Isolation and identification of phospholipids of bovine rhodopsin

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ABSTRACT Phospholipids present in digitonin solutions of bovine rhodopsin have been identified and assayed. Digitonin interferes with extraction of lipids by the usual methods; digitonin was therefore removed from the preparation as an ergosterol digitonide, soluble in absolute ethanol but precipitated in 80% ethanol. The supernatant 80% ethanol contained one portion of the phospholipid, mainly choline and ethanolamine phosphoglycerides with traces of serine phosphoglyceride and sphingomyelin. The rhodopsin residue (free from digitonin) was extracted with chloroform–methanol 2:1; this extract contained the rest of the phospholipid, which consisted only of choline and ethanolamine phosphoglycerides. Plasmalogens were not found, but could have decomposed during the procedures.

SUPPLEMENTARY KEY WORDS retina · digitonin · ergosterol complex · precipitation

It has long been assumed (1) that phospholipids, which are present in bovine retinal rod outer segments to the extent of 30–40% of the dry weight (2, 3), constitute part of the membrane structure of the discs forming the rod segments (4). Rhodopsin, the pigment of dark adaptation, is likely to be found in these membranes (5) either as a protein layer between two layers of phospholipid (1) or as a lipoprotein (6). In either case, interaction between rhodopsin protein and lipid in the membrane could have a role in the photochemical process or its immediate thermal consequence, the membrane change that results in depolarization and eventual nerve excitation (7). Any association of phospholipids with rhodopsin in situ has not been established and there are no adequate published methods for definitively relating the position of rhodopsin to that of phospholipids in membranes. Rhodopsin can be isolated for study only by solubilizing it in aqueous digitonin (8) or other surfactants which form micelles. Rhodopsin–digitonin preparations have been reported to contain up to 81% of the phospholipids found in bovine rods (6), and such solutions can be examined for specific phospholipids.

Aqueous digitonin apparently brings rhodopsin into solution by the formation of a 180–200 digitonin molecule micelle (9) in which the rhodopsin molecule is enclosed. It is not possible to remove the digitonin by dialysis because concentration of the solution causes the micelles to change size and finally precipitate as a gelatinous mass (9). Nor can the micellar structures be destroyed by organic solvents that would extract lipids from the freed rhodopsin (6). However, it is possible to form a mole-for-mole complex between digitonin and ergosterol. This complex is soluble in absolute ethanol (10) and is quantitatively precipitated by addition of water. I have found this to be an effective method for the separation of digitonin, both from rhodopsin protein and from the associated phospholipids.

The purpose of this work was to determine the composition of phospholipids associated with rhodopsin in digitonin solutions as a preliminary to further investigation of their function in the retina. The technique developed affords a new tool for the analysis of retinal phospholipids.

MATERIALS AND METHODS

All chemicals, except as noted, were reagent grade and used as received.

Digitonin purchased from Merck & Co., Inc. (Rahway, N.J.) was used exclusively. Other brands

Abbreviations: CPG, EPG, and SPG—choline, ethanolamine, and serine phosphoglycerides, respectively; SM, sphingomyelin; and GPC, GPE—glycerophosphoryl choline and ethanolamine, respectively.

tended to precipitate more readily when the solution was cooled and the resultant changes in concentration interfered with the stoichiometry of the digitonin–ergosterol reaction.

Ethanol, 99.5% (USPHS, Perry Point, Md.) was further dehydrated by storage over Molecular Sieve, type 4A (Fisher Scientific Co., Silver Spring, Md.) and, when required, deoxygenated by the passage of dry nitrogen.

Ergosterol (Aldrich Chemical Co., Milwaukee, Wis.) was twice crystallized from absolute ethanol immediately before use.

Phospholipids for standards were obtained from Mann Research Laboratories, Inc., New York (CPG, EPG, their lyso-forms, and SPG) and from Applied Science Laboratories, Inc., State College, Pa. (sphingomyelin, CPG, and SPG).

Assays

Rhodopsin concentration was calculated from the absorbance of the solution at 498 nm, using the molar absorptivity of 40,600 given by Wald and Brown (11).

The ergosterol content of the solutions was determined from absorbance measurements based on a molar absorptivity of 12,100 at 282 nm. Phosphorus was assayed as described by Marinetti (12), nitrogen according to Sloane-Stanley (13), and serine by the procedure outlined by Frisell and MacKenzie (14).

Isolation of Rod Outer Segments

Eyes of freshly slaughtered cattle, purchased from a local abattoir, were dissected under dim red light (25-watt Ruby darkroom lamp at 60 cm). Retinas were removed from the eyes and dropped into a 500 ml Erlenmeyer flask (immersed in ice), containing about 100 ml of glass beads (mixed sizes 2, 3, and 4) wet with 50 ml of 0.067 M KCl. The flask was swirled gently for 1 min or longer to form a slurry, which was then poured into a funnel made of 20-mesh stainless steel screen. The detached rods and cellular elements due to contamination with inner segments were carried through the mesh by three washings with 50-ml portions of 0.067 M KCl, with gentle stirring. This left much of the neural debris behind on the beads.

Centrifugation of the filtrate at 125 *g* for 5 min removed cell nuclei and small pieces of neural material from the supernatant suspension of rods, rod particles, and any incidental microsomes and mitochondria. The suspension was then centrifuged at 5000 *g* for 15 min to pack the rods into the bottom of the tube. The pellet was resuspended in 50 ml of a 40% solution of sucrose in 0.067 M KCl and centrifuged at 8000 *g* for 15 min to remove more cellular debris, black pigment particles, and any microsomes and mitochondria. The supernatant suspension was diluted to 8% sucrose with 0.067 M KCl, then centrifuged at 10,000 *g* for 20 min to pack the rods; since the resultant supernatant fraction would have retained microsomes and mitochondria in suspension (15, 16), it was discarded. The rods were washed once with distilled water, then hardened in 5% aqueous aluminum potassium sulfate (alum) for 30 min at 2°C (17). The preparation was freed of alum by resuspending it twice in distilled water and once in 0.067 M KCl, centrifuging each time at 1600 *g* for 10 min. At this point, 5.0 ml of 0.067 M KCl was added for each 50 retinas and an aliquot was examined under a microscope. Consistently, there was no gross contamination; only rod particles and whole rods appeared to be present. The KCl suspension was frozen and lyophilized.

Solubilization of Rhodopsin

Lyophilized retinal rods were twice suspended in 100-ml portions of petroleum ether (bp 40–50°C) and allowed to stand at 2°C in the dark for 4 and 10 hr, respectively, to remove unbound phospholipids and fatty acids. After centrifugation, the bulk of the petroleum ether was decanted and discarded and the remainder removed by evaporation under reduced pressure. After being thoroughly dispersed in 1.0 ml of 2% aqueous digitonin for each six retinas by use of a freely moving (loose-fitting) Tenbroeck homogenizer, the rods were allowed to remain at room temperature (24°C) for 30 min before centrifugation at 16,000 *g* for 30 min at 2°C. The resulting supernatant solution of rhodopsin was clear, with an absorbance always greater than 1.0 at 498 nm. Each preparation of rhodopsin was assayed for phosphorus and nitrogen, then frozen and lyophilized.

Digitonin Precipitation

Ergosterol, 1.5 mM in nitrogen-saturated anhydrous ethanol, was added to lyophilized rhodopsin–digitonin preparations in slight (2%) excess of 1 mole/mole of digitonin (using an average mol wt for digitonin of 1226). After the mixture had stood for 18 hr in the dark at 2°C, it was centrifuged at 3000 *g* for 10 min and a clear supernatant solution of ergosterol–digitonin complex was separated. The residue was twice suspended in absolute ethanol and centrifuged each time; these washes were added to the first ethanol solution which was then called fraction A (see flow sheet, Fig. 1). The residue, fraction B, was frozen and set aside for further treatment. Fraction A, containing ergosterol–digitonin complex and some digitonin-associated lipids, was diluted to 80% ethanol with distilled water. A copious white precipitate formed at once and, after 12 hr in the dark at 2°C, spectroscopic examination of the supernatant solution showed by the change of ergosterol absorbance that precipitation was complete. The

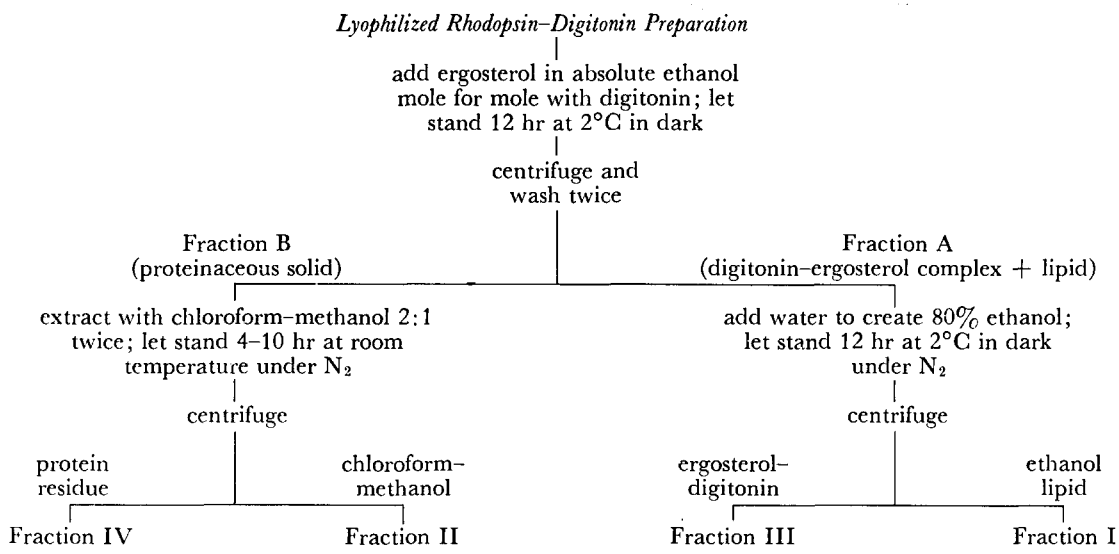

FIG. 1. Diagrammatic representation of preparation procedures.

precipitated complex was centrifuged, resuspended twice in 80% ethanol, and dried under reduced pressure; it is called fraction III. The ethanol washes were combined with the supernatant ethanolic digitonin–ergosterol solution and designated fraction I.

Fraction B was thawed, dried under vacuum, and extracted for 4 hr with chloroform–methanol 2:1 at room temperature (24°C) in the dark, under nitrogen. The extraction was repeated and the extracts were pooled as fraction II. The proteinaceous residue was dried (fraction IV).

All fractions were assayed for phosphorus, as described previously, and fraction IV was also assayed for serine. For chromatography, fractions I and II were diluted with chloroform–methanol 1:1 to a concentration of about 1.0 μg of phosphorus per μl.

Chromatography

Two-dimensional chromatograms were developed on glass-fiber sheets containing silicic acid (ITLC-SAF, Gelman Instrument Company, Ann Arbor, Mich.). Solvent systems were chloroform–methanol–water 70:25:4 in the first direction, and chloroform–methanol–8 M ammonium hydroxide 50:35:5 in the second (18). Solvent mixtures were freshly prepared for each chromatographic series and 50 mg/liter of butylated hydroxytoluene was added (19) to minimize oxidation of phospholipids during the drying phase between solvents. Samples of the fractions, containing 15 μg of P, were placed upon the chromatographic sheets under flowing nitrogen. (For comparison, standard solutions of known phospholipids were chromatographed in the same manner, as were digitonin and ergosterol solutions.) After development and thorough drying, phospholipids were stained by the techniques used by Marinetti (12) for paper chromatograms, except that phosphorus was made visible with the spray reagent described by Dittmer and Lester (20). The tentative identities were confirmed by comparison with chromatograms of known phospholipids.

The amount of each identified phospholipid was estimated by measuring the phosphorus content of the chromatographic spot. Areas of phospholipid that had been made visible by exposing fresh, dry chromatograms to iodine vapor were circumscribed in pencil. After the iodine had evaporated, the marked spots were excised with a cork borer (diameter = 1.067 inches) and a second disc was cut from an adjacent area with the same instrument to serve as a blank. The discs were broken into small pieces and digested with 72% perchloric acid in 155 × 25 mm Pyrex tubes in preparation for the application of Marinetti's (12) modification of Bartlett's method for the colorimetric determination of phosphorus. The glass fibers had no measurable effect on the accuracy of the method, as shown in recovery experiments, and were easily removed by centrifugation before the absorbance of the solutions was measured in the Cary 14 spectrophotometer.

RESULTS

Assays of aqueous rhodopsin solutions, and the fractions derived from them in the process of removing digitonin, produced the data listed in Table 1. There is good agreement between the total phosphorus content of the fractions and the corresponding figure for the parent solutions. Nitrogen-to-phosphorus ratios of nearly 1.0 for fractions I and II (0.83–1.11) and the high nitrogen content of fraction IV indicate that phospholipids were principally in fractions I and II, with the

TABLE 1 P AND N CONTENTS OF RHODOPSIN FRACTIONS

Rhodopsin Preparation	Parent Solution		Fraction I		Fraction II		Fraction IV		Totals		Purity Criteria*	
	P	N	P	N	P	N	P	N	P	N	K_{400}	K_{278}
1	83.1	—	47.0	—	31.2	29.5	7.0	—	85.2	—	0.45	7.10
2	100	700	—	61.0	—	27.0	—	600	—	688	0.42	9.69
3	78.0	—	40.1	—	34.5	31.0	5.1	—	79.7	—	0.41	7.42
4	84.0	630	39.1	61.7	33.0	30.1	5.2	580	77.3	672	0.41	4.30
6	116.1	841	79.4	72.8	31.4	35.0	6.0	733	116.8	841	0.33	6.68
7	81.0	520	56.1	48.7	30.0	27.3	4.3	500	90.4	576	0.31	3.33
8	77.0	—	43.8	40.1	29.3	—	6.2	—	79.3	—	0.27	5.01
9	85.9	541	48.6	45.1	28.7	27.5	3.7	469	81.0	542	0.26	3.24
10	76.4	392	45.1	42.5	33.0	27.3	5.0	322	83.1	392	0.19	2.78

Fraction III contained no P or N and is omitted.

All P and N determinations were performed in duplicate or triplicate and the values are means with maximum variations of ±2%. Recovery experiments showed 97–101% recovery of P and 96–98% of N.

* Purity criteria K_{400} and K_{278} are the ratios of absorbance of a rhodopsin solution at 400 nm and 278 nm, respectively, to its absorbance at 500 nm; the smaller the numbers, the "purer" the preparation (31).

protein moiety remaining in fraction IV. There were 3.7–7.0 moles of phosphorus/mole of rhodopsin present in fraction IV but, considering the extensive extraction, it is probably not lipid, especially since sufficient RNA phosphorus was found by Collins, Love, and Morton (2) (about 6% of total phosphorus) in digitonin extracts of retinal rods to account for the phosphorus measured in fraction IV. If one considers fraction IV as nonlipid, then in digitonin extracts of retinal rods total phospholipids ranged from 72.1 to 110.8 moles/mole of rhodopsin, exceeding the 54 moles Krinsky (6) found in digitonin extracts of alum-treated rod outer segments. Recalculation of the data reported by Collins et al. (2), using Hubbard's (9) values of 14 g of rhodopsin/100 g of dry retinal rods and a mol wt of 40,000, showed that their rhodopsin preparation from alum-treated rods contained 88–98 moles of phospholipid/mole of rhodopsin.

The molar concentrations presented in Table 2 are the products of percentage of phosphorus for a particular phospholipid, as identified on a chromatogram, and the molar phosphorus concentration of its parent fraction shown in Table 1. As the preparation of chromatograms required repeated opening and stirring of the diluted (1.0 μg/μl) fractions I and II, unavoidable concentration changes occurred. Another source of error was the drying of solvent on the tip of the spotting device (a Hamilton syringe). However, changes in concentration do not affect the percentage of phospholipid composition of the fractions and that information was readily obtained as previously described. Summation of the molar concentrations of phospholipid, and the calculation of each as percentage of the total, led to the following results: CPG 44.2%, monoacyl GPC 19.5%, EPG 25.3%; monoacyl GPE 9.0%, SPG 0.8%, and SM 1.2%. There are no comparative data in the literature. I was unable to detect any plasmalogens (12) on chromatograms containing as much as 30.0 μg of phosphorus. It should be noted that Eichberg and Hess (21) found very little plasmalogen in rod lipid extracted from frog retinas and that I found none in lipid extracts from retinal rods of cattle in earlier work (3). Possibly the long extraction procedures used in each case allowed the hydrolysis of plasmalogens to lyso forms.

On a molar basis, the distribution of the phospholipids in fraction I differs from that of fraction II since the SPG, SM, and most of the monoacyl GPE are in the former (Table 2). Fraction II had about the same composition ratio in each preparation; fraction I varied from one rhodopsin preparation to the next.

Because the serine-based phospholipids occurred only in fraction I and made up only 0.8% of the total phosphorus, it was possible that more might be found in fraction IV, and assays of fraction IV showed 4–6 moles of serine/mole of rhodopsin to be present. Shields et al. (22) found rhodopsin protein to have a serine content of 17.0 moles/mole of rhodopsin, so it appears unlikely that the serine I found was in SPG. No attempts were made to account for the discrepancy between Shields' serine values and mine; this would have required amino acid analysis.

DISCUSSION

The variations of up to 32% (equivalent to 39 moles/mole of rhodopsin) in the phosphorus contained in rhodopsin–digitonin solutions are not accounted for by the presence of impurities; the correlation coefficient between phosphorus content and K_{400} (Table 1) was 0.43 and, in a population of 9, can occur by chance alone more than 75% of the time. Neither are the 3–4% errors inherent in the phosphorus assay sufficient to cause the

TABLE 2 PHOSPHOLIPID COMPOSITIONS OF FRACTIONS I AND II

Rhodopsin Preparation	CPG		Monoacyl GPC		EPG		Monoacyl GPE		SPG		SB	
	I	II	I	II	I	II	I	II	I	II	I	II
	moles of P/mole of rhodopsin in parent rhodopsin–digitonin solution											
1	15.0 (31.9)*	17.9 (57.3)	8.4 (17.9)	7.0 (22.3)	12.8 (27.1)	6.1 (19.5)	7.7 (16.4)	0.3 (0.8)	0	0	3.1 (6.6)	0
3	12.8 (31.9)	20.4 (59.1)	7.8 (19.6)	7.3 (21.3)	13.3 (33.3)	6.5 (18.8)	4.6 (11.5)	0.3 (0.8)	0.7 (1.8)	0	0.8 (1.9)	0
6	30.8 (38.7)	18.9 (60.2)	22.0 (27.7)	6.8 (21.8)	16.9 (21.2)	5.7 (18.1)	8.8 (7.0)	0		0	2.8 (3.5)	0
7	18.5 (33.0)	18.0 (60.1)	11.0 (19.7)	6.5 (21.5)	14.4 (25.6)	5.5 (18.4)	11.5 (20.4)	0	0.7 1.3	0	0	0
8	16.3 (37.3)	17.4 (59.4)	8 (20.2)	6.5 (22.2)	11.4 (25.9)	5.0 (17.0)	6.4 (14.7)	0.4 (1.4)	0.8 (1.8)	0	0	0
9	17.1 (35.2)		2.9 (5.9)		19.3 (39.7)		8.2 (16.9)		1.2 (2.4)	0	0	0
10	17.5 (38.8)	19.5 (59.2)	4.2 (9.3)	6.9 (20.9)	15.0 (33.3)	6.3 (19.0)	7.5 (16.6)	0.3 (0.8)	0.9 (2.0)	0	0	0

* Figures in parentheses represent P as percentage of total P in the particular fraction. Each parenthetical number is an average of three chromatographic analyses with a maximum variation of ±2.0%. The relationship between molar ratio and percentage of P/fraction is explained in the text.

differences. It may be that the phospholipid content fluctuates with the various diets among cattle from different parts of the countryside. Although I am not aware of any studies on cattle in this regard, there is evidence that diet profoundly affects the fatty acids of the rat retina (23). For whatever reason the variation in phospholipid occurs, one would expect it to be reflected in the fractions derived from the parent solutions; such is the case for fraction I but not fraction II.

The phospholipids were divided between the two fractions because some were removed by ethanol (fraction I), whereas others required the more polar chloroform–methanol (fraction II) for extraction. It is clear that binding had to exist between phospholipid and either digitonin or protein. After the removal of digitonin and its associated lipids into fraction I, only protein remained to bind the phospholipids that were then extracted to form fraction II. The phospholipid of fraction II was therefore closely associated with rhodopsin protein. It is not possible, without further data, to determine whether the relationship exists in situ or how much of the phospholipid in fraction I is also bound to the protein in situ. Shields et al. (22) have reported that there may be two types of binding between rhodopsin protein and phospholipids in digitonin solutions of native rhodopsin from cattle.

The two phospholipids found most closely associated with rhodopsin protein—CPG, EPG, and their lyso forms—are both implicated in activities that one would expect to find in intact retina: (*a*) lecithins (CPG) are well-established as membrane-forming substances (24); and (*b*) EPG have recently been shown to form complexes with retinal (25–27), one of which has been extracted from retinal rods (26) as well as being formed in vitro between retinal and EPG extracted from bovine rods (27). The EPG complex has the absorption maximum of a bleaching product of bovine rhodopsin (26).

Retinal rods are neural in nature by virtue of their origin from the plasma membrane of the rod cell (21, 29). The EPG from bovine brain extracts contains up to 21% plasmalogens (30) and one might expect to find similar values in retinal rods and perhaps in the extracts from those rods. As was previously stated, neither this work nor the other study cited (21) have ruled out plasmalogens in retinal rods and an attempt should be made to prove their presence or confirm their absence definitively.

Manuscript received 27 June 1968 and in revised form 6 January 1969; accepted 15 April 1969.

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