Effect of cycloheximide on palmitylation of PO protein of the peripheral nervous system myelin

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Incubation of rat sciatic nerve slices with Krebs–Ringer bicarbonate buffer containing [^3H]palmitic acid resulted in the acylation of the PO glycoprotein and a 24 kDa protein of the peripheral nerve myelin. Radioactivity was removed from PO after treating PO with hydroxylamine (83 %) and methanolic KOH (97 %). These results provided evidence that the radioactivity incorporated into PO was not due to the metabolic conversion of [^3H]palmitic acid into amino acids or sugars. PO was more heavily labelled in the homogenate than in the myelin membrane in 8-day-old rat nerve between 5 min and 2 h of incubation. These results suggested that PO may be primarily acylated in the cell body. Incubation of purified myelin with [^{14}C]palmitoyl-CoA resulted in the non-enzymic acylation of PO. This provided evidence of the absence of fatty acyltransferase from the purified peripheral nerve myelin. Glycosylation of PO has been shown to occur in the Golgi complex, and monensin inhibited glycosylation of PO in the homogenate and myelin by 53 and 61 % respectively. These results suggest that the processing of PO in the Golgi complex and the assembly of PO into myelin is impaired by monensin. However, fatty acylation of PO was unimpaired by monensin, suggesting that the addition of fatty acids may not occur in the Golgi complex. There was a progressive decrease in the acylation of PO between 5 min (28 %) and 2 h (61 %) in the presence of cycloheximide, as the pool of previously synthesized PO was gradually depleted. These results also provide evidence that palmitylation of PO is not coupled to protein synthesis, and acylation of this protein probably occurs in the early subcompartment of the Golgi complex, which appears to be insensitive to monensin.

INTRODUCTION

The covalent linkage of fatty acids to proteins has been demonstrated in various cell types [1–6]. Palmitic acid is attached to the proteins by an ester or thioester bond, whereas myristic acid is exclusively linked to the polypeptide by an amide bond to *N*-terminal glycine [4–6]. The PO glycoprotein of the peripheral nervous system (PNS) myelin is sulphated [7] and phosphorylated [8–13]. We reported that PO was acylated after intraneural injection of [^3H]palmitic acid into rat sciatic nerve and that fatty acids were covalently bound to PO by a thioester and/or ester linkage [14]. Subsequently, Sakamoto *et al.* [15] isolated four peptides containing fatty acids from bovine PO with an identical *N*-terminal sequence and fatty acid composition. On a molar basis, each peptide contained 0.9 mol of fatty acids, and the relative molar ratio of palmitate, stearate and oleate was 8:3:2. However, the site of acylation of PO is unknown. The present study was initiated to ascertain if fatty acids are attached to PO (1) after synthesis (endoplasmic reticulum), (2) after processing in the Golgi apparatus or (3) after insertion of PO into the myelin by myelin-associated fatty acyltransferase. Our results suggest that acylation of PO occurs some time after the translation of protein in the early subcompartment of the Golgi complex.

MATERIALS AND METHODS

Reagents were obtained from the following sources: [$9,10\text{-}^3\text{H}$]palmitic acid (28.2 Ci/mmol), [$1\text{-}^{14}\text{C}$]palmitoyl-

CoA (58.5 mCi/mmol), [$\text{U-}^{14}\text{C}$]amino acid mixture, and L-[5,6- ^3H]fucose (48.4 Ci/mmol) from New England Nuclear; fatty-acid free bovine serum albumin (BSA), ATP, CoA, hydroxylamine, dimethyl sulphoxide, cycloheximide from Sigma; 2,5-diphenyloxazole (PPO), Soluene-350 and Instafluor from Packard Instruments, reagents for SDS gel electrophoresis from Bio-Rad, and monensin (highly purified) and tunicamycin from Calbiochem. Eight-day-old Sprague–Dawley rats with their mother were purchased from Sasco, O'Fallon, MO, U.S.A. The average body weight of the rats was 19.3 ± 1.4 g (mean $\pm$ S.D.; $n = 63$). Rats were killed by decapitation and sciatic nerves were exposed and excised from the sciatic notch to the knee (average length 2.5 cm). Nerves from two (24 ± 3 mg of tissue) or four (51 ± 3 mg of tissue) rats were sliced into 0.4 mm transverse sections with a McIlwain chopper. Monensin and tunicamycin were dissolved in 10 % ethanol and 0.1 M-NaOH respectively. Slices were incubated in 1.0 ml of Krebs–Ringer bicarbonate buffer, pH 7.4, containing 11 mM-glucose, 1 % (w/v) fatty-acid free BSA, 500 μCi of [^3H]palmitic acid or 50 μCi of ^{14}C -labelled amino acid mixture; the tubes were flushed with O_2/CO_2 (19:1) and immersed in a shaking water bath at 37 °C [16]. The incubation was stopped by adding 4 ml of ice-cold Krebs–Ringer bicarbonate buffer containing 1 % fatty-acid free BSA and the slices were pelleted by centrifugation at 1500 *g*. The pellets were washed three more times with 4 ml of buffer. Nerves were homogenized in 0.29 M-sucrose and a portion of the homogenate was centrifuged at 100 000 *g* for 1 h. The pellet was suspended

Abbreviations used: BSA, bovine serum albumin; RSA, relative specific activity; PPO, 2,5-diphenyloxazole; PNS, peripheral nervous system; CNS, central nervous system; PLP, proteolipid protein.

Fig. 1. Effect of hydroxylamine and methanolic KOH on PO protein labelled with [^3H]palmitic acid *in vitro*

Sciatic nerve slices ($n = 8$) from 8-day-old rats were incubated in 1.0 ml of Krebs–Ringer bicarbonate buffer containing 500 μCi of [^3H]palmitic acid for 2 h. Myelin was isolated, extracted with chloroform/methanol (2:1, v/v) and 50 μg of myelin proteins was examined by SDS/gel electrophoresis. Gels were stained with Coomassie Blue (lane 1), or treated with 1 M-Tris/HCl, pH 10 (lane 2), 1 M-hydroxylamine, pH 10 (lane 3), or 0.2 M-KOH in 20% methanol (lane 4). Abbreviations: LBP, large basic protein; SBP, small basic protein.

in water and freeze-dried. The rest of the homogenate was used for the isolation of myelin [14] and the purified myelin was freeze-dried. Lipids from the homogenate and myelin were removed by three successive extractions with chloroform/methanol (2:1, v/v) and once with diethyl ether. The resulting pellets were dissolved in 2% SDS/1.5% dithiothreitol/8% sucrose/0.01% Bromophenol Blue and heated in a boiling water bath for 2 min. Proteins (50 μg) were separated in 15% polyacrylamide/SDS slab gels according to Laemmli [17]. The gels were stained with Coomassie Blue, destained [18] and then scanned at 570 nm. The entire gel was cut into 2 mm sections, which were heated with 1 ml of Soluene-350 at 50 $^{\circ}\text{C}$ for 18 h. After the addition of 10 ml of Instafluor, radioactivity was determined by liquid scintillation spectrometry. Relative specific radioactivity (RSA) of PO (d.p.m./unit of PO) was determined as described [19]. The gels were also fixed in 40% methanol/10% acetic acid for 12 h, washed with water and then treated with 1 M-hydroxylamine (pH 10) or 1 M-Tris/HCl buffer, pH 10. Similarly, gels were also treated with 0.2 M-KOH in 20% methanol for 90 min at room temperature [20]. The gels were infiltrated in dimethyl sulphoxide containing PPO (22.2%, w/v) and then fluorographed [21].

Cell-free acylation of PO

Fresh myelin was incubated at 37 $^{\circ}\text{C}$ for 1 h in 20 mM-Tris/HCl buffer, pH 7.4, containing 2 mM-MgCl₂, 0.1% Triton X-100, 1 mM-dithiothreitol and 1 μCi of [$1\text{-}^{14}\text{C}$]palmitoyl-CoA. The reaction was terminated by adding 3 ml of ice-cold acetone followed by centrifugation at 10000 g for 20 min at 4 $^{\circ}\text{C}$. Lipids from myelin

were removed, and myelin proteins were dissolved in SDS and prepared for gel electrophoresis as described above.

RESULTS AND DISCUSSION

The PO and 24 kDa proteins were acylated after incubation of nerve slices with [^3H]palmitic acid (Fig. 1, lane 2). Retention of radiolabelled palmitate with PO after extraction of myelin with chloroform/methanol (2:1, v/v) followed by boiling with SDS and dithiothreitol provides evidence that fatty acids are covalently linked. Release of radioactivity from PO after treatment with 1 M-hydroxylamine, pH 10 (Fig. 1, lane 3) or 0.2 M-KOH in 20% methanol (Fig. 1, lane 4) confirms that fatty acids are linked by ester and/or thioester bonds. When palmitate-labelled PO was treated with methanolic 0.2 M-NaOH [22], methylated fatty acids were separated by reverse-phase h.p.t.l.c., 95% of the radioactivity was recovered as fatty acids (results not shown). Thus the release of radioactivity from acylated PO in nerve slices is identical with that obtained *in vivo* [14]. These results are consistent with the previous findings that palmitic acid is attached to most cellular, viral and cell-specific proteins by ester or thioester bonds [4–6].

Synthesis and acylation of PO

It has been reported that there is a delay of 30 min between the synthesis and appearance of PO in myelin membrane [23]. Similar results were obtained after incubating nerve slices from 8-day-old rats with a ^{14}C -amino acid mixture for between 5 min and 2 h (results not shown). Nerve slices from 8-day-old rats were also incubated with [^3H]palmitic acid for between 5 min and 2 h to determine the time course of acylation of PO in the homogenate and the myelin membrane. PO was acylated in both the homogenate and myelin within 5 min, and the specific radioactivity of PO increased with time (Fig. 2). The specific radioactivity of PO in the homogenate at all time points was consistently higher than in the myelin membrane.

The concentration of PO in the Schwann cells relative to the myelin membrane is not known, but it can be assumed to be minuscule because newly synthesized PO is rapidly incorporated into the myelin membrane during the active period of myelination. If this assumption is correct, most of the PO in the nerve homogenate should be of myelin origin. Since the RSA of acylated PO in the nerve homogenate is consistently higher than that in the myelin membrane (Fig. 2), the pool of acylated PO in the cell body must be higher. Therefore, it may be reasonable to conclude that PO is primarily acylated in the cell body.

Acylation of PO in a cell-free system

Isolated rat brain myelin has been shown to contain both fatty acid CoA ligase [24] and fatty acyltransferase [24,25] which catalyses the enzymic transfer of fatty acids into proteolipid protein (PLP) and DM-20 in a cell-free system. Incubation of myelin with [^3H]palmitic acid and ATP, CoA and MgCl₂ [24] resulted in the non-specific labelling of all myelin proteins. However, when myelin was incubated with [$1\text{-}^{14}\text{C}$]palmitoyl-CoA, only PO was acylated (Fig. 3, lane 1) and fatty acids from PO were removed after treatment with hydroxylamine (Fig. 3, lane 2). However, acylation of PO with palmitoyl-CoA in a cell-free system is not an enzymic reaction because its

Fig. 2. Acylation of PO in the developing nerves

Sciatic nerve slices ($n = 8$) from 8-day-old rats were incubated in 1 ml of Krebs-Ringer bicarbonate buffer containing 500 μ Ci of [³H]palmitic acid. The specific radioactivity of PO in the homogenate (○) and myelin (●) was determined as described [19]. Three separate experiments were carried out at each time point.

acylation was not inhibited by incubation of heated myelin (boiling water bath for 5 min) with palmitoyl-CoA or by incubating myelin in the presence of 0.1–0.5 % SDS. These results suggest the absence of fatty acid CoA ligase as well as fatty acyltransferase in isolated PNS myelin. Non-enzymic acylation of viral E₂ protein [26] and rhodopsin [27] has also been demonstrated.

Effects of monensin and tunicamycin on acylation and glycosylation of PO

Incubation of nerve slices from 8-day-old rats with monensin (0.1 μ M) had little or no effect on the acylation of PO in the homogenate or myelin (Fig. 4). However, monensin inhibited the incorporation of [³H]fucose into the homogenate and myelin PO by 53 and 61 % respectively (Fig. 4). These results provide evidence that monensin interferes with the glycosylation of PO, presumably in the Golgi complex, and severely inhibits the assembly of glycosylated PO into myelin [16]. The incorporation of [¹⁴C]amino acids into PO was slightly inhibited (15 %) by monensin under identical conditions, which is consistent with previous findings [16,28]. Higher concentrations of monensin (1–10 μ M) severely inhibited protein synthesis and acylation. Marked inhibition of protein synthesis at higher concentrations of monensin corroborate the findings of previous investigators [29,30]. The available morphological evidence suggests that the primary site of action of monensin in the non-neural and neural cells is the Golgi complex [16,31–40]. The lack of perturbation of fatty acylation of PO by monensin suggests that the Golgi complex may not be the site of palmitylation. However, it is feasible that the acylation of PO occurs in the early subcompartment of the Golgi complex [36,41–43], which appears to be insensitive to

Fig. 3. Non-enzymic cell-free acylation of PO

Fresh myelin (250 μ g of protein) was incubated at 37 °C for 1 h in 250 μ l of 20 mM-Tris/HCl, pH 7.4, containing 2 mM-MgCl₂, 1 mM dithiothreitol, 0.1 % Triton X-100 and 1 μ Ci of [¹⁴C]palmitoyl CoA. The reaction was stopped by adding 3 ml of ice-cold acetone, followed by centrifugation at 12000 g for 20 min. The pellet was suspended in water, freeze-dried and extracted with chloroform/methanol (2:1, v/v). Proteins were solubilized in SDS sample buffer containing 1.5 % dithiothreitol. These samples were heated in a boiling water bath for 2 min before SDS/gel electrophoresis. Gels were treated with 1 M-Tris/HCl, pH 10 (lane 1) or 1 M-hydroxylamine, pH 10 (lane 2).

monensin. PO [44] and PLP [45,46] have been localized immunocytochemically to the Golgi vesicles, but the acylation of PLP [47] is also not affected by monensin. In addition, tunicamycin (25 μ g/ml) did not inhibit the acylation of PO but severely inhibited (66 ± 3 %) the incorporation of [³H]fucose into PO (results not shown). The latter finding is consistent with the previously reported inhibition of *N*-glycosylation of PO by tunicamycin [16,48]. Acylation of the transferrin receptor with palmitate has been shown to occur in the presence of tunicamycin [49]. Similarly, palmitylation and myristylation of the chick embryo fibroblast proteins was affected neither by tunicamycin nor by monensin [50].

Effect of cycloheximide on the acylation of PO

Cycloheximide (1 mM) inhibited the incorporation of [¹⁴C]amino acids into PO protein by 90 and 97 % (Fig. 4) after 5 min and 2 h respectively. However, palmitylation of the homogenate PO and the myelin-associated PO was blocked by 61 and 56 % respectively (Fig. 4), after incubating nerve slices with cycloheximide for 2 h. These studies were extended to determine the degree of inhibition of palmitylation of PO by cycloheximide between 5 min and 1 h. Nerve slices were preincubated for 30 min

Fig. 4. Effects of monensin and cycloheximide on the synthesis, acylation and fucosylation of PO

Sciatic nerve slices ($n = 8$) from 8-day-old rats were preincubated in 1 ml of Krebs–Ringer bicarbonate (KRB) buffer containing monensin ($0.1 \mu\text{M}$) or cycloheximide (1 mM) for 30 min. The slices were then incubated for 2 h in 1.0 ml of fresh KRB buffer containing $500 \mu\text{Ci}$ of $[^3\text{H}]$ palmitic acid and $50 \mu\text{Ci}$ of $[^{14}\text{C}]$ amino acid mixture and monensin ($0.1 \mu\text{M}$) or cycloheximide (1 mM), or KRB containing $100 \mu\text{Ci}$ of $[^3\text{H}]$ fucose and monensin ($0.1 \mu\text{M}$). Nerve slices were washed six times with buffer, and the radioactivity associated with PO was determined as described in the Materials and methods section. The relative specific radioactivity of PO (d.p.m./unit of PO) labelled with $[^3\text{H}]$ palmitic acid and ^{14}C -amino acids in the control nerve homogenate was 3693 ± 1067 and 2490 ± 756 respectively, and in myelin it was 290 ± 15 and 862 ± 26 respectively. The relative specific radioactivity of PO labelled with $[^3\text{H}]$ fucose in the control nerve homogenate and myelin was 2319 ± 391 and 404 ± 61 respectively. Results are means $\pm$ S.D. of at least three separate experiments. ■, control; ▨, monensin; □, cycloheximide.

Fig. 5. Effect of cycloheximide on the acylation of PO

Sciatic nerve slices ($n = 4$) from 8-day-old rats were preincubated in 0.5 ml of Krebs–Ringer bicarbonate (KRB) buffer containing cycloheximide (1 mM) for 30 min. The slices were then incubated for between 5 and 60 min in 0.5 ml of fresh KRB buffer containing $500 \mu\text{Ci}$ of $[^3\text{H}]$ palmitic acid and cycloheximide (1 mM). The nerve slices were washed six times with buffer, homogenized in water and freeze-dried. The freeze-dried nerves were extracted successively three times with chloroform/methanol (2:1, v/v) and once with diethyl ether. The pellet was dried under N_2 , solubilized in 4% (w/v) SDS for 1 h at 37°C , centrifuged in a microfuge for 15 min, and the clear supernatant was used to determine the protein concentration. An equal volume of a sample buffer containing 3% (w/v) dithiothreitol, 16% (w/v) sucrose and 0.01% Bromophenol Blue was added to the nerve proteins and heated for 2 min in a boiling water bath. Proteins ($150 \mu\text{g}$) were separated in 15% polyacrylamide/SDS slab gels, and the radioactivity associated with PO was determined by liquid scintillation spectrometry. Results are means of 2–3 separate experiments.

with cycloheximide, then incubated with $[^3\text{H}]$ palmitic acid and cycloheximide. The radioactivity associated with PO was determined after gel electrophoresis. It is apparent from Fig. 5 that there is a progressive decrease in the acylation of PO in the presence of cycloheximide as the pool of previously synthesized PO was gradually depleted. These results also provide evidence that palmitylation of PO is not coupled to protein synthesis. The acylation of PO does not occur in myelin, and it is not co-translational. Therefore, PO must be acylated either in the endoplasmic reticulum or in the early subcompartment of the Golgi complex. Incorporation of palmitate into some cellular proteins [50–52] continues at a reduced rate in the absence of protein synthesis. Esterification of palmitic acid to the guanine-nucleotide-binding-protein [41], transferrin receptor [49], rat brain myelin PLP and DM-20 [53], rhodopsin [54] and $\text{p}^{21\text{N-ras}}$ [55] has also been shown to occur in the presence of cycloheximide. The continued incorporation of palmitic acid into proteins, after inhibition of protein synthesis, indicates the presence of a large pool of previously made protein which is available for palmitylation [52,53] or the rapid turnover of palmitate bound to proteins [51,52]. The latter phenomenon has in fact been demonstrated for the transferrin receptor [49], ankyrin [56], $\text{p}^{21\text{N-ras}}$ [55] and other cellular proteins [51,57]. The incorporation of palmitate into proteins in the presence of cycloheximide and the turnover of protein-bound fatty acids may be a unique feature of palmitylated protein, because these metabolic events do not occur in proteins acylated with myristic acid.

A large number of proteins of the central nervous system (CNS) and PNS myelin are glycosylated and phosphorylated. However, only PLP and DM-20 [19,22,53,58,59] of the CNS myelin and PO [14,15] of the PNS myelin are acylated. These results suggest the highly specific nature of fatty acid acylation with respect to myelin proteins. The functional significance of acylation of PO is not known; however, it is possible that the addition of fatty acids to PO may be necessary for the

assembly and the organization of this protein into the myelin membrane.

This research was supported by a grant from the National Institutes of Health NS-13464. The authors would like to thank Frances Manzella for typing the manuscript and Dr. Joyce Benjamins for helpful discussion.

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Received 8 December 1988/17 May 1989; accepted 19 May 1989