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Inhibition of protein synthesis and early protein processing by thapsigargin in cultured cells

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Thapsigargin, a tumour-promoting sesquiterpene lactone, selectively inhibits the Ca^{2+}-ATPase responsible for Ca^{2+} accumulation by the endoplasmic reticulum (ER). Mobilization of ER-sequestered Ca^{2+} to the cytosol and to the extracellular fluid subsequently ensues, with concomitant alteration of cellular functions. Thapsigargin was found to serve as a rapid, potent and efficacious inhibitor of amino acid incorporation in cultured mammalian cells. At concentrations mobilizing cell-associated Ca^{2+} to the extracellular fluid, thapsigargin provoked extensive inhibition of protein synthesis within 10 min. The inhibition in GH_3 pituitary cells involved the synthesis of almost all polypeptides, was not associated with increased cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), and was not reversed at high extracellular Ca^{2+}. The transient rise in $[Ca^{2+}]_i$ triggered by ionomycin was diminished by thapsigargin. Polysomes failed to accumulate in the presence of the drug, indicative of impaired translational initiation. With longer (1–3 h) exposures to thapsigargin, recovery of translational activity was observed accompanied by increased synthesis of the ER protein glucose-regulated stress protein 78 or immunoglobulin heavy-chain binding protein ('GRP78/BiP') and its mRNA. Such inductions were comparable with those observed previously with Ca^{2+} ionophores which mobilize the cation from all intracellular sequestered sites. Actin mRNA concentrations declined significantly during such treatments. In HepG2 cells processing and secretion of the glycoprotein α_1-antitrypsin were rapidly suppressed by thapsigargin. Ca^{2+} sequestered specifically by the ER is concluded to be essential for optimal protein synthesis and processing. These rapid effects of thapsigargin on mRNA translation, protein processing and gene expression should be considered when evaluating potential mechanisms by which this tumour promoter influences cellular events.

INTRODUCTION

The central role of transient increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in intracellular signalling related to stimulus–response coupling is well established. Such signalling requires the entrance of Ca^{2+} to the cytosol from the extracellular fluid and/or mobilization of the cation from intracellular sequestering sites. In contrast with cytosolic free Ca^{2+}, intracellular Ca^{2+} sequestered within organelles such as the endoplasmic reticulum (ER) is generally perceived as being non-regulatory. As described in recent reviews [1,2], however, a variety of evidence supports a role for sequestered Ca^{2+} in maintenance of high rates of protein synthesis in intact mammalian cells. Mobilization of sequestered cation consequent to the application of specific hormones or drugs results in the rapid suppression of translational initiation, regardless of changes in $[Ca^{2+}]_i$. Early glycoprotein processing has also been proposed to depend on adequate degrees of Ca^{2+} sequestration [3,4]. Since disruption of protein processing following mobilization of sequestered Ca^{2+} was consistently associated with strongly suppressed rates of amino acid incorporation, putative coupling of the rate of translational initiation to the functional status of the ER was proposed [4].

The ER functions prominently in early protein processing and is currently accepted as a major Ca^{2+}-storing organelle. Nonetheless the organelle is sufficiently heterogeneous in terms of its biochemical, functional and morphological properties that characterization of its role in Ca^{2+} homoeostasis has proven elusive and controversial [5–7]. The apparent lack of specificity with which most Ca^{2+}-mobilizing agents perturb various Ca^{2+} pools of non-muscle cells has precluded accurate appraisal of the role of ER-sequestered Ca^{2+} in any given process. By contrast, the tumour-promoting sesquiterpene lactone thapsigargin is believed to deplete the ER-sequestered Ca^{2+} pool specifically [8]. Initial effects of the drug are attributable to selective inhibition of the Ca^{2+}-ATPase of the ER [8–10]. Since depletion of ER Ca^{2+} stores by thapsigargin is usually accompanied by a significant rise in $[Ca^{2+}]_i$ [8], effects of this tumour promoter on cellular parameters such as proliferation and survival are most commonly interpreted to result directly from mobilization of Ca^{2+} to the cytosol. For example, the cytotoxic effects of thapsigargin on cultured keratinocytes were proposed to result from a long-lasting flooding of the cytosol with Ca^{2+} [11]. In the present paper thapsigargin, at concentrations that mobilize cell-associated Ca^{2+}, is shown to suppress protein synthesis within minutes. In GH_3 pituitary cells the inhibition, which involved a broad spectrum of polypeptide species and was attributable to impaired translational initiation, was not associated with an increase in $[Ca^{2+}]_i$. Within 1–3 h significant induction of the ER-resident protein glucose-regulated stress protein 78 or immunoglobulin heavy-chain binding protein (GRP78/BiP), which has been proposed to support protein synthesis and processing [12–15], and its mRNA were observed. Early glycoprotein processing by HepG2 cells was susceptible to rapid blockade by thapsigargin. We suggest that the effects of thapsigargin on mRNA translation and protein processing, as well as those on Ca^{2+} sequestered

Abbreviations used: ER, endoplasmic reticulum; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; IP_3, *myo*-inositol 1,4,5-trisphosphate; PMA, phorbol 12-myristate 13-acetate; Cbz-Gly-Phe-NH_2, benzyloxycarbonyl-glycyl-phenylalanyl-amide; GRP78/BiP, glucose-regulated stress protein 78 or immunoglobulin heavy-chain binding protein.

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specifically by the ER, must be considered when evaluating potential mechanisms by which this tumour promoter influences events such as cell proliferation and survival.

EXPERIMENTAL

General methodology

Procedures for culture and harvest of GH_3 [16] and HepG2 [4] cells, for analysis of ribosome size distribution [16] and for determination of cell-associated ^{45}Ca [14] have been previously described. Amino acid incorporation was measured as described previously [17] for incubations conducted in triplicate. Values for replicate samples routinely varied within 5% or less. Typical variation, expressed as the average ± range of values obtained, is presented in Table 1. For other experiments, average values are provided and the number of times findings were reproduced is given in the legends. One-dimensional PAGE of detergent-solubilized extracts of [^{35}S]methionine-labelled cells was conducted in 10% acrylamide gels as described by Laemmli [18]. Two-dimensional PAGE was performed as described previously [19]. Protein was determined by a Coomassie Blue dye procedure [20]. ATP and GTP contents were determined in 10% trichloroacetic acid extracts of variously treated preparations by h.p.l.c. as described [4].

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined by a procedure modified for GH_3 cells [21]. Cells were harvested by centrifugation, washed and resuspended to 10^7/ml in 2 ml of assay buffer (118 mM NaCl, 4.6 mM KCl, 1 mM $CaCl_2$, 10 mM glucose, 20 mM Hepes, pH 7.2), and loaded with Quin-2 acetoxymethyl ester (50 μM) for 30 min at 37 °C. Cell suspensions were then diluted to 20 ml, washed twice by centrifugation, and resuspended in 2 ml of assay buffer. Fluorescence was recorded with a Perkin–Elmer MPF-66 fluorescence spectrophotometer with excitation at 339 nm and emission at 492 nm, slit widths each 5 nm. Cells were maintained in suspension with a magnetic stirrer during measurements, which were performed on freshly loaded cells. Treatment agents were added directly from concentrated stock solutions to cells in the cuvette. Quin-2 was calibrated by addition of 0.5% Triton X-100 for $F_{max.}$, 20 mM Tris, pH 8.3 and 5 mM EGTA for $F_{min.}$, and 6 mM $CaCl_2$ to verify $F_{max.}$. Calculation of $[Ca^{2+}]_i$ was as described in [22].

Pulse-labelling, immunoprecipitation and electrophoresis of α_1-antitrypsin and albumin

HepG2 cells were suspended in serum-free medium identical with Ham's F-10 with the concentration of methionine adjusted to 1 μM and with Ca^{2+} adjusted to 1 mM and were pretreated for 15–20 min at 37 °C. [^{35}S]Methionine (60 μCi/ml) was then added and the incubation continued for 10 min. Cells were washed twice by centrifugation at 600 *g* and resuspension in Ham's F-10 medium containing 33 μM unlabelled methionine and 50 μM added Ca^{2+}. Treatment agents were then added and the chase period was continued for various time periods. The incubation was terminated by placing samples of cell suspension on ice. Samples of cell suspensions were centrifuged briefly at 12400 *g* in a Beckman microfuge, and pellets (cells) were separated from the supernatants (medium). Cells were washed once in ice-cold phosphate-buffered saline (PBS) and lysed in PBS, pH 7.2, containing 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40 and 1 mM phenylmethanesulphonyl fluoride. Lysates were clarified by centrifugation at 12400 *g*. Samples of lysate or of extracellular medium were incubated with formaldehyde-fixed *Staphylococcus* A membranes (IgGSorb; Enzyme Center, Malden, MA, U.S.A.) and centrifuged to remove material that bound non-specifically to *Staphylococcus* Protein A. Portions of supernatants were incubated with antibodies to α_1-antitrypsin or albumin at 1:100 dilution, and antigen–antibody complexes were precipitated by incubation with IgGSorb and were collected by centrifugation. Pellets were washed twice by centrifugation and resuspension in PBS and were then resuspended in Laemmli [18] sample buffer. Immunoprecipitated proteins were removed from antigen–antibody–Protein A complexes by boiling for 2–3 min. Electrophoresis was conducted with 7.5%-acrylamide gels. Identification of the two immunoprecipitable species of α_1-antitrypsin displayed in Figure 4 as the high-mannose and complex forms of the glycoprotein has been described previously [3,4].

Preparation of DNA probes and extraction and analysis of mRNA

CsCl-purified recombinant plasmids p3C5 (GRP78) and pA1 (β-actin) were linearized by digestion with an appropriate restriction enzyme, extracted with phenol/chloroform and precipitated with ethanol. Polythymidylate probe was prepared by using poly(A)$^+$ RNA as template, oligo(dT) as primer, thymidine 5′-[α-[^{35}S]thio]triphosphate, and AMV reverse transcriptase as described by Hollander and Fornace [23]. Total cellular RNA was isolated by the acid guanidinium isothiocyanate method [24], except for inclusion of one additional phenol/chloroform extraction. RNA concentrations were estimated by A_{260}, and measurement of various mRNAs by dot-blot analysis using labelled cDNA or poly(T) probes was performed as previously described [25].

Materials

Modified Ham's F-10 medium was prepared by omitting Ca^{2+}, leucine and methionine and was stored frozen. Unless indicated otherwise, the following adjustments were made before each experiment: leucine to 100 μM, methionine to 33 μM, and Ca^{2+} to 0.5 mM. Thapsigargin was purchased from LC Services Corp., and ionomycin and A23187 were from Calbiochem. Goat antiserum to human α_1-antitrypsin and rabbit antiserum to human albumin were obtained from U.S. Biochemical Corp. Plasmid carrying the cDNA for hamster GRP78 was generously provided by Dr. A. S. Lee, University of Southern California Medical School, and plasmid carrying the cDNA for β-actin was generously provided by Dr. D. Cleveland, Johns Hopkins University.

RESULTS

Rapid inhibition of protein synthesis by thapsigargin

Amino acid incorporation in both GH_3 and HepG2 cells was rapidly suppressed by thapsigargin at low concentrations (Figure 1). In GH_3 cells, which have been found particularly susceptible to Ca^{2+} depletion with either EGTA or Ca^{2+}-mobilizing agents [1,2], leucine incorporation was completely inhibited by thapsigargin within 15 min (Figures 1a and 1c). HepG2 cells, which retain sequestered Ca^{2+} more tenaciously [4], required 20–30 min exposure to the drug for complete suppression of incorporation (Figures 1b and 1d). The onset of inhibition was rapid for both cell types, with GH_3 requiring approx. 10 min of exposure and HepG2 about twice as long. Thapsigargin was especially potent in inhibiting incorporation in GH_3 cells. Although 1 nM drug was not inhibitory in these cells, a 75% decrease in incorporation

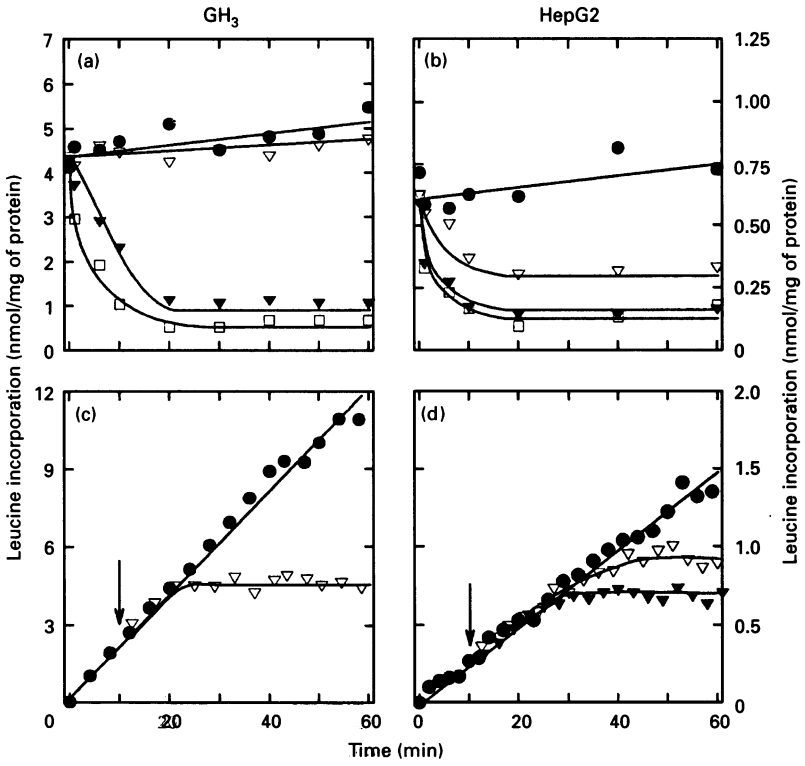

Figure 1 Inhibition of amino acid incorporation by thapsigargin in GH_3 and HepG2 cells

(**a**) Leucine incorporation in GH_3 cells after various times of exposure to thapsigargin. Cell suspensions were adjusted with dimethyl sulphoxide (●) or with 1 nM (▽), 2 nM (▼) or 3 nM (□) thapsigargin and incubated for the times indicated. [^{3}H]Leucine was then added, and pulse incorporation into protein was determined after incubation for 15 min. (**b**) Leucine incorporation in HepG2 cells after various times of exposure to thapsigargin. Preparations were treated with solvent (●), 10 nM (▽), 100 nM (▼) or 1000 nM (□) thapsigargin, and pulse incorporation was determined as in (**a**). (**c**) Time-dependence of thapsigargin inhibition in GH_3 cells. After addition of [^{3}H]leucine (2×10^6 c.p.m./0.5 ml), samples were removed at the indicated times throughout 1 h of incubation for measurements of incorporation. At 11 min (arrow), 30 nM thapsigargin (▽) was added to half of the preparation, and sampling of both preparations continued through 1 h. (**d**) Time-dependence of thapsigargin inhibition in HepG2 cells. [^{3}H]Leucine was added to the cell suspension, and samples were removed at the times indicated for incorporation measurements. Solvent (●), 10 nM (▽) or 100 nM (▼) thapsigargin were added at 11 min (arrow), and sampling of all preparations was continued through 1 h. Findings of panels (**a**)–(**d**) have been reproduced on three separate occasions.

was attained with 2 nM thapsigargin (Figure 1a). By contrast, drug concentrations greatly in excess of 10 nM were required for comparable degrees of inhibition in HepG2 cells (Figures 1b and 1d). Thapsigargin inhibited incorporation of methionine into a broad spectrum of polypeptides in GH_3 cells (Figure 2a). Inhibition comparable with that observed with the Ca^{2+} ionophores A23187 and ionomycin at 100 nM was attained with thapsigargin at 2 nM.

Inhibition of protein synthesis by agents that mobilize Ca^{2+} from intracellular sequestered sites has been attributed to an impairment of translational initiation [1,2,16,26]. The polysomal contents of thapsigargin-treated GH_3 cells were therefore examined to ascertain whether the specific depletion of the ER-sequestered Ca^{2+} pool altered the initiation step. Thapsigargin was found to inhibit polysome accumulation similarly to A23187, which mobilizes Ca^{2+} from all intracellular sequestered sites (Figure 2b). Results (not shown) were identical for preparations incubated at 2 mM or 75 μM extracellular Ca^{2+}. The decrease in polysome content, accompanied by an enrichment in 80 S ribosomes, indicated that translational initiation, rather than peptide-chain elongation or termination, was specifically affected.

Thapsigargin at concentrations in excess of those observed to provoke significant inhibition of leucine incorporation did not decrease the ATP or GTP contents of GH_3 cells (Table 1). Decreases in these nucleotides were also not observed in preparations treated with 1 μM ionomycin or with Ca^{2+}-free medium containing 1 mM EGTA. By contrast, arachidonic acid and the metalloendoproteinase inhibitor benzyloxycarbonyl-glycyl-phenylalanyl-amide (Cbz-Gly-Phe-NH_2), each of which mobilize sequestered Ca^{2+} from GH_3 cells [25,26], lowered ATP and GTP contents in a concentration-dependent manner and over the range of concentrations wherein leucine incorporation was inhibited.

Perturbation of Ca^{2+} homoeostasis in GH_3 cells by thapsigargin

GH_3 cells preloaded with ^{45}Ca were challenged with 3 nM thapsigargin for various times, and the amount of Ca^{2+} remaining cell-associated was determined (Figure 3a). Thapsigargin mobilized Ca^{2+} rapidly, with maximal effects observed at 10–15 min of incubation. In contrast with Ca^{2+} ionophores and unsaturated fatty acids, which provoke extensive Ca^{2+} mobilization [26,27], only 40% of cell-associated cation could be mobilized with thapsigargin. Cell-associated Ca^{2+} and leucine incorporation were decreased at similar thapsigargin concentrations, with half-maximal and full inhibitions obtained at 0.9 and 3 nM respectively (Figure 3c). Increased extracellular Ca^{2+} concentrations, which tend to reverse the inhibition of leucine incorporation by other Ca^{2+}-mobilizing agents, were unable to overturn the inhibition imposed by thapsigargin (Figure 3b). At 3 mM extra-

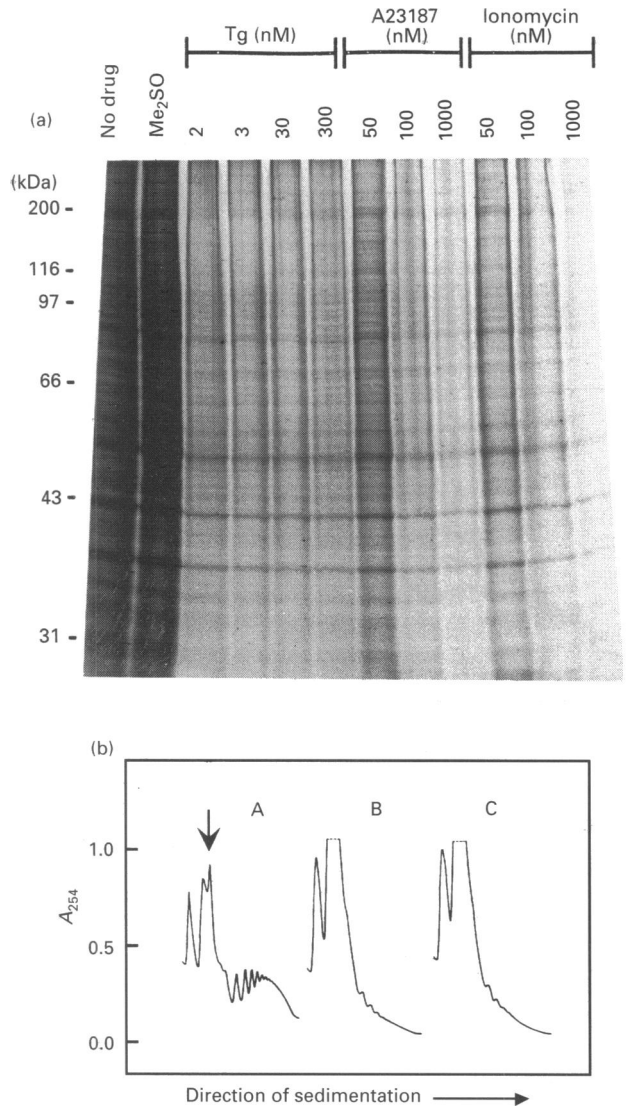

Figure 2 Inhibition of methionine incorporation into polypeptides and of polysome accumulation in GH_3 cells treated with thapsigargin, A23187 or ionomycin

(**a**) Methionine incorporation into various polypeptide species. Cells pretreated for 30 min with no additions, dimethyl sulphoxide (Me_2SO) or various concentrations of thapsigargin (Tg), A23187 or ionomycin were labelled with [^{35}S]methionine and prepared for SDS/PAGE (10% gels) and autoradiography. (**b**) Polysome accumulation. Cells in medium containing 2 mM Ca^{2+} were pretreated for 15 min with solvent (A), 300 nM thapsigargin (B) or 1 μM A23187 (C). Lysates of treated preparations were subjected to sucrose-density-gradient centrifugation for analysis of ribosomal size. The arrow indicates the position of 80 S ribosomes. Findings in (**a**) have been reproduced on two separate occasions; results identical with those described in (**b**) have been obtained with lower (3 nM) concentrations of thapsigargin.

cellular cation, for example, 10 nM thapsigargin was fully inhibitory.

Mobilization of Ca^{2+} from GH_3 cells to their extracellular fluids in the presence of thapsigargin was not accompanied by an increase in $[Ca^{2+}]_i$. At 10 nM drug $[Ca^{2+}]_i$ was not altered (Figure 3d, upward arrow). At concentrations of 90 nM or greater, thapsigargin significantly lowered $[Ca^{2+}]_i$ (result not shown). Additionally, pretreatment with 10 nM thapsigargin was found to diminish the rise in $[Ca^{2+}]_i$ provoked by 1 μM ionomycin. In the absence of thapsigargin, ionomycin raised $[Ca^{2+}]_i$ to approx. 1 μM (Figure 3d, lower tracing), whereas the ionophore increased

Table 1 ATP and GTP contents of GH_3 cells treated with various Ca^{2+}-mobilizing agents or EGTA

Cells suspended in medium containing 0.5 mM Ca^{2+} were treated for 15 min with thapsigargin, ionomycin, arachidonic acid or Cbz-Gly-Phe-NH_2 at the indicated concentrations. Additional preparations were incubated for 45 min in medium lacking Ca^{2+} and containing 1 mM EGTA. Samples were taken for measurements of leucine incorporation or for preparation of extracts for analysis of ATP and GTP contents by h.p.l.c. Findings regarding ATP and GTP contents have been reproduced on three separate occasions. Values for leucine incorporation are expressed as the average ± range of values obtained for triplicate incubation samples.

Treatment	Content (nmol/mg of protein) ATP	GTP	Leucine incorporation (nmol/mg of protein)
None	24.0	4.9	3.25 ± 0.05
Thapsigargin (100 nM)	28.6	5.2	0.34 ± 0.01
Ionomycin (1 μM)	29.3	5.3	0.37 ± 0.02
Arachidonic acid (10 μM)	14.0	4.2	2.14 ± 0.13
Arachidonic acid (20 μM)	10.8	3.8	0.42 ± 0.02
Cbz-Gly-Phe-NH_2 (1.35 mM)	20.4	4.2	0.97 ± 0.04
Cbz-Gly-Phe-NH_2 (3 mM)	16.6	3.1	0.23 ± 0.01
EGTA (1 mM)	23.9	5.0	0.36 ± 0.01

$[Ca^{2+}]_i$ to 800 nM after thapsigargin treatment (Figure 3d, upper tracing). Ionomycin effects on $[Ca^{2+}]_i$ were attenuated within 50 s regardless of thapsigargin pretreatment.

Inhibition of glycoprotein processing and secretion in HepG2 cells by thapsigargin

A variety of evidence favours the proposal that sequestered Ca^{2+} maintains post-translational processing [3,4,28,29]. In HepG2 human hepatoma cells, which constitute a popular model for the study of protein processing and secretion [30], ionomycin blocks conversion of α_1-antitrypsin from the high-mannose into the complex form and dramatically suppresses export of the glycoprotein in a manner reversible by Ca^{2+} [4]. HepG2 cells were therefore employed to ascertain whether the specific depletion of ER-sequestered Ca^{2+} by thapsigargin retarded the processing and secretion of newly synthesized α_1-antitrypsin. Cells were pulse-labelled with [^{35}S]methionine and chased with unlabelled amino acid for 30 or 60 min in the absence or presence of thapsigargin or ionomycin. This protocol permits evaluation of the effects of drug treatments on post-translational events independently of their effects on protein synthesis. α_1-Antitrypsin was quantitatively immunoprecipitated from lysates and extracellular fluid, and immunoprecipitates were analysed by SDS/PAGE and autoradiography (Figure 4). In untreated preparations the complex (upper band) form of the protein was detected intracellularly and in the medium, indicating that the glycoprotein had been processed and secreted efficiently. Conversion of α_1-antitrypsin from the high-mannose (lower band) into the complex form, as well as export of the glycoprotein to the medium, were considerably delayed in the presence of thapsigargin or ionomycin. By contrast, secretion of albumin, a non-glycoprotein, into the medium proceeded efficiently in the absence or presence of the drugs (Figure 4).

Modulation of gene expression in GH_3 cells by thapsigargin

GRP78/BiP, a resident protein of the lumen of the ER, is thought to have an important role in protein processing in metabolically stressed and non-stressed mammalian cells

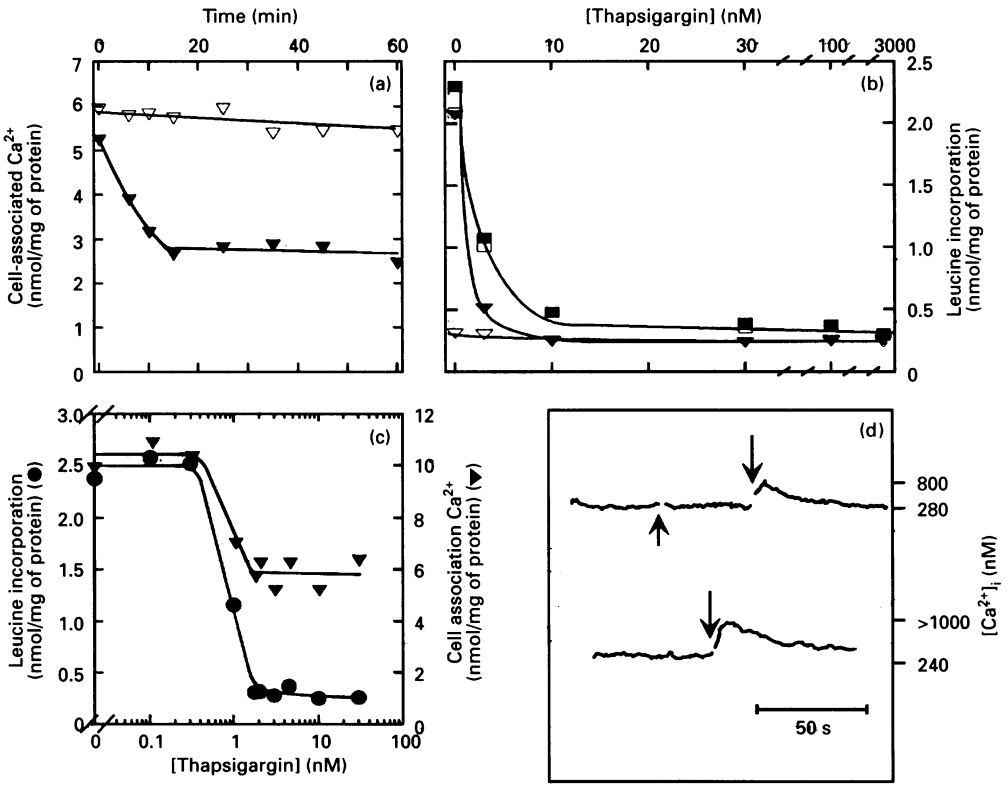

Figure 3 Cell-associated Ca^{2+} and $[Ca^{2+}]_i$ in thapsigargin-treated GH_3 cells and effect of extracellular Ca^{2+} on thapsigargin inhibition of protein synthesis

(**a**) Cell-associated Ca^{2+} after treatment with thapsigargin. Cells in medium containing 75 μM Ca^{2+} were preloaded for 90 min with $^{45}CaCl_2$ (8×10^6 c.p.m./ml) and then challenged with solvent (▽) or 3 nM thapsigargin (▼). At specified times, samples of cell suspension were removed for measurement of cell-associated ^{45}Ca. (**b**) Leucine incorporation at various concentrations of extracellular Ca^{2+} and thapsigargin. After pre-equilibration with 3 mM (■), 0.5 mM (□) or 0.05 mM (▼) Ca^{2+} or with 1 mM EGTA (▽), thapsigargin was added at the indicated concentrations and leucine incorporation determined. (**c**) Amino acid incorporation and Ca^{2+} mobilization at various thapsigargin concentrations. $^{45}CaCl_2$-preloaded or non-preloaded cells were incubated with various concentrations of thapsigargin and analysed for cell-associated ^{45}Ca (▼) or leucine incorporation (●) respectively. (**d**) Effects of thapsigargin and ionomycin on $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ of quin-2 loaded cells was assayed with a Perkin–Elmer MPF-66 fluorescence spectrophotometer. Cells were challenged with 1 μM ionomycin (downward arrow) in the presence (upper tracing) or absence (lower tracing) of 10 nM thapsigargin (upward arrow). Findings described in (**a**) and (**c**) have been reproduced on two separate occasions, those of (**b**) on three separate occasions, and those of (**d**) on four separate occasions.

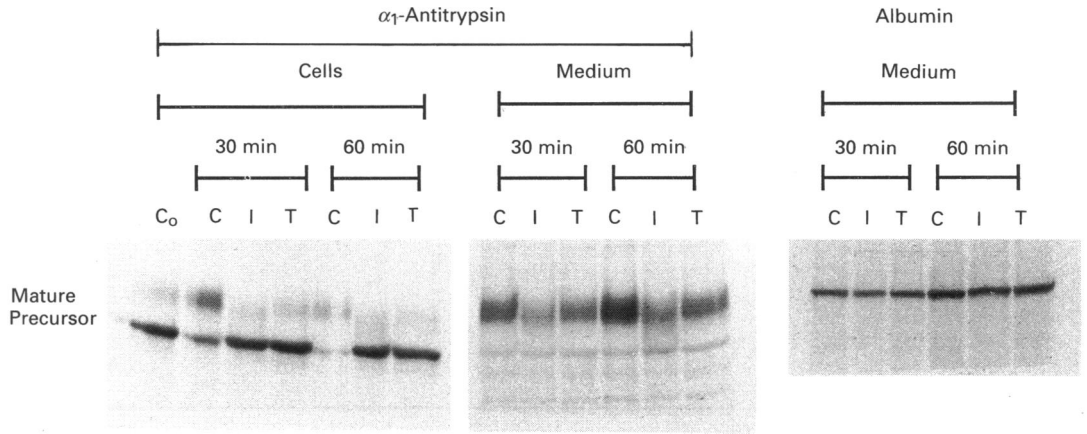

Figure 4 Effects of thapsigargin and ionomycin on processing and secretion of α_1-antitrypsin and on secretion of albumin

HepG2 cells were pulse-labelled with [^{35}S]methionine and chased in the absence or presence of 1 μM thapsigargin or 3 μM ionomycin. Samples of cells and of their respective media were collected at the start of the chase (zero time) or after 30 and 60 min of chase for immunoprecipitation with antibodies to α_1-antitrypsin. Immunoprecipitates were analysed by SDS/PAGE (7.5% gels) and autoradiography. Key: C_0, zero-time control; C, untreated control; I, ionomycin; T, thapsigargin. Upper and lower bands represent the complex (mature) and high-mannose (precursor) forms of α_1-antitrypsin respectively. Samples of media were also collected after 30 and 60 min of incubation for immunoprecipitation with antibodies to albumin, and immunoprecipitates were analysed as above. Findings of this experiment have been reproduced on two separate occasions.

[12,13,31–34]. It has been suggested that GRP78 functions to assist in the translocation and proper folding of polypeptides and in the assembly of polypeptides into stable quaternary structures, as well as to prevent the formation and secretion of abnormal oligomeric protein complexes. Agents that mobilize sequestered Ca^{2+} stores induce GRP78 and its mRNA within hours in a

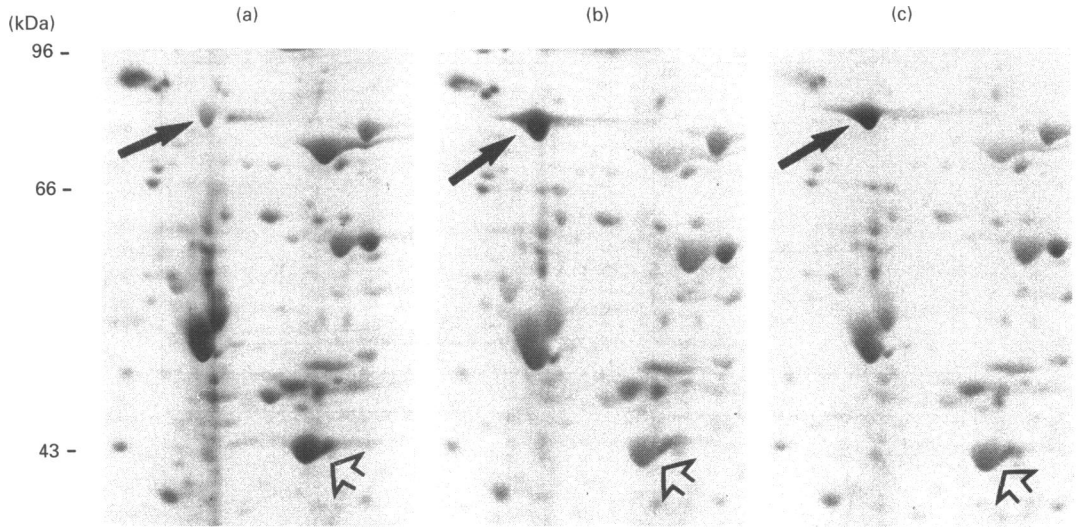

Figure 5 Relative methionine incorporation into various polypeptide species of GH_3 cells after extended exposure to thapsigargin or ionomycin

Cells were treated for 3 h with 0.6 μM PMA and 50 ng/ml cholera toxin in the absence or presence of 10 nM thapsigargin or 1 μM ionomycin. After resuspension in fresh medium containing treatment additives and 10 μM methionine, pulse-labelling with [^{35}S]methionine (25 μCi/ml) was conducted for 1 h. Lysates adjusted to contain equal amounts of radioactivity were subjected to two-dimensional PAGE, and autoradiography was performed. A segment of each autoradiogram is presented: (**a**) control; (**b**) thapsigargin-treated; (**c**) ionomycin-treated. Species migrating at the positions of authentic GRP78 and actin are indicated by the black and white arrows, respectively. Migration of acidic species is to the left. Radioactivity associated with actin and GRP78 in each preparation was quantified with a PhosphorImager (Molecular Dynamics); results appear below. Findings have been reproduced on three separate occasions.

	10^{-3} × Measured radioactivity (c.p.m.)		
	Control	Thapsigargin	Ionomycin
Actin	680	240	269
GRP78	120	802	537

Table 2 Relative mRNA concentrations of GH_3 cells incubated in the absence or presence of thapsigargin, phorbol ester and cholera toxin

Cells were incubated for the indicated times with 0.6 μM PMA and 50 ng/ml cholera toxin in the absence or presence of 15 nM thapsigargin. Total RNA was extracted and equal amounts were applied to nitrocellulose. Filters were hybridized to radioactive probes specific for GRP78, actin and poly(A)$^+$ mRNAs. Radioactivity was quantified with a PhosphorImager. The content of each mRNA sample in untreated cells at zero time was assigned a value of 1.0, and the results are expressed as the relative concentration at each incubation time. Results are presented as the average ± range of values obtained for triplicate samples. Findings have been reproduced on two separate occasions.

Time (h)	Thapsigargin	PMA + cholera toxin	GRP78 mRNA	Actin mRNA	Poly(A)$^+$ mRNA
0	−	−	1.0	1.0	1.0
1.5	+	−	1.37 ± 0.29	0.70 ± 0.07	1.03 ± 0.03
3	+	−	3.62 ± 0.60	0.47 ± 0.02	1.46 ± 0.01
5	+	−	3.67 ± 0.16	0.34 ± 0.01	1.28 ± 0.06
1.5	+	+	7.81 ± 0.07	0.78 ± 0.09	1.52 ± 0.08
3	+	+	8.92 ± 0.29	0.46 ± 0.01	1.20 ± 0.02
5	+	+	9.56 ± 0.14	0.39	1.28 ± 0.04
5	−	−	1.43 ± 0.13	0.95 ± 0.04	1.55 ± 0.02
5	−	+	1.05 ± 0.19	0.75 ± 0.02	0.95 ± 0.06

manner accompanied by expression of translational tolerance to such agents [12–15,35]. The GRP78 gene possesses a highly conserved promoter region that confers Ca^{2+}-ionophore inducibility and binds *trans*-acting transcription factors [36–38]. Activation of such factors may be necessary for transcription of the gene in that optimal induction of GRP78 mRNA in ionophore-treated GH_3 cells is known to require the elevation of cyclic AMP concentration and a phorbol ester. In the presence of these promoters optimal induction in GH_3 cells is achieved within 3 h [15].

Protein synthesis was initially suppressed when GH_3 cells were treated with 10 nM thapsigargin in the presence of cholera toxin and phorbol myristate acetate (PMA), but substantial (80–90 %) recovery was observed after 2.5 h (results not shown). At such extended incubation times, preferential synthesis of GRP78 was also observed (Figure 5). Cells were exposed for 3 h to PMA and cholera toxin alone or in the presence of thapsigargin or ionomycin and then pulse-labelled with methionine, and fractions of equal radioactivity were analysed by two-dimensional PAGE and autoradiography. A 78 kDa polypeptide, previously

identified as GRP78 [14], was preferentially labelled in the thapsigargin- and ionomycin-treated preparations. Such preferential labelling, which was obvious on inspection of the autoradiograms (Figure 5), was confirmed by quantification with a PhosphorImager. By contrast, the relative incorporation of methionine into actin was decreased in treated as compared with control preparations.

The relative amount of mRNA for GRP78 or actin did not change notably during extended incubation in Ham's F-10 medium ([15]; Table 2). After 3 h incubation with thapsigargin alone, a significant increase in the amount of GRP78 mRNA was detected. However, much greater (8–10-fold) inductions of GRP78 mRNA were observed when PMA and cholera toxin were included in the incubations with thapsigargin. These large inductions, which were apparent as early as 1.5 h of incubation, were not observed with PMA and cholera toxin alone. The amount of actin mRNA, by contrast, was observed to decline in a time-dependent fashion in thapsigargin-treated preparations throughout 5 h of incubation. The rate of decay of actin mRNA was not influenced by the presence of PMA and cholera toxin. The modest variation in relative amounts of total poly(A^+) mRNA recovered from the variously treated preparations could not account for the dramatic effects of thapsigargin on GRP78 mRNA and actin mRNA levels.

DISCUSSION

Translational initiation in mammalian cells is suppressed by extraction of cellular Ca^{2+} with EGTA [16] or by Ca^{2+}-mobilizing agents including hormones that generate inositol 1,4,5-trisphosphate (IP_3), Ca^{2+} ionophores, arachidonic acid, and peptide inhibitors of metalloendoprotease activity [25–27,39]. Inhibition by these agents is reversed either directly upon addition of Ca^{2+} at high extracellular concentrations or by washing procedures that include high extracellular Ca^{2+}. At concentrations that inhibit amino acid incorporation by 85–95%, Ca^{2+} chelators and ionophores mobilize > 90% of cell-associated Ca^{2+} without affecting ATP or GTP concentrations. By contrast, arachidonic acid and metalloendoprotease inhibitors were observed to mobilize only 40% of cell-associated Ca^{2+} at concentrations that impair translational initiation, consistent with a requirement for a specific intracellular Ca^{2+} pool such as the ER. The specific pool of Ca^{2+} mobilized by these agents was not identified, however. Studies with these agents are complicated by their capacity to decrease ATP concentrations and thereby to slow protein synthesis through additional mechanisms.

Thapsigargin, a potent and selective inhibitor of the Ca^{2+}-ATPase of isolated ER preparations, antagonizes both Ca^{2+}-pumping activity and Ca^{2+} uptake by the organelle [8–10]. In intact non-muscle cells thapsigargin provokes a rapid release of Ca^{2+} from IP_3-sensitive and IP_3-recruitable compartments [40–42]. Accordingly this tumour promoter represents a valuable tool to probe the importance of ER-sequestered Ca^{2+} in various cellular functions. The studies with thapsigargin reported here provide compelling evidence that Ca^{2+} sequestered by the ER is required for optimal translational initiation. Thapsigargin was found to suppress amino acid incorporation within minutes in GH_3 and HepG2 cells. Full inhibition, which occurred with 2–3 nM drug in GH_3 cells, involved a 40% loss of cell-associated Ca^{2+} with no change in $[Ca^{2+}]_i$, and was characterized by a loss of polysomal content. No decreases in ATP or GTP contents were observed under such conditions; in fact, a modest sparing of ATP was noted (Table 1).

Processing of α_1-antitrypsin to the mature form in HepG2 cells requires synthesis of the appropriate precursor form of the glycoprotein in the ER, followed by transport of the precursor from ER to Golgi [43]. A Ca^{2+} requirement for this conversion has been established [4], but the site of the Ca^{2+} requirement was not previously identified. In the present paper, thapsigargin was found as effective as a Ca^{2+} ionophore in slowing the processing of the high-mannose precursor form of α_1-antitrypsin and the subsequent export of the glycoprotein. It is apparent, therefore, that Ca^{2+} sequestered specifically by the ER is essential for efficient glycoprotein processing and secretion in these cells.

In most cell types examined, including GH_4C_1 pituitary cells transfected with a plasmid containing the cDNA for proinsulin [44], the discharge of sequestered Ca^{2+} by thapsigargin is associated with a transient increase in $[Ca^{2+}]_i$ [8]. This increase develops more slowly than that provoked by hormones, and is frequently followed by sustained Ca^{2+} influx from the extracellular medium consequent to increased permeability of the plasma membrane to the cation. These observations are consistent with a 'capacitative' coupling model [45,46], which specifies that plasmalemmal permeability to Ca^{2+} is controlled by the degree of filling of the sequestered Ca^{2+} pool. Results from the present study indicate that GH_3, like neuroblastoma [47], cells belong to a subset of cells that do not respond to thapsigargin with an elevation of $[Ca^{2+}]_i$. No change in this parameter was observed at thapsigargin concentrations that maximally suppressed protein synthesis. Thapsigargin was also found to attenuate the rise in $[Ca^{2+}]_i$ caused by ionomycin and, at high (> 90 nM) concentrations, to provoke a decrease in $[Ca^{2+}]_i$. It is therefore evident that the rapid translational suppression, the induction of GRP78, and the decline in actin mRNA after treatment of GH_3 cells with thapsigargin do not require that $[Ca^{2+}]_i$ be elevated even briefly. Furthermore, it is unlikely that the inhibitory effects of thapsigargin on early glycoprotein processing in HepG2 cells require increased $[Ca^{2+}]_i$, since EGTA, which lowers $[Ca^{2+}]_i$, provided inhibition qualitatively similar to that with thapsigargin [4]. It should be noted that non-cytosolic compartmentalization of certain fluorescent dyes such as fura-2 has proved responsible for discrepant reports regarding cytosolic Ca^{2+} signalling in thapsigargin-treated hepatocytes [48]. It is improbable, however, that the findings with thapsigargin reported here are attributable to compartmentalization of dye, since quin-2 was employed for these experiments and loading of the dye was conducted at 37 °C [49,50]. Why $[Ca^{2+}]_i$ in GH_4C_1 cells increases in response to thapsigargin, whereas that in GH_3 cells, a related pituitary tumour clone, does not, remains to be explained.

The increase in $[Ca^{2+}]_i$ that invariably follows exposure to Ca^{2+} ionophores has been repeatedly observed to attenuate unusually rapidly in GH_3 cells as compared with other cell types [25,51,52]. Furthermore, arachidonic acid and the metalloendoprotease antagonist Cbz-Gly-Phe-NH_2, which elevate $[Ca^{2+}]_i$ in other cell types [53–55], either produce no change in or lower $[Ca^{2+}]_i$ in GH_3 cells [25,56]. Nevertheless ionomycin, arachidonate and Cbz-Gly-Phe-NH_2 effectively mobilize Ca^{2+} associated with the GH_3 cell to the extracellular fluid. GH_3 cells are also observed to release their Ca^{2+} stores relatively rapidly in response to EGTA and to become largely depleted of Ca^{2+} within 15–30 min, whereas other cell types are relatively resistant to depletion by this procedure [1,2,17]. Collectively these findings support the proposal that the plasmalemmal Ca^{2+}-ATPase, which transports cytosolic free Ca^{2+} to the extracellular fluid, is present in atypically high concentrations or is unusually efficient in the GH_3 cell. This Ca^{2+} pump is thought to be present ordinarily in very small amounts and to possess a K_m for Ca^{2+} approximating to that of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (0.2 μM) [57]. Thapsigargin is reported to promote Ca^{2+} efflux from

various cell types with an ED_{50} of approx. 10–80 nM [8]. The potency with which thapsigargin was found in the present work to mobilize cell-associated Ca^{2+}, as well as the inability of thapsigargin to raise $[Ca^{2+}]_i$, in GH_3 cells are clearly consistent with unusually active plasmalemmal Ca^{2+} pumping in this cell type.

In contrast with the inhibition of protein synthesis observed with other Ca^{2+}-mobilizing agents, inhibition by thapsigargin could not be prevented or reversed even partially by increasing the extracellular Ca^{2+} concentration. In investigations of the mechanism by which this highly lipophilic agent inhibits the Ca^{2+}-ATPase of isolated sarcoplasmic reticulum, it was concluded that the thapsigargin–enzyme complex, which can be formed in the absence or presence of Ca^{2+}, albeit at different rates, represents a 'dead-end' structure incapable of binding Ca^{2+} [58]. If thapsigargin interacts similarly with the ER Ca^{2+}-ATPase, then irreversible depletion of ER Ca^{2+} stores would be expected.

Although not a member of the phorbol ester class, the behaviour of thapsigargin is characteristic of a tumour promoter. For example, the drug provokes an inflammatory response, followed by hyperplastic growth of mouse epidermis [11], and in certain cultured cells induces cytotoxic phenomena [11,59]. The rapid induction of the proto-oncogenes *c-fos* and *c-jun* in NIH 3T3 cells by thapsigargin has been concluded to indicate that Ca^{2+} signalling itself controls expression of growth- and transformation-related genes [60]. The persistent emptying of ER Ca^{2+} stores of smooth-muscle cells by continued treatment with thapsigargin was recently hypothesized to signal cytotoxic events [59]. An alternative to Ca^{2+} signalling, based on the findings of this and other reports, is the rapid suppression of translational activity that occurs consequently to depletion of ER-sequestered Ca^{2+}. It is believed, for example, that enhanced degradation of short-lived repressors of proto-oncogenes follows exposure to non-specific inhibitors of protein synthesis such as cycloheximide and puromycin [61–64]. Furthermore incubation of quiescent NIH 3T3 cells with cycloheximide or puromycin is reported to be necessary and sufficient to induce DNA synthesis in these cells [65]. Rapid inhibition of glycoprotein processing, followed by induction of the ER-resident protein GRP78 in thapsigargin-treated cells, represent additional early events that could conceivably signal important changes in patterns of cell proliferation. In this regard it is of interest that competitive inhibition of genes coding for a set of ER proteins including GRP78 retards cell growth and lowers viability after ionophore treatment [66].

GRP78 is rapidly induced in mammalian cells following depletion of sequestered Ca^{2+} and other treatments that impair early protein processing [31]. Although the stimuli triggering induction of the protein are well understood, the nature of the signal that must be relayed from the ER to the nucleus to activate transcription of the GRP78 gene remains elusive. Inhibition of translation is not required, since significant induction of the protein is observed with ionophores at low concentrations that do not change the rate of protein synthesis [35]. Translational initiation of GRP78 mRNA has been found to be exceptionally efficient, such that the message resides almost exclusively with polysomes regardless of treatments that deplete sequestered Ca^{2+} [15]. Such efficient initiation may relate to the unusual 5′ leader sequence of the mRNA, which can directly confer internal ribosome binding independent of the 5′ end of the message [67]. By contrast, other messages, such as actin mRNA, loaded poorly into polysomes of ionophore-treated GH_3 cells and tended to distribute with ribonucleoprotein particles, ribosomal subunits and 80 S monosomes [15]. It is conceivable that the decay of actin mRNA observed in the present work during extended treatment with thapsigargin reflects a lack of protection by polysomes against destabilizing influences or nucleases that degrade this mRNA. Further investigations with thapsigargin should prove helpful in identifying and characterizing the pathways through which modifications in the functional status of the ER signal changes in mRNA translation, glycoprotein processing and gene expression.

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