A New Type of Non-Ca$^{2+}$-buffering Apo(a)-based Fluorescent Indicator for Intraluminal Ca$^{2+}$ in the Endoplasmic Reticulum*

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Genetically encoded Ca$^{2+}$ indicators are outstanding tools for the assessment of intracellular/organelle Ca$^{2+}$ dynamics. Basically, most indicators contain the Ca$^{2+}$-binding site of a (mutated) cytosolic protein that interacts with its natural (mutated) interaction partner upon binding of Ca$^{2+}$. Consequently, a change in the structure of the sensor occurs that, in turn, alters the fluorescent properties of the sensor. Herein, we present a new type of genetically encoded Ca$^{2+}$ indicator for the endoplasmic reticulum (ER) (apoK1-er (W. F. Graier, K. Osibow, R. Malli, and G. M. Kostner, patent application number 05450006.1 at the European patent office)) that is based on a single kringle domain from apolipoprotein(a), which is flanked by yellow and cyan fluorescent protein at the 3′- and 5′-ends, respectively. Notably, apoK1-er does not interact with Ca$^{2+}$ itself but serves as a substrate for calreticulin, the main constitutive Ca$^{2+}$-binding protein in the ER. ApoK1-er assembles with calreticulin and the protein disulfide isomerase ERp57 and undergoes a conformational shift in a Ca$^{2+}$-dependent manner that allows fluorescence resonance energy transfer between the two fluorophores. This construct primarily offers three major advantages compared with the already existing probes: (i) it resolves perfectly the physiological range of the free Ca$^{2+}$ concentration in the ER, (ii) expression of apoK1-er does not affect the Ca$^{2+}$ buffering capacity of the ER, and (iii) apoK1-er is not inactivated by binding of constitutive interaction partners that prevent Ca$^{2+}$-dependent conformational changes. These unique characteristics of apoK1-er make this sensor particularly attractive for studies on ER Ca$^{2+}$ signaling and dynamics in which alteration of Ca$^{2+}$ fluctuations by expression of any additional Ca$^{2+}$ buffer essentially has to be avoided.

The ER represents the main Ca$^{2+}$ store in most mammalian cells and is crucially involved in cellular Ca$^{2+}$ signaling. In particular, by its ability to release Ca$^{2+}$ through receptor channels, such as inositol 1,4,5-trisphosphate receptors or ryanodine receptors, as well as by its large Ca$^{2+}$ sequestration capacity, the ER is crucial for cellular Ca$^{2+}$ homeostasis. However, the ER not only considerably contributes to cytosolic Ca$^{2+}$ signaling, but ER Ca$^{2+}$ release is also important for mitochondrial Ca$^{2+}$ signaling (1) and activation of Ca$^{2+}$-activated plasma membrane ion channels (2, 3). Moreover, the free ER Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{ER}$) is a key regulator for the activity of ER resident enzymes that are involved in fundamental processes, such as protein folding or lipid biosynthesis (4).

In view of such pivotal role of the ER Ca$^{2+}$ homeostasis for cell function, reliable measurements of intraluminal Ca$^{2+}$ dynamics are important for our understanding of Ca$^{2+}$-dependent signaling. Initially, synthetic fluorescent chelators with low Ca$^{2+}$-affinity (e.g. Mag-fura-2) have been established, which are loaded into the ER under certain conditions thus being suitable for intraluminal Ca$^{2+}$ measurements in the ER (5). Nevertheless, the targeting of these dyes is not specific, and the large proportion of fluorescence that accumulates in the cytosol influences measurements of [Ca$^{2+}$]$_{ER}$. The introduction of the genetically encoded bioluminescent protein aequorin allowed specific targeting to the ER and excellent measurements of ER Ca$^{2+}$ concentration (6). However, aequorin requires the incorporation of coelenterazine and is irreversibly consumed by Ca$^{2+}$ and, thus, long term measurements in the ER are difficult. Moreover, because of its limited light emission, this sensor is hardly suitable for image analysis, an emerging necessity for studies focused on subcellular Ca$^{2+}$ homeostasis or intraorganelle Ca$^{2+}$ cross-talk.

In an additional approach, intramolecular fluorescence resonance energy transfer (FRET) between two GFP mutants that were initially bridged by the calmodulin-binding domain from smooth muscle light chain kinase (M13), which undergoes structural changes upon binding of constitutive Ca$^{2+}$/calmodulin, was utilized (7, 8). This concept was further improved by the additional insertion of calmodulin between the two GFP mutants (9, 10) and the design of circularly permuted GFP Ca$^{2+}$ sensors (e.g. pericam, 11). However, the latter could not be successfully targeted into the ER so far. In a similar approach, fragments of the chicken skeletal muscle or human cardiac muscle troponin C were inserted between two fluorophores (12). These so-called S- and C-troponine monitor Ca$^{2+}$ independently of M13 and calmodulin.

Despite the diverse concepts of these fluorescent Ca$^{2+}$ sensors, in all types the binding of Ca$^{2+}$ to specific Ca$^{2+}$-binding domains subsequently alters the fluorescent properties of the protein. The latter sensors most frequently use calmodulin or troponin C as binding sites. Because these proteins are naturally found predominantly in the cytosol, their affinity for Ca$^{2+}$ is suitable to monitor Ca$^{2+}$ in compartments with similar Ca$^{2+}$ concentration dynamics than its natural environment (e.g. cytosol, mitochondria). Particularly for the ER, where the free Ca$^{2+}$ concentration exceeds that in the cytosol by more than 3 orders of magnitude, the Ca$^{2+}$-bindings site of the sensor needed to be redesigned to adapt its Ca$^{2+}$ sensitivity to this high Ca$^{2+}$ environment (for review see Ref. 13). Recently, a new sensor was
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introduced (i.e. D1ER) in which not only calmodulin but also M13 was mutated, and, thus, interference with constitutive calmodulin is strongly diminished (14).

However, despite these significant improvements, all sensors may affect the free Ca²⁺ concentration of the compartment/organelle as they conceptually act as Ca²⁺ chelators. Notably, an elevation of intralu-
minal Ca\textsuperscript{2+} binding capacity in the ER by overexpression of calretilcin has been found to modulate Ca\textsuperscript{2+} uptake and release in the ER and mitochondria as well as to reduce store-operated Ca\textsuperscript{2+} entry in HEK 293 cells (15).

Accordingly, we have designed a genetically encoded FRET sensor that combines the advantages of the existing tools and offers excellent targeting into the ER and a Ca\textsuperscript{2+} sensitivity that is suitable for Ca\textsuperscript{2+} measurements in the ER environment but does not contain a Ca\textsuperscript{2+} binding domain, which may affect cellular/subcellular/interorganellar Ca\textsuperscript{2+} signaling.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and substitutes, pBudCE4.1, primers, and DNA standards were from Invitrogen. pcDNA3 was from Clontech. Fetal calf serum was from PAA (Linz, Austria), and cell culture plasticware was purchased from Bertioli (Vienna, Austria). TransFast\textsuperscript{TM}, restriction enzymes, and T4 ligase were obtained from Promega. 2,5-Di-(tetr-butyl)-1,4-hydroquinone (BHQ), Dl-buthionine-[SR] sulfoximine (BSO), digitonin, histamine, MES, nigericin, monensin, and ionomycin were from Sigma-Aldrich. Hot Fire DNA Polymerase I was from Solis BioDyne (Tartu, Estonia). Antibodies and luminal reagent were obtained from Santa Cruz (Szabo Scandic, Vienna Austria). X-ray films were from Siemens (Graz, Austria). Nitrocellulose, protein standards, dNTPs, and all other chemicals were purchased from Roth (Lactan, Graz, Austria).

Cell Culture and Transfection—EA.hy 926 cells at passage ≥40 were grown on glass coverslips to ~80% confluence and transiently transfected with 2 \( \mu \)g of plasmid 2 days prior to experiments using TransFast\textsuperscript{TM}. To yield stable expression, HEK 293 cells transfected with 5 \( \mu \)g of pDNA per 6-cm dish were propagated in medium containing 100 \( \mu \)g/ml G418, and fluorescent colonies were consequently subcloned.

DNA Cloning—One and two kringles of apo(a) were amplified by PCR, thereby introducing SphI and SacI restriction sites at their 5\textsuperscript{-} and 3\textsuperscript{-}ends, respectively. YC4er and YC2.1 (10) were transferred into pUC19 (New England Biolabs, Frankfurt, Germany) as full-length HindIII/EcoRI fragments, and the calmodulin-M13 regions were exchanged for the restricted PCR products. The resultant constructs, termed apoK1/2-cyto/er, were confirmed by automated sequencing. The same PCR-based strategy was used to generate apoK1-er, containing 12 kringles of apo(a). To create ER-targeted sensors devoid of all 6 cysteines or both putative N-glycosylation sites, respectively, site-directed mutagenesis with primers bearing the intended mutations was performed. By use of internal restriction sites as indicated in Fig. 1c, apoK1-er was substituted sequentially for the respective mutated fragments. To allow introduction of the constructs via NotI/EcoRI into the mammalian expression vector pcDNA3, an additional NotI site was introduced between HindIII and EcoRI, and all following restriction sites were removed by blunt end ligation. For double transfection apoK1-er and mt-DsRed were inserted into the two multiple cloning sites of the transfection vector pBudCE4.1.

Immunohistochemistry—Following fixation by 4.5% paraformaldehyde, EA.hy 926 cells grown on glass coverslips were permeabilized with 0.6% Triton X-100 and incubated with specific antibodies against calretilcin, calnexin, or ERp57. A Cy3-labeled secondary antibody (Clontech) was used for visualization.

Confocal Microscopy—Image acquisition was performed on an array confocal laser scanning microscope (VoxCell Scan, Visitech) consisting of a Zeiss Axiovert 200 M (Vienna, Austria) and a QLC laser confocal scanning module (VisiTech, Visitron Systems, Puchheim, Germany) controlled by Metamorph 5.0 (Universal Imaging, Visitron Systems) as described previously (16, 17).

FRET Measurement—Experiments were performed on a Nikon inverted microscope (Eclipse 300TE; Nikon, Optotome, Vienna Austria) equipped with a CFI Plan Fluor 40× oil immersion objective, an epifluorescence system (Opti Quip, Highland Mills, NY), computer controlled z-stage and filter wheel (Ludl Electronic Products, Hawthorne, NY), and a liquid-cooled CCD-camera (Quantix KAF 1400G2, Roper Scientific, Acton, MA) and controlled by Metafluor 4.0 (Visitron Systems). As described previously (3, 16), FRET-based sensors were excited at 440 nm (440AF21; Omega Optical, Brattleboro, VT), and emission was monitored at 480 and 535 nm using an optical beam splitter (Dual View Micro-Imager\textsuperscript{TM}; Optical Insights, Visitron Systems). Heps-buffered solutions containing 145 mM NaCl, 5 mM KCl, 1 mM MgCl\textsubscript{2}, and 10 mM Heps acid (pH adjusted to 7.4) with 2 mM CaCl\textsubscript{2} or 1 mM EGTA were used.

pH and Ca\textsuperscript{2+} Titration—To characterize the pH sensitivity of the FRET-based ER Ca\textsuperscript{2+} sensors, a series of buffers with pHs ranging from 5 to 9 were prepared that contained 10 mM nigericin and 10 mM monensin and consisted of 140 mM KCl, 1 mM MgCl\textsubscript{2}, and either 20 mM MES (for pH 5–6.5), 20 mM HEPES (for pH 7–7.5) or 20 mM Tris-HCl (for pH 8–9). For pH/Ca\textsuperscript{2+} titrations, cells were permeabilized with digitonin (10 \( \mu \)M) and ionomycin (1.5 \( \mu \)M) in the respective buffer. The free Ca\textsuperscript{2+} concentrations ranged from 10\textsuperscript{-6} to 10\textsuperscript{-1} M and were set according to the Buffercalc program (Stanford, CA).

Redox Sensitivity—Cells were depleted by GSH by preincubation in GSH-free medium containing 5 mM BSO for 12 h (18). Cells were permeabilized as described above, and the change in fluorescence upon
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the switch between \(10^{-6}\) and \(10^{-3}\) M free Ca\(^{2+}\) was recorded in the presence of GSH from 0 to 10 mM.

Co-immunoprecipitation—Lysates of HEK 293 cells stably expressing apoK1-er were incubated with specific antibodies against calreticulin, calnexin, or ERp57, respectively, and the immune complexes were pre-precipitated with polyclonal anti-calreticulin antibody immobilized to Protein G-Sepharose beads. HEK 293 cells stably expressing apoK1-er were lysed and the sensor co-precipitated with polyclonal anti-calreticulin antibody immobilized to Protein G-Sepharose beads. HEK 293 cells stably expressing apoK1-er were lysed and the sensor co-precipitated with polyclonal anti-calreticulin antibody immobilized to Protein G-Sepharose beads.

Statistics—Analysis of variance and Scheffe's post hoc F test were used for evaluation of the statistical significance. \(p < 0.05\) was determined to be significant.

RESULTS

To achieve a high resolution and dynamic readout of ER Ca\(^{2+}\) concentration, we took advantage of the structural shift from a linear extended to a compact kringle conformation during the maturation of apo(a) (19) and designed a fluorescent protein sensor for [Ca\(^{2+}\)]\(_{ER}\). Consequently, a defined number of kringle IV sequences of apo(a) were flanked by the cDNA coding for the cyan (CFP) and yellow (YFP) variants of green fluorescent protein at the 5'- and 3'-ends, respectively (Fig. 1, a and b). By assembly of a complex with the chaperone and protein disulfide isomerase (i.e., calreticulin/ERp57, calnexin/ERp57) (20) the formation of the kringle(s) was assumed to occur. This structural shortening should result in FRET between the 5'-flanking donor (CFP) and the 3'-located acceptor (YFP) (Fig. 1a).

FRET efficiency decreases by the sixth order of magnitude of the distance between donor and acceptor fluorophores and depends on their relative orientation, which is unpredictable and therefore assumed to be constant (21). Thus, two constructs with either one (apoK1) or two (apoK2) kringle IV domain(s) between the two fluorophores were designed and tested for FRET efficiency upon protein folding (Fig. 1a).

Upon overexpression in endothelial cells the respective proteins localized in the cytosol (Fig. 2a). Because targeting of the sensors into the lumen of the ER is prerequisite for the Ca\(^{2+}\)-dependent maturation of kringle IV, the calreticulin signal sequence, and KDEL, which were successful for ER-targeting (10, 22), were introduced at the 5'- and 3'-ends of the constructs, respectively (Fig. 1c). As a consequence, successful targeting of the kringle IV containing sensors to the ER was achieved (Fig. 2b).

The structural integrity of the ER, the mitochondrial organization, and the well established network of ER and mitochondria (Fig. 2b) were not affected by overexpression of apoK1-er. Immunohistopa...
chemistry revealed co-localization of apoK1-er with calreticulin, calnexin, and ERp57 (data not shown).

To investigate whether the appearance of FRET from the apoK sensors is based on successful protein processing and folding that achieves a structural protein shortening and, thus, facilitates energy transfer between the two narrow fluorophores, several apoK variants were designed and targeted into the ER lumen. First, the two glycosylation sites of apoK1-er at positions 42 and 82 of kringle IV were mutated by exchange of the constitutive L-asparagine to L-alanine (ΔNapoK1-er; Fig. 1c). Such an approach has been used frequently to prevent protein glycosylation inside the ER, which is a prerequisite for further processing, and, thus, to preclude that the respective protein is subject for the protein folding machinery (23, 24). Second, the cysteines at position 9, 30, 58, 69, 81, and 86 of kringle IV were exchanged by site-directed mutagenesis to L-serine (CSapoK1-er; Fig. 1c). Because binding of ERp57 to its substrate as well as the folding/shortening of the apo(a)/apoK sensors are thought to depend on the formation of disulfide bridges that built up the known kringle structure (Fig. 1a), such a construct may not be suitable for Ca\textsuperscript{2+}-dependent folding at the calreticulin/ERp57 complex. Third, 12 copies of the kringle IV domain were cloned between CFP and YFP (apoK12-er, Fig. 1c) to enlarge the distance between the fluorescence donor and the putative energy acceptor and, thus, to prevent FRET from occurring even after the protein was folded properly. Notably, all constructs targeted nicely into the ER and did not exhibit any differences in intracellular localization compared with apoK1-er.

In HEK 293 cells that stably expressed apoK1, the sensor exhibited the expected spectrum representing the overlay of the two fluorescent proteins CFP and YFP (data not shown). In contrast to apoK1-cyto, the fluorescence profile of apoK1-er was similar to YC4er and changed upon chelating intraluminal Ca\textsuperscript{2+} indicating the loss of FRET by protein unfolding (Fig. 3a). ApoK1-er was isolated from stably expressing HEK 293 cells using an affinity column carrying an antibody against apo(a) as described previously (25). The isolated protein did not reveal any sensitivity to Ca\textsuperscript{2+} ions up to 2 mM free Ca\textsuperscript{2+} thus indicating that the designed protein per se does not sense Ca\textsuperscript{2+}. In addition, no changes in the fluorescence properties of the isolated and reduced apoK1-er protein were found if the redox potential was alternated stepwise from 0.1 mM GSSG/0 mM GSH to 0.1 mM GSSG/10 mM GSH. Thus, at least in the physiological redox range of the ER (26), the fluorescence of the isolated apoK1-er protein was not sensitive to changes in redox state of its environment (data not shown). In line with our immunohistochemical data described above, apoK1-er protein was co-immunoprecipitated with calreticulin (Fig. 3b), calnexin, and ERp57 (data not shown), suggesting that inside the ER lumen this putative folding sensor is recognized by the prominent Ca\textsuperscript{2+}-dependent chaperones and forms a complex with the respective disulfide isomerase.

To verify whether the concept of apoK1-er allows measurement of the Ca\textsuperscript{2+} concentration in the ER lumen of a living cell, the appearance of FRET due to the shortened distance between the donor and acceptor proteins was monitored initially in situ by a conventional Ca\textsuperscript{2+} calibration assay. Endothelial cells were transiently transfected with either
ApoK1-er or apoK1-cyto. After 48 h, the cells were permeabilized and intraluminal Ca\(^{2+}\) concentration was stepwise elevated by addition of 1 mM to 10 mM free Ca\(^{2+}\) (calculated according Buffercalc for MacOS 7–9). In single permeabilized cells, apoK1-er was visualized at 440 nm (excitation) and 535 nm (FRET) emission (Fig. 1a). Inside the ER lumen, apoK1-er folded in a Ca\(^{2+}\)-dependent manner where the apparent KD was 124.2 (120.7–127.7) μM with a Hill coefficient of 1.148–1.245 (Fig. 3b, left panel). In comparison, the frequently used ER Ca\(^{2+}\) sensor, YC4er was found to have a KD of 344.6 (325.4–365.0) μM in the same protocol (Fig. 3b, right panel), which is similar to that published in HeLa cells (27).

Furthermore, the pH sensitivity of apoK1-er was compared with that of YC4er, which also contains CFP and YFP, and the improved ER Ca\(^{2+}\) probe D1ER, where YFP has been replaced by citrine (Fig. 3d). As expected the pH sensitivities of the sensors containing YFP were identical and more pronounced than the citrine-containing probe (Fig. 3d). Nevertheless, all probes allowed ER Ca\(^{2+}\) measurements in a pH range of at least 6.5–8 (data not shown).

The kinetics of Ca\(^{2+}\)-dependent FRET appearance and disappearance in apoK1-er was evaluated in permeabilized cells, while intraluminal Ca\(^{2+}\) was changed rapidly (within 0.2 min from 1 μM to 1 mM free Ca\(^{2+}\), Fig. 4a). Herein, the on- and off-set kinetics of this sensor were calculated and revealed similar association and dissociation kinetics of apoK1-er (τ\(_{on}\) = 0.244 and τ\(_{off}\) = 0.249 min) that was comparable to that of YC4er (Fig. 3e). Considering such on/off kinetics, we assume that FRET occurs upon the Ca\(^{2+}\)-dependent binding of apoK1-er to the calreticulin-ERp57 complex that results in protein folding, whereas no FRET can be further measured once the sensor dissociates from the chaperone complex. In cells transiently expressing apoK1 in the cytosol (i.e. apoK1-cyto, Fig. 2a), no change in the fluorescence of the protein was found by switching from high to low Ca\(^{2+}\) conditions (Fig. 4a).

Because under these conditions apoK1-cyto remained inside the cells, these data indicate that the predicted Ca\(^{2+}\)-dependent protein shortening that facilitated the appearance of FRET between the flanking fluorophores needs the environment of the ER, presumably calreticulin and ERp57.

To elucidate to what extent the redox state of the ER affects the Ca\(^{2+}\)-dependent folding of apoK1-er, the effect of the reduction of intraluminal free Ca\(^{2+}\) from 1 mM to 1 μM was assessed in cells that were depleted from GSH by preincubation with 5 mM BSO for 12 h in the absence of GSH. In GSH-depleted cells, the Ca\(^{2+}\) sensitivity of apoK1-er was reduced and could be restored by elevation of intraluminal GSH (Fig. 4b). The concentration dependence of apoK1-er revealed an optimum around 1 mM intraluminal GSH (Fig. 4c).
Exhaustive ER depletion by 15 μM BHQ (16, 28, 29), an inhibitor of sarco/endoplasmic reticulum Ca\(^{2+}\) ATPases (SERCAs), resulted in a strong reduction of the FRET signal of apoK1-er and, to a lesser extent, that of apoK2-er (data not shown) but not that of apoK1-cyto (Fig. 5a). Moreover, in cells expressing apoK12-er and the mutants Δ\(α\)apoK1-er and Δ\(\gamma\)apoK1-er, which were designed to avoid either protein folding or FRET (apoK12-er), no effect of BHQ was observed (Fig. 5b).

To further test the sensor for measurements of [Ca\(^{2+}\)]\(_{\text{ER}}\) under physiological conditions, human endothelial cells were transiently transfected with apoK1-er. In the presence of extracellular Ca\(^{2+}\), stimulation with 100 μM histamine resulted in partial ER depletion that was more pronounced compared to the baseline (Fig. 5c).
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pronounced by removal of extracellular Ca\(^{2+}\) (Fig. 5c). Readout of extracellular Ca\(^{2+}\) resulted in recovery of the ER Ca\(^{2+}\) content despite continuous presence of the agonist and returned to basal level after histamine washout (Fig. 5c). These experiment resembles data obtained previously using YC4er (30) and, thus, confirm the suitability of apoK1-er for dynamic measurements of [Ca\(^{2+}\)]\(_{\text{ER}}\) in single living cells.

**DISCUSSION**

The approach for monitoring [Ca\(^{2+}\)]\(_{\text{ER}}\) by the visualization of Ca\(^{2+}\)-dependent changes of protein structure presented herein matches the most prominent advantages of existing techniques, whereas it is not claimed that this new technique replaces them but may provide further important information. Because of the fast on/off kinetics, apoK1-er allows measurements of [Ca\(^{2+}\)]\(_{\text{ER}}\) with a similar time resolution normally achieved by conventional Ca\(^{2+}\) imaging techniques. Thus, dynamic regulation of intraluminal Ca\(^{2+}\) signaling can be assessed even under fast fluctuations of cellular/ER Ca\(^{2+}\) concentration and, at least with the experimental setup used in this study, time resolution was only restricted by the limitations in detection efficiency of the system rather than by the kinetics of the sensor. Second, apoK1-er, like the cameleons, is not irreversibly consumed by Ca\(^{2+}\), and thus, [Ca\(^{2+}\)]\(_{\text{ER}}\) can be repetitively monitored in one given cell under various conditions, and long term measurements are possible. Third, as apoK1-er is suitable for use in high resolution microscopy it allows the analysis of [Ca\(^{2+}\)]\(_{\text{ER}}\) in a subset of the ER in one given cell. Fourth, in contrast to the cameleons, which compete with the constitutive calmodulin for their effector proteins, the sensing properties of apoK1-er are actually the result of its interaction with constitutive chaperons. Fifth, as shown in Fig. 2, the utilization of apoK1-er as an Ca\(^{2+}\)-sensor for the ER is not affected by co-expressed with additional e.g. DsRed-labeled proteins. Thus, apoK1-er is suitable for studies that are designed to follow spatial Ca\(^{2+}\) imaging in the ER simultaneously with e.g. mitochondrial movement (16, 31).

Moreover, compared with the existing genetically encoded Ca\(^{2+}\) sensors for the ER, apoK1-er offers also considerable advantages. As it follows the activity of constitutive Ca\(^{2+}\)-binding proteins in the ER (i.e. calreticulin), apoK1-er perfectly monitors physiological Ca\(^{2+}\) fluctuations in the ER environment. Notably, the observed reduction of apoK1-er FRET by BHQ reached ~70% FRET reduction compared with that found at the removal of extracellular Ca\(^{2+}\) in ionomycin-permeabilized cells. These data are in line with the Ca\(^{2+}\)-sensitivity curve obtained for apoK1-er (Fig. 3c, left panel) that indicates that the concentration for Ca\(^{2+}\) to regulate apoK1-er folding ranges between 10\(^{-5}\) and 10\(^{-3}\) M free Ca\(^{2+}\), which is in the physiological range of the intraluminal ER Ca\(^{2+}\) concentration (27, 32).

Notably, in the presence of extracellular Ca\(^{2+}\), endothelial cells are capable to retain most of the Ca\(^{2+}\) content of ER even upon stimulation with 100 µM histamine because of strong Ca\(^{2+}\) refilling that is fueled by Ca\(^{2+}\) entry (Fig. 5, Ref. 30). Under such conditions, the drop of apoK1-er FRET was 36% of that achievable with histamine in the absence of extracellular Ca\(^{2+}\) and, thus, was almost 1.6-fold higher as if conventional YC4er was used in the same set of experiments (30). This enhanced sensitivity of apoK1-er is because of its lower apparent K\(_D\) (i.e. 124.2 (120.7–127.7) µM, Fig. 3c, left panel) compared with YC4er. Importantly, in the physiological range of the ER Ca\(^{2+}\) concentration (i.e. 10–700 µM), apoK1-er exhibits a more steep Ca\(^{2+}\) dependence compared with YC4er and thus, may be even more sensitive to small Ca\(^{2+}\) changes/fluuctuations inside the ER (Fig. 3c).

These characteristics are very close to D1\(_{\text{ER}}\) that has been recently developed and provides outstanding suitability for measurements of [Ca\(^{2+}\)]\(_{\text{ER}}\) (14). Nevertheless, although D1\(_{\text{ER}}\) is an excellent tool for biological research, it shares the disadvantage of incorporating an additional Ca\(^{2+}\)-binding protein into the lumen of the ER with the Ca\(^{2+}\) sensors introduced so far and, thus, also elevates ER Ca\(^{2+}\)-buffering capacity inside this organelle. Because such intervention enhances the overall Ca\(^{2+}\) storage capacity of the ER, it may secondarily affect Ca\(^{2+}\)-mediated functions inside the ER (33). Moreover, elevating the Ca\(^{2+}\) buffering capacity of ER might influence intraluminal Ca\(^{2+}\) homeostasis and interorganelle Ca\(^{2+}\) signaling to mitochondria for example (15).

Particularly in view of the proposed regulatory function of intraluminal Ca\(^{2+}\) for activation/termination of the so-called capacitative Ca\(^{2+}\) entry (34–36) and the importance of interorganelle Ca\(^{2+}\) cross-talk (16, 27, 37), Ca\(^{2+}\) sensors that affect intraluminal Ca\(^{2+}\) buffer capacity might be critical. Importantly, apoK1-er does not contain a Ca\(^{2+}\)-binding domain but interacts with constitutive calreticulin/ERp57 in a Ca\(^{2+}\)-dependent manner and, thus, measures ER Ca\(^{2+}\) concentration by monitoring the action of luminal Ca\(^{2+}\) on its constitutive effector proteins. Therefore, this property can be further utilized to monitor the free Ca\(^{2+}\) concentration inside the ER without manipulating the Ca\(^{2+}\)-buffering/ storage capacity of this organelle. On the other hand, although apoK1-er lacks the cysteines essential for binding to apoB (38), a modulation of the properties of the sensor by binding ER components other than calreticulin/ERp57 cannot be excluded.

In addition, comparison of the pH sensitivities of D1\(_{\text{ER}}\) and apoK1-er indicate that the latter one can be significantly improved by exchanging its fluorophores to more stable ones. Therefore, such improved apoK1-er variants are currently constructed in our laboratory.

Accordingly, the concept to visualize [Ca\(^{2+}\)]\(_{\text{ER}}\) by FRET based on the Ca\(^{2+}\)-dependent structural shortening of a designed substrate protein for the given Ca\(^{2+}\)-dependent protein folding machinery shares the intriguing advantages of the existing sensors for measuring [Ca\(^{2+}\)]\(_{\text{ER}}\) provides excellent targeting into the ER, and offers a Ca\(^{2+}\) sensitivity that is suitable for Ca\(^{2+}\) measuring in the ER environment but does not contain a Ca\(^{2+}\)-binding domain that may affect cellular/subcellular/interorganelle Ca\(^{2+}\) signaling. These unique characteristics may make this new type of sensor the first choice in studies where the contribution of ER Ca\(^{2+}\) content to cellular, subcellular, and/or interorganelle Ca\(^{2+}\) homeostasis or Ca\(^{2+}\)-dependent processes in the cell has to be assessed.

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