Proc. Natl. Acad. Sci. USA
Vol. 78, No. 2, pp. 1293–1297, February 1981
Neurobiology

Two calcium/calmodulin-dependent protein kinases, which are highly concentrated in brain, phosphorylate protein I at distinct sites

(synaptic vesicles/phosphoproteins)

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Contributed by Paul Greengard, December 1, 1980

ABSTRACT **Two calcium-stimulated protein kinase activities (ATP:protein phosphotransferase, EC 2.7.1.37) that phosphorylate protein I, a specific synaptic protein, have been identified in homogenates of rat brain. One of these is found in both the particulate and cytosolic fractions and phosphorylates a region of protein I that is phosphorylated in intact synaptosomes in response to calcium but not to cyclic AMP. The stimulation by calcium of the particulate enzyme and of the partially purified cytosolic enzyme requires the addition of calmodulin. It is not yet known whether the particulate and cytosolic enzymes are related. A second calcium-stimulated protein I kinase is found only in the cytosol and phosphorylates a region of protein I that is phosphorylated in intact synaptosomes in response to either calcium or cyclic AMP. The calcium stimulation of this latter kinase is probably mediated by calmodulin, judging from its inhibition by low concentrations of trifluoperazine. Both of the calcium-stimulated protein I kinases are more highly concentrated in brain than in other tissues. The two cytosolic kinases are distinguishable from each other and from myosin light chain kinase and phosphorylase *b* kinase by their substrate specificities and their chromatographic behavior on DEAE-cellulose.**

Previous studies from this laboratory demonstrated that calcium influx into rat brain synaptosomes stimulates the phosphorylation of a set of synaptosomal proteins (1). The most prominent of these proteins is protein I, a doublet composed of two closely related peptides that are also phosphorylated by cyclic AMP (cAMP)-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) (2, 3). Protein I is present only in nervous tissue, where it is highly concentrated at synaptic terminals and appears to be associated primarily with synaptic vesicles (2, 4–7). Recent studies have shown that two distinct regions of the protein I molecule can be phosphorylated; one region is phosphorylated in the presence of either cAMP or calcium, whereas the other region is phosphorylated only in the presence of calcium (8, 9). Because protein phosphorylation is a regulatory mechanism in many physiological systems (10, 11), these studies suggest that protein I is involved in a synaptic function that is regulated both by cAMP and by calcium.

It is known that cAMP stimulates protein I phosphorylation by activating cAMP-dependent protein kinase (2, 12); however, the mechanism by which calcium ion stimulates protein I phosphorylation has been less clear. A general mechanism for calcium regulation of protein phosphorylation was suggested by a recent study showing that the particulate fraction from brain, as well as from a variety of other tissues, contains calcium/calmodulin-dependent protein kinase activity that catalyzes the phosphorylation of endogenous substrates (13, 14). In that study, protein I, although present in the particulate fraction from brain, was not a prominent substrate for calmodulin-dependent kinase. In order to clarify the mechanism by which calcium regulates protein I phosphorylation, we have used purified protein I as a substrate to characterize brain calcium-dependent kinases that may be responsible for the phosphorylation of protein I seen in response to calcium influx into synaptosomes.

METHODS

Materials. [γ-^{32}P]ATP (5–10 × 10^7 cpm/nmol) was prepared by the method of Glynn and Chappell (15) from ATP (Sigma) and [^{32}P]phosphate (New England Nuclear). The specific activity was adjusted with unlabeled ATP. Calmodulin was prepared from bovine brain by the method of Watterson *et al.* (16). Protein I was a gift of Louis DeGennaro or was prepared from bovine brain by a modification of the procedure of Ueda and Greengard (2). Trifluoperazine was purchased from Smith, Kline & French. Pure phosphorylase kinase from skeletal muscle was a gift of Jerry Wang, and pure myosin light chain kinase from turkey gizzard was a gift of Mary Conti and Robert Adelstein. Phosphorylase *b* was purchased from Sigma. Whole myosin light chain fraction from rabbit skeletal muscle was a gift of Angus Nairn, and partially purified Walsh inhibitor, prepared through step 3 of the procedure of Walsh *et al.* (17), was a gift of Wieland Huttner. *Staphylococcus aureus* V8 protease was purchased from Miles.

Preparation of Rat Brain Homogenates, Cytosol, and Membrane Fractions. Brains were removed from 150- to 200-g male Sprague–Dawley rats. The tissue was homogenized by 12 up-and-down strokes with a Teflon/glass homogenizer at 1200 rpm in 10 vol of 20 mM Tris/1 mM imidazole (pH 7.3)/1 mM $MgCl_2$/1 mM dithioerythritol/0.1 mM $CaCl_2$ (buffer A), containing 0.1 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$) (Sigma). In initial experiments, cellular debris and nuclei were removed by centrifugation at 900 × *g* for 10 min, and then large organelles and large membrane pieces were removed by centrifugation at 10,000 × *g* for 20 min; when this was done, the 10,000 × *g* supernatant showed 3–5 times more enzyme activity than either the 900 × *g* supernatant or the 10,000 × *g* pellet obtained from the 900 × *g* supernatant. For this reason, in later experiments, the cellular debris and large organelles were removed from the homogenized tissue by a single centrifugation at 10,000 × *g* for 20 min. The supernatant from this spin is referred to in *Results*

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Abbreviations: cAMP, cyclic AMP; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; kDal, kilodalton.

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as "homogenate." This 10,000 × *g* supernatant was separated into "cytosol" and "membrane" fractions by centrifugation at 170,000 × *g* for 1 hr. The membrane pellet was resuspended in the original volume of homogenization buffer. Protein concentration was determined by the method of Lowry *et al.* (18).

Protein I Kinase Assay. Calcium-stimulated protein I kinase was assayed at 30°C in a reaction mixture (final volume 0.1 ml) containing 50 mM 1,4-piperazinediethanesulfonic acid (Pipes) (pH 7.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, either 0.4 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) (minus calcium) or 0.4 mM EGTA and 0.7 mM $CaCl_2$ (plus calcium), 0.5–1 μg of calmodulin, 5–10 μg of protein I, 25 μM [γ-^{32}P]ATP (1–3 × 10^3 cpm/pmol), and various amounts of enzyme. After preincubation for 30 sec, the reaction was initiated by the addition of [γ-^{32}P]ATP and carried out for 30 sec, which ensured measurement of initial rates. The reaction was terminated by addition of 50 μl of a "stop solution" containing 9% $NaDodSO_4$, 6% (vol/vol) 2-mercaptoethanol, 15% (wt/vol) glycerol, 0.186 M Tris·HCl (pH 6.7), and a trace of bromphenol blue. The solution was then boiled for 2 min. Labeled proteins were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis as described (2). The position of protein I was determined either by staining the gels with Coomassie brilliant blue or by autoradiography. For quantitation, the labeled band was cut from the dried gel and placed in a vial containing liquid scintillation fluid, and its radioactivity was determined by liquid scintillation spectrometry.

Peptide Mapping of Phosphorylated Protein I. Peptide mapping was carried out by the method of Cleveland *et al.* (19), as described by Huttner and Greengard (8). Gel pieces containing phosphorylated protein I were rinsed with ether to remove the liquid scintillation counting fluor. The pieces were reswollen in 0.125 M Tris·HCl (pH 6.8)/0.1% $NaDodSO_4$, then placed into the slots of a $NaDodSO_4$/polyacrylamide gel (3.1% stacking gel, 4.5 cm long; 15% separating gel, 11 cm long). Seventy microliters of swelling buffer containing 15% glycerol, 14 μg of S. *aurens* protease, and a trace of pyronin Y was layered over the gel pieces. Electrophoresis was carried out at 40–60 V. Radioactive peptides were located in the dried gel by autoradiography. The percentage of the total radioactivity in each peptide was determined by cutting out gel pieces containing the peptides and determining their radioactivity by liquid scintillation spectrometry.

DEAE-cellulose Chromatography. Rat brain cytosol (60–100 ml, 0.2–0.3 g of protein) was applied to a 1.5 × 10 cm column of DEAE-cellulose (Whatman DE52). The column was washed with two column volumes of buffer A, then two column volumes of buffer A containing 0.05 M NaCl. Proteins were eluted from the column with a linear gradient of NaCl (0.05–0.3 M) in buffer A. Fractions (1 ml) were collected and 10- to 40-μl aliquots were assayed, in the absence and presence of calcium, for protein I kinase, myosin light chain kinase, or phosphorylase *b* kinase activity.

Pooled fractions were adjusted to a final concentration of 0.1 M Tris·HCl (pH 7.4)/1 mM dithioerythritol/0.1 mM $PhMeSO_2F$. Solid ammonium sulfate was then added to 70% saturation. The precipitated protein was collected by centrifugation at 27,000 × *g* for 30 min and resuspended in 20 mM Pipes (pH 7.2)/2 mM dithioerythritol/0.02% NaN_3/0.1 mM $PhMeSO_2F$.

Assays of Myosin Light Chain Kinase and Phosphorylase *b* Kinase. Myosin light chain kinase activity was assayed at 30°C in a reaction mixture (final volume, 0.1 ml) containing 30 mM Tris·HCl (pH 7.2), 10 mM $MgCl_2$, either 0.4 mM EGTA (minus calcium) or 0.4 mM EGTA and 0.9 mM $CaCl_2$ (plus calcium), 1 μg of calmodulin, 60 μg of whole myosin light chain fraction, 25 μM [γ-^{32}P]ATP (1–3 × 10^3 cpm/pmol), and various amounts of enzyme. Phosphorylase *b* kinase was assayed in a reaction mixture (final volume, 0.1 ml) containing 50 mM Tris, 50 mM sodium glycerophosphate (pH 8.2), 10 mM $MgCl_2$, 5 mM dithioerythritol, either 0.4 mM EGTA (minus calcium) or 0.4 mM EGTA and 0.7 mM $CaCl_2$ (plus calcium), 1 μg of calmodulin, 50 μg of phosphorylase *b*, 25 μM [γ-^{32}P]ATP (1–3 × 10^3 cpm/pmol), and various amounts of enzyme. Both reactions were carried out for 5 min and stopped by the addition of $NaDodSO_4$ stop solution. Incorporation of phosphate into myosin light chain, or into phosphorylase *b*, was determined by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography as described for the protein I kinase assay.

Preparation of Homogenates from Various Tissues. The tissues listed in Table 3 were removed from male Sprague–Dawley rats and minced with scissors. Heart and skeletal muscle were further minced with a Brinkmann tissue chopper. The minced tissues were then homogenized in 10 vol of buffer A containing 0.1 mM $PhMeSO_2F$, by 12 up-and-down strokes with a Teflon/glass homogenizer at 1200 rpm. Cellular debris and nuclei were removed by centrifugation at 900 × *g* for 10 min. The tissues were then separated into pellet and supernatant fractions by centrifugation at 10,000 × *g* for 20 min. The 10,000 × *g* pellets were resuspended in the original volume of buffer A. Calcium-dependent protein I kinase activity was determined as described above. The total activity of the 10,000 × *g* pellets was between 10% and 39% of that of the 10,000 × *g* supernatants of the various tissues. As in the case of brain, the 10,000 × *g* supernatant is referred to in *Results* as the tissue homogenate. Protein concentration was determined by the method of Lowry *et al.* (18).

RESULTS

When purified protein I was incubated with homogenates of rat brain under the conditions described in the legend to Fig. 1, the rate of its phosphorylation was stimulated 20- to 40-fold by the addition of calcium ion. Under these conditions the assay was reliably linear with time for about 1 min and with protein concentration up to about 30 μg of homogenate per assay tube; the maximal rate of phosphorylation of protein I in the presence of calcium was 2.5 nmol/min per mg of protein. Dephosphorylation of protein I occurred in the homogenate at about a sixth of the rate of phosphorylation and was unaffected by the presence of calcium ion (data not shown).

When the homogenates were separated into cytosolic and particulate fractions by centrifugation, 30–45% of the recovered calcium-stimulated protein I kinase activity was in the cytosolic fraction; the rest was associated with the particulate fraction. The total recovery of activity in the two fractions ranged from 60% to 110% of the activity in the homogenate. An example of an autoradiograph obtained in such an experiment is shown in Fig. 1.

Trifluoperazine abolished the calcium-stimulated activity. Levin and Weiss (20) have shown that phenothiazine antipsychotics, such as trifluoperazine, bind to calmodulin in a calcium-dependent manner and inhibit its ability to activate enzymes. Thus, the inhibition of the calcium-dependent protein I kinase activity by trifluoperazine suggested that its calcium dependence was mediated by calmodulin.

It has previously been shown, in both intact and lysed synaptosomes, that calcium stimulates the incorporation of phosphate into two distinct regions of the protein I molecule (8, 9). Upon digestion of protein I with S. *aureus* protease, these regions are contained in two different peptides, a 30-kilodalton (kDal) and a 10 kDal fragment. Incorporation of phosphate into the region of protein I yielding the 30-kDal fragment is stimulated only by calcium, whereas incorporation of phosphate into the region of protein I yielding the 10-kDal fragment is stim-

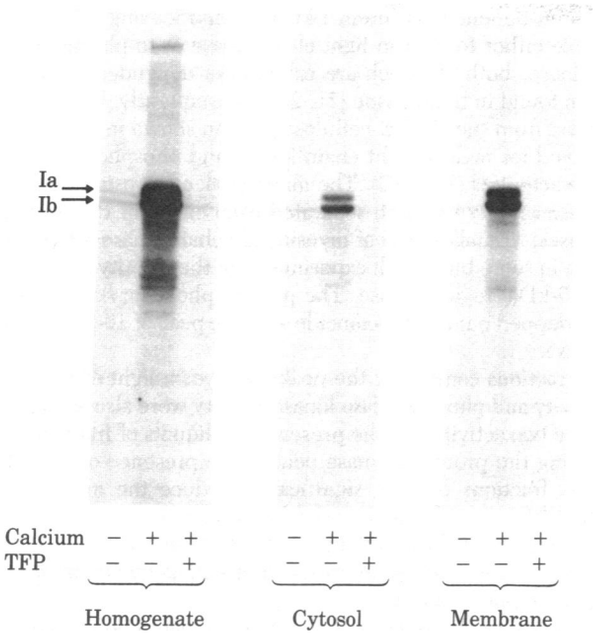

FIG. 1. Autoradiograph illustrating the phosphorylation of protein I catalyzed by fractions of a rat brain homogenate. An aliquot of the homogenate (23 μg of protein) and equivalent volumes of cytosol (13 μg of protein) and membrane (7 μg of protein) fractions were incubated under standard assay conditions with 5 μg of protein I and 0.5 μg of calmodulin in 0.4 mM EGTA (minus calcium), 0.4 mM EGTA and 0.7 mM $CaCl_2$ (plus calcium), or calcium plus 150 μM trifluoperazine (TFP). The rate of incorporation of phosphate into protein I was determined as described in *Methods*. In the experiment illustrated, incorporation catalyzed by the homogenate was 2.0 pmol/min in the absence of calcium, 51.0 pmol/min in the presence of calcium, and 1.4 pmol/min in the presence of calcium plus trifluoperazine. The corresponding values with the cytosol were 0.94, 9.4, and 1.2 pmol/min, and the values with the membranes were 0.85, 19.5, and 0.41 pmol/min.

ulated either by calcium or by cAMP. These two peptides can be separated by $NaDodSO_4$ gel electrophoresis so that incorporation of phosphate into the two regions can be measured separately. In order to determine which regions of protein I were phosphorylated by the calcium-stimulated kinases present in the cytosolic and membrane fractions, protein I that had been phosphorylated by these two fractions was digested with *S. aureus* protease and analyzed by $NaDodSO_4$ gel electrophoresis according to the procedure of Cleveland *et al.* (19) (Fig. 2). The cytosolic kinase activity catalyzed the incorporation of phosphate at approximately the same rate into both regions of protein I. In contrast, the membrane-associated activity catalyzed the incorporation of phosphate primarily into the region recovered in the 30-kDal fragment. The inclusion of the inhibitor of cAMP-dependent protein kinase in the assay mixture reduced phosphorylation of the 10-kDal region in the absence of calcium and had no effect on calcium-stimulated phosphorylation of either region of protein I.

Fractionation of the cytosol by DEAE-cellulose chromatography indicated that the two regions of the protein I molecule were phosphorylated by different calcium-dependent protein kinases (Fig. 3). Calcium-stimulated protein I kinase activity eluted as a broad peak between 0.13 M and 0.23 M NaCl (Fig. 3*A*). When the regions of protein I that had been phosphorylated by the individual column fractions were determined, it became apparent that there were two separate peaks of protein I kinase activity (Fig. 3*B*). One peak contained kinase activity that was specific for the region recovered in the 30-kDal fragment ("30-kDal region kinase"). A second peak contained activity specific for the region recovered in the 10-kDal fragment

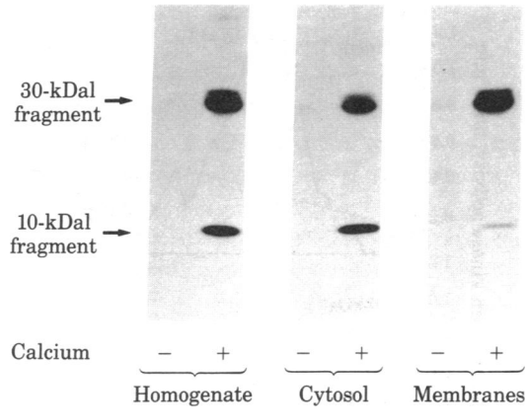

FIG. 2. Autoradiograph showing the phosphopeptides produced by digestion with *S. aureus* protease of protein I phosphorylated by fractions of rat brain homogenate. Aliquots of homogenate (29 μg), cytosol (25 μg), and membrane fraction (7 μg) were incubated as described in the legend of Fig. 1, except that 10 μg of protein I, 1 μg of calmodulin, and 47 μg of Walsh inhibitor were used. The Walsh inhibitor was included in the assays to eliminate phosphorylation by the catalytic subunit of cAMP-dependent protein kinase. The rate of incorporation of phosphate into protein I catalyzed by the homogenate was 1.6 pmol/min in the absence of calcium and 60.0 pmol/min in the presence of calcium. The corresponding values with cytosol were 1.7 and 39.6, and with the membranes the values were 1.5 and 45.3. The phosphorylated protein I bands were cut from the dried gels, assayed for radioactivity, then processed for digestion by *S. aureus* protease. Sixty-eight to 70% of the radioactivity in the protein I bands was recovered in the 30-kDal and 10-kDal fragments. Of the radioactivity recovered from protein I, 66% of the phosphate was found in the 30-kDal fragment and 34% in the 10-kDal fragment with homogenate, 52% in the 30-kDal fragment and 48% in the 10-kDal fragment with the cytosol, and 93% in the 30-kDal fragment and 7% in the 10-kDal fragment with the membranes.

("10-kDal region kinase"). A portion of calcium-stimulated activity was usually eluted in the 50 mM NaCl wash, and this activity was also specific for the region containing the 10-kDal fragment. Results similar to those described in Fig. 3 *A* and *B* were obtained in five experiments. For characterization of the two cytosolic kinase activities, fractions eluting from the DEAE-cellulose column between 0.125 M and 0.18 M NaCl were pooled, concentrated, and used as a source of the 30-kDal region kinase, and fractions eluting between 0.18 M and 0.23 M NaCl were pooled, concentrated, and used as the source of the 10-kDal region kinase.

The calcium and calmodulin dependence of the membrane kinase and that of the two cytosolic kinases were examined directly. The results are shown in Table 1. The calcium-stimulated kinase activity in membranes was nearly completely lost after one wash of the membrane pellet. This activity could be restored by the addition of pure calmodulin to the assay. Similarly, the cytosolic 30-kDal region kinase was stimulated only slightly by calcium in the absence of added calmodulin, but full stimulation was restored by the addition of pure calmodulin to the assay. In contrast, the 10-kDal region kinase was stimulated by calcium in the absence of added calmodulin. Moreover, the portion of kinase activity that was removed from the column by 50 mM NaCl was also fully stimulated by calcium in the absence of added calmodulin (data not shown). The calcium stimulation of the partially purified 10-kDal region kinase observed in the absence of added calmodulin was more than 98% inhibited by 50 μM trifluoperazine.

These results provide direct evidence that the membrane-associated and cytosolic calcium-stimulated 30-kDal region kinases are calmodulin dependent. The inhibition of the 10-kDal region kinase by 50 μM trifluoperazine suggests that it also is calmodulin dependent but that it has a higher affinity for cal-

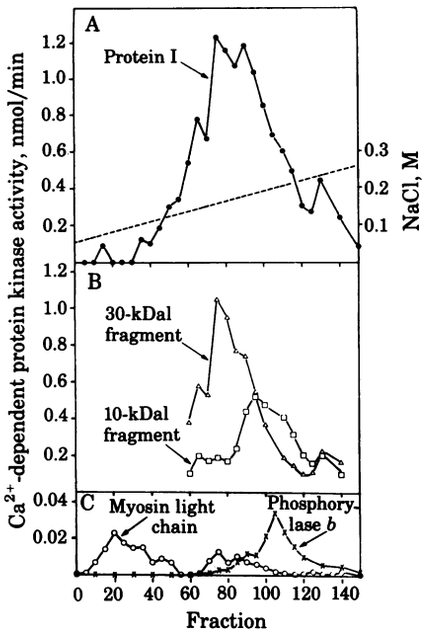

FIG. 3. DEAE-cellulose chromatography of calcium-dependent protein kinases in cytosol pooled from five rat brains. (*A*) Calcium-dependent protein I kinase activity. (*B*) Calcium-dependent 30-kDal region kinase (△) and 10-kDal region kinase (□) activity. The bands of protein I phosphorylated by every fifth column fraction from 60 to 140 were digested with *S. aureus* protease and the fragments were separated in a Cleveland–Laemmli $NaDodSO_4$ gel. Sixty to 70% of the radioactivity in the original protein I bands was recovered in the two fragments. The amount of ^{32}P incorporated into each of the two regions was calculated by correcting for this recovery. (*C*) Calcium-dependent myosin light chain kinase (○) and phosphorylase *b* kinase (×) activities. Note expanded scale of the ordinate in *C*.

modulin than does the 30-kDal region kinase and thus retains bound calmodulin during chromatography on DEAE-cellulose in the presence of 0.1 mM $CaCl_2$. Further purification of the 10-kDal region kinase will be necessary to prove conclusively that it is calmodulin dependent.

It was of interest to know whether either of the two cytosolic, calcium-dependent protein I kinase activities might be attributable either to myosin light chain kinase or to phosphorylase *b* kinase, both of which are calmodulin dependent and have been found in brain tissue (21, 22). Consequently, the fractions eluted from the DEAE-cellulose column shown in Fig. 3 were assayed for myosin light chain kinase and phosphorylase *b* kinase activities (Fig. 3*C*). The major peak of myosin light chain kinase activity was well separated from either of the protein I kinases. A smaller peak of myosin light chain kinase activity was seen in some but not all experiments in the vicinity of the peak of 30-kDal region kinase. The peak of phosphorylase *b* kinase overlapped but did not coincide with the peak of 10-kDal region kinase.

Fractions containing the peaks of myosin light chain kinase activity and phosphorylase kinase activity were also assayed for these two activities in the presence of aliquots of fractions containing the protein I kinase peaks. The presence of the latter peak fractions did not significantly reduce the myosin light chain kinase or phosphorylase *b* kinase activities, indicating that the absence of these activities in the protein I kinase peak fractions was not due to the presence of endogenous inhibitors or specific phosphatases.

These results suggested that the two cytosolic protein I kinases were distinct from myosin light chain kinase and phosphorylase *b* kinase. This conclusion was supported by experiments in which the substrate specificities of the various enzymes were compared. In these studies, myosin light chain kinase phosphorylated myosin light chain about 5 times faster than protein I under the conditions used, and all of the phosphorylation of protein I by myosin light chain kinase occurred in the region recovered in the 10-kDal fragment (Table 2). These results indicate that the small amount of myosin light chain kinase in fractions 65–105 of Fig. 3 cannot account for the 30-kDal region kinase present in those fractions. Similarly, phosphorylase *b* kinase phosphorylated phosphorylase *b* 10 times faster than protein I under the conditions used, and all of the phosphorylation of protein I by phosphorylase kinase occurred in the region recovered in the 30-kDal fragment. These results indicate that the phosphorylase *b* kinase present in fractions 80–140 cannot account for the 10-kDal region kinase present in those fractions.

Because protein I is specific to neural tissue (2, 4–7), it was of interest to examine the tissue distribution of calcium-stim-

Table 1. Effect of calcium and calmodulin on protein I kinases from rat brain

		Kinase activity, pmol/min		
		Particulate	Cytosolic	
Calcium	Calmodulin	30-kDal region	30-kDal region	10-kDal region
−	−	2.0	0.4	8.6
+	−	3.5	0.7	42
−	+	2.3	0.4	8.2
+	+	104	63	51

The incorporation of phosphate into protein I by various preparations of protein I kinase was measured in the absence and presence of calcium and calmodulin as indicated. The particulate kinase was prepared as described in *Methods*, washed once in buffer A plus 2 mM EGTA, then resuspended in buffer A. The two cytosolic kinases were prepared by DEAE-cellulose chromatography as described in the legend to Fig. 3. Fractions eluting between 0.125 M and 0.18 M NaCl were pooled and used as the source of partially purified 30-kDal region kinase. Fractions eluting between 0.18 M and 0.23 M NaCl were pooled and used as the source of partially purified 10-kDal region kinase. Both preparations were concentrated by ammonium sulfate precipitation. The rate of phosphorylation of the appropriate region of protein I by each pool was determined as described in the legend to Fig. 3. The assay tubes contained 26 μg of membrane protein, 17 μg of 30-kDal region kinase, or 36 μg of 10-kDal region kinase. The values are the average of duplicate determinations, which agreed within 5% or less.

Table 2. Phosphorylation of protein I, myosin light chain, and phosphorylase *b* by myosin light chain kinase and phosphorylase kinase

	Kinase activity on indicated substrate, pmol/min			
	Protein I		Myosin	
Kinase	30-kDal region	10-kDal region	light chain	Phosphorylase *b*
Myosin light chain	<0.1	5.7	27	—
Phosphorylase *b*	1.3	<0.1	—	14

The rates of calcium-stimulated phosphorylation of protein I and myosin light chain by purified myosin light chain kinase (1 μg) were determined under the standard protein I kinase assay conditions except that either 30 μg of protein I or 30 μg of whole myosin light chain fraction was used. The rates of calcium-stimulated phosphorylation of protein I and phosphorylase *b* by purified phosphorylase *b* kinase (0.5 μg) were determined under the conditions described in *Methods* for the assay of phosphorylase kinase except that either 30 μg of protein I or 50 μg of phosphorylase *b* was used and the reactions were carried out for 30 sec. The values represent the differences between rates of phosphorylation in the presence and absence of calcium. Similar results were obtained in three separate experiments.

Table 3. Calcium-dependent protein I kinase activity in homogenates of several tissues

Tissue	Specific activity, pmol/min per mg protein
Brain	2204
Spleen	542
Heart	254
Adrenal	191
Skeletal muscle	83
Liver	<2
Kidney	<2

ulated protein I kinase activity (Table 3). Calcium-stimulated protein I kinase activity was 4 times greater in brain than in spleen and 9 or more times greater in brain than in any of the other tissues examined. Liver and kidney showed no detectable activity. Protein I kinases in brain homogenates were also assayed in the presence of homogenates of the other tissues. The presence of the other homogenates did not significantly reduce the brain protein I kinase activities, indicating that the low levels of protein I kinase present in the other tissues were not due to the presence of endogenous inhibitors or phosphatases. In the tissues that had activity, the proportion of 30-kDal region kinase activity to 10-kDal region kinase activity was similar to that seen in brain.

DISCUSSION

The results presented here suggest that brain tissue contains at least four distinct calmodulin-dependent protein kinases: two protein I kinases, the previously described myosin light chain kinase (21–23), and phosphorylase *b* kinase (24, 25). This multiplicity of calmodulin-regulated kinases contrasts with the uniformity of the cyclic nucleotide-dependent protein kinases. There appear to be only two types of cAMP-dependent protein kinase, which are very similar in all tissues. The two cAMP-dependent kinases differ in their regulatory subunits but have identical catalytic subunits and therefore show the same substrate specificity. Similarly, it appears that cyclic GMP-dependent protein kinases from different tissues are similar, if not identical, to one another.

Payne and Soderling (26) have recently demonstrated the existence of a calmodulin-dependent glycogen synthase kinase in liver that is distinct from phosphorylase *b* kinase or myosin light chain kinase. Yamauchi and Fujisawa (27) have presented evidence suggesting the existence of three different calmodulin-dependent protein kinases in brain. These observations as well as the data presented in this paper support the notion that calmodulin-dependent protein kinases may, in general, be more diverse and substrate specific than the cyclic nucleotide-dependent protein kinases.

The presence of low levels of calcium-dependent protein I kinase activities in tissues other than brain can be interpreted in different ways. The enzymes in the tissues that phosphorylate protein I may be identical to those in brain but present in lower concentrations. This would indicate that different tissues contain different substrates for these enzymes, because protein I is not present in other tissues examined. Another possibility is that the other tissues contain calcium-dependent kinases that are relatively specific for the substrates present in those tissues, but that those kinases are capable of phosphorylating protein I as well.

The present findings, together with earlier studies from this laboratory (2, 12), provide evidence that protein I, which is located in presynaptic terminals and is associated primarily with synaptic vesicles, can be phosphorylated by three distinct protein kinases, all of which are found in brain homogenates: one (a type II) cAMP-dependent protein kinase (12) and two calcium-dependent protein kinases. Moreover, phosphorylation occurs in two different regions of the molecule (9). The phosphorylation of one region is regulated by a calcium-dependent kinase activity (the 30-kDal region kinase) that is found in both cytosolic and particulate fractions. It will be interesting to see whether the enzymes in these two fractions are related to each other and whether they regulate protein I phosphorylation under the same or different physiological conditions. The phosphorylation of the other region of protein I is regulated by cAMP-dependent protein kinase and a second calcium-dependent kinase that is found only in the cytosol (the 10-kDal region kinase). The fact that the state of phosphorylation of protein I is regulated by at least three distinct protein kinases strongly suggests that this synaptic vesicle protein plays a major role in the regulation of the physiology of the presynaptic nerve terminal.

We thank Teresa McGuinness, Rodrigo Andrade, and Penelope E. Miller for help with some of the experiments and Annette E. Gwardyak and Karen J. Hageman for help in preparing the manuscript. This work was supported by U.S. Public Health Service Grants MH-17387 and NS-08440 (to P.G.) and a grant from the McKnight Foundation (to P.G.). This work was done during the tenure of a Muscular Dystrophy Association Research Fellowship awarded to M.B.K.

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