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STUDIES ON PHOSPHOINOSITIDE METABOLISM IN

RETINAL ROD OUTER SEGMENTS

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BARRY DEAN GEHM

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Major professor

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STUDIES ON PHOSPHOINOSITIDE METABOLISM IN RETINAL ROD OUTER SEGMENTS

By

Barry Dean Gehm

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ABSTRACT

STUDIES ON PHOSPHOINOSITIDE METABOLISM IN

RETINAL ROD OUTER SEGMENTS

By

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Phosphoinositides are an important class of phospholipids that help mediate signal transduction in many types of eukaryotic cells. They are believed to act as second messengers in the response of invertebrate photoreceptors to light. A similar role for them has been suggested in vertebrate phototransduction as well. The present work was undertaken to determine whether isolated mammalian photoreceptors (bovine rod outer segements, ROS) were capable of synthesizing and hydrolyzing phosphoinositides, and if so, how synthesis and hydrolysis might be regulated.

Synthesis of phosphoinositides was demonstrated by the incorporation of radioactively labeled precursors. ROS incubated with [γ-^{32}P]ATP produced labeled phosphatidic acid (PA), phosphatidyl-inositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2). When Mn^{2+}, CTP and inositol were added, labeled phosphatidylinositol (PI) was also produced. [^{3}H]Inositol was incorporated into PI, PIP and PIP_2, although prolonged incubation was required for detectable incorporation into PIP_2. Incorporation of [^{3}H]inositol was dependent on CTP, indicating that labeling proceeded via synthesis, not base exchange. Incubation with [α-^{32}P]CTP

produced labeled CDP-diacylglycerol, an intermediate in PI synthesis. Incorporation of labeled precusors was stimulated by Mg^{2+}, Mn^{2+} and spermine, but unaffected by light.

Phosphoinositide hydrolysis was measured using exogenous ^{3}H-labeled substrates. PI, PIP, and PIP_2 were all hydrolyzed but most attention was devoted to PIP_2. Hydrolysis occured via the phospholipase C (PLC) reaction, producing inositol trisphosphate and diacylglycerol. PLC was found in particulate and soluble ROS fractions, and displayed multiple forms on ion-exhange columns. PLC activity was Ca^{2+}-dependent in the micromolar range. Crude enzyme preparations contained an endogenous inhibitor whose effects were Ca^{2+}-dependently relieved by calmodulin antagonists. This inhibitor does nor appear to be calmodulin but may be a novel Ca^{2+}-binding regulatory protein. No evidence for regulation of PLC activity by light or G-proteins was obtained. The effects on PLC activity of Mg^{2+}, Mn^{2+}, spermine, pH and detergents were also characterized.

To my parents,

sine qua non.

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ABBREVIATIONS

ATP, ADP, AMP: adenosine triphosphate, diphosphate and monophosphate

BAPTA: 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid

BSA: bovine serum albumin

CaM: calmodulin

CDP-DG: cytidine diphosphate-diacylglycerol

CTP: cytidine triphosphate

EC_{50}: 50% effective concentration

EGTA: ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid

GDPβS: guanosine-5'-O-(2-thiodiphosphate)

GMPPCP: guanylyl-(β,γ-methylene)-diphosphonate

GMPPNP: guanylylimidodiphosphate

GTP, GDP, GMP: guanosine triphosphate, diphosphate and monophosphate

GTPγS: guanosine-5'-O-(3-thiotriphosphate)

HEPES: *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

IBMX: 3-isobutyl-1-methylxanthine

IC_{50}: 50% inhibitory concentration

IP: inositol-1-phosphate

IP_2: inositol-1,4-bisphosphate

IP_3: inositol-1,4,5-trisphosphate

M_r: relative molecular weight

PI: phosphatidylinositol

PIP: phosphatidylinositol-4-phosphate

PIP_2: phosphatidylinositol-4,5-bisphosphate

PLC: phospholipase C

ROS: retinal rod outer segments

SDS: sodium dodecyl sulfate

Tris: tris(hydroxyethyl)aminomethane

W-5: N-(6-aminohexyl)-1-naphthalenesulfonamide

W-7: N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide

W-12: N-(4-aminobutyl)-1-naphthalenesulfonamide

W-13: N-(4-aminobutyl)-5-chloro-1-naphthalenesulfonamide.

WROS: isotonically washed ROS

INTRODUCTION

Vision is mediated by specialized photoreceptor cells in the retina. Two morphologically distinct types of photoreceptors, rods and cones, are found in vertebrate retinas. Cones operate in bright light and comprise several subtypes with differing spectral sensitivities; they are responsible for color vision. Rods are more sensitive to light than cones and are responsible for dim-light vision. Because rods are larger and more numerous than cones in most retinas, and do not introduce the complicating factor of multiple spectral types, most work on the biochemistry of vision has been done with rods.

As can be seen in Figure 1, the rod cell is divided by a narrow constriction into an inner and outer segment. The inner segment contains the normal appurtenances of animal cells, such as mitochondria, endoplasmic reticulum, and the nucleus. A synaptic terminus connects the proximal end of the inner segment to other retinal neurons. (The morphology of photoreceptors varies considerably from species to species, and some workers do not consider the nucleus and synaptic terminus as parts of the inner segment.)

The outer segment is a specialized structure in which the energy of light is absorbed, transduced and amplified to produce an electrical signal. Hundreds of flattened membranous vesicles (disks) form an orderly stack, surrounded by but not continuous with the cell's plasma membrane. The disks contain the visual pigment, rhodopsin. Rhodopsin

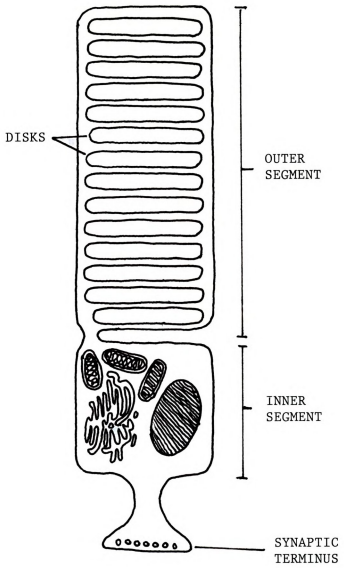

Figure 1. A rod cell.

This schematic drawing of a typical rod cell is oriented so that upwards represents the distal direction, *i. e.*, towards the back of the eye. Light enters from below.

consists of a protein, opsin, and a covalently attached lipid chromophore, 11-*cis*-retinal. Absorption of a photon causes retinal to isomerize to its all-*trans* form, causing a loss of color ("bleaching") and producing a series of conformational changes in the rhodopsin molecule.

This ultimately results in electrical hyperpolarization of the rod cell. In the dark, a constant current of positive ions (primarily Na^+, but some Ca^{2+} as well) exits the cell from the inner segment and re-enters through "sodium channels" in the plasma membrane of the outer segment. Bleaching of rhodopsin indirectly causes the sodium channels to close, preventing influx of Na^+ into the outer segment. The ATP-driven efflux of Na^+ from the inner segment, without a balancing influx, drives the membrane potential from its resting value of -30 mV to as much as -70 mV. This hyperpolarization triggers firing of the synapse connecting the rod cell to retinal neurons.

Internal messengers: How does the bleaching of rhodopsin, which is located in the disk membranes, bring about the closing of the sodium channels in the plasma membrane? One or more water-soluble messengers must carry information across the cytoplasm. The identity of the messenger molecule(s) has been one of the principal problems in visual biochemistry for more than a decade. The two major candidates that have been proposed for the role of internal messenger are cyclic GMP and Ca^{2+} (Pugh and Cobbs, 1986; Lamb, 1986).

cGMP: It has been known for more than a decade that rod outer segments (ROS) of frogs and cattle contain a light-activated cylic GMP phosphodiesterase (PDE) (Miki *et al.*, 1973; Manthorpe and McConnell, 1975). Activation is mediated by a GTP-binding protein that Fung *et*

al. (1981) named "transducin" but which earlier vision investigators (Godchaux & Zimmerman, 1979) called ROS G-protein. More recently, it has been demonstrated that cGMP can increase the Na^+ conductance of isolated patches of ROS plasma membrane, apparently by opening the sodium channels (Fesenko *et al.*, 1985). cGMP is now the leading candidate for internal messenger in vertebrate rods. In the "cGMP hypothesis", cGMP holds the sodium channels open in the dark. Photobleaching of rhodopsin activates PDE via ROS G-protein. Hydrolysis of cGMP by the activated PDE then causes the sodium channels to close.

Ca^{2+}: Large amounts of calcium are stored inside the disks. The "calcium hypothesis" proposed that, on illumination, Ca^{2+} is released from the disks into the cytoplasm and blocks the sodium channels (Hagins, 1972). This idea gained support from numerous experiments showing Ca^{2+}-regulation of photoreceptor electrical behavior (summarized in Pugh and Cobbs, 1986). Alterations in external Ca^{2+} concentration, which are believed to bring about concomitant changes in internal concentration via Na^+/Ca^{2+} exchange and/or entry of Ca^{2+} through the "sodium" channels, alter membrane conductance. Specifically, high $[Ca^{2+}]$ decreases dark current and increases membrane potential, and low $[Ca^{2+}]$ has opposite effects. These effects are enhanced by calcium ionophores. Microinjections of Ca^{2+} or EGTA into toad rods cause hyperpolarization and depolarization respectively (Brown *et al.*, 1977).

However, introduction of chelators into the ROS cytoplasm does not prevent the photoresponse (Matthews *et al.*,1985), and recent studies of Ca^{2+} fluxes in rods (reviewed in Pugh and Cobbs, 1986, and Lamb,

1986) indicate that cytoplasmic Ca^{2+} concentration may not increase in response to light as previously thought -- in fact, it may decrease. Additionally, experiments with isolated patches of outer segment membranes show no regulation of conductance by physiological levels of Ca^{2+}, in contrast to cGMP (Fesenko *et al.*, 1985). Hence, the calcium hypothesis in its original form has been largely abandoned as a model for vertebrate phototransduction.

Nevertheless, Ca^{2+} is still believed to play a role in the function of the rod cell, possibly in adjusting sensitivity at different light levels or terminating the photoresponse. Treatment of frog photoreceptors with EGTA and the calcium ionophore A23187 decreases sensitivity to low light levels (Nicol *et al.*, 1987). Infusion of the Ca^{2+} chelator BAPTA into salamander rods extends the duration of the photoresponse (Matthews *et al.*, 1985) and slows light-adaptation (Torre *et al.*, 1986). These findings suggest that Ca^{2+} may act as a modulator, rather than a transmitter, of the photoresponse.

Phosphoinositides: The phosphoinositides are a family of phospholipids found in membranes of eukaryotic cells. They contain an inositol moiety in their polar head groups, and comprise phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2).

Evidence for the involvement of phosphoinositides in signal transduction was first uncovered twenty-five years ago by Hokin and Hokin (1953) in blowfly salivary gland. As the presence of this pathway has been revealed in a host of different cell types in recent years, interest in it has increased almost explosively, and has focused

on the hydrolysis of PIP_2 by phospholipase C (PLC) (for reviews, see Berridge, 1984, Berridge and Irvine, 1984; Nishizuka, 1984, Joseph, 1984; Hokin, 1985; Majerus *et al.*, 1985, Majerus *et al.*, 1886, Berridge, 1987). This hydrolysis produces diacylglycerol, an activator of protein kinase C, and inositol-1,4,5-trisphosphate (IP_3), which mobilizes Ca^{2+} from intracellular stores. A portion of the inositol trisphosphate may be produced in the form of a 1,2-cyclic isomer (Wilson *et al.*, 1985); it appears to be similar to the non-cyclic isomer in its effects but is metabolized more slowly (Majerus *et al.*, 1986).

Figure 2 presents a simplified schematic of phosphoinositide metabolism. PI is synthesized in a two-step, CTP-requiring process from phosphatidic acid (PA) and inositol. Some of it is phosphorylated to produce PIP and PIP_2. PI, PIP and PIP_2 are substrates for one or more PLCs, producing diacylglycerol and the respective inositol phosphates. A series of phosphatases degrade the inositol phosphates back to inositol, which can then re-enter the synthetic pathway. (IP phosphatase is inhibited by Li^+.)

For the sake of simplicity, some reactions have been omitted from Figure 2. The 1,2-cylic isomers of the inositol phosphates are not shown, as they are beyond the scope of the present work. Similarly, the phosphorylation of IP_3 to IP_4, IP_5 and IP_6, which has been reported in some types of cells (Batty *et al.*, 1985; Vallejo, M. *et al.*, 1987) has not been demonstrated in photoreceptors and is omitted from Figure 2. Phosphatases which dephosphorylate PIP and PIP_2 are not shown; they have not been studied in photoreceptors previously and are touched on only briefly in this dissertation. Although a

Figure 2. Phosphoinositide metabolic pathways.

This diagram presents a simplified schematic of phosphoinositide synthesis and breakdown. Abbreviations:

DG, diacylglycerol;

PA; phosphatidic acid;

CDP-DG, CDP-diacylglycerol (also called phosphatidyl-CMP);

PI, phosphatidlyinositol;

PIP, phosphatidylinositol-4-phosphate;

PIP_2, phosphatidylinositol-4,5-bisphosphate;

IP_3, inositol-1,4,5-trisphosphate;

IP_2, inositol-1,4-bisphosphate;

IP, inositol-1-phosphate;

Ins, inositol.

The hydrolysis of PI, PIP, and PIP_2 may be carried out by the same or different PLCs.

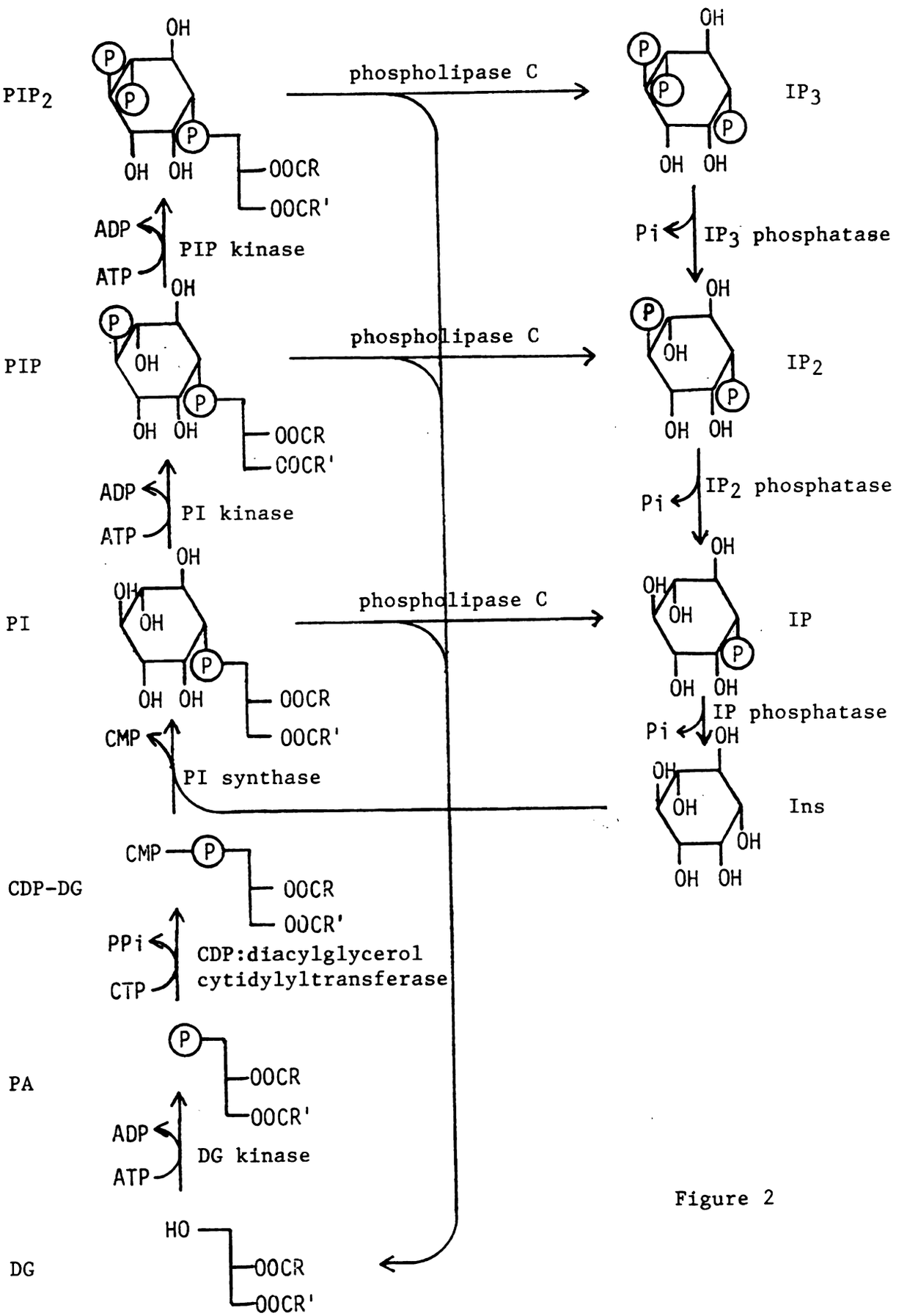
PIP2
phospholipase C
IP3
OH
P
OOCR
OOCR'
ADP
ATP
PIP kinase
Pi
IP3 phosphatase
PIP
IP2
IP2 phosphatase
PI
IP
IP phosphatase
Ins
CMP
PI synthase
CDP-DG
PPi
CTP
CDP:diacylglycerol
cytidylyltransferase
PA
DG kinase
DG
HO

Figure 2

phosphoinositide-specific phospholipase A_2 has been reported in ROS (Jelsema, 1987), it was not examined in these experiments and is not depicted in Figure 2.

Phosphoinositides and phototransduction: Interest in the possible involvement of phosphoinositides in visual transduction was a logical outgrowth of the calcium hypothesis. An increase in cytoplasmic Ca^{2+} is found in many kinds of cells when they respond to external stimuli. A common mechanism appears to underlie this response: binding of an agonist to a receptor protein causes activation of PLC via a GTP-binding protein (G protein). As described above, the PLC hydrolyses PIP_2 to IP_3 and diacylglycerol (DG) which then function as second messengers, DG activating protein kinase C and IP_3 mobilizing Ca^{2+}. With the substitution of bleached rhodpsin for the receptor-agonist complex, this mechanism provided an attractive model for mobilization of Ca^{2+} in response to light, and interest in phosphoinositide metablolism in photoreceptors has persisted despite the demise of the original calcium hypothesis.

Strong evidence now exists that a light- and G-protein-regulated PLC plays a major role in transduction in invertebrate photoreceptors (see Chapter II literature review). Increased hydrolysis of PIP_2 in response to light, and/or light-like effects of IP_3 injection, have been reported in insects, crustaceans and mollusks. Evidence for a similar role in vertebrate photoreceptors is less clear-cut, but results similar to those obtained in invertebrates have been reported in amphibians. Parallels between vertebrate and invertebrate phototransduction must be drawn cautiously, however. Invertebrate photoreceptors increase membrane permeabilty and depolarize in response

to light, the reverse of the vertebrate response; the role of calcium appears to be much more prominent in invertebrates, and that of cyclic nucleotides small or non-existent (Payne, 1986). There are also major differences in microanatomy between vertebrate and invertebrate photoreceptors.

Choice of experimental system: The experiments presented in this dissertation were performed on isolated bovine ROS, which offer several advantages:

1. Outer segments are specialized for photoreception. Use of isolated ROS helps reduce the likelihood of confounding processes secondary to photoreception (*e. g.* synaptic firing) with those central to it.

2. Our laboratory has long experience in preparation of large quantities of bovine ROS with very little contamination by other organelles (McConnell, 1965; McConnell *et al.*, 1969).

3. Light-regulation of cGMP phosphodiesterase in isolated ROS has been demonstrated by several laboratories, including ours (Kohnken *et al.*, 1981), indicating that light-regulated enzyme pathways are likely to remain functional.

4. Use of bovine ROS offers the opportunity to examine the role of phosphoinositides in mammalian photoreceptors. The bulk of work on vertebrates has been done in amphibians.

On the other hand, the choice of isolated ROS is attended by some possible problems. The homeostatic mechanisms that maintain physiological conditions inside the rod cell are lost. Some components of the phosphoinositide metabolic or regulatory pathways may be labile

to the rigors of isolation. Interpretation, especially of negative results, must be made with caution.

Research goals: The aim of this research was to determine if an active phosphoinositide metabolism is present in ROS, and if so, to characterize its regulation. Specific questions to be answered included: Are phosphoinositides synthesised in ROS, and what conditions are necessary to demonstrate synthesis? Is synthesis regulated by light? Do ROS contain phosphoinositide phospholipase C activity? If so, what conditions are necessary for hydrolysis? Is there more than one form of the PLC? What regulates PLC activity? Specifically, is it regulated by light and/or a GTP-binding protein? Does Ca^{2+} affect PLC activity? If so, is calmodulin involved?

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CHAPTER I

SYNTHETIC PATHWAYS

LITERATURE REVIEW

Most studies of retinal phospholipid synthesis have employed whole retinas. Urban *et al.* (1972) studied the incorporation of ^{32}P into phospholipids of rat and calf retinas *in vitro*. In both species they found that PA and PI were much more intensely labeled than the other phospholipids, although PI composed only ~4% of the total lipid phosphorus and PA less than 1%. Long (2 hrs) exposure to light decreased labeling of phosphatidylserine, but no other significant effects of light were found.

Bazan and his coworkers have examined glycerolipid (phospholipid and acylglycerol) synthesis in retinas using a variety of labelled precursors. Cattle retinas incubated with [^{3}H]arachidonate incorporated the bulk of the label into di- and triacylglycerols, PI and phosphatidylcholine, but PI and PA displayed the highest specific activity (Bazan and Bazan, 1975); similar results were obtained with [^{14}C]glycerol (Bazan *et al.*, 1977). Toad retinas incorporated [^{14}C]glycerol in a similar pattern, either *in vitro* or *in vivo* (Bazan and Bazan, 1976; Giusto and Bazan, 1979). Flashes of light increased labeling of total retinal phospholipids with [^{14}C]glycerol (Bazan and Bazan, 1977). Fractionation of retinas preincubated with [^{3}H]glycerol showed most of the labeled lipids in retinal fractions other than ROS, however (Bazan *et al.*, 1981).

Anderson *et al.* (1980) injected frogs with labeled precursors and, after periods ranging hours to months, extracted and fractionated the retinas. Labeled phospholipids appeared more rapidly in microsomes than in ROS. PI was labeled more rapidly and to higher specific

activity than PC, PS, or PE (other phospholipids were not collected). In addition to this evidence of *de novo* synthesis, Anderson and Kelleher (1981) demonstrated phospholipid base-exchange reactions in retinal microsomes, using labeled choline, serine and ethanolamine. Lower levels of base-exchange activity were found in ROS.

These results, and the post-labeling fractionations discussed above, suggest that retinal microsomes, probably derived from inner segment endoplasmic reticulum (ER), are the major site of retinal phospholipid synthesis. This was confirmed by the micro-autoradiographic work of Mercurio and Holtzman (1982), showing that [^{3}H]glycerol and [^{3}H]choline incorporation in frog rod cells occurs primarily in the inner segment rough ER. It is therefore unwise to use results obtained with whole retinas to test models of phospholipid involvement in phototransduction.

This point is further illustrated by the results of Anderson and Hollyfield (1981) who found that light increased *in vitro* incorporation of [^{3}H]inositol into PI in *Xenopus* retinas. Autoradiography showed the increased incorporation was localized in horizontal cells, a class of retinal neurons, and not in photoreceptors. [^{3}H]Inositol incorporation was also regulated by two neurotransmitters, acetylcholine and glycine (Anderson and Hollyfield, 1984). The post-synaptic involvement of phosphoinositide turnover in neurotransmission has also been demonstrated in other neural tissues (Fisher and Agranoff, 1987), and evidence for pre-synaptic involvement has been reported (Yandrasitz, *et al.*, 1985) . Light-enhanced [^{3}H]inositol incorporation into PI was also demonstrated in rat retinas, by Schmidt (1983a). This increase in labeling was enhanced by

cytidine, and localized in photoreceptor inner segments. Such a location suggests that the light-effect is indirect. Light-stimulated conversion of PI to PIP_2 in rat retinas was localized in the inner, *i. e.* non-photoreceptor, retinal layers (Schmidt, 1983b).

Use of isolated ROS holds out the possibility of a less ambiguous assessment of the role of phosphoinositides in phototransduction, since the reactions observed can be ascribed to the specialized photoreceptor organelle. Yoshioka *et al.* (1983), using a preparation of octopus photoreceptor microvilli membranes (analogous to isolated vertebrate ROS) observed that light decreased [^{32}P]incorporation into PA, PIP and PIP_2. Isobutylmethylxanthine (IBMX), a cyclic nucleotide phosphodiesterase inhibitor, decreased phosphoinositide labeling even more effectively than light, but increased PA labeling. The effect of IBMX appeared not to be due to increased cGMP or cAMP concentrations.

In squid photoreceptor membranes, light decreased [^{32}P]labeling of PIP and PIP_2, but increased that of PA (Vandenberg and Montal, 1984). The reason for the discrepancy in the effect on PA between squid and octopus is unclear. The results in squid are easier to rationalize, since light-induced hydrolysis of PIP_2 would be expected to increase PA labeling by making more diacylglycerol available for phosphorylation. The octopus membrane preparation was obtained from frozen retinas and exposed to light continuously during labeling (2-30 min), whereas the squid preparation was made from freshly dissected retinas and exposed to light for 1 min prior to labeling, but there is no *a priori* reason to expect divergent effects on PA from these methodological differences.

Despite these provocative results in cephalopods, relatively few studies of phospholipid precursor incorporation in isolated vertebrate ROS have been performed. Seyfred *et al.* (1984) observed rapid incorporation of label from [γ-^{32}P]ATP into PA. This project was undertaken to expand on their work. After most of the labeling experiments presented herein had been completed, and a summary thereof published in the form of an abstract (Gehm and McConnell, 1985), Giusto and Ilincheta de Boschero (1986) also reported labeling of PA, PIP and PIP_2 in isolated bovine ROS incubated with [γ-^{32}P]ATP. Labeling of the polyphosphoinositides was increased by the addition of PI in the presence of Triton X-100, or by the detergent alone. No examination of the effects of light was reported.

MATERIALS AND METHODS

Materials: [γ-^{32}P]ATP and [α-^{32}P]CTP were purchased from New England Nuclear. [^{3}H]Inositol was purchased from NEN or American Radiolabeled Chemicals. Labeled ATP and CTP were adjusted to the indicated specific activities with unlabelled compounds purchased from Sigma.

ROS Isolation: ROS were isolated from bovine retinas by the method of Kohnken *et al.* (1981b). Briefly, retinas were removed from cattle eyes and vigorously shaken in buffered 1.32 M sucrose-0.15M KCl, causing the outer segments to shear off at the ciliary connection to the inner segment. After centrifugation at 1100 x *g*, the supernatant was diluted with 3 volumes of buffered 0.15 M KCl and centrifuged at 2100 x *g*. The crude ROS pellet was resuspended in ρ=1.10 buffered sucrose-KCl and applied to a ρ=1.12 to 1.16 sucrose density gradient. Best results, in terms of yield and purity, were usually obtained with continuous gradients in winter and step (1.12-1.14-1.16) gradients in summer. ROS formed a band at a density between 1.12 and 1.14 g/ml. All operations were performed under dim red light at 0° to 4° C. ROS were stored at -196° C. until use.

Phospholipid labeling: In early experiments, ^{32}P labeling was carried out 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), which allowed assay of label incorporation into PA, PI, PIP and PIP_2 simultaneously. However, measurement of [^{32}P]PI was made difficult by comparatively low level of label incorporation and its proximity on TLC plates to the "tail" of

the intensely labeled PA spot. Consequently, PI synthesis was later assayed in a medium containing 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 [^{3}H]*myo*-inositol (~16 mCi/μmol). This produced more counts in PI, owing to the higher specific activity of the substrate, and did not label PA.

Since PI labeling could be measured more easily with [^{3}H]inositol, later ^{32}P labeling experiments used "minimal" ^{32}P labeling medium, which lacked CTP and inositol but was otherwise identical to the complete medium. This produced labeled PA, PIP and PIP_2.

Unless otherwise indicated, all phospholipid labeling reactions were carried out at 30° in a volume of 100 μl using illuminated ROS (100 - 300 μg Lowry protein), for 30 min in the case of ^{32}P labeling and 3 h in the case of ^{3}H labeling. Reactions were terminated by acidic chloroform:methanol extraction (Schacht, 1981). Extracts were evaporated with nitrogen, redissolved in chloroform:methanol (2:1) and spotted on TLC plates.

Analysis of labled lipids: Thin layer chromatography was performed on E. Merck silica gel 60 plates. 1-dimensional (^{3}H) TLCs were developed with $CHCl_3$:MeOH:H_2O:conc.NH_4OH (98:78:19:4). 2-dimensional (^{32}P) TLCs were developed in the 1st dimension with $CHCl_3$:MeOH:H_2O: conc.NH_4OH (98:78:19:5) and in the 2nd dimension with $CHCl_3$: acetone:MeOH:HOAc:H_2O (10:4:2:2:1). Labeled lipids were visualized by autoradiography or fluorography using Kodak X-O-Mat AR-5 film. ^{32}P-labeled plates were exposed for 18-72 hr at -20°. ^{3}H-labeled plates were dipped in a fluorographic enhancer containing 0.4% 2,5-diphenyloxazole (PPO) in 2-methylnaphthalene: toluene (9:1)

(Bonner and Stedman, 1978) and exposed overnight or longer at -20° to film that had been hypersensitized by strobe flash (Laskey and Mills, 1977). Labeled lipids were scraped from the plates, treated with 0.1 N NaOH, neutralized, and counted by liquid scintillometry. NaOH treatment enhanced release of label from silica gel, presumably by saponifying the lipids.

Miscellaneous methods: Protein concentrations were determined by the Lowry method (Lowry *et al.*, 1951). Rhodopsin concentrations were determined by ΔA_{500} in Emulphogene (McConnell *et al.*, 1981).

RESULTS

Synthesis of phosphoinositides in ROS: If ROS use IP_3 as an internal messenger, they must have a way of replenishing PIP_2. Transport of phospholipids from inner to outer segment has been reported (Mercurio and Holtzman, 1982), but synthesis of phosphoinositides *in situ* would permit more rapid replacement. Earlier workers (Seyfred *et al.*, 1984) had reported that bovine ROS incubated with [γ-^{32}P]ATP rapidly incorporated label into phosphatidic acid (PA), a precursor of phosphoinositides, but no labeled PI, PIP or PIP_2 were detected.

As noted in the introduction, synthesis of PI from PA occurs via condensation of PA with CTP to form CDP-diacylglycerol, which then reacts with inositol to form PI. The enzyme catalyzing the latter reaction requires Mn^{2+}. Inositol, CTP, and Mn^{2+} are small, water-soluble, and may be lost during ROS isolation. Inclusion of these substances in the labeling medium, and use of a different chromatographic system to separate the products, allowed demonstration of ^{32}P incorporation into CDP-diacylglycerol (CDP-DG), PI, PIP and PIP_2, although PA was still the major product (Figure 1). Traces of labeled phosphatidylglycerol were occasionally detected.

These lipids represent a very small fraction of ROS phospholipids by mass. The major phospholipids of the ROS are phosphatidyl-ethanolamine, phosphatidylcholine, and phosphatidylserine (Nielsen *et al.*, 1970). No ^{32}P incorporation into these lipids was detected, even when exogenous precursors were supplied (Table 1).

Figure 1. Autoradiogram of ^{32}P-labeled ROS lipids.

ROS (1.2 nmol rhodopsin) were incubated in complete ^{32}P labeling medium for 30 minutes. Extracted lipids were subjected to two-dimensional thin layer chromatography and autoradiography as described in 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.

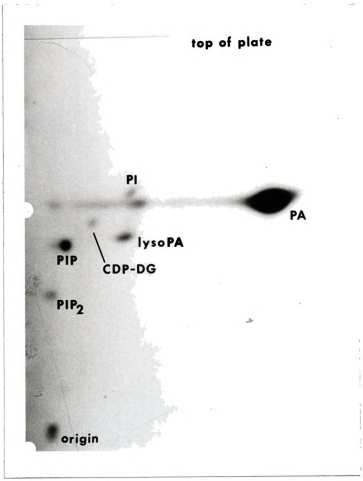

Figure 1

Table 1. ^{32}P labeling of ROS phospholipids.

Lipid	Additions to standard "complete" ^{32}P labeling mixture			
	None	Choline	Ethanolamine	Serine + Ethanolamine
PE	16 ±15	16 ±10	3 ±2	1 ±1
PC	2 ±1	0 ±1	0 ±5	1 ±5
PS	19 ±11	21 ±10	4 ±3	12 ±3
PA	21861 ±931	17809 ±3194	22224 ±650	23542 ±212
CDP-DG	102 ±13	115 ±6	121 ±7	139 ±26
PI	104 ±33	116 ±9	101 ±12	199 ±121
PIP	1601 ±112	1256 ±351	1569 ±23	1636 ±21
PIP_2	169 ±19	133 ±44	200 ±4	194 ±31

ROS (0.85 nmol rhodopsin/assay) were incubated in complete ^{32}P labeling medium as described in Materials and Methods, with the addition of the indicated bases (0.5 mM final concentration). All assays contained 0.5 mM inositol. Lipids were extracted, spotted on TLC plates and developed as described. Phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylserine (PS) were visualized by I_2 vapor staining, PA, CDP-DG, PI, PIP and PIP_2 by autoradiography. ^{32}P incorporation was measured by liquid scintillation counting of material scraped from TLC plates. Results (less backgound of 50 cpm) are expressed as means ± standard error of duplicate assays.

The spot identified CDP-DG in Figure 1 only appeared when CTP was present in the [γ-^{32}P]ATP labeling mix. Since accumulation of detectable amounts of labeled CDP-DG in ^{32}P labeled neural tissue is unusual (Fisher and Agranoff, 1986), formation of this compound was confirmed by use of [α-^{32}P]CTP. [^{32}P]CDP-DG was diminished but not eliminated by the addition of inositol (Table 2). Figure 2 shows a time course for ^{32}P labeling of PA and the phosphoinositides. The routine length was 30 min. Prolongation of the assay to as long as 3 hr did not result in conversion of substantial amounts of labeled PA into PI.

Labeling of ROS phosphoinositides was also demonstrated using [^{3}H]inositol as a precursor. ^{3}H-labeling of PI was detected after as little as 15 sec (Figure 3), but PIP and PIP_2 required several hours to incorporate detectable amounts of label. PI labeling continued to increase during this time. LysoPI was also observed.

In contrast to ^{32}P, which labeled PI and PIP_2 at similar rates and PIP more rapidly than either, [^{3}H]inositol labeled PIP more slowly (by more than an order of magnitude) than PI, and PIP_2 more slowly still. However, the rates of [^{3}H]inositol and ^{32}P incorporation into PI agreed well (typically *ca.* 0.5 pmol/min/mg protein). This is consistent with the presumption that ^{32}P can be incorporated directly into all three phosphoinositides (in the cases of PIP and PIP_2, by radiophosphorylating PI and PIP already present in the membranes), but [^{3}H]inositol must be incorporated into PI before appearing successively in PIP and PIP_2. For measuring PI synthesis, [^{3}H]inositol is preferable to [γ-^{32}P]ATP, since a higher specific

Table 2. Formation of labeled CDP-DG from [α-^{32}P]CTP.

	Additions			
	None	Inositol	$MnCl_2$	Inositol + $MnCl_2$
[^{32}P]CDP-DG, cpm:	1114	757	1219	535
	±15	±37	±134	±52

ROS (1.4 μmoles rhodopsin) were incubated for 30 min at 30° in 50 mM NaHEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM ATP and 1 mM [α-^{32}P]CTP (100 μCi/μmol), to which was added either 0.5 mM inositol, 1 mM $MnCl_2$, both, or neither. Lipids were extracted, separated by one-dimensional TLC and autoradiographed as described in materials and methods. CDP-DG was the principal labeled product. Faint traces of other labeled lipids were visible above and below CDP-DG on the autoradiogram. CDP-DG was scraped from the plate and counted by liquid scintillometry. Results are expressed as means ± standard errors for duplicate assays.

Figure 2. Time course of ^{32}P-labeling of ROS phospholipids.

ROS (1.2 nmol rhodopsin per assay) were incubated in complete ^{32}P labeling medium for the times indicated. Labeled lipids were extracted, separated and quantitated as described in Materials and Methods. Note separate scale for PA (♦), on right.

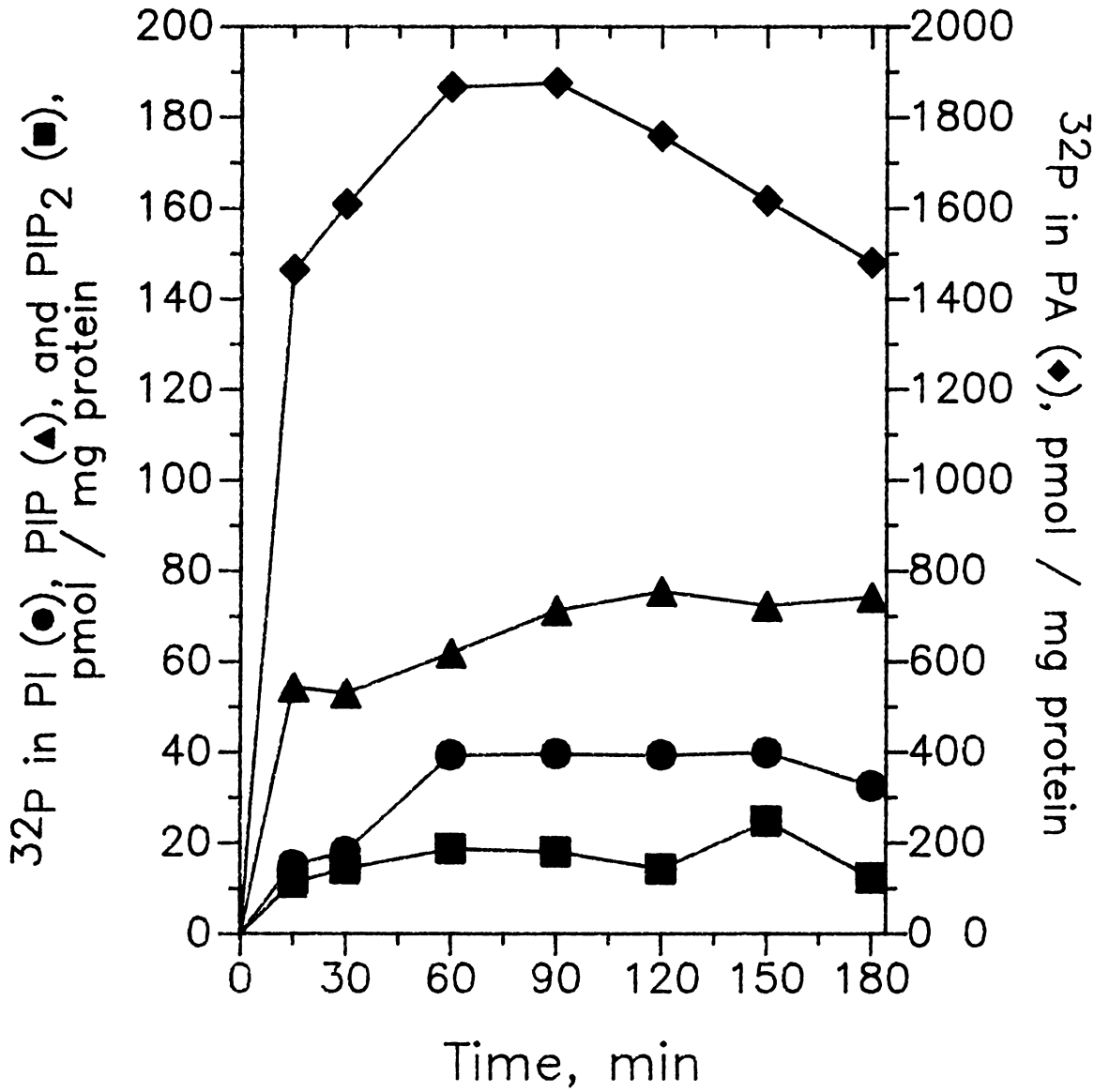
200
180
160
140
120
100
80
60
40
20
0
2000
1800
1600
1400
1200
1000
800
600
400
200
0 0
0 30 60 90 120 150 180
Time, min
32P in PI (●), PIP (▲), and PIP2 (■), pmol / mg protein
32P in PA (◆), pmol / mg protein

Figure 2

Figure 3. Time course of [^{3}H]inositol incorporation into PI.

ROS were incubated in ^{3}H labeling medium. 100 μl aliquots (containing 1.3 nmol rhodopsin) were taken at the times indicated and analyzed as described in Materials and Methods.

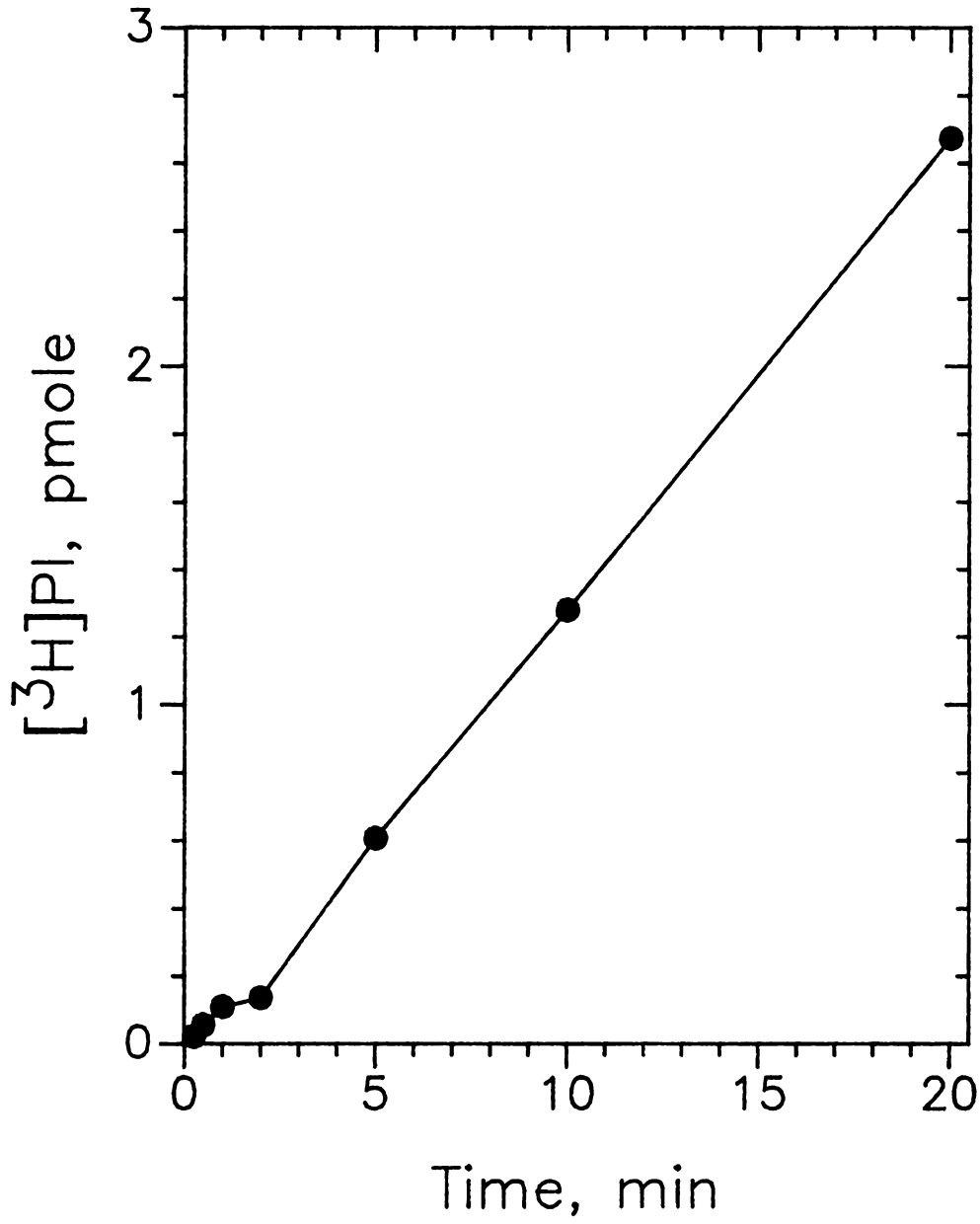

Figure 3

activity can be used, and labeling of PA, which produces a chromatographic tail near PI, is avoided.

Requirement for CTP: CTP was required for [^{3}H]inositol incorporation (Figure 4), indicating that the label was incorporated via *de novo* PI synthesis, and not by a base-exchange reaction between PI and free inositol (Eisenberg and 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 or CDP-DG.

In [γ-^{32}P]ATP-labeling experiments, neither PI nor CDP-DG were labeled in the absence of CTP.

Divalent metal ions and spermine: High magnesium concentrations appeared to stimulate PI kinase selectively, as Figure 5 shows. It has been reported that divalent metal cations non-enzymatically catalyze phosphorylation of PA and polyphosphoinositides (Gumber and Lowenstein, 1986). No labeled lipids were detected when ROS were 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 non-enzymatic labeling.

Spermine, a polycationic amine, had a more general stimulatory effect on phosphoinositide labeling (Figure 6). Spermine has been reported to inhibit PI-specific phospholipase C (Eichberg *et al.*,1981), but the effect reported here would appear not to be due to inhibited hydolysis, since ROS phosphoinositide PLC is inactive under the conditions used in labeling experiments (see Chapter II).

Figure 4. Effect of CTP on [^{3}H]inositol incorporation into PI.

ROS (1 nmol rhodopsin/assay) were incubated with [^{3}H]inositol for 3 hr as described in Materials and Methods, except that 1 mM CTP was omitted from some reaction mixtures. Lipids were extracted and separated and [^{3}H]PI quantitated by liquid scintillation counting. Means ± standard errors are shown for duplicate assays. [^{3}H]PIP was detected only when CTP was included.

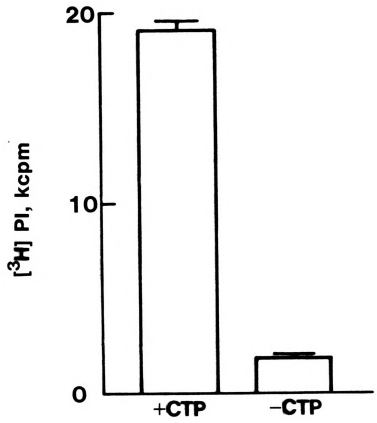

Figure 4

Figure 5. Effect of Mg^{2+} on ROS phospholipid labeling.

ROS (1.2 nmol rhodopsin/assay) were incubated with labeled substrates as described in Materials and Methods, except that the concentration of $MgCl_2$ was varied as indicated. Since 5mM 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 mM and 50 mM; error bars (± s. e.) are shown at 50 mM but omitted at 5 mM for clarity (s. e. < 10% for all 4 lipids). Only PIP showed a statistically significant ($p < .01$) difference between 5 mM 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.

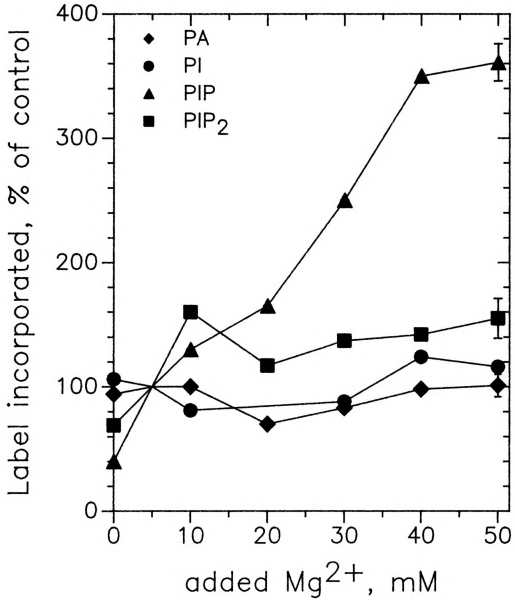
Label incorporated, % of control
400
300
200
100
0
PA
PI
PIP
PIP2
0
10
20
30
40
50
added Mg2+, mM

Figure 5

Figure 6. Effect of spermine on ROS phospholipid labeling.

ROS (1.2 nmol rhodopsin/assay) were incubated with labeled substrates as described in Materials and Methods, except that spermine tetrahydrochloride was added in the concentrations indicated. Label incorporation is shown as percent of control (no added spermine). 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. Triplicate assays were performed at added spermine concentrations of 0 mM and 2.5 mM; error bars (± s. e.) are shown at 2.5 mM but omitted at 0 mM for clarity. The effect of 2.5 mM spermine was statistically significant ($p < .01$) for all three phosphoinositides but not for PA.

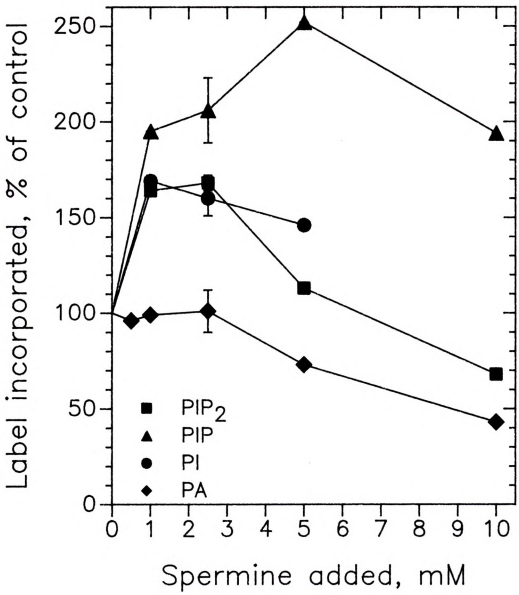
Label incorporated, % of control
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10
Spermine added, mM
PIP2
PIP
PI
PA

Figure 6

Manganese is a known cofactor of PI synthase and increases [^{3}H]inositol incorporation into PI (Figure 7). Millimolar Mn^{2+} also stimulates ^{32}P labeling of PIP (Figure 8).

Millimolar Ca^{2+} markedly inhibited labeling of phosphoinositides, although it had little or no effect on PA (Table 3). Lower concentrations had no effects. Chelation of endogenous calcium by 1 mM EGTA had no effect on ^{32}P-labeling of PA, PIP or PIP_2.

Addition of unlabeled PIP: Hayashi and Amakawa (1985) observed ^{32}P-labeling of PIP_2 in isolated frog ROS only in the presence of added PIP. In our preparation, labeling of PIP_2 occured even in the absence of added PIP, but was approximately doubled by addition of 50 μM or more unlabeled PIP (Figure 9). These results suggest that PIP phosphorylation is regulated at least in part by substrate availability.

Effects of washing ROS membranes: ROS contain peripheral membrane proteins, for instance cGMP PDE and G-protein (Kohnken *et al.*, 1981a), that can be removed by hypotonic washing. The effects of isotonic and hypotonic washing on phospholipid labeling patterns are shown in Table 4. PA labeling was most affected, being reduced by two-thirds after one isotonic washing, although more washing did not reduce it further. Two isotonic washings reduced [^{3}H]inositol incorporation into PI by about one-third; subsequent hypotonic washings had no effect.

Light and nucleotides: We examined the effect of light on phospholipid labeling under a variety of conditions. ROS were bleached prior to, at the start of, in the middle of and immediately before the end of labeling, using brief flashes or continuous illumination.

Figure 7. Effect of Mn^{2+} on [^{3}H]inositol incorporation.

ROS (2.1 nmol rhodopsin/assay) were incubated with [^{3}H]inositol as described in 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.

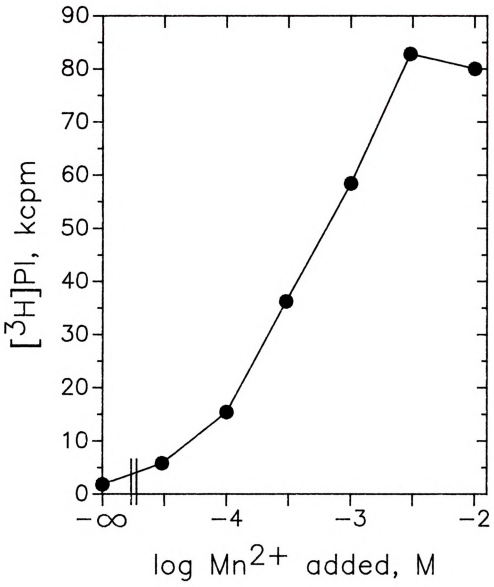

Figure 7

Figure 8. Effect of Mn^{2+} on ^{32}P labeling of ROS lipids.

ROS (2.6 nmol rhodopsin/assay) were incubated in minimal ^{32}P labeling medium as described in 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+}).

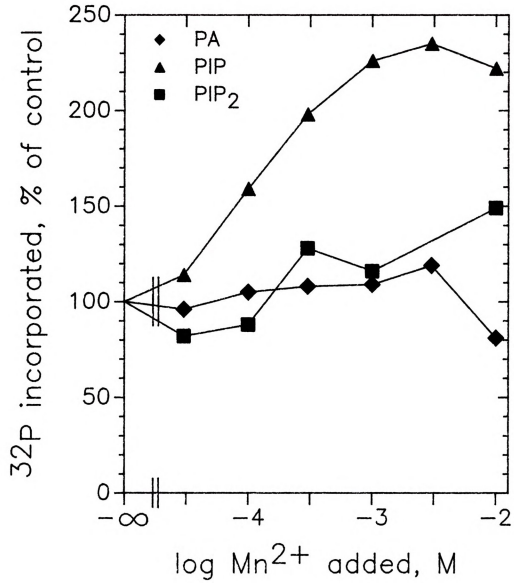
250
200
150
100
50
0
PA
PIP
PIP2
32P incorporated, % of control
−∞
−4
−3
−2
log Mn2+ added, M

Figure 8

Table 3. Effect of Ca^{2+} on phospholipid labeling.

	^{32}P incorporated, cpm			
$[Ca^{2+}]$, mM	PA	PI	PIP	PIP_2
0	39721 ±4094	1060 ±89	1702 ±50	627 ±169
1	41396 ±4500	1018 ±272	937 ±137	274 ±10
2	43119 ±3011	880 ±18	905 ±71	226 ±108

ROS (1.3 nmol rhodopsin/assay) were incubated in complete ^{32}P labeling medium as described in Materials and Methods, with the addition of the indicated concentration of $CaCl_2$. Labeled lipids were separated by TLC and quantitated by liquid scintillation counting. Results are shown as means ± standard errors for duplicate assays.

Figure 9. Effect of added PIP on ^{32}P incorporation.

ROS (1 nmol rhodpsin/assay) were incubated in minimal ^{32}P labeling mixture as described in Materials and Methods, with the addition of the indicated concentration of unlabeled PIP (sodium salt, purchased from Sigma and added as a 1mM aqueous suspension). Results are expressed as % of no-added-PIP control.

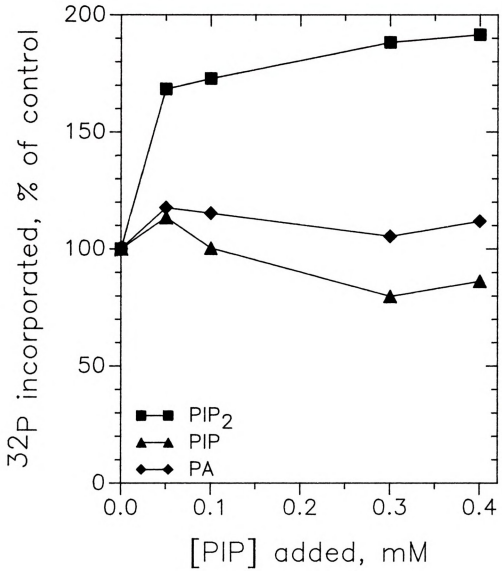
200
150
100
50
0
32P incorporated, % of control
PIP2
PIP
PA
0.0
0.1
0.2
0.3
0.4
[PIP] added, mM

Figure 9

Table 4. Effect of washing on phospholipid labeling.

Product	Relative Labeling (ROS = 100)				
	ROS	Isol	Iso2	Hypol	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

ROS were washed twice isotonically and twice hypotonically, as described in Methods. Samples of the pellets were retained at each step. Aliquots of the original ROS, the 1st isotonic pellet (Isol), the 2nd isotonic pellet (Iso2), the 1st hypotonic pellet (Hypol) and the 2nd 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 (± s.e.) of duplicate assays.

Various calcium concentrations were tried. No effects of light were found on label incorporation from either [^{32}P]ATP or [^{3}H]inositol. Table 5 shows the results of a representative experiment.

No effects of cyclic nucleotides on ^{32}P or ^{3}H labeling were observed, nor did the cyclic nucleotide phosphodiesterase inhibitor IBMX, reported to decrease phosphoinositide labeling in octopus photoreceptors (Yoshioka *et al.*, 1983), have any effect.

GTP (1mM) and its non-hydrolyzable analog, guanylylimidodiphosphate, produced a modest decrease in labeling of PA, PIP and PIP_2 in minimal ^{32}P labeling medium (Table 6). This is probably attributable to competition with ATP for the active sites of the kinases.

Comparison of ROS and microsomes: Although our ROS preparations are estimated to contain <1% microsomal contamination as determined by glucose 6-phosphatase (McConnell, 1965), NADPH-cytochrome C reductase and electron microscopy (McConnell *et al.*, 1969), the possible contribution of microsomes to phospholipid labeling was examined. Retinal microsomes were assayed for incorporation of labeled precursors into phospholipids. As shown in Table 7, the patterns of labeling in ROS and microsomes are quite different. PI synthesis is about 10-fold higher (on a pmole/min/mg protein basis) in microsomes than in outer segments, but microsomes cannot account for all the PI synthesis exhibited by ROS, as this would require *ca.* 10% microsomal contamination in our ROS preparations.

Table 5. Absence of light effect on ^{32}P labeling.

	^{32}P incorporated, cpm			
	PA	PI	PIP	PIP_2
Dark	20677 ±956	276 ±5	1361 ±189	320 ±63
Light	19726 ±175	292 ±44	1340 ±22	311 ±12

ROS (1.2 nmol rhodopsin/assay) phospholipids were labeled with ^{32}P as described in Materials and Methods, except that the incubation was done under dim red light. ROS were either maintained in darkness or exposed to normal laboratory illumination before labeling. Results are expressed as means ± standard errors for duplicate assays.

Table 6. Guanine nucleotide effects on phospholipid labeling.

	^{32}P incorporated, cpm		
Nucleotide	PA	PIP	PIP_2
None	46385	2930	144
	±883	±124	±7
GTP	32483	2272	105
	±1506	±117	±4
GMPPNP	40208	2371	116
	±2180	±169	±18

ROS (2.1 nmol rhodopsin) were incubated in minimal ^{32}P labeling medium, as described in Materials and Methods, with the addition of the indicated nucleotides (1mM). Results are expressed as means ± standard errors for duplicate determinations.

Table 7. Comparison of phospholipid labeling in ROS and microsomes.

Product	Rate of synthesis, pmol/min/mg protein		Ratio ROS/Microsomes
	ROS	Microsomes	
[^{32}P]PA	20	2.6	8.0
[^{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

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

DISCUSSION

These experiments establish that ROS contain a complete pathway for synthesis of phosphoinositides. Phosphoinositdes and PA incorporate radioactive phosphate from ATP much more rapidly than do the "major" phospholipids. Turnover of these lipids is metabolically expensive: synthesis of PIP_2 from inositol and diacylglycerol requires hydrolysis of four high-energy phosphate bonds. Based on the experiments presented here, it is logical to presume that phosphoinositides have some functional role in ROS.

The disproportionately high level of PA synthesis is somewhat puzzling. It may be that high levels of diacylglycerol accumulate in ROS membranes after slaughter, due to lack of perfusion (Matthys *et al.*, 1984). If so, it could be expected that PA would be rapidly synthesized when ATP became available.

^{32}P-labeling experiments were less than ideal for measuring PI synthesis, due to the proximity, on thin-layer chromatograms, of the PI to the tail of the intensely labelled PA spot. Drying the plates *in vacuo* between the first- and second-dimension developments decreased but did not eliminate tailing. Use of [^{3}H]inositol provided a much better way to measure PI synthesis. However, [^{3}H]inositol can also be incorporated into PI by a non-synthetic base-exchange reaction. Base exchange can be distinguished from *de novo* synthesis by the latter's requirement for CTP (Eisenberg and Hasegawa, 1981). Omission of CTP from ^{3}H-labeling experiments greatly reduced labeling of PI, which indicates that the bulk of [^{3}H]inositol incorporation represents PI synthesis.

PI synthesis was strongly dependent on Mn^{2+}. Routine phospholipid labeling reactions were carried out in the presence of 1 mM $MnCl_2$. Later work (Chapter II) showed that this concentration completely inhibits ROS PIP_2 phospholipase C. This simplifies interpretation of some labeling experiments by lessening the possibility that decreased labeling attributed to diminished synthesis is actually due to increased hydrolysis of labeled phosphoinositides. However, it raises the possibility that light-dependent decreases in labeling were not observed because of inhibition of the putative light-activated PLC.

Since their substrates are lipids and presumably confined to membranes, it might well be expected that the enzymes involved in phosphoinositide synthesis would also be tightly membrane-bound. Generally, this was found to be so. Washing of ROS produced a modest reduction in PI labeling and a more substantial reduction in PA labeling. These decreases in label incorporation may be due to removal of the relevant enzymes (diglyceride kinase for PA, phosphatidate cytidylyltransferase and/or PI synthase for PI) or loss of polar lipid precursors.

After the phospholipid labeling experiments presented here had been completed, Giusto and Ilincheta de Boschero (1986) reported labeling of PA, PIP and PIP_2 in bovine ROS and isolated disks incubated with [γ-^{32}P]ATP. Label incorporation into PA was ca. 2 orders of magnitude greater than into PIP. No experiments examining the effects of metal ions, spermine, or light were reported.

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CHAPTER II

ROS PHOSPHOLIPASE C

LITERATURE REVIEW

Published research on phospholipase C in photoreceptors has focused almost exclusively on its regulation by light and possible involvement in phototransduction. As noted above, much of the evidence for this involvement has been developed in invertebrates. Light-induced decreases in labeled PIP_2 in squid (Vandenberg and Montal, 1984) and octopus (Yoshioka *et al.*, 1983a) photoreceptors were discussed in the review of labeled precursor incorporation into retinal phospholipids (Chapter 1), but are also consistent with increased PLC activity.

Most reports of light-stimulated phosphoinositide hydrolysis employ substrates that are labeled *in situ* by incorporation of radioactive precursors. Brown *et al.* (1984) reported that light increased $[^3H]IP_3$ and decreased ^3H- and ^{32}P-labeled PIP_2 in *Limulus* (horseshoe crab) ventral eyes that had been preincubated with labeled precursors. Light flashes produced transitory increases in $[^3H]IP_3$ in squid retinas prelabeled with [^{3}H]inositol (Szuts *et al.*, 1986). In both cases essentially intact retinas were used; cells distal to photoreceptors (*i. e.* neurons) may account for some or all of the phosphoinositide turnover.

Devary *et al.* (1987) labeled fly eyes with [^{3}H]inositol, homogenized them, and prepared a crude membrane fraction which, even after frozen storage, displayed light-stimulated release of tritiated IP_3 and IP_2. This light-stimulation was enhanced by GTPγS and inhibited by GDPβS, indicating regulation by a G-protein. The use of a homogenized preparation instead of intact retinas would seem to reduce the possibility that light-stimulated PIP_2 hydrolysis is due simply

to neurotransmitter-stimulated neurons, but considerable non-photoreceptor material must be present in this preparation.

Yoshioka *et al.* (1983b), working with blind *Drosophila* mutants (*norpA*) whose photoreceptors lack normal electrophysiological response to light, reported decreased incorporation of [32P] into PA and increased labeling of PIP and PIP_2 compared to wild types. This was originally interpreted as an abnormality of phospholipid synthesis, but can also be interpreted as evidence of decreased PLC activity: less phosphoinositide hydrolysis resulting in decreased availability of DG to be phosphorylated to PA. The latter interpretation was confirmed by later work from the same laboratory (Inoue *et al.*, 1985) showing that homogenates of the eyes of wild-type *Drosophila*, but not *norpA* mutants, diplayed PIP_2 PLC activity against exogenous [^{32}P]PIP_2. No attempt to measure the effect of light on PLC activity was reported.

Recently, Baer and Saibil (1988) reported that light stimulated hydrolysis of exogenous [^{3}H]PIP_2 by isolated squid photoreceptor outer segments in the presence of GTP and Ca^{2+}. Hydrolysis was measured by formation of water-soluble labeled products, primarily IP_3. The experimental system used by these experimenters (exogenous substrate and isolated outer segments) is quite similar to that used in the present work, although there are some methodological differences as well as the difference in organisms.

Another type of experiment that supports a role for PLC in phototransduction involves injection of various substances into photoreceptors, whose electrical responses are measured with microelectrodes. The comparatively large size of *Limulus* photoreceptors makes them popular for such experiments. Injections of

IP_3 mimicked the effect of light (Fein *et al.*, 1984; Brown *et al.*, 1984). 1,2-cyclic IP_3 was also effective (Wilson *et al.*,1985). Although response to light was blocked by injection of GDPβS, response to IP_3 was not (Fein, 1986). IP_3 injection increased intracellular Ca^{2+} concentration (Brown and Rubin, 1984) apparently via release from stores within the photoreceptor (Payne and Fein, 1987). Injections of calcium salts also produced a light-like response (Payne *et al.*, 1986), as did injections of phospholipase C (Rubin *et al.*, 1986). These results all strongly support a model of G-protein mediated light-activation of PIP_2 PLC producing IP_3 which releases Ca^{2+}, which then (in the invertebrate version of the Ca^{2+} hypothesis) directly or indirectly increases Na^+ conductance and depolarizes the cell.

The picture in vertebrate photoreceptors is less clear-cut, but a few reports have appeared suggesting a role for PIP_2 hydrolysis, at least in amphibians. Ghalayini and Anderson (1984), using prelabeled frog retinas, found that ROS isolated from retinas exposed to a flash of light contained less [^{3}H]PIP_2 than those isolated from retinas kept in darkness. Hayashi and Amakawa (1985) obtained similar results using frog ROS labeled and illuminated after isolation. Using prelabeled toad retinas flash-frozen at timed intervals after light stimulation, Brown *et al.* (1987) found that [^{3}H]IP_3 in ROS rose 50-80% within 250 msec but returned to the unstimulated level by 1 sec. Levels of the breakdown products of IP_3 were not reported.

In experiments analogous to those performed in *Limulus*, Waloga and Anderson (1985) found that microinjection of IP_3 into outer

segments of salamander rods caused hyperpolarization and decreased the rods' response to dim light. Conversely, light decreased the response to IP_3 injection. No analysis of the response kinetics was presented, but inspection of receptor potential recordings suggest that the response elicited by IP_3 is considerably slower than the photoresponse.

The experiments that follow were undertaken to determine the presence of PIP_2 PLC in mammalian rod outer segments and to characterize its properties and regulation. The possibility of regulation by light or G protein was of significant but not exclusive interest.

MATERIALS AND METHODS

Materials: Tritiated phospholipids were purchased from New England Nuclear. Calmodulin antagonists, nucleotides and unlabeled phosphoinositides were purchased from Sigma. Non-hydrolyzable GTP analogs were purchased from Boehringer Mannheim. BAPTA (1,2-bis(2-aminophenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid) was purchased from Fluka.

Preparation of particulate and soluble PLC fractions: ROS were isolated as described in Chapter 1. Isolated unbleached ROS were washed 4 times at 100,000 x *g* in 10 - 20 volumes of 10 mM Tris-HCl, pH 7.8, 150 mM KCl, 1 mM dithiothreitol, to yield the particulate fraction (washed ROS = WROS). The soluble fraction (isotonic supernate) consisted of the supernate of the first washing; alternatively, supernate from washing at 15,000 x *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: [^{3}H]PIP_2 (2.5 μCi/μmole) was prepared from [*inositol*-2,3-^{3}H]PIP_2 and PIP_2 sodium salt, and used as a 1 mM aqueous solution. [^{3}H]PIP (5.0 μCi/μmol) and [^{3}H]PI (9.1 μCi/μmol) were prepared analogously, but required sonication to produce 1 mM dispersions. Unless otherwise indicated, assays were performed at 30° C. under normal room illumination in 200 mM potassium HEPES, pH 7.5, 50 mM KCl, 30 μM $CaCl_2$ and 10 μM tritiated phosphoinositide plus indicated additions. Total assay volume was 500 μl. After 30 min, reactions were terminated by addition of 500 μl 10% trichloracetic acid

followed by 100 μl 5% bovine serum albumin (Inoue *et al.*, 1985). After centrifugation and neutralization, total acid-soluble ^{3}H in the supernate was determined by liquid scintillometry, with subtraction of no-enzyme blanks.

Miscellaneous methods: Protein concentrations were determined by the method of Lowry (1951) or by the Read and Northcote (1981) modification of the Bradford (1976) method. ROS and washed ROS 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 supenatant was treated with pertussis toxin by the method of Manning *et al.* (1984).

RESULTS

A. Demonstration of Phospholipase C in ROS.

Hydrolysis of phosphoinositides labeled *in situ*: Breakdown of phospholipids can be measured by disappearance of labeled lipids or formation of water-soluble products. The work of Hayashi and Amakawa (1985) illustrates the first method. They incubated isolated frog ROS with [γ-^{32}P]ATP and PIP, producing labeled PA, PIP and PIP_2. A five-second flash of light immediately before termination of reaction and extraction of lipids resulted in a 20% decrease in labeled PIP_2. Table 1 shows the results of a similar experiment with bovine ROS. No effects of light were found.

Initial experiments examining formation of water-soluble products from labeled phosphoinositides employed ROS pre-incubated with [^{3}H]inositol. ([^{32}P]ATP was not used because of the large number of labeled products possible in addition to inositol phosphates.) After several hours incubation, ROS were pelleted by centrifugation and resuspended in fresh buffer containing no label. Various concentrations of metal ions and other effectors were used in the resuspension buffer, and the resuspended membranes were incubated with or without illumination. Water-soluble products were analyzed by ion-exchange chromatography (Downes and Michell, 1981).

The amount of labeled IP_3 recovered from such experiments was very small (<0.001% of original ^{3}H). As noted in Chapter I, [^{3}H]inositol incorporation into PIP_2 proceeds quite slowly, and it was concluded that the amounts of [^{3}H]PIP_2 produced by this method

Table 1. No decrease in labeled phospholipids upon illumination.

	^{32}P, cpm		
	PA	PIP	PIP_2
Dark	84560	3728	801
	±3524	±170	±33
Light	89536	3801	812
	±3474	±64	±11

ROS (1 nmol rhodopsin/assay) were incubated under dim red light in 100 μl minimal ^{32}P labeling medium with the following modifications: [γ-^{32}P]ATP concentration was 20 μM, with a specific activity of 5 mCi/μmol; unlabeled PIP was added to a final concentration of 100 μM. After 5 min, reactions were terminated by addition of 50 μl 1N HCl. Some tubes were exposed to a 5 sec flash of white light from an incandescent bulb immediately before termination. Labeled phospholipids were separated and quantified as described in Chapter 1. Results are shown as means ± standard errors for 3 (light) or 4 (dark) replicates.

were inadequate. It was therefore decided to use exogenous [^{3}H]PIP_2 as a substrate for measuring phospholipase C activity.

Hydrolysis of exogenous [^{3}H]PIP_2: ROS incubated with [^{3}H]PIP_2 released ^{3}H that remained in solution when protein and unhydrolyzed substrate were precipitated with trichloracetic acid. Analysis of the acid-soluble label by ion-exchange chromatography (Figure 1) indicated it was primarily [^{3}H]IP_3 with smaller amounts of [^{3}H]IP_2, [^{3}H]IP and [^{3}H]inositol. (This technique will not resolve isomers.) Since ion-exchange analysis of the products of every assay would be extremely time-consuming and limit the number of experiments that could be performed, PLC activity was routinely measured by determining total acid soluble ^{3}H.

B. Characterization of ROS PLC Activities.

Soluble and particulate forms of ROS phospholipase C: PLC activity was found both in soluble and 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 several washings (Table 2). However, repeated washing failed to eliminate PLC activity in the supernates, suggesting that washed ROS membranes (WROS) continued to release small amounts of soluble PLC. This may represent leakage of cryptic PLC from compartments in the ROS, or an equilibrium between soluble and membrane-bound forms of PLC. As a result of this slow release of soluble activity, assays of WROS do not represent purely membrane-bound PLC. Unless otherwise noted, assays of soluble PLC were performed using the 1st isotonic supernate from a washing such as that shown in Table 2. Particulate material was removed by 100,000 x *g* ultracentrifugation or 0.45 μm filtration.

Figure 1. Identification of labeled products of PIP_2 hydrolysis.

ROS (320 μg protein) were incubated with 10 uM [^{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 minutes at 30°in a final volume of 500 μl. After precipitation of unhydrolyzed PIP_2 with trichloracetic 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 H_2O. Inositol phosphates were eluted with a series of formic acid-ammonium formate solutions as described by Downes and Michell (1981). 1-ml fractions were collected and counted by liquid scintillometry. For the flowthrough (F) and water wash (W), 1-ml aliquots were counted and the results (less background) multiplied by 10. (Ins = inositol.)

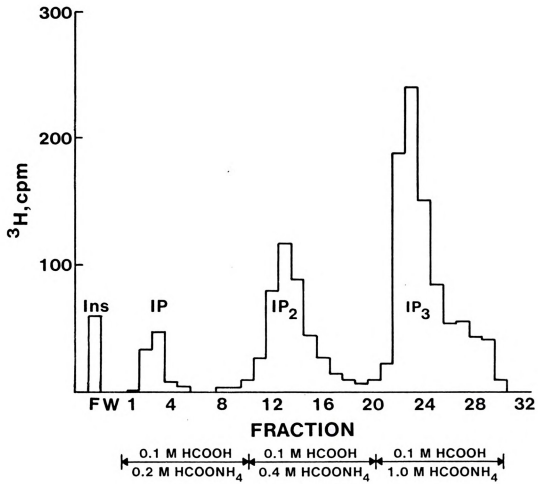
300
200
100
^{3}H, cpm
Ins
IP
IP$_2$
IP$_3$
FW 1 4 8 12 16 20 24 28 32
FRACTION
0.1 M HCOOH
0.2 M HCOONH$_4$
0.1 M HCOOH
0.4 M HCOONH$_4$
0.1 M HCOOH
1.0 M HCOONH$_4$

Figure 1

Table 2. Removal of phospholipase C from ROS by isotonic washing.

Fraction	Protein Concentration	Volume Assayed	[^{3}H]PIP_2 hydrolyzed
	mg/ml	µl	dpm
ROS	13.3	50	4663 ± 52
1st isotonic supernate	0.13	100	2423 ± 33
2nd isotonic supernate	0.15	100	843 ± 26
3rd isotonic supernate	0.03	100	493 ± 30
4th isotonic supernate	0.02	100	323 ± 49
Final pellet	6.6	50	1537 ± 76

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

The apparent specific activities of ROS and washed pellet are 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 unlabelled substrate in the membranes, the particulate fraction did not show a linear relationsip between volume of enzyme and $[^3H]PIP_2$ hydrolysis. Activity in the soluble fraction was also non-linear with respect to volume of enzyme, suggesting the presence of an endogenous inhibitor. Detergent (0.3% octylglucoside) relieved this inhibition, producing several-fold increases in activity when large amounts of crude enzyme were used (Figure 2). The nature of the endogenous inhibitor is discussed in more detail below. With respect to time, assays of unwashed ROS and isotonic supernate were approximately linear for 30 minutes, but activity in WROS began to decrease after ~15 minutes.

Comparison of substrates: ROS hydrolyzed all three phosphoinositides (Table 3), as did the soluble and particulate fractions (Table 10, columns 1 and 3). In separate experiments using $[^3H]$phosphatidylcholine as a substrate, no hydrolysis could be detected in ROS or isotonic supernate.

Effects of divalent metal ions: PLC in both isotonic supernate and WROS displayed an absolute requirement for Ca^{2+}. Treatment with EGTA abolished activity, which was restored by $CaCl_2$. Both fractions

Figure 2. Effect of octylglucoside on quantity-activity relationship of soluble PLC.

Varying amounts of isotonic supernate were assayed for PIP_2 PLC activity as described in Materials and Methods, with the addition of 0.3% octylglucoside to some tubes. Results are presented as means ± std. errors for duplicate assays.

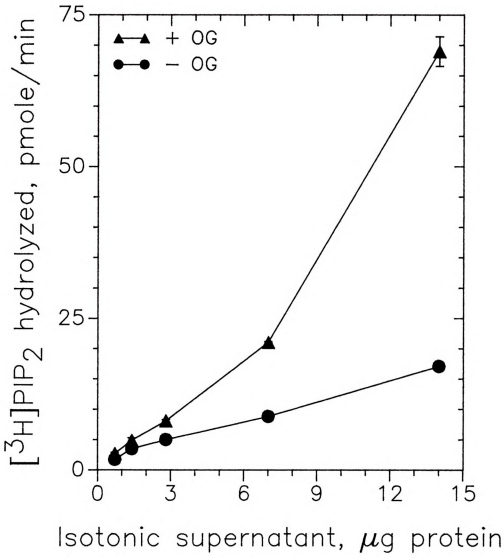
75
50
25
0
+ OG
– OG
[3H]PIP2 hydrolyzed, pmole/min
0
3
6
9
12
15
Isotonic supernatant, μg protein

Figure 2

Table 3. Comparison of substrates for ROS phospholipase C.

Substrate	Specific Activity μCi/μmol	Substrate Hydrolyzed dpm
[^{3}H]PI	10	31987 ± 2444
[^{3}H]PIP	5	33897 ± 169
[^{3}H]PIP_2	5	41610 ± 1537

ROS (0.9 mg protein/assay) were incubated with tritiated phosphoinositides as described in Materials and Methods. Results are shown as means ± standard errors for duplicate assays. [^{3}H]PIP_2 specific activity in this experiment was twice that used routinely. Dilution of substrate radioactivity by endogenous phosphoinositides in ROS was not considered in calculating specific activities.

contained sufficient endogenous Ca^{2+} to display substantial activity in the absence of added Ca^{2+} or chelators, but were usually stimulated by additional Ca^{2+}. This stimulation was potentiated by isotonic K^+ or Na^+ (Table 4). High ($\geq$ 300 μM) Ca^{2+} concentrations were inhibitory, however. Half-maximal activity was obtained from isotonic supernate at ~0.1 μM free Ca^{2+}, from WROS between 1 and 10 μM (Figure 3).

Mg^{2+} at concentrations above 0.1 mM strongly inhibited the soluble PLC. In contrast, PLC activity in WROS was stimulated by 1 mM Mg^{2+} but inhibited at higher concentrations (Figure 4).

Mn^{2+} at concentrations above 30 μM strongly inhibited both soluble and particulate activity (Figure 5).

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 water-soluble ^3H was observed.

Effect of spermine: Spermine at low concentrations (0.1 mM) stimulated the particulate activity but had no effect on the soluble. Higher (millimolar) concentrations inhibited both soluble and particulate activity (Figure 6).

Effects of monovalent metal ions: Low ionic strengths produced submaximal activity, as seen in Table 4, but no specific effects of K^+, Na^+ or Li^+ were observed; replacement of one of these ions by another at constant ionic strength had no significant effect.

Effect of pH: Both the soluble and particulate activities were maximal at pH 6.5. Activity at pH 7.5 was 40% - 50% less than at 6.5; nevertheless, assays were routinely performed at the higher pH to

Table 4. Effect of ionic strength and Ca^{2+} on PLC activity.

Fraction	Added Salt	[^{3}H]PIP_2 hydrolyzed, dpm	
		No added $CaCl_2$	30 μM $CaCl_2$
Particulate	none	929 ± 13	933 ± 120
	140 mM KCl	1346 ± 109	2344 ± 151
	140 mM NaCl	1457 ± 13	2223 ± 25
Soluble	none	1099 ± 21	643 ± 47
	140 mM KCl	1184 ± 8	1807 ± 47
	140 mM NaCl	982 ± 25	1584 ± 156

WROS and isotonic supernate (250 and 32 μg protein/assay, respectively) were incubated with [^{3}H]PIP_2 (10 μM, 2.5 μCi/μmol) for 15 minutes in 50mM Tris-HEPES pH 7.5 plus the indicated additions. Enzyme aliquots contributed an additional 7.5 mM KCl to each assay. Results are presented as means ± std. errors for duplicate assays.

Figure 3. Effect of Ca^{2+} on PIP_2 PLC activity.

Particulate (▪, 250 μg protein/assay, 10 min incubation) and soluble (●, 2.8 μg protein/assay, 30 min incubation) fractions were incubated with [^{3}H]PIP_2 as described in 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 using a value of 107 nM for the Ca-BAPTA dissociation constant (Tsien, 1980). Results are presented as means ± std. errors for duplicate assays.

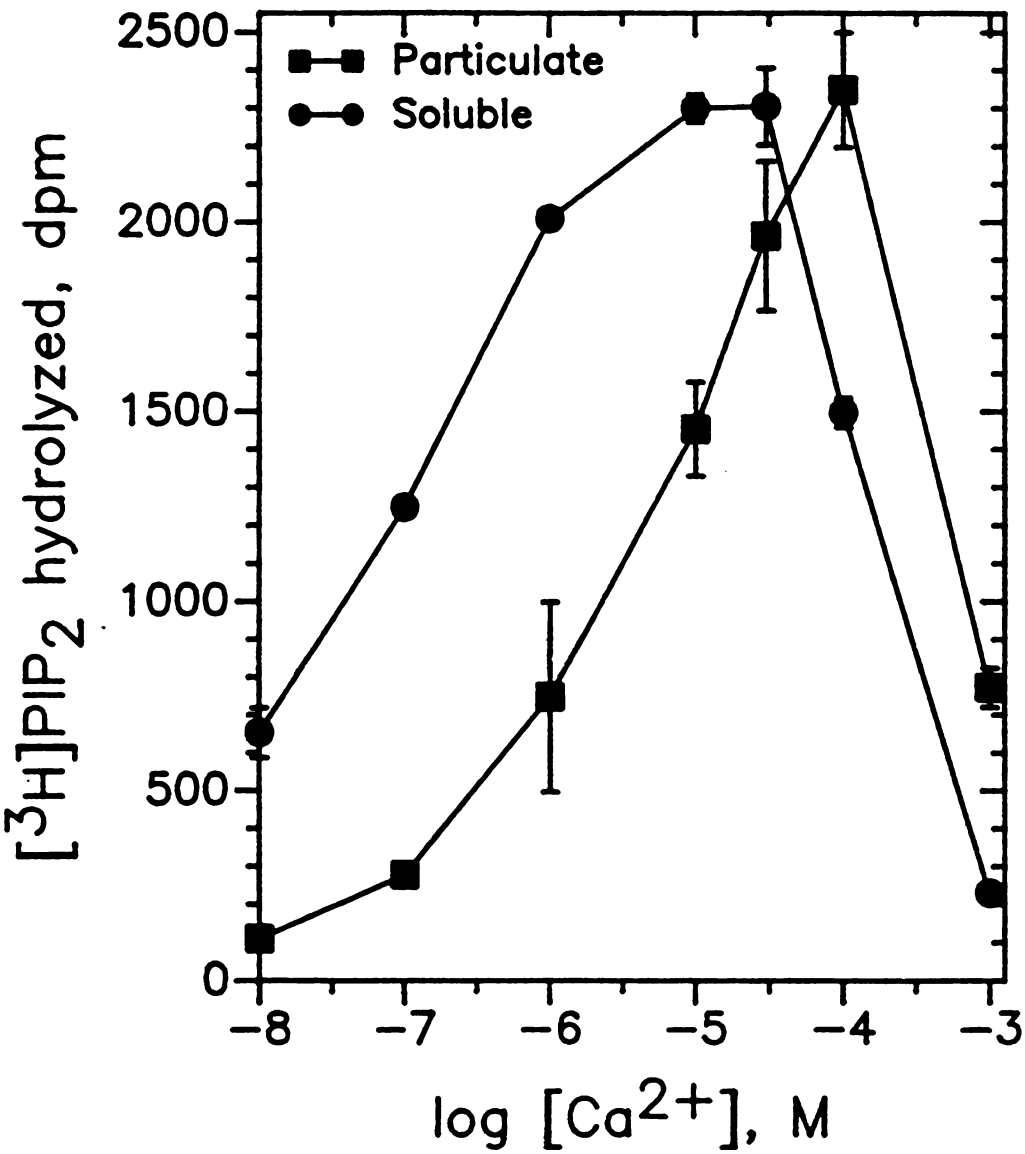
Particulate
Soluble
[3H]PIP2 hydrolyzed, dpm
2500
2000
1500
1000
500
0
−8
−7
−6
−5
−4
−3
log [Ca2+], M

Figure 3

Figure 4. Effect of Mg^{2+} on PIP_2 PLC activity.

Soluble (●, 30 min assays) and particulate (▪, 10 min assays) fractions were assayed for PIP_2 phospholipase C as described in 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 ± std. error for duplicate assays.

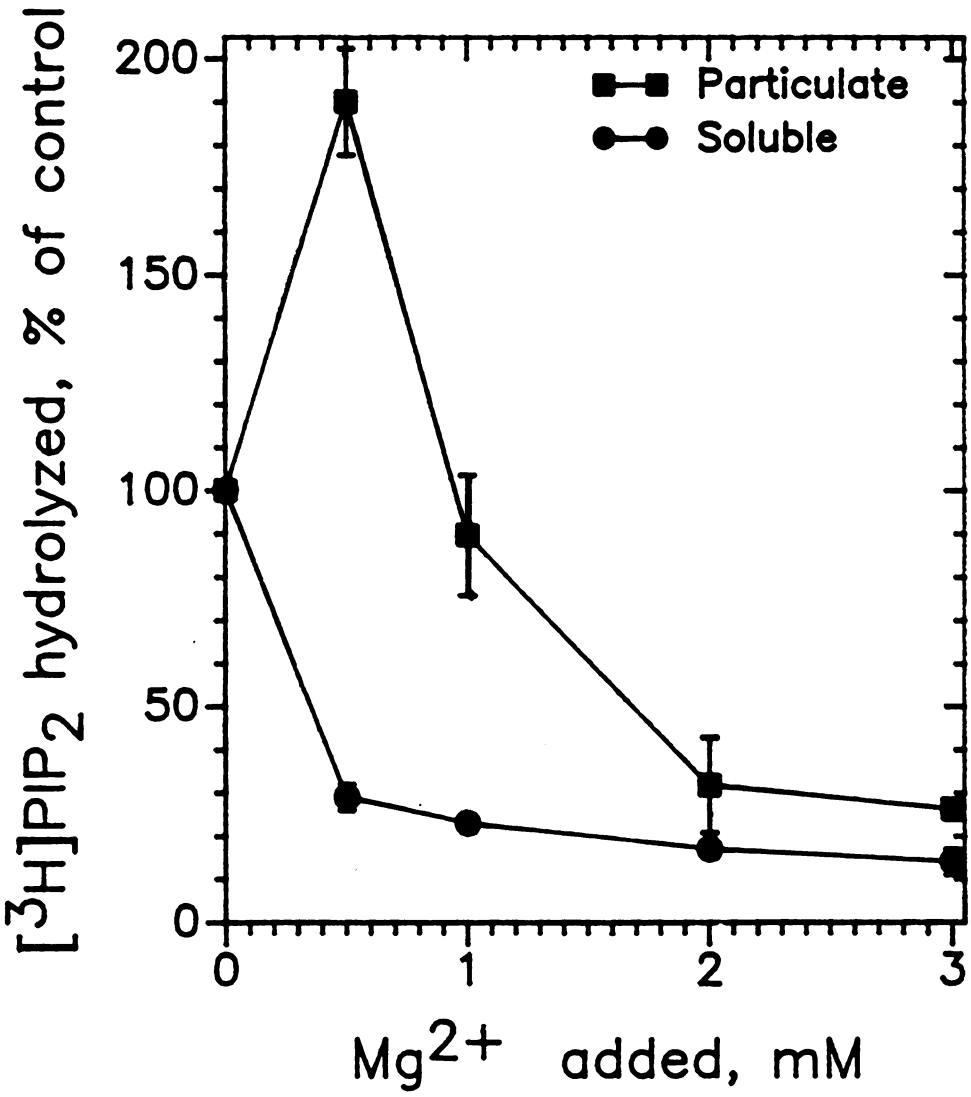
[3H]PIP2 hydrolyzed, % of control
200
150
100
50
0
0
1
2
3
Mg2+ added, mM
Particulate
Soluble

Figure 4

Figure 5. Effect of Mn^{2+} on PIP_2 PLC activity.

Soluble (●) and particulate (■) fractions were assayed for PIP_2 phospholipase C as described in Materials and Methods, with the addition of the indicated concentrations of $MnCl_2$. Activity at each concentration is expressed as mean ± std. error for duplicate assays.

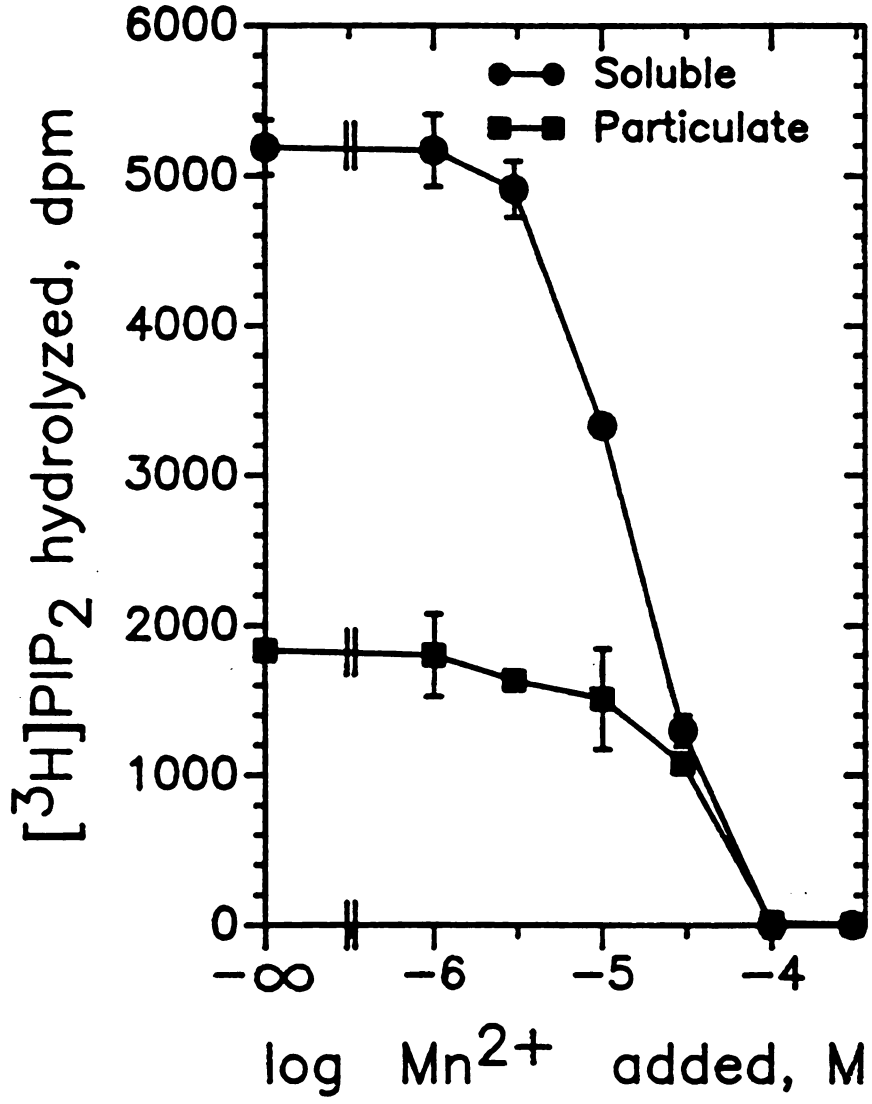
6000
5000
4000
3000
2000
1000
0
Soluble
Particulate
[3H]PIP2 hydrolyzed, dpm
−∞
−6
−5
−4
log Mn2+ added, M

Figure 5

Figure 6. Effect of spermine on PIP_2 PLC acitivity.

Particulate (▪, 10 min assays) and soluble (●, 30 min assays) fractions were assayed for PIP_2 phospholipase C as described in Materials and Methods, with the addition of the indicated concentrations of spermine tetrahydrochloride. Activity at each concentration is expressed as percent of no-spermine control, mean ± standard error for duplicate determinations.

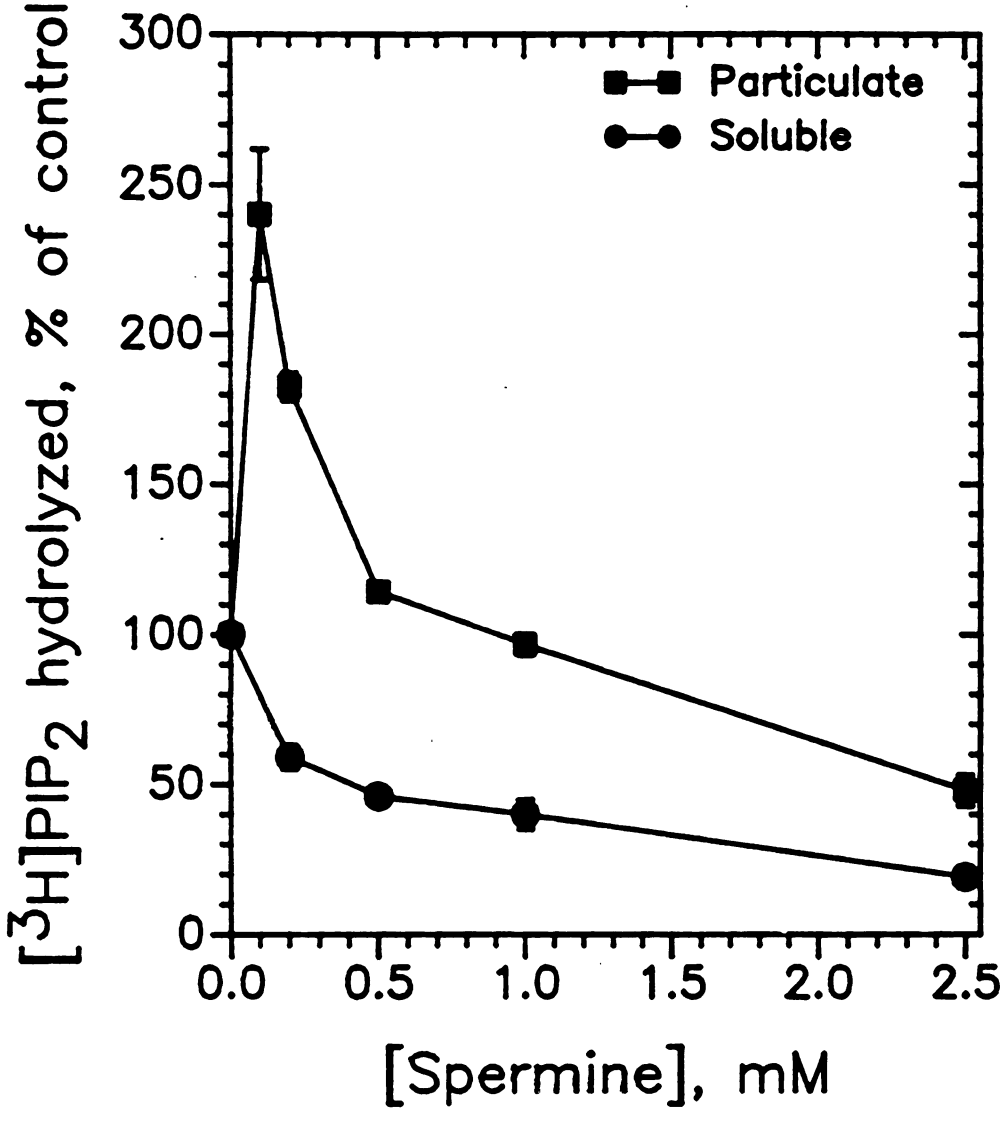
300
250
200
150
100
50
0
0.0
0.5
1.0
1.5
2.0
2.5
Particulate
Soluble
[3H]PIP2 hydrolyzed, % of control
[Spermine], mM

Figure 6

maintain consistency with experiments done before the pH optimum was determined.

Chromatographic characterization: Gel filtration of isotonic supernate produced a broad activity peak, suggesting poorly resolved multiple forms of PLC (Figure 7). The position of the peak's center corresponded to a M_r of ~160,000, however, the peak is wide enough to include proteins of twice or half that M_r. This may represent a multimer/monomer equilibrium.

Ion-exchange chromatography of isotonic supernate produces two peaks of PLC activity, typically eluting from DE-52 at 0.22 and 0.35 M NaCl (Figure 8).

C. Possible Regulators of ROS PLC.

1.Nucleotides and GTP Binding Proteins.

GTP had no effect on either the soluble or particulate form of the PLC. Since ROS contain GTPase activity (Godchaux and Zimmerman, 1979), non-hydrolyzable GTP analogs were also used. In the presence of Mg^{2+}, high (> 100 μM) concentrations of guanylyl-(β,γ-methylene)-diphosphonate (GMPPCP) inhibited both the soluble and particulate activities (Figure 9). At similar concentrations, guanosine-5'-O-(2-thiodiphosphate) (GDPβS) was found to be inhibitory and guanylyl-imidodiphosphate (GMPPNP) moderately stimulatory, but these effects were variable, possibly due to seasonal differences in the ROS. The concentrations required for these effects were considerably higher than those usually associated with G-protein-mediated effects. Inclusion of detergent (0.3% octylglucoside) in assays eliminated the effects of GTP analogs.

Figure 7. Gel filtration of soluble PLC.

Isotonic supernate (500 mg protein) in a volume of 1.5 ml was applied to a 100 x 1 cm G-150 column and eluted with 10 mM NaHEPES, pH 7.5, 250 mM NaCl, 30 μM $CaCl_2$. 4-ml fractions were collected and assayed for PLC activity by adding 5 μl 1mM [^{3}H]PIP_2 (2.5 μCi/μmol) to 495 μl fraction aliquots. After 30 min, reactions were terminated and acid soluble ^{3}H determined. Solid line = activity; dashed line = A_{280}.

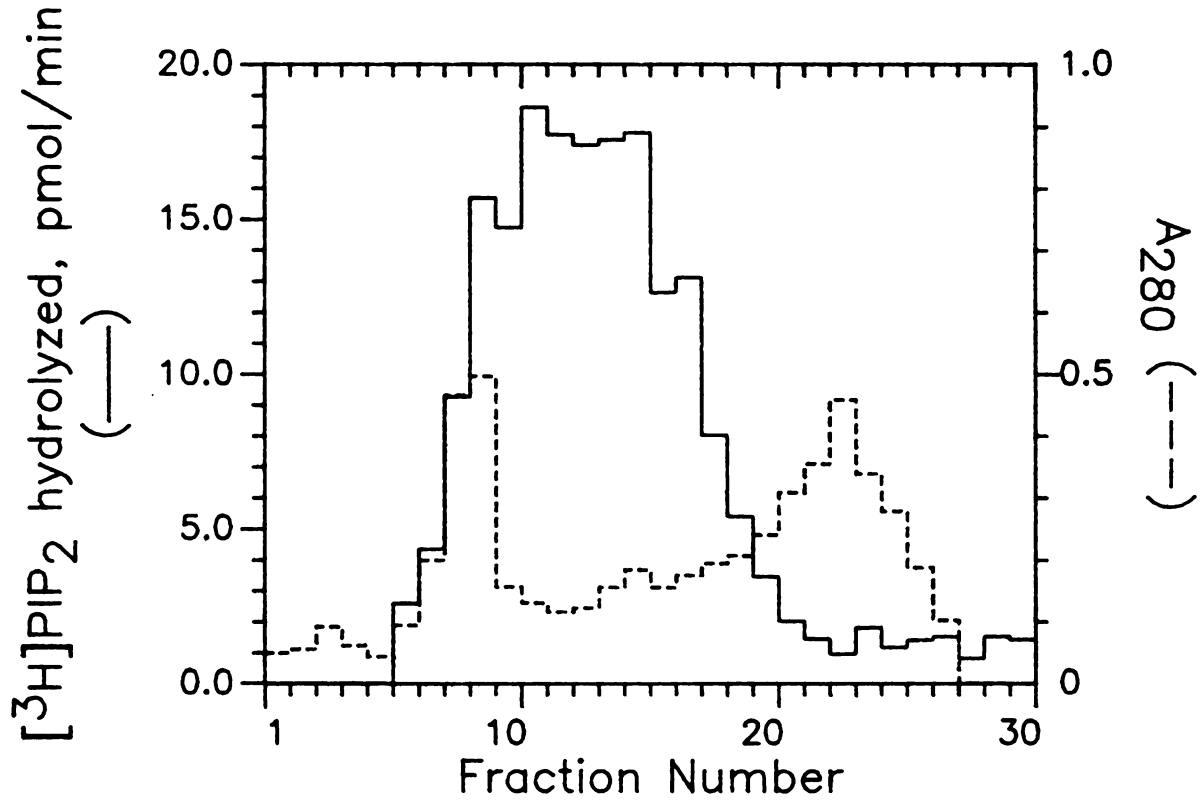
[3H]PIP2 hydrolyzed, pmol/min (——)
A280 (– – –)
20.0
15.0
10.0
5.0
0.0
1.0
0.5
0
1
10
20
30
Fraction Number

Figure 7

Figure 8. Ion-exchange chromatography of soluble PLC.

Isotonic supernate (*ca.* 20 mg protein) was applied to a 10-ml DE-52 column and eluted with a linear 0 - 1.0 M NaCl gradient (dotted line) in 10 mM Tris-HCl, pH 7.8, 1 mM DTT. 5-ml fractions were collected and assayed for PIP_2 phospholipase C activity (solid line) and A_{280} (dashed line).

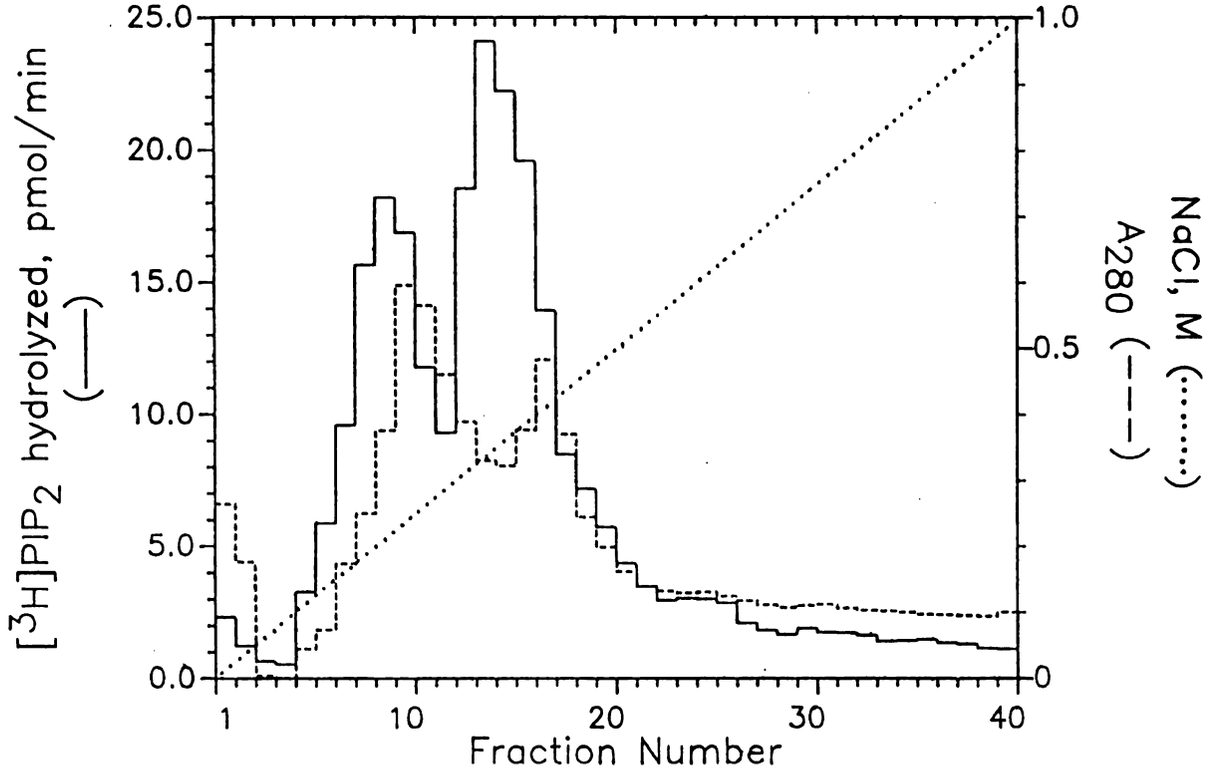
25.0
20.0
15.0
10.0
5.0
0.0
1
10
20
30
40
Fraction Number
[3H]PIP2 hydrolyzed, pmol/min (——)
1.0
0.5
0
A280 (– – –)
NaCl, M (.....)

Figure 8

Figure 9. Effect of GMPPCP on PIP_2 PLC activity.

Particulate (▪, 3.4 mg protein/assay) and soluble (●, 0.1 mg protein/ assay) fractions were assayed as described in Materials and Methods, with the addition of 3 mM $MgCl_2$ and the indicated concentrations of GMPPCP. Activities are shown as means ± std. errors for triplicate (particulate) or duplicate (soluble) assays.

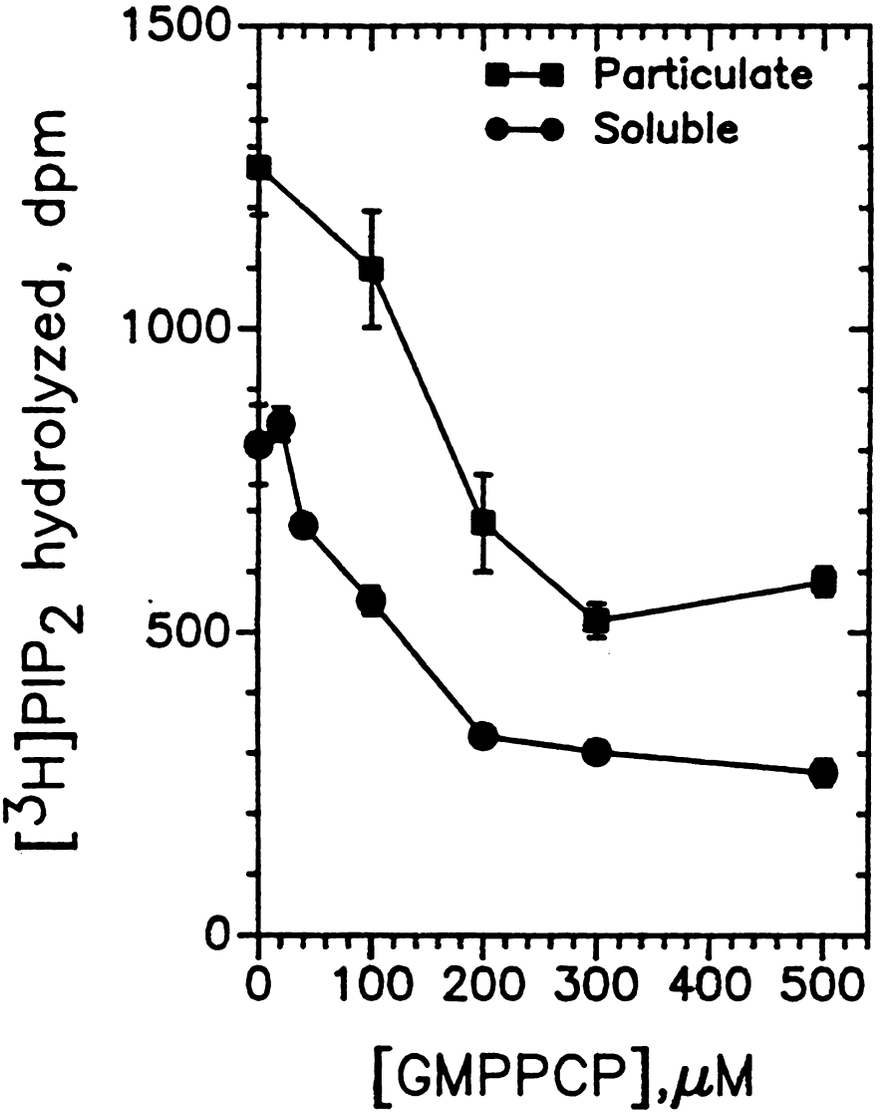
1500
1000
500
0
Particulate
Soluble
[^{3}H]PIP$_2$ hydrolyzed, dpm
0
100
200
300
400
500
[GMPPCP],μM

Figure 9

Pertussis toxin inhibits G-protein-mediated stimulation of phospholipase C in some cells (Cockcroft, 1987). Pertussis toxin ADP-ribosylated G-protein (Figure 10) but did not affect PLC activity (Table 5). The effects of GMPPCP, GDPβS and GMPPNP were not affected by pertussis toxin treatment. ROS G-protein can also be ADP-ribosylated by cholera toxin; this toxin too had no effect on PLC activity.

Guanosine-5'-*O*-(3-thiotriphosphate) (GTPγS) stimulates G-protein-regulated PLCs in many cell types (Banno *et al.*, 1986; Huque and Bruch, 1986; Hepler and Harden, 1986; Uhing *et al.*, 1985). In at least some cases this activation results from decreasing the PLC's Ca^{2+} requirement (Smith *et al.*, 1986; Deckmyn *et al.*, 1986). GTPγS at concentrations from 0.1 to 100 μM had no effect on particulate PIP_2 PLC activity in the presence of added Ca^{2+} (Figure 11). A modest inhibition by 100 μM GTPγS observed in the absence of added Ca^{2+} is probably due to chelation of endogenous Ca^{2+} by the nucleotide. GTPγS in concentrations from 0.1 to 100 μM was entirely without effect on the soluble PLC in the presence or absence of added Ca^{2+}. GTPγS was also without effect at low Ca^{2+} concentrations maintained by Ca-EGTA buffers, in the presence of added transducin, in whole (unwashed) ROS and under various conditions of illumination.

Fluoride ion activates G-protein-regulated PLCs (Martin *et al.*, 1986). The activation is enhanced by aluminum ion, presumably via formation of AlF_4^-, which appears to be the true activator (Cockcroft and Taylor, 1987). ROS G-protein is similarly affected (Kanaho *et al.*, 1985; Bigay *et al.*, 1985). ROS PLC activity was unaffected by NaF at concentrations up to 5 mM. Al^{3+} was not used because it inhibited ROS PLC at concentrations (~10 μM) used in the above papers.

Figure 10. ADP-ribosylation of ROS fractions by pertussis toxin.

ROS, particulate fraction, and soluble fraction were incubated for 30 min with (lanes 1, 3, and 5) or without (lanes 2, 4, and 6) pertussis toxin using the conditions described in the caption of Table 7, except that unlabelled NAD was replaced with 5 μCi (soluble fraction) or 10 Ci (ROS and particulate fraction) of [α-^{32}P]NAD (7 Ci/mmol). Incubation was terminated by addition of SDS (soluble fraction) or trichloracetic acid (ROS and particulate fraction). Labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis. An autoradiogram of the dried gel is shown. The autoradiogram was deliberately overexposed in lanes 3 and 5 to secure adequate exposure of lane 1.

Lanes 1 and 2: Soluble fraction (6 μg protein/lane). Lanes 3 and 4: particulate fraction (6 nmol rhodopsin/lane). Lanes 5 and 6: ROS (8 nmol rhodopsin/lane). Lane 7: purified ROS G protein (marker indicates position of α subunit).

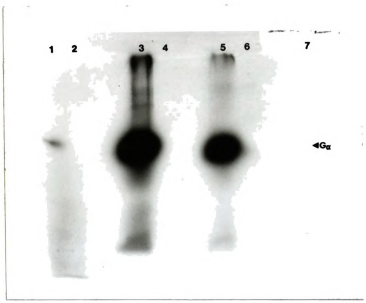

Figure 10

Table 5. Pertussis toxin does not affect PIP_2 PLC activity.

	[^{3}H]PIP_2 hydrolyzed, dpm	
Treatment	Particulate	Soluble
Control	5199 ± 330	12823 ± 167
Pertussis toxin	5097 ± 413	12955 ± 1220

Particulate fraction was treated with pertussis toxin by the method of van Dop *et al.* (1984). Briefly, WROS (25 nmol 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, 2mM $MgCl_2$, 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 in Materials and Methods, with the addition of 2.4 mM $MgCl_2$. The [^{3}H]PIP_2 used in these assays had a specific activity of 10 μCi/μmol. Results are shown as means ± std. 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 Lowry protein were incubated at 30 °C in 100 ul 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 hr, the total contents of each tube were assayed for PIP_2 PLC activity as described in Materials and Methods. Results are shown as means ± std. errors of duplicate assays. Pertussis toxin by itself had no PLC activity.

Figure 11. Effect of GTPγS on particulate PIP_2 PLC activity.

WROS (7 pmol rhodopsin/assay) were assayed for PLC activity as described in Materials and Methods, with the following modifications: GTPγS was added in the indicated concentrations, and $CaCl_2$ was omitted from some assays (filled circles) and present in others at a concentration of 10 μM (open circles).

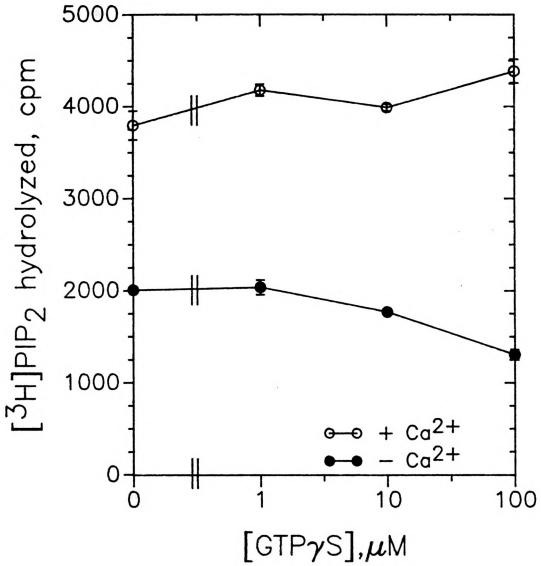
5000
4000
3000
2000
1000
0
[3H]PIP2 hydrolyzed, cpm
+ Ca2+
– Ca2+
0
1
10
100
[GTPγS],μM

Figure 11

Ghalayini and Anderson (1987) report inhibition of PIP_2 PLC in bovine ROS by 2.5 mM ATP in the presence of 5 mM $MgCl_2$ and 100 μM $CaCl_2$. As shown in Table 6, this inhibition also occurs in washed ROS but is not specific for ATP. The regulatory significance of such inhibition is unclear.

2. Light.

The possibility that light might regulate ROS PIP_2 PLC was examined under a variety of conditions, including the use of washed and unwashed ROS; exposure to light in advance of, at the start of, and during the assay; presence of GTP and non-hydrolyzable analogs; and varying concentration of added Ca^{2+} and Mg^{2+}. Although occasional modest increases in activity apparently caused by bleaching have been observed, these have not been consistently reproducible. Table 7 illustrates unresponsiveness of PLC activity to illumination; in contrast to PLC, cyclic nucleotide phosphodiesterase activity in isolated ROS is stimulated an order of magnitude or more by light in the presence of Mg^{2+} and GTP (Kohnken *et al.*, 1981a).

It is possible that the ROS PLC loses the capacity to respond to light during purification. The PLC activity of total retinal homogenates was compared in dark and light; no difference was found. Intact retinas cannot be assayed for PLC using exogenous substrate, but ROS and other retinal fractions isolated from retinas that were exposed to light were compared to those isolated from unbleached retinas (Table 8). The differences detected were minor at most; considering that the bleached and unbleached materials were necessarily handled separately during processing, small disparities do not provide significant support for light effects.

Table 6. Effect of nucleotides on particulate PIP_2 PLC activity.

Added Nucleotide	[^{3}H]PIP_2 hydrolyzed, dpm	
None	1033 ± 62	(4)
ATP	286 ± 13	(3)
ADP	323 ± 48	(3)
AMP	394 ± 55	(3)
GTP	818 ± 17	(3)
GDP	814 ± 40	(2)
GMP	603 ± 42	(2)

WROS (0.3 mg protein/assay) were assayed for PLC activity as described in Materials and Methods, except that each assay contained 100 μM $CaCl_2$, 5 mM $MgCl_2$ and 2.5 mM of the indicated nucleotide. Assay duration was 15 min. Results are shown as means ± std. errors for *n* replicates (*n* = number in parentheses).

Table 7. ROS PLC activity unaffected by light.

	[^{3}H]PIP_2 hydrolyzed, cpm	
Nucleotide	Dark	Light
None	4503 ± 39	4761 ± 167
GTP 100 μM	4184 ± 364	4720 ± 141
GMPPCP 100 μM	3027 ± 131	3110 ± 23

ROS (6.6 pmol rhodopsin/assay) were assayed for PIP_2 PLC activity as described in Materials and Methods, except that 100 μM $MgCl_2$, or 200 μM $MgCl_2$ and the indicated nucleotide, were added to the reaction mixture. Assays were performed under dim red light ("dark") or normal laboratory illumination ("light"). Results are shown as means ± std. errors of duplicate assays.

Table 8. PLC activity in fractions from bleached & unbleached retinas.

ROS were isolated as described in Chapter I. 100 retinas were exposed to normal laboratory illumination ("bleached") after excision but before shaking. These were processed in parallel with 200 retinas that were exposed to dim red light only. Fractions derived from the bleached retinas are labeled "(l)", those from the unbleached, "(d)". Volumes of the unbleached fractions were twice those of the corresponding bleached fractions, to maintain equivalent volume per retina. The supernants from the 1100 x *g* centrifugations (which pellets crude ROS) were centrifuged at 100000 x *g* for 1 hr to produce a pellet of microsomes. "Microsomal super" is the supernate from this ultracentrifugation. Aliquots of bleached and unbleached ROS were washed as described in Materials and Methods to produce WROS and isotonic supernates. Fractions produced from the two sets of retinas were assayed for PIP_2 PLC activity as described in Materials and Methods. ROS and WROS were assayed under dim red light. 0.3% octylglucoside (OG) was added to some assays to release cryptic or inhibited enzyme. (W-7 was used instead of OG in assays of isotonic supernates). Results are shown as dpm of [^{3}H]PIP_2 hydrolyzed in 15 min, means ± standard errors for duplicate assays.

Table 8. PLC Activity in Fractions from Bleached & Unbleached Retinas

Fraction	Lowry Protein mg/ml	Volume Assayed µl	[^{3}H]PIP_2 hydrolyzed, dpm - OG	+ OG
ROS (d)	14	5	2624 ± 139	5003 ±1385
		0.5	743 ± 20	781 ± 23
ROS (l)	13	5	2214 ± 195	5266 ± 723
		0.5	840 ± 86	944 ± 17
Microsomes (d)	8.4	5	3580 ± 354	8778 ± 322
		0.5	3457 ± 131	5722 ± 296
Microsomes (l)	8.4	5	3156 ± 277	8697 ± 565
		0.5	3746 ± 33	6986 ±1152
Microsom. super(d)	4.9	5	2502 ± 201	12279 ± 828
		0.5	1624 ± 171	1592 ± 52
Microsom. super(l)	4.6	5	2950 ± 36	11546 ± 444
		0.5	1749 ± 35	1607 ± 54
WROS (d)	8.6	5	1153 ± 53	728 ± 11
WROS (l)	9.4	4.5	1353 ± 21	933 ± 12
Isotonic super (d)	0.02	50	699 ± 17	1714 ± 24[a]
Isotonic super (l)	0.02	50	842 ± 16	2337 ± 60[a]

[a] 100 µM W-7 was used instead of 0.3% OG in these assays.

3. Calmodulin.

Sensitivity of ROS PLCs to Ca^{2+} suggested possible regulation by calmodulin (CaM). Although its target enzymes have not been identified, CaM is present in ROS (Kohnken *et al.*, 1981c). Because of the presence of endogenous CaM, initial experiments probing the possibility of CaM-regulation did not use added CaM but instead examined the effects of CaM antagonists. These are compounds that antagonize the effects of CaM by interfering with its binding to target proteins. Chemically they are quite diverse. The effects of a number of CaM antagonists from several different chemical categories are described below. Unexpectedly, CaM antagonists stimulated PIP_2 PLC.

Phenothiazines: The phenothiazines, a family of antipsychotic tranquilizers, were the first category of CaM antagonists to be discovered. Chlorpromazine (2-chloro-10-(3-dimethylaminopropyl)-phenothiazine) and trifluoperazine (10-[3-(4-methylpiperazin-1-yl)-propyl]-2-(trifluoromethyl)-10*H*-phenothiazine) inhibit CaM-stimulated cyclic nucleotide phosphodiesterase with IC_{50}s of 10 and 42 μM respectively (Weiss and Levin, 1978). Figure 12 shows the concentration-dependent stimulation of soluble PLC by the phenothiazines. The apparent EC_{50}s were somewhat higher than those for phosphodiesterase inhibition. Gietzen (1986) points out that reported IC_{50}s for CaM antagonists are highly variable, and depend greatly on the concentration of CaM and any other hydrophobic materials (such as proteins or lipids) present. Disparities of an order of magnitude or more for IC_{50}s of these and other CaM antagonists can be found in the literature. Excessive weight should therefore not be given to differences between experimental and literature values for effective concentrations; neither should CaM

Figure 12. Effect of phenothiazines on soluble PIP_2 PLC activity.

Isotonic supernate (30 μg protein/assay) was assayed for PIP_2 PLC activity as described in Materials and Methods, but with the addition of the indicated concentration of trifluoperazine (▲) or chlorpromazine (♦). Results are presented as % of no-drug control, mean ± std. error of duplicate determinations.

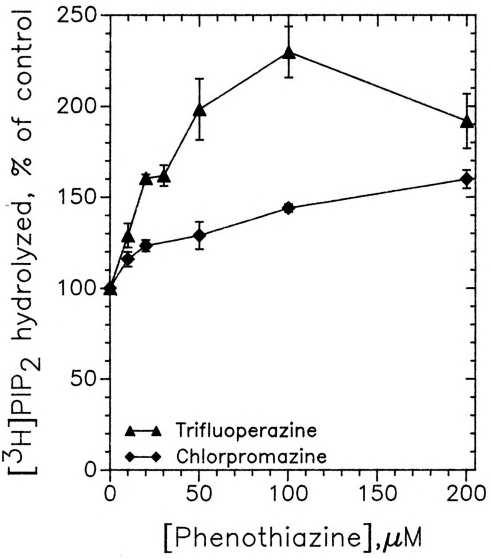
250
200
150
100
50
0
0
50
100
150
200
[^{3}H]PIP$_2$ hydrolyzed, % of control
[Phenothiazine],μM
Trifluoperazine
Chlorpromazine

Figure 12

involvement be inferred simply from similarities. Concentration curves for these drugs were not performed on WROS, but in separate experiments, WROS PLC was activated 31% by 200 μM chlorpromazine ($p < .05$) and 104% by 100 μM trifluoperazine ($p < .001$).

Haloperidol: Although unlike the phenothiazines structurally, haloperidol (4-[4-(4-chlorphenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone), like them, is used clinically as an antipsychotic tranquilizer and *in vitro* as a CaM antagonist, with an IC_{50} of 60 μM for CaM-stimulated phosphodiesterase (Weiss and Levin, 1978). Haloperidol at concentrations in the 10^{-4} M range stimulates PIP_2 PLC in both isotonic supernate and WROS (Figure 13), the effect being greater on the supernate.

Calmidazolium: Calmidazolium (Compound R24571, 1-[bis(4-chlorophenyl)methyl]-3-[2,4-dichloro-β-(2,4-dichlorobenzyl-oxy)phenethyl]-imidazolium chloride) is reportedly a highly specific CaM antagonist, with an IC_{50} for inhibition of erythrocyte CaM-dependent Ca^{2+}-ATPase of ~0.4 μM (Gietzen *et al.*, 1981). Table 9 shows the effects of 1 μM and 10 μM calmidazolium on PLC activity in isotonic supernate and WROS.

Melittin: Melittin is a 26-amino-acid polypeptide found in bee venom and is a potent CaM antagonist. Its reported K_i for CaM-dependent protein kinase is 80 nM (Katoh *et al.*, 1982). Melittin is not highly specific for CaM, however, and also stimulates phospholipase A_2 (Mollay *et al.*, 1976), inhibits protein kinase C (Katoh *et al.*, 1982) and interacts directly with phospholipid membranes (Dawson *et al.*, 1978).

Figure 13. Effect of haloperidol on PIP_2 PLC activity.

WROS (▪, 7 pmol rhodopsin/assay) and isotonic supernate (●, 30 μg protein/assay) were assayed for PLC activity as described in Materials and Methods, with the addition of the indicated concentration of haloperidol. Results are presented as % of no-drug control, means ± standard errors for duplicate assays.

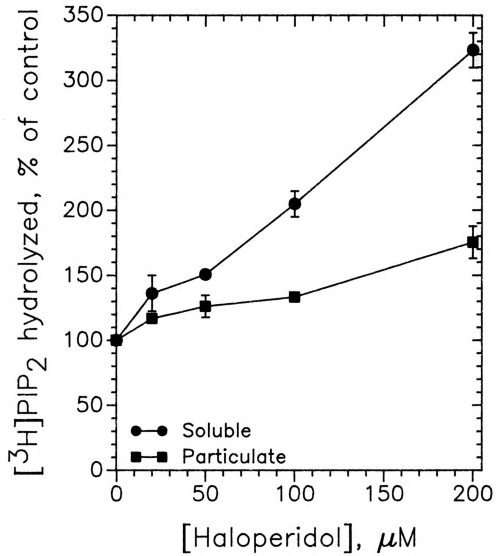
[^{3}H]PIP$_2$ hydrolyzed, % of control
350
300
250
200
150
100
50
0
0
50
100
150
200
[Haloperidol], μM
Soluble
Particulate

Figure 13

Table 9. Effect of calmidazolium on PIP_2 PLC activity.

[Calmidazolium], μM	[^{3}H]PIP_2 hydrolyzed, dpm	
	Soluble	Particulate
0	9348 ± 342	2155 ± 85
1	12400 ± 73	2293 ± 120
10	25007 ±1734	3234 ± 67

Isotonic supernate and WROS were assayed for PIP_2 PLC activity as described in Materials and Methods, with the addition of the indicated concentration of calmidazolium. Ethanol (solvent for calmidazolium) was present in all assays at a concentration of 1% v/v. Results are shown as means ± std. errors of duplicate (soluble) or triplicate (particulate) assays.

Figure 14 shows the effect of melittin on PLC activity in WROS and isotonic supernate. The soluble activity was activated by concentrations comparable to those that inhibit CaM-activated enzymes, but inhibited at higher concentrations. WROS required higher concentrations for activation.

Compound 48/80: Compound 48/80 is actually a mixture of compounds produced by the condensation reaction of *N*-methyl-*p*-methoxyphenethylamine and formaldehyde (Baltzy *et al.*, 1949). It is reportedly a highly specific CaM antagonist with an IC_{50} of 0.85 μg/ml for CaM-dependent Ca^{2+}-ATPase (Gietzen *et al.*, 1983). Compound 48/80 is more hydrophilic than other CaM antagonists and therefore less likely to exert non-specific hydrophobic effects on enzymes or substrates (Gietzen and Bader, 1985). The response of PLC to Compound 48/80 was biphasic: stimulation at concentrations up to 2.5 μg/ml, inhibition at higher concentrations. This pattern was seen in both WROS and isotonic supernate (Figure 15).

Bronner *et al.* (1987) report that Compound 48/80 inhibits PI PLC from human platelets. IC_{50}s were 2 μg/ml for soluble PLC and 5 μg/ml for the particulate fraction. Phospholipase A_2 gave a biphasic response, similar to those shown for PIP_2 PLC in Figure 15. They interpret their results as non-CaM mediated action of Compound 48/80 on the phospholipases or their substrates, but present no experimental evidence against CaM-mediation. Inhibition of PI hydrolysis is not necessarily inconsistent with stimulation of PIP_2 hydrolysis: the CaM-antagonist W-7 produced both effects in ROS isotonic supernate (see below).

Figure 14. Effect of melittin on PIP_2 PLC activity.

WROS (▪) and isotonic supernate (●) were assayed for PLC activity as described in Materials and Methods, with the addition of the indicated concentration of melittin. Results are presented as % of no-drug control, means ± std. errors for duplicate (particulate) or triplicate (soluble).

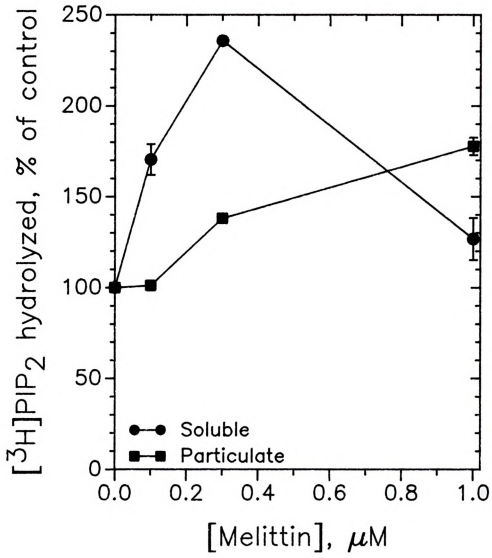
250
200
150
100
50
0
[^{3}H]PIP$_2$ hydrolyzed, % of control
Soluble
Particulate
0.0
0.2
0.4
0.6
0.8
1.0
[Melittin], μM

Figure 14

Figure 15. Effect of compound 48/80 on PIP_2 PLC activity.

WROS (▪) and isotonic supernate (●) were assayed for PLC activity as described in Materials and Methods, with the addition of the indicated concentration of compound 48/80. Results are presented as % of no-drug control, means ± std. errors for duplicate assays.

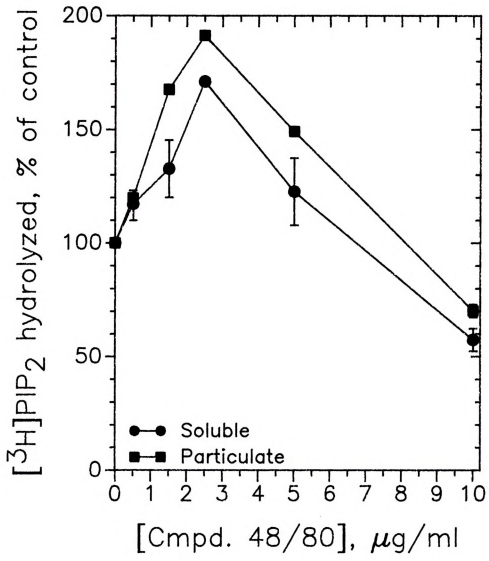
[^{3}H]PIP$_2$ hydrolyzed, % of control
200
150
100
50
0
Soluble
Particulate
0 1 2 3 4 5 6 7 8 9 10
[Cmpd. 48/80], μg/ml

Figure 15

Naphthalenesulfonamides: Hidaka and Tanaka (1983) have prepared a series of *N*-aminoalkyl-naphthalenesulfonamides that are widely used as probes of CaM regulation. Among the most useful are *N*-(6-amino-hexyl)-1-naphthalenesulfonamide (W-5), *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), *N*-(4-aminobutyl)-1-naphthalenesulfon-amide (W-12), and *N*-(4-aminobutyl)-5-chloro-1-naphthalenesulfonamide (W-13).

W-7 and W-13 are much more potent CaM antagonists than their non-chlorinated analogs, W-5 and W-12, but are similar to them in hydrophobicity. Comparing W-13 with W-12 and W-7 with W-5 has been proposed as a means of distinguishing CaM-mediated from non-CaM-mediated effects of CaM antagonists (Hidaka *et al.*, 1981; Chafouleas *et al.*, 1982). Figures 16 and 17 show concentration curves for the naphthalensulfonamides applied to isotonic supernate and WROS. W-13 and W-7 stimulated PLC activity much more effectively than W-12 and W-5.

Since W-7 is one of the most widely used and well-characterized CaM antagonists, it was selected for further experiments to examine the effects of these drugs in more detail.

W-7 eliminated the tendency of soluble PLC activity to "shoulder off" with increasing enzyme concentration, making activity approximately linear with volume of enzyme (Figure 18). This is consistent with antagonism of an endogenous inhibitor. Figure 18 illustrates another property of CaM-antagonist PLC stimulation: the fold activation obtained was dependent on the quantity of enzyme used in the assay. This was true not only of W-7 but also of the other CaM antagonists examined.

Figure 16. Effects of naphthalenesulfonamides on soluble PIP_2 PLC activity.

Isotonic supernate (32 μg protein/assay) was assayed for PIP_2 PLC activity as described in Materials and Methods, with the addition of the indicated concentrations of W-series compounds.

A: W-13 (▲) and W-12 (■).

B: W-7 (▲) and W-5 (■).

Results are presented as means ± std. errors for duplicate assays.

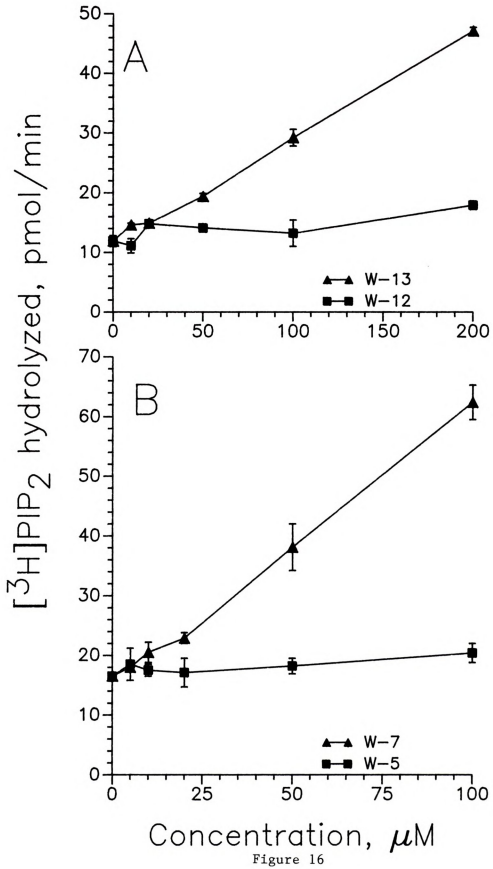
A
B
W-13
W-12
W-7
W-5
[3H]PIP2 hydrolyzed, pmol/min
Concentration, μM

Figure 16

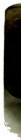

Figure 17. Effects of naphthalenesulfonamides on particulate PIP_2 PLC activity.

WROS (0.26 mg protein/assay) was assayed for PIP_2 PLC activity as described in Materials and Methods, with the addition of the indicated concentrations of W-series compounds.

A: W-13 (▲) and W-12 (■).

B: W-7 (▲) and W-5 (■).

Results are presented as means ± std. errors for duplicate assays.

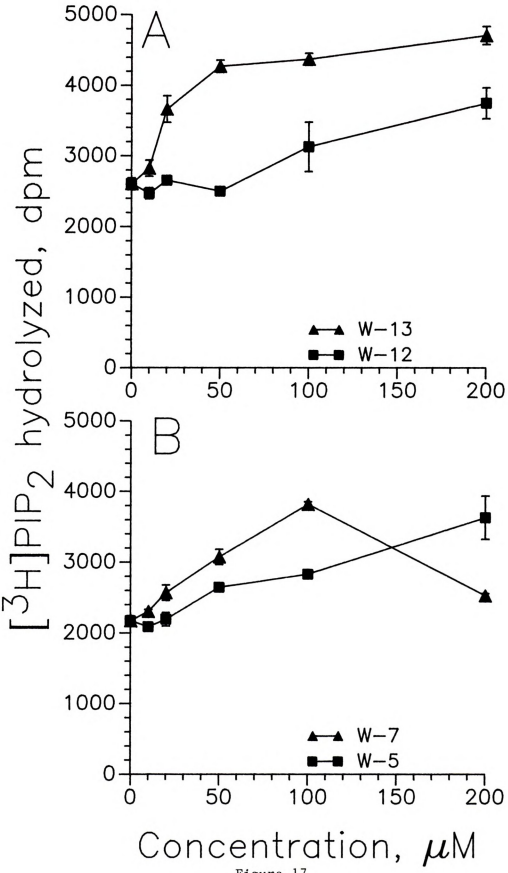
A
B
W-13
W-12
W-7
W-5
[3H]PIP2 hydrolyzed, dpm
Concentration, μM
0
1000
2000
3000
4000
5000
50
100
150
200

Figure 17

Figure 18. Effect of W-7 on activity-volume linearity.

The indicated volumes of soluble fraction (0.13 mg protein/ml) were assayed for PIP_2 PLC activity in the presence (♦) or absence (●) of 100 μM W-7. Results are presented as means ± std. errors of duplicate assays.

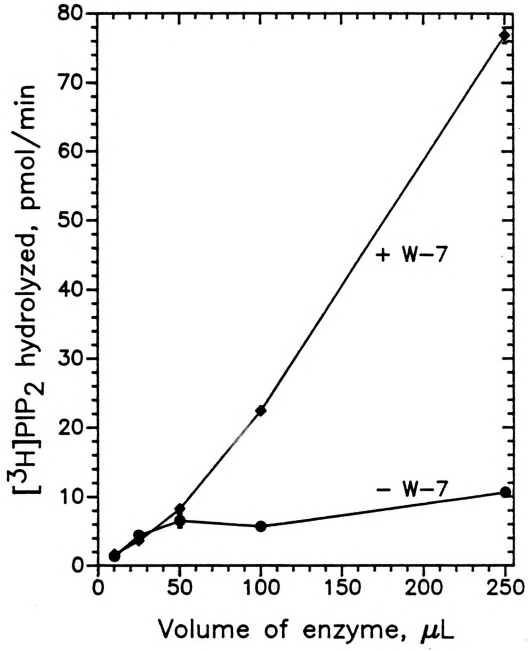
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
[3H]PIP2 hydrolyzed, pmol/min
Volume of enzyme, μL
+ W-7
- W-7

Figure 18

W-7 affected hydrolysis of all three phosphoinositides (Table 10), but in different ways. It appears to alter the specifity of the PLCs in favor of the polyphosphoinositides at the expense of PI. PI hydrolysis was inhibited in the soluble fraction and only slightly activated in the particulate fraction, in contrast to PIP and PIP_2. Similar results were obtained with trifluoperazine. Inhibition of PI PLC by CaM antagonists has been reported previously (Wightman *et al.*, 1981; Craven and DeRubertis, 1983; Benedikter *et al.*, 1985; Schwertz *et al.*, 1987; Bronner *et al.*, 1987) but these studies did not attempt to determine the role of CaM, if any, in this inhibition.

CaM increases the V_{max} of adenylate cyclase and cyclic nucleotide phosphodiesterase without altering their K_ms, although conflicting results have also been reported (Cheung *et al.*, 1978). W-7 increased the V_{max} of the soluble PLC without altering the apparent K_m of approximately 6 μM for PIP_2 (Figure 19). The linearity of the double-reciprocal plots is somewhat surprising considering the presence of multiple PLCs as revealed by ion-exchange chromatography. The different PLCs may be similar kinetically, or different forms of a single species.

CaM antagonists and Ca^{2+}: Stimulation by W-7 was dependent on Ca^{2+} concentration, with half maximal stimulation occuring between 0.1 and 1 μM (Figure 20). This figure also shows that the effects of W-7 and Ca^{2+} are dependent on the quantity of crude enzyme assayed. W-7 is most effective in the presence of concentrated enzyme, as noted above. In the absence of W-7, calcium's effect is largest in the presence of dilute enzyme. The latter observation can be interpreted as indicating the presence of a Ca^{2+}-dependent inhibitor which

Table 10. Effect of W-7 on phosphoinositide hydrolysis.

	Specific	Acid-soluble label released, dpm			
	Activity	Particulate		Soluble	
Substrate	μCi/μmol	- W-7	+ W-7	- W-7	+ W-7
[^{3}H]PI	9.1	4235 ± 75	4808 ± 53	1022 ± 62	348 ± 40
[^{3}H]PIP	5.0	6102 ± 388	11322 ± 390	4652 ± 475	5548 ± 65
[^{3}H]PIP_2	2.5	5348 ± 277	7868 ± 902	1558 ± 118	2898 ± 30

WROS and isotonic supernate (400 and 36 μg protein/assay, respectively) were assayed for phospholipase C activity as described in Materials and Methods, with or without the addition of 100 μM W-7. Results are shown as means ± standard errors for triplicate (particulate) or duplicate (soluble) assays. Values for specific activities of substrates do not reflect dilution of the label by endogenous lipids in the enzyme preparations, which could be significant for the particulate fraction. Similar results were obtained using trifluoperazine instead of W-7.

Figure 19. Effect of W-7 on kinetics of soluble phospholipase C.

Soluble phospholipase C activity was assayed as described in Materials and Methods, but with varying concentrations of [^{3}H]PIP_2 and in the presence (♦) or absence (●) of 100 μM W-7. Data are presented in a double-reciprocal plot; each point represents the mean of two assays. Data were analyzed by the method of Wilkinson (1961) and numerical results are shown below:

	- W-7	+ W-7
K_m	5.8 ± 0.9	6.5 ± 0.7
V_{max}	11.8 ± 0.6	28.2 ± 1.1

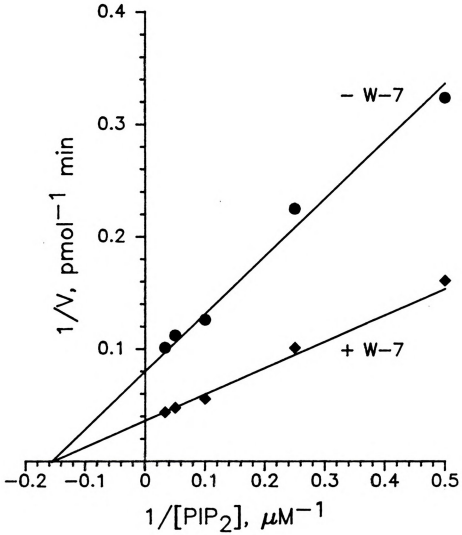

Figure 19

Figure 20. Calcium dependence of W-7 stimulation of PLC.

Isotonic supernate was assayed for PIP_2 PLC activity in the presence (triangles) and absence (circles) of 100 μM W-7. Ca^{2+} concentrations were set with a Ca-BAPTA buffer as described in Figure 3. Assays were performed on 15 μg (filled symbols) or 1.5 μg (open symbols) of isotonic supernate protein.

Inset: replot (with expanded y axis) of 1.5 μg results, showing activation by Ca^{2+} even in absence of W-7.

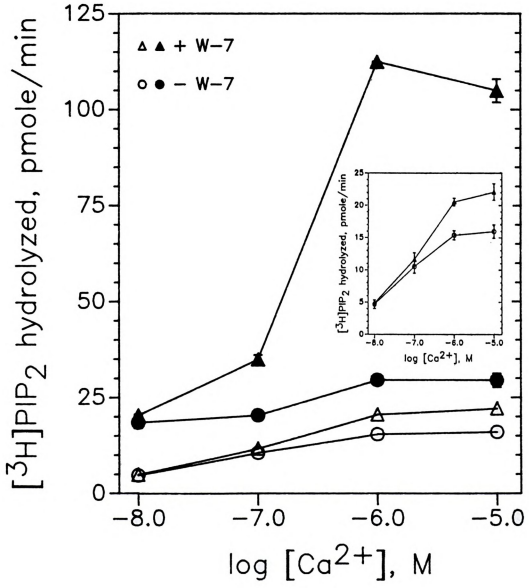
△ ▲ + W-7
○ ● – W-7
[3H]PIP2 hydrolyzed, pmole/min
125
100
75
50
25
0
–8.0
–7.0
–6.0
–5.0
log [Ca2+], M
[3H]PIP2 hydrolyzed, pmole/min
25
20
15
10
5
0
–8.0
–7.0
–6.0
–5.0
log [Ca2+], M

Figure 20

dissociates from the enzyme on dilution; this is also consistent with the response of the enzyme to W-7. This point is discussed in more detail below.

CaM antagonists and pH: The Ca^{2+}-dependent, high affinity binding of trifluoperazine to CaM is pH-dependent, and decreases sharply above pH 7.5. As shown in Table 11, the effect of trifluoperazine on soluble PLC activity increases in the interval from pH 7.5 to 8.5: PLC activity was less inhibited by increasing pH in the presence of trifluoperazine. Similar effects were observed with W-7, mellitin and compound 48/80.

CaM antagonists and BSA: Addition of bovine serum albumin to PLC assays in concentrations above 10 μg/ml resulted in decreased activity, which was initially interpreted as a non-specific protein effect. However, as shown in Table 12, this inhibition appeared to be specific to BSA. Inhibition by BSA was antagonized by W-7 but not by W-5 (Figure 21).

Albumin binds hydrophobic compounds in serum, and BSA preparations contain bound lipids and other non-polar substances. The inhibitory effect of BSA may be due to such contaminants, but a commercial preparation of BSA that had been specially processed to decrease such contamination (Sigma A-7030) also inhibited PLC. It is possible that substrate or the PLC bind to hydrophobic sites on BSA molecules.

On SDS-polyacylamide gels, isotonic supernate shows a minor (<1%) component with an M_r equal to BSA's. Even if this is BSA, its quantity is too small to account for the observed endogenous inhibiton, which typically appears at total isotonic supernate protein concentrations of 5 μg/ml in assay or higher.

Table 11. Effect of pH on trifluoperazine stimulation of PLC.

	[^{3}H]PIP_2 hydrolyzed, dpm	
pH	- TFP	+ TFP
7.5	4324 ± 466	5928 ± 332
8.0	3606 ± 105	5419 ± 32
8.5	2369 ± 112	4207 ± 300

Isotonic supernate (5 μg protein/assay) was assayed for PIP_2 PLC activity as described in Materials and Methods, in the presence or absence of 50 μM trifluoperazine (TFP). The pH of the HEPES buffer was adjusted to the indicated values with KOH. Results are expressed as means ± std. errors for duplicate determinations.

Table 12. Effect of added proteins on soluble PIP_2 PLC activity.

Protein	[^{3}H]PIP_2 hydrolyzed, dpm
None	2566 ± 94
BSA	498 ± 169
Ovalbumin	2257 ± 98
Sheep IgG	2386 ± 233
Parvalbumin	2594 ± 319
Gelatin	2505 ± 185
Calmodulin	2570 ± 94

Isotonic supernate (3.3 μg protein/assay) was assayed for PLC activity as described in Materials and Methods, with the addition of 25 μg of the indicated proteins. Gelatin (bacteriological grade) was purchased from Difco, calmodulin from Calbiochem, the others from Sigma. Results are shown as means ± standard errors for triplicate assays.

Figure 21. Effect of W-7 and W-5 on inhibition of soluble PLC by BSA.

Soluble PIP_2 phospholipase C activity was assayed in the presence of the indicated concentration of BSA and either 100 μM W-7 (♦), 100 μM W-5 (▲), or no drug (●). Protein concentration in assays due to enzyme solution was 15 μg/ml. Activities are shown as percent of no-BSA control for each drug treatment. Each point represents the mean ± std. error of duplicate assays.

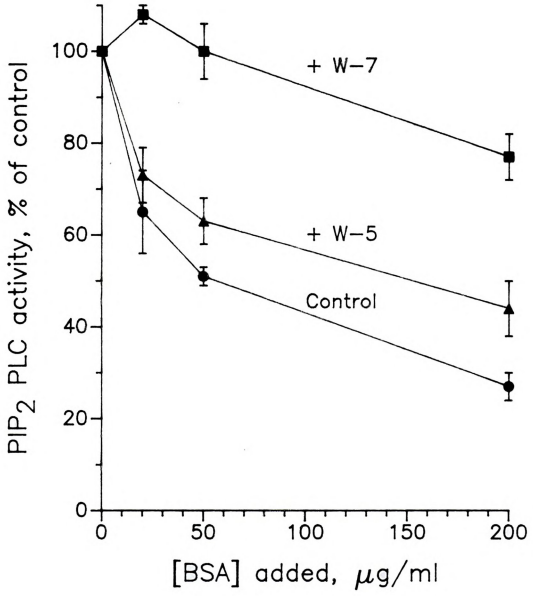
PIP2 PLC activity, % of control
100
80
60
40
20
0
0
50
100
150
200
[BSA] added, μg/ml
+ W-7
+ W-5
Control

Figure 21

CaM antagonists and detergent: 100 μM trifluoperazine and 0.3% octylglucoside (10 mM) produced equal stimulation of soluble PLC (Table 13). Together they had no greater effect than either one alone, suggesting that both prevent the same inhibitory interaction.

Calcineurin: Calcineurin is a CaM-dependent protein phosphatase that binds CaM extremely tightly in the presence of Ca_{2+}. Because of its tight binding it has been used as a CaM antagonist: by binding CaM to itself, calcineurin prevents it from binding to other proteins (Klee *et al.*, 1983). Calcineurin added in up to 1000-fold excess over CaM (estimated from radioimmunoassays described below) had no effect on soluble or particulate PIP_2 PLC activity (data not shown), although a low level of PLC activity in a commercial (Sigma) calcineurin preparation was initially mistaken for activation of the enzyme.

CaM antagonists and PLC purification: Preliminary attempts to render soluble PLC insensitive to CaM antagonists by purifying it away from CaM have been unsuccessful. Ion exchange and gel filtration chromatography yielded peaks of PLC activity that could still be stimulated by trifluoperazine. Isotonic supernate was applied to an affinity column of W-7-agarose, to which CaM binds in a Ca^{2+}-dependent manner (Endo *et al.*, 1981). It was hoped that CaM would bind to the column and CaM-depleted enzyme would flow through; instead, the PLC activity bound tightly to the column in a non-Ca^{2+}-dependent fashion. EGTA did not elute it, but about half the activity could be eluted with octylglucoside. The eluted enzyme could still be stimulated by W-7.

The failure of these chromatographic techniques to produce a CaM-antagonist-insensitive preparation would seem to argue for

Table 13. PLC activation by detergent and CaM antagonist not additive.

Addition	[^{3}H]PIP_2 hydrolyzed, dpm
None	9065 ± 489
Trifluoperazine (100 μM)	18276 ± 463
Octylglucoside (10 mM)	17301 ± 1448
Trifluoperazine + Octylglucoside	17162 ± 537

Isotonic supernate (45 μg protein/assay) was assayed for PIP_2 PLC activity as described in Materials and Methods, with the indicated additions. Results are shown as means ± std. errors for duplicate determinations.

CaM-independent stimulation of PLC, but radioimmunoassays performed in the laboratory of Dr. James Chafouleas (at the Centre Hospitalier de l'Université Laval in Ste. Foy, Quebec) indicated that the PLC preparations produced by these techniques were not depleted of CaM. These RIAs were not part of the original experimental designs, and in some cases no unfractionated starting material was available for analysis, but it appears that some of the PLC fractions were enriched in CaM during partial purification, suggesting co-purification. CaM content of isotonic supernate is ~4 ng CaM/ μg protein as measured by RIA (in the experiments described here and in Kohnken *et al.*, 1981c).

Addition of CaM to PLC assays: If CaM is the endogenous inhibitor, excess CaM should inhibit PLC activity in isotonic supernate that is sufficiently dilute. Sufficiently dilute enzyme would display PLC activity that is proportional to volume assayed and unresponsive to CaM antagonists (Figure 18, lower left corner). Table 14 shows the results of such an experiment. No effects of added CaM were found.

Table 14. Added CaM does not affect PLC activity.

	[^{3}H]PIP_2 hydrolyzed, dpm	
CaM added, μg	- W-7	+ W-7
0	2875 ± 310	2525 ± 56
0.2	2741 ± 95	2477 ± 98
2.0	2498 ± 150	2701 ± 60

Isotonic supernate was incubated overnight at 4° C in the presence of 10 μM $CaCl_2$ and varying amounts of added CaM (from bovine brain, purchased from Calbiochem). Aliquots containing 1.2 μg of isotonic supernate protein and the indicated quantity of CaM were assayed for PIP_2 PLC activity as described in Materials and Methods. Results are shown as means ± std. errors for triplicate determinations.

DISCUSSION

Demonstration of phosphoinositide hydrolysis complements demonstration of phosphoinositide synthesis. Analysis of the labeled products of PIP_2 hydrolysis shows IP_3 as the major product. The smaller amounts of IP_2, IP and inositol detected presumably result from the action of phosphatases on IP_3, although hydrolysis of [^{3}H]PIP and [^{3}H]PI (produced from [^{3}H]PIP_2 by phosphatases) may also contribute. Experiments using [^{3}H]PIP and [^{3}H]PI as substrates indicate that they can be hydrolyzed by soluble and membrane-bound ROS PLCs, although less rapidly than PIP_2. Their hydrolysis may be due to multiple enzymes or broad substrate specificity of PIP_2 PLC.

Although the particulate PLC can be ascribed to the ROS with confidence, the attribution of the soluble activity is less certain. It can be found throughout the sucrose gradients used for isolation of ROS (see Methods), and in the supernate of the crude ROS pellet. ROS prepared by our method have osmotically leaky plasma membranes [R. E. Kohnken, unpublished results, using the method of Yoshikami *et al.* (1974)] and it is possible that a cytosolic enzyme could escape during preparation. Retinal cells other than rods may also be damaged during shaking, and these could release cytoplasmic enzymes as well. Taking into account the dilution at each step, the activity in the isotonic supernate cannot be accounted for by carry-over from the crude supernate, indicating that soluble PLC continues to leak out of ROS. At least a portion of the soluble PLC activity can therefore by ascribed to the ROS.

In terms of the amount of label released by hydrolysis during assay, the total soluble activity would seem to be much greater than the total particulate activity. However, quantitative comparison of the soluble and particulate activities is difficult, due to the presence of endogenous PIP_2 in the membranes, which may decrease the specific radioactivity of the substrate. There is also the problem of accessibility of the particulate enzyme to an exogenous substrate. $[^3H]PIP_2$ added to WROS in the presence of Ca^{2+} chelators (to prevent hydrolysis) was quickly inserted into membranes as judged by centrifugation (data not shown), but it has been reported that some cells maintain discrete pools of phosphoinositides -- one pool available to agonist-stimulated PLC and the other not (Monaco and Woods, 1983). The mechanism of such segregation is unclear, but it further complicates interpretation of results obtained with ROS and WROS.

Another question arises concerning the soluble PLC activity: how does it function *in vivo*? The sensitivity to Mg^{2+} and spermine suggest that it would be inhibited under cytoplasmic conditions.

Majerus *et al.* (1986) propose that cytosolic PLCs may associate reversibly with cell membranes and that this may be a part of their regulation. The soluble PLC activity may represent a pool of inactive enzyme that can be activated by association with membranes (plasma or diskal) under the right conditions. If so, the loss of large amounts of this enzyme during preparation could account for failure to observe regulation by light.

Some cations appear to have opposite effects on the anabolic and catabolic pathways of phosphoinositide metabolism. Mg^{2+}, Mn^{2+} and

spermine stimulated precursor incorporation and inhibited hydrolysis, especially by soluble PLC. Ca^{2+} at micromolar concentrations has no effect on phosphoinositide synthesis but is required for hydrolysis. Al^{3+} inhibited PLC; its effects on synthesis were not examined.

Eichberg *et al.* (1981) have suggested that polyamines and metal ions form complexes with the negatively charged head groups of phosphoinositides. The observed effects of Mg^{2+}, Mn^{2+}, Ca^{2+}, Al^{3+} and spermine may be due in whole or in part to formation of such complexes, and not to the effects of the ions on the enzymes.

Hormonal regulation of PIP_2 PLC via G-proteins occurs in a wide variety of cells (reviewed by Cockcroft, 1987), and provides an obvious paradigm for possible regulation by light in ROS. The G-protein that mediates light-activation of cGMP phosphodiesterase constitutes a major part of the non-rhodopsin protein in ROS. The non-hydrolyzable GTP analog GTPγS is highly effective in stimulating G-protein regulated PIP_2 PLC in several cell types (Uhing *et al.*, 1985; Deckmyn *et al.*, 1986; Smith *et al.*, 1986; Hepler and Harden, 1986), and cGMP PDE in ROS (Yamanaka, *et al.*, 1986). The absence of any effect by GTPγS on ROS PLC, either soluble or particulate, argues against regulation by G-protein. Inhibition by GMPPCP, and the inconstant effects of GDPβs and GMPPNP, occur at concentrations considerably higher than those usually associated with G-protein activation. Pertussis toxin inactivates phospholipase-regulating G proteins in many, but not all, cell types (Cockscroft, 1987); both pertussis and cholera toxin inactivate transducin. Neither affected ROS PLC's activity or sensitivity to GMPPCP. In sum, these results provide little support for regulation of ROS PLC by a G-protein.

Nor do they provide any persuasive evidence of regulation by light. It is difficult to compare these results directly with those obtained by some other workers claiming light-stimulation of PLC, due to differences in assay techniques. Many workers, instead of using an exogenous labeled substrate, label retinal lipids *in situ*, *e. g.* by intraocular injection of [^{3}H]inositol or ^{32}Pi. Phospholipase activation is then inferred from a decrease in labeled PIP_2 in response to light. This technique is impractical with live cattle. Early in this project, analogous experiments were done with isolated ROS; these were not promising and led to the adoption of the exogenous-substrate assay. The recent successful use of exogenous [^{3}H]PIP_2 to detect light-stimulated PLC in isolated squid photoreceptor segments (Baer and Saibil, 1988) suggests that this choice of methodology should not have prevented detection of light-regulation.

If light does regulate ROS PLC, the regulatory system must be more labile than that of the cGMP PDE, since we have failed to detect any light-activation of PLC under conditions that result in a several-fold activation of PDE.

It is also possible that light-regulation of ROS PIP_2 PLC is indirect -- that changes in activity result from, rather than cause, the changes in ionic concentrations that attend the rod photoresponse. Isolated ROS are of course separated from the ion pumps of the inner segment; furthermore, ROS isolated in sucrose have leaky plasma membranes. Secondary light-regulation of PLC by ionic changes could hardly be preserved in such a preparation.

The stimulatory effects of CaM antagonists are especially provocative, suggesting as they do inhibition by CaM. This would be doubly novel, as PLC has not previously been shown to be CaM-regulated, and those enzymes whose regulation by CaM is well established are all activated by it, not inhibited. However, as Hartshorne (1985) has observed, "there is no *a priori* reason whey calmodulin interaction must activate enzymatic activity, and it is conceivable that calmodulin-dependent inhibition may occur."

The following observations are consistent with CaM inhibition of PIP_2 PLC:

1. CaM is present in ROS, both in the soluble and particulate fractions, as determined by radioimmunoassay (Kohnken *et al.*, 1981c).

2. The apparent specific activity of crude soluble PLC decreases with increasing amounts of enzyme, suggesting the presence of an endogenous inhibitor.

3. All anti-CaM drugs tested, including two that are reportedly highly specific, stimulated PLC activity.

4. The relative potencies of W-7 compared to W-5, and W-13 compared to W-12, for stimulating PLC are consistent with their potencies as CaM antagonists.

5. Stimulation of PLC activity by W-7, a representative CaM antagonist, was dependent on the quantity of enzyme; at high enzyme concentrations W-7 appeared to release the enzyme from the inhibition noted in 2.

6. The effect of W-7 was Ca^{2+} dependent, increasing sharply between 0.1 and 1 μM Ca^{2+}.

Although these observations seem persuasive, pharmacolgical probes of CaM regulation are seldom unambigous. No CaM antagonist is perfectly specific. Most of them are fairly hydrophobic and can interact with lipids and hydrophobic proteins. Phenothiazines and haloperidol inhibit protein kinase C (Schatzman *et al.*, 1981), as do naphthalenesulfonamides (Tanaka *et al.*, 1982) and melittin (Katoh *et al.*, 1982), which also activates phospholipase A_2 (Mollay *et al.*, 1976). Other results argue against CaM regulation:

1. The concentrations of the CaM antagonists required for full effectiveness are somewhat higher than those reported for CaM-activated enzymes; W-7 and W-13, for example, show no sign of saturation in isotonic supernate at concentrations up to 100 and 200 μM, respectively. Their respective IC_{50}s for CaM-stimulated cylic nucleotide phosphodiesterase are 26 and 66 μM, respectively (Hidaka and Tanaka, 1983). Gietzen (1986), warns against attaching great significance to effective concentrations, however.

2. Calcineurin, which binds CaM with high affinity, did not affect PLC activity. It is arguable that the affinity of PLC for CaM may also be very high.

3. The effect of pH on trifluoperazine stimulation of PIP_2 PLC does not match that reported for its binding to CaM.

4. Most significantly, addition of CaM to PLC assays does not produce inhibition. Initially this point was not considered compelling, due to the presence of endogenous CaM and the possibility that PLC was saturated with CaM under assay conditions. As the relationship between CaM antagonist stimulation and enzyme concentration became clearer, it became apparent that the enzyme is not

saturated with inhibitor when small quantities are assayed. The addition of CaM under such conditions should inhibit PLC activity if CaM is the endogenous inhibitor; it does not.

Nevertheless, the behavior of the CaM antagonists are difficult to reconcile with non-specific hydrophobic interactions with PLC or its substrate. A possible solution to this dilemma appears in the form of non-CaM regulatory proteins. Although CaM is the best characterized Ca^{2+}-dependent regulatory proteins, others are also known, some structurally similar to CaM (*e. g.* troponin C) and some quite dissimilar. Many of these proteins interact with CaM antagonists in a Ca^{2+}-dependent manner; this property has been exploited in their isolation via affinity chromatography. For instance, W-7 affinity resin has been used to purify S-100 (Endo *et al.*, 1981) a CaM-like protein originally isolated from nervous tissue. Moore and Dedman (1982) isolated several proteins that bind Ca^{2+}-dependently to phenothiazine and W-7 affinity columns; they later coined the name "calcimedins" to describe these. Unlike S-100 and troponin C, these proteins differ considerably from CaM in size. Phenothiazine resins also bind S-100 and troponin C (Marshak *et al.*, 1981). Calmidazolium and compound 48/80 apparently have not been employed as affinity-resin substituents and their effects on these non-CaM proteins is unknown. It should be borne in mind that these antagonists are described as "highly specific" on the basis of the relative concentrations required to inhibit a CaM-dependent reaction and a similar CaM-independent reaction,*e. g.* the CaM-stimulated and basal activities of phosphodiesterase. Selectivity for CaM *vs.* related proteins is not implied.

Except for troponin C, the physiological functions of most of these proteins are unknown. The endogenous inhibitor of PIP_2 PLC may be such a protein. Definitive testing of this hypothesis will entail purification of the PLC and inhibitor, followed by reconstitution.

Laying aside the question of identity, there is also the question of why a Ca^{2+}-activated enzyme should have a Ca^{2+}-dependent inhibitor. Two possible functions (neither mutually exclusive nor exhaustive) that have suggested themselves are negative feedback and substrate switching.

Negative feedback: The Ca^{2+}-dependence of PIP_2 PLC has been a topic of some controversy. Some laboratories find that PIP_2 hydrolysis is not affected by variations in $[Ca^{2+}]$ in the physiological range, others report that it is (Berridge, 1984). These differnces may result from differences in tissues and/or assay techniques. *In vitro*, at least, the ROS-derived PLC responds to physiogical Ca^{2+} concentrations. As a Ca^{2+}-stimulated enzyme one of whose products elevates cytoplasmic Ca^{2+}, it would seem to possess a potential for positive feedback, allowing it to function as a "Ca^{2+} amplifier." The release of many Ca^{2+} ions could be triggered by a few. Such an amplifier would require "damping" -- some form of negative feedback to prevent runaway hydrolysis. A Ca^{2+}-dependent inhibitor could provide such feedback.

Substrate switching: The CaM antagonists promote hydrolysis of PIP_2 and inhibit hydrolysis of PI. If these effects are produced by antagonizing a Ca^{2+}-dependent regulatory protein, that protein would presumably have the opposite effect: switching the substrate preference from PIP_2 to PI. Just such a switch has been proposed in

agonist-stimulated phosphoinositide hydrolysis to account for production of diacylglycerol in excess of IP_3 (Majerus *et al.*, 1986). PI hydrolysis appears to follow PIP_2 hydrolysis. Diacylglycerol activates protein kinase C, which can produce comparatively long-term changes in cell function by protein phosphorylation. In ROS, protein kinase C phosphorylates rhodopsin, inhibiting its interaction with ROS G-protein (Kelleher and Johnson, 1986). This suggest a means of controlling light-sensitivity via protein kinase C.

It is also possible that the inhibition is exerted not by a separated protein but by the PLC itself. Formation of less-active or inactive multimers from active monomers could also account for the results presented here if the multimerization were prevented by CaM antagonists. Purification of the PLC, which is being undertaken by another worker in our laboratory, should help decide this issue.

Regardless of the identity of the endogenous inhibitor, the effects of CaM antagonists on PIP_2 PLC are significant to anyone using these compounds as probes of CaM function, especially in whole cells or crude fractions. Additionally, the stimulation of PIP_2 hydrolysis may play a role in the clinical effects of the anti-psychotic tranquilizers. Another worker in our laboratory (Frank Wilkinson) has shown that PIP_2 PLC from bovine brain is also stimulated by CaM antagonists.

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SUMMARY

The experiments presented here demonstrate the presence of a complete pathway for synthesis and hydrolysis of phosphoinositides in isolated vertebrate photoreceptor outer segments, which had not previously been demonstrated. Radioactively labeled precursors were incorporated into phosphoinositides much more rapidly than into the major phospholipids. Phosphoinositides were also hydrolyzed preferentially. Phosphoinositide synthesis and hydrolysis were also powerfully influenced by divalent metal ions and spermine, a polyamine.

Phosphoinositides are believed to play a central role in invertebrate phototransduction, and the presence of this pathway in bovine ROS suggests that they have a role in vertebrate photoreceptor function as well. However, regulation of phosphoinositide turnover in ROS appears to be unlike that in invertebrate photoreceptors, suggesting that the function of phosphoinositides in the two systems may be quite different.

In invertebrates, hydrolysis of PIP_2 is stimulated by light via a G-protein which activates PLC. No regulation by light of PIP_2 PLC was observed in ROS, nor was any clear indication of regulation by G-protein obtained in experiments using GTP, non-hydrolyzable GTP analogs, fluoride ion, added ROS G-protein, or pertussis toxin. In contrast, evidence for a previously unexplored regulatory mechanism was obtained from experiments examining the effects of calmodulin antagonists on PLC activity. ROS were found to contain an endogenous inhibitor of PIP_2 PLC, the effects of which were opposed in a Ca^{2+}-dependent fashion by calmodulin antagonists. This inhibitor appeared not to be calmodulin *per se*, but may be a member of a recently-described class of Ca^{2+}-binding proteins.

Investigation of the function and regulation of PIP_2 PLC in ROS will be complicated by the presence of multiple forms of the enzyme. PLC activity was found in soluble and particulate fractions derived from ROS, and both fractions contained multiple forms as revealed by ion exchange chromatography. Resolution and purification of the different forms of ROS PLC, along with the endogenous inhibitor, would seem to be the next logical step in the elucidation of the role of phosphoinositides in vertebrate vision.