Supplemental Data

Inhibition of the sarco/endoplasmic reticulum (ER) Ca^{2+}-ATPase by thapsigargin analogs induces cell death via ER Ca^{2+} depletion and the unfolded protein response

Pankaj Sehgal[1,2,3†], Paula Szalai[4†], Claus Olesen[1,3], Helle A Praetorius[1], Poul Nissen[3,5], Søren Brøgger Christensen[6], Nikolai Engedal[4*], Jesper V. Møller[1,3*]

[1] From the Department of Biomedicine, Aarhus University, DK-8000 Aarhus, Denmark [2] Biological Sciences platform, Sunnybrook Research Institute, and Department of Biochemistry, Toronto, Ontario, [3] Centre for Membrane Pumps in Cells and Disease (Pumpkin), Danish Research Foundation, DK-8000 Aarhus, Denmark, [4] Centre for Molecular Medicine Norway (NCMM), Nordic EMBL Partnership for Molecular Medicine, University of Oslo, Norway, [5] Danish Research Institute of Translational Neuroscience (DANDRITE), Nordic EMBL Partnership for Molecular Medicine, Department of Molecular Biology and Genetics, DK-8000 Aarhus, Denmark, [6] Department of Drug Design and Pharmacology, University of Copenhagen, DK-2100.

Running title: Thapsigargin Analogs: Effects on SERCA and Cancer Cells

[†] These authors contributed equally

** To whom correspondence should be addressed: Jesper V. Møller, Department of Biomedicine, Aarhus University, DK-8000 Aarhus, Denmark (jvm@biomed.au.dk, Telephone: 45871 67754) or Nikolai Engedal, Centre for Molecular Medicine Norway (NCMM), Nordic EMBL Partnership for Molecular Medicine, University of Oslo, P.O. Box 1137 Blindern, 0318 Oslo, Norway (k.n.engedal@ncmm.uio.no, Telephone: +47 22840765 Fax: +47 22840598)*

List of Material included:

Figure S1

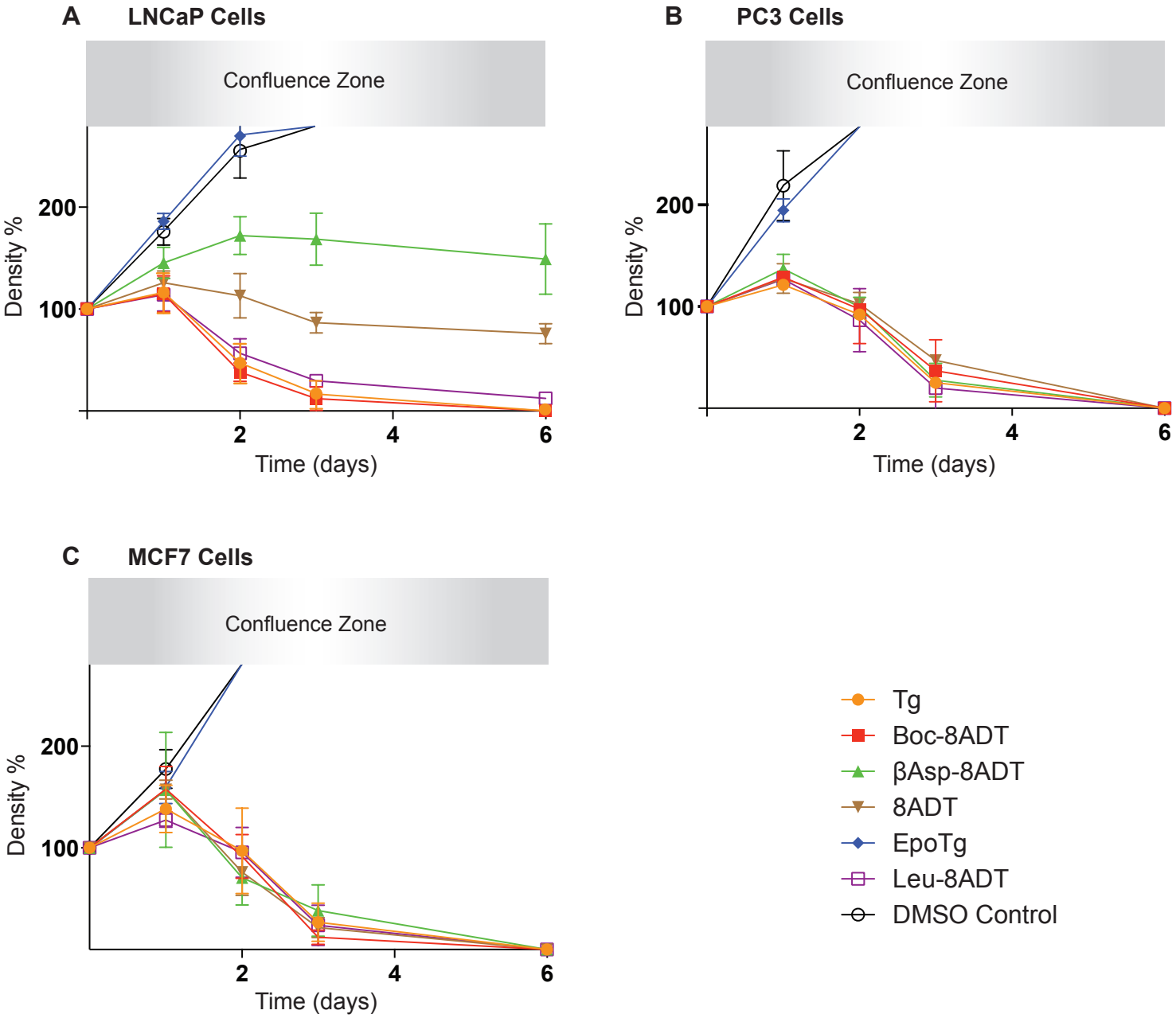

Fig. S1. Survival of prostate and breast cancer cell lines exposed to Tg and Tg analogs for an extended period of time. LNCaP androgen sensitive (Panel A) and insensitive PC3 (Panel B) prostate cancer cells, and MCF7 breast cancer cells (Panel C) were grown to around 35% confluence in NUNC 24-well plates and then exposed to 0.1 µM concentrations of Tg or Tg analogs. Cell survival was estimated by daily measurements of changes in cell densities (number of surviving (normal or affected) cells counted daily over the same eye field with a phase contrast Zeiss ID03) for a period of 6 days. Cells with moderate morphological changes such as cell rounding and a smooth outline, but without a shrunken or distended and disintegrated intracellular morphology, were included in the counts. The control (no inhibitor) contained 0.4% DMSO as a substitute of the DMSO added with Tg and the analogs. The data represent means ± SD of 4 independent experiments. Note that in cases when the cells reached confluency (for cells treated with DMSO or EpoTg for 3 days or more (LNCaP), or for 2 days or more (PC3 and MCF7), the cells become so tightly packed and/or start to grow on top of each other, making it impossible to evaluate their morphology or accurately count them. Consequently, in these cases the data points are set to the border of the confluence zone (indicated by the grey boxes), wherein no further data can be shown. After the cells had reached confluence, they remained confluent in all cases.

Figure S2

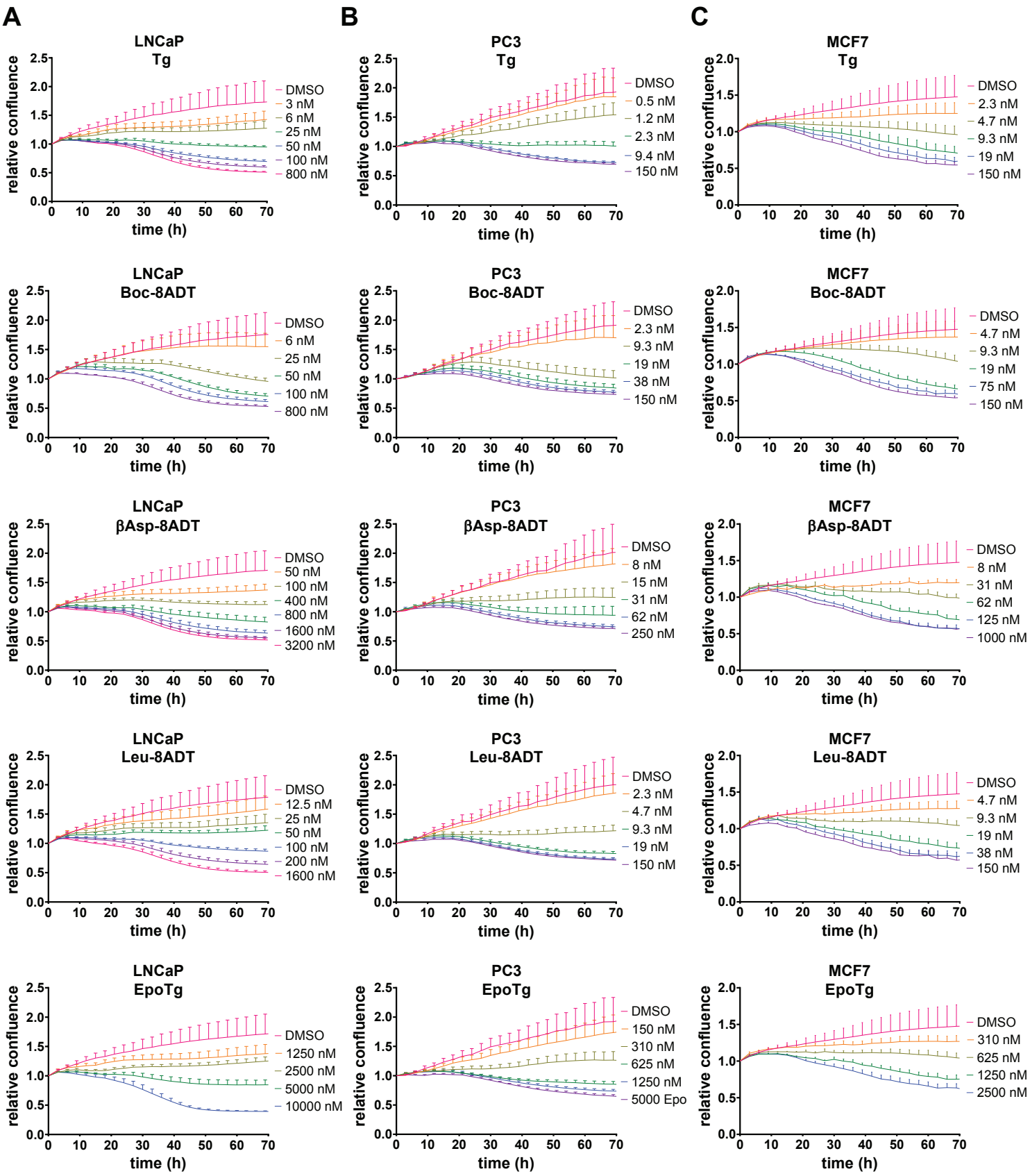

Fig. S2. Dose-dependent effects of Tg and Tg analogs on the cell density of LNCaP, PC3 and MCF7 cells. LNCaP (A), PC3 (B) or MCF7 (C) cells were seeded in 96-well plates and incubated for 2 days. Subsequently, the cells were treated with 0.1% DMSO control or the indicated concentrations of Tg, Boc-8ADT, βAsp-8ADT, Leu-8ADT or EpoTg together with 2.5 µg/ml propidium iodide in complete medium. Cell density was assessed every 3 hours using an Incucyte instrument (Essen Bioscience) for automated live-cell imaging and calculation of cell confluencies (whereas cell death was assessed at 60 hours (LNCaP) or 72 hours (PC3 and MCF7), as shown in Figure 4). Cell density was plotted over 3 days as relative confluence compared to the 0 h time point. Mean values ± SEMs from 4 (LNCaP and MCF7) or 5 (PC3) independent experiments are shown.

Figure S3

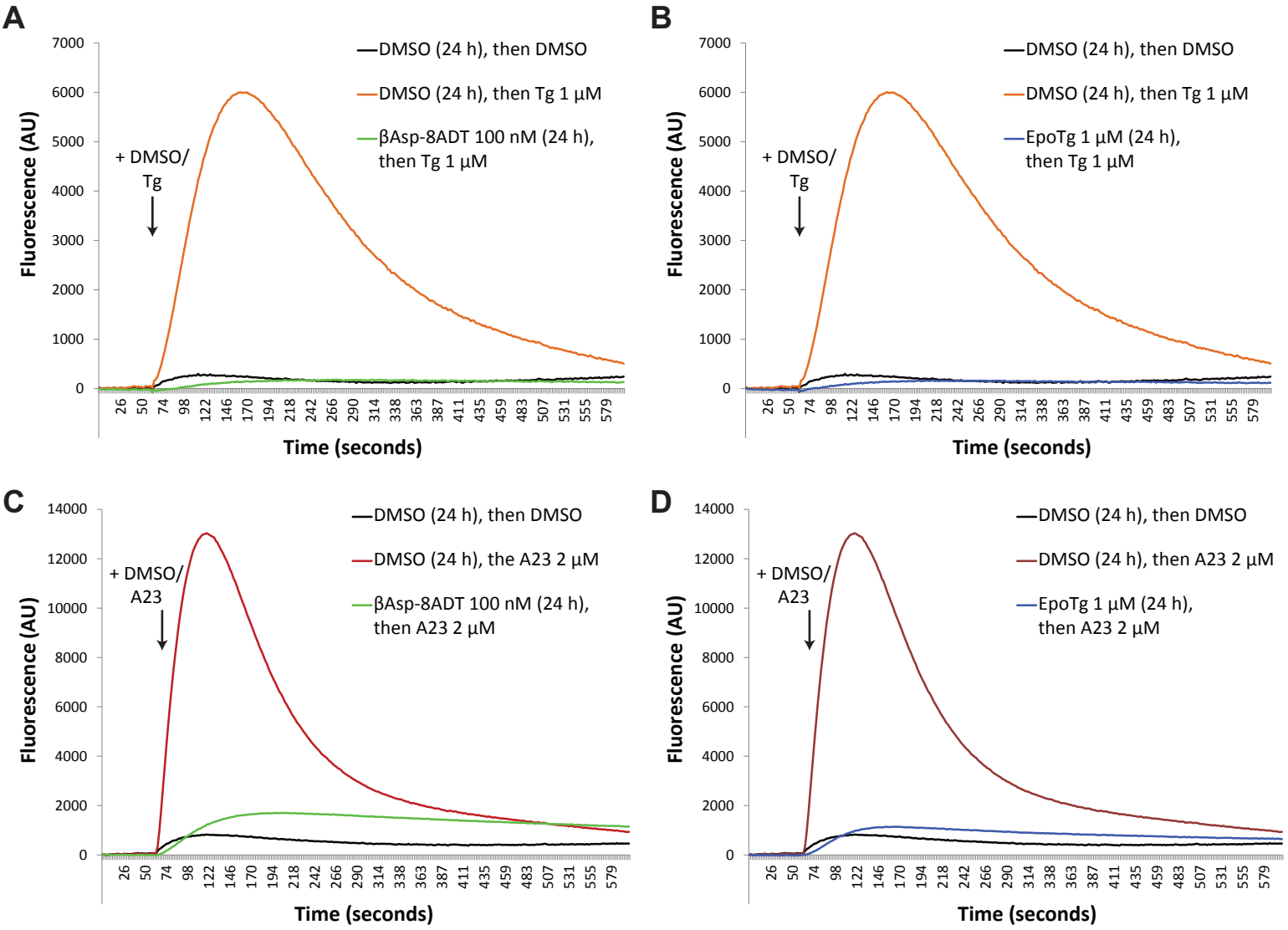

Fig. S3: 24 hour treatment of PC3 cells with 100 nM βAsp-8ADT or 1 µM EpoTg depletes ER calcium stores. PC3 cells were seeded in a 384-well plate and allowed to settle overnight. Subsequently, the cells were treated with 0.01% DMSO, 100 nM ßAsp-8ADT or 1 µM EpoTg for 24 hours, followed by loading with a Ca2+-sensitive dye using the FLIPR calcium 5 assay kit (Molecular Devices, R8185) as previously described (Engedal et al., 2013, Autophagy 9(10); 1475-90), but with the presence of 1 mM EGTA in the HBSS (Gibco 14025)/20 mM HEPES buffer, to keylate extracellular Ca2+, and with the continued presence of the treatments to maintain SERCA inhibition. After loading, the cells were challenged with either 0.01% DMSO (as a control), or with 1 µM Tg or 2 µM A23187 (A23) to release any remaining Ca2+ from the ER. Cytosolic Ca2+ levels were recorded every 2 seconds with a FLIPR384 Fluorometric Imaging Plate Reader instrument (Molecular Devices Corp.). The arrow indicates the time point for addition of DMSO/Tg/A23 challenge (added at the time point of 60 seconds). As shown, 24 hour pre-treatment of PC3 cells with 100 nM βAsp-8ADT (A) or 1 µM EpoTg (B) completely disables the ability of 1 µM Tg to increase cytosolic Ca2+ levels (confirming the results from Figure 6). This was due to ER Ca2+ depletion in the βAsp-8ADT/EpoTg pre-treated cells rather than an inability of the 1 µM Tg to bind and block SERCA after βAsp-8ADT/EpoTg pre-treatment, because also the ability of the calcium ionophore A23187 (A23) to release Ca2+ was severely disabled after the 24 hour pre-treatment with 100 nM βAsp-8ADT (C) or 1 µM EpoTg (D).

TABLE S1

Delays in the rise of cytosolic Ca^{2+} upon exposure of prostate cancer cells to Tg analogs, compared the rise obtained upon exposure to Tg (related to Figure 5).

The table shows the delay time to maximal Ca^{2+}-dependent fluorescence response as well as the fluorescence amplitudes obtained upon exposure of PC3 or LNCaP cells to 1 μM of Tg analogs, relative to those obtained upon exposure to 1 μM Tg. Delays to maximal fluorescence response (in minutes) were obtained by subtracting the values obtained with the Tg analogs from those obtained with Tg. Relative fluorescence amplitudes are expressed as percentage of those obtained with Tg. All results are given as means ± SD of n = 5 or 3 experiments, as indicated.

PC3 cells (n = 5)	βAsp-8ADT	Leu-8ADT	EpoTg
Delay to maximal fluorescence response (min)	6.2 ± 1.3	10.5 ± 2.4	2.1 ± 0.4
Relative fluorescence amplitude (%)	27 ± 8	36 ± 8	53 ± 19
LNCaP cells (n = 3)	βAsp-8ADT	Leu-8ADT	EpoTg
Delay to maximal fluorescence response (min)	13.8 ± 3.9	24.4 ± 2.9	8.5 ± 3.2
Relative fluorescence amplitude (%)	27 ± 8	34 ± 17	25 ± 15