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STUDIES OF cGMP PHOSPHODIESTERASES AND PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASES C IN BOVINE RETINAL ROD OUTER SEGMENTS

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By

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ABSTRACT

STUDIES OF cGMP PHOSPHODIESTERASES AND PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASES C IN BOVINE RETINAL ROD OUTER SEGMENTS

By

Jeffrey Robert Leipprandt

In vertebrate photoreceptors cGMP phosphodiesterase (PDE) is involved in mediating and amplifying the visual signal. Phosphoinositide-specific phospholipases C (PLCs), are implicated in signal transduction in many cell-types including invertebrate photoreceptor transduction, and may play a role in light adaptation in vertebrates as well. Evidence suggests both PDE and PLC have multiple isoforms in the bovine rod outer segment (ROS). This work was undertaken to identify these isoforms.

Peptide sequences obtained from isolated PDE α and β subunits revealed distinct differences from published sequences, suggesting multiple isoforms. Work was initiated to identify cDNA clones that code for the corresponding PDE isoforms.

Five bovine retinal PLC isoforms were identified from polymerase chain reaction products and cDNA clones. One of these is likely to have an important role in vertebrate vision because it bears striking amino acid sequence resemblance to *norp*A, a mutant β-PLC gene that results in blindness in *Drosophila*.

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ABBREVIATIONS

BHT: butylated hydroxytoluene

BME: β-mercaptoethanol

cDNA: complementary DNA

cGMP: cyclic guanosine monophosphate

DAG: diacylglycerol

EDTA: ethylenediaminetetraacetic acid

G_t: transducin

GDP: guanosine diphosphate

GTP: guanosine triphosphate

HPLC: high pressure liquid chromatography

IP_3: inositol-1,4,5-trisphosphate

MW: molecular weight

P_α: phosphodiesterase α-subunit

P_β: phosphodiesterase β-subunit

P_γ: phosphodiesterase γ-subunit

PAGE: polyacrylamide gel electrophoresis

PDE: phosphodiesterase

pfu: plaque forming unit

PIP_2: phosphotidylinositol-4,5-bisphosphate

PLC: phospholipase C

PMSF: phenylmethylsulfonylfluoride

ROS: retinal rod outer segment

SDS: sodium dodecyl sulfate

SM: phage suspension medium

sscDNA: single-stranded cDNA

TEA: tetraethylamine

TFA: trifluoroacetic acid

Tris: tris(hydroxyethyl)aminomethane

INTRODUCTION

In the retina specialized photoreceptor cells absorb light and convert it to an electrical signal by a process termed visual phototransduction. Vertebrate retinas have two morphologically distinct types of photoreceptor cells, cones and rods. Cones operate in bright light and are of three types characterized by their spectral sensitivity. Cones are responsible for color vision. Rods are of a single type and operate at low light levels. Visual biochemistry has been studied most extensively in rods because they: 1) are larger and outnumber cones in most retinas; 2) are easier to isolate and, 3) do not have the complication of multiple types.

The rods are long cells that can be divided into four defined regions (see Figure 1). The outer segment contains numerous stacked membrane disks and is the site of photo-transduction. It is attached to the inner segment by a slender ciliary connection. The inner segment contains typical cell structures such as mitochondria and endoplasmic reticulum. Next is the nucleus and finally, the synaptic terminus, which connects the rod to other neural cells of the retina [Rodieck, 1973].

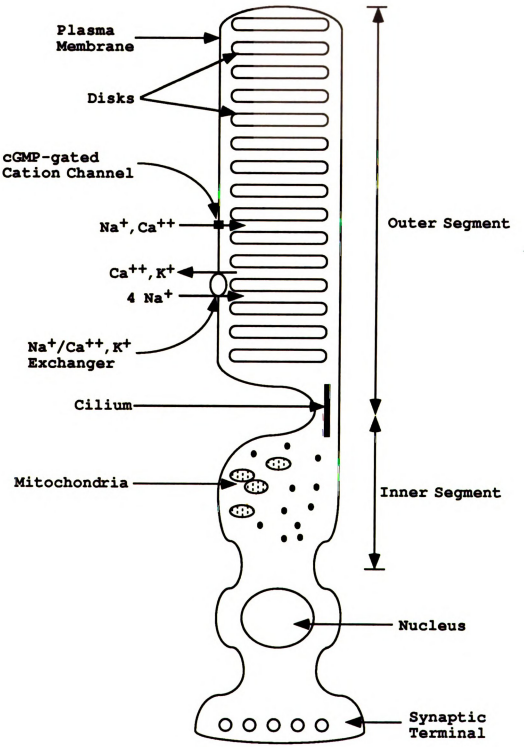
Phototransduction is initiated in the disks of the outer

Figure 1. A Rod Cell

This schematic diagram illustrates the major components of a rod cell. The cell is elongated and has four major divisions. The outer segment, the site of phototransduction, contains numerous stacked membrane disks which are not continuous with the plasma membrane. The photon receptor, rhodopsin, lies in these disk membranes. In the dark Ca^{++} and Na^{+} enter the outer segment via the cGMP-gated cation channels. Upon photon absorption, rhodopsin initiates a cascade that results in depletion of cGMP and subsequent closure of cGMP-gated cation channels. This leads to hyperpolarization of the plasma membrane and initiation of neural signaling. It also causes a decrease in cytosolic Ca^{++} which continually exits through a light-insensitive $Na^{+}/Ca^{++},K^{+}$ exchanger.

The outer segment is attached to the inner segment by a constricted ciliary connection. The inner segment contains many of the typical cellular structures and is the site of most of the biosynthetic pathways. Additional cellular constrictions separate the inner segment from the nucleus and the nucleus from the synaptic terminal.

Figure 1.


Plasma Membrane
Disks
cGMP-gated Cation Channel
Na^+, Ca^{++}
Ca^{++}, K^+
4 Na^+
$Na^+/Ca^{++}, K^+$ Exchanger
Cilium
Mitochondria
Outer Segment
Inner Segment
Nucleus
Synaptic Terminal

segment when light is absorbed by the visual pigment, rhodopsin. Rhodopsin consists of an integral membrane protein, opsin, and a covalently attached chromophore, 11-*cis*-retinal. When a photon is absorbed by 11-*cis*-retinal, it isomerizes to all-*trans*-retinal, activating the rhodopsin molecule by causing conformational changes [Dratz & Hargrave, 1983].

In its active form rhodopsin activates a GTP-binding protein known as transducin or G_t. This enzyme is a peripheral membrane protein composed of three subunits, $G_{t\alpha}$(40 kDa), $G_{t\beta}$(35 kDa), and $G_{t\gamma}$(8 kDa). It is a member of a well characterized family of GTP-binding proteins including the G_s and G_i regulatory proteins of the hormone-sensitive adenylate cyclase. Rhodopsin activates G_t by mediating the exchange of GDP for GTP on $G_{t\alpha}$ and dissociating it from the $G_{t\beta\gamma}$ complex. A single activated rhodopsin can activate as many as 500 G_t molecules [Stryer, 1986].

The active $G_{t\alpha}$-GTP complex in turn activates the cGMP phosphodiesterase (PDE). This enzyme is also a peripheral membrane protein that contains three different types of subunits, P_α(88 kDa), P_β(85 kDa), and P_γ(12 kDa) [Kohnken *et al*. 1981]. The PDE is activated when the $G_{t\alpha}$-GTP complex removes the inhibitory P_γ [Stryer, 1986].

The active PDE hydrolyzes the 3′-5′ phosphodiester bond of cGMP to form 5′-GMP. A single PDE is capable of hydrolyzing over 1000 cGMP molecules per second. Thus in this

entire cascade the signal can be amplified as much as 10^6 [Baehr & Applebury, 1986]. In phototransduction cGMP has the role of a soluble second messenger between the disk membranes and the plasma membrane. In the dark cGMP levels are high and the molecules act as allosteric effectors keeping open cGMP-gated cation channels in the plasma membrane. In the light the active PDE depletes the cGMP thus causing the closure of the cation channel. This closure in turn results in the hyperpolarization of the rod cell membrane and the initiation of the neural relay to the brain [Lamb, 1986].

CHAPTER I

cGMP PHOSPHODIESTERASES

LITERATURE REVIEW

The light activated cGMP PDE from the ROS plays a central role in the visual cascade [Pober and Bitensky, 1979]. It consists of three different subunits denoted P_α, P_β, and P_γ, which are 88, 85, and 12 kDa respectively [Baehr *et al.* 1979; Kohnken *et al.* 1981a]. In darkness there is very little PDE activity in the ROS but when light impinges on the photoreceptor, PDE activity is dramatically increased. This activation has been demonstrated to be caused by removal of the inhibitory P_γ, mediated by the active $G_{t\alpha}$-GTP complex [Hurley and Stryer, 1982]. Though the stoichiometry of the isolated PDE has been determined to be 1:1:2, P_α:P_β:P_γ [Deterre *et al.* 1988], this may not strictly be the case for the enzyme *in vivo*. Hurwitz *et al.* [1985] presented evidence that $P_{\alpha\gamma}$ or $P_{\beta\gamma}$, complexed with $G_{t\alpha}$-GTP, retained PDE activity. While this report did not indicate the subunit makeup of the PDE holoenzyme, it did suggest that P_α and P_β may be subunits of two distinct PDE isoforms, or at the very least are able to act independently.

Complementary DNA sequences have been published for all three of the PDE subunits, P_α [Ovchinnikov *et al.* 1987; Pittler *et al.* 1990], P_β [Lipkin *et al.* 1990], and P_γ

[Ovchinnikov *et al.* 1986]. The cDNAs for P_α and P_β code for quite similar proteins (72% sequence identity); however, the evidence that the cDNAs actually code for their designated subunits is not completely convincing. First, both cDNAs predict subunits of significantly higher MW than either P_α or P_β as revealed by SDS-PAGE, 99 *vs.* 88 kDa for P_α, and 98 *vs.* 85 kDa for P_β. Second, neither sequence is consistently corroborated by peptide sequences. At first glance the amino acid sequences deduced from the cDNAs appear to be well supported by numerous direct peptide sequences, 38 for P_α [Ovchinnikov *et al.* 1987] and 27 for P_β [Lipkin *et al.* 1990]. However, careful reading of the texts reveal that the vast majority of those peptides were derived from undifferentiated $P_{\alpha\beta}$. In fact only four peptides were derived from purified subunits, of which only two were actually sequenced, both from P_β. One of these was almost identical with its P_α counterpart. The other two peptides, from P_α, in too low a yield for sequencing merely had compositions from amino acid analysis that matched deduced peptides from the cDNA. However, these also were almost identical with their P_β counterparts. Thus confirmation that these cDNAs code for P_α and P_β hinges almost exclusively on the fact that a single sequenced P_β peptide could not be found in a P_α digest from which no peptides were sequenced. Nor do the authors mention if any peptide sequences were obtained that did not match either the P_α or P_β deduced amino acid sequences.

The authenticity of the cDNAs is further cast in doubt when considering the evidence for multiple PDE isoforms in the ROS. Hurwitz *et al.* [1985] first indicated that multiple isoforms exist on the basis of HPLC fractionated PDE activity and immunological crossreactivity. Their existence is also implied by two cDNA clones reported by Pittler [Ph.D. Thesis, 1989; Pittler *et al.* 1990]. These clones, which are not full length, are almost identical to the Ovchinnikov/Pittler P_α sequence, but each codes for a different C-terminal region diverging at amino acid 841, approximately the same place the published sequences diverge.

The research reported on the following pages was performed at the time that much of this information was coming to light. As originally proposed, part of this project was to be in conjunction with the work done by Steve Pittler, providing peptide sequence information from purified P_α and P_β by which cDNA clones could be identified. However, when differences between peptide and cDNA sequences arose that were not easily rectified the two projects were separated. Continuation of this project proved to be quite challenging but insightful, leading to proposals for research that may reconcile sequence discrepancies and provide an assured basis for subsequent research.

MATERIALS AND METHODS

Materials. Radiolabeled compounds were purchased from New England Nuclear. DE-52 cellulose was purchased from Whatman. HPLC grade acetonitrile was purchased first from Aldrich and later from Burdick and Jackson. TFA and TEA were purchased from Pierce. Oligo d(T) cellulose was purchased from Stratagene. *Taq* polymerase was purchased from Cetus. DNA modifying enzymes were purchased from either New England Biolabs, Bethesda Research Labs, or Boehringer Mannheim. Reagents for media preparation were purchased from Difco. All other chemicals were purchased from Sigma.

ROS Isolation. Bovine eyes were obtained from Murco, Inc. of Plainwell, MI. They were removed within minutes of slaughter, packed on ice, and kept dark. All subsequent operations were performed under dim red light and on ice. Within 24 hours the retinas were dissected from the eyes. ROS were prepared by the method of Kohnken *et al.* [1981a]. Briefly, isolated retinas were vigorously shaken in buffered 1.32 M sucrose, 150 mM KCl, shearing the ROS at their ciliary connection, to the inner segments. After centrifugation at 1100 x g the supernatant was diluted three-fold with buffered 150 mM KCl and centrifuged at 2100 x g. The pellet was

resuspended in buffered sucrose-KCl, ρ=1.10, and applied to a ρ=1.12 to 1.16 sucrose density step gradient. After centrifugation at 90,000 x g, ROS were collected from bands at the interface of gradient steps ρ=1.10 to 1.12 and ρ=1.12 to 1.14 and stored in liquid nitrogen until use.

cGMP PDE Isolation. The PDE was purified by the methods of Kohnken *et al.* [1981a] with modifications inspired by Kühn [1982]. ROS was diluted with 10 volumes of 250 mM sucrose, 150 mM KCl, 10 mM Tris-Cl, pH 8.0, 10 mM BME, 1 mM EDTA, 0.1 mM PMSF, 0.01% BHT (solution A, isotonic) and centrifuged at 15,000 x g. The pellets were resuspended in a minimal amount of 10 mM Tris-Cl, pH 8.0, 1 mM $MgCl_2$, 1 mM DTT (solution M, hypotonic), mixed, diluted to 500 ml with solution A, and centrifuged at 15,000 x g. The pellets were resuspended in minimal solution M, homogenized, bleached with bright light, diluted to 300 ml with solution M, and centrifuged at 90,000 x g. The supernatants were collected and the pellets were resuspended, homogenized, diluted, and centrifuged twice more pooling the supernatants.

A Whatman DE-52 cellulose column (1 x 8 cm) was equilibrated with solution M. The supernatants were applied and then the proteins were eluted with a 200 ml linear NaCl gradient (0-500 mM) in solution M. Fractions (5 ml) were collected and assayed for absorbance at 280 nm. Peak fractions containing the PDE were pooled and concentrated to 2 ml with an Amicon concentrator and YM-5 membrane.

A Sephadex G-100 column (1.2 x 115 cm) was equilibrated with solution M. Then the concentrated DE-52 pool was applied to the column and eluted with solution M. Fractions (3 ml) were collected and assayed for absorbance at 280 nm. A_{280} peak fractions with elution times the characteristic of the PDE were pooled and concentrated to 1.5 ml. The fractions were analyzed by analytical SDS-PAGE and protein content determined by the method of Lowry *et al.* [1951].

Gel Filtration HPLC was conducted using a Waters HPLC system employing a Zorbax Bio-Series GF-250 gel filtration column (DuPont). Material applied to the column was eluted with 10 mM Tris-Cl, pH 7.4, 1 mM DTT at a flow rate of 1 ml/min. The eluants were monitored for absorbance at 280 nm. Freshly prepared, soluble samples of PDE applied to the column eluted in the column's void volume indicating an aggregate greater than 500 kDa was present. There was essentially no material that eluted at a retention time indicative of the separate P_α or P_β. Neither was there a peak that might suggest a few subunits binding together as a holoenzyme. There was, however, a sharp peak with the retention time of a 10 kDa protein, most likely representing P_γ. Considering that only fairly harsh denaturing conditions could solubilize the PDE, preparative SDS-PAGE seemed like the best technique for $P_{\alpha\beta}$ separation.

SDS-PAGE. Some early analytical SDS-PAGE was performed in 10% acrylamide/0.267% bis-acrylamide as described by

Kohnken *et al.* [1981a]. The proteins were visualized with Coomassie brilliant blue [Gordon, 1975] or by silver staining [Wray *et al.* 1981]. To improve the separation of P_α and P_β, preparative and subsequent analytical SDS-PAGE were performed in 15% acrylamide/ 0.08% bis-acrylamide as described by Baehr *et al.* [1979] (see Figure 1A). Preparative gels were stained by gentle shaking in 0.25 M KCl at 0° for 10 to 30 min. Viewing was aided by a dark background and strong fluorescent side lighting. Bands were cut from the gel with a scalpel or sharp scissors.

Electroelution. The device used was an Elutrap electro-separation chamber (Schleicher & Schuell). It was set up so that when gel strips were placed in one of the Elutrap's chambers and voltage was applied, proteins migrated from the gel into a second, low volume chamber (the trap). The trap was bounded on the far end by a BT-1 membrane which allowed only small ions, driven by the voltage drop, to pass. It was bounded on the near end by a BT-2 membrane which allowed any charged, soluble ions, including proteins, to pass, under an applied voltage.

The Elutrap was placed in a horizontal gel electro-phoresis chamber filled with buffer (192 mM glycine, 25 mM Tris base). The Elutrap's chambers were also filled with buffer and gel slices containing individual KCl-stained bands were placed in the larger of the two chambers. A potential of 200 V was applied for about six hours with the positive pole

Figure 1. Separation of P_α and P_β.

A. This photograph depicts a SDS-polyacrylamide gel with a typical separation of preparative amounts of P_α and P_β which were identified by their characteristic mobilities. Between 0.5 and 1.0 mg of purified bovine ROS cGMP PDE was applied to a preparative 15% polyacrylamide, 0.08% bis-acrylamide gel (15 x 15 cm, 1.5 mm thick). The optimum amount of $P_{\alpha\beta2\gamma}$ applied to the gel was 500-1000 μg. More material overloaded the gel and hindered the separation and less material resulted in poor visualization by KCl-staining. It was run at 150V until P_α and P_β had migrated more than halfway down the gel, ~24 hr. The KCl-stained bands were viewed against a dark background. The bands were cut apart with a scalpel, trimmed away from the rest of the gel, and eluted as described in the Methods section.

B. The eluted P_α and P_β were analyzed for the completeness of their separation from each other by subjecting samples to analytical SDS-PAGE using the same gel composition as above. The bands on this gel, however, are visualized using the much more sensitive silver staining techniques as described in Methods. Lanes 1 and 7 are material from a DE-52 fraction of the PDE isolation protocol which contains both the PDE and G_t, used here as MW indicators. Lanes 2 and 6 contain well-separated P_α and P_β fractions, respectively. Lanes 3 and 4 show P_α fractions that were not well separated from P_β and lane 5 shows a P_β fraction that was not well separated from P_α. Only fractions that were very pure, like those in lanes 2 and 6 were used in subsequent analyses.

Figure 1A.

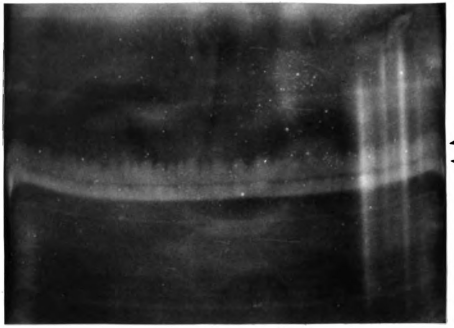
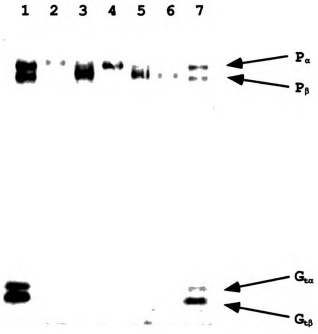


Figure 1B.



toward the trap end of the Elutrap. The contents of the trap were then put directly into dialysis tubing (10-12 kDa cutoff) and dialyzed against distilled water.

The yield of the elution was never very good, 50% at best and sometimes as low as 10%. Eluted P_α and P_β were analyzed for the quality of separation from each other by SDS-PAGE (see Figure 1B).

Carboxymethylation. The separated P_α and P_β were carboxymethylated or ^{3}H-carboxymethylated as described by Glazer *et al.* (1975).

Cyanogen Bromide Cleavage. The protocol was essentially that of Erhard Gross [1967]. The carboxymethylated protein was dissolved in 0.1 N HCl. CNBr was added in a 100-fold excess of the estimated methionine content of the sample and the reaction was allowed to proceed for 24 hr. at room temperature in the dark. The solution was then frozen and lyophilized. For P_α and P_β, this resulted in a yellow, largely insoluble material in both cases. These were intractable tars that yielded no detectable protein upon amino acid analysis and could not be resolved by 2-dimensional electrophoresis.

Tryptic Digestion. Carboxymethylated proteins in 10 mM NH_4HCO_3 were incubated at 37°. Bovine trypsin was dissolved in 0.4 M NH_4HCO_3 at a concentration of 100 μg/ml. An amount of trypsin, equal to 1% of the weight of the carboxymethylated protein, was added and the mixture was incubated at 37° for 3 hr. An equal amount of trypsin was added again and the 37°

incubation was continued overnight.

HPLC Purification of Tryptic Peptides. Initial fractionations of the P_α and P_β tryptic digests were performed using a Waters μBondapak C_{18} column with an 80 min. linear 1%/min gradient of acetonitrile/0.1% TFA in H_2O/0.1% TFA. Fractions were collected by hand, changing Eppendorf tubes between A_{214} peaks. Both digests yielded similar but not identical chromatographic profiles with numerous peaks. Individual tryptic peptides were purified in one or more additional chromatographic steps using the Waters μBondapak C_{18} column or a narrow bore Vydac Protein and Peptide C_{18} column. Fractions were rechromatographed in one of two different organic gradients: 1)a 1%/min. gradient of acetonitrile/0.1% TEA in H_2O/0.1% TEA, or 2)a 1%/min. gradient of 65:20:15 acetonitrile:2-propanol:H_2O in H_2O/0.1% TFA. Some peptides were further purified using one or more of the above solvent systems. When chromatograms revealed sharp isolated peaks without shoulders, peptides were considered pure. Fractions were dried on a Speed Vac concentrator and submitted for sequencing.

Peptide Sequencing. Some of the peptides were submitted to the Macromolecular Sequence, Synthesis, and Structure Facility (MSSSF), Department of Biochemistry, Michigan State University, where they were sequenced on a Beckman System 890 M protein sequencer. Other peptides were submitted to the Protein Structure Lab, University of California, Davis, where

they were sequenced on an Applied Biosystems 470 gas phase protein sequencer.

Oligonucleotide Synthesis and Purification. Degenerate oligonucleotides based on peptide sequences were synthesized by the MSSSF, Department of Biochemistry, Michigan State University on an Applied Biosystems 380B DNA synthesizer. The crude synthetic oligonucleotides were purified by HPLC using a Zorbax Bioseries Oligo column (DuPont). The column was equilibrated with 80% 50 mM $NaPO_4$, pH 7.0, 20% acetonitrile, 0.1 M NaCl. The oligonucleotides were applied and then eluted with a linear 0.1 to 0.8 M NaCl gradient in the same solvent in 70 min. Peak A_{260} fractions were pooled and desalted using C_{18} Sep Paks (Waters) as described by Atkinson and Smith [1984].

RNA Isolation. RNA was isolated by the method of Chirgwin *et al.* [1979] with modifications by Dr. Bill Helferich [personal communication, 1989]. Bovine eyes were obtained from Murco, Inc of Plainwell, MI. Within 3 min. of slaughter the retinas were removed from the eyes and dropped into liquid nitrogen. Frozen retinas were crushed in a supercooled tissue grinder and homogenized in a solution of 4 M guanidine isothiocyanate, 25 mM sodium citrate, pH 7.0, 100 mM BME, 0.5% N-lauryl sarcosine, and then centrifuged at 5,000 x g. The supernatant was put into ultracentrifuge tubes layered on top of pads of 5.7 M CsCl, 100 mM EDTA and then centrifuged in a SW 55 TI rotor at 35,000 rpm for 20 hr. The

pellets were resuspended in 7 M guanidine HCl, 20 mM sodium acetate, pH 7.0, 1mM DTT, 10 mM iodoacetic acid, 1 mM EDTA, and then ethanol precipitated. Pellets were washed with 3 M sodium acetate, 10 mM iodoacetamide, pH 5.0; 66% ethanol, 33 mM sodium acetate, pH 5.0, and then 100% ethanol. Absorbance spectra, 320 nm to 220 nm, were taken from samples of the RNA to assess purity. A_{260}/A_{280} ratios were routinely >1.8. The integrity of the RNA was assessed by formaldehyde-1% agarose gel electrophoresis as described by Fourney *et al.* [1988].

Poly A^+ RNA Isolation. Poly A^+ RNA was isolated from total RNA by oligo d(T) cellulose chromatography as described by Sambrook *et al.* [1989].

cDNA Synthesis. Single stranded cDNA (sscDNA) was initially synthesized using bovine retinal poly A^+ RNA, oligo $d(T)_{12-18}$ (Pharmacia), and M-MLV reverse transcriptase (BRL) applying protocols provided by BRL. Subsequently sscDNA was synthesized using specific antisense primers as described by Lee and Caskey [1990].

Polymerase Chain Reaction. Thermal cycling was performed with a DNA Thermal Cycler (Perkin Elmer Cetus). PCR was carried out by protocols developed by Perkin Elmer Cetus with empirically determined adjustment made for particular amplifications. Typical reaction conditions were 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM each dNTP, 1 μM sense and antisense primers, 40 units/ml *Taq* polymerase, and 1-200 ng sscDNA. The reaction mixtures were layered with

mineral oil and put through a heating regimen that typically included a 3-5 min. 94° initial denaturation step, followed by 25-30 thermal cycles containing a 1 min. 94° denaturation step, a 2 min. 37° primer annealing step, and a 3 min. 72° elongation step.

Agarose Gel Electrophoresis was performed essentially as described by Sambrook *et al.* [1989] except that agarose concentration as high as 5% was routinely used to provide resolution of DNA as small as 50 bp. Such gels were made as lower percentage gels but required careful heating in a microwave oven. Gels and chamber buffers were 1 x TAE, 0.5 μg/ml ethidium bromide.

Recovery of DNA from Agarose Gels was accomplished by the band interception technique originally developed by Winberg and Hammarskjold [1980] using NA-45 DEAE membranes (Schleicher & Schuell). After DNA was sufficiently separated on an agarose gel, the gel was cut just ahead of the band of interest so that a pre-equilibrated NA-45 membrane could be inserted. Then the gel was returned to the buffer chamber and electrophoresed until the DNA ran into the membrane. The membrane was briefly washed in 0.15 M NaCl, 0.1 mM EDTA, 20 mM Tris-Cl, pH 8.0. and then the DNA was eluted by incubating the membrane at 60° for 30 min. in two washes of 1.0 M NaCl, 0.1 mM EDTA, 20 mM Tris-Cl. The solution was extracted with three volumes of water-saturated n-butanol to remove residual ethidium bromide and precipitated by adding 2.5 volumes

ethanol followed by immersion of the tube in crushed dry ice for 10 min.

DNA Cloning. DNA was digested with restriction enzymes as described by Sambrook *et al.* [1989] or as recommended by vendors. Purified PCR products were ligated, with ends either blunt or 5′ overlapped, into pUC19 using protocols from Ausubel *et al.* [1987]. Recombinant DNA was used to transform DH5α *E. coli* and DNA was prepared from the resulting transformants by methods of Sambrook *et al.* [1989]

DNA Sequencing. The nucleotide sequence of cloned DNA was determined using Sequenase version 2.0 modified bacteriophage T7 DNA polymerase and its accompanying reagents and protocols with [^{35}S]-dATPαS and M13 -40 primer. Reaction products were separated on a 60 cm, 7 M urea, 8% polyacrylamide gel.

λgt10 cDNA Library Plating. A bovine retinal λgt10 cDNA library (Clontech) was diluted with SM to 2.5 x 10^5 pfu/ml. 100 μl aliquots were mixed with 300 μl SM and 600 μl of an overnight culture of Y1090 *E. coli* grown in LB broth plus 0.2% maltose. The mixture was incubated at 37° for 20 min. Then 10 ml aliquots of molten (45°) LB soft top agarose were added, mixed, and poured onto dry, 37°, 150 mm LB agar plates. After the top agarose hardened the plates were inverted and incubated at 37° until the plaques were 0.1-0.2 mm in diameter (typically 4-5 hr.). Then the plates were stored at 4°.

Library Replicas. BA85 (Schleicher & Schuell) nitro-

cellulose filters (132 mm) were wetted in 5x SSC, blotted on filter paper, and placed for 5 min on the surface of plates at 4° containing plaques. Second and third lifts were left for 7 and 9 min, respectively. Filters were removed and placed plaque-side-up on 2 sheets of 3MM paper saturated with 0.5 N NaOH, 1.5 M NaCl for 5 min. Filters were transferred to 2 sheets of 3MM paper saturated with 0.5 M Tris-Cl,pH 8.0, 1.5 M NaCl for 5 min, then to 2 sheets of 3MM paper saturated with 2x SSC for 5 min. Filters were blotted and air dried on 3MM paper, then dried in a vacuum oven at 80° for 1 hr. Finally, the filters were stored at room temperature in a vacuum desiccator.

Probe Labeling. Oligonucleotides were end-labeled using T4 polynucleotide kinase and [γ-^{32}P] ATP by the method of Ausubel *et al.* [1987].

Hybridization with Oligonucleotide Probes. Nitrocellulose filters containing plaque replicas were wetted and washed 3 times for 10 min each in 3x SSC, 0.1% SDS at room temperature, and once in the same solution at 65° for 1 hr. Southern blots were simply wetted in the same solution at room temperature. All filters, regardless of type, were next prehybridized for 1 hr at 37° with gentle shaking in 6x SSC, 5x Denhardt's solution, 0.05% Na pyrophosphate, 100 μg/ml denatured herring sperm DNA, and 0.5% SDS. Filters were removed from the prehybridization solution and put into heat-sealable bags with 6x SSC, 1x Denhardt's solution, 100 μg/ml

yeast tRNA, 0.05% Na pyrophosphate, and approximately 1 ng/ml end-labeled oligonucleotide at >5 x 10^5 cpm/ml. Filters were hybridized 24-48 hr at 50°. Then they were washed 3 times at room temperature in 6x SSC, 0.05% Na pyrophosphate for 10 min each and 1 time at 65° in the same solution for 30 min. Damp filters were resealed in heat-sealable bags, mounted on filter paper, and fluorographed. This protocol was adapted from Ausubel *et al.* [1987].

Southern Blot Analysis was performed by the methods of Sambrook *et al.* [1989].

RESULTS

Peptide Sequences. As the P_α and P_β tryptic peptides were purified they were submitted to either the Macromolecular Sequence, Synthesis, and Structure Facility, Department of Biochemistry, Michigan State University or to Alan J. Smith, Protein Structure Lab, University of California, Davis, for N-terminal sequence analysis. The first three peptides submitted, α-43-6, α-29-9, and α-42-16, did not yield any sequence, each appearing to be obscured by a contaminant. The source of the contaminant turned out to be a bad batch of "HPLC grade" acetonitrile. After switching vendors there was considerably more success at sequencing peptides. There were an additional nine α and nine β peptides submitted. Of those, 12 yielded peptide sequence, one yielded two independent peptide sequences, and one yielded a very unsure three residues (see Table 1). Of the other four, three yielded no detectable sequence and one was lost from the sequencer during a power failure at MSU.

The ^3H labeled peaks from the chromatograms were easily located but were significantly smaller than other peaks. When sequencing was attempted on some of these the amounts proved to be too small. This seemed to indicate that labeling by

Table 1. Peptide Sequence Similarity

This table reveals the sequences of numerous P_α and P_β tryptic peptides and their similarity with published P_α, P_β, and $P_{\alpha'}$ deduced amino acid sequences. The peptides are identified in the two left-hand columns. The α- and β- prefixes indicate that the peptides were derived from P_α and P_β respectively, which were physically separated on denaturing gels, stained with KCl, and cut apart with a razor blade. After electroeluting from the gel slices the subunits were carboxymethylated and then digested with trypsin. Numerous tryptic peptides were separated by C_{18} reverse phase HPLC and several of those were sequenced by Edman degradation. Residues that could not be determined are designated by a dash (-). Residues that could not be determined with complete confidence are bracketed. In the three right hand columns the peptides are compared with the published deduced amino acid sequences for P_α, P_β, and cone cell $P_{\alpha'}$ from Ovchinnikov *et al.* [1987], Lipkin *et al.* [1990], and Li *et al.* [1990] respectively. Comparisons without significant similarity between published and peptide sequences were marked with two dashes (--).

* Tentative partial match with P_α residues 483-502. At residue 5 a determination between proline or leucine could not be made.

Table 1.

Peptide ID	Sequence	Sequence Similarity Rod P_α	Rod P_β	Cone $P_{\alpha'}$
α-24-3-6	DFQDNLQAEK	100%	40%	--
α-26-6-7	EINFYK	100%	83%	66%
α-28-16-9	VIDYILHGK	100%	100%	89%
α-32-6-5-3*	VFEQ[P,L]NMILQGDAIV[TS-QS]	30%	--	--
α-36-12-4	FLDQNPGFADQY	67%	100%	50%
α-44-1,2-5-2	VALLVAAEF-E	100%	100%	90%
α-44-6-8	[VIV]	--	--	--
β-23-4	SQNPLAK	100%	100%	57%
β-23-5	AAELPK	83%	100%	66%
β-23-7	V[T]FK	--	--	--
β-30-9a	LFNVHK	100%	50%	33%
β-30-9b	AFLNCDR	100%	67%	57%
β-41-1	VAAEFWEQGDLER	100%	100%	92%
β-41-2R	FHEEI-PM	100%	100%	86%
β-46-12	VLAD[T]ELWI	--	--	--

carboxymethylation did not occur as expected. In addition one of the unlabeled peaks, β-30-9-5, yielded a sequence with an unlabeled carboxymethylcysteine.

The comparisons in Table 1 reveal that while some of these peptides corresponded to published sequences of either the P_α and P_β, some, including the longest P_α peptide and the second longest P_β peptide, corresponded to neither. These nonmatching peptides were isolated as major HPLC peaks. They cannot be dismissed as peptides from contaminating proteins, nor do they match P_γ [Ovchinnikov *et al.* 1986], cone cell $P_{\alpha'}$ [Li *et al.* 1990], or bovine trypsin [Keil, 1971].

In addition, two of the P_β peptides matched the published P_α sequence but only partially matched the published P_β sequence. The converse was true for one of the P_α peptides. These results indicated that while the parent subunits of these peptides were closely related to the published sequences, neither one was actually encoded by them. Even though the published P_α and P_β cDNAs almost certainly code for authentic cGMP PDE subunits, other isoforms most likely exist in significant quantities.

<u>Polymerase Chain Reaction.</u> Three PCR primers were synthesized corresponding to the peptide, α-32-6-5-3 (see Figure 2A), and used in PCRs as described in Methods. This resulted in a product of the expected size, 60 bp, seen as a minor band on an agarose gel (see Figure 3). Interestingly, the two major products, 120 and 180 bp, were two and three

Figure 2. PCR Primers.

A. The initial PCR primers were designed corresponding to opposite ends of peptide α-32-6-5-3, VFEQ[PorL]NMILQGDAIV[TS-QS]. It was chosen because it was the longest peptide and it had only limited similarity to the published P_α deduced amino acid sequence [Ovchinnikov *et al.* 1987]. The primers were designed with restriction sites to aid in subsequent cloning of the products and 14 bases of degenerate sequence corresponding to amino acid sequence from the peptide as was done by Lee *et al.* [1988]. To reduce the amount of degeneracy in these mixed oligonucleotide primers a bovine rod cell codon utilization table (2,511 codons from 8 rod cell cDNA sequences) was created (see Table A1 in the Appendix, p.98) and used to eliminate some of the least often-used codons. This table used only rod cell sequences to eliminate the effects of tissue-specific differences in codon usage [Newgard *et al.* 1986]. The sense primers corresponded to the five N-terminal amino acids of the peptide. Because of the ambiguity of the fifth amino acid 2 different sense primers were made. On the 5′ end of each there was a *Hin*dIII restriction site and both had a 16-fold degeneracy. The antisense primer was made corresponding to amino acids 11 though 15 of the peptide, the last 5 certain amino acids. On the 5′ end there was an *Eco*RI restriction site. It had a 24 fold degeneracy. All the primers are lined up with the corresponding amino acid sequence above them. Nucleotides below the primer line designate the sites of oligonucleotide degeneracy.

B. From several P_α peptides additional PCR primers were designed. In an effort to make these primers more specific for their target sequences the restriction sites on the 5′ ends were omitted, leaving each oligomer with 20 bases that corresponded to the peptides. It was thought these 5′ noncomplementary portions of the primers might hinder initial annealing to the target templates thus favoring nonspecific interaction resulting in more artifacts. Sense and antisense primers were again produced corresponding to α-32-6-5-3, but this time, both corresponded to the internal region. One primer, Sense$^{\alpha\text{-}26 \text{ and } \text{-}28}$, was produced corresponding to two peptides, α-26-6-7 and α-28-16-9, that were found to be next to each other in the published sequence [Ovchinnikov *et al.* 1987].

Figure 2A. Initial PCR Primers

Sense Primer #1		Val Phe Glu Gln Pro
	5′ CAAGCTTG	GTC TTC GAA CAA CC 3′
	Hind III	G T G G
Sense Primer #2		Val Phe Glu Gln Leu
	5′ CAAGCTTG	GTC TTC GAA CAA CT 3′
	Hind III	G T G G
Antisense Primer		Val Ile Ala Asp Gly
	5′ GGAATTCC	AC AAT AGC ATC CCC 3′
	Eco RI	G G G G
		T

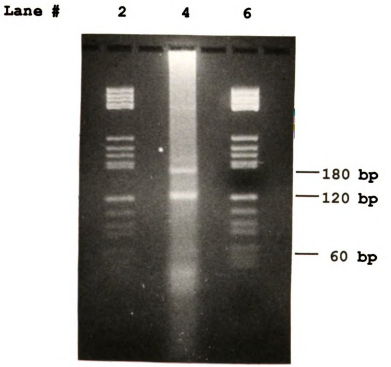
Figure 2B. Additional PCR Primers

1) Sense$^{\alpha\text{-}32\text{-}6\text{-}5\text{-}3}$		Asn Met Ile Leu Gln Gly Asp Ala
	5′ AAC ATG ATC CTC CAG GGC GAC GC 3′	
		G G
2) Antisense$^{\alpha\text{-}32\text{-}6\text{-}5\text{-}3}$		Ala Asp Gly Gln Leu Ile Met Asn
	5′ GC GTC CCC CTG CAG GAT CAT GTT 3′	
		G G
3) Sense$^{\alpha\text{-}36\text{-}12\text{-}4}$		Gln Asn Pro Gly Phe Ala Asp Gln Tyr
	5′ CAG AAT CCC GGC TTT GCC GAC CAG TA 3′	
		G G
4) Sense$^{\alpha\text{-}24\text{-}3\text{-}6}$		Asp Phe Gln Asp Asn Leu Gln Ala
	5′ GAC TTT CAG GAC AAC CTG CAG GC 3′	
5) Sense$^{\alpha\text{-}26 \text{ and } \text{-}28}$		Asn Phe Tyr Lys Val Ile Asp Tyr
	5′ AAC TTT TAC AAG GTC ATT GAC TA 3′	
6) Antisense$^{\alpha\text{-}44\text{-}1,2\text{-}5\text{-}2}$		Glu Trp Phe Glu Ala Ala Val Leu Leu
	5′ TC CCA GAA TTC AGC AGC AAC CAG CAG 3′	

Figure 3. Initial PCR Products

Three PCR primers, two sense and one antisense, were created corresponding to peptide α-32-6-5-3 and used with oligo d(T)-primed sscDNA in PCR. The reaction mixture was extracted with phenol/$CHCl_3$, ethanol precipitated, dissolved in H_2O, and fractionated on a 5% agarose gel in 1x TAE, 0.5 μg/ml ethidium bromide in lane 4. Molecular weight markers (1 μg pBR322 *Hae*III digest) were run in lanes 2 and 6. Visualization revealed a faint band at 60 bp, the size of the expected product, and predominant bands at 120 and 180 bp.

Figure 3.



times the length of the expected product. There also was a smear of background products and some other minor products. Three bands, 60, 120, and 180 bp, were intercepted with, and then eluted from NA-45 membranes (Schleicher and Schuell). This material was cloned into pUC19 using both cohesive end and blunt end ligations. Several different clones were found with approximately 60 bp inserts but upon sequencing none was found to code for the peptide sequence of interest. No clones were recovered containing the 120 bp insert and only one clone from the 180 bp ligations was found. However its sequence did not code for the peptide nor did it have any ORF coding for a PDE-like sequence.

Six additional primers were synthesized corresponding to various P_{α} peptides (see Figure 2B). Three different bovine retinal sscDNA templates were used in these PCRs, one primed with oligo d(T) and the others primed with one of the two antisense primers. Many different combinations of primers and templates were tried, each yielding a variety of products, most under 500 bp long (see Figure 4). Those PCRs that utilized primers matching the published sequences did not yield products of the expected sizes (2190, 2034, and 1293 bp in Figure 4, lanes 3, 5, and 7 respectively). Still, several bands from other PCRs were isolated from agarose gels. Direct sequencing attempts of the bands were frustrated by the presence of multiple templates. It was concluded that this approach could not succeed unless PCR products were

Figure 4. PCR Using Additional Primers

The PCR products fractionated on a 1% agarose gel illustrate some of the numerous combinations of primers. In this case the PCR primers listed in Figure 2B were used in the relevant combinations with sscDNA primed with the same antisense primer used in the PCR. Lane#1 contains 500 ng λ DNA-*Eco*RI-*Hin*dIII digest. The following lanes contain PCR products created using primers identified in Figure 2B:

Lane#2- Sense$^{\alpha\text{-}32\text{-}6\text{-}5\text{-}3}$ and Antisense$^{\alpha\text{-}44\text{-}1,2\text{-}5\text{-}2}$

Lane#3- Sense$^{\alpha\text{-}36\text{-}12\text{-}4}$ and Antisense$^{\alpha\text{-}44\text{-}1,2\text{-}5\text{-}2}$

Lane#4- Sense$^{\alpha\text{-}36\text{-}12\text{-}4}$ and Antisense$^{\alpha\text{-}32\text{-}6\text{-}5\text{-}3}$

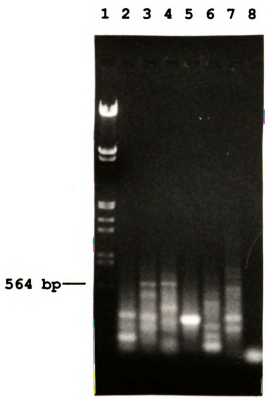
Lane#5- Sense$^{\alpha\text{-}24\text{-}3\text{-}6}$ and Antisense$^{\alpha\text{-}44\text{-}1,2\text{-}5\text{-}2}$

Lane#6- Sense$^{\alpha\text{-}24\text{-}3\text{-}6}$ and Antisense$^{\alpha\text{-}32\text{-}6\text{-}5\text{-}3}$

Lane#7- Sense$^{\alpha\text{-}26 \text{ and } \text{-}28}$ and Antisense$^{\alpha\text{-}44\text{-}1,2\text{-}5\text{-}2}$

Lane#8- Sense$^{\alpha\text{-}26 \text{ and } \text{-}28}$ and Antisense$^{\alpha\text{-}32\text{-}6\text{-}5\text{-}3}$.

Figure 4.



individually trapped by cloning, as was successfully accomplished later on with the phospholipase C probes.

Oligonucleotide "Guessmer" Probe. Without immediate success at PCR, a longer, less degenerate oligonucleotide "guessmer" probe, also based on peptide α-32-6-5-3, was synthesized. Such probes have been touted as a better alternative to using short highly degenerate mixed probes [Ausubel *et al.* 1987; Wood, 1987]. The best codons were determined using the bovine rod cell codon utilization table (see Table A1, p.98). This resulted in a synthetic 41 base oligonucleotide that was degenerate only at the position where the amino acid was uncertain (see Figure 5).

This probe was used on Southern blots of genomic DNA and even on lifts of a bovine retinal cDNA library without gaining any reliable results. However, background hybridization appeared unusually high, and this led to the discovery that the probe bound very well to λ-DNA. Considering that the libraries on hand were both in λ vectors this probe was not going to be immediately useful.

```
                Leu
   Phe Glu Gln Pro Asn Met Ile Leu Gln Gly Asp Ala Ile Val
5′ TTT GAG CAG CCC AAC ATG ATC CTG CAG GGC GAT GCC ATT GT 3′
                TG
```

Figure 5. Oligonucleotide "Guessmer" Probe

A 41 base oligonucleotide was designed corresponding to the α-32-6-5-3 peptide following the recommendations of Ausubel *et al.* [1987] and Wood [1987]. For each amino acid the single best codon was determined from a bovine rod cell codon utilization table (see Table A1 in the Appendix, p.98).

DISCUSSION

PDE Subunit Separation. The long-range purpose of this research was to better understand the function and regulation of the cGMP PDE of vertebrate photoreceptors. As initially proposed, my role was to isolate the large PDE subunits, P_α and P_β, and to use these purified subunits to: 1)obtain peptide sequence information that would in turn be used by Steve Pittler to find the corresponding cDNAs, and then 2)study their functional roles in the visual cascade through reconstitution experiments. Because reconstitutions were planned it was initially hoped that the P_α and P_β could be separated under nondenaturing conditions. However, soon after isolation from the ROS, the PDE invariably started to aggregate yielding a white precipitate. This phenomenon was also reported earlier by Kohnken *et al*. [1981]. This material could only be solubilized again in the presence of strong denaturants like SDS, urea, or guanidine hydrochloride. Even then not all of the material could be dissolved. As a result of this aggregation, HPLC gel filtration using nondenaturing conditions was found to be unsuitable for separation of P_α and P_β, leading to the successful separation by SDS-PAGE.

Peptide Sequences. Amino acid sequences could not be

determined directly from the intact P_α and P_β because their N-terminal amino groups were blocked, preventing Edman degradation [Ovchinnikov *et al.* 1986]. To obtain sequencable peptides it was decided to trypsinize the subunits. At the time preparations for trypsinization of P_α and P_β were being made, Steve Pittler requested that cysteine-containing peptides specifically be searched for in order to verify which of his cDNA clones actually coded for a PDE subunit. He had recently identified three cDNA clones that presumably coded for a PDE subunit but were not full length. The three clones were identical except that they diverged at the 3′ end of their ORFs. The clone that Steve suspected represented a "major" PDE isoform had in its deduced peptide sequence a C-terminal CAAX motif commonly associated with isoprenylation of peripheral membrane proteins [Hancock *et al.* 1989]. The predicted C-terminal tryptic peptide from this clone contained two cysteine residues.

To aid in the search for cysteine-containing peptides, P_α and P_β were ^{3}H-carboxymethylated using tritiated iodoacetic acid. However the resulting tryptic digest yielded only very minor labeled peaks that proved to contain too little material to sequence. It also yielded at least one unlabeled peptide that contained a carboxymethylated peptide. One possible explanation for these results is that the labeled iodoacetic acid may have been degraded, such as by exposure to light.

During the time the 14 sequenceable peptides in Table 1

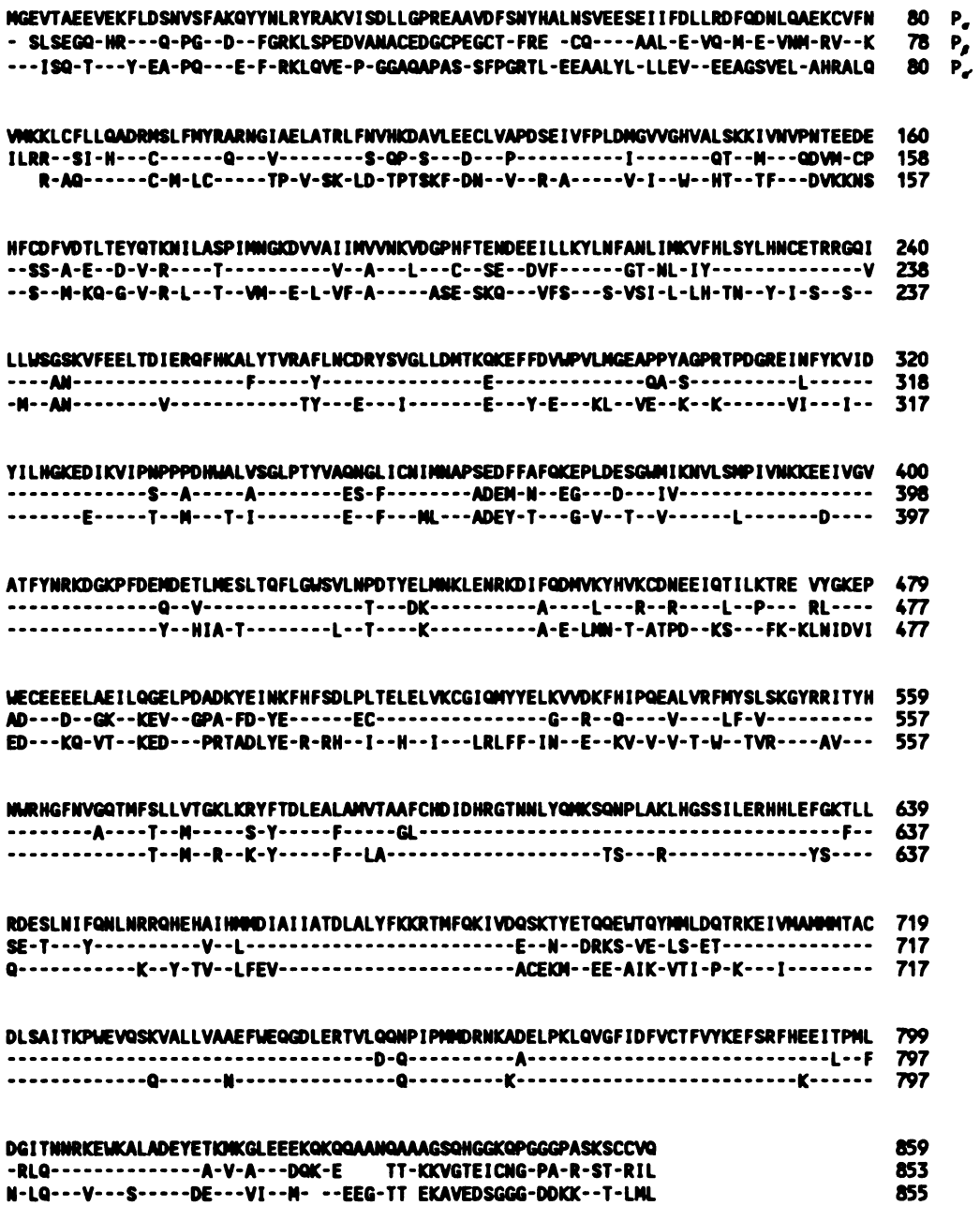
were being generated, Ovchinnikov's group published it's P_α cDNA and deduced amino acid sequence [1987]. Other bovine retinal PDE sequences followed: another P_α from Pittler *et al.* [1989], P_β from Lipkin *et al.* [1990], and the cone cell $P_{\alpha'}$ from Li *et al.* [1990]. To find cDNA coding for the missing isoforms it was decided to use the polymerase chain reaction. Comparison of the deduced amino acid sequences of P_α, P_β, and the cone $P_{\alpha'}$ reveals that the emerging family of photoreceptor cGMP PDEs has extended consensus regions and only very narrow hypervariable (HV) regions (see Figure 6). It is anticipated that the source of Table 1's non-matching peptides is/are one or more HV regions of predominant PDE subunit isoforms other than those published. In fact the longest peptide, α-32-6-5-3, does exhibit limited sequence similarity (30%) to the P_α deduced amino acid sequence at residues 483-502 (see Table 1 and Figure 6). This is a variable region where P_β and $P_{\alpha'}$ also have limited sequence similarities with P_α of 45% and 40%, respectively. Another peptide, β-46-12, has no apparent similarity to published P_α or P_β sequences. It pre-sumably comes from either the N- or C-terminal ends of its parent protein where the different isoforms diverge almost entirely.

Polymerase Chain Reaction. After noting the discrepancies between the peptide sequences and the deduced amino acid sequence from published P_α cDNA [Ovchinnikov *et al.* 1987, the only one published at the time], a DNA probe was designed to preferentially identify, from a cDNA library, P_α clones

Figure 6. Sequence Comparisons of P_α to P_β and $P_{\alpha'}$

The amino acid sequences deduced from the published cDNAs for P_α [Ovchinnikov *et al.* 1987], P_β [Lipkin *et al.* 1990], and $P_{\alpha'}$ [Li *et al.* 1990] were lined up and gaps were introduced to reveal their greatest sequence overlap. Dashes (-) in the P_β and $P_{\alpha'}$ sequences represent identity with the P_α residue directly above it. Substantial hypervariable regions are seen near the C- and N-termini (approximately residues 25-80 and 830-850) but only in limited areas in the internal regions.

Figure 6.


MGEVTAEEVEKFLDSNVSFAKQYYNLRYRAKVISDLLGPREAAVDFSNYHALNSVEESEIIFDLLRDFQDNLQAEKCVFN 80 P
- SLSEGQ-HR---Q-PG--D--FGRKLSPEDVANACEDGCPEGCT-FRE -CQ----AAL-E-VQ-H-E-VNH-RV--K 78 P
---ISQ-T---Y-EA-PQ---E-F-RKLQVE-P-GGAQAPAS-SFPGRTL-EEAALYL-LLEV--EEAGSVEL-AHRALQ 80 P
VHKKLCFLLQADRHSLFHYRARNGIAELATRLFNVHKDAVLEECLVAPDSEIVFPLDHGVVGHVALSKKIVNVPNTEEDE 160
ILRR--SI-H---C------Q---V--------S-QP-S---D---P----------I-------QT--H---QDVH-CP 158
R-AQ------C-H-LC-----TP-V-SK-LD-TPTSKF-DN--V--R-A-----V-I--W--HT--TF---DVKKNS 157
HFCDFVDTLTEYQTKNILASPIHNGKDVVAIIHVVNKVDGPHFTENDEEILLKYLNFANLIHKVFHLSYLHNCETRRGQI 240
--SS-A-E--D-V-R----T----------V--A---L---C--SE--DVF------GT-NL-IY-------------V 238
--S--H-KQ-G-V-R-L--T--VH--E-L-VF-A-----ASE-SKQ---VFS---S-VSI-L-LH-TN--Y-I-S--S-- 237
LLWSGSKVFEELTDIERQFHKALYTVRAFLNCDRYSVGLLDHTKQKEFFDVWPVLHGEAPPYAGPRTPDGREINFYKVID 320
----AN---------------F-----Y--------------E-------------QA-S----------L------ 318
-H--AN--------V-----------TY---E---I-------E---Y-E---KL--VE--K--K------VI---I-- 317
YILHGKEDIKVIPNPPPDHWALVSGLPTYVAQNGLICNIHNAPSEDFFAFQKEPLDESGWHIKNVLSHPIVNKKEEIVGV 400
------------S--A-----A--------ES-F--------ADEH-N--EG---D---IV----------------- 398
-------E-----T--H---T-I--------E--F---ML---ADEY-T---G-V--T--V------L-------D---- 397
ATFYNRKDGKPFDEHDETLHESLTQFLGWSVLNPDTYELHNKLENRKDIFQDHVKYHVKCDNEEIQTILKTRE VYGKEP 479
-------------Q--V--------------T---DK----------A----L---R--R----L--P--- RL---- 477
-------------Y--HIA-T--------L--T----K----------A-E-LMN-T-ATPD--KS---FK-KLNIDVI 477
WECEEEELAEILQGELPDADKYEINKFHFSDLPLTELELVKCGIQMYYELKVVDKFHIPQEALVRFMYSLSKGYRRITYH 559
AD---D--GK--KEV--GPA-FD-YE------EC---------------G--R--Q----V----LF-V---------- 557
ED---KQ-VT--KED---PRTADLYE-R-RH--I--H--I---LRLFF-IN--E--KV-V-V-T-W--TVR----AV--- 557
NWRHGFHVGQTMFSLLVTGKLKRYFTDLEALAMVTAAFCHDIDHRGTNNLYQMKSQNPLAKLHGSSILERHHLEFGKTLL 639
--------A----T--M-----S-Y-----F-----GL-------------------------------------F-- 637
------------T--M--R--K-Y-----F--LA-------------------TS---R------------YS---- 637
RDESLNIFQNLNRRQHEHAIHMMDIAIIATDLALYFKKRTMFQKIVDQSKTYETQQEWTQYMNLDQTRKEIVMAMMMTAC 719
SE-T---Y----------V--L-------------------------E--N--DRKS-VE-LS-ET------------- 717
Q----------K--Y-TV--LFEV---------------------ACEKM--EE-AIK-VTI-P-K---I-------- 717
DLSAITKPWEVQSKVALLVAAEFWEQGDLERTVLQQNPIPMMDRNKADELPKLQVGFIDFVCTFVYKEFSRFHEEITPML 799
----------------------------------D-Q----------A--------------------------L--F 797
------------Q------N--------------Q---------K------------------------K------ 797
DGITNNRKEWKALADEYETKMKGLEEEKQKQQAANQAAAGSQHGGKQPGGGPASKSCCVQ 859
-RLQ-------------A-V-A---DQK-E TT-KKVGTEICNG-PA-R-ST-RIL 853
N-LQ---V---S-----DE---VI--M- --EEG-TT EKAVEDSGGG-DDKK--T-LML 855

coding for the alternative sequences. It was to be created by PCR techniques adapted from Lee *et al.* [1988] using primers corresponding to both ends of peptide α-32-6-5-3 (see Table 1 and Figure 2A). It was chosen first because it was the longest peptide sequence obtained and it did not have significant sequence similarity with the published P_α.

When it was found that identifying PCR products corresponding to peptide α-32-6-5-3 from a mixture including many artifacts was going to be exceedingly difficult, additional PCR primers were prepared. These corresponded to several of the shorter isolated P_α peptides (see Figure 2B). However, with these primers the subsequent PCRs, like the earlier ones, yielded complex mixtures of products. Long products that were expected in some of the PCRs did not show up, in retrospect because reactions with degenerate primers and complex template mixtures favor shorter products. Still, the number of PCR products obtained gave promise that with sufficient care and persistence, the underlying variant P_α sequences, probably multiple, are very likely accessible by PCR amplification.

A Focus for Future Research. Future research should be directed at identifying the various isoforms of the photoreceptor PDE, verifying their localization in the photoreceptor cell, and determining their relative expression levels. With those items accomplished, the functional role of each isoform can be examined. An important first step for any future research is to obtain considerable additional peptide

sequence from separated P_α and P_β to further verify the alternative isoforms. This will yield more information about the predominant isoform(s) and allow the production of probes for cDNA libraries.

When producing peptides, trypsin could be used again which may be useful in verifying already identified peptides. However the purified P_α and P_β subunits could also be cleaved with other proteases that will yield fewer and longer sequenceable peptides. Enzymes are commercially available that cut exclusively at arginine or at lysine rather than at both as trypsin does. Using published cDNAs the following table was created giving the number of peptides theoretically produced, followed by the average peptide length:

	Rod PDE-α	Rod PDE-β	Cone PDE-α'
Trypsin	97/ 8.9	98/ 8.7	108/ 7.9
Endoproteinase Lys-C[a]	63/13.6	55/15.5	73/11.7
Submaxillaris Protease[b]	35/24.5	44/19.4	37/23.1

[a] Boehringer Mannheim Biochemicals
[b] Pierce.

From such alternative digests it will potentially be easier to isolate purified peptides of which many should yield more sequence information than those from a tryptic digest.

There are nonenzymatic methods that could also be used to fragment the subunits. Peptides were successfully isolated from cyanogen bromide digests of undifferentiated $P_{\alpha\beta}$

[Ovchinnikov et al, 1987, Lipkin et al, 1990]. Early on in these studies I attempted cyanogen bromide cleavage of purified P_α and P_β but ended up in both instances with mostly intractable tars. Possibly with greater diligence we can successfully employ this cleavage method.

A second chemical method that could be utilized is treatment with 70% formic acid. This method cleaves the relatively uncommon aspartate-proline peptide bond as was done with $G_{t\gamma}$ [McConnell *et al*. 1984]. No such sites exist in the deduced amino acid sequences for P_α and P_β but two are found in the closely related cone $P_{\alpha'}$. This method may be able to reveal very quickly the existence of multiple isoforms within an apparently homogeneous PDE-subunit. If after treatment with 70% formic acid only a portion of the material remains uncleaved as revealed by SDS-PAGE then that argues that more than one sequence is present. It is also a potential way to purify a formic acid insensitive isoform away from a sensitive one. Formic acid fragments may be directly sequenced or digested further by other means to obtain manageable peptides.

The P_α variants identified by Pittler suggest that the C-terminal region of the subunits could be important in identifying the predominant isoforms. Not one of the published cDNAs, nor Pittler's two variants, have yet had these C-terminal regions confirmed with any direct peptide sequence. A technique for quickly purifying C-terminal tryptic peptides has recently been offered commercially

(Immobilized Anhydrotrypsin Kit, Pierce). The anhydrotrypsin binds tryptic peptides with C-terminal arginine or lysine residues while C-terminal peptides, lacking these residues, elute through the column. These peptides can then be resolved and sequenced confirming which, if any, of the C-terminal regions represents the predominant isoform.

With additional peptide sequence in hand we will be in a better position to develop successful probes for screening bovine retinal cDNA libraries for clones that code for various PDE isoforms. One set of probes could be produced by PCR using primers corresponding to some of the more complete peptide information. Cloning and screening PCR products has been made much easier with the introduction of the TA Cloning kit [Invitrogen] as was later demonstrated with PCR products coding for PLCs (Chapter II). Individual clones can be sequenced to verify that they code for the peptides in question and to identify likely variants amplified from the cDNA of different isoforms.

Another potential set of probes could be antibodies raised against synthetic peptides based on the generated amino acid sequences. The specificity of the antibodies can be tested on Western blots of total ROS protein and purified PDE and then used to screen an expression library.

One strategy for identifying multiple PDE isoforms would be to screen a bovine retinal λgt11 expression library (Clontech) with a set of PCR produced probes corresponding to

the conserved catalytic domain of PDEs [Li *et al.* 1990]. The numerous clones that are likely to be identified by this screening can then be further probed with oligonucleotide or antibody probes which correspond to unique peptides coming from hypervariable regions.

After the variant PDE subunit isoforms have been identified and their sequences revealed by cDNA clones, the expression level of each isoform can be examined. A first approximation of the transcript levels could come from Northern blot analysis of total RNA and poly A^+ RNA [Sambrook *et al.* 1989] using probes that correspond to unique regions of each isoform. Next, a more accurate measurement might be made by ribonuclease protection assays [Ausubel *et al.* 1987; Sonnenburg *et al.* 1991]. Labeled antisense RNA probes, also corresponding to unique regions of each isoform, are hybridized to the target RNA and then treated with RNases which digest single-stranded RNA. Transcript levels are then calculated from the amounts of incorporated label remaining. If the transcript level of any isoform proves to be too low to be detected accurately by Northern blot analysis or ribonuclease protection assays, it can be determined after amplification by PCR using the methods of McConnell and Linz [unpublished studies]. Knowing the expression levels of the variant isoforms will aid in subsequent research examining their localization and functional roles in vision, and in other forms of cellular signaling.

CHAPTER II

PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASES C

LITERATURE REVIEW

The Role and Control of Calcium in the ROS. In the vertebrate ROS, modulation of [Ca^{++}] is believed to mediate light-adaptation [Matthews *et al.* 1988]; however its mode of action has not been established. It is known that in the dark Ca^{++} enters the ROS along with Na^{+} through the cGMP-gated cation channel [Koch, 1992] (see Figure 1, p. 3). Simultaneously Ca^{++} is extruded via a $Na^{+}/Ca^{++},K^{+}$ exchanger, harnessed to an outward K^{+} gradient [Cervetto *et al.* 1989]. Upon illumination the cGMP is depleted and the cGMP-gated cation channel shuts stopping the influx of Ca^{++} and Na^{+} while Ca^{++} efflux via the exchanger continues [Koch, 1992]. One possible explanation of Ca^{++}-mediated light adaptation is the activation of guanylate cyclase at low [Ca^{++}]. It has been shown that a 23 kDa protein named recoverin activated ROS guanylate cyclase *in vitro* when free [Ca^{++}] was lowered from 450 nM to 20 nM [Dizhoor *et al.* 1991]. However, this has been recently challenged by a report that purified bovine *recoverin*, as well as homologs from chicken and Gecko lizard retina, added to intact Gecko ROS were shown to *delay* recovery after illumination [Gray-Keller *et al.* 1993].

Another possible role for Ca^{++} is the activation of cGMP

PDE. A 26 kDa protein from frog ROS reportedly binds to ROS disks and stimulates cGMP hydrolysis at 1 μM Ca^{++} level [Kawamura and Murakami, 1991].

The mechanisms for the modulation of [Ca^{++}] have not been established either. Certainly calcium flux via the cGMP-gated cation channel and the $Na^{+}/Ca^{++},K^{+}$ exchanger plays an important role [Koch, 1992]. But so too must the calcium stores associated with ROS disks where most of the ROS calcium is found. These stores are sensitive to cGMP and calcium ionophore A23187 [Devenny and Clack, 1991]. It is also likely that these stores are sensitive to inositol 1,4,5-trisphosphate (IP_3) as are Ca^{++} stores in most cell types [Berridge & Irvine, 1989].

Background of Phospholipases C (PLCs) Phosphatidylinositol 4,5-bisphosphate (PIP_2) phospholipases C (PLCs) are a class of enzymes involved in various signal transductions. They are activated by transmembrane receptors, either directly or via a G-protein or a kinase [Majerus *et al.* 1990]. They in turn cleave the PIP_2 phosphodiester linkage to yield two second messengers: diacylglycerol (DAG) which activates protein kinase C, and IP_3 which has the ability to release Ca2+ stores in many different cell types [Berridge and Irvine, 1989]. Considering that both the activators and the substrate are membrane-associated it follows that PLCs need to bind to a membrane to be functional. However, PLC activity has been demonstrated in both particulate and cytosolic fractions [Rhee

et al. 1989; Gehm and McConnell, 1990b] and at least one isoform, a bovine brain β-PLC, has been isolated from both [Lee *et al.* 1987]. Certainly one attractive model for PLC activation is translocation from the cytosol to the membrane.

In the ROS, PLCs likely play a role in calcium modulation via IP_3 production. Rapid PIP_2 synthesis and multiple PLC isoforms have been detected in the bovine ROS [Gehm and McConnell, 1990a&b] although light activation has not yet been established.

A number of PLC cDNAs have been cloned and sequenced by S. G. Rhee's laboratory at NIH and by others. They have been categorized based on immunological cross-reactivity and extended sequence similarities into four different families denoted α, β, γ, and δ [Rhee *et al.* 1989]. With the exception of the α-PLCs, all the families have consensus regions denoted X and Y. Outside of these regions the PLC families have little sequence similarity among them. Additionally, within the γ-PLC family are highly conserved regions between X and Y. These have been designated SH2 and SH3 (*src* homology 2 and 3) and have significant sequence similarity with the *src*-encoded family of tyrosine kinases. One or both of these sequences have also been found in *crk* oncogene from avian sarcoma virus CT10, the GTPase activating protein (GAP) which activates the *ras* gene product p21, and the α-subunit of spectrin [Rhee *et al.* 1989].

The work presented in this chapter was initiated to

complement the continuing ROS PLC purification and characterization studies in our laboratory. It has led to some provocative insights and has provided some powerful tools, not before available, with which to further analyze these enzymes.

MATERIALS AND METHODS

Materials. Commercial suppliers were the same as in Chapter I.

Oligonucleotide Synthesis and Purification. Oligonucleotides were synthesized by the Macromolecular Structure, Synthesis, and Sequence Facility, Department of Biochemistry, Michigan State University on an Applied Biosystems 380B DNA synthesizer. The crude synthetic oligonucleotides were purified by HPLC using a Zorbax Bioseries Oligo column (DuPont). The column was equilibrated with 80% 50 mM $NaPO_4$, pH 7.0, 20% acetonitrile, 0.1 M NaCl. The oligonucleotides were applied and then eluted with a linear 0.1 to 0.8 M NaCl gradient in the same solvent in 70 min. Peak A_{260} fractions were pooled and desalted using C_{18} Sep Paks (Waters) as described by Atkinson and Smith [1984].

RNA Isolation. Bovine eyes were obtained from Murco, Inc. of Plainwell, MI. Within 3 min. of slaughter fresh retinas were removed from the eyes and dropped into liquid nitrogen. Poly A^+ RNA was isolated from the frozen retinas using the Fast Track kit (Invitrogen). This kit yielded poly A^+ RNA with A_{260}/A_{280} ratios consistently >1.8 in significantly less time than the traditional protocols used earlier. It

also had less contaminating non-poly A^+ RNA as judged by the relatively low intensity of rRNA bands seen on formaldehyde-1% agarose gels [Fourney *et al.* 1988].

cDNA Synthesis was performed as described in Chapter I.

Polymerase Chain Reaction. Thermal cycling was performed with a DNA Thermal Cycler or a Gene Amp PCR System 9600 (both Perkin Elmer Cetus). The reactions were carried out as described in Chapter I.

Agarose Gel Electrophoresis was performed as described in Chapter I.

Recovery of DNA from Agarose Gels was performed as described in Chapter I.

Cloning PCR Products. The TA Cloning kit (Invitrogen) was utilized. It came with a precut vector, pCR 1000, which has 5′ single T overhangs. This allowed direct ligation of the PCR products without having to resort to restriction cutting and subsequent cleanup. The PCR products, which inherently have 5′ single A overhangs as catalyzed by *Taq* polymerase, were ligated to the vector with T4 ligase. The recombinant DNAs were used to transform competent INV 1αF′ *E. coli* which were provided with the kit. Transformants were then screened by their ability to catabolize the chromogenic substrate, X-gal (blue/white screening).

Oligonucleotide End-labeling was performed by the methods of Ausubel *et al.* [1987] using T4 polynucleotide kinase and [γ-^{32}P] ATP.

DNA Sequencing was performed using two different methods. PCR products were sequenced both directly and after being cloned using Sequenase version 2.0 modified bacteriophage T7 DNA polymerase (USB) and its accompanying reagents and protocols with [^{35}S]-dATPαS and with either the M13 -40 forward primer or a PCR primer. The PCR-amplified inserts of λgt10 clones were sequenced by cycle sequencing using the dsDNA Cycle Sequencing Kit (BRL) and ^{32}P-end labeled primers (see above).

Probe Labeling. Double-stranded DNA probes were labeled in three different ways: by the method of random primers [Sambrook *et al.* 1989]; by T4 PNK end-labeling [Ausubel *et al.* 1987], and by PCR techniques. To label by PCR the reactions were set up and run the same as standard PCR except that the normal 200 μM dGTP was replaced with [α-^{32}P] dGTP diluted with an equal amount of unlabeled dGTP for a final concentration of approximately 1 μM [α-^{32}P] dGTP. The quality of the reactions was monitored from autoradiograms of products on agarose gels and the total amount and specific activity of the products were calculated on the basis of the total amount of incorporated label.

λgt10 cDNA Library Plating and Replicas were performed as described in Chapter I.

Southern and Northern Blot Analyses were performed by the methods of Sambrook *et al.* [1989].

Hybridization with Oligonucleotide Probes was performed

as described in Chapter I.

Hybridization with Double-stranded DNA Probes. Nitrocellulose filters containing plaque replicas or Southern-blotted DNA were wetted in 6x SSC, 0.5% SDS, then inserted into heat-sealable bags and incubated at 42^0 for 1 hr with 6x SSC, 5x Denhardt's solution, 0.5% SDS, 100 μg/ml denatured herring sperm DNA, and 50% formamide. Double-stranded, random primer- or PCR-labeled DNA probes were denatured by boiling for 5 min, chilled on ice, and injected into the bags. After resealing, the bags were incubated at 42° with gentle shaking for 2-3 times the $Cot_{1/2}$. Label concentrations were 2×10^5 to 1×10^6 cpm/ml with a probe specific activity $>5 \times 10^7$ cpm/μg. After incubation, filters were washed with four times in 2x SSC, 0.1% SDS for 10 min at rm temp, then once in 1x SSC for 1 hr at 68°. Filters were either dried or resealed still damp in heat sealable bags, and then fluorographed. This protocol was adapted from Sambrook *et al*. [1989].

Computer Analysis of Sequences was done on IBM-compatible PC equipment in our laboratory, using software obtained from other laboratories and modified to our own needs.

RESULTS

Polymerase Chain Reaction. Initially three PCR primer pairs were synthesized corresponding to the X-region of three published PLC cDNA sequences: one from a bovine brain γ-PLC [Stahl *et al.* 1988], and two from rat brain sources, β- and δ-PLCs [Suh *et al.* 1988] (see Figure 1A). These were used in PCRs as described in methods but had only limited success. To start, the individual primer pairs did not yield any product at all. Only when all six primers were used together were PCR products formed. When they were used in PCRs with bovine retinal oligo d(T)-primed sscDNA as the template no discernible bands were seen, only a smear of material between 0.2 and 4.0 kbp. However when the sscDNA had been primed using the antisense PCR primers the reactions yielded several distinct bands including one (or more) of approximately the expected size, 450 bp, though the yields were low and there were numerous artifacts of various sizes (see Figure 2). The PCR products were blotted onto nitrocellulose and probed with an internal oligonucleotide probe. The 450 bp product hybridized to the internal probe but nothing in the smear of DNA did (not shown). This suggested that at least some small amount of a PLC X-region had been amplified. In an attempt to improve the

Figure 1. PCR Primers

A. The initial three PCR primer pairs were complementary to 1)the bovine brain γ-PLC X-region cDNA [Stahl *et al.* 1988], 2)the rat brain β-PLC X-region cDNA [Suh *et al.* 1988], and 3) the rat brain tumor δ-PLC X-region cDNA [Suh *et al.* 1988]. They were constructed similar to those used by Lee *et al.* [1988]. Each one was 23 bases long, had a complementary region 15 bases long and an 8 base region at the 5′ end that contained a 6 base restriction site and a 2 base "clamp" to aid in subsequent cloning of PCR products [Scharf, 1990].

B. The second set of primers (1 sense and 2 antisense) were mixed oligonucleotides that were complementary to both the bovine brain γ-PLC X-region cDNA [Stahl *et al.* 1988] and the bovine adrenal cortex δ-PLC X-region cDNA [Suh *et al.* 1988]. They were 20 bases long and complementary to the published sequences for their full length. The mixed nucleotide sites are denoted with an additional base below the main line.

Figure 1A. Initial PCR Primer Pairs

1) Bovine Brain γ-PLC X-Region:

Sense primer corresponding to bases 973-987

*Xba*I P L S H Y
5′ TGTCTAGA CCC CTG TCC CAC TAC 3′

Antisense primer corresponding to bases 1,372-1,385

*Bam*HI K I L I K
5′ TTGGATCC CTT GAT AAG GAT CTT 3′

2) Rat Brain β-PLC X-Region:

Sense primer corresponding to bases 961-975

*Xba*I P L S H Y
5′ TGTCTAGA CCC CTG TCT CAC TAT 3′

Antisense primer corresponding to bases 1,381-1,395

*Bam*HI K V L I K
5′ TTGGATCC TTT CAC CAA GAT TTT 3′

3) Rat Brain Tumor δ-PLC X-Region:

Sense primercorresponding to bases 901-915

*Xba*I P L S H Y
5′ TGTCTAGA CCA CTG AGT CAC TAC 3′

Antisense primer corresponding to bases 1,300-1,314

*Bam*HI K L L I K
5′ TTGGATCC TTT CAA CAG GAT CTT 3′

Figure 1B. Secondary PCR Primers

Sense, γ-PLC Bases 994-1,014, δ-PLC Bases 922-942

S S S H N T Y
5′ TCC TCC TCG CAC AAC ACC TA 3′
G G

Antisense, γ-PLC 1,093-1113, δ-PLC 1,021-1,041

G D W C D L E
5′ CC ATC CCA GCA GTC CAA CTC 3′
G G G

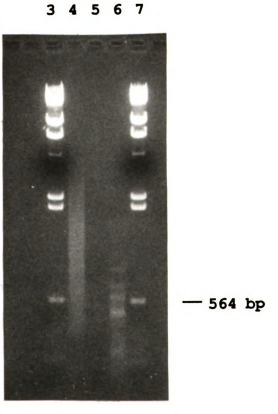
Antisense2, γ-PLC 1,219-1,239, δ-PLC 1,147-1,167

I S L I V P Y
5′ AA GGA CAG GAT GAC CGG GTA 3′
T G

Figure 2. PCR Products Using Initial Primers

All six of the initial PCR primers were combined in PCRs using either sscDNA primed with oligo d(T) (lane 4) or sscDNA primed with all three antisense primers together (lane 6). They were fractionated on a 1% agarose gel in 1 x TAE, 0.5 μg/ml ethidium bromide and compared with MW markers (lanes 3 and 7, 1 μg λ-*Hin*dIII digest). A product of the expected (~450 bp) size was seen in lane 6. The DNA was blotted onto Nytran and hybridized with a labeled internal probe (the secondary sense PCR primer, Figure 1B). Fluorograms revealed a faint signal at the ~450 bp level of lane 6 and no signal elsewhere (not shown).

Figure 2.



product yield the PCR conditions were manipulated. This led to the discovery that an approximately 450 bp product could be formed with just antisense primers present, suggesting that purification of the desired product(s) from the artifacts would be difficult.

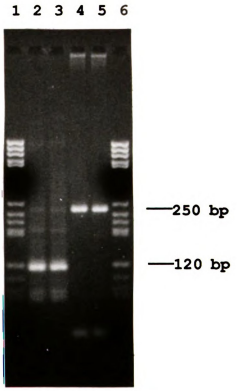
A second set of primers was more successful. The internal probe mentioned was also used as one of three new PCR primers. These primers, one sense and two different antisense, each corresponded to sequences between the initial PCR primer pairs. They were mixed oligonucleotides complementary to the bovine brain γ-PLC and the bovine adrenal cortex δ-PLC (see Figure 1B). These had greater specificity and when used in PCRs yielded fewer artifacts and larger amounts of the appropriate products, 120 or 250 bp depending on the antisense primer used (see Figure 3). Products were formed equally well whether oligo d(T)-primed or antisense-primed cDNA was used as the template, arguing that primer specificity was the reason the initial PCR primers were less successful.

Direct Sequencing. The 120 and 250 bp PCR products were gel-purified and samples were directly sequenced using dideoxynucleotides and Sequenase (USB). In separate reactions the sense and appropriate antisense PCR primers for each product were used as sequencing primers. The resulting sequences had ambiguities and were not full length but did reveal two different PLC X-region-like sequences (data not shown). There also were underlying sequences, possibly

Figure 3. PCR Products Using Secondary Primers

The secondary PCR primers (Figure 1B) were combined in PCRs using, in this case, oligo d(T)-primed sscDNA. The products were fractionated on a 3% agarose gel and compared with MW markers (lanes 1 and 6, 500 ng pBR322-*Hae*III digest). The PCRs used either the sense and antisense#1 primers (lanes 2 and 3) yielding a predominant band of the expected 120 bp size, or used the sense and antisense#2 primers (lanes 4 and 5) yielding a predominant band of the expected 250 bp size. These products were isolated by the band interception technique and subsequently cloned and sequenced.

Figure 3.



related, consistent with the proposal that PCR would amplify multiple PLC cDNAs if the primers matched closely enough to anneal. The predominant 120 bp sequence had the most sequence similarity with the bovine brain γ-PLC [Stahl *et al.* 1988]. Where the 120 bp sequences were unambiguous they had 89% identity with the γ-PLC. The predominant 250 bp sequence was most like the bovine adrenal cortex δ-PLC [Suh *et al.* 1988]. Likewise they were 60% similar. One result that was not immediately understood was that the 250 bp product yielded two different predominant sequences depending on which primer was used for sequencing: one, the δ-PLC-like product using the sense primer, and the other, an unrelated sequence using the antisense primer. Only after cloning and sequencing multiple PCR products was it determined that there was a major PCR artifact of approximately the same size that had antisense primers at both ends.

Cloning PCR Products. The 120 and 250 bp PCR products were cloned using the TA Cloning Kit (Invitrogen). This system takes advantage of the fact that Taq polymerase catalyzes the addition of a single A to the 3′ end of the newly amplified cDNA yielding a single base overhang. The supplied vector, pCR 1000, comes precut and has a complementary 3′ single T overhang allowing direct ligation of PCR products using T4 ligase. The INV 1αF′ *E. coli* transformed with these constructs theoretically should yield white colonies when grown on LB agar plus 50 μg/ml kanamycin and

spread with 1 mg X-gal, whereas those transformants that have self-ligated vectors should yield blue colonies denoting α-complementation. Some transformants with recombinant DNA were initially identified by this blue/white screening method. However, additional transformants with recombinant plasmids were discovered in subsequent screenings that still had either functional or partially functional β-galactosidases that in the presence of X-gal yielded deep blue and light blue colonies.

Streaks of 63 transformants from the 120-mer ligation and 261 from the 250-mer ligation (these included all of the white transformants as well as numerous blue transformants) were grown on duplicate sets of nitrocellulose filters. One set was probed with the labeled 250 bp PCR products to identify all the transformants containing a 250 bp PCR product, or 120 bp-containing transformants with the same or closely related sequences. The other set was probed with a labeled PCR oligonucleotide antisense primer to identify transformants with a 120 bp PCR product, and 250 bp-containing transformants that had significant sequence similarity to the primer. In tandem these two probes filtered out PCR artifacts unrelated to the two PLC-derived PCR products. However, they were not expected to, and in fact did not identify all PLC types present. The 250 bp PCR products hybridized to 244 out of 261 clones from the 250 bp transformation and to 15 out of 63 clones from the 120 bp transformation. The PCR primer hybridized to 26 out of

63 clones from the 120 bp transformation and 8 out of 261 clones from the 250 bp transformation. Thus this double screening identified 15 120-mer clones and 8 250-mer clones that possibly contained PLC sequences.

These 23 transformants and selected others that had been identified by just one hybridization were grown up in culture and their plasmids were purified by methods of Sambrook *et al.* [1989]. The desire at this point was to check the insert size by restriction cutting and then sequence many potential PLC-containing clones so as to obtain the maximum number of different PLC-like sequences. The plasmids were analyzed, first by restriction cutting, to determine insert size. However not all of them yielded the expected inserts. Those plasmids which showed the correct-size inserts sequenced well and many revealed PLC-like sequences. A few plasmids had PLC-like inserts plus unknown foreign DNAs that were of neither PCR nor vector origin. Some other clones had PLC-like inserts in an aberrant vector which was missing over 300 bp including half of the polylinker region. This was puzzling at first since no insert could be removed from them using restriction enzymes but it became clear after sequencing that they did indeed have PLC inserts. Still others could not be sequenced at all using the M13 forward (-40) primer leading to suspicion that this part of the vector was missing here as well. Although these clones were not pursued further they could potentially be sequenced using the M13 reverse primer or

a PCR primer.

Of the 23 clones identified by the double screening, 17 contained PLC-like sequences of 3 different types, 3 were unsequenceable, and 3 had unidentified DNA inserts of non-PCR origin. There were another 12 clones from the 120 bp ligation that were identified as PCR products but did not hybridize with the 250-mer PCR product. Five of them contained PLC-like sequences of two additional types, 3 were unsequenceable, and 4 were unidentified DNAs. Of the 236 250-mer clones that were identified as PCR products but which did not hybridize with the internal oligonucleotide probe, 4 were sequenced and 8 others were analyzed by restriction cutting and they all appeared to be the same 246 bp artifact. This artifact was a PCR product of close to the expected length but had antisense primers at each end and did not resemble the PLC X-region cDNAs. This indicates that a majority of the 250 bp PCR products were non-PLC artifacts, vindicating individual cloning of PCR products to sort them.

The 5 different PLC types discovered among the sequenced clones were designated PLC#1-5. They were compared with each other and with those of published PLC sequences for the levels of sequence similarity at both the cDNA level and the deduced amino acid level (see Tables 1A and 1B). From the comparisons it seemed likely that PLC#2 represented a γ-PLC X-region cDNA, perhaps the same clone that was purified from bovine brain cDNA library by Stahl *et al.* [1988]. PLC#3 likely represented

Table 1. Comparisons of Cloned PLC Sequences

These tables show the percent similarity of both the cDNA and the deduced amino acid sequences amongst the five identified bovine retinal PLC X-regions (Table 1A) and with those of published PLCs [A,C, and D from Suh *et al.* (1988), B from Stahl *et al.* (1988)] (Table 1B). All of the comparisons were made excluding the PCR primers.

Table 1A. Comparison of Clones with Each Other

	PLC≠2		PLC≠3		PLC≠4		PLC≠5	
	cDNA	AA	cDNA	AA	cDNA	AA	cDNA	AA
PLC≠1	47%	50%	48%	50%	44%	54%	53%	50%
PLC≠2			52%	46%	51%	54%	60%	54%
PLC≠3					67%	77%	61%	50%
PLC≠4							57%	54%

Table 1B. Comparison of Clones with Published Sequences

	A. β-PLC from Rat Brain		B. γ-PLC from Bovine Brain		C. δ-PLC from Bovine Adrenal Cortex		D. δ-PLC from Rat Brain Tumor	
	cDNA	AA	cDNA	AA	cDNA	AA	cDNA	AA
PLC≠1	57%	51%	59%	59%	73%	59%	68%	59%
PLC≠2	49%	62%	100%	100%	57%	54%	57%	54%
PLC≠3	90%	100%	52%	46%	49%	50%	48%	50%
PLC≠4	38%	77%	51%	54%	39%	46%	38%	46%
PLC≠5	64%	47%	66%	53%	63%	57%	62%	57%

a β-PLC X-region and possibly the bovine analog of a rat brain β-PLC characterized by Suh *et al.* [1988] with which it shared 89.9% cDNA and 100% amino acid sequence similarity. It was not as clear into which category the others fell comparing these limited sequences. Non-X-region sequences will probably be required to differentiate them.

Probe Labeling. When the cloned PCR products were first to be tested as probes on blots they were cut out of the vector with restriction enzymes and labeled by random primer extension. However subsequent attempts at labeling by this method were inconsistent and unsatisfactory. An alternative labeling method was needed that could consistently attain the desired levels of total counts and specific activity. End-labeling with T4 polynucleotide kinase was attempted but proved no better than random primer-labeling. Calculations showed that even using 6,000 Ci/mmole [γ-^{32}P] ATP and getting 100% end-labeling the probes would be below the recommended 10^9 dpm/μg [Sambrook *et al.* 1989] simply because they were too long. Committee member Jerry Dodgson agreed that the length of my probes made them less than ideal for the established methods of random primer and end-labeling, and suggested that labeling by PCR might be the easiest way to bring these probes to a sufficiently high specific activity. This turned out to be sound advice. PCR labeling has consistently yielded probes of a very high specific activity (>2x10^9 dpm/μg).

Test of Cloned PCR Products as Probes. The 5 cloned PCR

products were trimmed from their vectors, labeled by either random primer (initially) or PCR (later) methods, and tested as probes. On Southern blots of EcoRI-cut bovine genomic DNA, each probe hybridized to one or more bands, none of them the same size from probe to probe (see Figure 4). Similar results were seen with probe hybridization to Northern blots of bovine retinal poly A+ RNA (see Figure 5). The five probes were also tested for cross-hybridization among each other. Slot blots of the unlabeled probes were hybridized to the individual labeled probes. The results showed that there was little or no cross-hybridization between pairs of different probes (see Figure 6). This implied that in a library screening all of the probes would be required to identify clones representing each PLC even though all of them code for some part of the PLC X-consensus regions.

<u>λgt10 Library Screening.</u> After some initial attempts at library screening failed (in retrospect most likely due to using random primer-labeled probes) I wanted to test the screening protocols to determine if they were being correctly executed and to develop a positive control probe for comparison to the PLC probes. The probe chosen was a cloned PCR product developed by Carol Mindock and Cindy Wheeler, corresponding to the first exon of bovine rhodopsin [Nathans and Hogness, 1983]. Its attributes for this work were that: 1) it was 260 bp, close in length to some of the PLC probes and could be labeled similarly; and 2) it was expected to identify

Figure 4. Southern Analyses

Bovine genomic DNA was digested with *Eco*RI and then fractionated on a 1% agarose gel with 7.5 μg DNA/lane. It was transferred to a Nytran filter by standard blotting techniques. The cloned PCR products, PLC#1-5, were individually labeled by PCR and hybridized to filter strips, each containing two lanes of DNA. After washing, the filter strips were fluorographed, revealing the following bands (in bp):

PLC#1	PLC#2	PLC#3	PLC#4	PLC#5
5,850	>21,000*	3,850	>21,000*	>21,000*
4,450**	6,300**			7,400**
3,850**				
1,420**.				

* Although the size of these bands could not be measured accurately from this fractionation they were clearly not identical bands.

** These bands were faint and may be the result of cross-hybridization of closely related isoforms.

Figure 4.

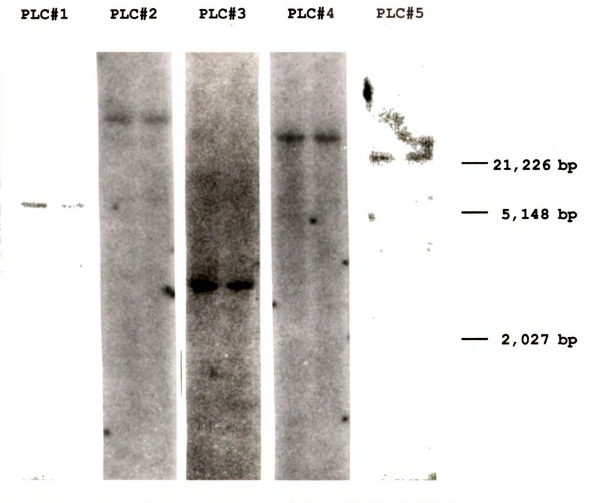


Figure 5. Northern Analyses

Bovine retinal poly A^+ RNA was fractionated on a formaldehyde 1% agarose gel with 5 µg/lane. It was transferred to a Nytran filter by standard blotting techniques. The cloned PCR products, PLC#1-3 and #5 were individually by the method of random primers. (PLC#4 was not included in this analysis due to difficulties associated with its aberrant vector.) The others were hybridized to filter strips each containing a lane of RNA. After washing, the strips were fluorographed, revealing the following bands (in bases):

PLC#1	PLC#2	PLC#3	PLC#5
5,800	6,100	7,250	8,030
4,390	2,620		6,460
3,630			5,200
3,130.			

Figure 5.

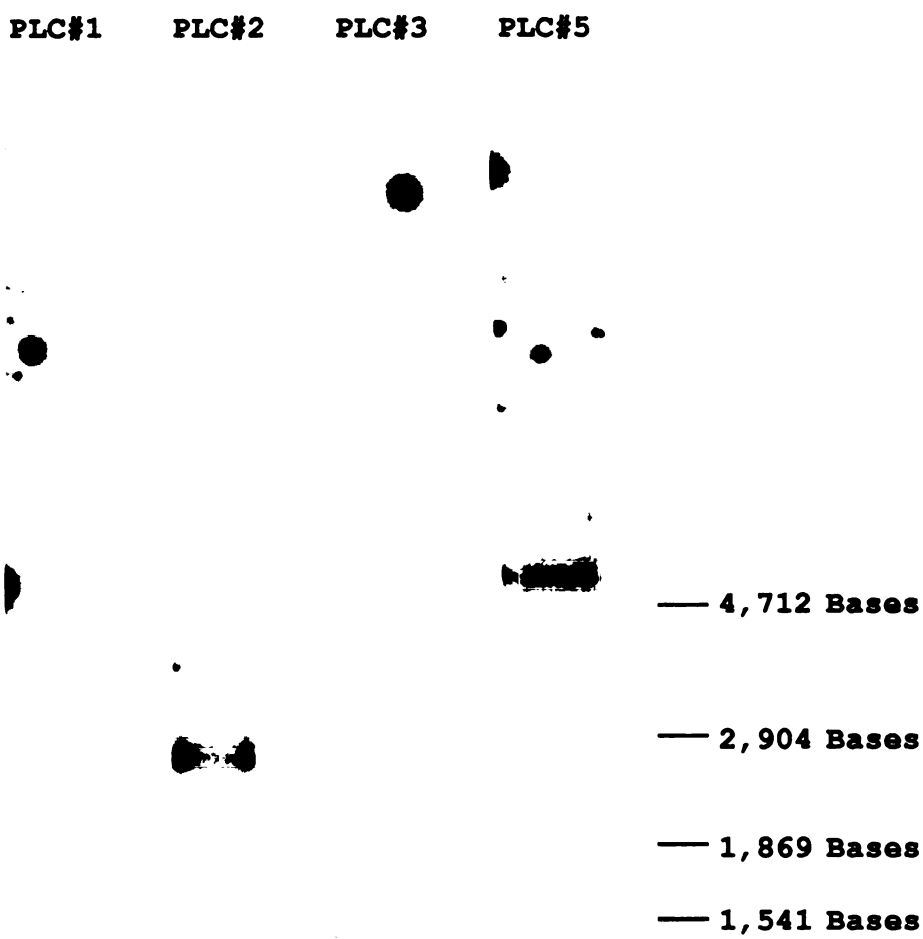

PLC#1
PLC#2
PLC#3
PLC#5
4,712 Bases
2,904 Bases
1,869 Bases
1,541 Bases

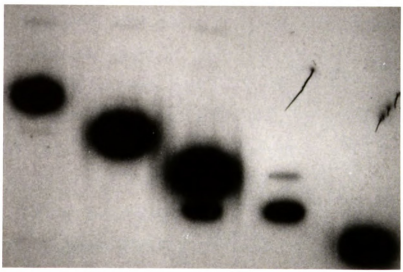
Figure 6. Cross-hybridisation of Cloned PCR Products

The cloned PCR products, PLC#1-5, were each slot-blotted five times onto a Nytran filter in equimolar amounts (0.6 pmoles). Also blotted were herring sperm DNA and λ DNA in 6 μg aliquots. Additional amounts of PLC#1-5 were individually labeled by the method of random primers and hybridized to strips of the filter containing one blot each of the DNAs. After washing, the filter strips were autoradiographed. As expected, each of the probes hybridized most strongly to itself. PLC#1 and #5 cross-hybridized to each other at ~20% of the level that they self-hybridized as determined by densitometric scans of the autoradiogram. Other pairs did not significantly cross-hybridize.

Figure 6.

Probes

PLC#2 PLC#3 PLC#5 PLC#1 PLC#4



Fish Sperm DNA

λ-DNA

PLC#2

PLC#3

PLC#5

PLC#1

PLC#4

Blotted DNA

the most abundant clone type in the library, as rhodopsin is the most abundant protein in the retina. Triplicate plaque lifts of 10^5 pfus were hybridized and washed by standard methods [Sambrook *et al.* 1989]. Fluorograms revealed 10 plaques that hybridized to the rhodopsin probe at varying intensities.

Next the library was screened with the five cloned PLC probes applied simultaneously. Triplicate plaque lifts of $7.5x10^5$ pfus were hybridized with the probes and the resulting fluorograms revealed 10 plaques that hybridized to the PLC probes to varying degrees. The plaques were subjected to standard plaque purification protocols (see Methods) where eight of the original ten hybridized again to the five pooled probes. For the other two, upon purification attempts no hybridizing plaques could be found and they were presumed to be artifacts of the first hybridization and were not pursued any further.

Characterization of λgt10 Clones. Thus far the clones have been characterized by which probe they hybridize to, determining the size of their inserts, and sequencing. When the clones were first plaque-purified, samples of the purified stocks were applied to separate sectors of a single plate with a Y1090 *E. coli* lawn and incubated until clear areas were visible. Then five plaque lifts were taken from the plate and separately hybridized with one of the five PLC probes. The fluorograms indicated that PLC≠4 identified six of the eight

clones and PLC#1 and PLC#3 each identified a single clone. These results suggest that the six PLC#4-hybridizing clones come from transcripts of a single gene or multiple closely related genes and that it/they are likely transcribed at a higher level than those genes represented by the other clones and/or probes. The fact that neither PLC#2 nor PLC#5 hybridized to any plaques in this particular library screening indicates only that they may be transcribed at a lower level than those of other PLC. Because there were PCR products generated it can be assumed that they were transcribed at some level. Presumably clones that hybridize to these probes could be identified by a more extensive screening of the λgt10 or an alternative library.

To determine the size of the inserts of the clones the initial plan was to produce large phage stocks, isolate the phage DNA, cut the DNA with *Eco*RI, and separate it on an agarose gel. When this was carried out using four of the clones, 1-1, 3-2, 4-1, and 4-2 it was found that only 3-2 and 4-2 actually yielded any insert fragments, of approximately 900 and 1,350 bp respectively. The other two clones had high MW λ-DNA but no discernable *Eco*RI insert fragments. This problem had been encountered previously by Steve Pittler when he was isolating PDE clones from this same library. At that time Ron Davis offered that when the library was constructed there may have been some *Eco*RI* activity which gave rise to some erroneous *Eco*RI restriction sites in the recombinant DNA.

Such sites were confirmed later upon sequencing (see below).

In order to rescue these clones a plan was devised to use PCR to amplify the insert cDNA, with primers specific for the λgt10 vector which flank the *Eco*RI insertion site. It was quickly discovered that this was a much quicker and easier way to obtain large quantities of very pure insert DNA. Hence it was employed for all the clones. The resulting PCR products were sized by agarose gel electrophoresis taking into account that they contained the inserts plus 81 bp of vector DNA. The clones are identified in Table 2. Six clones hybridized to PLC♯4. Three of these had inserts of 1000 bp, two had inserts of 1900 bp, and one had an insert of 1400 bp. It was suspected that the inserts of identical length were identical clones. This was confirmed later by sequencing (see below). Comparing the length of published PLC cDNAs and the inserts it was considered unlikely that any of the clones represented a full length open reading frame.

The PCR-amplified clone inserts were also used for DNA sequencing. This DNA was purer and yielded more readable sequence than the phage DNA isolated by traditional methods. To sequence the DNA a relatively new technique called cycle sequencing was employed (dsDNA Cycle Sequencing Kit, BRL). It uses multiple cycles of labeled primer annealing and elongation with *Taq* polymerase in the presence of dideoxy-nucleotides to yield a readable sequence. There are some advantages of cycle sequencing over traditional dideoxy-

Table 2. λgt10 Clones

Eight clones were identified by the screening of a bovine retinal λgt10 cDNA library provided to us by Jeremy Nathans [Nathans & Hogness, 1983] with PCR-labeled PLC≠1-5 together. Then each of the plaque lifts of the purified phage was tested with each of the probes independently to determine which probe hybridized to it. The inserts of the clones were amplified by PCR using λgt10 forward and reverse primers and then sized on a 1% agarose gel. These PCR products included 81 bp of vector DNA that was also amplified using these primers.

Table 2.

Clone ID.	Size (in bp)	Probe that hybridizes to it
1-1	1,900	PLC#4
3-2	1,000	PLC#4
4-1	800	PLC#1
4-2	1,400	PLC#4
7-1	1,000	PLC#4
11-1	1,900	PLC#4
13-1	1,800	PLC#3
15-1	1,000	PLC#4.

sequencing. One is that template secondary structure is less of a problem because of the elevated temperatures at which *Taq* polymerase operates. Another is that less template is needed overall because the cycling yields a linear amplification of dideoxy-termination products (as compared to the logarithmic amplification of PCR). Using this technique sequences over 500 bp have been routinely read.

To date all 8 of the amplified clone inserts have been partially or fully sequenced. All of these clones were sequenced using the λgt10 forward and reverse primers originally used to amplify the inserts. However, clones 4-1 and 13-1 could also be sequenced using the PLC sense and antisense primers originally used to create the PCR probes. The other 6 clones, which all hybridized to PLC♯4 and are closely related, could not be sequenced with either of those 2 PCR primers. Clone 3-2, which was fully sequenced using the λgt10 forward and reverse primers, revealed a lower sequence similarity to the PLC primers than clones 4-1 and 13-1 (see Figure 7).

As suspected, clones 3-2, 7-1, and 15-1, which all hybridized to PLC♯4 and had approximately 1000 bp inserts (see Table 2), were identical. Clones 1-1 and 11-1 have 1900 bp inserts and may also be identical although they have not yet been fully sequenced. It was confirmed that all six of the clones that hybridized to PLC♯4 coded for very similar PLCs. In fact through the regions that have been sequenced and

Figure 7. Comparison of Primers with Clones

Two of the PCR primers, originally used to create PLC#1-5, are shown at the top (shown is the inverse complement of the antisense primer, see Figure 1B). In those original PCRs the primers were able to anneal to the corresponding sequences below them and amplify the intervening sequences. When the same primers were tried for thermal cycle sequencing clones 4-1 and 13-1 could be read, however, neither primer yielded detectable sequence for the identical clones 3-2, 7-1, or 15-1, most likely due to the lower degree of sequence similarity. Clones 1-1, 4-2, and 11-1, which are closely related to clones 3-2, 7-1, and 15-1, also could not be sequenced with the PCR primers.

Figure 7.

Clones	PLC Sense Primer C C TCGTCCTCGCACAACACGTA	PLC Antisense Primer C C GAGTTGGACTGCTGGGACGG
3-2, 7-1, 15-1	--AGGTATCTG---A-----	--A--T-----T-----T--
4-1	--------A----------	----------G--
13-1	AAT---C----------	----------CG---A----

overlap, they are identical except that clones 3-2, 7-1, and 15-1 (1000 bp) contain a 36 bp insert missing from the 1400 bp and two 1900 bp clones. This suggests that these 6 clones may be members of a subfamily of PLCs. To simplify the subsequent comparisons these 6 sequences were put together in a preliminary composite.

The deduced amino acid sequences of the composite and of the other two clones, 4-1 and 13-1 were compared with the deduced amino acid sequences of selected published PLCs that contain X-regions and with each other. To follow the comparisons, refer to Figure 8 and Table 3 which follows it.

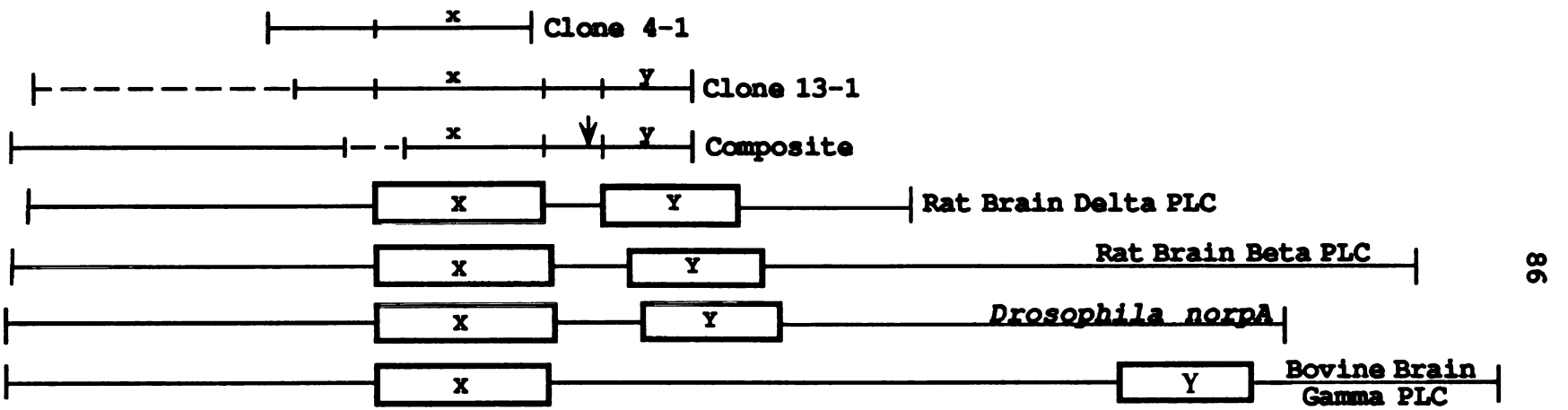
Clone 4-1 was the only one that did not have a 100% amino acid sequence match with the probe that hybridized to it (97% with PLC≠1). It also did not match predominantly with any of the PLC families, ranging between 38 and 54 overall sequence identity. This clone was, however, relatively short and sequence from overlapping clones may firmly place it in one of the established classes. Alternatively it may represent a PLC class that has not yet been characterized.

Clone 13-1, like the PCR probe (PLC≠3) that hybridized to it, was almost identical to a rat brain β-PLC cDNA isolated by Suh *et al.* [1988] with 97% identity of the deduced amino acid sequences. This clone potentially represents the bovine analog of that enzyme that might be found in both the brain and the retina or a closely related brain-like PLC expressed only in the retina. It had much less sequence similarity to

Figure 8. Overlap of Clones with Published PLCs

Schematic linear representations of the deduced amino acid sequences of the λgt10 clones and four published PLCs are presented. Dashed lines represent regions that have not yet been sequenced. Heavy boxes represent the PLC X and Y consensus regions. Larger vertical lines represent the ends of the ORFs or the clone inserts. Smaller vertical lines within the clones represent the limits of known sequence or the presumed X and Y regions (here denoted x and y). The line marked "Composite" is the compilation of six overlapping clone sequences. Where they overlap they are identical except for a 12-base insert in three of the clones, marked by an arrow. All of the sequences are aligned relative to their greatest sequence overlap within the PLC X consensus region. For the sake of clarity gaps needed to maximize the overlap in the actual sequences are not shown. At the bottom are shown the approximate extent of the designated regions compared in Table 3.

Figure 8.



Regions Compared:

| 1 | 2 (X) | 3 | 4 (Y) |

Table 3. Comparisons of Cloned Sequences

The deduced amino acid sequences of the λgt10 clones were compared with those of selected published PLCs and with each other. To better illustrate PLC family resemblance, the sequences were divided into numbered regions defined by the limits of the X and Y consensus regions and identified in the bottom line of Figure 8. Percentages in the table represent sequence identity. Comparisons without any significant sequence identity were denoted with two dashes (--). The published sequences used are *Drosophila norp*A [Bloomquist *et al*. 1988], rat brain β-PLC and δ-PLC [Suh *et al*. 1988], and bovine brain γ-PLC [Stahl *et al*. 1988].

Table 3.

Sequence/Regions	1	2 (X)	3	4 (Y)	Overall
Regions Compared with Composite of Clones 1-1, 3-2, 4-2, 7-1, 11-1, and 15-1					
norpA	46	72	37	62	61
β-PLC	30	58	--	42	35
γ-PLC	--	45	--	28	20
δ-PLC	--	44	--	15	17
Clone 13-1	49	58	--	42	43
Clone 4-1	--	47	NA	NA	32
Regions Compared with Clone 13-1					
β-PLC	97	97	100	92	97
norpA	44	58	--	40	42
δ-PLC	25	50	--	25	32
γ-PLC	13	50	--	35	29
Clone 4-1	19	48	NA	NA	36
Regions Compared with Clone 4-1					
δ-PLC	41%	62	NA	NA	54
β-PLC	28	52	NA	NA	42
norpA	24	50	NA	NA	40
γ-PLC	12	55	NA	NA	38

*norp*A, a β-PLC from *Drosophila* [Bloomquist *et al.* 1988], and even less to mammalian γ- and δ-PLCs (see Table 3).

The six related clones that hybridized to PLC#4, however, were the most intriguing. Their greatest deduced amino acid sequence similarity was to the *norp*A β-PLC, 61% overall. Comparisons with published mammalian PLCs revealed significantly lower sequence identities (see Table 3). Although the match is not as close as that between clone 13-1 and the rat brain β-PLC, the match is provocative considering the evolutionary distance between cattle and *Drosophila*.

DISCUSSION

Design of Primers. The X and Y regions of four published PLC cDNA sequences were compared to locate consensus regions to which PCR primers could be fashioned. It was anticipated that properly chosen primers would be able to amplify multiple retinal PLC sequences. Among the three X and Y containing classes β, γ, and δ, the published X-regions had the most extensive sequence similarity. The initial three PCR primer pairs were complementary to 1) the bovine brain γ-PLC X-region cDNA [Stahl *et al.* 1988], 2) the rat brain β-PLC X-region cDNA [Suh *et al.* 1988], and 3) the rat brain tumor δ-PLC X-region cDNA [Suh *et al.* 1988]. They corresponded to homologous positions in the three PLC sequences and they bracketed the majority of the X-region of those sequences. The design of the primers was similar to those of Lee *et al.* [1988]. They were each 23 bases long with 15 bases at the 3′ end that were complementary to the published cDNA sequences (Figure 1A). They also had an additional 8 bases including 6-base restriction sites to facilitate cloning of the subsequent PCR products and 2 bases at the 5′ end of each primer which were added as a "clamp" for the restriction sites because their respective enzymes cut inefficiently when the

recognition sequences are at the end of the DNA [Scharf, 1990]. In addition, those 2 bases were complementary to the cDNAs in hopes of increasing the specificity of the primers.

The second set of PCR primers was designed differently for several reasons. First they corresponded to regions between the initial primer pairs so they could function as internal probes of the initial PCR products. Second, because the first set of primers had only limited success in PCR, the new primers were complementary to areas of greater sequence similarity between the published cDNAs, hopefully to increase their specificity for the template cDNAs. Also to increase specificity, the primers were complementary for their full length, 20 bases (see Figure 1B). The restriction sites were eliminated because of the possibility that the noncomplementary regions destabilized the initial primer-cDNA hybrid. In addition, a new product, the TA Cloning Kit (Invitrogen), was available for direct cloning of unmodified PCR products (see below) eliminating the need for restriction sites in the primers.

Polymerase Chain Reaction. As described in the results section the initial set of three primer pairs had only limited success, low expected product yield and numerous artifacts, whereas the second set of primers yielded much more satisfying results. These results could have resulted from a low specificity of the initial primers for the cDNA templates. Having only a 15-base complementary region and then a

noncomplementary restriction site could limit the formation of the primer-cDNA hybrid during the PCR annealing step. Having 20 bases of complementary DNA without noncomplementary regions increased the specificity enough to enhance the PCRs.

Cloning of PCR Products. The 120 and 250 bp PCR products were cloned as reported in Results. The system utilized (TA Cloning kit, Invitrogen) employs the established technique of blue/white screening where blue colonies denote the presence of and white colonies denote the lack of α-complementation of the *lacZ* gene. Theoretically white colonies contain an insert in the *lacZ* gene and blue colonies do not. This, however, was not always the case as some dark blue transformants were found to have recombinant DNA. In addition some of the white colonies when transferred and grown in streaks displayed varying shades of blue. It was anticipated that these results might occur. A likely explanation for this is that some inserts do not significantly disrupt the *lacZ* gene. This could happen if the insert did not cause a shift in the reading frame and did not affect the overall structure of the resulting β-galactosidase.

*norp*A-like PLCs in Bovine Photoreceptors. In the invertebrate photoreceptor a G protein-linked PLC is light activated and plays a central role in the visual cascade [Rayer *et al.* 1991]. The *norp*A mutant *Drosophila* has a defective photoreceptor PLC (the product of the *norp*A gene) and as a result is blind [Bloomquist *et al.* 1988]. The fact

that a bovine retinal PLC is more closely related to the *norp*A PLC than it is to other mammalian PLCs is quite suggestive. Considering that this particular PLC has been conserved between *Drosophila* and cattle it follows that some of its functions and regulatory mechanisms may have been conserved as well. Almost certainly, distinct differences will be revealed upon detailed examination, but the similarities between invertebrate and vertebrate visual phototransduction may be greater than previously recognized, and a unified theory may in fact center around the role of PLC in both systems.

There are two important implications suggested by the relationship of this bovine retinal PLC to *norp*A. First is that the bovine retinal *norp*A-like PLC is very likely of photoreceptor origin as opposed to coming from other retinal cells. *Drosophila norp*A has been localized to the fly's photoreceptor cells [Bloomquist *et al*. 1988]. Bovine retinal cDNA libraries were expected to be biased toward photoreceptor clones due to the higher levels of gene expression than in other cell types [Hall *et al*. 1969]. This means that most, if not all the clones identified were expected to be represented in photoreceptors, and those that are represented by a greater number of clones are more likely expressed in photoreceptors.

The second implication is that like *norp*A this enzyme is quite possibly light-activated via a G protein. There are numerous reports of light-activation of various amphibian photoreceptor PLCs [Ghalayini and Anderson, 1984; Hayashi and

Amakawa, 1985; Waloga and Anderson, 1985; Brown *et al*. 1987]. However, in bovine ROS such activation has not yet been established likely because of the numerous PLC isoforms present [Gehm and McConnell, 1990b]. The *norp*A-like enzyme in bovine photoreceptors may be the PLC that is analogous to the lower vertebrate PLCs.

These implications assure that in future research the *norp*A-like bovine retinal PLC will be pursued most aggressively.

Proposals for Future Research. The future of this line of research lies in expression of PLCs from full length cDNAs and the verification of their comparability with native photoreceptor PLCs. It is very much intertwined with the current work of Rich Pinke, purifying multiple ROS PLC isoforms. Ultimately the aims are to verify the number, primary structure, subcellular location, and expression levels of native PLCs in bovine photoreceptors; to determine the regulatory mechanisms of each PLC *in vitro*, and its possible role in vision.

Since none of the present cDNA clones contains a full length ORF the λgt10 library will be rescreened and a bovine retinal λgt11 cDNA library (Clonetech) will be screened with labeled restriction fragments from these clones. DNA from positive plaques will be subjected to restriction analysis to establish apparent overlap with the present clones, and then sequenced to confirm the overlap.

If clones containing full length ORFs cannot be found in the libraries then perhaps composites can be constructed by PCR using retinal sscDNA or by ligation of restriction fragments of shorter clones. Another alternative may be in constructing an expression library using cDNA derived from photoreceptor-enriched poly A^+ RNA. This is obtainable by isolating RNA not from whole retinas but from the first supernatant of ROS preparation (see PDE Methods). This material has been shown to be enriched in polysomes from the inner segment of rod cells [McConnell *et al.* 1969].

Full length ORFs will be linked to strong promoters and put into an expression system. It is likely that no single expression system will work equally well with all ORFs and several may have to be tried. But with persistence one that yields the proper post-translational modifications and/or protein folding will be identified for each ORF.

The expressed products will be assayed for PLC activity directly in the crude homogenates of cultured cells and again after purification [Gehm and McConnell, 1990b]. They will be assayed more specifically to determine if they share characteristics of native ROS PLCs such as activation by W-7 [Gehm *et al.* 1991] and arrestin [Ghalayini and Anderson, 1992]. Also limited peptide sequencing will be done to insure that the proper cDNA is expressed, and apparent MW of expression products, determined by denaturing gel electrophoresis, will be compared with MW of their presumed native counterparts.

Next polyclonal antibodies will be raised against the recombinant protein and/or unique peptides from the protein. These will first be applied to dot blots and Western blots of native and recombinant ROS PLCs to determine their cross-reactivity. Those that appear to be specific for a single species can then be used to localize the enzyme by immuno-cytochemistry.

When correspondence between the recombinant PLCs and their native counterparts has been firmly established, regulatory mechanisms of each PLC can be explored by *in vitro* reconstitution experiments. Such experiments will initially involve, co-reconstituting the PLCs in phospholipid vesicles with a variety of potential regulatory/regulated proteins such as rhodopsin, G_t or other G proteins, arrestin, cGMP PDE, and PKC. Ultimately, understanding of regulatory mechanisms will demand site-directed mutagenesis studies, which are probably several years away in the case of bovine retinal PLCs.

APPENDIX

Table A1. Bovine Rod Cell Codon Utilization Table

The codon usage for 8 genes specifically expressed in the bovine rod photoreceptor cells were compiled from published cDNA sequences. The number of times each codon was seen in the 8 sequences was totaled with a grand total of 2,511 codons. The frequency, expressed as a percentage, of each codon in representing its corresponding amino acid was then calculated. The 8 cDNA sequences were: 1) $G_{t\alpha}$ [Yatsunami *et al.* 1985a]; 2) $G_{t\beta}$ [Sugimoto *et al.* 1985]; 3) $G_{t\gamma}$ [Yatsunami *et al.* 1985b]; 4) P_{α} [Ovchinnikov *et al.* 1987]; 5) P_{γ} [Ovchinnikov *et al.* 1986]; 6) Rhodopsin [Nathans and Hogness, 1983]; 7) Arrestin [Wistow *et al.* 1986]; and 8) Peripherin [Connell and Molday, 1990].

Table A1.

AA	Codon	Total#	%Freq.
Arg	CGT	11	10.19
	CGC	32	29.63
	CGA	7	6.48
	CGG	24	22.22
	AGA	12	11.11
	AGG	22	20.37
Leu	CTT	20	8.89
	CTC	56	24.89
	CTA	9	4.00
	CTG	116	51.56
	TTA	5	2.22
	TTG	19	8.44
Ser	TCT	22	16.30
	TCC	37	27.41
	TCA	15	11.11
	TCG	12	8.89
	AGT	13	9.63
	AGC	36	26.67
Thr	ACT	22	14.67
	ACC	71	47.33
	ACA	27	18.00
	ACG	30	20.00
Pro	CCT	19	18.81
	CCC	45	44.55
	CCA	17	16.83
	CCG	20	19.80
Ala	GCT	33	19.64
	GCC	90	53.57
	GCA	21	12.50
	GCG	24	14.29
Gly	GGT	18	12.24
	GGC	55	37.41
	GGA	27	18.37
	GGG	47	31.97
Val	GTT	11	6.43
	GTC	59	34.50
	GTA	6	3.51
	GTG	95	55.56

AA	Codon	Total#	%Freq.
Ile	ATT	34	23.78
	ATC	103	72.03
	ATA	6	4.20
Lys	AAA	51	31.68
	AAG	110	68.32
Asn	AAT	31	27.19
	AAC	83	72.81
Gln	CAA	18	18.00
	CAG	82	82.00
His	CAT	13	22.81
	CAC	44	77.19
Glu	GAA	59	31.55
	GAG	128	68.45
Asp	GAT	46	31.72
	GAC	99	68.28
Tyr	TAT	21	23.86
	TAC	67	76.14
Cys	TGT	19	31.67
	TGC	41	68.33
Phe	TTT	40	31.75
	TTC	86	68.25
Met	ATG	91	100.00
Trp	TGG	34	100.00

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