Two distinct calcium pools in the endoplasmic reticulum of HEK293T cells

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Short title: Two distinct ER calcium pools in HEK cells

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SYNOPSIS

Agonist-sensitive intracellular Ca^{2+} stores may be heterogeneous and exhibit distinct functional features. We have studied the properties of the intracellular Ca^{2+} stores using targeted aequorins for selective measurements in different subcellular compartments. Both, HEK293T and HeLa cells accumulated Ca^{2+} into the endoplasmic reticulum (ER) to near milimolar concentrations and the IP_3-generating agonists carbachol and ATP were able to mobilize this Ca^{2+} pool. We find in HEK, but not in HeLa cells, a distinct agonist-releasable Ca^{2+} pool insensitive to the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor, 2,5-di(tert-butyl)-1,4-benzohydroquinone (TBH). Thapsigargin and cyclopiazonic completely emptied this pool whereas lysosomal disruption or manoeuvres collapsing endomembrane pH gradients did not. Q-RT-PCR analysis showed that SERCA3d is 20 times more abundant in HEK than in HeLa cells. Importantly, we find that SERCA3 ATPase activity was not fully sensitive to TBH and that expression of SERCA3d in HeLa cells generated a TBH-resistant, agonist-mobilizable compartment in the ER. These findings indicate that differential distribution of SERCA isoforms may originate heterogeneity of the ER Ca^{2+} stores. The question of whether different stores may subserve diverse functions will require further research.

KEYWORDS:

Endoplasmic reticulum, Calcium, intracellular calcium stores, aequorin, calcium microdomains, SERCA, Sarco/endoplasmic Ca2+ ATPase

ABBREVIATION FOOTNOTE:

Abbreviations used are: GFP, green fluorescent protein; GA, chimeric GFP-AEQ fusion protein; ER, endoplasmic reticulum; SERCA, Sarco(endo)plasmic reticulum Ca^{2+} ATPase, $[Ca^{2+}]_C$, cytosolic free Ca^{2+} concentration; $[Ca^{2+}]_{ER}$, Ca^{2+} concentration inside ER; TBH, 2,5-di-*tert*-butyl-benzohydroquinone; TG, thapsigargin; DTS, dense tubular system; GPN, glycylphenylalanine-2-naphthylamide; AEQ, aequorin; erGA, ER-targeted GA; IP_3, inositol 1,4,5, trisphosphate; CCh, carbachol.

INTRODUCTION

Changes of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_C$)[3] 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 second messenger-mobilizable Ca^{2+} seems to be stored into the endoplasmic reticulum (ER), but other organelles such as the Golgi network [2] the lysosomes [3-5]or the secretory granules [6-8] may also release Ca^{2+} to the cytosol and contribute to Ca^{2+} signaling.

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 weights, phosphorylation patterns and sensitivity to the sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor 2,5-di(tert-butyl)-1,4-benzohydroquinone (TBH) [9]. These two platelet SERCA isoforms, which are also expressed in lymphoid cells, resulted to be SERCA2b and SERCA3 [10]. A contemporary functional study in platelets [11] also revealed the existence of two Ca^{2+} stores able to accumulate Ca^{2+} with differential sensitivity to inhibitors; the Ca^{2+} pumping activity expressed in one of the stores was fully inhibited by 10 nM thapsigargin (TG) and was not sensitive to TBH while the one expressed in the other store was less sensitive to TG but blocked by 10 μM 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, as that it is emptied by the lysosome-disrupting peptide glycylphenylalanine-2-naphthylamide (GPN), by the vacuolar H^+-ATPase inhibitor bafilomycin or by the H^+/K^+ ionophore nigericin. The TBH-sensitive store contains about half as much Ca^{2+} as the TBH-insensitive [12] and is mobilizable by NAADP [13]. The TBH-insensitive store of platelets would coincide with the dense tubular system (DTS, the equivalent to the ER in platelets) and would be mobilizable through inositol-trisphosphate (IP_3) receptors [13].Thrombin releases Ca^{2+} from both stores while ADP or vasopressin release Ca^{2+} only from DTS [14]. In platelets, it has been proposed that many of the differences in behavior are due to the differential expression of SERCA isoforms in the different stores, a TBH-sensitive SERCA3 in the acidic stores and a TBH-insensitive SERCA2b in DTS [13, 15].

The presence of a TBH-insensitive SERCA in smooth muscle, skeletal muscle and heart, which express mainly SERCA1 and SERCA2 isoforms [16], was also proposed in the platelets studies [10]. However, later studies have generally reported that SERCA1, 2 and 3 are all similarly sensitive to TBH, with IC_{50} in the micromolar range [17-20]. The origin of these contradictory observations is unclear. There may be differences in behavior between different cell types or between isoforms within the same SERCA family. For SERCA3, for example, six different isoforms, a to f, have been described [19].

Inhomogeneities of the intracellular Ca^{2+} stores have also been reported in other cell types. In some cases the differences may be due to the contribution of other organelles such as the Golgi network [2, 21], the lysosomes [3-5] or the secretory granules[6-8] to Ca^{2+} homeostasis. Inhomogeneities in the ER Ca^{2+} store itself have also been proposed on the basis of different Ca^{2+} contents or sensitivity to TG [22], but the mechanisms involved have not been investigated in detail.

In the present paper we analyze the homogeneity of intracellular Ca^{2+} stores in HEK293T and HeLa cells and their response to mobilizing agonists. Using ER-targeted aequorins to study the different Ca^{2+} stores, we have been able to identify a TBH-resistant Ca^{2+} pool in the ER of HEK cells, which can be mobilized by IP_3-producing agonists. On the contrary, the whole Ca^{2+} pool stored in the ER of HeLa cells is homogeneous and can be emptied by TBH.

EXPERIMENTAL PROCEDURES

Cell culture and gene transfection

HEK293T (ATCC CRL-11268) and HeLa (CCL-2) cells were maintained in DMEM (Invitrogen) supplemented with 10% (v/v) foetal bovine serum, 2 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin at 37°C, under an air/ 5% CO_2 mixture. A stable HEK293 clone expressing erGFP-aequorin (erGA) was generated by transfecting the erGA cDNA and selecting the neomycin

resistant clones in 800 μg/ml G-418 (Invitrogen) by limited dilution. The clone was routinely cultured in 100 μg/ml G-418.For aequorin measurements, cells were seeded on 12 mm diameter poly-L-lysine-coated coverslips at $7x10^4$ cells/coverslip. HeLa cells were transfected with 0.1μg 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 cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_C$)

The procedure was as described previously [23, 24]. Briefly, cells, attached to 12 mm diameter coverslips, were loaded with 4 μM fura2-acetoxymethyl ester (fura-2/AM, Molecular Probes) for 1 h at room temperature in standard incubation medium of the following composition (in mM): NaCl, 145; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 1; glucose, 10; sodium-HEPES, 10, pH, 7.4. The cell-coated coverslips were then mounted under a 20x 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 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 software and calibrated in $[Ca^{2+}]_C$ by comparison with fura-2 standards [23].

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

The GFP-aequorin (GA) fusion protein targeted to the ER (erGA) containing the mutated, low Ca^{2+} affinity, aequorin was used [25]. Cells expressing erGA were incubated for 1 h at room temperature with 1 μM of 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 [26]. When aequorin is reconstituted with coelenterazine n the affinity for Ca^{2+} is still smaller and measurements up to the milimolar range are possible [26-28]. The reconstitution medium contained also 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 Ca^{2+}-free medium and perfused as described in each case. Aequorin photoluminescence was measured as described previously in a luminometer constructed by Cairn Research Ltd [29], and calibrations in $[Ca^{2+}]$ were done using the constant values published before [30]. All the measurements were performed at 22°C.

SERCA3 activity determination

Determination of SERCA activity was perfortmed using an enzyme-coupled assay in samples purified by SERCA3 immunoprecipitation [31, 32]. Briefly, the cell suspension (6×10^6 cells/ml) was mixed 1:1 with 2xRIPA containing no phosphatase or kinase inhibitors, immunoprecipitated using anti-SERCA3 (PL/IM430) antibody and purified using the immunoprecipitation kit Dynabead® protein G (Invitrogen, Madrid, Spain). ATPase activity was determined at 37 ^oC 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 added.

Real-time quantitative (RT-PCR)

Relative expression levels of h-SERCA3d in HEK293T and HeLa cells were assessed by probe-based real-time quantitative RT-PCR (qRT-PCR). Total RNA was extracted with Trizol^R (Invitrogen) and quantified in a NanoDrop apparatus. 1-2 μg RNA were reverse transcribed with the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems using MultieScribe-RT and Oligo dT as primers. Amplifications of 1-100 ng cDNA were performed in triplicates using the kit 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'- GAC CAC ACC GGG GCC AGG GAC ACA-3' and reverse 5'- GCC TGT CAT TTA TCC GGC G -3' for h-SERCA3d and forward 5'-TAC CTC CGC TGC ATC TCC -3' and reverse 5'- GCC TGT CAT TTA TCC GGC -3' for RPL18 (housekeeping gene).The PCR was run on an LightCycler 480 (Roche, Germany). Relative gene expression was determined with the $2^{-\Delta\Delta CT}$ method [33].

Statistics

Data are expressed as mean±S.E.M. Statistical significance was evaluated by Student's t-test.

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. 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 pool was inferred from the size of the $[Ca^{2+}]_C$ increase. Both, cholinergic and purinergic agonists are known to stimulate PLC in both, HEK [34] and HeLa cells [35]. We found in preliminary experiments (not shown) that maximum releasing effect was best obtained when carbachol (CCh) and ATP were applied together, so that this stimulus was routinely used in further experiments. Figure 1 shows mobilization of the stored Ca^{2+}, visualized as the increase of $[Ca^{2+}]_C$, by stimulation with 100 µM CCh + 100 µM ATP in HEK (A-D) and HeLa (E-H) 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 (A, E). The second peak was obtained after 12 min incubation in Ca^{2+} free medium (B, F). This $[Ca^{2+}]_C$ increment was about 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 and HeLa cells. In another series of experiments, reuptake of Ca^{2+} into the ER during the incubation in Ca^{2+}-free medium was prevented by adding the SERCA inhibitor TBH [36]. $[Ca^{2+}]_C$ increased transiently during the incubation with TBH in Ca^{2+}-free medium, suggesting that there is a slow Ca^{2+} release from the stores under these circumstances (C, G). After 12 min incubation with TBH in Ca^{2+} free medium there was a nearly complete emptying of the IP_3-sensitive Ca^{2+} store in HeLa cells, where the $[Ca^{2+}]_C$ peak induced by CCh+ATP was under 2% of the control (Figure 1G). In contrast, the $[Ca^{2+}]_C$ peak obtained in HEK cells by stimulation with CCh+ATP after 12 min incubation with TBH in Ca^{2+}-free medium was quite substantial (64% of the control value; Figure 1C). Treatment with 1 µM thapsigargin, another SERCA inhibitor [37], completely prevented responses to CCh+ATP, both in HeLa and in HEK cells (Figure 1D and 1H) indicating that the intracellular Ca^{2+} stores sensitive to IP_3 are completely emptied by treatment with this inhibitor. The same results were obtained with another SERCA inhibitor [18, 38] cyclopiazonic acid (CPA; traces not shown). The averaged results of several similar experiments are shown in panels I and J of Figure 1. To summarize, it seems clear from our results that there is a substantial part of the IP_3 sensitive Ca^{2+} pool of HEK 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 shows that the TBH-resistant Ca^{2+} pool of HEK 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+ATP were applied. The first stimulus produced a large $[Ca^{2+}]_C$ peak, the effect of the second one was much smaller and the third response was hardly detectable, indicating that the Ca^{2+} store was completely empty. On readdition of Ca^{2+} (in the continuous presence of TBH) $[Ca^{2+}]_C$ increased very much and then declined slowly. The first step reflects rapid Ca^{2+} entry through capacitative mechanisms (store-operated Ca^{2+} entry, SOCE), which are activated by the emptying of the intracellular Ca^{2+} stores [39]. As the intracellular stores refill, store-operated channels (SOC) deactivate and $[Ca^{2+}]_C$ decreases. After 9 min., external Ca^{2+} was removed again and the cells were stimulated with CCh+ATP. The stimulus produced a Ca^{2+} peak that

was 80% as high as the initial one, indicating that the TBH-resistant intracellular Ca^{2+} stores had refilled substantially. The second stimulus had little effect, suggesting that the Ca^{2+} content of the intracellular stores was almost completely mobilized by the first stimulus.

Accumulation of Ca^{2+} into acidic [15] or alkaline [40] intracellular compartments has been described in different cell systems. Collapsing the pH gradient empties these Ca^{2+} stores and abolishes the release in both cases. Figure 3 shows the results of experiments designed to assess a role of acidic or alkaline intracellular compartments in the accumulation of Ca^{2+} inside TBH-resistant compartments. HEK cells were treated for 5 min with TBH in Ca^{2+} free medium and then the effects of several treatments collapsing pH gradients were studied by following the $[Ca^{2+}]_C$ peaks induced by CCh+ATP. The effects of substances able to change the cytoplasmic pH should be regarded with some caution as the pH changes modify also the affinity of the Ca^{2+} probes and could then disturb the measurements. Here we tested the effect of a weak base, trimethylamine (TMA) (Figure 3B), which would accumulate into acidic compartments and thus collapse the pH gradient; a weak acid, propionate (Figure 3C), which should accumulate inside alkaline compartments; and the H^+/K^+ exchanger nigericin, which should collapse both acidic and alkaline pH gradients (Figure 3D). Even though all the three maneuvers produced some effects on the apparent increase of $[Ca^{2+}]_C$ (Figure 3A-D), these were relatively small and similar for all the three inhibitors, suggesting that they are no specifically attributable to collapsing the pH gradient of a subcellular compartment. Finally, the lysosomal disruptor GPN did not inhibit the $[Ca^{2+}]_C$ peak (Figure 3E); as a matter of fact, the height of the peak was usually increased by GPN, the contrary outcome to the expected one if acidic granules contributed to the TBH-resistant agonist-sensitive Ca^{2+} pool of HEK cells. These results suggest that the TBH-resistant store of HEK cells is not (or not only) inside either acidic or alkaline granules.

We investigated directly the implication of the ER as a TBH-resistant store by measuring its Ca^{2+} content with an ER-targetedd aequorin [26, 41]. The results of these experiments are shown in Figure 4; erGA-transfected HEK (A) and HeLa cells (B) whose Ca^{2+} stores had been emptied were allowed to refill with Ca^{2+}, either in control medium with 1 mM Ca^{2+} (CONT.) or in medium containing 1 mM Ca^{2+} and 10 μM TBH (+TBH). After 5-min refilling, the cells were stimulated with CCh+ATP. In the control condition the ER refilled to levels approaching 500 μM in both cell types and stimulation with CCh+ATP produced Ca^{2+} release. Emptying was more complete in HeLa than in HEK cells. In the presence of TBH there was hardly any refilling in HeLa cells whereas in HEK cells ER refilled to about 20% of the control value and CCh+ATP produced a near-complete Ca^{2+} release.

As AEQ is burnt during light emission on Ca^{2+} binding [28, 42] the fractional consumption at equilibrium measures the fraction of the AEQ-containing space that is occupied by Ca^{2+}. Figure 5 compares the time courses of the consumption of the ER-targeted aequorin in HEK and HeLa cells during refilling of the intracellular Ca^{2+} stores in the presence of TBH (Ca^{2+}+TBH bar). After 5 min, TBH was removed to allow rapid refilling of the TRH-sensitive stores (Ca^{2+} bar). In HeLa cells burning of AEQ was extremely slow in the presence of TBH and seemed to be circumscribed to a small fraction (about 10%) of the total AEQ pool. In HEK cells, the AEQ pool that burned in the presence of AEQ was much larger, over 60% during the 5-min observation period. In order to measure the size of the ER-AEQ pools more precisely, a new series of experiments was designed reconstituting erAEQ with native coelenterazine. Under these conditions the affinity of AEQ for Ca^{2+} increases and consumption is much faster and more sensitive to smaller Ca^{2+} concentrations. Results are shown in Figure 6, where the time courses of the consumptions either with Ca^{2+} alone or in the presence of TBH are compared. In HEK cells (Figure 6A) the consumption was almost complete (>80%) in both cases, 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 6B). Refilling in the presence of TBH (dotted trace) was very slow but, in addition, it seemed to happen into a limited fraction of the total Ca^{2+} pool amounting less than 20% 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 very beginning (continuous trace) erAEQ was consumed quickly and completely.

It has been reported that the expression of SERCA2b by HeLa and HEK cells is similar, but HEK cells express higher SERCA3 levels than HeLa cells [43] and that the major isoform is SERCA3d [43]. We have determined and compared the relative abundance of the SERCA3d isoform in HEK and HeLa cells by quantitative RT-PCR and confirm the differences between both cell types.

We found that the relative expression of SERCA 3d is (mean±S.E.M.; n=3) 19±5 fold higher in HEK cells than in HeLa cells.

In order to investigate whether the differences found in ER Ca^{2+} transport between HEK and HeLa cells could be related to differences in the expression of SERCA isoforms, we determined the Ca^{2+}-dependent ATPase activity linked to SERCA3 in HEK and HeLa cells, and compared the sensitivity to TBH. Figure 7 shows the averaged values of ATPase activity obtained with and without Ca^{2+} and in absence and presence of TBH. The Ca^{2+}-dependent activity was larger in HEK than in HeLa cells, nominally (mean±S.E.M) 0.217±0.023 vs 0.093±0.003 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ (compare panels A and B) ($p<0.001$). Even more striking was the fact that whereas TBH inhibited completely (87-99%) the Ca^{2+} stimulated ATPase activity in HeLa cells (Figure 7B), there was a substantial fraction (32-46%) of TBH-resistant activity in HEK cells (Figure 7A).

Finally, we studied the effects of expressing SERCA3d on the TBH-resistant Ca^{2+} storage in HeLa cells. The results are shown in Figure 8. The Ca^{2+} content of the intracellular stores was estimated either from the size of the $[Ca^{2+}]_C$ peak induced by stimulation with agonists in fura-2-loaded HeLa cells (A and B) or by directly measuring the Ca^{2+}uptake into the ER with targeted aequorin (C and D). As shown above (Figure 1 and Figure 4), HeLa cells did not show any indication of TBH-resistant storage of Ca^{2+} into the ER: there was not $[Ca^{2+}]_C$ increase on stimulation with ATP+CCh (Figure 8A) and the ER refilled very little with Ca^{2+} (below 0.1 mM, Figure 8C). In contrast, upon SERCA3d overexpression the release of Ca^{2+} by stimulation with CCh+ATP was sharply increased (Figure 8B) and direct measurement of ER content showed increased refilling (to about 6 fold more than in the control (Figure 8D). Moreover, this stored Ca^{2+} was completely released by stimulation with CCh+ATP.

DISCUSSION

Our results reveal heterogeneity of Ca^{2+} stores in HEK cells, where a TBH-resistant store 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 thapsigargin and cyclopiazonic acid were able to empty completely the TBH-resistant Ca^{2+} store (Figure 1D; Figure 1I). In contrast with HEK cells, HeLa cells did not show evidence for a TBH-resistant Ca store (Figure 1G; Fig1J). 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 cells refilled by incubation with external Ca^{2+}, even in the presence of TBH (Figure 2).

What may be the structural basis that justifies the differences in intracellular Ca^{2+} storage in the various cell models? In platelets it seems clear that the TBH-sensitive and the TBH-resistant Ca^{2+} stores correspond to two different Ca^{2+} pools, located either inside the acidic dense granules and lysosomes or in the dense tubular system, respectively [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 or the vacuolar H^+-ATPase inhibitor bafilomycin, and with the osmotic lysosomal disruptor GPN. In the case of HEK cells these maneuvers did not abolish the TBH-resistant Ca^{2+} release from the stores (Figure 3) suggesting that, in these cells ,Ca^{2+} comes from another store. The effects of substances collapsing acidic and alkaline gradients, such as trimethylamine and propionate (Figure 3 B and C), were relatively small and similar, suggesting that ΔH^+ of the store is not essential for TBH-resistant Ca^{2+} accumulation.

In addition, we have direct evidence showing that the TBH-resistant Ca^{2+} pool accumulates inside the ER, as revealed by the ER targeted-AEQ (Figure 4B). The Ca^{2+} concentration attained by the TBH-resistant accumulation mechanism in this pool seemed smaller (about 1/5) than $[Ca^{2+}]_{ER}$ normally reached in the bulk of the store (Figure 4A). 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 cells (Figure4A and B). Consistently with the results obtained with fura-2 (Figure 1) ER-targeted AEQ 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 (Figure 4B).

Since AEQ is burned out in the presence of Ca^{2+}, the relative AEQ consumption at equilibrium informs us on 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 AEQ consumption, is very different in HEK and in HeLa cells (Figure 5). The test can be made more sensitive by increasing the affinity of AEQ for Ca^{2+}. We repeated the measurements using native coelenterazine instead of coelenterazine n to increases the affinity of AEQ for Ca^{2+} [26-28]. This makes consumption much faster and more sensitive to smaller Ca^{2+} concentrations and allows a more precise and astringent estimation of the TBH-resistant pool. In HEK cells the TBH-resistant AEQ pool was >80% of the total pool (Figure 6A) suggesting that there is a communication between the TBH-resistant and the TBH-sensitive pools or that the mechanism responsible for Ca^{2+} uptake distributes along the whole ER. On the contrary, in HeLa cells the TBH-resistant pool, probably overestimated by using an AEQ-coelenterazine system with higher-affinity for Ca^{2+}, amounted only 10-20% (Figure 6B).

In platelets, the TBH-sensitive uptake of Ca^{2+} into the acidic granules seems to be related with a distinct SERCA isoform [9], which has been later identified as SERCA3 [10], present also in several other tissues [19, 44]. On the other hand, SERCA2b would be resistant to TBH in platelets [9, 10]. However SERCA2b (as well as and SERCA1) activity and ER Ca^{2+} uptake have been consistently reported to be sensitive to TBH in other tissues, including skeletal muscle, heart, smooth muscle, and lymphocytes [17-20, 45, 46]. The dominant SERCA isoform in HEK and HeLa cells is the 2b [43], which according to our results is sensitive to TBH (Figure 4). It has been reported that HEK, but not HeLa cells, express, in addition a SERCA3d isoform [43] and we confirm it here by quantitative RT-PCR. In addition, HEK but not HeLa cells showed TBH-resistant SERCA3-mediated ATPase activity (Figure 7). Finally, overexpresion of SERCA3d in HeLa cells generated a TBH-resistant intracellular Ca^{2+} pool in the ER (Figure 8D), 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 cells and opens the question on whether differential expression of SERCA isoforms [44] could modify the properties of intracellular calcium stores and to allow coexistence of different pools, perhaps fulfilling different functions, in the same cell.

The presence of two distinct ER-derived Ca^{2+} compartments in HEK cells might have functional relevance as described in other cells, where function-specific Ca^{2+} compartments have been reported to regulate different cellular functions by 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, 47]. Similarly, in goldfish somatotropes, two different Ca^{2+} stores may differentially regulate growth hormone storage and secretion [48]. Therefore, our findings further advance our understanding of organellar Ca^{2+} stores underlying the generation of differential Ca^{2+} signals by different Ca^{2+}-mobilizing agonists.

AUTHOR CONTRIBUTION

Francisco J. Aulestia, Pedro C. Redondo and Arancha Rodríguez-García , performed most of the experiments. 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, and gave final approval.. Javier García-Sancho put together all data and wrote the final form of the manuscript.

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FIGURE LEGENDS

Figure 1. Comparison of calcium mobilization from the intracellular calcium stores in HEK293T (A-D) and HeLa cells (E-H). Measurements were performed with fura-2. Ca^{2+} was removed as indicated by the bars (EGTA) and 10 μM TBH or 1 μM thapsigargin was added as shown. The CCh+ATP bars indicate stimulation with Ca^{2+}-free solution containing 100 μM of each agonist. Each trace is the average of 3 similar experiments. Values are expressed as the ratio of the fluorescences excited at 340 and 380 nm. Note calibration in $[Ca^{2+}]_C$ at right. Panels **I** (HEK cells) and **J** (HeLa cells) show mean ± 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 thapsigargin in Ca^{2+}-free medium before the stimulus); CPA (12 min. treatment with 15 μM cyclopiazonic acid in Ca^{2+}-free medium before the stimulus). TBH + S3d, TBH condition in Hela cells overexpressing SERCA3d (See *Experimental Procedures* and Figure 8).

Figure 2. Emptying and refilling of the TBH-resistant intracellular calcium store of HEK cells. Experiment representative of three similar ones. Other details as in Figure 1.

Figure 3. Effects of several treatments for disrupting pH gradients in endomembranes on the TBH-resistant intracellular calcium store of HEK cells. TMA, 6 mM trimethylamine; PROP., 6 mM sodium propionate; NIGER., 10 μM nigericin ; GPN, 10 μM GPN. Each trace is the average of 3-6 experiments. Other details as in Figure 1.

Figure 4. Comparison of the Ca^{2+} refilling and the agonist-induced emptying of ER in HEK (A) and HeLa (B) cells. Cells transfected with erGA and reconstituted with 1 μM coelenterazine n (see Experimental Procedures) were washed with Ca-free medium and, at the time shown, perfused with standard medium containing 1 mM Ca^{2+} with (CONT., dotted line) or without (+TBH, continous line) 10 μM TBH. At the time shown the cells were challenged with carbachol + ATP (100 μM of each one). Values are plotted as L/L_{TOTAL}. Calibration in $[Ca^{2+}]$ is shown at right. Every trace is the mean of

3 individual experiments. The values attained at the steady state were (in $L/L_{TOTAL} \cdot 10^3$; Control vs TBH; mean±S.E.M.): 9.0±0.4 vs 1.5±0.5 in HEK cells and 7.7±0.9 vs 0.2±0.1 in HeLa cells. The values for the decrease induced by stimulation with CCh+ATP were (in $\Delta L/L_{TOTAL} \cdot 10^3$; Control vs TBH; mean±S.E.M.): 4.3±0.1 vs 1.3±0.2 in HEK cells and 7.7±0.9 vs non-measurable in HeLa cells.

Figure 5. Comparison of the aequorin consumption in HEK (dotted trace) and in HeLa cells (continous trace) on filling the TRH-resistant calcium stores. Cells treated as in Figure 4 were washed with Ca-free medium and then incubated first with standard medium containing 1 mM Ca^{2+} and 10 μM TBH (Ca^{2+}+TBH) and later with the same medium without TBH (Ca^{2+}). Results are expressed as percent of the total aequorin remaining in the cells at a given time. Each trace is the average of three experiments.

Figure 6.. **Comparison of the aequorin consumption in HEK (A) and in HeLa cells (B) on filling the intracellular calcium stores in the presence or in the absence of TBH** Cells treated as in Figure 4 except that aequorin was reconstituted with native coelenterazine instead of coelenterazine n. This increases the affinity for Ca^{2+} about one order of magnitude [26, 27, 30]. After 2-min washing with Ca^{2+}-free medium, 1 mM Ca^{2+} was added, as shown. Results expressed as percent of the 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.

Figure 7. SERCA3 ATPase activity in HEK (A) and in HeLa cells (B). Cell extracts were purified by immunoprecipitation and ATPase activity determined by an enzyme-coupled assay (see *Experimental Procedures*). The values in the absence (EGTA) and in the presence of 1 μM Ca^{2+} (+Ca^{2+}), and in the absence (open bars) and in the presence of TBH (black bars) are shown. Each value is the mean±S.E.M. of 4 individual data. The values obtained with 20 μM TBH (not shown for clarity) were the same as with 10 μM TBH.

Figure 8.. **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 as in Figure 1. The sizes of the Ca^{2+} peaks (in ΔRatio 340/380) were (mean±s.e.m): control, 0.000±0.030 (n=3); +S3b, 0.042±0.020 (n=9). **C.** and **D.** Aequorin measurements. Details as in Figure 4. Each trace is the average of 4 experiments. The $[Ca^{2+}]_{ER}$ levels reached as the steady state were (in $L/L_{TOTAL} \cdot 10^3$; mean±S.E.M.): Control 0.4±0.2; S3d, 2.4±0.2.

FIG. 1

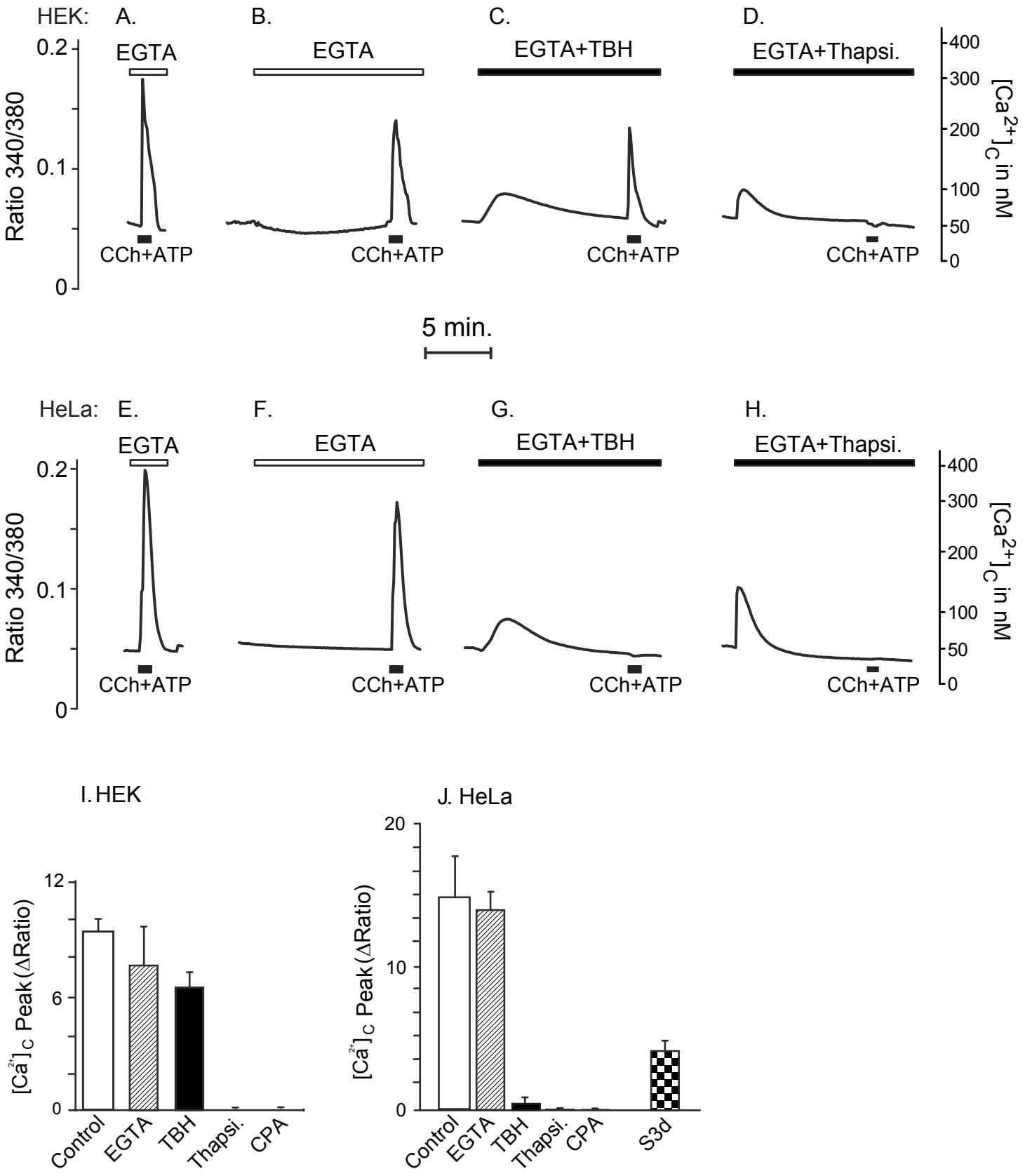

HEK:
A.
B.
C.
D.
EGTA
EGTA
EGTA+TBH
EGTA+Thapsi.
Ratio 340/380
0.2
0.1
0
CCh+ATP
[Ca2+]C in nM
400
300
200
100
50
0
5 min.
HeLa:
E.
F.
G.
H.
I. HEK
J. HeLa
[Ca2+]C Peak (ΔRatio)
12
6
20
10
Control
EGTA
TBH
Thapsi.
CPA
S3d

FIG. 2

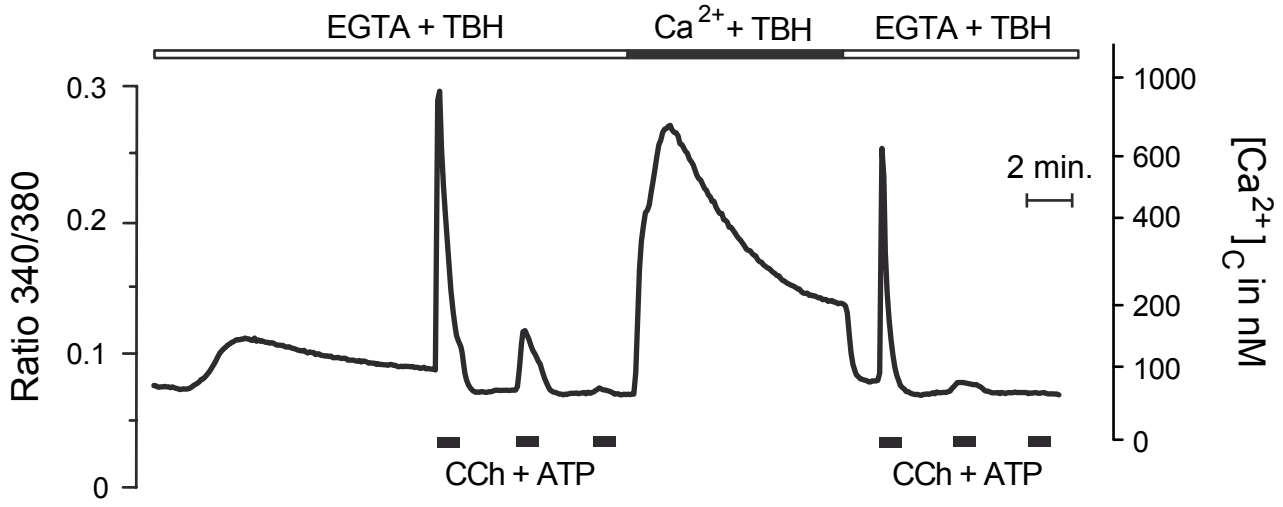

EGTA + TBH
Ca 2+ + TBH
EGTA + TBH
Ratio 340/380
0.3
0.2
0.1
0
2 min.
1000
600
400
200
100
0
[Ca2+]C in nM
CCh + ATP
CCh + ATP

FIG. 3

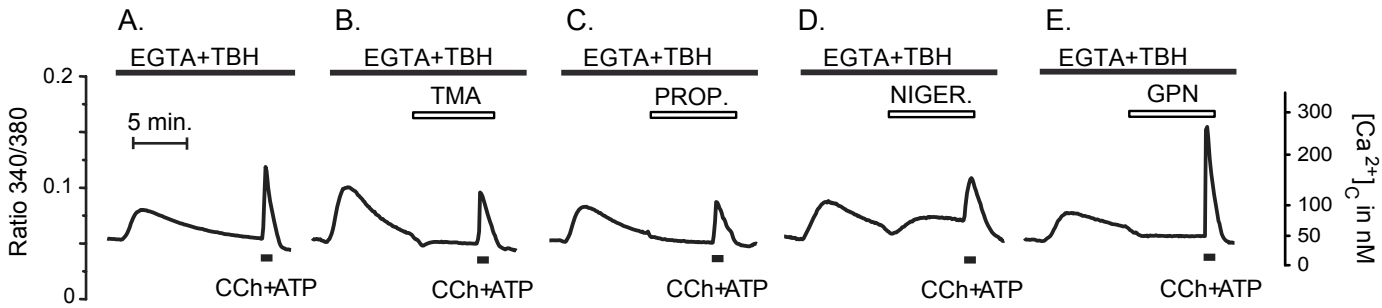

A.
B.
C.
D.
E.
EGTA+TBH
EGTA+TBH
EGTA+TBH
EGTA+TBH
EGTA+TBH
TMA
PROP.
NIGER.
GPN
5 min.
Ratio 340/380
0.2
0.1
0
CCh+ATP
CCh+ATP
CCh+ATP
CCh+ATP
CCh+ATP
300
200
100
50
0
[Ca2+]C in nM

FIG. 4

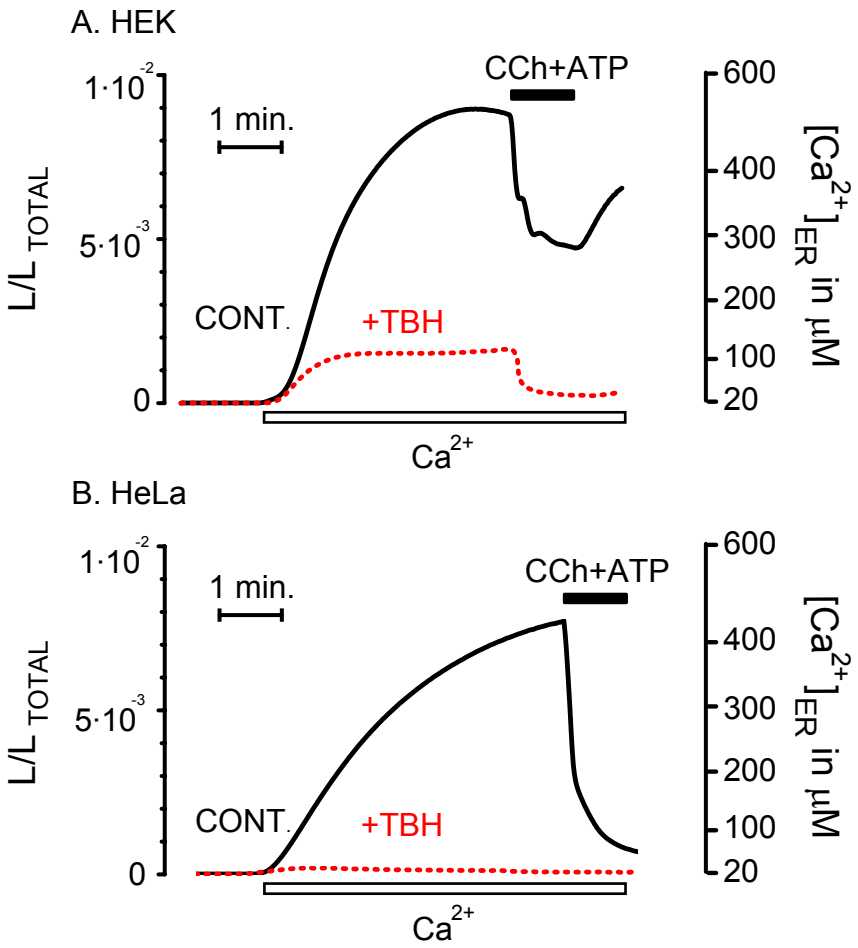

A. HEK
CCh+ATP
1 min.
1·10-2
5·10-3
0
L/L TOTAL
CONT.
+TBH
Ca2+
600
400
300
200
100
20
[Ca2+]ER in µM
B. HeLa
CCh+ATP
1 min.
1·10-2
5·10-3
0
L/L TOTAL
CONT.
+TBH
Ca2+
600
400
300
200
100
20
[Ca2+]ER in µM

Fig. 5

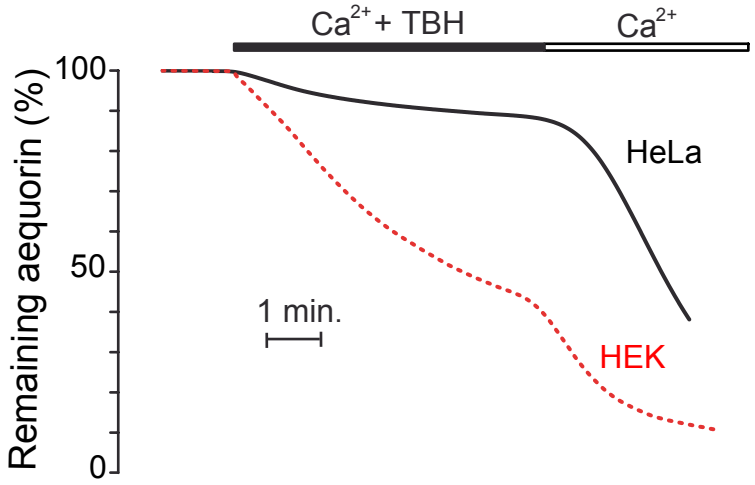


Fig.6

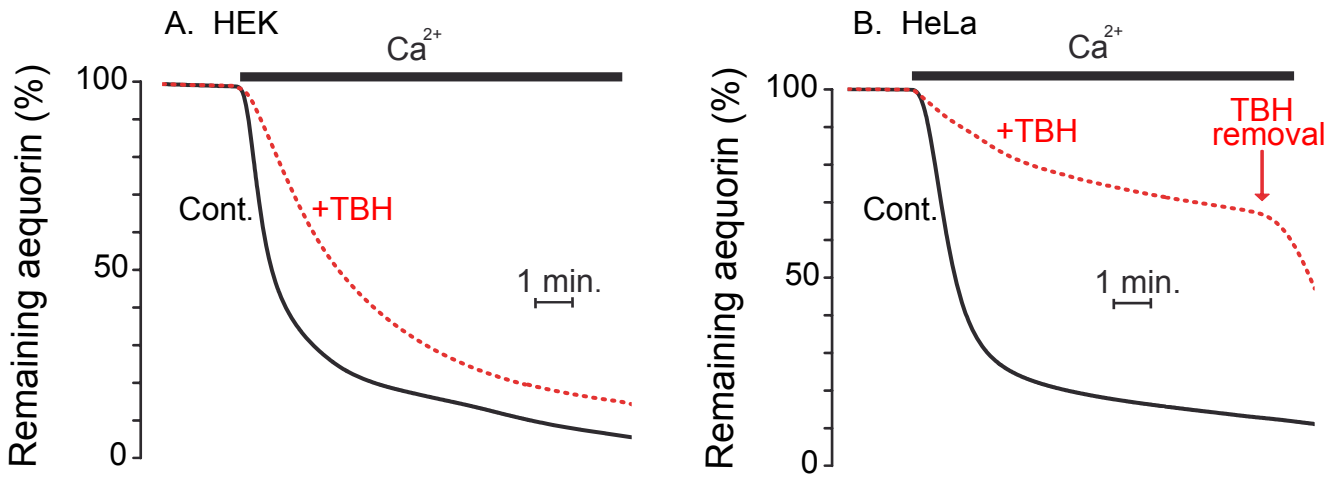

A. HEK
Ca2+
100
50
0
Remaining aequorin (%)
Cont.
+TBH
1 min.
B. HeLa
Ca2+
100
50
0
Remaining aequorin (%)
+TBH
TBH
removal
Cont.
1 min.

Fig. 7

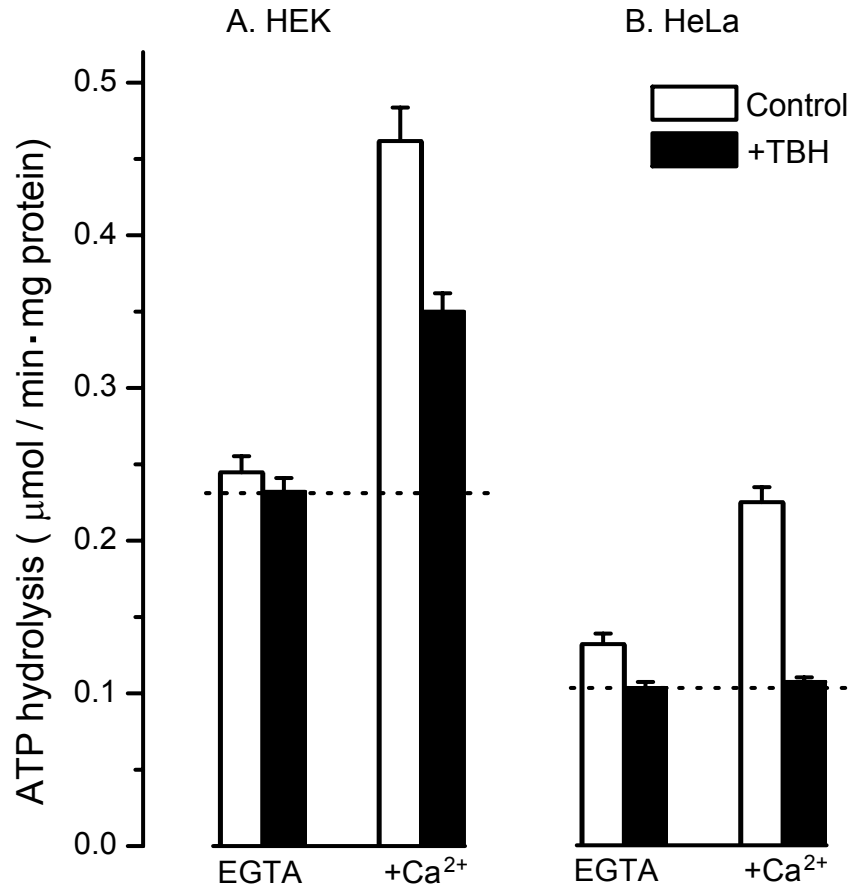
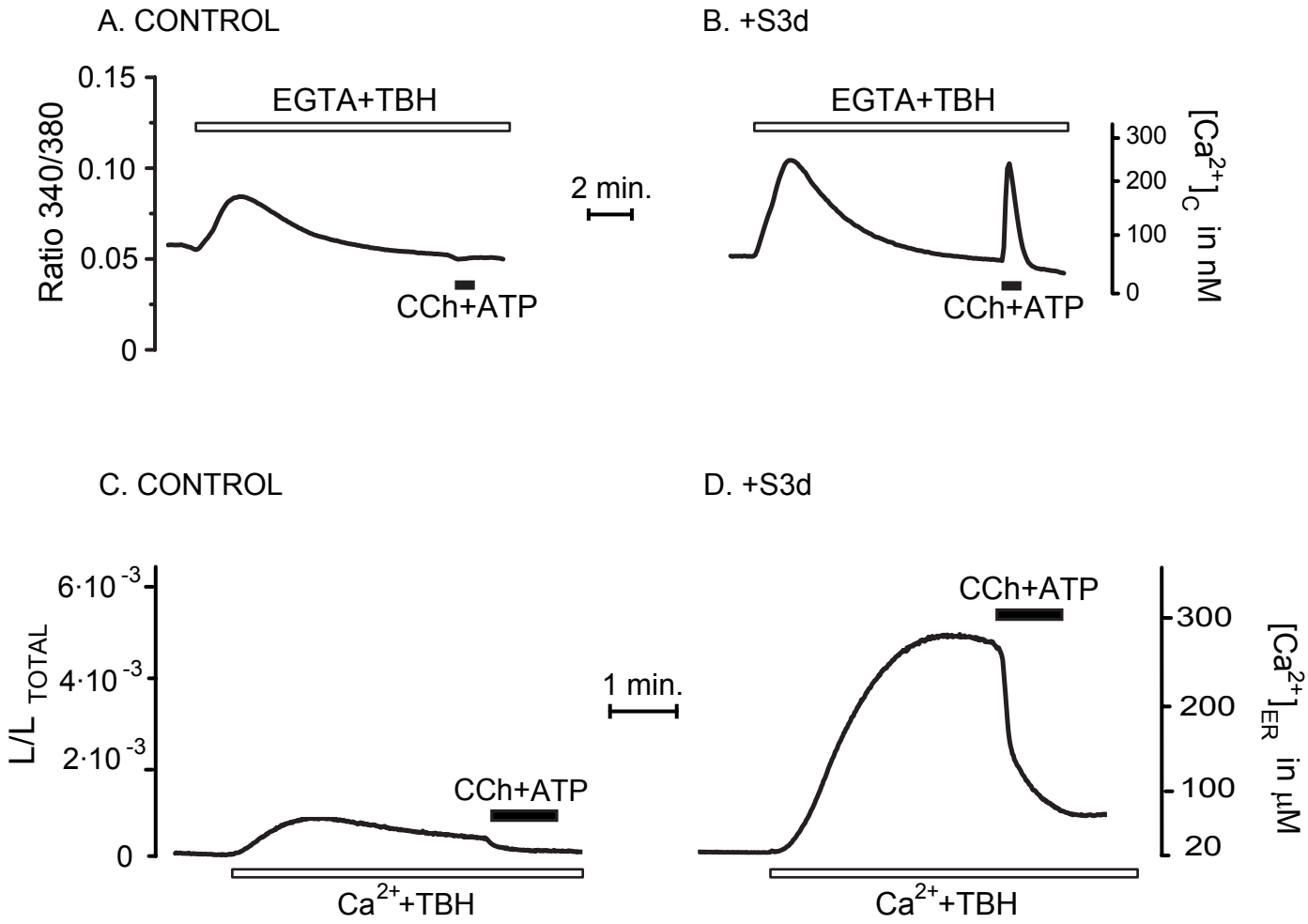

A. HEK
B. HeLa
Control
+TBH
ATP hydrolysis (μmol / min • mg protein)
0.5
0.4
0.3
0.2
0.1
0.0
EGTA
+Ca2+
EGTA
+Ca2+

FIG. 8


A. CONTROL
B. +S3d
EGTA+TBH
EGTA+TBH
Ratio 340/380
0.15
0.10
0.05
0
2 min.
CCh+ATP
CCh+ATP
300
200
100
0
[Ca2+]C in nM
C. CONTROL
D. +S3d
6·10-3
4·10-3
2·10-3
0
L/L TOTAL
1 min.
CCh+ATP
CCh+ATP
300
200
100
20
[Ca2+]ER in µM
Ca2+ +TBH
Ca2+ +TBH