Effect of CCK and intracellular calcium to regulate eIF2B and protein synthesis in rat pancreatic acinar cells

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Sans, Maria Dolors, Scot R. Kimball, and John A. Williams. Effect of CCK and intracellular calcium to regulate eIF2B and protein synthesis in rat pancreatic acinar cells. Am J Physiol Gastrointest Liver Physiol 282: G267–G276, 2002.—Pancreatic secretagogues enhance acinar protein synthesis at physiological concentrations and inhibit protein synthesis at high concentrations. We investigated the potential role in this process of the eukaryotic translation initiation factor (eIF)2B. Cholecystokinin (CCK) at 10–100 pM did not significantly affect eIF2B activity, which averaged 35.4 nmol guanosine 5′-diphosphate exchanged per minute per milligram protein under control conditions; higher CCK concentrations reduced eIF2B activity to 38.2% of control. Carbamylcholine chloride (Carbachol, CCh), A-23187, and thapsigargin also inhibited eIF2B and protein synthesis, whereas bombesin and the CCK analog JMV-180 were without effect. Previous studies have shown that eIF2B can be negatively regulated by glycogen synthase kinase-3 (GSK-3). However, GSK-3 activity, as assessed by phosphorylation state, was inhibited at high concentrations of CCK, an effect that should have stimulated, rather than repressed, eIF2B activity. An alternative mechanism for regulating eIF2B is through phosphorylation of the α-subunit of eIF2, which converts it into an inhibitor of eIF2B. CCK, CCh, A-23187, and thapsigargin all enhanced eIF2α phosphorylation, suggesting that eIF2B activity is regulated by eIF2α phosphorylation under these conditions. Removal of Ca2+ from the medium enhanced the inhibitory action of CCK on both protein synthesis and eIF2B activity as well as further increasing eIF2α phosphorylation. Although it is likely that other mechanisms account for the stimulation of acinar protein synthesis, these results suggest that the inhibition of acinar protein synthesis by CCK occurs as a result of depletion of Ca2+ from the endoplasmic reticulum lumen leading to phosphorylation of eIF2α and inhibition of eIF2B.

CCK is a major gastrointestinal hormone regulator of exocrine pancreatic function (45). In addition to its role of stimulating pancreatic exocrine secretion, it has been shown to regulate pancreatic protein synthesis, mitogenesis, and gene expression (44). In vivo, CCK exerts both short-term stimulatory effects on pancreatic protein synthesis and long-term trophic effects on pancreas size and digestive enzyme content (4, 6, 38). In vitro, in addition to its action to acutely stimulate pancreatic acinar cell secretion, CCK regulates the synthesis of pancreatic protein in a dose-dependent manner, with a characteristic biphasic response (2, 24, 25). These observations suggest that hormonal modulation of enzyme synthesis may be coordinated with the regulation of secretion. With short-term stimulation, there is no change in mRNA levels for digestive enzymes, and the major regulatory effect appears to be at the level of polysomes associated with the rough endoplasmic reticulum actively translating digestive enzyme mRNAs (27, 31, 32). Recent studies have begun to elucidate the signaling pathways regulating translational control in the pancreas. We have demonstrated that CCK activates the regulatory step in translation initiation that is critically dependent on the mRNA binding protein eIF4E that binds to the cap structure at the 5′ end of the mRNA and mediates assembly of an initiation-factor complex termed eIF4F. Assembly of this complex can be regulated by eIF4E-binding proteins (4E-BPs), which inhibit eIF4F complex assembly. In normal rats in vivo, both exogenous and endogenous CCK enhance phosphorylation of eIF4E and, more importantly, the release of eIF4E from its binding protein PHAS-I (4E-BP1) (2, 3). CCK also activates the 70-kDa ribosomal protein S6 kinase (p70[^6k]) (1) as a result of the same signaling pathway leading to the phosphorylation of PHAS-I.

The other major regulatory step in the translation initiation pathway is the binding of the initiator methionyl-tRNA<sub>i</sub> to the 40 S ribosomal subunit regulated by the eukaryotic initiation factor-2 (eIF2) (13, 34). eIF-2 is a heterotrimer composed of α, β, and γ subunits and forms a ternary complex with initiator methionyl-tRNA<sub>i</sub> and GTP. This complex binds to the 40 S ribosomal subunit forming the 43 S species. After the 60 S ribosomal subunit joins the 43 S complex, the eIF2-bound GTP is hydrolyzed to guanosine 5′-diphosphate (GDP) and the eIF2-GDP complex is released from the ribosome (13). For eIF2 to promote another round of initiation, GDP must be exchanged for GTP, a
reaction catalyzed by the guanine nucleotide exchange factor, eIF-2B (13). In some cells, including skeletal muscle and Swiss 3T3 cells, the global stimulation of protein synthesis is mediated by enhancing eIF2B activity. Insulin can regulate this activity through activation of the phosphatidylinositol 3-kinase (PI3K) pathway that leads to phosphorylation and inactivation of glycogen synthase kinase-3 (GSK-3) (21, 43) and finally to the dephosphorylation (activation) of eIF2Bε (10, 42). A second regulatory pathway for eIF2B involves the phosphorylation of serine residue 51 in eIF2α. Once this site has been phosphorylated, eIF2 has an increased affinity for eIF2B, converting eIF2 from a substrate to a competitive inhibitor of eIF2B (36, 39). Phosphorylation of eIF2α is also a major mechanism whereby environmental stress (12, 36), nutrient deprivation (5, 13, 30, 41), or viral infection (9) shuts off protein synthesis. Depletion of calcium from the endoplasmic reticulum also correlates with inhibition of protein synthesis and increased eIF2α phosphorylation (18, 39).

In the current study, we evaluated the activity of eIF2B in isolated pancreatic acini in vitro in response to CCK and other acinar cell secretagogues. Although no evidence was found for eIF2B stimulation contributing to secretagogue stimulation of protein synthesis, enhanced phosphorylation of eIF2α and inhibition of eIF2B were found to correlate with inhibition of protein synthesis induced by high doses of CCK and alterations in intracellular calcium.

MATERIALS AND METHODS

Materials. Sulfated CCK octapeptide was from Research Plus (Bayonne, NJ); bombesin (BBS) was from Bachem (Torrance, CA); and carbamoylcholine chloride (Carbachol, CCh), soybean trypsin inhibitor (SBTI), wortmannin, GDP, and dithiothreitol were obtained from Sigma (St. Louis, MO). Chromatographically purified collagenase was from Worthington Biochemicals (Freehold, NJ); goat anti-rabbit and anti-mouse IgG conjugated to horseradish peroxidase and enhanced chemiluminescence (ECL) reagent were from Amersham Pharmacia Biotech (Piscataway, NJ); minimal essential amino acids were from Gibco (Grand Island, NY); 10 and 12% Tris-HCl precast gels and low range prestained SDS-PAGE standard markers were from Bio-Rad (Hercules, CA); nitrocellulose membranes were from Schleicher & Schuell (Keene, NH); and A-23187, thapsigargin, and cycloheximide were from Calbiochem (La Jolla, CA), l-[35S]methionine (1,175 Ci/mM) and l-[3H]GDP (11.3 Ci/mM) were from Research Products International (Mount Prospect, IL) and National Diagnostics (Atlanta, GA), respectively; 25 mm nitrocellulose disks (HAWP) were from Millipore (Bedford, MA); and A-23187, thapsigargin, and cycloheximide were from Calbiochem (La Jolla, CA). GSK-3 monoclonal antibody was originally developed by Dr. E. C. Henshaw, that recognizes both phosphorylated and unphosphorylated forms of eIF2α was also used. Purified eIF2 and eIF2B were prepared from rat liver as described (15, 19).

Preparation of pancreatic acini. Pancreatic acini were prepared by collagenase digestion (1) of pancreases of 125- to 150-g male Sprague-Dawley rats. Acini were suspended in incubation buffer, consisting of a HEPES-buffered Ringer solution supplemented with 11.1 mM glucose, Eagle’s minimal essential amino acids, 0.1 mg/ml SBTI, and 10 mg/ml BSA, and equilibrated with 100% O2.

Methionine incorporation into pancreatic protein. To measure total net protein synthesis in acinar cells, l-[35S]methionine incorporation into protein was evaluated as described previously (2). After 1-h preincubation in HEPES-buffered Ringer, aliquots of isolated acini (1 ml) were incubated with agonists for 60 min at 37°C with gentle shaking. During the last 15 min of incubation, 2 μCi/ml of l-[35S]methionine were added to the incubation medium. Incubation was terminated by dilution with 2 ml of 154 mM NaCl at 4°C. After centrifugation at 300 g for 3 min, acinar pellets were resuspended in 0.5 ml water and sonicated. The precipitated was washed twice with ice-cold 10% TCA and dissolved in 200 μl of 0.1 N NaOH, and radioactivity in the insoluble material was measured in BioSafe II scintillation medium. All samples contained an equal amount of water and NaOH to ensure equal quenching. Background samples contained NaOH to control for chemiluminescence.

Measurement of eIF2B activity. Determination of eIF2B activity in pancreatic acini was performed as described by Kimball et al. (14), who measured the rate of exchange of [3H]GDP, present in an exogenous eIF2-[3H]GDP complex for free, nonradiolabeled GDP. To prepare acinar samples for experimental treatment, acini were rinsed in ice-cold PBS, centrifuged, and resuspended in 150 μl of lysis buffer consisting of 45 mM HEPES, pH 7.4, 0.375 mM magnesium acetate, 95 mM potassium acetate, 0.075 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium vanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The samples were then sonicated for 10 s and centrifuged for 15 min at 12,000 rpm at 4°C in a microcentrifuge. Supernatants were assayed for guanine nucleotide exchange activity. Briefly, 35 μl of a prepared binary complex, which was assembled by incubation of purified eIF2 (14) with 1.3 μM [3H]GDP, was combined with a mixture consisting of 35 μl of acini "homogenate," 87.5 μl of water, and 140 μl of buffer A (62.5 mM MOPS, pH 7.4, 209 μM GDP, 2.5 mM magnesium acetate, 125 mM potassium chloride, 1.25 mM dithiothreitol, and 250 μg/ml bovine serum albumin). The reaction was initiated by concentration of these reactants and transferred to a 30°C water bath. At four time points (0, 1, 2, and 3 min), a 60-μl aliquot was removed and placed into tubes containing 2.5 ml of ice-cold wash buffer (50 mM MOPS, pH 7.4, 2 mM magnesium acetate, 100 mM potassium chloride, 1 mM dithiothreitol). The contents were then mixed and immediately filtered under suction through a nitrocellulose filter disk and rinsed with 5 ml of ice-cold wash buffer. Filters were dissolved in 7 ml of Filtron-X. The guanine nucleotide exchange activity was measured as a decrease in eIF2-[3H]GDP complex bound to the filters and expressed as nanomoles GDP exchanged per minute per milligram acinar protein or as a percentage of the control group.

Evaluation of the phosphorylation state of eIF2α. The phosphorylation state of eIF2α was determined by two different methods. In the first method, the relative amount of eIF2α in the phosphorylated form was quantitated by protein immunoblot analysis using an affinity-purified antibody that spe-
specifically recognizes eIF2α phosphorylated at Ser51. For this analysis, aliquots of acinar lysates were heated for 5 min in a SDS sample buffer at 100°C, and after cooling they were resolved in a 10% SDS-PAGE gel, transferred to nitrocellulose followed by Western blot analysis using the anti-phospho eIF2α polyclonal antibody (1:1,500), and detected by ECL. Quantification was performed using Multi-Analyst software (Bio-Rad). To ensure equal loading, the same membranes were stripped and reprobed for total eIF2α using a monoclonal antibody to eIF2α diluted 1:500, which was also detected by ECL.

In the second method, the proportion of eIF2α present in the phosphorylated form was determined by protein immunoblot analysis after separation of the phosphorylated and unphosphorylated forms of the protein using vertical slab gel isoelectric focusing electrophoresis (17). To prepare acinar samples after experimental treatment, acini were rinsed in ice-cold PBS, centrifuged, and resuspended in 150 μl of lysis buffer consisting of (in mM) 20 Tris·HCl, pH 7.5, 250 sucrose, 250 potassium chloride, 0.090 EDTA, 1 dithiothreitol, 40 sodium fluoride, 70.8 β-glycerophosphate, 100 phenylmethylsulfonyl fluoride, and 0.2 sodium vanadate, with 10 μg/ml leupeptin and 10 μg/ml aprotinin. Samples were then sonicated for 10 s and centrifuged for 15 min at 12,000 rpm at 4°C in a microcentrifuge. Aliquots of 75 μl of the supernatant were mixed with 42.9 mg of urea and 300 μl of urea buffer (9.5 M urea, 2% Nonidet P-40, 4% ampholytes, and 5% β-mercaptoethanol) and stored at −70°C. Samples were resolved by isoelectric focusing followed by protein immunoblot analysis using the monoclonal anti-eIF2α antibody as described above.

**Quantification of eIF2α and eIF2Be.** The contents of eIF2 and eIF2B in pancreatic acinar cells were determined by protein immunoblot analysis using as standards eIF2α and eIF2Be expressed in and purified from Sf21 cells (7, 16). The eIF2Be antibody was raised against eIF2B, which was purified from rat liver as described previously (19).

**Evaluation of the phosphorylation state of GSK-3.** As an indicator of GSK-3 activity, acinar samples in SDS buffer were resolved in a 10% SDS-PAGE gel followed by Western blot analysis using an anti-phospho GSK-3α/β (Ser9/19) polyclonal antibody (1:1,000) and detected by ECL. Quantification was performed using Multi-Analyst software. To ensure equal loading, the same membranes were stripped and reprobed with a GSK-3α/β monoclonal antibody (1:1,000), which was also detected by ECL. GSK-3β is the predominant form in acinar cells and was the form whose phosphorylation was quantitated. Similar qualitative changes were seen in GSK-3α. A decrease in GSK-3 activity is indicated when phosphorylation is increased with respect to basal samples.

**Statistical analysis.** Data are presented as means ± SE and were obtained from at least four separate experiments. Statistical analysis was completed by Student’s *t*-test, unless otherwise indicated, as calculated by the SigmaStat program. Differences with *P* < 0.05 were considered significant.

**RESULTS**

**CCK stimulation and inhibition of protein synthesis.** To study the effect of CCK on pancreatic protein synthesis, we first evaluated the levels of L-[35S]methionine incorporation into TCA-precipitable protein at different concentrations of CCK. As in previous studies of acini prepared from diabetic rats (2, 25), CCK stimulated protein synthesis of rat acini in a dose-dependent manner (Fig. 1), describing a biphasic curve similar to the one described for in vitro secretory studies with acute preparations of pancreatic acini. Low concentrations of CCK, from 3 pM and above, increased L-[35S]methionine incorporation into TCA-precipitable protein with a maximum effect at 100 pM, where synthesis was 157.8 ± 12.8% of control. Higher concentrations of CCK (1 and 10 nM) decreased the incorporation of radiolabeled amino acid to 60.4 ± 9.4% of basal at 10 nM (Fig. 1).

**CCK effects on translation initiation factors eIF2B and eIF2α.** Because modulation of eIF2B activity is known to be one of the most important regulatory points in translation initiation (13), we studied the effect of different concentrations of CCK on eIF2B activity. Extracts of control pancreatic acini exhibited an eIF2B activity of 35.4 ± 2.7 nmol·min⁻¹·mg⁻¹ protein (*n* = 10). A small, but not significant, increase in eIF2B activity was seen at CCK concentrations that were stimulatory for protein synthesis (10 and 100 pM) (Fig. 2A). This insignificant increase was also observed when data for 100 pM CCK were pooled from all of the experiments carried out for this study (41.0 ± 2.4 nmol·min⁻¹·mg⁻¹, *n* = 18). By contrast, a clear and significant reduction in eIF2B activity was seen at concentrations of CCK (from 1 to 100 nM) that inhibited protein synthesis, reaching a minimum of 14.4 ± 2 nmol·min⁻¹·mg⁻¹ at 100 nM CCK (Fig. 2A).

The best-characterized mechanism for regulating eIF2B activity is phosphorylation of eIF2a, because eIF2B has a higher affinity for phosphorylated compared with unphosphorylated eIF2α (13, 16, 34). We therefore studied the effect of different concentrations of CCK on eIF2α phosphorylation to see whether such an increase might explain the results obtained for eIF2B activity. CCK increased eIF2α phosphorylation in a concentration-dependent manner from 10 pM to 100 nM, reaching a maximum value of 222 ± 22% of

![Fig. 1. Effect of CCK octapeptide on L-[35S]methionine incorporation into acinar protein.](http://ajpgi.physiology.org/Downloadedfrom)
the basal at 10 nM (Fig. 2B). This increase in phosphorylation was correlated with a dose-dependent increase in the amount of eIF2α in the phosphorylated form (Fig. 2C and Table 1) reaching values of ~30% or higher at 10 and 100 nM CCK. The increase in eIF2α phosphorylation at high concentrations of CCK was correlated with the inhibition of eIF2B activity at these doses (Fig. 2 and Table 1). Thus inhibition of protein synthesis at higher CCK concentrations likely occurs, at least in part, through an inhibition of eIF2B activity induced by eIF2α phosphorylation.

**CCK effects on GSK-3 phosphorylation.** Another mechanism by which eIF2B activity can be regulated is through phosphorylation of eIF2Be by GSK-3, which results in inhibition of its guanine nucleotide exchange activity. In the present study, GSK-3 regulatory phosphorylation increased in response to 100 pM to 100 nM CCK, reaching a maximum of 432 ± 51% (Fig. 3). This increased phosphorylation would be expected to inhibit GSK-3 activity and might thereby increase eIF2B activity. However, we observed either a nonsignificant increase or a reduction in eIF2B activity at these concentrations of CCK (Fig. 3). Therefore, GSK-3 is not likely to be involved in the inhibition of eIF2B. Moreover, acini treated with wortmannin to stimulate GSK-3 also showed no change in eIF2B activity (100.0 ± 14.4% in basal, 91.7 ± 17.4% at 100 pM CCK, 58.3 ± 0.1% at 1 nM CCK, and 30.6 ± 5.6% at 10 nM CCK, which is comparable to samples stimulated with CCK alone).

**Effects of high concentrations of various agonists on protein synthesis, eIF2B activity, and eIF2α phosphorylation.** To establish whether there is a parallelism between secretagogue agonists mediating inhibition of eIF2B activity and protein synthesis, we studied the effect of high concentrations of other well-known pancreatic secretagogues, including CCh, BBS, and the CCK analog JMV-180 on protein synthesis, eIF2B activity, and eIF2α phosphorylation. As expected, and like in previous studies (25), CCh at 1 mM had an inhibitory effect on protein synthesis, inhibiting L-[35S]methionine incorporation to pancreatic protein to 44.8 ± 7.0% (Fig. 4A). CCh at this concentration also inhibited eIF2B activity to 50% of the basal, comparable to the effect of CCK at 10 nM (Fig. 4B). CCh also increased both eIF2α phosphorylation and the proportion of eIF2α in the phosphorylated form, although these were

**Table 1. Percentage of phosphorylated eIF2α compared to total eIF2α in pancreatic acini in normal and calcium free media under different stimuli**

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<th>Basal</th>
<th>CCK 10 pM</th>
<th>CCK 100 pM</th>
<th>CCK 10 nM</th>
<th>CCK 100 nM</th>
<th>BBS 1 μM</th>
<th>CCh 1 μM</th>
<th>JMV 1 μM</th>
<th>Tg 1 μM</th>
<th>2 μM A-23187</th>
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<tr>
<td>n</td>
<td>8</td>
<td>3</td>
<td>6</td>
<td>6</td>
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<tr>
<td>Normal media</td>
<td>10.3 ± 0.7</td>
<td>12.2 ± 3.0</td>
<td>19.0 ± 4.4*</td>
<td>28.0 ± 3.7*</td>
<td>39.1 ± 7.9#</td>
<td>15.9 ± 6.1</td>
<td>25.1 ± 9.6</td>
<td>14.6 ± 1.1</td>
<td>58.5 ± 2.4*</td>
<td>47.5 ± 3.0*</td>
</tr>
<tr>
<td>Ca²⁺ free media</td>
<td>24.5 ± 2.0*</td>
<td>21.2 ± 1.6*</td>
<td>35.4 ± 9.3#</td>
<td>46.1 ± 8.7*</td>
<td>48.5 ± 12.3</td>
<td>30.1 ± 8.2</td>
<td>22.5 ± 6.7</td>
<td>17.6 ± 3.8</td>
<td>47.5 ± 3.0*</td>
<td>45.1 ± 3.0*</td>
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Values are means ± SE from Western blotting of IEF gels, similar to Fig. 2C. Calcium free media also contained 100 μM EGTA. *P < 0.05 vs. control basal in normal incubation media by Student t-test when data passed the normality and equal variance test, or by the Mann-Whitney rank sum test when it did not (#). CCK, cholecystokinin; BBS, bombesin; CCh, carbamylcholine chloride (Carbachol); JMV, CCK analog JMV-180; Tg, thapsigargin.
lower than the effect of CCK at 10 nM (Fig. 4C and Table 1). In agreement with previous studies (28, 29), neither 1/100 M of BBS nor 1/100 M of JMV-180 inhibited acinar protein synthesis (Fig. 4A). These later agents also had no effect on either eIF2B activity (Fig. 4B) or eIF2α phosphorylation (Fig. 4C and Table 1). Together, these results suggest that the inhibition of protein synthesis caused by CCK and CCh likely occurs through a common pathway that involves an increase on eIF2α phosphorylation and inhibition of eIF2B activity.

Calcium effects on protein synthesis, eIF2B activity, and eIF2α phosphorylation. CCK and CCh have similar effects on protein synthesis, eIF2B activity, and eIF2α phosphorylation at high concentrations. These secretagogues share a common intracellular mechanism to increase free cytoplasmic calcium by mobilizing calcium from intracellular stores and by activating the influx of extracellular calcium. Because it is known that depletion of calcium from intracellular stores increases eIF2α phosphorylation and inhibits protein synthesis in other cell types (18, 33, 39), we first studied the effect of the ionophore A-23187 and the inhibitor of the microsomal Ca2+-ATPase thapsigargin on protein synthesis, eIF2B activity, and eIF2α phosphorylation. The results show that both agents strongly inhibited protein synthesis to 4.9 ± 0.2% (thapsigargin) and to 9.4 ± 3.5% (A-23187) of the basal value (n = 3–5). They also inhibited eIF2B activity to <30% (Fig. 5A) and increased severalfold eIF2α phosphorylation (Fig. 5B) and the percentage of phosphorylated eIF2α to ~50% (Table 1), compared with basal values. Thus the inhibition of protein synthesis caused by releasing calcium from intracellular stores could be due to the inhibition of eIF2B activity and an increase on eIF2α phosphorylation.

Acinar protein synthesis was also inhibited when calcium was removed from the media by addition of the calcium chelator EGTA (100 μM) to the medium. In this case, basal protein synthesis was reduced to 46.5 ± 9.9% of control and was further reduced in the presence of both CCK and EGTA to a low of 9.0 ± 2.6% at 10 nM (n = 4). To determine whether this inhibition of acinar protein synthesis could be due to an effect on eIF2B activity and/or on eIF2α phosphorylation, acini were incubated with different concentrations of CCK (10 pM to 10 nM) in an incubation medium with a normal concentration of calcium (1.28 mM) or in a Ca2+-free medium containing 100 μM EGTA. In the

![Fig. 3. Effect of CCK octapeptide on glycogen synthase kinase-3 (GSK-3) phosphorylation. Acini were incubated for 60 min with CCK at different concentrations and processed as described in MATERIALS AND METHODS for detecting and quantitating the levels of GSK-3 phosphorylation. Results are expressed as a percentage of basal levels. Each column represents the mean of at least 4 different experiments ± SE. *P < 0.05 vs. control (basal group).]

![Fig. 4. Effect of high doses of CCK octapeptide, carbamylcholine chloride (Carbachol, CCh), bombesin (BBS), and the CCK analog (JMV-180) on protein synthesis (A), eIF2B activity (B), and eIF2α phosphorylation (C). Acini were incubated for 60 min with CCK at 10 nM, CCh at 1 mM, and BBS and JMV-180 at 1 μM and processed as described in MATERIALS AND METHODS for L-[35S]methionine incorporation, eIF2B activity, and eIF2α phosphorylation. Results are expressed as a percentage of basal levels. Each column represents the mean ± SE of 3–4 different experiments. *P < 0.05 vs. control (basal group). The blots in (C) are representative for eIF2α phosphorylation levels and were then stripped and reprobed for total eIF2α.]

AJP-Gastrointest Liver Physiol • VOL 282 • FEBRUARY 2002 • www.ajpgi.org
absence of CCK, EGTA reduced eIF2B activity to 50% of the value observed in calcium-containing medium (Fig. 6A). The inhibition of eIF2B activity caused by EGTA was maintained in CCK-treated acini, reaching a minimum of 10% of basal values at 100 nM CCK. Conversely, eIF2α phosphorylation was increased 2.5-fold in both basal and CCK-treated acini (Fig. 6B). The calcium-free medium + EGTA increased about two times the percentage of phosphorylated eIF2α in basal and all other CCK concentrations.

To evaluate the reversibility of calcium depletion, calcium was added back into the incubation medium after 1 h of preincubation in the calcium-free + EGTA medium. Protein synthesis recovered ~50%, similar to data previously reported by Korc (24). In parallel, adding calcium back into the medium for 1 h only partially restored eIF2B activity (63.2 ± 10.5 vs. 49.5 ± 9.7% of basal) and decreased the phosphorylation levels of eIF2α, compared with the calcium-free ones (159 ± 21 vs. 263 ± 49% of basal) (Fig. 6, B and C).

The aggregate results of the above experiments suggest that calcium is necessary for CCK stimulation of protein synthesis. Depletion of sequestered calcium from the endoplasmic reticulum and intracellular stores, either due to a chelation of extracellular calcium or a direct intracellular mobilization with the ionophore A-23187 and thapsigargin, rather than an increase in cytoplasmic calcium is the likely inhibitory mechanism of eIF2B activity and acinar protein synthesis. Further studies are necessary to better define the reversibility of these effects that most likely depends on the dephosphorylation of eIF2α.

Relative amount of eIF2B and eIF2α in pancreatic acinar cells. The mechanism through which eIF2α phosphorylation causes an inhibition of eIF2B activity involves an increased affinity of eIF2B for phosphorylated compared with unphosphorylated eIF2. The increased affinity of eIF2B for phospho-eIF2α results in the effective sequestration of eIF2B into an inactive complex, with phospho-eIF2α inhibiting eIF2B on an
approximately equimolar basis. Thus to inhibit eIF2B activity completely, an equimolar amount of phospho-eIF2α must be present. To establish whether the changes in eIF2α phosphorylation shown in Figs. 2B, 4C, 5B, and 6B could account for the observed changes in protein synthesis and eIF2B activity, the molar concentrations of eIF2 and eIF2B were determined. As shown in Fig. 7, the molar ratio of eIF2/eIF2B is 3:1 in acinar cells as determined by Western blot analysis using purified eIF2α and eIF2Be as standards (Fig. 7, A and B). The molecular mass of the proteins used as standards is greater than the native proteins, because the eIF2α and eIF2Be were expressed in Sf21 cells with an 8-amino acid extension at the NH2 terminus to aid in purification. None of the agonists or treatments of the acini modified the quantities of either eIF2α or eIF2Be (Fig. 7A) (data not shown for BBS, thapsigargin, or A-23187 treatments). These results suggest that a significant portion of the inhibitory effect on eIF2B activity could be related to the phosphorylated eIF2α.

**DISCUSSION**

The present study was designed to evaluate the regulation of the guanine nucleotide exchange factor eIF2B and its competitive inhibitor, phosphorylated eIF2α, by CCK and other pancreatic secretagogues.

![Fig. 7. Contents of eIF2 and eIF2B in pancreatic acinar cells. The contents of eIF2 and eIF2B in pancreatic acinar cells were determined by protein immunoblot analysis using eIF2α and eIF2Be as standards, as described in MATERIALS AND METHODS. A: immunoblots for eIF2α and eIF2Be. Lanes 1, 2, and 3 include 100, 50, and 25 ng of pure eIF2α or eIF2Be, respectively, whereas lanes 4–8 correspond to five different acinar treatments (basal, 100 pM CCK, 1 mM CCh, 1 µM JMV, basal in calcium-free medium and 100 pM CCK in a calcium-free medium) of 30 μg total acinar protein. B: quantification in control acini with conversion to molar units for both proteins, based on the molecular mass of 36 and 92 kDa for eIF2α and eIF2Be, respectively, as the mean ± SE of 3 different experiments.](https://www.ajpgi.org/doi/10.1152/ajpgi.01127.2002)
related to the stimulation of other regulatory steps in translation initiation, as seen in our previous studies (2). From these studies, we know that CCK at stimulatory doses increases the phosphorylation and activation of the p70S6K, the phosphorylation of PHAS-I, and the formation of the eIF4F initiation complex. These mechanisms are known to be downstream of PI3K and mTOR. However, eIF2B is not regulated through the PI3K/mTOR pathway in acinar cells, because neither the PI3K inhibitor worthmannin nor the mTOR inhibitor rapamycin (data not shown) had an effect on eIF2B activity, despite the fact that they blocked protein synthesis (2). The stimulatory effect of CCK in pancreatic acinar protein synthesis could thus be related to a stimulation of the PI3K pathway, thereby activating p70S6K and eIF4F assembly, without a significant effect on eIF2B activity. In fact, GSK-3 (one of the known eIF2B kinases) is phosphorylated and inactivated by CCK (Fig. 3) through the PI3K-PKB pathway (data not shown). In our case, however, GSK-3 inhibition would not activate eIF2B at stimulatory doses of CCK, because first, eIF2B was already activated in basal acini and second, stimulatory doses of CCK didn’t significantly increase eIF2B activity. eIF2B could be constitutively activated by another kinase such as casein kinase-I or -II (20, 23, 35) or through changes in the redox state of pyridine dinucleotides, because reduced pyridine dinucleotides upregulate eIF2B activity (11).

Inhibition of protein synthesis is characterized by disaggregation of polysomes, with a concomitant increase in free ribosomal subunits and monomers and a decrease in the rate of peptide-chain initiation (18, 32). At the first regulatory step of translation, phosphorylation of eIF2α immediately reduces the level of functional eIF2 and limits initiation events on all cellular mRNAs within the cell. Control through reversible phosphorylation of eIF2α provides the cell with an efficient and rapid means to respond to a variety of different stimuli (12). In this study on unstimulated pancreatic acini in vitro, we show how the eIF2-eIF2B complex is involved in the inhibition of protein synthesis by high doses of CCK or CCh. The inhibition seen in the protein synthesis experiments is correlated with the inhibition of eIF2B activity and an increase in the phosphorylation of eIF2α, transforming eIF2α into an inhibitor of eIF2B (20, 34). The molar ratio of eIF2 to eIF2B in pancreatic acini is 3:1 (Fig. 7), similar to most eukaryotic cells, where this ratio is 2–5:1 (17). Because eIF2α(P) inhibits eIF2B on an approximately equimolar basis (17), and we found that there are three molecules of eIF2α per one of eIF2B, we could suggest that phosphorylation of eIF2α accounts for most of the inhibitory effect seen in eIF2B activity. In fact, the proportion of eIF2α in the phosphorylated state increases with increasing concentrations of CCK until a value (at 10 nM CCK) almost three times the one in the basal group (Fig. 2C). Overall, these results suggest that phosphorylation of eIF2α can account completely for the observed inhibition of eIF2B activity (Fig. 2B) at 10 nM CCK. As a result, the inhibition of protein synthesis at 10 nM CCK to ~60% of control could be directly related to the inhibition of eIF2B, although eIF2B activity was inhibited to a greater extent (40% of basal). That could indicate that another mechanism (i.e., through regulating the eIF4F complex formation) compensates some of the eIF2B inhibitory effect at this concentration of CCK. An apparent conundrum is that eIF2α is also partially phosphorylated at 10 and 100 PM CCK (Fig. 2B), although no inhibition of eIF2B activity or protein synthesis is observed. Instead, eIF2B activity and protein synthesis started decreasing at 1 nM CCK, suggesting that at low concentrations of CCK, some other pathway(s) is activated that could be affecting eIF2B activity. One possible mechanism could involve changes in the phosphorylation of the ε-subunit of eIF2B, which might counteract the inhibition caused by eIF2α phosphorylation (17). The putative kinase activity involved is not known but is unlikely to be GSK-3. If GSK-3 were the only regulatory mechanism for eIF2B activity, we would expect that the activation of GSK-3 occurring at high concentrations of CCK would inhibit, rather than stimulate, eIF2B activity.

Protein synthesis is inhibited in a variety of cell types as a result of an increase in calcium release from ER or other intracellular stores (12, 18, 33, 39). For some types of cells, the release of calcium from the ER is sufficient to inhibit protein synthesis (18). The triggering mechanism for the inhibition of protein synthesis in acini could be the release of calcium from intracellular stores induced by some agonists. In unstimulated pancreatic acinar cells, intracellular calcium concentration ([Ca2+]i) is maintained by a balanced array of influx, efflux, and intracellular sequestration mechanism. High concentrations of CCK rapidly release (within 1–2 s) intracellular calcium and increase [Ca2+]i from 100 to 500–1,000 nM (45). In our study, we demonstrate that, in addition to increasing [Ca2+]i, high concentrations of CCK (1–100 nM) and CCh (1 mM) inhibited protein synthesis and eIF2B activity and also increased eIF2α phosphorylation. These results give clear evidence for a calcium-related regulation of protein synthesis. Results obtained with high concentrations of BBS and the CCK analog JMV-180 confirm this calcium-related theory, because BBS initiates similar intracellular messengers, as does CCK, but is ~10-fold less potent than CCK and does not induce as large a rise in intracellular calcium (29). In the case of the CCK analog JMV-180, it's effect on calcium release is also only 50–60% as efficacious as CCK and may be induced through a different pathway (44).

Depletion of intracellular calcium inhibits protein synthesis in a variety of cell types (18, 33, 39). We have shown that depleting calcium from the ER or from other intracellular stores, either by A-23187, thapsigargin, and calcium mobilizing agonists or chelation of extracellular calcium inhibits protein synthesis and eIF2B activity. The release of calcium induced by thapsigargin and A-23187 induced a severalfold increase in eIF2α phosphorylation and phosphorylation of ~50% of total eIF2α. Since the ratio eIF2α/eIF2B is 3:1, these...
results clearly show that a direct effect of the phosphorylation of eIF2α can account for the inhibition of eIF2B activity. Moreover, calcium-free media plus EGTA also inhibited protein synthesis at all assayed CCK concentrations (from 10 pM to 10 nM), having a stronger inhibitory effect when combined with high concentrations of CCK. Thus the trigger for the inhibition of protein synthesis could be the depletion of the ER-sequestered calcium (18). In general, alterations in [Ca²⁺], alter ER homeostasis and induce ER stress. This phenomenon induces a short-term response at the translational level and promotes a long-term adaptation to survival or apoptotic cell death through changes in gene expression (12). Our results suggest that pancreatic acinar cells adapt to short-term stress induced by reduction in calcium stores by inhibiting protein synthesis of pancreatic enzymes. The inhibition of protein synthesis associated with high concentrations of CCK could be an adaptive and protective mechanism. One of the most frequently used mechanisms for translational control is the reversible phosphorylation of eIF2, and the most likely kinase associated to phosphorylation of the eIF2α in pancreas would be one of the ER stress-signaling kinase such as pancreatic eIF2α kinase, known alternatively as PEK or PERK (8, 37). Although PEK/PERK could have a direct role in pancreatic acinar cell ER stress, it is possible that multiple eIF2α kinases, including PKR (double-stranded RNA-dependent protein kinase) may signal in response to separate as well as overlapping ER stresses (12, 40).

In summary, we conclude that CCK inhibition of protein synthesis in rat pancreatic acinar cells is mediated through the inhibition of eIF2B activity, and this effect is most likely related to depletion of intracellular calcium and activation of an eIF2α kinase. This phenomenon could be related to pathophysiological effects in the case of acute pancreatitis, where the inhibition of pancreatic digestive enzyme synthesis could stop, to some extent, the proteolytic enzyme activation and autodigestion cascade.

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