Calcineurin is required for translational control of protein synthesis in rat pancreatic acini

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Sans, Maria Dolors, and John A. Williams. Calcineurin is required for translational control of protein synthesis in rat pancreatic acini. Am J Physiol Cell Physiol 287: C310–C319, 2004. First published March 24, 2004; 10.1152/ajpcell.00534.2003.—CCK increases the rate of net protein synthesis in rat pancreatic acini by activating initiation and elongation factors required for translation. The immunosuppressant FK506 inhibits the Ca^{2+}-calmodulin-dependent phosphatase calcineurin in pancreatic acinar cells and blocks pancreatic growth induced by chronic CCK treatment. To test a requirement for calcineurin in the activation of the translational machinery stimulated by CCK, we evaluated the effects of FK506 on protein synthesis and on regulatory initiation and elongation factors in rat pancreatic acini in vitro. CCK acutely increased protein synthesis in acini from normal rats with a maximum increase at 100 pM CCK to 170 ± 11% of control. The immunosuppressant FK506 dose-dependently inhibited CCK-stimulated protein synthesis over the same concentration range that blocked calcineurin activity, as assessed by dephosphorylation of the calcineurin substrate calcium-regulated heat-stable protein of 24 kDa. Another immunosuppressant, cyclosporin A, inhibited protein synthesis, but its effects appeared more complex. FK506 also inhibited protein synthesis stimulated by bombesin and carbachol. FK506 did not significantly affect the activity of the initiation factor-2B, or the phosphorylation of the initiation factor-2α, ribosomal protein protein S6, or the mRNA cap binding protein eukaryotic initiation factor (eIF) 4E. Instead, blockade of calcineurin with FK506 reduced the phosphorylation of the eIF4E binding protein, reduced the formation of the eIF4F complex, and increased the phosphorylation of eukaryotic elongation factor 2. From these results, we conclude that calcineurin activity is required for protein synthesis, and this action may be related to an effect on the formation of the mRNA cap binding complex and the elongation processes.

exocrine pancreas; cholecystokinin; translation initiation factors; protein phosphatase 2B; immunosuppressants

Calcineurin, also known as protein phosphatase 2B (PP2B), is a serine/threonine protein phosphatase (48) that is highly regulated by Ca^{2+}-calmodulin (30). Calcineurin has been found in the highest concentrations in the brain, but it has also been detected in many other mammalian tissues (48, 53), including the pancreas (6, 21, 54). It is believed to be relatively inactive in cells under basal conditions of low intracellular calcium but becomes active after stimulation with calcium-mobilizing agonists (30). Calcineurin is the target of the immunosuppressive drugs FK506 and cyclosporin A (CsA), which, after binding to their respective intracellular binding proteins (12-kDa FK506 binding protein and cyclophilin A), inhibit calcineurin phosphatase activity (11, 42). The endogenous phosphatase inhibitors 1 and 2, as well as chemical inhibitors of PP1 and PP2A, fail to inhibit calcineurin, whereas immunosuppressants do not block these other phosphatases (48). The use of FK506 and CsA has implicated calcineurin in a number of cellular processes, including pancreatic endocrine (14, 23) and exocrine secretion (13, 21, 56); calcium-stimulated gene transcription (11, 24); cell growth (19, 36); cell cycle regulation (33); apoptosis (53); endocytosis (12); cytoskeletal organization (26); and neurite outgrowth (30). Moreover, study of the side effects of CsA and FK506 in organ transplant therapy has implicated calcineurin in the protein synthesis mechanisms of some tissues, including kidney and liver (7, 8). However, in contrast to the apparent multitude of cellular substrates for the type 1 and 2A serine/threonine phosphatases, relatively few cellular targets of calcineurin have been described (30, 53).

Because of the importance of increased intracellular calcium as a signaling mechanism in pancreatic acinar cells, the potential role of calcineurin in acinar cell signaling has been studied (21). Using CsA and FK506 as inhibitors, our laboratory previously identified a novel calcineurin substrate of unknown function named calcium-regulated heat-stable protein of 24 kDa (CRHSP-24), based on its calcium regulation, heat stability, and apparent molecular weight of 24 kDa (22). Whereas high concentrations of CsA were found to inhibit amylase secretion (21, 56), it is not clear whether FK506 inhibits pancreatic exocrine secretion (13, 56) or not (unpublished observations). More recently, both FK506 and CsA were found to block pancreatic growth in response to chronic elevation of CCK induced by feeding trypsin inhibitor to mice (55), indicating a possible role for calcineurin in pancreatic growth. An obligatory requirement for cell growth in all cells is the activation of protein synthesis (41). Associated with growth is an increase in protein translation, initially of regulatory and later structural proteins (36) that could be regulated by calcineurin.

Translational control of protein synthesis in the pancreas is important in regulating growth and also in the synthesis of digestive enzymes (51). Regulation of translation is primarily directed at initiation and elongation steps and involves reversible phosphorylation of initiation [eukaryotic initiation factors (eIFs)] and elongation [eukaryotic elongation factors (eEFs)] and ribosomal proteins. The assembly of the eIF4F mRNA cap binding complex, the activity of guanine nucleotide exchange factor eIF2B, the activity of ribosomal S6 kinase (S6K), and the activity of eEF2 are some of the potential regulatory sites (Fig. 1) (45, 51). Stimulation of protein synthesis in pancreatic
acinar cells is primarily mediated by the phosphatidylinositol 3-kinase (PI3K)-mammalian target of rapamycin (mTOR) pathway and involves both release of eIF4E from its binding protein and activation of the S6K (2, 3). Inhibition of acinar protein synthesis can be mediated by inhibition of eIF2B (Fig. 1) following phosphorylation of eIF2α (50).

In the present study, we evaluated the involvement of calcineurin in pancreatic protein synthesis by using FK506 and CsA to block calcineurin in suspensions of freshly isolated rat pancreatic acini in vitro. Following the initial observation that FK506 and CsA profoundly inhibited secretagogue-stimulated protein synthesis, we evaluated the key regulatory steps in translational control, including eIF2B activity, the formation of the eIF4F complex, the activation of the S6K, and the phosphorylation of eEF2. FK506 selectively blocked some but not all of these steps, indicating a requirement for calcineurin in activation of translation in acinar cells.

### EXPERIMENTAL PROCEDURES

**Materials.** Sulfated CCK octapeptide was from Research Plus (Bayonne, NJ). Bombesin (BBS) was from Bachem (Torrance, CA). Carbamylcholine chloride [carbachol (CCh)] and soybean trypsin inhibitor were obtained from Sigma Chemical (St. Louis, MO). Chromatographically purified collagenase was from Worthington Biochemicals (Freehold, NJ). FK506, CsA, and a polyclonal antibody to eIF4E-binding protein 1 (eIF4E-BP1) (PHAS-1) were from Calbiochem-Novabiochem (San Diego, CA). Pharmalyte ampholytes (3–10), goat anti-rabbit, and anti-mouse IgG antibodies, 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). Tris-HCl precast gels (7.5, 10, 12, 15, and 4–20%), high- and broad-range prestained SDS-PAGE standard markers, and other isolectricfocusing reagents were from Bio-Rad (Hercules, CA). Nitrocellulose membranes were from Schleicher and Schuell (Keene, NH). Bio-Mag goat anti-mouse IgG was from Transduction Laboratories (Lexington, KY).

**Preparation of pancreatic acini.** Pancreatic acini were prepared by collagenase digestion of pancreas from 125- to 150-g male Sprague-Dawley rats (2). Acini were suspended in incubation buffer, consisting of an N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid-buffered Ringer solution supplemented with 11.1 mM glucose, Eagle’s minimal essential amino acids, 0.1 mg/ml soybean trypsin inhibitor, and 1 mg/ml BSA and was equilibrated with 100% O2. In the assays where the immunosuppressants FK506 or CsA were used, acini were preincubated 1 h and then incubated with FK506 or CsA in the buffer at the specified concentrations.

**Incorporation of amino acid into protein.** To measure total net protein synthesis in acinar cells, [1-13C]methionine incorporation into protein was evaluated as described previously (3). Following 1 h preincubation in supplemented N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid-buffered Ringer solution, aliquots of isolated acini (1...
FK506 inhibits pancreatic protein synthesis

FK506 and CsA inhibit calcineurin activity and protein synthesis in pancreatic acinar cells. FK506 and CsA were initially used to evaluate the role of calcineurin in the regulation of pancreatic acinar protein synthesis. We first determined the concentrations of FK506 and CsA that would inhibit calcineurin in pancreatic acini by analyzing the changes in the phosphorylation state of the calcineurin substrate CRHSP-24 (22). CRHSP-24 is phosphorylated on multiple Ser residues in nonstimulated (basal) acini, resulting in one or two bands at the acidic pole of the IEF gel; CCK at 100 pM induced dephosphorylation of CRHSP-24, indicated by the shift to more alkaline forms (Fig. 2). FK506 blocked this CCK-induced

Measurement of eIF2B activity. Determination of eIF2B activity in pancreatic tissue samples was performed, as described previously, by measuring the rate of exchange of [3H]GDP present in an exogenous eIF2-[3H]GDP complex for free nonradioabeled GDP (28, 50). The guanine nucleotide exchange activity was measured as a decrease in the eIF2-[3H]GDP complex bound to nitrocellulose filters and expressed as nanomoles of GDP exchanged per minute per milligram of acinar protein or as a percentage of the control group (50).

Statistical analysis. Data are represented as means ± SE and were obtained from at least four separate experiments. Statistical analysis was carried out by one-way ANOVA and post-hoc Fisher’s protected least significant differences test on the Stat View program (SAS Institute, Cary, NC). Differences with P < 0.05 were considered significant.

RESULTS

FK506 and CsA inhibit calcineurin activity and protein synthesis in pancreatic acinar cells. FK506 and CsA were initially used to evaluate the role of calcineurin in the regulation of pancreatic acinar protein synthesis. We first determined the concentrations of FK506 and CsA that would inhibit calcineurin in pancreatic acini by analyzing the changes in the phosphorylation state of the calcineurin substrate CRHSP-24 (22). CRHSP-24 is phosphorylated on multiple Ser residues in nonstimulated (basal) acini, resulting in one or two bands at the acidic pole of the IEF gel; CCK at 100 pM induced dephosphorylation of CRHSP-24, indicated by the shift to more alkaline forms (Fig. 2). FK506 blocked this CCK-induced

fig. 2. Concentration-dependent effect of FK506 (A) and cyclosporin A (CsA; B) on calcium-regulated heat-stable protein of 24 kDa (CRHSP-24) phosphorylation state. Pancreatic acini were incubated with (+) or without (−) 100 pM CCK following preincubation with different concentrations of FK506 (A) or CsA (B). The different CRHSP-24 phosphorylated states (arrows) were separated by isoelectric focusing and visualized by Western blotting. The experiments shown are representative of 4 independent experiments.
CRHSP-24 dephosphorylation over the concentration range from 1 nM to 1 μM with full inhibition at 10 nM and higher concentrations (Fig. 2A). CsA, on the other hand, partially inhibited CRHSP-24 dephosphorylation at 100 nM and only fully inhibited CCK-induced CRHSP-24 dephosphorylation at 1 μM (Fig. 2B).

Next, the effects of FK506 and CsA on acinar protein synthesis measured as the incorporation of [35S]methionine into TCA precipitable protein were analyzed. Protein synthesis in rat pancreatic acini, in vitro, has been shown to be stimulated by CCK in a dose-dependent manner, describing a biphasic curve, which is maximal at 100 pM CCK, similar to stimulation of in vitro amylase secretion (3, 50). Addition of FK506 to acinar suspensions had only a minimal effect on basal [35S]methionine incorporation, which was not significant. CCK stimulated acinar [35S]methionine incorporation into protein by 170.0 ± 10.8%. This stimulation was inhibited in a concentration-dependent manner by FK506 with partial inhibition at 100 pM and maximal total inhibition at 10 nM and higher concentrations (Fig. 3A). The effect of 100 nM FK506 on different concentrations of CCK on pancreatic acini was also tested. FK506 significantly inhibited the stimulation of protein synthesis at all concentrations of CCK without affecting the inhibitory effect of 10 nM CCK on protein synthesis (Fig. 3B).

CsA also inhibited CCK-stimulated protein synthesis in acini in a dose-dependent manner, being significant from 10 nM to 1 μM CsA (Fig. 4). However, all CsA concentrations inhibited basal protein synthesis, reaching maximal inhibition at 1 μM CsA (the only dose that inhibits CRHSP-24 dephosphorylation), which could indicate a nonspecific effect of CsA on acinar cells. From these results, we conclude that both FK506 and CsA can inhibit calcineurin activity and protein synthesis in acinar cells. However, we chose to use FK506 for the rest of our studies to avoid possible nonspecific effects of CsA.

FK506 inhibits protein synthesis stimulated by other agonists. To demonstrate that the inhibitory effect of FK506 was not limited to CCK stimulation of protein synthesis, we also used the secretagogues BBS and CCh at concentrations that stimulate acinar protein synthesis (3, 32) and treated the acini with 100 nM FK506. A similar inhibitory effect of FK506 was seen when all three stimulatory agents were used (Fig. 5); stimulation of [35S]methionine incorporation into protein with 100 pM CCK was decreased from 162.7 ± 13.7 to 84.9 ± 14.7%, with 10 nM BBS from 145.0 ± 9.4 to 73.5 ± 16.8%, and with 30 μM CCh from 134.6 ± 9.7 to 82.9 ± 15.4% of basal incorporation. Together, these results show that the mechanisms underlying stimulation of protein synthesis by all
of these three secretagogues require calcineurin activity and that the effect of FK506 is not receptor specific.

FK506 inhibits the phosphorylation of the eIF4E binding protein (4E-BP1) but not the ribosomal protein S6. Previous work from our laboratory has established that stimulation of protein synthesis in acinar cells is primarily mediated by the PI3K-mTOR pathway (3). mTOR is activated by Akt/protein kinase B by phosphorylation on Ser-2448 (18, 39) and induces the phosphorylation of both the eIF4E binding protein 4E-BP1 (also called PHAS-1) and the phosphorylation of ribosomal S6 protein with the later mediated by activating S6K (2, 3). We first studied the effects of FK506 on mTOR phosphorylation at Ser-2448, and we found that FK506 had no effect on its phosphorylation when acini were stimulated by 100 pM CCK (401 ± 49% of basal for CCK + FK506 vs. 371 ± 26% of basal for CCK alone; n = 3–4). This confirms the activation of this pathway by CCK and shows that FK506 does not block the early steps in this pathway.

Next, we analyzed the phosphorylation state of 4E-BP1, which is usually phosphorylated when protein synthesis is stimulated, releasing eIF4E that binds the 5′-cap mRNA and joins with eIF4G to form the eIF4F complex (Fig. 1) (17, 18). Stimulation of acini by CCK increased 4E-BP1 phosphorylation in a biphasic manner. CCK at 100 pM maximally stimulated the phosphorylation of 4E-BP1, expressed as the percentage of total 4E-BP1 in its γ-form (the more slowly migrating form) from 6.1 ± 2.9 to 21.0 ± 5.2% of total, with this effect being decreased at higher concentrations of CCK (Fig. 6A). Incubation of acini with 100 nM FK506 had no effect on basal 4E-BP1 phosphorylation but strongly inhibited CCK-stimulated 4E-BP1 phosphorylation from 20.8 ± 2.5 to 12.3 ± 2.2% of total compared with FK506 alone (Fig. 6B).

The other important translational mechanism downstream of mTOR is the activation of the S6K that activates the ribosomal protein S6 and stimulates the translation of terminal oligopyrimidine-mRNAs (2, 34). Previous studies have demonstrated that S6K (also known as p70S6K) is activated by CCK in rat pancreatic acinar cells (2). In the present study, we analyzed the phosphorylation of the ribosomal protein S6 on Ser-240/244, by Western blotting, as a read-out of S6K activity. FK506 had no significant effect on basal or stimulated S6 phosphorylation (437 ± 66% of basal for CCK + FK506 vs. 399 ± 69% of basal for CCK alone) (Fig. 7). From these results, we conclude that 4E-BP1 phosphorylation is calcineurin dependent at a locus downstream of mTOR, but calcineurin seems not to be required for the phosphorylation of the ribosomal protein S6.

FK506 inhibits the formation of the eIF4F complex. We next studied the effects of CCK and FK506 on the formation of the eIF4F complex, which utilizes eIF4E released from the 4E-BP1. It has been previously demonstrated that CCK stimulates the formation of the eIF4F complex in rat pancreas in vivo (4). In the present study, we demonstrated that CCK stimulates the formation of this complex in vitro in a dose-dependent manner (Fig. 8A). The in vitro stimulation of eIF4F complex formation showed a biphasic pattern, increasing at 10 PM CCK, reaching a maximum (more than 4 times the basal levels) at 100 pM CCK, and decreasing at 1 and 10 nM CCK (Fig. 8A). This biphasic pattern correlates with the biphasic response to total protein synthesis due to CCK stimulation at different concentrations (3, 50).

After establishing the effects of CCK on eIF4F formation, we treated acini with 100 nM FK506 and then stimulated with 100 pM CCK. The results showed a reduction of the eIF4F complex formation due to FK506 treatment (68.3 ± 16.4% of basal) compared with the stimulated control samples (190.4 ± 31.1% of basal) (Fig. 8B), without reduction in the total eIF4E protein determined by Western blotting (Fig. 8B). These results follow from, and are in concert with, the effect of FK506 on 4E-BP1 phosphorylation and indicate that calcineurin activity is required for the eIF4F complex formation.

FK506 does not affect the phosphorylation state of the initiation factor 4E. Phosphorylation of the initiation factor eIF4E on Ser-209 often occurs when protein translation is stimulated (15, 18), and our laboratory has previously shown that CCK stimulation in vivo increases eIF4E phosphorylation (4). In the present study, we first analyzed its phosphorylation state in isolated acini, in vitro, after stimulation with different
FK506 inhibits the phosphorylation of eEF2. The effects of FK506 on the activity of eEF2, one of the main regulators of the elongation process, were also studied. eEF2 is a phosphoprotein with a single important regulatory site (Thr-56); the protein is active when this site is dephosphorylated. This dephosphorylation is known to reflect both from the action of an eEF2 phosphatase (5) and from the inhibition of a Ca2+/calmodulin-dependent kinase (eEF2K) (45), which itself is inactive when phosphorylated on its Ser-366 residue (5). Because calcineurin is a Ca2+/calmodulin-dependent phosphatase that could be potentially involved in the regulation of both proteins, we tested whether FK506 had an effect on their phosphorylation status. CCK at stimulatory (100 pM) and higher concentrations dephosphorylated eEF2 to ~25–30% of basal levels in pancreatic acinar cells in vitro (51a). In the present study, the blockade of calcineurin with FK506 partially reversed the dephosphorylation induced by 100 pM CCK from 25.7 ± 5.0 to 66.0 ± 17.4% of control level (Fig. 11A). The same samples were also analyzed for the phosphorylation state of the eEF2 kinase. CCK at 100 pM increased eEF2K phos-

Concentrations of CCK. Our results showed that ~60% of the total eIF4E was phosphorylated in nonstimulated (basal) acini (Fig. 8A) (as indicated by the stronger band in the acidic pole), and this fraction increased with different increasing doses of CCK, reaching a plateau of 95% phosphorylation of the total protein from 100 pM to 10 nM CCK (Fig. 9A). Treatment with 100 nM FK506 had no effect on basal or 100 pM CCK-stimulated eIF4E phosphorylation, as indicated in the pooled data and in the representative IEF gel image (Fig. 9B). Thus calcineurin is not likely to be involved in the regulation of the phosphorylation/dephosphorylation events on eIF4E stimulated by CCK.

FK506 inhibits basal eIF2B activity and increases basal eIF2α phosphorylation. We also studied the effects of FK506 on eIF2B, the guanine-nucleotide exchange factor for eIF2, another key regulatory point of translation initiation (52). A previous study using isolated pancreatic acini showed that CCK did not increase eIF2B activity at stimulatory concentrations, but it decreased eIF2B activity at higher concentrations that inhibit protein synthesis (50). It has also been shown that the phosphorylation of eIF2α can account for the inhibition of eIF2B activity seen at high concentrations of CCK (50). Because eIF2B can be regulated by phosphorylation/dephosphorylation mechanisms on its own subunits and on the α-subunit of eIF2, we determined whether calcineurin could be involved in the regulation of these proteins by analyzing eIF2B activity and eIF2α phosphorylation state in acini treated with 100 nM FK506 before stimulation with CCK. Basal eIF2B activity averaged 0.13 ± 0.01 pmol/min and was not significantly affected by CCK. However, eIF2B activity was reduced to 55.2 ± 6.4% of control in the presence of FK506 in basal acini (Fig. 10A). After CCK stimulation in the presence of FK506, it was 73 ± 5.5% of control, which did not differ from results with FK506 alone. In correlation with these results, FK506 increased basal eIF2α phosphorylation to 172.8 ± 11.2% of control (Fig. 10B). These results suggest that calcineurin may be involved in the regulation of this important regulatory point of translation initiation in the basal state. However, this does not appear to result in a significant reduction in basal protein synthesis.

Fig. 7. Effects of FK506 on S6 phosphorylation (P). Phosphorylation of S6 on Ser-240/244 is in response to CCK in the absence or presence of 100 nM FK506. Inset: representative Western blots of phosphorylated and total S6. Values are means ± SE of 5 independent experiments. *P < 0.05 vs. basal group.

Fig. 8. Effects of FK506 on the formation of the eIF4F complex, measured as communoprecipitation of eIF4E and eIF4G. A: effect of different concentrations of CCK (from 10 pM to 10 nM) on eIF4E-eIF4G association. B: effect of 100 nM FK506 on stimulated (100 pM CCK) and nonstimulated acini. Insets: representative Western blots for eIF4G (A and B) and total eIF4E (B). A: Western blot shows duplicate samples for each CCK concentration except for 1 nM. Values are means ± SE of 4–6 independent experiments. *P < 0.05 vs. basal group; #P < 0.05 vs. control CCK-stimulated group.
Phosphorylation on Ser-366 to ~225% of basal. Treatment with FK506 did not modify phosphorylation levels in either basal or stimulated acini (Fig. 11B). These results indicate that calcineurin phosphatase activity is involved in CCK’s action to activate (dephosphorylate) eEF2 by a means other than the Ser-366 site on eEF2K.

**DISCUSSION**

The present study was designed to evaluate the role of calcineurin in the regulation of pancreatic acinar protein synthesis. The effects of the calcineurin inhibitors FK506 and CsA were studied in suspensions of dissociated rat pancreatic acini at concentrations shown to inhibit calcineurin in these cells. Acinar protein synthesis was maximally stimulated at 100 pM CCK, and both FK506 and CsA inhibited protein synthesis in a dose-dependent manner. Calcineurin inhibition with FK506 inhibited the phosphorylation of the eIF4E-binding protein (4E-BP1) and the formation of the eIF4F complex and reversed the dephosphorylation of eEF2, while not having significant effects on the activity of several other translation factors. Thus calcineurin appears to be required for the stimulation of pancreatic acinar protein synthesis through the regulation of the eIF4F complex formation and the activity of eEF2 (Fig. 1).

Pancreatic protein synthesis is mainly stimulated through the activation of the PI3K/Akt/mTOR pathway (51, 57), but it can likely be modulated through other pathways. The involvement of calcineurin in protein synthesis has been reported in other tissues as a result of the side effects of the use of immunosuppressants to block organ transplant rejection (7–10). In addition to their effect inhibiting the production of interleukin 2 and other lymphokines by T-helper cells (10), CsA and FK506 induce a series of secondary effects in different tissues that have been the object of study. The blockade of renal, cardiac, or hepatic protein synthesis has been described (8, 9), but the activation of S6K and the induction of a hypertrophic response after chronic treatments have also been observed (35). The effects of CsA and FK506 on protein synthesis appear to be organ and immunosuppressant dependent. Thus CsA inhibits translation in microsomes from rat kidney and heart, stimulates it in microsomes from rat liver (8, 9), and has no effect on vascular smooth muscle protein synthesis (49).

In this study, using pancreatic acinar cells in suspension, we established that the immunosuppressants FK506 and CsA inhibited calcineurin activity and the concentration at which this occurred. For that, we used CRHSP-24, the only known substrate for calcineurin in pancreatic acinar cells (21, 22), as an additional tool to study the role of calcineurin in this model.
intracellular indicator. CRHSP-24 function is unknown, and preliminary experiments have shown no effect on basal or serum-stimulated protein synthesis when transfected into HEK293 cells (S.-H. Lee and J. A. Williams, unpublished observations). Our results showed that both FK506 and CsA inhibited CRHSP-24 dephosphorylation in a dose-dependent manner, with FK506 being at least 10-fold more potent.

Both FK506 and CsA inhibited protein synthesis at concentrations that inhibited calcineurin, but there were several major differences in their inhibitory profile. FK506 significantly inhibited CCK-stimulated protein synthesis while having only minimal effects on basal protein synthesis. This suggests a role for calcineurin in the stimulatory mechanism rather than the basal translational machinery. CsA at 100 nM partially blocked CCK-stimulated protein synthesis with minimal (but significant) effect on basal synthesis, consistent with partial inhibition of calcineurin at this concentration. At higher concentrations, 10 nM to 1 μM, CsA significantly inhibited basal protein synthesis and blocked CCK stimulation. This suggests the possibility of an action of CsA distinct from that of FK506; effects of CsA on LDH release, K⁺ channels, and the mitochondrial permeability transition pore have been reported (1, 20, 59). Thus FK506 appears to be a more specific tool to selectively inhibit calcineurin in acinar cells.

We confirmed that FK506 was not inhibiting protein synthesis through blockade of the CCK receptor by using other pancreatic secretagogues, known to stimulate secretion and protein synthesis in acini in vitro through distinct membrane receptors (16, 51). Both CCh, which acts via m3 muscarinic receptors, and BBS, which acts through a neuromedin C receptor, generally activate similar intracellular mechanisms, as does CCK in pancreatic acinar cells, increasing cytoplasmic Ca²⁺ and diacylglycerol (16, 58). Because their stimulation of protein synthesis was blocked by FK506, similar to the blockade of CCK, FK506 then seems to block distinct intracellular events related to calcineurin activation rather than initial steps in transmembrane signaling by the CCK receptor. This is consistent with an earlier report, where it was concluded that FK506 did not inhibit the CCK receptor in rat pancreas (13). Moreover, the fact that FK506 does not block signaling pathway intermediates, such as mTOR, S6K, and the eIF4E kinase, indicates that much of the intracellular signaling pathways are unaffected.

After establishing that acinar protein synthesis was dependent on calcineurin activity, we analyzed which key regulatory translation factors were affected by calcineurin. Surprisingly, only a few studies have evaluated the regulation of the translational machinery by calcineurin (9, 37), despite the fact that calcineurin has been involved in several processes of cell growth (19, 36) and activation of protein synthesis is required for growth. It has been demonstrated that the PI3K/Akt/mTOR pathway is a major stimulatory pathway for protein synthesis, and that it stimulates the phosphorylation and activation of the ribosomal protein S6K (46), the phosphorylation of the eIF4E binding protein 4E-BP1 (17), and the formation of the eIF4F complex (Fig. 1) (2, 3, 47). Our results indicated no effect of FK506 on the mTOR phosphorylation status (downstream of Akt) or on the ribosomal protein S6 phosphorylation (Fig. 7). This is different from the rabbit heart, in which chronic FK506 treatment increases S6K activity (35), but similar to other cell types, in which no effect on S6K was seen (40, 44). The lack of effect of FK506 on acinar mTOR and ribosomal protein S6 phosphorylation indicates that this pathway is independent of calcineurin action in pancreatic acinar cells.

On the other hand, the phosphorylation of 4E-BP1, which is also downstream of mTOR (Fig. 1), was inhibited by FK506. This result differs from studies in T cells in which this immunosuppressant was reported not to have any effect on 4E-BP1 (37). The role of calcineurin in regulating the phosphorylation of 4E-BP1 in acinar cells is not obvious. It has been described that mTOR may stimulate phosphorylation of 4E-BP1 indirectly by inactivating some phosphatases (PP2A, PP4, PP6) that would lead to the phosphorylation of 4E-BP1 (47), but it is not known how PP2A or these other phosphatases are regulated. It has also been described that 4E-BP1 phosphorylation can be dependent on calcium and calmodulin activation, independently of the PI3K/Akt/mTOR pathway activation (47), which could account for calcineurin activation in this process. Alternatively, FK506 could inhibit protein synthesis and translation effectors through an increase of intracellular Ca²⁺. FK506 binding protein and calcineurin interact under physiological conditions to modulate Ca²⁺ flux in
Calcineurin (PP2B) has an effect on eEF2 phosphorylation has not yet been established. Inactivated eEF2 is a substrate for PP2A (45), but whether calcineurin or eEF2K (Fig. 1) (5, 45). It has been described that phosphorylation after blockade of calcineurin by FK506 has no effect on basal protein synthesis (Fig. 3A). This research was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-52860 and DK-59578 (to J. A. Williams) and by the Michigan Gastrointestinal Peptide Center (DK-34933).

**REFERENCES**

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