Mass Spectrometric Identification of Phospholipids in Human Tears and Tear Lipocalin

Austin W. Dean and Ben J. Glasgow

PURPOSE: The purpose of this article was to identify by mass spectrometry phosphocholine lipids in stimulated human tears and determine the molecules bound to tear lipocalin or other proteins.

METHODS: Tear proteins were separated isocratically from pooled stimulated human tears by gel filtration fast performance liquid chromatography. Separation of tear lipocalin was confirmed by SDS tricine gradient PAGE. Protein fractions were extracted with chloroform/methanol and analyzed with electrospray ionization MS/MS triple quadrupole mass spectrometry in precursor ion scan mode for select leaving groups. For quantification, integrated ion counts were derived from standard curves of authentic compounds of phosphatidylcholine (PC) and phosphatidylserine.

RESULTS: Linear approximation was possible from integration of the mass spectrometrically obtained ion peaks at 760 Da for the PC standard. Tears contained 194 ng/mL of the major intact PC (34:2), m/z 758.6. Ten other monoisotopic phosphocholines were found in tears. A peak at 703.3 Da was assigned as a sphingomyelin. Four lysophosphatidylcholines (m/z 490–540) accounted for about 80% of the total integrated ion count. The $[M+H]^+$ compound, m/z 496.3, accounted for 60% of the signal intensity. Only the tear lipocalin-bearing fractions showed phosphocholines (104 ng/mL). Although the intact phospholipids bound to tear lipocalin corresponded precisely in mass and relative signal intensity to that found in tears, we did not identify phosphocholines between m/z 490 and 540 in any of the gel-filtration fractions.

CONCLUSIONS: Phospholipids, predominantly lysophospholipids, are present in tears. The higher mass intact PCs in tears are native ligands of tear lipocalin. (*Invest Ophthalmol Vis Sci*. 2012;53:1773–1782) DOI:10.1167/iovs.11-9419

The tear film provides a transparent, refractive, and protective coating over the ocular surface to promote clear vision. The historical model of the tear film architecture includes 3 layers. The outermost layer of the tear film is presumably lipid, but the exact composition and structure remain elusive.¹ Interference fringes observed at the surface

have been attributed to lipid multilayers with a dynamically changing thickness measured in the range of 15 to 370 nm.^{1–4} Tear proteins, particularly tear lipocalin (TL), are surface active^{5–7} and may intercalate with lipids at the air-tear interface.⁸ The underlying aqueous layer of tears has an estimated thickness of 1.7 to 46.0 μ m, which contains many proteins and soluble mucins.^{1,9–11} The aqueous layer envelops a transmembrane glycocalyx, which may project 500 nm from the surface epithelium.^{12,13}

Lipids have a number of potential functions in the tear film. A simple lipid monolayer film spread over aqueous retards evaporation by more than 50%. The reduction is related to alkyl chain length.¹⁴ Water reclamation departments have exploited this principle with C16–C18 lipids (similar in alkyl chain length to meibum lipids) applied on reservoirs for more than 60 years.¹⁵ Numerous ophthalmic publications posit that accelerated evaporation of tear fluid worsens dry-eye diseases in which lipids are deficient.^{16–18} Several therapeutic tear substitutes for dry eye contain phospholipids so as to replenish the depleted phospholipids presumed to be absent in meibomian deficiency in dry eye^{19–21}; however, the presence of phospholipids in normal tears is still contested.^{8,22–24}

Lipids and proteins in tears may work in concert as antimicrobial agents. The ocular surface is the interface of a hostile environment that is assaulted by eyelid microbial flora, including potential pathogens. The major protein components of tears, lysozyme, lactoferrin, lipocalin, phospholipase A2, immunoglobulins, and defensins all possess antimicrobial function with varying targets, actions, and specificity.^{25–30} Various types of lipids in tears have been shown to possess antimicrobial activity in other systems. Fatty acids and glycolipids are active against a variety of bacteria, viruses, and fungi.^{31–33} Lauric acid is bactericidal to *Propionibacterium acnes*.³⁴ Oleic acid is bactericidal to *Staphylococcus aureus* and disrupts enveloped viruses.^{35,36} Some fatty acids are fungicidal.³⁷ Phospholipids show antimicrobial activity to *S. aureus*.³³ The presence and exact type of phospholipids in tears have been debated and require further elucidation.^{1,38}

Phospholipids may also function to stabilize the tear film by lowering surface energy. In particular, phospholipids interact with specific tear proteins to contribute to stability in a non-Newtonian fashion.^{39,40}

The sources of lipids in tears include meibomian gland secretion, lacrimal gland secretion, and cellular degradation from the ocular surface. The studies investigating the lipid components in meibum for the past 50 years were recently reviewed.¹ There is accord that meibum contains an assortment of neutral and polar lipids, including wax esters, hydrocarbons, triacylglycerides, glycolipids, and fatty acids. Recent advances in mass spectrometric techniques have enhanced specification of individual lipid molecules.^{24,41–46} Nonetheless, the presence of phospholipids remains controversial. Although several remote^{9,47–49} as well as recent studies²⁴ using a variety of techniques have shown significant phospholipids in meibum, some studies^{22,43–45,50} challenged

From the Departments of Ophthalmology, Pathology, and Laboratory Medicine, David Geffen School of Medicine at the University of California Los Angeles, Jules Stein Eye Institute, Los Angeles, California.

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Corresponding author: Ben J. Glasgow, 100 Stein Plaza, B-269, Jules Stein Eye Institute, Los Angeles, CA 90095; bglasgow@mednet.ucla.edu.

TABLE 1. Studies of the Lipid Composition in Human Tears

Reference	DAG	TAG	Wax Ester	Steryl Ester	Cholesteryl Ester	Cholesterol	Fatty Alcohol	Fatty Acid	Glycolipid	Phospholipid
47	+	+	+	+		+	+	+		
55						5 mM				
56						0.8 mM				
58		6.9			9.7	7.1		18.3	55.0	0.9
57						1.45 mM				
54		+		45		15	+	<15	+	+
51		+			+	+	+	+	+	+
59								+		
8,38,60	+	+	+	+	+	0.5%–1.0%				negative†/+ small
24,53										+
22	+	+		+						negative
23		0.01 mM			0.02 mM					88

* + = positive but not further specified.

† Extraction performed on purified lipocalin rather than tears using plastic ware.

these findings because significant quantities of phospholipids failed to be identified in meibum by electrospray ionization mass spectrometry and atmospheric pressure ionization mass spectrometry. Classes of lipids in human tears have been identified that are similar to those in meibum.^{22,24,41,51–59} Although studies in tears are often limited to specific types of lipids, phospholipid results are the most discrepant and need clarification (Table 1).

Many tear lipids contain long alkyl chains, which would render them essentially insoluble in aqueous. The lipids are considered to reside at the tear film–air interface or are bound to protein. The major lipid-binding protein in tears is TL, also known as von Ebner gland protein or sometimes imprecisely referred to by the name for the gene, lipocalin-1 (*LCN-1*).⁵¹ Sixteen years ago, our laboratory extracted lipids from tears and showed by thin-layer silica chromatography that a number of types of lipids were present, including fatty acid, phospholipid, fatty alcohol, glycolipid, cholesterol, and cholesterol ester classes. All of these components, except cholesterol esters, were discovered in extracts of purified TL.⁵¹ Abundant palmitic and stearic acids were further confirmed by mass spectrometry, but specific phospholipid molecules were not further analyzed. More sensitive and specific methods of identification in both TLC and mass spectrometry are now available; however, in studies of phospholipids in tears and bound to TL, the results are discordant.^{8,22–24,60} Some have proposed that phospholipids are not found in tears because phospholipases degrade them into phosphatidic acid and fatty acids.²² Phospholipases A2 and C are known to be extant in tears. Others have claimed that tears are replete with phospholipids that interact with phospholipid-specific proteins, such as phospholipid transfer protein.²³

Given the potential significance of phospholipids in tears and the polemic debate over their presence, we used electrospray ionization tandem mass spectrometry to extend our prior chromatographic findings. In addition, we searched for phosphocholines bound to other proteins, and further specified the native phospholipid ligands of TL.

MATERIALS AND METHODS

Lipid standards, L- α -phosphatidylcholine type XVI from egg yolk, L- α -phosphatidyl-L-serine and L- α -phosphatidylethanolamine from bovine brain, chloroform, and methanol, spectrophotometric grade, were obtained from Sigma-Aldrich (St Louis MO); formic acid and 2-propanol were purchased from Fisher Scientific (Fair Lawn, NJ); and ammonium

acetate was a product of J.T. Baker Chemical Company (Phillipsburg, NJ).

Collection of Human Tears

The research was performed in accordance with the tenets of the Declaration of Helsinki. Informed consent was obtained from donors of human tears after explanation of the nature and possible consequences of the study; the procedures were approved by the institutional review board. Stimulated human tears were collected from healthy volunteers and pooled as previously described,^{61,62} except that the samples were collected with polished glass tips and glass transfer pipettes, then placed in polytetrafluoroethylene-lined glass vials to be stored under nitrogen at -80°C .

Gel Filtration Chromatography

Fractionation of tear proteins was performed by liquid chromatography (ÄKTA purifier Versatile FPLC) over a Superdex 75 HiLoad 16/60 preparative grade column with a bed volume of 120 mL (GE Healthcare, Piscataway, NJ). The column was prewashed in 30% acetonitrile and equilibrated in 0.050 M Tris-HCl, pH 8.4, 0.1 M NaCl. In a typical purification experiment, 0.8 mL of tears were loaded for each run and separated under isocratic conditions at a flow rate of 1 mL per minute. Protein peak fractions were monitored at 280 nm; fractions corresponding to individual peaks were collected and pooled for lipid extraction. Samples from pooled fractions of the chromatographic separation were analyzed by SDS tricine gel polyacrylamide electrophoresis as previously described, except a 4% to 12% polyacrylamide gradient gel was used.^{3,4}

Lipid Extraction

Lipid extraction was performed on whole tears, pooled column fractions, and column elution buffer, as well as blank buffer, according to previously published methods using chloroform/methanol (volume fraction = 0.5) to extract 0.8 mL of each solution.⁶³ Chloroform and water were then added to form a chloroform, methanol, and water mixture $\phi_A = 0.4$, $\phi_B = 0.4$. After extraction, the chloroform phase was collected and evaporated under ultrapure nitrogen gas (Air Liquide America L.P., Houston, TX). Samples were then redissolved in 100 μL of the appropriate organic solvent for mass spectrometry (see the following section).

Mass Spectrometry

Mass spectrometry was performed with an Agilent 6460 LC/MS/MS triple quadrupole (Agilent Technologies, Santa Clara, CA) and an

Choline leaving group $m/z=184$

FIGURE 1. Structure of phospholipid standard, L- α -phosphatidylcholine (760 Da). Curved dashed gray line represents the site of collision-induced dissociation of the choline leaving group.

electrospray ionization source (ESI). To reduce the surface contact with the interior of the system, samples were directly infused via the Agilent 1290 automated injection system, with the column adaptation bypassed; 20 μ L of each sample were infused at 100 μ L/min. Between each run, the syringe and insert tubing were cleaned using buffer and chloroform to avoid sample-to-sample contamination.

Phospholipids were identified by detection of specific leaving groups from precursor ions after collision-induced dissociation (precursor ion scan mode).⁶⁴ For example, the leaving group of fragmented phosphatidylcholine (PC) and sphingomyelin (SM) in positive ion mode is choline, with a neutral loss of 184 Da (Fig. 1). For phosphatidylethanolamine (PE), precursor ion scans were performed to detect the neutral loss of 141 Da in positive ion mode. Precursor ions of 87 Da for phosphatidylserine (PS) were detected in negative ion mode. The instrument parameters for the identification of PC included gas temperature (ultrahigh-purity nitrogen) 300°C, gas flow 5 L/min, 45 psi nebulizer, sheath gas temp 250°C, sheath gas flow

11 L/min, capillary 3500 positive V, 3500 negative, 9 nA nozzle voltage. The fragmentation and collision energy were optimized for each individual phospholipid class. The m/z range for each run was set from 200 to 1000. Ion counts were calculated by integration of extracted chromatographs using Agilent Masshunter software. To ensure that the full sample traversed the length of the tubing, the respective sample period was set for 30 minutes. Before each sample run, appropriate sample buffer, as well as negative controls for the lipid extracts from column purification, were infused to exclude sample contamination.

Limit of Detection of Phospholipid Standards

The lower limit of detection was characterized for phospholipid standards for each class of phospholipid. Six separate serially diluted samples (molarity was gravimetrically verified) were run for each class to ensure accuracy. PC was dissolved in a mixture of methanol $\phi_A = 0.78$, chloroform $\phi_B = 0.2$, and formic acid $\phi_C = 0.02$, pH 3.6, and both PS and PE were dissolved in 0.01 M ammonium acetate solution, pH 8.2, and infused in varying amounts to find the minimum quantity per injection that could be detected. Standard curves were constructed from the intensities of the $[M+H]^+$ peaks of the phospholipid standards at various concentrations and fit to a linear regression model and the correlation coefficient, R^2 , calculated using Excel software.

RESULTS

Phospholipid Detection-Standard Curves

The direct infusion electrospray ionization MS/MS method detected the authentic standard L- α -phosphatidylcholine at a lower limit of 0.293 ng/ μ L infusion, total 5.9 ng, predominant mass 760 Da. The methodologies were less sensitive for the detection of PE and PS with lower limits of detection of 3 ng/ μ L (60 ng) and 12 ng/ μ L (24 ng) infusion, respectively. The logarithmic plot of concentration versus total ion count from mass spectrometry of serial dilutions of phospholipid standards are shown in Figure 2. The plot of the means of 6 samples for PC approaches linearity ($R^2 = 0.99$ coefficient of linear

FIGURE 2. Standard curves of L- α -phosphatidylcholine (760 Da) (solid line) and PS (dashed line). Circle and triangle represent the concentration of a single PC, m/z 758.6, extracted from tears and TL, respectively. Error bars represent the SD of the means ($n = 6$). Arrow indicates detection limit of another study²² (see text).

TABLE 2. Phosphocholine Containing Lipids in Tears and Tear Lipocalin

Lipid	[M+H]	Tears	TL
PC(16:0)	496.3	Yes	
PC(18:2)	520.3	Yes	
PC(18:1)	522.3	Yes	
PC(18:0)	524.3	Yes	
SM(16:1)	703.3	Yes	Yes
PC(34:2)	758.6	Yes	Yes
PC(34:1)	760.6	Yes	Yes
PC(36:4)	782.6	Yes	Yes
PC(36:3)	784.6	Yes	Yes
PC(36:2)	786.6	Yes	Yes
PC(38:4)	810.6	Yes	Yes

determination), allowing an approximation of concentration from the ion count for experimental samples. The SD for each point is also quite small in this range. Although the plot for PS also appears roughly linear ($r^2 = 0.9677$), only three concentrations were assessed because the level of detection was deemed insensitive for the predicted biologic prevalence of PE and PS compared with PC.

Analysis of Phosphocholines in Stimulated Tears

Using the linear approximation provided from integration of the ion peaks at m/z 760 for the phosphocholine standard, the amount in the peak m/z 758.6 in tears was 194 ng/mL (Fig. 2). Mass spectrometric analysis revealed 11 monoisotopic phosphocholines in stimulated tears (Table 2, Figs. 3 and 4). The major PC in the 700- to 800- m/z range was m/z 758.6, consistent with PC (34:2). The peak at 759.6 Da showed intensity compatible with the predicted biologic prevalence of the C13 isotope for the monoisotopic form at 758.6 Da.⁶⁵ A

peak with m/z of 703.3 Da was assigned to an SM. Four major peaks of phosphocholines with relatively greater signal intensity than intact PC were discovered in the 490- to 540- m/z range (Fig. 4 and Table 2). The 4 peaks together accounted for about 80% of the signal intensity and total integrated ion count. The $[M+H]^+$ molecule at m/z 496.3 accounted for almost 60% of the signal intensity.

Protein-Bound Phosphocholines in Tears

The gel filtration profile of tears at 280 nm absorbance is shown in Figure 5. Fractions eluting at 41 to 55 minutes contained at least 3 overlapping individual peaks. These peaks correspond to bands on polyacrylamide gel electrophoresis of high molecular weight proteins in tears, including lactoferrin approximately 83 kDa (Fig. 5 inset, lane 2, tears). Under the conditions chosen, TL elutes in size-exclusion chromatography as a nearly symmetric peak and is the major protein seen from the gel electrophoresis (2 isoforms: 17.5 and 16.9 kDa).⁶⁶ Lysozyme makes up most of the third peak.^{2,4,9} Because our previously published TLC experiments demonstrated that only TL contained phospholipids,⁵¹ the fractions without lipocalin were pooled for lipid extraction for maximum sensitivity. The SDS-PAGE gel electrophoresis confirms the separation of TL from other components. Only the band for TL is clearly discernible on the Coomassie-stained gel of the pooled fractions (Fig. 5 inset, lane 4). The mass spectrum of lipids extracted from the pooled gel filtration fractions without lipocalin revealed no detectable signal intensity. These pooled fractions included an assortment of proteins, most notably lactoferrin, phospholipid transfer protein, lysozyme, and phospholipase A2. The only fraction to bear extractable lipid that was detectable by mass spectrometry was that containing TL. Linear regression ($R^2 = 0.9919$) of the integrated ion count revealed that the extracted TL fraction contained about 104 ng/mL (Fig. 2). The molecules identified and the corresponding mass spectrum are shown in Table 2 and Figure 6,

FIGURE 3. Mass spectrum (scan range m/z 700–820) of lipids extracted from human tears. Spectrum obtained in precursor ion scan for neutral loss of m/z 184.

FIGURE 4. Mass spectrum (scan range m/z 400–820) of lipids extracted from human tears in precursor ion mode for the neutral loss m/z 184 using a triple quadrupole mass spectrometer.

respectively. Interestingly, the only phosphocholines detected were m/z 700 to 800; no molecules between m/z 490 and 540 were found in any of the fractions extracted.

DISCUSSION

The 2 most significant findings in this study were the positive identification of phosphocholines in tears and the specification of those bound to TL. The presence and amounts of phospholipids in tears and meibum have recently been debated. Complicating the interpretation of data is the variation and complexity of methods used to collect and analyze the tear extracts (Table 3); however, the presence of phospholipids in tears are concordant with most prior studies,^{23–24,51,54,58,60} but discordant with others.^{8,22} Campbell et al.²² used both TLC and liquid chromatography mass spectrometry, but phospholipids were undetectable in tears by both techniques. For TLC,⁵¹ the volume of stimulated tears

that we applied was 50-fold greater than the volume of unstimulated tears applied by Campbell et al.²² Figure 2 shows the log of concentrations of the phospholipids in standard sample, as well as the average of those ($n = 2$) found by direct infusion of concentrated extracts from tears. From this graph, the limits of detection ($0.39 \mu\text{M}$) for m/z 760 can be derived, as well as the concentration ($0.26 \mu\text{M}$) of the predominant molecule m/z 758.6 in the more dilute original tear sample. The limit of detection of $4 \mu\text{g/mL}$ for PC reported by Campbell et al.²² corresponds with $5.2 \mu\text{M}$ (Fig. 2, at arrow on log-scale plot for our standard curve). The detection limit of Campbell et al.²² is above the levels of PC in tears and may account for the negative results.

Phosphatidylcholine

The major PCs seen in tears in this study match those found in tears and meibum in 2 other mass spectrometry studies.^{23,24} A unique feature of the current study is the elucidation of PCs

FIGURE 5. Gel filtration chromatography (Superdex 75) of 0.8 mL tears run at 1 mL/min. Inset, Coomassie-stained SDS tricine PAGE, of fractions. Lane 1, Molecular weight markers, sizes shown at the left; Lane 2, human tears 5 μL ; Lane 3, pooled fractions 40–55 and 80–95, lactoferrin (Lf) and lysozyme (Ly) positions are indicated at the right; Lane 4 pooled fractions 62–72 containing TL.

FIGURE 6. Mass spectrum in precursor ion mode for the neutral loss m/z 184 using a triple quadrupole mass spectrometer of extracted gel filtration fractions (62–72, Fig. 5), enriched in TL.

bound to TL. The mass spectrum profile of lipocalin-associated phospholipids is strikingly similar to those extracted from meibum and tears in all studies. The relative amounts of the major compounds compared with less-abundant forms closely match in these studies. This observation is evident whether computed from the total ion count of individual peaks or simple inspection of the spectra from each study. Taken together, the data suggest that the major source of tear lipids is probably meibum and that most of the intact phospholipids are bound to TL. Slightly less overall lipid was seen bound to TL, but some loss is expected in column chromatography. Similarly, our prior study illustrated a decreased intensity of PC spots observed on molybdenum blue-stained TLC plates.⁵¹ Both Saville et al.²⁴ and Rantamäki et al.²³ found more phosphocholines between m/z 700 and 800. There are several possible explanations that may explain these differences. Variation of instrument response function could account for most of the differences. The apparent greater overall sensitivity of the methods used by Rantamäki et al.²³ for detecting PEs and PSs reflect greater fidelity for lipids that were below level of detection in this study and others.^{22,24} In addition, application of a strict electronic limit of detection precludes interpretation of the peak at m/z 732 (PC 32:1), which was evident in our chromatograms and those of Saville et al.²⁴ Another difference is that we collected stimulated tears, which are considered more dilute and are expected to have a shorter residence time on the tear film to gather lipids. We purposely chose this method to avoid direct contact of the pipette with conjunctival tissue. Stimulated tears produce a large volume and create a relatively deep pool in the inferior cul de sac. Unstimulated tears are collected more slowly and tissue contact is difficult to avoid with epithelial cells whose membranes are undoubtedly replete with phospholipids; however, the concentration of TL

does not differ significantly between stimulated and unstimulated tear samples.⁶⁷

Minor differences emerge between studies in the assignment of some ion peaks of PCs. For example, Saville et al.²⁴ made unique assignments to $M+1$ phospholipid molecules that showed 30% to 40% of the intensity of M . We conservatively assigned these peaks as isotopes of the monoisotopic form. The predicted prevalence of $C13$ is roughly 1% for each carbon atom. In a study proffering an isotope-correction algorithm for blood PCs, the $M+1$ isotope accounted for a relative ion count of approximately 40% intensity of the monoisotopic form.⁶⁸ In the supplemental table provided by Rantamäki et al.,²³ some of the putatively identified lipids differed by less than 1 Da and would also affect the predicted number of compounds; however, neither of these differences in assignment would affect the calculations of overall yield of phospholipid.

Lysophosphatidylcholine

The most abundant phospholipid in tears reported here is lysophosphatidylcholine. This finding is consistent with prior TLC and recent mass spectrometric studies,^{23,58} but contrasts with other recent studies.^{22,24} All of the major peaks reported in tears by Rantamäki et al.²³ were verified in this study and the relative intensity of each peak found is strikingly concordant (Table 1). Apparent disparity to other mass spectrometric studies probably can be attributed to differences in the instrument response function, the sample sizes loaded, and the mass range scanned. Rantamäki et al.²³ also found peaks in tears that were beneath our limit of detection.²³ Campbell et al.²² did not find phospholipids in tears but astutely pointed out that lysophosphatidylcholine is a presumed enzymatic degradation product of phospholipid that would be predicted

TABLE 3. Comparison of Studies on Phospholipids in Tears

Reference	Phospholipid ng/uL (% Tear Lipids)	Analytical Methods	Types of Phospholipids ng/uL of Tears (% of Total Phospholipid) Phosphatidyl-				Tear Collection	Tears (uL) Equivalent per Run	LOD (pg/uL) of Standard Sample
			Choline	Serine	Ethanolamine	Sphingomyelin			
47	NS (small)	TLC, GC	Positive	NS	Positive	Positive preliminarily	U	NS	NS
58	1.179 (0.9)	Silicic acid, TLC	(15.6%)*	NS	NS	13.30%	NS	NS	NS
54	(15)	TLC	Several spots but not distinguished				S	NS	NS
51	Positive	TLC	NS	NS	NS	NS	S	2000	NS
8,60	Positive	ESI, MALDI	Positive	NS	NS	Positive	NS	NS	NS
24	Positive	ESI	4.6	<LOD	<LOD	3.8	U	NS	NS
22	Negative	LCMS, ESI, ion trap	Negative	Negative	Negative	Negative	U	5-40	TLC-(1.3) LCMS (4)
23	Positive (88)	UPLC, ESI, TLC, enzymatic	20.9 μM*	0.1 μM	5.2 μM	0.9 μM	U	80-150	
Dean current	Positive	ESI	5.2 μM*	Below LOD	Below LOD	0.063	S	160	(0.228)

* Lysophosphatidylcholine was predominant.
ESI, electrospray ionization mass spectrometry; GC, gas chromatography; LCMS, liquid chromatography mass spectrometry; LOD, limit of detection; MALDI, matrix-assisted laser desorption ionization; NS, not stated; S, stimulated; TLC, silica thin layer chromatography; U, unstimulated; UPLC, ultrahigh-pressure liquid chromatography.

from action of phospholipases that exist in tears. Both our results and those of Rantamäki et al.²³ are concord that the major lysophosphatidylcholine is m/z 496.3Da. This is the predicted mass for the intact SN-1 (C16:0) configuration product derived by action of phospholipase A2 on the major PC, 758.6 Da (Fig. 7). The products from other phospholipases would not be detected by our methods. No lysophosphatidylcholines were found in extracts from any of the protein fractions of tears. Possible explanations include retention of molecules on the column resin, elution in a micellar or unbound state, and degradation on the column. We suspect interaction with the column resin as the most likely cause. Removal from the resin was obviated because the concentration of organic solvents needed for elution would likely cause permanent damage to the resin. One might expect lysophosphatidylcholines to bind less strongly to TL than intact parent PCs if the remaining chain is shorter, or contains alkenic bonds or polar functional groups. Lipid-binding affinity to TL varies as a function of alkyl chain length⁵¹ up to 18 carbons.⁶⁹ The hydration free energy is reduced with increasing alkenic bonds.⁷⁰ As noted previously, the putative chain length of the major lysophosphatidylcholines would have a predicted alkyl chain length of C16. The 522-Da and 520-Da lysophosphatidylcholines could have a maximum chain length of C18 with 1 or 2 double bonds, respectively. Clarification of actual structures using ion trap mass spectrometry may clarify the apparent lower affinity of lysophosphatidylcholines for TL.

Phospholipids in Tears and Tear Lipocalin

We could unambiguously identify 1 SM, d181:16, m/z 703, in tears and bound to TL. This molecule is apparently identical to that of most abundant SM identified in tears by Rantamäki et al.²³ Ham et al.,⁶⁰ however, found other SMs using a solid ionic crystal matrix for assisted laser desorption ionization. Putative SMs of m/z 659, 719, and 747 were identified; the latter 2 were confirmed by Saville et al.²⁴ Ham et al.⁶⁰ also designated the molecule, m/z 678, as the protonated 1,2-(rac)-dimyristoyl phosphatidylcholine, but this molecule was not appreciated by us or others.^{23,24} Saville et al. ²⁴ found putative SMs SM 24:2, SM 24:1, and SM 24:0 of corresponding m/z, 811, 813, and 815. We also noticed small peaks in this region that were below the strict electronic limit of detection of 10% of the m/z signal. These SMs were not reported by others.²³

FIGURE 7. Putative cleavage sites (curved lines) and products for the hydrolysis by ocular surface phospholipases on a generic PC. R1 and R2 are the stereochemically specified SN 1 and 2 positions of the esterified fatty acids, respectively.

Phosphatidic Acid

Studies that used TLC and/or silica gel chromatography of extracted phospholipids from tears reported relative abundance of phosphatidic acid (Table 3). Phosphatidic acid lacks choline and would not be detectable in the current study or others that depend on the identification of the collision-induced dissociation of the choline group m/z 184. Inspection of our prior studies using TLC reveals a spot with a retardation factor consistent with phosphatidic acid, although this standard was not applied.⁵¹ Phosphatidic acid is one of the products of phospholipase D, which is present in conjunctiva and corneal epithelial cells.⁷¹

Phospholipid Carrier Proteins

Only the gel fraction of TL contained detectable phospholipids in this study. This finding is concordant with our previous TLC study.⁵¹ The results of Millar et al.⁸ are discordant in that extracts from TL analyzed by ESI revealed no phospholipid, but rather other lipids as well as a contaminant from plastic tubes used for the extraction. The amount of lipocalin used for the extraction in their study was not specified. Plentiful data exist that phospholipids bind TL. Fluorescent competitive binding assays show that TL has a high affinity for α -lysophosphatidylcholine (K_i 1.2 μ M).⁷² Only TL in human tears was associated with retrieval of the fluorescent phospholipid analog, 2-(6-(7-nitrobenz-2-oxa,1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine, from both normal and abnormal corneas.⁶² Another candidate, phospholipid transfer protein (apparent MWt 160–170 kDa in size exclusion chromatography) would be expected to elute in the first complex of peaks (Fig. 5) and migrate under reduced conditions in SDS tricine PAGE to about the same position as lactoferrin; both have MWts of about 80 kDa.¹⁰ The corresponding gel filtration fractions did not contain extractable phosphocholines in this study or in prior work.^{51,62} These data appear to exclude phospholipid transfer protein as a major carrier of phosphocholines in tears. Another negating argument is the relatively low concentration of phospholipid transfer protein in tears (0.130 μ M) compared with the estimated concentration of choline-derived lipids (48 μ M), both calculated by the same group.^{23,73} Although not clear, phospholipid transfer protein may have a role in facilitating transfer of some phospholipid components from cell membranes. The epithelial cells in the cornea and conjunctiva turn over rapidly. The apoptotic cells with their membrane components are continually degraded. The interaction of specific phospholipids, phospholipid transfer protein, TL, and phospholipases in the tear film requires further clarification.

Reports vary as to the quantity of phospholipids in tears. The sensitivity of various methodologies and collection may account for the differences; however, the major phospholipid molecules extracted from meibum, tears, and TL match in most of the recent studies. Mass spectrometric analyses show molecules with C16–C18 alkyl chain lengths predominate in tear lipids and are known to effectively retard evaporation. Our estimates of the concentration of a single intact PC m/z 758.6 (194 ng/mL) in tears with an ocular surface volume of about 10 μ L approaches the published estimates required to effect a monolayer film ($\sim$ 2–5 ng).²² Our estimated and perhaps underestimated concentration of phosphocholines (5.2 μ M) greatly exceeds that required for a monolayer film even before considering PE, PS, and the abundance of phosphatidic acid.

Exempting a few studies that could not demonstrate phospholipids in meibum and tears, there is general agreement spanning almost 30 years. The evidence supports specific

conclusions regarding the tear film. Phospholipids exist in tears in enough quantity to retard evaporation. The major PCs are consistent in meibum and tears and many are native ligands of TL. Matching mass spectrum profiles link the major PCs found in tears and TL to the probable origin in meibum.

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