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Delayed Activation of the Store-operated Calcium Current Induced by Calreticulin Overexpression in RBL-1 Cells

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Calreticulin (CRT) is a high-capacity, low-affinity Ca^{2+}-binding protein located in the lumen of the endoplasmic reticulum (ER) of all eukaryotic cells investigated so far. Its high level of conservation among different species suggests that it serves functions fundamental to cell survival. The role originally proposed for CRT, i.e., the main Ca^{2+} buffer of the ER, has been obscured or even casted by its implication in processes as diverse as gene expression, protein folding, and cell adhesion. In this work we seek the role of CRT in Ca^{2+} storing and signaling by evaluating its effects on the kinetics and amplitude of the store-operated Ca^{2+} current (I_{CRAC}). We show that, in the rat basophilic leukemia cell line RBL-1, overexpression of CRT, but not of its mutant lacking the high-capacity Ca^{2+}-binding domain, markedly retards the I_{CRAC} development, however, only when store depletion is slower than the rate of current activation. On the contrary, when store depletion is rapid and complete, overexpression of CRT has no effect. The present results are compatible with a major Ca^{2+}-buffering role of CRT within the ER but exclude a direct, or indirect, role of this protein on the mechanism of I_{CRAC} activation.

INTRODUCTION

The endoplasmic reticulum (ER) is the most relevant source of mobilizable Ca^{2+} in mammalian cells. The free intralumenal Ca^{2+} concentration has been estimated to range between 0.2 and 2 mM (Hofer *et al.*, 1995; Montero *et al.*, 1995, 1997; Hofer and Schulz 1996; Miyawaki *et al.*, 1997). Among Ca^{2+}-binding proteins that are known to be resident ER proteins, calreticulin (CRT) is among the most abundant (Lytton and Nigam, 1992) and may account for up to ~1–2% of total ER proteins. CRT has been considered the best candidate to function as an ER Ca^{2+} buffer, because it mostly resembles calsequestrin, the well-known Ca^{2+}-storing protein of the sarcoplasmic reticulum. The overall biochemical and functional properties of CRT have been thoroughly characterized. CRT is a soluble, small glycoprotein of 417 amino acids that contains three functional domains (Nash *et al.*, 1994; Pozzan *et al.*, 1994; Meldolesi *et al.*, 1996; Krause and Michalak, 1997). In particular, the C-terminal domain binds Ca^{2+} with high capacity (25–50 mol/mol of protein) and low affinity ($K_d \cong 0.5$–1 mM) through a negatively charged region (Ostwald and MacLennan, 1974; Macer and Koch, 1988; Damiani *et al.*, 1989; Treves *et al.*, 1990).

It has been shown previously that CRT overexpressed by transient transfection in HeLa cells is specifically targeted to the ER and selectively increases the Ca^{2+} content of thapsigargin-sensitive stores (Bastianutto *et al.*, 1995). Along the same line, treatment of cells with antisense oligonucleotides reduces the peak level of $[Ca^{2+}]_i$ increase caused by receptor stimulation (Liu *et al.*, 1994). Recent findings, however, have challenged the role of CRT as a major Ca^{2+}-storing protein (Meldolesi *et al.*, 1996; Krause and Michalak, 1997). The effect on Ca^{2+} homeostasis has in fact been suggested to be independent of the C domain and instead to be attributable to an interaction with the inositol 1,4,5-trisphosphate ($InsP_3$) receptor or the sarcoendoplasmic reticulum Ca^{2+} ATPases mediated by the high-affinity Ca^{2+}-binding site and/or the lectin

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region of the central P domain (Camacho and Lechleiter, 1995). In addition, other unique functions have been attributed to this protein, including modulation of gene expression and cell adhesiveness (Dedhar, 1994; Opas *et al.*, 1996; Coppolino *et al.*, 1997) and involvement in protein folding as a selective chaperone of glycosylated proteins (Helenius *et al.*, 1997).

A picture is emerging from all of these studies in which CRT is a multifunctional protein with a role in Ca^{2+} homeostasis, if any, that appears at the moment undetermined. Contradictions also exist concerning the effects of CRT on Ca^{2+} release and influx across the plasma membrane, particularly on the store-operated Ca^{2+} influx, which is the major pathway for Ca^{2+} entry in nonexcitable cells (Penner *et al.*, 1993; Fasolato *et al.*, 1994; Berridge, 1995). For instance, in CRT-overexpressing cells (Mery *et al.*, 1996), as well as in CRT knockout embryonic stem cells (Coppolino *et al.*, 1997), $[Ca^{2+}]_i$ changes induced by sarcoendoplasmic reticulum Ca^{2+} ATPase inhibitors are similar to those measured in control cells. In CRT knockout cells the $[Ca^{2+}]_i$ changes induced by agonists appear unchanged (Coppolino *et al.*, 1997; but see Liu *et al.*, 1994). Finally, Ca^{2+} influx induced by store depletion is unaffected in CRT knockout cells (Coppolino *et al.*, 1997), whereas in CRT stably transfected cells, Mery *et al.* (1996) found that capacitative Mn^{2+} influx is drastically reduced even on full depletion of the stores.

In view of the importance of CRT in so many cellular activities, we decided to reinvestigate its role in cellular Ca^{2+} handling, in particular trying to definitively establish whether it serves as an important lumenal Ca^{2+} buffer within $InsP_3$-sensitive Ca^{2+} stores. To this end, we transiently transfected cells with wild-type (wt) CRT or its C domain–deleted mutant and monitored the effect of the expressed proteins on the Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}) (Hoth and Penner, 1992, 1993; Zweifach and Lewis, 1993), a dynamic and highly sensitive reporter of free intralumenal $[Ca^{2+}]$ (Hofer *et al.*, 1998). The data obtained support the notion that CRT can efficiently buffer Ca^{2+} within the stores, whereas its overexpression has no direct effect on the mechanism activating this current.

MATERIALS AND METHODS

Construction of CRT cDNAs (tCRT and tCRTΔ)

The cDNA coding for the cytosolic green fluorescent protein (cytGFP bright mutant S65T) in the expression vector VR1012 was kindly provided by Dr. T. MacDonald (Albert Einstein University, New York, NY). CRT cDNA including the HA1 tag at amino acid 225 of the wtCRT sequence (tCRT) (Bastianutto *et al.*, 1995) was excised from the expression vector pcDNAI by *Eco*RI-*Xba*I digestion and cloned in pBluescript SK^+ (pBSK+; Stratagene, La Jolla, CA). The whole coding sequence of tCRT was then cloned in the expression vector VR1012 using *Xba*I and *Eco*RV sites. The sequence encoding the tCRT deletion mutant without the C domain (tCRTΔ) was obtained by amplifying by PCR the vector tCRT/pBSK+ (see above) with the following primers: forward, 5′-GTAATACGACTCACTATAGGGC-3′; reverse, 5′-CTACAGCTCGTCCTTATAGGCATAGATACTGG-3′.

The forward primer is the T7 primer sequence present into the pBSK+ vector; the reverse primer corresponds, from 5′ to 3′, to the antisense orientation of nucleotides 971–988 and 1306–1320 of the wtCRT cDNA (McCauliffe *et al.*, 1990). This primer hybridizes with the sequence coding for the amino acids (aa) SIYAY in position 304–308 and the KDEL sequence plus the stop codon, present in the end of the coding sequence of the wtCRT cDNA (aa 414–417), thus removing the C domain–coding region (aa 309–413) of CRT in the wt cDNA.

The PCR amplification was performed over 30 cycles (1 min at 95°C, 2 min at 55°C, and 1 min at 72°C), using 2 ng of template DNA. The PCR product, cloned in pCR2.1 (Invitrogen, San Diego, CA) and controlled by DNA sequencing, was excised via the *Sal*I site present in the PCR product (between the CRT sequence and the T7 primer) and *Xba*I site located in the vector sequence downstream of the insert. This *Sal*I-*Xba*I 1000 kb fragment was then cloned in the expression vector VR1012 and used together with the cytGFP bright/VR1012 for transfection experiments.

Cell Culture and Transfection

The rat basophilic leukemia cell line RBL-1 (from Dr. R. Penner, Max-Planck Institute, Göttingen, Germany) was cultured in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin. Transient transfection with recombinant cytGFP (bright mutant S65T) and CRT constructs (tCRT or tCRTΔ) was performed by electroporation (Kodak, Rochester, NY). Cells were harvested and resuspended in fresh medium in 4-mm cuvettes in the presence of 10 μg of cytGFP/VR1012 and tCRT/VR1012 (or tCRTΔ/VR1012) plasmids/2×10^6 cells. Cells were subjected to a single pulse characterized by an electric field of 300 V, 1500 microfarads. Cells were transferred to poly-L-lysine-coated glass coverslips (2.5×10^5 cells/coverslip, 13-mm diameter). After overnight incubation, the medium was changed, and after 24–48 h of incubation, the cells were used for immunofluorescence and patch-clamp experiments.

Current Measurements

Patch-clamp experiments were performed in the tight-seal whole-cell configuration in a standard external solution containing 145 mM NaCl, 10 mM $CaCl_2$, 11 mM glucose, and 10 mM HEPES (pH 7.4 at 25°C); 5 mM CsCl was routinely added to block inwardly rectifying K^+ channels (Hoth, 1995). Sylgard-coated patch pipettes had resistance between 2 and 4 MΩ after filling with the standard intracellular solution, which contained 145 mM Cs-glutamate, 8 mM NaCl, 1 mM $MgCl_2$, 0.5 mM MgATP, 12 mM bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), and 10 mM HEPES (pH 7.2, 25°C). Drugs were added by local pressure from a wide-tipped micropipette (5–10 μm). Patch-clamp experiments were performed with an inverted microscope (Axiovert 100; Zeiss, Milan, Italy) equipped for epifluorescence and photometry (TILL Photonics, Planegg, Germany). The light source was a Xenon short-arc lamp (75X-O; Ushio Inc., Tokyo, Japan) and a diffraction grating mounted on a high-speed scanner, providing monochromatic light. For detection of the bright GFP fluorescence, the excitation light (470 nm) was directed through a quartz glass fiber to a gray filter (Oriel, Milan, Italy) before entering the microscope and then deflected by a 505DRLP (XF73; Omega Optical, Milan, Italy) dichroic mirror into the 40x oil immersion objective (Fluar, NA 1.3, Zeiss). The emitted light was directed through a 500- to 530-nm emission filter (Zeiss) to a photomultiplier tube (R928; Hamamatsu, Tokyo, Japan). To collect fluorescence from a single cell, a pinhole was placed in the image plane of the phototube. Standard transfection protocols usually resulted in GFP fluorescence intensity ranging from 2 to 20 times the

background cell signal. Routinely, cells were selected with intensities between 5- and 10-fold of the background, to avoid either too low and too high CRT-overexpressing cells. High-resolution current recordings and GFP fluorescence were acquired by a computer-based patch-clamp amplifier system (EPC-9; HEKA, Lambrecht, Germany) controlled by Pulse software (HEKA). All voltages were corrected for a liquid junction potential of 8 mV between external and internal solutions. High-resolution currents were acquired at a sampling rate of 10 kHz, low-pass filtered at 2.3 kHz, and digitally filtered to 1 kHz for presentation. The holding current, the holding potential, the fluorescence, and other parameters were synchronously recorded, at low resolution (2 Hz), by X-Chart software (HEKA). Voltage ramps of 50 ms duration, from −100 to +100 mV, were delivered at 0.5 Hz. Capacitative currents were canceled before each voltage ramp using the automatic capacitance compensation of the EPC-9. Uncompensated series resistance was in the range of 5–12 MΩ.

Immunolocalization and Fluorescence Detection

RBL-1 cells were fixed and immunostained as described previously (Brini *et al.*, 1995). The antibodies (Abs) used were mouse monoclonal anti-HA1 Ab (Boehringer Mannheim, Milan, Italy) and rabbit polyclonal anti-CRT Ab (a kind gift from, Dr. E. Clementi, University of Catanzaro, Catanzaro, Italy), both used at 1:100 dilution. Ab binding was revealed with Texas Red-labeled anti-mouse and anti-rabbit IgG Ab, respectively. Fluorescence was analyzed with the Nikon RCM 8000 confocal microscope using a krypton ion laser at the 488-nm excitation band for GFP fluorescence (emission, 520 nm) and the 532-nm excitation band for Texas Red-conjugated secondary Abs (emission, 590 nm).

Materials

Culture media and sera were from Technogenetics (Milan, Italy); $InsP_3$ was from Calbiochem (San Diego, CA); other chemicals were from Sigma (St. Louis, Missouri, USA).

Statistical Analysis

Analysis has been performed by Pulse (HEKA) and Igor-Pro3 (Wavemetrics, Lake Oswego, OR) software. The reported traces and data are the average ± SE (mean ± SE) of five to eight experiments for each condition.

RESULTS

Although the Ca^{2+}-binding properties of CRT have been well characterized in vitro, its role in vivo as an intralumenal Ca^{2+} buffer is still highly debated. To solve this important issue we decided to investigate the effect of overexpressing CRT on the kinetics and amplitude of I_{CRAC}, the selective Ca^{2+} current that is activated by depletion of intracellular Ca^{2+} stores (for reviews, see Penner *et al.*, 1993; Fasolato *et al.*, 1994; Berridge, 1995). The exquisite sensitivity of I_{CRAC} to the Ca^{2+} content of the intracellular stores makes it ideally suited to monitor the role of alterations in Ca^{2+} handling within this compartment (Hofer *et al.*, 1998).

RBL-1 cells, which are known to express a robust I_{CRAC} (Fasolato *et al.*, 1993; Hoth, 1995; Innocenti *et al.*, 1996; Parekh and Penner, 1995; Parekh *et al.*, 1997), were thus cotransfected by electroporation with CRT-cDNA and, as a marker of transfection, with the bright mutant S65T-cDNA of cytGFP, as described in MATERIALS and METHODS. Twenty-four to 48 h after transfection, cells positive for the GFP signal were identified and selected for patch-clamp experiments as described below.

Figure 1 shows an example of RBL-1 cells positive for the bright mutant of cytGFP (Figure 1A) and decorated with the Ab specific for the HA1-tag inserted in the sequence of wtCRT (tCRT) (Figure 1B). Although the overall efficiency of transfection was rather low (8–10%), practically all cells positive for GFP were also positive for the tCRT. The same batch of cotransfected cells were decorated with anti-CRT Ab to track the subcellular distribution of the endogenous and transfected proteins. The cell positive for GFP (Figure 1C) had a more intense staining with the anti-CRT Ab (Figure 1D). As shown in Figure 1E, there is a good correlation in signal intensities when comparing GFP fluorescence and CRT overexpression. It is worth noting that both the Ab signals indicate a clear nuclear exclusion and a distinct reticular pattern distributed to a large part of the cytoplasm (cf. Figure 1, B and D). A rough estimation of the increase in CRT expression can be obtained by comparing the staining with the anti-CRT Ab in both GFP-positive and -negative cells. On average, CRT-overexpressing cells show an increase in fluorescence of 5.4 ± 1.4-fold over the basal signal because of the endogenous CRT (n = 14; cf. Figure 1, D and E).

Cells with comparable GFP signal (normalized to the cell size) were selected for patch-clamp experiments. Two main protocols were initially used to compare I_{CRAC} activation in controls (expressing only cytGFP) and tCRT-cotransfected cells. RBL-1 cells were voltage clamped at 0-mV holding potential in the whole-cell configuration of the patch-clamp technique with an internal solution containing a high concentration of the Ca^{2+} chelator BAPTA (12 mM; $[Ca^{2+}]_i \leq 10^{-9}$ nM) to prevent current inactivation (see MATERIALS AND METHODS). A rapid depletion of intracellular stores was achieved either with a maximal concentration of $InsP_3$ (20 μM) added to the intracellular solution or with a maximal dose of the Ca^{2+} ionophore ionomycin (1 μM) applied from a puff pipette close to the cell surface. As shown in Figure 2, A and B, the average time course of I_{CRAC} activation, under these conditions, was similar in kinetics and amplitudes in cells transfected with cytGFP alone or together with tCRT. In particular, when I_{CRAC} was activated by $InsP_3$ (Figure 2A), the current density (measured at −40 mV from fast voltage ramps acquired every 2 sec; see Figure 2A, inset, and MATERIALS AND METHODS) was −4.19 ± 0.32 and −4.37 ± 0.45 pA/pF in controls and CRT-overexpressing cells, respectively. Similarly, when ionomycin was applied within 40–50 sec from the establishment of the whole-cell configuration (Figure 2B), the current was maximally activated and reached −3.15 ± 0.31 and

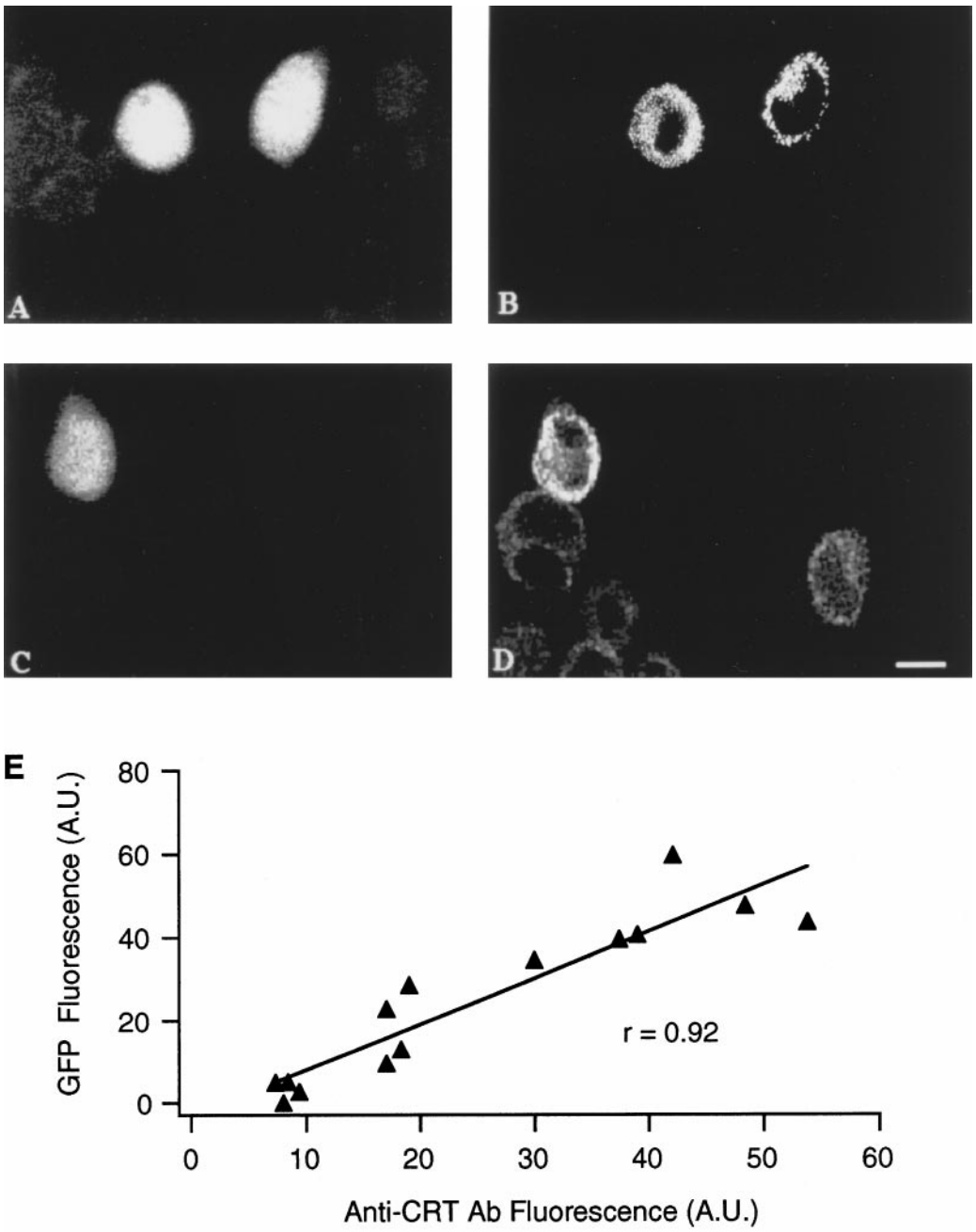

Figure 1. Immunofluorescence of cotransfected RBL-1 cells. CytGFP- and tCRT-cotransfected cells were analyzed for GFP (A and C), anti-tag (B), and anti-CRT (D) fluorescence signals by confocal microscope as described in MATERIALS AND METHODS. Note that the GFP-positive cell (C) shows a CRT signal clearly higher compared with control cells, which exhibit a moderate reticular signal attributable to the endogenous CRT (D; bar, 15 μm). E shows the correlation between fluorescence of the cytGFP and the antiCRT Ab in cotransfected cells. Each point represents the fluorescence intensity of a cell, obtained by averaging the signal of a standard digital box positioned on different parts of the cell. The fluorescence of GFP was subtracted from the signal intensity of a parallel batch of untransfected cells. The average increase in CRT expression was 5.4 ± 1.4-fold over the basal signal (n = 14 cells). A.U., arbitrary units of fluorescence.

−3.2 ± 0.51 pA/pF in controls and CRT-transfected cells, respectively. (Note also that the current density obtained with optimal doses of ionomycin is smaller than that obtained with maximal doses of $InsP_3$ [−3.2 and −4.4 pA/pF, respectively]. This reduction is likely attributable to the washout caused by the whole-cell dialysis [also see DISCUSSION].) Noteworthy, expression of cytGFP alone did not modify the kinetics and

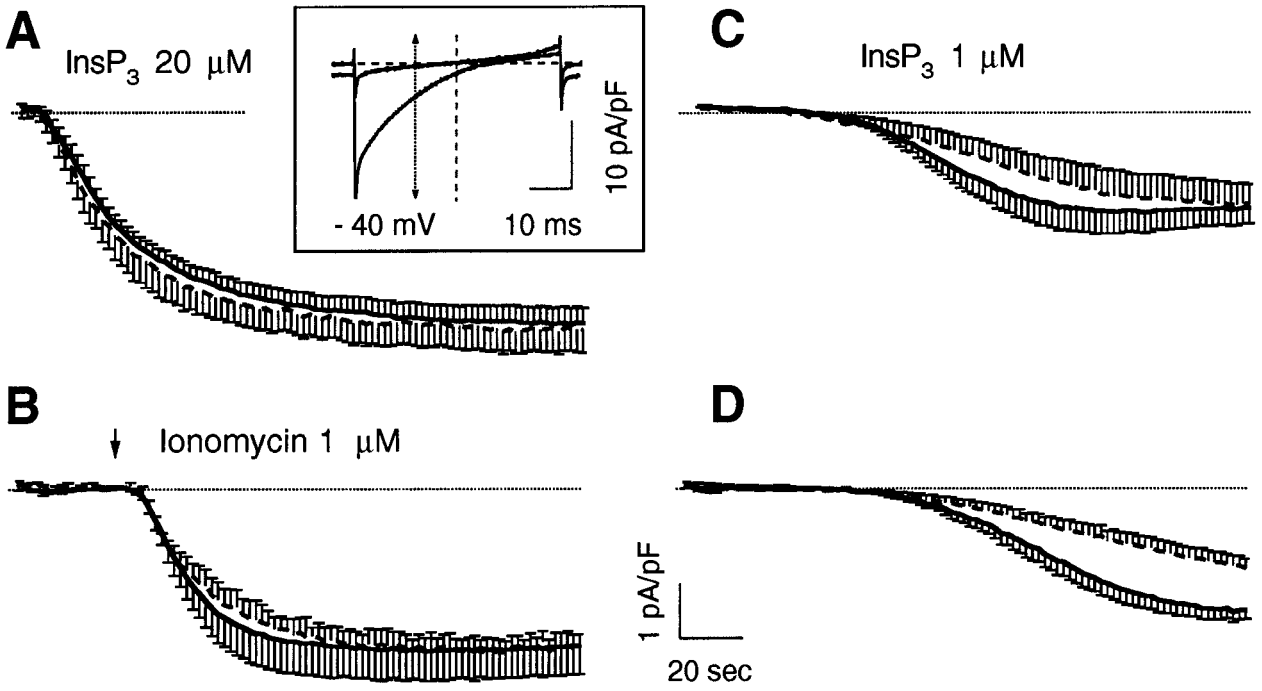

Figure 2. Activation I_{CRAC} by different procedures in control and CRT-overexpressing cells. RBL-1 cells transfected with the cDNA for cytGFP alone (control) or together with the cDNA for human tCRT (tCRT), as described in MATERIALS AND METHODS, were selected for GFP fluorescence and voltage clamped at 0 mV holding potential with an internal solution based on Cs-glutamate, containing 12 mM BAPTA, as described in MATERIALS AND METHODS. In A and C, $InsP_3$ was also included at the specified concentrations. In B, current activation was boosted by application of ionomycin (1 μM) from a wide-tipped pipette positioned close to the cell. In D, I_{CRAC} activation was induced simply by the presence of the Ca^{2+} chelator BAPTA. Traces represent the time courses of I_{CRAC} activation measured at -40 mV from the instantaneous voltage ramps, used to monitor I_{CRAC} development every 2 s, as shown in the inset. Currents from single cells were normalized to the cell capacitance, as indicator of the cell size, and plotted as average ± SE (from five to eight cells averaged for each condition); continuous trace, control; dashed trace, tCRT-transfected cells. The dotted line shows the zero current level. Inset, normalized current-voltage relationships, obtained by voltage ramps from -100 to $+100$ mV, lasting 50 ms and delivered at 0.5 Hz. The traces show the normalized current recorded before and after maximal activation in a typical experiment. The arrow shows the normalized current values measured at -40 mV and used to plot the time course of I_{CRAC} activation.

the overall properties of I_{CRAC} if compared with untransfected cells (Fasolato *et al.*, 1993; Innocenti *et al.*, 1996). A protocol such as that presented in Figure 2, A and B, however, causes such a rapid and complete discharge of the Ca^{2+} pools that the Ca^{2+}-buffering effect of CRT might be obscured (see DISCUSSION).

To address the problem of whether CRT can significantly buffer the lumenal Ca^{2+}, we designed a protocol in which the release of stored Ca^{2+} is much slower. Figure 2, C and D, shows that, when current activation was obtained by including in the patch pipette a submaximal dose of $InsP_3$ (1 μM; Figure 2C), or when store depletion was allowed to occur spontaneously, as an effect of the prolonged intracellular perfusion with high BAPTA concentrations (Figure 2D), CRT overexpression grossly modified the kinetics of I_{CRAC} activation. In particular, Figure 2D shows that, when depletion was caused by BAPTA alone, the time required to approximate the maximal current amplitude was doubled, from ~1 to 2 min in CRT overexpressers versus control cells. Given that the difference between controls and tCRT-transfected cells was best appreciated using the passive depletion protocol, the latter was routinely used in the experiments described below, although qualitatively similar data were obtained with low $InsP_3$ concentrations in the pipette (our unpublished data).

In the experiment presented in Figure 2D, it is clear that CRT overexpression modified not only the kinetics of I_{CRAC} but also the extent of the current. Figure 3 shows a few examples of the time course of I_{CRAC} activation in single cells during a typical experiment. It can be noted that in CRT-overexpressing cells the steady-state current, obtained by passive store depletion, did not reach the same level of control cells (-2.21 ± 0.10 and -1.75 ± 0.19 pA/pF in control and CRT overexpressers, respectively). Moreover, subsequent addition of a maximal dose of ionomycin (1 μM) did not result in recovery of the maximal current (-3.44 ± 0.41 and -2.37 ± 0.38 pA/pF in control and CRT overexpressers, respectively).

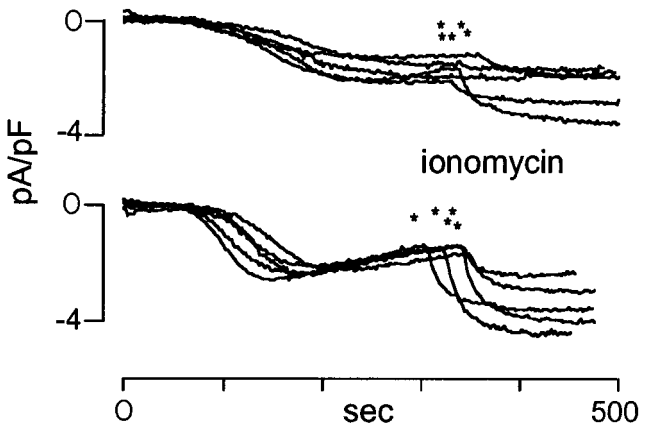

Figure 3. Time course of I_{CRAC} activation in single cells induced by passive store depletion. RBL-1 cells were transfected as described in Figure 1 and MATERIALS AND METHODS and voltage-clamped at 0 mV holding potential. I_{CRAC} was slowly activated by BAPTA diffusing inside the cell as shown in Figure 2D. At the times indicate by the asterisks, ionomycin (1 μM) was applied from the external solution to induce maximal current activation. The traces show the normalized current measured as described in Figure 2.

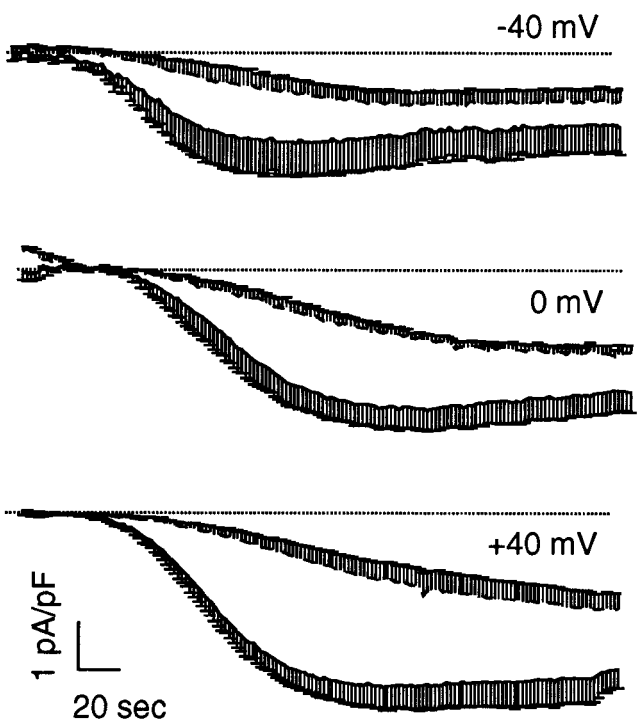

Figure 4. Effect of the holding potential on I_{CRAC} kinetics in CRT-overexpressing cells. RBL-1 cells were transfected and voltage clamped at different potentials. I_{CRAC} was activated as described in Figure 2D. Traces are the average ± SE (n = 5 for each condition) of the normalized current, measured at −40 mV from the instantaneous voltage ramps delivered at 0.5 Hz. Continuous traces, control; dashed traces, tCRT-transfected cells; the dotted line shows the zero current level.

The effect induced by CRT overexpression was also tested in cells voltage clamped at either negative (−40 mV) or positive (+40 mV) holding potentials. Figure 4 shows the average time course of current activation at the three different holding potentials. Note that, as in the previous figures, the traces show the average current measured at −40 mV from the fast voltage ramps. It appears that the slowdown induced by CRT overexpression can be appreciated at each holding potential.

The reduction of I_{CRAC} amplitude by CRT overexpression (Figure 3) is not easily explained by a simple buffering role of CRT. To determine whether CRT affects I_{CRAC} by mechanisms other than, or in addition to, its capacity of buffering ER Ca^{2+}, experiments were performed as presented in Figure 5.

We generated a tCRT deletion mutant (tCRTΔ) from which the C domain with its low-affinity and high-capacity Ca^{2+}-binding region had been removed. The mutant was further engineered to maintain, at the C terminus, the KDEL ER retention motif to ensure proper localization (see MATERIALS AND METHODS). As shown in Figure 5, cotransfected cells positive for cytGFP (Figure 5A) were also decorated with the anti-tag Ab against the mutant tCRTΔ (Figure 5B). A clear nuclear exclusion and a distinct reticular pattern confirm that the mutant was correctly targeted to the ER. Induction of I_{CRAC} by passive store depletion was then followed with the protocol described in Figure 2D. Figure 5C shows the average time course of I_{CRAC} activation in cells cotransfected with tCRTΔ (or tCRT) and cytGFP. The development of I_{CRAC} was clearly delayed only by tCRT overexpression but not by transfection of its mutant, tCRTΔ. In cells transfected with tCRTΔ there was a marginal, statistically not significant, reduction in the extent of I_{CRAC} (-3.06 ± 0.24, -2.67 ± 0.22, and -2.04 ± 0.21 pA/pF in control, tCRTΔ-, and tCRT-transfected cells, respectively). To test whether this small effect was indeed attributable to tCRTΔ or simply to the overexpression of a protein within the ER, cells were transfected with a recombinant protein targeted to the ER but with no Ca^{2+}-binding capacity, that is, a GFP specific for the ER (ER-GFP; De Giorgi *et al.*, 1998). The amplitude (as well as the kinetics) of I_{CRAC} in ER-GFP–expressing cells was indistinguishable from that of tCRTΔ (-2.77 ± 0.38 pA/pF).

DISCUSSION

In the last years the role of CRT has been thoroughly investigated, and new functions for this protein have been implicated in a variety of diverse biological processes (Meldolesi *et al.*, 1996; Helenius *et al.*, 1997; Krause and Michalak, 1997).

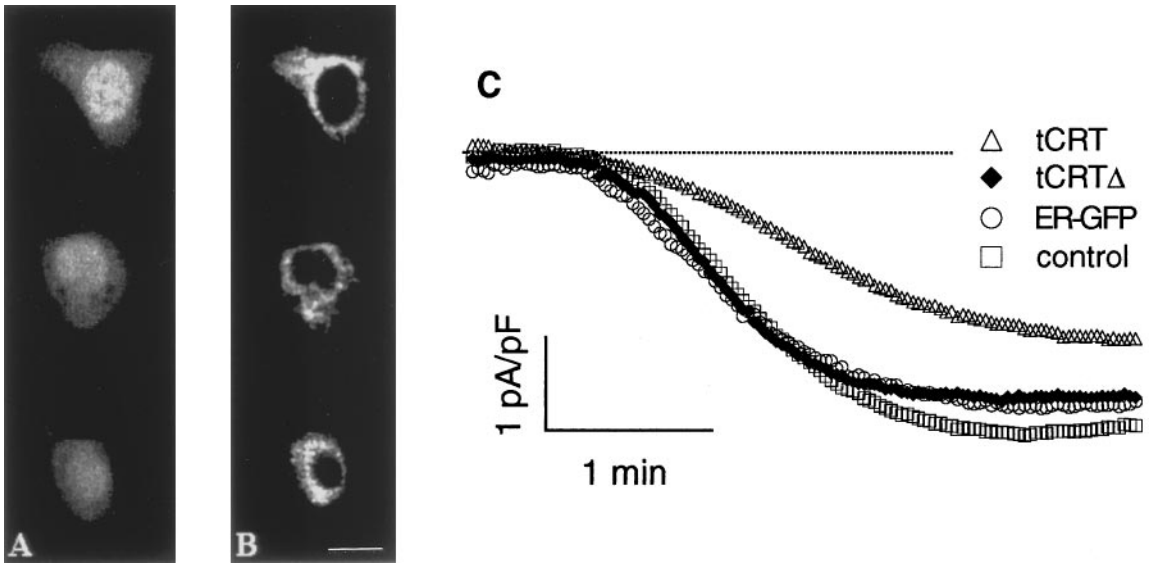

Figure 5. Immunolocalization of tCRTΔ cotransfected with cytGFP and its effect on I_{CRAC}. Confocal images of RBL-1 cells cotransfected with cytGFP (A) and tCRTΔ cDNAs (B, anti-tag Ab) show that the tCRT deletion mutant was correctly expressed in the ER. Bar, 15 μm. C, I_{CRAC} induced by passive store depletion, as described in Figure 2D, was monitored in cells transfected with cytGFP alone (control) or together with, respectively, tCRT, tCRTΔ, and ER-targeted GFP (ER-GFP) as described in MATERIALS AND METHODS. The traces show the normalized current, plotted as the pool average from three transfections; the dotted line shows the zero current level. For legibility SE is not shown (see RESULTS).

In this study we show that I_{CRAC} activation, induced by high doses of $InsP_3$ or ionomycin, reaches maximal amplitudes at almost identical rates in control and CRT-overexpressing cells. Thus, a drastic increase in ER CRT does not interfere per se with the signaling pathway that activates the CRAC channels. On the contrary, procedures that slowly deplete the stores, such as simply buffering the cytosolic Ca^{2+} at nanomolar levels with or without submaximal doses of $InsP_3$, result in a marked delay and slowdown of I_{CRAC} activation that is significantly higher in CRT-overexpressing cells. The slowdown of I_{CRAC}, induced by CRT overexpression, was consistently observed when cells were clamped at different holding potentials, confirming that the phenomenon is not attributable to current modulation but is causally linked to the depletion process.

The question thus arises of whether these observations are compatible with a simple Ca^{2+}-buffering role of CRT, or whether CRT affects I_{CRAC} by other means. To answer this question, let us first consider the basic features of ER Ca^{2+} handling.

To a first approximation, the steady-state [Ca^{2+}] of the ER is the result of a pump and leak equilibrium, whereby an increase in Ca^{2+} buffering, by CRT or any other means, results in an increase in the total Ca^{2+} content with no effect on the steady-state free lumenal Ca^{2+} concentration. Given that the rate and extent of I_{CRAC} activation depends on the free Ca^{2+} concentration in the ER (Hofer *et al.*, 1998), one would have expected that the kinetics of current activation should be slower in CRT-overexpressing cells, independently of the rate at which ER depletion occurs. To account for the experimental observations, we propose a simple model that takes into account a few established facts but makes no assumptions about the molecular mechanism of I_{CRAC} activation. In this model we assume that the kinetics of I_{CRAC} development follow, to a first approximation, the cellular concentration of the I_{CRAC}-activating factor. If the production of this factor is directly correlated with the [Ca^{2+}] of the ER, the maximal rate of its production will be reached faster at

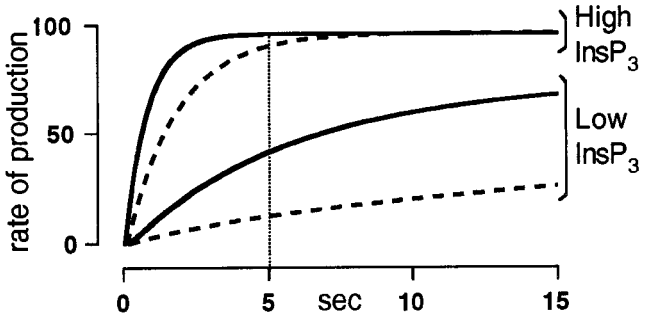

Figure 6. Model for the production of the I_{CRAC}-activating factor. Hypothetical rate of production of the I_{CRAC}-activating factor, at low and high concentrations of $InsP_3$, in control (continuous line) and tCRT-overexpressing cells (dashed line). The vertical line represents the lag time for I_{CRAC} activation for fast depletion protocols (Hofer *et al.*, 1998).

high $InsP_3$ concentrations and will be lowered by overexpression of CRT, as depicted in Figure 6. The cellular level of I_{CRAC}-activating factor will also increase with time in a similar manner, reaching a steady state when the rate of production equals that of degradation. Using these two simple assumptions and taking into account that, even at maximal rates of store depletion, there is an intrinsic lag time of 5 sec between the drop in the ER [Ca^{2+}] and the development of I_{CRAC} (Hofer *et al*., 1998), we can easily explain the experimental results. In fact, if store depletion occurs within the intrinsic lag time required for I_{CRAC} activation, the rate of production of the factor will approach its maximal value before current development. Accordingly, at high $InsP_3$ concentrations maximal rate of its production will be achieved also in tCRT-overexpressing cells. The kinetics of current activation will be indistinguishable from control cells despite an initial reduced rate (Figure 6). On the other hand, procedures that induce a slow depletion, such as cell perfusion with low $InsP_3$ concentrations or BAPTA alone, will take a much longer time to approach the maximal rate of production of the I_{CRAC}-activating factor, allowing the difference in lumenal Ca^{2+} buffering between control and CRT-overexpressing cells to become obvious. This is indeed what we observed experimentally.

The conclusion that CRT affects I_{CRAC} because of its Ca^{2+}-buffering role and not because of other functions of this protein is further strengthened by the demonstration that tCRTΔ, which lacks the C-terminal Ca^{2+}-binding domain, does not mimic the slowdown of the current observed with intact tCRT.

The model presented in Figure 6 also predicts that eventually the extent of I_{CRAC} should be identical, independent of the rate at which store depletion occurred. The finding that a slow activation of the current results in a reduction of current amplitude is in large part attributable to the fact that I_{CRAC} is subjected to a washout effect; i.e., procedures that activate I_{CRAC} at longer times after the establishment of the whole-cell configuration are known to produce currents of progressively reduced size (Fasolato *et al*., 1993). Also in this work, the current density in control cells is smaller if activation is triggered by passive store depletion compared with activation by maximal doses of $InsP_3$. Passive store depletion in CRT-overexpressing cells is even more affected, because channel recruitment occurs at a much slower rate.

The washout phenomenon, however, does not account entirely for the difference in I_{CRAC} amplitude between control and CRT-overexpressing cells when, after partial emptying of the stores by passive depletion, ionomycin is added to both cells (Figure 3). In this case, a simple washout effect would predict that the current density should be identical in the two conditions. We have no simple explanation for this finding. A plausible hypothesis is that the rate of channel recruitment dictates the extent of the current that can be eventually activated. This phenomenon cannot, however, be attributed to an effect of CRT overexpression per se, given that 1) it is not observed with tCRTΔ; and 2) it can be induced by other depletion protocols that imply a delay in current activation (Fasolato and Innocenti, unpublished observations).

How can one reconcile the present data (and the previous data along the same line) with those 1) negating a Ca^{2+}-buffering role of CRT and 2) sustaining a direct effect of CRT on the mechanism of I_{CRAC} activation? The simplest explanation is that in this study (and that of Bastianutto *et al*., 1995) control cells are compared with cells acutely transfected with CRT (or with cells in which CRT had been acutely down-regulated, as in the study by Liu *et al*., 1994). On the contrary, Coppolino *et al.* (1997) and Mery *et al.* (1996) used knockout or stably transfected cells, thus allowing for intrinsic homeostatic mechanisms to develop and for differences between clones to appear. In those studies, therefore, the lack of effects of CRT down-regulation, or the apparent inhibition of I_{CRAC} by CRT overexpression, most likely reflects adaptive mechanisms to CRT expression levels rather than being indicative of CRT functions.

Admittedly, our data provide evidence only in favor of a Ca^{2+}-buffering role of overexpressed CRT and do not address the question of whether endogenous CRT plays an important role as an intralumenal Ca^{2+} buffer. In fact, van de Put and Elliott (1997) have recently questioned this role in pancreatic acinar cells. A few considerations, however, suggest that in RBL-1 cells, as well as in other cells, endogenous CRT is a significant component of the ER Ca^{2+}-buffering system. In particular: 1) CRT is ~1–2% of total ER proteins (Krause and Michalak, 1997), and thus its lumenal concentration approaches 50–100 μM; 2) given that CRT can account for up to 50 Ca^{2+}-binding sites per mole of protein, its total Ca^{2+}-binding capacity could be as high as 2.5–5 mM in ER water; and 3) Considering that the free Ca^{2+} concentration in the ER lumen is ~0.3–0.6 mM, and the total mobilizable Ca^{2+} is 3–6 mM in ER (Pizzo *et al*., 1997), it derives that endogenous CRT has the potential to buffer a substantial amount of it.

A final question concerns whether CRT overexpression is only an experimental tool to investigate the function of this protein or whether it can offer some paradigms to understand more physiological or pathological conditions. In this respect, it is worth mentioning that CRT is up-regulated during proliferation, and high levels of CRT expression were detected in B16 melanoma cells (Gersten *et al*., 1990). Noteworthy, the promoter region of CRT contains a retinoblastoma control element (Krause and Michalak, 1997). In addition, up to a fourfold increase in the level

of CRT can be induced by prolonged exposure to Ca^{2+}-depleting agents and other stress treatments (Conway *et al.*, 1995; Nguyen *et al.*, 1996; Llewellyn *et al.*, 1996; Tsutsui *et al.*, 1997; Waser *et al.*, 1997). Thus, overexpression of CRT might be a widespread phenomenon with important consequences on Ca^{2+} homeostatic mechanisms.

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