The Sarco/Endoplasmic Reticulum Calcium-ATPase 2b Is an Endoplasmic Reticulum Stress-inducible Protein* 

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The sarco/endoplasmic reticulum calcium-ATPase (SERCA) translocates Ca\(^{2+}\) from the cytosol to the lumen of the endoplasmic reticulum. This Ca\(^{2+}\) storage is important for cellular processes such as calcium signaling and endoplasmic reticulum (ER)-associated post-translational protein modifications. We investigated the expression of the SERCA2 and SERCA3 isoforms in PC12 cells exposed to agents interfering with different aspects of the posttranslational protein processing within the ER, thereby activating the ER stress-induced unfolded protein response (UPR). All agents increased the SERCA2b mRNA level 3–4-fold, in parallel with increasing mRNA levels for the ER stress marker proteins BiP/GRP78 and CHOP/GADD153. In contrast, SERCA3 mRNA levels did not change. SERCA2b mRNA stability was not changed, indicating that the mechanism of its up-regulation was transcriptional, in accordance with the presence of ER stress response elements in the promoter region of the SERCA2 gene. SERCA2b was also increased at the protein level upon ER stress treatments. Induction of ER stress by tunicamycin, dithiothreitol, or L-azetidine 2-carboxylic acid did not result in depletion of ER calcium, showing that such depletion was not necessary for up-regulation of SERCA2b expression or UPR activation in general. We conclude that the SERCA2b expression can be controlled by the UPR pathway independently of ER Ca\(^{2+}\) depletion.

The Ca\(^{2+}\) storage of endoplasmic reticulum (ER)\(^1\) is crucial for a variety of Ca\(^{2+}\)-dependent processes, such as Ca\(^{2+}\)-mediated cytosolic signaling in response to external stimuli (1), cell growth, and proliferation (2, 3), as well as synthesis, posttranslational processing, folding and export of proteins synthesized on ER-associated ribosomes (4–6). The maturation of the newly translocated polypeptide chains within the ER lumen is accomplished by transient interactions with ER chaperones (such as calreticulin, calnexin, BiP/GRP78, GRP94, and others) (7, 8). The requirement for Ca\(^{2+}\) as a co-factor for proper ER protein processing and maturation presumably results from the Ca\(^{2+}\) binding capacities and Ca\(^{2+}\)-dependent interactions of the chaperones in the ER lumen (9).

The resting intra-ER free Ca\(^{2+}\) concentration is 3–4 orders of magnitude higher than the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) (10). This gradient is generated by the sarco/endoplasmic reticulum calcium-ATPase (SERCAs), encoded by three homologous genes (SERCA1, SERCA2, and SERCA3) (11). Transcripts from all three genes undergo alternative splicing in a developmentally and tissue-specific manner, giving rise to a number of isoforms differing in their C-terminal regions. The splicing of the primary transcripts from SERCA1 and SERCA2 genes produces SERCA1a-b (12) and SERCA2a-b isoforms (13, 14). SERCA1a-b and SERCA2a expression is restricted to skeletal and cardiac muscles, whereas the SERCA2b isoform is ubiquitously expressed in all nonmuscle tissues (15). Recently, SERCA3 transcripts were found to be spliced too, resulting in three isoforms in mice and humans (SERCA3a, SERCA3b, and SERCA3c) (16) and two isoforms in rats (SERCA3a and SERCA3b) (11, 17). SERCA3 is expressed in a number of nonmuscle tissues at variable levels, always coexpressed with SERCA2b (15, 18, 19). At present, the full physiological significance of this heterogeneity among the SERCA isoforms remains unclear.

When unfolded proteins accumulate in the ER, a conserved eukaryotic stress response pathway known as unfolded protein response (UPR) is activated (8, 20, 21). This response amplifies the protein folding capacity in the ER through an enhanced expression of the ER-resident chaperones (such as BiP), thus countering congestion of misfolded protein intermediates and promoting cell survival (22, 23). Ire1p, residing in the ER membrane and displaying both serine/threonine kinase and endonuclease activities, appears to function as the sensor of the accumulation of unfolded proteins and to activate the signal that enhances the transcription of chaperone genes in yeast (24) as well as mammals (25, 26). Recently, stress inducibility of the chaperone genes in higher eukaryotes was shown to require the presence of a 19-base pair long regulatory elements termed ER stress response elements (ERSEs) in their promoters (27, 28). In addition to chaperone up-regulation, several other signals emerge from the stressed ER, including induction of a growth arrest/cell death-promoting transcription factor C/EBP homolog protein (CHOP, also known as growth arrest and DNA damage protein 153) (29), and in a number of cases, protein synthesis is transiently down-regulated because of phosphorylation of eukaryotic initiation factor 2α by the dedi-
cated kinases PKR-like ER kinase (30) and possibly PKR (31).

Here we present results showing a strong correlation between induction of the UPR pathway and an elevated expression of SERCA2b, but not SERCA3, at the mRNA and protein levels. Depletion of ER calcium was not a necessary condition for UPR activation nor for the up-regulation of SERCA2b. Together with the presence of putative functional ERSE elements in the promoter region of SERCA2, these results provide evidence for a new aspect of SERCA2b function, manifested by its ability to act as a member of the ER stress protein family.

EXPERIMENTAL PROCEDURES

Materials and Reagents—PC12 cells were provided by Dr. S. Gammeltoft (Copenhagen County Glostrup Hospital). RINm5F cells were provided by Dr. K. Jørgensen (Bartholin Institute, Copenhagen). Thapsigargin was a gift from Dr. S. B. Christensen (Royal Danish School of Pharmacy). Immune sera against SERCA2b and SERCA3 were generously provided by Dr. Frank Wytsac (Leuven, Belgium).

1-[4,5-3H]-leucine, methyl-[3H]thymidine, [α-32P]dCTP, [γ-32P]ATP, and autoradiography films were from Amersham Pharmacia Biotech.

Cell Culture—PC12 cells were cultured in Dulbecco's modified Eagle's medium with 25 mM Hepes, 4500 mg/liter glucose supplemented with 6% (v/v) heat-inactivated fetal calf serum, 6% (v/v) heat-inactivated horse serum, 18 mM L-glutamine, 100 IU/ml penicillin/G, and 100 mg/ml streptomycin (Gambro). The medium was prepared as stock solutions concentrated 1000-fold in water (cycloheximide, DTT). The EGTA stock was adjusted to pH 7.2 with 1 M NaOH.

ProProtein Synthesis Rate—2 ml of cells were centrifuged at 100 × g for 10 min at room temperature. The supernatant was discarded, and the remaining cells were centrifuged in leucine-free Dulbecco's modified Eagle's medium and resuspended in 2 ml of leucine-free Dulbecco's modified Eagle's medium with the same stress agent as used prior to harvesting, and 2.5 μl (18 pmol) of 1-[4,5-3H]-leucine. Cells were labeled for 7 min at 37 °C. Labeling was stopped by addition of 1 ml of 0.1 M NaOH, after which the tube was cooled on ice. Macromolecules were precipitated by cold 10% trichloroacetic acid, and the pellet was washed three times with 5 ml of ice-cold 5% trichloroacetic acid. Finally the pellet was solubilized in 0.25 M NaOH and counted by liquid scintillation.

DNA Synthesis Rate—1 ml of cells were labeled with 2 μl (80 pmol) of methyl-[3H]thymidine. The labeling reaction was stopped after 15 min at 37 °C by 1 ml of 0.1 M NaOH, after which the tube was transferred to 70% (v/v) ethanol for 2 min at 37 °C. The cells were harvested by scraping, and the sample was precipitated with cold 70% (v/v) ethanol. The pellet was washed twice with 5% trichloroacetic acid. Macromolecules were extracted with 0.3 M NaCl, 0.1 M NaOH, and 0.1% SDS, washed, and the radioactivity was counted as described above.

Cell Viability—A 100-μl aliquot was taken out for cell counting and viability test. Cells were mixed 1:1 with 0.4% (w/v) trypan blue in PBS. After 2 min at room temperature, dead (blue) and live (not stained) cells were counted in a hemocytometer.

Cytosolic Calcium Measurements—PC12 cells cultured on polylysine-coated (100 μg/ml) glass coverslips were loaded for 30 min at 37 °C with 5 μM fura-2 acetoxymethyl ester and 0.04% pluronic F-127 (Molecular Probes) in EM solution (145 mM NaCl, 5 mM KCl, 20 mM Hepes, 3 mM CaCl2, 1 mM MgCl2, 10 mM glucose, and 100 μM sulfinpyrazone). The coverslip was then transferred to a solution consisting of 75% (v/v) EM solution with 25% (v/v) culture medium for additional 30 min at 37 °C. The coverslip was then placed in a 37 °C thermostated chamber (PHI and TC344A, Warner Instruments, Hamden, CT) with 5% CO2. Macromolecules were extracted with cold 10% trichloroacetic acid, and the pellet was washed twice with 5 ml of ice-cold 5% trichloroacetic acid. Finally the pellet was solubilized in 0.25 M NaOH and counted by liquid scintillation.

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sion filter. Cells were imaged using a 40× lens (Zeiss Fluar, 40/1.30 oil). Emitted light was collected by a high resolution image intensifier coupled to a video camera (GenIIsys, CCD-72E2X, DAGE-MTI, Michigan City, IN). The signal output from the camera was connected to a digital image-processing board (Matrox, MVP-AT) controlled by IMAGE-1/FL software (Universal Imaging, West Chester, PA). The digitized signal output from cells excited at each wavelength with an interval of 5 s was processed on-line (four frame average) to yield a ratio image map. 

Measurement of the SERCA Phosphorylated Intermediate—Following harvesting and centrifugation, cells were washed once in PBS and resuspended in 20 mM Tes (pH 7.0) and sucrose 300 mM (HB). Cells were broken by means of a cell cracker from the European Molecular Biology Laboratory workshop (38), employing the clearance of 12 μm and 10 passages. The homogenates were centrifuged for 10 min at 10,000 × g, followed by centrifugation of the supernatants for 60 min at 100,000 × g. The microsomal pellets were resuspended in HB and stored at −80 °C. ATPase phosphorylation was carried out as described earlier (“standard assay”) (39).

RESULTS

SERCA2b mRNA Is Induced by ER Stress—We have challenged PC12 cells with different agents sharing the ability to induce the mammalian UPR and measured the level of SERCA2b and SERCA3 mRNAs on Northern blots. The SERCA2b probe hybridized with the 4.5s-, 6s-, and 8-kb alternative splice variants of SERCA2b mRNA, differing in their 3′-untranslated region (11, 13). The SERCA2a mRNA was not expressed in PC12 cells (not shown). Fig. 1A shows that after a 6-h exposure, the agents known to perturb and deplete intra-ER Ca2+ (thapsigargin, A23187, or EGTA) all induced an increase in the SERCA2b mRNA relative to control cells exposed to the appropriate vehicle (Me2SO or water). Thapsigargin is an inhibitor of SERCA pumps (40), A23187 is an ionophore known to collapse divalent cation gradients (41), whereas EGTA treatment chelates extracellular Ca2+, thereby promoting Ca2+ efflux from the cell and ER. The level of SERCA3 mRNA was not changed by these agents (Fig. 1B). In addition, tunicamycin, DTT, L-azetidine-2-carboxylic acid (Aze), and brefeldin A (BFA) also induced an increase in the SERCA2b, but not the SERCA3, mRNA level (Fig. 1, A and B). Tunicamycin is an inhibitor of N-linked glycosylation (42). DTT perturbs the unique oxidative milieu of the ER lumen, thereby inhibiting the disulphide bond-dependent protein folding (43). Aze is a proline analog that is incorporated into proteins and causes their misfolding (44). BFA is an inhibitor of the transport from ER to Golgi, leading to an absorption of the Golgi apparatus into the ER (45). The treatments did not cause any major changes in the yield of total isolated RNA, except for Aze, which decreased the RNA yield to 50–60% of controls. Fig. 1C shows the quantitation of the relative levels of mRNA for SERCA2b and SERCA3, as normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

As a positive control of the UPR induction, we monitored BiP and CHOP mRNA levels in PC12 cells exposed to stressors. Panels D and E in Fig. 1 show Northern blots of the same total RNA as analyzed in panels A and B, hybridized to BiP and CHOP probes. The pattern of BiP induction resembled the induction of SERCA2b mRNA, except for the induction magnitude (up to 10-fold, quantitation not shown) compared with 3–4-fold of SERCA2b mRNA (Fig. 1C). CHOP mRNA was not detectable in control cells but was induced by all the stressors, with the level of CHOP mRNA induction highly dependent on the particular stressor. CHOP induction by DTT at 6 h was very weak and is better appreciated at 1 or 2 h of DTT treatment (Fig. 2D).

SERCA2b and BiP mRNA Induction Follows Similar Kinetics—To further characterize the SERCA2b mRNA induction we compared the time course with that of BiP and CHOP. Fig. 2A shows an autoradiogram of a SERCA2b-probed Northern blot with total RNA isolated from cells treated for 1, 2, 4, or 8 h with EGTA or DTT. Fig. 2C shows a corresponding Northern blot hybridized with a BiP probe. Both BiP and SERCA2b were induced in 1–2 h, consistent with the described pattern of BiP induction (46). For both SERCA2b and BiP maximum was reached in about 2 h with DTT and 4 h with EGTA. The same overall kinetics was found for tunicamycin and A23187 (not shown). Longer stress periods (up to 24 h) did not increase SERCA2b mRNA level further. Fig. 2B shows an autoradiogram of SERCA3-probed Northern blot, demonstrating that SERCA3 mRNA level did not change in response to DTT or EGTA after 1, 2, 4, or 8 h.

The time course of the CHOP mRNA induction (Fig. 2D) appeared faster than for the BiP or SERCA2b mRNA. CHOP mRNA level reached a maximum in the presence of DTT within 1 h and was decreasing thereafter to become undetectable after 8 h. However, addition of fresh DTT after 4 h led to an increase in the CHOP mRNA after 8 h to the same level as at 4 h (not shown), indicating that the decreasing CHOP mRNA response to DTT, at least in part, was the result of the inactivation of DTT.

The SERCA2b Induction Is Reversible and Reinducible—To test whether the increase in SERCA2b mRNA levels was reversible and reinducible, cells were treated with EGTA for 4 h, after which the medium was changed to control medium for 20 h, to be followed again by EGTA treatment for 4 h. As shown in Fig. 3, SERCA2b, BiP, and CHOP responses were reversible and could be reinduced after 20 h of reconstitution in control medium. Both BiP and SERCA2b mRNA levels remained elevated for 2 and 4 h after the withdrawal of the stressor (Fig. 3, time points 6–8 and 30–32 h), after which they returned to the basal level (time point 24 h).

In contrast to BiP and SERCA2b, CHOP mRNA level responded much more promptly to the application and withdrawal of EGTA (Fig. 3). Notably, for all three mRNA types, the second response to the stressor appeared less pronounced than the first one.

The SERCA2b mRNA Increase Is Dependent on Protein Synthesis—A characteristic feature of the UPR is the requirement of protein synthesis for the transcriptional induction of BiP mRNA (46). Fig. 4A shows that neither tunicamycin nor A23187 were able to induce the SERCA2b mRNA in the presence of cycloheximide, demonstrating that protein synthesis was necessary for stress-mediated SERCA2b mRNA induction.

The Stress-induced Increase in SERCA2b mRNA Level Is Dependent on Transcription, and SERCA2b mRNA Is Not Stabilized under Conditions of ER Stress—Fig. 4B shows that actinomycin D, an inhibitor of transcription (47), could prevent the A23187 or tunicamycin-mediated elevation of SERCA2b mRNA. To test whether SERCA2b mRNA is stabilized under the conditions of ER stress, cells were incubated in the presence of tunicamycin or the vehicle for 4 h, following which (Fig. 4C, time 0) each medium was replaced with a similar one except for the addition of actinomycin D. Fig. 4C shows that after 6 h in the presence of actinomycin D, the SERCA2b mRNA decreased in both control and tunicamycin-treated cells. Quantitation showed a decline to about 40% of the level before addition of actinomycin D. Without the addition of actinomycin D the SERCA2b mRNA was increasing throughout the tunicamycin treatment (not shown). Total RNA yield decreased after 6 h of actinomycin D treatment to 60–80% of control in both the vehicle and the tunicamycin-treated cells. Thus, SERCA2b mRNA was not stabilized in response to tunicamycin-induced
mRNA splice variants are indicated at 8, 6, and 4.5 kb). For EGTA, Tg, and A23187, autoradiographic bands were normalized to those for GAPDH. Fold increase refers to the ratios between values representing each treatment and the control to which appropriate vehicle was added (H2O or Me2SO).

Total RNA was isolated from PC12 cells after 6 h of exposure to the indicated agents: Tg (200 nM), A23187 (5 μM), Tun (10 μg/ml), DTT (0.8 mM), BFA (5 μg/ml), and Azc (5 mM). 10 μg of RNA was loaded on each gel lane. Except for C, autoradiograms are shown of Northern blots hybridized with the probes for the indicated mRNA species (size in kb). In this and other figures, GAPDH mRNA was unaffected by various treatments and was used to control for equal loading of gel lanes. For each condition, n indicates number of experiments represented by the figure. A, SERCA2b mRNA (alternative mRNA splice variants are indicated at 8, 6, and 4.5 kb). For EGTA, Tg, and A23187, n = 6. For Tun and DTT, n = 5. For BFA and Azc, n = 3. B, SERCA3 mRNA (4.8 kb), n values are as in A. C, quantitation of SERCA2b and SERCA3 mRNA levels in response to ER stressors, as shown in A and B of this figure. Optic density values of SERCA2b and SERCA3 mRNA autoradiographic bands were normalized to those for GAPDH. Fold increase refers to the ratios between values representing each treatment and the control to which appropriate vehicle was added (H2O or Me2SO). Bars represent standard deviations. D, BIP mRNA (2.7 kb), n = 3. E, CHOP mRNA (1.1 kb), n = 3. Statistical significance of differences relative to control for SERCA2b mRNA, p < 0.02 (**). Student’s t test was used. DMSO, dimethyl sulfoxide.

**ER Stress.**

**Up-regulation of SERCA2b, but Not SERCA3, mRNA in RINm5F Insulinoma Cells in Response to ER Stress—**To test whether the differential effect of ER stress on the 2 SERCA isoforms was present in other cell types, we tested RINm5F insulinoma cells (derived from rat pancreatic 74 cells) known to have a high expression level of SERCA3 (48). Fig. 5 shows that the various stress-inducing agents caused an elevation of SERCA2b, but not of SERCA3, mRNA in RINm5F cells, suggesting that the selective SERCA2b mRNA induction under ER stress conditions is a general response displayed by cells of different lineages. An elevation of SERCA2b mRNA in response to ER stressors was also found in EL4 lymphoma cells (not shown).

**SERCA2b Expression Is Down-regulated by Heat Shock—**A response to unfolded/malfolded proteins in the cytosolic compartment is another major stress-induced pathway in the cell. This pathway is highly activated upon heat exposure and is known as the heat shock response (49), whereas the UPR is normally not induced by heat shock. We tested whether SERCA2b mRNA level changed upon activation of the heat shock response, PC12 cells were incubated for 4 h at 42 °C, and RNA was isolated. Fig. 6 shows that the SERCA2b mRNA was down-regulated upon heat shock, whereas mRNA for the heat shock protein 70 (HSP70), a classical heat stress-responsive chaperone, was highly induced. On the other hand, HSP70 was not induced upon treatment of the cells with A23187 and tunicamycin (not shown), showing a clear dissociation of the ER stress and heat shock-activated response pathways.

**Stressed Cells Show Higher Levels of SERCA2b, but Not of SERCA3 Protein—**Western blots of PC12 cell lysates stained with the SERCA2b-specific antibody (35) showed one band at 115 kDa in accordance with the known size of SERCA2b (Fig. 7A, upper part). In PC12 cell lysates, proteins with molecular mass of 105 and 116 kDa were stained with an antibody directed against an N-terminal epitope shared by, and specific for, all SERCA3 isoforms (36) (Fig. 7A, lower part). The 105-kDa immunoreactive protein exhibited a tissue distribution profile in agreement with that described for SERCA3 mRNA (Ref. 15 and results not shown), most likely reflecting the presence of SERCA3a (999 amino acids) isoform. The size of the 116-kDa band suggests that it corresponds to the novel SERCA3b/c (1067 amino acids), recently identified (17). Fig. 7 (A and C) shows that the SERCA2b immunostaining increased after exposure to thapsigargin, A23187, EGTA, tunicamycin, and DTT for 8 h, whereas SERCA3 staining intensity remained at a steady level. Longer stress treatments (16 h) did not enhance the level of SERCA2b further. No effect was found of BFA or Azc on the SERCA2b level at 8 h (Fig. 7C).

**Total Active SERCA Is Up-regulated after 7 h of Stress—**An impaired folding and processing of proteins is at the heart of the ER stress, with an obvious potential for an impairment of function. To test whether the stress-induced increase in the synthesis of SERCA2b resulted in a functional protein, we measured the level of the phosphorylated Ca2+−ATPase intermediate in microsomes from PC12 cells. We have previously shown that the single radioactive band obtained in this phosphorylation assay represents a sum of SERCA2b and SERCA3 E−Ps (Ref. 39 and results not shown). Fig. 7 (B and C) shows that treatment of PC12 cells with A23187, EGTA, or DTT for 7 h did produce an increase in the amount of the phosphorylatable enzyme intermediate.

**Protein and DNA Synthesis Are Differentially Inhibited by ER Stressors in PC12 Cells—**The ER stress response has been shown to be accompanied by an inhibition of translation because of the activation of PKR-like ER kinase and/or PKR
species are shown. PC12 cells were treated with DTT (1 mM) or EGTA (2.5 mM) for 1, 2, 4, or 8 h, as indicated, and total RNA was isolated. H2O denotes addition of an equivalent volume of water to control cells. 10 μg of RNA was loaded on each gel lane. The mRNA species and the number of experiments represented were as follows. A, SERCA2b, n = 4. B, SERCA3, n = 3. C, BiP; n = 3. D, CHOP; n = 4.

Fig. 3. Reversible and repeated induction of SERCA2b, BiP and CHOP mRNAs. Autoradiograms of Northern blots hybridized with the probes for the indicated mRNA species are shown. PC12 cells were treated with DTT (1 mM) or EGTA (2.5 mM) for 1, 2, 4, or 8 h, as indicated, and total RNA was isolated. H2O denotes addition of an equivalent volume of water to control cells. 10 μg of RNA was loaded on each gel lane. The mRNA species and the number of experiments represented were as follows. A, SERCA2b, n = 4. B, SERCA3, n = 3. C, BiP; n = 3. D, CHOP; n = 4.

Depletion of ER Calcium Is Not an Obligatory Step for Activation of UPR or Up-regulation of SERCA2b by ER Stress—Although depletion of Ca2+ from the ER by thapsigargin, A23187, or EGTA is known to activate UPR (8), it has not been established whether initiation of the UPR response is always associated with such depletion. Resolution of this issue was of interest for at least two reasons. First, if induction of UPR were invariably accompanied by ER Ca2+ loss, one might suspect that such loss was obligatorily linked to the UPR activation. Second, independently of the precise relationship between UPR initiation and ER Ca2+ loss, such loss might be expected to be the key factor controlling the up-regulation of SERCA2b, in view of the obvious homeostatic rationale for such a mechanism. We therefore carried out experiments to estimate the size of the luminal ER Ca2+ pool following treatment with various stress-inducing agents, emphasizing those agents whose ability to interfere with ER Ca2+ homeostasis has not been generally established, i.e., DTT, tunicamycin, BFA, and Azc. In these experiments (Fig. 9), the cells were exposed to the stressor, followed by a removal of extracellular Ca2+ and an application of a test stimulus (a Ca2+-mobilizing agonist or thapsigargin) to release the ER Ca2+. PC12 cells in a resting state had a cytosolic Ca2+ concentration ([Ca2+]i) between 40 and 50 nM. They responded to stimulation with acetylcholine with a transient increase in [Ca2+]i (Fig. 9A, panel a), demonstrating the operation of a functional Ca2+ signaling system.

Treatment with thapsigargin (Fig. 9, A, panel b, and B, panel b, Tg addition at ~4 h) elicited a long lasting elevation of [Ca2+]i, as expected from the activation of store-operated Ca2+ channels in the plasma membrane (51). Fig. 9A (panels d and e) shows that no perturbation of [Ca2+]i within 18–20 min from addition of tunicamycin or Azc was observed, and addition of thapsigargin followed by ionomycin in Ca2+-free medium produced increases in the cytosolic free Ca2+ not different from those observed in control cells (Fig. 9A, panel c). The use of DTT as stressor gave analogous results. Therefore, tunicamycin, Azc, or DTT did not act as immediate or direct releasers of ER Ca2+. To test whether any such Ca2+ releasing action of these compounds would occur in the time frame necessary for ER protein kinases (30, 31). DNA synthesis and cell cycle progression were also found to be perturbed in stressed cells through an inhibition of cyclin D1 mRNA translation (50). To see whether the increased synthesis of SERCA2b protein during the ER stress occurred against a background of a decrease in the global protein and DNA synthesis, we tested whether these processes were perturbed in stressed PC12 cells. From Fig. 8 it appears that the inhibition of protein synthesis was very dependent on the particular type of stressor. Treatment with A23187 and thapsigargin resulted in only little or no reduction in the global protein synthesis rate. Treatments with DTT, tunicamycin, EGTA, or Azc resulted in an intermediate inhibition of protein synthesis to about 50–70%, whereas BFA inhibited protein synthesis to about 30% of the control. In contrast, the rate of DNA synthesis was depressed by all ER stressors, decreasing to about 20–40% of the controls.
stress on protein synthesis and transcription, and SERCA2b mRNA is not stabilized in stressed cells. Autoradiograms of Northern blots probed for SERCA2b and GAPDH mRNAs are shown. Total RNA was isolated, and 10 μg/lane was analyzed on agarose gels. A, PC12 cells were treated for 4 h with 10 μg/ml Tun, 5 μM A23187, or an equivalent volume of the vehicle (dimethyl sulfoxide), in the presence (+) or absence (−) of 20 μM cycloheximide (representative of two experiments). B, PC12 cells were treated for 4 h with 10 μg/ml Tun, 5 μM A23187, or an equivalent volume of the vehicle (dimethyl sulfoxide) in the presence (+) or absence (−) of 2 μM actinomycin D (representative of two experiments). C, PC12 cells were preincubated with control medium or 10 μg/ml tunicamycin-containing medium for 4 h. The medium was then replaced with one of the same composition (indicated by DMSO or Tun), except for the addition of 2 μM actinomycin D, and the incubation was continued for additional 3 or 6 h (representative of three independent experiments). DMSO, dimethyl sulfoxide.

stress induction, cells were treated with these compounds for 4 h. Fig. 9B shows that 4-h exposures to tunicamycin (panel c), DTT (panel d), or Azc (panel e) did not result in a significant depletion of Ca^{2+} from ER. Resting [Ca^{2+}], before removal of extracellular Ca^{2+}, was at a level equal to that in the control (Me_{2}SO-exposed) cells or slightly lower, indicating that store-operated Ca^{2+} channels were not activated, as would have been the case had the ER Ca^{2+} been depleted and as seen in Fig. 9 (A, panels a and b, and B, panel b). Importantly, in comparison with controls, thapsigargin was able to release similar amounts of Ca^{2+} (corresponding to a rise in [Ca^{2+}], of about 20 nM) in Azc-, tunicamycin-, or DTT-treated cells. In contrast, Fig. 9B (panel b) shows that an equivalent 4-h exposure to thapsigargin as a stressor abolished a subsequent effect of this drug applied as a test stimulus. Panel b also shows that thapsigargin-mediated emptying of ER Ca^{2+} store resulted in the expected capacitative Ca^{2+} entry, evidenced by an elevated [Ca^{2+}], (−75 nM, a value before introduction of Ca^{2+}-free medium), when compared with control cells as well as to cells exposed to Azc, tunicamycin, or DTT (−40–45 nM, before introduction of Ca^{2+}-free medium). The response to acetylcholine appeared somewhat smaller following these treatments in some experiments. It should be noted that the results with BFA (Fig. 9B, panel f) present a special case; although there was a clearly elevated [Ca^{2+}], level, the ER Ca^{2+} store was not depleted, as evidenced by a large increase of [Ca^{2+}], upon application of thapsigargin test stimulus (Fig. 9B, panel f, open arrowhead). (The reasons for this BFA-mediated elevation of [Ca^{2+}], were not investigated.)

DISCUSSION

The parameters that need to be set correctly and coordinated for the ER-luminal protein processing to proceed successfully include the total available capacity of the chaperones and folding enzymes and the physico-chemical parameters within the ER lumen, such as the oxidative milieu, oligosaccharide avail-

**Fig. 4.** Stress-mediated SERCA2b mRNA induction is dependent on protein synthesis and transcription, and SERCA2b mRNA is not stabilized in stressed cells. Autoradiograms of Northern blots probed for SERCA2b and GAPDH mRNAs are shown. Total RNA was isolated, and 10 μg/lane was analyzed on agarose gels. A, PC12 cells were treated for 4 h with 10 μg/ml Tun, 5 μM A23187, or an equivalent volume of the vehicle (dimethyl sulfoxide), in the presence (+) or absence (−) of 20 μM cycloheximide (representative of two experiments). B, PC12 cells were treated for 4 h with 10 μg/ml Tun, 5 μM A23187, or an equivalent volume of the vehicle (dimethyl sulfoxide) in the presence (+) or absence (−) of 2 μM actinomycin D (representative of two experiments). C, PC12 cells were preincubated with control medium or 10 μg/ml tunicamycin-containing medium for 4 h. The medium was then replaced with one of the same composition (indicated by DMSO or Tun), except for the addition of 2 μM actinomycin D, and the incubation was continued for additional 3 or 6 h (representative of three independent experiments). DMSO, dimethyl sulfoxide.

**Fig. 5.** SERCA2b mRNA induction in RINm5F cells with ER stressors. Autoradiograms of Northern blots analyzed for SERCA2b (A), SERCA3 (B), and GAPDH (A and B) are shown. RINm5F cells were exposed for 5 h to the stress agents as follows: Tg (200 nM), A23187 (5 μM), EGTA (2.5 mM), Tun (10 μM/ml), DTT (0.8 mM), BFA (5 μg/ml), Azc (5 μM), and total RNA was isolated. 7 μg of RNA was loaded/lane (representative of three (A) or two (B) experiments).
ability, and the high Ca\textsuperscript{2+} concentration. A number of agents and conditions may interfere with these parameters to evoke the ER stress response (8).

The main findings of this study focus on the regulation of ER

Ca\textsuperscript{2+}-ATPase expression under conditions evoking the ER stress response pathway known as UPR. We found a 3–4-fold selective up-regulation of the SERCA2b mRNA level in response to an array of UPR inducing agents. In contrast, there was no change in the SERCA3 mRNA level (Fig. 1). An earlier study has suggested that the intra-ER Ca\textsuperscript{2+} content may be an important determinant of SERCA expression, because depletion of ER Ca\textsuperscript{2+} resulted in an increased production of SERCA mRNA (however, no distinction was made between SERCA2 and SERCA3 mRNAs) (52). Our present findings agree with these earlier results. However, the present study substantially enlarges the context of SERCA expression regulation to that of the ER stress response in general, because of the inclusion of several stress conditions not expected a priori to affect the ER Ca\textsuperscript{2+} content. We confirmed such lack of any significant ER Ca\textsuperscript{2+} perturbation upon either short term or longer exposure of PC12 cells to DTT, tunicamycin, or Azc, by direct measurements of the thapsigargin-releasable ER Ca\textsuperscript{2+} (Fig. 9). Therefore, we propose that the induction of SERCA2b mRNA was linked to the activation of the UPR by the stressed ER rather than representing an adjustment to the depletion of the ER Ca\textsuperscript{2+}. More generally, these results also indicate that although the Ca\textsuperscript{2+}-releasing agents have been employed in studies of the UPR (8), ER Ca\textsuperscript{2+} depletion is not a necessary requirement to trigger this pathway.

Several independent lines of evidence support the up-regulation of SERCA2b mRNA as reflecting the activation of the UPR pathway. First, several features of the SERCA2b mRNA up-regulation corresponded closely to those observed for the mRNA of BiP, the well studied UPR-induced ER chaperone measured in parallel in our system. Thus, the increase in SERCA2b mRNA occurred at a time scale similar to that for BiP (2–4 h), was similarly reversible upon the removal of the stress agent, and could be reinduced upon the reintroduction of this agent. Second, it is well established that the ER stress-induced increase of BiP mRNA requires protein synthesis (46) and is due to a transcriptional activation (53). We have shown that these characteristics did apply to the increase in the amount of the SERCA2b mRNA, based on the inhibition of the response by cycloheximide and actinomycin D and the lack of mRNA stabilization. Third, an analysis of the promoter region of SERCA2 genes revealed, within the first 1000 nucleotides upstream to the transcription initiation site, up to three copies of the nucleotide sequences showing at least 80% identity with the recently identified ERSE consensus sequence (Fig. 10A).
ERSE regulatory elements have recently been shown to be sufficient and necessary for the induction of the ER stress genes in higher eukaryotes (27, 28). The ERSE consensus sequence encompasses 19 base pairs and has an overall structure CCAAT(N9)CCACG (Fig. 10A), proposed on the basis of promoter comparison between ER stress-regulated genes as well

**Fig. 9. The stressed ER is not calcium-depleted.** Single cell free cytosolic Ca\(^{2+}\) [Ca\(^{2+}\)]\(_{i}\), measurements were carried out with fura-2 ratio technique. The ordinate indicates nM [Ca\(^{2+}\)]\(_{i}\), and the abscissa indicates time in seconds. Means of recordings from 20–40 cells in each microscopic field are shown. A, panels a and b, validation of the assay system. PC12 cells show transient raise in [Ca\(^{2+}\)]\(_{i}\), upon stimulation with acetylcholine (panel a, ACh, arrow), or upon SERCA blockade by Tg (panels a and b, 300 nm, open arrowhead). Panels c–e, short term exposure to ER stressors does not release ER Ca\(^{2+}\). Agents (panel c, Me\(_2\)SO 0.1% v/v), Tun (panel d, 10 μg/ml) or Azc (panel e, 5 mM) were added at 180 s (long arrow). After 18–20 min, PC12 cells were washed with Ca\(^{2+}\)-free EM solution supplemented with 25 μM EGTA (filled arrowhead), and thapsigargin was added (open arrowhead, 300 nM), followed by ionomycin (diamond, 2 μM). Values are representative of three independent experiments.

B, long term exposure to ER stressors does not release ER Ca\(^{2+}\). Cells were treated for 4 h with the indicated stressors, loaded with fura-2, and subjected to calcium imaging. At 180 s (filled arrowhead) extracellular Ca\(^{2+}\) was removed by three washes in nominally Ca\(^{2+}\)-free EM solution. At 360 s cells were stimulated by 100 μM acetylcholine (arrow). At 600 s 300 nM thapsigargin was added (open arrowhead). Values are representative of three independent experiments.
A rat BiP UPRE (56).

from the transcription initiation site.

found are numbered 1–3 in the order of increasing distance upstream indicated by a negative number. For each gene, up to three ERSEs

count of the first C from the initiation of transcription (set to

SERCA3, Y15724. M33834.1; rat, AF031937; yeast BiP, S40310.1; rat BiP M14866.1; numbers: human, SERCA2, AC006088.1; murine, AF029982.1; rabbit,

sequences of SERCA2 and SERCA3 genes with those of yeast and rat BiP. Similarity, the presence of only a single ERSE-like element in

the single ERSE-like element identifiable within the SERCA3 gene, in contrast to the multiple copies in SERCA2, may also constitute a functionally important difference (27, 28).

as functional studies (27, 28). It may be seen in Fig. 10A that the SERCA2 ERSE1 was conserved between species and agreed with the consensus ERSE, except for the substitution of adenine for guanine in position 19. ERSE position 19 is also substituted to adenine in the ERSE-like element of GRP58 (54) (Fig. 10A) and is the least conserved position in the ERSE consensus sequence. Several ERSE2 and ERSE3 sequences of SERCA2 genes showed additional divergences from the ERSE consensus. Importantly, SERCA2 ERSE1 from all species analyzed contained the GGC motif within the nine intervening nucleotides. On the other hand, this GGC motif was missing in the single ERSE-like element identifiable within the SERCA3 gene, presumably invalidating the SERCA3 ERSE, because the functional importance of this nucleotide triplet has been demonstrated by mutagenesis of the rat the BiP promoter (28).

Similarly, the presence of only a single ERSE-like element in SERCA3 gene, in contrast to the multiple copies in SERCA2, may therefore be regarded as a "housekeeping" Ca²⁺-ATPase present in all tissues except cardiac and skeletal muscle, whereas SERCA3 displays a somewhat narrower distribution (11, 15, 16).

From the point of view of Ca²⁺ homeostasis it seems intriguing that significant up-regulation of SERCA2b should take place with no apparent change in the ER content of Ca²⁺ upon ER stress induced by agents like tunicamycin, DTT, or Azc. It may be speculated that an enhanced Ca²⁺ uptake activity into the ER might act to shorten the period of a relative ER Ca²⁺ depletion subsequent to a stimulus-induced ER Ca²⁺ release, in this way responding to an increased demand for Ca²⁺ by the ER chaperone system under conditions where unfolded proteins are accumulated.

One example of a possible pathophysiological relevance of the SERCA2b regulation by the UPR came from the recent identification of the presenilin-1 as a regulator of the mammalian UPR. Presenilin-1 mutations, known to underlie a large proportion of familial Alzheimer's disease cases, down-regulate UPR (57). Interestingly, such a presenilin-1 mutation was also shown to cause a destabilization of cellular Ca²⁺ homeostasis (58). It is tempting to speculate that this destabilization of Ca²⁺ homeostasis resulted from a low SERCA2b level, because of the ER-mediated regulation of SERCA pumps in an activity-dependent manner (39). As seen in Fig. 7C, the four tested ER stress-inducing agents did cause a statistically significant increase in SERCA E–P, albeit by a factor smaller than the corresponding increase in the immunoactive SERCA2b. This discrepancy was at least in part due to the fact that the acid SDS-PAGE system used for the E–P analysis (as distinct from the SDS-PAGE according to Laemmli (34)) was unable to resolve SERCA2b and SERCA3 E–Ps. Hence, the stress-unresponsive SERCA3 contribution to the E–P band at 105 kDa (Fig. 7C) must have partly obscured the stress-induced response because of SERCA2b. In addition, the level of SERCA E–P was measured in microsomes, in contrast to whole cell extracts used to measure SERCA2b immunostaining.

In summary, the experimental data (Figs. 1–6), as well as the analysis of the SERCA2 gene promoters (Fig. 10) strongly suggest that SERCA2 transcription is selectively activated by the UPR pathway, along with other ER stress-inducible genes and that this gives rise to an elevated level of active SERCA2b protein in the cell following ER stress (Fig. 7).

Given the universal nature of UPR, the observation of the specific association of SERCA2b, rather than SERCA3, with the ER stress-induced UPR agrees well with the ubiquitous nature of SERCA2b distribution. Indeed, SERCA2b has been regarded as a "housekeeping" Ca²⁺-ATPase present in all tissues except cardiac and skeletal muscle, whereas SERCA3 displays a somewhat narrower distribution (11, 15, 16).

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of a down-regulated UPR, in agreement with SERCA2b expression being linked to the UPR as reported here.

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