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The ionization behavior of retinoic acid in lipid bilayers and in membranes

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The ionization behavior of retinoic acid (RA) incorporated in unilamellar vesicles of different lipid compositions and in biological membranes was studied. Titration of RA in the various membranes was followed by monitoring the red shift in the absorption maximum of RA that occurred upon deprotonation. It was found that, similar to other hydrophobic carboxylic acids, the protonated form of RA is stabilized by incorporation into bilayers vs. RA monomers in an aqueous phase. The p*K* of RA in bilayers comprised of neutral phospholipids was approximately 7 regardless of the composition of the fatty acyl chains. Incorporation of RA in bilayers comprised of negatively charged phospholipids stabilized the protonated form to a larger extent vs. neutral lipids, resulting in p*K*'s that were about 1 pH unit higher. The ionization behavior of RA in plasma membranes from rat liver and in erythrocyte membranes was similar to its behavior in negatively charged bilayers. The data indicate that RA incorporated in membranes is predominantly protonated at physiologic pH.

Introduction

Retinoids are known to affect the properties of lipid bilayers and membranes. These compounds increase the permeability of membranes to ions and to non electrolytes [1–5], modify the gel to liquid crystalline phase transition of bilayers [4,6], and their presence increases the molecular ordering within lipid bilayers and membranes [6–9]. Few studies in which the location of retinoids within bilayers and the interactions of these ligands with phospholipids were reported. It was concluded from NMR and ESR studies of all-*trans*-retinol and -retinoic acid incorporated in lipid bilayers that the end groups of the retinoids are anchored at the polar group region of the bilayers and that the polyene chain extends into the bilayer along the lipid chains [6,9]. A recent study of the kinetic parameters of the interactions of retinol with lipid bilayers suggested that a rate determining step for dissociation of retinol from lipid bilayers involves breaking a hydrogen bond between the hydroxyl end group of retinol and the PC head group region [10]. This conclusion agrees with the positioning of the end group of retinoids close to the head group of phospholipids within a bilayer.

Some discrepancies exist in the literature regarding the details of the interactions of retinol and retinoic acids with lipid bilayers. It was concluded from the NMR study mentioned above [6] that the perturbation of bilayer packing by retinol is more pronounced than by retinoic acid, an observation that was interpreted by the authors to indicate that the solubility of retinoic acid in lipid bilayers is lower than the solubility of retinol. On the other hand, EPR measurements showed that retinoic acid displayed more pronounced membrane effects vs. retinol [9,11]. It was suggested in that study [9] that due to the strongly hydrophilic nature of the end group of retinoic acid, when retinoic acid is incorporated in bilayers comprised of lipids with saturated acyl chains, it is placed higher within the membrane as compared to retinol, so that the carboxyl group projects into the aqueous interface. It was further suggested [11,12] that when incorporated in bilayers comprised of lipids with unsaturated acyl chain (18:1 Δ9,*cis*), the 'bend' associated with the *cis* configuration double bond of the lipid acyl chain creates a space into which the cyclohexane ring of a retinoid is constrained to fit, and that in such bilayers, a lower location will be imposed on retinoic acid.

An important question regarding the interactions of retinoic acid (RA) with bilayers is whether the carboxyl group of this retinoid is protonated or whether it is negatively charged at physiological pH. The ionization

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state of RA will influence the interactions of this retinoid with the head groups of phospholipids and with water at the membrane interface, which may, in turn, affect the location of this retinoid within the bilayer and its interactions with the hydrophobic core. In the present study, the ionization state of RA incorporated in unilamellar vesicles comprised of different phospholipids and in membranes was investigated.

Materials and Methods

Lipids were obtained from Avanti Polar Lipids. All-*trans*-retinoic acid was obtained from Kodak. All other chemicals were from Sigma Chemical Co. Male Wistar rats weighing about 300 g were obtained from Charles River Breeding Laboratories.

Membranes. Unilamellar vesicles were formed by sonication [13] prior to the addition of RA. Phospholipids in chloroform solution were pipetted into a flask and the organic solvent was removed in a rotary evaporator. The lipid residue was further dried under vacuum for 2 h and hydrated for 1 h in 50 mM Hepes (pH 7.5) containing 100 mM KCl. Lipids were frozen and thawed 10 times to obtain a fully hydrated suspension of multilamellar vesicles (MLV's). Sonicated vesicles were prepared from the MLV suspension using a Heat-System sonicator (W350 cup horn) in a sealed tube under a nitrogen atmosphere, and centrifuged at 100000 × *g* for 15 min to pellet multilamellar vesicles. Rat liver plasma membranes were isolated on a discontinuous sucrose gradient as described in Ref. [14]. Erythocyte ghosts were prepared from rabbit red blood cells (Pel-Freeze, Rodgers, AK). Cells were lysed according to Dodge et al. [15]. Erythocyte ghosts were prepared using a Milipore Pellicaon Cassette system with a HVLP 0.45 μm filter [16]. Ghosts were washed twice with 0.15 M NaCl in 5 mM Na_2HPO_4 (pH 8.0) to remove the spectrin and the last traces of hemoglobin, re-suspended in 36 mM Na_2HPO_4 (pH 7.5) and frozen rapidly. Concentrations of phospholipids in vesicles and in membranes were determined by the phosphorus content [17].

Titration of retinoic acid incorporated in membranes. Vesicles or membranes were diluted with water to a final concentration of about 150 μM. The final mixture contained 0.5 mM Hepes (pH 7.5) and 1 mM KCl. RA was added from a concentrated solution in ethanol to a concentration of 2 mol% RA/lipids. This low concentration of RA was chosen because it is not expected to affect the properties of the bilayers significantly [6,9,11] but is high enough to be observed by its absorption. The mixture was incubated for 30 min, and the titration was carried out by addition of dilute solutions of either HCl or NaOH, the pH was measured by a Sigma Tris electrode using a Radiometer Research Grade pH meter. The absorption of the mixture at various pH values was measured using a computer driven Cary-14 spectrophotometer (On-Line Instruments). Buffers and assay mixtures were purged with argon before use to minimize oxidation of RA, and mixtures were kept in the dark whenever possible. Titrations and absorption measurements were carried out in dim light.

Calculation of theoretical Henderson-Hasselbach curves. To calculate the theoretical Henderson-Hasselbach curves, the limiting ratios of 340 nm/380 nm or 340 nm/360 nm (R_{max} and R_{min}) were used to express the fractions of ionized RA.

$$[RA^-]/([RA^-]+[RAH]) = (R - R_{min})/(R_{max} - R_{min}) \quad (1)$$

RA^- and RAH in expression 1 represent the concentrations of the ionized and the protonated RA, respectively. Expression 1 was solved for the ratio $[RA^-]/[RAH]$. This ratio was substituted into the Henderson-Hasselbach equation and Eqn. 2 was obtained.

$$pH = pK + \log[(R - R_{min})/(R_{max} - R)] \quad (2)$$

The experimentally obtained pK values was used in Eqn. 2 to calculate pH values for given R values.

Results

Effect of pH on the absorption spectrum of RA incorporated in lipid bilayers

The absorption spectra of RA incorporated in vesicles of 1-palmitoyl,2-oleoylphosphatidylcholine (POPC) at pH values 4 and 10 are shown in Fig. 1. The absorption maxima were at 336 nm and at 360 nm at pH 10.5 and at pH 3.0, respectively. λ_{max} shifted continuously as a function of the pH between the constant

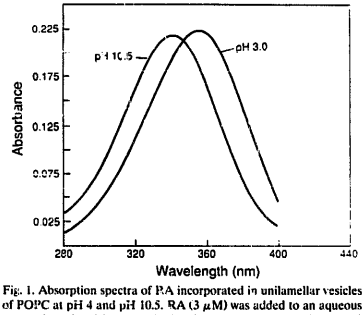

Fig. 1. Absorption spectra of RA incorporated in unilamellar vesicles of POPC at pH 4 and pH 10.5. RA (3 μM) was added to an aqueous suspension of vesicles comprised of POPC (150 μM lipids). The pH of the mixture was adjusted by the addition of either NaOH or HCl.

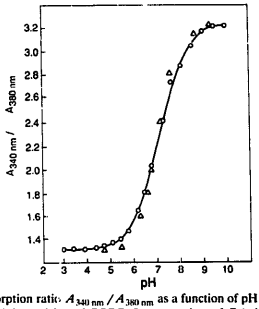

Fig. 2. Absorption ratio $A_{340\,nm}/A_{380\,nm}$ as a function of pH for RA incorporated in vesicles of POPC. Incorporation of RA into the vesicles and titration were carried out as described in Methods. Circles and triangles represent, respectively, experimental points and points calculated for the theoretical Henderson-Hasselbach curve (see Methods).

values reached at low and at high pH ranges. The shift in λ_{max} was completely reversible when the pH of the solution was adjusted from 10 to 4 and back. The progression of the shift in λ_{max} was monitored by following the absorption ratio $A_{340\,nm}/A_{360\,nm}$. This ratio as a function of pH was a sigmoidal function that reached constant values at both low and high pH values (Fig. 2). At these extremes of pH, it was assumed that RA existed as the fully protonated form or as the anion, respectively. An apparent pK for RA could thus be obtained at half the maximal change. The value of the pK for RA in bilayers of POPC was 7.1 (Table I). The experimental titration curve was compared with the theoretical Henderson-Hasselbach curve (calculated as detailed in the Methods) and showed very good agreement (see Fig. 2). Incorporation of RA into lipid bilayers thus seems to stabilizes the protonated form of RA as compared with RA monomers in an aqueous solution, the pK of which is < 6.1 (accompanying manuscript, Ref. 33). This effect of lipid bilayers on the ionization behavior of RA is similar to the effect of phospholipids on the ionization of fatty acids and bile acids [18–20]; e.g. it was found that the pK values of cholic acid monomers in water and of cholic acid in egg yolk phosphatidylcholine vesicles were 4.98 and 7, respectively [18].

The pK of RA in unilamellar vesicles comprised of neutral phospholipids

The shift in the absorption maximum of RA upon ionization was utilized to measure the pK of RA incorporated in unilamellar vesicles comprised of different phospholipids. Shown in Fig. 3 are representative titration curves of RA incorporated in the bilayers of vesicles comprised of PC's with acyl chains with 18 carbons and a single double bond: dioleoylPC (18:1 Δ9,*cis*), dielaidoylPC (18:1 Δ9,*trans*), and dipetroselinoyl (18:1 Δ6,*cis*). The apparent pK values of RA incorporated in these bilayers are given in Table I. The data show that the pK of RA in all of these bilayers is in the narrow range 6.8–7.1. The presence of a 'bend' in the lipid acyl chain, which is present in the lipids with a *cis*-configuration double bond and is absent in the *trans*-configuration (dielaidoylPC), and

TABLE I

Apparent pK values of retinoic acid incorporated in unilamellar vesicles of different phospholipids and in membranes

The pK values were obtained from titration curves as described under Methods and in the legends to the figures. Values presented are means of two determinations for each membrane population. Variations between measurements were 0.05–0.1 pH units.

Bilayer	pK
Neutral lipids	
DioleoylPC (18:1 Δ9,*cis*)	6.9
DielaidoylPC (18:1 Δ9,*trans*)	6.8
Dipetroselinoyl PC (18:1 Δ6,*cis*)	6.8
Palmitoyl,oleoylPC (16,18:1 Δ9)	7.1
70% dioleoylPE + 30% dioleoylPE	6.9
Anionic lipids	
Dioleoylphosphatidic acid (DOPA)	8.0
90% dioleoylPC + 10% DOPA	8.0
Palmitoyl,oleoylphosphatidylglycerol	8.4
Membranes	
Rat liver plasma membranes	8.4
Erythocytes	7.8

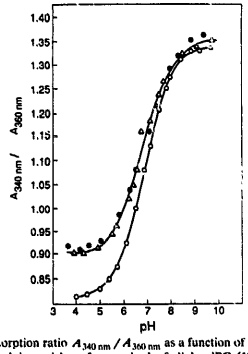

Fig. 3. Absorption ratio $A_{340\,nm}/A_{360\,nm}$ as a function of pH for RA incorporated in vesicles of comprised of dioleoylPC (18:1 Δ9,*cis*) (○), dielaidoylPC (18:1 Δ9,*trans*) (△) and dipetroselinoylPC (18:1 Δ6,*cis*) (●).

the exact location of such a 'bend' ($\Delta 9$ in DOPC and $\Delta 6$ in dipetroselinoylPC) did not affect the pK of RA incorporated in these bilayers (see Discussion). RA was also titrated following its incorporation into vesicles comprised of the mixture 70% dioleoylPC and 30% dioleoylphosphatidylethanolamine. The value of the apparent pK values for RA in bilayers comprised of this mixture and in bilayers comprised of POPC discussed above (Table I) indicate that the presence of an asymmetric phospholipid (POPC) or of another neutral phospholipid (DOPE) also does not affect the pK of RA incorporated in bilayers.

The ionization behavior of RA in unilamellar vesicles comprised of anionic lipids

In Fig. 4 are shown representative titration curves of RA incorporated in unilamellar vesicles comprised of phospholipid which carry a net negative charge: dioleoylphosphatidic acid (DOPA) and 1-palmitoyl,2-oleoylphosphatidylglycerol (POPG). The pK values of RA incorporated in these bilayers (Table I) were 1–1.5 pH units higher vs. RA incorporated in neutral phospholipids, indicating that the interactions between RA and lipids in these bilayers stabilize the protonated form of RA to a greater extent than the interactions in neutral lipids. When RA was incorporated in vesicles comprised of 10% DOPA and 90% DOPC, the pK was 8.0, which was identical to the pK of RA in pure DOPA bilayers. This observation indicates that RA in the mixed bilayer is preferentially associated with DOPA (see Discussion).

The ionization behavior of RA in intact membranes

In Fig. 5 are shown representative titration curves for RA incorporated in plasma membranes isolated from rat liver and in membranes isolated from erythocytes. The pK values derived from these titrations (Table I) indicate that the ionization behavior of RA in these membranes is similar to the behavior in bilayers comprised of negatively charged phospholipids. The shift in pK to higher values was more pronounced in the liver plasma membranes than in the erythocytes membranes (see Discussion).

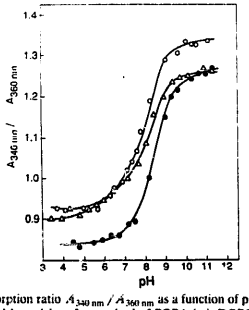

Fig. 4. Absorption ratio $A_{340\,nm}$ / $A_{360\,nm}$ as a function of pH for RA incorporated in vesicles of comprised of POPA (○), DOPC+POPA (△) and POPG (●).

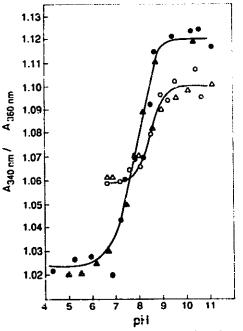

Fig. 5. Absorption ratio $A_{340\,nm}$ / $A_{360\,nm}$ as a function of pH for RA incorporated in plasma membranes isolated from rat liver (○, △) and from erythocytes (●; ▲). Circles represent experimental points and triangles represent points calculated for the theoretical Henderson-Hasselbach curve (see Methods).

Discussion

The data obtained in the present study clearly indicate that incorporation of RA into lipid bilayers and into membranes stabilizes the protonated form of RA vs. RA monomers in an aqueous phase. Thus, the values of the pK of RA in various bilayers and membranes were in the range 6.8–8.5 while the pK of monomeric RA in water is lower that 6.1 (accompanying paper [33]). A shift in the pK values of amphipathic carboxylic acids to higher values by incorporation into phospholipid bilayers has been reported for fatty acids and for bile acids [18–21] and seems then to be a general outcome of the interactions of such acids within phospholipid bilayers. These phenomena most likely reflect the partial shielding of the carboxyl group from the bulk aqueous phase by the less polar environment of the lipid bilayer.

The ionization behavior of RA incorporated in bilayers of phosphatidylcholines with different acyl chain compositions was studied. The acyl chains tested included 18:1 $\Delta 9$,*cis* and 18:1 $\Delta 9$,*trans*. These were studied in order to investigate the effect of the presence of a 'bend' in the acyl chain, which is introduced

by the *cis*-configuration double bond and is absent in the *trans*-configuration, on the accessibility of the carboxyl group of RA to the aqueous phase, as can be monitored by its pK. Thus, if the presence of a *cis*-configuration double bond imposes a lower (more shielded) location within the membrane on retinoic acid vs. the presence of a straight chain, as was postulated (Ref. 11, see Introduction), the pK of RA would be higher in bilayers with a *cis*- vs. a *trans*-double bond. The data (Table I) show that this is not the case. Neither the presence of a *cis*-configuration double bond nor the location of such a bond had any significant effect on the pK of RA incorporated in the bilayers. In addition, titration of RA incorporated in bilayers with asymmetric acyl chains (POPC), and in bilayers with mixed head groups (DOPC + DOPE) showed that these variations also did not affect the accessibility of the RA end group to the aqueous phase, i.e. the positioning of RA within the bilayers. In fact, the pK values of RA incorporated in all the bilayers comprised of neutral (zwitterionic) phospholipids studied were found to be in the narrow range 6.8–7.1.

The ionization behavior of RA incorporated into bilayers of the negatively charged phospholipids DOPA and DOPG was also studied. The data show that the protonated form of RA is stabilized to a larger extent within bilayers of the acidic vs. the neutral phospholipids studied, as is evidenced by the shift in the pK of RA to higher values (Table I). Theoretically, the pK of acids incorporated in lipid bilayers will be influenced by: (1) the local H^+ concentrations; and (2) the environment of the ionizable group, i.e. its location within the bilayer [21]. The negative surface charge of bilayers comprised of anionic lipids creates a H^+ concentration gradient so that concentrations of H^+ are higher at the lipid/water interface vs. the bulk water [22]. This concentration gradient may affect the pK of carboxylic acids incorporated in the bilayers. The shift in the pK of RA in bilayers comprised of acidic phospholipids may also reflect a deeper location within these bilayers as compared to its location within bilayers of neutral lipids. We would like to suggest that the observations may be explained, at least partially, by the difference between the preferred molecular conformation of zwitterionic phospholipids [23–26] and the conformation of acidic phospholipids [27,28]. As is shown in Fig. 6, the hydrocarbon chains in both zwitterionic and acidic phospholipids are stacked parallel to each other and are oriented perpendicular to the plane of the bilayer. However, the glycerol backbone is oriented approximately perpendicular to the plane of the bilayer in zwitterionic lipids (Fig. 6a) and parallel to the plane of the bilayer in acidic lipids (Fig. 6b). Consequently, the head group of the lipid (R in Fig. 6) is extended further into the aqueous phase in neutral vs. in acidic lipids. Since, as was discussed in the Introduction, RA within bilayers is positioned with the end group close to the phospholipids head groups, the carboxyl group will be extended further into the aqueous phase along the head group of a zwitterionic lipid and will be located deeper in a bilayer comprised of acidic lipids. The observations reported here, indicating that the protonated form of RA is stabilized to a larger extent upon incorporation of this ligand in acidic vs. neutral phospholipids, can thus be understood in terms of a lower, more shielded, location of RA in the negatively charged bilayers.

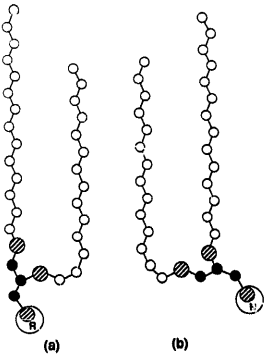

Fig. 6. Preferred molecular conformations of dimyristoylphosphatidylcholine (a) and dimyristoylphosphatidic acid (b) showing the difference in glycerol back bone orientation (see text).

The data in Table I show that incorporation of 10 mol% of the negatively charged lipid DOPA in vesicles of DOPC containing RA, was sufficient to result in a pK of RA that was identical to the pK in pure DOPA bilayers. This pK value was 1 pH unit higher that the pK of RA in pure DOPC bilayers. This observation may reflect stabilization of the protonated form of RA by the negative surface charge of bilayers containing acidic lipids as discussed above. If this interpretation is correct, it seems that a maximal effect was reached with 10 mole% of acidic lipid in an otherwise neutral bilayer. Alternatively, the data can be understood by specific interactions of RA with acidic lipids resulting in preferential association of RA with negatively charged lipids within a mixed bilayer. Such an association may be stabilized by formation of a hydrogen bond between the negative charge of the oxygens of the phosphate in the end group of DOPA and the proton of the RA carboxyl group.

Further studies are needed to clarify the origin of the apparent increase in pK of RA by the presence of acidic lipids. The results, however, indicate that these

effects may have important physiological implications. Thus, the pK values of RA incorporated in plasma membranes isolated from rat liver and from erythocytes (Table I) show that although a large fraction of the lipids in intact membranes is comprised of neutral lipids (PC's and PE's), RA, in these membranes, displayed a pK which was shifted to higher values. Based on the results of the present study, these observation are likely to reflect the presence of negatively charged lipids. It is interesting to note, in regard to this, that liver plasma membranes, in which the pK of RA to was shifted 8.45, contain a larger fraction of acidic lipids than erythocyte membranes [29] in which the pK of RA was found to be 7.75. Since most biological membranes carry some negatively charged lipids, a general conclusion that can be drawn from the data in the present work is that RA in membranes will be predominantly protonated at physiological pH. One important implication of this conclusion is that biological membranes are not likely to constitute a barrier for transport of RA between cells and subcellular organelles. The rate by which amphipathic carboxylic acids cross lipid bilayers is dramatically affected by their ionization state. RA in membranes, at physiological pH, will be a neutral compound and can be expected to traverse membranes in a manner similar to another neutral retinoid, all-*trans*-retinol, that was shown to rapidly and spontaneously cross membranes [10].

Since the ionization behavior of other amphipathic carboxylic acids like fatty acids and bile acids, when incorporated in bilayers comprised of zwitterionic phospholipids, is similar to the behavior of RA reported here [18–20], we would like to suggest that the presence of acidic lipids will affect these compounds in the same manner it affects RA, i.e. that their pK will be shifted to higher values and that, within biological membranes a predominant fraction of amphipathic carboxylic acids will be protonated.

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