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Identification of calmodulin-dependent protein kinase III and its major M_r 100,000 substrate in mammalian tissues

(calcium/pancreas/PC-12 cells/GH$_3$ cells)

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ABSTRACT **A major substrate, M_r 100,00 (100 kDa), for a Ca^{2+}/calmodulin (CaM)-dependent protein kinase found in many mammalian tissues has been purified from rat pancreas. The purified substrate was used to identify and partially purify a CaM-dependent protein kinase (CaM kinase III) from rat pancreas. The physical properties and substrate specificity of CaM kinase III were distinct from those of all known CaM-dependent protein kinases. Only CaM kinase III was able to phosphorylate the 100-kDa protein; synapsin I, phosphorylase *b*, myosin light chain, and histone were poor substrates for this enzyme. Polyclonal antibodies, raised against the purified 100-kDa protein, recognized the protein in a variety of mammalian tissues and cell lines. Immunoassay revealed that the 100-kDa protein made up 0.3–1.7% of the total cytosolic protein in these samples. Analysis of CaM kinase III revealed that the enzyme had a similar widespread tissue distribution. These results demonstrate the existence of a fifth CaM-dependent protein phosphorylation system present in high levels in animal cells.**

Calcium (Ca^{2+}) is widely recognized as an important intracellular messenger in mammalian tissues, achieving its effects by a number of different mechanisms (1, 2). In many instances, the actions of Ca^{2+} are mediated by Ca^{2+}-binding proteins, for example, calmodulin and troponin C (3, 4). Strong evidence indicates, in turn, that many of the effects of calmodulin may be mediated through the regulation of protein phosphorylation (5–7). Several distinct Ca^{2+}/calmodulin (CaM)-dependent protein kinases have been identified. These include myosin light-chain kinase (8–10); phosphorylase kinase (11, 12); a multifunctional CaM-dependent protein kinase referred to as CaM kinase II (13–19) that phosphorylates several proteins, among which are synapsin I (site II), glycogen synthase, microtubule-associated protein 2, and tyrosine hydroxylase; and CaM kinase I, which phosphorylates synapsin I (site I) (20, 21). In addition, a CaM-dependent phosphoprotein phosphatase, calcineurin, has been identified (22). In contrast to these well-characterized enzymes, several uncharacterized examples of CaM-dependent protein phosphorylation have also been described (23–27). We have previously detected a M_r 100,000 (100-kDa) substrate for a CaM-dependent protein kinase in a variety of mammalian tissues (28) and cell lines (29). In the present study the 100-kDa substrate has been purified to homogeneity and used to identify a specific CaM-dependent protein kinase, CaM kinase III, in mammalian tissues that is distinct from CaM-dependent kinases heretofore described.

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MATERIALS AND METHODS

Materials. CaM kinase I was purified from bovine brain (20), CaM kinase II was purified from rabbit heart (30) or rat brain (17) (the gift of Yvonne Lai), phosphorylase kinase was purified from rabbit skeletal muscle (31), myosin light-chain kinase was purified from rabbit skeletal muscle (10), and the catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart (32). The inhibitor of cAMP-dependent protein kinase (Walsh inhibitor) was purified by a minor modification of the procedure of Mcpherson *et al.* (33). Rabbit brain CaM (34), bovine brain synapsin I (35), and rabbit skeletal muscle and bovine heart myosin light chain (36) were prepared by established procedures. Smooth muscle myosin light-chain kinase and smooth muscle myosin light chain (turkey gizzard) were the gift of David Hathaway. Protein kinase C was the gift of Katherine Albert. [γ-^{32}P]ATP was from New England Nuclear and ^{125}I-labeled staphylococcal protein A was from Amersham. Histone f1 (type III), trypsin, phosphoserine, phosphothreonine, and phosphorylase *b* were obtained from Sigma and histone f2b was from Worthington. Leupeptin, antipain, and chymostatin were obtained from Chemicon. Trasylol was obtained from Mobay, FBA Pharmaceuticals (New York). Phenylmethylsulfonyl fluoride was obtained from Calbiochem–Behring.

Phosphorylation Assays. Assays were performed at 30°C in a reaction mixture (final volume 100 μl) containing: 50 mM Hepes (pH 7.6), 10 mM magnesium acetate, 5 mM dithiothreitol, CaM at 10 μg/ml, either 1 mM EGTA (minus Ca^{2+}) or 1 mM EGTA/1.5 mM $CaCl_2$ (plus Ca^{2+}), leupeptin at 10 μg/ml, Walsh inhibitor at 2 μg/ml, and 50 μM [γ-^{32}P]ATP (200–500 cpm/pmol). Endogenous phosphorylation assays were performed with 40–80 μg of cytoplasmic extract. Tissue distribution of CaM kinase III was determined with 10 μg of purified 100-kDa protein and 5 μg of cytoplasmic extract. Substrate specificity assays and assays of myosin light-chain kinase, CaM kinase I, and CaM kinase II were performed with 10 μg of the respective substrate and amounts of purified enzyme or column fraction that gave linear activity with respect to enzyme concentration. Reactions were initiated by the addition of [^{32}P]ATP, carried out for 1–2 min, terminated by the addition of a $NaDodSO_4$-containing "stop" solution, and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. For quantitation of phosphorylation of substrates, proteins were localized by Coomassie blue staining and autoradiography and were excised from the dried gel, and ^{32}P radioactivity was measured by liquid scintillation spectrometry.

Preparation of Tissue Extracts. Cytoplasmic extracts were prepared from various rat tissues as described (28) except that the buffer contained 20 mM Tris·HCl (pH 7.6), 1 mM EDTA, 1 mM EGTA, 15 mM 2-mercaptoethanol, 0.02%

Abbreviation: CaM, calmodulin.

NaN_3, 100 mM NaCl, leupeptin at 10 μg/ml, antipain at 10 μg/ml, Trasylol at 10 μg/ml, and 100 μM phenylmethylsulfonyl fluoride.

Preparation of Antibodies to the 100-kDa Protein. Purified pancreatic 100-kDa protein was emulsified with Freund's complete adjuvant and injected at multiple intradermal sites into female New Zealand White rabbits (100 μg per animal). Injections were repeated at 2-week intervals and antiserum was obtained after 5 weeks. The specificity of the antiserum was tested by immunoblotting using the method as described (37) (see Fig. 4).

Other Procedures. $NaDodSO_4$/polyacrylamide gel electrophoresis was carried out by using the method of Laemmli (38). Isoelectric focusing and two-dimensional polyacrylamide gel electrophoresis were carried out by using the method of O'Farrell (39). Two-dimensional thin-layer phosphopeptide mapping and phospho-amino acid analysis were performed as described (28).

RESULTS

Identification of the 100-kDa Protein and CaM Kinase III in Rat Pancreas. Pancreas was chosen as source material because of the high levels of CaM-dependent phosphorylation of the 100-kDa protein in this tissue (these studies) compared to all other tissues previously examined (28). The 100-kDa protein was identified by Coomassie blue stain, after two-dimensional isoelectric focusing/$NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1*A*), as a prominent cytosolic protein that migrated with an isoelectric point of ≈7.0. Incubation of rat pancreas cytosol in the presence of Mg[^{32}P]ATP, Ca^{2+}, and CaM resulted in phosphorylation of the 100-kDa protein. All of the ^{32}P label was associated with a Coomassie blue-stained species of the 100-kDa protein that migrated with a more acidic isoelectric point of ≈6.8. Maximal phosphorylation (Fig. 1*C*) and change in migration of the 100-kDa protein (Fig. 1*B*) were obtained in less than 2 min. The change in isoelectric point of the phosphorylated form of the 100-kDa protein was presumably a result of the incorporation of stoichiometric amounts of phosphate into the protein. This assumption was supported by studies using the purified 100-kDa protein (see below).

These results showed the presence in rat pancreas cytosol of high levels of a CaM-dependent protein kinase that could phosphorylate the endogenous 100-kDa protein. It became clear, from reconstitution experiments using partially purified 100-kDa protein, purified CaM-dependent protein kinases, and tissue extracts, that this enzyme was distinct from previously characterized CaM-dependent protein kinases. As described below, we have purified the 100-kDa protein to homogeneity and have used the purified protein to identify and partially purify this protein kinase, which we have named CaM kinase III.

Purification of the 100-kDa Protein. The 100-kDa protein was assayed either by its ability to be phosphorylated by endogenous and exogenously added partially purified CaM kinase III, or by Coomassie blue staining of $NaDodSO_4$/polyacrylamide gels. In brief, the purification of the 100-kDa protein was as follows (further details of this procedure will be described elsewhere). A crude supernatant fraction from rat pancreas was fractionated with ammonium sulfate, and the proteins precipitating between 33% and 60% saturation were collected. This fraction was subsequently purified by chromatography on hydroxylapatite, DEAE-Sephacel, Affi-Gel blue, and Ultrogel AcA 44. The 100-kDa protein, which eluted with a M_r of 95,000 on the gel filtration column, was judged pure by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 2*A*). The 100-kDa protein did not bind to CaM-Sepharose and does not appear to be a CaM-binding protein (data not shown).

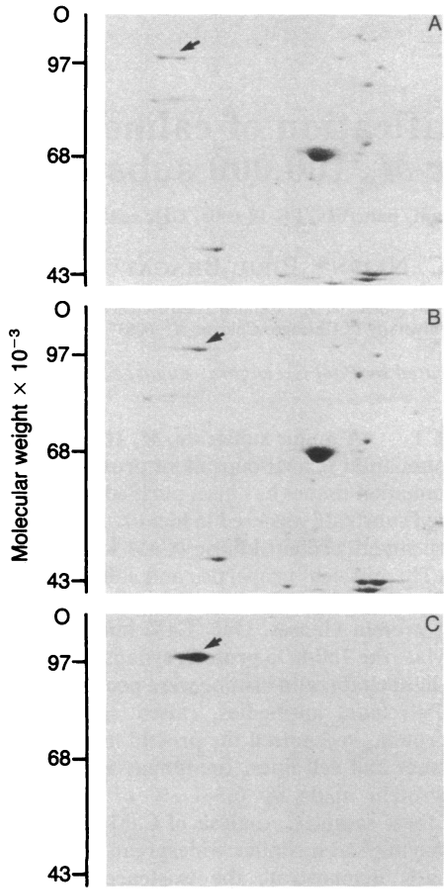

FIG. 1. Two-dimensional isoelectric focusing/$NaDodSO_4$/polyacrylamide gel of cytosol from rat pancreas, after incubation for 2 min with MgATP: Coomassie blue stain in the absence (*A*) and the presence (*B*) of Ca^{2+} and CaM, and corresponding autoradiogram (*C*) in the presence of Ca^{2+} and CaM. The arrow indicates the 100-kDa protein. The pH gradient ranged from 7.5 on the left of the gel to 5.5 on the right. O, origin.

The final product was identified on the basis of its comigration with the endogenous 100-kDa protein from pancreas cytosol when two-dimensional gel electrophoretic methods were used (e.g., Fig. 1) and by its ability to be phosphorylated in a CaM-dependent manner by extracts from a number of tissues (Table 2) or by partially purified CaM kinase III (Fig. 2*A*). The purified 100-kDa protein could be phosphorylated by partially purified CaM kinase III with a stoichiometry of ≈1 mol/mol (data not shown). Phosphorylation of the purified protein, like that found in the cytosolic extract (Fig. 1), resulted in a species that migrated with a more acidic isoelectric point on two-dimensional isoelectric focusing/$NaDodSO_4$/polyacrylamide gel electrophoresis (data not shown). In addition, the phosphopeptide generated by trypsin from the purified 100-kDa protein migrated with that derived from the protein phosphorylated in crude extracts (Fig. 2*B*; cf. ref. 28) and was found to contain only phosphothreonine as previously described (Fig. 2*C*; cf. ref. 28).

The purified 100-kDa protein was assayed as a substrate for a variety of purified CaM-dependent protein kinases (Table 1). No significant phosphorylation of the 100-kDa protein was obtained with smooth muscle or skeletal muscle myosin light-chain kinase, phosphorylase kinase, CaM kinase I, and CaM kinase II. In addition, the 100-kDa protein was not a substrate for cAMP-dependent protein kinase or protein kinase C.

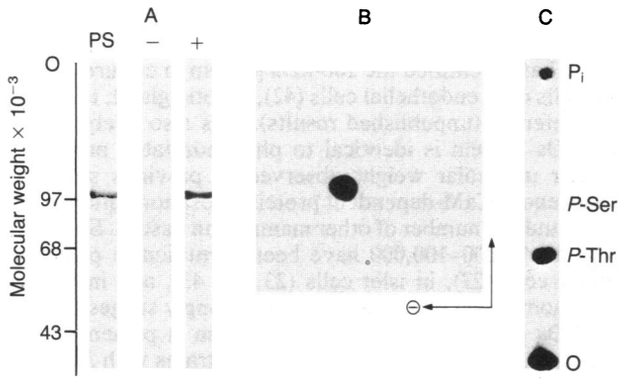

FIG. 2. (*A*) Coomassie blue stain of NaDodSO$_4$/polyacrylamide gel of purified 100-kDa protein from rat pancreas and corresponding autoradiogram of the protein phosphorylated with partially purified CaM kinase III. PS, protein stain; − and +, autoradiogram after phosphorylation in the absence and presence of Ca^{2+} plus CaM, respectively. (*B*) Autoradiogram of two-dimensional tryptic phosphopeptide map of purified 100-kDa protein phosphorylated as in *A*. Electrophoresis was in the horizontal dimension (negative pole, left) and ascending chromatography was in the vertical dimension (o, origin). The single major phosphopeptide had a mobility identical to that of the 100-kDa protein phosphorylated in crude cytosol fractions (28). (*C*) Autoradiogram after one-dimensional phosphoamino acid analysis of the 100-kDa protein phosphorylated as in *A*; *P*-Ser, phosphoserine; *P*-Thr, phosphothreonine; only threonine residues were phosphorylated.

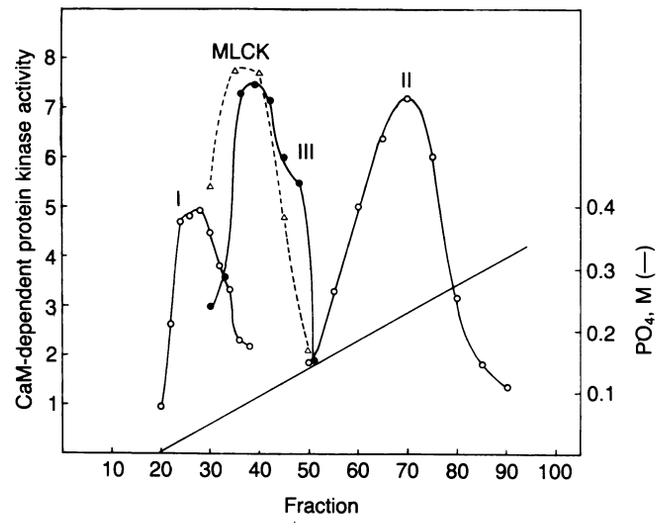

FIG. 3. Hydroxylapatite chromatography of CaM-dependent protein kinase activities present in rat pancreas cytosol. Crude cytosol was applied to a hydroxylapatite column, which was then washed with 2 M NaCl and developed with a 0–0.4 M gradient of KP_i. The four CaM-dependent kinase activities were assayed with purified 100-kDa protein (●—●), synapsin I (○—○), and smooth muscle myosin light chain (△---△). Protein kinase activities are also indicated as follows: I, CaM kinase I; II, CaM kinase II; III, CaM kinase III; MLCK, myosin light-chain kinase. The activities of the four protein kinases were normalized for convenience and do not reflect the relative activities. Fractions were diluted such that there was no phosphorylation of endogenous substrates. All kinase activities were dependent on Ca^{2+} and CaM. The phosphorylation of site I and site II of synapsin I by CaM kinase I and CaM kinase II, respectively, was measured after one-dimensional peptide mapping of synapsin I with *Staphylococcus aureus* V8 protease (40).

Partial Purification and Substrate Specificity of CaM Kinase III. A crude cytosolic fraction from rat pancreas was applied to a hydroxylapatite column and the proteins were eluted with a gradient of potassium phosphate (KP_i). A peak of CaM kinase III was obtained that eluted at ≈0.1 M KP_i (Fig. 3); at this and all later stages of purification, enzyme activity was completely dependent on the addition of both Ca^{2+} and CaM. The elution profile of CaM kinase III was distinct for that of CaM kinases I and II (assayed with synapsin I) and myosin light-chain kinase (assayed with smooth muscle myosin light chain) (Fig. 3). The elution of the kinase was also distinct from that of the 100-kDa protein, which eluted at ≈0.15 M KP_i (data not shown).

CaM kinase III from rat pancreas was further purified by chromatography on DEAE-Sephacel, Ultrogel AcA 44, and CaM-Sepharose. The enzyme was completely separated from the 100-kDa protein after chromatography on DEAE-Sephacel and eluted with a M_r of 140,000 on the gel filtration column (data not shown). While substantial enrichment of CaM kinase III was obtained by using this procedure, the final enzyme preparation contained a number of proteins, the identities of which have not yet been determined.

Investigation of the substrate specificity of partially purified CaM kinase III revealed that of the exogenous substrates tested, only the 100-kDa protein was phosphorylated (Table 1). Significantly, synapsin I and phosphorylase *b* were completely inactive as substrates, and smooth muscle myosin light chain was a poor substrate.

Quantitation of the 100-kDa Protein and CaM Kinase III Activity in Mammalian Tissues. Polyclonal antibodies, raised against purified 100-kDa protein, recognized a M_r 100,000 protein in immunoblot analysis of a variety of mammalian tissues and cell lines (Fig. 4). The antibodies also recognized species of lower molecular weight in some tissue extracts (e.g., see pancreas in Fig. 4, lane 2); aging of extracts on ice led to an increase in these species, suggesting that they were

Table 1. Comparison of substrate specificity of CaM kinase III with that of other Ca^{2+}-dependent protein kinases

	Kinase activity, % of maximum						
Substrate	CaM kinase III	Smooth muscle myosin light-chain kinase	Skeletal muscle myosin light-chain kinase	Phosphorylase kinase	Protein kinase C	CaM kinase I	CaM kinase II
100-kDa	100	0.03	1.0	1.1	2.1	0	1.2
Synapsin I	0.75	0.9	1.1	—	—	100	100
Phosphorylase *b*	0.1	—	—	100	—	—	—
Smooth muscle myosin light chain	5.5	100	11.6	—	—	—	—
Skeletal muscle myosin light chain	0.25	7	100	—	—	—	—
Cardiac muscle myosin light chain	0.72	18.7	54.7	—	—	—	—
Histone f1	0.79	—	—	—	100	—	—
Histone f2b	0.56	—	—	—	—	—	—

Enzyme activities were determined under optimal and linear conditions; 10 μg of each substrate was used and ATP was present at a final concentration of 50 μM. Assays were performed at 30°C for 2 min and terminated with NaDodSO$_4$ stop solution. Substrate phosphorylation was assayed by gel electrophoresis. Results are expressed as percent of the activity with the best substrate for each enzyme. —, Not determined.

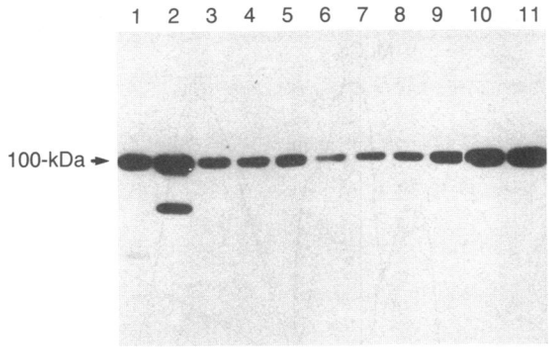

FIG. 4. Immunological identification of the 100-kDa protein in various rat tissues: total cytosolic protein from various tissues was separated on $NaDodSO_4$/polyacrylamide gels and transferred to nitrocellulose. The nitrocellulose sheet was incubated with buffered 0.25% gelatin to block nonspecific binding sites prior to exposure to antiserum against 100-kDa protein (diluted 1:100) followed by exposure to ^{125}I-labeled protein A. The lanes contained 30 μg of cytosolic protein from the following sources: 1, spleen; 2, pancreas; 3, heart; 4, skeletal muscle; 5, liver; 6, lung; 7, kidney; 8, cerebrum; 10, PC-12 cells; 11, GH_3 cells. Lane 9 contained 0.2 μg of pure 100-kDa protein.

proteolytic products of the original 100-kDa protein (data not shown). The antibodies to 100-kDa protein were used with standard ELISA techniques to measure the levels of the 100-kDa protein in cytosol from a number of mammalian tissues (Table 2). The level of the 100-kDa protein was highest in pancreas (≈1.7% of total cytosolic protein) with the levels in other tissues being relatively even; of the tissues so far investigated only erythrocytes appeared to be devoid of immunoreactivity (data not shown). The activity of CaM kinase III was also measured in cytosol from various mammalian tissues (Table 2). Enzyme activity was highest in pancreas, and the level in other tissues generally paralleled that of the 100-kDa protein. In all tissues examined the exogenously added 100-kDa protein was phosphorylated on a single threonine residue (data not shown). In addition, the levels of both the 100-kDa protein and CaM kinase III in particulate fractions were less than 5% of the levels found in cytosolic fractions.

DISCUSSION

A protein of M_r 100,000 (the 100-kDa protein), which is a major substrate for a CaM-dependent protein kinase in many mammalian tissues, has been purified to homogeneity from rat pancreas. The purified substrate has been used to identify and partially purify a CaM-dependent protein kinase that specifically phosphorylates this protein. The substrate specificity, physical properties, and tissue distribution suggest that this enzyme is distinct from the previously described CaM-dependent protein kinases, namely myosin light-chain kinase, phosphorylase kinase, CaM kinase I, and CaM kinase II. We have named this enzyme CaM-dependent protein kinase III (CaM kinase III).

The 100-kDa protein and CaM kinase III are widely distributed in mammalian tissue, with particularly high concentrations of both substrate and enzyme being present in pancreas. The identification of the 100-kDa protein in rat pancreas cytosol, by Coomassie blue staining of $NaDodSO_4$/polyacrylamide gels, confirms that the 100-kDa protein is present in high concentrations. The results of the tissue distribution studies (Table 2), and the fact that the 100-kDa protein is rapidly and stoichiometrically phosphorylated in tissue extracts from pancreas (Fig. 1*A*), suggest that CaM kinase III is also present in high concentration in pancreas and other tissues. Given the substrate specificity of CaM kinase III, and the failure of other protein kinases to phosphorylate the 100-kDa protein, it is probable that the interaction between these two proteins is relatively specific.

We have identified the 100-kDa protein in cultured epithelial cells (41), endothelial cells (42), parotid gland, testis, and vas deferens (unpublished results). It is also likely that the 100-kDa protein is identical to phosphorylated proteins of similar molecular weight observed in previous studies of endogenous CaM-dependent protein phosphorylation in pancreas and in a number of other mammalian tissues. Substrates with M_r 92,000–100,000 have been identified in pancreatic acinar cells (27), in islet cells (23, 24, 43), and in hamster insulinoma cells (44). These results strongly suggest that the 100-kDa protein–CaM kinase III system is present in both endocrine and exocrine pancreas. Substrates with M_r 94,000 have also been identified in rabbit ileum (25), rat lacrimal gland (26), and bovine chromaffin cells (45). The differences in molecular weight values in these studies probably reflect the different values taken for the molecular weight of phosphorylase *b*, which is 97,000, as determined from amino acid sequence (46). Our previous studies have shown conclusively that the 100-kDa protein is distinct from phosphorylase *b* (28).

We have also found that several mammalian cell lines contain the 100-kDa protein–CaM kinase III system (29). Recent work using immunoassay has confirmed that high levels (≈1–2% of total soluble protein) of the 100-kDa protein are present in GH_3 cells, PC-12 cells, and NG108-15 cells (unpublished results). It is probable that a phosphoprotein of M_r 97,000, termed S97, which was previously identified in GH_3 cells as a substrate for a CaM-dependent protein kinase (47), is the same as the 100-kDa protein. A M_r 100,000 phosphoprotein (termed Nsp 100), whose phosphorylation is inhibited by treatment with nerve growth factor, has also been found in PC-12 cells (48, 49). The physical properties of Nsp 100, which is phosphorylated on a threonine residue, and its specific protein kinase (48, 50) are, respectively, very similar to those described in this study for the 100-kDa protein and CaM kinase III. However, Guroff and his co-workers have not shown an effect of Ca^{2+} or CaM on the phosphorylation of Nsp 100 (48, 50). In view of the high levels of the 100-kDa protein in PC-12 cells and the similar physical properties of the two proteins, it is possible that Nsp 100 may be the same as the 100-kDa protein.

The widespread distribution of the 100-kDa protein and CaM kinase III suggests that this phosphorylation system may play an important role in mediating the intracellular effects of Ca^{2+} in mammalian cells. Phosphorylation of the 100-kDa protein appears to be under physiological control. Increases in intracellular Ca^{2+}, stimulated in intact cells by depolarizing agents and ionophore A23187, result in phos-

Table 2. Levels of the 100-kDa protein and CaM kinase III in rat tissues

Tissue	100-kDa protein, μg/mg of protein	CaM kinase III, pmol/min per mg of protein
Pancreas	16.5	470
Skeletal muscle	9.5	160
Adrenal gland	ND	60
Liver	5.3	37
Spleen	3.1	31
Forebrain	3.4	26
Lung	4.6	25
Heart	5.1	18
Kidney	3.1	17
Cerebellum	ND	13

Levels of the 100-kDa protein were determined by using a standard ELISA assay. CaM kinase III activity was determined by the standard phosphorylation assay, using 10 μg of purified 100-kDa protein and 5 μg of cytosol protein. ND, not determined.

phorylation of the protein (ref. 29; unpublished results). In addition, in GH_3 cells, thyrotropin-releasing hormone, through inositol trisphosphate-mediated Ca^{2+} release, has been found to stimulate phosphorylation of the S97 protein (47). Purification of CaM kinase III and further characterization of both the enzyme and the 100-kDa protein will be necessary to elucidate the function of this CaM-dependent phosphorylation system.

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