Biochimica et Biophysica Acta, 603 (1980) 130—141

BBA 79016

TRANSBILAYER DISTRIBUTION OF PHOSPHOLIPIDS IN PHOTORECEPTOR MEMBRANE STUDIED WITH TRINITROBENZENESULFONATE ALONE AND IN COMBINATION WITH PHOSPHOLIPASE D *

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(Received April 8th, 1980)

Key words: Phospholipid distribution; Photoreceptor membrane; Trinitrobenzenesulfonate; Phospholipase D; (Rod outer segment)

Summary

In a further study of the transbilayer distribution of phospholipids in rod disk membranes, the amino group reagent, trinitrobenzenesulfonate, and the phospholipid-hydrolyzing enzyme, phospholipase D, have been used alone and in combination.

Under carefully defined conditions (1 mM trinitrobenzenesulfonate, pH 7.4, 20° C, darkness), trinitrobenzenesulfonate yields limited final levels of modification of phosphatidylethanolamine and phosphatidylserine, suggesting only minor reagent penetration and membrane disturbance under these conditions.

Treatment of stacked disks with trinitrobenzenesulfonate under these conditions leads to a biphasic modification of the aminophospholipids. Relatively fast (less than 1 h) modification of 50% phosphatidylethanolamine and 40% phosphatidylserine occurs, slowly rising (approx. 3 h) to 60 and 50%, respectively.

Extensive treatment of stacked disks with phospholipase D leads to the hydrolysis of 55% phosphatidylcholine and 50% phosphatidylethanolamine, while phosphatidylserine is hardly attacked by this enzyme.

Treatment of stacked disks with trinitrobenzenesulfonate after prior treatment with phospholipase D leads to no further modification than that maximally obtained with either reagent alone: about one-half of the three major phospholipid classes is accessible. Although both reagents differ greatly in molecular size, mode of action and other properties, they apparently see the

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* No. XLIII in the series 'Biochemical Aspects of the Visual Process'.
Abbreviation: Mops, 3-(*N*-morpholino)propanesulfonic acid.

same pool of phosphatidylethanolamine, their joint substrate. Considering that we start with the original right-side-out configuration, that all phospholipids can in principle be modified (no shielding) and that the membrane remains essentially intact, we conclude that the accessible lipid pool represents the outer face of the disk membranes.

These results confirm our earlier conclusions from treatment with three phospholipases that the three major phospholipids are nearly symmetrically distributed over the two faces of the disk membrane.

The divergence with the conclusions of other investigators is most likely explained by their use of disk membranes (disk vesicles) in which the original phospholipid distribution had not been maintained and/or of conditions under which trinitrobenzenesulfonate markedly penetrates the membrane.

Introduction

In a previous study [1] we have used three phospholipases in a study of the phospholipid distribution over the two faces of the disk membrane in bovine rod outer segments. The results suggested an approximately symmetrical distribution of the three major phospholipids: 40—45% of the phosphatidylcholine, 55—60% of the phosphatidylethanolamine and 50% of the phosphatidylserine in the outer (cytoplasmic) face. Our conclusions were conditioned by the consideration that the possible occurrence of preferential transbilayer movement (flip-flop) of a given phospholipid as well as shielding of outer face phospholipids against phospholipase D had yet to be disproved.

The conclusions disagree with those of other investigators [2—4] who had used amino group reagents like trinitrobenzenesulfonate. They reported predominant (between 65 and 100%) location of phosphatidylethanolamine in the outer face of the membrane. The location of phosphatidylserine remained unclear with values of 25% [4] and 100% [3] at the outer face. Phosphatidylcholine, which has no primary amino group, cannot be localized by this method.

In order to resolve this discrepancy, we decided to study in more detail the reaction between rod disk membranes and trinitrobenzenesulfonate, alone and in combination with phospholipase D. The results strengthen our earlier conclusion of a nearly symmetrical distribution of the three major phospholipids in the disk membrane and offer a possible explanation for the divergent earlier reports.

Materials and Methods

Materials. 2,4,6-Trinitrobenzene-1-sulfonic acid tetrahydrate has been obtained from BDH Chemicals Ltd., Poole, U.K. Phospholipase D (EC 3.1.4.4.) from Savoy cabbage has been purchased from Boehringer, Mannheim, F.R.G. All other chemicals are of analytical grade.

Isolation of rod outer segment disk membranes. Stacked bovine rod outer segment disk membranes ('stacked disks') and 'disk vesicles' are prepared as previously described [5,6]. Electron-microscopic observation shows regularly piled disks partially surrounded by plasma membrane in the former prepara-

tion, and closed vesicular structures in the latter preparation.

The rhodopsin concentrations of the suspensions subjected to treatment with trinitrobenzenesulfonate and phospholipase D are 3—4 and 30—40 μM, respectively, as determined by using the method of van Breugel et al. [7].

Treatment with trinitrobenzenesulfonate. Optimal conditions for the application of trinitrobenzenesulfonate as an impermeable reagent with respect to disk membranes have first been established (see Results). In the final procedure, the membrane preparations (3—4 μM rhodopsin) are suspended in 40 mM Mops buffer (pH 7.4), 2 mM $CaCl_2$, 3 mM $MgCl_2$, 140 mM NaCl. Trinitrobenzenesulfonate is added as a freshly prepared solution in the same buffer to a final concentration of 1 mM. Incubation is performed at 20°C in darkness.

The total number of modified amino groups after treatment with trinitrobenzenesulfonate is determined by stopping the reaction through addition of 1 M HCl (final pH 2.5) and Triton X-100 (final concentration 1%, w/v) and reading the 340 nm absorbance against appropriate blanks [8].

The number of modified aminophospholipids (phosphatidylethanolamine and phosphatidylserine) is determined as follows. The reaction with trinitrobenzenesulfonate is stopped by adding excess ice-cold 0.2 M sodium acetate buffer (pH 5.5). After centrifugation of the diluted suspension (0°C, 100 000 × *g*, 30 min), the phospholipids are extracted with $CHCl_3/CH_3OH$ (2 : 1, v/v), washed with aqueous 0.1 M KCl and separated by two-dimensional thin-layer chromatography [1]. Trinitrophenylated aminophospholipids are identified by their yellow color [9], the other phospholipids are located by means of iodine vapor. The spots are carefully scraped off and their phosphorus contents are determined as P_i after acid destruction. The accuracy of the determination of phosphatidylcholine and (modified) phosphatidylethanolamine is about 5% (relative standard error). In the case of (modified) phosphatidylserine, the accuracy is only 10% (relative standard error) due to the lower concentration of this phospholipid.

The total number of primary amino groups present is determined by a modification of the method of Habeeb [10] by performing the reaction with trinitrobenzenesulfonate in the presence of 1% (w/v) Triton X-100 for 3—4 h and reading the 340 nm absorbance against appropriate blanks.

Treatment with phospholipase D. Stacked disks are resuspended in a medium containing 600 mM sucrose, 5% (w/w) Ficoll 400 and 20 mM Tris-maleate buffer (pH 6.0), while disk vesicles are resuspended in 0.16 M Tris-maleate buffer (pH 6.0). The suspension (rhodopsin concentration 30—40 μM) is incubated with an appropriate amount of phospholipase D (solubilized in distilled water) at 30°C in darkness in the presence of 40 mM $CaCl_2$. Hydrolysis is stopped by adding excess ice-cold buffer (pH 6.0) containing 10 mM EDTA. Phospholipid and product analyses are carried out as described in the previous section.

Combined treatment with phospholipase D and trinitrobenzenesulfonate. The incubations with phospholipase D and trinitrobenzenesulfonate are carried out as described above. When the first treatment is with trinitrobenzenesulfonate, the reaction is stopped by addition of an excess of ice-cold buffer (pH 6.0), the mixture is centrifuged (0°C, 10 000 × *g*, 30 min), and the pellet is

resuspended in the buffer used for phospholipase D treatment. When the first treatment is with phospholipase D, the incubation mixture can be diluted directly by addition of the medium used for modification with trinitrobenzenesulfonate (e.g., without prior centrifugation).

Results

General

In accordance with previous results from our laboratory [8], analysis of the primary amino groups in disk membranes with trinitrobenzenesulfonate in detergent solution shows the presence of 52 ± 2 mol/mol rhodopsin, consisting of 16 ϵ-amino groups of lysine, 27 amino groups of phosphatidylethanolamine and 9 amino groups of phosphatidylserine.

Treatment of disk vesicles with trinitrobenzenesulfonate

Disk vesicles have primarily been used to establish reaction conditions leading to a limited final level of modification, which would represent minimal reagent penetration and membrane disturbance. These conditions have then been used in our further studies with disk vesicles and stacked disks. In addition, inclusion of disk vesicles in these studies allows comparison of our results with those of other investigators [2—4] who have exclusively used this type of preparation.

Previously, it has been observed that temperatures substantially above 20°C and illumination lead to complete modification with trinitrobenzenesulfonate [8,11]. Hence, we have chosen to work at 20°C and in darkness. Buffer composition, pH and concentration of trinitrobenzenesulfonate have been varied. The buffer composition hardly influences the final degree of modification, except that the reaction proceeds rather slowly in a medium containing sucrose and Ficoll 400.

The effect of the reagent concentration has been studied by spectrophotometric determination of total amino group modification as a function of time at pH 7.4 and 20°C (Fig. 1). The trinitrobenzenesulfonate concentration has been varied from 1 to 5 mM, representing a 5- to 25-fold molar excess with

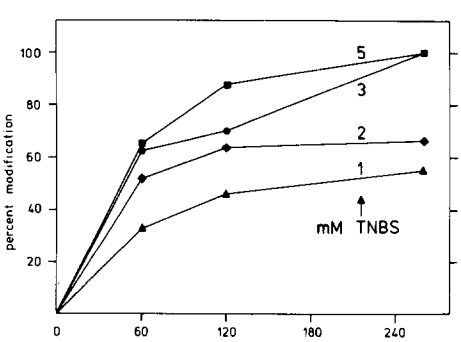

Fig. 1. Amino group modification in disk vesicles at different trinitrobenzenesulfonate (TNBS) concentrations. Results are expressed as percent modification of total amino groups, determined by measuring the 340 nm absorbance. Conditions: pH 7.4; darkness; 20°C; 3—4 μM rhodopsin.

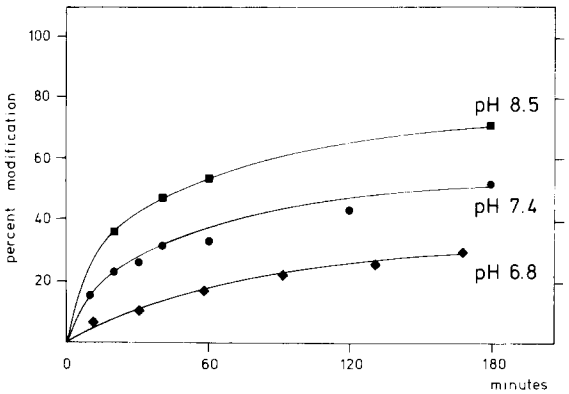

Fig. 2. Amino group modification in disk vesicles at three pH values. Results are expressed as percent modification of total amino groups, determined by measuring the 340 nm absorbance. Conditions: darkness; 20°C; 1 mM trinitrobenzenesulfonate; 3—4 μM rhodopson. Inductions at pH 6.8 and 7.4 are carried out in 40 mM Mops buffer containing 2 mM $CaCl_2$, 3 mM $MgCl_2$ and 140 mM NaCl; incubations at pH 8.5 in 120 mM $NaHCO_3$ and 40 mM NaCl.

regard to the total number of primary amino groups. Both the course and the final level of modification are greatly influenced by the reagent concentration. The complete modification occurring at 3—5 mM trinitrobenzenesulfonate shows that in principle all amino groups are reactive with the reagent and suggests that at these concentrations trinitrobenzenesulfonate penetrates the membrane. However, at 1—2 mM trinitrobenzenesulfonate the final level of modification remains restricted to 50—60% of the amino groups. Therefore, we have further investigated the course of the reaction at 1 mM trinitrobenzenesulfonate.

Variation of pH also appears to have a large influence (Fig. 2). After 3 h of incubation, approx. 70, 50 and 30% of the amino groups are found to be modified by 1 mM trinitrobenzenesulfonate at pH 8.5, 7.4 and 6.8, respectively. Analysis of the individual amino groups, modified at pH 7.4 and 8.5, shows that after 3 h of incubation the higher overall modification at pH 8.5 is

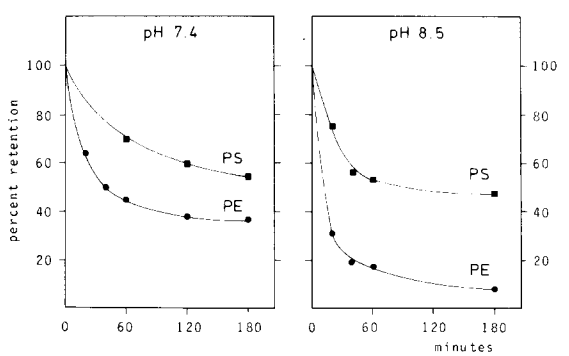

Fig. 3. Treatment of disk vesicles with trinitrobenzenesulfonate at pH 7.4 and 8.5. Percent retention of phosphatidylethanolamine (●) and phosphatidylserine (■) is shown. Conditions: see legend of Fig. 2. PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; TNP-PE, trinitrophenyl phosphatidylethanolamine; PA, phosphatidic acid.

largely due to almost complete modification of phosphatidylethanolamine: 92% at pH 8.5 as compared to 63% at pH 7.4 (Fig. 3). Phosphatidylethanolamine is the compound of most interest to us, since in contrast to phosphatidylserine, it is also a substrate of phospholipase D. Considering the limited final level of phosphatidylethanolamine modification at pH 7.4, we have chosen this pH for our further experiments.

Under the standard conditions for limited final modification thus established (20°C, darkness, 1 mM trinitrobenzenesulfonate, pH 7.4), complete modification can be induced within 1 h by addition of Triton X-100 to a final concentration of 1% (w/v). Thus, even at 1 mM trinitrobenezenesulfonate and pH 7.4, all amino groups can in principle be modified.

Combined treatment of disk vesicles with trinitrobenzenesulfonate and phospholipase D

We have previously noticed [1] that phospholipase D also shows a limited final hydrolysis level of disk membrane phospholipids. Therefore, it seemed of interest to test the complementarity of the reactions of trinitrobenzenesulfonate and phospholipase D with disk vesicles.

Aliquots of a single disk vesicle preparation are treated with trinitrobenzenesulfonate for either 30 or 60 min. This leads to trinitrophenylation of 30 or 43% of phosphatidylethanolamine and 28 or 39% of phosphatidylserine, respectively. A third aliquot, incubated for 60 min without reagent, serves as the control. After removal of excess reagent by centrifugation and washing, these samples are incubated with phospholipase D during 2 h, leading to a plateau level of hydrolysis. The reaction products are analyzed by thin-layer chromatography, and the results are shown in Fig. 4. The trinitrophenylated phospholipids are found not to be hydrolyzed by phospholipase D.

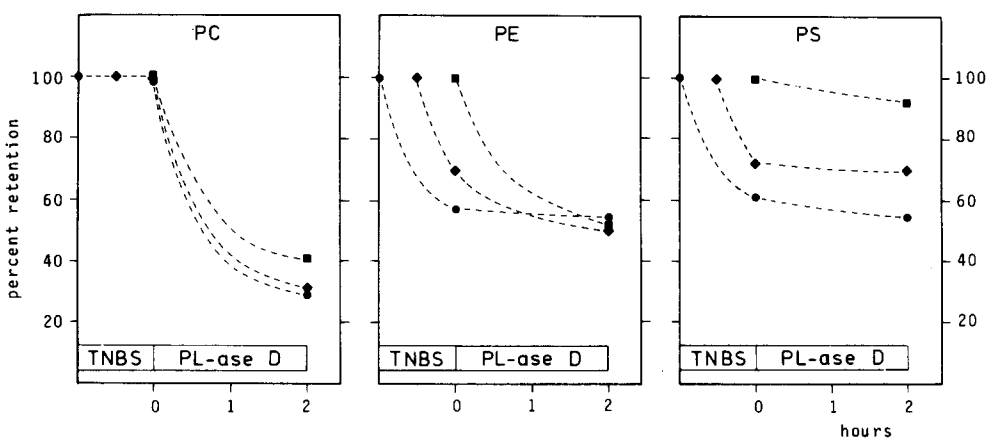

Fig. 4. Successive treatment of disk vesicles with trinitrobenzenesulfonate (TNBS) and phospholipase D. Disk vesicles are first treated with trinitrobenzenesulfonate during 0 (■), 30 (◆) and 60 (●) min. After removal of trinitrobenzenesulfonate, each preparation is treated with phospholipase D during 120 min. The results for each phospholipid are expressed as percent retention: 100% — % modified — % hydrolyzed. Conditions: for trinitrobenzenesulfonate treatment, pH 7.4; darkness; 20°C; 1 mM trinitrobenzenesulfonate; 3—4 μM rhodopsin; for phospholipase D treatment, pH 6.0; darkness; 30°C; 40 mM $CaCl_2$; 30—40 μM rhodopsin; 0.7 mg/ml phospholipase D. Abbreviations as in Fig. 3.

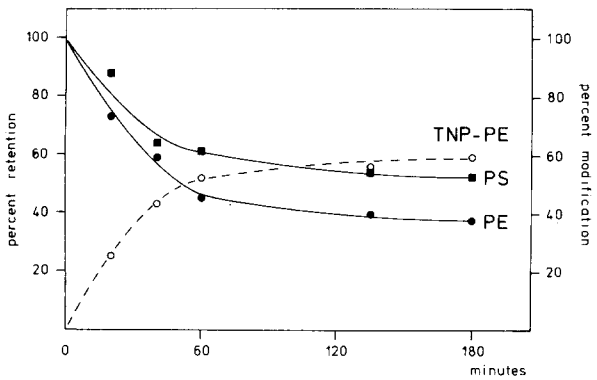

Fig. 5. Treatment of stacked disks with trinitrobenzenesulfonate. Percent retention of phosphatidylethanolamine (•) and phosphatidylserine (■) and percent modification (○) of phosphatidylethanolamine are shown. Conditions: pH 7.4; darkness; 20°C; 1 mM trinitrobenzenesulfonate; 3—4 μM rhodopsin. Abbreviations as in Fig. 3.

With respect to phosphatidylcholine (a good substrate) and phosphatidylserine (a poor substrate), the action of phospholipase D appears to be largely independent of the pretreatment with trinitrobenzenesulfonate. The most relevant results concerns phosphatidylethanolamine, for which the cumulative, final level of modification (trinitrophenylation + hydrolysis) is equal for all three preparations.

Treatment of stacked disks with trinitrobenzenesulfonate

Reaction of stacked disks with trinitrobenzenesulfonate under our conditions leads to the results shown in Fig. 5. Quantitative thin-layer chromatography shows that 50% of phosphatidylethanolamine is rather rapidly modified within 1 h. During the next 2 h an additional 10% is slowly modified. These results are confirmed by analysis of the product: trinitrophenyl phosphatidylethanolamine. Phosphatidylserine reacts rapidly with 40% modification in 1 h and a further 10% reacting slowly during the next 2 h. In this case, analysis of

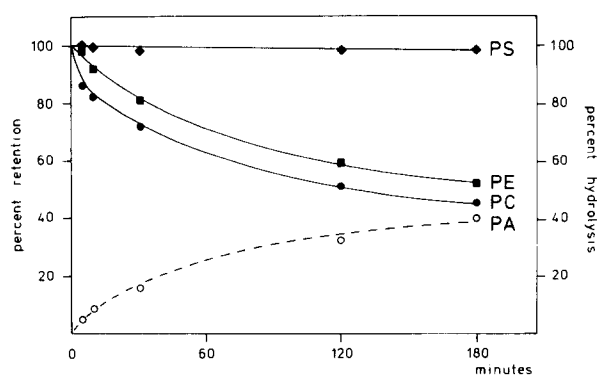

Fig. 6. Treatment of stacked disks with phospholipase D. Percent retention of individual phospholipids (solid symbols) and percent hydrolysis (open symbols) are shown. Conditions: pH 6.0; darkness; 30°C; 40 mM $CaCl_2$; 30—40 μM rhodopsin; 0.7 mg/ml phospholipase D. Abbreviations as in Fig. 3.

the product, trinitrophenyl phosphatidylserine, gives unsatisfactory results due to difficulties with the exact location of the less intensely colored spot on the thin-layer plate, leading to recoveries varying from 50 to 100%.

Addition of Triton X-100 (final concentration 1%, w/v) to the incubation mixture leads again to complete modification of both phospholipids, indicating that also in stacked disks all aminophospholipids can in principle be modified with 1 mM trinitrobenzenesulfonate at pH 7.4.

Combined treatment of stacked disks with phospholipase D and trinitrobenzenesulfonate

The complementarity of the action of trinitrobenzenesulfonate and phospholipase D is investigated by treating stacked disks first with phospholipase D, followed by treatment with trinitrobenzenesulfonate. Treatment of stacked disks with phospholipase D alone for 3 h causes maximally 40% phospholipid hydrolysis, comprising 55% hydrolysis of phosphatidylcholine and 50% hydrolysis of phosphatidylethanolamine, while phosphatidylserine is almost resistant to the enzyme (Fig. 6). This confirms our earlier experiments [1].

The results obtained after treatment with phospholipase D, followed by trinitrobenzenesulfonate, are shown in Fig. 7. Aliquots of a single stacked disk preparation are incubated for either 30 or 120 min with phospholipase D, leading to the hydrolysis of 20 or 40% total phospholipid, viz., 20 or 50% phosphatidylethanolamine, 1 or 3% phosphatidylserine and 29 or 55% phosphatidylcholine, respectively. A third aliquot, incubated for 120 min without enzyme, serves as the control. Subsequently, these preparations are incubated with trinitrobenzenesulfonate during 3 h and the course of the reaction is followed by phospholipid analysis. For phosphatidylethanolamine, the same cumulative, final level of nearly 60% modification (hydrolysis + trinitrophenyl-

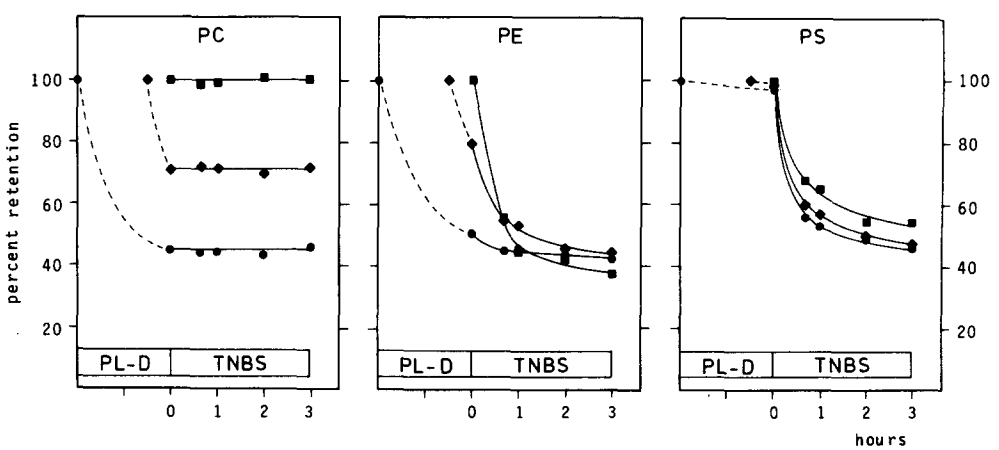

Fig. 7. Successive treatment of stacked disks with phospholipase D and trinitrobenzenesulfonate. Stacked disks are first treated with phospholipase D during 0 (■), 30 (◆) and 120 (●) min (- - - - -). After removal of the enzyme each preparation is treated with trinitrobenzenesulfonate during 180 min (———). The results for each phospholipid are expressed as percent retention: 100% — % hydrolyzed — % modified. Conditions: for phospholipase D treatment as in Fig. 6, for trinitrobenzenesulfonate treatment as in Fig. 5. Abbreviations as in Fig. 3. PL-D, phospholipase D.

ation) is obtained, regardless of the degree of previous hydrolysis by phospholipase D. The final level of modification of phosphatidylserine (50—52%, largely trinitrophenylation), and even the rate at which this level is reached, appear to be almost independent of the previous treatment with phospholipase D. Hence, successive treatment with the two reagents again does not lead to greater modification than that maximally obtained with either reagent alone, taking into account their different specificities.

The overall morphological appearance of the stacked disk preparations is maintained during all these incubations, as observed by phase-contrast microscopy.

Discussion

During the last 5 years, it has become evident that studies of the transbilayer distribution of phospholipids in biological membranes should be executed and interpreted with great caution [12]. Maintenance of the original lipid orientation, exclusive reactivity of the outer face of the membrane and complete accessibility of all outer face lipids to the reagent should be firmly established before valid conclusions can be drawn. We will discuss our results with trinitrobenzenesulfonate and phospholipase D treatment of disk membranes in the light of these conditions.

Trinitrobenzenesulfonate and disk vesicles

The conditions under which trinitrobenzenesulfonate has previously been used as a supposedly impermeable amino group reagent vary considerably, especially with respect to reagent concentration and pH. Since differences in membrane permeability of trinitrobenzenesulfonate have been noticed for different membrane systems [12], it is essential to establish conditions under which its permeability is restricted as much as possible. One of the difficulties with disk membranes is the lack of a well defined endogenous marker, becoming detectable in the case of membrane leakage, like hemoglobin in erythrocytes. Insertion of an exogenous marker, if at all possible, presents unknown risks of loss of the right-side-out orientation in the resulting vesicles. Therefore, we have confined ourselves to finding incubation conditions which lead to a clearly limited modification level of the amino groups in disk vesicles.

Figs. 1—3 suggest that at 1 mM concentration and pH 7.4, trinitrobenzenesulfonate behaves as a non-permeant probe with respect to phosphatidylethanolamine. The lower reactivity of phosphatidylserine at pH 7.4 is probably due to the higher pK_a of its amino group and a possible shielding effect of the carbonyl group. This is suggested by the observation that trinitrophenylation of phosphatidylserine rapidly reaches a plateau level at pH 8.5 only (Fig. 3). The reactivity of the lysine ϵ-amino group is even lower than that of the phospholipid amino groups due to its higher pK_a [8], but this substance can be left out of consideration for our present purpose.

The fact that, even at pH 7.4, all amino groups of the membrane can be modified in the presence of detergent or at high (3—5 mM) trinitrobenzenesulfonate concentration shows that in principle all amino groups are available for modification with trinitrobenzenesulfonate and that appreciable shielding

effects due to protein-lipid interactions do not seem to occur.

The effects of combined treatment of disk vesicles with trinitrobenzenesulfonate and phospholipase D (Fig. 4) indicate that the pool of phosphatidylethanolamine, susceptible to the action of phospholipase D, coincides with that accessible to trinitrobenzenesulfonate under the conditions used. Since trinitrophenylation of phosphatidylethanolamine and phosphatidylserine does not seem to influence seriously the action of phospholipase D towards residual substrate, indicating little perturbation of the membrane, this strongly suggests that the accessible phosphatidylethenolamine represents the outer face of the disk membrane.

Trinitrobenzenesulfonate and stacked disks

In studies of the phospholipid transbilayer distribution, stacked disks are preferable to disk vesicles, since disk vesicles may show membrane inversion [13] which is unlikely to occur in the native structure of stacked disks.

Treatment of stacked disks with tribitrobenzenesulfonate under our standard conditions results in modification of phosphatidylethanolamine in a way similar to that in disk vesicles. The reaction course suggests a biphasic reaction, in which about 50% of the total phosphatidylethanolamine belongs to a fast-reacting pool and this phosphatidylethanolamine must almost certainly be located at the outer face of the membrane. The slow second phase of trinitrophenylation could then represent penetration of the reagent or perturbation of the membrane, leading to modification of originally inside-located phosphatidylethanolamine. A similar biphasic pattern of trinitrophenylation is observed for phosphatidylserine, although percentually slightly less phosphatidylserine than phosphatidylethanolamine is susceptible to modification.

As already mentioned, the behavior of trinitrobenzenesulfonate towards disk vesicles is rather similar to that found towards stacked disks. Therefore, it is very unlikely that in stacked disks phospholipids are not accessible due to spatial restrictions like residual plasma membrane and juxtaposition of disks, which are not present in disk vesicles.

Combined treatment of stacked disks with phospholipase D and trinitrobenzenesulfonate

In our earlier experiments we have already obtained evidence that phospholipase D does not seem to penetrate the disk membrane [1]. In agreement with this, we now find that exhaustive treatment of stacked disks with this enzyme shows (Fig. 6) that approximately one-half of the phosphatidylethanolamine and phosphatidylcholine present is susceptible to enzymatic hydrolysis.

The experiments with combined treatment of stacked disks with phospholipase D and trinitrobenzenesulfonate (Fig. 7) show that only the phosphatidylethanolamine, which is susceptible to phospholipase D action, can be modified by trinitrobenzenesulfonate. Although we cannot exclude the possibility of a rapid 1 : 1 exchange between the pools of non-accessible and accessible unmodified phosphatidylethanolamine, this would not change the overall conclusion of a symmetrical distribution of phosphatidylethanolamine.

The maximal extent of trinitrophenylation of phosphatidylserine is virtually the same, regardless of whether 0, 20 or 40% of the accessible phospholipid

pool is hydrolyzed. This indicates that no net transfer of phosphatidylserine occurs from one pool to the other due to the treatment with phospholipase D. The independence of the rate of the phosphatidylserine trinitrophenylation of prior phospholipase D treatment can only mean that the membrane is not seriously disturbed by either reagent.

Thus, we find that the combined action of phospholipase D and trinitrobenzenesulfonate gives the same results as those obtained with these reagents separately: about one-half of the three major phospholipid classes is accessible in stacked disks. For phosphatidylethanolamine we find that both reagents see the same pool, even though they differ very much in molecular size, mode of action and other properties. Since (1) we start with the original right-side-out configuration, (2) disk structure remains essentially intact, and (3) all phospholipids can in principle be modified (no shielding), we conclude that the accessible phospholipid pool represents the outer face of the disk photoreceptor membrane. This implies phospholipid symmetry in these membranes.

Comparison with previous studies

Our conclusions differ from those of earlier studies on the phospholipid distribution in rod outer segment membranes with amino group reagents. Raubach et al. [2] find 70% of the aminophospholipids on the outer surface when isothionylacetimidate is used as a modifying reagent. Smith et al. [3] find complete modification of all amino groups by trinitrobenzenesulfonate, while more recently, Crain et al. [4] conclude that 63—72% of the phosphatidylethanolamine is located at the outer surface, 18—27% at the inner surface and that 6—14% is not readily available to labelling with trinitrobenzenesulfonate. Phosphatidylserine was found to be located on the outer face at an amount of 25—31% and on the inner face at 25—35% with 35—50% resistant to labeling.

However, these studies have been conducted under conditions, which in our hands give evidence of reagent penetration and membrane disturbance. In all cases, a pH of 8.5 or higher was used, higher trinitrobenzenesulfonate concentrations (4.9 and 2 mM) were used in two cases, and in all three studies disk vesicles were used, which in the trinitrobenzenesulfonate studies were prepared from frozen retinas. Freeze-thawing of disk vesicles causes inversion of rhodopsin molecules, as detected by concanavalin A-labeling studies [13], indicating serious disturbance of the membrane. The results obtained in these experiments agree with our own results for disk vesicles at similar trinitrobenzenesulfonate concentrations and pH (Figs. 1—3). The low level of phosphatidylserine modification in the experiments of Crain et al. [4] at 0° C may be due to the difference in reactivity between it and phosphatidylethanolamine, which may be even more pronounced at low temperature.

Recent experiments of Bishop et al. [14] with monolayers show that in this case, the rate and extent of trinitrophenylation of phosphatidylethanolamine are greatly affected by the fatty acid composition of this phospholipid. Saturated phosphatidylethanolamine reacts much more slowly than dioleoyl phosphatidylethanolamine at the same surface pressure. However, in the highly unsaturated disk membranes (at least six double bonds per phospholipid molecule), the individual phospholipid molecules are probably so far apart that their trinitrophenylation does not cause the spatial restrictions for further

reactivity observed in other membranes.

Another factor, which may interfere with trinitrophenylation of membrane aminophospholipids, could be the presence of negatively charged phospholipids [12,14]. However, a negative surface charge introduced in stacked disk membranes by prior phospholipase D treatment, leading to up to 40% phosphatidic acid, does not seem to influence the rate and extent of trinitrophenylation of the enzyme-resistant phosphatidylserine molecules (Fig. 7). This may be due to the spacing of the phospholipids in the disk membrane, but it is also conceivable that Ca^{2+}, present in high concentration, shields the extra negative charge of the phosphatidic acid.

In our previous paper [1], we have stated that our tentative conclusions on the transbilayer phospholipid distribution rested on the assumption that no induced preferential transbilayer movement of a specific phospholipid and no shielding of outer face phospholipids against phospholipase D occur. The results of the combined treatment with phospholipase D and trinitrobenzenesulfonate indicate that this assumption is valid. Hence, the present findings confirm and strengthen our earlier conclusions obtained with three different phospholipases of a nearly symmetrical distribution of the three major phospholipids, viz., 55–60% of the phosphatidylethanolamine, 40–45% of the phosphatidylcholine and approx. 50% of the phosphatidylserine located on the outer (cytoplasmic) surface of the disk membrane. The question as to whether this symmetrical distribution represents a static or a dynamic situation remains open. Hence, the rate of transbilayer movement of phospholipids in intact disk membranes needs further investigation.

Acknowledgements

The authors wish to thank Dr. J. Olive (Laboratoire de Microscopie Electronique, Université Paris VII, France) for the electron-microscopic analysis of many samples. This investigation has been carried out with financial support from the Netherlands Organization for Basic Scientific Research (ZWO) through the Netherlands Foundation for Chemical Research (SON).

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