Calmodulin-stimulated Protein Kinase Activity from Rat Pancreas

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ABSTRACT Previous work from our laboratory has demonstrated that neurohumoral stimulation of the exocrine pancreas is associated with the phosphorylation of the Mr 29,000 ribosomal protein S6. In a cell-free system using pancreatic postmicrosomal supernatant as the kinase donor, we found that the following co-factors stimulate the phosphorylation of the Mr 29,000 ribosomal protein: calcium with calmodulin, calcium with phosphatidyl serine, and cAMP. These findings suggest that the pancreas contains a calcium-calmodulin-dependent protein kinase (CaM-PK) that can phosphorylate the Mr 29,000 ribosomal protein. A CaM-PK activity was partially purified sequentially by ion exchange, gel filtration, and calmodulin-affinity chromatography. Phosphorylation of the Mr 29,000 ribosomal protein by the partially purified CaM-PK was dependent on the presence of both calcium and calmodulin and not on the other co-factors. The CaM-PK fraction contained a phosphoprotein of Mr 51,000 whose phosphorylation was also dependent on calcium and calmodulin. When 125I-calmodulin-binding proteins from the CaM-PK fraction were identified using electrophoretic transfers of SDS-polyacrylamide gels, a single Mr 51,000 protein was labeled. The preparation enriched in CaM-PK activity contained an Mr 51,000 protein that underwent phosphorylation in a calcium-calmodulin-dependent manner and an Mr 51,000 calmodulin-binding protein. It is therefore possible that the CaM-PK may comprise a calmodulin-binding phosphoprotein component of Mr 51,000.

Neurohumoral stimulation of the exocrine pancreas by agents such as carbachol and cholecystokinin results in alterations in protein phosphorylation (1-4) and intracellular calcium levels (5, 6). Recent studies from this laboratory have demonstrated that an Mr 29,000 ribosomal protein which has been putatively identified as S6 in pancreas undergoes phosphorylation in close association with secretagogue action. This phosphorylation is stimulated in situ in 32P-labeled pancreatic lobules by carbachol and cholecystokinin and in pancreatic homogenates by calcium and cAMP using [γ-32P]ATP as the phosphate donor (2-4). In this report we describe a calcium-calmodulin-dependent protein kinase (CaM-PK) in rat pancreatic postmicrosomal supernatant that phosphorylates the Mr 29,000 ribosomal protein. Partial purification of this CaM-PK activity yielded both an Mr 51,000 calcium-binding protein (CBP) and an Mr 51,000 phosphoprotein which underwent phosphorylation in a calcium-calmodulin-dependent manner. This CaM-PK activity resembled calmodulin-regulated kinases that have recently been characterized in liver using glycogen synthase as substrate (7, 8) and in brain using synapsin I (9) and tubulin (10) as substrates.

MATERIALS AND METHODS

Tissue Preparation: Pancreata were obtained from fed, male, Sprague-Dawley rats (150-200 g) following decapitation. The pancreata were homogenized in 0.3 M sucrose containing 0.1% soybean trypsin inhibitor (Worthington Biochemical Corp., Freehold, NJ) and 0.3 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO); postmicrosomal supernatant and deoxycholate-solubilized ribosomes were then prepared as previously described (2). Protein concentrations were determined fluorometrically using fluorescamine with BSA as a standard (11).

Assay for Protein Kinase Activity: Pancreatic ribosomes (25 µg) were used as a substrate. Ribosomes were mixed at 4°C with 20 mM Tris-maleate (pH 6.8), 2 mM EDTA, 1 mM EGTA, 10 mM MgCl₂, and, in some assays, with calmodulin (3.0 µM), final concentration, Calbiochem-Behring Corp., San Diego, CA) or phosphatidyl serine (~1 mM). [γ-32P]ATP (t1/2 ~10 µM, 2.0 Ci/mmol; New England Nuclear, Boston, MA) was then added to all tubes followed by either calcium (0.5 µmol added to the 100-µl incubation volume; estimated final calcium activity = 1 mM), 5 µM cAMP, 5 µM cGMP, or water. After a 60-s incubation at 37°C, the reaction was terminated by adding a solution of 5% SDS containing 30 mM EDTA and heating to 90°C, as previously described (3). Proteins were then resolved under reducing conditions by SDS-PAGE using 9.5% polyacrylamide (3); the degree of 32P-incorporation into different proteins was determined by autoradiography of dried gels.

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1 Abbreviations used in this paper: CaM-PK, calcium-calmodulin-dependent protein kinase; CBP, calmodulin-binding protein.
excision of labeled bands, and scintillation counting as described in reference 2.

Partial Purification of Protein Kinase Activity: Pancreatic postmicrosomal supernatant (~100 mg protein) was adjusted to a final concentration of 2 mM EDTA and 5 mM HEPES pH 7.4, and applied to a column of DEAE-cellulose (10-ml bed volume, DE52; Pierce Chemical Co., Rockford, IL) equilibrated with 5 mM HEPES containing 2 mM EDTA at pH 7.4. All subsequent buffers containing HEPES were 5 mM and pH 7.4. In preliminary studies, it was noted that calcium chelation improved the resolution of protein kinases eluted from DEAE cellulose. Proteins were eluted using solutions containing 0, 60, 100, 200, 300, and 500 mM NaCl in HEPES and 2 mM EDTA in 30-ml aliquots. The fraction from ion-exchange chromatography exhibiting maximal CaM-PK activity was concentrated (10-15 mg protein in 3-4 ml) by ultrafiltration (Amicon PM10, 20 psi N2; Amicon Corp., Scientific Systems Div, Danvers, MA) and further purified by gel filtration using a Biogel A1.5 m column (1.6 x 50 cm, flow rate 40 ml/h, 3-ml fractions; Bio-Rad Laboratories, Richmond, CA) eluted with 0.1 mM EDTA, 200 mM NaCl, and HEPES. Molecular weight standards for gel filtration included thyroglobulin, β-galactosidase, catalase, and ovalbumin. The addition of phenylmethylsulfonyl fluoride to pancreata prior to homogenization appeared to be necessary to preserve protein kinase activity during gel filtration.

Gel filtration fractions containing CaM-PK activity (<500 μg protein) were brought to a final calcium concentration of 0.5 mM and applied to a calmodulin-affinity column containing 6 mg of calmodulin (Calbiochem-Behring Corp.) coupled to 2 ml of agarose (Affigel 15, Bio-Rad Laboratories). Proteins were eluted from this column using solutions in sequence of 0.5 mM CaCl₂ (5 ml), 0.02 mM CaCl₂ (10 ml), and 2 mM EDTA (3 ml)—all in HEPES with 200 mM NaCl. Proteins were undetectable in the final kinase preparation by the fluorometric assay (11).

Identification of Calmodulin-binding Proteins: Iodinated calmodulin was prepared from Iodobenzene affinity-purified calmodulin using [125I]Bolton-Hunter reagent (2,000 Ci/mmol, New England Nuclear) as described by Chafouleas et al. (12). This preparation was selected because the product retains biologic activity measured by phosphodiesterase activation.

Proteins were resolved by SDS PAGE and electrophoretically transferred from polyacrylamide gels to a positively charged nylon membrane filter (Zeta-bind, AMG Specialty Materials Group/CUNO Division, Meriden, CT) under conditions described by Gershoni and Palade (13). The membrane filters were then quenched using 1% hemoglobin, 0.1% gelatin in quenching buffer (0.1 mM CaCl₂, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4) for 12 h at 45°C, and incubated with [125I]calmodulin (~5 x 10⁻¹⁵ mol or 1 μCi) in quenching buffer (100 ml) with 0.1% gelatin (12 h, room temperature). The filter was then washed extensively with quenching buffer, and CBP were demonstrated by autoradiography of the filter. One advantage of this method compared with identification of CBP directly in polyacrylamide gels (14) is that the specificity of [125I]calmodulin binding to immobilized proteins can be tested by sequentially incubating a single filter. CBP that bind [125I]calmodulin specifically release the probe when the filter is subsequently incubated in the absence of calcium (15).

Other Methods: Proteins were resolved on SDS PAGE and stained with Coomassie Blue as described in reference 2 or silver stain as described by Merril et al. (16).

RESULTS

Co-factor Dependence of Protein Kinase Activity in Postmicrosomal Supernatant

When pancreatic ribosomes and postmicrosomal supernatant were incubated together with different potential second messengers, there were striking changes in the pattern of protein phosphorylation, as depicted in Fig. 1. Previous studies of protein phosphorylation in pancreatic homogenates and lobules have demonstrated that [³²P]-incorporation into the M₉ 29,000 phosphoprotein is closely associated with secretagogue action and that this M₉ 29,000 protein is ribosomal protein S6 (3). As shown in Fig. 1, [³²P]-incorporation into the M₉ 29,000 polypeptide in our reconstituted membrane-free system is markedly increased in the presence of calcium with calmodulin, calcium with phosphatidyl serine, and cAMP. Increased individually, calmodulin, phosphatidyl serine, calcium, or cGMP had no significant effect. Several other protein bands including a principal M₉ 51,000 protein also exhibit phosphorylation which is activated by these different co-factors. We will focus on the protein kinase activity that phosphorylates the M₉ 29,000-protein because this corresponds to an identified protein whose phosphorylation is closely associated with secretagogue action in situ and is calcium-dependent in pancreatic homogenates (4). This M₉ 29,000 phosphoprotein represents ribosomal protein substrate, while the M₉ 51,000 protein is derived from postmicrosomal supernatant (see below) and may represent the CaM-PK.

Partial Purification of CaM-PK Activity

Fig. 2 depicts the pattern of [³²P]-incorporation during incubation of ribosomes with the maximally active kinase fractions following each of the chromatographic steps described in Materials and Methods. Each panel exhibits the kinase activity in the presence and absence of the combination of calcium and calmodulin. Ion-exchange chromatography permitted partial resolution of protein kinase activities with different co-factor dependencies. Both the calcium-calmodulin- and the cAMP-dependent components of kinase activity responsible for the phosphorylation of the M₉ 29,000 protein eluted from DEAE cellulose at 200 mM NaCl, whereas other
FIGURE 2 Autoradiography of CaM-PK activity obtained by sequential chromatography steps. Fractions containing the peak of the CaM-PK directed toward the Mr 29,000 ribosomal protein from each chromatography step were mixed with ribosomes (25 µg) and reacted as described in Materials and Methods in the presence or absence of calcium (~1 mM) with calmodulin (3.0 µM) for 60 s. Phosphorylated proteins were separated under reducing conditions and autoradiography was performed as described in Materials and Methods. The arrow indicates the Mr 29,000 ribosomal phosphoprotein and the arrowhead the Mr 51,000 phosphoprotein contained in the fractions with CaM-PK activity. Protein loaded per lane: ribosomes, 8 µg; DEAE, 3 µg; A1.5 and CaM-protein undetectable, 15 µl added. Exposure: 22 h at room temperature.

Protein kinase activities dependent on calcium-phosphatidyl serine or independent of calcium eluted at higher NaCl concentrations (not shown). The active fraction after ion-exchange chromatography exhibits calcium-calcmodulin-dependent phosphorylation of several protein bands, in addition to the Mr 29,000 phosphoprotein.

The 200-mM NaCl fraction was then used to further resolve the CaM-PK by gel filtration on Biogel A1.5 m. Maximal calcium-calcmodulin-dependent Mr 29,000 protein kinase activity eluted with an apparent Mr of ~500,000. This value closely agrees with that obtained for a CaM-PK from liver (7). As depicted in Fig. 2, this fraction contains the Mr 29,000 ribosomal protein which, in addition to the Mr 29,000 ribosomal protein, undergoes phosphorylation in the presence of calcium and calcmodulin. This phosphorylation of both the Mr 29,000 and the Mr 51,000 bands did not occur in the presence of calcium or calcmodulin alone (not shown).

Affinity chromatography was the final step in the partial purification of this protein kinase activity. The kinase-containing fraction following gel filtration was applied to the calmodulin-affinity column and equilibrated as described in Materials and Methods to a concentration of 20 µM calcium. CaM-PK activity was eluted with 2 mM EDTA. It was again noted (Fig. 2) that an Mr 51,000 protein exhibiting calcium-calcmodulin-dependent phosphorylation was present in the affinity-purified fraction that phosphorylated the Mr 29,000 ribosomal protein.

The CaM-PK assay was adjusted so that it was linear with respect to time and CaM-PK concentration (data not shown). The enzyme activity was calculated on the basis of 32P incorporation into the Mr 29,000 ribosomal protein (see Materials and Methods). Based on the CaM-PK activity in postmicrosomal supernatant, the recovery was 47, 5, and 3% from ion exchange, gel filtration, and affinity chromatography, respectively (see Table I).

The CaM-PK eluted from the calmodulin-affinity column was lyophilized and subjected to SDS PAGE under reducing conditions. Silver stains (16) of this preparation demonstrated three principal polypeptide bands of Mr 51,000, 56,000, and 68,000 (not shown). In some preparations, bands of Mr 43,000 and 53,000 were also observed.

**Summary of the Partial Purification of CaM-PK from Pancreas**

| Fraction              | Protein | Total activity (µmol P/min) | Specific activity (µmol P/mg/min) | Recovery |
|-----------------------|---------|----------------------------|----------------------------------|----------|
| Postmicrosomal supernatant | 130     | 540                        | 4.1                              | 100      |
| DEAE                  | 8.4     | 250                        | 30                               | 47       |
| A1.5m                 | 0.45    | 27                         | 60                               | 5        |
| Calmodulin affinity   | *       | 15                         | *                                | 3        |

*Protein not detectable by fluorescamine assay but estimated to be 0.01 mg by silver stain; approximate specific activity 1,500 µmol P/mg/min.

Co-factor Dependence of the Phosphorylation of the 29,000- and 51,000-dalton Proteins by the Partially Purified Protein Kinase

When the kinase preparation was incubated with ribosomes, phosphorylation of the Mr 29,000 protein and of other proteins occurred in the presence of the combination of calcium and calcmodulin, but not in the presence of calcium alone, calcium with phosphatidyl serine, cAMP (Fig. 3, left), or cGMP (not shown). During these incubations, phosphorylation of the Mr 51,000 protein was also dependent on calcium and calcmodulin; the combination of calcium and phosphatidyl serine stimulated 32P incorporation into the Mr 51,000 protein to a small degree. When the kinase preparation was incubated without ribosomes, phosphorylation of the Mr 51,000 protein exhibited the same co-factor dependence (Fig. 3, right). The Mr 51,000 phosphoprotein was a wide band, extending to Mr 53,000 and appearing as a doublet in some experiments. Since this protein kinase activity was both activated by calcmodulin and adhered to a calcmodulin-affinity column, it is likely that this protein kinase specifically bound
calmodulin. For this reason the kinase-containing fractions were examined for CBP.

**CBP in Pancreatic Postmicrosomal Supernatant and Kinase-containing Fractions**

Preliminary experiments demonstrated that pancreas homogenate and postmicrosomal supernatant contain many proteins that bind calmodulin, five of which were specific (i.e., calmodulin binding was reversible during incubation of filters with EDTA following $^3$H-calmodulin binding). Of these five specific CBP, two CBP ($M_r$ 51,000, $M_r$ 55,000) were predominant in the kinase fraction derived from the ion-exchange column chromatography (Fig. 4). Following gel filtration and affinity chromatography, a single CBP at $M_r$ 51,000 was detected during the kinase preparation. This $M_r$ 51,000 CBP co-migrated (by SDS PAGE) with the lower portion of the $M_r$ 51,000 phosphoprotein contained in the CaM-PK preparation (not shown).

**DISCUSSION**

This report describes the partial purification of a CaM-PK from rat pancreas. These investigations were undertaken because of the observation in our laboratory (2-4) and others (1) that neurohumoral stimulation of the exocrine pancreas is associated with a specific pattern of protein phosphorylation. This pattern of phosphorylation can be produced in pancreatic homogenates by the addition of calcium or cAMP (4), indicating that different classes of protein kinases may be active in this system. Since the protein calmodulin appears to mediate many aspects of calcium-directed cellular metabolism, we examined its potential role as a regulator of pancreatic protein kinase activity.

The CaM-PK from pancreas has been detected using a membrane-free preparation of pancreatic ribosomes as substrate. Ribosomes were chosen as a model substrate because ribosomal protein S6 undergoes phosphorylation in close association with in situ neurohumoral stimulation of the exocrine pancreas (2, 3). In addition, purified pancreatic ribosomes possess negligible endogenous protein kinase activity.

When pancreatic postmicrosomal supernatant and ribosomes were combined, we observed calcium-calmodulin-, calcium-phospholipid-, and cAMP-dependent protein kinase activities. Previous studies have demonstrated cAMP (17) and cGMP (18) protein kinase activities that can utilize ribosomal proteins as substrate. This study demonstrates that ribosomal proteins can be phosphorylated by calcium-dependent protein kinases that are mediated by calmodulin and by phosphatidyl serine. The predominant ribosomal protein substrate for these kinase activities had a mass of $M_r$ 29,000; on the basis of
previous studies from this laboratory, we believe that this Mr 29,000 protein is ribosomal protein S6 (3).

Protein kinase activity in the pancreas has been examined by Wrenn et al. (19). While that group detected a calcium-phospholipid-dependent protein kinase activity in rat pancreas, they did not detect a calcium-calmodulin-stimulated protein kinase activity. The apparent discrepancy between that study and our findings may be due to differences in use of protease inhibitors, rapidity of tissue preparation, or ATP concentrations during kinase assays.

The purification protocol used for the protein kinase described here is based on the properties of calmodulin-dependent protein kinases that have been characterized from brain and liver. These kinases are slightly acidic, are of masses of 200,000 to 500,000, and apparently bind calmodulin in a calcium-dependent manner (7–10). With this in mind, we selected ion exchange using DEAE cellulose, gel filtration using Biogel A1.5 m, and finally affinity chromatography using immobilized calmodulin for our partial purification of the calmodulin-dependent kinase activity from rat pancreatic postmicrosomal supernatant. This protocol resulted in kinase activity that is stimulated by calcium with calmodulin but that is not stimulated by calcium with phosphatidyl serine or by cAMP.

The partially purified protein kinase fraction from the calmodulin-affinity column has two important properties. First, the fractions exhibiting CaM-PK activity contained a Mr 51,000 polypeptide that underwent phosphorylation in a calcium-calmodulin-dependent manner. Second, this partially purified CaM-PK fraction contained an Mr 51,000 protein that bound 45Ca-calmodulin in a calcium-dependent manner. Since the CaM-PK was specifically retained on a calmodulin-affinity column and since its activity exhibited calmodulin dependence, it is likely that a portion of the CaM-PK can bind calmodulin. The single Mr 51,000 CBP of this CaM-PK fraction may represent the calmodulin-binding portion of this CaM-PK and in fact may be the Mr 51,000 phosphoprotein contained in our CaM-PK fraction. This suggests that calcium-calmodulin-dependent phosphorylation and calmodulin binding may occur on the same polypeptide of this CaM-PK. Although the importance of this apparent calcium-calmodulin-dependent phosphorylation of the CaM-PK itself is unknown, one possibility is that it is involved in the regulation of the CaM-PK. An example of such a regulation has been described for heme-regulated eIF-2α kinase whose activity appears to be modulated through phosphorylation of a portion of the kinase (20).

Preliminary investigations from this laboratory and others suggest that a similar CaM-PK is found in tissues other than pancreas. We found a CaM-PK activity in rat parotid that can utilize an Mr 29,000 protein (presumably S6) as a substrate (data not shown). In addition, CaM-PK have been described that demonstrate physical characteristics similar to those of the CaM-PK from pancreas investigated here. These CaM-PK include a synapsin 1 kinase that is associated with both membrane and soluble fractions of the brain (9), a brain-derived tubulin kinase activity (10), and liver-derived glycogen synthase kinase activities (7, 8). The properties shared by these CaM-PK and our pancreatic CaM-PK are an apparent net positive charge, similar molecular weights (see Results), and phosphoproteins that range in Mr from 51,000 to 55,000 and appear to represent a portion of the CaM-PK undergoing autophosphorylation. Similar to the CaM-PK described here, the tubulin kinase activity demonstrates a Mr 55,000 calmodulin-binding protein (10). Moreover, the synapsin 1 kinase described by Kennedy et al. (9) and the glycogen synthase kinase of Payne et al. (8) are similar in their substrate recognition. Based on these observations it is possible that the CaM-PK activities described in liver, brain, and pancreas represent a closely related group of protein kinases with a widespread tissue distribution.

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**REFERENCES**

1. Burnham, D. B., and J. A. Williams. 1982. Effects of carbachol, cholecystokinin, and insulin on protein phosphorylation in isolated pancreatic acini. *J. Biol. Chem.* 257:10523–10528.
2. Freedman, S. D., and J. D. Jamieson. 1982. Hormone-induced protein phosphorylation. I. Relationship between secretogogue action and endogenous protein phosphorylation in intact cells from the exocrine pancreas and parotid. *J. Cell Biol.* 95:903–908.
3. Freedman, S. D., and J. D. Jamieson. 1982. Hormone-induced protein phosphorylation. II. Localization to the ribosomal fraction from rat exocrine pancreas and parotid of a 29,000-dalton protein phosphorylated in situ in response to secretogogues. *J. Cell Biol.* 95:909–917.
4. Freedman, S. D., and J. D. Jamieson. 1982. Hormone-induced protein phosphorylation. III. Regulation of the phosphorylation of the secretagogue-responsive 29,000-dalton protein by both Ca2+ and cAMP in vitro. *J. Cell Biol.* 95:1889–1923.
5. Schatz, I. 1980. Messenger role of calcium in function of pancreatic acinar cells. *Annu. Rev. Physiol.* 42:215–237.
6. Dormer, R. L., and J. A. Williams. 1981. Secretagogue-induced changes in subcellular Ca2+ distribution in isolated pancreatic acini. *Annu. Rev. Physiol.* 43:249–270.
7. Ahmad, Z., A. A. DePaul-Rouc, and D. M. Roue. 1982. Purification and characterization of a rabbit liver calmodulin-dependent protein kinase able to phosphorylate glycogen synthase. *J. Biol. Chem.* 257:8348–8355.
8. Payne, M. E., C. M. Schwartz, and T. R. Soderling. 1983. Purification and characterization of rabbit liver calmodulin-dependent glycogen synthase kinase. *J. Biol. Chem.* 258:2376–2382.
9. Kennedy, M. B., T. McGuiness, and P. Greenberg. 1983. A calmodulin/calmodulin-dependent protein kinase from mammalian brain that phosphorylates synapsin 1: partial purification and characterization. *J. Neurosci.* 3:818–831.
10. Goldenring, J. R., B. Gonzalez, and R. J. D’Lorenzo. 1982. Isolation of brain Ca2+-calmodulin tubulin kinase containing calmodulin-binding proteins. *Biochim. Biophys. Acta.* 720:331–335.
11. Ueda, S., S. Stein, P. Bohlen, W. Dairman, W. Leimgruber, and M. Weigle. 1972. Application of fluorescamine, a new reagent for assay of amino acids, peptides, protein and other primary amines in the picomole range. *Science (Wash. D.C.)* 178:871–872.
12. Chaouloff, J. G., J. R. Redman, R. P. Munjap, and A. R. Means. 1979. Calmodulin: development and application of a sensitive radioimmunoassay. *J. Biol. Chem.* 254:10262–10267.
13. Gershoni, J. M., and G. E. Palade. 1982. Electrophoretic transfer of proteins from sodium dodecyl sulfate—polyacrylamide gels to a positively charged membrane filter. *Anal. Biochem.* 124:396–405.
14. Carlin, R. R., D. J. Grab, and P. Sievelk. 1981. Function of calmodulin in postlysosomal densities. III. Calmodulin-binding proteins of the postlysosomal nascent. *J. Biol. Chem.* 256:489–495.
15. Gershoni, J. M., and G. E. Palade. 1983. Protein blotting: principles and applications. *Annu. Rev. Biochem.* 52:111–155.
16. Freedman, S. D., T. McGuiness, A. S. Sedman, and M. H. Fieret. 1981. Ultrafastive stain for proteins in polyacrylamide gels shows regions variation in centrifugoplal fluid proteins. *Science (Wash. D.C.)* 211:1437–1438.
17. Traugh, J. A., and G. T. Porter. 1976. A comparison of ribosomal proteins from rabbit reticulocytes phosphorylated in situ and in vitro. *Biochemistry.* 15:610–616.
18. Issinger, O. G., and H. Besser. 1980. Comparison of phosphorylation of ribosomal proteins from HeLa and Krebs II ascites-tumour cells by cyclic AMP-dependent and cyclic GMP-dependent protein kinases. *Biochemistry.* 19:883–99.
19. Honig, D. W., N. Ratoh, and J. F. Kuo. 1981. Stimulation by phospholipid of calcium-dependent phosphorylation of endogenous proteins from mammalian. *Biochem. Biophys. Acta.* 676:266–269.
20. London, M., V. Ernst, R. Fagard, A. Lenzou, D. H. Levin, and R. Petryshyn. 1981. Regulation of protein synthesis by phosphorylation and hem. *In Protein Phosphorylation* O. M. Rosen and E. G. Krebs, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 941–957.