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Recognition of Different Pools of Phosphatidylglycerol in Intact Cells and Isolated Membranes of *Acholeplasma laidlawii* by Phospholipase A_2†

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ABSTRACT: Phospholipase A_2 (EC 3.1.1.4) from pig pancreas hydrolyzes phosphatidylglycerol in intact cells and isolated membranes of *Acholeplasma laidlawii.* Complete degradation of phosphatidylglycerol in intact cells at 37 °C does not result in lysis as shown by the retention of intracellular K^+ ions and the cytoplasmic glucose-6-phosphatase, as well as the inability to detect activity of membrane-bound intracellular NADH-oxidase. *A. laidlawii* was grown on linoleic acid. Phospholipase A_2 treatment of these cells at 5 °C, at which temperature the lipids are still in the liquid-crystalline state, results in a rapid breakdown of 50% of the phosphatidylglycerol. The residual phosphatidylglycerol can be hydrolyzed only at elevated temperatures and at much smaller rates, depending strongly on the incubation temperature. When membranes isolated from these cells are incubated at 5 °C, 70% of the phosphatidylglycerol is hydrolyzed immediately. The hydrolysis of the residual 30% is again strongly temperature dependent. Cells were grown on palmitate, elaidate, or oleate to investigate possible effects of the lipid phase transition on the accessibility of phosphatidylglycerol for phospholipase A_2. Under conditions in which all the lipid is in the solid state, no hydrolysis occurs. When solid and liquid-crystalline lipid phases coexist, a limited hydrolysis of phosphatidylglycerol can be observed. The results demonstrate the disposition of phosphatidylglycerol in three different pools in the membrane of *A. laidlawii.* Phospholipase A_2 has been used to discriminate between these pools and to estimate the amount of phosphatidylglycerol which is present in the liquid-crystalline phase. The present data, however, do not allow a definite localization of the phosphatidylglycerol pools.

Phospholipases have been used to determine the distribution of phospholipids between both layers of three types of natural membranes. Zwaal and co-workers (1973) demonstrated an asymmetric distribution of human erythrocyte phospholipids using a number of purified phospholipases of different origin. Using a similar approach, an asymmetric phospholipid distribution was found in rat liver microsome preparations by Depierre and Dallner (1975), in influenza virus membrane (Tsai and Lenard, 1975) and in other species of erythrocytes, although caution should be exercised in the application of phospholipases to elucidate membrane structure, as was shown by Martin et al. (1975).

Not only the localization, but also some physicochemical parameters of phospholipids in membranes, can be revealed by the action of phospholipases. Studying the surface pressure dependency of a number of enzymes for phosphatidylcholine spread on the air-water interface and extrapolating their results to the erythrocyte membrane, Demel et al. (1975) proposed a packing of phospholipids in this membrane which corresponds to a surface pressure between 31 and 34.8 dynes/cm. In model membranes, such as phosphatidylcholine liposomes, phospholipase A_2 can reveal the phase transition (Op den Kamp et al., 1975) and, finally, the inability of phospholipases to hydrolyze membranous phospholipids can point to a protective disposition of membranous proteins (Rottem et al., 1973; Gazitt et al., 1976).

Bearing these possibilities in mind, we studied the hydrolysis of phosphatidylglycerol in *Acholeplasma laidlawii.* This unicellular organism offers several advantages. It has no cell wall nor intracellular membrane systems (Razin, 1969); lysis is easily accomplished by washing in distilled water and the membranes do not vesiculate (Razin, 1969); only one of the lipids, phosphatidylglycerol, is susceptible to phospholipases (see Results); the lytic hydrolysis product of phospholipase A_2 action (lysophosphatidylglycerol) does not accumulate but is instantaneously hydrolyzed by a membrane bound lysophospholipase (van Golde et al., 1971); finally, the fatty acid composition of the lipids can be manipulated by the addition of fatty acids to the growth medium (McElhaney, 1974).

Materials and Methods

Cultivation of the Organism and Isolation of Membranes. Acholeplasma laidlawii strain B was grown on a lipid-depleted tryptose medium supplemented with palmitic acid, elaidic acid, oleic acid, or linoleic acid at a concentration of 0.12 mM (de Kruyff et al., 1972). Radioactive labeling of the lipids was obtained by the addition of 100 μCi of [^{32}P]orthophosphate per 100 mL of medium or by adding the fatty acids as 1-

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TABLE I: Fatty Acid Composition of Total Lipid Extracts from *Acholeplasma laidlawii*.

Fatty Acid Composition	Fatty Acids Added to the Medium			
	18:2	$18:1_{cis}$	$18:1_{tr}$	16:0
12:0	0.3	1.9	0.9	3.2
14:0	4.0	5.7	2.4	12.4
15:0	1.3	2.0	0.8	1.1
16:0	27.6	27.4	14.5	70.6
18:0	4.9	5.8	2.1	2.7
18:1	7.9	52.5	77.6	7.7
18:2	53.9	4.7	1.7	2.3

^{14}C-labeled derivatives. Cells from 100-mL cultures were harvested in the late logarithmic growth phase by centrifugation at 5000*g*, washed once in a buffer containing 0.05 M tris(hydroxymethyl)aminoethane, 0.15 M NaCl, and 0.025 M $CaCl_2$, adjusted to pH 7.2 with HCl, and suspended in 8 mL of the same buffer. Membranes were isolated according to the procedure of van Golde et al. (1971) and also suspended in the buffer described above at a concentration of 5 mg of protein per mL.

Phospholipase Treatment. Cells or membranes were incubated with purified phospholipase A_2 from pig pancreas, generously provided by Dr. G. H. de Haas. Incubations were carried out with various amounts of enzymes and at several temperatures as indicated in the text.

Lipid Analysis. One-milliter samples were taken from the incubation mixture and mixed with 0.5 mL of 0.2 M EDTA[1] to stop the enzymatic activity. Six milliliters of chloroform–methanol (1:2, v/v) was added and extraction was carried out as described before (Op den Kamp et al., 1972). The lipids were separated on silica gel H plates with chloroform–methanol–water–acetic acid (65:25:4:1, by volume) at 4 °C. Compounds were visualized by I_2 vapor and autoradiography. Quantitative measurements were carried out by scraping off the silica gel and counting the radioactivity in a liquid scintillation counter. Fatty acid analysis was carried out as described before (Haest et al., 1972). For the results of fatty acid analysis, see Table I.

Enzyme Assays. Glucose-6-phosphatase was assayed in a double-beam Varian spectrophotometer at 37 °C by the method of Pollack et al. (1965). Samples (100 μL) of the incubation mixtures were diluted ninefold with the buffer described above, glucose 6-phosphate (50 mM) and $NADP^+$ (26 mM) were added, and the reaction was followed at 340 nm. Intracellular enzyme activity could be measured after lysis of the cells with 5 μL of a 20% Triton solution (de Kruyff et al., 1974). NADH oxidase was measured by a similar technique. The reaction mixture contained 100 μL of cell suspension, 0.9 mL of buffer, and 5 μL of NADH (20 mM). Total activity is found after lysing the cells as described above.

K^+ Leakage. Samples of 100–200 μL are taken from the incubation mixture and injected into 5 mL of a buffer containing 0.1 M $CaCl_2$ and 0.01 M Tris, pH 7.2, at 37 °C. A K^+-sensitive electrode was used to measure the extracellular K^+ and, after lysing the cells with 5 μL of Triton X-100 (20% solution), also the total K^+ content of the cells.

Results

Effects of Phosphatidylglycerol Hydrolysis on the Integrity of A. laidlawii Cells. Phosphatidylglycerol is the only phospholipid in the membrane of *A. laidlawii* which can be hydrolyzed by phospholipase A_2. Figure 1 demonstrates that phospholipase A_2 treatment at 37 °C of membranes, which were isolated from cells grown on [^{14}C]oleic acid, results in the breakdown of the majority of phosphatidylglycerol and a corresponding increase in the free fatty acid content. No lysophosphatidylglycerol is accumulated, which has to be ascribed to the presence of a membrane-bound lysophospholipase (van Golde et al., 1971). A quantitative analysis of the effects of phospholipase A_2 shows that the breakdown of phosphatidylglycerol at 37 °C is nearly complete and that none of the other lipids is hydrolyzed (Table II). Complete hydrolysis of phosphatidylglycerol can be obtained also at 37 °C with intact cells of *A. laidlawii* (Figure 2). Breakdown of phosphatidylglycerol, which accounts for 30% of the total lipids, does not result in lysis of the cells. A slightly increased leakage of K^+ ions can be observed during phospholipase treatment but, even after 30 min of incubation when most of the phosphatidylglycerol is hydrolyzed, a substantial amount of K^+ is present in the cells. Additional evidence for the intactness of the cells is obtained by electron microscopy (results not shown) and

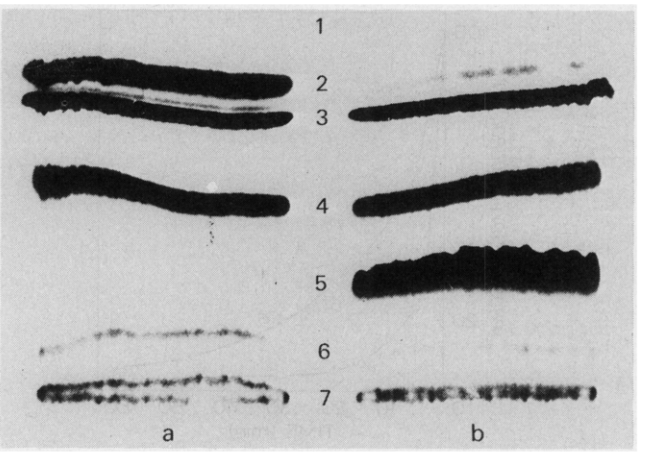

FIGURE 1: Autoradiogram of *A. laidlawii* lipids from membranes before (b) and after (a) treatment with phospholipase A_2. Membranes were isolated from *A. laidlawii* grown on [1-^{14}C]oleic acid and incubated with (a) or without (b) an excess of phospholipase A_2 for 60 min at 37 °C. Compounds are: (1) diglycerides; (2) free fatty acids; (3) monoglucosyldiglyceride; (4) diglucosyldiglyceride; (5) phosphatidylglycerol; (6) diglucosyldiglyceride glycero-3-phosphate; (7) unidentified.

TABLE II: Effect of Pig Pancreatic Phospholipase A_2 on the Lipid Composition of *A. laidlawii Membranes*.[a]

Lipid Compound	Untreated Membranes	Phospholipase A_2 Treated Membranes
1. Diglycerides	1	1
2. Free fatty acids	2	33
3. Monoglucosyldiglyceride	38	38
4. Diglucosyldiglyceride	18	18
5. Phosphatidylglycerol	31	1
6. Diglucosyldiglyceride glycero-3-phosphate	4	3
7. Unidentified	6	6

[a] Data are expressed as percentages of the total lipid content of the membrane. Membranes were isolated from cells grown on [1-^{14}C]-oleate and incubated with excess phospholipase A_2 at 37 °C for 60 min.

[1] Abbreviation used: EDTA, ethylenediaminetetraacetic acid.

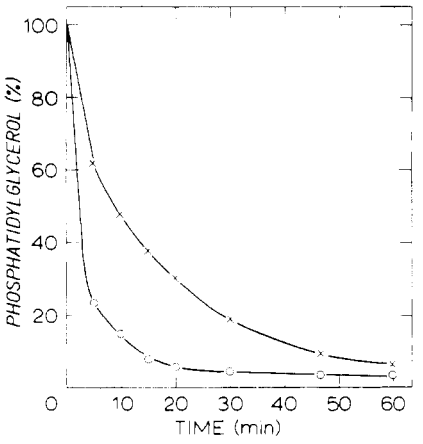

FIGURE 2: Phosphatidylglycerol hydrolysis in cells and isolated membranes of *A. laidlawii*. Intact cells of *A. laidlawii* grown in [1-^{14}C]oleic acid and membranes isolated from these cells were incubated with phospholipase A_2 at 37 °C. Phosphatidylglycerol hydrolysis in intact cells (X) and isolated membranes (O) is expressed as percentage of the initial amount.

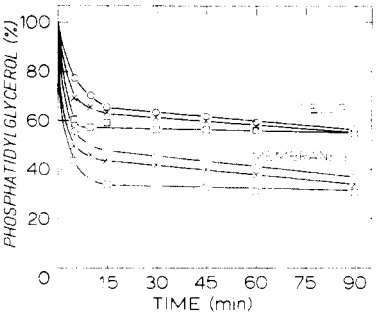

FIGURE 3: Hydrolysis of phosphatidylglycerol in intact cells and isolated membranes of *A. laidlawii* at low temperature. Intact cells and isolated membranes from *A. laidlawii* grown on linoleic acid and [^{32}P]H_3PO_4 were incubated at 5 °C with 1 unit (O), 10 units (X), and 200 units (Δ) of phospholipase A_2. Data are expressed as in Figure 2.

furthermore by assaying both a soluble cytoplasmic enzyme, glucose-6-phosphatase (Pollack et al., 1965), and the NADH oxidase which is bound to the inner membrane surface and therefore not accessible for endogenous substrate (Ne'eman and Razin, 1975). Glucose-6-phosphatase does not leak from the cells during phospholipase A_2 treatment. At zero time and after 60 min the amount of free enzyme that can be measured is about 10% of the total enzyme activity, indicating that 10% of the cells are lysed during preparation of the cell suspension. Also, 10% from the membrane-bound NADH oxidase can be measured in suspensions of intact cells before and after phospholipase A_2 treatment. Both enzymatic assays show that large molecular weight molecules are retained within the cell but also that low molecular weight substrates such as NADH and glucose 6-phosphate do not permeate into the cell. The membrane still functions as a permeability barrier despite the loss of phosphatidylglycerol.

The complete hydrolysis of phosphatidylglycerol in intact cells at 37 °C suggests that this lipid is localized in the outer leaflet of the bilayer membrane. Additional experimental evidence, however, casts some doubt on the validity of this assumption. A comparison was made between the hydrolysis rate of phosphatidylglycerol in intact cells and isolated membranes at 37 °C (Figure 2). The results suggest that hydrolysis is enhanced when the enzyme is in contact with both sides of the membrane, although it cannot be excluded that alterations in the structure of the membrane, which are caused by osmotic shock of the cells and successive washings of the membranes, are responsible for the observed increase in hydrolysis rate. Nevertheless, this observation might imply that part of the phosphatidylglycerol is localized in the cytoplasmic layer. Furthermore, hydrolysis at lower temperatures shows, as is described below, that the phosphatidylglycerol is present in different pools in the membrane and it cannot be excluded that these pools are localized in both the outer and the cytoplasmic layer of the membrane.

Effect of Temperature on Phosphatidylglycerol Hydrolysis. As shown in the preceding section all the phosphatidylglycerol in isolated membranes as well as in intact cells is available for hydrolysis with phospholipase A_2 when the incubation is carried out at 37 °C. At low temperature, however, a substantial amount of phosphatidylglycerol appears to be inaccessible for hydrolysis. Cells were grown with linoleic acid in order to prevent a lipid phase transition at low temperature, which affects the phospholipase A_2 activity (see next section). Isolated membranes were incubated at 5 °C with increasing amounts of phospholipase A_2 and it is obvious from Figure 3 that 70% of the phosphatidylglycerol is hydrolyzed immediately, whereas 30% is not available. Addition of another 200 units of phospholipase A_2 after 30 min of incubation does not increase the amount of phosphatidylglycerol hydrolyzed. Furthermore, raising the temperature from 5 to 37 °C after 30 min of incubation immediately results in the hydrolysis of the remaining phosphatidylglycerol. These results demonstrate that, although enough enzyme is present and active, 30% of the phosphatidylglycerol is not accessible. When intact cells grown on linoleic acid are incubated under these conditions, only 50% of the total phosphatidylglycerol can be hydrolyzed (Figure 3).

The phosphatidylglycerol which is not readily hydrolyzed with an excess of phospholipase A_2 at 5 °C (about 50% in cells, 30% in isolated membranes) becomes available for hydrolysis at elevated temperatures. Figure 4 shows that in isolated membranes from cells grown on oleic acid again about 70% of the phosphatidylglycerol is hydrolyzed immediately. The hydrolysis of the residual phosphatidylglycerol is strongly temperature dependent. Whereas at 37 °C hydrolysis is nearly completed within 30 min, the hydrolysis of all the phosphatidylglycerol at 5 °C takes at least 4 h. Similar observations have been made with whole cells grown on oleate (Figure 5). Again a rapid hydrolysis of about 40% of the total phosphatidylglycerol followed by a much slower, temperature-dependent breakdown of the remaining phosphatidylglycerol is observed. The results demonstrate that three pools of phosphatidylglycerol are present in membranes of *A. laidlawii*, one of which contains phosphatidylglycerol which is, especially at lower temperatures, very effectively protected against enzymatic hydrolysis. This protection is overcome by a temperature-dependent process which occurs in the membrane and is independent of the available phospholipase A_2. The second minor pool of phosphatidylglycerol (20%) can be readily hydrolyzed at low temperatures, but only in isolated membranes and is not seen by phospholipase A_2 in intact cells. Finally, the phosphatidylglycerol which is hydrolyzed readily in intact cells is most likely located in the outer monolayer of the membrane. Definite conclusions, however, on the localization of the three pools of phosphatidylglycerol cannot be drawn. The data do not exclude the possibility that one or both minor pools are located in the outer monolayer.

Effect of the Lipid Phase Transition on Phosphatidylglycerol Hydrolysis. A. laidlawii was grown on palmitate, ela-

TABLE III: Effect of Temperature on the Amount of Phosphatidylglycerol Which Can Be Readily Hydrolyzed in Cells and Isolated Membranes of *A. laidlawii* Grown on Different Fatty Acids.[a]

Incubation Temp (°C)	Percent Phosphatidylglycerol Hydrolyzed in Cells[b] 18:2	$18:1_{cis}$	$18:1_{tr}$	16:0	Membranes[b] 18:2	$18:1_{cis}$	$18:1_{tr}$	16:0
0	45	30	0	0	65	55	3	5
5	45	35	4	0	65	65	18	5
10	45	38	15	5	65	65	38	10
15	50	50	30	10	70	70	55	15
20	50	50	40	20	70	70	65	25
Transition range (°C)					−30 ↓ −10	−22 ↓ −4	5 ↓ 32	20 ↓ 50
Transition midpoint (°C)					−19	−13	21	38

[a] Cells grown with different fatty acids in the presence of $[^{32}P]H_3PO_4$ and their derived membranes were incubated at the temperature indicated with excess phospholipase A_2 exactly as shown in Figure 4. The size of the readily accessible pool of phosphatidylglycerol is estimated by extrapolation of the time curves of hydrolysis to zero time. The data which show the approximate temperature range of the phase transition are taken from McElhaney (1974) and are in agreement with the results obtained in our department (de Kruijff, personal communication). [b] Fatty acid in medium.

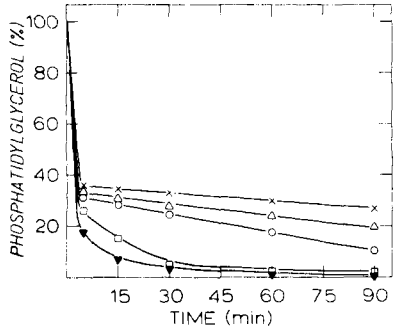

FIGURE 4: Effect of temperature on the hydrolysis of phosphatidylglycerol in membranes of *A. laidlawii*. Membranes were isolated from cells grown with oleic acid and $[^{32}P]H_3PO_4$. Incubations with excess phospholipase A_2 were carried out at 5 (X), 10 (△), 20 (○), 30 (□), and 37 °C (▼). Data are given as in Figure 2.

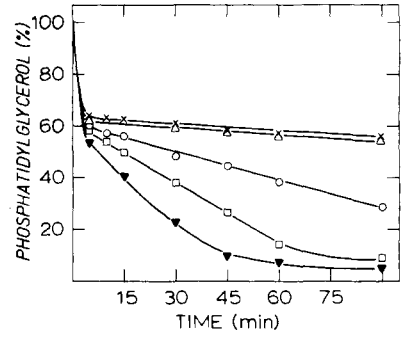

FIGURE 5: Effect of temperature on the hydrolysis of phosphatidylglycerol in intact cells of *A. laidlawii*. Cells grown with oleic acid and $[^{32}P]H_3PO_4$ were incubated with excess phospholipase A_2 as described in the legends to Figure 4.

idate, oleate, or linoleate in the presence of $[^{32}P]H_3PO_4$. Cells and isolated membranes were incubated with excess phospholipase A_2 at 0, 10, 15, and 20 °C. All the results were plotted as shown in Figures 4 and 5 which allows an extrapolation to zero time, indicating the approximate size of the phosphatidylglycerol pool which is immediately available for hydrolysis in cells and membranes. Table III summarizes these data together with the temperatures between which the phase transition occurs. The fatty acid composition of the total lipids of these cells is shown in Table I. A comparison of the data presented in Tables I and III leads to several conclusions. At temperatures well above the phase transition, half of the phosphatidylglycerol is accessible in intact cells. None of this phospholipid is available in cells in which all the lipid is in the frozen state. Within the transition temperature range, increasing amounts of phosphatidylglycerol become available when the temperature is raised. Thus the amount of phosphatidylglycerol hydrolyzed in intact cells at any temperature within the temperature range of the phase transition corresponds most likely to that part of the phosphatidylglycerol which is in a liquid environment in the outer monolayer of the membrane. A comparison of the results on isolated membranes with those on intact cells shows that the phosphatidylglycerol in isolated membranes is hydrolyzed to a somewhat larger extent than in intact cells. That this observation points to a difference in lipid fluidity between both membrane leaflets is merely speculative as long as the distribution of phosphatidylglycerol over the inner and outer layer is obscure.

Discussion

Phosphatidylglycerol is the only glycerophospholipid and therefore the only substrate for phospholipases in the membrane of *Acholeplasma laidlawii*. This observation has prompted us to investigate the localization and possible functions of this phospholipid using a selective hydrolysis with phospholipase A_2, C, and D. A first approach to the problem of localization is described in this paper and it is obvious that a number of still unknown parameters prevent a straightforward interpretation of the results.

The first remarkable observation is that in *A. laidlawii* the lipids of the cytoplasmic membrane are accessible for exogenous lipolytic enzymes. Other microorganisms which have been investigated before are protected against lipolytic enzymes by a cell wall, as in gram positive microorganisms (Op den Kamp et al., 1972), or other membrane constituents, such as the lipopolysaccharide in *Escherichia coli* (Duckworth et al., 1974). Another way of protection is found in the erythrocyte where a tight packing of lipid molecules in the membrane prevents hydrolysis, from the outside at least, by a number of

phospholipases (Demel et al., 1975). *A. laidlawii,* however, is surrounded by a membrane in which one of the lipids is readily hydrolyzed by phospholipases A_2 from pig pancreas, *Naja naja* venom, and bee venom, phospholipase C from *Bacillus cereus,* phospholipase D from cabbage and even pancreatic lipase. (The results obtained with some of the enzymes mentioned above will be described elsewhere.)

The complete hydrolysis of phosphatidylglycerol in intact cells at 37 °C might indicate that this phospholipid is localized entirely in the outer layer of the membrane. Martin et al. (1975), however, demonstrated already that the use of phospholipases to localize phospholipids in a membrane requires a careful interpretation and the present data, although not excluding the possibility that all the phosphatidylglycerol is in the outside layer, allow an interpretation in which 20% (maybe even 50%) of this lipid is localized in the inner layer. This interpretation is supported by the experiments on cells and isolated membranes at low temperatures. The latter interpretation assigning part of the phosphatidylglycerol to the inner membrane leaflet implies the presence of a translocation mechanism (flip-flop) which enables the phosphatidylglycerol to move from the inner layer of the membrane to the outside in order to account for complete hydrolysis. Such a translocation of lipids between two membrane layers has been shown to occur in liposomes (Kornberg and McConnell, 1971; McNamee and McConnell, 1973) and natural membranes (Renooy et al., 1976; Bloj and Zilversmit, 1976). The reported flip-flop times, however, are quite long, varying between several hours and many days. On the other hand, it might be important to realize that these values were obtained studying systems which were close to or in equilibrium with respect to the distribution of lipids over both membrane layers. One can speculate that a quick depletion of a given phospholipid in one layer activates the translocation of this compound from the other layer, especially when a complete polar head group is lost as is the case in our experiments.

An interesting phenomenon which is revealed by the incubation at low temperatures is the protection of part or all of the phosphatidylglycerol against hydrolysis. Such a protection might arise first of all by a close packing of lipid molecules, because the enzymatic activity is effectively prevented at high surface pressures in the lipid interfaces (Demel et al., 1975; Op den Kamp et al., 1975; Verger et al., 1973). It is evident that the results obtained with elaidate- and palmitate-grown cells and their derived membranes can be interpreted this way. In or below the lipid phase transition, part or all of the lipid is in the solid state, being more tightly packed than in the liquid crystalline phase, and therefore partly or completely inaccessible to the enzyme.

In cells grown on linoleic acid, however, the lipids are completely in the liquid-crystalline state at 0 °C. The observed protection of 30% of the phosphatidylglycerol against hydrolysis cannot be explained, therefore, with a close packing due to a lipid-phase transition into the gel phase. This was confirmed by fatty acid analysis of this protected pool. The residual phosphatidylglycerol after hydrolysis at 0 °C contained the same high content of linoleic acid (50% of the total fatty acids) as was found for the total phosphatidylglycerol before hydrolysis. The possibility that Ca^{2+} ions are responsible for the formation of this inaccessible pool via a lateral phase separation of the acidic phosphatidylglycerol is very unlikely. Identical results as described here, using 25 mM $CaCl_2$ in the incubation mixture, can be obtained with Ca^{2+} concentrations between 1 and 200 mM. Protection against hydrolysis has to be due, therefore, to the interaction of phosphatidylglycerol with another membrane constituent. The present results do not allow any conclusion with respect to the nature of this protective interaction, but several observations suggest that membrane bound (intrinsic or surface) proteins might play a role. Protein protects phosphatidylglycerol in *Mycoplasma hominis* against phospholipase C degradation (Rottem et al., 1973). Physicochemical studies have shown the presence of domains of lipid surrounding proteins in several types of membranes (Jost et al., 1973; Träuble and Overath, 1973; Warren et al., 1974), and also in *A. laidlawii* proteins may have an effect on the physical state of the membrane lipids (Rottem et al., 1970; Rottem and Samuni, 1973). The lateral mobility of this lipid may be restricted due to a close interaction with the proteins, which might in turn prevent penetration of phospholipase A_2 in these areas. The relative amounts of such "rigid" lipid components were found to increase upon treatment of sarcoplasmic reticulum membranes with phospholipase A_2 (Nakamura and Onishi, 1975). The present data do not allow firm conclusions with respect to the above discussed parameters of phosphatidylglycerol localization, flip-flop, binding to protein or lateral mobility. It is obvious, however, that phospholipases, especially in the membrane which contains only one substrate, can be useful tools in elucidating the role of phospholipids in membrane structure and function.

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Photochemical Functionality of Rhodopsin–Phospholipid Recombinant Membranes†

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ABSTRACT: Purified rhodopsin was incorporated into phospholipid bilayers to give recombinant membranes. The photochemical functionality in these systems was examined by low-temperature spectroscopy and by kinetic spectrophotometry. Changes in the absorption spectra of glycerol–water mixtures of rhodopsin–egg phosphatidylcholine and rhodopsin–asolectin recombinants were monitored after the sample was cooled to −196 °C, presented with light of wavelength greater than 440 nm, and then warmed gradually to room temperature. Absorption characteristics indicative of the spectral intermediates prelumirhodopsin, lumirhodopsin, metarhodopsin I, and metarhodopsin II were observed. The kinetics of the metarhodopsin I to metarhodopsin II transition in these recombinants was studied by flash photolytic observation of the decay of meta I and the formation of meta II. Recombinants prepared from unsaturated phospholipids, e.g., asolectin, egg phosphatidylcholine, egg phosphatidylethanolamine, and dioleoylphosphatidylcholine, showed first-order kinetics for the transition with rates comparable to that of rod outer segment membranes. Recombinants prepared from saturated phosphatidylcholines have a retarded rate of conversion from meta I to meta II and are considered to be nonfunctional. The photochemical functionality of rhodopsin–phospholipid recombinants is dependent upon the presence of phospholipid unsaturation and the fluidity of the phospholipid hydrocarbon chains, and is independent of the polar head group of the phospholipid.

The vertebrate rod outer segment is the site of light reception and energy transduction to electrical activity in the visual system. Rhodopsin is the major protein component of ROS[1] membranes (Daemen, 1973) and its chromophore, 11-*cis*-retinal, serves as the light receptor (Wald, 1968a,b). The subsequent role of rhodopsin in excitation remains to be clearly defined, although it has been proposed that rhodopsin mediates the permeability of disk membranes in ROS to transmitter substances (Yoshikami and Hagins, 1971; Hagins, 1972).

Rhodopsin is an integral protein in the ROS membrane. Data from x-ray diffraction (Blaise, 1972; Blaurock and Wilkins, 1972; Chabre, 1975) and freeze fracture electron microscopy experiments (Chen and Hubbell, 1973) suggest part of the protein molecule is in contact with the hydrophobic interior of the membrane. Thus, the properties of rhodopsin in the membrane should be partly dependent upon the physical and chemical nature of the lipids. Phospholipids comprise the major portion (80–85%) of the lipid content of ROS membranes (Daemen, 1973). The kind and composition of the phospholipids are known, as well as the fatty acid composition of the acyl groups. The chief phospholipids in cattle ROS are PC (41% of lipid phosphorus), PE (39%), and PS (13%) (Anderson and Maude, 1970). Nearly half of the fatty acids in ROS are polyunsaturated, which is high for natural membranes, although many membranes are composed of unsaturated phospholipids. The phospholipids in many native membranes exhibit rapid translational and rotational motion at room and physiological temperatures (Hubbell and McConnell, 1971; Devaux and McConnell, 1972). The dynamic properties of the polyunsaturated ROS membranes are demonstrated by the observed rotational (Brown, 1972; Cone,

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[1] Abbreviations used are: ROS, rod outer segment; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; di-12:0-PC, 1,2-didodecanoylphosphatidylcholine; di-14:0-PC, 1,2-ditetradecanoylphosphatidylcholine; di-(18:1^c)-PC, 1,2-di(*cis*-9-octadecanoyl)-phosphatidylcholine; DTAB, dodecyltrimethylammonium bromide; meta I, metarhodopsin I; meta II, metarhodopsin II; DDAO, dodecyldimethylamine oxide; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TrTAB, tridecyltrimethylammonium bromide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance.