Overexpression of Calreticulin Increases the Ca^{2+} Capacity of Rapidly Exchanging Ca^{2+} Stores and Reveals Aspects of Their Lumenal Microenvironment and Function

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Abstract. A molecularly tagged form of calreticulin (CR), a low affinity-high capacity Ca^{2+} binding protein that resides in the ER lumen, was transiently transfected into HeLa cells to specifically modify the Ca^{2+} buffering capacity of the intracellular Ca^{2+} stores. Fluorescence and confocal microscope immunocytochemistry revealed the tagged protein to be expressed by over 40% of the cells and to overlap in its distribution the endogenous CR yielding a delicate cytoplasmic network, i.e., the typical pattern of ER. In contrast, no signal was observed associated with the plasmalemma (marked by ConA) and within the nucleus. One- and two-dimensional Western blots revealed the transfected to exceed the endogenous CR of ~3.5-fold and to maintain its Ca^{2+} binding ability, whereas the expression of other ER proteins was unchanged. Ca^{2+} homeostasis in the transfected cells was investigated by three parallel approaches: (*a*) ^{45}Ca equilibrium loading of cell populations; (*b*) $[Ca^{2+}]_c$ measurement with fura-2 followed by quantitative immunocytochemistry of single cells and iii) $[Ca^{2+}]_c$ measurement of cell population upon cotransfection with the Ca^{2+}-sensitive photoprotein, aequorin. The three approaches revealed different aspects of Ca^{2+} homeostasis, yielding results which were largely complementary. In particular, the following conclusions were established: (*a*) both endogenous and transfected CR participate in Ca^{2+} buffering within the IP3-sensitive, rapidly exchanging, Ca^{2+} stores; the other pools of the cells were in contrast unaffected by CR transfection; (*b*) the Ca^{2+} capacity of the stores is not the main limiting factor of individual IP_3-mediated Ca^{2+} release responses triggered by receptor agonists; (*c*) in control cells, the contribution of CR to Ca^{2+} buffering within the IP_3-sensitive stores accounts for ~45% of the total, the rest being probably contributed by the other lumenal (and also membrane) Ca^{2+} binding proteins; (*d*) the free $[Ca^{2+}]$ within the lumen of the IP_3-sensitive stores, revealed by the degree of Ca^{2+} binding to the transfected CR protein, amounts to values in (or approaching) the millimolar range; and (*e*) Ca^{2+} influx across the plasmalemma activated by depletion of the stores is directly dependent on the lumenal $[Ca^{2+}]$.

THE intracellular rapidly exchanging stores of Ca^{2+} have attracted increasing interest during the last several years (for reviews see Carafoli, 1987; Berridge, 1993; Pozzan et al., 1994). These structures are responsible for a fundamental step in many types of cell activation, the release of Ca^{2+} to the cytosol taking place not at the surface but at multiple sites within the cytoplasm. Among cytoplasmic organelles, the ER is commonly identified as the cytological counterpart of the stores. Results in various cell types have however indicated that not the entire ER, but discrete areas (e.g., the sarcoplasmic reticulum of striated and smooth muscle fibers) are specialized in Ca^{2+} uptake and release (for review see Pozzan et al., 1994). Moreover, although many of the various molecular actors of the stores have been identified (a family of Ca^{2+} pumps, the sarcoplasmic–endoplasmic reticulum Ca^{2+} ATPases, [SERCAs],[1] for uptake [MacLennan, 1990]; a group of lumenal Ca^{2+} binding proteins characterized by low affinity and high capacity, for storage [Lytton and Ni-

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1. *Abbreviations used in this paper*: Ab, antibody; $[Ca^{2+}]_c$ and $[Ca^{2+}]_{er}$, concentration of Ca^{2+} in the cytosol and in the lumen of rapidly exchanging Ca^{2+} stores; CR, tCR, and tCR cells, calreticulin, tagged calreticulin, and cells transfected with tagged calreticulin; IP_3, inositol 1,4,5-trisphosphate; PDI, protein disulfide isomerase; SERCA, sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase; Tg, thapsigargin.

gam, 1992]; and the channels of two families, the inositol 1,4,5 trisphosphate, IP_3, and the ryanodine receptors, for release [Furuichi et al., 1989; Takeshima et al., 1989]) the mechanisms that underlie their coordinate functioning are still incompletely understood.

An approach that can provide direct information on the in vivo regulation of complex biological processes is the study of cells in which the expression level and/or the molecular properties of appropriately chosen component(s) have been modified. Up until now, except for studies with SERCA blockers, this approach has been used only in a few cases to investigate Ca^{2+} stores. The present investigation was carried out in HeLa epithelial cells transiently transfected with calreticulin (CR) molecularly modified by the insertion of the nonapeptide tag HA1, derived from influenza virus hemoagglutinin (Field et al., 1988). CR is an ubiquitous Ca^{2+} binding protein recently proposed to play multiple roles as diverse as buffering of the cation within the ER lumen (Michalak et al., 1992; Pozzan et al., 1994), regulation of transmembrane signaling at the plasmalemma (Rojiani et al., 1991) and regulation of transcription in the nucleus (Dedhar et al., 1994). Our study, carried out by the combination of morphological, biochemical, and Ca^{2+} measurement techniques, failed to reveal any surface and nuclear distribution of the protein. In contrast, ample and direct evidence emerged not only about the role of the protein in intralumenal Ca^{2+} buffering, but also about the nature and the mechanisms of functioning of the agonist-sensitive Ca^{2+} stores, revealed by CR acting as a specific reporter of its own microenvironment.

Materials and Methods

Materials

HeLa cell line was obtained from American Type Culture Collection (Rockville, MD); fura-2 and ionomycin from Calbiochem-Behring Corp. (San Diego, CA); thapsigargin (Tg) from L.C. Service Corp. (Woburn, MA). The antibodies (Abs) used have been described elsewhere: anti-CR, a rabbit polyclonal Ab (pAb), by Perrin et al. (1991); anti-protein disulfide isomerase (PDI), a rabbit pAb, by Villa et al. (1993); anti-SERCAs, a mouse mAb, by Colyer et al. (1989); anti-inositol 1,4,5-trisphosphate (IP_3) receptor, by Villa et al. (1992). The anti-tag monoclonal Ab, mAb12CA5 was purchased from the Berkeley Antibody Co. (Berkeley, CA); [^{125}I] protein A and $^{45}Ca^{2+}$ from Amersham Int. (Amersham, UK); culture sera and media from GIBCO-BRL (Basel, Switzerland); monensin, ATP, bradykinin, histamine, carbachol, A23187, and the remaining chemicals from Sigma-Aldrich (Milano, Italy). The used PCR modification of the original aequorin cDNA (kindly provided by Dr. Y. Sakaki, University of Tokyo, Tokyo, Japan), has been described elsewhere (Brini et al., 1995). The cDNA of human CR was obtained from Dr. D. P. McCauliffe (Southwestern Medical Center, Dallas, TX).

Construction of the Tagged Calreticulin (tCR) cDNA

The sequence encoding the HA1 epitope of hemagglutinin (Field et al., 1988) was inserted downstream of the ClaI site located at nucleotide 739 of the wild type (wt) CR cDNA (McCauliffe et al., 1990) by the procedure summarized in Fig. 1. The portion of the wt CR cDNA downstream of the ClaI site was first amplified by PCR with the following primers:

forward:

5′-ATCGATTATGATGTTCCTGATTATGCAAGCTTAATTGATGATCCCACAGACTCCAAGCCT-3′

reverse:

5′-TCTCTACAGCTCGTCCTTGGC-3′

The reverse primer corresponds to the antisense orientation of nt 1303-1323 of the CR cDNA; the forward primer specifies, from 5′ to 3′, a ClaI site, the sequence encoding the HA1 epitope tag (YDVPDYASL) and nt 739-765 of the CR cDNA, starting from the endogenous ClaI site, which was eliminated by introducing a silent mutation in codon 225 (ATC^{Ile}-ATT^{Ile}). The sequences within the primer specifying the new ClaI site and the mutated ClaI site of the CR cDNA are underlined.

The PCR amplification was done over 30 cycles (1 min at 95°, 2 min at 55°C, 1 min at 72°C), using 2 ng of template DNA. The following cloning steps were carried out according to standard procedures (Sambrook et al., 1989). The PCR product, cloned in pBSK$^+$ (Stratagene Corp., La Jolla, CA) and controlled by DNA sequencing, was excised via the ClaI site and an XbaI site located in the vector sequence immediately downstream of the insert. This ClaI/XbaI 0.65-kb fragment was then utilized to replace the endogenous ClaI/XbaI fragment of the CR cDNA (thus removing a large part of the 3′ non-coding region); the final cDNA encodes a CR polypeptide (tCR) which includes the HA1 tag at amino acid (aa) 225 of the CR sequence. The whole coding sequence of tCR was excised by EcoRI/XbaI digestion and cloned in the expression vector pcDNAI (InVitrogen). The recombinant vector (tCR/pcDNAI) was then employed in all the transfection experiments.

Cell Culture and Transfection

HeLa cells were grown in DMEM, supplemented with 10% FCS, in 75 cm^2 Falcon flasks. For the experiments on immunofluorescence, Western blotting, ^{45}Ca release and single cell $[Ca^{2+}]_i$ measurement, purified plasmids containing either the vector alone or the tCR cDNA were introduced into subconfluent cells using the lipofectin protocol as described by Muller et al. (1990). For the aequorin experiments, the cells were plated onto 13-mm-diam round coverslips and transfection with 4 μg/well of plasmid DNA (cytAEQ/pcDNAI and tCR/pcDNAI in 1:1 ratio) was carried out with the Ca^{2+} phosphate technique as described by Rizzuto et al. (1994*b*). Aequorin measurements and calibration of the aequorin signal were carried out 36 h after transfection, as previously described (Brini et al., 1995).

Immunofluorescence

For CR and tag immunolabeling cell monolayers were fixed with a mixture of 4% paraformaldehyde and 0.25% glutaraldehyde in 125 mM phosphate buffer, pH 7.4, detached mechanically and centrifuged. The pellet was washed with the buffer and infiltrated in 0.6 M sucrose mixed with 7% polyvinylpyrrolidone. By successive concentration increases of the infiltrating solutions the samples were brought to 1.86 M sucrose–20% polyvinylpyrrolidone and then frozen in a 3:1 mixture of propane and cyclopentane cooled with liquid nitrogen. 1-μm-thick sections were cut in an Ultracut ultramicrotome equipped with a FC4 cryosection apparatus (both from Reichert-Jung, Vienna, Austria). Sections were then flattened over glass slides and covered with 2% liquid gelatin in 125 mM Na phosphate buffer, pH 7.4. After a short treatment with 1% Na borohydrate (to eliminate glutaraldehyde fluorescence) they were washed and then exposed for 30 min to the permeabilizing solution containing 0.3% Triton X-100, 15% filtered goat serum, 0.45 M NaCl, and 10 mM phosphate buffer, pH 7.4. After washing, the sections were exposed (1 h at 37°C) to either one of the primary Abs diluted in the above Triton X-100 and goat serum–containing solution, or to a mixture of the two. They were then washed thoroughly and treated with the appropriate rhodamine-labeled sheep anti-first Ab (1:20–1:40 in the Triton X-100, goat serum solution, 30–60 min, 37 °C), or with a mixture of fluorescein-labeled anti–rabbit IgG and rhodamine-labeled anti–mouse IgG (Technogenetics, Milano, Italy). The Con A–tag dual labeling was carried out in cell monolayers that were fixed with 4% buffered formaldehyde for 15 min, washed, and immediately exposed for 30 min to biotinylated Con A (Pierce, Rockford IL; 5 μg/ml). After thorough washing, they were exposed for 30 min to fluoresceinated streptavidin (Amersham Int.; diluted 1/300). After further washing they were exposed for 30 min to the Triton X-100 containing permeabilizing solution and then immunolabeled with the anti-tag Ab exactly as described above. After another thorough washing, the sections were mounted in glycerol to be examined in a Zeiss Photomicroscope III apparatus (Carl Zeiss Inc., Oberkochen, Germany). For additional details see Villa et al. (1993).

One and Two-dimensional Polyacrylamide Gel Electrophoresis and Western Blotting

Control and tCR-transfected HeLa cells were cultured, harvested, washed in PBS, and lysed in 150 mM NaCl, 15 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 50 mM Hepes-KOH, 10% glycerol, 1% Triton X-100, pH 7.5. In

some experiments scale amounts of a purified CR standard were prepared in parallel. For one-dimensional analysis, after addition of SDS and β-mercaptoethanol, the samples were boiled, and 50 μg protein (assayed by the bicinchoninic acid procedure) were loaded per lane onto either 5–8% gradient or 10% SDS polyacrylamide minigels which were run as described elsewhere (Villa et al., 1992). Two-dimensional gel electrophoresis was performed essentially as described by O'Farrel (1977). Briefly, samples were additioned with SDS, β-mercaptoethanol, urea, and carrier ampholyte (2.5–5; Pharmalyte), then 250 μg protein were analyzed by non-equilibrium pH electrophoresis in the first dimension, followed by 8% SDS polyacrylamide gel electrophoresis in the second dimension. High transfer of proteins onto nitrocellulose membranes was carried out at 200 mA for 18 h in a buffer containing: 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3. After transfer, both the gels and the blots (one and two dimensional) were routinely stained with Ponceau red. Labeling with ^{45}Ca (Ca overlay) was carried out as described by Damiani et al. (1989). When needed, bound ^{45}Ca was removed from the blots by extensive washing with 1 mM EGTA-Tris, pH 7.4. For Ab immunodecoration the nitrocellulose sheets were processed at room temperature, first for 1 h with PBS + 3% BSA, then for 2 h with appropriate concentrations of the specific Abs in the same buffer. After washing five times for 5 min with 150 mM NaCl, 50 mM Tris-HCl, 0.05% Tween-20, 5% powdered milk, pH 7.4, the blotted bands were decorated with [^{125}I] protein A. Blots were then washed five times for 10 min with the above buffer, dried and finally autoradiographed at −80°C for variable periods of time. Microdensitometry of the relevant bands was carried out using a Molecular Dynamics Imagequant apparatus (Raichman et al., 1995). Results shown are representative of three to five separate experiments.

$^{45}Ca^{2+}$ Release

HeLa cells were grown as described above, except that during the last 48 hours their incubation medium was additioned with $^{45}Ca^{2+}$ (1.0 μCi/ml). Labeled cells were rapidly washed in Krebs-Ringer-Hepes (KRH) medium (containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM glucose, 25 mM Hepes-NaOH, pH 7.4), and then resuspended at 37°C in 3 mM EGTA-containing, Ca^{2+}-free KRH medium and quickly additioned with Tg (0.1 μM) followed 5 min later by ionomycin (0.5 μM) and, after 5 more min, by monensin (1 μM). Immediately before the additions and 5 min after monensin, equal aliquots containing ~0.5 mg of cell protein were centrifuged, and the $^{45}Ca^{2+}$ recovered into the medium estimated in a Beckman β-counter (for further details see Fasolato et al., 1991). Results shown are means ± SD of two separate, highly consistent experiments run in triplicate.

Combined $[Ca^{2+}]_i$ and CR Measurements in Single Cells

Coverslips were incubated at 37°C for 30 min with culture medium containing fura-2 AM (5 μM). At the end of loading the coverslips were mounted on the recording chamber of an inverted microscope (Zeiss IM) and incubated in EGTA-containing Ca^{2+}-free KRH medium. Single bright fields were selected and pictures taken before starting the fluorescence analyses. For the assay, a modified Jasco CAM-230 dual wavelength microfluorimeter was used as a light source. Images were collected by a low light level CCD camera (Photonic Science), digitized and integrated in real time in an image processor developed by the laboratory (Grohovaz et al., 1991). Image files were processed off line to convert fluorescence data in $[Ca^{2+}]_c$ maps according to the 340/380 nm excitation wavelength ratio method (Grynkyewicz et al., 1985). At the end of each fluorescence experiment the analyzed field was marked by a diamond tip mounted on the objective lens revolver to facilitate recognition. The coverslip was then washed three times in PBS 120 mM, pH 7.4, and then fixed at room temperature with paraformaldheyde (4%) in the same buffer. 40 min later the coverslip was extensively washed with PBS (four passages, 30 min) and then covered with 2 ml of a solution containing 5 vol of a phosphate-buffered, 0.45 M Na Cl solution mixed with 1 vol of non-immune goat serum. After dual indirect immunolabeling for CR and the tag as described in the immunocytochemistry section, the same cells analyzed for $[Ca^{2+}]_c$ were examined in the computerized imaging apparatus for a quantitative assay of both anti-CR and anti-tag immunofluorescence. Control preparations were processed in parallel but the first Abs were omitted.

Results

Fig. 1 shows the map of the tCR cDNA and a summary of its construction strategy. The nucleotide sequence coding the nine aminoacids of the immunogenic epitope of influenza virus hemagglutinin, HA1 (Field et al., 1988), was inserted in the cDNA coding for human CR (McCauliffe et al., 1990) at nt 740, i.e. before the sequence encoding the strongly acidic COOH-terminal domain, where low affinity Ca^{2+} binding is known to take place. The modified CR cDNA was inserted in the expression plasmid pcDNAI and employed for transient transfection of HeLa cells (tCR cells), while controls received the plasmid alone.

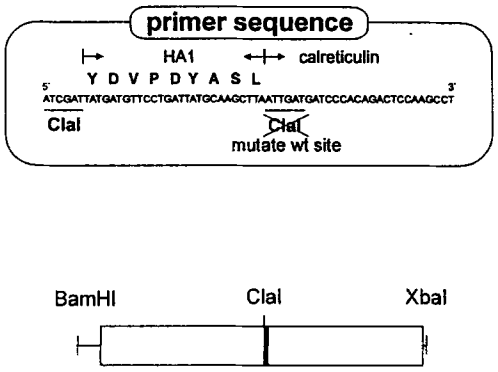

Figure 1. Construction strategy of HA1-tagged calreticulin. On the top is shown the sequence of the upstream primer which includes the modification to the wild type CR cDNA (McCauliffe et al., 1990); on the bottom, a schematic map of the tCR cDNA. Dark and light boxes indicate the portions of the cDNAs encoding the epitope and the Ca^{2+}-binding protein, respectively, while the non-coding regions are represented as lines. The position of the relevant restriction sites is also indicated.

The subcellular distribution of the endogenous and tagged CR, as revealed by immunofluorescence microscopy of 1-μm-thick sections prepared from tCR and control cells, is illustrated in Fig 2. As can be seen, all the analyzed cells were found to be positive with the anti-CR Ab, however in the controls (Fig. 2 *A*) the signal was moderate and the variability among cells minimal, whereas in a fraction of the tCR cells (on the average 42%, analyzed fields = 12) the signal was stronger and variable (cfr Fig. 2, *A* and *B*). When the same preparations were decorated with the anti-tag Ab, control cells remained consistently negative, as expected, whereas a fraction of the transfected cells, corresponding to those exhibiting the strong CR signal, appeared distinctly positive (Fig. 2, *C* and *D*). With both Abs the signal (where present) revealed a delicate reticulum distributed to a large part of the cytoplasm. This is the well known pattern typical of the ER. Dual labeling of tag-positive cells with anti-CR Abs revealed overlapping distribution (not shown). At variance with previous reports (Michalak et al., 1992; Dedhar, 1994), neither with anti-CR, nor with anti-tag Ab was any immunolabeling revealed within the nucleus (Fig. 2, *A–D*). Moreover, confocal microscopy analysis of tCR cells first surface labeled with Con A and then immunostained with the anti-tag Ab revealed neither colocalization nor even contiguity of the two signals, which remained largely separated by a clear space (Fig. 2, *E–G*). The above results exclude the free cytosolic localization of appreciable amounts of tCR and their binding to the inner surface of the plasmalemma (Rajiani et al., 1991).

A biochemical characterization of ER and intracellular

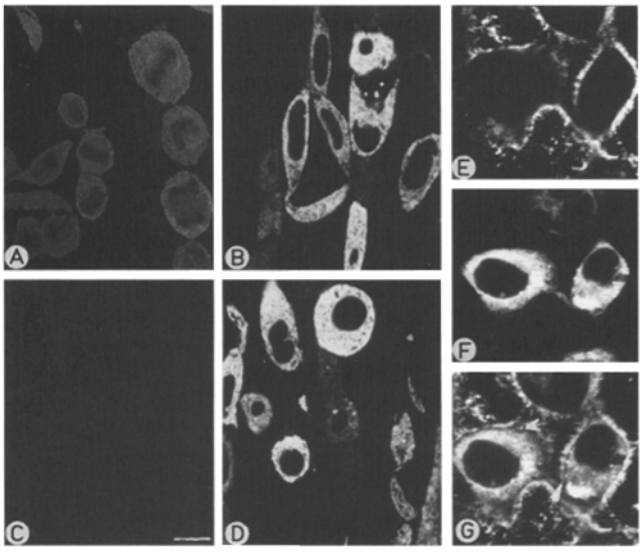

Figure 2. Immunofluorescence of 1 μm sections of control and tCR HeLa cells indirectly labeled with anti-CR (*A* and *B*), anti-tag (*C* and *D*) and with ConA + anti-tag (*E–G*), respectively. Notice that the CR signal is uniformly moderate in control cells, revealing a delicate network throughout the cytoplasm (*A*). In the tCR cells the signal is clearly higher in a large subpopulation (~40% of the total), exhibiting a considerable variability in intensity and maintaining the intracellular CR distribution (*B*). No tag signal appears in the control cells (*C*), whereas in the tCR cells (*D*) it resembles closely, as far as frequency, variability and distribution, the increased signal observed with the anti-CR Abs. In contrast nuclei are consistently negative (compare *B* and *D*). In *E* and *F* (confocal images of the same field) ConA decorates the dense coat located at the external surface of the cells whereas the anti-tag Ab reveals the delicate cytoplasmic network in transfected cells already described in panel *D*. Superimposition of *E* and *F* (*G*) reveals the presence of a linear space separating the ConA and tag images (*arrows*), consistent with the conclusion that, even in the overexpressing cells, CR is located within the ER, with no appreciable evidence for its binding at the cytosolic surface of the plasmalemma. Bars: (*A–D*) 10 μm; (*E–G*) 12 μm.

Ca^{2+} store markers of control and tCR HeLa cells was carried out by Western blotting. Fig. 3, upper panels, shows the anti-CR and anti-tag immunolabeling of the two preparations. As expected, positivity for the anti-tag Ab was restricted to the tCR cells which, in addition, exhibited an anti-CR signal much higher than that of controls (on the average +250%; $n = 5$). In the gel, tCR exhibited a slightly slower mobility compared to native CR, with ensuing appearance of a band doublet, visible however only after short autoradiogram exposure. By making reference to scale amounts of an authentic CR standard (Raichman et al., 1995) run in parallel, the total CR values in control and tCR cells were calculated to be on the average 1.6 and 5.6 μg/mg of cell lysate protein, respectively. Other markers of the ER and Ca^{2+} stores, i.e., the lumenal PDI, the SERCA and the IP_3 receptor, appeared unchanged in the tCR preparations with respect to controls (Fig. 3).

Additional information on the native and transfected CR was obtained by the study of two-dimensional blots. Fig. 4, *A* and *B* and *D* and *E*, compares the Ponceau red and the immunostaining of parallel CR Western blots of a control and a tCR cell preparation. As can be seen, the wild type and tagged proteins, separate now as discrete spots, were both found to run in the acidic range, however with a slight difference of their apparent isoelectric point (4.42 and 4.50, respectively). By microdensitometry the CR values in tCR cells were found on the average to exceed the controls of 3.2- and 3.8-fold for Ponceau red and immunolabeling, respectively. Fig. 4, *C* and *F*, illustrate the results obtained when the same blots were labeled with ^{45}Ca to reveal the binding of the cation to the protein spots (Ca^{2+} overlay test). Both the native and the transfected recombinant CRs were found to be positive, with a labeling ratio similar to that mentioned above for Ponceau red and immunodecoration.

The study of Ca^{2+} homeostasis in the two types of cell preparations was carried out in parallel by three different experimental approaches. In the first, the cells were labeled at the equilibrium (48 h) with ^{45}Ca, washed, and then exposed, while bathed in the nonradioactive, Ca^{2+}-free medium, to three 5 min treatments applied in sequence: with the SERCA blocker, Tg (Thastrup et al., 1990), followed by the Ca^{2+} ionophore ionomycin, which is known to exchange 1 Ca^{2+} for 2 H^+, and then by monensin, another ionophore, however of different specificity (Na^+ or K^+ for H^+). Preliminary experiments revealed this protocol to be appropriate to release to the incubation medium the ^{45}Ca contained in the three distinct pools previously characterized in the PC12 cell line (Fasolato et al., 1991). These pools correspond functionally to: (*a*) the rapidly ex-

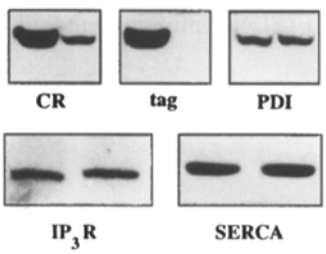

Figure 3. One-dimensional Western blot of endogenous and tagged calreticulin, as well as of other ER proteins, in tCR and control HeLa cells. Specific Abs were employed to reveal the expression of the different proteins. 50 μg protein aliquots of cell lysates of tCR and control cells were run in parallel in 5–8% gradient (IP_3R and SERCA) or 10% (all the others) polyacrylamide gels. Labels are as follows. *CR*, polyclonal Ab against CR; *tag*, monoclonal Ab against the HA1 tag; *PDI*, polyclonal Ab against PDI; *IP_3R*, polyclonal Ab against IP_3R; *SERCA*, monoclonal Ab against SERCA. For each Ab, the left lane refers to tCR cells and the right lane to control cells. Blot transfer and immunodecoration were as described in Materials and Methods.

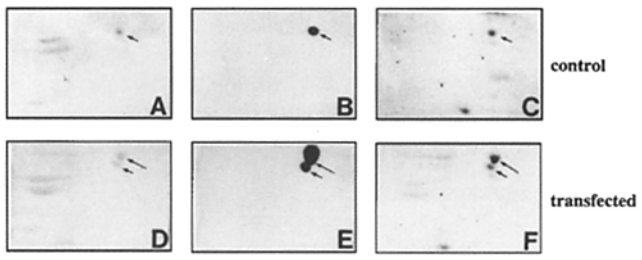

Figure 4. Single two-dimensional calreticulin Western blot from control and tCR HeLa cells revealed with Ponceau red staining (*left*), anti-CR immunodecoration (*center*) and ^{45}Ca overlay (*right*). The position of the endogenous and tCR is indicated by short and long arrows, respectively. For details see Materials and Methods.

changing Ca^{2+} store sensitive to agonists, most likely located in the ER lumen; (*b*) a group of other organelles (including mitochondria) characterized by a neutral or alkaline lumenal pH; and (*c*) the acidic pools of the cell (secretory granules, endosomes, lysosomes, etc.) (Fasolato et al., 1991). The results obtained by the ^{45}Ca labeling approach are shown in Fig. 5. Only the Tg-releasable pool was considerably different (+118%) in the tCR vs control cells. In terms of Ca^{2+} content the values in the two preparations correspond to 1.05 and 0.48 nmoles/mg protein, respectively. The pool released by ionomycin appeared only moderately (+13%) larger in the tCR cells, while the monensin pool was unaffected. Thus, the change in Ca^{2+} content induced by CR overexpression appeared concentrated almost exclusively in the Tg-sensitive pool. These results not only confirm the identification of the first Ca^{2+} pool as the ER, but also suggest that the ionomycin sensitive pool, at least in HeLa cells, is primarily accounted for by non-ER organelles.

In the second approach, monolayers of the control and tCR cells were loaded with fura-2 and then investigated by computerized image analysis. At the end of the $[Ca^{2+}]_c$ experiments the preparations were fixed with aldehydes and dually immunolabeled with Abs against CR and the tag. Thus, the same cells previously analyzed for $[Ca^{2+}]_c$ could be individually assayed quantitatively for expression of the two proteins. By this approach correlations between CR expression and $[Ca^{2+}]_c$ responses were expected to be revealed. When however the cells were exposed to maximal stimulation by IP_3-generating agents (a mixture of 100 μM histamine + 500 μM carbachol + 0.1 μM bradykinin) administered in Ca^{2+}-free medium to exclude any contribution of influx from the extracellular environment, the $[Ca^{2+}]_c$ response differences observed between tCR and control cells were small and the CR/$[Ca^{2+}]_c$ correlations remained below significance (six experiments, 126 cells, data not shown).

The inconclusive results obtained by the single cell $[Ca^{2+}]_c$ approach may be due to experimental drawbacks. Given the heterogeneity of the cell populations, the variability among cells responses and among experiments was in fact quite high. This was not unexpected since a variety of parameters, such as fura-2 concentration (and thus Ca^{2+} buffering capacity), dye sequestration, etc., can contribute to cell heterogeneity and could thus mask the differences between tCR and control cells. In order to bypass these problems, a different experimental approach was developed based on the cotransfection of either tCR or a control plasmid together with the Ca^{2+} sensitive photoprotein, aequorin. Unless specific targeting sequences are added, the photoprotein remains localized in the cytosol and can therefore reveal $[Ca^{2+}]_c$ (Brini et al., 1995). In cotransfection experiments, the same subset of cells receives the two cDNAs together and does therefore express both recombinant proteins (Brini et al., 1995), offering advantages for our purpose. In particular: (*a*) the protocol is very rapid and reproducible; (*b*) aequorin is well suited for detecting $[Ca^{2+}]_c$ changes in the low micromolar range; (*c*) the Ca^{2+} indicator is exclusively located in the cytosol; (*d*) the signal is averaged over the whole population of transfected cells; and (*e*) the extra Ca^{2+} buffering capacity is negligible. To validate the use of aequorin in our experimental system we first carried out a simple experiment in which tCR and control cells were exposed not to receptor agonists but to a high concentration (10 μM) of the Ca^{2+} ionophore, A23187. As already mentioned for ionomycin in the ^{45}Ca experiments, exposure to ionophores releases to the cytosol (although with different kinetics) not only IP_3-sensitive, but also any non-acidic Ca^{2+} stores of the cell. The effect induced by A23187 on the aequorin-loaded tCR and control cells suspended in Ca^{2+}-free medium is shown in Fig. 6. As can be seen, drastic differences appeared both in $[Ca^{2+}]_c$ peak height and in total Ca^{2+} release, calculated from the integral of the curve (1.4- and 2.8-fold increases, respectively, Fig. 6).

The next question investigated by the use of aequorin was whether the increased Ca^{2+} content of tCR cells revealed by A23187 treatment was indeed releasable by IP_3. When tCR cells were stimulated with histamine (100 μM, administered in the Ca^{2+}-free medium), the peak $[Ca^{2+}]_c$ response of tCR cells was increased compared to control cells, however only modestly (1.8 ± 0.1 vs. 1.7 ± 0.1 μM), with a more drastic alteration of the kinetics of the $[Ca^{2+}]_c$ transients. In particular, the time required to return to the prestimulatory level was two to three times longer in the tCR cells, with an average (n = 5) increase of almost twofold (1.8 ± 0.1) in the integral of the curve expressing the overall $[Ca^{2+}]_c$ change (see Fig. 7 *A*, *left peak*).

To establish whether these differences were due to a different Ca^{2+} content of the IP_3-sensitive Ca^{2+} stores, a two response protocol was developed in which cell preparations suspended in Ca^{2+}-free, EGTA-containing medium

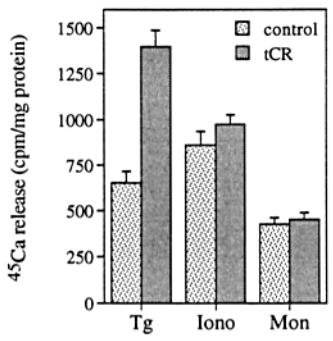

Figure 5. ^{45}Ca release from control and tCR HeLa cells suspensions treated in sequence with Tg (0.1 μM), ionomycin (Iono 0.5 μM), and monensin (Mon, 1 μM). Cell suspensions in Ca^{2+}-free, EGTA-containing KRH medium were incubated at 37°C and parallel aliquots were collected and centrifuged immediately before the addition of Tg and 5 min after the addition of each drug. Values shown are averages ±SD of the cpm recovered in the media, subtracted of those recovered in the preceding collection, normalized to the protein in the corresponding cell pellets.

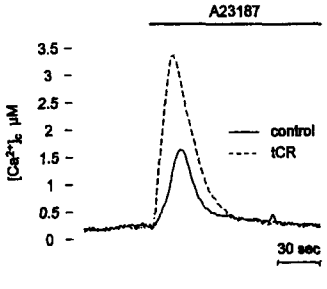

Figure 6. Effect of A23187 on $[Ca^{2+}]_c$ in tCR and control HeLa cells. The cells were trypsinized, plated on 13 mm coverslips, transfected with cytosolic aequorin together with HA1-tagged CR or pcDNAI, and left in culture for 2 d, as described in the Materials and Methods section. At the beginning of the experiment the Ca^{2+}-free KRH medium was supplemented with 100 μM EGTA. The dotted and solid traces refer, respectively, to tCR and control cells. Where indicated the cells were challenged with 10 μM A23187.

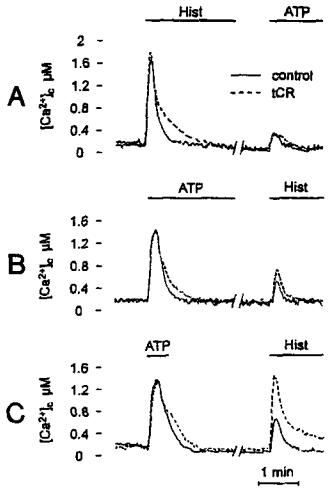

Figure 7. Measurement with cytosolic aequorin of $[Ca^{2+}]_c$ changes in tCR and control HeLa cells upon stimulation with two different agonists. Experimental conditions were as in Fig. 6. (*A* and *B*) changes of $[Ca^{2+}]_c$ induced by stimulation with histamine (*Hist*, 100 μM) followed by ATP (100 μM) (*A*) or vice versa (*B*). (*C*) changes of $[Ca^{2+}]_c$ induced by histamine administered after a short (30 s) stimulation with ATP followed by washing. Labels as in Fig. 6.

(to prevent Ca^{2+} influx and refilling of the stores) were first challenged with an agonist and then, after exhaustion of the first $[Ca^{2+}]_c$ response, with a second agonist, addressed however to a different receptor to escape the risk of homologous desensitization. Initial experiments were carried out with stimulations a few min long, by histamine (100 μM) first and ATP (100 μM) second. Under these conditions, after the first response, a second $[Ca^{2+}]_c$ response appeared which however was small and again not very different in height in the two types of cell preparations (Fig. 7 *A*). Reversing the order of the additions (Fig. 7 *B*), i.e., administering ATP first and histamine second however with no change of the stimulant exposure times, did not modify the overall picture, except that the height of the first peak was indistinguishable in the two cell populations, while the second small peak, induced by histamine, was usually bigger in tCR cells.

Clear evidence of increased Ca^{2+} content in IP_3-sensitive stores of tCR cells emerged only when stimulation with the first agonist was shortened, i.e., when the stimulus was washed out 30 s after its addition (Fig. 7 *C*). In this case, the difference with respect to controls concerned not the amplitude of the first peak (induced by ATP), which was unaffected, but the second $[Ca^{2+}]_c$ response (to histamine). In control cells, in fact, this response was only moderately bigger than that observed in the long-treated preparations, whereas in tCR cells it appeared as big as the first response, i.e., almost twofold bigger (1.54 ± 0.17, $n = 6$) in peak height and almost fourfold (3.90 ± 0.87, $n = 6$) as integral of the curve with respect to its counterpart in control cells (Fig. 7 *C*). Further experiments revealed that the appearance of a larger second response in tCR with respect to control cells depended critically on the duration of the first stimulus and not on the time interval between the stimuli, at least in the 2–10 min range (not shown). Taken together, the aequorin results document that, compared to controls, tCR cells contain more Ca^{2+} in the lumen of their IP_3-sensitive stores. This difference, however is revealed clearly not by a simple stimulation, whose $[Ca^{2+}]_c$ peak response appears limited primarily by factors other than the store capacity, but by the two stimulation protocol, provided that the first agonist is applied for a short period, enough to elicit a drastic depletion of the stores in controls but not in tCR cells.

In a final series of experiments, the aequorin cotransfection approach was employed to investigate whether expression of tCR had an effect also on the Ca^{2+} influx induced by receptor activation. Previous experiments in HeLa and in a variety of other cells demonstrated that part of this influx is sustained by a current (indicated with the acronym I_{CRAC}, Hoth and Penner, 1992) activated in relation to the state of emptying of the agonist-sensitive stores (for review see Fasolato et al., 1994). If indeed stimulation of Ca^{2+} influx depends on the free $[Ca^{2+}]$ of the ER lumen $[Ca^{2+}]_{er}$ short-term receptor activations would be expected to stimulate influx more intensely in control than in tCR cells. To investigate the problem, cell preparations were exposed first for 30 s to histamine while suspended in the Ca^{2+}-free EGTA-containing medium, and then excess (3 mM) $CaCl_2$ was added to induce a rapid and transient overshoot of $[Ca^{2+}]_c$ sustained by influx. As can be seen in Fig. 8, the overshoot, well visible in controls, was drastically reduced in tCR-cotransfected cells. Averages of the maximal overshoot values obtained in three consistent experiments were 0.43 ± 0.16 and 0.83 ± 0.16 μM, respectively. These results confirm that in tCR cells the degree of store emptying induced by the short histamine treatment is less profound than in control cells, thus generating a weaker signal for activation of I_{CRAC}.

Discussion

The present knowledge on the functioning of rapidly exchanging Ca^{2+} stores is the result of studies carried out primarily by two experimental approaches. The first, in intact cells, consists of the study of the $[Ca^{2+}]_c$ rises triggered by receptor stimulation, while the second is based on the study of permeabilized cells, microsomal fractions or individual store components: SERCA pumps, lumenal Ca^{2+} binding proteins, IP_3, and ryanodine receptor channels.

A third approach that can combine the advantages of the first two can be developed working with intact cells, modified however specifically in one of their store components. Up until now, however, no studies of this type had been reported with lumenal Ca^{2+} binding proteins except for the recent demonstrations that the decrease of CR levels, induced in NG-108-15 cells by specific antisense oligonucleotides (Liu et al., 1994), and the accumulation of transfected calsequestrin within the ER cisternae of differentiated L6 myotubes (Raichman et al., 1995) result in attenuation and reinforcement of the receptor-induced, IP_3-dependent $[Ca^{2+}]_c$ responses, respectively.

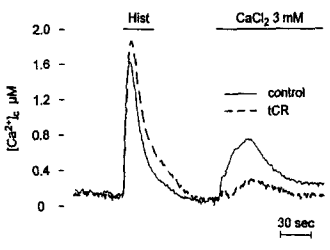

Figure 8. Effect of tCR overexpression on receptor-triggered Ca^{2+} influx measured with cytosolic aequorin in tCR and control HeLa cells. Where indicated the cells were challenged with 100 μM histamine (*Hist*) in the Ca^{2+}-free KRH medium supplemented with 100 μM EGTA. Ca^{2+} influx was initiated by perfusing KRH medium supplemented, where indicated, with 3 mM $CaCl_2$. All other conditions as in Fig. 6.

In order to gain further insight into Ca^{2+} storage problems, we have carried out experiments in HeLa cells transiently expressing tCR. The latter protein (which includes one high affinity and numerous, possibly 20, low affinity Ca^{2+} binding sites; see Michalak et al., 1992) was used not only to modify cell Ca^{2+} homeostasis, but also to serve as a reporter of the lumenal store microenvironment. As a whole, our approach was based on the assumption that tCR resembles in subcellular distribution, Ca^{2+} binding and molecular properties its endogenous counterpart. Indeed, the overlapping distribution patterns revealed by anti-CR and anti-tag Abs and the parallel ^{45}Ca binding data revealed by blot overlay strongly support this assumption, even if moderate changes in affinity and number of the Ca^{2+} binding sites cannot be formally excluded until equilibrium binding studies with purified tCR are carried out. However, (*a*) the lack of effect of the tag in the ^{45}Ca overlay test; and (*b*) the knowledge that the low affinity Ca^{2+} binding to CR occurs in the COOH-terminal, acidic domain (while the tag was inserted upstream of it), make our assumption quite likely.

The ^{45}Ca results should be considered together with the $[Ca^{2+}]_c$ results obtained in stimulated cells by either fura-2 or aequorin. In these latter two cases, however, the changes of $[Ca^{2+}]_c$ are not simply related to the content of Ca^{2+} within the stores. Rather, they result from dynamic equilibria among the stores themselves, the Ca^{2+} buffering of the cytosol, the uptake and release of other organelles such as mitochondria and, most relevant, the transport of Ca^{2+} across the plasmalemma.

In spite of these problems, important conclusions can be drawn from the results we have obtained. First, the increased ^{45}Ca accumulation of the tCR cell population was almost entirely released by Tg, the well known, irreversible blocker of the SERCA pumps (Thastrup et al., 1990). CR and SERCAs appear therefore to colocalize in the same compartment, the ER. This observation excludes that major areas of the latter compartment are insensitive to Tg and can be emptied by the subsequent administration of ionomycin, as previously hypothesized (Fasolato et al., 1991). Second, CR expression in the whole tCR-transfected cell population was found to exceed the levels in controls of 3.5-fold, while the corresponding increase of the Tg-releasable ^{45}Ca pool was lower, i.e., 2.18-fold. The increased ^{45}Ca accumulation of tCR cells does demonstrate the role of CR in Ca^{2+} storage within the ER lumen, however the contribution of the protein seems to account for only a fraction of the total Ca^{2+} binding capacity. Residual storage is presumably contributed by other ER lumenal Ca^{2+} binding proteins: PDI, BiP, endoplasmin, CaBP1 and CaBP2, calstorin, reticulocalbin, and possibly also by some membrane proteins, such as calnexin (see Van et al., 1989; Lucero et al., 1994; Racchetti et al., 1994; for review see Pozzan et al., 1994). In our present study, expression of PDI, (together with SERCA and IP_3 receptor) was found to be unchanged in tCR cells, and we have no reason to expect different situations for the other ER lumenal and membrane proteins. If this assumption is correct, the contribution of CR to rapidly exchanging Ca^{2+} storage of control cells can be calculated (by simply dividing for each other the percent increases of Tg-induced ^{45}Ca release and CR expression in tCR cells) to be around 47%. Interestingly, this value is not far from a previous estimate based on the observation that, at 1 mM $[Ca^{2+}]$, CR accounts for ~57% of the total Ca^{2+} binding to liver microsomal extracts (Treves et al., 1990). As to the physiological responses, the height of the first peak induced by receptor stimulation in individual cells and cell populations expressing high levels of tCR (revealed by fura-2 or aequorin) was almost unchanged with respect to the controls expressing only the endogenous protein (and thus much less Ca^{2+} stored in the ER). These results demonstrate that the main limiting factor determining the $[Ca^{2+}]_c$ peak amplitude is not the Ca^{2+} content of the stores but other properties, most likely including the concentration of IP_3 receptors and/or their modulation. Support to this interpretation comes from the demonstration that, when IP_3 receptors were bypassed by an ionophore, the peak rise in $[Ca^{2+}]_c$ was much larger in tCR than in control cells. Taken together, the release results concur therefore with the ^{45}Ca and A123187 data to indicate in tCR cells a gross increase of the lumenal ER Ca^{2+} buffering capacity, sustained by CR overexpression.

The comparison of Ca^{2+} storage in control and tCR cells provides indirect information also on $[Ca^{2+}]_{er}$. Under resting conditions such a parameter is expected to be set by the equilibrium between the rates of Ca^{2+} uptake and leak, independently of the buffering capacity of the stores. However, the fact that overexpression of CR (a protein that, in addition to 1 high affinity includes numerous binding sites with k_D ~1 mM: Treves et al., 1989; Michalak et al., 1992) induces a large increase of bound Ca^{2+} (at least 9 moles/mole of tCR, as revealed by the ^{45}Ca release and Western blot data) strongly suggests the resting $[Ca^{2+}]_{er}$ to be at (or to approach) mM values. Consistent with these conclusions are results with another ER lumenal Ca^{2+} binding protein, calsequestrin, obtained in differentiated L6 cells (Raichman et al., 1995) and also recent data of direct lumenal monitoring with ER-segregated aequorin, obtained however using not Ca^{2+} but the divalent cation with lower affinity for the photoprotein, Sr^{2+}(Montero, M., M. Brini, R. Marsault, J. Alvarez, R. Sitia, T. Pozzan and R. Rizzuto, manuscript submitted for publication). In our present experiments the equilibrium conditions are expected to be differentially changed in tCR and control cells upon receptor stimulation, i.e. upon release to the cytosol of the same amount of Ca^{2+}, since the drop of free $[Ca^{2+}]_{er}$ is expected to be bigger in control than in tCR cells. These considerations explain why clear differences of the $[Ca^{2+}]_c$ responses in tCR cells with respect to controls emerged with the aequorin experiments only when the store had been partially depleted. Moreover, our results provide evidence concerning two additional hot aspects of Ca^{2+} store physiology. In particular: (*a*) consistent with recent conclusions of Hirose and Iino (1994), free lumenal Ca^{2+} appears not to be a major determinant of the kinetic properties of the IP_3 receptor, otherwise one would have expected larger $[Ca^{2+}]_c$ peaks in cells overexpressing CR; and (*b*) Ca^{2+} influx through I_{CRAC}-type channels appears indeed inversely correlated to the level of free Ca^{2+} in the store lumen. This conclusion had been postulated since the initial proposal of the capacitative model for Ca^{2+} influx (Putney, 1986), based however on only indirect evidence.

Taken together the results that we have obtained by studying HeLa cells transiently transfected with tCR have revealed a number of new aspects of the ER-located, rapidly exchanging Ca^{2+} stores, concerning the storage activity of CR itself and the free $[Ca^{2+}]$ existing within the lumen. In spite of the high levels of overexpression obtained in the transfected cells, CR was apparently located only within the ER, as documented by its appearance as a delicate cytoplasmic network in the microscopic images, distinct from the nucleus and the plasmalemma, and by the fact that the extra Ca^{2+} accumulation of tCR was almost entirely released by Tg. Thus, our results do not support the possibility of a multiple localization and function of the protein, as recently suggested (Rojiani et al., 1991; Dedhar et al., 1994). Application to other cell types, using again CR or, alternatively, other appropriate lumenal proteins and store components, might ultimately yield valuable information about other problems of Ca^{2+} storage, that still remain open.

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