Two distinct calcium pools in the endoplasmic reticulum of HEK293T cells

Francisco J Aulestia, Pedro C Redondo, Arancha Rodriguez-Garcia, Juan A. Rosado, Gines M Salido, Maria Teresa Alonso, Javier Garcia-Sancho

Two distinct calcium pools in the endoplasmic reticulum of HEK293T cells

Francisco J. AULESTIA[*], Pedro C. REDONDO[†], Arancha RODRÍGUEZ-GARCÍA[*], Juan A. ROSADO[†], Ginés M. SALIDO[†], Maria Teresa ALONSO[*] and Javier GARCÍA-SANCHO[*1]

[*]*Instituto de Biología y Genética Molecular (IBGM), Universidad de Valladolid y Consejo Superior de Investigaciones Científicas (CSIC), c/ Sanz y Forés s/n, 47003 Valladolid, Spain and* [†]*Cell Physiology Research Group, Department of Physiology, Universidad de Extremadura, Av. de la Universidad s/n, 10071 Cáceres, Spain*

Short title: Two distinct ER calcium pools in HEK cells

[1]To whom correspondence should be addressed at: Dr. J. García-Sancho, IBGM, c/Sanz y Forés, 3, 47003-Valladolid, Spain. Phone: 34-983-423084; Fax: 34-983-421800; e-mail: jgsancho@ibgm.uva.es

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 millimolar concentrations and the IP_3-generating agonists carbachol and ATP mobilized 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. Our results indicate that SERCA3d is important for filling the TBH-resistant store as: i)SERCA3d is more abundant in HEK than in HeLa cells; ii)SERCA 3 ATPase activity of HEK cells is not fully blocked by TBH; and iii)expression of SERCA3d in Hela cells generated a TBH-resistant, agonist-mobilizable compartment in the ER. Therefore distribution of SERCA isoforms may originate 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.

KEYWORDS:

Endoplasmic reticulum, Calcium, intracellular calcium stores, aequorin, calcium microdomains, SERCA, Sarco/endoplasmic Ca^{2+} ATPase

ABBREVIATION FOOTNOTE:

Abbreviations used are: GFP, green fluorescent protein; GA, chimeric GFP-aequorin fusion protein; ER, endoplasmic reticulum; SERCA, sarco/endoplasmic 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; GPN, glycylphenylalanine-2-naphthylamide; erGA, ER-targeted GA; IP_3, inositol 1,4,5, trisphosphate; CCh, carbachol.

INTRODUCTION

Changes of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_C$) 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 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 sarco/endoplasmic 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, were found to be SERCA2b and SERCA3 [10]. Another contemporary 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 thapsigargin (TG) and 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 glycylphenylalanine-2-naphthylamide (GPN), by the vacuolar H^+-ATPase inhibitor bafilomycin or by the H^+/K^+ ionophore nigericin. The calcium content of this TBH-sensitive store would be half of the TBH-insensitive one [12]. Interestingly, NAADP 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 inositol-trisphosphate (IP_3) 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 SERCA isoforms in the two stores, a TBH-sensitive SERCA3 in the acidic store and a TBH-insensitive SERCA2b in dense tubular system [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. SERCA3 is the most recent member and differs structurally and functionally from the other isoforms [19]. SERCA3 is insensitive to phospholamban and has a five fold lower affinity for cytosolic Ca^{2+} [16, 19]. The $K_{0.5}$ value for SERCA2b is about 0.2 µM compared to 1.1 µM for SERCA3 [21]. This means that activity of SERCA3 is very low at the resting Ca^{2+} levels of 50-100 nM but it increases very much when $[Ca^{2+}]_C$ reaches high levels 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]. Expression of SERCA3 may change during different conditions. It behaves as a differentiation marker during Xenopus laevis development [23] or during differentiation of vascular endothelial cells and colon mucosa [19]. On the other hand, SERCA3 increases during ER stress in 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+} homeostasis 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]. Emptying of the different stores could eventually evoke a differential Ca^{2+} influx by activation of the store-operated Ca^{2+} entry (SOCE), 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, HEK293T 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 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 [28, 29]. 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 [28].

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

Measurements inside ER were performed using the ER-targeted low $[Ca^{2+}]$ affinity probe erGFP-mutated-aequorin (erGA) which has been previously described [30]. 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 [31]. When aequorin is reconstituted with coelenterazine n the affinity for Ca^{2+} is about 6 fold smaller than when it is reconstituted with the native coelenterazine and measurements up to the millimolar range are possible [31-33]. 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. All the measurements were performed at 22°C. Aequorin photoluminescence was measured as described previously in a luminometer constructed by Cairn Research Ltd [34], and calibrations in $[Ca^{2+}]$ were done using the formula and the constant values published before [35, 36]:

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

where $R = (L/\lambda \cdot L_{TOTAL}))^{(1/n)}$, $K_R = 8.47 \cdot 10^7$; K_{TR}, $157 \cdot 10^3$; n=1.20; λ=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 x 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 ^{o}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 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 [39].

Statistics

Data are expressed as mean±S.E.M. Statistical significance was evaluated by Student's t-test or one-way ANOVA using CraphPad 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. 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 [40] and HeLa cells [41]. 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. Since HEK 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, but this should be a very minor effect, most probably completely masked by fura-2 (see Figure 5 in [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 + 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 [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 (C, G). After 12 min incubation with TBH in 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+ATP was less than 2% of the control (Figure 1G). By contrast, the $[Ca^{2+}]_C$ peak obtained in HEK cells by stimulation with CCh+ATP in the same conditions was quite substantial (64% of the control value; Figure 1C). Treatment with 1 µM thapsigargin, another SERCA inhibitor [44], 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. Comparable results were obtained with another SERCA inhibitor, namely cyclopiazonic acid (CPA; traces not shown) [18, 45]. 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 2A 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 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. On re-addition 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 SOCE, which is activated by the emptying of the intracellular Ca^{2+} stores [46]. 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 another series of three pulses of CCh+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 into 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. Figure 2B-F shows 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 2G summarizes the average values obtained in three similar experiments. HEK cells were first treated for 5 min with TBH in 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+ATP. The effects of substances able to change the cytoplasmic pH should be regarded with some caution as these 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 2C), which accumulates into 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 maneuvers was able to abolish the peak induced by CCh+ATP (compare Figure 2C-E to control, Figure 2B), although the $[Ca^{2+}]_C$ peaks were somewhat reduced in all the 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 (Figure 2F; Figure 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 cells is not (or not only) inside either acidic or alkaline granules.

We next investigated directly the implication of the ER in TBH-resistant storage by measuring its Ca^{2+} content with an ER-targeted aequorin [31, 48]. In these experiments (Figure 3) 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., continuous lines) or in medium containing 1 mM Ca^{2+} and 10 µM TBH (+TBH; dotted lines). After 5-min refilling, the cells were stimulated with CCh+ATP. In the control condition the ER refilled to $[Ca^{2+}]_{ER}$ 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, where it amounted about 50% of the total calcium pool. 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. A summary of the averaged estimated sizes of the different calcium pools is presented in Figure 3C, where the whole bars stand for the levels attained at the end of the refilling period and the black boxes for the fraction of the pool released by the agonists. Note again the striking difference in the behavior of HEK 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 and HeLa cells during refilling of the calcium stores in the presence of TBH (Ca^{2+}+TBH bar). In the 6-min period shown in the figure, aequorin consumption was less than 2% in HeLa cells and near 40% in HEK cells. In addition, the rate of consumption tended to decrease with time in the first case but remained unchanged in the second, 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 cells (see Supplemental Figure 1). After 6 min, TBH was removed to allow rapid refilling of the TBH-sensitive stores (Ca^{2+} bar). Under these conditions, Ca^{2+} consumption was similarly quick in both cases and seemed to affect to 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 er-GA 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. 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 cells (Figure 5A) the consumption was almost complete (>80%) by the end of the incubation period 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 5B). Consumption in the presence of TBH (dotted trace) was very slow (10 fold slower than the control) but, in addition, it seemed to occur into 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 very beginning (continuous trace) ER-aequorin was consumed quickly and completely. The results of the experiments illustrated in panels A and B were quantified as percentage of ER-aequorin consumption at the plateau and $t_{1/2}$ for consumption (see Figure 5 legend and supplemental Figure 2 for details). The average values of 3 similar experiments are plotted in Figures 5C and 5D. It is clear that the % ER-aequorin consumption at equilibrium was, in the presence TBH, much smaller in HeLa than in HEK cells (Figure 5C; 25±3 *vs.* 66±4; $p<0.001$). In addition, in the presence of TBH consumption was slower both in HEK and in HeLa cells as shown by the increase of the $t_{1/2}$ values (Fig. 5D).

In order to explore further the mechanism responsible for the TBH-resistant Ca^{2+} storage in HEK cells, we looked for 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 cells, but, interestingly, HEK cells express higher SERCA3 levels than HeLa cells [50] and the major isoform is SERCA3d [50]. We have determined and compared the relative abundance of the SERCA3d mRNA in HEK and HeLa cells by quantitative RT-PCR and confirm the differences between both cell types. In our hands the relative expression of SERCA 3d mRNA is (mean±S.E.M.; n=3) 19±5 fold higher in HEK cells than in HeLa cells.

We next investigated 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. For this purpose, we determined the Ca^{2+}-dependent ATPase activity linked to SERCA3 in HEK and HeLa cells, and compared the sensitivity to TBH. Figure 6 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 reasoned that if SERCA3 was responsible for the TBH-insensitive activity in HEK 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 (+S3d in Figure) 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 (A and B) or by directly measuring with ER-aequorin the Ca^{2+} accumulation into the ER (C and D). As shown above (Figure 1 and Figure 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+CCh (Figure 7A) and the ER refilled very little with Ca^{2+} (Figure 7C). In contrast, upon SERCA3d overexpression (+S3d) the release of Ca^{2+} by stimulation with CCh+ATP was sharply increased (Figure 7B) and direct measurement of ER content showed increased refilling (to about 6 fold more than in the control (Figure 7D). In addition, this stored Ca^{2+} was almost completely released by stimulation with CCh+ATP (Figure 7D). Figures 7E and 7F shows the average values obtained in three similar experiments for the agonist-induced $[Ca^{2+}]_C$ peaks (7E) and for ER refilling (7F).

DISCUSSION

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

What may 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 to 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]. In 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 by the osmotic lysosomal disruptor GPN. In contrast with these observations, our results in HEK cells indicate that maneuvers that collapse the pH gradient of intracellular organelles did not abolish the TBH-resistant Ca^{2+} release (Figure 2B-G) 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 (about 1/5) 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 cells (Figure 3A and C). 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 (Figure 3B and C).

Since aequorin is burned out in the presence of Ca^{2+}, the relative aequorin 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 aequorin consumption, is very different in HEK and in HeLa cells (Figure 4; Figure 5). In HEK 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 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 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, 52, 53]. The dominant SERCA isoform in HEK and HeLa cells is the 2b [50], which according to our results is sensitive to TBH (Figures 3 and 4). It has been reported that HEK, but not HeLa cells, also express a SERCA3d isoform [50] and we confirm it here at the mRNA expression level by quantitative RT-PCR. In addition, HEK 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 (Figure 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 cells and raises the interesting question of whether differential expression of SERCA isoforms [51] could modify the properties of intracellular calcium stores and to allow 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 cells might have functional relevance as proposed in other cells, where function-specific Ca^{2+} compartments have been reported to regulate different cellular mechanisms though 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 HEK293 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, and gave final approval. Javier García-Sancho put together all data and wrote the final form of the manuscript.

ACKNOWLEDGEMENTS

We are grateful to Dr. Jocelyne Enouf, Inserm, U 689, Paris, for the human SERCA 3d cDNA. We thank Carmen Galán for assistance in some experiments and Jesús Fernández and Miriam García Cubillas for technical help.

FUNDING

This work was supported by grants from The Spanish Ministerio de Ciencia e Innovación [MICINN-FEDER BFU2007-60157, BFU2007-60104, BFU2010-17379, BFU2010-21043-C02-01 and RD06/0010/0000] and the Junta de Castilla y León [GR175]. Francisco J Aulestia was supported by a fellowship from the Spanish MICINN.

REFERENCES

1 Clapham, D. E. (2007) Calcium signaling. Cell. **131**, 1047-1058

2 Lissandron, V., Podini, P., Pizzo, P. and Pozzan, T. (2010) Unique characteristics of Ca2+ homeostasis of the trans-Golgi compartment. Proc Natl Acad Sci U S A. **107**, 9198-9203

3 Galione, A. (2006) NAADP, a new intracellular messenger that mobilizes Ca2+ from acidic stores. Biochem Soc Trans. **34**, 922-926

4 Galione, A., Evans, A. M., Ma, J., Parrington, J., Arredouani, A., Cheng, X. and Zhu, M. X. (2009) The acid test: the discovery of two-pore channels (TPCs) as NAADP-gated endolysosomal Ca(2+) release channels. Pflugers Arch. **458**, 869-876

5 Patel, S., Marchant, J. S. and Brailoiu, E. (2010) Two-pore channels: Regulation by NAADP and customized roles in triggering calcium signals. Cell calcium. **47**, 480-490

6 Gerasimenko, J. V., Sherwood, M., Tepikin, A. V., Petersen, O. H. and Gerasimenko, O. V. (2006) NAADP, cADPR and IP3 all release Ca2+ from the endoplasmic reticulum and an acidic store in the secretory granule area. J Cell Sci. **119**, 226-238

7 Petersen, O. H. and Tepikin, A. V. (2008) Polarized calcium signaling in exocrine gland cells. Annu Rev Physiol. **70**, 273-299

8 Yoo, S. H. (2009) Secretory granules in inositol 1,4,5-trisphosphate-dependent Ca2+ signaling in the cytoplasm of neuroendocrine cells. FASEB J. **24**, 653-664

9 Papp, B., Enyedi, A., Paszty, K., Kovacs, T., Sarkadi, B., Gardos, G., Magnier, C., Wuytack, F. and Enouf, J. (1992) Simultaneous presence of two distinct endoplasmic-reticulum-type calcium-pump isoforms in human cells. Characterization by radio-immunoblotting and inhibition by 2,5-di-(t-butyl)-1,4-benzohydroquinone. Biochem J. **288 (Pt 1)**, 297-302

10 Wuytack, F., Papp, B., Verboomen, H., Raeymaekers, L., Dode, L., Bobe, R., Enouf, J., Bokkala, S., Authi, K. S. and Casteels, R. (1994) A sarco/endoplasmic reticulum Ca(2+)-ATPase 3-type Ca2+ pump is expressed in platelets, in lymphoid cells, and in mast cells. J Biol Chem. **269**, 1410-1416

11 Cavallini, L., Coassin, M. and Alexandre, A. (1995) Two classes of agonist-sensitive Ca2+ stores in platelets, as identified by their differential sensitivity to 2,5-di-(tert-butyl)-1,4-benzohydroquinone and thapsigargin. Biochem J. **310 (Pt 2)**, 449-452

12 Juska, A., Jardin, I. and Rosado, J. A. (2008) Physical properties of two types of calcium stores and SERCAs in human platelets. Mol Cell Biochem. **311**, 9-18

13 Lopez, J. J., Redondo, P. C., Salido, G. M., Pariente, J. A. and Rosado, J. A. (2006) Two distinct Ca2+ compartments show differential sensitivity to thrombin, ADP and vasopressin in human platelets. Cell Signal. **18**, 373-381

14 Jardin, I., Ben Amor, N., Bartegi, A., Pariente, J. A., Salido, G. M. and Rosado, J. A. (2007) Differential involvement of thrombin receptors in Ca2+ release from two different intracellular stores in human platelets. Biochem J. **401**, 167-174

15 Lopez, J. J., Camello-Almaraz, C., Pariente, J. A., Salido, G. M. and Rosado, J. A. (2005) Ca2+ accumulation into acidic organelles mediated by Ca2+- and vacuolar H+-ATPases in human platelets. Biochem J. **390**, 243-252

16 Periasamy, M. and Kalyanasundaram, A. (2007) SERCA pump isoforms: their role in calcium transport and disease. Muscle Nerve. **35**, 430-442

17 Lape, M., Elam, C., Versluis, M., Kempton, R. and Paula, S. (2008) Molecular determinants of sarco/endoplasmic reticulum calcium ATPase inhibition by hydroquinone-based compounds. Proteins. **70**, 639-649

18 Mason, M. J., Garcia-Rodriguez, C. and Grinstein, S. (1991) Coupling between intracellular Ca2+ stores and the Ca2+ permeability of the plasma membrane. Comparison of the effects of thapsigargin, 2,5-di-(tert-butyl)-1,4-hydroquinone, and cyclopiazonic acid in rat thymic lymphocytes. J Biol Chem. **266**, 20856-20862

19 Vangheluwe, P., Sepulveda, M. R., Missiaen, L., Raeymaekers, L., Wuytack, F. and Vanoevelen, J. (2009) Intracellular Ca2+- and Mn2+-transport ATPases. Chem Rev. **109**, 4733-4759

20 Wootton, L. L. and Michelangeli, F. (2006) The effects of the phenylalanine 256 to valine mutation on the sensitivity of sarcoplasmic/endoplasmic reticulum Ca2+ ATPase (SERCA) Ca2+ pump isoforms 1, 2, and 3 to thapsigargin and other inhibitors. J Biol Chem. **281**, 6970-6976

21 Chandrasekera, P. C., Kargacin, M. E., Deans, J. P. and Lytton, J. (2009) Determination of apparent calcium affinity for endogenously expressed human sarco(endo)plasmic reticulum calcium-ATPase isoform SERCA3. Am J Physiol Cell Physiol. **296**, C1105-1114

22 Dally, S., Monceau, V., Corvazier, E., Bredoux, R., Raies, A., Bobe, R., del Monte, F. and Enouf, J. (2009) Compartmentalized expression of three novel sarco/endoplasmic reticulum Ca2+ATPase 3 isoforms including the switch to ER stress, SERCA3f, in non-failing and failing human heart. Cell calcium. **45**, 144-154

23 Pegoraro, C., Pollet, N. and Monsoro-Burq, A. H. (2010) Tissue-specific expression of Sarcoplasmic/Endoplasmic Reticulum Calcium ATPases (ATP2A/SERCA) 1, 2, 3 during Xenopus laevis development. Gene Expr Patterns. **in press**

24 Chaabane, C., Dally, S., Corvazier, E., Bredoux, R., Bobe, R., Ftouhi, B., Raies, A. and Enouf, J. (2007) Platelet PMCA- and SERCA-type Ca2+ -ATPase expression in diabetes: a novel signature of abnormal megakaryocytopoiesis. J Thromb Haemost. **5**, 2127-2135

25 Brouland, J. P., Gelebart, P., Kovacs, T., Enouf, J., Grossmann, J. and Papp, B. (2005) The loss of sarco/endoplasmic reticulum calcium transport ATPase 3 expression is an early event during the multistep process of colon carcinogenesis. The American journal of pathology. **167**, 233-242

26 Pinton, P., Pozzan, T. and Rizzuto, R. (1998) The Golgi apparatus is an inositol 1,4,5-trisphosphate-sensitive Ca2+ store, with functional properties distinct from those of the endoplasmic reticulum. EMBO J. **17**, 5298-5308

27 Ong, H. L., Liu, X., Tsaneva-Atanasova, K., Singh, B. B., Bandyopadhyay, B. C., Swaim, W. D., Russell, J. T., Hegde, R. S., Sherman, A. and Ambudkar, I. S. (2007) Relocalization of STIM1 for activation of store-operated Ca(2+) entry is determined by the depletion of subplasma membrane endoplasmic reticulum Ca(2+) store. J Biol Chem. **282**, 12176-12185

28 Nunez, L., De La Fuente, M. T., Garcia, A. G. and Garcia-Sancho, J. (1995) Differential Ca2+ responses of adrenergic and noradrenergic chromaffin cells to various secretagogues. Am J Physiol. **269**, C1540-1546

29 Villalobos, C., Nunez, L., Montero, M., Garcia, A. G., Alonso, M. T., Chamero, P., Alvarez, J. and Garcia-Sancho, J. (2002) Redistribution of Ca2+ among cytosol and organella during stimulation of bovine chromaffin cells. FASEB J. **16**, 343-353

30 Chamero, P., Manjarres, I. M., Garcia-Verdugo, J. M., Villalobos, C., Alonso, M. T. and Garcia-Sancho, J. (2008) Nuclear calcium signaling by inositol trisphosphate in GH3 pituitary cells. Cell calcium. **43**, 205-214

31 Alonso, M. T., Barrero, M. J., Michelena, P., Carnicero, E., Cuchillo, I., Garcia, A. G., Garcia-Sancho, J., Montero, M. and Alvarez, J. (1999) Ca2+-induced Ca2+ release in chromaffin cells seen from inside the ER with targeted aequorin. J Cell Biol. **144**, 241-254

32 Barrero, M. J., Montero, M. and Alvarez, J. (1997) Dynamics of [Ca2+] in the endoplasmic reticulum and cytoplasm of intact HeLa cells. A comparative study. J Biol Chem. **272**, 27694-27699

33 Shimomura, O., Musicki, B., Kishi, Y. and Inouye, S. (1993) Light-emitting properties of recombinant semi-synthetic aequorins and recombinant fluorescein-conjugated aequorin for measuring cellular calcium. Cell calcium. **14**, 373-378

34 Manjarres, I. M., Chamero, P., Domingo, B., Molina, F., Llopis, J., Alonso, M. T. and Garcia-Sancho, J. (2008) Red and green aequorins for simultaneous monitoring of Ca2+ signals from two different organelles. Pflugers Arch. **455**, 961-970

35 Montero, M., Alonso, M. T., Carnicero, E., Cuchillo-Ibanez, I., Albillos, A., Garcia, A. G., Garcia-Sancho, J. and Alvarez, J. (2000) Chromaffin-cell stimulation triggers fast millimolar mitochondrial Ca2+ transients that modulate secretion. Nat Cell Biol. **2**, 57-61

36 Villalobos, C., Alonso, M. T. and Garcia-Sancho, J. (2009) Bioluminescence imaging of calcium oscillations inside intracellular organelles. Methods in molecular biology (Clifton, N.J. **574**, 203-214

37 Chandrasekera, C. P. and Lytton, J. (2003) Inhibition of human SERCA3 by PL/IM430. Molecular analysis of the interaction. J Biol Chem. **278**, 12482-12488

38 Munch, G., Bolck, B., Karczewski, P. and Schwinger, R. H. (2002) Evidence for calcineurin-mediated regulation of SERCA 2a activity in human myocardium. J Mol Cell Cardiol. **34**, 321-334

39 Livak, K. J. and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. **25**, 402-408

40 Short, A. D., Winston, G. P. and Taylor, C. W. (2000) Different receptors use inositol trisphosphate to mobilize Ca(2+) from different intracellular pools. Biochem J. **351 Pt 3**, 683-686

41 Smit, M. J., Leurs, R., Bloemers, S. M., Tertoolen, L. G., Bast, A., De Laat, S. W. and Timmerman, H. (1993) Extracellular ATP elevates cytoplasmatic free Ca2+ in HeLa cells by the interaction with a 5'-nucleotide receptor. Eur J Pharmacol. **247**, 223-226

42 Alonso, M. T., Chamero, P., Villalobos, C. and Garcia-Sancho, J. (2003) Fura-2 antagonises calcium-induced calcium release. Cell calcium. **33**, 27-35

43 Moore, G. A., Kass, G. E., Duddy, S. K., Farrell, G. C., Llopis, J. and Orrenius, S. (1990) 2,5-Di(tert-butyl)-1,4-benzohydroquinone--a novel mobilizer of the inositol 1,4,5-trisphosphate-sensitive Ca2+ pool. Free Radic Res Commun. **8**, 337-345

44 Thastrup, O., Dawson, A. P., Scharff, O., Foder, B., Cullen, P. J., Drobak, B. K., Bjerrum, P. J., Christensen, S. B. and Hanley, M. R. (1989) Thapsigargin, a novel molecular probe for studying intracellular calcium release and storage. Agents Actions. **27**, 17-23

45 Seidler, N. W., Jona, I., Vegh, M. and Martonosi, A. (1989) Cyclopiazonic acid is a specific inhibitor of the Ca2+-ATPase of sarcoplasmic reticulum. J Biol Chem. **264**, 17816-17823

46 Parekh, A. B. and Putney, J. W., Jr. (2005) Store-operated calcium channels. Physiological reviews. **85**, 757-810

47 Fonteriz, R. I., Sanchez, A., Mollinedo, F., Collado-Escobar, D. and Garcia-Sancho, J. (1991) The role of intracellular acidification in calcium mobilization in human neutrophils. Biochim Biophys Acta. **1093**, 1-6

48 Montero, M., Brini, M., Marsault, R., Alvarez, J., Sitia, R., Pozzan, T. and Rizzuto, R. (1995) Monitoring dynamic changes in free Ca2+ concentration in the endoplasmic reticulum of intact cells. EMBO J. **14**, 5467-5475

49 Shimomura, O., Johnson, F. H. and Morise, H. (1974) Mechanism of the luminescent intramolecular reaction of aequorin. Biochemistry. **13**, 3278-3286

50 Martin, V., Bredoux, R., Corvazier, E., Van Gorp, R., Kovacs, T., Gelebart, P. and Enouf, J. (2002) Three novel sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) 3 isoforms. Expression, regulation, and function of the membranes of the SERCA3 family. J Biol Chem. **277**, 24442-24452

51 Hadri, L., Pavoine, C., Lipskaia, L., Yacoubi, S. and Lompre, A. M. (2006) Transcription of the sarcoplasmic/endoplasmic reticulum Ca2+-ATPase type 3 gene, ATP2A3, is regulated by the calcineurin/NFAT pathway in endothelial cells. Biochem J. **394**, 27-33

52 Kabbara, A. A. and Stephenson, D. G. (1997) Effects of 2,5-di-tert-butylhydroquinone on rat cardiac muscle contractility. Am J Physiol. **272**, H1001-1010

53 Luo, D., Nakazawa, M., Yoshida, Y., Cai, J. and Imai, S. (2000) Effects of three different Ca(2+) pump ATPase inhibitors on evoked contractions in rabbit aorta and activities of Ca(2+) pump ATPases in porcine aorta. Gen Pharmacol. **34**, 211-220

54 Ben Amor, N. B., Zbidi, H., Bouaziz, A., Isaac, J., Hernandez-Cruz, J. M., Salido, G. M., Rosado, J. A. and Bartegi, A. (2009) Acidic-store depletion is required for human platelet aggregation. Blood Coagul Fibrinolysis. **20**, 511-516

55 Johnson, J. D., Klausen, C., Habibi, H. R. and Chang, J. P. (2002) Function-specific calcium stores selectively regulate growth hormone secretion, storage, and mRNA level. Am J Physiol Endocrinol Metab. **282**, E810-819

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 7).

Figure 2. (A) 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. **(B-G) 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. The values shown in G are means±S.E.M. of three determinations.

Figure 3. 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. **(C)** Bars represent means±SEM (n=3) of the Ca^{2+} levels attained at the steady state (in $L/L_{TOTAL} \cdot 10^3$) in the different conditions. The black boxes correspond to the fraction of the pool released by stimulation with CCh+ATP.

Figure 4. Time course of the aequorin consumption in HEK and in HeLa cells on filling the calcium stores. Cells treated as in Figure 3 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+}). Finally, the cells were lysed with digitonin in the presence of 10 mM 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 and the vertical bars correspond to S.E.M. values.

Figure 5. 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 3 except that aequorin was reconstituted with native coelenterazine instead of coelenterazine n. This increases the affinity for Ca^{2+} about one order of magnitude [31, 32, 35]. 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. **(C)** Estimations of the % 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 referred to % consumption calculated in (C). Bars in (C) and (D) correspond to the means±S.E.M. of three independent experiments.

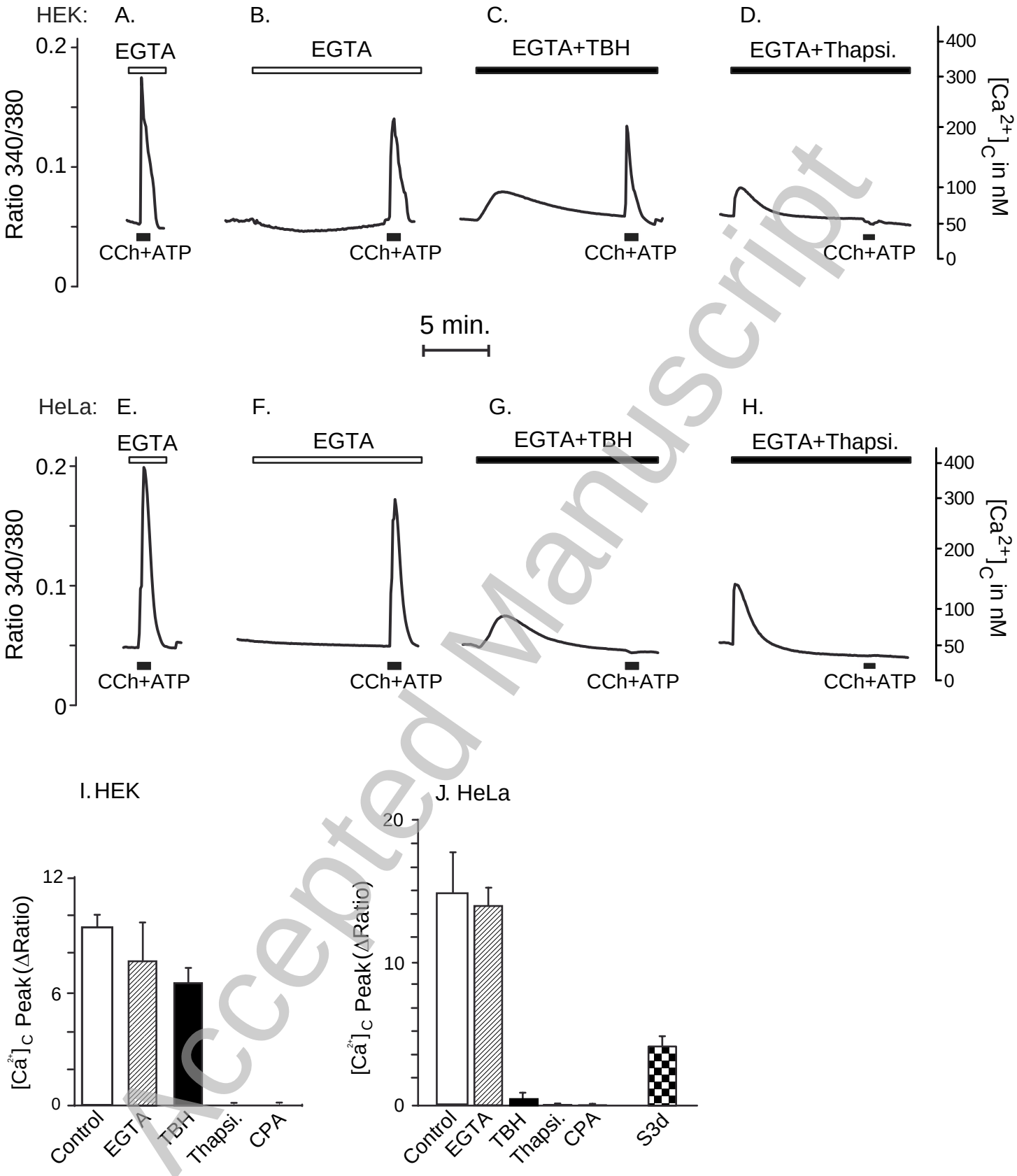
Figure 6. SERCA3 ATPase activity in HEK (A) and in HeLa cells (B). Cell extracts were purified by immunoprecipitation and ATPase activity determined by an enzyme-coupled assay (see *Experimental Procedures*). The values in the absence (EGTA) and in the presence of 1 µM Ca^{2+} ($+Ca^{2+}$), and in the 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 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 as in Figure 1. **C.** and **D.** Aequorin measurements. Details as in Figure 3. Results are representative of 3-4 similar experiments. Average values (±S.E.M) are shown in **E** (size of the Ca^{2+} peak in and B) and **F** ($[Ca^{2+}]_{ER}$ levels reached as the steady state were (in $L/L_{TOTAL} \cdot 10^3$). 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),

See supplemental figures in next page

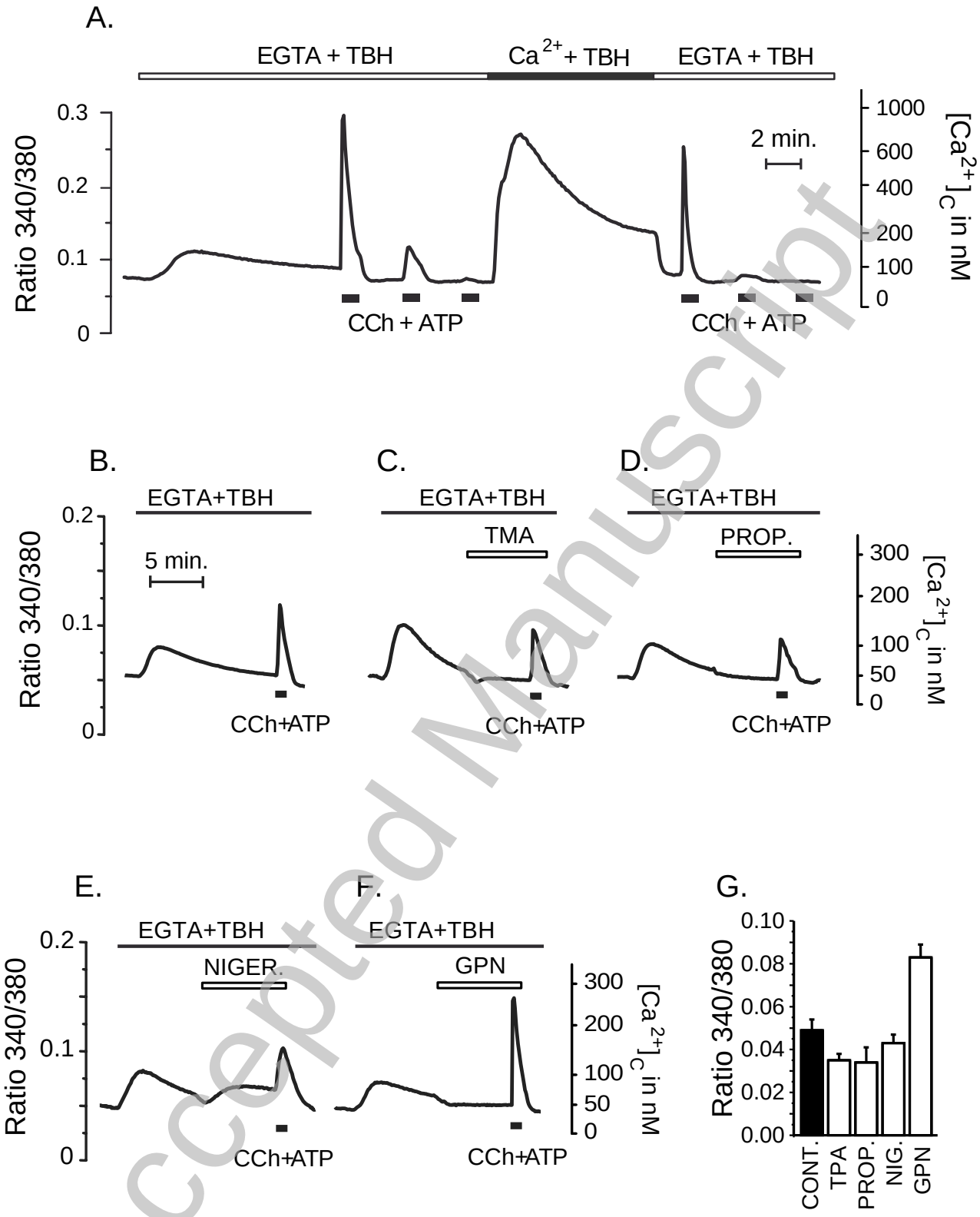
Biochemical Journal Immediate Publication. Published on 10 Jan 2011 as manuscript BJ20101427

FIG. 1



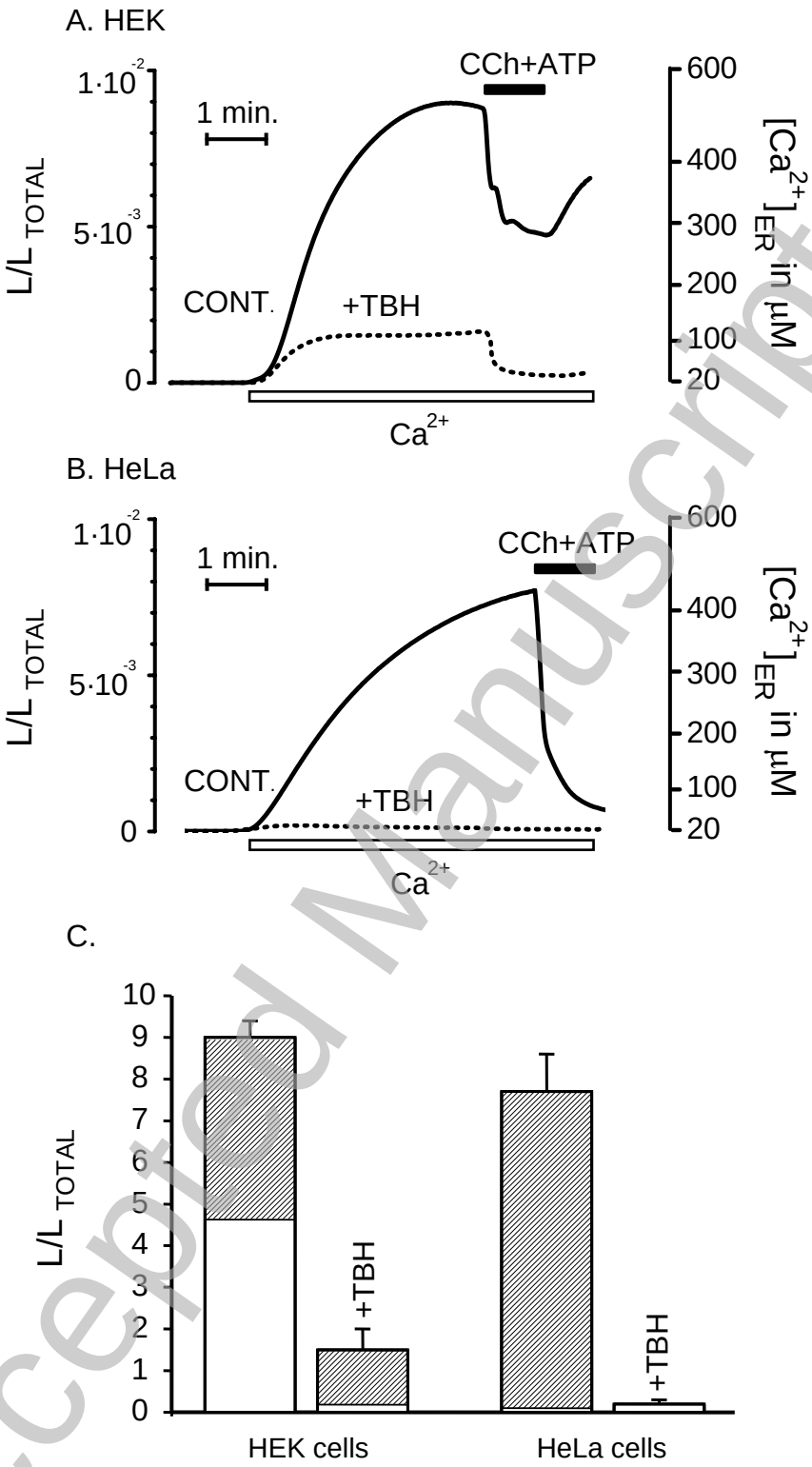
Biochemical Journal Immediate Publication. Published on 10 Jan 2011 as manuscript BJ20101427

FIG. 2



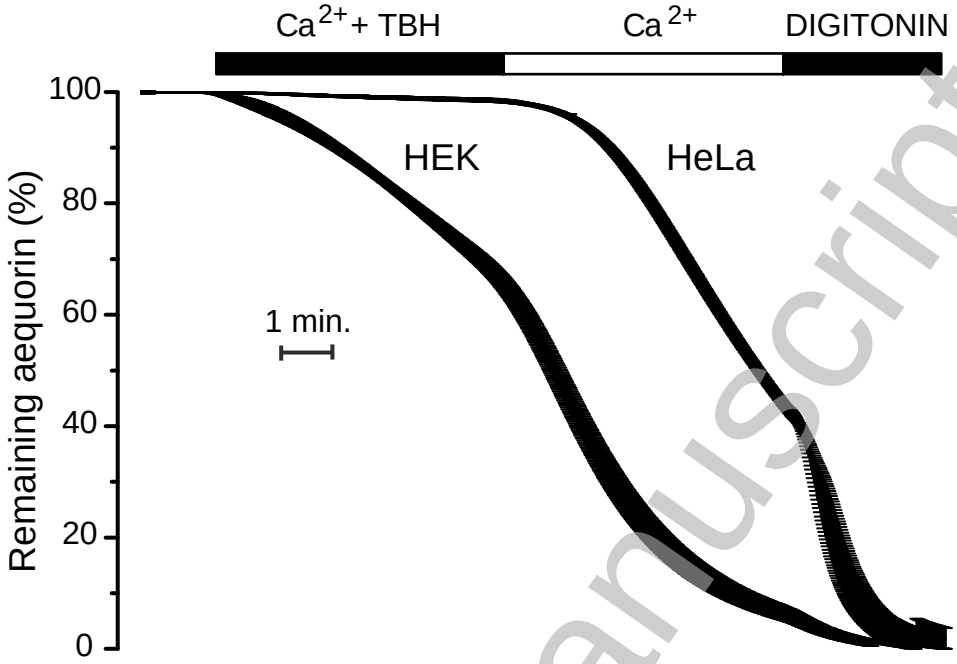
Biochemical Journal Immediate Publication. Published on 10 Jan 2011 as manuscript BJ20101427

FIG. 3



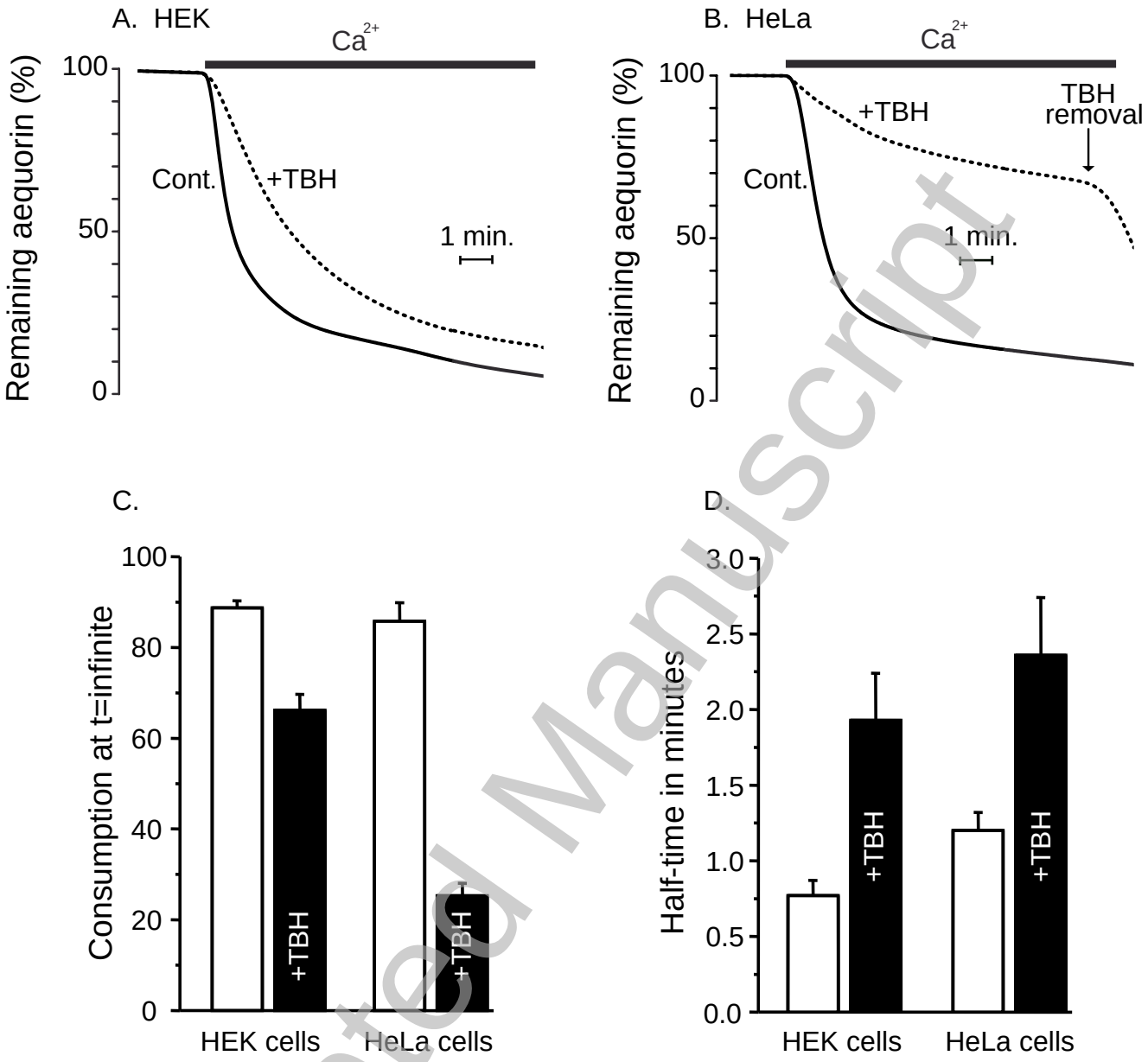
Biochemical Journal Immediate Publication. Published on 10 Jan 2011 as manuscript BJ20101427

Fig. 4



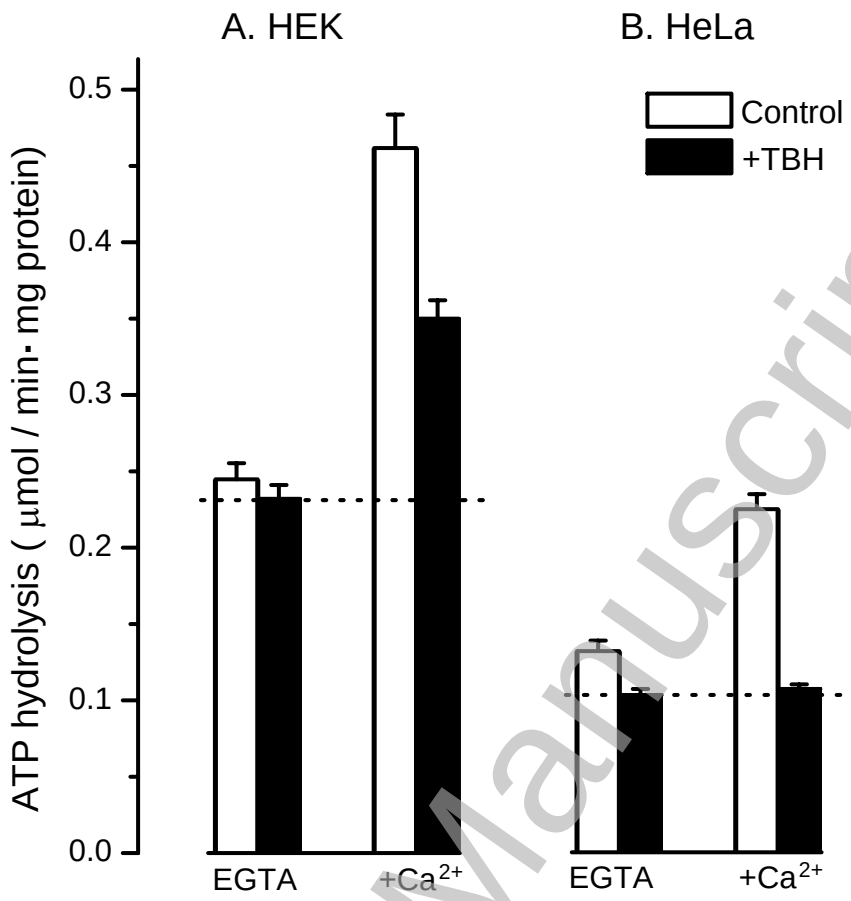
Biochemical Journal Immediate Publication. Published on 10 Jan 2011 as manuscript BJ20101427

Fig.5



Biochemical Journal Immediate Publication. Published on 10 Jan 2011 as manuscript BJ20101427

Fig. 6



Biochemical Journal Immediate Publication. Published on 10 Jan 2011 as manuscript BJ20101427

FIG. 7