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Calcium- and cyclic AMP-regulated protein kinases of bovine central-nervous-system myelin

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Bovine central-nervous-system myelin was found to contain both Ca^{2+}-activated and cyclic AMP-dependent protein kinases. Each enzyme possesses unique solubility and substrate-specificity characteristics. The Ca^{2+}-activated enzyme, like its substrate (basic protein), is probably deeply embedded in the neural membrane, whereas the cyclic AMP-dependent kinase appears to be much less tightly associated with myelin. Treatment of insoluble myelin fraction housing the Ca^{2+}-activated kinase with phospholipase A_2 and phospholipases A_2+C causes a decrease in its ability to become activated by Ca^{2+}. This can be countered by phosphatidylserine and phosphatidylethanolamine. Whereas the activity of the Ca^{2+}-activated membrane-associated kinase is inhibited by chlorpromazine, dibucaine, melittin and Triton X-100, it is activated by certain phorbol diesters (4β-phorbol 12-myristate 13-acetate, 4β-phorbol 12,13-didecanoate, 4β-phorbol 12,13-dibenzoate and 4β-phorbol 12,13-diacetate), which appear to exert this effect by lowering the concentration of Ca^{2+} normally required for the activation of this enzyme. Together these results suggest that the activation of the membrane-associated kinase by Ca^{2+} most probably requires certain lipids, perhaps those already present in the membrane.

Brain tissue contains a variety of effector-specific protein kinases. In addition to the cyclic AMP-dependent and cyclic GMP-dependent protein kinases, both the cytosolic and membrane fractions of neural origin contain Ca^{2+} + calmodulin-activated and Ca^{2+} + phospholipid-activated phosphotransferases (Takai *et al.*, 1977; Wrenn *et al.*, 1980; Greengard, 1981). The distribution of phosphate-acceptor proteins and the enzymes capable of removing phosphate moieties (phosphoprotein phosphatases) from the phosphoproteins closely parallels that found for the protein kinases (Ueda *et al.*, 1975). Thus post-translational control of protein function may occur in a cellular compartment that houses both converter enzymes (protein kinases and phosphoprotein phosphatases) and their substrates.

Myelin is a neural membrane. Its lipid–protein composition gives it characteristic hydrodynamic properties that allow the rapid isolation of myelin of a high degree of purity by rather simple procedures (Uyemura *et al.*, 1972; Norton, 1974). Highly purified myelin preparations from a variety of sources have been found to contain both the protein kinase and phosphoprotein phosphatase activities that add phosphate to and remove it from basic protein, a major myelin constituent (Carnegie *et al.*, 1973, 1974; Miyamoto & Kakiuchi, 1974, 1975; Steck & Appel, 1974; McNamara & Appel, 1977; Yourist *et al.*, 1978; Wu *et al.*, 1980; Sulakhe *et al.*, 1980; Wu *et al.*, 1983).

Not only does basic protein undergo multi-site phosphorylation under appropriate conditions *in vitro*, but it is also phosphorylated (under physiological conditions) when the animals are injected with [^{32}P]orthophosphate (Miyamoto & Kakiuchi, 1974; Steck & Appel, 1975; Agrawal *et al.*, 1982). The oscillation of myelin basic protein between phospho and dephospho forms would potentially constitute a futile ATPase cycle unless the activities of the enzymes catalysing these interconversions are rigidly controlled. We have previously shown the presence of a basic-protein-specific phosphatase in human central-nervous-system myelin preparations (Yourist *et al.*, 1978; Wu *et al.*, 1980). Both the purified and partially purified preparations of this phosphatase are activated markedly by certain bivalent cations (especially by Mn^{2+}), raising the possibility that changing concentrations of these cations may modulate the

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activity of this enzyme in myelin. The present paper deals with the regulation of the phosphotransferases found in bovine central-nervous-system myelin. The results obtained show that bovine myelin contains at least two different protein kinases. In addition to a cyclic AMP-dependent kinase, there is present another kinase whose activation requires the presence of both Ca^{2+} and certain phospholipids, presumably already present in the myelin membrane. Its solubility characteristics suggest that the Ca^{2+}-activated kinase may be deeply embedded in the membrane whereas the cyclic AMP-dependent enzyme is peripherally oriented. The substrate specificities as well as the effects of a number of compounds on the activities of these enzymes are described.

Materials and methods

Materials

Benzamidine, various histone fractions, phosphatidylserine, phosphatidylethanolamine, palmitoylcarnitine, different brain extracts, chlorpromazine, dibucaine, mellitin, protein kinase inhibitor and phorbol and its different derivatives were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Calmodulin was purchased from Calbiochem–Behring Corp. (San Diego, CA, U.S.A.). 4β-Phorbol 12-myristate 13-acetate was a gift from Dr. R. Zucker of this Institute. Triton X-100 was from Polysciences (Warrington, PA, U.S.A.) and [γ-^{32}P]ATP (sp. radioactivity 1–3 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, MA, U.S.A.). Trifluoperazine was a gift from Dr. H. Green, Smith, Kline and French, Philadelphia, PA, U.S.A. All other chemicals used were of the highest purity commercially available.

Isolation of myelin

Myelin was isolated from bovine white matter essentially by the method of Uyemura *et al.* (1972). This method has been described in detail elsewhere (Wu *et al.*, 1983).

Myelin purified through at least three successive sucrose density gradients was used in the experiments described below. Such myelin preparations are mostly white in appearance. When examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, besides a few minor protein bands, the most prominent components are basic protein, proteolipid and the Wolfgram protein. In a number of myelin preparations we have observed the presence of two closely migrating components of M_r 18000–19000. This suggests that (*a*) basic protein had undergone partial degradation or (*b*) bovine myelin contains more than one basic protein, as is the case for myelin from other species (Barbarese *et al.*, 1978; Agrawal *et al.*, 1981, 1982; Carnegie *et al.*, 1983).

No significant differences either in sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic patterns or in the activities of different protein kinases were observed between myelin isolated from freshly dissected white matter or from the white matter that had been kept at −70°C for up to 2 months, after homogenization in 0.32 M-sucrose.

Isolation of the unmodified form of basic protein

Purification of basic protein from bovine central-nervous system tissue was achieved by applying certain modifications to the published procedures (Deibler & Martenson, 1973; Chou *et al.*, 1976). Myelin partially purified through one sucrose-gradient centrifugation was delipidated successively with diethyl ether/methanol (3:2, v/v) followed by acetone, washed with water and then extracted overnight with a dilute solution of HCl (approx. 1 mM, pH 3.0). The suspension was centrifuged. The supernatant was fractionated on a Sephadex G-50 (superfine grade) column (4 cm × 90 cm). Fractions containing protein were pooled, concentrated and then applied to a CM-52 CM-cellulose column (2 cm × 15 cm) previously equilibrated with 6 M-urea/0.08 M-glycine/NaOH buffer, pH 10.4. The protein was eluted by establishing a linear gradient of 0–0.3 M-KCl in 2 M-urea/0.08 M-glycine/NaOH buffer, pH 10.4. The major protein peak, emerging last, contained dephospho basic protein. Fractions comprising this peak were pooled, desalted and then concentrated. When examined by polyacrylamide-gel electrophoresis under denaturing (sodium dodecyl sulphate) and non-denaturing (pH 10) conditions, only a single Coomassie Blue-staining protein component was detectable. Similar preparations are known to contain basic protein that has undergone no post-translational modification, except for methylation of the arginine residue at position 109 (Smith, 1982).

Extraction of protein kinases from myelin

The buffers employed were buffer A, containing 20 mM-Tris/HCl buffer, pH 8.0, 20 mM-benzamidine, 50 mM-2-mercaptoethanol and 0.15% (v/v) Triton X-100, and buffer B, containing all of the components of buffer A except Triton X-100.

Bovine myelin was suspended in buffer A at a final protein concentration of 2–3 mg/ml. After being stirred for 16 h at 4°C, the myelin suspension was centrifuged at 100000 *g* for 1 h. This procedure was found to give optimal cyclic AMP-dependent protein kinase activity in the supernatant fraction. The Ca^{2+}-activated kinase remained with the 100000 *g* pellet. The pellet constitutes the insoluble

myelin fraction. It was suspended in buffer A and then assayed for the kinase activity.

In contrast with insoluble myelin, myelin suspended in buffers A and B is referred to as the undisrupted myelin.

Protein kinase assay

Cyclic AMP-dependent protein kinase activity was assayed in a total volume of 100 μl containing 50mM-Tris/HCl buffer, pH8.0, 10mM-$MgCl_2$, 50mM-2-mercaptoethanol, 10μM-cyclic AMP, 100μg of basic protein or other substrate proteins, 110μM-[γ-^{32}P]ATP (100c.p.m./pmol) and an appropriate amount of protein kinase. The Ca^{2+}-activated protein kinase was assayed under identical conditions except that cyclic AMP was replaced by that concentration of $CaCl_2$ required to provide 10 μM free Ca^{2+} or such concentrations as shown in the legends to Figures and Tables. During these assays, Triton X-100 was present at a final concentration of 0.015%. Higher concentrations of the detergent are inhibitory (see the Results section). After incubation at 37°C for 5min the reactions were terminated by the addition of acetic acid. The incorporation of radioactivity into the acceptor proteins was quantified as described previously (Wu *et al.*, 1983). One unit of enzyme activity represents the transfer of 1 nmol of P_i from [γ-^{32}P]ATP to the substrate per min at 37°C.

Identification of sites phosphorylated by myelin kinases

Basic protein (20mg) was phosphorylated separately by the cyclic AMP-dependent protein kinase (0.4mg of protein present in the 100000*g* supernatant) and the Ca^{2+}-activated kinase (insoluble myelin equivalent to 1.6mg of protein) under the conditions described above except that the incubation volume was 4.0ml and the duration of phosphorylation was extended to 4h. After incubation at 37°C, the phosphorylation mixture (15000*g* supernatant in the case of the protein phosphorylated by Ca^{2+}-activated kinase) was adjusted to pH10 with NaOH and applied to a CM-52 CM-cellulose column. Elution was achieved with a linear gradient of 0–0.3M-NaCl in 2M-urea/0.08M-glycine buffer, pH10.4. Fractions containing phospho basic protein, located by radioactivity, were pooled, desalted over a Sephadex G-25 column with 0.1M-acetic acid, and then freeze-dried. The freeze-dried powder was dissolved in 4ml of 20mM-NH_4HCO_3. This was digested with 400μg of trypsin at 37°C for 4h, and tryptic peptides were separated first by chromatography on a silica gel G thin-layer plate with the solvent system chloroform/methanol/30% (w/v) NH_3 (2:2:1, by vol.) and then by electrophoresis at 90° to the direction of chromatography in a buffer system containing pyridine/acetic acid/water (200:7:1800, by vol.). Peptides were located by spraying with ninhydrin, and the phosphopeptides were located by autoradiography. The radioactive spots were removed by scraping, and the silica gel was extracted with 10mM-NH_3. After acid hydrolysis their amino acid compositions were determined with a Beckman 120C amino acid analyser. Since the complete covalent structure of bovine basic protein is known (Eylar *et al.*, 1971), the amino acid composition of the phosphopeptide provided information sufficient to identify the site(s) phosphorylated by myelin kinases.

Other methods

Phospholipid vesicles were prepared as described by Wu *et al.* (1983). Protein was measured by the method of Bradford (1976). Protein concentrations of myelin preparations (undisrupted and insoluble fractions) were determined by the method of Lowry *et al.* (1951) after digestion of the membrane in 0.5% sodium deoxycholate/0.5M-NaOH (Petrali *et al.*, 1980*a*). Bovine serum albumin was used as a standard. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed by the method of Weber & Osborn (1969).

Results

Evidence for the presence of Ca^{2+}-activated and cyclic AMP-dependent protein kinases in bovine central-nervous-system myelin preparations

When a suspension of myelin in Triton-containing buffer (buffer A) was incubated with [γ-^{32}P]ATP, phosphorylation of a number of endogenous proteins was observed. The phosphorylation of basic protein was most prominent. After sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis, autoradiographic procedures allowed identification of endogenous proteins phosphorylated by myelin kinases as basic protein and 25000-M_r and 45000-M_r proteins (N. C. Wu & F. Ahmad, unpublished work). When purified basic protein was included in the reaction mixture containing myelin, it was rapidly phosphorylated. Optimal phosphorylation of exogenous basic protein occurred in the presence of 10mM-Mg^{2+}. Cyclic AMP, but not cyclic GMP, modestly enhanced the rate of phosphorylation of exogenous basic protein, whereas the addition of EGTA markedly decreased its phosphorylation. These results suggest that, besides Mg^{2+} (to chelate ATP), Ca^{2+} was probably required for the activation of the phosphotransferase reaction catalysed by myelin kinase(s).

When certain histone fractions replaced basic protein as substrate, the presence of cyclic AMP

enhanced the activity of the undisrupted myelin kinase(s) about 2–3-fold (Table 1). The rate of phosphorylation of histone V in the presence of cyclic AMP was nearly equal to the rate at which basic protein was phosphorylated by the myelin kinase(s). This cyclic AMP stimulation of kinase activity was almost completely blocked by addition of a heat-stable protein known to inhibit the cyclic AMP-dependent kinases (Traugh *et al.*, 1974).

With undisrupted myelin as enzyme source, phosphorylation of exogenously added basic protein was activated by Ca^{2+}. Activation of the phosphotransferase reaction by cyclic AMP was usually observed in histone phosphorylation (Table 1). Thus substrate selection played an important role in unravelling the nature of the regulators of different kinases associated with bovine central-nervous-system myelin.

Solubility characteristics of myelin kinases were then investigated. Myelin was suspended in buffer lacking Triton X-100 (buffer B). After dispersal, the suspension was centrifuged at 100000*g*. Distribution of kinase activities in both the supernatant and the insoluble fractions was examined with basic protein and histones as the substrates. Essentially no kinase activity was found in the supernatant fraction. Both the cyclic AMP-dependent and the Ca^{2+}-activated kinases remained with the insoluble myelin fraction (results not shown). When a similar set of experiments was performed with the use of buffer A (containing detergent) as the extractant, enzymic activities were present in both the fractions (Table 1). The supernatant derived from the first extraction contained most (about 85%) of the cyclic AMP-dependent kinase, whereas the Ca^{2+}-activated enzyme remained in the insoluble fraction. Since these enzymes displayed a reasonable degree of substrate-specificity, the soluble enzyme (cyclic AMP-dependent) may be referred to as histone kinase and the membrane-associated Ca^{2+}-activated enzyme as basic-protein kinase. Similar solubility and substrate-specificity characteristics have been noted previously for the human myelin kinases. However, the basic-protein-specific human myelin kinase was not activated by Ca^{2+} (Wu *et al.*, 1983). The reasons for this difference between the human and bovine myelin preparations are not clear.

Characterization of the basic-protein kinase

Effect of Ca^{2+}. The results discussed here deal with the Ca^{2+}-activated kinase of bovine myelin. In most of the experiments described below, myelin previously extracted with buffer A (insoluble fraction) served as the source for the Ca^{2+}-activated kinase. [Phosphorylation of basic protein catalysed by the enzyme(s) present in insoluble myelin fraction was not inhibited by the inhibitor protein of the cyclic AMP-dependent protein kinase.] The effect of varying the concentration of Ca^{2+} on the rate of phosphorylation of exogenous-

Table 1. *Solubility characteristics of bovine myelin protein kinases*

Phosphorylation of basic protein and histone V was measured in the presence and in the absence of cyclic AMP (10 μM) under the conditions detailed in the Materials and methods section. Undisrupted myelin, insoluble myelin fractions (pellets insoluble in buffer A) and their corresponding high-speed supernatants provided the enzyme source. On the basis of the units of activity found in various fractions, nearly 85% of the cyclic AMP-dependent kinase activity (histone V as substrate) was solubilized from myelin during the first extraction.

Enzyme source	Cyclic AMP (10 μM)	Activity (units/mg of protein) With histone V	With basic protein
Undisrupted myelin	−	1.0	2.4
	+	2.4	2.5
1st extraction with a buffer containing Triton X-100			
100000*g* supernatant	−	2.2	0.2
	+	4.9	0.5
Pellet	−	0.7	2.4
	+	1.1	2.3
2nd extraction with a buffer containing Triton X-100			
100000*g* supernatant	−	0.8	0.1
	+	1.4	0.1
Pellet	−	0.7	2.4
	+	1.0	2.6
3rd extraction with a buffer containing Triton X-100			
100000*g* supernatant	−	0.4	0.1
	+	0.6	0.1
Pellet	−	0.7	2.3
	+	1.0	2.4

ly added basic protein is given in Fig. 1. This effect was biphasic, with maximum activation occurring at approx. 100μM-Ca^{2+}. When the concentration of Ca^{2+} approached 1mM, however, phosphorylation of basic protein was inhibited. The most marked increase in the rate of phosphorylation of basic protein was observed in the concentration range 1–10μM, and less than 5μM-Ca^{2+} was required for half-maximal activation. This sensitivity to Ca^{2+} may make the basic-protein kinase responsive to fluctuations in Ca^{2+} concentrations believed to occur in neural tissue *in vivo* (Kretsinger, 1979).

Substrate specificity. Among the various substrate proteins tested, basic protein appeared to be the best substrate for the Ca^{2+}-activated myelin enzyme (Table 2). In a number of myelin preparations examined, the rate of phosphorylation of basic protein was increased 2–4-fold by 10μM-Ca^{2+}. Under optimal assay conditions, about 30–60pmol of phosphate was incorporated/min per 0.1mg of basic protein.

Presence of Ca^{2+} affected the phosphorylation of histones minimally when undisrupted myelin served as the enzyme source. However, with insoluble myelin as the enzyme source, phosphorylation of various histone fractions was stimulated by this cation (Table 2). Therefore removal of the cyclic AMP-dependent kinase from myelin was needed to observe activation by Ca^{2+} of histone phosphorylation catalysed by the basic-protein kinase.

Effects of calmodulin, phospholipids and certain chemical agents on activity. Sulakhe and co-workers provided evidence suggesting that calmodulin or a calmodulin-like protein was involved in Ca^{2+}-stimulated phosphorylation of basic protein by the rat myelin enzyme (Petrali *et al.*, 1980*a*,*b*; Sulakhe *et al.*, 1980). However, the data of Kuo and co-workers did not provide support for this suggestion (Turner *et al.*, 1982). Phosphorylation of basic protein catalysed by the bovine myelin enzyme was also not affected by calmodulin (Table 3), indicating that (*a*) Ca^{2+} + calmodulin-regulated kinase was absent and/or (*b*) bovine myelin contained saturating concentrations of calmodulin, so that further addition of this cofactor was not required. To distinguish between these possibilities, insoluble myelin was extracted with a buffer containing EGTA to remove traces of calmodulin known to be present in undisrupted myelin (Grand & Perry, 1980). When this preparation was used as a source for the Ca^{2+}-activated enzyme, addition of calmodulin did not affect the rate of phosphorylation of basic protein. These results support the notion that Ca^{2+} + calmodulin-regulated kinase is not present in bovine myelin, or if it is present then it must have an extremely low specificity towards basic protein.

A new class of Ca^{2+}-dependent protein kinases, recognized in recent years, required certain phospholipids as cofactors (Takai *et al.*, 1977; Wrenn *et al.*, 1980). Table 3 compares the effects of phospholipids on basic-protein phosphorylation. Neither pure phospholipids nor those present in various brain extracts produced any noticeable effects over those found with Ca^{2+} alone.

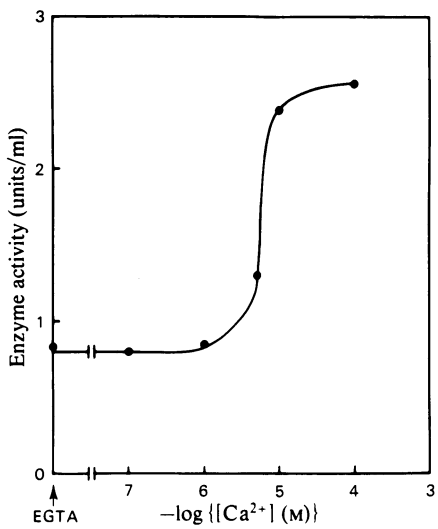

Fig. 1. *Effect of Ca^{2+} on protein kinase activity*
Protein kinase activity was measured with basic protein as substrate under the assay conditions described in the Materials and methods section. Each assay mixture contained 0.5mM-EGTA and an amount of $CaCl_2$ sufficient to give the concentration of free Ca^{2+} indicated. The arrow marked EGTA corresponds to no exogenous addition of $CaCl_2$. Insoluble myelin provided the enzyme source.

Table 2. *Substrate-specificity of Ca^{2+}-activated bovine myelin protein kinase*

Phosphorylation of different proteins was measured as given in the Materials and methods section. Insoluble myelin was the enzyme source. Assays were performed in the absence and in the presence of 10μM-Ca^{2+} with the substrates shown. The presence of 0.5mM-EGTA during assays provided Ca^{2+}-free conditions.

	Activity (units/mg of protein)	
Substrate	$-Ca^{2+}$	$+Ca^{2+}$
Bovine basic protein	2.2	5.3
Histone II	0.6	1.2
Histone III	1.7	4.0
Histone V	1.6	2.4
Histone VII	1.3	1.9

Table 3. *Effects of calmodulin and phospholipids on the phosphorylation of basic protein catalysed by Ca^{2+}-activated bovine myelin protein kinase*

Assays were performed in the presence and in the absence of 10 μM-Ca^{2+}, with insoluble myelin as the enzyme source. The concentrations of various effectors tested are shown against individual experiments. 'None' indicates activity in the absence of exogenously added Ca^{2+}.

Additions	Relative activity (%)
None	42
Ca^{2+}	100
Ca^{2+} + calmodulin (25 μg)	94
Ca^{2+} + trifluoperazine (200 μM)	53
Ca^{2+} + trifluoperazine (200 μM) + calmodulin (25 μg)	57
Ca^{2+} + trifluoperazine (200 μM) + calmodulin (50 μg)	53
Ca^{2+} + trifluoperazine (200 μM) + calmodulin (75 μg)	49
Ca^{2+} + phosphatidylserine (0.2 mg/ml)	102
Ca^{2+} + phosphatidylethanolamine (0.2 mg/ml)	102
Ca^{2+} + palmitoylcarnitine (0.1 mM)	100
Ca^{2+} + brain extracts type I (0.2 mg/ml)	102
Ca^{2+} + brain extracts type III (0.2 mg/ml)	104
Ca^{2+} + brain extracts type V (0.2 mg/ml)	96

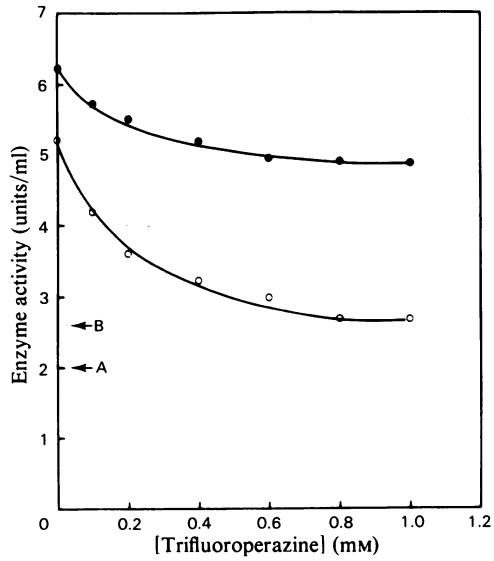

Fig. 2. *Effect of trifluoperazine on protein kinase activity*
Protein kinase activity was measured at various concentrations of trifluoperazine in the presence (20 μg; ●) and in the absence (○) of phosphatidylserine. Arrows marked A and B indicate enzyme activities in the presence of EGTA (0.5 mM) and EGTA (0.5 mM) + phosphatidylserine (20 μg) respectively. Assays were performed with basic protein as substrate and insoluble myelin as the enzyme source.

Trifluoperazine has been employed as a diagnostic tool in detecting the involvement of calmodulin in calmodulin-regulated reactions (Cheung, 1979), but this drug also inhibits the Ca^{2+}-activated phospholipid-dependent kinase activities (Schatzman *et al.*, 1981). The basic-protein kinase was likewise inhibited by trifluoperazine, and the inhibition was concentration-dependent. Trifluoperazine virtually blocked the activation induced by Ca^{2+} (Fig. 2 and Table 3). Although this inhibition was not reversed by calmodulin (Table 3), it was countered, to a large extent, by phosphatidylserine (Fig. 2).

In addition to trifluoperazine, a number of other compounds, such as chlorpromazine, dibucaine and mellitin, have previously been found to inhibit the Ca^{2+}-activated phospholipid-dependent protein kinases of brain, spleen, heart etc. (Schatzman *et al.*, 1981; Katoh *et al.*, 1982). Ca^{2+}-activated bovine myelin enzyme was also inhibited by these compounds (Table 4).

Besides the compounds mentioned above, Triton X-100 inhibited the reaction catalysed by the Ca^{2+}-activated myelin kinase. At concentrations exceeding 0.15%, the stimulation caused by Ca^{2+} was almost completely abolished by the detergent (Fig. 3). A similar deleterious effect of Triton X-100 was observed previously on the Ca^{2+}-activated phospholipid-dependent kinase (Katoh & Kuo, 1982).

Table 4. *Effect of chemical agents on the phosphorylation of basic protein catalysed by Ca^{2+}-activated protein kinase*

Assays were performed, with insoluble myelin as enzyme source, in the absence and in the presence of 10 μM-Ca^{2+} as given in the legend to Fig. 1. The concentrations of drugs tested are given in parentheses.

Addition	Activity (units/mg of protein) $-Ca^{2+}$	$+Ca^{2+}$
None	1.7	7.1
Chlorpromazine (1 mM)	0.7	1.1
Dibucaine (1 mM)	0.9	1.3
Melittin (52 mM)	1.1	4.1

In the absence of exogenously added Ca^{2+}, 4β-phorbol 12-myristate 13-acetate, 4β-phorbol 12,13-didecanoate, 4β-phorbol 12,13-dibenzoate and 4β-phorbol 12,13-diacetate markedly enhanced the activity of the basic-protein kinase, whereas α- and β-phorbol and a number of their derivatives exerted marginal effects. The rate of phosphorylation of basic protein in the presence of 4β-phorbol

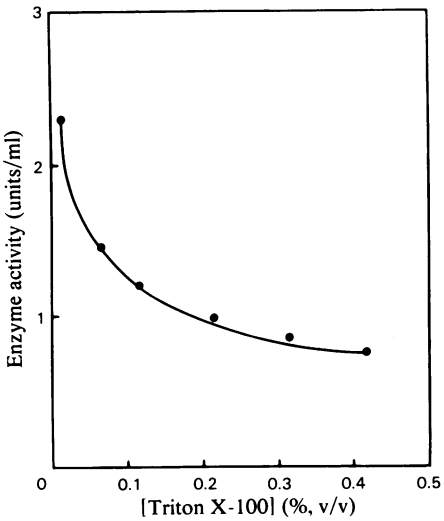

Fig. 3. *Inhibition of myelin protein kinase activity by Triton X-100*

Protein kinase activity was determined in the presence of 10 μM free Ca^{2+} and at various concentrations of Triton X-100. Basic protein was the substrate and insoluble myelin served as the enzyme source.

Table 5. *Effect of phorbol esters on the phosphorylation of basic protein catalysed by Ca^{2+}-activated protein kinase*

Insoluble myelin was the enzyme source. Each compound, except 4β-phorbol 12-myristate 13-acetate (12 μg/ml), was tested at a final concentration of 20 μg/ml. Phosphorylation of exogenously added basic protein was measured as given in the Materials and methods section.

Phorbol ester	Ca^{2+} (10 μM)	Relative activity (%)
None	+	100
4β-Phorbol 12-myristate 13-acetate	–	71
4β-Phorbol 12,13-didecanoate	–	70
4α-Phorbol 12,13-didecanoate	–	0
4β-Phorbol 12,13-dibenzoate	–	52
4β-Phorbol 12,13-diacetate	–	41
4β-Phorbol 13-acetate	–	11
4β-Phorbol 12-myristate	–	4
4β-Phorbol 13,20-diacetate	–	0
4β-Phorbol	–	0
4α-Phorbol	–	0

12-myristate 13-acetate approached almost 70% the value observed with optimal Ca^{2+} concentration (Table 5). During these experiments precautions to exclude Ca^{2+} from buffers and other solutions were not taken. Therefore some of the phorbol esters caused the enzyme to become activated at relatively low (contaminating) concen-

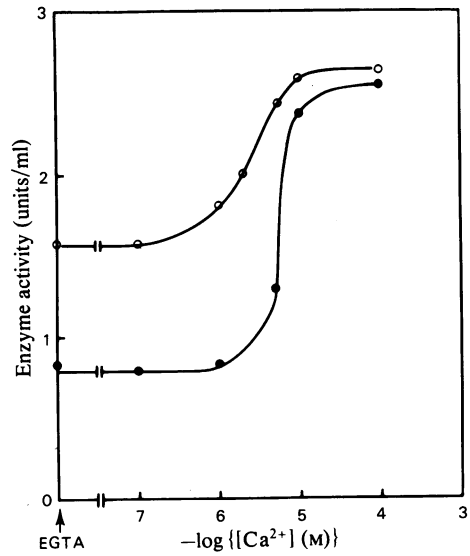

Fig. 4. *Effect of 4β-phorbol 12-myristate 13-acetate on protein kinase activity*

Assays were performed in the presence (120 ng/assay; ○) and in the absence (●) of 4β-phorbol 12-myristate 13-acetate. The phosphorylation of basic protein catalysed by the insoluble myelin-associated enzyme was measured as given in the legend to Fig. 1. The arrow marked EGTA corresponds to no exogenous addition of $CaCl_2$.

trations of Ca^{2+}. The results of kinetic analysis (Fig. 4) supported this suggestion, since in the presence of 4β-phorbol 12-myristate 13-acetate a decrease (5- 10-fold) in the concentration of Ca^{2+} normally required to achieve half-maximal activation was observed. 4β-Phorbol 12-myristate 13-acetate has also been found to increase greatly the affinity of the Ca^{2+} + phospholipid-dependent kinase for both Ca^{2+} and phospholipids (Castagna *et al.*, 1982).

Effect of phospholipase treatment on activity. Ca^{2+} alone could cause maximum enhancement in the rate of phosphorylation of basic protein catalysed by the Ca^{2+}-dependent enzyme present in the insoluble myelin fraction. In addition to Ca^{2+}, the presence of pure or a mixture (brain extracts) of lipids did not augment the rate at which basic protein was phosphorylated by this enzyme (Table 3). However, activation engendered by Ca^{2+} decreased markedly when insoluble myelin previously treated with phospholipase A_2 or a mixture of phospholipases A_2 + C was employed as the enzyme source. Phosphatidylserine could partly counter the deleterious effects of phospholipases (Table 6). Phosphatidylethanolamine and 4β-phorbol 12-myristate 13-acetate were more potent in restoring activation of the phospholipase-

Table 6. *Effects of phospholipase treatment on the catalytic activity of the Ca^{2+}-activated protein kinase*

Insoluble myelin was incubated (total volume 1 ml) with 250 units of phospholipase A_2 or a mixture of phospholipase A_2 (250 units) plus phospholipase C (65 units) at 4°C for 18h. The phospholipase-treated and control insoluble myelin suspensions were centrifuged at 27000*g* for 20min. Pellets were washed twice with 1 ml portions of buffer A to remove the hydrolytic enzymes from myelin. Each pellet was suspended separately in 1 ml of buffer A, and then phosphorylation of basic protein was measured in the absence and in the presence of 10 μM-Ca^{2+} as described in the Materials and methods section. Phosphatidylserine (25 μg), phosphatidylethanolamine (25 μg) or 4β-phorbol 12-myristate 13-acetate (1.2 μg) was present where indicated.

	Protein kinase activity (munits/ml)							
	No phospholipid added		Phosphatidylserine added		Phosphatidylethanolamine added		4β-Phorbol 12-myristate 13-acetate added	
Pretreatment	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$
None (control)	2.5	6.0	3.1	7.0	3.4	6.9	5.2	6.8
Phospholipase A_2	2.0	2.5	2.4	3.7	3.2	5.2	3.6	5.5
Phospholipases A_2 + C	2.5	3.1	2.6	4.4	3.4	5.4	3.4	5.1

treated enzyme by this cation. These findings suggest that the Ca^{2+}-regulated enzyme of bovine central-nervous-system myelin may also require certain myelinic lipid(s) as a cofactor. Somewhat similar observations have been made on the Ca^{2+}-dependent kinase that was solubilized from rat myelin (Turner *et al.*, 1982).

Identification of sites phosphorylated by myelin kinases

When unmodified basic protein was phosphorylated by the cyclic AMP-dependent kinase solubilized from myelin, analyses of the radioactive tryptic peptides indicated phosphorylation to have occurred at two sites. The major site (serine) was contained in the peptide comprising residues 105–112, namely Gly-Arg-Gly-Leu-Ser-Leu-Ser-Arg, and the minor site (serine) was located within residues 5–9, namely Arg-Pro-Ser-Gln-Arg. Methylation is known to occur on arginine-106 (Baldwin & Carnegie, 1971; Brostoff & Eylar, 1971), which prevents cleavage by trypsin at this site. Although the exact site of phosphorylation in this peptide (residues 105–112) is not obvious, previous workers have shown that serine-109 is phosphorylated both *in vitro* and *in vivo* (Carnegie *et al.*, 1974; Martenson *et al.*, 1983).

When unmodified basic protein was phosphorylated by the Ca^{2+}-dependent kinase of insoluble myelin, phosphorylation occurred mainly on residue 7 of basic protein. Evidence for the modification of this position of basic protein under conditions *in vivo* has been provided by Martenson *et al.* (1983). Since in the present study, however, the sequence of the phosphopeptides has not been determined by independent means, these assignments remain tentative.

Discussion

In the present study, the phosphotransferase system of bovine central-nervous-system myelin with exogenous proteins (purified basic protein and various histone fractions) as substrates was examined. The data presented show that at least two distinct protein kinases differing in solubility properties and substrate-specificity reside in this membrane. Basic protein was phosphorylated preferentially by the Ca^{2+}-activated enzyme, whereas, unlike certain histone fractions, it was a relatively poor substrate for the cyclic AMP-dependent kinase. In view of these findings, conditions [e.g. substrate protein, presence of positive effector (Ca^{2+} or cyclic AMP), or inhibitor (EGTA)] may be selected such that either the Ca^{2+}-activated or the cyclic AMP-dependent kinase is predominantly active. Therefore several properties of each of the individual enzymes can be investigated by using undisrupted myelin as enzyme source.

A single extraction with buffer containing Triton X-100 was sufficient to solubilize most of the cyclic AMP-dependent kinase activity. In contrast, even repeated extractions with the same buffer (or a buffer containing Triton + EGTA) failed to release the Ca^{2+}-activated enzyme. Thus the Ca^{2+}-activated enzyme, like its substrate (basic protein), appears to be deeply embedded, whereas the cyclic AMP-dependent kinase is associated much less tightly with bovine central-nervous-system myelin. Both the rat (Petrali *et al.*, 1980*b*) and the human (Wu *et al.*, 1983) myelin preparations house protein kinases of solubility characteristics similar to those found for bovine myelin enzymes.

Activation of certain protein kinases by Ca^{2+} is dependent on the presence of either calmodulin or certain phospholipids (e.g. phosphatidylserine and/or diacylglycerol etc.) (Takai *et al.*, 1977; for a review see Cheung, 1979). In contrast with the Ca^{2+}-activated kinase that is firmly bound to bovine (the present paper) and rat (Petrali *et al.*,

1980*b*) myelin preparations, yet another Ca^{2+}-activated enzyme has been found in rat myelin that can be readily extracted and partially purified by conventional protein fractionation procedures (Turner *et al.*, 1982). While the present studies were in progress, Turner *et al.* (1982) reported that activation of the partially purified enzyme by Ca^{2+} required certain phospholipids, such as phosphatidylserine. After incubation of bovine insoluble myelin fraction with phospholipase A_2 or phospholipases A_2 + C, nearly all the activation engendered by Ca^{2+} was lost. Phosphatidylserine and phosphatidylethanolamine countered the effects of the phospholipases (Table 6). These results, together with the effects of various inhibitors and activators examined (Tables 4 and 5), suggest that the activation of bovine myelin enzyme by Ca^{2+} may also require certain lipid(s), perhaps those already present in the neural membrane.

Castagna *et al.* (1982) reported a marked activation of the purified Ca^{2+}-activated phospholipid-sensitive kinase by 4β-phorbol 12-myristate 13-acetate and some of its analogues. In human platelets, 4β-phorbol 12-myristate 13-acetate replaced the diacylglycerol normally required for the activation of this enzyme (Sano *et al.*, 1983). Interestingly, the Ca^{2+}-activated kinase of the insoluble myelin fraction was likewise activated by phorbol diesters but not by 4α- or 4β-phorbol and some of their mono- and di-esters used in the present study (Table 5). The mechanism by which these compounds activate the insoluble myelin kinase is not understood. As shown previously by Castagna *et al.* (1982), 4β-phorbol 12-myristate 13-acetate appears to lower the concentration of Ca^{2+} normally required for the activation of myelin enzyme (Fig. 4). It has been proposed that the activation of the Ca^{2+}-activated phospholipid-sensitive kinase may involve an intercalation of 4β-phorbol 12-myristate 13-acetate with lipid lamellae or micelles, thereby changing the interaction between phospholipids and the enzyme. Whether or not activation of the myelin enzyme by 4β-phorbol 12-myristate 13-acetate occurs by an analogous mechanism provides avenues for future endeavours.

Regulation of a number of key enzymes by a phosphorylation–dephosphorylation cycle is well established (for a review see Krebs & Beavo, 1979). The study of reversible membrane protein phosphorylation now promises to offer new insights into the regulation of membrane structure and function.

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