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Ca^{2+}-CALMODULIN DEPENDENT PHOSPHORYLATION OF MYELIN ISOLATED FROM RABBIT BRAIN

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SUMMARY

The phosphorylation of myelin (basic protein) purified from rabbit brain was markedly stimulated by exogenously added calmodulin in the presence of calcium and inhibited by W-7(N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide), a calmodulin interacting agent, in a dose-dependent fashion. However, exogenously added myelin basic protein free from protein kinase activity could not serve as a substrate of this calmodulin dependent protein kinase, suggesting that this kinase catalyzes the phosphorylation of the enzyme-substrate complex. These results suggest that a calmodulin-dependent protein kinase complex with the substrate (basic protein) is located in the myelin membrane of the central nervous system.

INTRODUCTION

Myelin isolated from the central nervous system contains myelin basic protein (M.W.=18,400), which constitutes about 30% of the total protein in myelin (1). The protein is unique in inducing experimental allergic encephalomyelitis in laboratory animals when injected with adjuvant. Recently, central nervous system myelin membrane was shown to contain an endogenous, cAMP-independent protein kinase which proved to be mainly responsible for the phosphorylation of myelin basic protein (2, 3). Calmodulin, a heat stable, small (M.W.=16,500) acidic Ca^{2+}-binding protein (4) is known to mediate a number of Ca^{2+}-mediated effects in a variety of cellular reactions (5-11). Among these, glycogen metabolism and smooth muscle contraction are regulated by calmodulin

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The abbreviations used are : EGTA; ethyleneglycol-bis-(β-aminoethyl ether)-N,N'-tetraacetic acid.
SDS; sodium dodecyl sulfate

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via phosphorylation of glycogen phosphorylase and phosphorylation of the myosin light chain (12-15).

W-7(N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide) reportedly inhibits Ca^{2+}-dependent phosphodiesterase (16), Ca^{2+}-Mg^{2+} ATPase from erythrocyte ghost (17) and myosin light chain kinase from chicken gizzard (18, 19) by interacting with calmodulin in the presence of calcium.

In the present paper, we demonstrate that central nervous system myelin contains calmodulin-dependent protein kinase which catalyzes the phosphorylation of myelin basic protein and that W-7 inhibits this protein kinase. Substrate specificity of this calmodulin dependent protein kinase was also investigated.

MATERIALS AND METHODS

Preparation of myelin fraction : Myelin fraction was prepared from rabbit (male New Zealand white rabbit) brain by the procedure of Miyamoto et al.(2).

Protein preparation : Calmodulin was purified from bovine brain by the method of Teo et al.(20), myelin basic protein by the method of Oshiro and Eylar (21), and myosin light chain of chicken gizzard by the method of Klee (22). Calf thymus histone (Type II), protamine and casein were purchased from Sigma.

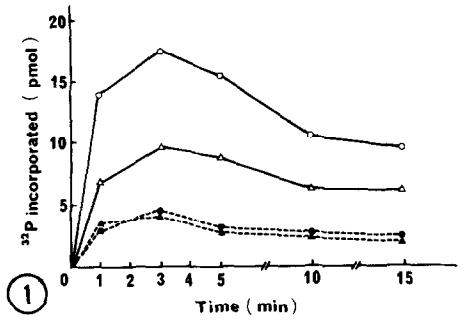
Enzyme assays : Phosphorylation of the myelin proteins was assayed as described previously (18) in a reaction mixture containing in a final volume of 0.3 ml, Tris-HCl buffer (pH 7.5), 15 μmol; $MgCl_2$, 3 μmol; [γ-^{32}P]ATP, 1.5 nmol (0.5-1.5·10^6 c.p.m.); EGTA, 0.6 μmol or $CaCl_2$, 30 nmol; 12.5 μl of the myelin fraction containing 50 μg of protein; various amounts of calmodulin as indicated. The standard incubation was carried out at 30°C for 3 min. The reaction was terminated by the addition of 1 ml of ice cold 20% trichloroacetic acid following addition of 500 μg of bovine serum albumin, as a carrier protein. The sample was centrifuged at 3,000 rpm for 10 min, the pellet resuspended in ice cold 10% trichloroacetic acid solution and the centrifugation-resuspension cycle was repeated three times. The final pellet was dissolved in 1 ml of 0.5 N NaOH and the radioactivity was measured by liquid scintillation counter.

Gel electrophoresis and autaradiography : The standard enzyme assay reaction was stopped by the addition of 10% SDS solution and the addition of bovine serum albumin was omitted. SDS-polyacrylamide gel electrophoresis was performed on a 15% acrylamide slab gel containing 0.1% SDS by the procedure of Laemmli (23). Autoradiography was carried out by using Kodak X-Omat RP film.

Materials : W-7 was synthesized according to methods of Hidaka et al.(24).

RESULTS AND DISCUSSION

The time course of endogenous phosphorylation of the myelin fraction is shown in Fig. 1. Incorporation of phosphate into the myelin fraction was observed both in the presence and absence of Ca^{2+}. When 5 μg/ml of calmodulin



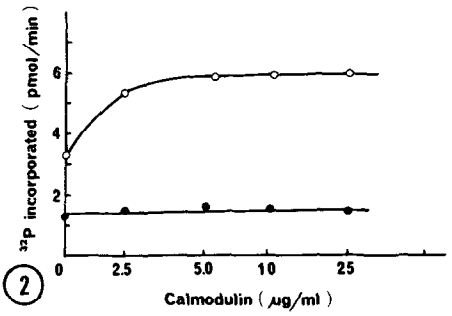
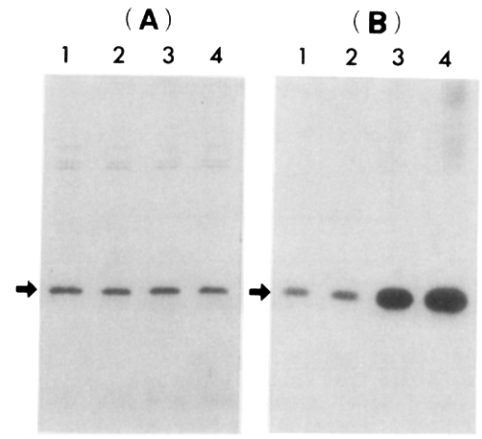


Fig. 1 Time course of endogenous phosphorylation of myelin fraction
Myelin fraction (50 μg of protein) obtained from rabbit brain was incubated with (○●) or without (△ ▲) 5 μg/ml calmodulin in the presence (——) or absence (------) of Ca^{2+}. Reaction was carried out as described in the text.

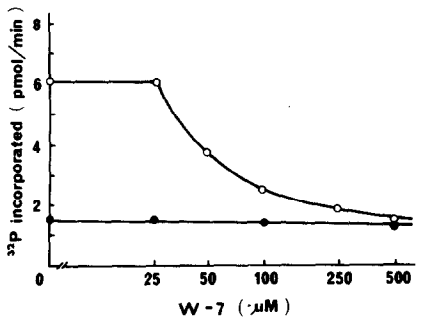
Fig. 2 Concentration dependence of calmodulin stimulation of endogenous myelin phosphoryaltion
Myelin fraction (50 μg of protein) obtained from rabbit brain was incubated with various amounts of calmodulin in the presence (○) or absence (●) of Ca^{2+}. Other incubation conditions were as described in the text.

was added to the reaction mixture in the presence of Ca^{2+}, there was a 3.8-fold stimulation of endogenous phosphorylation (Fig. 1). Addition of Ca^{2+} alone to the reaction mixture also stimulated phosphorylation of the myelin fraction, suggesting that this fraction contained endogenous calmodulin. Fig. 2 shows the activation as a function of the amount of calmodulin added. Maximal stimulation occurred with 5 μg/ml and half-maximal required about 1 μg/ml of calmodulin.

To determine the location of radioactivity incorporated into the myelin fraction, the phosphorylated myelin fraction was subjected to analytical slab gel electrophoresis after incubation for 3 min in the presence or absence of Ca^{2+}, with or without 5 μg/ml of calmodulin. One major band, myelin basic protein, and a few minor bands were observed by protein staining after electrophoresis (Fig. 3-A). An autoradiogram of the gel showed that radioactivity was mostly located in the band which corresponded to the myelin basic protein and that calmodulin in the presence of Ca^{2+} promoted incorporation of phosphate into the protein (Fig. 3-B).



(3)



(4)

Fig. 3 SDS-polyacrylamide gel electrophoresis (A) an its autoradiography (B) of phosphorylated myelin fraction
Incubation was performed as described in the text. Approximately 10 μg of protein were loaded in each line of 15% polyacrylamide gel using slab electrophoretic apparatus. Protein were stained with Coomassie brilliant blue. Lane 1, 2 mM EGTA; 2, 2 mM EGTA + 5 μg/ml calmodulin; 3, 100 μM $CaCl_2$; 4, 100 μM $CaCl_2$ + 5 μg/ml calmodulin. Arrow indicates myelin basic protein.

Fig. 4 Inhibition of endogenous myelin phosphorylation by W-7
Myelin fraction (50 μg of protein) obtained from rabbit brain was incubated with various concentrations of W-7 in the presence of 100 μM Ca^{2+} and 5 μg/ml calmodulin (○) or 2 mM EGTA (●). Other incubation conditions were as described in the text.

W-7 inhibited this calmodulin stimulated phosphorylation of myelin basic protein in a dose dependent manner (Fig. 4). A concentration of 70 μM produced 50% inhibition. Not only Ca^{2+}-calmodulin induced stimulation of myelin basic protein phosphorylation but the phosphorylation of the protein in the presence of Ca^{2+} alone was also inhibited by addition of W-7. In other words, W-7 inhibited Ca^{2+}-stimulated phosphorylation both in the presence and absence of exogenously added calmodulin, thereby suggesting that the myelin fraction used in this experiment contained calmodulin and moreover, Ca^{2+}-dependent activity of this myelin phosphorylation was mediated by calmodulin.

The extent to which myelin basic protein, myosin light chain, histone, casein and protamine could serve as a substrate when added exogenously to the reaction mixture of the phosphorylation was then investigated (Table 1).

TABLE 1

Phosphorylation of various substrates by rabbit brain myelin associated protein kinase

Addition	Protein kinase activity (pmol/min)		
	+EGTA (I)	+Ca^{2+}-calmodulin (II)	Ca^{2+}-calmodulin stimulation (II - I)
Myelin	2.1	6.7	4.6
Myelin plus myelin basic protein	4.1	6.4	2.3
Myelin plus myosin light chain	1.9	6.6	4.7
Myelin plus histone	3.1	2.9	-0.2
Myelin plus casein	2.3	6.5	4.2
Myelin plus protamine	8.6	12.1	4.5

Myelin fraction (50 μg of protein) obtained from rabbit brain was incubated with exogenously added proteins (100 μg) as indicated in the presence of 100 μM Ca^{2+} and 5 μg/ml calmodulin or 2 mM EGTA. Other incubation conditions were as described in the text.

Although myelin basic protein, histone and protamine were adequate substrates for the activity in the absence of Ca^{2+}(Mg^{2+}-dependent endogenous protein kinase), the phosphorylation of these three proteins was not stimulated with the addition of Ca^{2+}-calmodulin.

We have thus demonstrated that the myelin fraction from the central nervous system contained Ca^{2+}-calmodulin dependent protein kinase which catalyzed myelin basic protein. This Ca^{2+}-calmodulin dependent protein kinase seemed to form an enzyme-substrate complex in the myelin membrane as the isolated myelin basic protein could not serve as a substrate when added exogenously to the reaction mixture. Histone could serve as a substrate for Mg^{2+}-dependent protein kinase, as was also found by Miyamoto and Kakiuchi (2). However, this histone inhibited Ca^{2+}-calmodulin dependent protein kinase (Table 1), suggesting that this enzyme is not identical with Mg^{2+}-dependent protein kinase. Furthermore 10 mM NaF reportedly has no effect to Mg^{2+}-dependent protein kinase and inhibits ATPase activity in the myelin fraction (25). Ca^{2+}-calmodulin dependent protein kinase in our myelin preparation was markedly inhibited by 10 mM NaF (data not shown).

These results also suggest that Ca^{2+}-calmodulin dependent protein kinase in the myelin fraction differes from the Mg^{2+}-dependent protein kinase.

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