Functional specialization of calreticulin domains

Kimitoshi Nakamura, Anna Zuppini, Serge Arnaudeau, Jeffery Lynch, Irfan Ahsan, Ryoko Krause, Sylvia Papp, Humbert De Smedt, Jan B. Parys, Werner Müller-Esterl, Daniel P. Lew, Karl-Heinz Krause, Nicolas Demaurex, Michal Opas, and Marek Michalak

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Calreticulin is a Ca\(^{2+}\)-binding chaperone in the endoplasmic reticulum (ER), and calreticulin gene knockout is embryonic lethal. Here, we used calreticulin-deficient mouse embryonic fibroblasts to examine the function of calreticulin as a regulator of Ca\(^{2+}\) homeostasis. In cells without calreticulin, the ER has a lower capacity for Ca\(^{2+}\) storage, although the free ER luminal Ca\(^{2+}\) concentration is unchanged. Calreticulin-deficient cells show inhibited Ca\(^{2+}\) release in response to bradykinin, yet they release Ca\(^{2+}\) upon direct activation with the inositol 1,4,5-trisphosphate (InsP\(_3\)). These cells fail to produce a measurable level of InsP\(_3\) upon stimulation with bradykinin, likely because the binding of bradykinin to its cell surface receptor is impaired. Bradykinin binding and bradykinin-induced Ca\(^{2+}\) release are both restored by expression of full-length calreticulin and the N + P domain of the protein. Expression of the P + C domain of calreticulin does not affect bradykinin-induced Ca\(^{2+}\) release but restores the ER Ca\(^{2+}\) storage capacity. Our results indicate that calreticulin may play a role in folding of the bradykinin receptor, which affects its ability to initiate InsP\(_3\)-dependent Ca\(^{2+}\) release in calreticulin-deficient cells. We concluded that the C domain of calreticulin plays a role in Ca\(^{2+}\) storage and that the N domain may participate in its chaperone functions.

Introduction

Calreticulin is a Ca\(^{2+}\)-binding chaperone found in the lumen of the endoplasmic reticulum (ER)* (Michalak et al., 1999). The protein binds monoglucosylated oligosaccharides (Bergon et al., 1994; Helenius et al., 1997) and misfolded proteins (Saito et al., 1999), and it is believed to play a critical role in quality control processes during protein synthesis and folding (Helenius et al., 1997). Several studies indicate that increased expression of calreticulin increases the Ca\(^{2+}\) storage capacity of the ER (Michalak et al., 1999). It also appears to modulate store-operated Ca\(^{2+}\) influx (Bastianutto et al., 1995; Mery et al., 1996; Fasolato et al., 1998; Xu et al., 2000) and to alter Ca\(^{2+}\) transport by the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase, SERCA2b (John et al., 1998). Evidence now indicates that Ca\(^{2+}\)-binding chaperones in the lumen of the ER affect luminal Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_{ER}\)) (Meldolesi and Pozzan, 1998) and that agonist-induced changes in [Ca\(^{2+}\)]\(_{ER}\) affect ER function (Corbett and Michalak, 2000).

Mice homozygous for calreticulin gene disruption (crt\(^{-/-}\)) showed a marked decrease in ventricular wall thickness and in deep intertrabecular recesses in the ventricular walls, indicating that calreticulin is essential for proper cardiac development (Mesaeli et al., 1999; Rauch et al., 2000). Agonist-induced Ca\(^{2+}\) release via the inositol 1,4,5-trisphosphate (InsP\(_3\)) pathway is inhibited in crt\(^{-/-}\) cells as is Ca\(^{2+}\)-dependent translocation of nuclear factor in activated T cells (Mesaeli et al., 1999). These results indicate that calreticulin, in addition to being a chaperone, plays a critical role in Ca\(^{2+}\) homeostasis.

In these studies, we used calreticulin-deficient mouse embryonic fibroblasts to examine the in vivo function of calreticulin as a regulator of Ca\(^{2+}\) homeostasis. In cells without calreticulin, the ER has a lower capacity for storage of Ca\(^{2+}\),...
although the free \([\text{Ca}^{2+}]_{\text{ER}}\) as measured by an ER-targeted “cameleon” reporter is not changed. Bradykinin-induced \(\text{Ca}^{2+}\) release is inhibited in calreticulin-deficient cells, yet they release \(\text{Ca}^{2+}\) in response to \(\text{InsP}_3\). Apparently, \(\text{crt}^{-/-}\) cells fail to produce measurable levels of \(\text{InsP}_3\) in response to bradykinin, likely because of impaired binding of bradykinin to its cell surface receptor. Our findings suggest that in calreticulin-deficient cells altered folding of the bradykinin receptor adversely affects its ability to initiate \(\text{InsP}_3\)-dependent \(\text{Ca}^{2+}\) release and that different domains of calreticulin may play distinct functions in the lumen of ER.

**Results**

**Calreticulin-deficient mouse embryonic fibroblasts**

To determine the effects of calreticulin deficiency on ER function, we isolated mouse embryonic fibroblasts from \(\text{crt}^{-/-}\) and wild-type embryos. These cell lines were designated K41 for wild-type mouse embryonic fibroblasts and K42 for calreticulin-deficient mouse embryonic fibroblasts. Some of the calreticulin-deficient cells (K42) were also transfected with a calreticulin expression vector and were designated K42CRT. As expected, Western blot analysis revealed that the K41 and K42CRT cells contained immunoreactive calreticulin (Fig. 1 A, lanes 1 and 3), whereas the K42 \(\text{crt}^{-/-}\) cells did not (Fig. 1 A, lane 2). The morphological appearance of the wild-type (K41) and calreticulin-deficient (K42) cells was indistinguishable and typical of fibroblasts (Fig. 1 B). The cell lines all attached firmly to plastic, and we detected no differences in the kinetics of their long-term (14 d) growth (unpublished data). Fig. 1 C shows that K41 and K42CRT cells both expressed calreticulin and that the protein was localized to an ER-like network. Morphologically, at a light microscope level the ER appeared intact in all cell lines as judged by staining with antibodies against pro-
tein disulfide isomerase (PDI) and Grp94 (Fig. 1 C). We observed typical nuclear morphology in all of the cell lines and the actin cytoskeleton visualized by labeling with fluorescent phalloidin also appeared normal (unpublished data).

**[Ca\textsuperscript{2+}]\textsubscript{ER} capacity in calreticulin-deficient cells**

The overexpression of calreticulin leads to an increased total Ca\textsuperscript{2+} content in the lumen of the ER (Michalak et al., 1999). This indicates that calreticulin somehow alters the Ca\textsuperscript{2+} storage capacity of the ER. Therefore, we used \textsuperscript{45}Ca\textsuperscript{2+} to estimate the Ca\textsuperscript{2+} content of the ER in calreticulin-deficient cells (Mery et al., 1996). Wild-type cells (K41) contained 44 ± 3 pmol of Ca\textsuperscript{2+}/10\textsuperscript{6} cells, whereas the calreticulin-deficient cells (K42) contained 24 ± 4 pmol of Ca\textsuperscript{2+}/10\textsuperscript{6} cells. Thus, the absence of calreticulin resulted in a >1.8-fold reduction in cellular Ca\textsuperscript{2+} content.

Next, we used thapsigargin, an inhibitor of the Ca\textsuperscript{2+}-ATPase, to measure the amount of Ca\textsuperscript{2+} associated with rapidly exchangeable stores. To measure the residual amounts of Ca\textsuperscript{2+} contained within thapsigargin-insensitive stores, we added the Ca\textsuperscript{2+} ionophore ionomycin. In these experiments, cells were equilibrium loaded with \textsuperscript{45}Ca\textsuperscript{2+} and then resuspended in a nonradioactive Ca\textsuperscript{2+}-free medium (Mery et al., 1996). Fig. 2 shows that in response to thapsigargin the wild-type cells (K41) released almost twice as much \textsuperscript{45}Ca\textsuperscript{2+} as the calreticulin-deficient cells (K42). When the remaining \textsuperscript{45}Ca\textsuperscript{2+} was released with ionomycin, the amount released from K41 cells was greater than from K42 cells (Fig. 2 A). This indicates that calreticulin somehow alters the Ca\textsuperscript{2+} release in the presence of ionomycin compared with thapsigargin alone. This is likely due to ionomycin-dependent Ca\textsuperscript{2+} release from intracellular compartments other than ER including mitochondria. To confirm that the absence of calreticulin caused the decreased Ca\textsuperscript{2+} content of the K42 cells, some K42 cells were stably transfected with a calreticulin expression vector, creating the K42CRT cell line (Fig. 1). Fig. 2 A shows that the Ca\textsuperscript{2+} content of thapsigargin-sensitive stores was indistinguishable in K42CRT and wild-type (K41) cells. We conclude that the absence of calreticulin in K42 cells causes a significant decrease in the Ca\textsuperscript{2+} content of thapsigargin-sensitive stores.

**The C domain of calreticulin affects ER Ca\textsuperscript{2+} storage capacity in vivo**

K42 (crt\textsuperscript{−/−}) cells provide us with an excellent tool for investigating the role of calreticulin’s different domains in determining ER Ca\textsuperscript{2+} storage capacity in vivo. Calreticulin binds Ca\textsuperscript{2+} with high capacity to its COOH-terminal domain (Michalak et al., 1999), but the effects of this binding have not been documented in vivo. To investigate the effect of calreticulin’s C domain on the Ca\textsuperscript{2+} storage capacity of thapsigargin-sensitive stores, we transfected K42 (calreticulin-deficient) cells with expression vectors encoding different domains of the protein. cDNA encoding N + P + C domain of calreticulin contained HA epitope for immunological detection of the recombinant proteins. Using anti-HA tag antibodies, we showed that calreticulin-deficient cells transfected with expression vectors encoding N + P or P + C domain expressed similar levels of both recombinant proteins (Fig. 2 B). Despite numerous attempts, we were unable to create a K42 (calreticulin-deficient) cell line expressing the C domain of calreticulin alone. Attempts to create other cell lines that overexpress the C domain of calreticulin (HeLa, HEK293, CHO) have also failed. This is probably because the C domain, when expressed alone, is very unstable (Corbett et al., 2000). However, we can express the C domain of calreticulin when the central P domain is also included (Fig. 2 B, lane 3). Although the P do-
modulates thapsigargin-sensitive (ER) Ca\(^{2+}\) storage capacity. Our results show that calreticulin via its C domain plays a role in determining the Ca\(^{2+}\) storage capacity of the ER.

**Free [Ca\(^{2+}\)]\(_{ER}\) in calreticulin-deficient cells**

To determine the free [Ca\(^{2+}\)]\(_{ER}\) in calreticulin-deficient cells, we transfected wild-type (K41) and calreticulin-deficient (K42) mouse embryonic fibroblasts with the ER-targeted cameleon YC4\(_{ER}\) (Arnaudeau et al., 2001), a Ca\(^{2+}\) indicator, which relies on fluorescent proteins and calmodulin (Miyawaki et al., 1997). Fig. 3 A shows that the cameleon was expressed in both cell types and was localized in a reticular pattern consistent with labeling of the ER. Fig. 3 B shows that the free [Ca\(^{2+}\)]\(_{ER}\) did not differ significantly in the wild-type cells (290 ± 18 \(\mu\)M Ca\(^{2+}\); mean ± SE; \(n = 18\)) and calreticulin-deficient cells (288 ± 20 \(\mu\)M Ca\(^{2+}\); mean ± SE; \(n = 22\)). In further studies, we used thapsigargin and ionomycin to completely deplete cellular Ca\(^{2+}\) stores. Under these conditions, free [Ca\(^{2+}\)]\(_{ER}\) was reduced to 27 ± 3 \(\mu\)M Ca\(^{2+}\) (mean ± SE; \(n = 15\)) in the wild-type cells and to 23 ± 3 \(\mu\)M Ca\(^{2+}\) (mean ± SE) in the calreticulin-deficient cells (Fig. 3 B). Our results indicate that although the total Ca\(^{2+}\) content of the ER is significantly decreased in calreticulin-deficient cells, the free [Ca\(^{2+}\)]\(_{ER}\) is unaltered both when Ca\(^{2+}\) stores are full and when they are depleted.

**Bradykinin-induced Ca\(^{2+}\) release in calreticulin-deficient cells**

We next performed experiments with the Ca\(^{2+}\)-sensitive fluorescent dye fura-2. Fig. 4 shows that in wild-type (K41) cells, the resting free cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{c}\)) was \(\sim 100 \pm 12\) nM (mean ± SD; \(n = 3\)). In calreticulin-deficient fibroblasts (K42), the resting free [Ca\(^{2+}\)]\(_{c}\) was significantly increased (148 ± 10 nM; mean ± SD; \(n = 3\)). We also performed fura-2 analysis of [Ca\(^{2+}\)]\(_{c}\) in calreticulin-deficient mouse embryonic fibroblasts, which had been transfected with a calreticulin expression vector (K42CRT cells). These cells, which express recombinant calreticulin, had 108 ± 13 nM (mean ± SD; \(n = 3\)) basal [Ca\(^{2+}\)]\(_{c}\), indicating that reinduction of calreticulin to calreticulin-deficient cells lowered the basal [Ca\(^{2+}\)]\(_{c}\) to the levels observed in the wild-type (K41) cells (Fig. 4, A and B).

Next, we compared agonist-induced Ca\(^{2+}\) release in wild-type (K41) and calreticulin-deficient fibroblasts (K42). In preliminary experiments, we tested the effect of 100 \(\mu\)M carbachol, 1 \(\mu\)M angiotensin II, 50 nM bombesin, 200 nM bradykinin, and 25 ng PDGF/ml on Ca\(^{2+}\) release from the wild-type cells. Among all these agonists, only bradykinin resulted in Ca\(^{2+}\) release from the K41 cells, and so it was used in our subsequent experiments. Furthermore, in full agreement with our earlier report (Mesi et al., 1999) bradykinin caused a rapid and transient increase in the cytoplasmic Ca\(^{2+}\) concentration in wild-type (K41) cells but not in K42 cells, indicating that Ca\(^{2+}\) release via InsP\(_{3}\)-dependent pathways is impaired in calreticulin-deficient cells (Fig. 4, A and C). To show that calreticulin is involved in this impairment, we investigated bradykinin-induced Ca\(^{2+}\) release in K42CRT cells. Fig. 4, A and C, show that the expression of calreticulin in K42 cells restored bradyki-
nin-stimulated \( \text{Ca}^{2+} \) release. Importantly, the basal \( \text{[Ca}^{2+}]_c \) was also decreased to levels observed in wild-type cells (Fig. 4, A and B).

In further experiments, we used a cameleon reporter to make time-resolved measurements of free \( \text{[Ca}^{2+}]_\text{ER} \) in wild-type and calreticulin-deficient cells. Bradykinin by itself had little effects on \( \text{[Ca}^{2+}]_\text{ER} \) (unpublished data) but caused a rapid \( \text{[Ca}^{2+}]_\text{ER} \) decrease when thapsigargin was included to prevent ER refilling (Fig. 5 A). A pronounced \( \text{Ca}^{2+} \) release was observed in wild-type cells, the \( \text{[Ca}^{2+}]_\text{ER} \) decreasing to \( 80 \pm 12 \, \mu\text{M} \) within 150 s. (Fig. 5 C). Subsequent addition of ionomycin had little effects on the kinetics of the \( \text{[Ca}^{2+}]_\text{ER} \) response (Fig. 5 A), indicating the ER \( \text{Ca}^{2+} \) permeability was maximally activated by the combination of bradykinin and thapsigargin. In contrast, the addition of bradykinin and thapsigargin to calreticulin-deficient cells decreased \( \text{[Ca}^{2+}]_\text{ER} \) only to \( 114 \pm 18 \, \mu\text{M} \) (Fig. 5 C), and subsequent addition of ionomycin caused a further decrease in \( \text{[Ca}^{2+}]_\text{ER} \) (Fig. 5 A). No differences were observed when thapsigargin was added alone, the \( \text{[Ca}^{2+}]_\text{ER} \) decreasing with similar kinetics and to similar levels in wild-type and calreticulin-deficient cells (Fig. 5, B and C). The higher \( \text{[Ca}^{2+}]_\text{ER} \) measured in calreticulin-deficient cells stimulated with bradykinin and thapsigargin thus likely reflects the failure of bradykinin to increase the ER \( \text{Ca}^{2+} \) permeability. These observations are in keeping with the fura-2 measurements presented in Fig. 4 and indicate that bradykinin-induced \( \text{Ca}^{2+} \) release from the ER is impaired in the calreticulin-deficient K42 cells.

**SERCA, the InsP\(_3\) receptor (InsP\(_3\)R) and the bradykinin receptor in calreticulin-deficient cells**

There are several potential explanations for the observation that bradykinin-induced \( \text{Ca}^{2+} \) release is impaired in calreticulin-deficient cells. For example, in \( \text{crt}^{-/-} \) (K42) cells there could be changes in the expression and/or function of \( \text{Ca}^{2+} \) transport proteins in the ER or in the bradykinin receptor in the plasma membrane. To investigate these possibilities, we first compared expression of SERCA2 and the InsP\(_3\)R in wild-type and calreticulin-deficient cells. Fig. 6 A shows that the expression of SERCA2 was not altered in calreticulin-deficient cells. Further, the level of mRNA for SERCA was the same in wild-type (K41) and calreticulin-deficient (K42) cells (unpublished data). We also used Western blot analysis (Fig. 6 B) and reverse transcriptase PCR (Table I) to compare expression of the three isoforms of the InsP\(_3\)R (type 1, 2, and 3) in K41 and K42 cells. All three isoforms of the InsP\(_3\)R were expressed in wild-type and \( \text{crt}^{-/-} \) cells (Fig. 6 B and Table I). However, we found a 30–40% reduction in mRNA for the InsP\(_3\)R in calreticulin-deficient cells compared with the wild-type cells (Table I). Western blot analysis of types 1, 2, and 3 of the InsP\(_3\)R revealed that these cells contain all three types of InsP\(_3\)R at a ratio of 10:70:20 (Fig. 6). A significant decrease of \( \sim 20\% \) was found in the calreticulin-deficient K42

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**Table I. Quantitative analysis of mRNA encoding different isoforms of InsP\(_3\)Rs in calreticulin-deficient K42 cells**

| K41 wild-type cells | K42 calreticulin-deficient cells |
|---------------------|---------------------------------|
| InsP\(_3\)R type 1   | 100                             | 74.6 ± 17.6                     |
| InsP\(_3\)R type 2   | 100                             | 58.0 ± 17.8                     |
| InsP\(_3\)R type 3   | 100                             | 66.0 ± 7.0                      |
| GAPDH                | 100                             | 101.5 ± 4.2                     |
| InsP\(_3\)R 1/2/3 ratio (% of total level) | 25.0/13.7/61.2 | 28.8/13.0/58.1 |

Reverse transcriptase PCR was carried out as described in Materials and methods. GAPDH was used as an internal standard. Data are mean ± SD (n = 3).
cells for InsP_3R type 1 (3.1 ± 0.4 versus 2.6 ± 0.4; n = 4; P < 0.01) and for InsP_3R type 3 (9.4 ± 0.4 versus 7.5 ± 0.6; n = 4; P < 0.01). The relative level of InsP_3R type 2 was approximately equal in both cell types (0.2 ± 0.1; n = 4).

Next, we compared expression of the bradykinin receptor and its targeting to the plasma membrane in wild-type (K41) and calreticulin-deficient (K42) cells. Fig. 7 A shows that calreticulin-deficient cells had decreased level of bradykinin receptor protein. Quantitative analysis of Western blots indicated that there was ~50% less bradykinin receptor in calreticulin-deficient cells compared with K41 wild-type cells. These results indicate that calreticulin deficiency had some effect on expression and/or turnover of the bradykinin receptor. Using confocal microscopy and antibodies against the bradykinin receptor, we found that the receptor was distributed in a “dotty” pattern across the cell surface in K41, K42, and K42CRT cells (Fig. 7 B). We noticed no difference between the cell lines in distribution of the receptor (Fig. 7 B). Cell surface localization of the receptor was further confirmed by flow cytometry assay using antibradykinin receptor antibodies. We observed no significant difference in the antibradykinin antibodies surface labeling of K41 and K42 cells (Fig. 7 C), indicating the similar number of immunoreactive receptor protein molecules was present on cell surface. We concluded that in calreticulin-deficient cells the bradykinin receptor was properly targeted to and localized in the plasma membrane.

### InsP_3-induced Ca^{2+} release and InsP_3 synthesis in calreticulin-deficient cells

We have shown that expression of the InsP_3R is reduced in calreticulin-deficient (K42) cells, whereas expression of the bradykinin receptor is not. Therefore, we wanted to determine whether the altered expression of the InsP_3R was responsible for the impairment of bradykinin-induced Ca^{2+} release in these cells. To test this hypothesis, we loaded wild-type and calreticulin-deficient cells with fluo-3, and then we added saponin to permeabilize the plasma membrane (Favre et al., 1994). Subsequently, exogenous InsP_3 was added to
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The digitonin-permeabilized cells, and Ca\(^{2+}\) release from the ER was monitored by changes in fluo-3 fluorescence. Fig. 8 shows that InsP\(_3\)-induced Ca\(^{2+}\) release from the ER was indistinguishable in the wild-type (K41), calreticulin-deficient cells (K42), and calreticulin-deficient cells transfected with calreticulin expression vector (K42CRT). K42 mock-transfected control cells are also shown. In all cell lines, bradykinin receptor localizes to cell surface. Flow cytometry analysis of mouse embryonic fibroblasts was carried out with antibradykinin receptor antibodies. Results are presented as the relative mean fluorescence intensity after subtracting fluorescent values for the secondary antibodies alone. Results shown are representative of five experiments. K41, wild type cells; K42, calreticulin-deficient cells; BK, bradykinin receptor.

The digitonin-permeabilized cells, and Ca\(^{2+}\) release from the ER was monitored by changes in fluo-3 fluorescence. Fig. 8 shows that InsP\(_3\)-induced Ca\(^{2+}\) release from the ER was indistinguishable in the wild-type (K41) and calreticulin-deficient (K42) cells, indicating that there is no difference in function of the InsP\(_3\)Rs in the different cell types.

Since InsP\(_3\)-induced Ca\(^{2+}\) release in permeabilized \(crt^{-/-}\) cells is normal, the impaired bradykinin-induced Ca\(^{2+}\) release in intact cells could result from a deficiency in InsP\(_3\) synthesis. To investigate this possibility, we incubated wild-type and calreticulin-deficient cells with 200 nM bradykinin and then measured InsP\(_3\) levels. Fig. 9A shows that the incubation with bradykinin resulted in significant synthesis of InsP\(_3\) in the K41 cells. In contrast, when calreticulin-deficient K42 cells were treated with bradykinin there was no detectable synthesis of InsP\(_3\) (Fig. 9A). We conclude that impairment of bradykinin-induced Ca\(^{2+}\) release in \(crt^{-/-}\) K42 cells likely results from a failure to synthesize InsP\(_3\).
Bradykinin binding in calreticulin-deficient cells

Our results indicate that bradykinin-dependent signaling is impaired in calreticulin-deficient cells and that this results from impaired InsP₃ synthesis. Therefore, we measured bradykinin binding to calreticulin-deficient cells. Fig. 9B shows that binding of [¹H]bradykinin to wild-type K41 cells was saturable with a Bₘₐₓ of 2.5 ± 0.3 pmol/mg of total cell protein (n = 3). Scatchard analysis of the specific binding data gave an apparent dissociation constant (Kᵰ) of 230 ± 20 pM. These values are in agreement with previously reported Kᵰ and Bₘₐₓ values for bradykinin receptors (Marceau et al., 1998). In contrast, the binding of bradykinin to calreticulin-deficient cells was significantly reduced (Bₘₐₓ < 0.5 ± 0.01 pmol/mg of protein; n = 3) (Fig. 9B), which would explain why these cells show impairment of bradykinin-induced Ca²⁺ release (Fig. 4). K42CRT cells, which express calreticulin and exhibit normal bradykinin-induced Ca²⁺ release (Fig. 4), showed bradykinin binding comparable to that seen in wild-type cells (Bₘₐₓ 2.7 ± 0.3 pmol/mg of total cell protein; n = 3; Kᵰ of 225 ± 25 pM) (Fig. 9B). This was despite of a slightly lower level of expression of the bradykinin receptor in K42CRT cells (Fig. 7A). We concluded that bradykinin binding to its receptor is inhibited in calreticulin-deficient cells.

N + P domain of calreticulin restores bradykinin-induced Ca²⁺ release from K42 cells

The data presented in Figs. 4 and 9 indicate that the impairment of bradykinin-induced Ca²⁺ release in calreticulin-deficient cells results from the failure of bradykinin to bind to its receptor. This indicates that the bradykinin receptor may be misfolded and therefore unable to bind bradykinin. In preliminary experiments, we showed that calreticulin and bradykinin receptor form complexes, which can be immunoprecipitated (unpublished data), indicating that calreticulin may play a role in folding of the bradykinin receptor. This presented us with a unique opportunity to investigate the role of calreticulin’s different domains in its function as a chaperone. We used bradykinin-induced changes in [Ca²⁺]ₜ as a measure of the function of the bradykinin receptor. Cells were loaded with fluora-2, stimulated with 200 nM bradykinin, and changes in [Ca²⁺]ₜ were monitored. As shown in Fig. 10, in K42 CRT cells expressing calreticulin (K42CRT), bradykinin-induced Ca²⁺ release was restored to the levels seen in wild-type cells. This indicates that full-length calreticulin is required for normal ligand binding to the receptor. Bradykinin-induced Ca²⁺ release was also reestablished in K42 cells after transfection with the N + P domain of calreticulin (Fig. 10B, K42N+P). However, it was not reestablished in cells expressing the P + C domain of the protein (Fig. 10B, K42P+C). This indicates that the N and P domain of calreticulin may play a role in peptide binding and/or folding of the bradykinin receptor in mouse embryonic fibroblasts.
Discusión

En este estudio, tomamos ventaja de una nueva calreticulin-deficient cell line, que nos permitió abordar algunas de las muchas preguntas interesantes respecto a la función de calreticulin y sus diferentes estructuras en el lúmen del ER. Nos dimos cuenta de que calreticulin-deficient cells tienen un efecto bradykinin-induced Ca$^{2+}$ release, que resulta de una función bradykinin receptora disfuncional. La disfunción probablemente proviene porque el receptor está incorrectamente doblado y como resultado, es incapaz de unir su ligando. Cuando el receptor no puede unir el ligando, no puede iniciar el proceso de transducción de señales, lo cual normalmente conduce a la producción de InsP3 y Ca$^{2+}$. Estos cambios pueden explicar por qué la deficiencia de calreticulin es letal. El hecho de que la deficiencia de calreticulin afecte la capacidad de bradykinin-induced Ca$^{2+}$ release, que se ha sugerido que calreticulin deficiency has a profound effect on the Ca$^{2+}$ storage capacity of the ER. Para algunos, la función del calreticulin en el ER puede jugar un papel en determinar la sensibilidad celular a la apoptosis. En consecuencia, células que expresan Bcl-2 tienen un flujo intraruminal Ca$^{2+}$ reducido, lo que también se ha mostrado en modelos de calreticulin knockout cells, que tienen una disminución intraluminal en la concentración de Ca$^{2+}$. Estos hallazgos sugieren que la deficiencia de calreticulin puede afectar la capacidad de bradykinin-induced Ca$^{2+}$ release, lo cual podría explicarse por la función de calreticulin en el ER, y su efecto en la función del calreticulin en el ER.
induced Ca\(^{2+}\) release but fail to produce InsP\(_3\) in response to bradykinin. Although the calreticulin-deficient cells have somewhat lower levels of the InsP\(_3\), its function is apparent unaffected. This indicates that cells can tolerate some change in the expression of InsP\(_3\), without compromising the ER's ability to release Ca\(^{2+}\). It is not clear why expression of the InsP\(_3\) is decreased in calreticulin-deficient cells, but Ca\(^{2+}\) and calcineurin may play a role. Calcineurin affects expression of the InsP\(_3\) at the transcriptional level (Genazzani et al., 1999) and calcineurin-dependent transcriptional processes are impaired in calreticulin-deficient cells.

Although the level of the bradykinin receptor is reduced in \(crt^{-/}\) cells, its targeting to the cell surface is unchanged, indicating that the absence of calreticulin does not affect its intracellular trafficking. Decreased expression of the bradykinin receptor may be due to increased degradation of the receptor in calreticulin-deficient cells. Our results indicate that calreticulin-deficient cells do not show bradykinin-induced Ca\(^{2+}\) release because the bradykinin receptor is unable to bind its ligand. If the bradykinin cannot bind, the receptor is unable to stimulate phospholipase C activity and synthesis of InsP\(_3\). Bradykinin-induced Ca\(^{2+}\) release can be rescued in \(crt^{-/}\) cells by reintroduction of full-length calreticulin. Importantly, the N + P domain of calreticulin is involved in this restoration, whereas the P + C domain is not. We conclude that the N domain of calreticulin is somehow essential for enabling interaction between the bradykinin receptor and its ligand, most likely through assisting in proper folding of the receptor's ligand-binding domain. Although we do not have direct evidence for this, it is conceivable that the N domain may directly interact with bradykinin receptor or it may recruit other chaperones necessary for the receptor folding and/or posttranslational modification. For example, the N domain interacts in vitro with ERp57 (Corbett et al., 1999), and this may be critical for proper folding of the ligand-binding domain. At present, the chaperone function of the P domain is not clear. This study indicates that either the P domain plays less important chaperone role, or most likely its chaperone function requires the presence of the N domain. However, this is the first evidence that the chaperone function of calreticulin may be also contained in the N domain of the protein.

It is widely thought that the central P domain of calreticulin can act as a chaperone for glycosylated proteins because of its amino acid sequence similarities to calcnexin and its lectin-like activity. However, a series of in vitro experiments indicate that calreticulin may also function as a molecular chaperone for nonglycosylated proteins (Saito et al., 1999). Both ATP and Zn\(^{2+}\) enhance calreticulin's ability to complex with unfolded nonglycosylated substrates in vitro (Saito et al., 1999). This likely occurs because of the dramatic conformational change, which occurs in calreticulin in the presence of Zn\(^{2+}\) (Khanna et al., 1986). Calreticulin binds Zn\(^{2+}\) at its N domain (Michalak et al., 1999). The amino acid sequence of the N domain is extremely conserved among all calreticulins and is also unique to calreticulin (Michalak et al., 1999). This observation supports the suggestion that the N domain of calreticulin has a highly specific function, which may include the folding of specific substrates. The bradykinin receptor may be such a substrate. Calreticulin and calcnexin both interact with monoglycosylated carbohy-

### Materials and methods

**Cell culture and DNA constructs**

Mouse embryonic fibroblasts were isolated from calreticulin-deficient and wild-type embryos, immortalized, and designated K41 and K42, respectively (Nakamura et al., 2000). K42 \(crt^{-/}\) cells were transfected with the pcDNA3 expression vector containing cDNA encoding rabbit calreticulin to generate \(crt^{-}\) cell lines expressing recombinant calreticulin (designated K42CRT). K42 cells were also transfected with expression vectors encoding the N + P domain of calreticulin to generated K42N + P and K42P + C lines, respectively. cDNA encoding the N + P domain of calreticulin (amino acid residues 1-287) was synthesized by PCR-driven reaction using the following oligodeoxynucleotides with 5' flanking EcoR1 (primer N5 \(crt\)) and P3 \(crt\) arms) and transferred to nitrocellulose membrane (Mery et al., 1996). SDS-PAGE and Western blot analysis of InsP3R mRNA

Cells were lysed, and proteins were separated by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose membrane (Mery et al., 1996). Blots were also probed with goat and rabbit anti-calreticulin antibodies (Nakamura et al., 2000), antibradykinin B2 receptor antibodies (Blaukat et al., 1996), or rabbit anti-SERCA2 antibodies at a 1:1,000 dilution (Lyton et al., 1992).

To assess the level of InsP3R protein, microsomal membranes were isolated from wild-type (K41) and \(crt^{-}\) (K42) cells (Lytton et al., 1992) and from RBL-2H3 cells as control (Vanlingen et al., 1997). Membrane proteins were separated on SDS-PAGE, transferred to Immobilon-P and probed with isofom-specific antibodies against InsP3R type 1 (Rbit03, dilution 1:1,000), InsP3R type 2 (Rbit02, dilution 1:200), or InsP3R type 3 (I31220, dilution 1:2,000; Transduction Laboratories) (Parys et al., 1995; De Smedt et al., 1997). Quantitation of the immunoreactive bands was achieved after incubation with secondary antibodies coupled to alkaline phosphatase detection using Vistra™ enhanced chemiluminescence and fluorimaging as described before (Vanlingen et al., 1997). Statistical analysis of the InsP3R isoforms levels in the K41 and K42 cells was performed using the paired Student's t test after normalization of the levels to those found in microsomes of RBL-2H3 cells.

**Reverse transcriptase PCR analysis of InsP3R mRNA**

Relative levels of mRNA encoding different isoforms of InsP3R were determined by reverse transcriptase PCR (De Smedt et al., 1997). PCR products and restriction fragments were separated on a 6% acrylamide gel and visu-
alized by staining with Vistra Green (followed by fluorimaging and quantitative analysis on a Storm840 Fluoromager equipped with the ImageQuant 4.2 software.) GAPDH mRNA was used as an internal standard.

Total and free ER luminal Ca\(^{2+}\) concentrations

The total Ca\(^{2+}\) in ER Ca\(^{2+}\) stores was estimated using \(\text{Ca}^{2+}\) \(10 \mu\text{M}\) as described earlier (Mery et al., 1996). Free \(\text{Ca}^{2+}\) luminal was estimated by dual emission ratio imaging using the ER-targeted yellow cameleon (YC4 ER) described earlier (Mery et al., 1996). Free \([\text{Ca}^{2+}]_o\) was estimated using fura-2/AM (2 µM) (Mery et al., 1996). Cells were stimolated for 15 s with 200 nM bradykinin, and the reaction was terminated by a Becton Dickinson FACScan using CellQuest software. Results are presented as the relative mean fluorescence intensity of the population labeled with primary and secondary antibodies minus that obtained with secondary antibody alone. Results shown are representative of five experiments.

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References

Araujo, S., W.L. Kelley, J.V. Walsh Jr., and N. Demaurex. 2001. Mitochondria recycle calcium to the endoplasmic reticulum and prevent the depletion of neighboring ER regions. J. Biol. Chem. 276:29430–29439.

Bastianutto, C., E. Clementi, F. Codazzi, P. Podini, F. De Giorgi, R. Rizzuto, J. Meldolesi, and T. Pozzan. 1995. Overexpression of calreticulin increases the \(\text{Ca}^{2+}\) capacity of rapidly exchanging \(\text{Ca}^{2+}\) stores and reveals aspects of their luminal microenvironment and function. J. Cell Biol. 130:847–855.

Bergersen, J.J.M., M.B. Brenner, D.Y. Thomas, and D.B. Williams. 1994. Calnexin: a membrane-bound chaperone of the endoplasmic reticulum. Trends Biochem. Sci. 19:124–128.

Bers, D.M., C.W. Parton, and R. Nucettiell. 1994. A practical guide to the preparation of \(\text{Ca}^{2+}\) buffers. Methods Cell Biol. 40:5–29.

Blautak, S.A. All, M.J. Lohoe, and W. Muller-Esterl. 1996. Ligand-induced phosphorylation/dephosphorylation of the endogenous bradykinin B2 receptor from human fibroblasts. J. Biol. Chem. 271:32666–32674.

Coppolino, M.G., M.J. Woodside, N. Demaurex, S. Grinstein, S. Arnaud, and S. Dedhar. 1999. Calreticulin is essential for integrin-mediated calcium signalling and cell adhesion. Nature. 386:843–847.

Corbett, E.F., and M. Michalak. 2000. Calcium, a signaling molecule in the endoplasmic reticulum? Trends Biochem. Sci. 25:307–311.

Corbett, E.F., K. Oikawa, P. Francois, D.C. Tessier, C. Kay, J.J.M. Bergeon, D.Y. Thomas, K.-H. Krause, and M. Michalak. 1999. \(\text{Ca}^{2+}\) regulation of interactions between endoplasmic reticulum chaperones. J. Biol. Chem. 274:6203–6211.

Corbett, E.F., K.M. Michalak, K. Oikawa, S. Johnson, I.D. Campbell, P. Eggleston, C. Kay, and M. Michalak. 2000. The conformation of calreticulin is influenced by the endoplasmic reticulum luminal environment. J. Biol. Chem. 275:27177–27185.

Danilczyk, U.G., M.F. Cohen-Doyle, and D.B. Williams. 2000. Functional relationship between calreticulin, calnexin, and the endoplasmic reticulum luminal domain of calnexin. J. Biol. Chem. 275:13089–13097.

De Smedt, H., L. Missiaen, J.B. Pays, R.H. Henning, I. Sienaert, S. Vanliingen, A. Gijswijns, B. Himpens, and R. Casteels. 1997. Isoform diversity of the inositol trisphosphate receptor in cell types of mouse origin. Biochem. J. 322:575–583.

Dobalora, C., P. Pizzo, and T. Pozzan. 1998. Delayed activation of the store-operated \(\text{Ca}^{2+}\) current induced by calreticulin overexpression in RBL-1 cells. Med. Biol. Cell. 9:1513–1522.

Favre, C.J., D.P. Lew, and K.-H. Krause. 1994. Rapid heparin-sensitive \(\text{Ca}^{2+}\) release following \(\text{Ca}^{2+}\)-ATPase inhibition in intact HL-60 granulocytes. Evidence for \(\text{Ins}(1,4,5)\text{P}_3\)-dependent \(\text{Ca}^{2+}\) cycling across the membrane of \(\text{Ca}^{2+}\) stores. Biochem. J. 302:155–162.

Fvoyou-Yossifi, R., S. Araujo, C. Berner, W.L. Kelley, J. Tischhop, D.P. Lew, N. Demaurex, and K.-H. Krause. 2000. Bcl-2 decreases the free \(\text{Ca}^{2+}\) concentration within the endoplasmic reticulum. Proc. Natl. Acad. Sci. USA. 97:5723–5728.

Genazzani, A.A., E. Carafoli, and D. Guerini. 1999. Calcineurin controls inositol phospholipid metabolism. J. Biol. Chem. 274:27177–27185.

Hausen, M., Y. Nozawa, and H. Higashida. 1993. Bradykinin-induced cytosolic \(\text{Ca}^{2+}\) oscillations and inositol triphosphate-induced \(\text{Ca}^{2+}\) influx in voltage-clamped rat-transfected NIH/3T3 fibroblasts. J. Biol. Chem. 268:19403–19410.

Helenius, A., E.S. Trombeta, D.N. Hebert, and J.F. Simons. 1997. Calnexin, calreticulin and the folding of glycoproteins. Trends Cell Biol. 7:193–200.

Holaska, J.M., B.E. Black, D.C. Love, J.A. Hanover, J. Leszyk, and B.M. Paschal,
Calreticulin is a receptor for nuclear export. *J. Cell Biol.* 152:127–140.

John, L.M., J.D. Lechleiter, and P. Camacho. 1998. Differential modulation of SERCA isoforms by calreticulin. *J. Cell Biol.* 142:963–973.

Khanna, N.C., M. Tokuda, and D.M. Waisman. 1986. Conformational changes induced by binding of divalent cations to calregulin. *J. Biol. Chem.* 261:8883–8887.

Liu, N., R.E. Fine, E. Simons, and R.J. Johnson. 1994. Decreasing calreticulin expression lowers the Ca2+/H1001 response to bradykinin and increases sensitivity to ionomycin in NG108-15 cells. *J. Biol. Chem.* 269:28635–28639.

Lyton, J., M. Westlin, S.E. Burke, G.E. Shull, and D.H. MacLennan. 1992. Functional comparisons between isoforms of the sarcoplasmic or endoplasmic reticulum family of calcium pumps. *J. Biol. Chem.* 267:14483–14489.

Marceau, F., J.F. Hess, and D.R. Bachvarov. 1998. The B1 receptors for kinins. *Pharmacol. Rev.* 50:357–386.

Michalak, M., E.F. Corbett, N. Mesaeli, K. Nakamura, and M. Opas. 1999. Calreticulin: one protein, one gene, many functions. *Biochem. J.* 344:281–292.

Miyawaki, A., J. Llopis, R. Heim, J.M. McCaffery, J.A. Adams, M. Ikura, and R.Y. Tsien. 1997. Fluorescent indicators for Ca2+/H1001 based on green fluorescent proteins and calmodulin. *Nature.* 388:882–887.

Nakamura, K., E. Bosy-Wetzel, K. Burns, M. Fadel, M. Lozyle, I.S. Goping, M. Opas, R.C. Blackley, D.R. Green, and M. Michalak. 2000. Changes in endoplasmic reticulum luminal environment affect cell sensitivity to apoptosis. *J. Cell Biol.* 150:731–740.

Parys, J.B., H. De Smedt, L. Missiaen, M.D. Bootman, I. Sienaert, and R. Casteels. 1995. Rat basophilic leukemia cells as model system for inositol 1,4,5-trisphosphate receptor IV, a receptor of the type II family: functional comparison and immunological detection. *Cell Calcium.* 17:239–249.

Pinton, P., D. Ferrari, P. Magalhaes, K. Schulze-Osthoff, F. Di Virgilio, T. Pozzan, and R. Rizzuto. 2000. Reduced loading of intracellular Ca2+/H1001 stores and downregulation of capacitative Ca2+/H1001 influx in Bcl-2-overexpressing cells. *J. Cell Biol.* 148:857–862.

Rauch, F., J. Prud’homme, A. Arabian, S. Dedihr, and R. St-Arnaud. 2000. Heart, brain, and body wall defects in mice lacking calreticulin. *Exp. Cell Res.* 256:105–111.

Saito, Y., Y. Ichita, M.R. Leach, M.F. Cohen-Doyle, and D.B. Williams. 1999. Calreticulin functions in vitro as a molecular chaperone for both glycosylated and non-glycosylated proteins. *EMBO J.* 18:6718–6729.

VanLingen, S., J.B. Parys, L. Missiaen, H. De Smedt, F. Wuytack, and R. Casteels. 1997. Distribution of inositol 1,4,5-trisphosphate receptor isoforms, SERCA isoforms and Ca2+/H1001 binding proteins in RBL-2H3 rat basophilic leukemia cells. *Cell Calcium.* 22:475–486.

Xu, W., F.J. Longo, M.R. Wintermantel, X. Jiang, R.A. Clark, and S. DeLisle. 2000. Calreticulin modulates capacitative Ca2+/H1001 influx by controlling the extent of inositol 1,4,5-trisphosphate-induced Ca2+/H1001 store depletion. *J. Biol. Chem.* 275:36670–36682.