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RAPID DIGLYCERIDE PHOSPHORYLATION IN ISOLATED BOVINE ROD OUTER SEGMENTS*

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When isolated bovine rod outer segment fragments were incubated with [γ-^{32}P]ATP, ^{32}P, as revealed by autoradiography, was rapidly incorporated into rhodopsin bands on sodium dodecyl sulfate polyacrylamide gels, and into a low M_r lipid band. Incorporation of ^{32}P into rhodopsin was light-dependent, but labeling of the lipid band was not. A single phosphorylated product, phosphatidic acid, was identified by 2-dimensional thin layer chromatography and by high pressure liquid chromatography of the corresponding glycerophosphate ester. Incorporation of label into phosphatidic acid was detected as early as 15 sec following start of incubation and the product was stable for at least 30 min. No other products were detected, indicating that under the experimental conditions phosphatidic acid was not metabolized to other phospholipids. Up to 1 mol phosphatidic acid was formed per 18 to 40 mol rhodopsin present.

When light-dependent phosphorylation of rhodopsin is studied in vitro (1) and rod outer segments (ROS[1]) are analyzed for ^{32}P incorporation by sodium dodecyl sulfate (SDS[1]) polyacrylamide gel electrophoresis (PAGE[1]) it is common to see a low M_r radiolabeled band just above the tracking dye which does not stain with Coomassie Blue. Frank et al.(2) were among the earliest to suggest that this radioactive band contained lipid. We report here that isolated ROS incubated with [γ-^{32}P]ATP rapidly incorporated ^{32}P into phosphatidic

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[1] The abbreviations used are: ROS-rod outer segment(s); BHT-butylated hydroxytoluene; HEPES-4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HPLC-high pressure liquid chromatography; SDS-sodium dodecylsulfate; PAGE-polyacrylamide gel electrophoresis; PA-phosphatidic acid; PC-phosphatidylcholine; PE-phosphatidyl-ethanolamine; PS-phosphatidylserine; PI-phosphatidylinositol; TLC-thin layer chromatography.

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acid (PA[1]), apparently through the action of a diglyceride kinase on endogenous diacylglycerol.

METHODS

ROS were isolated from sucrose gradients as previously described (3) and either frozen in liquid nitrogen or used directly. [γ-^{32}P]ATP was prepared from [^{32}P]inorganic phosphate by the method of Glynn and Chapell (4) as modified by Walsh et al. (5). ROS containing 40-80 µg protein (6) were incubated at 30° for 15 sec to 30 min in a final vol of 60-100 µl containing 50 mM Tris-Cl or HEPES[1] buffer, pH 7.5; 5 mM $MgCl_2$, and 0.01-1.0 mM [γ-^{32}P]ATP (200-300 cpm/pmol). The reaction was carried out in darkness or room light, and was terminated by addition of either 1.5 ml chloroform:methanol (1:2) containing 0.01% BHT[1] (if lipid extraction followed) or 0.5 ml of 10% trichloroacetic acid/10 mM sodium pyrophosphate (if SDS PAGE followed). A Gelman ACD-18 scanning densitometer was used to quantitate autoradiograms, made on Kodak XAR-5 films.

Phospholipids were extracted from ROS with chloroform:methanol (1:2) or with acidic chloroform: methanol using the method of Schacht (7). The extracts were then spotted directly on TLC plates or deacylated by mild alkali treatment as described by Kates (8) if HPLC followed. Two-dimensional TLC employed Silica 60 plates (Merck) developed in the first dimension using chloroform:methanol:28% NH_4OH (13:7:1) and in the second dimension using chloroform:acetone:methanol: acetic acid:H_2O (10:4:2:2:1) as described by Sickotos and Rouser (9). The time course of ^{32}P incorporation was measured by densitometric scanning of autoradiograms of SDS PAGE gels, and by scintillation counting of areas scraped from TLC plates after autoradiography of one-dimensional chromatograms developed in the acid solvent only. Lipids were visualized by exposure of the TLC plates to iodine vapor. TLC phospholipid, standards were purchased from Boehringer-Mannheim or Sigma.

HPLC of deacylated glycerophosphate esters was performed with a Bio-Rad Aminex A-27 anion exchange column as previously described (10). A polyphasic gradient was used beginning with 20 mM ammonium borate, 100 mM ammonium formate, pH 9.5 and ending with 20 mM ammonium borate, 850 mM ammonium formate, pH 9.5.

SDS-PAGE was performed by the method of Fairbanks et al. (11). In some cases bands were visualized by staining with Coomassie Brilliant Blue R, and in others, dried gels were directly subjected to autoradiography.

RESULTS

^{32}P incorporation into ROS. ROS incubated with [γ-^{32}P]ATP incorporated ^{32}P into multiple bands of rhodopsin and into a low M_r band as shown in the autoradiogram in Fig. 1. The low M_r band was not present in gels stained with Coomassie Blue. Labeling of rhodopsin monomer and oligomers was light-dependent whereas that of the low M_r band was not (data not shown). No effect on labeling of the low M_r band was observed when 1 mM GTP, 1 mM cGMP, 1 mM Ca^{2+}, 1 mM EGTA, 3.3 µg/ml calmodulin, or 33.3 µg/ml antibody to calmodulin was included in the reaction mix (data not shown). Prior depletion of peripheral proteins from ROS by hypotonic washing in darkness (3) did not prevent

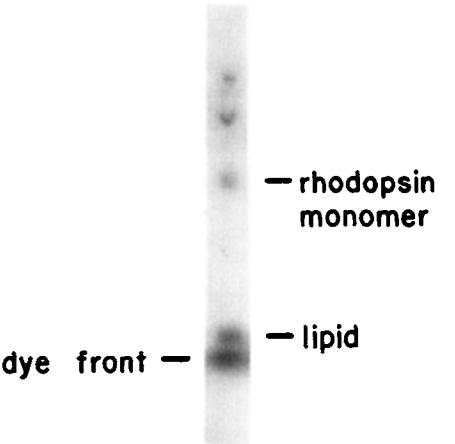

FIGURE 1. ^{32}P incorporation into ROS bands on SDS polyacrylamide gel. Autoradiogram of unstained SDS polyacrylamide gel following 1 min incubation at 30° of ROS (75 µg protein) depleted of peripheral proteins (3) with 100 µM [γ-^{32}P]ATP, 5 mM $MgCl_2$, 50 mM Tris-Cl pH 7.5. In parallel gels stained with Coomassie Blue, all three of the upper bands (rhodopsin monomer and oligomers) stained but not the lipid. The radioactive spot corresponding to the dye front contained ATP and phosphate.

labeling of the low M_r band, nor of rhodopsin. However, the presence of emulphogene or sodium cholate at 0.5% in the reaction mix inhibited labeling of the low M_r band by 90% (data not shown).

Identification of low M_r labeled product by TLC and HPLC. After incubation with [γ-^{32}P]ATP, ROS were extracted in acidic chloroform: methanol and chromatographed in 2 dimensions. The single labeled product was identified as PA by co-migration of added carrier (Fig. 2) and of PA run on a parallel chromatogram. The material remaining at the origin was most likely free phosphate.

After alkaline deacylation as described in Methods, the water-soluble products were separated by HPLC (Fig. 3). A single labeled product eluted at the same point as glycerol phosphate and inorganic phosphate standards. Peak fractions were treated with 0.4% ammonium molybdate, 0.5 N H_2SO_4 and then extracted 3 times with 1:1 isobutanol:benzene. Approximately 80% of the counts remained in the aqueous phase indicating their association with organic phosphate (12).

Time course and stoichiometry of ^{32}P incorporation. Figure 4A illustrates the time course of ^{32}P incorporation into PA in one experiment.

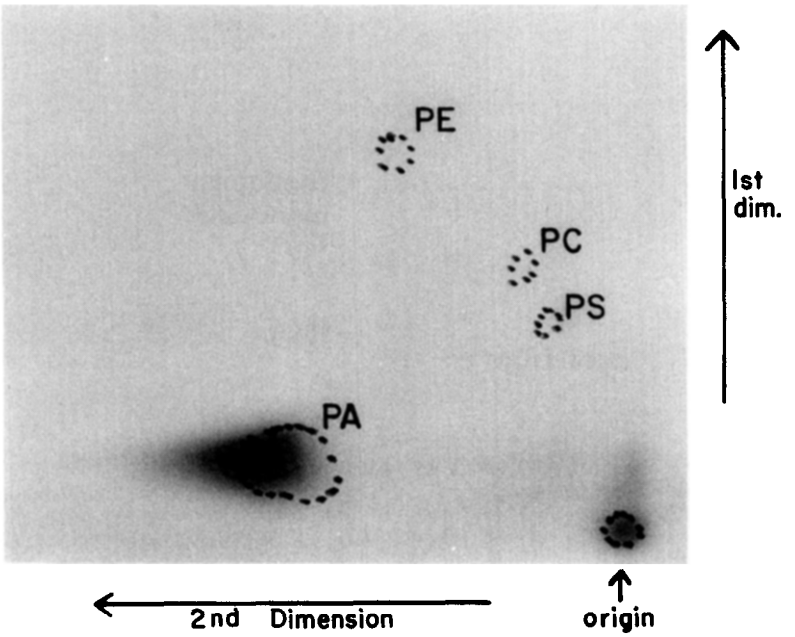

FIGURE 2. Autoradiogram of 2-dimensional thin-layer chromatogram of labeled ROS lipids. Lipids were extracted from ROS after 1 min incubation with [γ-^{32}P]ATP as described in Methods. Phosphatidic acid (PA) standard was added to the extract before chromatography. Dashed lines indicate lipid spots after development with iodine vapor. Had PI been detectable it would have appeared close to PS. Standards were run in a parallel chromatogram.

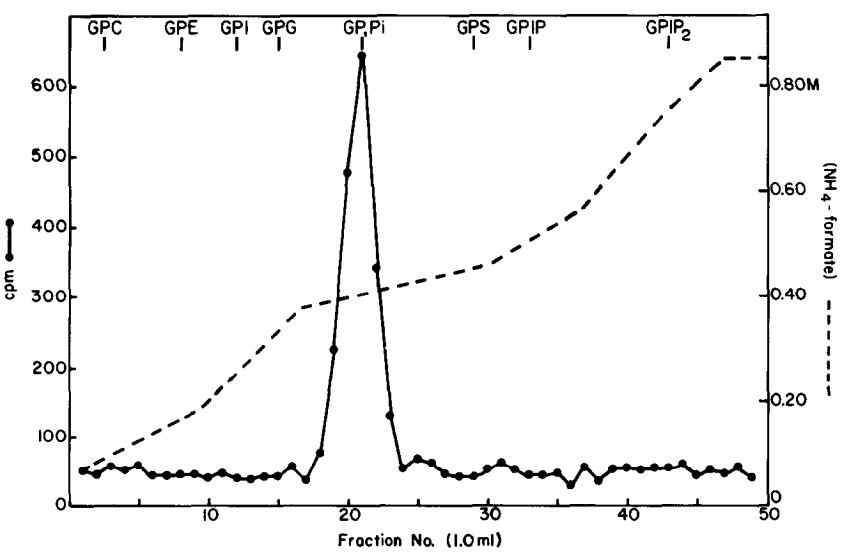

FIGURE 3. Separation of glycerophosphate esters by anion exchange HPLC. Incubation of ROS with [γ-^{32}P]ATP as before was followed by acidic chloroform/methanol extraction and mild alkaline deacylation as described in Methods. Along the top of the figure are indicated the points at which unlabeled standard samples of the indicated glycerophosphate esters eluted. GPC-glycerophosphorylcholine; GPE-glycerophosphorylethanolamine; GPI-glycerophosphorylinositol; GPG-glycerophosphorylglycerol; GP-glycerol phosphate; GPS-glycerophosphorylserine; GPIP-glycerophosphorylinositol 4-phosphate; $GPIP_2$-glycerophosphorylinositol 4,5-bisphosphate.

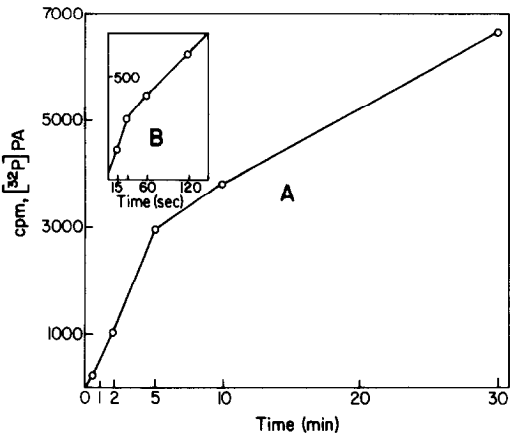

FIGURE 4A. Time course of ^{32}P incorporation into PA. After different incubation times with 1 mM [γ-^{32}P]ATP as before, ROS lipid extracts were spotted on thin layer chromatograms and run in one dimension with chloroform:acetone:methanol: acetic acid:H_2O (10:4:2:2:1). The chromatogram was exposed to X-ray film for 12 h. ^{32}P-positive areas were scraped off the plate, hydrated in 0.5 ml H_2O before addition of 7.5 ml scintillation cocktail and counted in a Beckman LS 7000 scintillation counter.

4B. Similar experiment in which incorporation of ^{32}P was assayed at shorter incubation times.

The inset (4B) shows a time course during another, shorter experiment. In 4A incorporation appeared linear for the first 5 min, reaching a final value in this experiment of about 1 mol PA labeled per 40 mol rhodopsin protein. In 4B final incorporation was calculated to be 1 mol phosphate in PA per 18 mol rhodopsin.

DISCUSSION

In isolated ROS, we observed a rapid rate of ^{32}P incorporation from ATP into phosphatidic acid. In a 30 min incubation, up to 1 mol of PA was synthesized per 18 mol rhodopsin or approximately 1 nmol PA per mg ROS protein. This incorporation was not dependent upon bleaching of the ROS by visible light. ^{32}P label could not be detected in any other lipids. Whether this reflects the absence of precursors and/or synthetic enzymes remains to be determined. However, these results indicate that diacylglycerol and a diacylglycerol kinase activity are present in isolated ROS.

Although rapid turnover of ROS lipids has been surmised for a decade or more (13,14), identification of enzymes involved has been elusive. Fliesler and

Anderson (15) have marshalled evidence that ROS lipids must be renewed in individual discs over and above disc replacement from the base of the rod. Phospholipids, especially PI[1], appear to diffuse freely from one disc to another and between discs and the ROS plasmalemma (15). In addition, slow decarboxylation of PS[1] to PE[1] in ROS has been observed (16) as well as transmethylation of PE to PC[1] (16). Anderson et al. reported a light-stimulated incorporation of both [^{3}H]inositol and ^{32}P into PI in Xenopus laevis retinas, and of [^{3}H]inositol into PI in toad retinas (17,18). However, the bulk of this incorporation was localized in the outer plexiform layer and not in the photoreceptor cells. Moreover, precursors for de novo phospholipid synthesis were not incorporated, suggesting that incorporation of inositol and phosphate reflected only the recovery of hydrolyzed, pre-existing PI. Schmidt (19) reported a light-stimulated incorporation of [^{14}C]glycerol, $^{32}PO_4$ or [^{3}H]inositol into PI in isolated rat retinas. Following fractionation, some labeled PI was detected in a crude ROS fraction (19). A low level of PA synthesis (50 x 10^{-18} mol ^{32}P per mg retina in 30 min) was also reported although this was not localized (19).

Production from endogenous PI by the action of a phospholipase may account for the presence of diacylglycerol in our isolated bovine ROS. The size of the PI pool (including PA) is restricted to 3% of the total phospholipids on a molar basis (15). The high rate of incorporation of ^{32}P and the strategic location of PA in the synthetic pathways for the major ROS phospholipids PC and PE as well as the minor components PS and PI raise the possibility that the phosphorylation observed here reflects a significant process in turnover and renewal of ROS disc phospholipids. Recently, Yoshioka et al. have reported absence of diglyceride kinase activity in photoreceptor cells of blind drosophila mutants (20), a finding which underlies the possible importance of this activity in vision.

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