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Two distinct calcium pools in the endoplasmic reticulum of HEK-293T cells

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Agonist-sensitive intracellular Ca^{2+} stores may be heterogeneous and exhibit distinct functional features. We have studied the properties of intracellular Ca^{2+} stores using targeted aequorins for selective measurements in different subcellular compartments. Both, HEK-293T [HEK (human embryonic kidney)-293 cells expressing the large T-antigen of SV40 (simian virus 40)] and HeLa cells accumulated Ca^{2+} into the ER (endoplasmic reticulum) to near millimolar concentrations and the IP_3-generating agonists, carbachol and ATP, mobilized this Ca^{2+} pool. We find in HEK-293T, but not in HeLa cells, a distinct agonist-releasable Ca^{2+} pool insensitive to the SERCA (sarco/endoplasmic reticulum Ca^{2+} ATPase) inhibitor TBH [2,5-di-(*t*-butyl)-benzohydroquinone]. TG (thapsigargin) and CPA (cyclopiazonic acid) completely emptied this pool, whereas lysosomal disruption or manoeuvres collapsing endomembrane pH gradients did not. Our results indicate that SERCA3d is important for filling the TBH-resistant store as: (i) SERCA3d is more abundant in HEK-293T than in HeLa cells; (ii) the SERCA 3 ATPase activity of HEK-293T cells is not fully blocked by TBH; and (iii) the expression of SERCA3d in HeLa cells generated a TBH-resistant agonist-mobilizable compartment in the ER. Therefore the distribution of SERCA isoforms may originate the heterogeneity of the ER Ca^{2+} stores and this may be the basis for store specialization in diverse functions. This adds to recent evidence indicating that SERCA3 isoforms may subserve important physiological and pathophysiological mechanisms.

Key words: aequorin, calcium microdomain, endoplasmic reticulum, intracellular calcium store, sarco/endoplasmic Ca^{2+} ATPase (SERCA).

INTRODUCTION

Changes in the $[Ca^{2+}]_C$ (cytosolic free-Ca^{2+} concentration) are key activation signals for many physiological processes [1]. In non-excitable cells, these $[Ca^{2+}]_C$ signals are usually generated by Ca^{2+} release from the intracellular Ca^{2+} stores, which can be triggered by a variety of intracellular messengers. Most of the mobilizable Ca^{2+} pool seems to be stored in the ER (endoplasmic reticulum), but other organelles such as the Golgi network [2], the lysosomes [3–5] or the secretory granules [6–8] may also release Ca^{2+} into the cytosol and contribute to Ca^{2+} signalling.

Functional heterogeneity of the intracellular Ca^{2+} stores was first proposed in platelets on the basis of the existence of two different organellar-type Ca^{2+}-ATPases, with different molecular masses, phosphorylation patterns and sensitivity to the SERCA (sarco/endoplasmic reticulum Ca^{2+}-ATPase) inhibitor TBH [2,5-di(*t*-butyl)-1,4-benzohydroquinone] [9]. These two platelet SERCA isoforms, which are also expressed in lymphoid cells, were found to be SERCA2b and SERCA3 [10]. Another previous functional study in platelets [11] revealed the existence of two intracellular stores able to accumulate Ca^{2+} with differential sensitivity to inhibitors; the Ca^{2+} pumping activity in one of the stores was fully inhibited by 10 nM TG (thapsigargin) and was not sensitive to TBH, whereas the second store was less sensitive to TG but blocked by TBH. Further investigation on this topic has shown that the TBH-sensitive Ca^{2+} store is probably an acidic subcellular compartment, such as the lysosome, since it is emptied by the lysosome-disrupting peptide GPN (glycylphenylalanine-2-naphthylamide), by the vacuolar H^+-ATPase inhibitor bafilomycin or by the H^+/K^+ ionophore nigericin. The calcium content of this TBH-sensitive store is half that of the TBH-insensitive one [12]. Interestingly, NAADP (nicotinic acid–adenine dinucleotide phosphate) has been shown to release Ca^{2+} from the acidic compartment [13]. The TBH-insensitive store of platelets would correspond to the dense tubular system, the equivalent to the ER in platelets, and is mobilizable through IP_3 (inositol 1,4,5-trisphosphate) receptors [13]. ADP or vasopressin release Ca^{2+} only from the dense tubular system, whereas thrombin does it from both the TBH-sensitive and -insensitive stores [14]. These results have been explained by the differential expression of the SERCA isoforms in the two stores, the TBH-sensitive SERCA3 in the acidic store and the TBH-insensitive SERCA2b in dense tubular system [13,15].

The presence of a TBH-insensitive SERCA in smooth muscle, skeletal muscle and the heart, which express mainly SERCA1 and SERCA2 isoforms [16], was also proposed in platelet studies [10]. However, later studies have generally reported that SERCA1, 2 and 3 are all similarly sensitive to TBH, with an IC_{50} in the micromolar range [17–20]. The origin of these contradictory observations is unclear. There may be differences in the behaviour between different cell types or between isoforms within the same SERCA family. SERCA3 is the most recent member and differs both structurally and functionally from the other isoforms [19]. SERCA3 is insensitive to phospholamban and has a 5-fold lower affinity for cytosolic Ca^{2+} [16,19]. The $K_{0.5}$ value for SERCA2b is approx. 0.2 μM compared with 1.1 μM for SERCA3 [21]. This means that the activity of SERCA3 is very low at

Abbreviations used: $[Ca^{2+}]_C$, cytosolic free-Ca^{2+} concentration; CCh, carbachol; CPA, cyclopiazonic acid; ER, endoplasmic reticulum; $[Ca^{2+}]_{ER}$, free-Ca^{2+} concentration inside the ER; GFP, green fluorescent protein; GA, chimaeric GFP–aequorin fusion protein; erGA, ER-targeted GA; GPN, glycylphenylalanine-2-naphthylamide; HEK-293T, HEK (human embryonic kidney)-293 cells expressing the large T-antigen of SV40 (simian virus 40); IP_3, inositol 1,4,5-trisphosphate; RT, reverse transcription; qRT–PCR, quantitative RT–PCR; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SOCE, store-operated Ca^{2+} entry; TBH, 2,5-di-(*t*-butyl)-benzohydroquinone; TG, thapsigargin; TMA; trimethylamine.

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the resting Ca^{2+} levels of 50–100 nM, but it increases greatly when $[Ca^{2+}]_C$ reaches high levels of near or above 10^{-6} M, e.g. after massive stimulation or during the peak of cytosolic Ca^{2+} oscillations. SERCA3 has been found in many different tissues including endocrine pancreatic cells, intestinal epithelial cells, salivary glands, endothelial cells, different types of white blood cells, platelets and in cerebellar Purkinje neurons. Six different splice variants (SERCA3a–f) have been described in human cells, although the significance of each protein is not clear at present [19,22]. The expression of SERCA3 may change during different conditions. It behaves as a differentiation marker during *Xenopus laevis* development [23] or during the differentiation of vascular endothelial cells and colon mucosa [19]. On the other hand, SERCA3 increases during ER stress in the heart [22] and it has been used as a marker of different diseases such as cardiac failure [22], diabetes [24] or colon cancer [25].

Inhomogeneities of the intracellular Ca^{2+} stores have also been reported in other cell types apart from platelets. In some cases, the differences may be due to the contribution to Ca^{2+} homoeostasis of other organelles such as the Golgi network [2,26], the lysosomes [3–5] or the secretory granules [6–8]. Inhomogeneities in the ER Ca^{2+} store itself have also been proposed on the basis of differential sensitivity to TG [27]. The emptying of the different stores could eventually evoke a differential Ca^{2+} influx by activation of the SOCE (store-operated Ca^{2+} entry), but the mechanisms involved have not been investigated in detail [27].

In the present paper we have tested whether the whole intracellular Ca^{2+} pool behaves homogeneously, both during mobilization by agonists and during refilling through different Ca^{2+}-storing mechanisms. Two different cell types, HEK-293T (human embryonic kidney-293 cells expressing the large T-antigen of simian virus 40) cells and HeLa cells, have been compared. Using ER-targeted aequorins to monitor the different Ca^{2+} stores, we have been able to identify a TBH-resistant Ca^{2+} pool in the ER of HEK-293T cells, which can be mobilized by IP_3-producing agonists. In contrast, the whole Ca^{2+} pool stored in the ER of HeLa cells is homogeneous and can be emptied by TBH.

EXPERIMENTAL

Cell culture and gene transfection

HEK-293T (ATCC CRL-11268) and HeLa (CCL-2) cells were maintained in DMEM (Dulbecco's modified Eagle's medium; Invitrogen) supplemented with 10 % (v/v) fetal bovine serum, 2 mM L-glutamine, 100 μg/ml streptomycin and 100 units/ml penicillin at 37°C, under an air/5 % CO_2 mixture. A stable HEK-293 clone expressing erGA {ER-targeted GA [chimaeric GFP (green fluorescent protein)–aequorin fusion protein]} was generated by transfecting erGA cDNA and selecting the neomycin-resistant clones in 800 μg/ml G418 (Invitrogen) by limited dilution. The clone was routinely cultured in 100 μg/ml G418. For aequorin measurements, cells were seeded on 12 mm diameter poly-L-lysine-coated coverslips at 7×10^4 cells per coverslip. HeLa cells were transfected with 0.1 μg of erGA cDNA using Lipofectamine™ 2000 (Invitrogen). The human SERCA3d cDNA (kindly provided by Dr Jocelyne Enouf, Inserm, U 689, Paris) was cotransfected together with erGA at a 3:1 ratio.

Measurements of the $[Ca^{2+}]_C$

The procedure was performed as described previously [28,29]. Briefly, cells, attached to 12 mm diameter coverslips, were loaded with 4 μM fura-2/AM (fura 2-acetoxymethyl ester; Molecular Probes) for 1 h at room temperature (22 °C) in a standard incubation medium of the following composition: 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose and 10 mM sodium-Hepes (pH 7.4). The cell-coated coverslips were then mounted under a × 20 Olympus PlanApoUV objective in a Nikon Diaphot microscope and washed with fresh medium. Test solutions were applied by continuous perfusion at 2–3 ml/min. For fluorescence measurements, cells were alternately epi-illuminated at 340 and 380 nm and the light emitted above 520 nm was recorded using a Hamamatsu Digital Camera C4742–98 handled by Simple PCI 6.6 Hamamatsu software. Consecutive frames obtained at 340 and 380 nm excitation were ratioed pixel by pixel using ImageJ (http://rsbweb.nih.gov/ij/) and calibrated in $[Ca^{2+}]_C$ by comparison with fura-2 standards [28].

Measurements of the $[Ca^{2+}]_{ER}$ (free Ca^{2+} concentration inside the ER)

Measurements inside the ER were performed using the ER-targeted low $[Ca^{2+}]$-affinity probe, erGA, which has been described previously [30]. Cells expressing erGA were incubated for 1 h at room temperature with 1 μM coelenterazine, either native or n, in a standard Ca^{2+}-free medium (same composition as the standard medium described above except that $CaCl_2$ was omitted and 0.5 mM EGTA was added) in order to reconstitute the aequorin [31]. When aequorin is reconstituted with coelenterazine n, its affinity for Ca^{2+} is approx. 6-fold less than when it is reconstituted with the native coelenterazine and measurements up to the millimolar range are possible [31–33]. The reconstitution medium also contained 10 μM of the SERCA inhibitor TBH in order to prevent refilling of the Ca^{2+} stores, which would lead to burning of the reconstituted aequorin. Finally, the cells were washed once with a Ca^{2+}-free medium and perfused as described in each case. All the measurements were performed at 22°C. Aequorin photoluminescence was measured as described previously in a luminometer constructed by Cairn Research [34], and the calibrations in $[Ca^{2+}]$ were performed using the formula and the constant values published previously [35,36]:

$$[Ca^{2+}]\ (\text{in M}) = [R + (R \times K_{TR}) + 1]/K_R - (R \times K_R) \qquad (1)$$

where $R = (L/\lambda \times L_{TOTAL})^{(1/n)}$, $K_R = 8.47 \times 10^7$, $K_{TR} = 157 \times 10^3$, $n = 1.20$ and $\lambda = 40.1$.

SERCA3 activity determination

Determination of SERCA activity was performed using an enzyme-coupled assay in samples purified by SERCA3 immunoprecipitation [37,38]. Briefly, the cell suspension (6×10^6 cells/ml) was mixed at a ratio of 1:1 with 2 × RIPA buffer [20 mM Tris/HCl (pH 7.2), 2 mM EGTA, 316 mM NaCl, 0.2 % sodium dodecyl sulfate, 2 % sodium deoxycholate and 2 % (v/v) Triton X-100] containing no phosphatase or kinase inhibitors, immunoprecipitated using anti-SERCA3 (PL/IM430) antibody and purified using the immunoprecipitation kit Dynabead® Protein G (Invitrogen). ATPase activity was determined at 37°C and pH 7.2, in the absence (4 mM EGTA) and in the presence of 1 μM Ca^{2+} and with and without 10 μM TBH.

Real-time qRT–PCR [quantitative RT (reverse transcription)–PCR]

Relative expression levels of h-SERCA3d in HEK-293T and HeLa cells were assessed by probe-based real-time qRT–PCR. Total RNA was extracted with TRIzol® (Invitrogen) and quantified in a NanoDrop apparatus. RNA (1–2 μg) was reverse transcribed with the High Capacity cDNA RT Kit

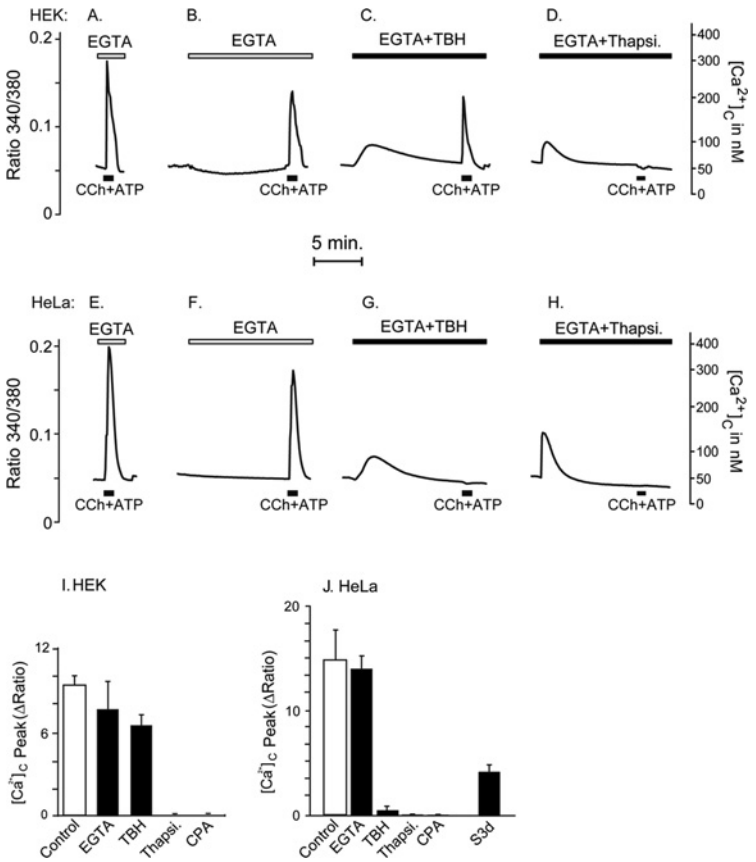

Figure 1 Comparison of calcium mobilization from the intracellular calcium stores in HEK-293T and HeLa cells

Measurements were performed on HEK-293T (**A–D**) and HeLa (**E–H**) cells with fura-2. Ca^{2+} was removed as indicated by the bars (EGTA) and 10 μM TBH or 1 μM TG was added as shown. The CCh and ATP bars indicate stimulation with Ca^{2+}-free solution containing 100 μM of each agonist. Each trace is the average of three similar experiments. Values are expressed as the ratio of the fluorescences excited at 340 and 380 nm. Note the calibration in $[Ca^{2+}]_C$ at the right. (**I**) HEK-293T cells and (**J**) HeLa cells show means $\pm$ S.E.M. ($n = 3$–6) values of the $[Ca^{2+}]_C$ peaks obtained in different conditions, as shown in the abscissa axis: Control (Ca^{2+} removal 30 s before the stimulus), EGTA (Ca^{2+} removal 12 min before the stimulus), TBH (12 min treatment with 10 μM TBH in Ca^{2+}-free medium before the stimulus), Thapsi. (12 min treatment with 1 μM TG in Ca^{2+}-free medium before the stimulus) and CPA (12 min treatment with 15 μM CPA in Ca^{2+}-free medium before the stimulus). TBH and S3d, TBH condition in HeLa cells overexpressing SERCA3d (see the Experimental section and Figure 7).

from Applied Biosystems using MultieScribe-RT and Oligo-dT as primers. Amplifications of 1–100 ng of cDNA were performed in triplicate using the provided SYBR Green PCR Master Mix in a 25 μl reaction mixture containing 100 nM primers and 100 nM probe (Applied Biosystems). Primers were as follows: forward 5′-GACCACACCGGGGCCAGG GACACA-3′ and reverse 5′-GCCTGTCATTTATCCGGCG-3′ for h-SERCA3d, and forward 5′-TACCTCCGCTGCATCTCC-3′ and reverse 5′-GCCTGTCATTTATCCGGC-3′ for RPL18 (housekeeping gene). The PCR was run on an LightCycler 480 (Roche). The relative gene expression was determined with the $2^{-\Delta\Delta CT}$ method [39].

Statistics

The results are expressed as means $\pm$ S.E.M. Statistical significance was evaluated by Student's *t* test or one-way ANOVA using GraphPad InStat software.

RESULTS

In order to assess the homogeneity of the calcium pools mobilized by agonists, we compared the size of the $[Ca^{2+}]_C$ peaks obtained on stimulation after treatment with different inhibitors. The results are summarized in Figure 1. In these experiments, Ca^{2+} mobilization from the intracellular Ca^{2+} stores was induced by IP_3-producing agonists and the size of the Ca^{2+} pool was inferred from the size of the $[Ca^{2+}]_C$ increase. Both cholinergic and purinergic agonists are known to stimulate PLC (phospholipase C) in both HEK-293T [40] and HeLa cells [41]. We found in preliminary experiments (results not shown) that the maximum releasing effect was best obtained when CCh (carbachol) and ATP were applied together. Therefore this stimulus was used routinely in further experiments. Since HEK-293T cells have been reported to have ryanodine receptors [42], we cannot exclude that CCh and ATP could also mobilize Ca^{2+} through ryanodine receptors activated indirectly by the Ca^{2+} released via IP_3 receptors; however, this should be a very minor effect, most probably completely masked by fura-2 [42]. Figure 1 shows the mobilization of the stored Ca^{2+}, visualized as the increase of $[Ca^{2+}]_C$, by stimulation with 100 μM CCh and 100 μM ATP in HEK-293T (Figures 1A–1D) and HeLa (Figures 1E–1H) cells. Stimulation was performed in Ca^{2+}-free medium to avoid contamination of the $[Ca^{2+}]_C$ peak with Ca^{2+} entry through the plasma membrane. The first peak was triggered 30 s after removing Ca^{2+}, a period long enough to allow complete washing of the extracellular Ca^{2+}, but short enough to avoid substantial emptying of the intracellular Ca^{2+} stores (Figures 1A and 1E). The second peak was obtained after 12 min of incubation in Ca^{2+}-free medium

(Figures 1B and 1F). This $[Ca^{2+}]_C$ increment was approx. 80 % of the first one, suggesting that passive emptying of the intracellular Ca^{2+} stores by removal of external Ca^{2+} is relatively slow in both HEK-293T and HeLa cells. In another series of experiments, the re-uptake of Ca^{2+} into the ER during incubation in a Ca^{2+}-free medium was prevented by adding the SERCA inhibitor TBH [43]. $[Ca^{2+}]_C$ increased transiently during the incubation with TBH in Ca^{2+}-free medium, indicating that there is a slow passive Ca^{2+} release by leak from the stores (Figures 1C and 1G). After 12 min of incubation with TBH in the Ca^{2+}-free medium the IP_3-sensitive Ca^{2+} store in HeLa cells was almost completely empty, since the $[Ca^{2+}]_C$ peak induced by CCh and ATP was less than 2 % of the control (Figure 1G). By contrast, the $[Ca^{2+}]_C$ peak obtained in HEK-293T cells by stimulation with CCh and ATP in the same conditions was quite substantial (64 % of the control value; Figure 1C). Treatment with 1 μM TG, another SERCA inhibitor [44], completely prevented responses to CCh and ATP, both in HeLa and in HEK-293T cells (Figures 1D and 1H), indicating that the intracellular Ca^{2+} stores sensitive to IP_3 are completely emptied by treatment with this inhibitor. Comparable results were obtained with another SERCA inhibitor, namely CPA (results not shown) [18,45]. The averaged results of several similar experiments are shown in Figures 1(I) and 1(J). To summarize, it seems clear from our results that there is a substantial part of the IP_3-sensitive Ca^{2+} pool of HEK-293T cells that is resistant to TBH, but sensitive to TG or CPA, and that this Ca^{2+} pool does not exist in HeLa cells.

Figure 2(A) shows that the TBH-resistant Ca^{2+} pool of HEK-293T cells can be emptied by stimulation with agonists and then filled again by incubation with Ca^{2+}. The cells were treated with TBH in Ca^{2+}-free medium for 12 min and then three consecutive pulses of CCh and ATP were applied. The first stimulus produced a much larger $[Ca^{2+}]_C$ peak than the second one and the response to the third was barely detectable. These results indicate that after the third stimulus the Ca^{2+} store was completely empty. Upon the re-addition of Ca^{2+} (in the continuous presence of TBH) $[Ca^{2+}]_C$ increased greatly and then declined slowly. The first step reflects a rapid Ca^{2+} entry through SOCE, which is activated by the emptying of the intracellular Ca^{2+} stores [46]. As the intracellular stores refill, the store-operated channels deactivate and $[Ca^{2+}]_C$ decreases. After 9 min, external Ca^{2+} was removed again and another series of three pulses of CCh and ATP was applied. The first stimulus evoked 80 % of the initial response, indicating that the TBH-resistant intracellular Ca^{2+} stores had refilled substantially. By contrast, the second stimulus had little effect, suggesting that the Ca^{2+} content of the intracellular stores was almost completely mobilized by the first stimulus.

It has been reported in different cell types that Ca^{2+} can accumulate in acidic intracellular compartments, such as lysosomes or secretory granules [15], or in alkaline organelles, such as mitochondria [47]. Collapsing the pH gradient empties these Ca^{2+} stores and abolishes the release in both cases. Figures 2(B)–2(F) show the results of a series of experiments designed to test whether acidic or alkaline intracellular compartments could be responsible for the TBH-resistant Ca^{2+} accumulation. Figure 2(G) summarizes the average values obtained in three similar experiments. HEK-293T cells were first treated for 5 min with TBH in the Ca^{2+}-free medium and then the effects of several treatments, aimed to collapse the organellar pH gradients, were studied by monitoring the $[Ca^{2+}]_C$ peaks induced by CCh and ATP. The effects of the substances able to change the cytoplasmic pH should be regarded with some caution as these pH changes also modify the affinity of the Ca^{2+} probes and could therefore disturb the measurements. In the present paper we tested the effect of a weak base,

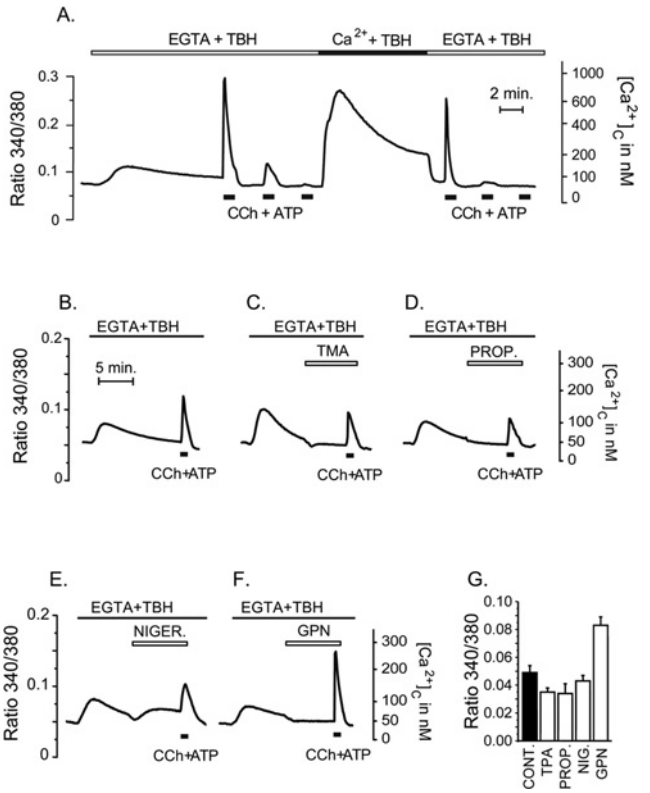

Figure 2 Emptying and refilling of the TBH-resistant intracellular store of HEK-293T cells

(**A**) Typical trace representative of three similar ones. Other details are as described in the legend to Figure 1. (**B**–**G**) The effects of several treatments for disrupting pH gradients in endomembranes on the TBH-resistant intracellular calcium store of HEK-293T cells. Each trace is the average of three to six experiments. Other details are as described in the legend to Figure 1. The values shown in (**G**) are means ± S.E.M. of three determinations. GPN, 10 μM GPN; NIGER, 10 μM nigericin; PROP, 6 mM sodium propionate; TMA, 6 mM trimethylamine.

TMA (trimethylamine) (Figure 2C), which accumulates in acidic compartments and thus collapses their pH gradient; a weak acid, propionate (Figure 2D), which accumulates inside alkaline compartments; and the H^+/K^+ exchanger nigericin, which should collapse both acidic and alkaline pH gradients (Figure 2E). None of the three treatments was able to abolish the peak induced by CCh and ATP (compare Figures 2C–2E with the control, Figure 2B), although the $[Ca^{2+}]_C$ peaks were somewhat reduced in all cases. The lysosomal disruptor GPN did not inhibit the $[Ca^{2+}]_C$ peak either (Figure 2F). In fact, the height of the peak obtained in the presence of GPN was larger than the control (Figures 2F and 2G), the opposite outcome to the one expected if acidic granules contributed to the TBH-resistant Ca^{2+} pool. These results suggest that the TBH-resistant store of HEK-293T cells is not (or not only) inside either acidic or alkaline granules.

We next investigated directly the implications of the ER in TBH-resistant storage by measuring its Ca^{2+} content with an ER-targeted aequorin [31,48]. These erGA-transfected HEK-293T (Figure 3A) and HeLa (Figure 3B) cells, whose Ca^{2+} stores had been emptied, were allowed to refill with Ca^{2+}, either in control medium with 1 mM Ca^{2+} or in medium containing 1 mM Ca^{2+} and 10 μM TBH. After 5 min refilling, the cells were stimulated with CCh and ATP. Under the control conditions, the ER refilled to $[Ca^{2+}]_{ER}$ levels approaching 500 μM in both cell types and stimulation with CCh and ATP produced Ca^{2+} release. Ca^{2+} store emptying was more complete in HeLa than in HEK-293T cells,

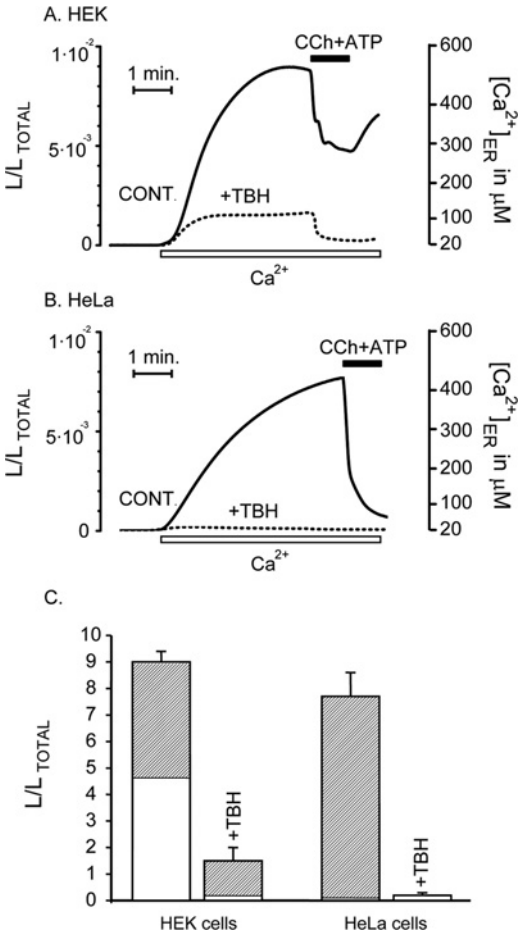

Figure 3 Comparison of the Ca^{2+} refilling and the agonist-induced emptying of ER in HEK-293T and HeLa cells

Cells [HEK-293T (**A**) and HeLA (**B**)] transfected with erGA and reconstituted with 1 μM coelenterazine n (see the Experimental section) were washed with Ca^{2+}-free medium and, at the time shown, perfused with standard medium containing 1 mM Ca^{2+} with (CONT., continuous line) or without (+TBH, broken line) 10 μM TBH. At the time shown the cells were challenged with CCh and ATP (100 μM of each one). Values are plotted as L/L_{TOTAL}. Calibration in $[Ca^{2+}]$ is shown at the right. Every trace is the mean of three individual experiments. (**C**) Bars represent means $\pm$ S.E.M. ($n = 3$) of the Ca^{2+} levels attained at the steady state (in $L/L_{TOTAL} \times 10^3$) in the different conditions. The hatched boxes correspond to the fraction of the pool released by stimulation with CCh and ATP.

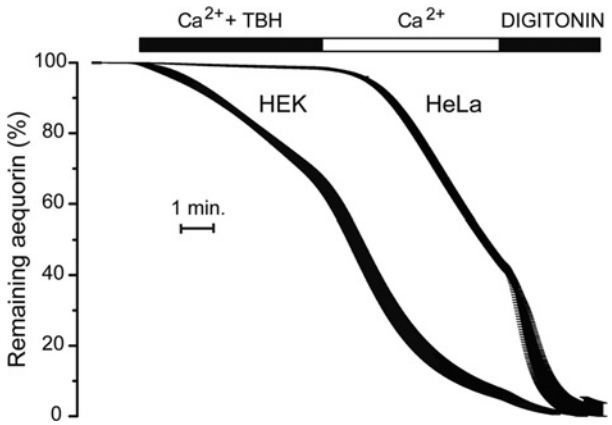

Figure 4 Time course of the aequorin consumption in HEK-293T and HeLa cells on filling the calcium stores

Cells, treated as described in the legend to Figure 3, were washed with Ca^{2+}-free medium and then incubated first with standard medium containing 1 mM Ca^{2+} and 10 μM TBH (Ca^{2+} and TBH) and later with the same medium without TBH (Ca^{2+}). Finally, the cells were lysed with digitonin in the presence of 10 mM Ca^{2+}. Results are expressed as a percentage of the total aequorin remaining in the cells at a given time. Each trace is the average of three experiments and the vertical bars correspond to S.E.M. values.

where it amounted to $\sim$50 % of the total calcium pool. In the presence of TBH, there was hardly any refilling in HeLa cells, whereas in HEK-293T cells the ER refilled to $\sim$20 % of the control value and CCh and ATP produced a near complete Ca^{2+} release. A summary of the averaged estimated sizes of the different calcium pools is presented in Figure 3(C). Note again the striking difference in the behaviour of HEK-293T and HeLa cells in the presence of TBH.

Since aequorin is burnt out during light emission on Ca^{2+} binding [33,49], the fractional consumption at equilibrium measures the relative size of the aequorin-containing space that is occupied by Ca^{2+} as the intact light-emitting aequorin remains only in the locations that have not taken up Ca^{2+}. Figure 4 compares the time courses of the consumption of the ER-targeted aequorin in HEK-293T and HeLa cells during refilling of the calcium stores in the presence of TBH. In the 6 min period shown in the Figure, aequorin consumption was less than 2 % in HeLa cells and near 40 % in HEK-293T cells. In addition, the rate of consumption tended to decrease with time for the former, but remained unchanged for the latter, suggesting that the Ca^{2+}-accesible aequorin pool was only a small fraction of the total in HeLa cells and most of the pool in HEK-293T cells (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/435/bj4350227add.htm). After 6 min, TBH was removed to allow rapid refilling of the TBH-sensitive stores. Under these conditions, Ca^{2+} consumption was similarly quick in both cases and seemed to affect most of the aequorin pool (Figure 4).

In order to measure the size of the ER-aequorin pools more precisely, a new series of experiments were designed where reconstitution of erGA was performed with native coelenterazine instead of coelenterazine n. Under these conditions, the affinity of aequorin for Ca^{2+} increases and consumption is much faster and more sensitive to smaller Ca^{2+} concentrations. These results are shown in Figure 5, where the time courses of the consumptions either with Ca^{2+} alone or in the presence of TBH are compared. In HEK-293T cells (Figure 5A), the consumption was almost complete (>80 %) by the end of the incubation period in both cases, although somewhat slower in the presence of TBH. This suggests that most of the ER pool is able to accumulate Ca^{2+} even in the presence of TBH. The results in HeLa cells were very different (Figure 5B). Consumption in the presence of TBH was very slow (10-fold slower than the control), but, in addition, it seemed to occur to a limited fraction of the total Ca^{2+} pool accounting for less than 25 % of the total aequorin. When TBH was removed, the rest of the Ca^{2+} pool was consumed quickly revealing the presence of the TBH-sensitive Ca^{2+} pumping mechanism. When Ca^{2+} was given in the absence of TBH from the outset, ER-aequorin was consumed quickly and completely. The results of the experiments illustrated in Figures 5(A) and 5(B) were quantified as the percentage of ER-aequorin consumption at the plateau and $t_{1/2}$ for consumption (for details see Figure 5 and Supplementary Figure S2 at http://www.BiochemJ.org/bj/435/bj4350227add.htm). The average values of three similar experiments are plotted in Figures 5(C) and 5(D). It is clear that the percentage of ER-aequorin consumption at equilibrium was, in the presence of TBH, much smaller in HeLa than in HEK-293T cells (Figure 5C; 25 ± 3 compared with 66 ± 4; $P < 0.001$). In addition, in the presence

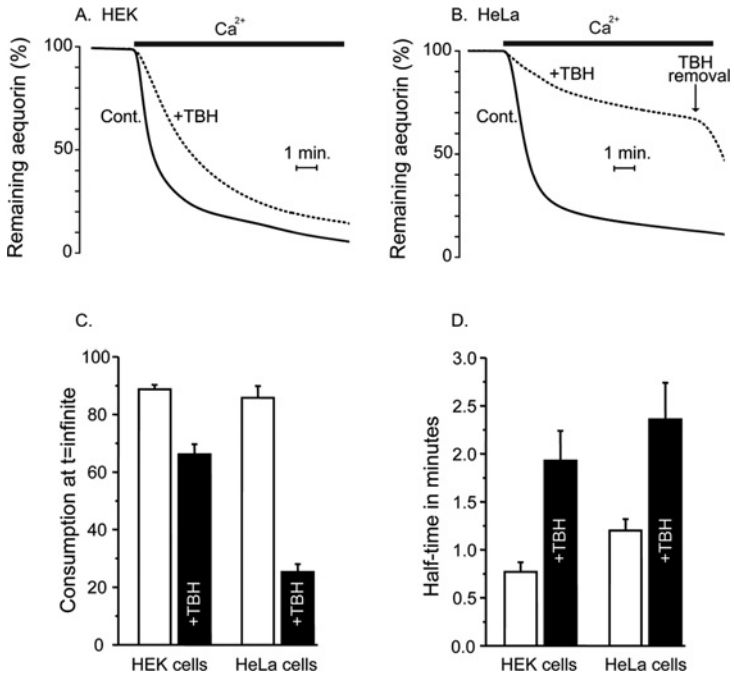

Figure 5 Comparison of the aequorin consumption in HEK-293T and HeLa cells on filling the intracellular calcium stores in the presence or in the absence of TBH

Cells [HEK-293T (**A**) and HeLa (**B**)] were treated as described in the legend to Figure 3 except that aequorin was reconstituted with native coelenterazine instead of coelenterazine n. This increases the affinity for Ca^{2+} approx. one order of magnitude [31,32,35]. After 2 min washing with the Ca^{2+}-free medium, 1 mM Ca^{2+} was added, as shown. Results are expressed as the percentage of total aequorin remaining in the cells at a given time. Each trace is the average of three experiments. In the case of HeLa cells refilled in the presence of TBH the effect of TBH removal is shown at the end of the trace. (**C**) Estimations of the percentage consumption of the aequorin pool at equilibrium in the different conditions studied in (**A**) and (**B**). The values were estimated by extrapolating the plateau of the curves to the time of Ca^{2+} addition. (**D**) Estimations of the $t_{1/2}$ values for aequorin consumption in the different conditions. Half-times were estimated in reference to the percentage consumption calculated in (**C**). Values in (**C**) and (**D**) correspond to the means $\pm$ S.E.M. for three independent experiments.

of TBH, consumption was slower in both HEK-293T and in HeLa cells, as shown by the increase of the $t_{1/2}$ values (Figure 5D).

In order to explore further the mechanism responsible for the TBH-resistant Ca^{2+} storage in HEK-293T cells, we looked for the differences in the expression of SERCAs between both cell types. It has been reported that the expression of SERCA2b is similar in HeLa and HEK-293T cells, but, interestingly, HEK-293T cells express higher levels of SERCA3 (principally isoform d) than HeLa cells [50]. We have determined and compared the relative abundance of the SERCA3d mRNA in HEK-293T and HeLa cells by qRT–PCR and confirm the differences between both cell types. Our results show that the relative expression of SERCA3d mRNA is (mean $\pm$ S.E.M.; $n = 3$) 19 ± 5-fold higher in HEK-293T cells than in HeLa cells.

We next investigated whether the differences found in ER Ca^{2+} transport between HEK-293T and HeLa cells could be related to differences in the expression of SERCA isoforms. For this purpose, we determined the Ca^{2+}-dependent ATPase activity linked to SERCA3 in HEK-293T and HeLa cells, and compared the sensitivity with TBH. Figure 6 shows the averaged values of ATPase activity obtained with and without Ca^{2+} and in the absence and presence of TBH. The Ca^{2+}-dependent activity was nominally larger in HEK-293T than in HeLa cells (mean $\pm$ S.E.M): 0.217 ± 0.023 compared with 0.093 ± 0.003 μmol $\cdot$ min^{-1} $\cdot$ mg of protein^{-1} (compare Figures 6A and 6B) ($P < 0.001$). Even more striking was the fact that whereas TBH inhibited completely (87–99 %) the Ca^{2+}-stimulated ATPase activity in HeLa cells (Figure 6B), there was a substantial fraction (32–46 %) of TBH-resistant activity in HEK-293T cells (Figure 6A).

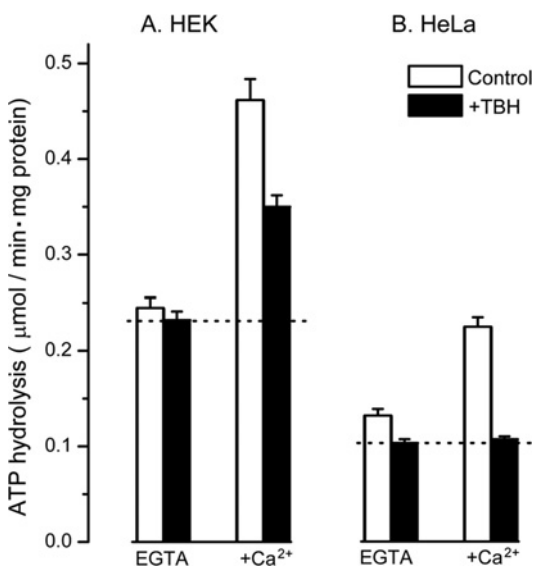

Figure 6 SERCA3 ATPase activity in HEK-293T and HeLa cells

Cell [HEK-293T (**A**) and HeLa (**B**)] extracts were purified by immunoprecipitation and ATPase activity was determined by an enzyme-coupled assay (see the Experimental section). The values in the absence (EGTA) and in the presence of 1 μM Ca^{2+} (and Ca^{2+}), and in the absence (open bars) and in the presence of TBH (solid bars) are shown. Each value is the mean $\pm$ S.E.M. for four individual experiments. The values obtained with 20 μM TBH (results not shown) were the same as with 10 μM TBH.

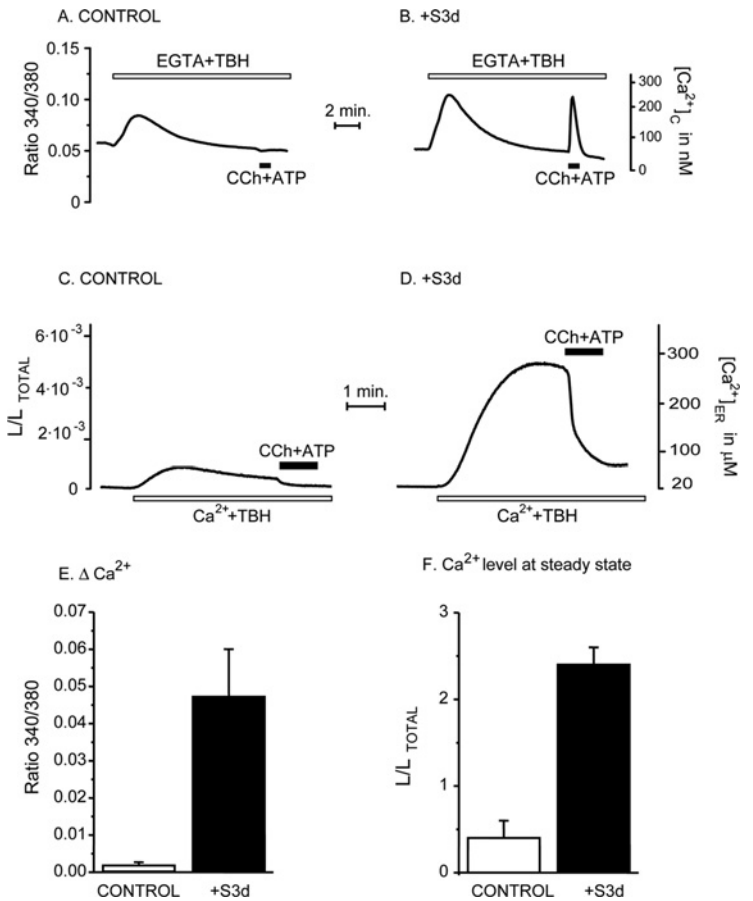

Figure 7 Expression of SERCA3d generates a TBH-resistant intracellular calcium store in HeLa cells

SERCA3d was cotransfected together with erGA at a 3:1 ratio; controls were transfected with the empty vector (pcDNA3). (**A** and **B**) Experiments with fura-2-loaded cells. Details are as described in the legend to Figure 1. (**C** and **D**) Aequorin measurements. Details are as described in the legend to Figure 3. Results are representative of three to four similar experiments. Average values (±S.E.M) are shown in (**E**) (size of the Ca^{2+} peak) and (**F**) ($[Ca^{2+}]_{ER}$ at the steady state measured in $L/L_{TOT} \times 10^3$). The differences between the control cells and the cells expressing SERCA3d (S3d) were statistically significant [$P < 0.02$ in (**E**) and $P < 0.0004$ in (**F**)],

Finally, we reasoned that if SERCA3 was responsible for the TBH-insensitive activity in HEK-293T cells, we should confer this activity to HeLa cells by expressing SERCA3d. The results of the experiments designed to test this point are shown in Figure 7. In these experiments the filling of the intracellular Ca^{2+} stores in the presence of TBH was compared in control and SERCA3d-overexpressing HeLa cells. The Ca^{2+} content of the stores was estimated either from the size of the $[Ca^{2+}]_C$ peak induced by stimulation with agonists in fura-2-loaded HeLa cells (Figures 7A and 7B) or by directly measuring with ER-aequorin the Ca^{2+} accumulation into the ER (Figures 7C and 7D). As shown above (Figures 1 and 3), control HeLa cells did not show any indication of TBH-resistant storage of Ca^{2+} into the ER: there was no $[Ca^{2+}]_C$ increase on stimulation with ATP and CCh (Figure 7A) and the ER refilled very little with Ca^{2+} (Figure 7C). In contrast, upon SERCA3d overexpression the release of Ca^{2+} by stimulation with CCh and ATP was sharply increased (Figure 7B) and direct measurement of ER content showed increased refilling (~6-fold more than in the control; Figure 7D). In addition, this stored Ca^{2+} was almost completely released by stimulation with CCh and ATP (Figure 7D). Figures 7(E) and 7(F) show the average values obtained in three similar experiments for the agonist-induced $[Ca^{2+}]_C$ peaks and for ER refilling respectively.

DISCUSSION

The results of the present study reveal the heterogeneity of the intracellular Ca^{2+} stores in HEK-293T cells, where a TBH-resistant pool was found (Figure 1C). This store was able to sustain the IP_3-induced $[Ca^{2+}]_C$ peak to values near 80 % of the control (Figure 1I). Both TG and CPA were able to empty completely this TBH-resistant Ca^{2+} store (Figures 1D and 1I). In contrast with HEK-293T cells, HeLa cells did not show evidence for a TBH-resistant Ca^{2+} store (Figures 1G and 1J). In lymphocytes, TBH has also been shown to empty completely the intracellular Ca^{2+} stores [18]. Once emptied by stimulation with agonists, the TBH-resistant store of HEK-293T cells refilled by incubation with external Ca^{2+}, even in the presence of TBH (Figure 2A).

What could be the structural basis that justifies the differences in intracellular Ca^{2+} storage among the various cell types? In platelets, it seems clear that the TBH-sensitive and the TBH-resistant Ca^{2+} stores correspond with two different Ca^{2+} pools, the former located in the acidic dense granules or the lysosomes, and the latter in the dense tubular system [9,11,14,15]. As a consequence, Ca^{2+} is released from the granules by substances collapsing the H^+ gradient, such as the K^+/H^+ exchanger nigericin, the vacuolar H^+-ATPase inhibitor bafilomycin or

the osmotic lysosomal disruptor GPN. In contrast with these observations, our results in HEK-293T cells indicate that treatments that collapse the pH gradient of intracellular organelles did not abolish the TBH-resistant Ca^{2+} release (Figures 2B–2G) suggesting that, in these cells, Ca^{2+} comes from another store.

In addition, we have direct evidence showing that the TBH-resistant Ca^{2+} pool accumulates inside the ER, as revealed by the ER-targeted aequorin (Figure 3). The Ca^{2+} concentration attained by the TBH-resistant accumulation mechanism in this pool seemed smaller (approx. one-fifth) than the $[Ca^{2+}]_{ER}$ normally reached in the bulk of the store (Figure 3A). Stimulation with the IP_3-producing agonists released Ca^{2+} from the TBH-resistant store as efficiently as, or even better than, from the bulk ER in HEK-293T cells (Figures 3A and 3C). Consistently with the results obtained with fura-2 (Figure 1) ER-targeted aequorin showed that, in HeLa cells, emptying of ER by TBH was virtually complete and that ER did not refill significantly when incubated with Ca^{2+} in the presence of TBH (Figures 3B and 3C).

Since aequorin is burned out in the presence of Ca^{2+}, the relative aequorin consumption at equilibrium informs us of the size of the Ca^{2+} pool involved in Ca^{2+} uptake. Using this strategy, we find that the relative size of the TBH-resistant ER Ca^{2+} pool, as indicated by the asymptotic fraction of aequorin consumption, is very different in HEK-293T and in HeLa cells (Figures 4 and 5). In HEK-293T cells the TBH-resistant aequorin pool was $>80\,\%$ of the total (Figures 5A and 5C), suggesting that there is a communication between the TBH-resistant and the TBH-sensitive pools or, alternatively, that the mechanism responsible for Ca^{2+} uptake distributes along the whole ER. Conversely, in HeLa cells the TBH-resistant pool, probably overestimated by using an aequorin–coelenterazine system with higher-affinity for Ca^{2+}, amounted to only 20–25 % (Figures 5B and 5C).

In platelets, the TBH-sensitive uptake of Ca^{2+} into the acidic granules seems to be related with a distinct SERCA isoform [9], which was later identified as SERCA3 [10], present also in several other tissues [19,51]. On the other hand, SERCA2b would be resistant to TBH in platelets [9,10]. However SERCA2b (as well as SERCA1) activity and ER Ca^{2+} uptake have been consistently reported to be sensitive to TBH in other tissues, including skeletal muscle, the heart, smooth muscle and lymphocytes [17–20,52,53]. The dominant SERCA isoform in HEK-293T and HeLa cells is the 2b isoform [50], which according to our results, is sensitive to TBH (Figures 3 and 4). It has been reported that HEK-293T, but not HeLa cells, also express a SERCA3d isoform [50] and we confirm it in the present study at the mRNA expression level by qRT–PCR. In addition, HEK-293T, but not HeLa, cells showed TBH-resistant SERCA3-mediated ATPase activity (Figure 6). Finally, overexpression of SERCA3d in HeLa cells generated a TBH-resistant intracellular Ca^{2+} pool in the ER (Figures 7D and 7F), which was released by stimulation with IP_3-producing agonists. These results suggest that SERCA3d may be responsible for the TBH-resistant Ca^{2+} pool of HEK-293T cells and raises the interesting question of whether differential expression of SERCA isoforms [51] could modify the properties of intracellular calcium stores and allow the coexistence of different pools, perhaps fulfilling different functions, in the same cell. It has been proposed recently that SERCA3 isoforms could participate in differentiation [23] and be related to transduction of both physiological and pathophysiological reactions [19,22,24,25,51].

The presence of two distinct ER-derived Ca^{2+} compartments in HEK-293T cells might have functional relevance as proposed in other cells, where function-specific Ca^{2+} compartments have been reported to regulate different cellular mechanisms through multiple agonists. For instance, in human platelets the TBH-sensitive acidic store is discharged upon occupation of high-affinity thrombin receptors and participates in aggregation [14, 54]. Similarly, in goldfish somatotropes, two different Ca^{2+} stores may differentially regulate growth hormone storage and secretion [55]. Therefore our findings further advance our knowledge of organellar Ca^{2+} stores underlying the generation of differential Ca^{2+} signals by different Ca^{2+}-mobilizing agonists.

AUTHOR CONTRIBUTION

Francisco J. Aulestia performed most of the experiments. Pedro C. Redondo carried out the SERCA3 determinations and Arancha Rodríguez–García created the stable HEK-293T clone. Juan A. Rosado, Ginés M. Salido, Maria Teresa Alonso and Javier García-Sancho provided conceptual input and designed the experiments. All authors participated in analysis, discussion and interpretation of data, revised the article prior to submission and gave final approval. Javier García-Sancho put together all results and wrote the final form of the manuscript prior to submission.

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online data

SUPPLEMENTARY ONLINE DATA

Two distinct calcium pools in the endoplasmic reticulum of HEK-293T cells

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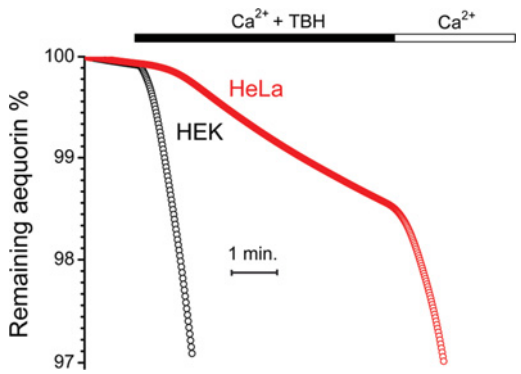

Figure S1 Comparison of the initial rates of aequorin consumption in HEK-293T and HeLa cells in the presence of TBH

For HEK -293T (black circles) and HeLa cells (red circles) the initial 3 % consumption has been expanded and a representative experiment of the same lot as in Figure 4 of the main text is shown. Note that the rate of consumption in HeLa cells decreases somewhat during the first 6 min of incubation with Ca^{2+} +TBH.

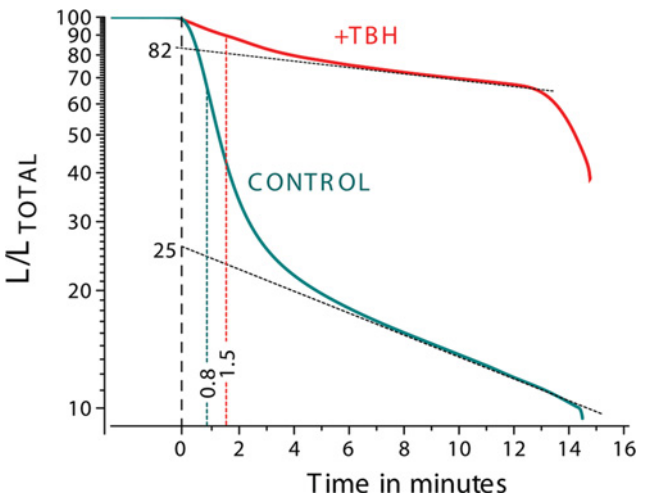

Figure S2 The procedure used to calculate the percentual aequorin consumption and the $t_{1/2}$

One representative experiment with HeLa cells is shown. Data were plotted in a logarithmic scale and the slope at the final part of the trace was extrapolated to zero time to estimate the aequorin consumption (broken lines with figures at left). The vertical broken lines illustrate calculations of the $t_{1/2}$ values.

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