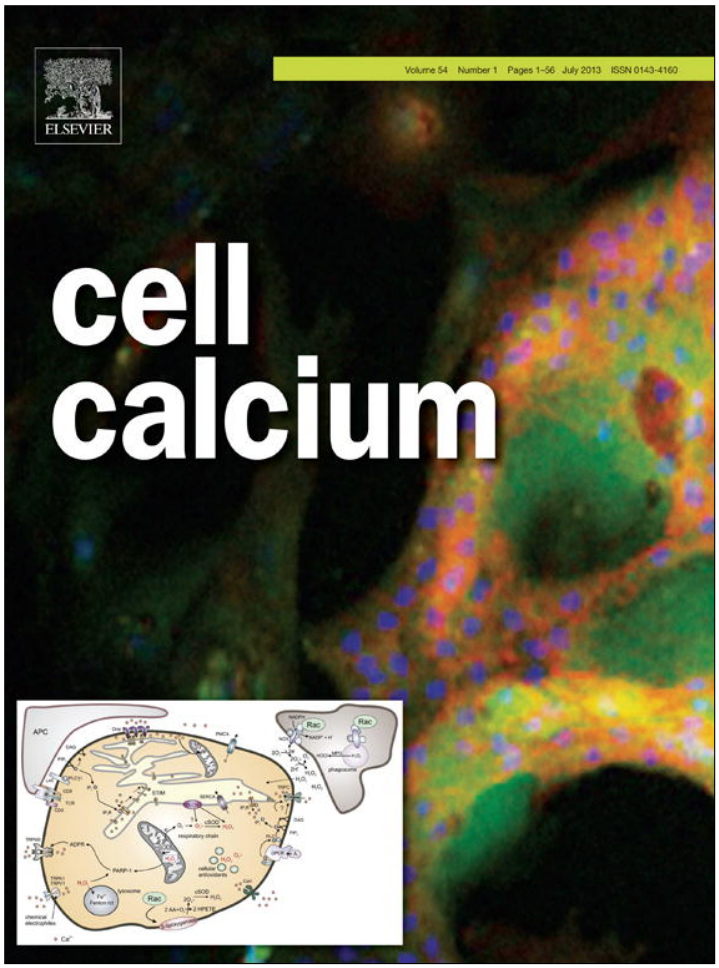

Cell Calcium 54 (2013) 37–45

Contents lists available at SciVerse ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Ca^{2+} homeostasis in the endoplasmic reticulum measured with a new low-Ca^{2+}-affinity targeted aequorin

Sergio de la Fuente, Rosalba I. Fonteriz, Mayte Montero, Javier Alvarez*

Instituto de Biología y Genética Molecular (IBGM), Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, Universidad de Valladolid and Consejo Superior de Investigaciones Científicas (CSIC), Ramón y Cajal 7, E-47005 Valladolid, Spain

ARTICLE INFO

Article history:
Received 9 January 2013
Received in revised form 27 February 2013
Accepted 4 April 2013
Available online 2 May 2013

Keywords:
Aequorin
Endoplasmic reticulum
Ca^{2+}
Ca^{2+}-affinity
Ca^{2+}-dynamics

ABSTRACT

We use here a new very low-Ca^{2+}-affinity targeted aequorin to measure the $[Ca^{2+}]$ in the endoplasmic reticulum ($[Ca^{2+}]_{ER}$). The new aequorin chimera has the right Ca^{2+}-affinity to make long-lasting measurements of $[Ca^{2+}]_{ER}$ in the millimolar range. Moreover, previous Ca^{2+}-depletion of the ER is no longer required. The steady-state $[Ca^{2+}]_{ER}$ obtained is 1–2 mM, higher than previously reported. In addition, we find evidence that there is significant heterogeneity in $[Ca^{2+}]_{ER}$ among different regions of the ER. About half of the ER had a $[Ca^{2+}]_{ER}$ of 1 mM or below, and the rest had $[Ca^{2+}]_{ER}$ values above 1 mM and in some parts even above 2 mM. About 5% of the ER was also found to have high $[Ca^{2+}]_{ER}$ levels but to be thapsigargin-insensitive and inositol trisphosphate insensitive. The rate of refilling with Ca^{2+} of the ER was almost linearly dependent on the extracellular $[Ca^{2+}]$ between 0.1 and 3 mM, and was only partially affected by mitochondrial membrane depolarization. Instead, it was significantly reduced by loading cells with chelators, and the fast chelator BAPTA was much more effective than the slow chelator EGTA. This suggests that local $[Ca^{2+}]$ microdomains connecting the store operated Ca^{2+} channels with the ER Ca^{2+} pumps may be important during refilling.

1. Introduction

We have recently described the use of a new double mutated form of aequorin with very low $[Ca^{2+}]$ affinity to measure mitochondrial $[Ca^{2+}]$. We use here this double mutated form of aequorin to measure the $[Ca^{2+}]$ in the endoplasmic reticulum ($[Ca^{2+}]_{ER}$). The first measurements of $[Ca^{2+}]_{ER}$ using mutated aequorin, reconstituted with a semi-synthetic coelenterazine with lower relative luminescence intensity (coelenterazine n), were made by us 15 years ago [1–3]. This combination of mutated aequorin and coelenterazine n (ER-mutAEQ-n) has been widely used to measure $[Ca^{2+}]_{ER}$ along these years [4–8]. However, it still has some problems that severely limit the results which can be obtained. These problems derive both from the Ca^{2+}-affinity, still too high for the large $[Ca^{2+}]$ present into the ER, and from the irreversible aequorin consumption that occurs at high $[Ca^{2+}]$. Reconstitution of aequorin with coelenterazine requires previous depletion of Ca^{2+} of the ER. Then, refilling of the ER with Ca^{2+} is accomplished prior to the experiments by perfusing cells with Ca^{2+}-containing extracellular medium. However, because of the still relatively high Ca^{2+}-affinity of the ER-mutAEQ-n, about 90% of the aequorin molecules are consumed within the first 3 min after Ca^{2+} addition, and therefore during the refilling process, when the experiments are performed at 37 °C. Thus, in order to see an effect, addition of agonists or effectors has to be made before refilling of the ER has been completed. In fact, as we show here, this problem has led to an under-estimation of the steady-state $[Ca^{2+}]_{ER}$ levels, because in those experiments ER-refilling could never reach steady-state $[Ca^{2+}]$ values before full aequorin consumption.

An important finding to facilitate the use of ER-mutAEQ-n was that reducing the temperature to 22 °C increased the amount of time available for the experiments to 10–15 min [2]. This is why many $[Ca^{2+}]_{ER}$ measurements have been made up to now at 22 °C. However, this temperature is not physiological, this time is still too short for many experimental protocols, and saturation of the probe with Ca^{2+} limits anyway the sensitivity at high $[Ca^{2+}]$.

In this paper we show measurements of $[Ca^{2+}]_{ER}$ in HeLa cells obtained with a new double-mutated aequorin form [9] targeted to the endoplasmic reticulum. This new probe allows making prolonged measurements at 37 °C of $[Ca^{2+}]_{ER}$ without problems of aequorin consumption, and provides much new information on the steady-state $[Ca^{2+}]_{ER}$ and on the dynamics of $[Ca^{2+}]_{ER}$ along different experimental maneuvers. In addition, contrarily to previous aequorin probes for the ER, it allows making $[Ca^{2+}]_{ER}$ measurements without previous Ca^{2+} depletion of the ER.

* Corresponding author. Tel.: +34 983 423085; fax: +34 983 423588.
E-mail addresses: jalvarez@ibgm.uva.es, javieramnd@gmail.com (J. Alvarez).

0143-4160/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ceca.2013.04.001

2. Methods

2.1. Cell culture and targeted mutated aequorin expression

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 100 i.u. ml^{-1} penicillin and 100 i.u. ml^{-1} streptomycin. PC12 rat pheochromocytoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 7.5% fetal bovine serum, 7.5% horse serum, 2 mM glutamine, 100 i.u. ml^{-1} penicillin and 100 i.u. ml^{-1} streptomycin. Description of the double mutated aequorin form has been made previously [9]. The same Asn28→Leu mutation was introduced in a pcDNA 3.1 plasmid containing ER-targeted 119Ala mut-AEQ. Transfections were carried out using Metafectene (Biontex, Munich, Germany).

2.2. $[Ca^{2+}]_{ER}$ measurements with aequorin

HeLa cells were plated onto 13 mm round coverslips and transfected with the plasmid for the ER-targeted double mutated aequorin (ER-2mutAEQ). Reconstitution of this aequorin type with coelenterazine i was carried out either with or without previous Ca^{2+}-depletion of the ER. In the first case, before reconstituting aequorin, $[Ca^{2+}]_{ER}$ was reduced by incubating the cells for 10 min at 20 °C with the sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPase inhibitor 2,5-di-tert-buthyl-benzohydroquinone (BHQ) 10 μM in standard external medium containing (in mM): NaCl, 145; KCl, 5; $MgCl_2$, 1; glucose, 10; HEPES, 10, pH 7.4, supplemented with 0.5 mM EGTA. Cells were then washed and incubated for 1–2 h at 20 °C in the same medium with 2 μM coelenterazine i. Then, the coverslip was placed in the perfusion chamber of a purpose-built thermostatized luminometer (at 37 °C), and standard medium containing 0.5 mM EGTA was perfused for 5 min prior to the experiment. For reconstitution in Ca^{2+}-containing medium, cells were incubated for 1 h at 20 °C in external medium containing (in mM): NaCl, 145; KCl, 5; $MgCl_2$, 1; glucose, 10; HEPES, 10, $CaCl_2$, 1, pH 7.4, supplemented with 2 μM coelenterazine i. The coverslip with the cells was then placed in the thermostatized luminometer (at 37 °C) and perfused with the same Ca^{2+}-containing external medium. In the case of using permeabilized cells (only Fig. 2e), standard medium containing 0.5 mM EGTA instead of Ca^{2+} was perfused for 1 min, followed by 1 min of intracellular medium (130 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM potassium phosphate, 0.5 mM EGTA, 1 mM ATP, 20 μM ADP, 5 mM L-malate, 5 mM glutamate, 5 mM succinate, 20 mM HEPES, pH 7) containing 20 μM digitonin. Then, intracellular medium without digitonin was perfused for 5 min, followed by a 100 nM $[Ca^{2+}]$ buffer prepared with EGTA. Temperature was set at 37 °C in all the experiments, except for that in Fig. 2f. Calibration of the luminescence data into $[Ca^{2+}]$ was made using an algorithm adjusted to the calibration, as previously described [10].

2.3. Calibration of ER-2mutAEQ inside the endoplasmic reticulum

HeLa cells expressing ER-2mutAEQ were reconstituted with 2 μM coelenterazine i in the presence of 1 μM thapsigargin (to avoid any possible interference from the endoplasmic reticulum Ca^{2+} store in the calibration). Then, cells were placed in the luminometer, permeabilized with digitonin and perfused with intracellular medium (130 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM potassium phosphate, 0.5 mM EGTA, 20 mM Hepes, pH 7) containing no ATP and no metabolic substrates, ionophores ionomycin (1 μM) and FCCP (2 μM), and 10 μM of the sarco-endoplasmic reticulum Ca^{2+}-ATPase inhibitor 2,5-di-tert-buthyl benzohydroquinone (BHQ). In this way, the endoplasmic reticulum becomes unable to accumulate Ca^{2+} and behaves simply as an aequorin container, freely permeable to Ca^{2+} and H^+, and ready to perform a calibration in the desired conditions. Cells were then perfused under these conditions with the same intracellular medium containing no EGTA and different unbuffered Ca^{2+} concentrations added, from 50 μM to 5 mM. The relationship between the relative luminescence data (luminescence/total residual luminescence or L/L_{max}) and the corresponding $[Ca^{2+}]$ was then plotted as shown in Fig. 1 below to obtain the calibration curve. Data were then fitted as shown in the figure and the curve obtained was then used to calibrate the experiments.

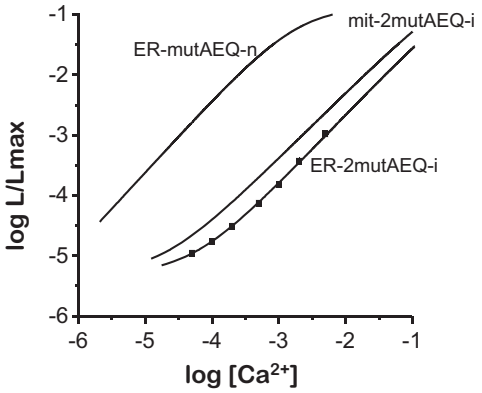

Fig. 1. In situ calibration of ER-targeted double mutated aequorin (ER-2mutAEQ-i). HeLa cells expressing ER-2mutAEQ were reconstituted with coelenterazine i in the presence of 1(M thapsigargin. Then, cells were placed in the luminometer, permeabilized with digitonin and perfused with intracellular medium containing no ATP and no metabolic substrates, 1 μM ionomycin, 2 μM FCCP, 5(M oligomycin and 10 μM BHQ, pH 7 (buffered with Hepes). Cells were then perfused under these conditions with different unbuffered Ca^{2+} concentrations, from 50 (M to 5 mM. In each experiment, the total amount of luminescence was obtained at the end by allowing full consumption of the probe at 20 mM $[Ca^{2+}]$. Each experimental point was obtained as the ratio between the luminescence obtained at a given $[Ca^{2+}]$ (L) and the total remaining luminescence at that moment (Lmax). These L/Lmax values were represented against the $[Ca^{2+}]$. Each point shows the mean ± S.E. obtained with 3–5 data of each kind. These experimental points were then fitted to the algorithm described previously [10]. The figure also shows for comparison the calibration curves corresponding to ER-mutAEQ reconstituted with coelenterazine n [1] and mit-2mutAEQ reconstituted with coelenterazine i [9]. All the data represented were obtained at 37 °C.

2.4. Measurements of mitochondrial membrane potential

Mitochondrial membrane potential was monitored using the fluorescent indicator tetramethylrhodamine ethyl ester (TMRE). HeLa cells were mounted in a cell chamber in the stage of a Zeiss Axiovert 200 microscope under continuous perfusion. Cells were then permeabilized with digitonin and perfused with intracellular medium containing 20 nM TMRE until steady-state fluorescence was reached (usually about 5 min). Single cell fluorescence was excited at 540 nm using a Cairn monochromator (200 ms excitation every 2 s) and images of the fluorescence emitted between 570 and 630 nm obtained with a 40× Fluar objective were recorded by a Hamamatsu ORCA-ER camera. Single cell fluorescence records were analyzed off-line using the Metafluor program (Universal Imaging). Experiments were performed at 37 °C using an on-line heater from Harvard Apparatus.

2.5. Measurements of intracellular $[Ca^{2+}]$ with fura-2

Cells were loaded with fura-2 by incubation in standard medium containing 2 μM fura-2-acetoxymethyl ester (fura-2-AM) for 1 h at room temperature. Simultaneously, cells were also loaded with either none, 10 μM EGTA-AM or 10 μM BAPTA-AM. Cells were then

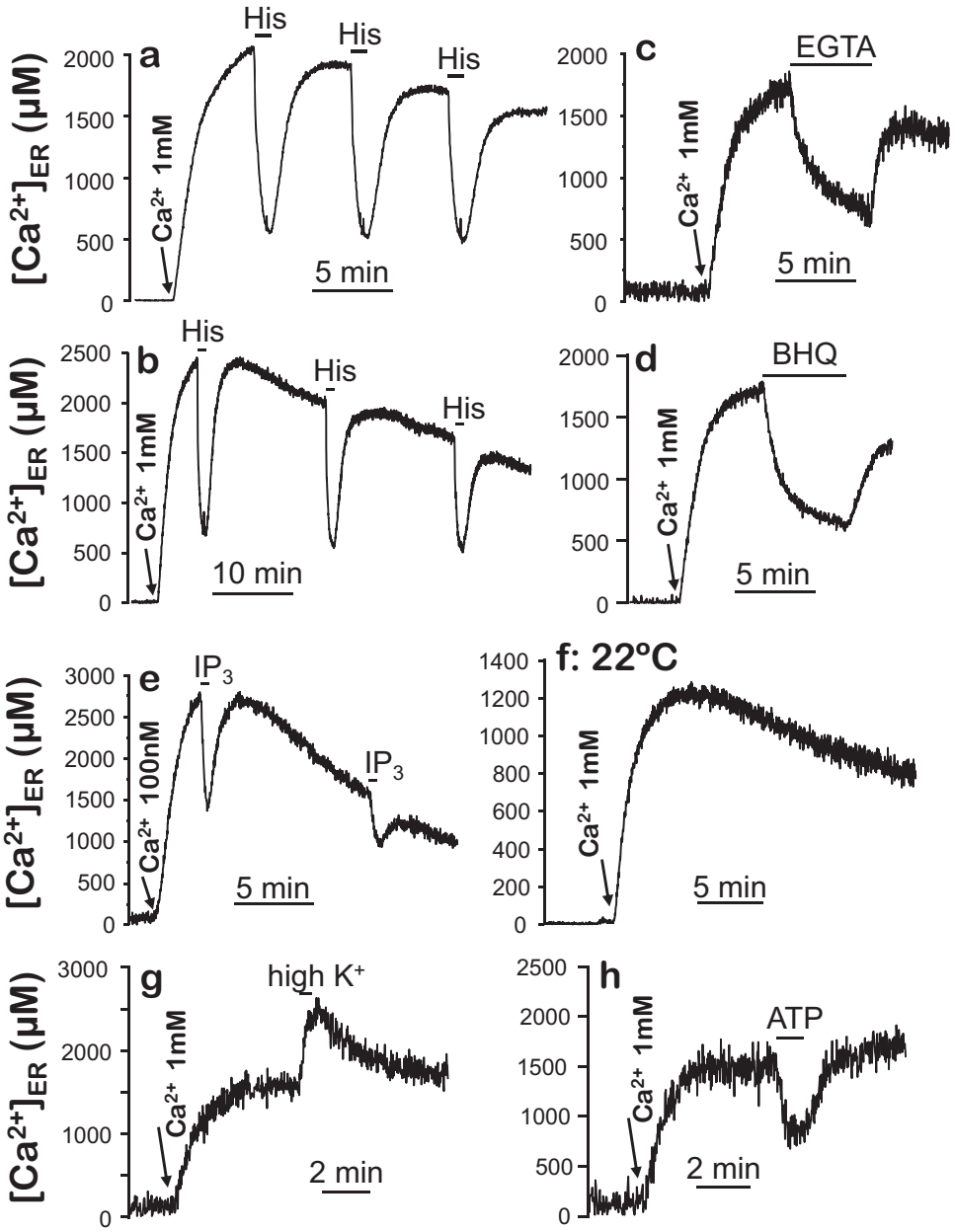

Fig. 2. $[Ca^{2+}]_{ER}$ dynamics measured with ER-2mutAEQ-i in Ca^{2+}-depleted cells. a-d: HeLa cells expressing ER-2mutAEQ and reconstituted with coelenterazine i were refilled with Ca^{2+} by addition of 1 mM Ca^{2+}-containing medium when indicated. Then, either 100 μM histamine, 0.5 mM EGTA or 10 μM BHQ were perfused as indicated. (e) HeLa cells expressing ER-2mutAEQ and reconstituted with coelenterazine i were permeabilized with digitonin and perfused when indicated with intracellular medium containing 100 nM $[Ca^{2+}]$ and 2 μM $InsP_3$. (f) HeLa cells expressing ER-2mutAEQ and reconstituted with coelenterazine wild type were refilled with Ca^{2+} by addition of 1 mM Ca^{2+}-containing medium at 22 °C. (g and h) Experiments performed with PC12 cells. When indicated, either medium containing 70 mM KCl (substituting for NaCl) or 100 μM ATP were perfused. The experiments shown are representative of 3–6 similar experiments of each kind.

washed with standard medium for 15 min at room temperature and mounted in a cell chamber in the stage of a Zeiss Axiovert 200 microscope under continuous perfusion. Single cell fluorescence was excited at 340 nm and 380 nm using a Cairn monochromator (200 ms excitation at each wavelength every 2 s, 10 nm bandwidth) and images of the emitted fluorescence obtained with a 40× Fluar objective were collected using a 400DCLP dichroic mirror and a D510/80 emission filter (both from Chroma Technology) and recorded by a Hamamatsu ORCA-ER camera. Single cell fluorescence records were ratioed and calibrated into $[Ca^{2+}]$ values using the Metafluor program (Universal Imaging). Experiments were performed at 37 °C using an on-line heater from Harvard Apparatus.

2.6. Materials

Coelenterazine i was obtained from Biotium, Inc., Hayward, CA, U.S.A. Fura-2 was from Molecular Probes, OR, U.S.A. Other reagents were from Sigma, Madrid, Spain or Merck, Darmstadt, Germany.

3. Results

Although this new aequorin form has been previous calibrated in mitochondria, we have shown previously that there are some small differences between the calibrations made in mitochondria and in the endoplasmic reticulum, which can be mainly attributed to the different pH [11]. Thus, we have calibrated the

probe also inside the endoplasmic reticulum. Fig. 1 shows the results obtained (curve ER-2mutAEQ-i) and compares this calibration curve obtained in the endoplasmic reticulum with that previously obtained inside mitochondria (curve mit-2mutAEQ-i, Ref. [9]). The difference can be attributed to the different pH (pH 7 in the ER vs pH 8 in mitochondria), as increasing the pH has been shown to increase luminescence [12]. Fig. 1 also includes for comparison the calibration curve of the ER-mutAEQ-n aequorin form [1], which is shifted by about two orders of magnitude of $[Ca^{2+}]$ to the left.

The new probe allows obtaining long-lasting measurements of $[Ca^{2+}]_{ER}$ without problems derived from aequorin consumption. Fig. 2 shows several measurements of $[Ca^{2+}]_{ER}$ obtained with the new aequorin probe. The experiments were always started with Ca^{2+}-depleted cells and then 1 mM Ca^{2+} was added to the extracellular medium to refill the stores. Addition of Ca^{2+} to the extracellular medium triggered a fast increase in $[Ca^{2+}]_{ER}$ which reached levels between 1.5 and 2.5 mM within 5 min of Ca^{2+} addition. Panels a and b show the effect of three consecutive additions of the inositol trisphosphate ($InsP_3$)-producing agonist histamine, separated by 5 min (panel a) or 10 min (panel b) intervals. A slow decrease with time in the steady-state $[Ca^{2+}]_{ER}$ can be appreciated in long-lasting experiments such as that shown in panel b. This point will be studied in further detail later. Panels c and d show that addition of either EGTA or the SERCA pump inhibitor BHQ to the extracellular medium also induced a decrease in $[Ca^{2+}]_{ER}$, though at a slower rate. These effects were reverted either by readdition of Ca^{2+} to the extracellular medium (panel c) or by washing the inhibitor (panel d).

Panel e of Fig. 2 shows an experiment performed in permeabilized HeLa cells, showing the refilling induced by perfusion of a 100 nM $[Ca^{2+}]$ buffer and the release induced by the addition of the second messenger $InsP_3$. In permeabilized cells, as in the intact ones, $[Ca^{2+}]_{ER}$ reaches also a maximum during refilling in 100 nM $[Ca^{2+}]$ and then slowly decreases. As mentioned above, we believe this behavior is due to the heterogeneity in $[Ca^{2+}]$ among different regions of the ER and will be discussed later.

Panel f of Fig. 2 shows the time course of $[Ca^{2+}]_{ER}$ refilling at 22 °C. We have included this experiment here because many of the previous data on $[Ca^{2+}]_{ER}$ had been obtained at this temperature, as mentioned above. To measure $[Ca^{2+}]_{ER}$ at 22 °C with the double mutated aequorin, we had to reconstitute it with wild type aequorin, as using coelenterazine i produced an aequorin form with too low Ca^{2+} affinity at this temperature. The kinetics of refilling at 22 °C was similar to that obtained at 37 °C, although the maximum $[Ca^{2+}]$ values obtained were somewhat smaller, just slightly above 1 mM.

Finally, panels g and h of Fig. 2 show results obtained in the neuroendocrine cell line PC12. Refilling of the ER in these cells led to similar steady-state $[Ca^{2+}]_{ER}$ values. Then, activation of voltage-dependent Ca^{2+} channels with high K^+ induced an increase in $[Ca^{2+}]_{ER}$ following the increase in cytosolic $[Ca^{2+}]$. Instead, the $InsP_3$-producing agonist ATP activated $InsP_3$ receptors and triggered a fast Ca^{2+} release from the ER.

Ca^{2+}-depletion of the ER has been always required to measure $[Ca^{2+}]_{ER}$ with aequorin, because the high $[Ca^{2+}]$ present in the ER under resting conditions precludes reconstitution with coelenterazine. However, the very low Ca^{2+}-affinity of ER-2mutAEQ-i allows making for the first time $[Ca^{2+}]_{ER}$ experiments with aequorin without previous depletion of Ca^{2+}. In the experiments shown in Fig. 3, reconstitution with coelenterazine i was carried out in 1 mM Ca^{2+}-containing medium. The results obtained in terms of both $[Ca^{2+}]_{ER}$ values and the effects of histamine, BHQ and EGTA addition, were the same as those found in Ca^{2+}-depleted cells (Fig. 2), indicating that Ca^{2+}-depletion does not significantly modify these responses in HeLa cells. In other cell types, however, the possibility to make $[Ca^{2+}]_{ER}$ measurements without previous Ca^{2+} depletion may represent a significant advantage.

We have then investigated the link between Ca^{2+}-entry and Ca^{2+} refilling of the ER, using three different experimental protocols. In

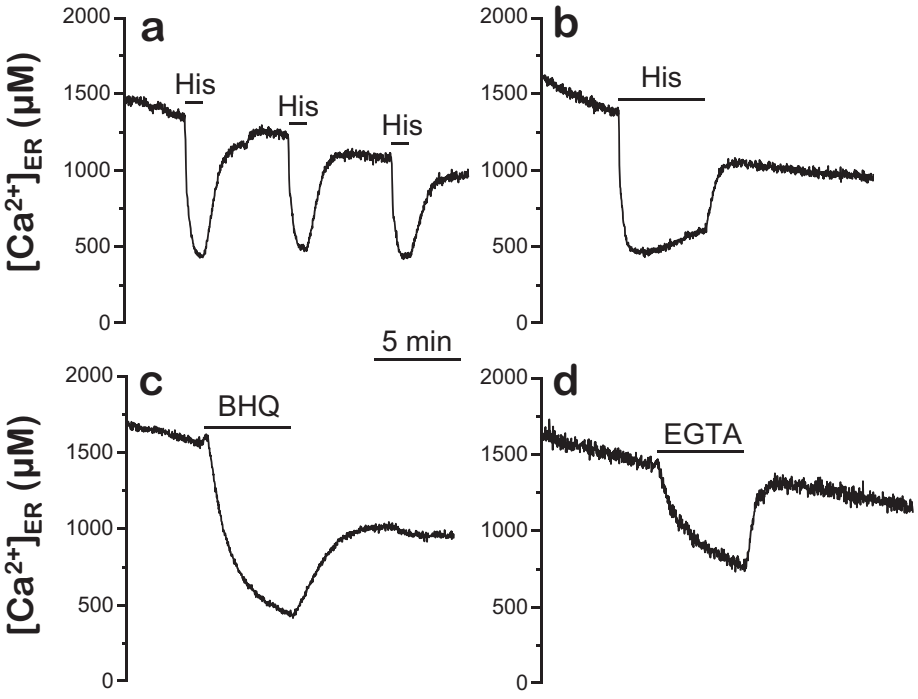

Fig. 3. $[Ca^{2+}]_{ER}$ dynamics measured with ER-2mutAEQ-i in intact (non-Ca^{2+}-depleted) cells. HeLa cells expressing ER-2mutAEQ were reconstituted with coelenterazine i in 1 mM Ca^{2+}-containing medium, without Ca^{2+} depletion. The figure shows the effect of additions of either 100 μM histamine, 10 μM BHQ or 0.5 mM EGTA. The experiments shown are representative of 3 similar experiments of each kind.

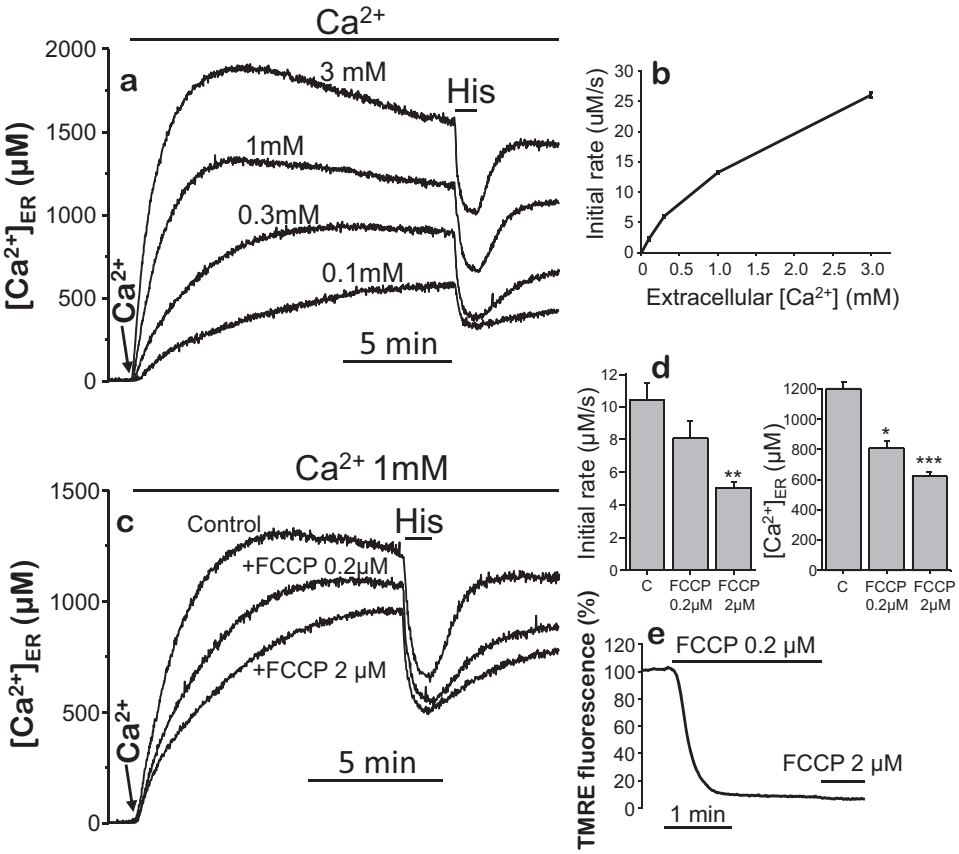

Fig. 4. Dependence of the rate of refilling with Ca^{2+} of the ER on the extracellular $[Ca^{2+}]$ and the mitochondrial membrane depolarization. Experiments were made in HeLa cells as described in Fig. 2, and 100 μM histamine was added when indicated. In panel a, different $[Ca^{2+}]$ were added to the extracellular medium to initiate refilling, from 0.1 to 3 mM, as indicated. The experiments shown are the mean of 3 different experiments of each kind. Panel b shows the relationship among the initial rate of refilling and the extracellular $[Ca^{2+}]$ (mean ± S.E.). In panel c, refilling was triggered by perfusing medium containing 1 mM $[Ca^{2+}]$ with or without the protonophore FCCP (0.2 μM or 2 μM, as indicated). The protonophore was also present 2 min before the addition of Ca^{2+}. The experiments shown are the mean of 3 different experiments of each kind. Panel d shows the effects and statistical significance of the addition of FCCP on both the initial rate of Ca^{2+} uptake and the $[Ca^{2+}]_{ER}$ level 3 min after Ca^{2+} addition. Data are mean ± S.E. ($n = 3$ data in each case). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$. Panel e shows the effect of the protonophore on mitochondrial membrane potential measured with TMRE.

panel a of Fig. 4, we have studied the effect of the extracellular $[Ca^{2+}]$ on the rate of refilling of the ER and the steady-state $[Ca^{2+}]_{ER}$. As shown in the figure, refilling with Ca^{2+} of the ER was triggered with different $[Ca^{2+}]$, from 0.1 mM to 3 mM. Increasing the extracellular $[Ca^{2+}]$ produced a large increase in the rate of ER Ca^{2+}-refilling and in the steady-state $[Ca^{2+}]_{ER}$. In fact, the rate of refilling was almost linearly dependent on the extracellular $[Ca^{2+}]$ (panel b). In panel c, we show experiments studying the effect of the protonophore FCCP on $[Ca^{2+}]_{ER}$. By abolishing mitochondrial membrane potential, this compound has been suggested to significantly modify $[Ca^{2+}]_{ER}$ dynamics [13] and Ca^{2+}-entry through store-operated Ca^{2+} channels [14,15]. Our data show that addition of FCCP reduced both the rate of ER $[Ca^{2+}]$ refilling and the steady-state $[Ca^{2+}]_{ER}$ values. We have used two FCCP concentrations, 0.2 and 2 μM. As shown in panel d, 0.2 μM FCCP reduced by 20–30% both the initial rate of Ca^{2+} accumulation in the ER and the $[Ca^{2+}]_{ER}$ values 3 min after Ca^{2+} addition, although only the last effect was significant. Regarding 2 μM FCCP, it reduced by about 50% both parameters with high statistical significance. Panel e shows the effect of these concentrations on mitochondrial membrane potential. The lower concentration, 0.2 μM FCCP, almost fully abolished mitochondrial membrane potential. Therefore, this concentration should be considered the most relevant to show the effects of mitochondrial membrane potential depolarization. The additional effects of higher concentrations of FCCP may be due to actions of the protonophore in other cell locations.

Fig. 5 shows the effect of loading the cells with Ca^{2+} chelators, either EGTA or BAPTA. We used these two chelators because they have different kinetic properties and can be useful to detect transient local high-Ca^{2+} microdomains. Cells were incubated with the AM-derivative of each chelator during aequorin reconstitution and then refilling was triggered by addition of Ca^{2+} to the extracellular medium. Panel a shows that refilling was significantly slowed by both chelators, but the fast chelator BAPTA was much more effective than EGTA to produce this effect. Panel b shows that the initial rate was reduced by 50% in cells loaded with EGTA and by 80% in cells loaded with BAPTA, in both cases with high significance. In addition, in the cells loaded with EGTA, the $[Ca^{2+}]_{ER}$ level reached the same steady-state values than the control after 10 min in Ca^{2+}-containing medium, while in BAPTA loaded cells these levels were significantly smaller. This suggests that local $[Ca^{2+}]$ microdomains may be involved in the process of refilling of the ER. Regarding cytosolic $[Ca^{2+}]$, the effect of the chelators in $[Ca^{2+}]_c$ measured by fura-2 imaging can be seen in panel c. The presence of any of the chelators slowed similarly the increase in $[Ca^{2+}]_c$ after Ca^{2+}-addition, but did not modify the final steady-state $[Ca^{2+}]_c$ (mean ± S.E., control, 66 ± 2 nM, $n = 14$; EGTA-AM, 66 ± 1 nM, $n = 22$; BAPTA-AM, 68 ± 2 nM, $n = 15$).

In Fig. 6 we make a more complete study of the $[Ca^{2+}]_{ER}$ values obtained after monitoring $[Ca^{2+}]_{ER}$ for more than 1 h. The upper panel shows the unprocessed luminescence record. 1 mM Ca^{2+} was perfused for 1 h and then digitonin was added together with 20 mM

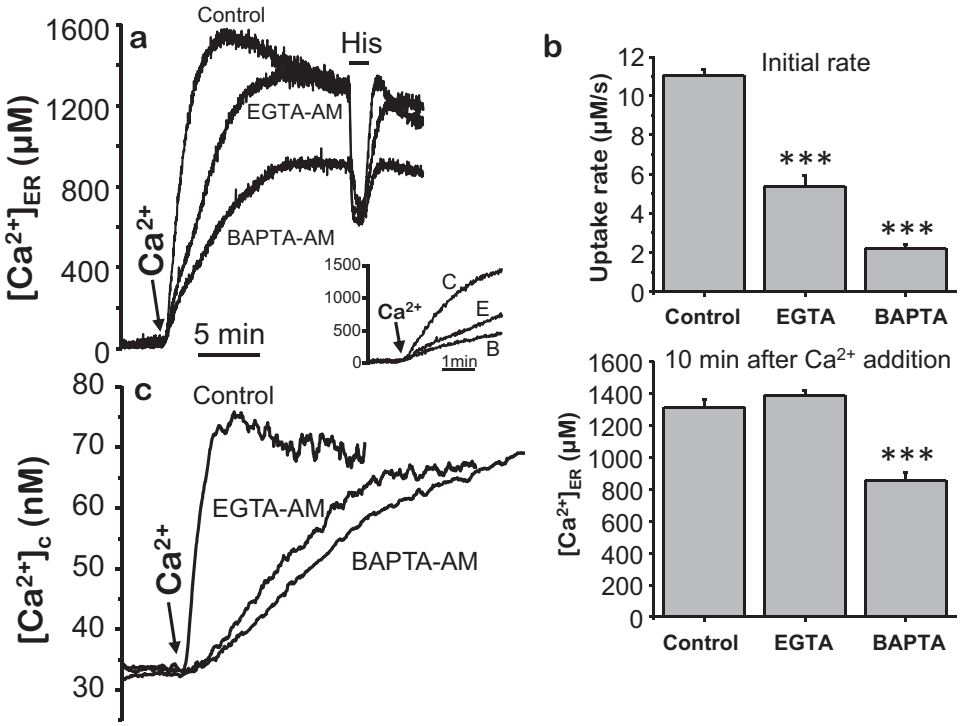

Fig. 5. Dependence of the rate of refilling with Ca^{2+} of the ER on the cytosolic buffering capacity. In panel a, cells were incubated during reconstitution with either none (control), 10 μM EGTA-AM or 10 μM BAPTA-AM, and refilling was started by addition of 1 mM Ca^{2+}-containing medium. The inset shows expanded the initial part of the graph. The experiments shown are the mean of 3 different experiments of each kind. Panel b shows the effects and statistical significance of loading with EGTA and BAPTA on both the initial Ca^{2+} uptake rate and the $[Ca^{2+}]_{ER}$ value obtained 10 min after Ca^{2+} addition. Data are mean ± S.E. (n = 6–8 data in each case). ***$p < 0.001$, ANOVA test. In panel c, cells were incubated during reconstitution with 2 μM fura-2 and either none (control), 10 μM EGTA-AM or 10 μM BAPTA-AM. Then, refilling was started by addition of 1 mM Ca^{2+}-containing medium. The data shown are the mean obtained from all the cells present in the microscope field (control, 14 cells; EGTA, 22 cells; BAPTA, 15 cells) in one of two similar experiments made.

Ca^{2+} to release any residual luminescence. The middle panel shows the calibrated record. $[Ca^{2+}]_{ER}$ increases rapidly up to about 2 mM and then decreases slowly to around 1 mM after 1 h. This phenomenon was not due to the overshoot in cytosolic $[Ca^{2+}]$ occurring after Ca^{2+} addition. As shown in the inset, this cytosolic $[Ca^{2+}]$ overshoot lasts only 1–2 min. An alternative explanation for the decaying kinetics of the $[Ca^{2+}]_{ER}$ curve relies in the heterogeneity of $[Ca^{2+}]_{ER}$ among different parts of the ER and will be discussed later. The lower panel of the figure shows the time course of aequorin consumption along the experiment. It can be observed that the percentage of aequorin consumption is still below 60% after more than 1 h of experiment, showing that the new aequorin probe can be used for long-lasting experiments and is little affected by aequorin consumption.

The presence of heterogeneity in $[Ca^{2+}]_{ER}$ had been described before using the ER-mutAEQ probe reconstituted with both coelenterazine wild type and n [16]. As we show in panel a of Fig. 7 (see also Refs. [16,17]), addition of Ca^{2+} to cells expressing ER-mutAEQ-n induced a very fast peak of luminescence which consumed about 95% of the luminescence. Then, later addition of digitonin and high calcium released the residual 5% of the luminescence. This implies that about 5% of the probe is present in a compartment with very low $[Ca^{2+}]$. On the other hand, and this has not been described before, inhibition of the SERCA pump with both thapsigargin and benzohydroquinone abolished most of the $[Ca^{2+}]_{ER}$ increase, except for a 5.4 ± 0.4% (mean ± S.E., n = 4) of the space, which was insensitive to the inhibitors (panel b). When we study then this compartment using the ER-2mutAEQ-i, we find that addition of Ca^{2+} to cells with the SERCA pump inhibited by both thapsigargin and BHQ, induced a small but persistent increase in luminescence. Calibration into $[Ca^{2+}]$ of this increase provided values around 300 μM. However, considering that the luminescence comes only from less than 10% of the total space, the real $[Ca^{2+}]_{ER}$ value for this compartment would be in the millimolar range. Addition of histamine did not produce any significant effect (data from 4 experiments), indicating that this space, which must accumulate Ca^{2+} through thapsigargin and BHQ insensitive Ca^{2+}-pumps, does not possess $InsP_3$ receptors.

4. Discussion

Measuring $[Ca^{2+}]_{ER}$ requires probes with a very low Ca^{2+}-affinity, able to have good sensitivity in the millimolar range. In the case of aequorin, the low Ca^{2+}-affinity is also essential to be able to monitor very high $[Ca^{2+}]$ levels for prolonged times without consumption. We have recently developed a double mutated aequorin probe able to measure the millimolar $[Ca^{2+}]$ levels which are reached in mitochondria during prolonged increases in the cytosolic $[Ca^{2+}]$ [9]. We have now used this probe targeted to the endoplasmic reticulum to investigate the dynamics of $[Ca^{2+}]_{ER}$. The specificity of targeting of this probe has been previously shown in HeLa cells by immunocytochemistry [17] and by electron microscopy, using immunogold labeling of ultrathin cryosections of HeLa cells expressing the targeted aequorin [16]. Both techniques showed that the protein is localized within the lumen of the ER and perinuclear cisternae.

Original measurements of $[Ca^{2+}]_{ER}$ had been made with mutated aequorin reconstituted with synthetic coelenterazine n. The values obtained in these original works for the steady-state $[Ca^{2+}]_{ER}$ in several cell types were between 300 and 800 μM [1–3,18]. Later works by other authors reported similar values [4–8]. However, our data here indicate that $[Ca^{2+}]_{ER}$ is higher. About 50% of the ER has $[Ca^{2+}]$ above 1 mM, and some parts even above 2 mM. The other 50% may have $[Ca^{2+}]_{ER}$ values of 1 mM and below, including a 5% of

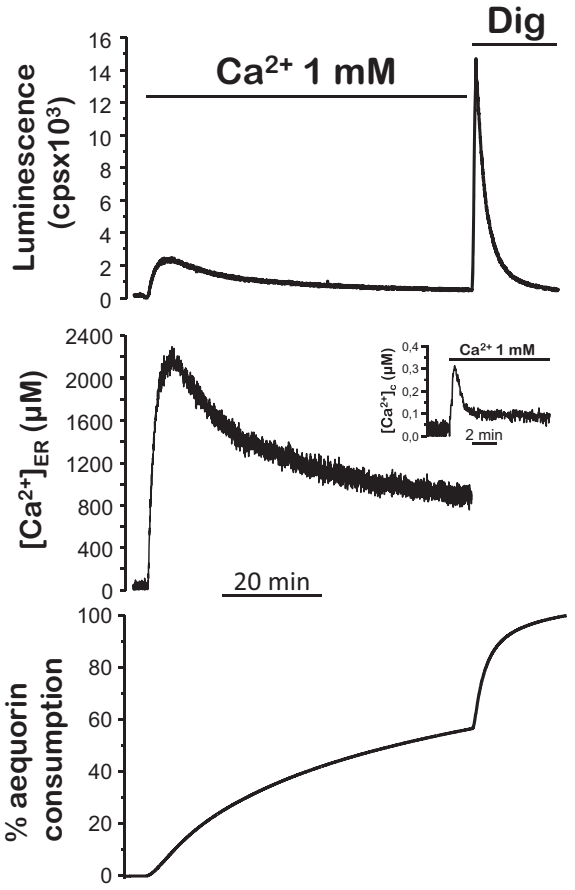

Fig. 6. Evidence for the heterogeneity of $[Ca^{2+}]$ in the endoplasmic reticulum. HeLa cells expressing ER-2mutAEQ and reconstituted with coelenterazine i were refilled with Ca^{2+} by addition of 1 mM Ca^{2+}-containing medium when indicated. Then, aequorin luminescence and subsequently $[Ca^{2+}]_{ER}$ was monitored for more than 1 h. The upper panel shows the unprocessed luminescence record. At the end of the experiment, 100 μM digitonin and 20 mM Ca^{2+} were added to release all the residual luminescence. The middle panel shows the calibrated signal. The inset shows a separate experiment made with the same protocol but in cells expressing cytosolic aequorin. The lower panel shows the time course of aequorin consumption. This experiment is representative of 5 similar experiments.

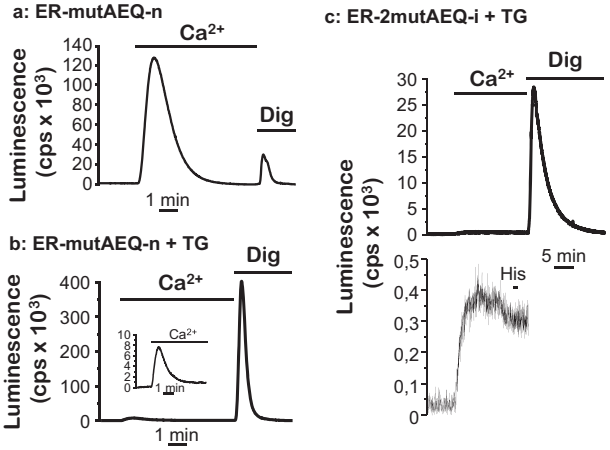

Fig. 7. Evidence for a thapsigargin-insensitive space in the ER. Panels a and b show experiments performed in HeLa cells expressing ER-mutAEQ reconstituted with coelenterazine n. In panel a, addition of Ca^{2+} to control cells triggered a large peak of luminescence which consumed more than 90% of the luminescence. In panel b, cells were treated with 1 μM thapsigargin during reconstitution and Ca^{2+} was added in the presence of 10 μM BHQ. The inset shows expanded the luminescence after Ca^{2+} addition. In panel c, the same protocol of panel b was applied to HeLa cells expressing ER-2mutAEQ reconstituted with coelenterazine i. The lower panel shows expanded the luminescence after Ca^{2+} addition. The experiments shown are representative of 3–5 similar experiments of each kind.

the space with very low $[Ca^{2+}]$. The reason for the difference with the previous data relies mainly in the higher Ca^{2+}-affinity of the old probe and particularly in its fast consumption during ER Ca^{2+} refilling. As can be seen in Fig. 5a, the time available for measuring $[Ca^{2+}]_{ER}$ during refilling with the old probe at 37 °C was only about 3 min. However, detailed study of the time course of refilling monitored with the new probe shows clearly that full refilling requires at least 5 min to reach the steady-state. Therefore, the data obtained with the old probe were necessarily an underestimation of the real value. Regarding the data obtained at 22 °C, $[Ca^{2+}]_{ER}$ values around 600 μM [2] were obtained with the old aequorin probe at this temperature, which slowed aequorin consumption increasing up to 10–15 min the time available to measure $[Ca^{2+}]_{ER}$ during refilling. Using the new probe, we show here (Fig. 2f) that $[Ca^{2+}]_{ER}$ is somewhat lower at 22 °C than at 37 °C, but still rises above 1 mM. The underestimation in the data obtained with the old aequorin probe at 22 °C probably comes from saturation of the probe with Ca^{2+}.

$[Ca^{2+}]_{ER}$ has been also measured using a variety of targeted fluorescent proteins. Values for the steady-state $[Ca^{2+}]_{ER}$ obtained with this approach have been quite variable, going from 50–100 μM [19] to near 1 mM [20], with most of the obtained figures around 400–800 μM [21–27]. On the other hand, NMR measurements using a BAPTA derivative provided values of 1.5 mM for sarcoplasmic reticulum $[Ca^{2+}]$ [28]. Measurements of $[Ca^{2+}]_{ER}$ have been carried out not only in cell lines, but also in primary cultures, such as chromaffin cells [21], skeletal muscle cells [25,27] and rabbit heart cells [28]. The reason for the higher $[Ca^{2+}]_{ER}$ values found here may rely in part in the heterogeneity of $[Ca^{2+}]_{ER}$ and the differential properties of aequorin with respect to fluorescent proteins. Because of the higher response of aequorin in high Ca^{2+} environments and the low Ca^{2+}-affinity of our new probe, the $[Ca^{2+}]_{ER}$ values we measure mainly correspond to the portions of the ER with higher $[Ca^{2+}]$. Instead, fluorescent probes provide a mean measurement of all the ER regions, where the presence of regions with fluorescence close to saturation with Ca^{2+} may be compensated with that of other regions with lower $[Ca^{2+}]$.

The new probe has also a very important technical and experimental advantage over the previous aequorin probes. All the $[Ca^{2+}]_{ER}$ measurements carried out before with these probes were performed in cells previously depleted of Ca^{2+}, because the high Ca^{2+} content of the ER precluded reconstitution with the coelenterazine cofactor. Instead, the very low Ca^{2+}-affinity of the ER-2mutAEQ probe allows reconstitution with coelenterazine i in intact non-Ca^{2+}-depleted cells. Most probably, this is possible thanks to the fact that the Ca^{2+}-affinity is further reduced during reconstitution at 20 °C, and little aequorin is consumed before the temperature is increased to 37 °C at the beginning of the experiment. In HeLa cells, the data obtained in non-Ca^{2+}-depleted cells were essentially identical to those obtained in Ca^{2+}-depleted cells. However, this advantage may be particularly important to perform $[Ca^{2+}]_{ER}$ measurements in other cells which may be more sensitive to Ca^{2+}-depletion treatments.

The new probe has allowed us to monitor the dynamics of $[Ca^{2+}]_{ER}$ under several experimental conditions. We have first determined the effect of the extracellular $[Ca^{2+}]$ on the rate of refilling with Ca^{2+} of the ER. Ca^{2+} entry to the cells takes place through the store-operated Ca^{2+} channels, which rapidly inactivate when the ER is refilled with Ca^{2+}. The $[Ca^{2+}]_{ER}$ required to completely close these channels is not well known, but it should be expected to be close to the steady-state values. Thus, we expected to find only

small differences among the rate of refilling obtained at extracellular [Ca^{2+}] around and above 1 mM. However, we found a nearly linear relationship among the extracellular [Ca^{2+}] and the rate of refilling. This means that at 0.3 mM extracellular [Ca^{2+}], refilling is slow and the channels are fully inhibited when [Ca^{2+}]$_{ER}$ reaches 1 mM. Instead, at 3 mM extracellular [Ca^{2+}], the store operated channels remain still active for some time even when [Ca^{2+}]$_{ER}$ has reached millimolar levels. The time required for uncoupling STIM from Orai may be responsible for this delay.

We have also studied the effect of mitochondrial membrane depolarization with FCCP on the rate of ER Ca^{2+} refilling. Mitochondria have been reported to control the activity of store operated Ca^{2+} channels by avoiding their Ca^{2+}-inactivation [14,15]. Under this view, mitochondrial membrane depolarization should lead to rapid channel inactivation during refilling and thus, to a significant reduction in the rate of refilling. Our data confirm that FCCP reduces the rate of Ca^{2+} uptake by the ER. However, we only find a relatively small (20–30%) reduction in the rate of refilling by using an FCCP concentration able to fully depolarize mitochondrial membranes. This means that, at least in these cells, the contribution of mitochondria to the control of store operated channels is only minor.

Loading the cells with the chelators EGTA and BAPTA significantly reduced the rate of refilling. Obviously, the increase in the buffering capacity of the cytosol transiently reduces the amount of Ca^{2+} available for pumping into the ER. However, the effect was much stronger with the fast chelator BAPTA than with EGTA, even though the effect of both chelators on the [Ca^{2+}]$_c$ increase was similar. The fact that a fast chelator is more efficient to reduce the rate of refilling of the ER suggests that a localized [Ca^{2+}] microdomain may be involved in the process of refilling. It has been proposed recently that store operated channels and SERCA are closely coupled [29], so that a pathway for fast Ca^{2+} transport from the extracellular medium to the ER may exist. Our data are consistent with this hypothesis and suggest that local cytosolic [Ca^{2+}] microdomains may be involved in ER Ca^{2+} refilling.

Our data show also evidence for the presence of heterogeneity in [Ca^{2+}] among different areas of the ER. As it has been reported before [16,30], aequorin has the ability to detect heterogeneities in [Ca^{2+}] because of its irreversible consumption. Faster consumption of aequorin in regions with high [Ca^{2+}] leads to a depletion of active aequorin molecules in those regions, allowing then to obtain data selectively from regions with lower [Ca^{2+}]. In our case, [Ca^{2+}]$_{ER}$ values go up to 2–2.5 mM in the first minute after Ca^{2+} addition and then decrease slowly. This effect is not due to an initial overshoot of the cytosolic [Ca^{2+}] after Ca^{2+} addition. This overshoot actually exists but only lasts for 1–2 min and then cytosolic [Ca^{2+}] remains stable. Instead, [Ca^{2+}]$_{ER}$ peaks at about 5 min after Ca^{2+} addition and then goes down very slowly for more than 1 h. Moreover, the same behavior of [Ca^{2+}]$_{ER}$ is observed in permeabilized cells (Fig. 2e), where the cytosolic [Ca^{2+}] is kept constant at 100 nM in the perfusion buffer for the whole refilling period. It is true that adaptation of the ER-Ca^{2+}-pumping and leak mechanisms could still lead to a genuine overshoot of [Ca^{2+}]$_{ER}$ after Ca^{2+} addition. However, a progressive decrease in [Ca^{2+}]$_{ER}$ is also observed in non-Ca^{2+}-depleted cells. In this case, reconstitution takes place at 20 °C, where the Ca^{2+} affinity of ER-2mutAEQ-i is lower, and it is the increase in the temperature to 37 °C what reveals the phenomenon. Although an adaptation to the temperature change could still be possible here, we think that the best explanation for this behavior relies in the heterogeneity. Aequorin is rapidly consumed first in ER regions having [Ca^{2+}] above 2 mM and then the luminescence from other regions having only about 1 mM [Ca^{2+}] predominates. According to the percentages of consumption obtained, high and low millimolar [Ca^{2+}] regions would contain each about half of the ER. Of course, this may be an oversimplification and a more continuous range of concentrations between 1 mM and about 3 mM appears more probable. Our data reveal also the presence of two small compartments with functional properties different to the rest. About 5% of the ER space containing our probe has very low [Ca^{2+}], unable to trigger aequorin luminescence, and another 5% of the ER space has high [Ca^{2+}], also in the millimolar range, but is thapsigargin-insensitive and has no $InsP_3$ receptors.

In summary, the new low-Ca^{2+} affinity aequorin used here allows studying [Ca^{2+}]$_{ER}$ dynamics with a much higher detail. It provides new higher values for the steady-state [Ca^{2+}]$_{ER}$, reveals the presence of heterogeneity in [Ca^{2+}] among different regions of the ER, and provides new information on the rate of refilling of the ER and its dependence on Ca^{2+} entry through the plasma membrane and cytosolic Ca^{2+} buffering.

Acknowledgements

This work was supported by grants from Ministerio de Ciencia e Innovación (BFU 2011-25763) and from Junta de Castilla y León (VA029A12-1). Sergio de la Fuente holds an FPI (Formación de Personal Investigador) fellowship from the Spanish Ministerio de Ciencia e Innovación. We thank Pilar Alvarez for excellent technical assistance.

References

[1] M. Montero, M.J. Barrero, J. Alvarez, [Ca^{2+}] microdomains control agonist-induced Ca^{2+} release in intact HeLa cells, FASEB Journal 11 (1997) 881–885.
[2] M.J. Barrero, M. Montero, J. Alvarez, Dynamics of [Ca^{2+}] in the endoplasmic reticulum and cytoplasm of intact HeLa cells. A comparative study, Journal of Biological Chemistry 272 (1997) 27694–27699.
[3] J. Alvarez, M. Montero, Measuring [Ca^{2+}] in the endoplasmic reticulum with aequorin, Cell Calcium 32 (2002) 251–260.
[4] P. Pinton, D. Ferrari, P. Magalhães, K. Schulze-Osthoff, F. Di Virgilio, T. Pozzan, R. Rizzuto, Reduced loading of intracellular Ca^{2+} stores and downregulation of capacitative Ca^{2+} influx in Bcl-2-overexpressing cells, Journal of Cell Biology 148 (2000) 857–862.
[5] G. Zatti, A. Burgo, M. Giacomello, L. Barbiero, R. Ghidoni, G. Sinigaglia, C. Florean, S. Bagnoli, G. Binetti, S. Sorbi, P. Pizzo, C. Fasolato, Presenilin mutations linked to familial Alzheimer's disease reduce endoplasmic reticulum and Golgi apparatus calcium levels, Cell Calcium 39 (2006) 539–550.
[6] D. Takei, H. Ishihara, S. Yamaguchi, T. Yamada, A. Tamura, H. Katagiri, Y. Maruyama, Y. Oka, WFS1 protein modulates the free Ca^{2+} concentration in the endoplasmic reticulum, FEBS Letters 580 (2006) 5635–5640.
[7] N.N. Kasri, S.L. Kocks, L. Verbert, S.S. Hébert, G. Callewaert, J.B. Parys, L. Missiaen, H. De Smedt, Up-regulation of inositol 1, 4,5-trisphosphate receptor type 1 is responsible for a decreased endoplasmic-reticulum Ca^{2+} content in presenilin double knock-out cells, Cell Calcium 40 (2006) 41–51.
[8] T. Calì, D. Ottolini, A. Negro, M. Brini, α-Synuclein controls mitochondrial calcium homeostasis by enhancing endoplasmic reticulum–mitochondria interactions, Journal of Biological Chemistry 287 (2012) 17914–17929.
[9] S. de la Fuente, R.I. Fonteriz, P.J. de la Cruz, M. Montero, J. Alvarez, Mitochondrial free [Ca^{2+}] dynamics measured with a novel low-Ca^{2+} affinity aequorin probe, Biochemical Journal 445 (2012) 371–376.
[10] M. Montero, C.D. Lobatón, A. Moreno, J. Alvarez, A novel regulatory mechanism of the mitochondrial Ca^{2+} uniporter revealed by the p38 mitogen-activated protein kinase inhibitor SB202190, FASEB Journal 16 (2002) 1955–1957.
[11] L. Vay, E. Hernández-SanMiguel, C.D. Lobatón, A. Moreno, M. Montero, J. Alvarez, Mitochondrial free [Ca^{2+}] levels and the permeability transition, Cell Calcium 45 (2009) 243–250.
[12] D.G. Moisescu, C.C. Ashley, The effect of physiologically occurring cations upon aequorin light emission. Determination of the binding constants, Biochimica et Biophysica Acta 460 (1977) 189–205.
[13] T.J. Collins, P. Lipp, M.J. Berridge, W. Li, M.D. Bootman, Inositol 1,4,5-trisphosphate-induced Ca^{2+} release is inhibited by mitochondrial depolarization, Biochemical Journal 347 (2000) 593–600.
[14] M. Hoth, C.M. Fanger, R.S.J. Lewis, Mitochondrial regulation of store-operated calcium signaling in T lymphocytes, Journal of Cell Biology 137 (1997) 633–648.
[15] A. Quintana, M. Pasche, C. Junker, D. Al-Ansary, H. Rieger, C. Kummerow, L. Nuñez, C. Villalobos, P. Meraner, U. Becherer, J. Rettig, B.A. Niemeyer, M. Hoth, Calcium microdomains at the immunological synapse: how ORAI channels, mitochondria and calcium pumps generate local calcium signals for efficient T-cell activation, EMBO Journal 30 (2011) 3895–3912.
[16] M. Montero, J. Alvarez, W.J. Scheenen, R. Rizzuto, J. Meldolesi, T. Pozzan, Ca^{2+} homeostasis in the endoplasmic reticulum: coexistence of high and low [Ca^{2+}] subcompartments in intact HeLa cells, Journal of Cell Biology 139 (1997) 601–611.

[17] M. Montero, M. Brini, R. Marsault, J. Alvarez, R. Sitia, T. Pozzan, R. Rizzuto, Monitoring dynamic changes in free Ca^{2+} concentration in the endoplasmic reticulum of intact cells, EMBO Journal 14 (1995) 5467–5475.
[18] M.T. Alonso, M.J. Barrero, E. Carnicero, M. Montero, J. Garcia-Sancho, J. Alvarez, Functional measurements of $[Ca^{2+}]$ in the endoplasmic reticulum using a herpes virus to deliver targeted aequorin, Cell Calcium 24 (1998) 87–96.
[19] A.E. Palmer, C. Jin, J.C. Reed, R.Y. Tsien, Bcl-2-mediated alterations in endoplasmic reticulum Ca^{2+} analyzed with an improved genetically encoded fluorescent sensor, Proceedings of the National Academy of Sciences of the United States of America 101 (2004) 17404–17409.
[20] J. Zou, A.M. Hofer, M.M. Lurtz, G. Gadda, A.L. Ellis, N. Chen, Y. Huang, A. Holder, Y. Ye, C.F. Louis, K. Welshhans, V. Rehder, J.J. Yang, Developing sensors for real-time measurement of high Ca^{2+} concentrations, Biochemistry 46 (2007) 12275–12288.
[21] M.T. Alonso, M.J. Barrero, P. Michelena, E. Carnicero, I. Cuchillo, A.G. García, J. García-Sancho, M. Montero, J. Alvarez, Ca^{2+}-induced Ca^{2+}release in chromaffin cells seen from inside the ER with targeted aequorin, Journal of Cell Biology 144 (1999) 241–254.
[22] R. Yu, P.M. Hinkle, Rapid turnover of calcium in the endoplasmic reticulum during signaling. Studies with cameleon calcium indicators, Journal of Biological Chemistry 275 (2000) 23648–23653.
[23] S. Arnaudeau, W.L. Kelley, J.V. Walsh Jr., N. Demaurex, Mitochondria recycle Ca^{2+} to the endoplasmic reticulum and prevent the depletion of neighboring endoplasmic reticulum regions, Journal of Biological Chemistry 276 (2001) 29430–29439.
[24] N. Demaurex, M. Frieden, Measurements of the free luminal ER Ca^{2+} concentration with targeted “cameleon” fluorescent proteins, Cell Calcium 34 (2003) 109–119.
[25] R. Rudolf, P.J. Magalhães, T. Pozzan, Direct in vivo monitoring of sarcoplasmic reticulum Ca^{2+} and cytosolic cAMP dynamics in mouse skeletal muscle, Journal of Cell Biology 173 (2006) 187–193.
[26] S. Tang, H.C. Wong, Z.M. Wang, Y. Huang, J. Zou, Y. Zhuo, A. Pennati, G. Gadda, O. Delbono, J.J. Yang, Design and application of a class of sensors to monitor Ca^{2+} dynamics in high Ca^{2+} concentration cellular compartments, Proceedings of the National Academy of Sciences of the United States of America 108 (2011) 16265–16270.
[27] M. Sztretye, J. Yi, L. Figueroa, J. Zhou, L. Royer, E. Ríos, D4cpv-calsequestrin: a sensitive ratiometric biosensor accurately targeted to the calcium store of skeletal muscle, Journal of General Physiology 138 (2011) 211–229.
[28] W. Chen, C. Steenbergen, L.A. Levy, J. Vance, R.E. London, E. Murphy, Measurement of free Ca^{2+} in sarcoplasmic reticulum in perfused rabbit heart loaded with 1,2-bis(2-amino-5,6-difluorophenoxy)ethane-N,N,N′,N′-tetraacetic acid by ^{19}F NMR, Journal of Biological Chemistry 271 (1996) 7398–7403.
[29] I.M. Manjarrés, M.T. Alonso, J. García-Sancho, Calcium entry-calcium refilling (CECR) coupling between store-operated Ca^{2+} entry and sarco/endoplasmic reticulum Ca^{2+}-ATPase, Cell Calcium 49 (2011) 153–161.
[30] M. Montero, M.T. Alonso, E. Carnicero, I. Cuchillo-Ibañez, A. Albillos, A.G. Garcia, J. Garcia-Sancho, J. Alvarez, Chromaffin-cell stimulation triggers fast millimolar mitochondrial Ca^{2+} transients that modulate secretion, Nature Cell Biology 2 (2000) 57–61.