Presence of a Light-independent Phospholipase A$_2$ in Bovine Retina but Not in Rod Outer Segments*

Michèle Jacob, Philip K. Weech, and Christian Salesse†

From the Centre de Recherche en Photobiophysique, Université du Québec à Trois-Rivières, Trois-Rivières, Quebec, Canada G9A 5H7 and the Merck Frosst Centre for Therapeutic Research, Pointe-Claire-Dorval, Quebec, Canada M9R 4P8

Rod outer segments (ROS) are responsible for the visual transduction process. Rhodopsin, which constitutes 85–90% of ROS proteins, absorbs light photons, changes its conformation, and then binds to a heterotrimeric G-protein called transducin. As a consequence, transducin dissociates into T$a$ and T$b$$g$ subunits. The presence in ROS of a phospholipase A$_2$ (PLA$_2$) stimulated by light and guanosine 5'-O-(3-thio)triphospho was first demonstrated in 1987 (Jelsema, C. L. (1987) J. Biol. Chem. 262, 163–168). This led that author to conclude that ROS PLA$_2$ could be involved in the phototransduction process, and raised the possibility of receptor-mediated activation of PLA$_2$ via G-proteins in cell types other than rods. However, the biochemical characteristics and the role of this PLA$_2$ have not been fully elucidated. We have tried to reproduce some of the results previously reported in order to further characterize this enzyme. We have found that, in our hands, there is neither light-dependent nor GTP-dependent PLA$_2$ activity in intact purified ROS. Also we failed to detect PLA$_2$ activity in those ROS preparations. Nevertheless, we detected significant amounts of PLA$_2$ activity in two subretinal fractions adjacent to ROS: RPE (enriched with retinal pigment epithelial cells) and P200 (presumably containing neuronal cells, Müller cells, and rod inner segments). The enzyme present in both RPE and P200 is light- and GTP-independent, Ca$^{2+}$- and Mg$^{2+}$-independent, and seems to be optimally active in the alkaline pH range. Our results suggest that there is, if any, vanishingly little PLA$_2$ in ROS, or PLA$_2$ activity in intact purified ROS and that the activity levels previously reported in the literature could have been due to a contamination by either RPE or P200. This is supported by our observation that some contaminated ROS preparations were "PLA$_2$ active."

In the mammalian eye, rod outer segments (ROS) consist of a stack of 1000–2000 disks which contain the visual pigment, rhodopsin. ROS are thus responsible for the phototransduction process. It has been clearly shown that following absorption of light, photoexcited rhodopsin binds to and activates transducin (T$a$b$g$), which is a member of the heterotrimeric GTP-binding protein family. During the activation of transducin, the GDP molecule (normally associated with the inactive state of the protein) is exchanged for GTP. As a consequence, transducin dissociates into T$a$ and T$b$$g$ subunits. $T_a$ is well known to activate a cGMP-phosphodiesterase whose activity eventually leads to hyperpolarization of the rod through closure of Na$^+$/Ca$^{2+}$ cGMP-dependent channels (see Refs. 1–4 for reviews). Jelsema reported in 1987 (5) that phospholipase A$_2$ (PLA$_2$) activity was present in "crude ROS" and "partially purified ROS," and that this activity was stimulated by light and a non-hydrolyzable analog of GTP, GTP$y$S. Moreover, Jelsema and Axelrod (6) demonstrated that T$b$$g$ was responsible for the activation of this ROS PLA$_2$. These results led them to conclude that ROS PLA$_2$ could be involved in the phototransduction process. However, conflicting results have been reported since that time. In fact, although Zimmerman and Kays (8) detected phospholipase A activity in their ROS preparations, the activity that they measured, either PLA$_2$ or PLA$_3$, was neither light-dependent nor GTP-dependent. It was rather stimulated by ATP and coenzyme A (CoA). Moreover, the maximum activity that they observed was approximately 1 order of magnitude lower than that reported by J elsema (5). In addition, Castagnet and Giusto (9) published data on ROS PLA$_2$ activity but the maximum activity that they obtained was almost 3 orders of magnitude lower than that reported by J elsema (5).

The mechanism of stimulus-response coupling between G-proteins and phospholipase A$_2$ could be important in many cells other than the retinal rods, given the wide distribution of PLA$_2$ in tissues (10–20) and its potential role in controlling the biosynthesis of prostaglandins, leukotrienes, and other inflammatory mediators (21–25), as well as the turnover of phospholipids. Moreover, the biochemical characteristics of this secreted PLA$_2$ have not been fully studied yet and its role is still unknown. We present here results showing that two subretinal fractions, namely RPE (enriched with retinal pigment epithelial cells) and P200 (presumably containing neuronal cells, Müller cells, and rod inner segments) are rich in PLA$_2$ activity having an alkaline pH optimum. Our results also point out that purified ROS isolated by different methods, vortexing, homogenizing or hand shaking, are devoid of significant PLA$_2$ activity. The varying levels of activity that have been reported in ROS preparations by the aforementioned authors (5, 8, 9)
could thus, in most cases, be accounted for by a contamination by adjacent retinal cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—1-Palmityl-2-[14C]arachidonoyl-phosphatidylcholine ([14C]PAPC), 1-hexadecyl-2-[14C]arachidonoyl-phosphatidylcholine ([14C]HAPC), 1-Palmityl-2-[14C]arachidonoyl-phosphatidylethanolamine ([14C]PEPA), 1'-[14C]oleoyl-2-[14C]oleoyl-phosphatidylcholine ([14C]OPPC), 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were purchased from Dupont Canada. 1-Oleoyl-
lysophospholipids and dipalmitoyl phosphatidylcholine (DPFC) were from Avanti Polar Lipids. Tris-HCl, 20% sucrose, 0.5 mM DTT, pH 7.4) and were either homogenized immediately or kept on ice in total darkness for about 2 h (to allow for reannealing (30). When [14C]CPAPE was used as the substrate, unlabeled DPPC ([80 μg/ml]) was added to [14C]CPAPE before the evaporation of solvent. The sonication and incubation steps were done at 42°C. Reactions were initiated by addition of 20 μl of the sonicated radiolabeled substrate to ROS aliquots containing 20–75 μg of proteins. The buffer used was either 30 mM Tris-HCl, pH 8.8, 8.5 mM CaCl2, 30 mM MgCl2, 0.6 mM NaCl, 4 μM glutathione as described by Zimmerman and Keys (8). The total volume was 250 μl. Incubations were done at 37°C under either dim red light or white light (1330 lx; we used a 250 watt tungsten lamp which practically does not emit in the UV range (31)). Reactions were stopped at specified times with 1.25 ml of Dole's reagent (isopropyl alcohol, n-heptane, 1 ν H2SO4, 40:10:1 v/v). After addition of 0.75 ml of n-heptane and 0.5 ml of water, samples were vortexed and allowed to stand for 5 min for phase separation. The enzymatically released [14C]oleic or [14C]arachidonic acids were separated from unreacted substrate by addition of 10 ml of 1 M NaOH, followed by the addition of 0.5 ml of 1 M NaOH and 0.5 ml of water, and 1 ml of Bio-Sil A silicic acid with 1 ml of diethyl ether. After addition of 7.5 ml of Econofluor-2 to the 1-ml eluates, radioactivity was measured using a Beckman scintillation counter programmed to correct for quenching and counting efficiency. PLA2 activity was expressed as nanomoles of [14C]arachidonic or [14C]oleic acid released/μg of proteins and was used directly for the determination of the radiolabeled substrate by the endogenous unlabeled phospholipids. This was done by using a phospholipid to rhodopsin weight ratio of 1:1 (32). Zero-time control values were subtracted. This method was also used to assay PLA2 activity in the particulate and soluble fractions collected during the ROS purification procedure, as well as in the RPE and P200 fractions. In these cases, we used phospholipid to protein weight ratios of 0.09:1 for RPE, which is in agreement with the data published by Berman (33), and 0.35:1 for P200 (as determined in our laboratory by measuring the phosphorus content of a P200-phospholipid extract (see below)).

**Measurement of ROS PLA2 or PLA1 Activity**—In vitro investigations of ROS PLA2 or PLA1 activity were done by HPLC. Purified ROS were incubated at 37°C for different periods of time (0.5, 1, 3, 6, 12, and 24 h) with 0.35 mM of H2SO4 or 0.35 mM of HClO4. All ROS preparations were phospholipid to protein weight ratios of 0.09:1 for RPE, which is in agreement with the data published by Berman (33), and 0.35:1 for P200 (as determined in our laboratory by measuring the phosphorus content of a P200-phospholipid extract (see below)).
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**RESULTS**

**Effect of Light on ROS PLA$_2$ Activity**—Considering the discrepancy between ROS PLA$_2$ activity levels reported in the literature and that its role is still unknown, we have attempted to reproduce some of the reported results in order to further characterize this enzyme. We first tried to measure PLA$_2$ activity in the conditions described by Jelsen (5) because she reported the highest level of activity. Surprisingly, we could not detect any significant light-dependent or light-independent PLA$_2$ activity in our ROS preparations (Fig. 1). In an attempt to detect PLA$_2$ activity, we have tried: 1) to preincubate the eyes on ice, in total darkness, for different periods of time (1, 2, or 4 h) prior to dissection, 2) to vary the composition of the ROS purification buffer, 3) to vary the method used to either collect the retinas or detach ROS from the retina (see the three procedures for ROS isolation and purification), and 4) to use mixed substrate vesicles ([$^{14}$C]PAPC + DPPC) in the activity assay, but none of these conditions allowed us to detect significant PLA$_2$ activity in ROS. Fig. 1 shows typical results that were obtained in all those diverse conditions.

**Effect of Different Substrates on ROS PLA$_2$ Activity**—We have tested ROS PLA$_2$ activity toward other exogenous radiolabeled substrates differing from [$^{14}$C]PAPC either by the type of fatty acid present in sn-1 and sn-2 positions ([$^{14}$C]DOPC), the type of polar headgroup ([$^{14}$C]HAPC), or the type of bond between the sn-1 fatty acid and the glycerol backbone ([$^{14}$C]PAPE). Since [$^{14}$C]DOPC was labeled on both fatty acid chains, it allowed for the detection of either PLA$_2$ or PLA$_1$ activity. However, even if we used [$^{14}$C]DOPC in the same conditions as described by Zimmerman and Keys (8) or Jelsen (5), we did not observe any PLA$_2$ or PLA$_1$ activity that could be considered significantly different from the background. In fact, as can be seen in Fig. 2, none of the substrates we used was significantly hydrolyzed by our ROS preparations.

**Intactness of ROS**—As we could not reproduce the results of Jelsen (5) and Zimmerman and Keys (8), we have investigated the intactness of our ROS preparations. Using electron microscopy of freshly purified v-ROS, we found that our preparations were in fact completely burst (Fig. 3). Since our negative results could be due to the loss of the PLA$_2$ (or PLA$_1$) enzyme itself or some soluble activating factors during the purification procedure, we changed our method of ROS preparation in order to get intact plasma membranes. We used homogenization (as described by Zimmerman and Godchaux (28)) and hand shaking (as described by McDowell and Kühn (29)) to isolate h-ROS and hs-ROS, respectively. Fig. 3, B and C, clearly show that hand shaking gives better results; only ROS isolated by hand shaking have densely packed discs as well as a sealed plasma membrane (Fig. 3C). Moreover, we measured the production of NADPH as a quantitative criteria for evaluating the intactness of v-ROS, h-ROS, and hs-ROS. Given that the NADPH-recycling enzyme required for the reduction of rhodopsin's chromophore is cytosolic (36), we measured the production of NADPH, in the presence of exogenously added substrates ([$\beta$-glucose 6-phosphate + $\beta$NADP]), prior to
and after solubilization of ROS membranes with Triton X-100 (36). The results were compared to negative controls where addition of both substrates was omitted. An increase in the absorbance at 340 nm after solubilization of the plasma membrane indicates that cytosolic NADPH-recycling enzymes were present in ROS and thus provides an indication that ROS were initially intact. Fig. 4 shows results obtained for the three types of ROS preparations. The three curves show a slow production of NADPH prior to membrane solubilization with Triton X-100. The addition of the detergent clearly leads to a large increase of NADPH production, mainly for h-ROS and hs-ROS. Obviously, v-ROS contain much less intact ROS as observed in Fig. 3A. From Figs. 3 and 4, the intactness of these ROS preparations can be assessed as hs-ROS ≫ h-ROS ≫ v-ROS. Nonetheless, no matter whether we used v-ROS, h-ROS, or hs-ROS preparations, we have failed to detect significant PLA2 activity. Fig. 1 shows typical results that we observed with each type of preparation.

Distribution of PLA2 Activity Among Particulate and Soluble Fractions Generated during the Purification of hs-ROS—To further investigate the possibility that PLA2 enzymes were lost during the purification procedure, we measured PLA2 activity in the particulate and soluble fractions generated at each step of ROS purification. As can be seen in Table I, there is no significant PLA2 activity in any of these fractions. Moreover, reincubation of the corresponding pellet and supernatant obtained after the centrifugation run at either 4,400 or 17,500 g was not sufficient to restore PLA2 activity. The only PLA2 active fraction was “P 3,000 g” which is equivalent to the P200 fraction (presumed to contain neuronal cells, Müller cells, and rod inner segments). These results argue strongly against the loss of PLA2 enzymes during ROS purification and supports our results showing the absence of endogenous PLA2 activity in intact ROS (see Figs. 1 and 2, and Table I).

Effect of Guanine Nucleotides on ROS PLA2 Activity—It has been well established that guanine nucleotides such as GTP and GDP influence the active-inactive state transition of G-proteins. Moreover, Jelsema and Axelrod (6) have shown that light-activation of ROS PLA2 occurred through the $T_{\alpha}$ complex of transducin ($T_{\alpha}$GDP). That is, photoexcited rhodopsin undergoes conformational changes which allow its binding to the inactive undissociated transducin $\alpha$-subunit containing a GDP molecule in its catalytic site ($T_{\alpha}$GDP). After photoexcited rhodopsin has bound to $T_{\alpha}$GDP, the GDP molecule is exchanged for GTP which confers the active state to transducin and allows it to dissociate into $T_{\alpha}$GTP and $T_{\beta\gamma}$ subunits. $T_{\beta\gamma}$ would then, according to the results of Jelsema and Axelrod (6), be able to...
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TABLE I
Distribution of PLA₂ activity among particulate and soluble fractions generated during the purification of hs-ROS

| Fractions assayed (see “isolation of ROS by Hand Shaking” for a description of each fraction) | PLA₂ activity* (nmol of [¹⁴C]arachidonic acid/mg protein/15 min) |
|---|---|
| | Dark | Light |
| | −GTP·S | +GTP·S | −GTP·S | +GTP·S |
| SN 3,000 × g (crude ROS) | -0.02 ± 0.00 | 0.00 ± 0.00 | -0.06 ± 0.08 | -0.02 ± 0.11 |
| SN 4,400 × g (waste) | 0.01 ± 0.03 | -0.02 ± 0.02 | 0.07 ± 0.13 | 0.03 ± 0.11 |
| P 4,400 × g (semi-purified ROS) | -0.14 ± 0.08 | -0.13 ± 0.05 | -0.10 ± 0.11 | -0.01 ± 0.09 |
| SN 140,000 × g (purified diluted ROS) | 0.00 ± 0.02 | 0.01 ± 0.03 | 0.05 ± 0.11 | 0.01 ± 0.09 |
| SN 17,500 × g (purified concentrated hs-ROS) | 0.03 ± 0.02 | 0.02 ± 0.02 | 0.05 ± 0.03 | 0.01 ± 0.03 |
| SN 4,400 × g + P 4,400 g | 0.06 ± 0.07 | 0.02 ± 0.09 | 0.02 ± 0.06 | 0.02 ± 0.04 |
| SN 17,500 × g + P 17,500 g | -0.02 ± 0.02 | -0.01 ± 0.02 | 0.07 ± 0.03 | 0.06 ± 0.06 |

*The assay was performed over 15 min as described in the legend to Fig. 1. P 3,000 g (not shown) is equivalent to the P200 fraction (see “Preparation of P200”). These results are representative of mean ± S.D. of triplicates from two separate experiments.

activate ROS PLA₂ until GTP is hydrolyzed to GDP by Tα and Tα-GDP reassociates with Tβγ. We thus have attempted to measure PLA₂ activity in the presence of a non-hydrolyzable GTP analog (GTP·S) or a GDP analog that cannot be phosphorylated (GDP·S) to promote, respectively, the permanent dissociation or association of transducin Tα and Tβγ subunits. As a consequence, light-stimulated ROS PLA₂ activity was expected to be enhanced while dark-adapted basal activity was expected to be lowered. We have also tested the effect of unmodified hydrolyzable GTP. Unfortunately, as shown in Fig. 5, we did not observe any significant light-dependent or light-independent PLA₂ activity in any of these conditions.

Effect of Mg²⁺ and Ca²⁺ Concentration, EGTA, and Detergent on ROS PLA₂ Activity—Since Mg²⁺ is an essential cofactor of G-proteins and since there are several types of PLA₂ differing in their Ca²⁺ requirement (see Refs. 37–39 for reviews), we have tested the effect of two Mg²⁺ concentrations on ROS PLA₂ activity as a function of Ca²⁺ concentration. Moreover, it has been shown by Marshall and MCarle-Roshak (40) that the addition of EGTA to the assay buffer can reduce the Ca²⁺ requirement of some PLA₂. So we have used two types of PLA₂ assay buffers in our experiments: one containing increasing concentrations of Ca²⁺ and the other one containing an increasing excess of Ca²⁺ over a fixed concentration of EGTA. We have also tested the effect of emulphogene on ROS PLA₂ activity since this detergent is efficient at extracting ROS proteins (41) and could thus favor the interactions between the substrate vesicles and ROS PLA₂. However, as shown in Fig. 6, we did not detect any significant PLA₂ activity in any of these conditions.

ROS PLA₂ or PLA₁ Activity Toward Endogenous Phospholipids—As can be seen in Figs. 1, 2, 5, and 6, we did not observe significant PLA₂ activity in ROS incubated with exogenous radiolabeled substrate, whatever the conditions used, and whatever the type of ROS preparations we used. Moreover, the results obtained with [¹⁴C]DOPC also suggest the absence of PLA₁ activity (see Fig. 2). We have thus tested ROS PLA₂ or PLA₁ activity toward endogenous unlabeled ROS phospholipids. Purified ROS were incubated for different periods of time under either white light or dim red light. A decrease in any phospholipid peak height and/or the appearance of lysophospholipid peak(s) on HPLC chromatograms, as compared to zero time controls, was considered to be indicative of ROS PLA₂ or PLA₁ activity. Fig. 7 represents a typical HPLC elution profile of ROS phospholipids. We did not find any significant decrease in any phospholipid peak height from samples incubated under light or in the dark. This result is in agreement with those shown in Figs. 1, 2, 5, and 6, and suggests that there is neither PLA₂ nor PLA₁ activity in intact ROS.

PLA₂ Activity in Subretinal Fractions Other Than ROS—In order to explain the discrepancy between the activity levels reported by Jelsema (5), Zimmerman and Keys (8), Castagnet and Giusto (9), and in the present study, we have measured PLA₂ activity in two subretinal fractions containing cell types which are found adjacent to ROS in vivo, RPE (enriched with retinal pigment epithelial cells) and P200 (presumably containing neuronal cells, Müller cells and rod inner segments). Significant levels of light-independent PLA₂ activity were detected in both fractions (Fig. 8). Considering the possibility that ROS PLA₂ activity detected by Jelsema (5), Zimmerman and Keys (8), and Castagnet and Giusto (9) is due to a contamination by adjacent cell types, our results on a light-independent PLA₂ activity would be in agreement with the characteristics deter-
mined by Zimmerman and Keys (8) but would contradict the results of Jelsema (5) and Castagnet and Giusto (9) who observed a 3.3-fold and a 35% increase upon light-stimulation, respectively.

**Influence of pH and Ca\(^{2+}\) on RPE and P200 PLA\(_2\) Activity**—In order to better identify the type of PLA\(_2\) present in RPE and P200, and because it is known that there are at least three types of PLA\(_2\) differing by their Ca\(^{2+}\) requirement and their pH optimum (37–39), we have tested the effect of pH and Ca\(^{2+}\) concentration on its activity. As shown in Fig. 9, the enzyme present in both fractions was found to be Ca\(^{2+}\)-independent and optimally active at alkaline pH. Moreover, Fig. 9, A and C, suggest that Tris-HCl is not a good buffer to measure PLA\(_2\) activity, at least in our systems, when used in conjunction with calcium. In fact, we always observed an inhibitory effect of this buffer on both RPE and P200 PLA\(_2\) activity.

**DISCUSSION**

In 1987, Jelsema (5) reported high levels of PLA\(_2\) activity in “crude” and “partially purified” ROS preparations. Moreover, the enzyme was shown to be stimulated 4.5- and 3.8-fold by light and GTP\(_{\text{G}}\), respectively, as compared to dark-adapted controls. Values of 133.6±24.0 and 110.5±12.7 nmol of [14C]arachidonic acid released/mg of proteins/10 min were reported for light- and GTP\(_{\text{G}}\)-stimulated samples, respectively, as compared to a value of 29.4±2.6 for dark-adapted controls. Thus, it was suggested that ROS PLA\(_2\) could be regulated by light through a GTP-binding protein, herein transducin (5). In 1993, Castagnet and Giusto (9) also published data on the presence of a light-stimulated PLA\(_2\) in ROS. However, the maximum activity level that they observed (see Table II) was more than 2 orders of magnitude lower than that reported by Jelsema (5), although using the same assay conditions. That is, Castagnet and Giusto (9) obtained 350 times less activity than Jelsema (5) for a 6-fold longer incubation period (see Table II). The reported effect of light and GTP\(_{\text{G}}\) was also much less important with only a 35 and 63% increase, respectively, over dark-adapted controls (9).

Zimmerman and Keys (8) also reported results on a ROS phospholipase A activity but, in their hands, it was found to be light-independent but CoA- and ATP-dependent. The substrate ([14C]DOPC) they used did not allow them to discriminate between PLA\(_2\) or PLA\(_3\) activity. Moreover, the maximum activity that they measured (see Table II) was approximately 1 order of magnitude lower than that reported by J elsema (5). In fact, Zimmerman and Keys (8) observed seven times less activ-
ity than Jelsema (5) for a 6-fold longer incubation period (see Table II). They reported activity values of 11.22 ± 2.11 nmol of [14C]oleic acid released/mol of phospholipid/h which, once expressed in the same units as Jelsema (5) and Castagnet and Giusto (9), represents 19.16 ± 3.60 nmol of [14C]oleic acid released/mg of proteins/h.

Recently, Jung and Remé (43) reported that light could stimulate the release of [3H]arachidonic acid in intact retinas by approximately 2-fold as compared to dark-adapted controls. It is, however, difficult for us to compare their activity values with those cited above as they were not expressed in terms of specific activity but rather as a ratio of [3H]arachidonic acid released/[3H]arachidonic acid preincorporated in retinal phospholipids (43). Moreover, they worked with whole retinas on a retinal light-damage model using lithium-treated albino rats. These conditions are very different from those used by Jelsema (5, 6), Zimmerman and Keys (8), Castagnet and Giusto (9), and in the present paper.

Considering the high levels of ROS PLA2 activity reported by Jelsema (5), its likely regulation by a G-protein (transducin) (6), the discrepancy between ROS PLA2 activity in the literature (see Table II), and that its biochemical characteristics and its role have not been fully elucidated, we have attempted to reproduce the aforementioned results. We were first interested in characterizing this enzyme because the mechanism of stimulus-response coupling between G-proteins and PLA2 could be important in many cells other than retinal rods. Moreover, the activation of PLA2 through G-protein-coupled receptors in cells is still a matter of controversy (7). Unfortunately, we did not find any significant PLA2 or PLA1 activity in ROS, whatever the conditions we used and whatever the type of ROS preparations we used (Figs. 1–7). We did not detect either light- and GTP-dependent (Fig. 5, Table I), or light-independent but CoA- and ATP-dependent PLA2 or PLA1 activity (Fig. 2). In our hands, there was no significant increase in ROS PLA2 or PLA1 activity over time (Figs. 1, 2, 5, and 7). This means that the amount of 14C-fatty acids collected after the enzymatic reaction had been quenched was, in all cases, equivalent to the level of

### Table II

| Authors               | Maximum activity reported (nmol of fatty acids/mg protein) | Experimental conditions                                                                 |
|-----------------------|----------------------------------------------------------|----------------------------------------------------------------------------------------|
| Jelsema (5)           | 133.6 ± 24.0/10 min                                      | 20 μg of dark-adapted ROS proteins; 4.5 μCi/ml [14C]PAPC + 80 μg/ml DPPC                |
| Castagnet and Giusto (9) | 0.38 ± 0.03/60 min                                     | 100 μg of dark-adapted ROS proteins; 4.5 μCi/ml [14C]PAPC + 100 μg/ml DPPC             |
| Zimmerman and Keys (8)   | 19.16 ± 3.60/60 min                                      | 300–500 μg of ROS proteins; 10 μCi/ml [14C]DOPC                                        |
| Jacob et al. (this publication) | 0.46 ± 0.55/5 min                                   | Same as Zimmerman and Keys (8) (result not shown)                                      |
|                       | 0.00 ± 0.01/60 min                                     | Same as Zimmerman and Keys (8) (result not shown)                                      |
free $^{14}$C-fatty acids originally contaminating the substrate preparation. In other words, we obtained the same results whether the substrate was incubated or not with ROS. So when we subtracted the background value due to those contaminating free $^{14}$C-fatty acids, the activity level became almost equal to zero (Figs. 1, 2, 5, and 6).

Considering that our negative results could be due to the loss of PL$A_2$ enzymes, or some soluble-activating factors, from ROS during the purification procedure, we investigated the intactness and PL$A_2$ activity of ROS isolated using three fundamentally different methods. Indeed, there are essentially three basic methods described in the literature for ROS purification, the major difference between them being the way ROS are separated from the retina: 1) vortexing (v-ROS), 2) homogenizing (h-ROS), and 3) hand shaking (hs-ROS). We thus prepared v-ROS, h-ROS, and hs-ROS as described (see "Experimental Procedures") and assayed each preparation for PL$A_2$ activity, after we looked at their intactness both qualitatively and quantitatively (Figs. 3 and 4). The intactness was quantitatively estimated as described by Schnetkamp and Daemen (36), by measuring the production of NADPH for each type of ROS preparations. This assay is based on the fact that sealed ROS plasma membranes prevent access of cytosolic NADPH-recycling enzymes to exogenously added substrates. Thus, a larger production of NADPH following solubilization of the membrane with Triton X-100 is expected to reflect a higher degree of intactness prior to solubilization.

Since we did not observe any contaminating mitochondria, which would give erroneously high results (36), in the electron micrographs of v-ROS, h-ROS, and hs-ROS preparations (Fig. 3 as well as other micrographs not shown), the extent of NADPH production effectively correlates with the extent of intactness. However, the highest production of NADPH (Fig. 4) by the most intact hs-ROS (Fig. 3C), did not correlate with a higher level of PL$A_2$ activity. None of the ROS preparations show significant levels of PL$A_2$ activity (Figs. 1, 2, 5, 6, and 7). We also failed to detect PL$A_2$ activity in the different particulate and soluble fractions that were generated during the purification of hs-ROS (see Table I). Incubating for longer (30 min) or shorter (1, 5, or 10 min) periods of time did not appear to be beneficial since no significant PL$A_2$ activity was detected (data not shown). Another important point is that we could not recover any significant PL$A_2$ activity by coincubating the corresponding pellet and supernatant generated after centrifugation at either 4,400 or 17,500 × g (Table I). These results thus strongly suggest that the absence of PL$A_2$ or PL$A_1$ activity in our ROS preparations is not due to the loss of PL$A_2$ or PL$A_1$ enzymes or some soluble-activating factors during ROS purification.

In order to understand why we did not find PL$A_2$ activity in ROS whereas others did (5, 8, 9), we have assayed PL$A_2$ activity in two other subretinal fractions and found that RPE and P200 contained reproducibly high levels of PL$A_2$ activity (Fig. 8). This result suggests that the varying levels of activity that have been reported for ROS preparations could be due, in most cases, to contamination to varying extent by adjacent retinal cell types. As a matter of fact, we found that there was a relationship between the level of PL$A_2$ activity in our h-ROS preparations and their purity coefficient ($A_{280}/A_{400}$) results not shown). That is, rhodopsin constitutes 85–90% of total ROS proteins (44) and has two principal absorption bands: 1) aromatic amino acids of the protein moiety at 280 nm and 2) the 11-cis-retinal Schiff base at 500 nm. Thus, measurement of this $A_{280}/A_{400}$ ratio for ROS preparations is indicative of their contamination by proteins from adjacent cell types other than ROS. Moreover, Salesse et al. (26) have demonstrated that this purity criterion is useful to evaluate the contamination of ROS by non-ROS proteins. In our hands, PL$A_2$ activity was detected only in contaminated h-ROS. Samples with the highest $A_{280}/A_{400}$ ratios being the most PL$A_2$ active ROS preparations. For example, we detected 12.5 ± 0.8 and 4.4 ± 0.7 nmol of hydrolyzed $^{14}$C-arachidonic acid/mg of proteins/10 min for ROS preparations having a $A_{280}/A_{400}$ ratio of 6.9 and 3.4, respectively (data not shown). As a comparison, results presented in Figs. 1–7 were all obtained with ROS preparations having $A_{280}/A_{400}$ ratios which were always lower than 3.

This observation supports the idea that the different levels of ROS PL$A_2$ activity reported in the literature could be due to a contamination by proteins coming from adjacent retinal cell types. However, it still does not explain why J elsema (5) observed such a strong light effect on PL$A_2$ activity whereas Castagnet and Giusto (9) only noted a slight effect, and Zimmerman and Keys (8) and our laboratory did not (see Figs. 1 and 5). We have tried to restore this light-stimulation by incubating RPE and P200 in the presence of ROS. Unfortunately, ROS were unable to induce light-activation of PL$A_2$ in either fraction. The activity detected in light-exposed samples containing ROS were equivalent to dark-adapted controls without ROS (not shown).

It could be suggested that the light-sensitive PL$A_2$ detected by J elsema (5) is normally associated with the outside of the plasma membrane and that, depending on the treatment during the purification, we lose it either partially or totally. This could explain why Castagnet and Giusto (9) saw only very low levels of PL$A_2$ activity whereas we saw none (Figs. 1, 2, and 5–7). We cannot exclude this possibility. However, given that PL$A_2$ hydrolyzes phospholipids, which account for up to 80% of total ROS lipids (45), it seems improbable to us that the enzyme is present on the outside of the plasma membrane since it certainly requires to be highly regulated. Moreover, it was shown (5, 6) to be regulated by G-protein and guanine nucleotide components of intracellular signal transduction. One alternative hypothesis is that the PL$A_2$ activity measured by J elsema (5) was in fact present in rod inner segments still present in her crude and partially purified ROS preparations and was activated secondary to light-activation of the ROS visual cascade. The extent of light-sensitive PL$A_2$ activity detected in ROS would thus be dependent on the extent of contamination by rod inner segments still attached to ROS (via the connective cilium). Since electron microscopy of our ROS preparations did not reveal the presence of mitochondria, this would mean that our preparations were essentially free of rod inner segments and this could thus explain why we did not observe any light-sensitive PL$A_2$ activity.

This latter hypothesis suggests that PL$A_2$ activity found in our P200 fractions could come, at least partly, from rod inner segments. The absence of a light-effect (Fig. 8) could then be due to the disruption of the connection between rod inner and outer segments. PL$A_2$ activity of rod inner segments would only be light-sensitive, although probably indirectly, when rod inner and outer segments are joined to each other by the connective cilium. Moreover, this light-stimulation could effectively be mediated by a G-protein as described by J elsema (6) but, since cross-reactivity between different G-proteins has been demonstrated in different systems (46, 47), the exogenously added transducin could have mimicked the role that should normally be held by a different but similar G-protein.

Considering the high PL$A_2$ activity levels that we detected in RPE and P200, we have tested the influence of pH and Ca$^{2+}$ concentration on this activity to identify the type of enzyme present in both fractions. As shown in Fig. 9, PL$A_2$ present in both RPE and P200 is optimally active in the alkaline pH range...
and is Ca\(^{2+}\) independent. However, care should be taken when interpreting the effect of Ca\(^{2+}\) because Reynolds et al. (48) have recently shown that, in the absence of Ca\(^{2+}\), high salt concentrations can overcome the requirement of PL\(A_2\) for divalent metals. They showed that 10 mM Mg\(^{2+}\) could stimulate human cytosolic PL\(A_2\) to almost the same extent as did 2 mM Ca\(^{2+}\). Since those pH- and Ca\(^{2+}\)-dependence measurements were made in the assay conditions described by J elsema (5) and thus contained a high concentration of Mg\(^{2+}\), additional experiments using different Mg\(^{2+}\) concentrations will be required to characterize this enzyme. As can be pointed out in Fig. 9, the maximum activity detected in RPE and P200 are, respectively, lower than those shown in Fig. 8. This might be explained by the fact that, for these experiments (see Fig. 9), we used buffers at 133 mM final concentration, instead of 30 mM (Fig. 8), to make sure that substrate and sample buffers would not significantly affect the final pH needed for the assay. It may be possible that such high buffer concentration partially inhibits PL\(A_2\) activity by affecting either the PLA\(2\)-substrate interactions or the enzyme itself. Otherwise, it is possible that part of the hydralyzed \(^{14}C\)arachidonic acids were further metabolized during the 1-h incubation period, thus rendering them unavailable for quantitation as free fatty acids. Another point is that Tris-HCl seems to inhibit the enzyme activity at pH 7, 8, and 9 when used in conjunction with calcium. This is evident both in RPE and P200 (see Fig. 9, A and C). We also had similar difficulties with cacodylate and imidazole buffers having a profound inhibitory effect on PL\(A_2\) activity at pH 5, 6, and 7, and 6, 7, and 8, respectively (results not shown). Although we were able to successfully replace cacodylate and imidazole buffers by Na\(^{+}\)acetate, pH 5 and 6, and bis-Tris, pH 6 and 7, buffers, we did not find an adequate combination of buffers which could be used at pH 7, 8, and 9 instead of Tris-HCl. Glycine seems to be a more “permissive” buffer since we repeatedly obtained higher levels of PL\(A_2\) activity with glycine, pH 9, compared to Tris-HCl, pH 9 (see Fig. 9). We also tested CHES and AMP buffers at pH 9 and we observed results which were similar to those obtained with glycine, pH 9. In fact, activity was greater with CHES, AMP, and glycine buffers compared to Tris-HCl (not shown). This suggests that Tris-HCl is not a good buffer to assay PL\(A_2\) activity at pH 7, 8, or 9 in RPE and P200.

Taken together, our results suggest that both RPE and P200 contain PL\(A_2\) activity which is light-independent, Ca\(^{2+}\)-independent, and optimally active at alkaline pH (Figs. 8 and 9). Considering that the maximum activity observed in RPE is less than that observed in P200, the possibility that RPE preparations are contaminated by P200 cells cannot be completely excluded. However, it seems unlikely that neuronal cells, Müller cells, and/or rod inner segments could have contaminated our RPE preparations since retinas were removed from the eyecup after incubation with buffer which allows ROS to retract from RPE microvilli, thus facilitating detachment of retinas (49). Moreover, it is well known that RPE cells and ROS are in intimate contact (50). Most of the contamination of RPE should thus come from ROS which, according to our results, does not contain PL\(A_2\). Berman et al. (33) have demonstrated that most RPE preparations purified by brushing out the cells are contaminated with varying amounts of ROS and red blood cells. Again, this should not affect our results since, as shown in Figs. 1–7, ROS do not contain PL\(A_2\) activity. Moreover, PL\(A_2\) activity present in RPE is Ca\(^{2+}\)-independent (Fig. 9, A and B) whereas that in red blood cells is Ca\(^{2+}\)-dependent (51). This suggests that RPE-PL\(A_2\) is different from red blood cell-PL\(A_2\) and probably not due to a contamination by P200. More experiments will be necessary to verify this hypothesis.

In summary, we have shown that there is no significant PL\(A_2\) or PL\(A_1\) activity in ROS and that the activity levels previously reported by J elsema (5), Castagnet and Giusto (9), and Zimmerman and Keys (8) could be accounted for by a contamination by adjacent retinal cell types. We identified two potential sources of such contaminating activity: RPE and P200. The enzyme present in both fractions is light- and Ca\(^{2+}\)-independent and is optimally active at alkaline pH. Other experiments are needed to further characterize the PL\(A_2\) present in RPE and P200. It will be particularly important to study PL\(A_2\) activity in P200 because of its cell composition. Indeed, we hypothesize that PL\(A_2\) activity found in our P200 fractions could come, at least partly, from rod inner segments. The absence of a light effect in our hands could then be due to the disruption of the connection between rod inner and outer segments. PL\(A_2\) activity of rod inner segments could be light-sensitive only when these two segments are joined by the connective cilium. It will thus be very interesting to isolate and purify rod cells where the inner and outer segments are still attached to test this attractive hypothesis.
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