Mechanism of Inhibition of Polypeptide Chain Initiation in Calcium-depleted Ehrlich Ascites Tumor Cells

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Abstract. Protein synthesis in Ehrlich ascites tumor cells is inhibited when cellular calcium is depleted by the addition of EGTA to the growth medium. This inhibition is at the level of polypeptide chain initiation as evidenced by a disaggregation of polyribosomes accompanied by a significant elevation in 80-S monomers. To identify direct effects of calcium on the protein synthesis apparatus we have developed a calcium-dependent, cell-free protein-synthesizing system from the Ehrlich cells by using 1,2-bis(O-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (BAPTA), a recently developed chelator with a high (>10³) selectivity for calcium (pKa = 6.97) over magnesium (pKa = 1.77). BAPTA inhibits protein synthesis by 70% at 1 mM and 90% at 2 mM. This effect was reversed by calcium but not by other cations tested. The levels of 43-S complexes (i.e., 40-S subunits containing bound methionyl-tRNAf, eIF-2·GTP) were significantly lower in the calcium-deprived incubations, indicating either inhibition of the rate of formation or decreased stability of 43-S complexes. Analysis of 43-S complexes on CsCl gradients showed that in BAPTA-treated lysates, 40-S subunits containing eIF-3, completely disappeared and the residual methionyl-tRNA-containing complexes were bound to 40-S subunits lacking eIF-3. Our results demonstrate a direct involvement of Ca²⁺ in protein synthesis and we have localized the effect of calcium deprivation to decreased binding of eIF-2 and eIF-3 to 40-S subunits.

Protein synthesis is a tightly regulated metabolic function in eukaryotic cells and is modulated in response to suboptimal nutrient conditions (13, 17, 21, 22, 39), which pose a direct stress on the cell, and in response to hormonal signals, which reflect the needs of the total organism (5, 20). We have studied the mechanisms whereby protein synthesis is inhibited in Ehrlich ascites tumor cells as a part of the cellular adaptation to several suboptimal growth conditions, including deprivation of glucose or an essential amino acid, and elevated temperatures. In these cases, inhibition has been localized to a reaction in polypeptide chain initiation (24, 39), but the intracellular effectors linking inhibition of initiation with altered nutrient supply remain unknown. In the present report we describe investigations of the mechanisms by which deprivation of another nutrient, calcium, inhibits protein synthesis in the Ehrlich cell and the cell-free system derived from Ehrlich cells. Calcium deprivation may offer new insights into regulation because calcium is not only a nutrient but also an intracellular effector mediating some of the actions of hormones, such as the polypeptide growth factors (6, 11, 28, 30), which affect the rate of cellular protein synthesis and cell growth (5, 20). Facets of the calcium-dependent hormonal responses and calcium deprivation response may occur by the same pathway, so that elucidation of the mechanism of response to calcium deprivation may throw light on the calcium-dependent hormonal mechanisms.

Eukaryotic cells have specific calcium requirements for maintaining an optimal growth rate in cell culture. The close coupling which is usually seen between rate of growth and rate of protein synthesis (5, 20) implies that suboptimal calcium concentrations will most likely inhibit protein synthesis. Brostrom et al. (3) have earlier demonstrated sensitivity of protein synthesis initiation to calcium deprivation in a wide variety of cultured cells. In the present communication, we report a similar inhibition of protein-synthesis initiation in the intact Ehrlich cell and present detailed studies of the mechanism. To distinguish direct effects of reduced calcium on protein synthesis from effects secondary to calcium-induced changes in the cytoskeleton, cellular metabolism, etc., we performed the studies of the mechanism in the cell-free protein-synthesizing system. We reduced free calcium concentration to the low nanomolar range in the cell-free system with 1,2-bis(O-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (BAPTA), a recently developed calcium chelator with a high selectivity for calcium over magnesium.

We find that the steady-state concentration of 43-S initiation complexes in the cell-free system is reduced dramatically by calcium depletion. We have shown previously that the reduction in 43-S complexes which occurs in cells under a variety of other stresses, including deprivation of an essen-

1. Abbreviation used in this paper: BAPTA, 1,2-bis(O-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid; GEF, guanine nucleotide exchange factor.
tial amino acid, exposure to heat shock, and deprivation of serum growth factors, is caused by an increase in the phosphorylation of the α-subunit of eIF-2, which leads to inhibition of guanine nucleotide exchange factor (GEF) function (32). We have been surprised, therefore, to find that the decrease in 43-S complexes in calcium-deprived cells is not caused by eIF-2 phosphorylation. Rather, eIF-3 normally is associated with and stabilizes 40-S methionyl-tRNAf•GTP complexes and calcium appears to be required for decrease in 43-S complexes in calcium-deprived cells. Calcium depletion has thus revealed a new mechanism for modulating the level of 43-S complexes.

Materials and Methods

Materials

The radioactive amino acids [35S]methionine (1,000-1,200 Ci/mmol) and [14C]leucine (300 mCi/mmol) and 45Ca2+ were obtained from New England Nuclear (Boston, MA). BAPTA was obtained from Calbiochem-Behring Corp. (San Diego, CA); Chelex-100 from Bio-Rad Laboratories (Richmond, CA); and parvalbumin from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Ehrlich ascites tumor cells (Ehrlich cells) were grown in suspension at 37°C in Eagle's minimal essential medium (F-14; Gibco Laboratories, Grand Island, NY), supplemented with 3 % NuSerum (Collaborative Research, Inc., Waltham, MA). The Ca2+ concentration in 3 % NuSerum-supplemented medium was 50-90 μM and rose during storage. Exponential growth was maintained by careful maintenance of the cell density between 1 × 105 and 1 × 106 cells/ml. On the day of use, cells were centrifuged at 1,000 g for 10 min, the cell pellet was resuspended in fresh medium plus 3 % NuSerum and nonessential amino acids, and the cultures were incubated at least 20 min at 37°C before use.

Ca2+ Measurements

The Ca2+ concentration in NuSerum was measured on a clinical atomic absorption spectrophotometer. Measurements of medium and medium supplemented with 3 % NuSerum, in which the Ca2+ concentrations are low, were performed using the fluorescent indicator quin 2. An excitation wavelength of 339 nm and emission wavelength of 500 nm were used. Ca2+ concentrations were calculated from the equation Ca2+ = Kd (F - Fmin)/Fmax - F. The Kd (dissociation constant for Ca2+ and quin 2) used was 117 mM (18). (F = fluorescence in presence of unknown; Fmin = fluorescence in presence of EGTA; no Ca2+; Fmax = fluorescence at saturating Ca2+.)

Measurement of Protein Synthesis Rates, Pulse Labeling

Small aliquots (100-200 μl) of cell suspension (5 × 106 cells/ml) were removed from larger cultures and placed in 1.5-mI microfuge tubes which contained [14C]leucine (Amersham Corp., Arlington Heights, IL; 342 mCi/mmol). The final [14C]leucine varied between 20 and 50 μCi/ml depending upon the length of incubation. All incubations, including the larger cultures, were at 37°C. Labeling periods were from 2 to 10 min (as stated in figure legends) during which time cells were held in suspension and CO2 levels maintained. After indicated time periods, the cell suspension was made 0.5 N NaOH. Protein was precipitated with the addition of 1 ml of 10% CoCl2. The precipitate was collected on Whatman Inc. (Clifton, NJ) GF A filters, and washed with 10 ml of 5% CoCl2.0H and 15 ml of 95% ethanol. The filters were dried under a heat lamp, and radioactivity determined by scintillation spectrophotometry.

45Ca2+ Uptake and Depletion Measurements

At time zero, 1.5 μCi/ml (31.5 mCi/mg Ca2+) 45Ca2+ was added to Ehrlich cell cultures at a cell density of 5 × 107 cells/ml. The pH of the cultured cells was kept constant with continuously flowing 5% CO2/95% air. 1-ml aliquots were removed at intervals and layered onto 0.5 ml of a Mazola corn oil/N-butyl phthalate (1:4) mixture in 1.5-ml microfuge tubes. After centrifugation in a microfuge for 30 s, the medium and the oil mixture were aspirated. Drops of oil above the cell pellet were carefully removed with paper towels and the tube inverted. The pellet-containing tip of the microfuge tube was cut and placed into a glass 20-ml scintillation vial. 2 ml of protocol (New England Nuclear) were added and the vials incubated at 55°C for 1.5 h. 10 ml of toluene-based scintillation fluid were added and the cell-associated 45Ca2+ determined in a liquid scintillation system. Controls for extracellular water trapped by the cell pellet were performed using [3H]inulin. At 5 × 105 cells/ml, 0.3 μl of extracellular medium could be accounted for in the cell pellet. Controls for cell recovery were performed using [3H]thymidine-labeled cells. Routinely, cell recovery was >96%.

Preparation of Ehrlich Cell Lysates

The procedure of Henschaw and Panniers (14) was used to prepare an initiation-dependent translation system from Ehrlich ascites tumor cells. Briefly, after Dounce homogenization (Kontes Glass Co., Vineland, NJ), the preparation was centrifuged at 15,000 g for 10 min to remove nuclei and mitochondria. This S-10 lysate was then preincubated at 37°C for 30 min under conditions unfavorable to initiation to allow the runoff of preexisting ribosomes and make synthesis dependent upon polypeptide chain initiation. The preincubated S-10 lysate was then gel filtered by Sephadex G-25 chromatography to remove small inhibitory molecules and slow completion of initiation conditions. The preincubated, gel-filtered S-10 lysate was stored in aliquots in liquid nitrogen. Fresh aliquots were thawed immediately before use for each experiment.

Conditions for In Vitro Protein Synthesis

A previous procedure was followed with minor modifications (14). The translation system contained 20 mM 3-(N-Morpholino)propane sulfonic acid (pH 7.5), 60 mM KCl, 1.75 mM magnesium acetate, 0.25 mM DTT, 0.1 mM EDTA, 1.2 mM ATP, 0.2 mM GTP, 3 mM phospho-enol pyruvate, 40 μg/ml pyruvate kinase, 19 amino acids at 40 μM and 5 μCi/ml [14C]leucine (14 μM). Lysate was added at a final concentration of 20% (vol/vol) and the reaction was incubated in a shaking water bath at 37°C. Incorporation of radioactivity into protein was measured by spotting 10-μl samples of incubation mixture onto Whatman Inc. No. 3 filter discs which were processed as described.

Conditions for Measuring Protein Synthesis in the Reticulocyte Lysate

The translation system contained 20 mM 3-(N-Morpholino)propane sulfonic acid (pH 7.5), 80 mM KCl, 0.75 mM magnesium acetate, 0.5 mM ATP, 0.1 mM GTP, 3 mM phospho-enol pyruvate, 100 μM of 20 amino acids, 0.2 M spermidine, 10 μg/ml pyruvate kinase, and 5 μCi/ml [14C]leucine. Lysate was added at a final concentration of 40% (vol/vol) and the reaction was incubated in a shaking water bath at 37°C. Incorporation of radioactivity into protein was measured by spotting 10-μl samples of incubation mixture onto Whatman Inc. No. 3 filter discs which were processed as described.

Measurement of [35S]methionyl–tRNAf in 43-S Initiation Complexes

The cell-free protein-synthesizing system used was identical to the one mentioned above except that 20 amino acids were used at 10 μM and 250-300 μCi/ml of [35S]methionine was added. The reaction was incubated at 37°C and at specified time points, 10 μl of the reaction was spotted onto Whatman Inc. No. 3 filter discs to monitor protein synthesis. For analysis of 43-S complexes, the reaction was stopped by the addition of an equal volume of formaldehyde (final concentration, 4%). The reaction was continued on ice until it was layered on a 20-40% linear sucrose gradient (25 mM sodium cacodylate, pH 6.9, 100 mM KCl, 2.5 mM magnesium acetate, 1 mM DTT, 0.1 mM EDTA). The gradients were centrifuged at 30,000 rpm for 16 h at 4°C in a Beckman Instruments Inc. (model SW41; Fullerton, CA) rotor. At least 20 fractions per gradient were collected. 1 ml of 0.5 M sodium acetate, pH 5.0, with 0.2 mg/ml yeast RNA as carrier was added to each fraction. The RNA was precipitated by the addition of 1 ml of ethanol.
Dependence of protein synthesis in Ehrlich cells on \( \text{Ca}^{2+} \). (A) \( \text{Ca}^{2+} \) uptake in Ehrlich cells. Cells were resuspended in fresh medium for 1 h. Then \( \text{Ca}^{2+} \) (1.5 \( \mu \text{Ci/ml} \); 60,000 cpm/nmol \( \text{Ca}^{2+} \)) was added to the medium and duplicate 1-ml samples were removed at the indicated times for the estimation of cell-associated \( \text{Ca}^{2+} \). Results are expressed as percent of control calcium levels (0.5 nmol/5 \( \times 10^5 \) cells) (n).

(B) Rates of protein synthesis are expressed as percent of control rates without EGTA (control). As described in B (except that rates of protein synthesis were estimated using 2-min pulses). After 2.5 h \( \text{Ca}^{2+} \) was added to the EGTA-treated culture. Rates of protein synthesis (m) and cellular \( \text{Ca}^{2+} \) levels (C) are expressed as percent of control.

Figure 1. Dependence of protein synthesis in Ehrlich cells on \( \text{Ca}^{2+} \). (A) \( \text{Ca}^{2+} \) uptake in Ehrlich cells. Cells were resuspended in fresh medium for 1 h. Then \( \text{Ca}^{2+} \) (1.5 \( \mu \text{Ci/ml} \); 60,000 cpm/nmol \( \text{Ca}^{2+} \)) was added to the medium and duplicate 1-ml samples were removed at the indicated times for the estimation of cell-associated radioactivity as described. Results are expressed as amount of external calcium taken up, which was calculated from the specific activity of \( \text{Ca}^{2+} \) as described in Materials and Methods. (B) Protein synthesis and calcium levels in Ehrlich cells treated with EGTA. Ehrlich cells were preloaded with \( \text{Ca}^{2+} \) (1.5 \( \mu \text{Ci/ml} \)) for 1 h at 37°C. EGTA (150 \( \mu \text{M} \)) was then added to one culture and 1-ml samples were removed from both cultures and cell-associated \( \text{Ca}^{2+} \) determined at the indicated times. Results are expressed as percent of control calcium levels (0.5 nmol/5 \( \times 10^5 \) cells) (C). Simultaneously rates of protein synthesis were estimated at the indicated times by pulse-labeling samples of cell suspension with [\(^{14}\text{C}\)]leucine for 5 min as described in Materials and Methods. Rates of protein synthesis are expressed as percent of control rates (5,400 cpm/5 \( \times 10^5 \) cells/5 min) at time zero (m). (C) Reversal of the inhibition of protein synthesis by addition of \( \text{Ca}^{2+} \). Protein synthesis and levels of \( \text{Ca}^{2+} \) were followed in cells incubated with or without EGTA (control) as described in B (except that rates of protein synthesis were estimated using 2-min pulses). After 2.5 h \( \text{Ca}^{2+} \) was added to the EGTA-treated culture. Rates of protein synthesis (m) and cellular \( \text{Ca}^{2+} \) levels (C) are expressed as percent of control.
characteristic of preferential inhibition of initiation, rather than elongation. There is a rapid recovery (in 10 min) of the control polysome pattern after addition of Ca\textsuperscript{2+} to Ca\textsuperscript{2+}-depleted cells, implying a rapidly reversible modification of polypeptide chain initiation (data not shown). Premature termination also results in an increase in 80-S monomers; however, this was ruled out by SDS-PAGE; Ca\textsuperscript{2+}-depleted cells synthesized the same molecular weight range of proteins as the control cells (data not shown).

Recently, we have shown that elF-2\textalpha phosphorylation increases in cells in which protein synthesis is inhibited by several stresses (32). We therefore examined the phosphorylation state of elF-2\textalpha in calcium-depleted cells. There was a small reproducible increase, EGTA-treated cells having 28\% of their elF-2 phosphorylated while control cells had 22\% (Table I). However, refeeding EGTA-treated cells with Ca\textsuperscript{2+} increased rates of protein synthesis back to control levels within 10 min, but had no effect on phosphorylation of elF-2\textalpha, which remained at 30\%.

### Inhibition of Protein Synthesis in the Cell-free Systems Depleted of Calcium

The Ehrlich cell-free system incorporated \[^{14}C\]leucine linearly for \( \approx 45 \) min (Fig. 2A). The system had a magnesium optimum of 1.25–1.5 mM (Fig. 2B) and a KCl optimum of 40–60 mM (Fig. 2C). When added to the cell-free system, 1 mM BAPTA inhibited protein synthesis 70\% and 2 mM inhibited 90\%, this inhibition being evident at the first time point measured (2 min; Fig. 2A). BAPTA increased the optimum for added magnesium by 0.15 and 0.3 mM for 1 mM and 2 mM BAPTA, respectively, consistent with its Mg\textsuperscript{2+}-chelating activity. BAPTA was, however, inhibitory at all magnesium concentrations (Fig. 2B). In all subsequent figures, a Mg\textsuperscript{2+} concentration of 1.75 mM was used. Inhibition by BAPTA demonstrated a considerable dependence upon the KCl concentration. Although BAPTA inhibited protein synthesis at all KCl concentrations tested (Fig. 2C), it was much more inhibitory at high KCl concentrations than at low KCl, so that the KCl optimum was shifted to 20 mM or below. KCl concentration of 60 mM was used in all subsequent experiments. There is no measurable change in free Ca\textsuperscript{2+} concentration with changes in KCl concentration over the ranges shown in Fig. 2C (Panniers, R., unpublished observations). Therefore, the affinity of BAPTA for Ca\textsuperscript{2+} does not detectably change over the range of KCl used for cell-free protein synthesis, indicating that the dependence of the lysate on Ca\textsuperscript{2+} varies with KCl concentrations. Although calcium chelation by BAPTA is considered to be relatively insensitive to pH, the inhibitory effect of BAPTA on protein synthesis is also increased with increasing pH; pH 7.5 was used in all experiments shown here.

If the inhibition by BAPTA results from the chelation of Ca\textsuperscript{2+}, the inhibition should be prevented upon addition of calcium. The data shown in Fig. 3A demonstrate that this indeed was the case. 1 mM calcium prevented the inhibition by 1 mM BAPTA, bringing it back to 80–92\% of control levels, and 250 \( \mu \)M increased synthesis to \( \approx 70\% \) of control levels. Added calcium inhibited the control incubation, which explains why Ca\textsuperscript{2+} concentrations greater than BAPTA concentrations were inhibitory (Fig. 3A). Calcium also reversed the inhibition by BAPTA when added during the incubation (Fig. 3B). Protein synthesis was restored to control rates when calcium was added to the inhibited system at 3 or 10 min, after a lag of \( \approx 2 \) min.

Other divalent and trivalent cations, Mn\textsuperscript{2+}, Cd\textsuperscript{2+}, Ba\textsuperscript{2+}, Fe\textsuperscript{3+}, and La\textsuperscript{3+}, did not reverse the inhibition by BAPTA. 1 mM Zn\textsuperscript{2+} did prevent the effect of 1 mM BAPTA. This

### Table I. Effect of EGTA on elF-2\textalpha Phosphorylation in Ehrlich Cells

|            | Protein synthesis (cpm) | elF-(P) % |
|------------|-------------------------|-----------|
| - EGTA     | 4,010                   | 22        |
| + EGTA     | 2,440                   | 28        |
| + EGTA + Ca\textsuperscript{2+} | 3,715            | 30        |

Cells were incubated with or without 1 mM EGTA as described for Fig. 2. 1 mM Ca\textsuperscript{2+} was added to one EGTA culture after 4 h, and 10 min later rates of protein synthesis were measured in all cultures, by pulse labeling with \[^{14}C\]leucine for 10 min as described. Samples for estimation of percent of total cell elF-2\textalpha that was phosphorylated were taken and analyzed as described (32).
Exogenous calcium prevents and reverses the inhibition of protein synthesis by BAPTA. Ehrlich cell lysates were incubated as described in Materials and Methods. (A) Prevention. Indicated concentrations of calcium were added to the reactions and protein synthesis estimated. Results are expressed as percentage of control without BAPTA or added calcium. No BAPTA (●); 1 mM BAPTA (○); or no BAPTA (■). Calcium (1 mM) was added to the BAPTA-inhibited system at 3 min (□); or 10 min (▲).

is expected because Zn$^{2+}$ is known to bind to BAPTA two orders of magnitude more tightly than Ca$^{2+}$ and will release bound Ca$^{2+}$ from BAPTA. However, at lower concentrations Ca$^{2+}$ reversed much more effectively than Zn$^{2+}$. EDTA also binds Zn$^{2+}$ six orders of magnitude greater than Mg$^{2+}$ (33); however this chelating agent does not inhibit protein synthesis until it is present in concentrations sufficient to reduce Mg$^{2+}$ significantly. Therefore, depletion of Zn$^{2+}$ does not explain inhibition of cell-free protein synthesis by BAPTA.

To examine whether this effect of calcium depletion was a more general phenomenon in mammalian systems, we studied the effect of BAPTA on the reticulocyte lysate. 1 mM BAPTA inhibited protein synthesis in the reticulocyte lysate by 50% and 2 mM BAPTA by 80% (Fig. 4 A). There was a slight shift in the Mg$^{2+}$ optimum as seen in the Ehrlich cell lysate, but BAPTA was inhibitory at all magnesium concentrations tested. As in Ehrlich cells, BAPTA was more inhibitory at high KCl concentrations (Fig. 4 B).

Reduction of the Level of 43-S Initiation Complexes by BAPTA

Modulation of eIF-2 function is a major mechanism of regulation of initiation in Ehrlich cells; modulation is reflected in changes in the level of 43-S initiation complexes. Therefore, we assessed the levels of 43-S initiation complexes, by measuring the amount of [$^{35}$S]methionyl-tRNA associated with the native 40-S subunits in the presence or absence of BAPTA. [$^{35}$S]Methionine was added to the cell-free system and the label was allowed to equilibrate with the methionine pool during 3 min of protein synthesis. BAPTA was then added and the reaction allowed to proceed for another 5 min and then terminated by fixing with formaldehyde (final concentration, 4%). Sucrose gradient analysis (see Materials and Methods) indicates a 70% reduction in levels of 43-S complexes in BAPTA-treated lysate compared to controls (Fig. 5, A and B). [$^{35}$S]Methionyl-tRNA, eIF-2·GTP ternary complexes are associated preferentially with the subfraction of native 40-S subunits which also contain bound eIF-3 (2, 25). Because of the large size of eIF-3 (>600 kD) these subunits sediment on the leading edge of the 40-S peak and are often visualized as a shoulder on the absorbance curve, as in Fig. 5 A. Thus, the majority of [$^{35}$S]methionyl-tRNA normally also sediments in this region, slightly ahead of this 40-S absorbance peak (Fig. 5 A). In the presence of BAPTA this shoulder disappears, concomitant with the loss of the majority of [$^{35}$S]methionyl-tRNA from this region (Fig. 5 B). This indicates that eIF-3 has also been lost from the 40-S subunits, and has been confirmed by CsCl gradient analysis (see below).

Our recent analysis of the GEF-dependent conversion of eIF-2-GDP to eIF-2-GTP indicated that under some conditions this reaction can proceed on ice (29). Therefore, we compared 43-S complexes in samples fixed simultaneously, as usual, with samples cooled rapidly in a dry ice-ethanol bath and then held on ice for various periods before fixing. The results were dramatic. After 3 min on ice, 43-S complexes had increased 135% in control incubations and 400% in BAPTA-treated incubations, and had reached the same level, BAPTA having started from a lower baseline (Fig. 5, C and D). At 10 min both control and BAPTA-treated incubations were still at the same level (Table I) indicating that they had reached a maximum level within 3 min. We also compared 43-S complexes in control and BAPTA-treated incubations in unfixed preparations, and found that they were identical (Table II). Since >3 min is obviously required for the sucrose gradients to be layered and for the particles to separate from the soluble fraction on the gradients, equilibration on ice is inevitable in unfixed preparations under our conditions. The fact that much lower levels are in fact seen in unfixed preparations than in preparations held on ice and then fixed means that in both samples there was also considerable, and equal, breakdown on the gradients.

We also estimated the levels of 43-S complexes at various points during protein synthesis. In the control incubation...
A Western blot assay that used a monoclonal antibody developed in our laboratory (Fig. 6 B). There was no significant difference in the levels of phosphorylation between control and BAPTA-treated incubation, despite a marked inhibition of protein synthesis in the latter. Thus, the effect of BAPTA on 43-S complexes is not due to increased phosphorylation of eIF-2α.

The increasing levels of eIF-2α phosphorylation in both control and BAPTA-treated lysates (Fig. 6 B) results from an alteration of the equilibrium between kinase and phosphatase during preparation of the lysates. Whereas in the reticulocyte lysate system inhibition of protein synthesis by phosphorylation of eIF-2α becomes manifest after a lag of only 5 or 10
min, synthesis in Ehrlich cell lysates is unaffected by the increased phosphorylation levels until 30-45 min. This is presumably because of the slow rate of initiation in the Ehrlich cell system, and the limited rounds of initiation which eIF-2 has to support in a given time period. The immediate onset of inhibition in both the Ehrlich cell (Fig. 2 A) and reticulocyte (Fig. 3 A) systems is therefore another strong indication that BAPTA is not inhibiting through enhancement of phosphorylation of eIF-2.

**CsCl Equilibrium Density-Gradient Analysis**

To confirm the loss of eIF-3 from the particles in the 40-S region, we isolated the 40-S subunits and analyzed them on CsCl equilibrium density gradients. Fig. 7, A and B, shows CsCl density gradient profiles of native 40-S subunits cut from sucrose gradients of control and BAPTA-inhibited lysates. As explained in detail in the Discussion section, two predominant subunit populations are seen, with buoyant densities of 1.49 and 1.40 g/cm$^3$. Ribosome subunits which do not contain eIF-3 sediment at 40-S in sucrose gradients and have a density of 1.49 g/cm$^3$ on CsCl gradients. The addition of eIF-3 (600 kD) to the subunits increases the protein/RNA ratio and decreases the buoyant density to 1.40 g/cm$^3$ (1, 15). In the control incubations, the major peak of $[^{35}S]$methionine radioactivity coincided with the subunit of density 1.40 g/cm$^3$ (Fig. 7 A) as demonstrated previously (1). A small fraction of the radioactivity appears to be associated with particles of intermediate density. The nature of these particles is unknown. With BAPTA treatment, the subunits of density 1.40 g/cm$^3$ disappeared, with a proportionate increase in subunits of density 1.49 g/cm$^3$ (optical density) (Fig. 7 B). Therefore, the level of subunits containing eIF-3 is very low in the BAPTA-treated incubations. Comparison of Fig. 7 B with 7 A shows a virtual disappearance of $[^{35}S]$methionine radioactivity from the region corresponding to buoyant density 1.40 g/cm$^3$, consistent with the disappearance of these particles. The remaining $[^{35}S]$methionyl-tRNA radioactivity is in the 1.49 g/cm$^3$ region.

The reduced level of label over the whole region 1.49-1.40 g/cm$^3$ confirms that BAPTA reduces levels of $[^{35}S]$methionyl-tRNA binding to 40-S subunits. The most marked effect of Ca$^{2+}$ depletion however is the reduction of 40-S subunits of buoyant density 1.40 g/cm$^3$ (optical density), indicating that lowering Ca$^{2+}$ strongly inhibits the binding of eIF-3 to 40-S subunits. The peak of $[^{35}S]$methionyl-tRNA counts at 1.3 g/cm$^3$ is coincident with the position of ternary complexes, $[^{35}S]$methionyl-tRNA-eIF-2-GTP, (Rowlands, A., unpublished observations). As a very broad cut is made on the sucrose gradients to obtain all 40-S subunits it is not surprising that some ternary complexes are retained from the soluble fraction.

**Discussion**

Brostrom et al. (3) have demonstrated an inhibition of protein synthesis in a wide variety of intact cells deprived of calcium by external EGTA. We have confirmed a similar inhibition in intact Ehrlich cells growing in suspension culture (Fig. 1 B) and we find that polypeptide chain initiation is inhibited preferentially, relative to chain elongation, as indicated by a large increase in proportion of monomeric ribosomes (data not shown). Recent reports (4, 9) indicate that monomeric ribosomes are elevated in calcium-deprived GH3 cells while ribosome transit times and ATP/ADP ratios are equal to control cells. Thus, inhibition of initiation by calcium depletion appears to be a general phenomenon.

We analyzed the site of inhibition in greater detail in the cell-free system. Calcium concentration was reduced with BAPTA. BAPTA is a modification of EGTA in which methylene links between oxygen and nitrogen are replaced by benzene rings (37). BAPTA has a high (>10$^5$) selectivity for Ca$^{2+}$ over Mg$^{2+}$ (pKa = 6.97) over Mg$^{2+}$ (pKa = 1.77). In comparison, the selectivity of EGTA is only 100 times greater for calcium than for magnesium at pH's optimal for protein synthesis. Use of BAPTA has allowed reduction of free Ca$^{2+}$ concentrations to the low nanomolar range while Mg$^{2+}$ is maintained at levels suitable for protein synthesis.

BAPTA inhibited protein synthesis in the cell-free system in a concentration-dependent manner. Inhibition is ~70% at 1 mM BAPTA and 90% at 2 mM, and is apparent at the earliest time point measured (Fig. 2 A). The effect of BAPTA is prevented by the addition of calcium at time zero and is reversed, after a short lag, by the addition of calcium during...
the incubation (Fig. 3 B). Of a series of metal ions tested, only calcium prevented inhibition in a manner consistent with a limiting species (Fig. 3 A). Brostrom et al. (3) found that the effect of EGTA on intact cells was reversed by calcium but not by any of eleven other divalent or trivalent metal ions.

A major intermediate in initiation is the 43-S initiation complex. To localize the site of inhibition by calcium depletion, we measured the steady-state concentration of 43-S initiation complexes in control and BAPTA-treated incubations. We found a 70% decrease in the concentration of 43-S complexes within 2½ min, suggesting that initiation is inhibited by a reduction in the level of these complexes (Figs. 5 B and 6 A). The concentration of 43-S complexes is reduced in a wide variety of physiological conditions in which protein synthesis is reduced. This reduction has been shown to be caused by an increase in phosphorylation of the α-subunit of eIF-2 in reticulocyte-deprived of heme (21), and in Ehrlich cells deprived of an essential amino acid, exposed to elevated temperatures, or made quiescent by withdrawal of serum growth factors (32). We were therefore surprised to find that the reduction in 43-S initiation complexes in calcium-deprived cells (Table I) and in the BAPTA-treated cell-free system (Fig. 6 B) was not caused by a change in eIF-2 phosphorylation.

An explanation for the reduction in 43-S complexes was suggested by the finding that the 40-S peak on sucrose gradient analysis of BAPTA-treated extracts (Fig. 5 B) was unusually narrow, lacking the shoulder normally seen on the leading edge (Fig. 5 A). This implies that there is no longer a fraction of 40-S subunits containing bound eIF-3, and this was confirmed by CsCl equilibrium density gradient analysis. A 43-S initiation complex is defined by the presence of ternary complex (eIF-2-GTP-methionyl-tRNA) on a 40-S ribosomal subunit. The complex is stabilized by the presence of eIF-3 (35, 36), an ~600-kD protein, and the majority of 43-S initiation complexes contain this factor. Because of the large size of eIF-3 then, eIF-3-containing complexes sediment on the leading edge of the 40-S subunit peak (thus the designation 43-S) in sucrose gradient analysis and at a buoyant density of 1.40 g/ml in CsCl gradient analyses (1). A minority of labeled methionyl-tRNA normally is found on subunits the buoyant density of which indicates that they do not contain eIF-3 (12, 15, 23). Chelation of calcium by BAPTA led to the virtually complete loss of 1.40 g/ml particles (Fig. 7 B), indicating that there was no longer a fraction of 40-S subunits with bound eIF-3. The remaining [35S]methionyl-tRNA was bound to denser particles and the total bound [35S]methionyl-tRNA was greatly reduced.

An interpretation which is consistent with our results and with our current understanding of the steps in initiation is that calcium is required for the binding of eIF-3 to the 40-S subunit and in the absence of eIF-3 the binding of ternary complexes is less stable so that the concentration of bound complexes is reduced. This is the first report to our knowledge of a possible modification of eIF-3 function. Alternatively, it is possible that the effect on eIF-3 binding is indirect. For instance, the binding of a factor which precedes eIF-3 binding may be modified. eIF-4C, for example, is also known to bind to 40-S subunits (34), and inactivation of this factor could also explain the data.

Currently it is not known in what order eIF-2 and eIF-3 bind 40-S subunits. It has generally been thought likely that eIF-3 binds ribosomes first. However, either factor can bind in the absence of the other in purified preparations, and as treatment with BAPTA leads to ribosomal complexes containing ternary complexes but lacking eIF-3 (Figs. 5 B and 7 B), it is suggestive that eIF-2 binds 40-S subunits sequentially before eIF-3 or that there normally is random order binding of eIF-2 and eIF-3.

We do not report 43-S complex levels in whole cells because of a problem in their measurement. Our data from analyses of formaldehyde-fixed and unfixed extracts indicate that 43-S complex formation can take place rapidly even in extracts held on ice. The fact that a large shift takes place in the cell-free system during incubation on ice (Table II) indicates that under steady-state flow conditions at 37°C, the reactions are not at equilibrium; when the incubation is chilled on ice, the utilization of 43-S complexes presumably ceases, and equilibration of the various forms of eIF-2 takes place, with a shift of free forms of eIF-2 and of ternary complexes to 43-S complexes. The factors determining the rate at which redistribution occurs are complex. However it is evident that the possibility of redistribution at 0°C must be taken into account in interpreting analysis of cell extracts, which cannot be rapidly fixed with formaldehyde. We are attempting to develop a method for stabilizing the in vivo concentration of 43-S complexes during the preparation of cell extracts.

We find that under normal, pH controlled, growth conditions total cellular calcium turns over in the Ehrlich cell within 1 h (Fig. 1 A). This is in disagreement with older measurements which have suggested that as much as 80% of total cellular calcium is only very slowly exchangeable (7, 8, 10, 18, 19). However, these measurements were performed under nongrowth conditions; cells were often suspended at growth-inhibitory concentrations (>107 cells/ml), in simple salt solutions lacking amino acids and sometimes glucose, and with inadequate pH control. More recent results have tended to resemble those reported here (16, 26, 31); for instance, cellular calcium has been found to turn over in 1 h in GH3 cells (28).

In intact cells, total cell calcium fell rapidly after addition of EGTA, and protein synthesis began to fall only after 1 h, when total cell calcium had fallen to ~20% of the control level. We assume that there was a decrease in free calcium as total calcium fell (27), but we do not know the relationship between the time course of total and free cellular calcium. If they fell in parallel, protein synthesis did not decline until free calcium had fallen to 20% of the value in growing cells. This would suggest that protein synthesis was responding to severe calcium deficiency but was not responding to changes in the normal intracellular range. On the other hand, reports indicate that the intracellular free calcium appears to be buffered by internal stores against external changes in calcium (38). If the free calcium was maintained by compensatory mechanisms until 80% of the total calcium was lost, protein synthesis might be responding to calcium over the physiological calcium range, implying that calcium could be an intracellular regulator of protein synthesis. We calculated the free calcium concentration in the cell-free system to be 50 nM in incubations containing 0.5 mM BAPTA, and 70—90 nM in incubations containing 0.25 mM BAPTA, both of which are inhibitory concentrations. The normal physiological calcium range is usually considered to be 100—1,000 nM.
An inhibitory effect at 90 nM is sufficiently close to the physiological range and hence, we cannot decide whether the inhibition of protein synthesis in EGTA-treated cells represents simply a response to severe calcium depletion or indicates that calcium is an intracellular regulator of protein synthesis under physiological conditions.

Calcium depletion could have multiple effects including effects on the cytoskeleton, altered energy metabolism (especially mitochondrial), altered intracellular concentrations of other ions, and a number of other effects. Our results from the cell-free system, both Ehrlich cells and reticulocytes, alleviate some of these concerns and suggest that the effect of calcium on protein synthesis is a direct effect and a generalized phenomenon true for all mammalian systems.

We would like to thank Eileen Stewart, Pamela Porter, and Peter Reichard for their expert technical assistance.

This work was supported by National Institutes of Health Grants ROI CA-21663, CA-11198, and T-32-CA-09363.

Received for publication 23 August 1988 and in revised form 31 January 1989.

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