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Effect of light and protein phosphorylation on photoreceptor rod outer segment acyltransferase activity

P.I. Castagnet and N.M. Giusto*

Institute de Investigaciones Bioquímicas, Universidad Nacional del Sur y Consejo Nacional de Investigaciones Científicas y Técnicas, B8000FWB Bahía Blanca, Argentina

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Abstract

Rod outer segments (ROS) exhibit high acyltransferase (AT) activity, the preferred substrate of which being lysophosphatidylcholine. To study factors possibly regulating ROS AT activity purified ROS membranes were assayed under conditions under which protein kinase C (PKC), cAMP-dependent protein kinase (PKA), and phosphatases were stimulated or inhibited. PKC activation produced a significant increase in the acylation of phosphatidylethanolamine (PE) and phosphatidylinositol (PI) with oleate, it inhibited phosphatidylcholine (PC) acylation, and phosphatidylserine (PS) and phosphatidic acid (PA) acylation remained unchanged. ROS PKA activation resulted in increased oleate incorporation into PS and PI while the acylation of PC, PE, and PA remained unchanged. Inhibition of ROS PKC or PKA produced, as a general trait, inverse effects with respect to those observed under kinase-stimulatory conditions. ROS phosphatase 2A was inhibited by using okadaic acid, and the changes observed in AT activity are described. These findings suggest that changes in ROS protein phosphorylation produce specific changes in AT activity depending on the phospholipid substrate. The effect of light on AT activity in ROS membranes was also studied and it is reported that acylation in these membranes remains unchanged independent of the illumination condition used.

Keywords: Acyltransferase; Phosphorylation; cAMP-dependent protein kinase; Protein kinase C; Rod outer segment

The outer segment of retinal rod photoreceptors (ROS)[1] is a membranous cellular organelle that consists of 50% protein and 50% lipid. ROS phospholipids and/or their components are renewed by molecular replacement as well as by membrane replacement. In the past 2 decades, phospholipase C (PLC) [1], phosphatidate phosphohydrolase (PAP), diacylglycerol lipase [2,3], diacylglycerol kinase (DAGK) [4,5], phosphatidylethanolamine *N*-methyltransferase (PEMT) [6], phospholipase D (PLD) [7], and enzyme activities catalyzing deacylation–reacylation have been reported to be present in ROS [8–13].

Fatty acids taken up by nucleated cells may be utilized either in deacylation–reacylation reactions, referred to as the Land's cycle [14], or in de novo phospholipid synthesis. Essentially, no phospholipid synthesis occurs in photoreceptor ROS, which therefore represent a convenient model for studying deacylation–reacylation reactions. These reactions are considered to be primarily involved in the establishment and maintenance of the molecular lipid species important for membrane function. They have also been proposed as the necessary mechanism for the removal of oxidized acyl groups [15] and the regulation of levels of lysophospholipids and unesterified fatty acids, including arachidonic acid [16].

Phospholipid molecules from isolated bovine ROS incorporate exogenous fatty acids at different rates [17],

* Corresponding author. Fax: +54-291-4861200.
E-mail address: ngiusto@criba.edu.ar (N.M. Giusto).

[1] *Abbreviations used:* AT, acyltransferase; DAG, diacyglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP2, phosphatidylinositol 4,5-biphosphate; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; ROS, rod outer segment; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; PLC, phospholipase C; DAGK, diacylglycerol kinase; PEMT, phosphatidylethanolamine *N*-methyltransferase; PAP, phosphatidate phosphohydrolase; DTT, dithiothreitol; PP2A, protein phosphatase 2A; OA, okadaic acid; St, staurosporine; PLA_2, phospholipase A_2; PI-3-K, phosphatidylinositol 3-kinase; PLD, phospholipase D.

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and lipid molecular species in rat ROS exhibit unique turnover rates which determine the steady-state level of each species in ROS [18].

The important role of signals arising from outside the cell in the modulation of cellular processes is being increasingly recognized. In photoreceptor cells, light is the main external signal triggering a complex cascade of conformational and biochemical transformations that result in the phototransduction process. Most of the work aimed at studying which modifications take place in photoreceptors due to light involves proteins directly participating in phototransduction. However, in the past decade it has also been reported that several enzymes related to ROS lipid metabolism, such as PLC [19,20], phospholipase A_2 (PLA_2) [11,12], phosphatidylinositol-3-kinase (PI-3-K) [21], DAGK [22], PAP [23], and PEMT [24], are modulated by light via a transducin-mediated mechanism. Furthermore, it has been recently demonstrated that PEMT and PAP activity is modulated by different association states of transducin [24].

In addition, it is now widely proven that messages carried by extracellular stimuli are transmitted via phosphorylation of the key functional proteins ultimately responsible for the cellular functions known to be affected by the various external agents. Several enzyme activities involved in phototransduction and in ROS lipid metabolism are modulated by light and/or protein phosphorylation in ROS membranes [25,26].

We have previously described an oleoyl-CoA:lysophosphatidylcholine acyltransferase in purified ROS [13]. In the present study, we have investigated whether AT activity in ROS membranes is modulated by a light- or phosphorylation-mediated mechanism. The experimental results presented herein suggest that this enzymatic activity is differently affected depending on whether ROS membranes are subjected to phosphorylation by protein kinases or dephosphorylation by protein phosphatases. Our data also suggest that such effects differ according to the kinase involved. Finally, we have observed no changes in acyltransferase activity under different illumination conditions.

Materials and methods

[^{14}C]Oleoyl-coenzyme A (56 mCi/mmol) was obtained from New England Nuclear–Dupont (Boston, MA). Adenosine 5′-triphosphate (ATP), L-α-phosphatidyl-L-serine (PS), 1,2-dioleoylglycerol (DAG), adenosine 3′,5′-cyclic monophosphate (cAMP), protein kinase A inhibitory protein, staurosporine, okadaic acid, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), and Tris were obtained from Sigma Chemical (St. Louis, MO). All other chemicals were of the highest purity available.

Bovine eyes were obtained from a local abattoir and placed on ice within 10 min of the animal's death. Retinas were dissected from the eyes after a a dark adaptation period of no less than 2 h. All subsequent isolation procedures were conducted under dim red light at 2–4 °C. Rod outer segments were purified by a discontinuous sucrose gradient as previously described [12]. Electron micrographs from purified ROS presented intact outer segments with their typical structures and no other membrane material was observed (data not shown). The purity of ROS membrane preparations was controlled also by the ratio of absorbance at 278 and 500 nm after solubilization in 70 mM potassium phosphate buffer (pH 7) containing 1% Emulphogene. Values of 2.3 ± 0.2 were typically obtained for this ratio. In addition, sodium dodecyl sulfate–polyacrylamide gel electrophoresis was used to check the purity of membranes. Even in heavily overloaded gels, rhodopsin and its oligomers comprised 80–85% of photoreceptor membrane proteins. Moreover, thin-layer chromatography of photoreceptor membrane lipids in overloaded plates showed no cardiolipin, suggesting nondetectable contamination with mitochondria. Instead, an enrichment of long and very long chain polyunsaturated fatty acids characteristic of bovine ROS, esterified to dipolyunsaturated molecular species of PC, was found. In addition, marker enzyme activities were determined in all fractions to assess possible mitochondrial or microsomal contamination of ROS membranes (band I) of the gradient. These results indicated that band I (purified ROS) contamination with microsomes or mitochondria was lower than 5%.

Incubation of purified ROS under light or dark conditions. Immediately after their isolation, ROS were assayed for acyltransferase activity in light or darkness, in the presence or absence of GTP, GDP, or their nonhydrolyzable analogs GTPγS or GDPβS, respectively. Experiments included assays in which ROS membranes were preincubated with the nonhydrolyzable analog before the labeled oleoyl-CoA was added and assays in which the membranes were co-incubated both with the analog and with the enzyme substrate. Concentration curves (1–500 μM) were done with all the drugs (GTP, GDP, and their nonhydrolyzable analogs).

Incubation of ROS under conditions favoring endogenous PKC activity. The incubation mixtures for ROS phosphorylation via PKC contained 3 mg ROS protein, 60 mM Tris–HCl buffer, pH 7.4, 10 mM KF, 0.5 mM DTT, 7.5 mM $MgCl_2$, 2.5 mM ATP, 20 μg/ml PS, 0.8 μg/ml 1,2-diolein, and 0.5 mM $CaCl_2$ in a total volume of 1.72 ml. The mixture was sonicated for 30 s to increase the accessibility of cofactors to the enzyme and the samples were incubated at 37 °C for 30 min in light in a shaking bath. ROS incubated with the buffer described above but without ATP, PS, and 1,2-diolein, to which Me_2SO was added at a final concentration of 0.01%, were used as controls. Alternatively, 0.5 mM EGTA was

added to control membranes to rule out any activation of PKC by endogenous calcium.

To study the effect of protein kinase C inhibitors on ROS AT, 40 nM staurosporine instead of DAG was added to the assay medium. Staurosporine was added in Me_2SO 0.1%, in a volume not exceeding 0.01% of the final assay volume (at this concentration Me_2SO was found not to affect the activities of the enzymes being studied). The assay mixture was incubated under light conditions at 37 °C for 30 min.

Incubation of ROS membranes under conditions favoring endogenous PKA activity. The incubation mixture for ROS phosphorylation by cAMP-dependent protein kinase contained 3 mg ROS protein, 60 mM Tris–acetate buffer, pH 6.8, 2.5 mM ATP, 10 mM KF, 50 μM cAMP, and 2 mM $MgCl_2$ in a total volume of 1.72 ml. The mixture was incubated in light at 37 °C for 15 min. The corresponding controls were ROS membranes incubated with all ingredients except ATP and cAMP to which Me_2SO was added at a final concentration of 0.01%.

To study the effect of protein kinase A inhibitors on ROS AT, ROS membranes were preincubated with 25 μM protein kinase A inhibitory protein, then cAMP was added, and the mixture was further incubated for 20 min under light conditions at 37 °C. Protein kinase A inhibitory protein was added in Me_2SO 0.1%, in a volume not exceeding 0.01% of the final assay volume (at this concentration Me_2SO was found not to affect the activities of the enzymes being studied).

Incubation of ROS membranes with protein phosphatase 2A inhibitor, okadaic acid. Control incubation mixtures for phosphatase activity contained 3 mg ROS protein, 60 mM Tris–HCl buffer, pH 7.5, 1 mM EDTA, 0.5 mM EGTA, 1 mg/ml BSA, 2 mM DTT, and 0.2 mM PMSF in a total volume of 1.72 ml. ROS membranes incubated with the buffer containing 1 μM okadaic acid were used to assess phosphatase 2A inhibition. Okadaic acid was added in Me_2SO 0.1% in a volume not exceeding 0.01% of the final assay volume. The reaction mixture was incubated in light at 37 °C for 30 min.

At the end of the incubation periods for protein phosphorylation or dephosphorylation, aliquots of the samples were diluted to 10 ml with the buffer used for assaying AT activity and centrifuged at 35,300*g* for 30 min. The supernatant was discarded and the various pellets were resuspended by vortexing in 60 mM Tris–HCl, pH 7.8, for the assay of AT activity.

Assay of ROS oleoyl-CoA:lysophospholipid acyltransferase activity. The activity of ROS acyl-CoA:lysophospholipid acyltransferase was determined by measuring the incorporation of [1-^{14}C]oleate from [1-^{14}C]oleoyl-CoA (56 mCi/mmol) into lysophospholipids. The standard incubation mixture contained 60 mM Tris–HCl, pH 7.8, 4 μM [1-^{14}C]oleoyl-CoA (10^5 dpm/assay), and 200 μg of ROS protein in a final volume of 250 μl. The reaction was started by the addition of radiolabeled substrate resuspended in assay buffer or by the addition of the membranes. The assay mixture was sonicated for 30 s and it was then incubated for 5 min with shaking at 37 °C. The reaction was stopped by addition of 5 ml chloroform/methanol (2/1, v/v). Blanks were prepared identically, except that chloroform/methanol was added to the membranes before the substrate was added. Alternatively, blanks were prepared by using membranes boiled for 10 min and incubated as described above. Both blank preparations yielded similar values of activity (less than 0.5% of the activity observed in the experimental samples), and the first preparation was there used on a regular basis. The lipids were extracted according to the methoi Folch et al. [27]. The lipid extract was dried under N_2, resuspended in chloroform/methanol (2/1, v/v), and spotted on precoated silica gel plates. Unlabeled phospholipids were used as standards. The chromatogram was developed in a solvent system made up of chloroform/methanol/acetic acid/water (50/37.5/3.5/2, v/v). Lipid spots were visualized by exposing the plates to iodine vapors. The regions corresponding to PC, PS, PI, PE, and PA were scraped off and transferred to vials where the silica was deactivated by addition of water. Then, 5 ml of 0.4% Omnifluor in toluene/Triton X-100 (4/1, v/v) was added. Radioactivity in lipid spots from the blanks (typically 100 cpm) was subtracted from that of the experimental samples. The activity of the AT is expressed in nanomoles (or pmol) of diacylphospholipid formed per milligram of protein per hour.

Other methods. Protein content was determined according to the method of Bradford [28]. Statistical analysis was performed using the Student *t* test. The difference was considered to be significant when $P < 0.05$.

Results

Acyltransferase activity in ROS membranes incubated under light or dark conditions

The effects of light or GTP, GDP, or GDP/GTP analogs on PC acylation by AT activity are shown in Fig. 1. Light produced no changes in AT activity nor did GTP or its nonhydrolyzable analog, GTPγS. This could be indicating that neither light nor G proteins are involved in the modulation of acyltransferase activity in ROS membranes under the experimental conditions described in this paper.

Acyltransferase activity toward lysophosphatidylcholine in ROS membranes preincubated under conditions stimulating phosphorylation by endogenous PKC or PKA

We have carried out ROS protein phosphorylation by PKA and PKC in the presence of [γ-^{32}P]ATP and

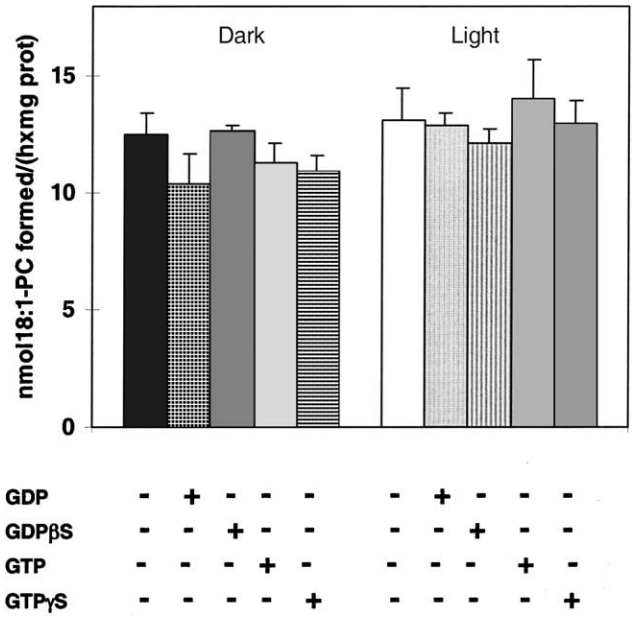

Fig. 1. Effects of light or G protein activation/inactivation on lyso-PC acylation. Purified ROS membranes were incubated in light or dark in the presence or absence of GDP, GTP, or their nonhydrolyzable analogs (GDPβS and GTPγS) at 37 °C for 5 min. Acyltransferase activity was assessed by measuring the incorporation of [^{14}C]oleate from [1-^{14}C]oleoyl-CoA into PC. The standard incubation mixture contained 60 mM Tris–HCl, pH 7.8, 4 μM [1-^{14}C]oleoyl-CoA (10^5 dpm/assay), and 200 μg of ROS protein in a final volume of 250 μl. The assay mixture was sonicated for 30 s and it was then incubated for 5 min with shaking at 37 °C. The activity of the AT is expressed as the mean nanomoles of diacylphospholipid formed per hour per milligram of protein ±SD ($n = 8$).

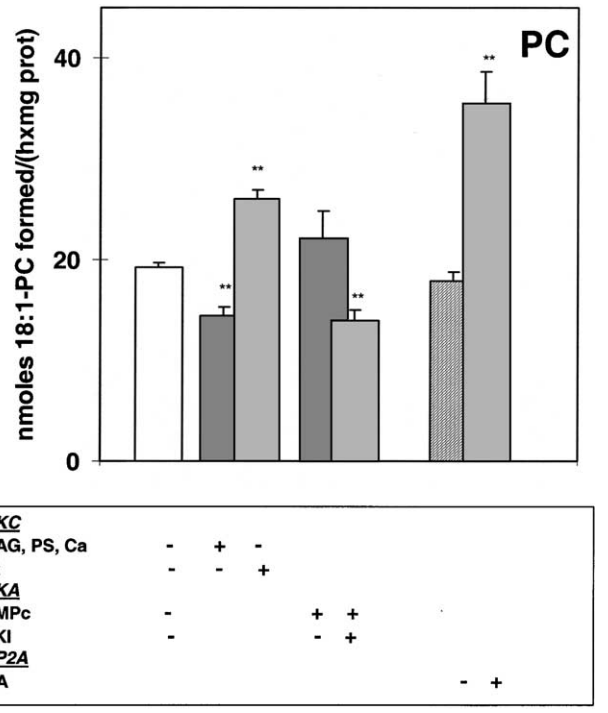

Fig. 2. Effects of ROS PKC, PKA, or PP2A stimulation or inhibition on lyso-PC acylation. Purified ROS membranes were incubated under conditions for the stimulation or inhibition of PKC or PKA or in the presence of okadaic acid (a PP2A inhibitor). Acyltransferase activity was measured in the phosphorylated or dephosphorylated membranes by measuring the incorporation of [^{14}C]oleate into PC, as described for Fig. 1. The blank bar represents acylation values obtained in control membranes resuspended in buffer containing 60 mM Tris–HCl, pH 7.4, 10 mM KF, 0.5 mM DTT, 7.5 mM $MgCl_2$, and 2.5 mM ATP and lacking cofactors necessary for phosphorylation by PKC and PKA. The hatched bar represents acylation values obtained in control membranes resuspended in 60 mM Tris–HCl buffer, pH 7.5, 1 mM EDTA, 0.5 mM EGTA, 1 mg/ml BSA, 2 mM DTT, and 0.2 mM PMSF. The results are expressed as the mean nmol × (h × mg prot)$^{-1}$ ± SD ($n = 3$). Statistical analysis was performed using Student's t test: *$P < 0.005$, **$P < 0.001$.

dephosphorylation in the presence of okadaic acid under the same conditions as those of the experiments carried out in the present work to ensure that under these experimental procedures, protein phosphorylation or dephosphorylation does occur [25]. The results obtained support the idea that the changes observed in the enzymatic activities studied after incubation of ROS membranes under phosphorylating and dephosphorylating conditions are caused mainly by phosphorylation or dephosphorylation of ROS proteins (data not shown).

The effects of preincubating ROS membranes under phosphorylating and dephosphorylating conditions on the acylation of PC are shown in Fig. 2. Under conditions favoring protein phosphorylation via endogenous PKC, a 26% inhibition was observed in PC AT activity with respect to the values obtained for control membranes incubated in the same buffer lacking the specific cofactors for phosphorylation to occur (Fig. 2).

Phosphorylation of ROS membrane proteins via PKA, in turn, produced no significant changes in PC AT activity. The PC AT activity in membranes previously incubated in the presence of the PKA-inhibitory protein inhibited PC AT activity by 30%.

In ROS membranes incubated in the presence of the protein phosphatase 2A inhibitor okadaic acid (OA) a onefold increase in PC AT activity was observed.

Acyltransferase activity toward lysophosphatidylethanolamine in ROS membranes preincubated under conditions stimulating phosphorylation by endogenous PKC or PKA

The effects of preincubating ROS membranes under phosphorylating and dephosphorylating conditions on the acylation of PE are shown in Fig. 3. Under conditions favoring protein phosphorylation via endogenous PKC, acylation of PE was stimulated by 32% with respect to the values obtained for control membranes (Fig. 3). The PE AT activity in membranes previously incubated in the presence of the PKC inhibitor staurosporine strongly inhibited PE AT activity by 55%, reaching values that were well below the values of the activity measured in control membranes.

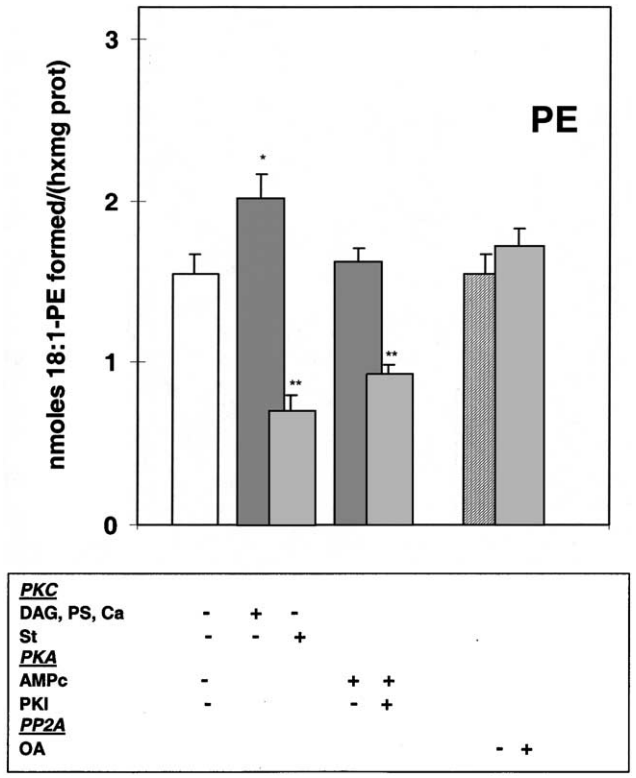

Fig. 3. Effects of ROS PKC, PKA, or PP2A stimulation or inhibition on lyso-PE acylation. Purified ROS membranes were incubated under conditions for the stimulation or inhibition of PKC or PKA or in the presence of okadaic acid (a PP2A inhibitor), and acyltransferase activity was assayed in the phosphorylated or dephosphorylated membranes as described for Fig. 1 except that the incorporation of [^{14}C]oleate into PE was measured. The blank and the hatched bars represent acylation values obtained in control membranes assayed as described for Fig. 2. The results are expressed as the mean nmol $\times$ (h $\times$ mg prot)$^{-1}$ $\pm$ SD ($n = 3$). Statistical analysis was performed using Student's t test: *$P < 0.005$, **$P < 0.001$.

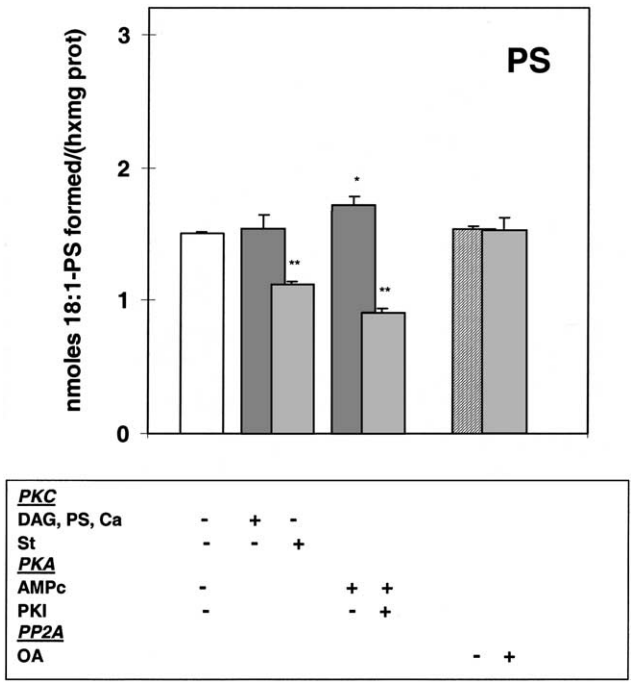

Fig. 4. Effects of ROS PKC, PKA, or PP2A stimulation or inhibition on lyso-PS acylation. Purified ROS membranes were incubated under conditions for the stimulation or inhibition of PKC or PKA or in the presence of okadaic acid (a PP2A inhibitor), and acyltransferase activity was assayed in the phosphorylated or dephosphorylated membranes as described for Fig. 1 except that the incorporation of [^{14}C]oleate into PS was measured. The blank and the hatched bars represent acylation values obtained in control membranes assayed as described for Fig. 2. The results are expressed as the mean nmol $\times$ (h $\times$ mg prot)$^{-1}$ $\pm$ SD ($n = 3$). Statistical analysis was performed using Student's t test: *$P < 0.005$, **$P < 0.001$.

Phosphorylation of ROS membrane proteins via PKA produced no significant changes in PE AT activity. However, when the membranes were incubated in the presence of PKA-inhibitory protein, PE AT activity was inhibited by 40% with respect to control value.

In ROS membranes incubated in the presence of the protein phosphatase 2A inhibitor OA, no significant changes in PE AT activity were observed.

Acyltransferase activity toward lysophosphatidylserine in ROS membranes preincubated under conditions stimulating phosphorylation by endogenous PKC or PKA

The effects of preincubating ROS membranes under phosphorylating and dephosphorylating conditions on the acylation of PS are shown in Fig. 4. Under conditions favoring protein phosphorylation via endogenous PKC, acylation of PS was unaffected (Fig. 4). The PS AT activity in membranes previously incubated in the presence of the PKC inhibitor staurosporine was slightly lower than the activity measured in control membranes.

Phosphorylation of ROS membrane proteins via PKA produced, in turn, a slight increase in PS AT activity (14%). This increase was reversed and the acylation of PS was inhibited by 40% when ROS membranes were incubated in the presence of the PKA-inhibitory protein.

In ROS membranes incubated in the presence of the protein phosphatase 2A inhibitor OA, no significant changes in PS AT activity were observed (Fig. 4).

Acyltransferase activity toward lysophosphatidylinositol in ROS membranes preincubated under conditions stimulating phosphorylation by endogenous PKC or PKA

The effects of preincubating ROS membranes under phosphorylating and dephosphorylating conditions on the acylation of PI are shown in Fig. 5. Phosphorylation of ROS membranes by both endogenous PKC and PKA resulted in a significant increase in PI acylation (by 80 and 63%, respectively) with respect to the values of

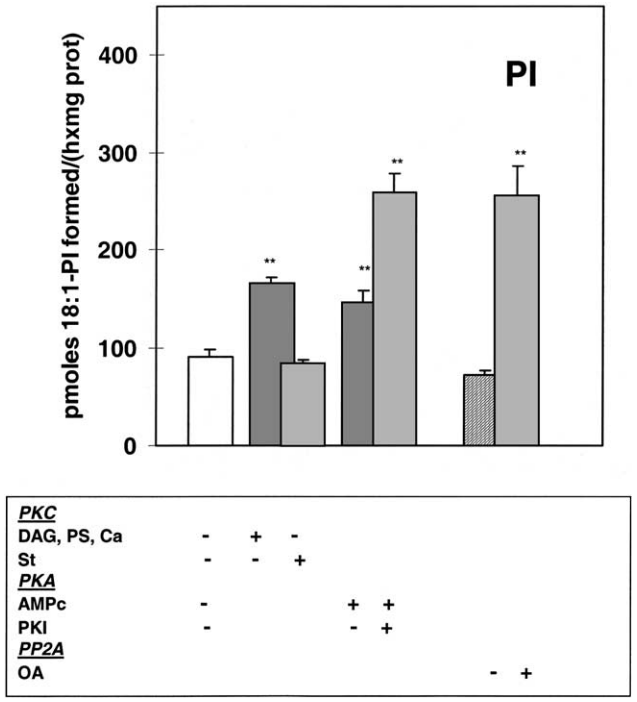

Fig. 5. Effects of ROS PKC, PKA, or PP2A stimulation or inhibition on lyso-PI acylation. Purified ROS membranes were incubated under conditions for the stimulation or inhibition of PKC or PKA or in the presence of okadaic acid (a PP2A inhibitor), and acyltransferase activity was assayed in the phosphorylated or dephosphorylated membranes as described for Fig. 1 except that the incorporation of [^{14}C]oleate into PI was measured. The blank and the hatched bars represent acylation values obtained in control membranes assayed as described for Fig. 2. The results are expressed as the mean pmol $\times$ (h $\times$ mg prot)$^{-1}$ $\pm$ SD ($n = 3$). Statistical analysis was performed using Student's t test: $^{*}P < 0.005$, $^{**}P < 0.001$.

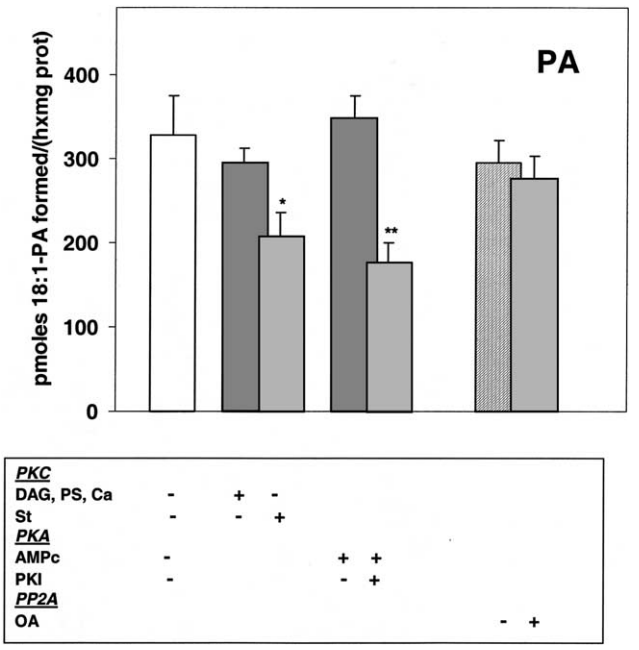

Fig. 6. Effects of ROS PKC, PKA, or PP2A stimulation or inhibition on lyso-PA acylation. Purified ROS membranes were incubated under conditions for the stimulation or inhibition of PKC or PKA or in the presence of okadaic acid (a PP2A inhibitor), and acyltransferase activity was assayed in the phosphorylated or dephosphorylated membranes as described for Fig. 1 except that the incorporation of [^{14}C]oleate into PA was measured. The blank and the hatched bars represent acylation values obtained in control membranes assayed as described for Fig. 2. The results are expressed as the mean pmol $\times$ (h $\times$ mg prot)$^{-1}$ $\pm$ SD ($n = 3$). Statistical analysis was performed using Student's t test: $^{*}P < 0.005$, $^{**}P < 0.001$.

oleate incorporation in PI in control membranes. However, when acylation in the presence of kinase inhibitors was assayed two situations were found. In membranes that had been incubated in the presence of staurosporine, a PKC inhibitor, values of activity returned to control values (Fig. 5), whereas a 187% increase in PI AT activity could be observed when membranes were incubated in the presence of the PKA inhibitor.

ROS membranes incubated in the presence of OA incorporated 2.5 times more oleate into PI than control membranes.

Acyltransferase activity toward lysophosphatidic acid in ROS membranes preincubated under conditions stimulating phosphorylation by endogenous PKC or PKA

The effects of preincubating ROS membranes under phosphorylating and dephosphorylating conditions on the acylation of PA are shown in Fig. 6.

We observed no significant changes in the incorporation of oleate into PA when membranes were incubated under phosphorylation conditions by PKC or PKA. However, in the presence of the inhibitors staurosporine and PKA-inhibitory protein, a decrease in PA acylation by 35 and 43%, respectively, was observed.

Discussion

Light is the most important physiological, extracellular signal affecting photoreceptor membranes. It exerts its effects on photoreceptor cells by acting on a G-protein-coupled "receptor": rhodopsin. Transducin, the photoreceptor's most abundant G protein, is a heterotrimeric G protein that transduces light interaction with rhodopsin into an intracellular response underlying the photoresponse of rods. Transducin is inactive in the GDP-bound, heterotrimeric state and it is activated by light through a rhodopsin-catalyzed guanine nucleotide exchange resulting in GTP binding to the α subunit. GTP binding leads to the dissociation of Gα-GTP from G$\beta\gamma$ subunits and the activation of downstream effectors by both Gα-GTP and free G$\beta\gamma$ subunits. G protein deactivation occurs when Gα subunit hydrolyzes GTP to GDP.

It has been reported that several enzymes related to ROS lipid metabolism such as PLC [19,20], PLA_2 [11,12], PI-3-K [22], PEMT [24], and PAP [23] are modulated by light, and transducin has been suggested to be involved in this mechanism.

Transducin activation and deactivation can be mimicked by using nonhydrolyzable GTP or GDP analogs, respectively. When we used this strategy to mimic light activation, or when we carried out acyltransferase assays in light or darkness to see whether the enzyme was under light control, it was not possible to detect significant changes in AT activity under the assay conditions we used.

One of the mechanisms most widely used by nature in signal transduction is reversible phosphorylation (and dephosphorylation) of proteins. This modification produces changes in the activity or function of the modified protein. In retinal photoreceptor ROS this mechanism plays a key role in putting an end to the phototransduction process by rhodopsin kinase-mediated rhodopsin phosphorylation. The presence of a variety of kinases in ROS, such as rhodopsin kinase [29], cyclic nucleotide-dependent protein kinase [30], and protein kinase C [31], has been reported. Yet, and even though some of their substrates have been identified, the function of this modification related to the physiological role of the protein has not been so far elucidated. Tyrosine phosphorylation has also been demonstrated to occur in vertebrate ROS [32]. Protein phosphatase activities involved in the dephosphorylation of the phosphoproteins have also been described in ROS [33].

Enzymes involved in the metabolic turnover of fatty acids through the deacylation–reacylation cycle must be stringently controlled since they generate lipid intermediates that, on the one hand, have potent detergent properties and, on the other, are involved in signal transduction mechanisms. Free fatty acids, acyl-CoA, and lysophospholipids are particularly known for their membrane-perturbing properties. Under normal conditions, only trace amounts of these compounds are present in the cell.

ROS membranes contain enzymes for fatty acid hydrolysis from phospholipids (phospholipases A) [8,10–12] as well as for the activation and transfer of fatty acids to lysophospholipids (acyltransferases) [8,9,13].

The results herein reported suggest the existence of different acyltransferases. The fact that phosphorylation by a given kinase produces different changes (incremental or decremental) in the acylation of the different lysophospholipids supports this view. Acyltransferases specific for a given lysophospholipid class have been described [34–36] and some of them have even been purified [34,35]. However, no reports have, to our best knowledge, focused on the mechanisms by which they are regulated. It cannot be ruled out that the binding of different substrates could change the conformational state of the enzyme, making it more or less susceptible to phosphorylation, or that phosphorylation of the enzyme produces either activation or inhibition depending on which substrate is bound to the enzyme.

Our results show that lipid metabolism in isolated ROS is modulated by mechanisms involving specific protein kinases and phosphatases. Activation of endogenous ROS PKC produced an increase in PE and PI acylation while oleate incorporation into PC was decreased, and PS and PA acylation remained unaltered. These changes in AT activity were reversed in the membranes incubated in the presence of staurosporine. Interestingly, PS and PA acylation in the presence of staurosporine decreased with respect to the acylation observed in control membranes, suggesting a partial activation of PKC in unstimulated membranes. Activation of endogenous ROS PKA produced an increase in the incorporation of oleate into PS and PI, while PC, PE, and PA acylation did not change. Incubation of ROS membranes in the presence of the PKA-inhibitory protein, however, produced a decrease in the acylation of PC, PE, PA, and PS, suggesting that PKA is partially activated in control membranes. PS acylation was the most markedly inhibited under this experimental condition. In contrast, PI acylation in the presence of the PKA-inhibitory protein was greatly increased. The role of PKA-inhibitory protein in vivo has not yet been completely elucidated [37], and this unexpected finding could be due to a specific effect of the PKA-inhibitory protein on the lyso-PI acyltransferase enzyme.

Okadaic acid is a known phosphatase 2A inhibitor. Incubation of ROS membranes in the presence of this agent produced an increase in PC and PI acylation and no further changes in the acylation of the rest of the phospholipids. These results suggest that phosphatase 2A specifically hydrolyzes phosphates incorporated by particular kinases as has been demonstrated in other systems [38], and they also suggest that there must be other phosphatases operative in ROS membranes. The presence of phosphatase isozymes 2Cα and 2Cβ in bovine ROS has been recently demonstrated by enzymatic analysis as well as by immunological techniques [39].

Lysophospholipids have been found to be important regulatory molecules involved in a series of physiologically important processes. These include increase in intracellular calcium concentration [40], activation of receptor internalization [40], membrane fusion events [41], and activation of MAP kinases [42]. These lysophospholipids may act on G-protein-coupled receptors through a variety of G proteins [42].

The organization of individual rods in a discontinuous lateral array distinguishes these cells from cones. The length of ROS remains constant through the coordinated processes of disk membrane formation at the base and disk shedding at the tip. New disk closure and disk membrane pack formation prior to shedding are consid-

ered to involve tightly controlled membrane fusion processes. While the specificity and timing of membrane fusion in diverse physiological reactions is determined by proteins, fusion always involves the merger of membrane lipid bilayers. Inhibition of fusion is mediated by inverted cone-shaped lysophospholipids (as lyso-PC and lyso-PA) and promotion of fusion is mediated by cone-shaped oleic acid, suggesting that fusion proteins begin membrane merger by promoting the formation of a bent, lipid-involving, stalk intermediate [43].

The effect of lysophospholipids on the cytoskeleton has also been described. Depletion of lysophosphatidic acid triggers a loss of oriented detyrosinated microtubules that support motility in fibroblasts [44]. In amphibian ROS the incisures mark the site of a cytoskeletal system containing longitudinal microtubules. This cytoskeleton is expected to be important for the normal structure, function, and renewal of ROS. Thus, modifications in cytoskeletal organization could affect, for example, the movement of disks from the base to the tip of the ROS.

Lysophospholipids have also been involved in the modulation of ion channel activity. The two pore domain K^+ channels TREK and TRAAK are opened by membrane stretch. Emerging work shows that these channels are opened by various lipids, including long chain polyunsaturated anionic fatty acids and neutral cone-shaped lysophospholipids [45]. TRAAK has been found to be present in brain, spinal cord, and retina [46].

Lyso-PA has also been involved in the regulation of PIP2 levels in NIH 3T3 cells. PIPKI, the kinase that phosphorylates phosphatidylinositol-4-P to yield PIP2, is a phosphoprotein whose phosphorylation state regulates its activity. The kinase is active in the unphosphorylated state, and dephosphorylation is activated by lyso-PA-induced modulation of protein phosphatase 1 activity [47]. PIP2 has been shown to modulate a series of enzyme activities, such as PLD, that have been suggested to participate in membrane fusion events, and its hydrolysis products upon PLC activation, inositol trisphosphate and diacylglycerol, are known second messengers involved in PKC- and Ca-mediated signaling pathways. These pathways have been shown to be operative in ROS membranes.

The fact that lysophospholipid acylation is regulated by phosphorylation/dephosphorylation of ROS membrane components and the fact that these molecules could be participating in many physiologically important processes that take place in these membranes opens the door for future research aimed at defining the role of specific lysophospholipids in retinal ROS physiology.

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