Lipid Analysis of the Glycoinositol Phospholipid Membrane Anchor of Human Erythrocyte Acetylcholinesterase

PALMITOYLATION OF INOSITOL RESULTS IN RESISTANCE TO PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C

(Received for publication, April 25, 1988)

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The glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase (EC 3.1.1.7) contains a novel inositol phospholipid which in this and the accompanying paper (Roberts, W. L., Sautkarn, S., Reinhold, V. N., and Rosenberry, T. L. (1988) J. Biol. Chem. 263, 18776-18784) is shown to be a plasmamninositol that is palmitoylated on the inositol ring. The inositol phospholipid was radiolabeled with the photoactivated reagent 3-(trifluoromethyl)-3-(m-[^26I]iodophenyl)diazirine and characterized by various chemical and enzymatic cleavage procedures whose products were analyzed by thin layer chromatography or autoradiography or gas chromatography. Acidic methanolysis of human erythrocyte acetylcholinesterase (E^AChE) revealed 18:0 and 18:1 alkylglycerols (0.55 and 0.20 mol/mol AChE, respectively). Acetylation was shown by TLC to release alkylacylglycerol acetates from E^AChE. Analysis by gas chromatography revealed that 83% of the alkylacylglycerol acetates contained an 18:0 or 18:1-1-alkyl group and a 22:4 (n-6), 22:5 (n-3), or 22:6 (n-3) 2-acyl group. The inositol phospholipid is linked to the anchor by a glucosamine in glycosidic linkage, and deamination with nitrous acid cleaved the glycosidic linkage and released the phospholipid. The deamination and acetylation products from E^AChE were purified by high performance liquid chromatography, and fatty acid analysis following acidic methanolysis of the purified products revealed that 2 fatty acid residues were associated with the deamination product and only one with the alkylacylglycerol acetylation product. The other fatty acid residue was primarily palmitate and was indicated to be in ester linkage to an inositol hydroxy1(s). This linkage was shown to be responsible for the resistance of the inositol phospholipid to cleavage by Staphylococcus aureus phospholipid-specific phospholipase. Decacylation of the inositol phospholipid deamination product by treatment with base removed this palmitoyl group and facilitated release of alkyl- and alkylacylglycerol species by phosphatidylinositol-specific phospholipase C with concomitant formation of inositol 1-phosphate. In contrast, digestion of E^AChE with a recently reported anchor-specific phospholipase D resulted in release of plasmamn acidic from the intact palmitoylated plasmamninositol.

Acetylcholinesterase (EC 3.1.1.7) exists in multiple molecular forms which are widely distributed in many tissues (for reviews, see Massoulié and Bon, 1982; Rosenberry 1985). These diverse molecular forms possess similar catalytic and antigenic properties but differ in their extent of oligomeric assembly and their mode of attachment to the cell surface. AChE^1 on the surface of mammalian erythrocytes is an amphipathic globular dimer (G2) (Ott and Brodbeck, 1978; Duttachoudhury and Rosenberry, 1984). These erythrocyte G2 forms as well as the G3 forms that are abundant in Torpedo electric organ and insect heads are anchored in the plasma membrane by a covalently attached glycoinositol phospholipid (Roberts et al., 1987; Futerman et al., 1986; Gnagay et al., 1986; Haas et al., 1988). Similar glycolipid anchors have been identified in many proteins with an extracellular orientation (Low, 1987; Ferguson and Williams, 1988) including trypanosome variant surface glycoproteins (Ferguson et al., 1985a), rodent Thy-1 (Tse et al., 1986), alkaline phosphatase (Low et al., 1987), decay accelerating factor (Davitz et al., 1986; Medof et al., 1986), merozoite and schizont proteins from Plasmodium falciparum (Braun-Breton et al., 1988; Haldar et al., 1985; Haldar et al., 1986), Sgp1 and Sgp2 from squid brain (Williams et al., 1988), and scarpie prion protein (Stahl et al., 1987). Common structural features of glycoinositol phospholipid anchors are an ethanolamine residue in amide linkage to the C terminus of the protein, phosphate groups, a mannose-containing glycan, and a nonacylated glucosamine residue.

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*This investigation was supported by Grants NS16577, DK38181, and GM33873 from the National Institutes of Health and by grants from the Muscular Dystrophy Association, the American Heart Association, the Medical Research Council of Canada, Ottawa, and the Heart and Stroke Foundation of Ontario, and the Muscular Dystrophy Association, the American Heart Association.

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†† The abbreviations used are: AChE, acetylcholinesterase; E^AChE, bovine erythrocyte; E^AChE, human erythrocyte; G2, a globular dimeric AChE form; GLC, gas-liquid chromatography; GC-MS, gas-liquid chromatography-mass spectrometry; HPLC, high performance liquid chromatography; [126I]TID, 3-(trifluoromethyl)-3-(m-[^26I]iodophenyl)diazirine; PIPLC, phosphatidylinositol-specific phospholipase C; VSG, variant surface glycoprotein from T. brucei.
PIPLC-resistant Inositol Phospholipid Anchor of E." AChE

One characteristic of most glycoinositol phospholipid-anchored proteins is their conversion from membrane-bound amphipathic forms to soluble hydrophilic forms by treatment with purified PIPLCs from bacteria or anchor-specific phospholipase C from trypanosomes (Slein and Logan, 1965; Ikezawa et al., 1976; Low and Finean, 1977a; Bulow and Overath, 1986; Hereid et al., 1986; Fox et al., 1986). Many proteins have been classified as having such anchors solely on the basis of their susceptibility to this conversion. However, a subclass of glycoinositol phospholipid anchors is apparently resistant to the action of PIPLC. AChE G3 forms can be released from ox, pig, and rat erythrocytes by the action of PIPLC, but AChE on mouse and human erythrocytes is largely resistant to cleavage by this enzyme (Low and Finean, 1977a; Puterman et al., 1985b). The structural basis for the resistance of E. AChE to PIPLC resides in the anchor structure itself since the highly purified protein is >90% resistant to the action of PIPLC (Roberts et al., 1987). This conclusion was supported by analysis of anchor fragments labeled with the photoactivatable lipophilic reagent [125I]TID, a radiolabel selective for the lipid portion of E. AChE membrane anchors which has little effect on lipid TLC mobilities (Roberts and Rosenberry, 1986; Roberts et al., 1987).

Treatment of anchored proteins with nitrous acid deaminates the anchor glucosamine and cleaves its glycosidic linkage to inositol (see Scheme 1; Ferguson et al., 1985a). Deamination of [125I]TID-labeled AChEs released a radiolabeled product from E' AChE which comigrated with phosphatidyl inositol on TLC, but the corresponding product from E" AChE had a mobility much greater than phosphatidylinositol (Roberts et al., 1987). This novel deamination product from E" AChE contained myo-inositol and fatty acids and therefore appeared to be a modified form of phosphatidylinositol that is resistant to PIPLC. In this paper the structural basis for the PIPLC resistance of E' AChE was pursued by several approaches which are summarized in Scheme 1. First, since alkylacylglycerols were identified in E' AChE (Roberts et al., 1988a), acidic methanolysis was used to liberate alkylglycerols from E' AChE for analysis by TLC and GLC. Second, acetylation, a procedure that cleaves the same phosphodiester bond as PIPLC digestion, was used to generate diradylglycerol acetates from E' AChE for further analysis. Third, the fatty acid composition of the deacetylation and deamination products from E' AChE were compared. Fourth, the structure of the deamination product was investigated in more detail using a combination of chemical and enzymatic techniques. Finally, both E' and E" AChEs were examined for susceptibility to a recently reported anchor-specific phospholipase D (Davitz et al., 1987; Low and Prasad, 1988). In the accompanying paper (Roberts et al., 1988a), fast atom bombardment mass spectrometry was employed for further structural characterization of the E' AChE anchor and its deamination product.

**EXPERIMENTAL PROCEDURES**

**Preparation of AChEs—E" and E" AChEs were prepared by affinity chromatography and, where required, enzyme was depleted of Triton X-100 detergent by a second cycle of affinity chromatography (Roberts et al., 1987). Moles of catalytic subunit were determined either from the myo-inositol content, measured here as 1.02 mol of inositol/mol subunit (also see Roberts et al., 1987), or from the enzyme activity in a modified Elman assay, assuming 410 units/nmol of catalytic subunit (Roseberry and Scoggin, 1984). Samples of E" and E" AChEs were radiolabeled with [125I]TID (Amersham Corp.) as described (Roberts and Rosenberry, 1986).

**Acidic Methanolysis for Fatty Acid and Alkylglycerol Analysis—**To a dried sample was added 1 M anhydrous methanolic HCl (100 µl) and the reaction mixture was heated at 65 °C for 16 h. For analysis only of fatty acid methyl esters, samples were extracted with 2,2,4-trimethylpentane (Roberts and Rosenberry, 1985). For combined analysis of fatty acid methyl esters and alkylglycerols chloroform (200 µl) and water (75 µl) were added to the sample, and after vortexing, the lower organic phase was recovered. Samples for subsequent analysis by TLC were dried at this point. For GLC analysis, the aqueous phase was reextracted with chloroform (100 µl) and the combined organic extracts were acetylated by incubation at 65-85 °C, and the solution examined by TLC analysis of fatty acid methyl esters and alkylglycerols.

**Acetylation of AChEs—**Samples of [125I]TID-labeled E' and E" AChEs (75-150 pmol) for subsequent TLC analysis were dried, acetic acid (acetic anhydride 3:2, 100 µl) was added, and the samples were acetylated by heating for 4 h at 105 °C (Renkonen, 1965; Ferguson et al., 1985b). After drying the reaction mixtures in vacuo, chloroform/methanol (2:1, 200 µl) were added, the samples were vortexed, and the lower phases were removed and dried. Samples of detergent-depleted [125I]TID-labeled E" AChE for subsequent GLC analysis (65-85 nmol) were dried and treated in similar fashion except on a 10-fold larger scale. The dried samples were resuspended in acetonitrile (1 ml), and the product was extracted with three portions of hexane (1 ml each). The combined hexane phases were dried, resuspended in hexane/2-propanol (HPLC grade, 99.85:0.15), and chromatographed on a 4.8 × 250-mm Supelcosil HPLC column (Supelco, Inc., Bellefonte, PA) in the same solvent at a flow rate of 1 ml/min. The UV absorbance at 206 nm was monitored, and 1-min fractions were collected for 3H determination.

**Base Methanolysis of E' AChE Acetylation Fragments—**Dried samples of the HPLC-purified products generated from E' AChE by acidic methanolysis were treated with 1 M sodium methoxide in methanol/toluene (3:2, 100 µl) for 15 min at 25 °C. Water (50 µl) was added, and after vortexing the upper phase was recovered. The lower aqueous phase was extracted with chloroform (100 µl) and after vortexing the lower phase was removed, combined with the previously removed upper phase, and dried for analysis.

**Nitrous Acid Deamination of E" AChE—**Detergent-depleted samples of [125I]TID-labeled E" AChE (15-50 nmol) were dialyzed against water and reduced in volume in a Speedvac concentrator. To the enzyme in 1.2 ml of 0.1 M sodium acetate (pH 3.5) was added 0.3 ml of 1 M sodium nitrite, and the pH of the solution was adjusted to 4.0 by the addition of 6 N HCl (6 µl). After incubation of the mixture at 50 °C for 4 h, the phospholipid product was removed by extraction with 4 ml of chloroform/methanol (2:1) and two 3-ml portions of chloroform. The combined organic phases were dried and yielded 50-60% of the initial [3H]radioactivity. The deamination product was purified by inorganic normal phase HPLC on a Supelcosil column using 2-propanol/hexane/water (6:8:6.0) mobile phase (Hax and Geurts van Kessel, 1977) at a flow rate of 0.5 ml/min. The absorbance at 206 nm was monitored, and 0.5-ml fractions were collected. The major peak (radioactivity concentration time 25 min) contained 60-75% of the radioactivity applied to the column.

**PIPLC Digestion of the E' AChE Inositol Phospholipid—**Samples of the E' AChE deamination product purified by HPLC were divided into 2
PIPLC-resistant Inositol Phospholipid Anchor of E\textsuperscript{hu} AChE

Alkylglycerols in E\textsuperscript{hu} AChE—The photoactivated reagent \([^{125}\text{I}]\text{TID}\) has been shown to label the lipid groups in the glycoinositol phospholipid anchor of E\textsuperscript{hu} AChE with high selectivity. Greater than 90% of the incorporated label could be released by purified S. aureus PIPLC (Roberts et al., 1987) and was associated with alkylacylglycerols whose individual molecular species were quantified by GLC analysis (Roberts et al., 1988a). Since E\textsuperscript{hu} AChE is largely resistant to PIPLC, alternate methods of chemical cleavage and analysis of alkyl- and alkylacylglycerols were employed. Samples of E\textsuperscript{bo} and E\textsuperscript{hu} AChEs were labeled with \([^{125}\text{I}]\text{TID}\) subject to acidic methanolysis under conditions which cleave oxysterols, thioesters, and phosphodiester bonds. The radiolabeled fragments were extracted and analyzed by TLC. We have previously shown that under these conditions fatty acid methyl esters are released from both AChEs and that E\textsuperscript{hu} AChE contains considerably more palmitic acid than E\textsuperscript{bo} AChE (Roberts and Rosenberry, 1985; Roberts et al., 1987). In the current experiments the more polar solvent chloroform was used in place of 2,2,4-trimethylpentane to extract quantitatively alkylglycerols as well as fatty acid methyl esters from the reaction mixture. The TLC autoradiograph in Fig. 1 indicates that acidic methanolysis liberates both fatty acid methyl esters and alkylglycerols from E\textsuperscript{bo} and E\textsuperscript{hu} AChEs. The alkylglycerol content of E\textsuperscript{hu} AChE was quantitated by GLC after acidic methanolysis as described under "Experimental Procedures." Two major alkylglycerol species, 1-stear-ylglycerol and 1-oleylglycerol, were observed at levels of 0.55 and 0.20 mol/mol of myo-inositol, respectively. No dimethylacetals, products of acid hydrolysis of alkylglycerols, were detected. A profile of the fatty acids and alkylglycerols released from E\textsuperscript{bo} by acidic methanolysis is shown in Fig. 2.

Acetylation of \([^{125}\text{I}]\text{TID}\)-labeled E\textsuperscript{bo} and E\textsuperscript{hu} AChEs—The identification of alkylglycerol and fatty acid components in E\textsuperscript{bo} and E\textsuperscript{hu} AChEs was shown to increase the enzyme's sensitivity as a result of a diradylglycerol acetate (Renkonen, 1965). This cleavage site is identical to that of PIPLC. Acetylation of

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**RESULTS**

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**Acidic Methanolyis**

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**FIG. 1.** Silica TLC analysis of fragments generated by acidic methanolysis of E\textsuperscript{bo} and E\textsuperscript{hu} AChE. Samples of \([^{125}\text{I}]\text{TID}\)-labeled, purified AChEs (50-100 pmol) were subjected to acidic methanolysis, and radiolabeled fragments were extracted and analyzed by TLC in solvent A and autoradiography as described under "Experimental Procedures." The standards were 1-hexadecylglycerol (a) and methyl palmitate (f). E\textsuperscript{bo} and E\textsuperscript{hu} designate their respective \([^{125}\text{I}]\text{TID}\)-labeled AChEs as starting material. Residual radioactivity at the origin may result from the degradation of \([^{125}\text{I}]\text{TID}\).
Acetylation was performed on Ehu AChE (5 nmol) to which heptadecanoic acid (17:0) had been added as an internal standard. After methanolysis, the lipids were extracted, acetylated, and determined by GLC Method 1. Peaks are designated by alkyl or acyl chain carbon number:double bond number, and species containing alkyl chains are indicated by a prime. Unidentified peaks are added as an internal standard. After methanolysis, the lipids were methanolysis blanks. Peaks were identified by comparison of retention times with standards and agreed to within ± 0.03 min.

Fig. 2. Polar column GLC analysis of alkylglycerols and fatty acids from Ehu AChE. Acidic methanolysis was performed on Ehu AChE (5 nmol) to which heptadecanoic acid (17:0) had been added as an internal standard. After methanolysis, the lipids were extracted, acetylated, and determined by GLC Method 1. Peaks are designated by alkyl or acyl chain carbon number:double bond number, and species containing alkyl chains are indicated by a prime. Unidentified peaks (*) at 10.0, 10.9, and 13.0 min also were observed in methanolysis blanks. Peaks were identified by comparison of retention times with standards and agreed to within ± 0.03 min.

Fig. 3. Silica TLC analysis of fragments generated by acetolysis of Ehu and Ebo AChEs. Panel A, samples derived from [125I]TID-labeled Ehu and Ebo AChE by acetolysis and extraction. Panel B, [125I]TID-labeled Ebo AChE was subjected to acetolysis, and products α and β were resolved by HPLC and treated with sodium methoxide. The extracts were analyzed by TLC (lane 1, α; lane 2, β). Solvent A was employed in panel A and solvent B, in panel B. The standards are 1-hexadecylglycerol (a), 1,2-dioleoylglycerol acetate (d), 1,3-dioleoylglycerol acetate (e), and methyl palmitate (f). The Rf of 1-hexadecylglycerol was 0.06 in solvent B. Residual radioactivity at the origin in panel A may result from degradation of [125I]TID.

[125I]TID-labeled Ebo and Ehu AChE permitted extraction of 72 and 73% of the radiolabel, respectively, into the lower chloroform phase. TLC autoradiographic patterns of these acetolysis products from the two enzymes were identical (Fig. 3A). The mobility of the major radioactive spot (β), near that of the unlabeled 1,3-dioleoylglycerol acetate standard (ε), was consistent with the migration of 1,2-alkylacylglycerol acetates (Renkonen, 1965). An additional unidentified spot (α) with greater mobility was generated from both enzymes by acetolysis. This additional product has not been reported previously (Renkonen, 1965; Ferguson et al., 1985b) nor was a corresponding spot observed when acetolysis was performed on unlabeled phosphatidylinositol (data not shown). HPLC analysis of the acetolysis products (see Fig. 7, Miniprint Supplement) resolved α from the major peak β, which comprised 60–75% of the total products. The separated products α and β were treated with sodium methoxide in methanol/toluene, and the products of this reaction were extracted with chloroform and analyzed by TLC and autoradiography (Fig. 3B). Lanes 1 (α) and 2 (β) both contained major radioactive spots which migrated as fatty acid methyl esters. Lane 1 indicates that base methanolysis of α also gave an unidentified spot with a Rf about 80% of that of the fatty acid methyl ester standard. In contrast, methanolysis of β in lane 2 revealed a radioactive spot which migrated with the alkylglycerol standard. Fig. 3B supports the assignment of β as an alkylacylglycerol acetate and indicates that the unidentified acetolysis product α does contain fatty acids and another component which is not an alkylglycerol.

Samples of β were analyzed directly by GLC on both nonpolar and polar capillary columns. A relatively simple profile was obtained on a nonpolar column (Fig. 4) where alkylacylglycerol acetate species were resolved solely on the basis of their carbon number. Most of the alkylacylglycerol acetates in β (83%) corresponded to species with 40 carbon atoms in their alkyl and acyl chains (Table 4, Miniprint Supplement). However, several peaks were resolved on a polar GLC column (Fig. 9, Miniprint Supplement), indicating considerable heterogeneity in the alkylacylglycerol acetates. Details of the alkylacylglycerol acetate molecular species analysis are presented in the Miniprint Supplement, and a summary is pro-
vided in Table I. In agreement with the nonpolar GLC analysis, 83% of the major species corresponded to alkylglycerol acetates composed of 18:0 and 18:1 alkylglycerols combined with 22:4, 22:5, and 22:6 acyl chains. The predominance of alkylglycerol species in both the Ehu and Ehu AChE anchor indicates that the anchor inositol phospholipid should properly be termed plasmalyinositol.

Fatty Acid Compositions of Intact AChEs and Fragments—We have previously reported 1.4 and 2.0 mol of fatty acids/mol of catalytic subunit for Ebo and Ebo AChE, respectively (Roberts and Rosenberry, 1985; Roberts et al., 1987). It was unclear whether or not these fatty acids were associated exclusively with a glycoinositol phospholipid. To estimate the quantity of fatty acids located outside this portion of the enzyme, Ebo AChE was cleaved with PIPLC to remove the diradylglycerols (Roberts et al., 1987), and Ebo AChE was digested with papain to remove the entire glycolipid with the C-terminal dipeptide (Roberts and Rosenberry, 1985, 1986). The resulting AChEs were repurified and analyzed for residual fatty acids (Table II). Complete removal of the glycolipid from Ebo AChE was indicated by the absence of 22:4 and 22:5 fatty acids. The hydrophilic forms of both Ebo and Ebo AChE retained about 0.3 mol of fatty acids/mol of subunit which appear to be either very tightly associated or possibly covalently attached to the AChEs at sites other than the glycoinositol phospholipid anchor. Fatty acylation of proteins is a well-documented post-translational modification (for reviews see Schmidt, 1983; Sefton and Buss, 1987). Other acyl groups including stearic and oleic acids in addition to palmitic and myristic acids can be involved in covalent linkage to protein (Schmidt et al., 1979), consistent with Table II. After correcting the total fatty acids per mol of Ebo AChE monomer (2.02 mol) for fatty acids not associated with the anchor (0.26 mol), we conclude that about 1.8 mol of fatty acids are associated with the Ebo AChE glycoinositol phospholipid anchor. Since only about one fatty acid is accounted for in the alkylglycerol analysis above, roughly 0.8 fatty acid residues must be present at another site on the glycoinositol phospholipid anchor of Ebo AChE.

To identify and localize these additional fatty acids, fatty acid compositions of lipophilic fragments generated from Ebo AChE by chemical cleavage at two sites are compared in Table III. Nitrous acid deamination, which cleaves the C-1 glycosidic bond of the anchor glucosamine to generate an inositol phospholipid (Roberts et al., 1987), released a product whose fatty acid composition very closely resembled that of intact Ebo AChE. In contrast, the fatty acids released in alkylglycerols (β) by acetolysis lacked most of the palmitate and small amounts of 14:0, 16:1, 18:1, and 18:2 fatty acids found in the intact enzyme, but the quantities of polysaturated fatty acids in the released acetolysis product were equal to or greater than those in either the released deamination product or the intact enzyme. The results indicate that an inositol hydroxyl group(s) is acylated, primarily with palmitate but also with smaller amounts of other 14, 16, and 18 carbon-containing fatty acids.

Basic Methanolysis Abolishes the Resistance of the Ebo AChE Deamination Product to PIPLC—We have noted previously that an endogenous modification of the inositol phospholipid in Ebo AChE correlates with its resistance to cleavage by PIPLC (Roberts et al., 1987). In this and the accompanying paper (Roberts et al., 1988b) we characterize this modification as fatty acid acylation of the inositol portion of the phospholipid. To test if this modification is sufficient to confer PIPLC

| Table I |
|---|
| **Alkylglycerol molecular species in Ebo AChE** |
| Molecular species | mol % |
| 18:0 | 18:0 | 8.3 |
| 18:1 | 18:0 | 2.7 |
| 18:0 | 20:4 (n-6) | <2.8 |
| 16:0 | 22:4 (n-6) | 2.0 |
| 18:0 | 22:4 (n-6) | 2.0 |
| 18:0 | 22:5 (n-3) | 25.2 |
| 18:1 | 22:5 (n-3) and 22:6 (n-3) | 11.0 |

* The alkyl chains, presumably in the sn-1 position, are indicated by a prime and shown on the left. The values for sn-1,2- and sn-1,3-isomers which were generated by acetolysis have been summed.

| Table II |
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| **Residual fatty acids on AChE catalytic subunits** |
| Fatty acid | Bovine | Human |
| 16:0 | 0.09 ± 0.02 | 0.07 |
| 16:1 | 0.02 ± 0.01 | ND |
| 18:0 | 0.02 ± 0.02 | 0.06 |
| 18:1 | 0.06 ± 0.07 | 0.05 |
| 18:2 | 0.03 ± 0.02 | 0.08 |
| Total | 0.32 ± 0.11 | 0.26 |

* The fatty acid content of the PIPLC-digested catalytic subunit of Ebo AChE (3.7 nmol) was determined by GLC Method 2 after acidic methanolysis, and the moles of fatty acids per mol of AChE were calculated based on myo-inositol content. The results are the mean and standard error of three determinations performed on three distinct enzyme preparations.

| Table III |
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| **Fatty acid composition of Ebo AChE acetolysis and deamination products** |
| Fatty acid | Acetolysis<sup>*</sup> | Deamination<sup>*</sup> | Intact<sup>4</sup> |
| 14:0 | ND | 0.04 ± 0.01 | 0.06 |
| 16:0 | 0.08 ± 0.04 | 0.82 ± 0.07 | 0.75 |
| 16:1 | ND | 0.10 ± 0.02 | 0.09 |
| 18:0 | 0.12 ± 0.04 | 0.14 ± 0.01 | 0.23 |
| 18:1 | 0.03 ± 0.01 | 0.13 ± 0.02 | 0.24 |
| 18:2 | ND | 0.05 ± 0.01 | 0.06 |
| 20:4 | 0.03 ± 0.01 | 0.04 ± 0.01 | 0.05 |
| 22:4 | 0.04 ± 0.01 | 0.25 ± 0.02 | 0.28 |
| 22:5 | 0.26 ± 0.01 | 0.19 ± 0.01 | 0.31 |
| 22:6 | 0.05 ± 0.01 | 0.04 ± 0.01 | 0.05 |
| Total | 1.00 | 1.54 ± 0.02 | 2.02 |

* Mean and S.E. of determinations on two independent preparations of acetolysis product β. One mole of total fatty acids per mol of β was assumed based on the observation of exclusively alkylglycerol products in β, and results were normalized to this total.

* Mean and S.E. from determinations on two independent preparations, expressed as moles of fatty acid per mol of myo-inositol in the deamination product.

* Mean from five enzyme preparations (from Roberts and Rosenberry, 1985), expressed as moles of fatty acid per mol of catalytic subunit based on enzyme activity.

<sup>4</sup> ND means not detected. The detection limit is 0.02 mol/mol of sample.
resistance, the $^{125}$I-labeled product generated from $E_{nu}$ AChE by nitrous acid deamination was first deacylated with methanolic KOH or methanolic NH$_3$ to remove the putative acyl group from inositol and then digested with PIPLC. As controls either PIPLC digestion or base methanolysis alone were performed. The results of this experiment are shown in Fig. 5. Methanolic KOH generated fatty acid methyl esters from the two samples which received this treatment. No alkylglycerols (a) were detectable in the control sample (lane 1), but digestion of the putative alkyllyso-plasmanylinositol generated by methanolic KOH with PIPLC did release alkylglycerols as shown in lane 2. Ammonia methanolysis liberated fatty acid methyl esters and a small quantity of alkylglycerols from the deamination product (lane 3) but more importantly permitted PIPLC digestion to release alkylacylglycerols (lane 4). PIPLC digestion alone produced no detectable cleavage of the deamination product since all radioactivity remained at the origin (lane 5). These results indicate qualitatively that alkyl- or alkylacylglycerols can be released from the $E_{nu}$ AChE inositol phospholipid by PIPLC digestion only after the inositol residue has been deacylated. The other product generated from alkyllyso-plasmanylinositol by PIPLC digestion is inositol 1-phosphate. A more quantitative estimate of the extent of PIPLC cleavage was obtained by using GC-MS to detect this product. Basic methanolysis and PIPLC digestion were conducted as in Fig. 5, and the samples were prepared for GC-MS analysis as described under "Experimental Procedures" (Sherman et al., 1986). Treatment with methanolic KOH followed by PIPLC digestion produced 1.1 ± 0.2 (n = 2) mol of inositol 1-phosphate/mol of myo-inositol. The levels of inositol 1-phosphate detected after base methanolysis or PIPLC digestion alone were less than 0.03 mol/mol myo-inositol. These results demonstrate inositol 1-phosphate in the deamination product and provide our strongest evidence that decylation of a hydroxyl group on myo-inositol permits the quantitative cleavage of the remaining inositol phospholipid by PIPLC.

**Anchor-specific Phospholipase D Digestion of $E_{nu}$ and $E_{bo}$ AChE**—Recently, an abundant anchor-specific phospholipase D enzyme activity has been detected in mammalian serum (Davitz et al., 1987; Low and Prasad, 1988). Aliquots of this enzyme partially purified from rabbit serum were mixed with $[125]$I-labeled $E_{nu}$ and $E_{bo}$ AChE to assess cleavage of the anchors in these enzymes. After digestion the hydrophobic products were extracted and subjected to TLC and autoradiography. The release of $[125]$I-TID label into the chloroform phase was 47 ± 5% for $E_{nu}$ AChE and 39 ± 11% for $E_{bo}$ AChE. This partial release is consistent with that observed for other substrates of the anchor-specific phospholipase D (Davitz et al., 1987; Low and Prasad, 1988). TLC analysis of the released products is shown in Fig. 6. The major radiolabeled fragment from both enzymes had a mobility very similar to that of a phosphatidic acid standard (g). These results indicate that the plasmanylinositols in both $E_{nu}$ and $E_{bo}$ AChE anchors are substrates for this phospholipase D activity. Furthermore, the observation that plasmanic acids with equivalent TLC mobilities are released from both AChEs is consistent with the conclusion above that palmitoylation of the inositol phospholipid in $E_{nu}$ AChE occurs proximal to the phosphate group linking inositol and the alkylglycerol. This palmitoylation, although conferring resistance to PIPLC, apparently has little or no effect on the action of this phospholipase D.
DISCUSSION

Although many proteins with glycoinositol phospholipid membrane anchors have been identified, the chemical structure of only one of these, the Trypanosoma brucei variant 117 VSG anchor, has been fully elucidated (Ferguson et al., 1985a; Ferguson et al., 1988). Characterization of the phospholipid portion of the VSG anchor phospholipid revealed the presence of dimyristoylglycerol, a diacylglycerol (Ferguson et al., 1985b). In contrast, other anchor or anchor-like glycolipids contain primarily alkyl- or alkyacylglycerols instead of diacylglycerols. Examples include an insulin-sensitive glycolipid from H35 hepatoma cells (Mato et al., 1987), a lipophosphoglycan from Leishmania donovani (Orlandi and Turco, 1987) and the membrane anchor of Eμ AChE (Roberts et al., 1987, 1988a). In all three cases, the alkyacylglycerol-containing component could be released by digestion with PIPLC. The identification of alkyacylglycerols as the main diradylglycerol species in Eμ AChE prompted us to examine Eμ AChE for similar constituents. Acidic methanolysis of Eμ AChE and GLC analysis revealed two major alkylglycerol species, 18:0 (0.55 mol/mol) and 18:1 (0.20 mol/mol), in Eμ AChE. This procedure illustrates quantitative analysis of fatty acid and alkyacylglycerol anchor components directly on a purified protein sample.

Further analysis of the molecular species released from Eμ AChE by acetylation revealed that alkyacylglycerols, predominantly combinations of 18:0 alkylglycerol with 22:4, 22:5, and 22:6 acyl groups but with additional heterogeneity, were the only type of diradylglycerol in the membrane anchor of Eμ AChE. These results, which account for only 1 mol of fatty acid/mol of anchor, raised a question of the location of the second mol of fatty acids previously shown to be associated with the Eμ AChE anchor (Roberts and Rosenberry, 1985). Three approaches illustrated in Scheme 2 were used to answer this question.

First, a comparison of the fatty acid compositions of the Eμ AChE anchor lipid products generated by acetylation and nitrous acid deamination indicated that the second fatty acid residue, primarily palmitate, is localized to a region of the deamination product outside the diradylglycerol. Second, digestion of Eμ and Eβ AChE with anchor-specific phospholipase D generated similar amounts of plasmanylglycerol from both enzymes. This result indicates that the second fatty acid residue on Eμ AChE is not attached to the plasmanylglycerol portion of the anchor and is consistent with a second fatty acid residue linked in some manner to inositol. Finally, data obtained by fast atom bombardment mass spectrometry and presented in the accompanying paper (Roberts et al., 1988b) confirmed direct palmitoylation of an inositol hydroxyl group as the site of attachment of a second fatty acid residue, although the technique provided no information about which inositol hydroxyl group is acylated. To our knowledge fatty acid acylation of inositol has not been documented previously in any biological system, although a triacylated glycerophosphoinositol in Corynebacterium xeroxis has been reported (Brennan, 1986).

To address our previous suggestion (Roberts et al., 1987) that a substituent on the inositol phospholipid could be responsible for the resistance of the Eμ AChE anchor to PIPLC, the anchor deamination product was decacylated by basic methanolysis and digested with PIPLC. Following methanolysis with KOH, alkyacylglycerol and myo-inositol 1-phosphate were produced quantitatively by PIPLC. Methanolysis with NH3 selectively removed the fatty acid from inositol with little release of fatty acid from glycerol, a result confirmed by fast atom bombardment mass spectrometry (Roberts et al., 1988b). PIPLC treatment of the resulting plasmainositol released alkyacylglycerols. This result provides specific evidence that acylation of the inositol ring is solely responsible for PIPLC resistance and ruled out any effect of the polyunsaturated fatty acids at the 2-position of the alkyacylglycerol on resistance to PIPLC. The presence of palmitic acid on the 2-hydroxyl of inositol would prevent the formation of a 1,2-cyclic phosphate intermediate by PIPLC (Ferguson et al., 1985a) and provide a mechanistic basis for PIPLC resistance. Palmitoylation at this position is currently under investigation.

Although the functions of glycoinositol phospholipid anchors in proteins currently are unknown, speculation focuses on the susceptibility of these proteins to release from the cell surface on activation of endogenous anchor-specific phospholipases (Low and Saltiel, 1988). Resistance to phospholipase C cleavage conferred by palmitoylation of the anchor inositol thus could provide a cellular mechanism for the regulation of phospholipase-induced protein release. Other glycoinositol phospholipid-anchored proteins are at least partially resistant to PIPLC release, including 5'-nucleotidase (Low and Finean, 1978; Shukla et al., 1980), decay accelerating factor (Davitz et al., 1986; Medof et al., 1986), and surface antigens from Dictyostelium discoideum (Sadeghi et al., 1988). Furthermore, the TLC mobility of the nitrous acid deamination product from [14C]TID-labeled decay accelerating factor was identical with that of the corresponding product from Eμ AChE (Medof et al., 1986; Walter et al., 1987). These observations support the speculation that fatty acylation of an inositol hydroxyl(s) may be a general modification of glycoinositol phospholipid-anchored membrane proteins, particularly in Eμ, that confers resistance to release by PIPLC. It is noteworthy, however, that decay accelerating factor purified from Eμ also can be converted to a hydrophilic form by phospholipase D (Davitz et al., 1987). This observation supports the conclusion that palmitoylation of inositol does not interfere with digestion by anchor-specific phospholipase D.

Despite the shortcomings of acetylation, which include production of an unidentified by-product (Fig. 3A) and formation of 1,3-diradylglycerol acetates (Fig. 9, see Miniprint Supplement), diradylglycerol acetates released from Eμ AChE by acetylation were chosen for molecular species analysis rather than phosphatic acids released by phospholipase D for several reasons. First, cleavage of diradylglycerols during acetylation is complete and nonselective (Renkonen, 1965), while phospholipase D from human serum removes phosphatic
acid from only about 70% of the apparently homogeneous VSG substrate molecules (Davitz et al., 1987). Second, it is more difficult to resolve a mixture of phosphatic acids than the less polar, more volatile diradylglycerol derivatives by either GLC or HPLC methods (Snyder, 1973). Finally, the chemical reagents used in acetylation are far less likely to introduce contaminants than the crude biological preparations of phospholipase D. The major drawbacks of the acetylation procedure, the isomerization of 1,2-diradylglycerols to 1,3-species and the generation of α, both can be circumvented. The 1,2- and 1,3-isomers were resolved and identified on a polar capillary column. The fatty acid composition of α was very similar to that of the alkylacylglycerol acetates so its production did not affect determination of the mole percentages of the alkylacylglycerol species. The exact nature of δ remains unclear. The data suggest that the major difference between α and δ involves their glycerol ether moieties. Since acetylation of E 

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PIPLC-resistant Inositol Phospholipid Anchor of \(E^{\text{III}}\) AChE

**Figure 1.** TLC analysis of \(\text{E}^{\text{III}}\) AChE - methylation products a and b. The TLC solvent was acetic anhydride in chloroform. The products were separated on TLC plates and visualized under UV light. The plates were then stained with ninhydrin. The retention times of the products were recorded and compared to the known standards.

**Table 4.** Direct TLC analysis of a and b on a chiral column.

**Table 5.** Fatty acid composition of \(E^{\text{III}}\) AChE products.

**Table 6.** Lipidomic analysis of \(E^{\text{III}}\) AChE - methylation products. The analysis was performed on a capillary column in a gas chromatograph. The retention times and molecular weights of the fatty acid methyl esters were recorded and compared to the known standards.

**Table 7.** Phospholipid composition of \(E^{\text{III}}\) AChE - methylation products. The analysis was performed on a capillary column in a gas chromatograph. The retention times and molecular weights of the phospholipids were recorded and compared to the known standards.

**Figure 2.** UV absorbance spectrum of the PIPLC-resistant Inositol Phospholipid Anchor of \(E^{\text{III}}\) AChE. The absorbance was measured at 280 nm. The spectrum was recorded and compared to the known standards.

**Figure 3.** Radioactivity analysis of the PIPLC-resistant Inositol Phospholipid Anchor of \(E^{\text{III}}\) AChE. The radioactivity was measured using a liquid scintillation counter. The results were recorded and compared to the known standards.

Carbon backbone and the fatty acid profiles of each were nearly identical. The possibility that e might contain aminopropanol was found to be unlikely by carbon backbone analysis. The aminopropanol was detected after methylation. However, the aminopropanol was not detected after hydrolysis. In addition, the aminopropanol was detected after hydrolysis with aminopropanol. The results were compared to the known standards. The aminopropanol was not detected after hydrolysis with aminopropanol. The results were compared to the known standards.

**Figure 4.** Gas chromatography-mass spectrometry analysis of the PIPLC-resistant Inositol Phospholipid Anchor of \(E^{\text{III}}\) AChE. The analysis was performed on a capillary column in a gas chromatograph. The retention times and molecular weights of the fatty acid methyl esters were recorded and compared to the known standards.

**Table 8.** Glycolipid analysis of \(E^{\text{III}}\) AChE - methylation products. The analysis was performed on a capillary column in a gas chromatograph. The retention times and molecular weights of the glycolipids were recorded and compared to the known standards.

**Figure 5.** Mass spectrometry analysis of the PIPLC-resistant Inositol Phospholipid Anchor of \(E^{\text{III}}\) AChE. The analysis was performed on a mass spectrometer. The mass spectra were recorded and compared to the known standards.

**Figure 6.** Negative ion electrospray ionization mass spectrometry analysis of the PIPLC-resistant Inositol Phospholipid Anchor of \(E^{\text{III}}\) AChE. The analysis was performed on a mass spectrometer. The mass spectra were recorded and compared to the known standards.

**Figure 7.** Positive ion electrospray ionization mass spectrometry analysis of the PIPLC-resistant Inositol Phospholipid Anchor of \(E^{\text{III}}\) AChE. The analysis was performed on a mass spectrometer. The mass spectra were recorded and compared to the known standards.

**Figure 8.** High-performance liquid chromatography analysis of the PIPLC-resistant Inositol Phospholipid Anchor of \(E^{\text{III}}\) AChE. The analysis was performed on a high-performance liquid chromatograph. The retention times and molecular weights of the fatty acid methyl esters were recorded and compared to the known standards.
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require activation prior to chromatographic analysis. In contrast to the glycerol ethers, from B, and they were eluted considerably earlier than the corresponding aldehyde ether cleavage on a polar stationary phase. The major glycerol ether obtained from D was identified in 18:0 (AA) but a significant amount of 18:1 (20%) was observed that was divided among several subfractions, some of which could not be precisely identified. The second resolved 18:1 species eluted with a [M+H] 18:1 (M+H) reference standard (methyl aldehyde) (1984). This is the principle isomer found in the aliphatic/glycerol fraction of the cation and, in other phospholipids from bovine erythrocytes (Mayer et al., 1980). The earlier 18:1 subfractions may be a glucosyl ester. In agreement with the elution characteristics of 18:2 and 18:3 (AA) standards of 18:2 fatty acids (Pate et al., 1987; Ahls and Baten, 1988). These qualitative and quantitative fatty acid and glycerol ether estimates for B were confirmed by catalytic hydrolysis and re-analysis of the second column (Table 9 and 10).

Analysis of E and F revealed a high level of glycerol ethers as a polar column. An attempt to resolve molecular species with varying degrees of unsaturation, A and B were eluted as a trienic/trisaturated fraction. A chromatogram was obtained from B (Fig. 9), but for unknown reasons no peaks were detectable when B was eluted under the same conditions. The chromatograph of E in Fig. 9 is more complex than one would expect on the basis of the glycerol ether and fatty acid composition of the aliphatic/glycerol fraction, probably due to the generation of both 18:0-2 and 18:0-3 isomers during analysis as has been observed previously for reactions of phospholipids/bilayer (Kermer and Baten, 1986). No resolution of these species was obtained in the polar column (Fig. 4). But the isomers were readily resolved on the polar column where almost every species occurred as diasteroisomers, identifiable 1,2,3-triisole. Standards were available only for diesters of 1,2 and 1,3 isomers of the 18:0-2 isomers. The resolution of species containing 18:0 and 18:0 fatty acids was not achieved under these chromatographic conditions. Of 12 components observed in Fig. 9, 10 were identified and are listed in Table 1. The aliphatic/glycerol molecular species in the polar bilayer phospholipid fraction (Table 1). Resolutions were reanalyzed from the molecular species resolved in Fig. 9 and summarized in Table 4 and the glycerol ether data in Table 5. Details of this type of reconstitution have been reported previously (Roberts et al., 1980).

Table 1: Aliphatic/glycerol species resolved on a polar column.

| Peak No. | Principal Species | Mole % |
|----------|------------------|--------|
| 12       | 1,3,18(18:3)     | 0.7    |
| 13       | 1,3,18(18:3)     | 1.2    |
| 14       | 1,3,18(18:3)     | 4.5    |
| 15       | 1,3,18(18:3)     | 6.5    |
| 16       | 1,3,18(18:3)     | 9.0    |
| 17       | 1,3,18(18:3)     | 11.5   |
| 18       | 1,3,18(18:3)     | 14.0   |
| 19       | 1,3,18(18:3)     | 16.5   |
| 20       | 1,3,18(18:3)     | 19.0   |

The peaks were identified by comparing their retention times and areas with those predicted from the fatty acid and aliphatic/glycerol composition of the aliphatic/glycerol fraction.

The peak numbers refer to peaks in Fig. 9 which were derived from MPLC fraction A from acetaminophen at pH 7.0.

The peaks were identified by catalytic hydrolysis and area with that predicted from the fatty acid and aliphatic/glycerol composition of the aliphatic/glycerol fraction.