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Calcium Ion-Stimulated Phosphorylation of Myelin Proteins

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Myelin isolated from the central and peripheral nervous system contains a Mg^{2+}-dependent protein kinase that catalyses phosphorylation of myelin-specific proteins. This phosphorylation is markedly stimulated by Ca^{2+} but not by cyclic AMP. Evidence was obtained that suggested an involvement of calmodulin-like protein in the stimulatory effects of Ca^{2+} on myelin phosphorylation.

Myelin isolated from the central and peripheral nervous system contains protein kinase(s) and phosphatase(s) that catalyse phosphorylation and dephosphorylation of myelin proteins (Carnegie *et al.*, 1973, 1974; Steck & Appel, 1974; Miyamoto & Kakiuchi, 1974, 1975; Miyamoto, 1975, 1976; Singh & Spritz, 1976; McNamara & Appel, 1977; Yourist *et al.*, 1978). With the central-nervous-system myelin, small and large basic proteins, and with the peripheral-nervous-system myelin, glycoprotein and basic proteins are reportedly phosphorylated under conditions both *in vitro* and *in vivo*. Whenever tested, cyclic AMP produced only a modest (25–30%) increase in the phosphorylation and only when Triton X-100-dispersed myelin was used in the assay. Despite the fact that cyclic nucleotide-stimulable kinase represents only a small fraction (6%) of the total myelin kinase activity (Petrali *et al.*, 1979), very little attention has been directed towards the study of cyclic nucleotide-independent kinase. We have reported that Ca^{2+} in micromolar concentrations profoundly stimulated endogenous Mg^{2+}-dependent protein kinase-catalysed phosphorylation of the rat central-nervous-system myelin basic proteins, the extent of stimulation being 2–10-fold depending on whether Triton X-100 was present in the phosphorylation assay (Sulakhe *et al.*, 1978; Petrali *et al.*, 1979). In the present paper we show that phosphorylation of the rat peripheral-nervous-system myelin proteins, like the central-nervous-system myelin proteins, is markedly stimulated by Ca^{2+} (but not cyclic AMP). We also present some evidence that calmodulin-like protein(s) are involved in the stimulatory effect of Ca^{2+} on the central-nervous-system-myelin phosphorylation.

Abbreviations used: SDS, sodium dodecyl sulphate.

Experimental

Isolation of myelin

Myelin was isolated [essentially by the method of Norton & Poduslo (1973)] from each of the following rat (Wistar, male, 190–240 g) tissues: brain-stem white matter, spinal cord, cauda equina and sciatic nerve. Briefly, 10% (w/v) homogenate (Teflon/glass homogenizer) in 0.25 M-sucrose/0.2 mM-dithiothreitol/10 mM-Tris/HCl, pH 7.8, was centrifuged at $10000 g_{av.}$ for 30 min to obtain a myelin-enriched particulate fraction, which after hypo-osmotic shock and sonication was subjected to discontinuous-sucrose-density-gradient centrifugation. The band (0.32/0.88 M-sucrose interface) was diluted with 5 mM-Tris/HCl, pH 7.8, and centrifuged twice to obtain the myelin pellet. This was again subjected to a second sucrose-density-gradient centrifugation and the band (0.32/0.65 M-sucrose interface) was removed, centrifuged, the pellet washed and finally suspended in the homogenizing buffer.

Polypeptide profiles of the central-nervous-system (brain stem and spinal cord) and peripheral-nervous-system (cauda equina and sciatic nerve) myelin were essentially in good agreement with those reported in the literature [see reviews by Norton (1977) and Braun & Brostoff (1977)]. The terminology used for the myelin proteins is that described by Greenfield *et al.* (1973).

Phosphorylation assay, electrophoresis and radioautography

Incubations at 30°C were carried out in the

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standard reaction mixture (0.15ml), which contained 30mM-Tris/HCl, pH 7.4, 1mM-$MgCl_2$, 1mM-EGTA and 50μm-[γ-^{32}P]ATP (specific radioactivity 100–300c.p.m./pmol) and 50–100μg of myelin protein. Whenever added, Triton X-100 was present at 2.2μmol/mg of protein, cyclic AMP at 5μM and $CaCl_2$ at 1.00mM (15μM free Ca^{2+}). Under these conditions, phosphorylation was linear up to 3min of incubation and thereafter increased steadily, reaching a maximal value by 15–20min incubation, and was proportional to protein concentration from 20–200μg in the assay. The reaction was terminated by addition of 30μl of 'solubilizing buffer' (Dunkley *et al.*, 1976) and the SDS-solubilized membrane proteins were subjected to slab-gel electrophoresis by the Laemmli (1970) procedure. The slab was stained with Coomassie Blue, destained in 10% (v/v) acetic acid, treated with 70% (v/v) methanol/3% (v/v) glycerol and dried on Whatman no. 1 filter paper under vacuum. The dried gel was placed in contact with Kodak X-omat R film for 1–3 days and the resulting radioautogram was scanned at 595nm. The Coomassie Blue-stained-polypeptide pattern was also obtained by scanning the gels at 595nm. All other details have been reported elsewhere (Petrali *et al.*, 1979).

When total phosphate incorporation was determined, the reaction was terminated with ice-cold 10% (w/v) trichloroacetic acid and the remaining procedure was essentially that described by Kuo & Greengard (1970) (also see Petrali *et al.*, 1979).

Essentially similar results were obtained with brain-stem-white-matter, spinal-cord, cauda-equina or sciatic-nerve myelin. The data for brain stem (central nervous system) and sciatic nerve (peripheral nervous system) are presented here.

Materials

The sources of these were described previously (Petrali *et al.*, 1979). Partially purified calmodulin-deficient cyclic AMP phosphodiesterase and purified calmodulin [both from bovine heart; see Teo & Wang (1973) and Teo *et al.* (1973)] and purified modulator-binding protein [from bovine brain; see Wang & Desai (1977)] were kindly supplied by Dr. J. H. Wang of the University of Manitoba, Winnipeg, Manitoba, Canada.

Results

Myelin isolated from the rat central (spinal cord or brain-stem white matter) and peripheral (sciatic nerve or cauda equina) nervous systems contained Mg^{2+}-supported endogenous protein kinase activity that effected phosphorylation of endogenous proteins. For central-nervous-system myelin, the substrate proteins were large and small basic proteins [relative molecular mass (M_r) 18000 and 16000 respectively] (Petrali *et al.*, 1979), whereas the major glycoprotein P_0 (M_r 28000), was the major phosphorylatable protein for peripheral-nervous-system myelin (results not shown). In the latter case, phosphorylation of polypeptides Y (M_r 26500), P_1 (M_r 18000) and P_2 (M_r 15000) was also observed. With either peripheral- or central-nervous-system myelin, cyclic AMP (1nM–100μM) caused a very weak stimulatory effect on phosphorylation (with or without Triton X-100 in the assay) (Table 1). On the other hand, Ca^{2+}, in micromolar concentrations, stimulated phosphorylation by 4–6-fold when Triton X-100 was included in the assay, and about 2–3-fold when the detergent was absent. Ca^{2+} action was biphasic, with stimulation at low (half-maximal at 5μM free Ca^{2+}) and inhibition at the higher (>100μM) concentrations. Amongst the bivalent cations tested, Sr^{2+}, Ba^{2+} and Mn^{2+} mimicked the Ca^{2+}-stimulatory action, but only with up to 20% efficacy. The phosphorylation reaction was optimum at pH 6.5 and 37°C, showed an apparent affinity towards $MgATP^{2-}$ of about 50–75μM, and required 15–20mM-Mg^{2+} for maximal activity (half-maximal at 3mM-Mg^{2+}). Ca^{2+} increased the rate of reaction but did not cause any detectable change in the apparent K_m for $MgATP^{2-}$. Kinetic analysis (Eadie–Hofstee and Scatchard plots) revealed the presence of two Mg^{2+}-binding sites characterized by high ($K_{app.}$ of 100μM, site A) and low ($K_{app.}$ of 3–5mM, site B) apparent affinity towards Mg^{2+}. Ca^{2+} showed no effect on the $K_{app.}$ of site A towards Mg^{2+}, but did cause an increase of approx. 4–10-fold in the $K_{app.}$ of site B. Triton X-100 caused an increase in the $V_{max.}$, but did not influence any of the other kinetic parameters tested (with and without Ca^{2+}). With either rat brain white matter or sciatic nerve, the Ca^{2+}-sensitive kinase was exclusively particulate and not found in the soluble fraction; the particulate Ca^{2+}-sensitive kinase was mostly recovered in the myelin. The soluble (cytosolic) fraction contained cyclic AMP-stimulable kinase activity, which effected phosphorylation of soluble proteins of mol.wt. higher than 45000.

With either central- or peripheral-nervous-system myelin, the phosphorylated product was hydroxylamine-insensitive, alkali-labile, acid-stable and phosphatase-labile. Serine residues and, to a small extent, threonine residues of the central-nervous-system myelin basic proteins, were phosphorylated by the endogenous kinase and Ca^{2+} promoted phosphorylation of serine residues.

When central-nervous-system myelin was exposed to EGTA and then centrifuged and washed, there was a marked decrease (40–60%) in the Ca^{2+}-stimulable phosphorylation of basic proteins compared with myelin treated identically but with-

Table 1. *Phosphorylation of myelin isolated from the central and peripheral nervous system: effects of cyclic AMP and Ca^{2+} in the absence and presence of Triton X-100*

Myelin was phosphorylated for 2 min (Expt. 1) or 15 min (Expt. 2) at 30°C under the standard assay conditions. When added, Triton X-100 was present at 2.2 μmol/mg of protein, cyclic AMP at 5 μM, and Ca^{2+} at 15 μM. The numbers in parentheses represent the difference between cyclic AMP or Ca^{2+} and basal (no addition) phosphorylation. Results represent the means ± S.E.M. of 8–13 experiments.

Assay additions	^{32}P incorporation (pmol/mg of protein): Peripheral-nervous-system myelin (sciatic nerve), Without Triton X-100	Peripheral-nervous-system myelin (sciatic nerve), With Triton X-100	Central-nervous-system myelin (brain-stem white matter), Without Triton X-100	Central-nervous-system myelin (brain-stem white matter), With Triton X-100
Expt. 1				
None	11 ± 1	29 ± 2	80 ± 7	149 ± 13
Cyclic AMP	15 ± 2 (4)	32 ± 3 (3)	82 ± 8 (2)	160 ± 20 (11)
Ca^{2+}	22 ± 2 (9)	70 ± 6 (41)	227 ± 21 (147)	494 ± 59 (345)
Expt. 2				
None	56 ± 4	137 ± 11	239 ± 15	564 ± 50
Cyclic AMP	58 ± 6 (2)	147 ± 16 (10)	272 ± 26 (33)	604 ± 30 (40)
Ca^{2+}	112 ± 10 (56)	851 ± 50 (714)	539 ± 51 (300)	1223 ± 90 (659)

Table 2. *Decrease in the Ca^{2+}-stimulable phosphorylation of myelin by its prior treatment with EGTA and restoration of Ca^{2+}-stimulable phosphorylation by addition of the EGTA extract or purified bovine heart calmodulin*

Control myelin (CM) was exposed to 2 mM-EGTA in 10 mM-Tris/HCl (pH 7.4)/0.25 M-sucrose at 0°C for 30 min and was then centrifuged at 40000 ***g*** for 1 h. The pellet, the EGTA-extracted myelin (EM), and the supernatant fluid (EGTA extract, EE) were removed. Samples CM, EM and EE were incubated under the standard phosphorylation assay conditions for 2 min with or without Triton X-100 and with and without cyclic AMP (5 μM) or Ca^{2+} (50 μM free cation) or calmodulin (CaM). EGTA extract (EE) was dialysed against 10 mM-Tris/HCl, pH 7.4, containing 1 mM-$MgCl_2$ before its use. Protein concentrations in the assay were: samples CM or EM, 80 ± 3 μg; sample EE, 13 ± 2 μg; calmodulin, 1.2 μg. The values in parentheses represent percentage activity, with 100% set according to respective controls used. Results are expressed as the means ± S.E.M. for three experiments.

Fraction used	Triton X-100 in assay	^{32}P incorporation (pmol/2 min): Basal	+Cyclic AMP	$+Ca^{2+}$	+Cyclic AMP minus basal	$+Ca^{2+}$ minus basal
CM	–	10.7 ± 0.4 (100)	11.9 ± 1.3	31.0 ± 3.0	1.2 ± 0.16 (100)	20.3 ± 2.8 (100)
EM	–	4.0 ± 0.3 (37)	6.3 ± 0.5	16.9 ± 1.7	2.3 ± 0.21 (138)	12.9 ± 1.8 (59)
EE	–	0.3 ± 0.02	0.2 ± 0.01	0.4 ± 0.03	0	0.1
CM + EE	–	11.1 ± 1.0 (104)	12.0 ± 1.0	34.0 ± 3.3	0.9 ± 0.12 (77)	22.9 ± 3.2 (112)
EM + EE	–	5.6 ± 0.4 (52)	6.9 ± 0.7	27.5 ± 2.6	1.3 ± 0.17 (108)	21.9 ± 3.1 (109)
EM + CaM	–	5.9 ± 0.5 (55)	7.0 ± 0.6	23.2 ± 2.2	1.1 ± 0.13 (77)	17.3 ± 2.4 (87)
CM	+	13.6 ± 1.1 (100)	17.8 ± 1.6	66.2 ± 6.0	4.2 ± 0.54 (100)	52.6 ± 2.9 (100)
EM	+	9.6 ± 0.8 (70)	14.4 ± 1.3	31.3 ± 2.9	4.8 ± 0.61 (117)	21.7 ± 2.6 (44)
EE	+	0.2 ± 0.01	0.2 ± 0.01	0.3 ± 0.01	0	0.1
CM + EE	+	13.9 ± 1.2 (102)	17.9 ± 1.7	60.0 ± 5.3	4.0 ± 0.52 (95)	46.1 ± 2.3 (125)
EM + EE	+	12.3 ± 1.3 (90)	17.4 ± 1.6	58.3 ± 4.8	5.1 ± 0.66 (119)	46.0 ± 2.5 (120)
EM + CaM	+	12.1 ± 1.0 (89)	16.4 ± 1.5	52.2 ± 4.9	4.3 ± 0.56 (95)	40.1 ± 3.8 (98)

out EGTA (Table 2). The EGTA extract, which had barely detectable phosphosphorylating activity, when added back to the assay mixture, restored the Ca^{2+}-stimulable phosphorylation. The EGTA extract, even after extensive dialysis and heating to 100°C for 15 min, was capabe of restoring the Ca^{2+}-stimulable phosphorylation of the EGTA-treated myelin to that observed in control myelin. Basal phosphorylation of the EGTA-treated myelin was also decreased, even though to a lesser extent than in the Ca^{2+}-stimulable reaction. The extract also restored to a large extent the decreased basal phosphorylation of the EGTA-treated myelin. Cyclic AMP-stimulable phosphorylation, which although modest in amount, was not decreased after EGTA treatment, but in fact was increased (up to 40%). Calmodulin (bovine heart) showed the same effect as the EGTA extract on both basal and Ca^{2+}-stimulable phosphorylation. Interestingly, the extract moderately increased the Ca^{2+}-stimulable phosphorylation of the control myelin as well. That the extract contains calmodulin-like protein(s) was established in two ways: (1) the extract contained a polypeptide that co-migrated with purified bovine heart calmodulin; (2) the EGTA extract restored the Ca^{2+}-sensitive phosphodiesterase activity of the calmodulin-deficient bovine heart cyclic AMP phosphodiesterase preparation to the same extent as calmodulin (results not shown).

Discussion

The present study clearly shows that myelin protein kinase-catalysed phosphorylation of myelin proteins is markedly stimulated by Ca^{2+} and only modestly by cyclic AMP. This is true irrespective whether central- or peripheral-nervous-system myelin is used in the investigation. Our previous work on cyclic AMP- and Ca^{2+}-stimulable phosphorylation of rat brain white matter (Petrali *et al.*, 1979) or sciatic-nerve homogenate(s) (E. H. Petrali & P. V. Sulakhe, unpublished work) and subcellular fractions derived from these indicated that the Ca^{2+}-sensitive kinase is exclusively particulate, mostly present in the myelin, whereas the cyclic nucleotide-sensitive kinase is a soluble enzyme in these tissues. In view of the considerable similarities in the effects of temperature of the assay and NaF (Petrali *et al.*, 1979) as well as EGTA treatment (the present study) on basal and Ca^{2+}-stimulable phosphorylation, it is tempting to suggest that most of the basal myelin kinase activity is due to the same enzyme that is stimulated by Ca^{2+}. The present evidence, although not conclusive, implies that calmodulin-like protein(s) are involved in the mechanism through which Ca^{2+} exerts its effects on the kinase. The likely mechanism involved in the Ca^{2+} effect on myelin phosphorylation appears similar to that proposed for the Ca^{2+} activation of the cyclic nucleotide phosphodiesterase. According to this [see review by Wang & Waisman (1979)], Ca^{2+} binds to calmodulin and the Ca^{2+}–calmodulin complex interacts with the inactive phosphodiesterase, with the resultant increase in enzyme activity. In the case of myelin, the Ca^{2+}–calmodulin complex interacts with the myelin kinase, leading to enhanced kinase activity, which is reflected in increased phosphorylation of basic proteins (central nervous system) or glycoproteins and other proteins (peripheral nervous system). Further support to this postulate comes from the observations that: (1) the apparent affinity of the calmodulin–Ca^{2+} binding site as well as of the myelin phosphorylation reaction towards Ca^{2+} is very similar (3–5μM); (2) myelin reportedly contains a high-affinity $^{45}Ca^{2+}$-binding site ($K_{app.}$ of about 10μM) (Hemminki, 1974); (3) the dependence of Ca^{2+}-stimulable cyclic nucleotide phosphodiesterase and that of myelin phosphorylation on Mg^{2+} (with and without Ca^{2+}) is also very similar; (4) calmodulin-binding protein, which selectively inhibits Ca^{2+}–calmodulin-activatable phosphodiesterase activity (see Wang & Waisman, 1979), also inhibits Ca^{2+}-stimulable myelin phosphorylation (results not shown).

A unique feature of myelin phosphorylation is that, under conditions both *in vitro* and *in vivo*, the same proteins of either central- or peripheral-nervous-system myelin are phosphorylated (Miyamoto & Kakiuchi, 1974; Steck & Appel, 1974; Singh & Spritz, 1976), a situation clearly different from most, if not all, of the studies on membranes from other mammalian sources, which reveal considerable differences in the proteins phosphorylated under conditions *in vivo* and *in vitro*. The observation that Ca^{2+}, but not cyclic AMP, significantly promotes phosphorylation of specific myelin proteins also provides an excellent opportunity to use myelin to investigate the properties of a membrane-bound Ca^{2+}-sensitive kinase. A detailed study of the changes in the Ca^{2+} concentrations or fluxes in sciatic nerve in relation to myelin phosphorylation may provide clues concerning a likely role of phosphorylation of (basic) proteins in myelin structure and function.

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References

Braun, P. E. & Brostoff, S. W. (1977) in *Myelin* (Morell, P., ed.), pp. 201–231, Plenum Press, New York
Carnegie, P. R., Kemp, B. E., Dunkley, P. R. & Murray, A. W. (1973) *Biochem. J.* **135**, 569–572
Carnegie, P. R., Dunkley, P. R., Kemp, B. E. & Murray, A. W. (1974) *Nature (London)* **249**, 147–150
Dunkley, P. R., Holmes, H. & Rodnight, R. (1976) *Biochem. J.* **157**, 661–666
Greenfield, S., Brostoff, S., Eylar, E. H. & Morell, P. (1973) *J. Neurochem.* **20**, 1207–1216
Hemminki, K. (1974) *Biochim. Biophys. Acta* **363**, 202–210
Kuo, J. F. & Greengard, P. (1970) *J. Biol. Chem.* **245**, 4067–4073
Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
McNamara, J. O. & Appel, S. H. (1977) *J. Neurochem.* **29**, 27–35
Miyamoto, E. (1975) *J. Neurochem.* **24**, 503–512
Miyamoto, E. (1976) *J. Neurochem.* **26**, 573–577
Miyamoto, E. & Kakiuchi, S. (1974) *J. Biol. Chem.* **249**, 2769–2777

Miyamoto, E. & Kakiuchi, S. (1975) *Biochim. Biophys. Acta* **384**, 458–465

Norton, W. T. (1977) in *Myelin* (Morell, P., ed.), pp. 161–199, Plenum Press, New York

Norton, W. T. & Poduslo, S. E. (1973) *J. Neurochem.* **21**, 749–757

Petrali, E. H., Thiessen, B. J. & Sulakhe, P. V. (1979) *Int. J. Biochem.* in the press

Singh, H. & Spritz, N. (1976) *Biochim. Biophys. Acta* **448**, 325–337

Steck, A. J. & Appel. S. H. (1974) *J. Biol. Chem.* **249**, 5416–5420

Sulakhe, P. V., Petrali, E. H. & Thiessen, B. J. (1978) *Abstr. Soc. Neurosci.* **4**, 249

Teo, T. S. & Wang, J. H. (1973) *J. Biol. Chem.* **248**, 5950–5955

Teo, T. S., Wang, T. H. & Wang, J. H. (1973) *J. Biol. Chem.* **248**, 588–595

Wang, J. H. & Desai, R. (1977) *J. Biol. Chem.* **252**, 4175–4184

Wang, J. H. & Waisman, D. M. (1979) *Curr. Top. Cell. Regul.* in the press

Yourist, J. E., Ahmad, F. & Brady, A. H. (1978) *Biochim. Biophys. Acta* **522**, 452–464