \pm SE from the number of cells (n) or fluctuations (N) indicated. Statistical significance compared to DMSO control is indicated by: n.s. not significant; * $p < 0.05$; ** $p < 0.01$; # $p < 0.001$.

	decreased cell viability	A: Ca ²⁺ homeostasis				B: fluctuations in [Ca ²⁺] _i				
		Ca ²⁺ influx (extracellular)	Ca ²⁺ release (ER)	Ca ²⁺ release (mitochondria)	NOEC	% of cells showing fluctuations	fluctuation frequency	fluctuation duration	fluctuation amplitude	NOEC
BDE-47	-	-	-	-	20 µM	-	2 µM	2 µM	-	1 µM (<)
BDE-49	-	-	-	-	20 µM	-	-	-	-	20 µM
BDE-99	-	-	-	-	20 µM	-	-	-	-	20 µM ^b
BDE-100	-	-	-	-	20 µM	-	-	-	-	20 µM ^b
BDE-153	-	-	-	-	20 µM	-	-	-	-	20 µM ^b
6-MeO-BDE-47	-	-	-	-	20 µM	-	-	-	-	20 µM
6-OH-BDE-47	20 µM	1 µM	1 µM	2 µM	0.2 µM	0.2 µM	-	0.2 µM	-	0.1 µM (<)
6'-OH-BDE-49	-	-	20 µM	-	2 µM	20 µM ^a	20 µM ^a	20 µM ^a	20 µM ^a	2 µM ^b
5-OH-BDE-47	20 µM	1 µM	1 µM	-	0.2 µM	1 µM	-	1 µM	-	0.2 µM ^b
3-OH-BDE-47	-	-	20 µM	20 µM	2 µM	20 µM ^a	20 µM ^a	20 µM ^a	20 µM ^a	2 µM
4'-OH-BDE-49	20 µM	20 µM	20 µM	20 µM	2 µM	2 µM	2 µM	2 µM	2 µM	1 µM (<)

b. Possible effects on parameters of fluctuations are obscured by release of Ca²⁺ from intracellular stores.

c. The number of data points to investigate duration and amplitude of the fluctuations in [Ca²⁺]_i is insufficient to ensure the NOEL.

Supplemental Material, Table 4. Table summarizes the LOECs of BDE-47 and hydroxylated metabolites on cell viability and different parameters of [Ca²⁺]_i investigated in PC12 cells in the present research. A: Below the mechanisms responsible for effects on Ca²⁺ homeostasis, LOEC values of effects causing a decrease of >25% in the specific Ca²⁺-free experiments (Figure 5) are indicated, as well as resulting NOEC levels. B: Below the investigated parameters of fluctuations in [Ca²⁺]_i, LOEC values are indicated, as well as resulting NOEC levels. When NOEC levels for effects on fluctuations in [Ca²⁺]_i are lower compared to NOEC values for effects on Ca²⁺ homeostasis related processes, this is indicated by (<). Values of [Ca²⁺]_i and fluctuation parameters are shown in Tables S2 and S3 of Supplemental Material.