Lipophosphonoglycan of the Plasma Membrane of Acanthamoeba castellani

FATTY ACID COMPOSITION

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SUMMARY

Approximately 14% of the mass of lipophosphonoglycan of the amoeba plasma membrane consists of three classes of long chain fatty acids: (a) normal, saturated and unsaturated and branched, saturated fatty acids C-16 to C-28, 4.5%; (b) normal and branched, saturated 2-hydroxy fatty acids C-22 to C-28, 8.4%; and (c) a group tentatively identified as normal and branched, saturated 2-hydroxy-3-methyl fatty acids, 0.9%. The branched fatty acids in the first two groups are tentatively identified as the anteiso series. Radioactive palmitic acid is incorporated by growing cells into the first two groups of fatty acids.

In the accompanying article (1) we described the isolation from whole amoebae of lipophosphonoglycan, demonstrated it to be identical with a major component of the amoeba plasma membrane, and characterized its water-soluble products of acid hydrolysis. One of the striking properties of the compound is that it is highly aggregated in aqueous buffers, partially dissociated in buffers saturated with 1-butanol, and extensively dissociated in dodecyl sulfate to give two rapidly migrating electrophoretic bands. These acrylamide electrophoretic bands were also shown to stain positively with oil red O. All of these properties suggested that the molecule might contain lipid constituents. In this paper we demonstrate that the isolated lipophosphonoglycan does indeed contain a relatively high percentage of long chain fatty acids whose composition differs significantly from the fatty acid composition of the amoeba's phospholipids and glycerides. The fatty acid composition of lipid-free plasma membranes is shown to be identical with that of lipophosphonoglycan isolated from whole cells providing further evidence that lipophosphonoglycan is a major component of the amoeba plasma membrane.

EXPERIMENTAL PROCEDURE

Lipophosphonoglycan and lipid-free plasma membranes were prepared as described in the accompanying article (1).

Fatty acids were extracted from acid hydrolysates of lipophosphonoglycan either with 2 equal volumes of heptane followed by 2 equal volumes of ether or by addition of 19 volumes of chloroform-methanol, 2:1, to the acid hydrolysate followed by 0.2 volume of water to separate the organic and aqueous phases. In the latter case the aqueous and organic phases were washed with clean opposite phase and the organic phases were pooled. The solvents were evaporated under a stream of nitrogen and the fatty acids were esterified by treatment with either diazomethane in diethyl ether containing 10% methanol at room temperature or with 10% BF3 in methanol at 100°. Methyl esters of fatty acids were qualitatively and quantitatively analyzed by gas chromatography on 3% OV-17 and 17% ethylene glycol succinate (Supelco, Bellefonte, Pa.). Fatty acid methyl esters were also separated into classes by thin layer chromatography on 5-mm-thick plates of Silica Gel H containing 1.95% potassium oxalate developed with a solvent of petroleum ether-diethyl ether-acetic acid, 90:10:1. Bands were detected by exposure to I2 vapor. After the I2 evaporated the fatty acid methyl esters were eluted from the silica gel by sequential extractions with 5 ml of chloroform-methanol-water (25:15:2.5) twice, 4 ml of 95% methanol, and 4 ml of absolute methanol. The pooled extracts were evaporated under a stream of nitrogen and dissolved in heptane or chloroform.

Mass spectral analysis was accomplished on an LKB 9000 mass spectrometer equipped with a gas chromatographic inlet at a nominal ionization voltage of 70 e.v. Proton magnetic resonance spectra were obtained with fatty acid methyl esters and internal standard dissolved in CDCl3 after 5000 scans at 100 MHz in a Varian XL-100.15 spectrometer with Fourier transform modification by Digilabs, Inc., Cambridge, Mass.

Fatty acid standards were obtained from Applied Science Laboratories, State College, Pa. and Supelco, Bellefonte, Pa.

RESULTS

Fatty Acid Composition of Lipophosphonoglycan—The presence of lipid constituents in lipophosphonoglycan was suggested by the dissociating effects of 1-butanol and the positive staining of electrophoretic gels with oil red O (1). Gas chromatography of presumptive fatty acid methyl esters derived from acid hydrolysates of lipophosphonoglycan resolved about 22 peaks (Fig. 1). Identification of these peaks was facilitated by prior thin layer chromatography which separated the material into four I2-positive bands in addition to that which remained at the origin (Fig. 1): Band I, Rf = 0.15; Band II, Rf = 0.35; Band III, Rf = 0.50; Band IV, Rf = 0.57. Bands I, II, and III + IV were eluted separately and analyzed by gas chromatography. Band III + IV was resolved into about 13 gas chromatographic peaks (Fig. 1). Eight of these were readily identified by their retention times on ethylene glycol succinate and OV-17 as methyl esters of the saturated and unsaturated normal fatty acids 16:0, 18:0, 18:1, and 18:2.
NEUTRAL METHYL ESTERS

Fig. 1. Gas chromatograms of the fatty acid methyl esters isolated from lipophosphoglycan and of Bands I and III + IV after separation by thin layer chromatography. Gas chromatography was on 3% OV-17 at 225°C. A schematic drawing of the thin layer chromatograph developed with I2 from which Bands I and III + IV were taken is shown.

Fig. 2. A plot of the gas chromatographic retention times of the fatty acid methyl esters isolated from hydrolysates of lipophosphoglycan versus the molecular weights of the compounds as determined by mass spectroscopy. In each case the fatty acids of Bands I, II, and III + IV segregate into two homologous series. ○, standard fatty acids; ●, lipophosphoglycan fatty acids.

Fig. 3. Mass spectra of the fatty acid methyl esters identified as Band III + IV, Peak 10. Except for the differences in molecular weight of the parent molecular ion the spectra of Band III + IV, Peaks 4, 6, 8, and 15 were identical with the spectrum of Band III + IV, Peak 10.

22:0, 25:0, 26:0, 27:0, 28:0, 18:1, and 20:1. The two unsaturated fatty acid methyl esters were further identified by their conversion to 18:0 and 20:0, respectively, when the mixture of fatty acid methyl esters was hydrogenated in the presence of PtO2 catalyst. Each of these identifications was confirmed by low resolution mass spectroscopy.

The remaining five fatty acid methyl esters of Band III + IV were tentatively characterized as follows. A plot of the log of their retention times versus their molecular weights as determined by mass spectroscopy produced a straight line that nearly parallels the line obtained by a similar plot for the saturated normal fatty acid methyl esters (Fig. 2) but that lies slightly below it. This suggested that the unknown compounds comprise a homologous series of saturated branched chain fatty acid methyl esters. The mass spectra of the five fatty acid methyl esters were indistinguishable from each other except for an increase of 14 in the mass of the parent molecular ion between adjacent pairs in the series. These mass spectra (Fig. 3) differed from the mass spectra of saturated normal fatty acid methyl esters only in a consistently higher ratio of fragment m/e 57 relative to m/e 43 (about 0.96 versus 0.71). Since standard methyl iso-eicosanoate had a ratio of 0.57 and standard methyl anteiso-heneicosanoate had a ratio of 0.98 for m/e 57:m/e 43, it is possible that the five unknown compounds are homologous saturated branched fatty acids in the anteiso series.

The fatty acid methyl esters in Band I were resolved into eight peaks by gas chromatography (Fig. 1). The mass spectra of the eight compounds were essentially identical with each other and to the mass spectra of authentic saturated 2-hydroxy fatty acid methyl esters differing only in the molecular weight of the parent ions. Plots of the logs of the retention times versus molecular weight showed that four of the unknown compounds fell on the straight line connecting the positions of standard C-22 and C-24, 2-hydroxy fatty acid methyl esters (Fig. 2) and these compounds are, therefore, identified as normal, 2-hydroxy C-25, C-26, C-27, and C-28 fatty acids. These identifications were confirmed by the retention times and mass spectra of the
BAND I. Peak 7  
BAND II. Peak 21

FIG. 4. Mass spectra of the fatty acid methyl ester identified as Band I, Peak 7 and of the trimethylsilyl derivative of Band I, Peak 12. The mass spectra of Band I, Peaks 9, 12, and 14, and of the trimethylsilyl derivatives of Band I, Peaks 7, 9, and 14 were essentially identical with those illustrated except for the molecular weights of the parent molecular ions.

trimethylsilyl derivatives of the four unknown compounds compared to two standards.

The other four peaks in the gas chromatogram of Band I were also identified as 2-hydroxy fatty acid methyl esters by their mass spectra and by the mass spectra of their trimethylsilyl derivatives (Fig. 4). These four compounds form a homologous series of C-22 to C-25 whose retention times are slightly less than those of the corresponding normal, 2-hydroxy fatty acid methyl esters (Fig. 2). These compounds are probably branched 2-hydroxy fatty acids possibly, by analogy to the branched saturated fatty acids in the anteiso series.

Band II, which comprises a minor percentage of the total fatty acids of lipophosphoglycan, was resolved into nine gas chromatographic peaks (not illustrated). By their retention times and mass spectra, the nine peaks fall into one group of five compounds and one group of four compounds. The two groups have retention times slightly less than the two groups of 2-hydroxy fatty acid methyl esters of corresponding masses in Band I (Fig. 2). The fatty acids comprising Band II have not been identified but some preliminary structural information has been obtained. The proton magnetic resonance spectrum of the mixture of compounds in Band II shows the presence of methoxy protons at 3.36 ppm and a doublet at 4.24 ppm suggestive of a single proton on C-2 coupled to a single proton on C-3 (in contrast to the spectrum of the 2-hydroxy fatty acids which show a triplet at 4.22 ppm for the single proton on C-2 coupled to the 2 protons on C-3). These data are compatible with a structural assignment of 2-methoxy-3-methyl fatty acid methyl esters. The mass spectra of the nine compounds in Band II are nearly identical differing primarily in the mass of the parent ions and would be compatible with the above structure (Fig. 5). Methylation of the 2-hydroxyl group probably occurred during preparation of the methyl esters. Substitution of diazomethane for diazomethane produced a homologous series of compounds whose masses corresponded to 2-ethoxy-3-methyl fatty acid ethyl esters. We do not know what structural difference causes the nine fatty acid methyl esters of Band II to fall into the two gas chromatographic groups illustrated in Fig. 2.

FIG. 5. Mass spectrum of fatty acid methyl ester Band II, Peak 21. Similar spectra were obtained for all of the compounds in Band II except for the molecular weights of the parent ions.

TABLE

Fatty acid composition of lipophosphoglycan

| Fatty acid | Thin layer band | Gas chromatographic peak | Concentration |
|------------|----------------|--------------------------|---------------|
|            |                |                          | umoles/mg     |
| Saturated  | II + IV        |                          | mg/100 mg     |
| 16:0       |                | 1                        | 0.013         |
| 22:0       |                | 5                        | 0.001         |
| 25:0       |                | 11                       | 0.002         |
| 26:0       |                | 13                       | 0.014         |
| 27:0       |                | 16                       | 0.001         |
| 28:0       |                | 18                       | 0.011         |
| Unsaturated| II + IV        | 2                        | 0.061         |
| 18:1       |                | 3                        | 0.011         |
| Branched saturated | II | 15                       | 0.009         |
| 22:0       |                | 4                        | 0.004         |
| 23:0       |                | 6                        | 0.009         |
| 24:0       |                | 8                        | 0.008         |
| 25:0       |                | 10                       | 0.004         |
| 27:0       |                | 15                       | 0.001         |
| 25:0       |                | 15                       | 0.008         |
| 26:0       |                | 17                       | 0.054         |
| 27:0       |                | 20                       | 0.037         |
| 28:0       |                | 21                       | 0.031         |
| 2-Hydroxy  | I              |                          | 0.008         |
| 22:0       |                | 7                        | 0.02          |
| 23:0       |                | 9                        | 0.031         |
| 24:0       |                | 12                       | 0.02          |
| 25:0       |                | 14                       | 0.007         |
| 2-Hydroxy-3-methyl | II | 7                        | 0.005         |
| 22:0       |                | 14                       | 0.001         |
| 23:0       |                | 17                       | 0.007         |
| 24:0       |                | 19                       | 0.007         |
| 25:0       |                | 21                       | 0.002         |
| Branched 2-hydroxy-3-methyl | II | 7                        | 0.0005        |
| 22:0       |                | 9                        | 0.001         |
| 23:0       |                | 12                       | 0.002         |
| 25:0       |                | 14                       | 0.001         |

* Fatty acids were identified by the gas chromatographic retention times and mass spectra of their methyl esters. The branched saturated fatty acids and branched 2-hydroxy fatty acids are tentatively assigned to the anteiso series. Less definitive information is available on the 2-hydroxy-3-methyl fatty acids (see text).
The fatty acid composition of lipophosphonoglycan is summarized in Table I.

Identification of Fatty Acids of Lipophosphonoglycan in Lipid-Free Plasma Membranes—Plasma membranes were isolated from amoebae grown in the presence of [1-14C]palmitic acid and the lipids were extracted with chloroform-methanol. About 95% of the added radioactivity was incorporated into the amoebae and about 3% was recovered in the isolated plasma membranes of which about 14% remained in the lipid-free residue (Table II). Aliquots of the lipid-free plasma membranes were subjected to dodecyl sulfate polyacrylamide gel electrophoresis on replicate gels which were stained for either protein (Coomassie blue) or carbohydrate (periodic acid-Schiff reagent) and analyzed for radioactivity. The distribution of radioactivity was coincident with the two periodic acid-Schiff-positive bands (Fig. 6) and 2-hydroxy fatty acids are typically present in glycolipids and glycans of the amoeba which are saturated and unsaturated fatty acid methyl esters.

Band I, although detectable by I2 staining, contained no detectable radioactivity. Another aliquot of the fatty acid methyl esters was analyzed by gas chromatography and shown to be essentially identical in composition with the fatty acids of isolated lipophosphonoglycan.

Quantification of the fatty acids by gas chromatography gave a value of 28 pg per mg of lipid-free residue of plasma membrane.

Fractionation of radioactivity in plasma membranes isolated from acanthamoebae grown on [14C]palmitic acid

Plasma membranes were isolated from cells grown in the presence of [14C]palmitic acid (1.9 x 10^6 cpm). The cells incorporated 1.8 x 10^6 cpm of which about 3% was recovered in the isolated plasma membrane.

| Fraction                  | Radioactivity | Percentage distribution |
|--------------------------|---------------|-------------------------|
| 1. Plasma membranes      | 5.45 x 10^6   | 100                     |
| 2. (a) Membrane lipids   | 4.2 x 10^6    | 77                      |
| (b) Lipid-free residue   | 7 x 10^4      | 13                      |
| 3. Fatty acid methyl esters from lipid-free residue | 5.8 x 10^4 | 11 |
| 4. Thin layer chromatography of fatty acid methyl esters | | |
| Origin                    | 1 x 10^4      | 2                       |
| Band I                    | 3.7 x 10^4    | 7                       |
| Band II                   | 6 x 10^4      | 0.1                     |
| Band III + IV             | 1.2 x 10^4    | 2.2                     |

On the assumption that the specific radioactivity of the fatty acids in the lipid-free residue was the same as the specific radioactivity of the phospholipid fatty acids (counts per min per 2 x 10^6 moles of phospholipid-P) the lipid-free residue was calculated to contain 31 µg of fatty acid per mg. The close agreement of these values confirms that, as revealed by the distribution of radioactivity on the thin layer chromatograms, the major fatty acids of the lipophosphonoglycan are synthesized by the amoeba.

**DISCUSSION**

It is certain that the isolated fatty acids are an integral part of the lipophosphonoglycan for the following reasons. They can be extracted from the purified compound only after acid or base hydrolysis: the fatty acids of the lipophosphonoglycan are entirely different from the fatty acids of the phospholipids and glycerides of the amoeba which are saturated and unsaturated C14 to C20 normal fatty acids (2, 3); the lipophosphonoglycan fatty acids remain in the residual plasma membrane after exhaustive extraction with lipid solvents, and the fatty acids co-migrate with the other components of lipophosphonoglycan in dodecyl sulfate-polyacrylamide gel electrophoresis. Indeed, it is the presence of the fatty acids in the molecule that rationalizes the disaggregating effects of 1-butanol and dodecyl sulfate (1).

The fatty acids of lipophosphonoglycan are interesting in their own right. Very long chain odd and even number fatty acids and 2-hydroxy fatty acids are typically present in glycolipids and sphingolipids. These are frequently components of cell surfaces and are particularly rich in nerve myelin (6). Hydroxy fatty acids have not previously been detected in protozoa (7). Branched chain fatty acids are rare in plants and higher animals (8); they are minor components of eukaryotic microorganisms but can be the major fatty acids of gram-positive bacteria (7), although usually of shorter chain length than the ones described in this paper. Branched chain 2-hydroxy fatty acids are even more unusual although 3-hydroxy fatty acids do occur in bacterial lipoplysaccharides to which the amoeba lipophosphonoglycan may bear some resemblance. Both contain fatty acids, including hydroxy fatty acids, and neutral and amino sugars; lipoplysaccharide contains ethanolaminephosphate, the ester analogue of 2-aminoethylphosphonate. Possibly the bacterial...
lipopolysaccharide, which occurs in the outer bacterial membrane, and the amoeba lipophosphonoglycan serve functions similar to those of the glycoproteins and glycolipids of the cell surfaces of metazoans.

That the lipophosphonoglycan is a true component of the amoeba plasma membrane seems well established. The same two periodic acid-Schiff-positive bands are seen upon electrophoresis of whole cells, isolated plasma membranes, lipid-free plasma membranes, and lipophosphonoglycan purified from whole cells (1, 4). The ratios of acid-stable phosphorus to total phosphorus and of total phosphorus to carbohydrate (1), and the compositions of the neutral sugars (1) and of the fatty acids are the same for the lipid-free plasma membranes and the purified lipophosphonoglycan. From the phosphorus content of lipophosphonoglycan (1) and the lipid, protein, and non-lipid phosphorus content of the membranes (4), it is readily calculated that actin-free plasma membranes (4, 5) consist of approximately 25% phospholipid, 13% sterol, 33% protein, and 29% lipophosphonoglycan. This composition can be confirmed by the distribution of radioactivity between the lipid and non-lipid fractions of plasma membranes isolated from amoebae grown in the presence of [1-14C]palmitic acid. Fatty acids comprise 12% of the mass of lipophosphonoglycan and about 70% of the mass of phospholipids. Assuming equal specific radioactivity of all of the fatty acids, from the membrane composition given above the lipophosphonoglycan fatty acids would contain about 16% of the total radioactivity. This compares favorably with the observed value of 14% of the recovered radioactivity (Table II).

It seems reasonable to assume that, whatever its state of aggregation, the lipophosphonoglycan is anchored in the plasma membrane by association of its fatty acid moieties with the phospholipid-sterol matrix of the membrane. Preliminary differential scanning calorimetric measurements have demonstrated interaction between lipophosphonoglycan and dipalmitoylphosphatidylcholine which supports this idea. Finally, electron microscopic cytochemical studies have provided clear evidence for the presence of acidic carbohydrates that react with concanavalin A on both sides of isolated plasma membrane and the plasma membranes of intact cells (9). These data suggest that at least some portion of lipophosphonoglycan is exposed at both surfaces of the plasma membrane.

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