Biochimica et Biophysica Acta, 972 (1988) 69–78
Elsevier

BBA 12341

The phosphorylation of calmodulin and calmodulin fragments by kinase fractions from bovine brain

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(Received 20 June 1988)

Key words: Phosphorylation; Calmodulin fragment; Protein kinase; (Bovine brain)

The phosphorylation of intact calmodulin and of fragments obtained by trypsin digestion was studied, using a protein kinase partially purified from bovine brain. Brain extracts were made in the presence of the detergent CHAPS (3-[3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate). The protein kinase catalyzed the incorporation of nearly 1 mol of ^{32}P from [γ-^{32}P]ATP into calmodulin fragment 1-106. Incorporation was exclusively into serine 101. With fragment 78–148, the extent of phosphorylation was somewhat less and ^{32}P appeared mainly in threonine residues. Fragment 1–90 was also a fairly good substrate, but the phosphorylation of intact calmodulin never exceeded 0.01 mol per mol. Little or no phosphorylation was seen with parvalbumin, the brain Ca^{2+}-binding protein (CBP-18) and intestinal calcium-binding protein. The protein kinase had no requirement for cAMP or phospholipids. High levels of Mg^{2+} (60–70 mM) stimulated phosphorylation of the fragments 20-fold. Millimolar concentrations of Ca^{2+} were inhibitory. It is suggested that the calmodulin fragments were in a conformation more favorable for phosphorylation than intact soluble calmodulin.

Introduction

In the course of an investigation of calmodulin-dependent phosphorylation reactions, we observed an extremely limited phosphorylation of calmodulin itself when incubated with [γ-^{32}P]ATP and extracts of bovine brain. The calmodulin had been isolated from ram testis, and it was contaminated with about 10 percent of a material whose mobility in SDS-gel electrophoresis suggested that it was the calmodulin proteolytic fragment 1–106 [1,2]. This polypeptide was more heavily phosphorylated than calmodulin. These preliminary observations led to an investigation of the phosphorylation of calmodulin and its fragments by brain kinase fractions which copurify with calmodulin-binding proteins [3]. A procedure for partial purification of kinase activity against calmodulin fragments was then developed, beginning with an extract of bovine brain made in the presence of CHAPS.

In this paper, we describe a kinase fraction from calf brain which brings about the incorporation of nearly 1 mol of ^{32}P from [γ-^{32}P]ATP into calmodulin fragment 1–106. Incorporation was exclusively into one site, namely serine 101. A

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Abbreviations: CBP-18, Ca^{2+}-binding protein (apparent M_r 18000); CHAPS, 3-[(3-cholamidodopropyl)-dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HPLC, high-performance liquid chromatography; TPCK, *N*-tosyl-L-phenylalaninechloromethylketone.

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substantial, but smaller, incorporation was noted with fragment 78–148. In this case, ^{32}P was located in two peptides obtained by exhaustive digestion with trypsin, and the only phosphorylated amino acid that could be detected was threonine. Fragment 1–90 was also a fairly good substrate, but the phosphorylation of calmodulin itself never exceeded 0.01 mol per mol. Cyclic AMP-dependent protein kinase was not able to phosphorylate calmodulin or its fragments.

Materials and Methods

Materials. Intestinal calcium-binding protein [4,5] was a gift from Dr. Keith Moffat, Cornell University, Ithaca, NY. The Ca^{2+}-binding protein, CBP-18, from bovine brain [6] was kindly provided by Dr. Allan S. Manalan, NCI, NIH. Parvalbumin was a gift from Dr. Jaques Haiech, Centre de Recherche de Biochimie Macromoleculaire, Montpellier, France. The purification of ram testis calmodulin and calmodulin fragments 1–106, 78–148 and 1–90 has been described [2,7]. The catalytic subunit of cAMP-dependent kinase [8] and purified rabbit muscle phosphorylate kinase [9] were gifts from Drs. E.H. Fischer and S. Pocinwong, University of Washington, Seattle.

All HPLC columns and solvents were from Waters Associates. PD-10 columns and DEAE-Sephacel were from Pharmacia Fine Chemicals. Ram testes and bovine brains were purchased fresh from a local slaughter house or frozen from Pel-freeze. Histones, type V-S, were from Sigma.

Hydroxylapatite was prepared according to Levin [10].

Other materials were commercially obtained and of this highest purity available.

Protein kinase purification. Bovine brains, stored at −70 °C, were thawed and homogenized as described [3]. CHAPS (final concentration, 10 mM) was added in order to solubilize membrane proteins. The crude extract from 100 g (wet weight) of brain was mixed with 24 g dry cellulose DE-23 and poured to give a 5 × 15 cm column. It was washed with 200 ml solution A (0.1 M NaCl, 1 mM CHAPS, 1 mM $MgCl_2$, 0.1 mM DTT, 1 mM EDTA, 1 mM EGTA, 75 μg/ml phenylmethylsulfonyl fluoride and 20 mM Tris-Cl (pH 7.5). Gradient elution was carried out with 500 ml solution A in the mixing chamber and 500 ml solution A containing 0.5 M NaCl in the reservoir. The fraction volume was 5 ml. The peak of protein kinase eluted just before the peak of calcineurin (Fig. 1). Fractions 38–60, containing both activities, were pooled, concentrated by a 0–60% ammonium sulfate precipitation and applied to a 5 × 78 cm Sephacryl 200 column equilibrated in 0.1 M NaCl, 1 mM EGTA, 1 mM $MgCl_2$, 0.25 mM DTT, 10% glycerol, 10 μg/ml soybean inhibitor, 1 μg/ml leupeptin, 0.1 mM benzamidine and 10 μg/ml TPCK in 20 mM Tris-Cl (pH 7.5) (Fig. 2).

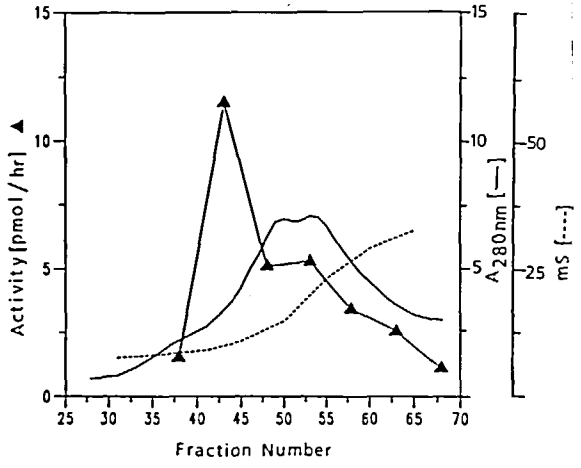

Fig. 1. Fractionation of calmodulin peptide kinase activity on a DEAE-cellulose, DE-23, column (see text for details). Activity is expressed as pmol ^{32}P incorporated into calmodulin peptides per 2 μl per h. Conductivity of the fractions was measured in mS (Sieman) units. The protein kinase assay is described in Materials and Methods.

Fractions 57–64 (10 ml each) from the Sephacryl 200 column were combined and dialyzed for 2 h against solution B (10 mM EDTA, 1 mM EGTA, 2.5 mM DTT and 20 mM Tris-Cl, pH 7.6). The dialyzed material was loaded on a 5 ml DEAE Sephacel column (1.5 cm × 3 cm) equilibrated with solution B. The kinase activity was eluted using a linear gradient of KCl (30 ml of solution B in mixing chamber; 30 ml of solution B containing 0.6 M KCl in the reservoir). Fractions of 1 ml were collected and kinase activity appeared in fractions 24–36. The peak fractions were able to incorporate 0.17 nmol ^{32}P/mg protein per min into fragment 1–106 at 37 °C. The

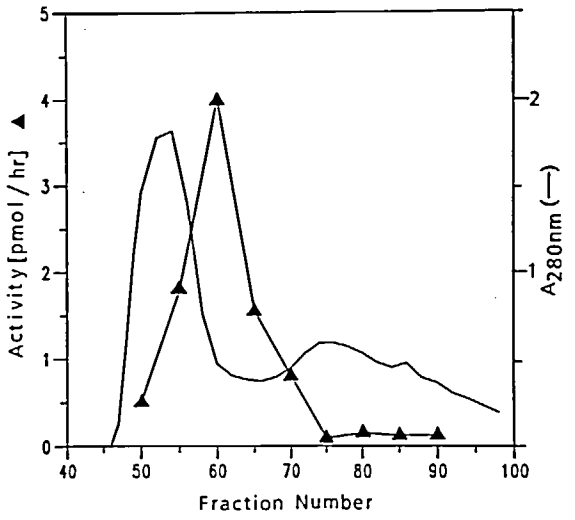

Fig. 2. Fractionation of calmodulin peptide kinase activity on a Sephacryl 200 column (see text for details). Units are as in Fig. 1.

rate of endogenous phosphorylation of the kinase fraction at this stage of purification was one fourth the rate of phosphorylation of added substrate. This amounted to a 10-fold purification over the DE-23 stage. It is difficult to compare DEAE Sephacel fractions with crude extract, since the latter had a very high blank rate and the rate showed poor linearity with enzyme concentration.

Protein kinase assay. The reaction mixture (40 μl) contained 50 mM Tris-Cl (pH 7.5), 64 mM $MgCl_2$, 1 mM EGTA, 0.15 mM [γ-^{32}P]ATP (500 cpm per pmol), enzyme fraction and 2 μl of a limited trypsin digest of calmodulin. (This trypsin digest was made by incubating a solution containing 8 mg/ml calmodulin, 2 mM EGTA, 0.05 M NH_4HCO_3 and 50 mM NaCl with 2 μg/ml of trypsin for 50 min at 30°C. The reaction was stopped with soybean trypsin inhibitor. Such a preparation, described in detail in Ref. 2, consists largely of fragments 1–106, 1–90 and 107–148.)

The kinase incubation was terminated after 1 h at 37°C. Carrier bovine serum albumin was added (5 μg of a 2 mg/ml solution), after which a 40 μl portion was applied to Whatman 3 MM filter discs which were immediately immersed in chilled 10% trichloroacetic acid containing 40 mM sodium pyrophosphate. The filter discs were washed three times with 5% trichloroacetic acid and one with 95% ethanol (15 min each time), and were counted in a Beckman LS335 scintillation spectrometer after addition of 7.5 ml Aquasol. A control incubation was treated in the same way except that it lacked the calmodulin digest. This assay showed good linearity over a 10-fold range of enzyme concentration.

On occasion, the reaction mixture was subjected to SDS-gel electrophoresis in slabs of 15% acrylamide. Proteins were located on Coomassie blue-stained gels, and appropriate sections of gel were cut out with a razor blade and treated with 30% hydrogen peroxide for 3 h at 85°C. The solubilized material was counted in a Beckman LS335 scintillation spectrometer after addition of 8.0 ml of Aquasol.

Preparation of phosphorylated fragments 1–106 and 78–148. In the case of fragment 1–106, the reaction mixture (1 ml) contained 50 mM Tris-Cl Tris-Cl (pH 7.5), 64 mM $MgCl_2$, 1 mM EGTA, 0.15 mM [^{32}P]ATP (500 cpm/pmol), 0.1% sodium azide, 28 nmol peptide 1–106 and 60 μl of a Sephacryl 200 fraction. Incubation was for 25 h at 37°C. For fragment 78–148, the reaction mixture (0.55 ml) contained 50 mM Tris-HCl (pH 7.5), 64 mM $MgCl_2$, 1 mM EGTA, 0.15 mM [γ-^{32}P]ATP (500 cpm/pmol), 0.01% sodium azide, 17 nmol peptide 78–148 and 60 μl of a Sephacryl 200 fraction. Incubation was at 37°C for 23.5 h. The extent of incorporation in this experiment was 0.72 and 0.25 mol ^{32}P per mol of peptide 1–106 and 78–148, respectively.

The labeled phosphorylated peptides were purified using HPLC [2]. Pooled fractions containing the purified fragments were flash evaporated, dissolved in 1 ml of 0.05 M NH_4HCO_3 and half was applied to each of two PD-10 columns to remove EGTA and phosphate. Elution was carried out with sequential 0.5 ml portions of 0.05 M NH_4HCO_3, and fractions were monitored for absorbance at 215, 235 and 280 nm. The purified labeled peptides were lyophilized and stored either as a dry powder or as a solution in 0.05 M NH_4HCO_3.

Phosphoamino acid analysis. Samples of labeled peptide were lyophilized to remove NH_4HCO_3 and hydrolyzed in 50 μl 6 M HCl at 110°C for 2 h [11]. Phosphoamino acids were separated by electrophoresis (1000 V, 60 min) on cellulose

thin-layer plates at pH 3.5 in pyridine/acetic acid/water (1 : 10 : 189, v/v) [12]. Standards, which had been added, were identified by spraying with ninhydrin and the labeled amino acids were located by autoradiography.

Amino acid analyses. Amino acid analyses were performed by a modification of the method of Spackman et al. [13] using a Waters high-performance liquid chromatography amino acid analysis system, equipped with a CAT-EX Resin column (0.4 cm × 25 cm). The ionic strength gradient system was used as described in the manufacturer's manual. At 59 °C, trimethyllysine elutes at 59 min between phenylalanine (retention time 51 min) and lysine (retention time 63 min).

Protein determination. Protein concentrations were determined by the method of Lowry et al [14] as well as by ultraviolet absorption.

Ultraviolet absorption spectra were measured with a Cary model 118C spectrophotometer.

SDS-gel electrophoresis was performed in slabs of 15% gel acrylamide using the Laemmli system [15]. To help characterize Ca^{2+} binding polypeptides, duplicate samples were run side by side, one in the presence of 2 mM $CaCl_2$ and the other in 2 mM EGTA. Molecular weight standards were phosphorylase (97000), bovine serum albumin (68000), catalase (58000), fumarase (48000), actin (42000), lactate dehydrogenase (36000) and β-lactoglobulin (17500).

Results

Phosphorylation of calmodulin

Freshly prepared fractions obtained during calmodulin-sepharose affinity chromatography and Sephadex G-200 chromatography when incubated with [γ-^{32}P]ATP exhibited a Ca^{2+}/calmodulin- as well as cAMP-stimulated phosphorylation of calmodulin-binding proteins. When a large amount of calmodulin was used, a faint density was observed to migrate with calmodulin after SDS-gel electrophoresis and autoradiography. This density showed the expected shift when its mobility in the presence of 2 mM Ca^{2+} was compared with its mobility in the presence of 2 mM EGTA. The maximum extent of incorporation of ^{32}P into calmodulin was somewhat less than 0.01 mol per mol. This low value was not improved by either testing other soluble kinase fractions or by using fractions obtained by fractionation of extracts made in the presence of CHAPS. Various conditions of phosphorylation were tried, including the presence of Ca^{2+} or of EGTA, presence of 8-bromo-cAMP, supplements of triolein and phosphatidylserine, all without any significant effect. Preincubation for up to 4 h to dephosphorylate possible sites for phosphorylation that were already occupied did not help either.

Phosphorylation of calmodulin fragments

Greater success was achieved when we examined the phosphorylation of fragments of calmodulin obtained by partial digestion with trypsin, especially peptide 1–106 [2]. With soluble

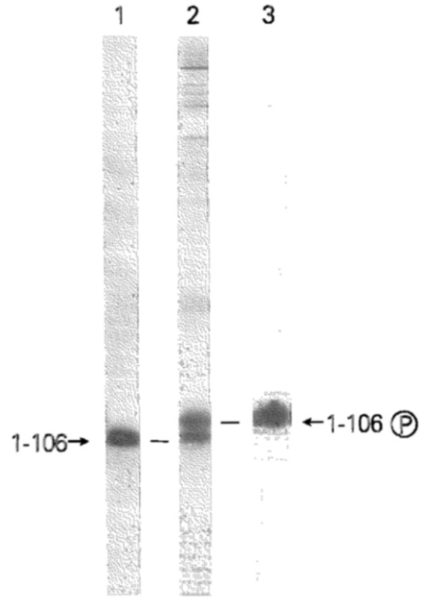

Fig. 3. The conversion of calmodulin fragment 1–106 to a phosphorylated form with reduced electrophoretic mobility. The reaction mixture (40 μl) contained 50 mM Tris-Cl (pH 7.5), 65 mM $MgCl_2$, 1 mM EGTA, 0.15 mM [γ-^{32}P]ATP (500 cpm per pmol), 0.1% sodium azide, 2 μg of purified protein kinase and 0.28 nmol peptide 1–106. Incubation was for 23 h at 37 °C. Electrophoresis, autoradiography and measurement of radioactivity in the gel sections are described in Materials and Methods. Lanes 1 and 2 depict Coomassie blue-stained gels of incubation mixtures in the absence or presence of enzyme, respectively. Lane 3 is an autoradiogram. Incorporation of ^{32}P amounted to 0.7 mol/mol peptide, and over 90% of the radioactivity appeared in a new Coomassie blue-staining band (see arrow).

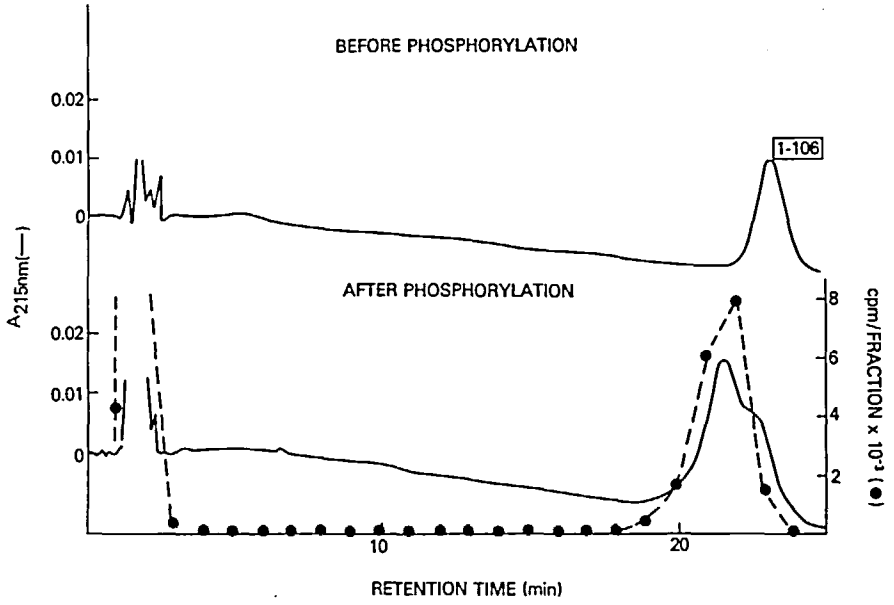

Fig. 4. Protein kinase from brain converts the bulk of the calmodulin fragment 1–106 to a phosphorylated derivative with different chromatographic properties. A detailed protocol for the incubation of peptide 1–106 with [γ-^{32}P]ATP and purification of the product is given in Materials and Methods. A sample was subjected to HPLC, using alkyl phenyl reverse-phase chromatography. A linear gradient from 80% buffer A (0.01 M potassium phosphate (pH 6.1), 2 mM EGTA) – 20% solvent B (100% CH_3CN) to 35% solvent B over 40 min was used. The rate of elution was 1.5 ml per min. Fractions of 0.75 ml each were obtained in a fraction collector whose operation was synchronized with the recording absorbance detectors which monitored absorbance at 280 and 215 nm.

kinase fractions, incorporation of ^{32}P of up to 0.3 mol per mol peptide 1–106 was noted. When bovine brain was extracted in the presence of CHAPS to solubilize membrane proteins (see Materials and Methods), fractions were obtained which enable the phosphorylation to reach 0.7 mol per mol of peptide 1–106 in large-scale incubations and 1.0 mol per mol in small-scale runs. With fragments 78–148 and 1–90, values of 0.25 mol per mol and 0.05 mol per mol, respectively, were observed. Peptides 1–106 and 78–148 have a greater mobility during SDS-acrylamide gel electrophoresis in the presence of Ca^{2+} than that in the presence of EGTA. The same was true for peptide 1–90. The Ca^{2+}-dependent shift in migration was also observed after phosphorylation. The conversion of 70% of peptide 1–106 to a phosphorylate form, whose electrophoretic mobility was reduced compared with unphosphorylated 1–106, is illustrated in Fig. 3.

Fig. 4 also provides evidence for conversion of the bulk of peptide 1–106 to a phosphorylated form. Highly purified peptide was treated with [γ-^{32}P]ATP as described in Materials and Methods. A sample was analyzed by HPLC, and another sample, of unphosphorylated peptide, was analyzed under the same conditions. It is apparent that most of original fragment 1–1006 was converted to a new material carrying ^{32}P, which elutes a little earlier, at pH 6, than the unphosphorylated fragment 1–106.

Location of the sites of phosphorylation in peptides 1–106

The labeled fragments 1–106 and 78–148 were prepared as described in Materials and Methods. Further treatment was as follows:

(A) For purposes of HPLC calibration, calmodulin itself was hydrolyzed. The incubation mixture (0.5 ml) contained 0.94 mg per ml calmodulin, 1 mM EGTA, 0.1% thiodiglycol and 0.05 M NH_4HCO_3. After 10 min at 37 °C, trypsin was added at a concentration of 100 μg per ml. Incubation was continued for 90 min, after which 25 μl (23.5 μg digested calmodulin) was subjected to HPLC. A linear gradient from 0 to 50% CH_3CN

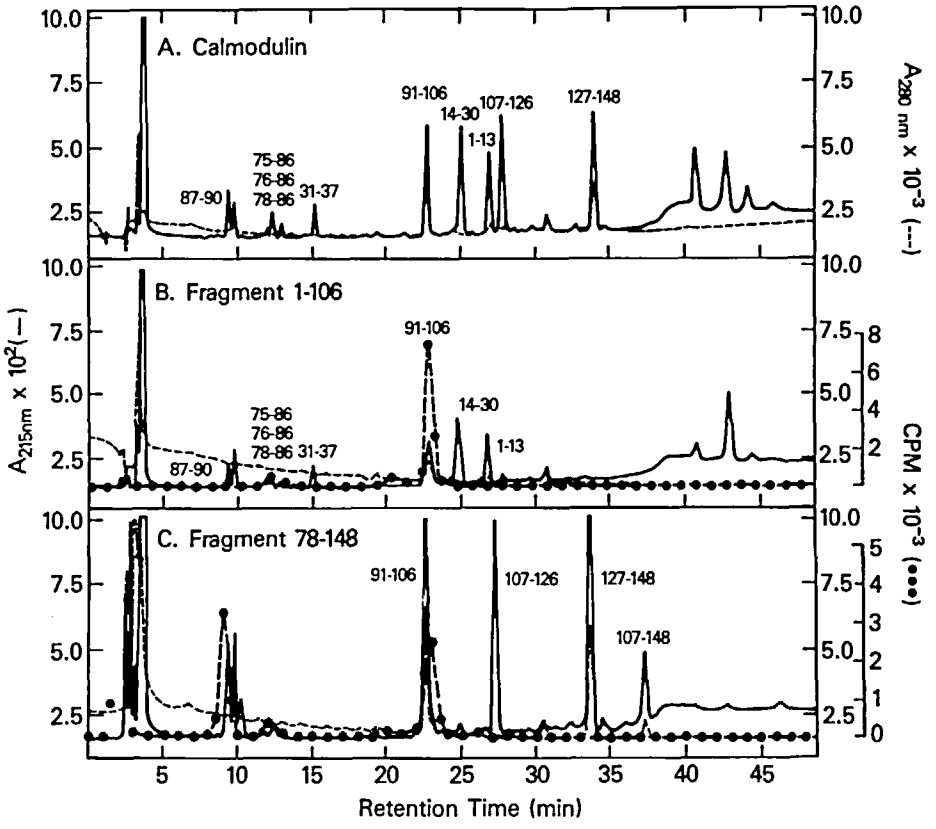

Fig. 5. Tryptic digests of (A) calmodulin (23.5 μg), (B) phosphorylated fragment 1–106 (14.1 μg, 12000 cpm) and (C) phosphorylated fragment 78–148 (24.1 μg, 18000 cpm) were analyzed by HPLC in a C18 column as described in the text. The recovery of radioactivity was 93% in a single peptide from fragment 1–106, identified as fragment 91–106. In the case of fragment 78–148, 37% of the radioactivity eluting at the origin was identified as a mixture of ATP and P_i; the remaining radioactivity eluted later (26% at 9 min and 31% at 23.5 min). The radioactive peptides were not identified, but the low recovery (20%) of peptide 78–86 suggests that they are derived from this peptide.

in 0.1% (w/v) PO_4H_3 (pH 2.0) over 50 min was used.

(B) The next step was to carry out an exhaustive digestion of the ^{32}P-labeled fragment 1–106 with trypsin and to separate the terminal peptides by HPLC. The incubation mixture (150 μl) contained 0.93 mg/ml fragment 1–106, 1 mM EGTA, 0.1% thiodiglycol, 0.05 M NH_4HCO_3 and 100 μg/ml of trypsin. After 90 min at 37°C, a pilot HPLC trial was carried out with 25 μl, using the same gradient elution conditions as in (A) above. Eluates were collected in a small fraction collector and the appearance of fractions was synchronized with the appearance of peaks monitored at 280 and 215 nm. The terminal peptides were unchanged with the exception of 91–106. Peptide 91–106, which absorbs at 280 and 215 nm, eluted as a double peak between 22.5 and 24 min. The applied radioactivity was quantitatively recovered (greater than 95%) in a single peak (RT = 23.5 min) coeluting with the second peak of peptide 91–106 (Fig. 5). Thus, at pH 2.0 phosphorylated peptide 91–106 elutes later than nonphosphorylated 91–106. A larger-scale run was then carried out.

Acid hydrolysis and electrophoresis under conditions designed for detection of phosphoamino acids [11,12] was performed. Phosphoserine was the only compound to be seen when several concentrations of ^{32}P-labeled peptide 1–106 were analyzed. Accordingly, we conclude that peptide 1–106 was phosphorylated by the brain kinase

fraction exclusively at serine 101, the only serine present in peptide 91–106.

(C) In the case of the ^{32}P-labeled peptide 78–148, the reaction mixture (96.4 μl) contained 0.35 mg/ml of 78–148, 1 mM EGTA, 0.1% thiodiglycol and 35.3 μg per ml trypsin. Incubation was at 37°C for 90 min. A pilot run on 25 μl digest was carried out using a linear gradient from 0 to 50% CH_3CN over 50 min. Peptide-bound radioactivity was recovered mainly in two peaks, eluting at 9 and 23.5 min. The peptides were not identified, but the low yield of peptide 78–86 and excellent recovery of the other peptides suggests that the major radioactive peptide is a derivative of peptide 78–86. A larger-scale run using 75 μl digest was then carried out, with similar results. Acid hydrolysis was performed under conditions designed to detect phosphoamino acids. This time only phosphothreonine was found.

General properties of the protein kinase

Substrate specificity. As already noted, peptide fragment 1–106 was the best substrate followed by peptides 78–148 and 1–90. Parvalbumin was tested at concentrations of up to 0.13 mM; it was phosphorylated at only 2% of the rate seen with peptide 1–106. The striking stimulation caused by high concentrations of Mg^{2+} (see below) was not observed with parvalbumin. Brain calcium-binding protein, CBP-18 [6], at a concentration of 0.14 mg/ml showed no detectable phosphorylation in the presence of either low or high concentrations of Mg^{2+}. The same was true for the intestinal calcium-binding protein [4,5].

Effect of cAMP, Ca^{2+} and lipids. There was no stimulation of phosphorylation by 25 μM 8-bromo-cyclic AMP. Neither did the presence of 2 mM $CaCl_2$ and a catalytic concentration of calmodulin (10^{-6} M) stimulate the phosphorylation of peptide 1–106. On the contrary, inhibition was observed (data not shown).

Effect of inorganic phosphate and fluoride. The protein kinase active against peptide 1–106 was more sensitive to inorganic phosphate than, for example, the catalytic subunit of cAMP-dependent protein kinase; 0.05 M inorganic phosphate inhibited 60%. The effect of inorganic phosphate was immediate. We considered the possibility that phosphatase activity was involved in the incorporation of ^{32}P from ATP into calmodulin peptides. Such a phosphatase might be inhibited by inorganic phosphate. However, there was no effect on the initial rate or extent of reaction by up to 4 h of preincubation before addition of [γ-^{32}P]ATP. Thus, we have no evidence that nonradioactive phosphate groups on the polypeptide must be hydrolyzed in order to free sites for incorporation of ^{32}P.

Other properties of the protein kinase fraction. The phosphorylation of fragment 1–106 was unusually sensitive to fluoride, being inhibited 97% by 0.02 M NaF and 95% by 0.01 M NaF. Histone phosphorylation by the catalytic subunit of cAMP-dependent kinase was inhibited only 23% by 0.02 M NaF.

Ammonium sulfate inhibited 55% at 0.025 M and 80% at 0.06 M. The preparation of kinase tolerated 0.01% sodium azide and the addition of 1/100 volume of toluene, which were required during long incubations.

Effect of divalent cations. The protein kinase showed a requirement for unusually high concentrations of Mg^{2+}. The rate of phosphorylation at 60–70 mM was 21-times greater than at 5 mM (Fig. 6). Sodium ion could not replace the higher

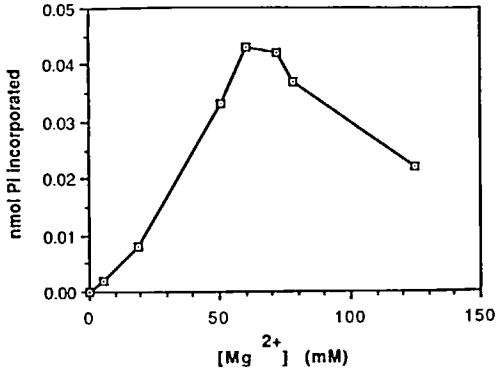

Fig. 6. Effect of Mg^{2+} concentration on the rate of phosphorylation of peptide 1–106. The reaction mixture (400 μl) contained 30 mM Tris-HCl (pH 7.5), 20 μg purified kinase, 90 μg of peptide 1–106, 0.16 mM [γ-^{32}P]ATP (500 cpm per pmol) and $MgCl_2$ as indicated. Incubation was for 45 min at 30°C, after which SDS-polyacrylamide gel electrophoresis was carried out. Gels were stained with Coomassie blue and sections corresponding to peptide 1–106 were cut out, dissolved in 30% H_2O_2 and counted (see Materials and Methods).

concentration of Mg^{2+}. Stimulation by these high levels of Mg^{2+} was observed with fragments 1–106, 78–148 and 1–90. The very low rate of phosphorylation of calmodulin itself showed no striking stimulation by high concentrations of Mg^{2+}. In the presence of 15 mM Mg^{2+}, 1 mM Mn^{2+} inhibited phosphorylation of peptide 1–106 by 80%, and 10 mM Mn^{2+} inhibited it by 93%. NiCl inhibited it completely at a concentration of only 0.01 mM.

Brain fractions also carried out a vigorous phosphorylation of histone, but this reaction was inhibited by 60–70% in the presence of 60 mM Mg^{2+} when compared with 4 mM Mg^{2+} and, in contrast to calmodulin peptides, no inhibition by $CaCl_2$ was observed. The major histone kinase elutes just before the peptide kinase during DE-23 column chromatography. Phosphorylation of histone by the catalytic subunit of cAMP-dependent protein kinase was also inhibited 60% by high levels of Mg^{2+}. Rabbit muscle phosphorylase kinase showed a 10-fold stimulation in the rate of phosphorylation of peptide 1–106 in the presence of high levels of Mg^{2+}. However, this rate of reaction was minute compared with the rate of self phosphorylation of phosphorylase kinase.

Discussion

The present data show that calmodulin is resistant to phosphorylation by fractions from bovine brain. In contrast, the calmodulin peptide fragment 1–106 can be phosphorylated stoichiometrically at a slow, but significant, rate and significant phosphorylation of other fragments has also been observed. Since it is known that secondary structure can affect protein phosphorylation [16], it is reasonable to suppose that this difference in rate may reflect partial unfolding of the peptides as opposed to the high degree of structure of calmodulin. Phosphorylation of different and specific residues in the two peptides that were analyzed in consistent with our present knowledge of the conformation of these peptides. Although the conformation of peptide 1–106 and 78–148 is very similar to that of the same peptides in the native protein, as judged by ^{1}H-NMR studies [17–20], once severed from the protein they melt at a lower temperature [21]. It is reasonable to assume that at least some portions of the peptide molecules, close to the cleavage sites, are less tightly folded than is calmodulin.

Calmodulin is folded as two apparently independent structural units each consisting of two adjacent Ca^{2+}-binding domains [22,23]. In EGTA, domain III of calmodulin is highly susceptible to proteolysis at arginine residues 106, 86 and 90 [1]. By contrast, the lysines in the amino terminus and Arg-74, Lys-75 and Lys-77 in the putative central helix are cleaved more slowly. Cleavage of the molecule at Arg-106, two residues away from the last Ca^{2+} ligand of the third loop, is likely to decrease the stability of this loop, which contains Ser-101, the site of phosphorylation of fragment 1–106.

Fragment 78–148, which has preserved its two complete Ca^{2+}-binding domains (III and IV), is apparently not phosphorylated at Ser-101. In this case, cleavage of the molecule in the middle of the central helix at Lys-77 is likely to destabilize the remaining part of this helix from residue 78 to residue 84. This would be consistent with phosphorylation of Thr-79. The experiments described in this paper suggest that such is indeed the case.

A structural basis for the phosphorylation of calmodulin and its fragments could also explain the requirement for a high concentration of Mg^{2+}. At these concentrations, Mg^{2+} binds calmodulin and induces specific conformational changes which could make sites available for protein kinases. A high requirement for Mg^{2+} was reported by Kilimann and Heilmeyer [24] for one of three separate enzymatic activities of phosphorylase kinase, called A_2. Presumably, under the best conditions that we were able to devise in vitro, specific conformational changes to make sites available for really effective phosphorylation of calmodulin itself were not induced. Plancke and Lazarides [25] found that chicken brain contains an alkali-labile, phosphorylated form of calmodulin present in an amount almost equivalent to the unphosphorylated form. However, they also were unable to demonstrate stoichiometric phosphorylation in vitro. The inhibition of phosphorylation by Ca^{2+} and Mn^{2+}, seen with our brain fractions, is consistent with the idea that cation-induced structural changes might alter the accessibility of phosphory-

lation sites. Finally, the phosphorylation of calmodulin by purified *src* kinase was strongly inhibited by Ca^{2+} [26], and this was accounted for by effects on the structure of calmodulin rather than direct inhibition of the *src* kinase.

If structural alteration of calmodulin exposes potential phosphorylation sites, it remains to be shown whether or not the availability of these sites is a reflection of partial denaturation (preceding protein degradation in vivo or occurring during isolation). Taking into account the recent reports of calmodulin phosphorylation under physiological conditions [25–31], a more attractive hypothesis would be that phosphorylation is part of the mechanism of action of calmodulin. Binding of target proteins, peptides or calmodulin antagonists induces conformational changes detected by ^{1}H-NMR, as in the case of the calmodulin-binding peptide of myosin light chain kinase [32] or lysine reactivity [33]. These changes are different for different targets. Thus, different lysines respond to binding of phenothiazines, β-endorphins, myosin light chain kinase [34] and calcineurin [33]. Under these conditions, only a fraction of calmodulin would become phosphorylated, consistent with the low level of phosphorylation which has been reported. In this connection, it is unfortunate that recent publications [26–31] give no quantitative data on the extent of phosphorylation, except for one study on casein kinase II [35].

In conclusion, it must be admitted that, while the phosphorylation reactions reported here and elsewhere [25–31] are of interest, their significance will depend on whether or not they effect some enzymatic reaction.

Acknowledgements

This work was done during a tenure of L.A.H. as a Fogarty Scholar.

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