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Mitochondrial and ER-targeted eCALWY probes reveal high levels of free Zn^{2+}

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Abstract

Zinc (Zn^{2+}) ions are increasingly recognised as playing an important role in cellular physiology. Whereas the free Zn^{2+} concentration in the cytosol has been established to be 0.1-1 nM, the free Zn^{2+} concentration in subcellular organelles is not well-established. Here, we extend the eCALWY family of genetically-encoded Förster Resonance Energy Transfer (FRET) Zn^{2+} probes to permit measurements in the endo(sarco)plasmic reticulum (ER) and mitochondrial matrix. Deployed in a range of mammalian cell types, these probes reveal resting mitochondrial free $[Zn^{2+}]$ values of ~ 300 pM, somewhat lower than in the cytosol but three orders of magnitude higher than recently reported using an alternative FRET-based sensor. By contrast, free ER $[Zn^{2+}]$ was found to be ≥5 nM, which is >5000-fold higher than recently reported, but consistent with the proposed role of the ER as a mobilisable Zn^{2+} store. Treatment of β-cells or cardiomyocytes with sarco(endo)plasmic reticulum Ca^{2+}-ATPase inhibitors, mobilization of ER Ca^{2+} after purinergic stimulation with ATP, or manipulation of ER redox, exerted no detectable effects on $[Zn^{2+}]_{ER}$. These findings question the previously proposed role of Ca^{2+} in Zn^{2+} mobilization from the ER and suggest that high ER Zn^{2+} levels may be an important aspect of cellular homeostasis.

Zinc is an essential trace element in all living cells, binding to as many as 3,000 proteins (~10% of human proteome).1 In addition to its structural role in proteins, zinc is also an important cofactor in more than 300 metalloenzymes2 and redox sensors.3 Because of its role in fundamental cellular processes such as transcription, cell proliferation, apoptosis, and possibly signal transduction,2,4 precise regulation of the uptake, storage and availability of zinc ions is vital. To regulate intracellular free Zn^{2+} concentrations, an elaborate system exists in mammalian cells involving metal binding proteins such as metallothionein (MT), 3,5 zinc transporters (ZnT, encoded by *Slc30a1-10*) and zinc importers (ZiP, Zrt- and Irt-like proteins encoded by *Slc39a1-14*).6 High Zn^{2+} concentrations (total and free) within the

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endoplasmic reticulum or other organelles may conceivably provide a mobilisable store (or buffer) for these ions which might be targeted by hormones or other stimuli to provoke changes in cytosolic free Zn^{2+} concentration.7–10 At present, however, evidence for such fluxes of zinc in individual cells is sparse and sometimes conflicting.

We have recently described a new generation of genetically-encoded Zn^{2+} probes termed "eCALWYs".11 These Förster Resonance Energy Transfer (FRET)-based probes consist of two cysteine-containing metal binding domains (ATOX1 and WD4) connected by a long, flexible glycine-serine linker and flanked by GFP-derived cerulean and citrine fluorescent domains (Figure 1a). In the absence of zinc an intramolecular complex is formed between cerulean and citrine which results in high FRET. Conversely, when Zn^{2+} binds between ATOX and WD4, the interaction between cerulean and citrine is disrupted, lowering the citrine:cerulean fluorescence intensity ratio. Shortening of the linker length between the metal binding domains and/or mutation of one of the metal binding cysteines in the WD4 domain yielded a series of sensor variants (eCALWY1-6) with Zn^{2+} affinities ranging between 2 pM and 5 nM, while all displaying at least a two-fold change in emission ratio. 11,12 Mid-range in this sensor toolbox, eCALWY-4 has a dissociation constant (K_d = 630 pM) that is appropriate for *in cellulo* measurements and has therefore been used successfully to determine cytosolic free zinc concentrations in HEK293, HeLa, primary and immortalised pancreatic β-cells.11–13

The high picomolar (400-1500 pM) values obtained for $[Zn^{2+}]_{cyt}$ are close to those measured in other secretory and non-secretory cell types using synthetic, intracellularly-trappable fluorescent probes such as FluoZin-3,1,14 but considerably higher than measurements made using carbonic anhydrase-(CA) based sensors in PC12 cells.15,16 Subsequent studies17,18 using the ZapCY2 FRET-sensor protein reported values that, albeit somewhat lower, are in the same range as those determined using the eCALWY probes. These free Zn^{2+} levels are buffered by the cytosolic metallothionein system and are sufficient to supply Zn^{2+} to endogenous zinc binding proteins, yet below those levels that are known to inhibitory to several metabolic enzymes.1

Recent reports using the ZapCY1 FRET sensor17,19 have suggested that concentrations of free Zn^{2+} may be far lower in the ER, Golgi (~1 pM) and mitochondria (~0.1 pM) than those reported in the cytosol. A value of 0.2 pM has also been reported using a mitochondria-targeted CA-based sensor,16 while a substantially higher concentration of 72 pM was recently determined using a small molecule ratiometric fluorescent probe targeted to the mitochondria of NIH3T3 cells.20 The low pM values would appear to argue against a role for the latter pools in regulating cytosolic zinc levels, or providing a mobilisable source of these ions whose release could be triggered in response to extracellular stimuli or other environmental changes. Organellar Zn^{2+} measurements with the eCALWY probes, which provide a large range of K_d values and robust FRET responses, have so far only been reported for the secretory granule.11 Here, we report targeting of the eCALWYs to the endoplasmic reticulum or mitochondrial matrix in a variety of mammalian cell types including primary pancreatic β-cells and cardiomyocytes. Surprisingly, the eCALWY probes show free Zn^{2+} concentrations in the mitochondria (300 pM) and ER (>5 nM) that are different from the cytosol and three orders of magnitude higher than those reported with the

ZapCY1 sensor. Importantly, these results suggest that ER/SR Zn^{2+} concentrations might be far higher and more resistant to the effects of cell stimulation and Ca^{2+} fluxes across the ER membrane, than proposed.17,19

Results and Discussion

Targeting eCALWY probes to the ER/SR and mitochondria

Examined in the murine pancreatic β-cells-derived line, MIN6, non-targeted eCALWY-4 displayed the expected cytosolic localisation (Figure 1b). We targeted the probe to the mitochondrial matrix by fusion with cytochrome *c* oxidase subunit VIII (Cox VIII) signal peptide,21 generating the new reporter mito-eCALWY-4. This sequence has previously been used to target other probes, including aequorin,21,22 to mitochondria and, after cleavage, is expected to lead to the liberation of the active soluble reporter in the matrix.

Co-expression of mito-eCALWY-4 with the genetically-encoded mitotracker DsRed-mito showed essentially complete overlap of fluorescence, confirming correct targeting of the zinc probe to this compartment (Figure 1c). Secondly, we targeted eCALWY-4 to the ER (ER-eCALWY-4) by the addition of an N-terminal preproinsulin (PPI) sequence as a signal peptide and a C-terminal Lys-Asp-Glu-Leu (KDEL) sequence, inducing probe retention within the ER lumen.23 Co-expression with a genetically-encoded ER-tracker (DsRed-ER) demonstrated precise localisation to the target compartment in MIN6 β-cells (Figure 1d). After packaging into adenoviral particles24, correct organellar localisation of the expressed targeted probes was also demonstrated in primary rat cardiomyocytes (Figure S1).

Figure 2 shows representative traces illustrating the behaviour of cytosolic eCALWY-4, mito-eCALWY-4 or ER-eCALWY-4/6 in primary murine β-cells (C57BL/6), primary rat cardiomyocytes and MIN6 cells. As discussed below, ER-eCALWY6 (K_d = 2.9 nM) was designed to extend the range of concentrations over which Zn^{2+} could be measured in this compartment. ER-eCALWY-6 probe localisation was identical to ER-eCALWY-4 localisation (not shown). Collected data from similar experiments in these and other cell types (Figure S2) are provided in Table 1.

Cells expressing eCALWY-4 and mito-eCALWY-4 displayed large changes in FRET ratio upon addition of the membrane permeable chelator TPEN and subsequent addition of excess $ZnCl_2$ in the presence of the ionophore pyrithione (Figure 2a-f). This calibration allowed determination of $[Zn^{2+}]_{cyt}$ for each individual cell, yielding values of 0.6-2.2 nM that are broadly consistent with previous reports with this probe in β-cell lines11 though slightly higher. A variable percentage of cells of each type analysed presented with a saturated response, meaning that in these cells $[Zn^{2+}]_{cyt}$ was ≥5 nM (given a K_d value of the probe of 630 pM).11

The occupancy of the mito-eCALWY-4 probes was found to be ~80% in all cells examined. The Zn^{2+} affinity of the eCALWY probes is pH dependent11 and their use in different organelles requires that the K_d value is determined at the relevant pH. Since intramitochondrial pH values are typically ~7.825 we re-determined the K_d value of eCALWY-4 at this pH and obtained a value of 60 pM (Figure S3). Using this value, the

mitochondrial zinc concentrations were determined to be 0.2-0.3 nM in each of the cell types interrogated (Table 1). While this concentration is somewhat lower than typically observed in the cytosol, these values are 3 orders of magnitude higher than recently reported using a mitochrondria-targeted ZapCY1 FRET sensor. In this case, the percentage of cells in which the Zn^{2+} probe was saturated was low, indicative of relatively low cell-cell variability (Table 1).

ER-eCALWY4 was found to be fully saturated at the start of the experiment in MIN6 (Figure 2i), HeLa and the great majority of HEK293 cells (80%) examined (Figure S2, Table 1), which means that $[Zn^{2+}]_{ER}$ was ≥ 5 nM. Even in the small percentage of HEK293 cells and primary cardiomyocytes in which ER-eCALWY-4 appeared not completely saturated, the average sensor occupancy was still > 80%, corresponding to $[Zn^{2+}]_{ER}$ = 2-4 nM. In each case, a population was also observed that did not respond to TPEN or Zn^{2+}/pyrithione, possibly reflecting mis-folding of the probe in the ER lumen.

Unfortunately, adenovirus-mediated expression of ER-eCALWY-4 failed to lead to the production of a Zn^{2+}-sensitive reporter in primary β-cells (61 cells examined from 5 mice; Figure 2g), and the adenovirally-delivered probe was similarly unresponsive in HEK293 and MIN6 cells. On the other hand expressed in primary cardiomyocytes, ER-eCALWY-4 responded to zinc changes in a substantial minority of cells examined (8/23 from 6 rats), whereas in the remaining cells the probe did not respond to TPEN or Zn^{2+}/pyrithione (again, likely to reflect mis-folding of the probe in this compartment). The above value provides an estimate for free Zn^{2+} in the ER and/or SR of cardiomyocytes of ~4 nM or above (Table 1).

Given the observation that $[Zn^{2+}]_{ER}$ values were close to, or above, levels where ER-eCALWY-4 was saturated in the majority of cell types examined, we turned next to the use of the less Zn^{2+}-sensitive (K_d = 2.9 nM) probe, ER-eCALWY-6. In this case, the proportion of cells displaying probe saturation was substantially lower (44, 50 and 0% in HEK293, MIN6 and cardiomyocytes, respectively, Table 1) returning $[Zn^{2+}]_{ER}$ values in the range 5-7 nM in each case. Of note, the change in emission ratio upon calibration was low with ER-eCALWY-6, limiting the precision of the measurements with this probe.

Free Zn^{2+} concentrations in the cytosol and ER remain stable in MIN6 cells, primary β-cells and cardiomyocytes during large excursions in ER calcium content and redox state

Using a zinc finger-based probe (ER-ZapCY1) Palmer and colleagues17 recently reported that $[Zn^{2+}]_{ER}$ is lowered by mobilisation of Ca^{2+} from this store in HeLa cells. Similarly, others26 reported an action of Ca^{2+} store depletion to increase cytosolic Zn^{2+} in cortical neurones. We therefore used simultaneous imaging of Zn^{2+} alongside cytosolic Ca^{2+}, monitored with Fura-2, to determine whether a similar phenomenon may be evident in excitable β-cells or heart cells.

Contrary to this expectation, cyclopiazonic acid (CPA), a potent inhibitor of sarco(endo)plasmic reticulum Ca^{2+}ATPase (SERCA) pumps, whose action leads to ER Ca^{2+} release via leak channels,27 exerted no significant effect on cytosolic Zn^{2+} in primary β-cells (Figure 3a,b) or myocytes (Figure 3c) whilst clearly mobilising ER Ca^{2+} (as measured indirectly via changes in cytosolic Ca^{2+} in β-cells [red traces] or via cell contraction in

myocytes; not shown). Thus, the stability of the cytosolic Zn^{2+} concentration under these conditions strongly suggests the absence of zinc release from the ER.

The latter result was unaffected by removal of calcium from the medium in primary β-cells (Figure 3b), a condition under which cytosolic calcium signals are not confounded by Ca^{2+} influx from the extracellular medium. Likewise, neither CPA nor thapsigargin, another SERCA pump inhibitor,27,28 substantially affected $[Zn^{2+}]_{cyt}$ (Figure 4a,c,g) or caused $[Zn^{2+}]_{ER}$ to fall below 5 nM (Figure 4b,d,h) in MIN6 β-cells. Again, similar observations were made in the absence of external calcium (Figure S4a,b). Furthermore, stimulation of MIN6 cells with the purinoreceptor agonist ATP,29 which prompts robust and rapid release of ER calcium through inositol 1,4,5 *tris*phosphate receptors, exerted no detectable effect on $[Zn^{2+}]_{cyt}$ or in apparent $[Zn^{2+}]_{ER}$, either in the presence (Figure 4e-h) or in absence of external calcium (Figure S4c,d).

The ER redox state is known to be substantially more oxidising than the cytosol,30 providing a permissive environment for disulphide bond formation and protein folding. We therefore also determined whether fluctuations in ER redox, which may conceivably occur during the stimulation of pancreatic β-cells with fuels31 and might influence $[Zn^{2+}]_{ER}$. To test this possibility, cells were treated with the reducing agent dithiothreitol (DTT), or with the oxidising agent 2-2'-dithiodipyridine (DPS), manoeuvres which led to the expected changes in ER redox state, as monitored with the targeted redox probe ERroGFPiE32 (Figure 5a). By contrast, excursions in $[Zn^{2+}]_{ER}$ elicited by the sequential addition of TPEN and Zn^{2+}/pyrithione had no impact on ER redox state (Figure 5b). Likewise, forced changes in redox had no evident impact on ER-eCALWY-4 ratio (Figure 5c). Considering that the probe has been shown to be zinc-saturated under these conditions (Figure 2i) this indicates that $[Zn^{2+}]_{ER}$ remains above 5 nM.

Discussion

Our major aim in the present study was to compare the free concentration of Zn^{2+} ions in three subcellular locations: the cytosol, ER lumen and mitochondrial matrix, in a variety of mammalian cell types. To this end, we addressed the recently developed and robust eCALWY zinc sensors11 to these compartments.

The current experiments revealed, firstly, that cytosolic $[Zn^{2+}]$ values can vary between different cell types when assessed using an identical probe and the same imaging system. Thus, previous reports1 of variations reported in separate studies and using different probes may represent genuine differences between cell types.18 Moreover, we12 recently reported that long term (24 h) culture at elevated glucose concentrations increased free cytosolic Zn^{2+}, as reported with eCALWY-4, showing that variations can exist in this parameter for a given cell type depending on culture conditions. We also noted during the course of the present studies that considerable cell-cell variation in cytosolic Zn^{2+} was apparent in cardiomyocytes, and was related to the apparent health of individual cells: those with abnormal (rounded) morphology tended to display higher $[Zn^{2+}]_{cyt}$ values (Tuncay *et al*, in preparation).

We did, however, find a higher cytosolic zinc concentration in HEK293 cells than previously determined with the same probe (1.8 vs 0.4 nM).11 We suspect that this difference is due to several factors including: the use here of a different batch of HEK293 cells, improvements in the transfection, acquisition, background correction and analysis protocols, and correction for photobleaching.

Next, we demonstrated that the limiting membranes of both mitochondria and the ER sustain significant gradients of Zn^{2+} ions *versus* the cytosolic compartment, with lower and higher free zinc levels respectively pertaining in these organelles. Across all the cell types examined here the mitochondrial free [Zn^{2+}] was ~2000-fold higher than the value of ~0.14 pM reported by Palmer and colleagues19 using the mito-ZapCY1 sensor in HeLa cells and the 0.2 pM obtained by McCranor et al using a CA-based sensor in PC12 cells.16 Our values are more in line with the 72 pM that was recently determined using a small-molecule ratiometric fluorescent probe targeted to the mitochondria of NIH3T3 cells.20 We based our calculation of mitochondrial free [Zn^{2+}] on a mitochondrial pH value of 7.8. This pH may be less in pancreatic β-cells where lower intramitochondrial pH values have been reported (~7.3), at least in clonal insulinoma-derived INS1E cells at low glucose concentrations.33 Should the latter pH values pertain then the present data would translate into an even higher concentration of free Zn^{2+}, suggesting that β-cells may be different from the other cell lines studies here in maintaining a smaller gradient (out *vs* in) for mitochondrial Zn^{2+}. Elevated concentrations of Zn^{2+} such as those that occur following ischemia34 are known to inhibit mitochondrial energy metabolism, and several enzyme systems in respiratory metabolism have been reported to be inhibited by Zn^{2+}, including the cytochrome bc1 complex and α-ketoglutarate dehydrogenase.35,36 Thus, the work of Brown and co-workers37 showed that the lipoamide dehydrogenase reaction catalysed by the latter enzyme is inhibited by Zn^{2+}, whereas the oxidase reaction that results in the production of hydrogen peroxide is enhanced 5-fold. However, the apparent inhibition and activation constants for Zn^{2+} (0.15 μM and 0.09 μM, respectively) are still much higher than the 300 pM mitochondrial Zn^{2+} concentrations obtained in this work. To the best of our knowledge, there is no evidence of mitochondrial enzymes that are inhibited by sub-nanomolar Zn^{2+} concentrations.

Whilst we show that our new ER probes function well in cell lines after conventional transfection of plasmids, delivery of these probes using adenoviral vectors (necessary for efficient expression of the probe in primary cells) was more problematic. Although the reasons behind this are not obvious, we were able to exclude proteolysis by the absence of degraded products in Western analysis (not shown). Notably, infection was also problematic in MIN6 cells (data not shown), strongly indicating that stresses induced by infection are likely to be the cause of the unresponsiveness. Interestingly, probe localisation to the ER was unaffected while the steady-state emission ratio was unusually low (~1), suggesting mis-folding of the probe.

Successfully deployed in the clonal MIN6 cell line upon transfection, as well as two other transformed cell lines (HEK293, HeLa), the ER-eCALWY probes were saturated with Zn^{2+} in the majority of cells. These observations suggested that the concentration of Zn^{2+} ions in the ER was likely to be in excess of ~5 nM (i.e. some 8-10-fold above the K_d of the probe for Zn^{2+}, ~600 pM). Measurements achieved with the less Zn^{2+}-sensitive, but more weakly

responding, ER-eCALWY-6, further indicated that ER/SR concentrations are likely to be around 5-8 nM in about 50 % of HEK293 and MIN6 cells, where the probe was not fully saturated and higher in the remaining cells. Likewise, in primary cardiomyocytes, $[Zn^{2+}]_{ER}$ was ≥ 6 nM (Table 1). Taken together, across all the cell types tested here, we conclude that ER/SR free zinc concentrations are substantially higher than the cytosolic zinc values. The values reported here for the ER are more compatible with the known coordination chemistry of thiols which, through metallothionein in particular, create high affinity Zn^{2+} binding sites in the cytosol. In the oxidising environment of the ER (and Golgi), free thiols are not available to provide these high affinity Zn^{2+} binding sites, which means that one would have to invoke other mechanisms (e.g. vigorous pumping/exchange across the ER membrane) to maintain free Zn^{2+} concentrations of 1 pM or lower.

Importantly, the $[Zn^{2+}]_{ER}$ values (5 nM or above) reported here seem more compatible with the view that the ER compartment may provide a sink or store for Zn^{2+} ions8 than might be surmised from measurements of <1 pM.17 Indeed, studies in the past few years have suggested that changes in cytosolic free Zn^{2+} may be an important means of transmitting signals generated at the plasma membrane across the cytoplasmic space, at least in immune cells.7–10 Similarly, an influx of Zn^{2+} across the plasma membrane has been reported in several studies,38 notably in response to depolarisation of excitable cells including primary β-cells39 (P.C., G.M., G.A.R., unpublished). However, more controversial has been the suggestion that the ER may serve as a mobilisable store of these ions in such cells. Indeed, the recent work by Qin et al17 using ER-ZapCY1 probe suggested that Zn^{2+} may decrease in the ER during Ca^{2+} mobilisation in HeLa cells (albeit an initial concentration lower than 1 pM). Moreover, a study26 reported an action of Ca^{2+} store depletion by thapsigargin to increase cytosolic Zn^{2+} in cortical neurones, and suggested a mobilisation of ER Zn^{2+}. The present report failed to replicate the findings of the latter study in β-cells and cardiomyocytes.

In these primary cell types, changes in $[Zn^{2+}]_{ER}$ were monitored indirectly by measuring $[Zn^{2+}]_{cyt}$ using eCALWY-4 sensor. However, in MIN6 cells (as in other cell lines), ER-eCALWY-4 was deployable in order to follow a potential decrease in ER Zn^{2+}.Thus, we found that a variety of manoeuvres causing drastic changes in cytosolic and/or ER calcium failed to decrease apparent $[Zn^{2+}]_{ER}$ below 5 nM in MIN6 and primary β-cells as well as cardiomyocytes (Figure 4). Whilst we have no clear explanation for this difference between the present results and previous reports,17,19,26 we cannot exclude the possibility of differences in this respect between the relationship between ER Zn^{2+} and Ca^{2+} in HeLa17 or cortical neurones 26 and the cell types examined here. Furthermore, we note that an increase in ER Zn^{2+} would be difficult to detect with the ER probes used in this study, given their apparent near-saturation under resting conditions.

Both ER and mitochondrial zinc concentrations were drastically higher than recent estimates performed with the targeted ZapCY1 probes.17,19 This discrepancy cannot simply be explained by an error in determining the Zn^{2+} affinities of one of the sensors, since the eCALWY sensors and ZapCY1/CY2 sensors give much more consistent results when applied in the cytosol. To exclude the possibility that the high Ca^{2+} concentration in the ER (~ 200 μM40) affected the eCALWY-4 sensor, we performed a Ca^{2+} titration in vitro.

However, no significant response was observed upon addition of up to 860 μM Ca^{2+} (Figure S5).

We presently do not have a convincing explanation as to why the eCALWY and ZapCY-based sensors behave so differently when targeted to the ER and mitochondria. We note, however, that in the case of either probe, the calculated values for free Zn^{2+} are dependent upon the K_d value *in situ* remaining identical to that measured *in vitro*. Whilst known variables such as pH can be accounted for, the binding of other species, or protein mis-folding, could affect these values. Unfortunately, precise measurements of *in situ* K_d for the organelle-targeted probes are very difficult to perform, as they would require equilibration of Zn^{2+} ions across two (or three in the case of mitochondria) separate membranes. Importantly, in the present study, we obtained no evidence that disulphide bridge formation in the oxidising environment of the ER may lead to a change in Zn^{2+} binding for ER-eCALWY4 (Figure 5 and PC, unpublished results). In addition, partial oxidation of the cysteines present in eCALWY4 would be expected to decrease its affinity for Zn^{2+}, and would therefore not provide an explanation for the differences observed with the ZapCY1-probe. One way to resolve these discrepancies might be to develop and test FRET-sensors that use alternative binding mechanisms.

In summary, the present report revises upwards recent estimates17,19 of Zn^{2+} concentrations in the mitochondrial matrix and the ER lumen in a range of mammalian cell types and casts doubt over the suggestions that significant Zn^{2+} fluxes occur across the ER membrane in response to Ca^{2+} mobilization from the latter organelle. Release of Zn^{2+} mediated by other forms of stimulation8 remains conceivable, especially given higher free ER Zn^{2+} concentrations than have latterly been assumed.17

Methods

Supplemental methods are available in Supporting Information.

Generation of organelles-targeted eCALWY-4 and eCALWY-6

Cloning strategies to address eCALWY- probes to the mitochondrial matrix and ER is described in Supporting Information. Adenoviral vectors were generated using pAdeasy system as described earlier.41

Imaging of [Zn^{2+}] by FRET measurement

Cells on coverslips were washed twice in Krebs-Hepes-bicarbonate (KHB) buffer (140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 10 mM Hepes, 25 mM $NaHCO_3$), which was warmed, bubbled with 95:5 O_2:CO_2, set to pH 7.4, and contained 3 mM glucose. Imaging of [Zn^{2+}] using eCALWY sensors was carried out as optimized before.11,12 Briefly, cells were maintained at 37°C throughout with a heating stage (MC60, LINKAM, Scientific Instruments), and KHB was perifused (1.5 to 2 ml/minute) with additions as stated in the Figures. Images were captured at 433 nm monochromatic excitation wavelength (Polychrome IV, Till photonics) using an Olympus IX-70 wide-field microscope with a 40x/1.35NA oil immersion objective and a zyla sCMOS camera (Andor Technology) controlled by Micromanager software.42 Acquisition rate was 20 images/

minute. Emitted light was splitted and filtered by a Dual-View beam splitter (Photometrics) equipped with a 505dcxn dichroic mirror and two emission filters (Chroma Technology - D470/24 for cerulean and D535/30 for citrine).

Image analysis was performed with ImageJ software43 using a home-made macro and the fluorescence emission ratios were derived after subtracting background. We observed that during acquisition, photobleaching gradually decreased the steady-state ratio with a linear kinetic (not shown). This drift was thus, when necessary, corrected in function of time with a constant factor. Steady-state fluorescence intensity ratio citrine/cerulean (R) was measured, then maximum and minimum ratios were determined to calculate free Zn^{2+} concentration using the following formula: $[Zn^{2+}] = K_d \times (R_{max}-R)/(R-R_{min})$. The maximum ratio ($R_{max}$) was obtained upon intracellular zinc chelation with 50 μM TPEN and the minimum ratio (R_{min}) was obtain upon zinc saturation with 100 μM $ZnCl_2$ in the presence of the Zn^{2+} ionophore, pyrithione (5 μM).11

Simultaneous imaging of Zn^{2+} and Ca^{2+}

eCALWY-expressing cells were preincubated with 3 μM Fura-2AM (Invitrogen) in KHB prior to imaging. eCALWYs signal was collected as described previously and Fura-2 signal was collected at 340 and 380 nm excitation wavelengths through the D535/30 emission filter. Fura-2 fluorescence emission ratio 340/380 was determined after subtracting background using a home-made macro. Acquisition rates are specified in the figure legends.

Statistics

Data are presented as mean ± S.E.M. unless otherwise stated. Differences were determined using Student's *t*-test with Bonferroni correction for multiple comparisons as required using GraphPad Prism. P-values <0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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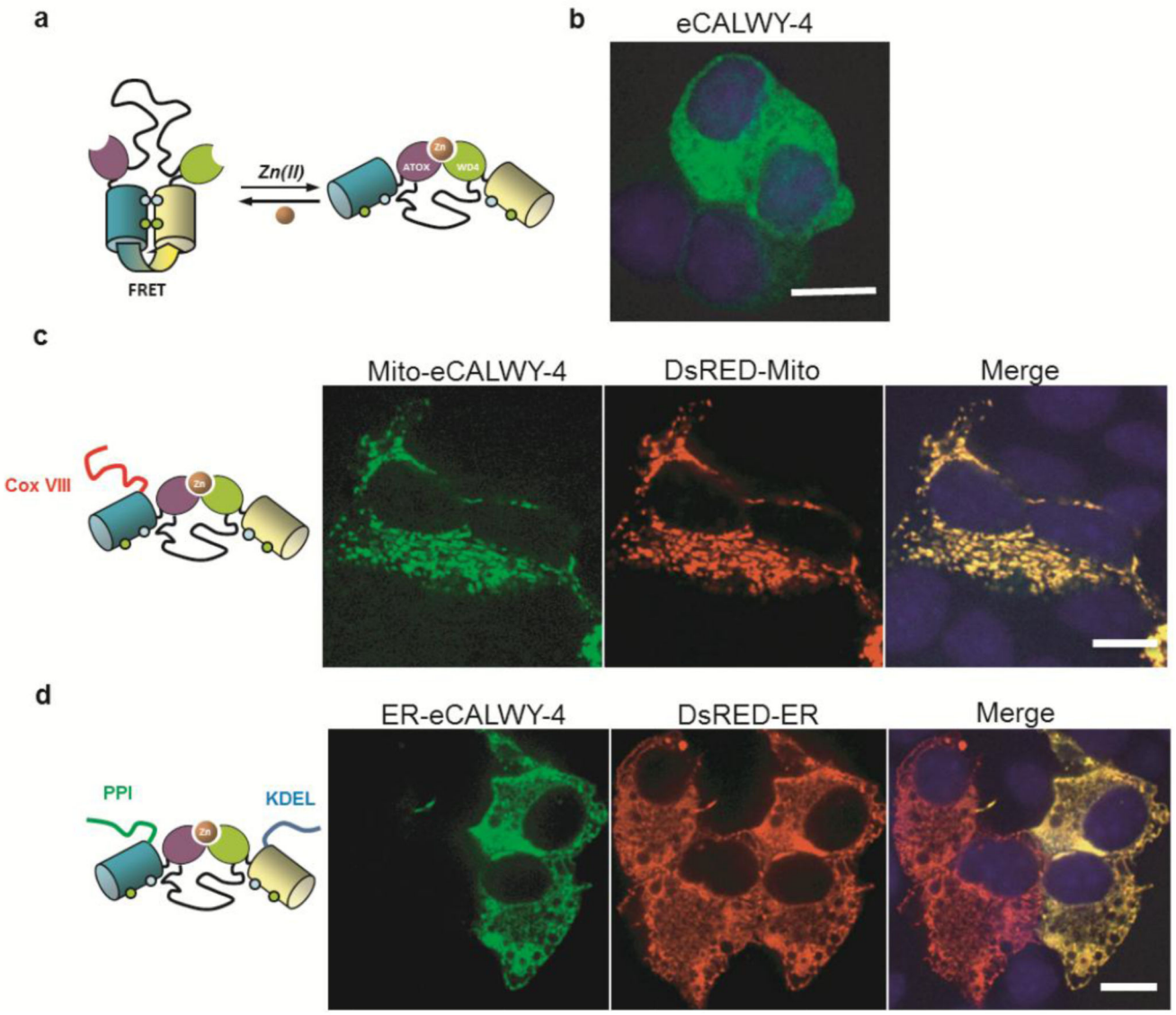

Figure 1. Design and subcellular localisation of mito-eCALWY-4 and ER-eCALWY-4 in MIN6 β-cells.

(a) eCALWY-4 sensor design11 and (b) localization in the cytosol in MIN6 cells. (c) MIN6 cells were co-transfected with an eCALWY-4 construct targeted to the mitochondria (mito-eCALWY-4) and with a mitochondrial marker (DsRED-Mito). (d) MIN6 cells were co-transfected with an eCALWY-4 construct targeted to the Endoplasmic Reticulum (ER-eCALWY-4) and with an ER marker (DsRED-ER). Images were acquired on a spinning-disk system with a 63x/NA1.4 objective. Merged images show a precise localisation in the targeted organelles. Scale bars represent 10 μm.

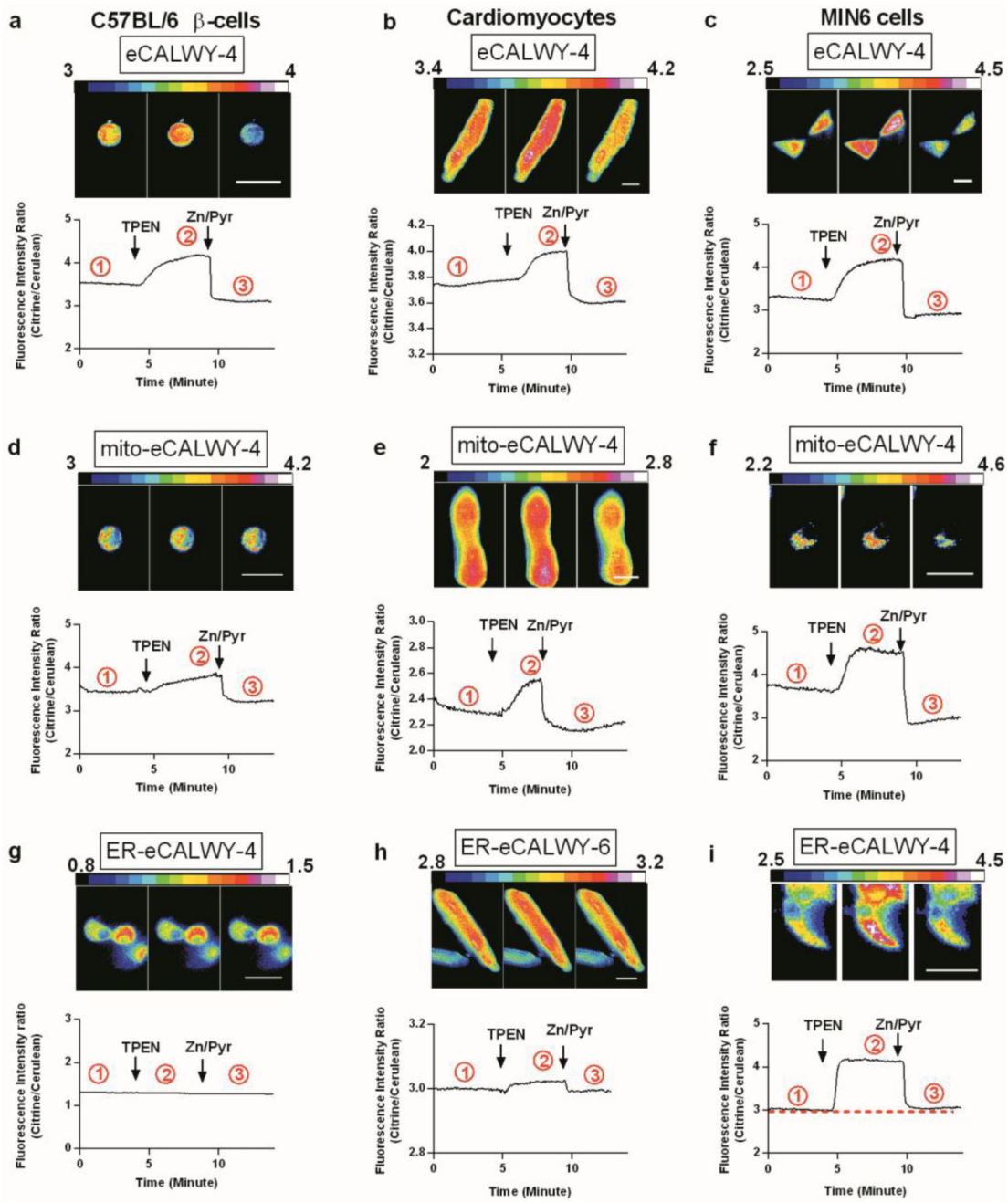

Figure 2. Measurement of resting compartmentalised free Zn^{2+} concentrations in primary murine β-cells, primary rat cardiomyocytes and MIN6 cells.

Representative traces and ratiometric images are shown for primary murine β-cells (a,d,g), ventricular cardiomyocytes (b,e,h) and MIN6 cells (c,f,i) expressing cytosolic, mitochondrial or ER-targeted sensors. Murine β-cells and rat cardiomyocytes were transduced with adenoviral particles and MIN6 cells were transfected with plasmid constructs. Acquisitions were performed 16-24 h after transfection or infection, and during perifusion with KREBS-Hepes-Bicarbonate (KHB) buffer. Steady state fluorescence intensity ratio (Citrine/Cerulean) was first measured before the maximum ratio was obtained under perifusion with

KHB buffer containing 50 μM TPEN (Zinc-free condition). Finally minimum ratio was obtained under perfusion with KHB buffer containing 5 μM pyrithione and 100 μM Zn^{2+} (Zinc-saturated condition). Ratio images were captured at the points (1,2,3) on the traces circled. Scale bars represent 20 μm.

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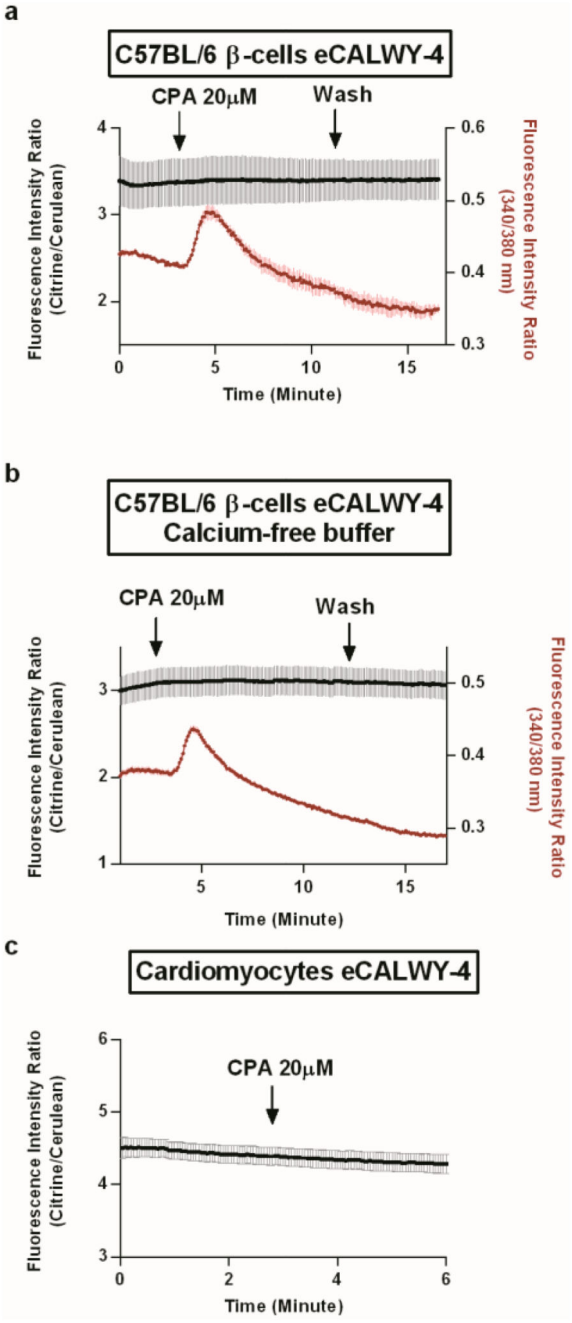

Figure 3. SERCA inhibition with CPA and ER calcium depletion with has no effect on cytosolic free Zn^{2+} in primary murine β-cells and primary rat cardiomyocytes.

(a,b) Primary β-cells expressing eCALWY-4 were preincubated with Fura-2 (3μM) in KHB for 20 minutes before acquisition. Acquisitions rate was 12 images per minutes and fluorescence intensity ratios were determined sequentially for eCALWY-4 and Fura-2. Representative results are shown (a) Cells were perifused with KHB and after 3 minutes were treated with CPA 20 μM (n = 3 cells). (b) Cells were perifused with KHB containing 100 μM EGTA (Calcium-free buffer) and treated in the same conditions (n = 7 cells). (c)

Cardiomyocytes expressing eCALWY-4 were perfused with KHB for 3 minutes then with KHB containing 20 μM CPA (n = 4 cells). Data are means ± S.E.M.

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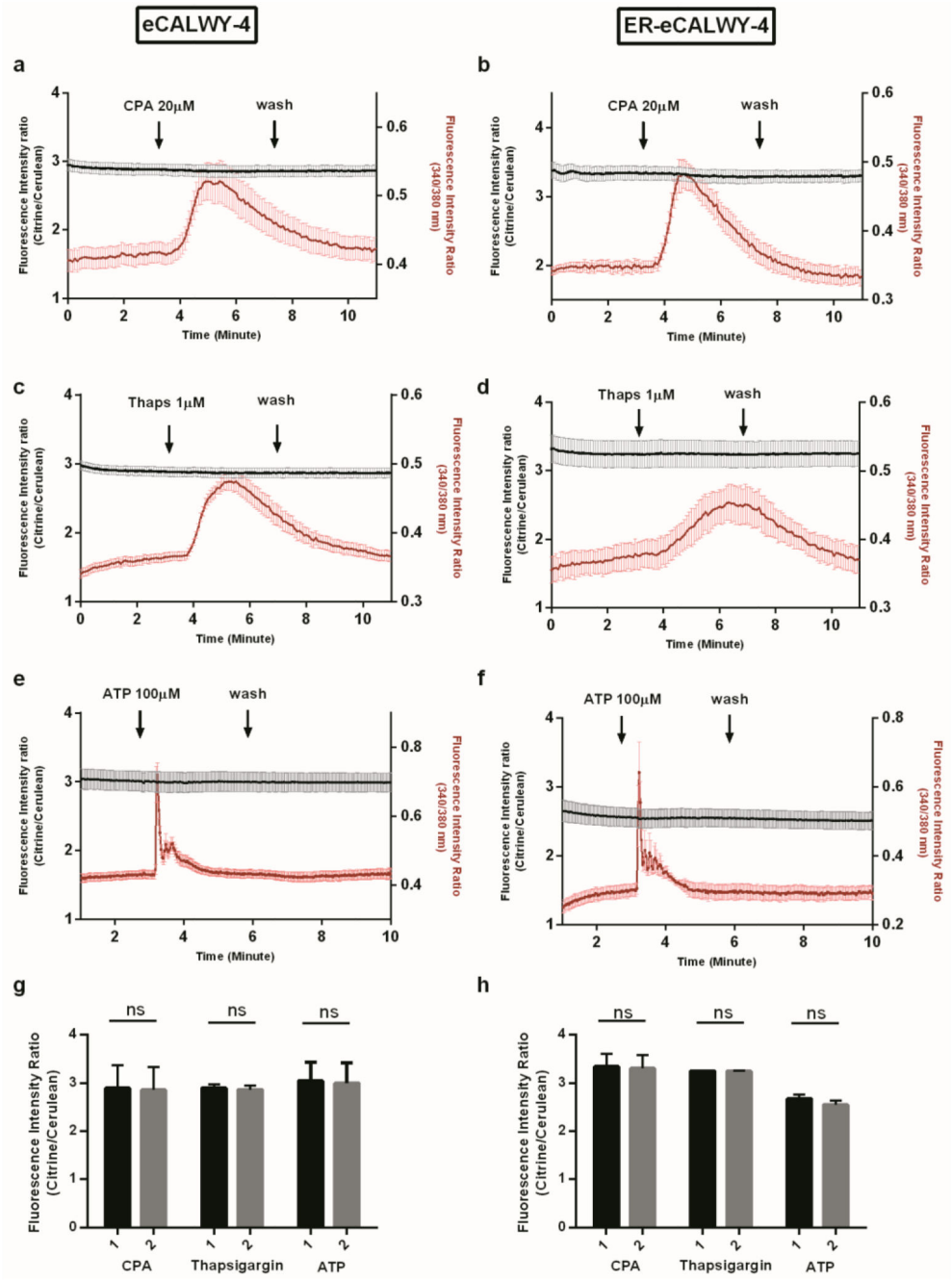

Figure 4. Simultaneous recording of free ER Zn^{2+} and cytosolic free Ca^{2+} in response to CPA, thapsigargin or ATP treatment of MIN6 cells.

MIN6 cells expressing eCALWY-4 (a,c,e) or ER-eCALWY-4 (b,d,f) were incubated with Fura-2 (3 µM) in KHB for 20 minutes before acquisition. Fluorescence intensity ratios were determined sequentially for eCALWY-4 sensors and Fura-2. After 3 minutes of perfusion with KHB, cells were treated with different drugs. Acquisition rates were 20 images per minutes for CPA and thapsigargin experiments, and 30 images per minute for ATP experiments. Results displayed are representatives of the responses obtained. (a) MIN6 cells expressing eCALWY-4 were treated with 20 µM CPA (n = 6 cells) (b) MIN6 cells

expressing ER-eCALWY-4 were treated with 20 μM CPA (n = 6 cells) (c) MIN6 cells expressing eCALWY-4 were treated with 1 μM thapsigargin (n = 9 cells) (d) MIN6 cells expressing ER-eCALWY-4 were treated with 1 μM thapsigargin (n = 4 cells) (e) MIN6 cells expressing eCALWY-4 were treated with 100 μM ATP (n = 9 cells) (f) MIN6 cells expressing ER-eCALWY-4 were treated with 100 μM ATP (n = 3 cells). Data are means ± S.E.M.

(g,h) Mean ratios were calculated before (1) and during (2) calcium response for represented traces and for an acquisition time of 1 minute. (g) Mean ratios upon CPA, thapsigargin and ATP treatment for MIN6 cells expressing eCALWY-4. (h) Mean ratios upon CPA thapsigargin and ATP treatment for MIN6 cells expressing ER-eCALWY-4. Error bars represent S.D.

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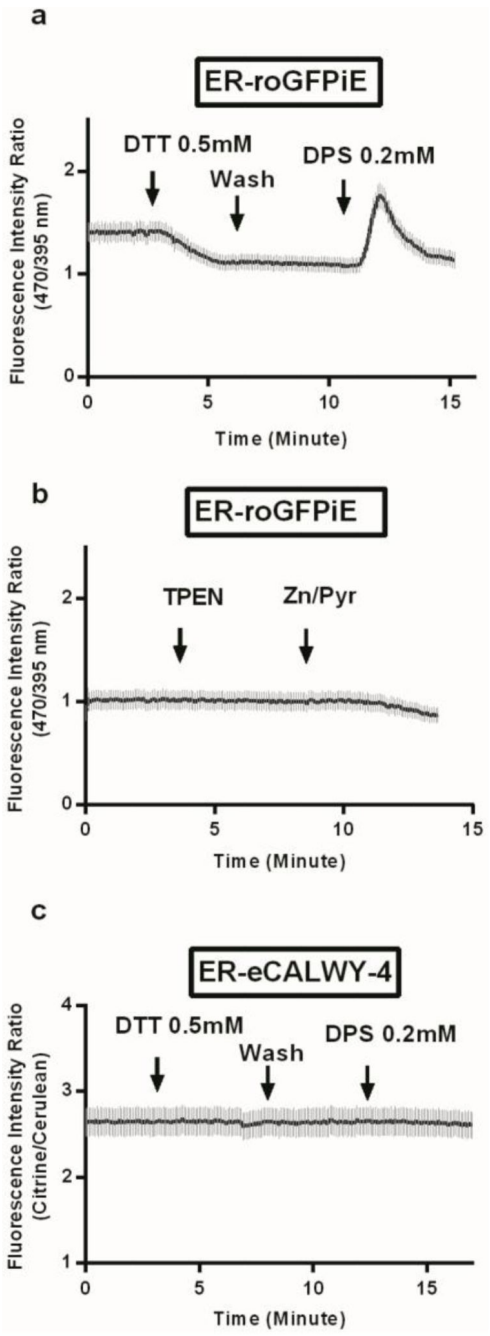

Figure 5. Forced changes in ER redox state do not impact ER free Zn^{2+} levels in HEK293 cells as reported with EReCALWY-4.

(a,b) HEK293 cells were transfected with a construct expressing the redox-sensitive GFP targeted to the ER, ERroGFPiE.32 (a) Redox status changes in the ER were monitored with ERroGFPiE: cells were perfused and treated as stated with reducing agent DTT and oxidising agent DPS (n = 8 cells). (b) TPEN and Zn^{2+}/pyrithione treatment on ER-roGFPiE-expressing cells showed no impact on the ER redox state (n = 7 cells). (c) ER-eCALWY4

expressing HeK cells were perfused and treated by DTT and DPS, which had no effect on fluorescence intensity ratio (n = 8 cells). Data are means ± S.E.M.

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Table 1

Cytosolic, mitochondrial and ER free Zn^{2+} concentrations measured in different cell types, using the indicated e-CALWY sensor.

N values indicate number of cells (non-saturated) used in quantitation of Zn^{2+} and total cells analysed (parentheses). Where 100 % of cells were saturated, values given are based on an estimate from the Kd of the probe used. Values are means (± S.E.M) - nd, not determined.

Cell type	Cytosolic Concentration (nM) e-CALWY-4	n value	Percentage of cells saturated	Mitochochondrial Concentration (nM) mito-eCALWY-4	n value	Percentage of cells saturated	ER Concentration (nM) ER-eCALWY-4	n value	Percentage of cells saturated	ER Concentration (nM) ER-eCALWY-6	n value	Percentage of cells saturated
HEK293	1.84 ± 0.18	37 (53)	30%	0.3 ± 0.04	5 (5)	0%	1.6 ± 0.26	7 (32)	78%	5.58 ± 0.7	6 (10)	44%
HeLa	1.18 ± 0.21	13 (20)	35%	0.23 ± 0.03	10 (12)	16%	> 5	0 (9)	100%	nd		
MIN6	1.89 ± 0.28	22 (42)	48%	0.25 ± 0.07	10 (13)	23%	> 5	0 (35)	100%	7.24 ± 1.0	2 (4)	50%
Rat cardiomyocytes	0.61 ± 0.07	42 (49)	14%	0.18 ± 0.04	18 (18)	0%	4.13 ± 1.75	3 (8)	62%	5.6 ± 1.1	6 (6)	0%
C57BL/6 beta cells	1.5 ± 0.3	13 (23)	43%	0.25 ± 0.03	10 (10)	0%	N/A			N/A		
Human beta cells	2.2 ± 0.6	18 (24)	25%	nd			N/A			N/A		