2',3'-Cyclic Nucleotide-3'-phosphodiesterase in the Central Nervous System Is Fatty-acylated by Thioester Linkage*

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Harish C. Agrawal†§, Terry Joe Sprinkle¶, and Daya Agrawal‡

From the †Department of Pediatrics, Washington University School of Medicine, St. Louis, Missouri 63110 and the §Veterans Administration, Medical College of Georgia, Augusta, Georgia 30910

2',3'-Cyclic nucleotide-3'-phosphodiesterase (CNP1, and CNP2 with M, of 46,000 and 48,000, respectively) is the major enzyme of central nervous system myelin. It is associated with oligodendroglial plasma membrane and uncompacted myelin (myelin-like fraction), which are in contact with glial cytoplasm. Proteins of the myelin-like fraction were labeled with [3H]palmitic acid in brain slices from 17-day-old rats and immunoprecipitated with anti-CNP antiserum. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; KRB, Krebs-Ringer bicarbonate.

and demonstrated that this fraction (a) floated at a higher density of sucrose than myelin, (b) contained predominantly single vesicles, instead of compact myelin, and (c) was almost devoid of cerebroside and was highly enriched in CNP. These results provide conclusive evidence that CNP1 and CNP2 are fatty acid acylated with palmitate through a thioester linkage and is posttranslational modification sometime after synthesis.

2',3'-Cyclic nucleotide-3'-phosphodiesterase (CNP, EC 3.1.4.37) accounts for approximately 2-5 and 0.5-1% of the total proteins in central nervous system and peripheral nervous system myelin, respectively (1-5). Davison and coworkers (4, 5) isolated a myelin-like fraction from developing brain and demonstrated that this fraction (a) floated at a higher density of sucrose than myelin, (b) contained predominantly single vesicles, instead of compact myelin, and (c) was almost devoid of cerebroside and was highly enriched in CNP. These investigators postulated that the myelin-like material is a form of myelin in continuity with the oligodendroglial plasma membrane and the compact myelin sheath in the CNS (5).

Subsequently, these results were confirmed (reviewed in Ref. 6). In some preparations, the specific activity of CNP in the myelin-like membranes was greater than in myelin (7, 8). Recent immunohistochemical studies at electron microscopic level provided conclusive evidence that CNP was localized to single membranes, loosely wrapped glial membranes, and structures enriched in glial cell cytoplasm (9). Purified enzyme from rat brain migrates with an M, of 46,000 (CNP1) and 48,000 (CNP2) on SDS-PAGE and the ratio of CNP1 to CNP2 is about 10:1 (10, 11). The complete amino acid sequence of CNP in four species have been derived from their cDNA sequences (12-16). Results of cDNA sequencing suggest that CNP is synthesized from a single gene and the two forms of CNP may result from alternative splicing of mRNA (12) and/or posttranslational modifications (2, 3, 12). We recently provided the first direct immunochromometric evidence that both CNP1 and CNP2 in the peripheral nervous system undergo phosphorylation. In this communication, we report that CNP in the myelin-like fraction is fatty acylated and the fatty acids appear to be linked to this enzyme by thioester linkage.

MATERIALS AND METHODS

Reagents obtained from the following sources: [9,10-3H]palmitic acid (56 Ci/mmol) and [14C]-amino acid mixture were obtained from Du Pont-New England Nuclear; cycloheximide, colchicine, [14C]hexadecanol, sodium borohydride, and tetrahydrofuran were obtained from Sigma; highly purified monensin and Trasylol from Calbiochem. Staphy A from IgG-Sorb, and reagents for SDS-gel electrophoresis were obtained from Bethesda Research Laboratories, Sprague-Dawley rats (17-day-old) with their mothers were obtained from Sasco, O'Fallon, MO.

Generation of Antibody to CNP—CNP from normal human brain was purified to homogeneity as described (17). Purified enzyme (5 mg) was emulsified with an equal volume of complete Freund's adjuvant (Difco) and injected on multiple sites into a goat. The goat was injected two more times with CNP (1 mg) emulsified in incomplete Freund's adjuvant at 3-week intervals as described above. The goat was bled 10 days after the last injection and the specificity of antiserum was determined by immunoprecipitation. Monoclonal antibodies to bovine CNP were generated and their specificity was determined by immunoblot and by enzyme-linked immunosorbent assay (18).

Acylation of CNP—Rats were anesthetized and killed by decapitation, brains were removed, and pons medulla (500 mg of tissue) was washed with Krebs-Ringer bicarbonate buffer, pH 7.4, containing 10 mCi of [3H]palmitic acid or 200 μCi of [14C]-amino acid mixture at 37 °C for 2.5 h under 95% O2/5% CO2 (19). Brain slices were preincubated with the drugs for 30 min, washed with Krebs-Ringer buffer at 4 °C, and then reincubated with the fresh buffer containing the isotope and drugs for 2.5 h as described in the legends to the figures. The brain slices were washed six times with 6
ml each of KRB buffer containing 1% fatty acid free bovine serum albumin and pelleted at 1,500 x g at 4 °C. All operations were carried out at 0-4 °C. Brain slices were homogenized in 0.88 M sucrose (5%, w/v). Aliquots were removed to determine protein concentration, incorporation of [14C]-amino acids into total proteins (20), and for immunoprecipitation. The remaining homogenate was overlayed with sucrose density gradient and washed with water as described (21). Myelin floating at the interface was collected, homogenized, diluted with ice-cold water, and pelleted at 44,000 x g for 30 min. Pellets were homogenized in ice-cold water, incubated in an ice chest for 30 min, and then centrifuged at 12,000 x g for 10 min (5). The turbid supernatant containing the myelin-like fraction was collected and centrifuged at 12,000 x g for 1 h. The pellets were homogenized again in water and the myelin-like fraction was sedimented by centrifugation at 93,000 x g for 1 h. Myelin was further purified by recycling on sucrose density gradient and washed with water as described (21). Both myelin and myelin-like fractions were each suspended in water, protein concentrations were determined (22), and aliquots were stored at -80 °C to measure CNP activity (23) and for immunoprecipitation. The specific activity of CNP is expressed as micromoles of 2'-AMP produced per mg protein/h. Results are the mean of three separate experiments ± S.D.

Immunoprecipitation and SDS-PAGE—For immunoprecipitation, SDS was added to brain homogenates (700 µg of proteins) or myelin-like fraction (100 µg of proteins) to a final concentration of 1% and then the samples were boiled for 5 min. Four to six volumes of immunoprecipitation buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.5 mM dithiothreitol, and 1% Triton X-100), 5 µl of Trastroyl (21,000 units), and polyclonal or monoclonal antibodies to CNP were added and samples were incubated for 18 h at 4°C with gentle rotation. When monoclonal antibodies to CNP were used, the Staph A was first coated with rabbit anti-mouse IgG (ICN Radiochemicals). The immunoprecipitation was performed as described (24) using 100 µl of Staph A. Precipitated proteins were eluted from the washed Staph A pellet by boiling for 5 min in 40 µl of sample buffer (125 mM Tris/HCl, pH 6.8, 2.5% SDS, 3% dithiothreitol, 10% glycerol, and 0.01% bromphenol blue) and analyzed on 12% polyacrylamide-SDS gels (25). Proteins were fixed by immersion of gels for 2 h in 10% trichloroacetic acid. Gels were then stained, destained, and prepared for fluorography (26). Stained gels were treated with 1 M hydroxylamine, pH 10, for 18 h (27) and then fluorographed. Absolute amount of radioactivity was determined by liquid scintillation spectrometry.

Immunoprecipitation, Extraction with Organic Solvents, and Treatment of Proteins with Hydroxylamine or Sodium Borohydride or Trichloroacetic Acid—Myelin-like fractions were immunoprecipitated and each immunoprecipitate was eluted by boiling in 5 min in 80 µl of sample buffer. Eluates were divided into 3 aliquots (640 µl each) after adding fatty acid-free bovine serum albumin (2 µg/µl). Each aliquot was successively extracted 3 times with 30 volumes of chloroform/methanol (2:1, v/v) and one time each with chloroform/methanol (1:1, v/v), chloroform/methanol/H2O (1:1:0.3, v/v/v) and acetone (28), and then dried under N2. Furthermore, extraction of the protein pellet with organic solvents did not release any radioactivity, indicating that noncovalently bound lipids were removed by this procedure. The lipid species linked to CNP was identified by treating the lipid specie linked to CNP was identified by treating the fraction (100 pg of proteins) to a final concentration of 1% and the samples were boiled for 5 min. Four to six volumes of immunoprecipitation buffer (50 mM Tris/HCl, pH 6.8, 2.5% SDS, 3% dithiothreitol, 10% glycerol, and 0.01% bromphenol blue) and analyzed on 12% polyacrylamide-SDS gels (25). Proteins were fixed by immersion of gels for 2 h in 10% trichloroacetic acid. Gels were then stained, destained, and prepared for fluorography (26). Stained gels were treated with 1 M hydroxylamine, pH 10, for 18 h (27) and then fluorographed. Absolute amount of radioactivity was determined by liquid scintillation spectrometry.

RESULTS AND DISCUSSION

The specific activity of CNP in the myelin-like fraction (296 ± 34) was 3.8-fold higher than myelin (764 ± 92) isolated from pons-medulla of 17-day-old rats. These results were further confirmed by immunostaining CNP from myelin and the myelin-like fraction by the immunoblot technique (33). The amount of CNP present in the myelin-like fraction is at least 3-4-fold higher than in purified myelin (data not shown). These findings are consistent with the immunohistochemical localization of CNP to the oligodendroglial plasma membrane and noncompacted regions of myelin membrane (9).

To characterize the fatty acid acylation of CNP, brain slices were incubated with [3H]palmitic acid, and the myelin-like fraction was prepared. CNP was isolated by immunoprecipitation from the myelin-like fraction labeled with [3H]palmitic acid with polyclonal antisera to CNP, and on fluorography, CNP, and CNPz were acylated (Fig. 1, lane 2). Analysis of 2-mm gel sections containing immunoprecipitated CNP with a monoclonal antibody also revealed a solitary peak of radioactivity (Fig. 2). Fatty acids were removed by treatment of immunoprecipitated CNP in the gel with 1 M hydroxylamine, pH 10 (Fig. 1, lane 3). These results provided strong evidence that fatty acids were tightly bound to CNP.

Treatment of immunoprecipitated CNP with 1 M hydroxylamine did not result in hydrolysis of the protein as determined by immunostaining of CNP (Fig. 3A, lane 2) and by the retention of radioactivity associated with CNP (Fig. 3B, lane 2). However, it is apparent from Fig. 3B, lane 2, that there was a slight reduction of radioactivity from CNP labeled with [14C]-amino acid mixture by hydroxylamine when compared to control (Fig. 3B, lane 1). The decrease is in all probability due to metabolic conversion of some [14C]-amino acids into fatty acids which were removed by hydroxylamine. In addition, 4, 17, 40, 76, 78, and 80% of the radioactivity was released after treatment of CNP in the gel section by hydroxylamine between pH 5-10, respectively. Immunoprecipitated CNP was exhaustively delipidated. The delipidated enzyme was also treated with hydroxylamine as described above and then examined by SDS-PAGE and fluorography.

![Fig. 1. Labeling of CNP with [3H]palmitic acid and effect of treatment of acylated CNP with hydroxylamine. Brain slices were labeled with 10 µCi of [3H]palmitic acid for 2.5 h as described under "Materials and Methods." The myelin-like fraction was isolated, immunoprecipitated, and analyzed by SDS-gel electrophoresis and the gels were fluorographed. Myelin-like fraction immunoprecipitated with (a) preimmune goat serum (lane 1), (b) anti-CNP antisemur and CNP in the gel was treated with 1 M hydroxylamine, pH 10 (lane 3). An immunoprecipitate of CNP labeled with [14C]-amino acid mixture is shown in lane 4 for comparison.](http://www.jbc.org/)
was then treated with 1 M hydroxylamine, pH 10, for varying periods of time at 23 °C to determine the kinetics of release of fatty acid. Lipids were extracted with chloroform/methanol and the radioactivity was determined. Forty percent of radioactivity was released from CNP in 50 min after treatment with hydroxylamine (Fig. 4).

The following experiments were performed to demonstrate covalent linkage of [3H]palmitic acid to CNP. First, fatty acids attached to CNP were not removed by repeated extraction of the immunoprecipitate with chloroform/methanol (2:1, v/v), chloroform/methanol (1:2, v/v), chloroform/methanol/H₂O (1:0.3, v/v/v) and acetone (28). Second, radioactivity associated with CNP was not released by boiling the immunoprecipitate for 5 min with 2.5% SDS and 3% dithiothreitol, followed by SDS-PAGE. These results, therefore, fulfilled two stringent criteria for covalent linkage of fatty acids to CNP (34–36). Finally, virtually all the radioactivity (80%) from immunoprecipitated CNP was removed following treatment of the SDS gels with 1 M hydroxylamine, pH 10.

The release of fatty acid from CNP by hydroxylamine suggests that [3H]palmitic acid may be conjugated to CNP by thioester and not by O-ester linkages (34–36).

To demonstrate directly the fatty acid bound to immunoprecipitated CNP and the nature of the protein-fatty acid bond, immunoprecipitated CNP was exhaustively extracted with organic solvents to remove noncovalently attached lipids as described above and then digested with 1 M hydroxylamine, pH 9.8 (29), sodium borohydride (30), or methanolic-KOH (37). The extracted radioactive materials were analyzed by TLC. The released radioactivity (85–95%) was associated with palmitohyroxamate and palmitic acid after treatment of CNP with hydroxylamine (Fig. 5A). The presence of free palmitic acid may result from hydrolysis of covalently bound palmitate under the alkaline conditions used (31).

CNP was also cleaved with sodium borohydride, which simultaneously hydrolyzes acylthioesters and reduces the acyl group to an alcohol (30, 34). The released radioactivity (83%) was extracted with toluene and all the radioactivity co-chromatographed with authentic hexadecanol (Fig. 5B, lane 2). These results further suggest the covalent linkage of palmitic acid with cysteine residues of CNP. Sodium borohydride cleaves fatty acids linked by thioester bond to cysteine in proteins (34, 38, 39). Finally, nearly all the radioactivity released from CNP after alkaline hydrolysis (97%) was identified as methyl palmitate (Fig. 5B, lane 5).

CNP is probably synthesized on free ribosomes and newly synthesized enzyme is rapidly incorporated into the glial cell plasma membrane (40, 41). Brain slices were incubated with 14C-amino acids in the presence or absence of colchicine and monensin to determine if the synthesis or transport of CNP was affected. Incorporation of label into CNP₁ or CNP₂ was not impaired by colchicine (Fig. 6, lane 2) or by monensin (Fig. 6, lane 3) suggesting that neither microtubule nor Golgi complex-mediated (40) transport of the newly synthesized enzyme occurred. Likewise, neither the incorporation of [3H] palmitic acid into CNP immunoprecipitated from the brain homogenate (Fig. 7) nor from the myelin-like fraction (data
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Fig. 5. Analysis of fatty acids released from CNP labeled with [3H]palmitic acid. Brain slices were incubated with [3H]palmitic acid, the myelin-like fraction was prepared, and CNP was immunoprecipitated. Immunoprecipitates were exhaustively extracted with the organic solvents to remove noncovalently associated lipids. The protein pellet was treated with a) 1 M hydroxylamine, pH 9.8 for 24 h and lipids were extracted with chloroform/methanol (2:1, v/v) and analyzed by TLC, (b) treated with sodium borohydride in tetrahydrofuran (30%) for 40 min at 37 °C, hexadecanol was extracted with toluene and analyzed by TLC, and (c) treated with methanolic-KOH (0.2 N) for 18 h at 25 °C, lipids were extracted with hexane and analyzed by TLC. A, palmitohydroxamate (PH) and palmitic acid (PA) released after treatment of CNP with hydroxylamine. B, hexadecanol released after treatment of CNP with sodium borohydride (lane 2) and methyl palmitate released after treatment of CNP with methanolic-KOH (lane 5). Migration of authentic [3H]palmitic acid, [14C]hexadecanol, and [3H]methyl palmitate is shown in lanes 1, 3, and 4, respectively. Identical results were obtained when gel sections containing immunoprecipitated CNP labeled with [3H]palmitic acid were cleaved and analyzed for fatty acids.

Fig. 6. Effect of colchicine and monensin on [14C]-amino acid incorporation into CNP1 and CNP2. Brain slices were preincubated for 30 min in KRB buffer containing colchicine (0.5 mM) or monensin (0.1 μM). Brain slices were washed and incubated with fresh KRB buffer containing 200 μCi of [14C]-amino acid mixture in the presence or the absence of colchicine (0.5 mM) and monensin (0.1 μM) for 2.5 h at 37 °C. Brain slices were washed with KRB buffer 6 times. Brain homogenates were solubilized in SDS, and CNP1 and CNP2 were immunoprecipitated and analyzed by SDS-PAGE and fluorography. Brain slices were incubated with [14C]-amino acids in the absence (lane 1) or in the presence of colchicine (lane 2) or in the presence of monensin (lane 3). Incorporation of [14C]-amino acids into total brain proteins was not affected at these concentrations of colchicine or monensin.

Fig. 7. Effect of colchicine, monensin, and cycloheximide on the acylation of CNP. Brain slices were preincubated with colchicine (0.5 mM), monensin (0.1 μM) or cycloheximide (0.4 mM) for 30 min as described above. They were washed and incubated with fresh KRB buffer containing 10 mCi of [3H]palmitic acid in the presence or in the absence of colchicine (0.5 mM), monensin (0.1 μM), or cycloheximide (0.4 mM) for 2.5 h at 37 °C as described above. CNP from the brain homogenate was immunoprecipitated, analyzed by SDS-PAGE, and fluorography. Brain slices were incubated with [3H]palmitic acid in the absence (lane 1), in the presence of colchicine (lane 2), in the presence of monensin (lane 3), in the presence of cycloheximide (lane 4). Identical results were obtained when myelin-like fraction was immunoprecipitated. The incorporation of [3H]palmitic acid into CNP was reduced by cycloheximide only 24–32% in three consecutive experiments.

The exact role of palmitoylation of CNP is unknown. However, linkage of fatty acids to CNP may be necessary for...
it to interact with specific lipids or proteins within the bilayer or glial cytoplasm. Furthermore, the posttranslational addition of fatty acids to CNP may be a means by which this enzyme acquires an affinity for oligodendroglial plasma membrane. It is also feasible that CNP is synthesized initially as a soluble protein and later becomes associated with membranes after fatty acylation, like proteins (48). Alternatively, hydrophobicity conferred by covalent linkage of palmitic acid to CNP might permit it to play specific roles in cell-cell interaction during development of the central nervous system as it has been postulated for fatty acylated membrane. It is tempting to speculate that acylation and/or phosphorylation of CNP may regulate its activity or facilitate differentiation of glial precursor (progenitor) cells into mature oligodendrocytes.

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