Phosphatidylinositol-4,5-bisphosphate Phospholipase C in Bovine Rod Outer Segments†

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ABSTRACT: Preparations of rod outer segments from cattle retinas contained soluble and particulate phospholipase C activities which hydrolyzed phosphatidylinositol 4,5-bisphosphate (PIP_2) and the other phosphoinositides. Ca^{2+} was required for PIP_2 hydrolysis, but high (>300 μM) concentrations were inhibitory. Mg^{2+} and spermine at low concentrations stimulated the particulate activity but inhibited the soluble. Mn^{2+} inhibited both. High (>100 μM) concentrations of the nonhydrolyzable GTP analogue guanylyl β,γ-methylenediphosphonate inhibited PIP_2 hydrolysis by both the soluble and particulate activities, but guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), fluoride, and cholera and pertussis toxins were without effect. Overall phospholipase C activity in ROS was unaffected by light. Evidence was found for multiple forms of the enzyme, requiring isolation and separate characterization before ruling out regulation by light or G-protein.

Many kinds of cells respond to external stimuli by activating a phosphoinositide-specific phospholipase C which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2),[1] to diacylglycerol and the calcium-mobilizing second messenger inositol 1,4,5-trisphosphate (IP_3) [reviewed by Berridge and Irvine (1984)]. In photoreceptors, this process may be central to control of calcium flux and has been implicated in response to light. Several lines of evidence [reviewed by Payne (1986)] suggest that phosphoinositide hydrolysis plays a crucial role in phototransduction in invertebrates. *Drosophila* bearing the *norpA* (no receptor potential A) mutation, which show no behavioral or electrophysiological response to light, are deficient in ocular PIP_2 phospholipase C (Inoue et al., 1985). Bloomquist et al. (1988) have now cloned and sequenced the *norpA* gene, revealing sequence similarities to vertebrate phospholipases C. In *Limulus* photoreceptors, light increases IP_3 levels (Brown et al., 1984), and injection of IP_3 produces the same electrophysiological response as light (Brown et al., 1984; Fein et al., 1984). Recently, Baer and Saibil (1988) reported light-stimulated hydrolysis of exogenous [^{3}H]PIP_2 by isolated squid photoreceptor outer segments in the presence of GTP and Ca^{2+}.

Evidence for a role in vertebrate phototransduction is less clear-cut, but light has been reported to decrease PIP_2 content in retinal rod outer segments (ROS) of frogs (Ghalayini & Anderson, 1984; Hayashi & Amakawa, 1985) and to produce a transient increase in IP_3 in toad ROS (Brown et al., 1987). Isolated frog ROS contain a phospholipase C activity that hydrolyzes exogenous phosphoinositides (Tarver & Anderson, 1988). Injection of IP_3 into salamander rods produces a transient hyperpolarization similar to that produced by light (Waloga & Anderson, 1985). Millar et al. (1988) report light-stimulated PIP_2 hydrolysis in dark-adapted chick and rat ROS, though they were unable to detect the IP_3 product. Finally, new evidence that calcium ion mediates light adaptation in vertebrate ROS (Matthews et al., 1988; Nakatani & Yau, 1988) makes an understanding of PIP_2 phospholipase C regulation in ROS increasingly important. In this paper, we examine bovine ROS phospholipase C activity and some possible regulators.

Materials and Methods

Materials. [^{3}H]PIP_2 (2.5 μCi/μmol) was prepared from [*inositol*-2,3-^{3}H]PIP_2 (New England Nuclear) and PIP_2 sodium salt (Sigma), and used as a 1 mM aqueous solution. [^{3}H]PIP (5.0 μCi/μmol) and [^{3}H]PI (10 μCi/μmol) were prepared analogously, but required sonication. Nucleotides and spermine were purchased from Sigma. GTP analogues were purchased from Boehringer Mannheim.

Preparation of Particulate and Soluble Fractions. ROS isolated under dim red light were purified by sucrose gradient ultracentrifugation (Kohnken et al., 1981c) and washed 4 times at 100000*g* in 10–20 volumes of isotonic washing solution (10 mM Tris-HCl, pH 7.8, 150 mM KCl, 250 mM sucrose, 1 mM DTT, and 0.1 mM phenylmethanesulfonyl fluoride) to yield the particulate fraction. Supernate from the first washing was retained as the soluble fraction; alternatively, supernate from washing at 15000*g* was clarified by filtration through Durapore-type HA 0.45-μm filter membranes (Millipore). No differences were observed between soluble fractions prepared by these two methods.

Phospholipase C Assays. Assays were performed at 30 °C in 200 mM K-HEPES, pH 7.5, 50 mM KCl, 30 μM $CaCl_2$, and 10 μM [^{3}H]PIP_2 (or other phosphoinositides as noted) plus indicated additions. Total assay volume was 500 μL. Assay duration was 30 min unless otherwise specified. Reactions were terminated by addition of 500 μL of 10% trichloroacetic acid followed by 100 μL of 5% BSA (Inoue et

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[1] Abbreviations: BAPTA, 1,2-bis(*o*-aminophenoxy)-*N*,*N*,*N*′,*N*′-ethanetetraacetic acid; BSA, bovine serum albumin; DTT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GMPPCP, guanylyl β,γ-methylenediphosphonate; GMPPNP, guanylyl imidodiphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IP, inositol 1-phosphate; IP_2, inositol 1,4-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; ROS, retinal rod outer segment; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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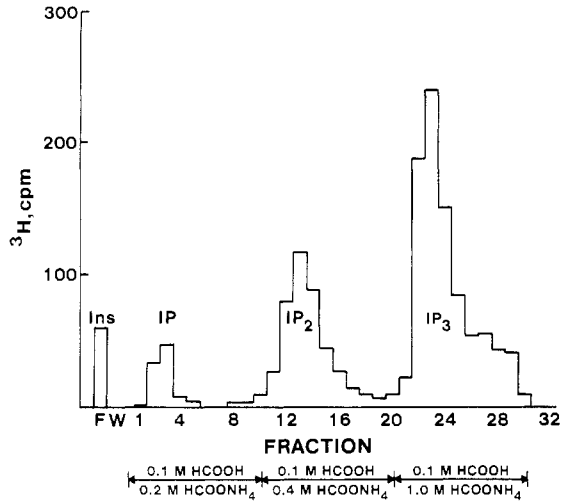

FIGURE 1: Identification of labeled products of PIP_2 hydrolysis. ROS (320 μg of protein) were incubated with 10 μM [^{3}H]PIP_2 (5 μCi/μmol) in 50 mM Tris–HEPES, pH 7.5, 150 mM KCl, and 50 μM $CaCl_2$ for 30 min at 30 °C in a final volume of 500 μL. After precipitation of unhydrolyzed PIP_2 with trichloroacetic acid and BSA, the supernate was neutralized, diluted to 10 mL, and applied to a 1-mL Dowex-1 (formate) minicolumn. The column was then washed with 10 mL of H_2O. Inositol phosphates were eluted with a series of formic acid–ammonium formate solutions as described by Downes and Michell (1981). One-milliliter fractions were collected and counted by liquid scintillometry. For the flow-through (F) and water wash (W), 1-mL aliquots were counted and the results (less background) multiplied by 10. (Ins = inositol.)

al., 1985). After centrifugation and neutralization, total acid-soluble ^{3}H in the supernate was determined by liquid scintillation counting, with subtraction of no-enzyme blanks. Except in experiments on light effects, assays were performed under normal room illumination. In preliminary experiments (Figure 1), labeled products from [^{3}H]PIP_2 were identified as inositol phosphates (primarily trisphosphate) by chromatography on Dowex-1 (Downes & Michell, 1981).

Miscellaneous Methods. ROS G-protein ("transducin") was purified by the method of Kohnken et al. (1981a). ROS and particulate fractions were treated with pertussis toxin (List Biological Laboratories, Campbell, CA) by the method of van Dop et al. (1984) and with cholera toxin (Sigma) by the method of Abood et al. (1982). Isotonic supernatant was treated with pertussis toxin by a modification of the method of Manning et al. (1984). Protein concentrations were determined by the method of Lowry et al. (1951). Rhodopsin concentrations were determined by ΔA_{500} in Emulphogene (McConnell et al., 1981).

RESULTS

Presence of Phospholipase C in ROS. ROS incubated with [^{3}H]PIP_2 released ^{3}H that remained in solution after protein and unhydrolyzed substrate were precipitated with trichloroacetic acid. Analysis of the acid-soluble label by ion-exchange chromatography (Downes & Michell, 1981) indicated it was primarily [^{3}H]IP_3 with smaller amounts of [^{3}H]IP_2, [^{3}H]IP, and [^{3}H]inositol (Figure 1). (This technique does not resolve isomers.) Since ion-exchange analysis of the products of every assay would have been extremely time-consuming, phospholipase C activity was routinely measured by determining the total acid-soluble ^{3}H produced during incubation. Unless otherwise specified, all references to phospholipase C in this paper refer to PIP_2 phospholipase C.

Soluble and Particulate Forms of Phospholipase C. Phospholipase C activity was found both in soluble and in particulate fractions derived from ROS. The soluble activity was released from ROS during isotonic washing, while particulate activity remained associated with the membranes even after repeated washing (Table I).

Table I: Removal of Phospholipase C from ROS by Isotonic Washing[a]

fraction	protein concn (mg/mL)	volume assayed (μL)	[^{3}H]PIP_2 hydrolyzed (dpm)
ROS	13.3	50	4663 ± 52
first isotonic supernate	0.13	100	2423 ± 33
second isotonic supernate	0.15	100	843 ± 26
third isotonic supernate	0.03	100	493 ± 30
fourth isotonic supernate	0.02	100	323 ± 49
final pellet	6.6	50	1537 ± 76

[a] ROS were washed 4 times with 20 volumes of isotonic washing solution. The final pellet (particulate fraction) was resuspended in 1 volume of the same solution. The pellet, supernates, and ROS were assayed for PIP_2 phospholipase C as described under Materials and Methods. Results are presented as means ± standard errors for duplicate assays.

The apparent specific activities of ROS and washed pellet were much lower than those of the supernates, due in part to the large amount of protein (rhodopsin) present in the membranes. Additionally, dilution of label by endogenous PIP_2 may produce a lower effective specific radioactivity for substrate in assays of ROS and particulate fraction. Comparison of activity in soluble and membranous preparations must therefore be made with caution.

Validation of Assay. As would be expected, due to the presence of unlabeled substrate in the membranes, the particulate fraction did not show a linear relationship between volume of enzyme and [^{3}H]PIP_2 hydrolysis. Activity in the soluble fraction was also nonlinear with respect to volume of enzyme, suggesting the presence of an endogenous inhibitor. This point will be discussed in more detail in a forthcoming paper on effects of calmodulin antagonists. Assays of phospholipase C in unwashed ROS and soluble fraction were approximately linear with respect to time for 30 min; however, particulate fraction activity began to decrease after 10–15 min.

Effects of Divalent Metal Ions. Phospholipase C in both soluble and particulate fractions displayed an absolute requirement for Ca^{2+}. Treatment with EGTA abolished activity, which was restored by Ca^{2+}. High (≥300 μM) Ca^{2+} concentrations were inhibitory, however. Half-maximal activity was obtained at Ca^{2+} concentrations of approximately 0.1 μM in the soluble fraction and between 1 and 10 μM in the particulate fraction (Figure 2). Both fractions contained sufficient endogenous Ca^{2+} to display substantial activity in the absence of added Ca^{2+} or chelators, but were usually stimulated by additional (30 μM) Ca^{2+}. This stimulation was potentiated by isotonic K^+ or Na^+; addition of Ca^{2+} produced no increase in activity at low ionic strengths.

Mg^{2+} at concentrations above 0.1 mM strongly inhibited the soluble phospholipase C. In contrast, the particulate activity was stimulated by 0.5 mM Mg^{2+} but inhibited at higher concentrations (Figure 3).

Mn^{2+} at concentrations above 30 μM strongly inhibited both soluble and particulate activity (Figure 4).

The possibility that inhibition by these ions was an artifact caused by precipitation of metal-IP_3 salts was examined by adding an inhibitory concentration of each ion at the end of an assay. No decrease in supernatant radioactivity was observed.

Effect of Spermine. The effect of spermine was qualitatively similar to that of Mg^{2+}. At low concentrations (100–200 μM), it stimulated the particulate activity but inhibited the soluble.

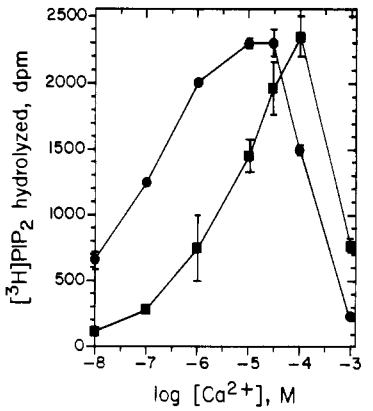

FIGURE 2: Effect of $[Ca^{2+}]$ on phospholipase C activity. Particulate [(■) 250 μg of protein/assay, 10-min incubation] and soluble [(●) 2.8 μg of protein/assay, 30-min incubation] fractions were incubated with $[^3H]PIP_2$ as described under Materials and Methods, except that Ca^{2+} concentration was adjusted to the indicated values with a Ca-BAPTA buffer. All assays contained 100 μM total BAPTA; free Ca^{2+} concentrations were calculated by using a value of 107 nM for the Ca-BAPTA dissociation constant (Tsien, 1980). Results are presented as means ± standard errors for duplicate assays.

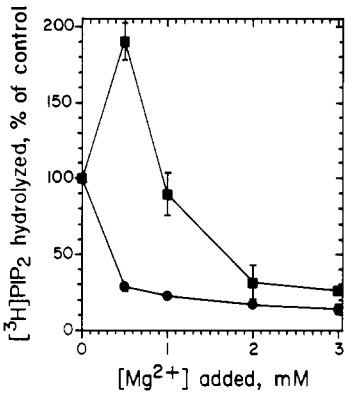

FIGURE 3: Effect of $[Mg^{2+}]$ on phospholipase C. Soluble [(●) 30-min assays] and particulate [(■) 10-min assays] fractions were assayed for PIP_2 phospholipase C as described under Materials and Methods, with the addition of the indicated concentrations of $MgCl_2$. Activity at each concentration is expressed as percent of no-Mg^{2+} control, mean ± standard error for duplicate assays.

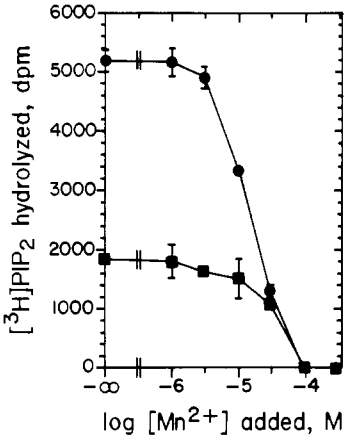

FIGURE 4: Effect of $[Mn^{2+}]$ on phospholipase C. Soluble (●) and particulate (■) fractions were assayed for PIP_2 phospholipase C as described under Materials and Methods, with the addition of the indicated concentrations of $MnCl_2$. Activity at each concentration is expressed as mean ± standard error for duplicate assays.

Higher (millimolar) concentrations inhibited both soluble and particulate activity.

Effects of Monovalent Metal Ions. Low ionic strengths decreased phospholipase C activity, but no specific effects of K^+, Na^+, or Li^+ were observed; replacement of one of these ions with another at constant ionic strength had no significant effect.

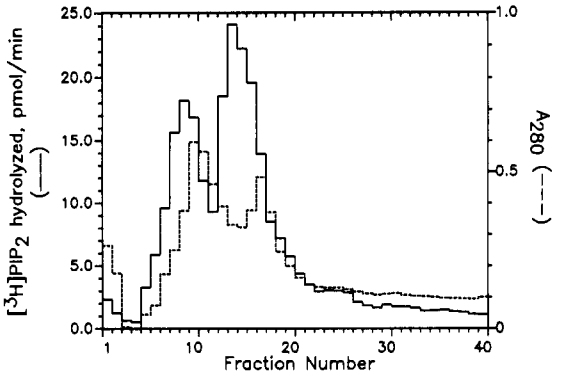

FIGURE 5: Ion-exchange chromatography of soluble phospholipase C. Soluble fraction (ca. 20 mg of protein) was applied to a 10-mL DE-52 column and eluted with a linear 0–1.0 M NaCl gradient in 10 mM Tris-HCl, pH 7.8, and 1 mM DTT. Five-milliliter fractions were collected and assayed for PIP_2 phospholipase C activity (solid line) and A_{280} (dashed line).

Table II: Effect of Nucleotides on Particulate PIP_2 Phospholipase C[a]

nucleotide	$[^3H]PIP_2$ hydrolyzed (dpm)	nucleotide	$[^3H]PIP_2$ hydrolyzed (dpm)
none	1033 ± 62 (4)	GTP	818 ± 17 (3)
ATP	286 ± 13 (3)	GDP	814 ± 40 (2)
ADP	323 ± 48 (3)	GMP	603 ± 42 (2)
AMP	394 ± 55 (3)		

[a] Particulate fraction (0.3 mg of protein/assay) was assayed for PIP_2 phospholipase C as described under Materials and Methods, except that each assay contained 100 μM $CaCl_2$, 5 mM $MgCl_2$, and a 2.5 mM sample of the indicated nucleotide. Assay duration was 15 min. Results are shown as means ± standard errors for n replicates (n = number in parentheses).

Effect of pH. Both the soluble and particulate activities were maximal at pH 6.5. Activity at pH 7.5 was ca. 30% less than at pH 6.5; nevertheless, assays were routinely performed at the higher pH to maintain consistency with experiments done before the pH optimum was determined.

Comparison of Substrates. Identical aliquots of ROS (each containing 0.9 mg of protein) incubated for 30 min with $[^3H]PI$, $[^3H]PIP$, or $[^3H]PIP_2$ produced 29%, 62%, and 76% hydrolysis, respectively. Soluble and particulate fractions of ROS were also able to hydrolyze all three substrates (data not shown).

Evidence for Multiple Forms. Chromatography of the soluble fraction on DE-52 revealed two peaks of phospholipase C activity (Figure 5). One of these has now been purified close to homogeneity.[2] Preliminary experiments on octyl glucoside solubilized particulate fractions also indicated the presence of multiple forms of the enzyme.

Effects of Nucleotides. Ghalayini and Anderson (1987) have reported inhibition of PIP_2 phospholipase C in bovine ROS by 2.5 mM ATP in the presence of 5 mM $MgCl_2$ and 100 mM $CaCl_2$. ROS particulate fraction was inhibited by 2.5 mM ATP, ADP, and AMP, as shown in Table II. GTP, GDP, and GMP were also inhibitory, although less effective than the adenine nucleotides. Lower (0.5–1.0 mM) concentrations were ineffective.

Since ROS contain GTPase activity (Godchaux & Zimmerman, 1979), the effects of nonhydrolyzable GTP analogues

[2] R. M. Pinke and D. A. Schultz, personal communication.

Table III: Effect of Pertussis Toxin on PIP_2 Phospholipase C[a]

treatment	$[^3H]PIP_2$ hydrolyzed (dpm) particulate	soluble
control	5199 ± 330	12823 ± 167
pertussis toxin	5097 ± 413	12955 ± 1220

[a] Particulate fraction was treated with pertussis toxin by the method of van Dop et al. (1984). Briefly, washed ROS membranes containing 25 nmol of rhodopsin were incubated at 30 °C under dim red light in 500 μL of 8 mM sodium 3-(*N*-morpholino)propanesulfonic acid, pH 7.4, 35 mM NaCl, 50 mM KCl, 2 mM $MgCl_2$, 2 mM DTT, and 0.2 mM NAD, with or without 12.5 μg of pertussis toxin. After 30 min, 75-μL aliquots were assayed for phospholipase C activity as described under Materials and Methods, with the addition of 2.4 mM $MgCl_2$. The $[^3H]PIP_2$ used in these assays had a specific activity of 10 μCi/μmol. Results are shown as means ± standard errors of triplicate assays. Soluble fraction was treated with pertussis toxin by a modification of the method of Manning et al. (1984). Aliquots of isotonic supernate containing 60 μg of Lowry protein were incubated at 30 °C in 100 μL of 100 mM Tris-HCl, pH 7.8, 2.5 mM $MgCl_2$, 10 mM thymidine, 2 mM DTT, 0.2 mM ATP, 0.1 mM GTP, and 0.1 mM NAD, with or without 1.2 μg of pertussis toxin. After 3 h, the total contents of each tube were assayed for phospholipase C. Results are shown as means ± standard errors of duplicate assays. ADP-ribosylation of ROS G-protein in both fractions was confirmed by use of $[\alpha\text{-}^{32}P]$NAD followed by SDS gel electrophoresis. Pertussis toxin, assayed separately, had no phospholipase C activity.

were investigated. In the presence of Mg^{2+}, GMPPCP at concentrations above 100 μM inhibited both the soluble and particulate activities (60–70% inhibition at 500 μM). At similar concentrations, GDPβS was inhibitory, and GMPPNP was moderately stimulatory, but these effects were variable, possibly due to seasonal differences in the ROS.

GTPγS (0.1–100 μM) had no effect on phospholipase C activity or Ca^{2+} dependence in either particulate or soluble fractions.

Addition of 5 μg of purified ROS G-protein per assay did not alter phospholipase C activity or confer GTPγS sensitivity in the particulate or soluble fractions. Addition of antibodies to ROS G-protein or its subunits (α and β) produced no consistent effects.

Pertussis toxin inhibits G-protein-mediated stimulation of PIP_2 phospholipase C in some cells (Cockcroft, 1987). Treatment with pertussis toxin ADP-ribosylated G-protein but did not affect phospholipase C activity in soluble or particulate fractions (Table III). The effects of GMPPCP, GDPβS, and GMPPNP were not altered by pertussis or cholera toxin.

cGMP, 8-bromo-cGMP, and cAMP, at concentrations of 0.1–1 mM, had no effect on phospholipase C activity of either soluble or particulate fractions.

No Effect of Light. The possibility that light might regulate ROS PIP_2 phospholipase C was examined under a variety of conditions, including the use of whole retinas, washed and unwashed ROS, and ROS with added soluble phospholipase C; exposure to light in advance of, at the start of, and during the assay; use of very short light flashes or continuous illumination; presence of GTP and nonhydrolyzable analogues; and varying concentration of added Ca^{2+} and Mg^{2+}. Although modest increases in activity apparently caused by light were occasionally observed, these were not consistently reproducible.

Discussion

Analysis of the labeled products of PIP_2 hydrolysis shows IP_3 as the major product, consistent with phospholipase C activity. The smaller amounts of IP_2, IP, and inositol detected presumably result from the action of phosphatases on IP_3, although hydrolysis of $[^3H]$PIP and $[^3H]$PI (produced from $[^3H]PIP_2$ by phosphatases) may also contribute. Experiments using $[^3H]$PIP and $[^3H]$PI as substrates indicated that they were hydrolyzed by soluble and particulate ROS phospholipases C, although less rapidly than PIP_2. Their hydrolysis may be due to multiple enzymes or broad substrate specificity of PIP_2 phospholipase C, a question which can be resolved by purification of the multiple forms of the PIP_2-specific enzyme.

Purified ROS which were repeatedly washed isotonically to remove soluble proteins still retained significant phospholipase C activity. This particulate activity was almost certainly native to the ROS. The soluble activity, which was found in the supernatant of the crude ROS pellet and throughout the final sucrose gradient used in purifying the ROS, may originate in ROS, rod inner segments, or elsewhere. Taking into account the dilution at each step, the activity in the isotonic supernate cannot be accounted for by carry-over from the crude ROS supernate, indicating that soluble phospholipase C continues to leak out of ROS. We therefore ascribe a significant portion of the overall soluble phospholipase C activity to the ROS. In any case, forms of the enzyme found in ROS may be identical with one or more forms found in the inner segment, especially since the outer segment has no known capability for protein synthesis.

The in vivo function of the soluble activity is also uncertain. The sensitivity to Mg^{2+} and spermine suggests that it would be inhibited under cytoplasmic conditions. Majerus et al. (1986) propose that cytosolic phospholipases C may associate reversibly with cell membranes as a part of their regulation. Soluble phospholipase C may represent a pool of inactive enzyme that can be activated by association with ROS plasmalemma or disk membranes under the right conditions. If so, the loss of large amounts of this enzyme from ROS during preparation might account for failure to observe regulation by light and/or G-protein.

PIP_2 hydrolysis was markedly sensitive to divalent cations. Although phospholipases are often assayed in millimolar Ca^{2+}, we assayed phospholipase C at 30 μM Ca^{2+}, which we believe to be closer to the physiological concentration. The inhibitory effect of millimolar Ca^{2+} (Figure 2) may account for the low level of ROS phospholipase C activity reported by van Rooijen and Bazan (1986). The difference between the Ca^{2+} requirements of the soluble and particulate activities is intriguing, but may be due to incorporation of the substrate into lipid bilayers in assays of the particulate fraction (Irvine et al., 1984) rather than to differences in the enzymes. Mn^{2+} inhibited phospholipase C in both fractions. At low (<1 mM) concentrations, Mg^{2+} inhibited the soluble activity but stimulated the particulate; higher concentrations inhibited both fractions. As is often the case, spermine, a polycationic amine, resembled Mg^{2+} in its effects. In contrast to their effects on phospholipase C, Mg^{2+}, Mn^{2+}, and spermine stimulated phosphoinositide synthesis in ROS, as described in the preceding paper (Gehm & McConnell, 1990). The effects of divalent cations on phosphoinositide metabolism in ROS are of special interest in view of the reported ability of Mg^{2+} and Ca^{2+} to control the conductance of the ROS plasma membrane (Stern et al., 1987), and the special role of Ca^{2+} in light/dark adaptation (Matthews et al., 1988; Nakatani & Yau, 1988).

PIP_2 phospholipase C is regulated by G-proteins in a wide variety of cells [reviewed by Cockcroft (1987)], possibly by alteration of its Ca^{2+} requirement (Smith et al., 1986). It has been proposed that in invertebrate photoreceptors, photoisomerized rhodopsin activates a G-protein which in turn activates phospholipase C (Fein, 1986; Tsuda, 1987). In vertebrate ROS, the G-protein that mediates light activation of cGMP phosphodiesterase constitutes a major part of the

non-rhodopsin protein. The nonhydrolyzable GTP analogue GTPγS is highly effective in stimulating G-protein-regulated PIP_2 phospholipase C in several cell types (Uhing et al., 1985; Deckmyn et al., 1986; Smith et al., 1986; Hepler & Harden, 1986), and cGMP phosphodiesterase in ROS (Yamanaka et al., 1986). The absence of any effect by GTPγS on phospholipase C from ROS at any Ca^{2+} concentration appears to argue against regulation by G-protein. Inhibition by GMPPCP and the inconstant effects of GDPβS and GMPPNP occurred at concentrations considerably higher than those usually associated with G-protein regulation.

Pertussis toxin ADP-ribosylates ROS G-protein, inhibiting its GTPase activity and its ability to stimulate cGMP phosphodiesterase (van Dop et al., 1984); it similarly inactivates phospholipase-regulating G-proteins in many, but not all, cell types (Cockcroft, 1987). Cholera toxin also ADP-ribosylates ROS G-protein (Abood et al., 1982). Neither toxin affected the activity or GMPPCP sensitivity of phospholipase C from ROS.

Nor have we obtained any evidence of regulation by light. It is difficult to compare our results directly with reports of light-stimulated phospholipase C in lower vertebrates (Ghalayini & Anderson, 1984; Hayashi & Amakawa, 1985; Millar et al., 1988) due to differences in preparations and in assay techniques. Tarver and Anderson (1988) have not reported light sensitivity of isolated frog ROS PIP_2-phospholipase C, in contrast with an earlier observation by Ghalayini and Anderson (1984) that flash illumination of intact, prelabeled frog retinas decreased the ROS content of $[^3H]PIP_2$. It may be the isolation process in the case of both frog and bovine ROS which severs a link between light and the enzyme. Nevertheless, squid outer segments isolated and assayed by essentially the same procedures as we use for bovine ROS exhibit light sensitivity (Baer & Saibil, 1988). If light does regulate vertebrate ROS phospholipase C in vivo, the regulatory system may differ from that of cGMP phosphodiesterase, since we have failed to detect any light activation of phospholipase C under conditions that result in a severalfold activation of phosphodiesterase (Manthorpe & McConnell, 1975; Kohnken et al., 1981b). It is possible that minimal bleaching fully activates the enzyme and that only totally dark-adapted outer segments display light regulation. Cattle eyes are seldom dark-adapted before removal, and we estimate our ROS preparations to be as much as 4–6% bleached (unpublished observations). Despite this, cGMP phosphodiesterase in these ROS is activated by light, and Jelsema (1987) has reported regulation of phospholipase A_2 by light and the βγ component of ROS G-protein in isolated bovine ROS.

It is also possible that light regulation of ROS PIP_2 phospholipase C is indirect—that changes in activity result from the changes in ionic concentrations that attend the rod photoresponse. The sensitivity of PIP_2 hydrolysis to physiological concentrations of Ca^{2+} and Mg^{2+} makes this an attractive possibility. Isolated ROS are of course separated from the ion pumps of the inner segment; furthermore, ROS isolated in sucrose have leaky plasma membranes. Indirect light regulation via ionic changes could hardly be preserved in such a preparation.

Our preliminary evidence suggests multiple forms of soluble phospholipase C in the ROS and possibly of the membranous enzyme also. This would be consistent with reports of multiple forms of the brain enzyme, which have been purified, cloned, and sequenced (Suh et al., 1988a,b), revealing some similarities, but major differences as well. Characterization of overall phospholipase C activity in ROS may obscure the intrinsic functions of individual forms. For example, one may be responsive to light or G-protein; another may not be, or not in the same fashion. Their Ca^{2+} requirements and substrate specificities could differ. A single form of the enzyme may have different subcellular locations which dictate its regulation. Together with uncertainty that ROS phospholipases C assayed in vitro even retain their native relationships to membranes, these considerations dictate purification, cloning, sequencing, and separate characterization of enzyme forms before assessment of their individual regulatory modes.

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S-Phase-Specific Expression of Mammalian Ribonucleotide Reductase R1 and R2 Subunit mRNAs†

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ABSTRACT: Ribonucleotide reductase in mammalian cells is composed of two nonidentical subunits, proteins R1 and R2, each inactive alone. The R1 protein is present in excess in proliferating cells, and its levels are constant during the cell cycle. Expression of the R2 protein, which is limiting for enzyme activity, is strictly S-phase-correlated. In this paper, we have used antisense RNA probes in a solution hybridization assay to measure the levels of R1 and R2 mRNA during the cell cycle in centrifugally elutriated cells and in cells synchronized by isoleucine or serum starvation. The levels of both transcripts were very low or undetectable in G_0/G_1-phase cells, showed a pronounced increase as cells progressed into S phase, and then declined when cells progressed into G_2+M phase. The R1 and R2 transcripts increased in parallel, starting slightly before the rise in S-phase cells, and reached the same levels. The relative lack of cell cycle dependent variation in R1 protein levels, obtained previously, may therefore simply be a consequence of the long half-life of the R1 protein. Hydroxyurea-resistant, R2-overproducing mouse TA3 cells showed the same regulation of the R1 and R2 transcripts as the parental cells, but with R2 mRNA at a 40-fold higher level.

Mammalian ribonucleotide reductase is composed of two nonidentical dimeric subunits, proteins R1 and R2, which are both essential for activity. It catalyzes the direct reduction of ribonucleotides to the corresponding deoxyribonucleotides, the first unique step in the reactions leading to DNA synthesis, to which its activity is strongly correlated (Thelander & Reichard, 1979; Reichard, 1988).

The mammalian R1 and R2 subunits have been purified to homogeneity, and the corresponding cDNAs have been cloned from mouse cells (Caras et al., 1985; Thelander & Berg, 1986). The R1 subunit binds the nucleoside triphosphate allosteric effectors and the ribonucleoside diphosphate substrates and provides redox-active sulfhydryl groups (Thelander & Reichard, 1979; Thelander et al., 1980). In analogy with the *Escherichia coli* enzyme, the R2 subunit most probably contains two non-heme binuclear iron centers, which each generate and stabilize a tyrosyl free radical essential for activity (Thelander et al., 1985; Lynch et al., 1989). Hydroxyurea specifically destroys the radical and has been used to select hydroxyurea-resistant human and rodent cells, which over-

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