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TRANSBILAYER DISTRIBUTION OF PHOSPHOLIPIDS IN PHOTORECEPTOR MEMBRANE STUDIED WITH VARIOUS PHOSPHOLIPASES *

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Summary

The distribution of the three major phospholipids of bovine rod outer segment disk membranes over the two faces of the membrane has been studied by means of treatment with phospholipase C, phospholipase A_2 and phospholipase D.

Two different preparations of rod outer segment disk membranes have been used, which are called 'stacked disks' and 'disk vesicles' on account of their morphological appearance.

The hydrolysis patterns obtained by phospholipase treatment of these preparations have been compared to those of a retinal lipid suspension or detergent-solubilized disk membranes, which serve as control preparations with a similar phospholipid composition but a random availability of the phospholipids.

Special attention is given to the early phase of enzyme treatment in order to eliminate secondary effects on the molecular organization of the membrane due to appreciable phospholipid hydrolysis. Analysis of the hydrolysis patterns for all three phospholipases in stacked disks, as compared to those in randomized control preparations, suggests a slightly asymmetrical distribution of phosphatidylcholine (40—45% at the outer face) and phosphatidylethanolamine (55—60% at the outer face) and a symmetrical distribution of phosphatidylserine in rod outer segment disk membranes.

Extensive treatment with phospholipases C and A_2 leads ultimately to nearly complete hydrolysis of all phospholipids, but with phospholipase D a final level of 40% phospholipid hydrolysis is observed in stacked disk prepa-

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rations. This suggests that in the latter case the inner face of the membrane is inaccessible to the enzyme.

Further work will be necessary in order to substantiate these conclusions.

Introduction

The question as to whether membrane lipids are distributed symmetrically or asymmetrically over the two faces of biological membranes is currently much studied because of its obvious significance for our understanding of the role of lipids in membrane function. It has recently been claimed that asymmetrical distribution of phospholipids occurs in various biological membranes [1], although in some cases controversy still exists [2—5].

Various independent methods are available to obtain information on the lipid arrangement in biological membranes and artificial bilayered systems. These involve the use of chemical modifying reagents, of lipolytic enzymes like phospholipases, of phospholipid exchange proteins and of physical techniques like NMR spectroscopy [6].

Relatively little is known in this respect about the photoreceptor membranes of rod outer segments, which contain 36% phosphatidylcholine, 45% phosphatidylethanolamine and 16% phosphatidylserine (together, 97% of total phospholipid; Ref. 7). So far, chemical labeling experiments, mainly with trinitrobenzenesulfonate, have suggested that at least phosphatidylethanolamine is preferentially located at the cytoplasmic (outer) face of the rod outer segment membranes [8—10].

It is, however, generally felt that more than one approach should be used before reliable conclusions can be drawn. Hence, we have carried out a study of the phospholipid distribution of rod outer segment membranes by means of three different phospholipases. The results suggest a nearly symmetrical distribution of the phospholipids.

Materials and Methods

Preparations. Three different types of bovine rod outer segment preparations and a retinal lipid extract have been used, always starting from fresh retinas.

'Stacked disks' are prepared in a sucrose-Ficoll 400 medium according to the method of Schnetkamp et al. [11]. Rod-like structures are clearly visible under the phase-contrast microscope. Electron-microscopic observation reveals stacked disks, partially surrounded by plasma membrane.

'Disk vesicles' are isolated according to the method of de Grip et al. [12], omitting the enrichment procedure, and finally washed three times with distilled water. Electron-microscopic observation shows that after washing with distilled water the flat disk structure has been converted to globular unilamellar vesicles. The term disk membranes is used to imply both stacked disk and disk vesicle preparations.

'Solubilized disks' are made by dissolving disk vesicles in 0.16 M Tris-maleate buffer (pH 6.0), containing 20 mM β-1-nonylglucose. The non-ionic detergent β-1-nonylglucose is prepared by Dr. W.J. de Grip in our laboratory [13].

'Retinal lipid suspension' is prepared by extracting the lipids from whole bovine retina by a modification of the procedure of Bligh and Dyer [14]. After evaporation of the organic solvent, the lipids are suspended in 0.16 M Tris-HCl buffer (pH 7.4) and subjected to sonic vibration at 0°C (Branson Sonifier B-12; three 1 min periods at half-maximal output with a 1 min delay between each sonic burst), and finally centrifuged (10 min at 10 000 × g) in order to remove metal (titanium) contamination and larger lipid particles.

The phospholipid composition of the retina very much resembles that of rod outer segments. Hence, a retinal lipid suspension can be used as a reference which reflects the substrate specificities of phospholipases A_2 and C for different phospholipids, present in a given ratio and randomly available. Phospholipase D requires 40 mM Ca^{2+} for good activity, a concentration which causes aggregation and flocculation of the retinal lipid suspension. Hence, solubilized disks are used as a reference for the substrate specificity of phospholipase D.

Phospholipase preparations. Phospholipase C (EC 3.1.4.3) is isolated from cultures of *Bacillus cereus* according to the method of Otnaess et al. [15], and is stored in 50% glycerol containing 1 mM Zn^{2+}.

Lyophilized phospholipase A_2 (EC 3.1.1.4) from porcine pancreas is a gift of Professor G.H. de Haas (Department of Biochemistry, University of Utrecht, The Netherlands).

Phospholipase D (EC 3.1.4.4) is isolated and partially purified from Savoy cabbage according to the method of Davidson and Long [16], or obtained commercially (Boehringer, Mannheim, F.R.G.). Both preparations give the same results.

Phospholipase treatment. The stacked disks are resuspended in a medium containing 600 mM sucrose, 5% (w/w) Ficoll 400 and 20 mM Tris-HCl buffer, pH 7.4 (for treatment with phospholipases C and A_2), or 20 mM Tris-maleate buffer, pH 6.0 (for treatment with phospholipase D). Disk vesicles are resuspended in 0.16 M Tris-HCl buffer, pH 7.4 (for treatment with phospholipases C and A_2), or 0.16 M Tris-maleate buffer, pH 6.0 (for treatment with phospholipase D).

Incubations with phospholipase C are carried out at 20°C, with phospholipase A_2 at 20°C in the presence of 10 mM $CaCl_2$, with phospholipase D at 30°C in the presence of 40 mM $CaCl_2$, all in darkness. The reactions are started by adding an appropriate amount of enzyme solubilized in distilled water (phospholipases A_2 and D) or in 50% glycerol containing 1 mM $ZnCl_2$ (phospholipase C). The reactions are stopped by adding an excess of ice-cold buffer containing 10 mM EDTA.

Retinal lipid suspension and detergent-solubilized disk membranes are incubated as described for disk vesicles, and the reaction is stopped by adding the $CHCl_3/CH_3OH$ extraction mixture to the incubation medium.

The entire procedure, starting with the isolation of rod outer segment membranes, takes less than 6 h. Freshly isolated rod outer segment membranes have been used throughout. Control incubations, omitting phospholipase, are always carried out and demonstrate the absence of endogenous phospholipase activity under the conditions used.

Phospholipid analysis. The suspensions, diluted with EDTA buffer, are

centrifuged (0°C, 30 min at 100 000 × *g*). The supernatant contains neither phospholipids nor rhodopsin. The phospholipids in the pellet are extracted [17], washed with acidified 0.1 M KCl [18] and separated by two-dimensional thin-layer chromatography [19]. The phospholipid spots, visualized by iodine vapor, are scraped off and their phosphate content is determined [19]. The error in the phosphate determination varies from 5 to 10% (relative standard error; $n \geqslant 3$) depending on the amount of lipid analyzed (range 150—30 nmol).

When the incubation is stopped with $CHCl_3/CH_3OH$, the organic extract is washed and analyzed as described above.

The aqueous upper layers do not contain phospholipids, except for minor amounts of lysophosphatidylserine. Phosphate analysis of the aqueous layers is performed, when water-soluble products of the enzymatic hydrolysis (phosphate esters or P_i) are expected. In this way the balance of phospholipid breakdown can be checked.

Rhodopsin determination. Rhodopsin determinations are performed spectrophotometrically by measuring the difference spectra before and after illumination in the presence of 50 mM NH_2OH and 1% Triton X-100 [20].

Electron microscopy. Stacked disk and disk vesicle suspensions are fixed in 2% glutaraldehyde in 0.16 M Tris-maleate buffer (pH 6.0), containing 10 mM EDTA, and post-fixed in 1% OsO_4. After dehydration the samples are embedded in Vestopal W. Thin sections are stained with uranyl acetate and lead citrate and examined in a Philips 300 or 301 electron microscope.

Results

Rhodopsin content

Determination of rhodopsin before and after incubation with any of the three phospholipases never shows a significant loss of rhodopsin absorbance, regardless of the degree of phospholipid hydrolysis reached.

Phospholipase C treatment

During phospholipase C treatment, quantitative agreement exists between the amount of phosphate ester, appearing in the aqueous supernatant and measured as P_i after acid destruction, and the disappearance of phospholipids from the disk membranes and the retinal lipid suspension.

TABLE I

LEVELS OF PHOSPHOLIPID HYDROLYSIS UPON PROLONGED TREATMENT WITH PHOSPHOLIPASE C, A_2 AND D

Results are expressed as percent of total phospholipids hydrolyzed in stacked disks, disk vesicles and reference preparations: retinal lipid suspensions (for phospholipases C and A_2) and detergent-solubilized disk membranes (for phospholipase D). Incubation conditions as in Figs. 1—3, except that a double concentration of phospholipase D as in Fig. 3 has been used; incubation time, 180 min. The results are an average of at least three experiments with a relative standard error of maximally 6%.

Phospholipase	Stacked disks	Disk vesicles	Reference
C	98	97	99
A_2	100	100	100
D	40	53	88

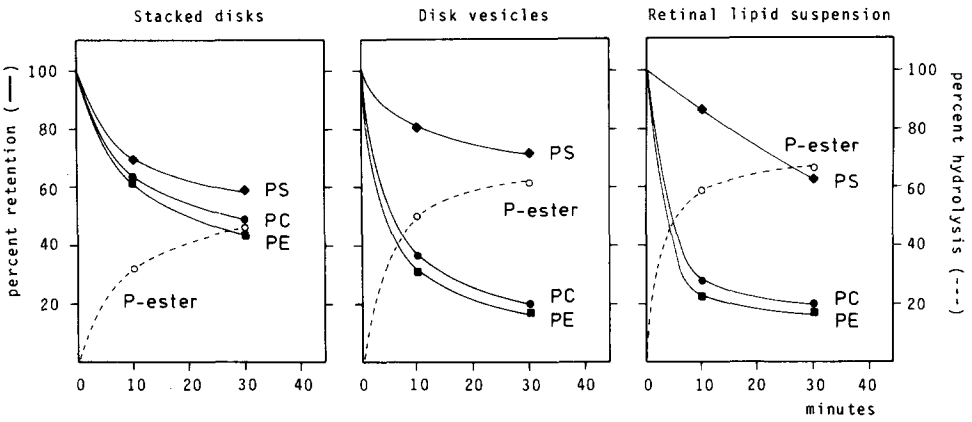

Fig. 1. Treatment with phospholipase C. Percent retention of individual phospholipids (solid symbols) and percent hydrolysis (open symbols) upon treatment of stacked disks, disk vesicles and retinal lipid suspensions are shown. Conditions: pH 7.4; darkness; 20°C; about 2 μmol/ml phospholipid and 2 units/ml phospholipase C (enzyme units as defined in Ref. 30). PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; P-ester, phosphate ester; LPL, lysophospholipid; PA, phosphatidic acid.

Exhaustive phospholipase C treatment of disk membranes and retinal lipid suspension results in nearly complete hydrolysis of the phospholipids (Table I). Residual phospholipid is almost exclusively phosphatidylserine, 5—20% of which is resistant to this enzyme.

The substrate preference of phospholipase C, as measured with the retinal lipid suspension, decreases in the order: phosphatidylethanolamine > phosphatidylcholine > phosphatidylserine (Fig. 1). The same order of preference is found for stacked disks and disk vesicles.

Phospholipase A_2 treatment

During phospholipase A_2 treatment, the decrease for each phospholipid is completely accounted for by the increase in the corresponding lysocompounds, except that some lysophosphatidylserine is lost, probably during the washing procedure.

Treatment of disk membranes with high concentrations of phospholipase A_2 completely modifies all phospholipids into their corresponding lysoproducts (Table I).

The observed order of preference of phospholipase A_2 using retinal lipid suspension is phosphatidylserine > phosphatidylethanolamine > phosphatidylcholine, which is also found with stacked disks and disk vesicles (Fig. 2).

Phospholipase D treatment

The optimal conditions for phospholipase D hydrolysis of phospholipids in rod outer segment disk membranes have been ascertained by varying pH, temperature, Ca^{2+} concentration and amount of enzyme. Maximal hydrolysis is obtained at pH 6.0 and 30°C in the presence of 40 mM $CaCl_2$. Under these conditions no transferase activity of phospholipase D is observed.

The decrease in each phospholipid after treatment with phospholipase D

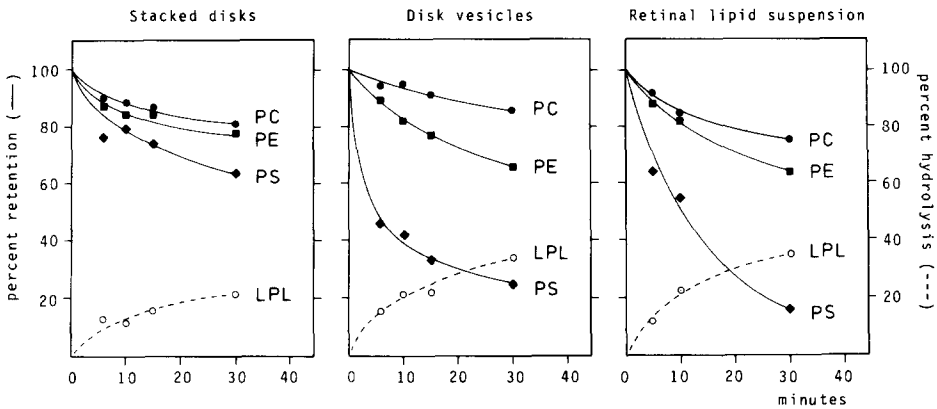

Fig. 2. Treatment with phospholipase A_2. Percent retention of individual phospholipids (solid symbols) and percent hydrolysis (open symbols) upon treatment of stacked disks, disk vesicles and retinal lipid suspensions are shown. Conditions: pH 7.4; darkness; 20° C; 10 mM Ca^{2+}; about 2 μmol/ml phospholipid and 0.7 μg/ml phospholipase A_2. Abbreviations as in Fig. 1.

should be equal to the amount of phosphatidic acid formed, but we always find less phosphatidic acid than expected. This is due to the presence in phospholipase D preparations of phosphatidate phosphohydrolase (EC 3.1.3.4), which hydrolyses phosphatidic acid to diglyceride and P_i [16]. Determination of P_i in the aqueous layers gives the contribution of phosphatidic acid breakdown, which is less than 20%. The sum of phosphatidic acid and P_i fully accounts for the amount of phospholipids hydrolyzed.

In contrast to the situation with phospholipases C and A_2, there is a limited final level of hydrolysis upon treatment of disk membranes with phospholipase D (Table I). Approx. 50% of phosphatidylcholine and phosphatidylethanolamine and nearly all phosphatidylserine, together 60% of the membrane phospholipids, are resistant to this enzyme in stacked disks. Removal of the water-soluble hydrolysis products, either by centrifugation followed by addition of fresh phospholipase D or by dialysis of the incubation mixture against fresh buffer, does not result in additional hydrolysis. Thus, inhibition by the water-soluble hydrolysis products does not play a role and the final level seems to represent a true limit to hydrolysis. Also, in disk vesicles an apparent final level of phospholipid hydrolysis is observed. This level is significantly higher (53%) than in stacked disks and will be discussed later.

When the membranes are solubilized in nonylglucose, phospholipid hydrolysis by phospholipase D proceeds much further, viz., to approx. 90% (Table I). Residual intact phospholipid consists of approximately equal amounts of phosphatidylethanolamine (9% retention) and phosphatidylserine (30% retention). Nearly complete hydrolysis (at least 95%) of the phospholipids can only be obtained at longer incubation times (up to 6 h) and higher concentrations of phospholipase D.

The substrate preference of phospolipase D decreases in the order: phosphatidylcholine > phosphatidylethanolamine >> phosphatidylserine in detergent-solubilized disk membranes. The same order of decreasing preference is found in stacked disks and disk vesicles (Fig. 3).

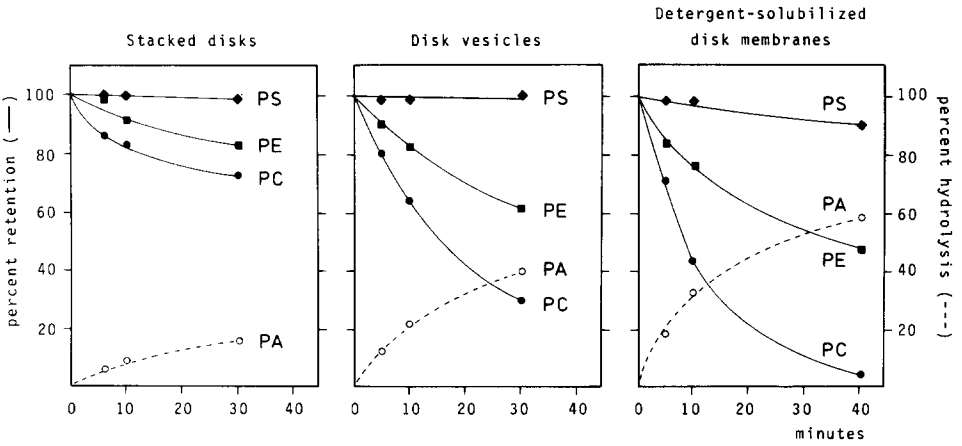

Fig. 3. Treatment with phospholipase D. Percent retention of individual phospholipids (solid symbols) and percent hydrolysis (open symbols) upon treatment of stacked disks, disk vesicles and detergent-solubilized disk membranes are shown. Conditions: pH 6.0; darkness; 40 mM Ca^{2+}; 30°C; about 2 μmol/ml phospholipid and 0.7 mg/ml phospholipase D. Disk membranes solubilized in 20 mM β-1-nonylglucose. Abbreviations as in Fig. 1.

Initial hydrolysis

Under identical incubation conditions, the rate at which the phospholipids of stacked disks are hydrolyzed in the first 10 min is always lower than that for disk vesicles and retinal lipid suspension. This is shown for phospholipase C in Fig. 1 and for phospholipase A_2 in Fig. 2. In the case of phospholipase D, this is true for phosphatidylcholine and phosphatidylethanolamine, of which the initial rates of hydrolysis decrease in the order: detergent-solubilized disk membranes > disk vesicles > stacked disks (Fig. 3). Phosphatidylserine is only attacked in detergent solution. The differences are most readily explained by the different degree to which the substrates are directly accessible to the enzymes.

The effects of the enzymes during the initial stage of the incubation are particularly important, since in this early period changes in the membrane organization due to phospholipid hydrolysis can be expected to be minimal. The most relevant results of 10-min incubations are, therefore, analyzed in more detail in Table II. In stacked disks about 30% overall hydrolysis is achieved with phospholipase C during this time and about 10% with the two other phospolipases at the enzyme concentrations used. The results are presented as the percent at which each phospholipid class contributes to the total fraction of phospholipids hydrolyzed in 10 min. In this way the substrate specificities of the phospholipases in the reference preparations are easily compared with those in stacked disks. A lower percentage indicates that the phospholipid is less available to enzyme action than in the randomized sample and vice versa.

The data of Table II clearly argue against a major asymmetrical distribution of any of the phospholipids in the disk membrane. If the phospholipids in the reference preparations are indeed randomly available (see Discussion), phosphatidylcholine would appear to occur at a somewhat lower, and phospha-

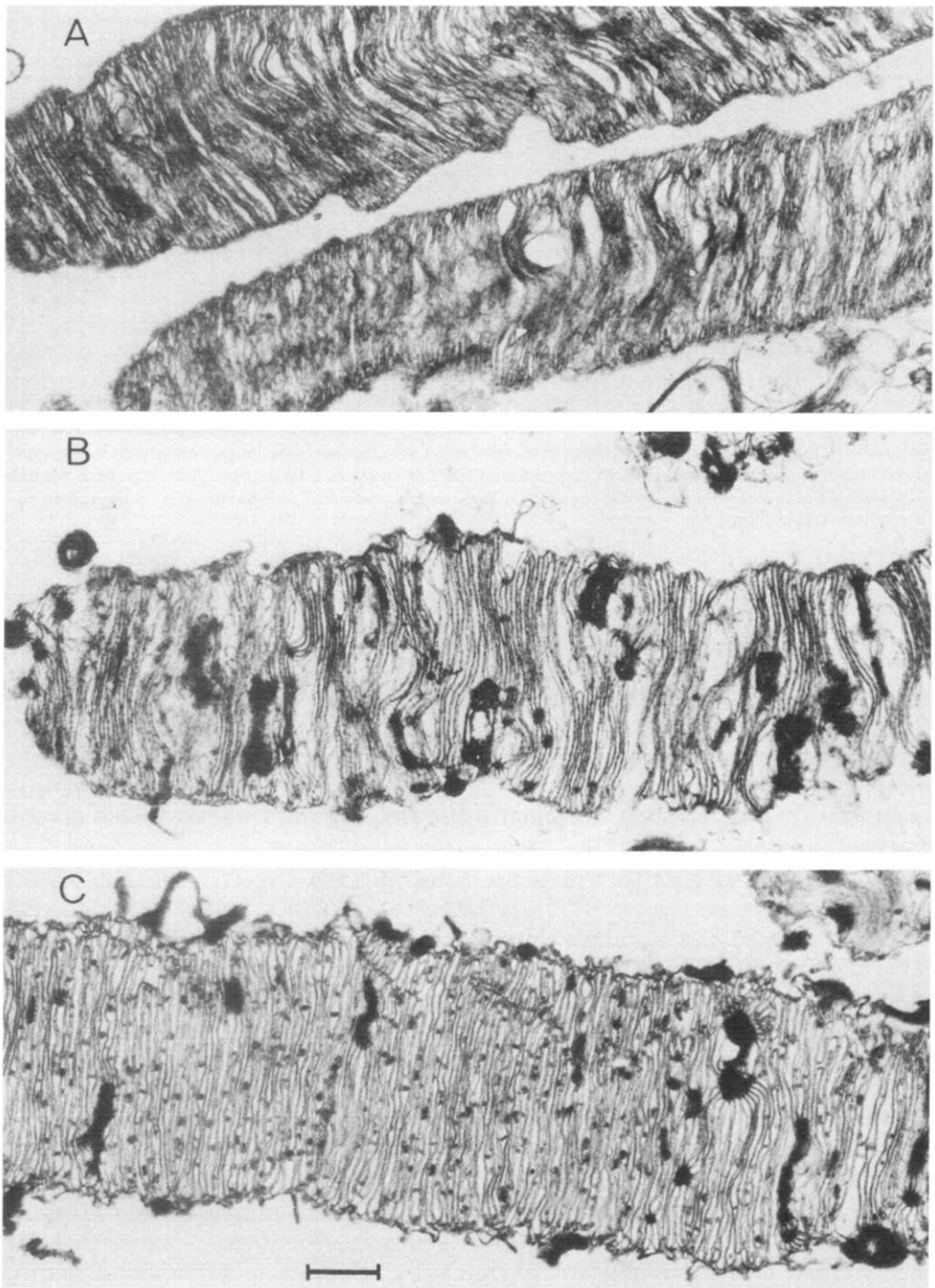

Fig. 4. Thin sections of stacked disks treated with phospholipase D. A, control sample incubated for 150 min in the absence of phospholipase D; B, sample incubated with phospholipase D during 40 min, resulting in 19% phospholipid hydrolysis; C, sample incubated with phospholipase D during 150 min, resulting in 41% phospholipid hydrolysis. The dark spots in B and C are presumably aggregates of phospholipase D (+ Ca^{2+}?), since they are absent if the suspensions are centrifuged and washed prior to fixation. Bar represents 0.4 μm.

TABLE II

PHOSPHOLIPID HYDROLYSIS DURING THE INITIAL STAGE OF TREATMENT WITH PHOSPHOLIPASE C, A_2 AND D

Results are expressed as the percent at which each phospholipid class contributes to the total fraction of phospholipids hydrolyzed after 10 min of incubation. Overall hydrolysis is the percent of initially present total phospholipid hydrolyzed at that time. Minor phospholipids (3% of total phospholipids) are neglected. Reference preparations as in Table I. Values are averages of two experiments with a relative standard error of maximally 10%.

Phospholipase		Stacked disks	Reference	Ratio disks: reference
C	phosphatidylcholine	38	49	0.75
	phosphatidylethanolamine	47	48	0.98
	phosphatidylserine	15	3	5
	overall hydrolysis	33	59	
A_2	phosphatidylcholine	30	38	0.79
	phosphatidylethanolamine	45	36	1.25
	phosphatidylserine	25	26	0.96
	overall hydrolysis	14	23	
D	phosphatidylcholine	54	61	0.89
	phosphatidylethanolamine	44	37	1.20
	phosphatidylserine	2	2	1
	overall hydrolysis	9	32	

tidylethanolamine at a somewhat higher percentage at the outer face of the disk membranes. Even after a 30 min incubation period, these percentages are fairly close to those after enzyme treatment for 10 min. This is particularly true for phospholipases A_2 and D, which have both produced about 20% overall phospholipid hydrolysis at that time.

Corresponding calculations for disk vesicles have been omitted from Table II, since they do not provide additional information.

Microscopy

Phase-contrast microscopy reveals that treatment of the stacked disk preparations with any of the three phospholipases does not seriously change their appearance in the earlier stages of incubation. Electron microscopy of thin sections (Fig. 4) convincingly shows that stacked disks retain their original morphology when 19 or even 41% of the phospholipids are hydrolyzed by phospholipase D. Similar results are obtained with phospholipases C and A_2 at relatively low (approx. 30%) phospholipid hydrolysis levels.

Discussion

Principles of the approach

The distribution of phospholipids over the two faces of biomembranes can, in principle, be deduced from their availability to the action of phospholipases on intact cells or cell organelles. However, if valid conclusions are to be drawn a number of conditions must be fulfilled: (1) the outer membrane face of the intact preparation should be the original outer face and this

outer face should remain exposed during incubation with phospholipase; (2) the membranes should not be significantly penetrated by the phospholipase; and (3) the specificity of the phospholipase towards different phospholipids should be taken into account by using random control preparations. In addition, the results with different phospholipases should agree.

Membrane orientation

Studies on the topography of phospholipids in rod outer segment disk membranes have, so far, only been conducted with disk vesicles [8—10]. In order to avoid the uncertainty about the maintenance of the original inside-outside arrangement in these structures [21], we have used isolated rod outer segments (stacked disks) as the primary experimental preparation. Their morphology resembles that of outer segments in situ so closely that membrane inversion must be considered to be extremely unlikely. Microscopic and electron-microscopic evidence indicates that the gross morphology of the stacked disks is hardly affected during the early stages of incubations with phospholipases. Therefore, it seems safe to assume that the stacked disk membranes retain a 'right-side-out' orientation under these conditions.

Penetration of phospholipases

Since phospholipases are water-soluble proteins with molecular weights of at least 14 000, it seems improbable that they would penetrate the intact hydrophobic core of a biological membrane. However, the application of phospholipases results in chemical alteration of part of the phospholipids, which might lead to abolition of the permeability barriers in the membrane. Unfortunately, an endogenous or exogenous internal marker, the release of which could indicate gross permeability of the membrane (as has been applied with erythrocytes), cannot be used very well. A well defined internal marker is lacking, whereas the introduction of an exogenous marker presents unknown risks of loss of the right-side-out orientation in the resulting vesicles. Since, however, secondary effects due to the action of phospholipases should be minimal in the early phase of enzymatic degradation [4], special attention has been given to the initial period of incubation (Table II).

Substantial disturbance of the membrane, leading to almost complete hydrolysis of the phospholipids, clearly happens in the later stages of incubation with phospholipases C and A_2 (Table I). The products of phospholipase C treatment, hydrophobic diglycerides and water-soluble phosphate esters, will not fit very well in a bilayer membrane, while the products of phospholipase A_2 treatment, lysophospholipids, are well known for their lytic activity. Therefore, it is not surprising that these enzymes upon extensive incubation induce serious disturbance of the membrane, resulting in complete accessibility of all phospholipids.

Phospholipase D, which hydrolyses glycerophospholipids to phosphatidic acid, would be expected to present less risk. Phosphatidic acid very much resembles the usual phospholipids with respect to its amphiphilic character and, therefore, serious membrane disturbance is unlikely [22,23]. This probably explains the limited final level of hydrolysis by phospholipase D, at least in stacked disks. It also suggests that in this preparation, even up to 180

min incubation, penetration of the enzyme, gross disturbance of the membrane and appreciable transbilayer exchange of phospholipids against phosphatidic acid * do not occur. The alternative explanation for the limited final level of hydrolysis, viz., shielding of outer face phospholipids either by their interaction with protein or by accumulation of negative charge, seems unlikely. The unrestricted action of phospholipase A_2 and, especially, phospholipase C argues against the first possibility and the presence of 40 mM Ca^{2+} during phospholipase D treatment against the second.

The reason for the different final hydrolysis levels in stacked disks (40%) and in disk vesicles (53%) after phospholipase D treatment is not immediately clear. A possible explanation is provided by the observation of Woodward and Zwaal [24] that resealed ghosts, prepared from intact erythrocytes by a hypotonic lysis procedure, are more easily hemolyzed by treatment with phospholipase C (*B. cereus*) than erythrocytes themselves. This shows that prior exposure to hypotonic conditions makes the membrane permeability barrier for macromolecules more vulnerable to phospholipases, which attack the polar head groups. Disk vesicles, obtained by washing disk membranes with distilled water, may similarly have become partially permeable to phospholipase D. This would imply another argument in favor of preferring stacked disks to disk vesicles in studying phospholipid distribution in the photoreceptor membrane.

Specificity of the phospholipases

The specificity of the phospholipases has been determined by measuring their effect on control preparations of phospholipid composition similar to rod outer segments, but with presumably random orientation of the phospholipids. The ^{31}P-NMR spectra obtained with retinal lipid suspensions indicate that the phospholipids in this preparation are present in hexagonal and inverted micellar phases, in which at least phosphatidylcholine and phosphatidylethanolamine are randomly available [25]. Disks solubilized in nonylglucose above its critical micelle concentration (6.5 mM; Ref. 13) must also have their phospholipids randomly available.

The specificity of phospholipase C decreases in the order: phosphatidylethanolamine > phosphatidylcholine > phosphatidylserine (Fig. 1). Roberts et al. [26] reported the same substrate preference of this enzyme in Triton X-100-solubilized phospholipid preparations.

The specificity of phospholipase A_2 decreases in the order: phosphatidylserine > phosphatidylethanolamine > phosphatidylcholine (Fig. 2). This specificity is in agreement with the preference of pancreatic phospholipase A_2 for negatively charged phospholipids (like phosphatidylserine) compared to neutral ones [27].

The substrate specificity of phospholipase D decreases in the order: phosphatidylcholine > phosphatidylethanolamine >> phosphatidylserine (Fig. 3). The specificity of this enzyme, which has not been obtained in a pure form so far, is not well established [16,28,29].

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* Although it has been shown that phospholipase D is able to induce transbilayer exchange of phosphatidic acid against phosphatidylcholine in unilamellar phosphatidylcholine vesicles in the absence of Ca^{2+} [33], this phenomenon was not found in the presence of 6 mM Ca^{2+} [34].

Tentative conclusions

The phospholipid hydrolysis patterns obtained from stacked disks and disk vesicles with phospholipase C (Fig. 1), phospholipase A_2 (Fig. 2) and phospholipase D (Fig. 3) do not substantially deviate from those obtained from randomized control preparations, apart from readily explained differences in reaction rate. They suggest symmetry rather than asymmetry in the distribution of the phospholipids over both faces of the disk membrane.

In view of the discussion in one of the previous sections, more refined conclusions might be derived from the comparison of the effects of a brief (10 min) phospholipase treatment on stacked disks and randomized control preparations (Table II). With respect to phosphatidylcholine, all three approaches show a slightly asymmetrical distribution with 40—45% of this phospholipid at the outer (cytoplasmic) face of the disk membrane. For phosphatidylethanolamine, a similar conclusion seems warranted, albeit with 55—60% of this phospholipid at the outer face of the membrane. For phosphatidylserine, we must rely primarily on the results with phospholipase A_2, since its high preference for this phospholipid compensates for the reduced analytical accuracy caused by the low phosphatidylserine concentration. The results obtained with phospholipase A_2 indicate a symmetrical distribution of phosphatidylserine over the two faces of the membrane.

Our present conclusions obtained with three phospholipases can only have a preliminary character. The occurrence of preferential transmembrane flip-flop of a specific phospholipid and the possibility of shielding of outer face phospholipids against phospholipase D, including inaccessibility due to the piled disk structure, cannot yet be excluded. In addition, our conclusions do not agree with those obtained by studies of the modification of phosphatidylethanolamine and phosphatidylserine by amino group reagents, where preferential (70—100%) location of both phosphatidylethanolamine and phosphatidylserine [8,9] or of phosphatidylethanolamine only (63—72%; Ref. 10) on the cytoplasmic side of the disk membrane has been concluded. We shall return to these aspects in a subsequent paper, which describes the effects of trinitrobenzenesulfonate alone and in combination with phospholipase D [31]. Such multiple approaches are necessary in order to avoid the many pitfalls that may occur in studies of the phospholipid distribution in biological membranes [32].

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