Vol. 150, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1988 Pages 271-277

ALKYLACYLGLYCEROL MOLECULAR SPECIES IN THE GLYCOSYLINOSITOL PHOSPHOLIPID MEMBRANE ANCHOR OF BOVINE ERYTHROCYTE ACETYLCHOLINESTERASE

William L. Roberts*, John J. Myher+, Arnis Kuksis+, and Terrone L. Rosenberry*

*Department of Pharmacology, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106; +Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario, Canada M5G 1L6

Received December 1, 1987

Summary: Bovine erythrocyte acetylcholinesterase, a glycosylinositol phospholipid anchored membrane enzyme, was digested with phosphatidylinositol-specific phospholipase C and the released glycerol-containing moieties were identified and quantitated. About 96% of the total was alkylacylglycerol, of which sn-1-stearyl-2-stearoylglycerol, sn-1-stearyl-2-oleoylglycerol and sn-1-oleyl-2-stearoylglycerol accounted for 69%, 13% and 10%, respectively. These alkylacylglycerols are in marked contrast to the exclusively diacylglycerol species present in phosphatidylinositol from bovine erythrocyte membranes. This difference suggests that assembly of the membrane anchor of E^{bo} AChE involves a selected cellular pool of diradylglycerols.

A functionally diverse class of membrane proteins possess a glycosylinositol phospholipid membrane anchor as their common structural feature (for a review see 1). Many of these proteins can be released from the cell surface after degradation of the anchor PI by treatment with PIPLC (EC 3.1.4.10). The well characterized anchor from trypanosome variant surface glycoproteins contains a diacylglycerol, specifically sn-1,2-dimyristoyl-glycerol, which is released by PIPLC treatment (2). Recently, the presence of alkylacylglycerols in an insulin-sensitive glycolipid has been proposed (3) and a lysoalkylphosphatidylinositol with saturated, unbranched C_{24} and C_{26} alkyl chains was found in the membrane anchor of Leishmania donovani lipophosphoglycan (4). The presence of alkylglycerols in membrane anchors of proteins is interesting since these lipid species are far less common in cell membrane phospholipids than their acylglycerol counterparts.

We have recently reported the usefulness of 3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine as a radiolabel for the characterization of lipid components of the glycosylinositol phospholipid membrane anchors of AChEs (EC 3.1.1.7) isolated from bovine and human erythrocytes (5). The radiolabeled

ABBREVIATIONS: E^{bo}, bovine erythrocyte; AChE, acetylcholinesterase; HPLC, high performance liquid chromatography; GLC, gas-liquid chromatography, TLC, thin layer chromatography; PI, phosphatidylinositol; PIPLC, PI-specific phospholipase C.

0006-291X/88 $1.50

fragment released from E^{bo} AChE by digestion with PIPLC does not migrate as a 1,2-diacylglycerol on TLC. In this report we describe the identification and quantification of the diradylglycerol species released from E^{bo} AChE by PIPLC.

METHODS

Preparation of AChE

E^{bo} AChE was obtained from fresh citrate-treated bovine blood by affinity chromatography as previously described (5). Enzyme was depleted of Triton X-100 detergent by a second cycle of affinity chromatography and eluted with 20 mM sodium phosphate buffer, pH 7, (buffer A) containing 250 mM NaCl and 10 mM decamethonium bromide. The enzyme was dialyzed against buffer A prior to use. The protein content was measured by a modified Ellman assay assuming 410 units/nmol of catalytic subunit (6).

PIPLC digestion of AChE

Purified E^{bo} AChE (3-12 nmol) was digested with PIPLC (5 ug/ml final concentration) from S. aureus (kindly provided by Martin Low, Columbia University, New York, NY) in 0.7-1.4 ml of buffer A containing 0.1% sodium deoxycholate for 90 min. at 37 °C. Diradylglycerols were extracted with 3-1 ml portions of hexane.

Analysis of diradylglycerol species

The crude diradylglycerols (2-5 ug) obtained by evaporation of the hexane extracts after PIPLC digestion were converted to trimethylsilyl ethers by reaction with pyridine/hexamethyldisilazane/trimethylchlorosilane (15:5:2) (7). The reaction mixture was evaporated to dryness and redissolved in hexane (50 ul). Aliquots of this solution (1-2 ul) were examined by GLC on a non-polar capillary column as described below. The remainder of the sample was subjected to normal phase HPLC on a silica column (Supelco, Inc., Bellefonte, PA) with 0.3% 2-propanol in hexane (1 ml/min) (8). Fractions corresponding to reference alkenylacyl- (retention time 3.8-4.8 min), alkylacyl- (4.8-6.3 min) and diacyl- (6.3-8.5 min) glycerol trimethylsilyl ethers were collected and evaporated to dryness. The residues were taken up in hexane and a portion was examined by polar and non-polar capillary column GLC with flame ionization detection. The non-polar column used was fused silica with bonded SE-54 phase (8 m x 0.3 mm id) and hydrogen carrier gas at 6 psi. The oven temperature was programmed from 40 to 150°C at 30°C/min, then to 230°C at 20°/min, to 280°C at 10°C/min and to 340°C at 5°C/min (9). The polar capillary column was fused silica with RT_x 2330 phase (15 m x 0.32 mm id, Restek Corp., Port Matilda, PA) operated isothermally at 250 °C with hydrogen carrier gas at 3 psi (7).

Analysis of alkylglycerols and fatty acids

Alkylglycerol trimethylsilyl ethers isolated by HPLC also were subjected to basic methanolysis with sodium methoxide (1M in methanol/toluene (3:2)) for 15 min at 25 °C. The reaction products were diluted with water, extracted into chloroform, taken to dryness and acetylated with acetic anhydride/pyridine. After evaporation of the reagent the products were subjected to normal phase HPLC on silica with 0.8% 2-propanol in hexane (1ml/min) (8). The fractions that corresponded to standard fatty acid methyl esters (4.3-7.3 min) and alkylglycerol diacetates (7.3-10.6 min) were collected and evaporated to dryness. The residues were taken up in hexane and examined by GLC on both polar and non-polar capillary columns as described (7,8).

Preparation of sn-1-stearyl-2-stearoylglycerol

Stearoyl chloride was prepared by the reaction of stearic acid with oxalyl chloride (10). 1-stearyl-2,3-distearoylglycerol was obtained by the pyridine-catalyzed reaction of batyl alcohol (Grade II, Sigma Chemical Co., St. Louis, MO) with stearoyl chloride and the product purified by adsorption chromatography on silica Sep-Pak cartridges (Waters Associates, Milford, MA) using hexane/chloroform (3:1). The alkyldiacylglycerol was partially reacted with ethylmagnesium bromide in ether to give sn-1-stearyl-2-stearoyl and sn-1-stearyl-3-stearoylglycerols, which were separated by TLC on borate impregnated silica gel with chloroform/methanol (96:4) as the developing solvent (11). The main component of batyl alcohol is 1-octadecylglycerol, but small amounts of 1-hexadecyl and 1-heptadecylglycerol are also present. These small components give rise to their respective C_{34} and C_{35} homologues in the final product.

RESULTS AND DISCUSSION

Preliminary GLC analysis of crude trimethylsilyl ether derivatives of the diradylglycerol species released from E^{bo} AChE by PIPLC revealed one major component corresponding to a C_{36} diradylglycerol. Fractionation by HPLC indicated that alkylacylglycerols comprised about 96% of the sample and diacylglycerols, 4%. A chromatogram of the alkylacylglycerol fraction obtained on a non-polar capillary GLC column is shown in figure 1. The major peak corresponds to a species with a total of 36 carbon atoms in the alkyl and acyl groups. No resolution of saturated from unsaturated species was achieved on this column. Further analysis on a polar capillary column permitted identification of the peaks in the alkylacylglycerol fraction (Figure 2A).

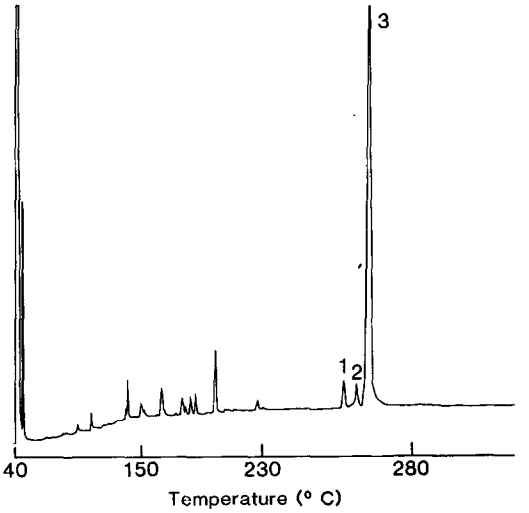

Fig. 1. Non-polar capillary column GLC analysis of the alkylacylglycerol fraction isolated by HPLC. Samples of the diradylglycerols released from E^{bo} AChE by PIPLC digestion were converted to trimethylsilyl ethers and the alkylacylglycerol HPLC fraction was recovered and subjected to GLC analysis on a bonded phase SE-54 column as described in the Methods. Peak 1 corresponds to species containing 34 carbon atoms, peak 2 to 35-carbon species, and peak 3 to 36-carbon species.

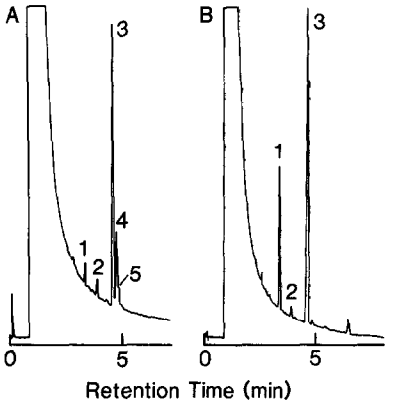

Fig. 2. Analysis of alkylacylglycerol trimethylsilyl ethers on a polar capillary column. An RT 2330 capillary column was operated isothermally at 250°C as described in the Methods. Panel A shows the chromatogram obtained from an aliquot of the same sample used in fig. 1. Peak 1 (retention time 3.45 min) is a 34:0 species, peak 2 (4.02 min) is a 35:0 species, peak 3 (4.74 min) is 36:0 species, and peaks 4 and 5 (4.9 min) are 36:1 species. Panel B is a chromatogram of standards prepared by the acylation of batyl alcohol with stearyl chloride as described in the Methods. The peaks are identified as in panel A. The retention times are peak 1, 3.44 min; peak 2, 3.99 min; peak 3, 4.73 min.

Monounsaturated components corresponding to 18:0' 18:1 and 18:1' 18:0 were resolved from the major, 18:0' 18:0 component. The identities of the major saturated species were confirmed on this polar column by comparison with a 18:0' 18:0 synthetic standard (Figure 2B). Also, the lower molecular weight homologues were found to be saturated species by comparison with retention times of standards. The relative proportions of the various diradylglycerol species determined by GLC are summarized in Table 1.

TABLE 1: Alkylacylglycerol Composition of E^{bo} AChE[a]

Peak No.[b]	Molecular Subclass[c]	Mole %[d]
1	34:0	3.8
2	35:0	3.8
3	36:0	69.1
4 & 5	36:1	23.3

[a]The alkylacylglycerol composition of the diradylglycerols released from E^{bo} AChE by digestion with PIPLC was determined by GLC.

[b]Refers to the peak number in figure 2A.

[c]Identified as carbon number:double bond number.

[d]The molar amounts were obtained by dividing the peak areas by the molecular weight of the corresponding components. The results are the mean of two determinations performed on two PIPLC-digested aliquots from one enzyme preparation.

Results of the analyses of alkylglycerols and fatty acids obtained after base methanolysis of the alkylacylglycerol trimethylsilyl ether HPLC fraction are presented in Table 2. The glyceryl ether content in Table 2 is in excellent agreement with the alkylacylglycerol composition in Table 1. In particular, the observation that ethers with 17:0 alkyl chains account for 4.2% of the total glyceryl ethers corresponds well to the finding that the 35:0 subclass of alkylacylglycerols accounts for 3.8% of the total alkylacylglycerol content. Furthermore, the sum of the 18:0 plus 18:1 glyceryl ethers (93.5%) agrees well with the sum of the 36:0 plus 36:1 alkylacylglycerols (92.4%). The determination of stearate (18:0) as the predominant fatty acid and of oleate (18:1) and palmitate (16:0) as the only other significant fatty acids also agrees qualitatively with the alkylacylglycerol composition in Table 1. However, the fatty acid determinations in Table 2 are quantitatively less consistent with those of the alkylacylglycerols, since the percentage of palmitate (8.4%) is considerably greater that the percentage of the 34:0 alkylacylglycerol subclass (3.8%) and the percentage of oleate (30.5%) is much larger than that of the entire 36:1 alkylacylglycerol subclass. It appears that the values for palmitate and oleate are inflated by contamination from solvents, equipment or glassware.

We interpret the agreement noted above between the distributions of alkylacylglycerols and that of the glyceryl ethers to indicate that the glyceryl ether determinations are much less susceptible to contamination errors than fatty acid determinations. Therefore, the glyceryl ether percentages were used to quantitate the entire set of alkylacylglycerol molecular species released from E^{bo} AChE by PIPLC derived as shown in Table 3. Three species,

TABLE 2: Glyceryl Ether and Fatty Acid Content of E^{bo} AChE Alkylacylglycerols[a]

Fatty Chains[b]	Glyceryl Ethers	Fatty Acids
16:0	2.3	8.4
17:0	4.2	--
18:0	83.3	61.2
18:1	10.2	30.5

[a]The glyceryl ether and fatty composition of HPLC purified alkylacylglycerol trimethylsilyl ethers were determined by polar capillary column GLC after base methanolysis. The results are the mean of two determinations performed on two PIPLC-digested aliquots from one enzyme preparation and are expressed as mole percent.

[b]Identified as carbon number:double bond number of alkyl chains in glyceryl ethers and of acyl chains in fatty acids.

TABLE 3: Alkylacylglycerol Molecular Species of E^{bo} AChE

Molecular Species[a]	Mole %[b]
16:0' 18:0	2.3
18:0' 16:0	1.5
17:0' 18:0	3.8
18:0' 18:0	69.1
18:0' 18:1	13.1
18:1' 18:0	10.2

[a]The alkyl chains, presumably in the sn-1 position, are indicated by a prime and shown on the left.

[b]The relative proportions of the 16:0' 18:0 and 18:0' 16:0 components that overlapped in peak 1 of figure 2A were calculated by taking the difference between the total amount of 34:0 species from Table 1 (3.8%) and the 16:0' ether content (2.3%). Similarly, 18:1' 18:0 and 18:0' 18:1 in peaks 4 and 5 of figure 2A were calculated from the difference between the total (23.3%) and the 18:1' ether content (10.2%).

18:0' 18:0, 18:0' 18:1, and 18:1' 18:0, accounted for 69, 13, and 10% respectively of the total alkylacylglycerol. Lesser amounts of 16:0' 18:0, 18:0' 16:0, and 17:0' 18:0 were also present. The slight splitting of the monoenoic species in peaks 4 and 5 (Figure 2A) does not correspond to the resolution of 18:0' 18:1 and 18:1' 18:0. This separation could be due to the resolution of $\omega 7$ and $\omega 9$ isomers of the 18:1 glyceryl ethers.

These results, which identify the PI covalently bound to E^{bo} AChE as predominantly alkylacylglycerol species, are in contrast with the exclusively diacylglycerol species found in bulk E^{bo} PI examined by similar analytical methods (data not shown) and suggest that the inositol in the glycolipid membrane anchor derives its diradylglycerols from a discrete cellular pool. The function of alkylglycerols in the glycosylinositol phospholipid of E^{bo} AChE is not clear since this enzyme is readily released from erythrocytes by treatment with PIPLC from S. aureus (12) and its diradylglycerols are readily removed in detergent solution by anchor-specific PLC isolated from T. brucei (T.L. Rosenberry, unpublished observations). The fact that a select pool of diradylglycerols is involved in anchor biosynthesis suggests that regulation of the anchor synthesis process may occur at the level of lipid addition.

ACKNOWLEDGEMENTS

We thank Todd Marshall for excellent technical assistance. This investigation was supported by Grants NS16577 and GM07250 from the National

Institutes of Health and by grants from the Muscular Dystrophy Association, the Medical Research Council of Canada and the Heart and Stroke Foundation of Ontario.

REFERENCES

1. Low, M.G. (1987) Biochem. J. 244, 1-13.
2. Ferguson, M.A.J., Haldar, K., and Cross, G.A.M. (1985) J. Biol. Chem. 260, 4963-4968.
3. Mato, J.M., Kelly, K.L., Abler, A., and Jarrett, L. (1987) J. Biol. Chem. 262, 2131-2137.
4. Orlandi, P.A., Jr. and Turco, S.J. (1987) J. Biol. Chem. 262, 10384-10391.
5. Roberts, W.L., Kim, B.H., and Rosenberry, T.L. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 7817-7821.
6. Rosenberry, T.L. and Scoggin, D.M. (1984) J. Biol. Chem. 259, 5643-5652.
7. Myher, J.J. and Kuksis, A. (1984) Can. J. Biochem. Cell Biol. 62, 352-362.
8. Kuksis, A. and Myher, J.J. (1986) In Fat Absorption (A. Kuksis, ed.) Vol. I, pp. 1-41, CRC Press, Boca Raton, Florida.
9. Myher, J.J. and Kuksis, A. (1984) J. Biochem. Biophys. Methods 10, 13-23.
10. Myher, J.J., Kuksis, A., Marai, L., and Yeung, S.K.F. (1978) Anal. Chem. 50, 557-561.
11. Thomas, A.E., III, Scharoun, J.E., and Ralston, H. (1965) J. Am. Oil Chem. Soc. 42, 789-792.
12. Low, M.G. and Finean, J.B. (1977) FEBS Lett. 82, 143-146.