Post-Golgi Vesicles Cotransport Docosahexaenoyl-Phospholipids and Rhodopsin during Frog Photoreceptor Membrane Biogenesis*

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Post-Golgi vesicles budding from the trans-Golgi network (TGN) are involved in the vectorial transport and delivery of rhodopsin to photoreceptor rod outer segments (ROS). We report here that newly synthesized docosahexaenoic (DHA) phospholipids are sequestered and cotransported by rhodopsin-bearing post-Golgi vesicles to ROS. Frog retinas were pulse-labeled with [3H]DHA and [35S]methionine/cysteine prior to ROS isolation and subcellular fractionation. After a 1-h pulse, relatively uniform [3H]DHA-lipid labeling (DPM/μg protein) was observed in all fractions enriched in post-Golgi vesicles, TGN, Golgi, and endoplasmic reticulum (ER) membranes. During the subsequent 2-h chase translocation of free [3H]DHA from ROS to the photoreceptor inner segment contributed to an additional overall increase in labeling of lipids. The specific activity (dpm/nmol DHA) in ER-enriched fraction was similar or higher than in other subcellular fractions after both the pulse and the chase, indicating that the bulk of [3H]DHA-lipids was synthesized in the ER. After the chase a 2-fold increase in labeling of lipids in the ER and Golgi and a 2.6-fold in lighter TGN-enriched fractions was observed. The highest labeling was in the post-Golgi vesicle fraction (4-fold increase), with [3H]DHA-phosphatidylcholine and [3H]DHA-phosphatidylethanolamine showing the greatest increase. At the same time, newly synthesized [35S]rhodopsin shifted from the ER and Golgi toward TGN and post-Golgi fractions. Therefore, sequestration and association of [35S]rhodopsin and [3H]DHA-lipids in a TGN membrane domain occurs prior to their exit and subsequent vectorial cotransport on post-Golgi vesicles to ROS. Labeling of ROS lipids was very low, with phosphatidylinositol and diacylglycerols displaying the highest labeling. This indicates that other mechanisms by-passing Golgi, i.e. facilitated by lipid carrier proteins, may also contribute to molecular replacement of disc membrane DHA-phospholipids, particularly phosphatidylinositol.

Vertebrate photoreceptors are polarized and compartmentalized cells, with a photosensitive outer segment and a synaptic terminal domain at opposite ends of the cell. These domains are attached through a connecting cilium and a short axon, respectively, to a central region, the inner segment. The inner segment is the site where lipid and protein synthesis, polarized sorting of molecules, and initiation of membrane biogenesis for both the outer segment and synaptic terminals take place. Disc membranes in rod outer segments (ROS)1 display a unique lipid-protein composition. The visual pigment rhodopsin, which accounts for more than 85% of disc membrane proteins (1), is embedded within a highly fluid lipid bilayer comprised of phospholipids (PLs) highly enriched in docosahexaenoic acid (DHA, 22:6n-3) (2–6).

Amphibian photoreceptor cells are a useful experimental model to study protein and lipid trafficking in a polarized cell. Photoreceptors actively synthesize proteins (mainly rhodopsin) and DHA-PLs to support the dynamic daily renewal of 50–80 large disc membranes in each rod cell that results in the addition of membrane at ~3 μm²/min (7, 8). ROS lack the capacity for de novo synthesis of PLs (4, 9). Therefore, they depend entirely on an external supply of PLs from the inner segment where they are synthesized mainly in the rough endoplasmic reticulum (ER) (10). How these highly unsaturated lipids become components of ROS membranes and at which stage of membrane biosynthesis and disc morphogenesis they become associated with rhodopsin is not yet clear.

Newly synthesized rhodopsin is vectorially transported from its site of synthesis in the rough ER to ROS by vesicles that bud from the trans-Golgi network (TGN), cluster beneath the connecting cilium, and fuse with the inner segment plasma membrane within the periciliary ridge complex (11, 12). A very low buoyant density (ρ = 1.09 g/ml) post-Golgi vesicular subcellular fraction carrying newly synthesized rhodopsin has been isolated and characterized from frog retinal photoreceptor cells (12–15). Whereas rhodopsin and the bulk of DHA-PLs are synthesized in the rough ER and both can follow a vesicle-mediated traffic through the biosynthetic pathway, other mechanisms can contribute to trafficking and selective delivery of PLs to intracellular organelles (16–18). For example, the rapid monomer transport of PLs is facilitated by transfer proteins (TP) through the cytosol (19). A transfer protein that with high affinity transfers PC to ROS membranes has also been described (20), and immunohistochemical analysis of chicken retinas at hatching revealed the presence of phosphatidylinositol (PI)-TP in retinal cells including the inner segment of photoreceptors (21).

Phospholipid renewal of ROS membranes involves both membrane replacement (as new disc membranes are assembled at the base of the ROS) and molecular replacement (i.e. PL

1 The abbreviations used are: ROS, rod outer segment(s); PLs, phospholipids; DHA, 22:6n-3, docosahexaenoic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; DAG, diacylglycerol; ER, endoplasmic reticulum; TGN, trans-Golgi network; TP, transfer proteins.

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transfer protein mediated and remodeling of disc PLs by turnover (22,23). Using various radiolabeled lipid precursors under experimental conditions that inhibit protein synthesis or vesicle-mediated transport, lipids can be transported to ROS by independent pathways by-passing the Golgi (24–28). However, sorting of DHA-PLs, vectorial transport to ROS, and the contribution of alternative pathways to their trafficking has not been experimentally addressed. This question is especially intriguing because biochemical and autoradiographic studies of [3H]DHA trafficking in frog photoreceptors after in vitro (29,30) and in vivo (31,32) labeling have disclosed that newly synthesized [3H]DHA-PLs display a polarized delivery to ROS, where they are incorporated at the base as new discs are formed, in a pattern paralleling incorporation of radiolabeled amino acids into disk membrane proteins.

The aim of the current study was to investigate if newly synthesized DHA-lipids could be, at least in part, segregated and cotransported with rhodopsin in vesicles budding from the TGN and then enter the ROS as new membranes are formed (membrane replacement). To address this question, we pulse-labeled retinas for 1 h in the presence of [3H]DHA and [35S]methionine/cysteine, followed by a 2-h chase in a cold buffer prior to subcellular fractionation (12,33). [3H]DHA was chosen because this precursor is actively esterified into PLs in the inner segment of photoreceptor (34–38) prior to their vectorial transport to the ellipsoid region at the base of ROS and also to synaptic terminals (29,30). Our results reveal that newly synthesized [3H]DHA-PLs, especially the main components of disc membranes (i.e., DHA-phosphatidylethanolamine (PC) and DHA-phosphatidylserine (PS)), are segregated and loaded together with newly synthesized [35S]rhodopsin in post-Golgi vesicles. Some lipids, i.e., [3H]DHA-PC and [3H]DHA-diacylglycerols (DAG), are very rapidly synthesized and delivered to ROS probably by alternative pathways that by-pass the Golgi and may be facilitated by carrier proteins.

EXPERIMENTAL PROCEDURES

Frogs, Rana berlandieri (100–250 g), were purchased from Rana Co. (Burlington, VT), maintained in a 12L:12D light:dark cycle, and fed crickets for a week prior to the experiment. [4,5-3H]DHA (specific activity 17 Ci/mmol) and [35S]Express protein labeling mixture (1,000 Ci/mmol) were from DuPont NEN. High performance thin layer chromatography plates (10 cm, 150 mm film thickness, Supelco, Bellefonte, PA) by using ethyl acetate as solvent. Fatty acid methyl esters were prepared in glass tubes by transesterification with 2 ml of toluene/methanol/sulfuric acid (100:100:4, v/v) at 4 °C, after flushing the tubes with nitrogen and capping with a Teflon-lined cap. The tubes were cooled at room temperature, and 1 ml of water, 3 ml of hexane, and a mixture of two internal standards (17:0 and 21:0 methyl esters) were added. Fatty acid methyl esters resuspended in hexane were separated onto a SP-2330 column (30 m, 0.25 mm inner diameter, 0.2-µm film thickness, Supelco, Bellefonte, PA) by using helium as a carrier gas, in a Varian Vista 401 gas chromatograph (Palo Alto, CA). The injector and detector temperatures were 220 and 250 °C, respectively, and the column temperature was programmed from 70 to 200 °C (42). The peaks were detected by flame ionization, identified by comparison of the retention times of authentic fatty acid methyl esters standard, and quantified using the internal standards.

Lipid Extraction and Analysis—Lipids were extracted from the fractions by adding 3 ml of chloroform:methanol (2:1, v/v) following the Folch procedure (41). Individual phospholipids and neutral lipids were isolated in the same TLC plate following a two-dimensional, three-step TLC procedure (6) as follows: an aliquot of the labeled lipid extract containing phospholipid and neutral lipid standards as a carriers was applied over the low right corner (1.5 cm from each hipofront) with hexane, 10 cm high performance thin layer chromatography plates previously sprayed with 3% magnesium acetate and activated for at least 1 h at 100 °C. The plate was developed in the first dimension twice using the Rouser I chromatographic system (chloroform/methanol/ammonia, 65:25:5, v/v) until the solvent front reached 2 cm from the top of the plate. After drying with cold air, the plate was turned to the right 90°, and neutral lipid standards (cholesterol, triacylglycerol, diacylglycerol, and monoacylglycerol) were spotted 1.5 cm from the bottom and 0.5 cm from the right border. Plates were then developed in hexane/ether (60:40, v/v) to isolate individual neutral lipids that had accumulated at the front of the first chromatographic system. The silica gel was cut with a vertical line to isolate neutral lipid (right) from phospholipid (left). After drying, the left side of the plate was scraped off from the bottom right corner prior to running the plates in the Rouser II system (chloroform/acetonitrile/methanol/acetate acid, 30:40:10:10.5, v/v). This third chromatographic step, run in the same direction as the second step, allows the isolation of individual phospholipid classes and free fatty acids that run with the solvent front above PE. Lipid spots were visualized by iodine staining, and the radioactivity was determined according to Fanger (40), using bovine serum albumin as a standard.

RESULTS

Post-Golgi Vesicles Are Enriched with Newly Synthesized [35S]Rhodopsin and [3H]DHA-Lipids—To determine whether newly synthesized [3H]DHA-PLs and [35S]rhodopsin are transported together in the same population of post-Golgi vesicles recovered in fraction 5 of the sucrose gradient, retinas were pulse-labeled for 1 h in the presence of both precursors and further incubated for 2 h (chase) in cold buffer prior to subcellular fractionation. This experimental protocol gives sufficient...
labeling of newly synthesized rhodopsin within 1-h pulse and a maximum labeling of the vesicles (fraction 5) during the following 2-h chase (12, 43). While the total [35S]rhodopsin labeling recovered from the combined 14 fractions was similar for pulse and chase samples (data not shown), total esterified [3H]DHA was increased by 2.6 ± 0.2-fold; from 1.2 × 10^6 dpm/21 retinas after a 1-h pulse to 3.2 × 10^6 dpm/21 retinas after a 2-h chase. After 1 h of pulse labeling, 58% of total [35S]rhodopsin was recovered in TGN and Golgi (fractions 7–11) (Fig. 1) as expected (12). After a 2-h “chase,” a shift toward post-Golgi fractions 4–6 was observed, with fraction 5 displaying the greatest increase (ratio chase/pulse: 2.4 ± 0.5). The profile of total esterified [3H]DHA among subcellular fractions was similar to that of [35S]rhodopsin (Fig. 1), with the highest percent values observed in those fractions that also accumulated the largest proportion of membranes (Fig. 2D). Remarkably after the 2-h chase only post-Golgi fraction 5 displayed significantly higher [3H]DHA percent labeling (ratio chase/pulse, 1.7 ± 0.2) at the time when newly synthesized [35S]rhodopsin accumulated in this fraction. Simultaneously, percent labeling in fractions 12 and 13 was lower than during the pulse. Labeling recovered from individual subcellular fractions based upon protein content is shown in Fig. 2 and reveals four features. First, after a 1-h pulse, all fractions displayed similar labeling of esterified [3H]DHA (Fig. 2B). Second, after the subsequent 2-h “chase,” labeling of DHA-lipids increased at least 2-fold in the heavy fractions that correspond to the density of ER (13–14), Golgi (11–12), and TGN (10), by 2.6-fold in lighter TGN fractions 7–9, and peaking at 4-fold higher labeling in post-Golgi vesicles recovered in fraction 5 as compared with pulse-labeled values (Fig. 2, B–C). Third, no significant difference between pulse and chase labeling was observed in the free [3H]DHA pool, indicating an equilibrium between the arrival of the precursor to these membrane compartments and its esterification into lipids either by de novo synthesis and/or turnover (Fig. 2A). The peak of labeling observed in fraction 8 may be the result of free [3H]DHA contributed by a small proportion of heavily labeled ROS cosedimenting between fractions 7 and 9 at a buoyant density of 1.13 g/ml (12). Fourth, most of the label recovered from ROS after pulse and chase labeling was found as free DHA (92 ± 1 and 76 ± 1%, respectively) (Fig. 2A), whereas ROS lipids labeling was the lowest among all subcellular fractions analyzed (Fig. 2B). A 2.5-fold increase in ROS [3H]DHA-lipids labeling after the “chase” (from 138 ± 25 to 350 ± 40 dpm/μg protein) accounted for by the concomitant loss of only 15% of free [3H]DHA (from 2600 to 1160 dpm/μg protein).

The specific activity of total [3H]DHA-lipids (dpm/nmol of endogenous DHA content) showed that fraction 14 (ER) displayed, after both the pulse (2790 ± 245 dpm/nmol DHA) and the “chase” (5060 ± 840 dpm/nmol DHA) labeling, similar or higher values than other fractions. After the pulse labeling, the specific activity of fraction 5 (1990 ± 115 dpm/nmol DHA) was significantly lower than that of fraction 14 (p < 0.05). After the chase, the specific activity of fraction 5 increased 3-fold (6580 ± 550 dpm/nmol DHA) but was not significantly different from the specific activity of fraction 14 (p > 0.19).
Phosphatidylcholine, Phosphatidylethanolamine, and Phosphatidylinositol Are Highly Labeled in All Subcellular Fractions; \([3H]\)DHA-PC and \([3H]\)DHA-PE Preferentially Shift toward Post-Golgi Vesicles (Fraction 5) after the Chase—The two most abundant PLs in retinal membranes, PC and PE, reveal a similar labeling profile after a 2-h “chase” (Fig. 3). \([3H]\)DHA-PC and \([3H]\)DHA-PE gradually increased from fractions enriched in ER to heavy fractions of the TGN, with a sharp peak in post-Golgi fraction 5 reaching a 5.2- and 4.6-fold increase, respectively, above pulse labeling. The profile of \([3H]\)DHA-PI was very similar to that of \([3H]\)diacylglycerol (DAG), and their labeling was significantly increased only in TGN fractions 7–9, with the highest increase in post-Golgi fraction 5 (3.9- and 2.5-fold, respectively). PS was the only phospholipid that did not show a peak of labeling in fraction 5 but displayed 3-fold increase between fractions 5 and 7. Phosphatidic acid (PA) labeling gradually increased from ER fractions to post-Golgi fraction 5 with no significant differences from the 1-h pulse labeling.

\([3H]\)DHA-PL labeling in ROS was very low (Fig. 3, insets) with a different pattern of distribution than all other subcellular fractions (Fig. 4). Although the small amount of ROS that is recovered in the gradient between fraction 7 and 9 may contribute to their highly free \([3H]\)DHA labeling (Fig. 2), it cannot contribute to but rather results in an underestimation of lipid labeling in these fractions that arise from inner segment membranes. \([3H]\)DHA-PL labeling, by far, the highest labeling in ROS, with a 7.9-fold increase after a 2-h “chase,” followed by DAG (4.8-fold), PC, and PS (3-fold each). No differences were observed between a 1-h pulse and 2-h “chase” labeling in \([3H]\)DHA-PE and \([3H]\)DHA-PA (Fig. 3, insets). This short-term incubation may reflect the labeling of disc membrane lipids by molecular replacement including (a) \([3H]\)DHA incorporation by turnover, (b) the fast transport from the inner segment of a portion of newly synthesized \([3H]\)DHA-lipids (i.e. \([3H]\)DHA-PL), and/or (c) further metabolism of newly incorporated \([3H]\)DHA-PLs into ROS such as N-methylation of \([3H]\)DHA-PE to \([3H]\)DHA-PC (4, 44).

The percent distribution of esterified \([3H]\)DHA displayed high values for PI, PC, and PE in all fractions except for ROS (Fig. 4). In fraction 5 they reached a similar value (28%), although in other fractions, PI labeling alone prevailed. The highest percent labeling of PI was observed in fraction 1 (48%) and to a lesser extent in fraction 2 (34%), probably associated with cytosolic proteins recovered at the top of the gradient that may sediment after the 40,000 centrifugation (12). The ratio PI to PC labeling was higher in TGN fractions as compared with post-Golgi fractions 4–6, showing the highest value in fraction 8 of the TGN after both pulse and chase labeling (Fig. 5).

The Content of Endogenous Fatty Acyl Chains of Lipids from Post-Golgi Vesicular Fractions—Total fatty acyl group content, reflecting mainly membrane phospholipids, increased gradually from the heaviest, ER-enriched fractions (2 nmol/μg protein) to the post-Golgi light vesicular fractions 4–5 (4 nmol/μg protein) (Fig. 6). The endogenous DHA content was very similar for all fractions (approximately 20% of total acyl groups) except for TGN fractions 7–9 where ROS, not completely removed prior to subcellular fractionation, cosedimented. In ROS, DHA accounted for 50% of total acyl groups. The lower % DHA content in fraction 5 as compared with ROS suggests that either the lipids from the vesicles bearing rhodopsin are less enriched in DHA-lipid and/or that lipids contributed by other vesicles with a lower degree of unsaturation are recovered in this fraction. The latter possibility is unlikely since immunolabeling of rhodopsin-bearing post-Golgi vesicles with anti-rhodopsin antibody indicated that they constitute >85% of the vesicles sedimenting in fraction 5 of the gradient (12). The net amount of DHA per protein in ROS (1.93 nmol/μg) was twice that of lipids from fraction 5 vesicles (0.85 nmol/μg protein). Although rhodopsin is the most abundant protein recovered in fraction 5, its contribution to the total proteins in the fraction

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**Fig. 3.** \([3H]\)DHA-PC and \([3H]\)DHA-PE are the phospholipids that display the highest increase of labeling in rhodopsin bearing post-Golgi vesicles after the chase. Total dpm recovered in individual lipids per μg protein are shown. Insets, labeling of ROS lipids (dpm per μg of protein) after pulse (open bars) and chase (closed bars). Other details as in Fig. 1 legend.

**Fig. 4.** \([3H]\)DHA-PI, \([3H]\)DHA-PC, and \([3H]\)DHA-PE display the highest percent labeling in all retinal subcellular fractions except for ROS which show the highest labeling in \([3H]\)DHA-PI and \([3H]\)DHA-DAG. Values represent percent labeling of individual lipids with respect to total \([3H]\)DHA recovered esterified into lipids. Other details as Fig. 1 legend.
...findings: (a) newly synthesized [3H]DHA-PLs although much less efficiently than when utilized in vivo labeling distribution observed among subcellular fractions. This rapid equilibrium [3H]DHA-lipids among all fractions was also reflected in a similar mol % content of endogenous DHA (20%). Only fractions 7–9, contaminated with ROS membranes, displayed higher mol % values. Thus, newly synthesized [3H]DHA-lipids were rapidly transported throughout the multiple compartments of the biosynthetic pathway either by vesicle budding and fusion, by carrier proteins, and/or by lateral diffusion through intermembrane bridges (17, 18). Incorporation of [3H]DHA by turnover in lipids trafficking along the transport pathway could also contribute to the uniform labeling distribution observed among subcellular fractions.

After the 2-h “chase” labeling in cold buffer, it became apparent that the high labeling of free [3H]DHA in ROS was not paralleled by an efficient esterification into disc membrane phospholipids but rather by a translocation to the inner segment where it was actively esterified. This could be accomplished by the presence of (a) DHA-fatty acid binding proteins in ROS (45) and in the cytosolic fraction of retinas (46, 47) and (b) DHA-CoA synthetase in microsomes. This enzyme that activates DHA prior to its esterification into lipids displays the highest activity in microsomes from frog retinas and very low activity in ROS (48). Although free DHA can be incorporated in disc membrane PLs by turnover of their acyl groups (49–51), our present results indicate that the bulk of DHA is incorporated into lipids in the inner segment prior to their delivery to ROS (52). Indeed, the similar or higher specific activity of total...
[\textsuperscript{3}H]DHA-lipids observed in the ER-enriched fractions as compared with other fractions enriched in membranes of the Golgi and post-Golgi supports this notion. An interesting observation after the 2-h cold chase incubation was that the increase in [\textsuperscript{3}H]DHA-lipids labeling was not of the same magnitude for all subcellular fractions (Figs. 2 and 3). It showed a clear trend from a 2-fold increase in ER and Golgi, to 2.5-fold in TGN, and the highest 4-fold increase in post-Golgi vesicles (gradient fraction 5). Because these fractions also became heavily labeled with newly synthesized \textsuperscript{[\textsuperscript{35}S]}rhodopsin after the chase, it appears that some newly synthesized [\textsuperscript{3}H]DHA-PLs are sorted, along with newly synthesized \textsuperscript{[\textsuperscript{35}S]}rhodopsin in transit toward the TGN exit. The highest [\textsuperscript{3}H]DHA-PLs labeling observed in \textsuperscript{[\textsuperscript{35}S]}rhodopsin-bearing post-Golgi vesicles suggests that they budded from microdomains in the TGN enriched in both [\textsuperscript{3}H]DHA-PLs and \textsuperscript{[\textsuperscript{35}S]}rhodopsin. In fact, rhodopsin shows a preference for association with more fluid lipids (53), and in ROS PLs with high DHA content are in closer association with rhodopsin than less unsaturated ones (54). That the highest [\textsuperscript{3}H]DHA-PLs labeling in post-Golgi vesicles observed after the 2-h “chase” could be the result of differences in lipid turnover in this fraction is unlikely since (a) labeling gradually increased from Golgi to post-Golgi vesicles, (b) no differences in the specific activity (total [\textsuperscript{3}H]DHA-PLs/endogenous DHA content) between post-Golgi vesicles and ER fraction was observed, and (c) after 1-h pulse labeling post-Golgi vesicles did not show higher labeling than other fractions. Taken together these data strongly argue in favor of a progression of label through a series of compartments. Moreover, the similar [\textsuperscript{3}H]DHA-lipid labeling observed after 1 h among all subcellular fractions also suggests an early association between newly synthesized DHA-PLs and newly synthesized rhodopsin rather than with older rhodopsin molecules already moving ahead in transit through the Golgi.

The profile of individual [\textsuperscript{3}H]DHA-lipids labeling in subcellular fractions after the 2-h “chase” was very similar for the two main membrane components PE and PC, which also displayed the highest increase of labeling in post-Golgi vesicles (Fig. 3). This observation and the very low labeling of [\textsuperscript{3}H]DHA-PC and [\textsuperscript{3}H]DHA-PE recovered in ROS (Figs. 3 and 4) suggests that their incorporation into disc membranes mainly occurs by membrane replacement. Our preliminary studies using brefeldin A, which perturbs rhodopsin trafficking, show that [\textsuperscript{3}H]DHA-PL and \textsuperscript{[\textsuperscript{35}S]}rhodopsin transfer into fraction 5, PE and PC, in particular, were successfully blocked, and also that ROS lipid labeling was reduced. \textsuperscript{2} At difference with PE and PC, [\textsuperscript{3}H]DHA-PI displayed a more sustained increase throughout TGN peaking in post-Golgi fraction 5. Although the labeling of [\textsuperscript{3}H]DHA-DAG was much lower than that of [\textsuperscript{3}H]DHA-PI (Fig. 4), both followed a very similar profile (Fig. 3), probably reflecting an active phosophodiesterase catabolism of PI with the consequent generation of labeled DAG along the TGN compart- ment. In ROS, [\textsuperscript{3}H]DHA-DAG and [\textsuperscript{3}H]DHA-PI displayed the highest labeling (Fig. 4), suggestive of their active translocation and incorporation in disc membranes by molecular replacement. Several lines of experimental evidence appear to indicate that PI, synthesized \textit{de novo} in the inner segment of photoreceptors, can actively be transferred to ROS by-passing the Golgi (24, 28). In ROS, PI can be further phosphorylated to phosphatidylinositol 4,5-bisphosphate (9, 55). Because ROS contains a light-stimulated phosphoinositide-specific phospholipase C (56, 57), the presence of a photoreceptor cytosolic PI-TP, possibly similar to the one found in rat brain cytosol (58), could contribute to sustain and modulate the inositol lipid-derived signals triggered by light.

Frog and primates retina labeled \textit{in vitro} and/or \textit{in vivo} with [\textsuperscript{3}H]DHA display an early high level of labeling of PI, reaching values similar to that of PC and PE (29, 30, 59). In the present study, we confirm and further extend our previous observation to show that [\textsuperscript{3}H]DHA-PI preferentially accumulates in the lightest fractions 1–3 of the gradient where cytosolic proteins are recovered (12), and also in ROS and in TGN fractions. The high ratio [\textsuperscript{3}H]DHA-PI to [\textsuperscript{3}H]DHA-PC found in TGN fractions 7–9 of frog retinas is the first evidence indicating a relative enrichment with newly synthesized [\textsuperscript{3}H]DHA-PI of membranes located at the exit from the TGN compartment. As previously shown in yeast (19) and on PC12 cells (60), the high PI/PC ratio may also be essential for budding of rhodopsin-bearing vesicles from TGN and further suggests the involvement of PI/PC-TP in the dynamics of Golgi function in photoreceptors. Moreover, PI-TP has been identified as a cytosolic factor that stimulates the formation of secretory vesicles in PC12 cells (60). Since membranes recovered in TGN-enriched fractions 6–11 also contain synaptophysin, a synaptic membrane protein (12), further studies are necessary to evaluate the possible contribution of [\textsuperscript{3}H]DHA-PI, in transit together with synaptophysin toward synaptic terminals, to the high [\textsuperscript{3}H]DHA-PI in TGN fractions 7–9. The overall contribution of synaptic protein biosynthesis in this fraction must be relatively minor, however, since rhodopsin synthesis greatly exceeds the rate of synthesis of all other retinal membrane proteins (61).

Post-Golgi vesicles recovered from the gradient fraction 5 (\(\rho = 1.09 \text{ g/ml}\)) display lower density than ROS which sediments in fractions 7–8 (\(\rho = 1.12–1.13\)) and therefore must have a higher lipid to protein ratio (12). This is also supported by freeze-fracture EM studies (62) showing that vesicles clustered around the connecting cilium display half the density of the intramembranous particle of ROS discs. Since in post-Golgi vesicles the total acyl group content, derived mainly from PLs (4.2 mmol/\(\mu\)g protein), was similar to that of the ROS (3.9 mmol/\(\mu\)g protein), other lipids such as sterols presumably contribute to their lower density. Cholesterol delivery to ROS may be accomplished either by a pathway(s) independent from that followed by integral plasma membrane proteins (18, 63) and/or together with rhodopsin and DHA-PL-containing post-Golgi vesicles. As vesicles fuse with the plasma membrane adjacent to the base of the connecting cilium, they could generate the cholesterol-enriched domains observed in frog photoreceptors surrounding the periciliary ridge complex (64, 65) and in nascent discs at the base of the ROS (66).

In summary, this study shows that newly synthesized [\textsuperscript{3}H]DHA-PLs, mainly [\textsuperscript{3}H]DHA-PC and [\textsuperscript{3}H]DHA-PE, are vectorially cotransported to ROS by rhodopsin-bearing post-Golgi vesicles, and other PLs such as [\textsuperscript{3}H]DHA-PI may also reach ROS and the TGN by-passing the Golgi carried by transfer proteins. Moreover, in the complex process of membrane biogenesis, addition of rhodopsin and DHA-PLs at the base of ROS could be “the driving force” for the incorporation of other PLs that do not contain DHA (approximately 40–50% of total PLs in disc membranes) possibly reaching the periciliary region by independent pathways. Current studies aim to delineate the mechanism(s) that contribute to the complex polarized trafficking of DHA-PLs either by vesicular and/or by transfer protein-mediated transport to ROS.

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