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Phosphoinositide Synthesis in Bovine Rod Outer Segments†

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ABSTRACT: Phosphoinositide turnover has been implicated in signal transduction in a variety of cells, including photoreceptors. We demonstrate here the presence of a complete pathway for rapid synthesis of phosphoinositides in isolated bovine retinal rod outer segments (ROS) free of microsomal contaminants. Synthesis was measured by the incorporation of label from radioactive precursors, [γ-^{32}P]ATP and [^{3}H]inositol. [γ-^{32}P]ATP also produced large amounts of labeled phosphatidic acid. Incorporation of [^{3}H]inositol required CTP and Mn^{2+}. Mn^{2+} and Mg^{2+} increased ^{32}P incorporation into phosphatidylinositol 4-phosphate, while spermine increased phosphoinositide labeling generally. ROS that had been washed to remove soluble and peripheral proteins incorporated less label than unwashed ROS into phosphatidic acid and phosphatidylinositol. No effects of light were detected. Inhibitory effects of high concentrations of nonhydrolyzable GTP analogues were probably due to competition with ATP.

Understanding of vertebrate visual transduction has been greatly advanced by the discovery that cyclic GMP maintains the vertebrate retinal rod outer segment (ROS)[1] sodium channel in the open state (Fesenko et al., 1985). Levels of cyclic GMP are regulated by a light-driven cascade involving bleached rhodopsin, a G protein [transducin (Fung et al., 1981; Godchaux & Zimmerman, 1979)], and a cGMP phosphodiesterase (Bitensky et al., 1978; Baehr et al., 1979; Kohnken et al., 1981a,b). The possible role of calcium in transduction or light/dark adaptation is also of great interest, especially because cyclic GMP levels have been shown to depend on Ca^{2+} (Cohen et al., 1978; Kilbride, 1980; Woodruff & Fain, 1982;

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[1] Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid; ROS, retinal rod outer segment(s); EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Koch & Stryer, 1988). Of particular interest are the mechanisms by which Ca^{2+} levels and flux are regulated (Matthews et al., 1988; Nakatani & Yau, 1988). In many cell types, phosphatidylinositol 4,5-bisphosphate (PIP_2)-specific phospholipase C brings about altered concentration or distribution of intracellular Ca^{2+} by catalyzing release of inositol 1,4,5-trisphosphate (IP_3). In some cells, phospholipase C is regulated by G proteins, which mediate effects of extracellular agonists.

PIP_2 phospholipase C is light-activated in the photoreceptors of *Limulus* and other invertebrates [reviewed by Payne (1986)] and has been implicated in amphibian phototransduction as well (Ghalayini & Anderson, 1984; Hayashi & Amakawa, 1985; Waloga & Anderson, 1985; Brown et al., 1987). In the following paper (Gehm & Mc Connell, 1990), we examine PIP_2 phospholipase C in bovine ROS.

In the present report, we explore the synthetic pathway for phosphoinositides in bovine ROS, using radioactive precursors. Incorporation of ^{32}P into polyphosphoinositides and phosphatidic acid (PA) has been reported in isolated photoreceptor membranes from octopus (Yoshioka et al., 1983), squid (Vandenberg & Montal, 1984), frog (Hayashi & Amakawa, 1985), and cattle (Giusto & Ilincheta de Boschero, 1986; van Rooijen & Bazan, 1986). Incorporation of [^{3}H]inositol into phosphatidylinositol (PI) has been reported in whole retinas from toad (Anderson & Hollyfield, 1981) and rat (Schmidt, 1983a). A regulatory role for phosphoinositides has been proposed in phosphorylation of frog ROS-soluble proteins (Hayashi et al., 1987). We previously reported that isolated bovine ROS rapidly incorporate ^{32}P into PA (Seyfred et al., 1984) and ^{32}P and [^{3}H]inositol into phosphoinositides (Gehm & Mc Connell, 1985). Here we demonstrate the presence in ROS of the complete pathway for rapid synthesis of PIP_2.

Materials and Methods

[γ-^{32}P]ATP and *myo*-[2-^{3}H]inositol were purchased from New England Nuclear. The former was diluted with unlabeled ATP to the specific activity indicated below. ATP, CTP, spermine, and phospholipid standards were purchased from Sigma.

Retinal Fractions. ROS were isolated as previously described (Kohnken et al., 1981a). Rhodopsin content was measured spectrophotometrically before and after bleaching in room light, in the presence of 0.1 M NH_2OH–HCl, pH 7.0, and 1% Emulphogene (Mc Connell et al., 1981). A_{280}/A_{500} ratios were between 2.3 and 2.7. Protein concentrations were determined by the method of Lowry et al. (1951).

Washing of ROS was performed as previously described (Kohnken et al., 1981a). Briefly, ROS were washed twice in an isotonic solution (10 mM Tris-HCl, pH 8.0, 250 mM sucrose, 150 mM KCl, 10 mM β-mercaptoethanol, 1 mM EDTA, 1 mM NaN_3, 0.1 mM phenylmethanesulfonyl fluoride, and 0.01% butylated hydroxytoluene) and then twice in a hypotonic solution (same as isotonic solution except KCl omitted).

Retinal microsomes were isolated by centrifuging the second supernate from the ROS isolation procedure at 100000*g* for 90 min. This produces an orange (unbleachable) pellet which contains microsomes identified both by electron microscopy and by enzyme assays (Mc Connell et al., 1969).

Labeling Reactions. ^{32}P labeling was carried out either in "complete" ^{32}P labeling medium [50 mM NaHEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM [γ-^{32}P]ATP (50–100 μCi/μmol), 1 mM CTP, and 0.5 mM *myo*-inositol] or in "minimal" ^{32}P labeling medium, which lacks CTP and inositol but is otherwise identical with the complete medium. Both media permit labeling of PA, PIP, and PIP_2, but labeled [^{32}P]PI is produced only with the complete medium. However, measurement of [^{32}P]PI was made difficult by its comparatively low level of label incorporation and its proximity on TLC plates to the "tail" of the intensely labeled PA spot.

[^{3}H]Inositol labeling was carried out in 50 mM NaHEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 1–1.5 mM ATP, 1 mM CTP, and 5–10 μCi of *myo*-[^{3}H]inositol (ca. 16 mCi/μmol). This technique produced more counts in PI than did ^{32}P labeling, owing to the higher specific activity of the precursor, and did not label PA.

Unless otherwise indicated, all labeling reactions were carried out at 30 °C in a volume of 100 μL using illuminated ROS (100–300 μg of Lowry protein), for 30 min in the case of ^{32}P labeling and for 3 h in the case of ^{3}H labeling. Reactions were terminated by acidic chloroform/methanol extraction using the method of Schacht (1981). Extracts were evaporated with nitrogen, redissolved in chloroform/methanol (2:1), and spotted on E. Merck silica gel 60 TLC plates. The labeled lipids were separated and quantitated as described below.

Analysis of Labeled Lipids. [^{3}H]Inositol-labeled lipids were resolved with $CHCl_3/CH_3OH/H_2O$/concentrated NH_4OH (98:78:19:4). ^{32}P-Labeled lipids were resolved by two-dimensional TLC, the first dimension using $CHCl_3/CH_3OH/H_2O$/concentrated NH_4OH (98:78:19:5) and the second using $CHCl_3/(CH_3)_2CO/CH_3OH/CH_3COOH/H_2O$ (10:4:2:2:1). Between dimensions, plates were dried in a vacuum desiccator. Labeled lipids were visualized by ^{32}P autoradiography or ^{3}H fluorography using Kodak X-O-Mat AR-5 film. Before fluorography, the film was hypersensitized by flashing (Laskey & Mills, 1977), and TLC plates were dipped in a solution of 0.4% 2,5-diphenyloxazole in 2-methylnaphthalene/toluene (9:1) (Bonner & Stedman, 1978). Autoradiograms/fluorograms were exposed 12–72 h at −20 °C. Labeled lipids were scraped from the plates into scintillation vials. Radioactivity was released from the silica gel by treatment with 0.1 N NaOH and measured by liquid scintillation counting after neutralization.

Results

Differential Labeling of ROS Phospholipids. Incubation of ROS with [γ-^{32}P]ATP results in labeling of PA, CDP-diacylglycerol, phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP_2). A representative autoradiogram is shown in Figure 1. Small amounts of the corresponding labeled lysolipids are also observed. PA, which has already been reported by our laboratory to incorporate ^{32}P rapidly (Seyfred et al., 1984), is the major labeled product. PIP is labeled more rapidly than PI or PIP_2 (Figure 2), and labeled PIP can be detected after incubations as short as 30 s. CDP-diacylglycerol is an intermediate in the synthesis of phosphatidylinositol from inositol and PA; it is also labeled when [α-^{32}P]CTP is used as a precursor. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine, the three principal phospholipids of bovine ROS (Nielsen et al., 1970, Fliesler & Anderson, 1983), are not labeled. Traces of labeled phosphatidylglycerol are occasionally detected.

Labeling of ROS phosphoinositides can also be demonstrated by using [^{3}H]inositol as a precursor. ^{3}H labeling of PI can be detected after as little as 15 s, but PIP and PIP_2 require several hours to incorporate detectable amounts of label. PI labeling continues to increase during this time. LysoPI is also observed.

Requirement for CTP. In the absence of added CTP, [^{3}H]inositol incorporation into PI is reduced to ~10% of that seen when CTP is present, and no [^{3}H]PIP can be detected.

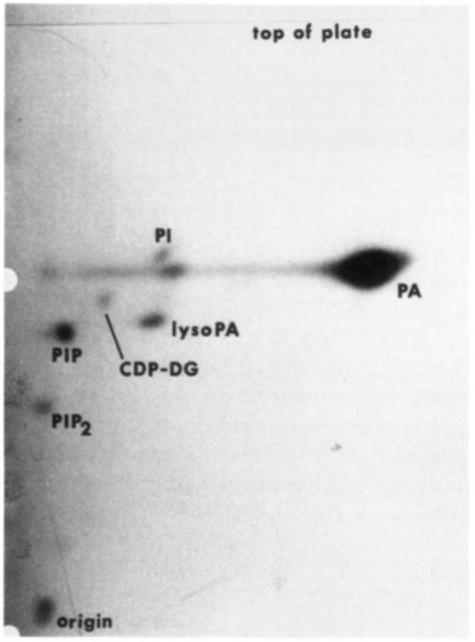

FIGURE 1: Autoradiogram of ^{32}P-labeled ROS lipids. ROS (1.2 nmol of rhodopsin) were incubated in complete ^{32}P labeling medium for 30 min. Extracted lipids were subjected to two-dimensional thin-layer chromatography and autoradiography as described under Materials and Methods. The unidentified spots at and near the origin are not lipids: they result from residual aqueous-phase contamination of the organic extract. LysoPA = monodeacylated PA; CDP-DG = CDP-diacylglycerol.

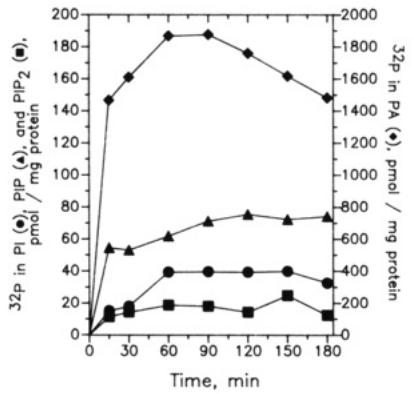

FIGURE 2: Time course of ^{32}P incorporation into ROS phospholipids. ROS (1.2 nmol of rhodopsin per assay) were incubated in complete ^{32}P labeling medium for the times indicated. Labeled lipids were extracted, separated, and quantitated as described under Materials and Methods. PI (●), PIP (▲), and PIP_2 (■) are plotted against the left scale and PA (♦) against the right.

This requirement for CTP indicates that the label is incorporated via de novo PI synthesis, and not by a base-exchange reaction between PI and free inositol (Eisenberg & Hasegawa, 1981). The small amount of label incorporated in the absence of added CTP may be due to a low level of base exchange or may reflect PI synthesis utilizing residual endogenous CTP.

In ^{32}P labeling experiments, neither PI nor CDP-diacylglycerol is labeled in the absence of CTP.

Divalent Metal Ions and Spermine. High magnesium concentrations appear to stimulate PI kinase selectively, as Figure 3 shows. It has been reported that divalent metal cations nonenzymatically catalyze phosphorylation of PA and polyphosphoinositides (Gumber & Lowenstein, 1986). No labeled lipids are detected if ROS are boiled before incubation with [γ-^{32}P]ATP, even in the presence of 50 mM $MgCl_2$; it is therefore unlikely that magnesium's effect is due to nonenzymatic labeling.

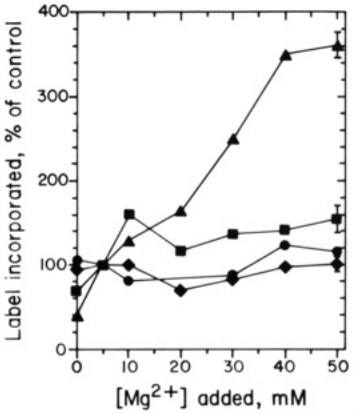

FIGURE 3: Effect of $[Mg^{2+}]$ on ROS phospholipid labeling. ROS (1.2 nmol of rhodopsin/assay) were incubated with labeled substrates as described under Materials and Methods, except that the concentration of $MgCl_2$ was varied as indicated. Since 5 mM Mg^{2+} was used routinely in other labeling experiments, label incorporation is shown as percent of 5 mM $MgCl_2$ control. Triplicate assays were performed at $[Mg^{2+}]$ of 5 and 50 mM; error bars (±SE) are shown at 50 mM but omitted at 5 mM for clarity (SE <10% for all four lipids). Only PIP showed a statistically significant ($p < 0.01$) difference between 5 and 50 mM. The PA (♦), PIP (▲), and PIP_2 (■) curves represent ^{32}P labeling; the PI (●) curve is a composite of ^{32}P and ^{3}H labeling experiments.

Spermine, a polycationic amine, has a more general stimulatory effect on phosphoinositide labeling; 2.5 mM spermine produces a ~100% increase in [^{32}P]PIP and 60–70% increases in [^{32}P]PIP_2, [^{32}P]PI, and [^{3}H]PI. ^{32}P labeling of PA is unaffected by 2.5 mM spermine but is inhibited by higher concentrations (10 mM). Spermine has been reported to inhibit PI-specific phospholipase C (Eichberg et al., 1981), but the effects reported here would appear not to be due to inhibited hydrolysis, since ROS phosphoinositide PLC is inactive under the conditions used in the labeling reactions (Gehm & Mc Connell, 1990).

Manganese is a known cofactor of PI synthase and increases [^{3}H]inositol incorporation into PI (Figure 4A). Millimolar Mn^{2+} also stimulates ^{32}P labeling of PIP more than 2-fold, but has little or no effect on PIP_2 or PA (Figure 4B).

Calcium is not required for phosphorylation, as indicated by the absence of effects of 1 mM EGTA on ^{32}P labeling of PA, PIP, and PIP_2. Furthermore, 1 mM Ca^{2+} markedly inhibits labeling of phosphoinositides, although it has little or no effect on PA.

Addition of Unlabeled PIP. Hayashi and Amakawa (1985) report ^{32}P labeling of PIP_2 in isolated frog ROS only in the presence of added PIP. In our preparation, labeling of PIP_2 occurs even in the absence of added PIP, but is approximately doubled by addition of 50 μM or more unlabeled PIP. These results suggest that PIP phosphorylation is regulated at least in part by substrate availability.

No Effect of Light or Nucleotides. Increased incorporation of labeled precursors into phosphoinositides in response to light has been reported in whole retinas from rats (Schmidt, 1983a,b) and *Xenopus* (Anderson & Hollyfield, 1981), although the increased labeling was not localized in outer segments. In isolated photoreceptor membranes from squid, light causes a decrease in ^{32}P incorporation into PIP and PIP_2 and an increase into PA (Vandenberg & Montal, 1984); in octopus, a similar pattern of changes is produced by the cyclic nucleotide phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), but light decreases labeling of all three lipids (Yoshioka et al., 1983). In more than 25 experiments, we have seen no consistent effects of light or cyclic nucleotides (at concentrations up to 2 mM) on phosphoinositide labeling in

Table I: Effect of Washing on Anabolic Label Incorporation[a]

product	relative labeling (ROS = 100)				
	ROS	Iso1	Iso2	Hypo1	Hypo2
[^{32}P]PA	100 ± 14.3	33.6 ● 3.8	46.5 ± 2.6	35.9 ± 0.3	36.7 ± 0.3
[^{3}H]PI	100 ± 3.5	78.0 ± 4.6	68.7 ± 4.1	69.9 ± 1.1	65.8 ± 5.5
[^{32}P]PIP	100 ± 10.7	99.0 ± 9.7	110.3 ± 8.0	112.5 ± 2.0	126.2 ± 2.8
[^{32}P]PIP$_2$	100 ± 8.4	88.9 ± 15.9	91.8 ± 4.6	88.9 ± 1.7	74.6 ± 16.2

[a] ROS were washed twice isotonically and twice hypotonically, as described under Materials and Methods. Samples of the pellets were retained at each step. Aliquots of the original ROS, the first isotonic pellet (Iso1), the second isotonic pellet (Iso2), the first hypotonic pellet (Hypo1), and the second hypotonic pellet (Hypo2), each containing 2 nmol of rhodopsin, were incubated in minimal ^{32}P labeling medium (to label PA, PIP, and PIP$_2$) and in ^{3}H labeling medium (to label PI). Labeling of each lipid in the washed membranes is expressed as a percentage of the labeling seen in ROS. Values shown are means (±SE) of duplicate assays.

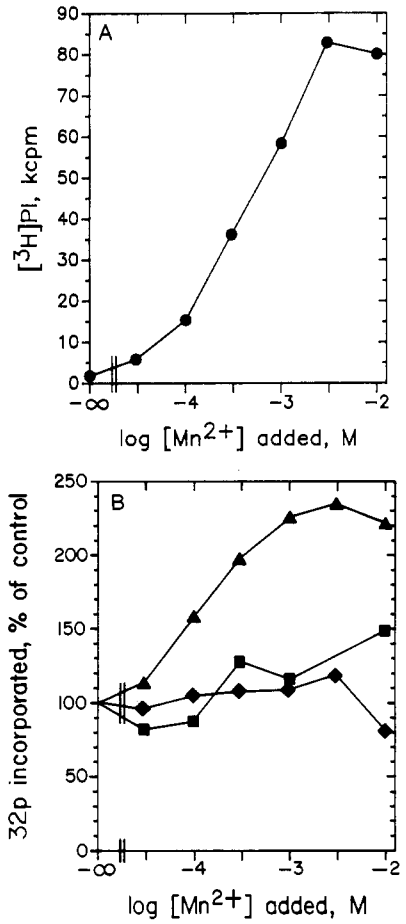

FIGURE 4: Effect of [Mn^{2+}] on ROS phospholipid labeling. (A) ROS (2.1 nmol of rhodopsin/assay) were incubated with [^{3}H]inositol as described under Materials and Methods, except that the Mn^{2+} concentration was varied as shown. ^{3}H incorporation into PI (●) is shown. [^{3}H]PIP was detected only at [Mn^{2+}] > 0.1 mM and is not shown on the graph. (B) ROS (2.6 nmol of rhodopsin/assay) were incubated in minimal ^{32}P labeling medium as described under Materials and Methods, except that the Mn^{2+} concentration was varied as shown. ^{32}P incorporation into PA (♦), PIP (▲), and PIP$_2$ (■) is shown as percent of control (no added Mn^{2+}). Each data point represents a single measurement; similar results were obtained in several experiments.

bovine ROS, either with ^{32}P or with ^{3}H. GTP and its nonhydrolyzable analogue guanylyl imidodiphosphate produce a modest (25–30%) decrease in labeling of PA, PIP, and PIP$_2$ when added in 1 mM concentration to minimal ^{32}P labeling medium. This is probably attributable to competition with ATP for the kinases' active sites.

Washing of ROS. ROS which have been washed to remove soluble and peripheral proteins have diminished ability to incorporate label into PA and PI (Table I). Labeling of PIP is less affected, and PIP$_2$ labeling is slightly increased. One interpretation of this is that PI and PIP kinases are tightly membrane-bound, but diglyceride kinase and PI synthase are more vulnerable to washing. Another possibility is that washing removes different amounts of the enzymes' subtrates (polar lipids) or cofactors.

Table II: Comparison of ROS and Microsomes[a]

product	rate of synthesis [pmol min^{-1} (mg of protein)$^{-1}$]		ROS/microsome ratio
	ROS	microsomes	
[^{32}P]PA	20	2.6	7.7
[^{32}P]PIP	0.95	3.0	0.32
[^{32}P]PIP$_2$	0.22	0.44	0.50
[^{3}H]PI	0.24	2.5	0.10

[a] ROS and retinal microsomes were incubated for 30 min with [^{32}P]ATP or for 45 min with [^{3}H]inositol. Labeled products were isolated and quantitated as described under Materials and Methods. Values shown are averages of duplicate determinations.

Comparison of ROS and Microsomes. Although our ROS preparations have been shown to be free of inner segment derived microsomes as determined by glucose 6-phosphatase (McConnell, 1965), NADPH–cytochrome *c* reductase, and electron microscopy (McConnell et al., 1969), we examined the possibility that anabolic labeling of phospholipids is due to microsomal contamination of the ROS. Retinal microsomes were assayed for incorporation of labeled precursors into phospholipids, and compared with ROS. As shown in Table II, the patterns of incorporation are quite different. PA labeling is 8-fold higher in ROS than in microsomes; labeling of the phosphoinositides is 2–10-fold higher in microsomes than in ROS.

Discussion

The ROS, site of the initial steps of photoreception, is the most specialized part of a highly specialized cell, comprising hundreds of stacked rhodopsin-laden membranous disks enveloped by the rod's plasma membrane. The inner segment, in contrast, contains the nucleus, mitochondria, endoplasmic reticulum, Golgi, etc., and is the site of synthesis for the proteins, carbohydrates, and most of the lipids that make up the ROS (Young, 1974). The presence in ROS of a complete pathway for phosphoinositide synthesis and breakdown (Gehm & McConnell, 1990) suggests that inositol lipids may play a role in vertebrate photoreceptor function.

The rates of ^{32}P and [^{3}H]inositol incorporation into PI agree well [typically ~0.5 pmol min^{-1} (mg of protein)$^{-1}$]. However, [^{3}H]inositol is preferable to [γ-^{32}P]ATP for measuring PI synthesis, since a higher specific activity can be used, and labeling of PA, which produces a chromatographic tail near PI, is avoided.

The two labeled precursors differ in their pattern of incorporation into the other phosphoinositides. [^{3}H]Inositol labels PIP more slowly than PI (by more than an order of magnitude), and PIP$_2$ more slowly still, whereas ^{32}P labels PI and PIP$_2$ at similar rates, and PIP more rapidly than either.

This is consistent with the presumption that [^{3}H]inositol must be incorporated into PI before appearing successively in PIP and PIP_2 but ^{32}P can be incorporated directly into all three phosphoinositides (in the case of PIP and PIP_2, by radiophosphorylation of PI and PIP already present in the membranes). The more rapid formation of [^{32}P]PIP than [^{32}P]PIP_2 probably reflects the larger amount of PI compared to PIP available for phosphorylation; in most animal cells, PI makes up the bulk of the phosphoinositides (Hokin, 1985). The large amount of [^{32}P]PA produced also may reflect high levels of precursor, i.e., diacylglycerol, accumulating in the membranes due to lack of perfusion (Matthys et al., 1984) after slaughter.

Van Rooijen and Bazan (1986) have previously reported ^{32}P labeling of PA and PIP in isolated ROS incubated with [γ-^{32}P]ATP, but found no labeled PI and only traces of labeled PIP_2. Labeled PI was detected only when whole retinas were incubated with ^{32}P. In contrast, we find that isolated ROS incorporate measurable radioactivity into PI and PIP_2 as well as PIP and PA. These discrepancies may be due to our use of more ROS protein and longer incubations (30 min vs 5 min) in labeling reactions. It is unlikely that our results could be due to microsomal contamination of our ROS preparation. On the basis of the relative rates of synthesis exhibited by the two membrane fractions, a 10% contamination of ROS by microsomes would be required to account for ROS [^{3}H]PI labeling if ROS lacked their own capability, and 50% contamination to account for PIP_2 labeling. Because the combined enzymatic and morphological determinations we employed can detect less than 1% contamination, we feel confident in excluding microsomes as an explanation, and attributing to ROS a complete pathway for phosphoinositide synthesis.

The presence of such a pathway in so specialized an organelle is provocative, but its function remains problematic, as we did not observe any evidence of regulation by light or guanine nucleotides. This does not completely rule out the possibility of such regulation, as cattle retinas are not completely dark-adapted before use. This point is discussed in more detail in the following paper (Gehm & McConnell, 1990). Divalent cations and/or polyamines may regulate the enzymes in this pathway, especially PI kinase.

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