HepG2 cells were employed as model system to investigate potential relationships between early protein processing and Ca\(^{2+}\) storage by the endoplasmic reticulum. Ca\(^{2+}\) was required for glycoprotein processing and export by intact cells. The processing and export of \(\alpha_1\)-antitrypsin and the secretion of complement factor 3, which are glycosylated proteins, were inhibited by the Ca\(^{2+}\) ionophore ionomycin whereas the export of albumin, a non-glycoprotein, was little affected. Ionomycin blocked processing of \(\alpha_1\)-antitrypsin at the conversion from the high mannose to the complex glycosylated form without affecting ATP or GTP contents. Pre-existing inhibition of intracellular processing of \(\alpha_1\)-antitrypsin by ionomycin was fully reversible upon removal of the ionophore with fatty acid-free bovine serum albumin. This reversal required Ca\(^{2+}\). After reversal the arrested form of \(\alpha_1\)-antitrypsin was fully converted to the mature form and exported to the medium. Inhibitions of \(\alpha_1\)-antitrypsin processing and complement factor 3 secretion by the metalloendoprotease antagonist Cbz-Gly-Phe-NH\textsubscript{2} (where Cbz is benzylxycarbonyl) were strongest at low extracellular Ca\(^{2+}\) but were reduced or prevented by high extracellular Ca\(^{2+}\). Processing and secretion of \(\alpha_1\)-antitrypsin were reduced upon incubation in low Ca\(^{2+}\) medium. Exposure to dithiothreitol reduced albumin export while affecting \(\alpha_1\)-antitrypsin export minimally. Suppression of amino acid incorporation into total cellular proteins from the ER of proteins that are folded, assembled, or glycosylated events performed within the organelle. Exit of proteins from the ER to the Golgi were reported to suppress translational initiation as a consequence of their capacity to mobilize sequestered Ca\(^{2+}\) stores (5). Reticulocytes, which lack an ER, do not exhibit Ca\(^{2+}\)-dependent initiation (6). Cultured cells also respond to mild reducing agents, which do not mobilize Ca\(^{2+}\), with an acute inhibition of translational initiation comparable with that observed with Ca\(^{2+}\) mobilization (7). On chronic exposure to either condition, transcriptionally dependent translational accommodation developed that appeared to depend on the rapid inhibition of GRP78/BiP, an ER resident protein that reportedly associates with malformed proteins in the lumen of the organelle (8-11). On these bases alterations of translational activity were proposed to emanate from perturbation of ER function.

The ER serves an essential role in the processing of newly synthesized secretory, lysosomal, and membrane proteins (12-14). Proteolytic cleavage of signal sequences, glycosylation of asparagine residues, formation of disulfide linkages, and assembly of subunits represent examples of post-translational processing events performed within the organellae. Exit of proteins from the ER is currently believed to constitute a rate-limiting step in intracellular transport and maturation (15, 16). Additionally mechanisms exist that allow retention by the ER of proteins that are folded, assembled, or glycosylated incorrectly (8, 17-19). Although cytosolic free Ca\(^{2+}\), rather than sequestered Ca\(^{2+}\), is conventionally considered to support regulated metabolic processes, certain lines of experimentation favor the possibility that sequestered Ca\(^{2+}\) maintains post-translational processing. For example, the activity in vitro of a1,2-mannosidase, a vesicular enzyme involved in the processing of N-linked oligosaccharides, is reported to be stimulated by Ca\(^{2+}\) (20). Depletion of cellular Ca\(^{2+}\) stores has been proposed to accelerate degradation of certain hetero-dimorphisms. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby sole to indicate this fact.

The abbreviations used are: ER, endoplasmic reticulum; DTT, dithiothreitol; EGTA, \(\text{ethylenbis(oxayethylene)nitrilo} \)tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; Eno H, endoglucosidase H; BSA, bovine serum albumin; Cbz, benzylxycarbonyl; PMPSF, phenylmethylsulfonyl fluoride; C3, complement factor 3; [Ca\(^{2+}\)]\textsubscript{i}, cytosolic free Ca\(^{2+}\) concentration; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

The endoplasmic reticulum (ER\(^{2}\)) functions prominently in early protein processing, phospholipid biosynthesis, drug metabolism, and Ca\(^{2+}\) homeostasis related to hormonal control mechanisms. In addition to sequestering high concentrations of Ca\(^{2+}\) the ER is thought to possess a more oxidizing environment than the cytosol. A growing body of evidence supports the proposal that Ca\(^{2+}\) sequestered within the ER is essential in the maintenance of high rates of mRNA translation in intact mammalian cells (1). Depletion of cellular Ca\(^{2+}\) by exposure to EGTA results in severely depressed rates of translational initiation in C6 glial and GH3 pituitary tumor cells and in isolated hepatocytes. These inhibitions are reversed within several minutes by the addition of supraphysiologic Ca\(^{2+}\) concentrations to the extracellular medium. Ca\(^{2+}\) ionophores, A23187 and ionomycin, which increase [Ca\(^{2+}\)], while releasing intracellular sequestered Ca\(^{2+}\) (2, 3), rapidly and completely inhibit the Ca\(^{2+}\)-dependent component of initiation in GH3 and other cell types (4). Recently metalloendoprotease antagonists that slow transport of secretory proteins from the ER to the Golgi were reported to suppress translational initiation as a consequence of their capacity to mobilize sequestered Ca\(^{2+}\) stores (5). Reticulocytes, which lack an ER, do not exhibit Ca\(^{2+}\)-dependent initiation (6). Cultured cells also respond to mild reducing agents, which do not mobilize Ca\(^{2+}\), with an acute inhibition of translational initiation comparable with that observed with Ca\(^{2+}\) mobilization (7). On chronic exposure to either condition, transcriptionally dependent translational accommodation developed that appeared to depend on the rapid inhibition of GRP78/BiP, an ER resident protein that reportedly associates with malformed proteins in the lumen of the organelle (8-11). On these bases, alterations of translational activity were proposed to emanate from perturbation of ER function.

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gous proteins in the ER (21). Of particular relevance are the findings of Lodish and Kong (22) that Ca\(^{2+}\) ionophores block the processing and secretion of α1-antitrypsin, and to a lesser extent other proteins, by HepG2 cells. Secretory proteins were found to accumulate in vesicles with the density of the rough ER in ionophore-treated preparations. These workers speculated that secretion was delayed because ionophores deplete ER Ca\(^{2+}\) stores requisite for correct folding of specific proteins. However, direct evidence that Ca\(^{2+}\) mobilization mediated the observed secretory arrest and that resumption of normal glycoprotein processing and export occurred upon removal of the stress was not provided. In the present investigation Ca\(^{2+}\) per se is shown to be needed for optimal processing and export of the secretory glycoproteins, α1-antitrypsin and complement factor 3 (C3), by HepG2 cells. Processing of albumin was not Ca\(^{2+}\)-dependent but was inhibited by mild reducing conditions not affecting glycoprotein secretion. Disruption of protein processing by either mobilization of sequestered Ca\(^{2+}\) or by reducing conditions not mobilizing the cation was associated with strongly suppressed rates of amino acid incorporation. A putative coupling of the rate of translational initiation to functional ER protein processing is discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ionomycin was purchased from Calbiochem. L-[3,4,5-\(^{3}H\)]Leucine and L-[\(^{35}S\)]methionine were obtained from ICN. Endoglycosidase H (Endo H) was from Genzyme, Boston, MA, and IgGSorb was from the Enzyme Center, Malden, MA. Phenylmethylsulfonyl fluoride (PMSF), DTT, Cbz-Gly-Phe-NH\(_2\), and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma. Goat antisera to human α1-antitrypsin and to the C3 component of the complement system and rabbit antiserum to human albumin were purchased from U. S. Biochemical Corp.

**Cell Culture**—Human hepatoma HepG2 cells were grown in monolayers in Eagle's minimal essential medium containing 10% fetal bovine serum (23). Cells were harvested by incubation with buffered saline containing 1 mM EGTA, followed by centrifugation and resuspension in serum-free medium.

**Measurement of Amino Acid Incorporation**—Aliquots (500 μl) of previously treated or control preparations were incubated with [\(^{3}H\)]leucine (1.5 μCi/0.5 ml) for 15 min at 37 °C. Incorporation of [\(^{3}H\)]leucine into trichloroacetic acid-precipitable, alkali-stable material was measured as previously described (24). Incubations were conducted in triplicate, and results are presented as the mean ± range of values obtained. Each experiment was reproduced at least twice and representative findings presented.

**Pulse Labeling of Cellular Proteins**—HepG2 cells were suspended in serum-free medium identical to 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 15-30 min at 37 °C. [\(^{35}S\)]Methionine (60 μCi/ml) was then added and the incubation continued for 10 min. Unless indicated otherwise, cells were washed twice by centrifugation at 600 × g and resuspended in Ham's F-10 containing 33 μM unlabeled methionine and 50 μM added Ca\(^{2+}\). Treatment agents were then added, and the chase period was continued for defined periods of time. The incubation was terminated by boiling aliquots of cell suspension on ice.

**Immunoprecipitation and Electrophoresis**—Following pulse labeling, aliquots of cell suspensions were centrifuged at 12,000 rpm in a Beckman microcentrifuge, and pellets (cells) were separated from the supernatants (medium). Cells were washed once in ice-cold PBS and lysed in PBS, pH 7.2, containing 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, and 1 mM PMSF. Lysates were clarified by centrifugation at 12,000 rpm. Samples were incubated with formaldehyde-fixed *Staphylococcus* A membranes (IgGSorb) and centrifuged to remove material that bound nonspecifically to *Staphylococcus* protein A. Aliquots of supernatants were incubated with specific antibodies at a 1/100 to 1/200 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. Washed pellets were then resuspended in Laemmli's sample buffer (25). Immunoprecipitated proteins were removed from antigen-antibody-protein A complexes by boiling for 2-3 min. Electrophoresis was conducted with 10 or 7.5% acrylamide gels as described (25).

**Deglycosylation with Endo H**—Following immunoprecipitation, α1-antitrypsin was removed from immune complexes and denatured by boiling in sodium phosphate buffer containing 0.2% SDS. Aliquots (30 μl) were incubated without or with 50 μlI/μl Endo H for 16 h at 37 °C in sodium phosphate buffer with 0.2% SDS and 1 mM PMSF. Samples were boiled for 3-5 min and subjected to SDS-PAGE and autoradiography. Sensitivity to Endo H was evidenced by increased electrophoretic mobility (26).

**Nucleotide Measurements**—ATP and GTP contents were determined on 10% trichloroacetic acid extracts of variously treated preparations by high pressure liquid chromatography with an Ultrasil SAX column eluted with 1 mM ammonium formate adjusted to pH 3.7 with phosphoric acid. Separation of these nucleotides occurs under this condition without employment of a gradient.

**RESULTS**

**Inhibition of Protein Processing and Secretion by Ionomycin and EGTA**—As described previously (1) translational initiation is inhibited in mammalian cells by EGTA, which gradually withdraws Ca\(^{2+}\) from sequestered stores via continual depletion of cytosolic free Ca\(^{2+}\) and by ionomycin, which facilitates diffusion of the cation into the cytosol across concentration gradients bounded by biological membranes. Ionomycin is generally more effective and rapid than EGTA treatment in suppressing translational initiation (4). To examine the effects of ionomycin and EGTA on the secretion of newly synthesized polypeptides, HepG2 cells were pulse-labeled with [\(^{35}S\)]methionine and chased with unlabeled amino acid for 1-2 h in the absence or presence of test agent. This protocol permits evaluation of the effects of the treatments on post-translational events independently of their effects on protein synthesis. Under comparable conditions without pulse-chase, leucine incorporation into total cellular proteins was reduced approximately 95% in the presence of 3 μM ionomycin and 50% in the presence of 1 mM EGTA (Table I, A). The spectrum of polypeptides secreted in the absence or presence of ionomycin or EGTA, as visualized by SDS-PAGE and autoradiography, is shown in Fig. 1. Albumin and α1-antitrypsin, the major HepG2 export products appearing as species of 66 and 52 kDa, respectively, were each secreted to lesser degrees in the presence of either ionomycin or EGTA (lanes 1-3) although less prominent effects were observed with the chelator. Ionomycin-treated preparations were also seen to export polypeptides that were not detected in the medium of untreated controls or EGTA-treated preparations.

Albumin and α1-antitrypsin were quantitatively immunoprecipitated from lysates and the extracellular fluid derived from cells incubated for 1 or 2 h with or without 3 μM ionomycin. These immunoprecipitates were subjected to SDS-PAGE and radioanalysis. Albumin secretion measured in the medium was decreased modestly (10%) at either time of incubation with ionomycin (Fig. 2, top, hatched bars) whereas secretion of α1-antitrypsin was reduced 76% within 1 h (Fig. 1, lanes 4 and 5, band B; Fig. 2, top). A 2.3- and 1.5-fold increase, respectively, in cellular accumulation of α1-antitrypsin and albumin accompanied inhibition of export in cells treated with ionophore for 1 h (Fig. 2, top, filled bars). Cellular accumulation of α1-antitrypsin increased to 3.7-fold over control after 2 h of treatment. Extracellular fluids were similarly analyzed for their glycoprotein C3 content. Two immunoreactive polypeptide species of 115 and 75 kDa were observed which corresponded to the α and β subunits of C3, respectively (27). Ionomycin treatment reduced the extent of release into the medium of these immunoreactive species by 50-66% within 1 h and 70% within 2 h (Fig. 2, top).

The high mannos and complex forms of α1-antitrypsin,
with apparent molecular weights of 50 and 52 kDa, respectively (28, 29), were readily detected in non-treated preparations (Fig. 1, lane 7, bands C and B). The high mannose form was deglycosylated to a 44-kDa species upon treatment with Endo H whereas the complex form was resistant to digestion significantly retarded by EGTA treatment. The phor could have exerted additional inhibitory mechanisms in Ca2+-containing medium were routinely observed to retain accumulated abundantly in ionomycin-treated preparations. Disturbance of cellular energetics did not explain the consistency of pulse-labeled proteins (18,30) such that the exposure period removal of ionophore, which is normally considered to be an irreversible perturbant of Ca2+ homeostasis in intact Cells. It was of interest to ascertain whether the arrest in processing would be abolished by the removal of ionophore (4). Alternatively, ionophore could have exerted additional inhibitory mechanisms not involving Ca2+-perturbations. To increase the degree of Ca2+ extraction by chelator, reduced temperatures were utilized to retard ER processing events and ER to Golgi transport of pulse-labeled proteins (18,30) such that the exposure period to EGTA could be greatly extended. Cells were placed on ice immediately following pulse labeling and were incubated in the absence of Ca2+ for 1 h. Preparations were then transferred to 37 °C, and the chase period continued for 2 h. Under these conditions, the intracellular retention of the 50-kDa high mannose form of α1-antitrypsin was at least 2-fold higher for EGTA-treated preparations in comparison with controls incubated with Ca2+ (Fig. 2, bottom). Intracellular retention of the precursor was accompanied by a 35% decrease in the amount of mature form secreted into the medium. Export of newly synthesized albumin was not significantly retarded by EGTA treatment.

ATP contents of HepG2 cells (Table II) were found to be similar to those reported for normal perfused rat liver (31). Disturbance of cellular energetics did not explain the consistently stronger inhibition of protein processing by ionomycin. Concentrations of ATP and GTP in ionomycin-treated HepG2 cells did not differ from those in EGTA-treated or control preparations even during extended incubations (Table II).

Ca2+-dependent Reversal of the Ionomycin Inhibition of α1-Antitrypsin Processing—HepG2 cells treated with ionomycin in Ca2+-containing medium were routinely observed to retain α1-antitrypsin in the high mannose form for more than 3 h without evidence of degradation. It was of interest to ascertain whether the arrest in processing would be abolished by the removal of ionophore, which is normally considered to be an irreversible perturbant of Ca2+ homeostasis in intact cells. It has recently proven feasible to reverse the inhibitory effects of Ca2+ ionophores on both amino acid incorporation and Ca2+ sequestration by a procedure involving exposures to medium containing fatty acid-free BSA.2 Reversibility of ionophore inhibition of incorporation was absolutely dependent on the presence of Ca2+ in the medium. Protein synthesis in HepG2

cells remained suppressed for up to 2 h if ionomycin-treated
cells were exposed to medium containing BSA and EGTA but
returned to normal rates upon re-addition of Ca\(^{2+}\) at concen-
trations in excess of chelator (Table III). This procedure was
therefore adapted to remove ionomycin from pulse-chase cells
arrested in the processing of \(\alpha_1\)-antitrypsin and the subse-
dquent disposition of the intracellular forms of the protein
observed (Fig. 3). Ionomycin-treated cells that were exposed to
BSA and Ca\(^{2+}\) regained the ability to process and export
\(\alpha_1\)-antitrypsin (panel A). Increasing degrees of conversion
from the high mannose to complex form were observed as a
function of time in cells relieved of ionomycin arrest by BSA
(lanes 6, 10, and 14). Conversion was perceptible within 45
min (lane 6) progressing to extensive loss of both intracellular
forms of the protein by 105 min (lane 14) that was accompa-
nied by export of mature \(\alpha_1\)-antitrypsin into the medium
(not shown). Ionophore-treated cells not exposed to BSA
remained in processing arrest (lanes 5, 9, and 13). Cells that
were never exposed to ionomycin processed \(\alpha_1\)-antitrypsin
equally well whether treated with BSA (lanes 4, 8, and 12) or
not (lanes 3, 7, and 11).

Reversal of the ionomycin-induced arrest of \(\alpha_1\)-antitrypsin
processing required Ca\(^{2+}\) (Fig. 3, panel B). Cells that were not
treated with ionophore largely completed processing of the
protein within 45 min (top section) when resuspended in fresh
medium without other additives (lane 3) or in medium con-
taining various combinations of EGTA and BSA (lanes 4–6).
In contrast cells exposed to ionophore exhibited complete
retention of the high mannose form of \(\alpha_1\)-antitrypsin through-
out this period whether incubated without change of medium
(lane 7) or resuspended in fresh medium without other addi-
tion (lane 8) or with 1 mM EGTA (lanes 9 and 10). Substantial
conversion of the high mannose form of \(\alpha_1\)-antitrypsin to the

**Table II**

**ATP and GTP contents of HepG2 cells treated with inhibitors of protein export**

Cells in Ham's F-10 were adjusted to the indicated Ca\(^{2+}\) concentrations and treated for the indicated times with 3 \(\mu\)M ionomycin, 1 mM EGTA, 2 mM Bz-Gly-Phe-NH\(_2\), or 1 mM DTT. Nucleotide measurements were performed as described under "Experimental Procedures."

<table>
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<tr>
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<th>Added Ca(^{2+})</th>
<th>Duration</th>
<th>ATP</th>
<th>GTP</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(\mu)M</td>
<td>min</td>
<td>nmol/mg protein</td>
<td></td>
</tr>
<tr>
<td>Exp. 1</td>
<td></td>
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</tr>
<tr>
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<td>15</td>
<td>6.0</td>
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</tr>
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<td>DTT</td>
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<td>5.3</td>
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**Table III**

**Ca\(^{2+}\)-dependent reversibility of ionomycin inhibition of protein synthesis in HepG2 cells**

Cells in medium containing 50 \(\mu\)M Ca\(^{2+}\) were pretreated for 20 min at 37°C and then incubated with or without 3 \(\mu\)M ionomycin for 15 min. Aliquots were removed for measurements of [\(^3\)H]leucine incor-
poration. The remainder of each preparation was then adjusted with BSA (2 mg/ml) and/or 1 mM EGTA, or carried as unadjusted controls. Following 15 min of incubation, cells were collected by centrifugation. The cells were resuspended in corresponding fresh media without ionomycin and incubated for an additional 90 min for a total exposure period of 105 min to ionophore-reversing additives. Forty-five min following resuspension, portions of the EGTA-treated preparations were adjusted with 2 mM Ca\(^{2+}\) in excess of chelator. Samples of each treated preparation were then removed for measurements of [\(^3\)H] leucine incorporation.

**First treatment** | **Leucine incorporation**
<table>
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<tr>
<td>Ionomycin</td>
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</tbody>
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**First treatment** | **Second treatment(s)** | **Leucine incorporation**
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<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>No additions</td>
<td>1.72 ± 0.23</td>
</tr>
<tr>
<td>Control</td>
<td>EGTA</td>
<td>1.30 ± 0.02</td>
</tr>
<tr>
<td>Control</td>
<td>BSA</td>
<td>1.47 ± 0.13</td>
</tr>
<tr>
<td>Control</td>
<td>BSA, EGTA</td>
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</tr>
<tr>
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<td>Ionomycin</td>
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<tr>
<td>Ionomycin</td>
<td>EGTA, then Ca(^{2+})</td>
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<td>BSA, EGTA, then Ca(^{2+})</td>
<td>1.59 ± 0.08</td>
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</table>

**Fig. 2.** Time dependence of effects of ionomycin and EGTA on export of \(\alpha_1\)-antitrypsin, albumin, and C3. Upper panels, effects of ionomycin. Cells pulse-labeled with \(^{35}\)S methionine were suspended in chase medium with or without 3 \(\mu\)M ionomycin for the indicated times. Albumin and \(\alpha_1\)-antitrypsin (\(\alpha_1\-AT\)) were immunoprecipitated from aliquots of cell lysate (filled bars) and medium (hatched bars). C3 was immunoprecipitated from aliquots of medium; the \(\alpha\) subunit is indicated by cross-hatched bars and the \(\beta\) subunit by vertically hatched bars. Immunoprecipitates were subjected to 7.5% SDS-PAGE, and protein-associated radioactivity was quantitated using the AMBIS radioanalytic imaging system. Results are expressed as percent of values obtained for untreated control preparations at each incubation time. Error bars express the range of values obtained for duplicate incubation samples. Lower panels, effects of EGTA. Pulse-labeled cells were resuspended in chase medium containing 50 \(\mu\)M Ca\(^{2+}\) and held on ice for 60 min with or without adjustment to 1 mM EGTA, followed by incubation at 37°C for the indicated times. Albumin and \(\alpha_1\)-antitrypsin were immunoprecipitated from aliquots of cell lysate (filled bars) and medium (hatched bars) and analyzed for protein-associated radioactivity as described above. Results are presented as percent of values obtained for untreated control preparations at each incubation time.
Radioactivity associated with intracellular \( \alpha \)-antitrypsin was analyzed by in vitro incubation of HepG2 cells with serum albumin and Ca\(^{2+} \). Cells pulsed with \(^{35}S\)methionine were incubated for 15 min in chase medium containing 50 \( \mu \)M Ca\(^{2+} \) either without (lanes 1) or with 3 \( \mu \)M ionomycin (lanes 2) preliminary to later incubations. Radioactivity associated with intracellular \( \alpha \)-antitrypsin was analyzed as in Fig. 1. Panel A, time dependence of reversal by BSA. Samples (500 \( \mu \)l) of ionomycin-treated and untreated cells at 15-min chase were either adjusted with fatty acid-free BSA (2 mg/ml) or carried as unadjusted controls, incubated for 15 min, and cells were collected by centrifugation. The cells were resuspended in corresponding fresh medium without ionomycin and incubated for an additional 15, 45, or 75 min for total exposure periods of 30, 60, and 90 min to BSA and overall methionine chase periods of 45 (lanes 3-6), 75 (lanes 7-10), and 105 min (lanes 11-14). Lanes 5, 6, 9, 10, 13, and 14 represent cells treated with ionomycin, and lanes 3, 4, 7, 8, 11, and 12 represent corresponding untreated controls. Lanes 4, 6, 8, 10, 12, and 14 represent BSA-treated cells and lanes 3, 5, 7, 9, 11, and 13 represent corresponding untreated controls. Panel B, Ca\(^{2+} \) dependence of reversal of processing inhibition. Ionomycin-treated and untreated cells sampled at 15-min chase were adjusted with BSA (2 mg/ml) and/or 1 mM EGTA, or carried as unadjusted controls, incubated for 15 min, and cells collected by centrifugation. The cells were resuspended in corresponding fresh medium without ionomycin and incubated for an additional 30 or 90 min for total exposure periods of either 45 min (top section) or 185 min (bottom section) to ionophore-reversing additives. Ionomophore-treated cells are represented by lanes 7-13 and controls without ionophore by lanes 3-6. Treatments involving BSA and/or EGTA at 45 min (top) were lanes 3 and 8 (buffer controls), lanes 4, 9, and 10 (EGTA), lanes 5 and 11 (BSA), and lanes 6, 12, and 15 (BSA and EGTA). Lane 7 represents an ionomycin-treated sample that was never washed. The bottom section represents longer exposure periods (105 min) for the additives of lanes 1-9, 11, and 12. *, samples adjusted with 2 mM Ca\(^{2+} \) in excess of EGTA at 45 min are represented in lanes 10 and 13.

Inhibition of post-translational processing of \( \alpha \)-antitrypsin by Cbz-Gly-Phe-NH\(_2\) was affected similarly by extracellular Ca\(^{2+} \) (Fig. 4, top, filled bars). High degrees of intracellular retention of the high mannose form of the protein (not shown) were observed for cells treated with dipeptide at low as compared with high extracellular Ca\(^{2+} \) concentrations. For example, in cells treated with 2 mM dipeptide at 1 mM EGTA approximately 90% of the precursor form was found to be cell-associated after incubation for 1 h whereas only 30% of the precursor form was retained in comparable incubations conducted at 2 mM extracellular Ca\(^{2+} \). Increased degrees of intracellular retention of \( \alpha \)-antitrypsin were faithfully reflected by corresponding decrements in the export of the mature form of the protein (not shown). Although cells incubated without the dipeptide inhibitor processed the protein efficiently at all extracellular Ca\(^{2+} \) concentrations, perceptibly higher rates occurred with increasing concentrations of the cation (Fig. 4, top, open bars). For example, approximately 20% of the pulse-labeled \( \alpha \)-antitrypsin remained cell-associated after 1 h of incubation in 1 mM EGTA as compared with 10% retention for incubations conducted at 2 mM Ca\(^{2+} \).

The processing of the C3 component of complement, as evidenced by the secretion of the \( \beta \) subunit into the medium, was also retarded by Cbz-Gly-Phe-NH\(_2\) in a manner reversed by Ca\(^{2+} \) (Fig. 4, bottom) but to lesser degrees than \( \alpha \)-antitrypsin (not shown). Cells incubated for 60 min in Ca\(^{2+} \)-free medium and 1 or 2 mM dipeptide, respectively, exhibited 25 and 50% inhibitions of export. These inhibitions were largely
suppressed as extracellular Ca\(^{2+}\) was increased to the millimolar values. Similar findings were made for the \(\alpha_1\) subunit of C3 (not shown). Significant reduction of C3 secretion was not consistently observed at low as opposed to high extracellular Ca\(^{2+}\) concentrations for incubations conducted without the dipeptide inhibitor.

**Inhibition by Reducing Conditions**—Treatment with DTT, a reversible inhibitor of translational initiation in glial and pituitary tumor cells (7), also markedly suppressed leucine incorporation into HepG2 cell proteins (Table I, A) without affecting ATP or GTP contents (Table II). This agent presumably creates a reducing environment within the ER that interferes with the formation of disulfide bonds required for the correct folding in the early processing of many proteins, including albumin. Exposure of HepG2 cells to 1 mM DTT reduced the secretion of newly synthesized albumin by approximately 50% over 2 h whereas the secretion of \(\alpha_1\)-antitrypsin, which does not contain disulfide linkages, was reduced no more than 10% during this period (Fig. 5). Although the inhibition of albumin export in this experiment was not accompanied by significant intracellular accumulation of 66-kDa polypeptide typical of albumin, considerable amounts of several cell-associated, immunoreactive polypeptides of differing molecular mass were invariably seen to develop in DTT-treated cells (Fig. 1, lanes 12 and 13). These peptides appeared to be aggregate/degradative forms of albumin collectively more than doubling cell-associated retention of the protein. Following subcellular fractionation and separation of vesicular fractions by density gradient centrifugation, these variant forms were observed to sediment at the density of the rough ER (not shown). For the purposes of comparison with DTT within the same experiment, the effects of ionomycin on the processing of albumin and \(\alpha_1\)-antitrypsin are also provided (Fig. 5). As described earlier ionomycin exerts minimal effects on albumin, which is not glycosylated, and strongly disrupts the processing of \(\alpha_1\)-antitrypsin.

**DISCUSSION**

Ionomycin and A23187 are remarkably effective in facilitating the transport of various divalent cations, including Ca\(^{2+}\), down concentration gradients established across biological membranes such as the plasmalemma and endoplasmic reticulum (34-36). The perturbant effects of these agents on intracellular Ca\(^{2+}\) homeostasis are so prominent that a tendency has developed to regard their biological actions as deriving exclusively from and synonymous with their properties as Ca\(^{2+}\) ionophores. Such specificity would, of course, be rare for any complex pharmacologic probe. Ionophores are known to interfere with protein processing (22, 37, 38). They may be capable of disrupting the integrity of organelles that function in protein processing. For example, prolonged exposure of NIH 3T3 cells to A23187 precipitates loss of resident ER proteins (37). Exposure of various cultured cells to the sodium ionophore monensin causes Golgi to become destabilized (37, 38). The present report confirms and extends the earlier observations of Lodish and Kong (22) that export of \(\alpha_1\)-antitrypsin, and to a lesser extent that of other newly synthesized secretory proteins, by HepG2 cells is blocked by both ionomycin (3 \(\mu\)M) or DTT (1 mM). Albumin and \(\alpha_1\)-antitrypsin (\(\alpha_1\)-AT) were immunoprecipitated from aliquots of medium and cell lysate and analyzed for radioactivity as in Fig. 2. Values represent means of two measurements, and the error bars represent the ranges of values obtained.
Three lines of direct evidence are now provided that Ca\(^{2+}\) _per se_ is required for processing and secretion. First, secretion of α1-antitrypsin and to a lesser degree other newly synthesized polypeptides was reduced upon incubation in low Ca\(^{2+}\) medium. Reductions were largest for cells pretreated with EGTA-containing medium on ice (Fig. 2) but were also observed for non-pretreated preparations (Fig. 4). Second, the effects of the metalloendoproteinase antagonist Cbz-Gly-Phe-NH\(_2\) on glycoprotein processing and export were found to be Ca\(^{2+}\)-dependent. Inhibitions of α1-antitrypsin processing and of C3 secretion by the dipetide were largest in Ca\(^{2+}\)-depleted medium and were either markedly reduced or prevented at elevated extracellular Ca\(^{2+}\). Third, it was demonstrated that arrest of α1-antitrypsin processing and export in the presence of ionomycin was fully reversed by Ca\(^{2+}\) following extraction of the drug with fatty acid-free albumin. In addition to establishing a Ca\(^{2+}\) involvement in the mechanism of ionophore inhibition, accomplishment of the reversal demonstrates (a) that ionomycin-treated preparations retained the normal precursor form of α1-antitrypsin rather than a malprocessed intermediate destined for degradation and (b) that both vesicular and protein processing were not permanently disrupted by ionophore treatment.

Although ER to Golgi transport in permeabilized cell preparations is sensitive to modest changes in extraluminal Ca\(^{2+}\) concentration (39), sequestered rather than cytosolic free Ca\(^{2+}\) appears essential for early protein processing in intact HepG2 cells. Ionomycin, which rapidly mobilizes sequestered cation while increasing [Ca\(^{2+}\)](i), (2, 3), and thapsigargin, which inhibits Ca\(^{2+}\) uptake into the ER, each provoked rapid accumulation of α1-antitrypsin in the high mannose form. The extracellular Ca\(^{2+}\) chelator EGTA, which depletes cation stores less rapidly than cytosolic Ca\(^{2+}\), reduced secretion and processing of HepG2 glycoproteins only following extended incubations. Treatment with Cbz-Gly-Phe-NH\(_2\), which depletes sequestered Ca\(^{2+}\) stores while altering [Ca\(^{2+}\)](i), marginally (5, 40), resulted in extensive intracellular accumulation of α1-antitrypsin in the high mannose form at low, but not at high, extracellular Ca\(^{2+}\), further favoring a processing requirement for sequestered cation.

Ca\(^{2+}\) susceptible to perturbation by the agents used in this study could act through several potential mechanisms to sustain protein processing and export. The cation has been proposed by others to favor the process through which secretions are formed. Treatment with Cbz-Gly-Phe-NH\(_2\), which depletes sequestered Ca\(^{2+}\) stores with EGTA, A23187, or Cbz-Gly-Phe-NH\(_2\) or continued exposure to DTT, both accommodation of translational initiation, characterized by tolerance to the stress, and prominent induction ofGRP78 were observed (5, 7). GRP78, a resident protein of the ER lumen, is currently believed to function in protein folding and assembly and to bind to certain misfolded proteins that accumulate with the lumen of the organelle during metabolic stress (8–11). Recent studies (7, 46) have provided evidence strongly favoring a role for the newly induced reticuloplasm in the expression of translational accommodation observed in chronically stressed preparations. An emerging picture from these observations is the ER may effectively coordinate rates of mRNA translation with protein processing. While translation obviously provides the nascent polypeptide substrates acted upon by the processing apparatus of the ER and Golgi, there is no evidence of any other influence of translation on processing (47). In contrast it is quite clear that inhibitors of protein processing interdict translational initiation, such that the synthesis of almost all proteins, including those not subject to processing, is curtailed.

REFERENCES

A Ca\textsuperscript{2+} Requirement for Protein Processing