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BIOCHEMICAL ASPECTS OF THE VISUAL PROCESS

IV. ALDEHYDES AND CATION PERMEABILITY OF ARTIFICIAL PHOSPHOLIPID MICELLES

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SUMMARY

1. Addition of retinaldehyde to phosphatidylethanolamine liposomes increased the leakage rate of $^{86}Rb^+$. At low concentrations retinol and retinoic acid did not show this effect, while the ion leakage from phosphatidylcholine liposomes was not influenced by any of these vitamin A compounds.

2. Similar results were obtained with the series benzaldehyde, benzylalcohol and benzoic acid; only the combination of benzaldehyde and phosphatidylethanolamine caused an increase in ion leakage.

3. Fluorodinitrobenzene caused an increase in ion leakage from liposomes only if phosphatidylethanolamine was present.

4. The interaction between retinaldehyde and phosphatidylethanolamine was shown to consist of Schiff base formation.

5. The relevance of these findings for the visual process is discussed.

INTRODUCTION

Recently BONTING AND BANGHAM[1,2] presented a hypothesis for the mechanism of the visual process, *i.e.*, the sequence of events between the absorption of a photon by the visual pigment rhodopsin and a nervous impulse.

Part of the evidence for this hypothesis rests on observations of the effects of all-*trans*-retinaldehyde and all-*trans*-retinol on the leakage of $^{42}K^+$ through artificial micelles (liposomes) composed of either phosphatidylcholine or phosphatidylethanolamine. The aim of the present study was to extend these observations and to determine more precisely the role of the aldehyde group in cation leakage of phospholipid liposomes.

MATERIALS AND METHODS

Phosphatidylcholine was isolated according to the method of PANGBORN[3] with minor modifications and characterized as described recently[4]. Phosphatidylethanolamine containing 50% palmitic acid, 30% oleic acid and 20% linoleic acid was syn-

thesized according to DAEMEN[5]. A synthetic compound was used because of the high degree of unsaturation of natural phosphatidylethanolamines compared with natural phosphatidylcholines, while the fatty acid composition of our phosphatidylethanolamine was chosen so as to resemble that of the natural phosphatidylcholine used in the ratio of saturated, unsaturated and polyunsaturated fatty acids.

^{86}RbCl was used as a substitute for ^{42}KCl, since its permeability characteristics are the same and its longer half-life makes it easier to use. All other reagents were of reagent grade. Storage of vitamin A derivatives was as described previously[6]. Benzyl alcohol and benzaldehyde were redistilled prior to use.

Liposome suspensions were prepared as follows. Organic solvent was removed from 40 μmoles of phospholipid in a rotating evaporator. The resulting thin film was suspended in 4 ml of a salt medium: NaCl, 120 mM; RbCl, 15 mM; and Tris–HCl (pH 7.4), 10.0 mM; containing 0.4–0.8 mC of ^{86}Rb. In the case of phosphatidylcholine 3 mg of stearic acid were added to the starting solution to make the liposomes sufficiently permeable to cations. Dispersion of the synthetic phosphatidylethanolamine in the salt medium was impossible at room temperature, but addition of 1.5 mg of the nonionic detergent Tween 80 to the starting solution redressed this inconvenience. In the experiments with FDNB, liposomes consisting of equimolar amounts of both phosphatidylcholine and phosphatidylethanolamine were used without addition of stearic acid or Tween 80. Before use, the liposome suspensions were equilibrated for at least 2 h. Storage of suspensions for over 2 days at 4° increased the cation permeability of the liposomes to undesirable levels.

In order to remove radioactive ions which had not been trapped by the liposomes, a gel filtration method was used[7]. Of the diluted liposome suspension thus obtained, free of nontrapped $^{86}Rb^+$, samples of 1 ml were placed in small dialysis bags, 10 μl of ethanolic reagent solution (experimental bags) or 10 μl ethanol (control bags) were added, and the closed dialysis bags were placed in reagent tubes containing 10 ml of salt medium without $^{86}Rb^+$. The closed tubes were attached to a vertically rotating disk (1 rev./min). After a certain period of dialysis, usually 30 min, the radioactivity of the fluid inside and outside the bags was measured. The amount of radioactivity which had leaked out of the bags may be considered to be equal to the amount which had leaked out of the liposomes[7] and thus is a measure for the leakage rate of cation through these artificial membranous structures. The amount of reagent used was based on the desired molar ratio to phospholipid present. The addition of 10 μl ethanol to the dialysis bag did not affect the $^{86}Rb^+$ leakage rate from the liposome suspension.

RESULTS

Phosphatidylcholine and phosphatidylethanolamine liposomes prepared by our procedure, appeared to sequester 7.6% (S.E. 0.3) and 3.9% (S.E. 0.2), respectively, of the $^{86}Rb^+$ added to the phospholipid suspensions. The control leakage rates from these various types of liposomes also differed to some extent. From phosphatidylcholine liposomes about 10% of sequestered radioactivity leaked out in 30 min, while 15% leaked out from the phosphatidylethanolamine liposomes in the same time.

Addition of all-*trans*-retinol, retinaldehyde or retinoic acid in a molar ratio of 1:100 (vitamin A derivative to phospholipid) to phosphatidylcholine liposomes did not influence cation leakage from these liposomes at all. The same was true for phos-

phatidylethanolamine liposomes, except that addition of retinaldehyde caused a statistically significant increase of the cation leakage of 27% as compared to controls (Fig. 1; Table I). When $^{22}Na^{+}$ was the radioactive cation captured, a significantly increased leakage of Na^{+} was observed upon additon of retinaldehyde to liposomes containing phosphatidylethanolamine in both experiments.

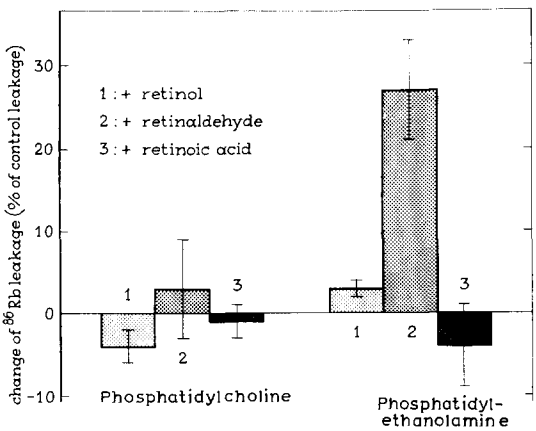

Fig. 1. Effect of vitamin A derivatives on cation leakage from phospholipid liposomes. Molar ratio of vitamin A derivative to phospholipid is 1 : 100. Leakage time, 30 min. For experimental details see MATERIALS AND METHODS.

TABLE 1

EFFECT OF (all-*trans*) VITAMIN A DERIVATIVES ON THE ION LEAKAGE FROM PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE LIPOSOMES

Ion leakage in per cent of control leakage, with standard error, and the number of independent experiments in parentheses (each experiment included 3 control and 3 experimental dialysis bags). Leakage time, 30 min. Molar ratio represents moles of vitamin A derivative added to moles of phospholipid.

Vitamin A derivative	*Phosphatidylcholine liposomes*		*Phosphatidylethanolamine liposomes*	
Molar ratio:	*1:100*	*1:33*	*1:100*	*1:33*
Retinol	$-4\pm2(3)$	$-7\pm3(3)$	$3\pm1(3)$	65 ± 2 (3)
Retinaldehyde	$3\pm6(4)$	$-9\pm5(3)$	$27\pm6(4)$	98 ± 7 (4)
Retinoic acid	$-1\pm2(3)$	$-13\pm6(3)$	$-4\pm3(3)$	$19\pm15(3)$

Increasing the molar ratio of added vitamin A derivatives to phospholipid present to 1:33 did not influence the cation leakage in the case of phosphatidylcholine liposomes, but affected the ion leakage from phosphatidylethanolamine liposomes rather strongly (Table I). While retinaldehyde continued to show the greatest effect, retinol and retinoic acid at this higher molar ratio also began to raise the cation permeability of phosphatidylethanolamine liposomes.

These results clearly suggest a specific interaction between retinaldehyde and phosphatidylethanolamine. In order to distinguish between the influence of the "vitamin A skeleton" and the functional group, the series benzyl alcohol, benzaldehyde, benzoic acid, was applied instead of the analogous vitamin A compounds in

TABLE II

EFFECT OF THE ADDITION OF ARYL DERIVATIVES ON THE CATION LEAKAGE FROM PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE LIPOSOMES IN PER CENT OF CONTROL LEAKAGE

Leakage time, 30 min. Equimolar amounts of aryl derivatives and phospholipid. Each entry represents one experiment with 2 experimental dialysis bags against 2 control dialysis bags.

Aryl derivative	*Phosphatidylcholine liposomes*	*Phosphatidylethanolamine liposomes*
Benzyl alcohol	5	24
Benzaldehyde	2	280
Benzoic acid	−5	28

ion-leakage experiments. An effect was only observed with the combination aldehyde–phosphatidylethanolamine at the high molar ratio of 1:1 (Table II).

In another series of experiments the influence of FDNB, the free amino group reagent of Sanger, was studied. Comparison of liposomes consisting of 50% phosphatidylethanolamine and 50% phosphatidylcholine and of phosphatidylcholine only, showed that at a final concentration of 2 mM FDNB (uncorrected for hydrolysis), an increase in permeability was observed for mixed liposomes only and not for phosphatidylcholine liposomes (Fig. 2). In contrast to the results with the other reagents added, this increase was clearly progressive with time, probably as a result of the rather slow reaction rate of FDNB. At lower final concentrations of FDNB no effects were seen in either type of liposome.

In ion-leakage experiments the actual concentration of the added compound at the lipid–water interphase is of course crucial. Therefore, we estimated the percentage of reagents left in the aqueous phase after centrifugation of the liposome suspensions at 100 000 × *g* for 1.5 h (ref. 8). Measurement of the absorbance of the supernatants at the wavelength of maximal absorption of the added compound against the corresponding control supernatant gave the concentration of each reagent. At a molar ratio of vitamin A derivative to phospholipid of 1:33, 92–95% of retinol, 95–98% of retinaldehyde

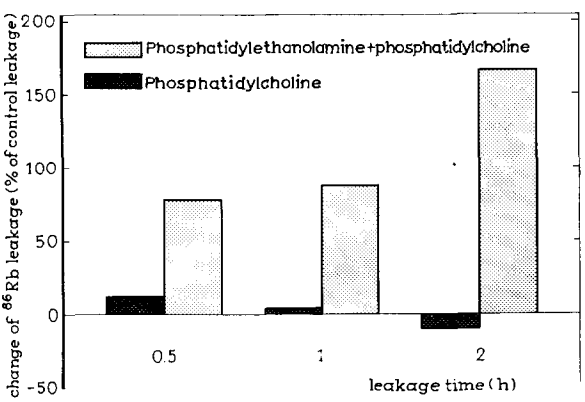

Fig. 2. Effect of 2 mM FDNB on cation leakage from phospholipid liposomes as a function of leakage time. Phosphatidylethanolamine + phosphatidylcholine liposomes consisting of 50% phosphatidylethanolamine + 50% phosphatidylcholine. For further experimental details see MATERIALS AND METHODS.

and 85–87% of retinoic acid was present in the lipid phase with both phosphatidylcholine and phosphatidylethanolamine. This means that the actual concentrations of the three vitamin A derivatives in both types of liposomes are nearly equal. Of the three aryl derivatives, on the other hand, more than 90% remained in the aqueous phase. This explains why benzaldehyde exerts the same ion-leakage effect only at a much higher molar ratio than retinaldehyde does. The same is true for FDNB, which additionally undergoes extensive hydrolysis.

Which type of interaction between phosphatidylethanolamine and an aldehyde is involved in the increased ion leakage? When difference spectra were measured between liposome suspensions of either phosphatidylcholine or phosphatidylethanolamine before and after addition of retinol or retinaldehyde, the absorption maxima of the free compounds were found, except for the combination of phosphatidylethanolamine and retinaldehyde. In the latter case a λ_{max} of 368 nm was found (Fig. 3a), although free retinaldehyde has a λ_{max} of 380 nm under these conditions. This shift to shorter wavelengths is a well-known phenomenon for Schiff bases of retinaldehyde. Further proof for the presence of a Schiff base in this case is supplied by the shift of absorption maximum to 440 nm upon acidification of the suspension by HCl (0.6 M final concentration), indicating the formation of a protonated Schiff base (Fig. 3b). No such shift was noticed upon acidification of the phosphatidylcholine suspension containing retinaldehyde.

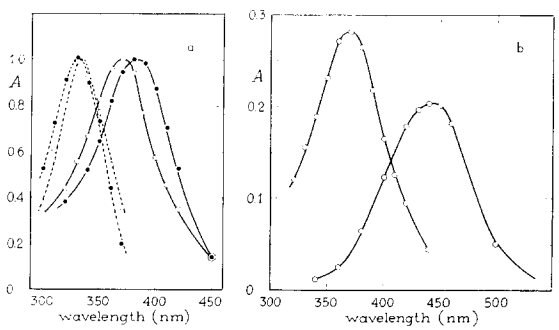

Fig. 3. Difference spectra of liposome suspensions before and after addition of retinol (– – – – – –) or retinaldehyde (———). To correct for light scattering, the blank cuvette contained the liposome suspension to which only solvent (alcohol) had been added. The liposome suspension in the salt medium used throughout the ion-leakage experiments contained as much phospholipid as after the Sephadex-dialysis procedure and was further treated as in those experiments. The molar ratio of vitamin A derivative to phospholipid was about 1:100. (a) The absorbance maxima of the suspension are arbitrarily set at 1.000 for the purpose of comparison. ●, phosphatidylcholine; ○, phosphatidylethanolamine. (b) Effect of addition of 10 μl concentrated HCl to 200 μl phosphatidylcholine suspension to which retinaldehyde had previously been added. The spectrum of the Schiff base with λ_{max} at 368 nm changes to a spectrum characteristic of a protonated Schiff base with λ_{max} at 440 nm.

DISCUSSION

The purpose of this investigation was to study the effects of vitamin A derivatives on cation leakage from phospholipid liposomes. Differences in behaviour between phosphatidylethanolamine and phosphatidylcholine liposomes, such as noted by PAPAHADJOPOULOS *et al.*[9,10], or possibly because of the presence of Tween 80 in our

phosphatidylethanolamine liposomes and stearic acid in our phosphatidylcholine liposomes, could make comparisons between these two types of liposomes difficult. However, the differences in tracer capture and control leakage rate between phosphatidylethanolamine and phosphatidylcholine liposomes in our experiments were not large. This may be due on the one hand to the effect of stearic acid of increasing ion leakage in our phosphatidylcholine liposomes, or on the other hand to our use of a synthetic phosphatidylethanolamine with a fatty acid composition resembling that of natural phosphatidylcholine. PAPAHADJOPOULOS *et al.*[9,10] used natural phosphatidylethanolamine with a much higher degree of unsaturation than in their other phospholipids. The influence of fatty acid unsaturation on liposome behaviour has been reported by DE GIER *et al.*[11]. In order to preclude all ambiguity, all experimental results in this study were referred to control experiments with the same liposome preparation to which no vitamin A derivative or other reagent was added.

Molar ratios of vitamin A derivative to phospholipid higher than 1:33 were not taken into consideration because they are not relevant from a physiological point of view. In the rod sac the molar ratio of vitamin A derivative to amino groups is smaller[4], and under physiological conditions more than 3% photolysis of rhodopsin does not normally occur.

The specific effect of retinaldehyde on the cation leakage (Na^+, K^+, Rb^+) in phosphatidylethanolamine liposomes, confirmed in this study, must be due to the aldehyde group. First of all the three vitamin A derivatives were all predominantly present in about the same concentration at the lipid–water interphase, thus ruling out the possibility that the retinaldehyde effect could be due to concentration differences at this interphase. In the second place in the experiment with the three aryl derivatives again only the combination aldehyde–phosphatidylethanolamine gave a large increase in cation leakage (Table II). The other essential factor is the presence of the amino group in the liposome membrane. This is confirmed by our finding that the amino group reagent FDNB also increased the cation leakage in phosphatidylethanolamine-containing liposomes, but not in liposomes containing solely phosphatidylcholine.

The attribution by BONTING AND BANGHAM[1,2] of the specific interaction between phosphatidylethanolamine and aldehydes to Schiff base formation could be conclusively confirmed. In addition to the blue shift in the absorption maximum to 368 nm at neutral pH (Fig. 3a), subsequent acidification caused a red shift to 440 nm (Fig. 3b) indicative of protonation of the Schiff base[12,13]. The overall equation for the Schiff base formation is:

$$R\text{–}NH_3^+ + O{=}\underset{H}{C}\text{–}R' \rightarrow R\text{–}N{=}\underset{H}{C}\text{–}R' + H_2O + H^+$$

At neutral pH the amino group of phosphatidylethanolamine is largely protonated, but the resulting Schiff base is not, resulting in the release of a proton and the removal of a positive charge from the membrane.

The blocking of the protonated amino group by Schiff base formation should therefore result in a more negative surface charge of the phosphatidylethanolamine liposome. This was shown to be the case by BONTING AND BANGHAM[1,2] by means of measurements of the electrophoretic mobility of the liposomes. The increasing negativity of the liposomal surface charge indeed leads to a greatly increased cation leakage

according to the observations of BANGHAM *et al.*[7]. Thus the increased cation leakage from phosphatidylethanolamine liposomes upon addition of retinaldehyde can be considered to be explained.

What is the relevance of these model experiments for our understanding of the mechanism of visual excitation? Considerable evidence, reviewed by BONTING[14], has accumulated, indicating that the transduction of the light impulse to the synaptic stimulation involves an ionic process. BONTING AND BANGHAM[1,2] have demonstrated a net Na^+ influx and a net K^+ efflux upon illumination of isolated cattle rod outer segments. A more direct proof of a sudden Na^+ influx and K^+ efflux in frog rod outer segments within 15 sec of illumination, has been obtained by DUNCAN *et al.*[15] by means of tracer studies in suspensions of isolated outer segments. A similar cation exchange was observed upon addition of all-*trans*-retinaldehyde to the isolated rod outer segments[1,2,15]. This effect was given by all-*trans*- and 11-*cis*-retinaldehyde, but not by retinol and retinoic acid[6]. These findings suggest that the process observed in the liposome experiments occurs in a similar form in rod outer segments.

Schiff bases of retinaldehyde play a crucial role in the visual process. During the photolytic transition of the visual pigment rhodopsin to metarhodopsin II, retinaldehyde moves from an attachment to phosphatidylethanolamine[16], where it is not affected by aqueous $NaBH_4$ or lipid oxidase, to a link with an ε-amino group of lysine, where it can be attacked by these agents[17,18]. Blocking of the latter group in erythrocytes by FDNB has been shown to lead to a large increase in Na^+ and K^+ leakage[19], suggesting that this group guards a Na^+ channel in the erythrocyte membrane and that blocking of the group opens the Na^+ channel. A similar role for this group in the rod sac membrane seems indicated. In the dark-adapted state of the rod sac membrane, retinaldehyde would be linked to phosphatidylethanolamine in the rhodopsin complex, and the ε-amino group of lysine would be free and keep the Na^+ channel closed. Illumination would switch retinaldehyde to this ε-amino group of lysine, thereby opening the Na^+ channel and allowing an influx of Na^+ along the concentration gradient, followed by an efflux of K^+. This would constitute a depolarizing current, which would stimulate the synapse with the bipolar cell.

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