Regulation of Type II Phosphatidylinositol Phosphate Kinase by Tyrosine Phosphorylation in Bovine Rod Outer Segments†

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ABSTRACT: Type II phosphatidylinositol phosphate kinase (PIPKII) is an enzyme responsible for the synthesis of phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2) from phosphatidylinositol-5-phosphate (PI-5-P). In this study, we demonstrate the presence of PIPKIIα in bovine photoreceptor rod outer segments (ROS) and the involvement of tyrosine phosphorylation in the regulation of its activity. PIPKII activity in bovine ROS was verified by the preferential conversion of synthetic dipalmitoyl PI-5-P to PI-4,5-P_2, lack of effect of phosphatidic acid, inhibition by heparin, immunoreaction with an anti-PIPKIIα antibody on Western blots, and immunocytochemical localization in bovine and rat ROS by anti-PIPKIIα. Immunoprecipitates of bovine ROS with the anti-PIPKIIα antibody possessed PIPK enzymatic activity and preferentially used PI-5-P as substrate for PI-4,5-P_2 biosynthesis. The activity of PIPKII was greatly increased under conditions favoring tyrosine phosphorylation in ROS, and PIPKII activity was immunoprecipitated with anti-phosphotyrosine (anti-PY) antibodies from tyrosine phosphorylated ROS. Preincubation of ROS with tyrosine kinase inhibitors almost abolished the kinase activity in the anti-PY immunoprecipitates. Immunoblot analysis showed that PIPKIIα was present in anti-PY immunoprecipitates from phosphorylated ROS but not from nonphosphorylated controls. We conclude that PIPKIIα is present in ROS and that its activity is regulated by tyrosine phosphorylation.

Hydrolysis of phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2)[1] by phospholipase C generates two second messengers, 1,2-diacylglycerol and inositol 1,4,5-trisphosphate, which activates protein kinase C (PKC) and raises cytosolic calcium concentration, respectively (*1*). PI-4,5-P_2 can also be phosphorylated by PI 3-kinase (*2*) to produce PI-3,4,5-trisphosphate (PI-3,4,5-P_3) in a receptor-dependent manner. This lipid second messenger has an intracellular function quite distinct from that of PI-4,5-P_2 and its hydrolytic products (*3*−*5*). Binding of PI-4,5-P_2 (*6*) and PI-3,4,5-P_3 (*7*) to pleckstrin homology domains in many intracellular signaling proteins links the proteins to the cellular membrane surface and stimulates their enzymatic activities. The multiple functions of PI-4,5-P_2 imply that the levels of this molecule must be tightly regulated in order to maintain the integrity of the signaling pathways in which it participates.

Phosphatidylinositol phosphate kinases participate in the synthesis of PI-4,5-P_2 by phosphorylation of PI-4-P and PI-5-P on the D-5 and the D-4 positions of inositol, respectively. Several PIP kinase enzymes have been purified and characterized (*8*−*10*), and some have been cloned and sequenced (*11*−*13*). The family of PIP kinases can be divided into two groups (type I and type II) according to their biochemical and immunological properties (*13*, *14*). Using PI-5-P or PI-4-P as substrates has revealed that PIPKI is predominantly a PI-4-P 5-kinase, while PIPKII is predominantly a PI-5-P 4-kinase (*15*). However, neither kinase family has perfect fidelity (*16*).

The mechanism of activation of the phosphoinositide kinases is not well understood. It has been reported that the activity PIPKI is stimulated by GTPγS (*17*), small G-proteins (*18*), PA (*19*), and heparin and spermine (*20*), while PIPKII activity is not affected by PA, is inhibited or not affected by spermine, and is inhibited by heparin (*20*). However, there is evidence that tyrosine phosphorylation may be involved in the control of the activity of some of the kinases.

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[1] Abbreviations: PA, phosphatidic acid; PI, phosphatidylinositol; gPI, deacylated product of PI; PI-3-P, phosphatidylinositol-3-phosphate; PI-4-P, phosphatidylinositol-4-phosphate; PI-5-P, phosphatidylinositol-5-phosphate; PI-4,5-P_2, phosphatidylinositol-4,5-bisphosphate; PI-3,4,5-P_3, phosphatidylinositol 3,4,5-trisphosphate; PIP kinase, phosphatidylinositol phosphate kinase; PIPKI, type I phosphatidylinositol-4-P 5-kinase; PIPKII, type II phosphatidylinositol-5-P 4-kinase; PKC, protein kinase C; ROS, rod outer segments; PY-ROS, tyrosine phosphorylated rod outer segments; N-ROS, non-tyrosine phosphorylated rod outer segments; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.

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Following EGF receptor activation, PI 4-kinase and PI-4-P 5-kinase activities were increased in proteins immunoprecipitated with anti-PY antibodies (*21*). Four tyrosine-phosphorylated proteins were found to be associated with the EGF receptor in vivo in cross-linking experiments, two of which were tentatively identified as PI 4-kinase and PI-4-P 5-kinase. More recently, Castellino and Chao (*22*) found that PIPKII was associated with the activated EGF receptor. Treatment of A431 and PC12−615 cells with EGF, followed by immunoprecipitating with anti-PIPKII antibody and western blotting with anti-PY or anti-EGF receptor antibodies, revealed the presence of tyrosine phosphorylated EGF receptor in the immunoprecipitates. EGF receptor specificity was shown by the absence of TrkA receptors in the immunoprecipitates. Treatment of MCF7 cells with GGF/neuregulin, which promotes tyrosine phosphorylation of ErbB2 and ErbB3, resulted in the immunoprecipitation of tyrosine phosphorylated ErbB2, but not ErbB3, by anti-PIPKII antibodies (*22*). Homogenates of MCF7 cells pretreated with GGF/neuregulin had a 5-fold increase in PIP kinase activity. However, these incubations were carried out on homogenates, rather that anti-PIPKII immunoprecipitates, and used PI-4-P as substrate, so it is not clear if type I or type II PIPK activity was measured. Also, these latter studies did not establish if PIPKII was tyrosine phosphorylated or recruited to a phosphorylated protein following receptor activation.

Vertebrate retinal photoreceptor cells have an active phosphoinositide metabolism and several steps in the PI cycle are stimulated by light (*23*−*27*). Activation of phospholipase C by light leads to the hydrolysis of PIP_2 (*23*, *24*) and the stimulation of protein kinase C activity (*28*, *29*). PI 3-kinase activity is present in bovine ROS membranes, and its activity is higher in membranes prepared from light-adapted retinas (*30*). DAG-kinase activity is higher in light-adapted bovine ROS (in vitro) and rat ROS (in vivo), due to increased binding of the kinase to the light-adapted membranes (*26*). Finally, PI synthesis from radiolabeled inositol and endogenous substrates is greater in light-adapted bovine ROS (*27*). Thus, the two pathways that deplete the small amounts of PIP_2 normally present in membranes are active in vertebrate ROS, although the physiological consequences of their activities are not yet fully appreciated.

Light stimulates tyrosine phosphorylation of several proteins in rat ROS in vivo (*31*). Incubation of bovine ROS under conditions favoring tyrosine phosphorylation leads to the phosphorylation of at least 10 proteins by an endogenous tyrosine kinase (*32*). Recently, we found that the activity of PI 3-kinase is 6 times higher in anti-PY immunoprecipitates from tyrosine phosphorylated bovine ROS than from nonphosphorylated ROS (*33*). PLCγ1, the enzyme responsible for hydrolysis of PI-4,5-P_2 and known to be stimulated by tyrosine phosphorylation (*34*), is present in bovine ROS (*35*, *36*). In the current study, we identified one of the enzymes in bovine ROS responsible for replenishing PI-4,5-P_2 in these membranes as the α-isoform of PIPKII. Its in vitro enzymatic activity is stimulated by tyrosine phosphorylation of ROS proteins, although PIPKII itself is not phosphorylated.

EXPERIMENTAL PROCEDURES

Materials. Dark-adapted frozen bovine retinas were purchased from Rockville Meat Co. (Rockville, MO). Goat polyclonal anti-PIPKIIα antibody (N-19; catalog no. Sc-1330) and blocking peptide (catalog no. Sc-1330P) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). This antibody has been shown by Boronenkov et al. (*37*) to be highly specific for PIPKIIα. Mouse monoclonal anti-phosphotyrosine (anti-PY20) antibody was purchased from Transduction Laboratories (Lexington, KY). Anti-PY69 antibody was also purchased from Transduction Laboratories (Lexington, KY). In our hands, anti-PY20 was better for immunoprecipitation reactions where recovery of PIPK was measured, and anti-PY69 was better for western blots. [^{3}H]-PI-4,5-P_2 and [^{3}H]PI-4-P were from American Radiolabeled Chemicals (St. Louis, MO). [γ-^{32}P]ATP was from Dupont NEN (Boston, MA). The tyrosine kinase inhibitors PP1, genestin, tyrphostin A-25, and tyrphostin A-46 were obtained from Calbiochem (San Diego, CA). PA was from Matreya (Pleasant, PA). ATP, orthovanadate (Na_3VO_4), and all other chemicals were from Sigma (St. Louis, MO). Echelon Research Laboratories Inc. (Salt Lake City, UT) provided synthetic dipalmitoyl-PI, PI-3-P, PI-4-P, and PI-5-P, which had >99% isomeric purity according to the manufacturer's analysis.

Preparation of Bovine ROS. ROS were prepared from frozen bovine retinas on a discontinuous sucrose gradient as described (*38*). The final ROS pellets were suspended in ROS buffer [10 mM MOPS (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, and 0.1 mM PMSF] containing 10% sucrose, aliquoted into tubes, and stored at −80 °C. Protein concentration was determined using the BCA kit according to the manufacturer's protocol (Pierce, Rockford, IL).

Preparation of Tyrosine Phosphorylated ROS. ROS prepared in the manner described above have an endogenous tyrosine kinase activity (*32*). PY-ROS were prepared by incubating ROS for 20 min at 37 °C in a phosphorylation buffer [50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 2 mM $MgCl_2$, 1.5 mM ATP, and 0.2 mM Na_3VO_4]. N-ROS were prepared by incubating ROS in a similar buffer without ATP and Na_3VO_4. After incubation, ROS membranes were pelleted by centrifugation for 20 min at 17000*g* and washed twice with phosphorylation buffer without ATP. The resulting ROS membranes were suspended in a kinase assay buffer [50 mM Tris-HCl (pH 7.4), 1 mM EGTA, 2 mM $MgCl_2$] for PIP kinase activity determination. For testing the effect of tyrosine kinase inhibitors on the activity of PIP kinase, ROS were preincubated for 15 min with 10 μM PP1, 80 μM of genestin, tyrphostin A-25, tyrphostin A-46, or 1% DMSO, in phosphorylation buffer without ATP and Na_3VO_4. Following preincubation, ATP and Na_3VO_4 were added, and the tyrosine phosphorylation reaction was conducted as described above.

Immunoprecipitation. PY-ROS and N-ROS (300 μg each) were solubilized at 4 °C for 30 min in a solubilization buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, and 10% glycerol]. Insoluble materials were removed by centrifugation at 17000*g* for 20 min, and the solubilized ROS proteins were precleared by incubation with 40 μL of protein A−Sepharose for 1 h at 4 °C with mixing. PY-ROS and N-ROS proteins were immunoprecipitated for 3 h at room temperature by incubating with 6 μg of anti-PY20 or 4 μg of anti-PIPKIIα. Controls were prepared using an equal amount of nonimmune IgG to immunoprecipitate PY-ROS. Immune complexes were incubated with 40 μL of protein

A−Sepharose for 2 h, followed by two washes with modified solubilization buffer (the 1% Triton X-100 was reduced to 0.1% and glycerol was removed) and two washes with phosphorylation buffer without ATP. Immunoprecipitates were assayed for PIP kinase activity or subjected to immunoblot analyses.

ROS PIP Kinase Assays. PY-ROS, N-ROS, and immunoprecipitates from these membranes were assayed for PIP kinase activity by the method of Bazenet et al. (*20*) with slight modifications. Briefly, the enzyme assay reaction was performed for 15 min at room temperature in 50 μL of kinase assay buffer plus 0.2 mM Na_3VO_4, 50 μM ATP, and 5 μCi of [γ-^{32}P]ATP, with or without 80 μM synthetic dipalmitoyl-PI, PI-3-P, PI-4-P, or PI-5-P. The reactions were terminated by the addition of 100 μL of 1 N HCl. Total lipids were extracted with 250 μL of chloroform/methanol (1/1 v/v), separated on thin-layer chromatography (TLC) plates in a solvent system of chloroform/methanol/ammonium hydroxide/water (70/100/25/15 by vol), and subjected to autoradiography overnight at −80 °C. The product PIP_2 was identified by comigration with a PI-4,5-P_2 standard, scraped from the TLC plates, and counted for radioactivity.

HPLC Analysis of PIP Kinase Products. PIP_2 prepared by TLC was deacylated with 1.8 mL of methylamine/methanol/1-butanol (42.8/45.7/11.4 by vol) for 50 min at 53 °C (*39*). Deacylated products were dried under nitrogen, suspended in 2 mL of H_2O, and extracted twice with 2 mL of 1-butanol/petroleum ether/ethyl formate (20/4/1 by vol). The aqueous phase containing deacylated products was dried under nitrogen and resuspended in 10 mM $(NH_4)_2HPO_4$ (pH 3.8). HPLC separation was achieved on a Partsphere 5 Sax anion-exchange column (Whatman, Clifton, NJ) using an elution scheme of water (10 min) followed by a linear gradient of $(NH_4)_2HPO_4$ (pH 3.8) from 0 to 0.25 M for 60 min at a flow rate of 1.0 mL/min and 3 mL/min of High Flash-Point LSC−Cocktail (Packard Instrument Co., Meriden, CT). Radioactivity was monitored on-line by a Flow-One A250 radioisotope detector (Radiomatic Instruments, Tampa, FL).

SDS−PAGE and Immunoblot Analysis. Proteins were resolved by 10% SDS−PAGE and transferred onto nitrocellulose membranes, and the blots were washed twice for 10 min with TTBS [20 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 0.1% Tween 20] and blocked with 10% bovine serum albumin (BSA) in TTBS overnight at 4 °C. Blots were then incubated with anti-PIPKIIα (0.5 g/mL), anti-PY20 (0.5 μg/mL), or anti-PY69 (0.3 μg/mL) antibodies at room temperature for 3 h. For neutralization of anti-PIPKIIα, the specific blocking peptide was preincubated with the antibody for 2 h at room temperature in a ratio 10:1 (by weight) in 500 μL of PBS, and the resulting antibody/peptide mixture was diluted and used to probe the blots. Following reaction with primary antibodies, the blots were washed three times for 5 min with TTBS and incubated for 1 h with HRP-conjugated anti-goat (0.05 μg/mL) or anti-mouse (0.05 μg/mL) IgG at room temperature. The nitrocellulose membrane was finally washed four times for 15 min with TTBS and developed by ECL according to the manufacturer's instructions.

Renaturation of PIP Kinase Following SDS−PAGE. ROS proteins (300 μg) were resolved by 10% SDS−PAGE, after which the gels were cut into 5 mm slices and renatured according to Jenkins et al. (*19*) with slight modifications. Briefly, gel slices were washed once with an elution buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.2 mM Na_3VO_4, and 0.1 mg/mL BSA], mashed with a glass pestle, and incubated at 4 °C for 18 h in 1.5 mL of elution buffer. Eluted proteins were collected after a brief centrifugation and precipitated with 10% trichloroacetic acid in elution buffer on ice for 10 min. Precipitated proteins were washed three times with a mixture of 80% acetone and 20% elution buffer that was precooled to −20 °C. The final products were dissolved in 20 μL of 6 M guanidine hydrochloride for 45 min. PIP kinase was renatured and assayed overnight at room temperature in 500 μL of assay buffer [50 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, 1 mM EGTA, and 0.2 mM Na_3VO_4] with 50 μM PI-5-P, 50 μM ATP, and 50 μCi of [γ-^{32}P]ATP. The assay reactions were terminated by the addition of 300 μL of 1 N HCl, and the assay products were extracted and analyzed as described above.

Immunocytochemical Localization of PIPKIIα in Bovine and Rat Retinas. Adult bovine eyes obtained from a local abattoir and rat eyes removed under anesthesia were opened by an encircling cut and prepared for immunocytochemistry as previously described (*32*). After fixation, bovine and rat eyecups were washed with PBS, dissected into retinal−scleral pieces, cryoprotected in 30% sucrose in PBS overnight, embedded in OCT medium, and frozen. Retinal pieces were sectioned at 14 μm on a Leica Cryocut 1800 and collected on gelatin-coated slides. After sections were hydrated with 10 mM PBS (pH 7.4) for 20 min at room temperature, the manufacturer's protocols were followed for peroxide and protein blocking (Biogenex, San Raman, CA). Sections were incubated with anti-PIPKIIα (1:70), antibody/blocking peptide mixture (1:100), or PBS at room temperature for 2 h. Secondary antibody linking (biotinylated), peroxide labeling (peroxidase-conjugated streptavidin), and diaminobenzidine substrate developing were performed as described. Slides were rinsed in distilled water, cover-slipped, and visualized on a Nikon Eclipse E800 digital microscope. Images were captured using Adobe Photoshop 5.5.

Data Analysis. PIP kinase activity assays were done on at least four independent ROS preparations. The data were analyzed by ANOVA or Student's *t*-test, and statistical significances were set at $P < 0.05$.

RESULTS

Identification and Substrate Specificity of PIPKIIα in Bovine ROS. PIP kinase activity in bovine ROS was established by standard assay using [γ-^{32}P]ATP and exogenous synthetic dipalmitoyl-PI-5-P as substrates. The enzyme assay products were extracted with chloroform/methanol, separated on TLC plates, and subjected to autoradiography. As shown in Figure 1A, ROS converted PIP to PI-4,5-P_2, most of which was synthesized from exogenous PI-5-P (Figure 1A, right lane), although some PI-4,5-P_2 was apparently formed from endogenous PIP (Figure 1A, left lane). The PI-4,5-P_2 formed from endogenous PIP migrated slightly faster than the PI-4,5-P_2 formed from synthetic dipalmitoyl-PI-5-P because of differences in the fatty acid side chains. The identities of PI-4,5-P_2 formed from endogenous and exogenous PIP were demonstrated by HPLC analysis of the deacylated lipids (Figure 1B). Both eluted in

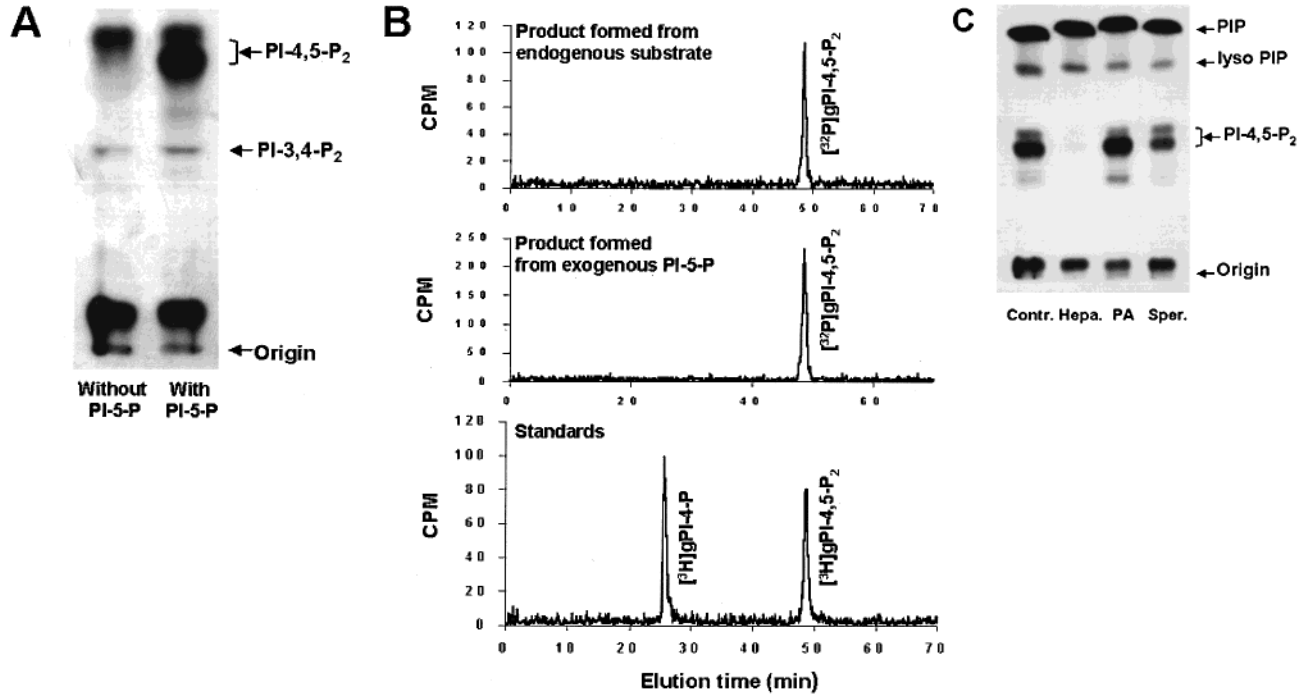

FIGURE 1: PIP kinase enzyme activity in bovine ROS. (A) TLC autoradiogram of ROS PIP kinase products formed when synthetic dipalmitoyl PI-5-P (80 μM) was used as substrate. Only the lower half of the TLC plate is shown, which amplifies the separation of PI-3, 4-P_2 and PI-4, 5-P_2. (B) HPLC analysis of the PIP kinase reaction product PI-4,5-P_2 formed from endogenous and exogenous substrates. (C) TLC autoradiogram of the effect of phosphatidic acid (PA, 100 μM), heparin (Hepa, 30 μg/mL), and spermine (Sper, 2 mM) on PIP kinase activity in bovine ROS.

about 48 min, the same time as the synthetic standard. In some experiments, sample and standard were mixed, and both eluted at exactly the same time.

PIPKI activity has been demonstrated to be stimulated by PA (*19*), heparin (*20*), and spermine (*20*), whereas PIPKII activity is not affected by PA, is inhibited or not affected by spermine, and is inhibited by heparin (*20*). Figure 1C shows that PIP kinase activity in bovine ROS was not affected by the presence of 100 μM PA in the kinase assay but was almost completely inhibited by 30 μg/mL heparin and reduced about 50% by 2 mM spermine. These results suggest that ROS contain PIPKII.

The presence of PIPKIIα in bovine ROS was demonstrated by immunoblot analysis utilizing a peptide-specific antiserum directed against the N-terminus (N-19) of PIPKIIα. Bovine ROS probed with anti-PIPKIIα (Figure 2A, lane 2) showed two immunoreactive bands, one with an apparent molecular mass of 41 kDa and another with a molecular mass of 93 kDa. Omission of the primary antibody resulted in the absence of immunoreaction in the 41 kDa band (Figure 2A, lane 1). Immunoreaction of the 41 kDa protein with the antibody could be specifically blocked by a control peptide corresponding to the epitope recognized by anti-PIPKIIα (Figure 2A, lane 3). These results demonstrate the presence of PIPKIIα in bovine ROS.

To further determine if the 41 kDa protein recognized by anti-PIPKIIα is a PIP kinase, PIP kinase assays were conducted using the kinase renatured from the SDS−PAGE as the enzyme source and PI-5-P as substrate. As shown in Figure 2B, only the slice containing the 41 kDa protein contained renaturable PIP kinase activity.

Phosphorylation of PI-3-P, PI-4-P, and PI-5-P by Anti-PIPKIIα Immunoprecipitates. The substrate specificity of PIPKIIα was tested by using the immunoprecipitates of anti-

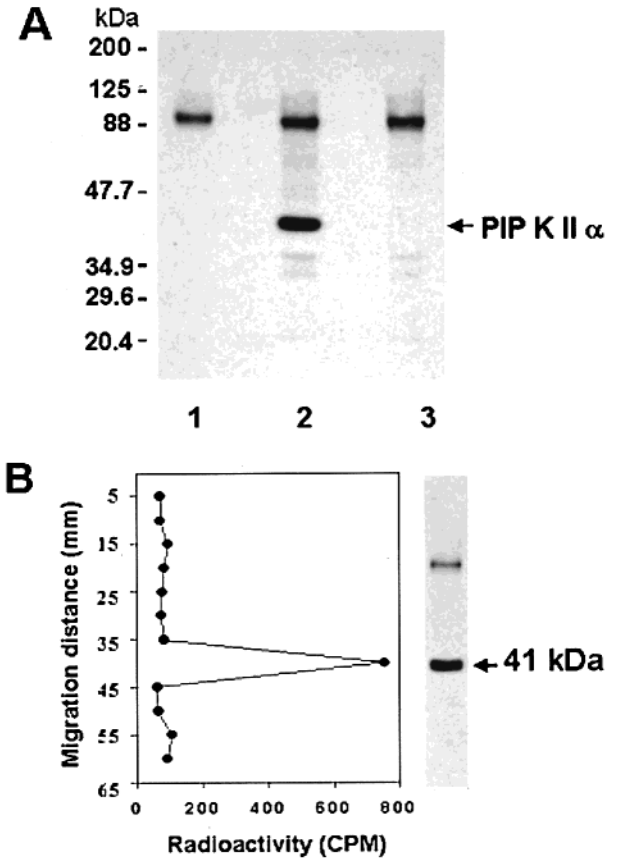

FIGURE 2: Identification of PIPKIIα in bovine ROS. (A) Western blot of ROS probed with anti-PIPKIIα (lane 2), secondary antibody only (lane 1), or primary antibody neutralized with peptide (lane 3). (B) PIP kinase activities following renaturation of proteins taken from 5 mm slices of the SDS−PAGE gel. The location of the 41 kDa protein identified by the anti-PIPKIIα antibody is shown on the gel.

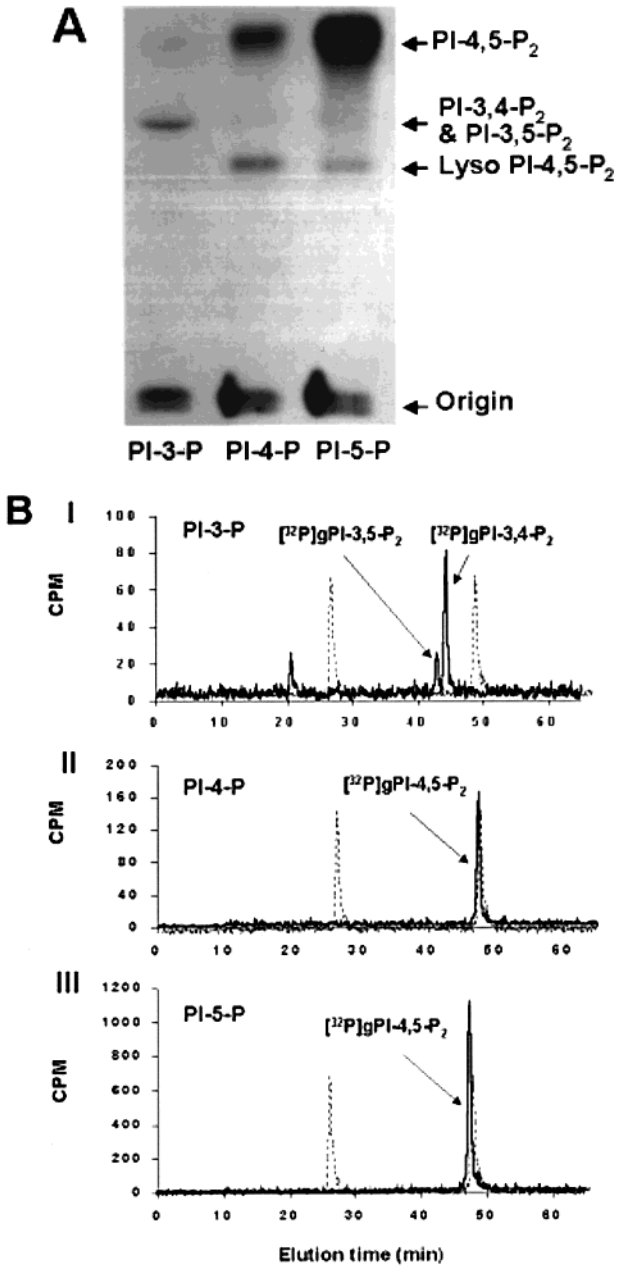

FIGURE 3: Substrate preference of PIPKIIα. (A) TLC autoradiogram of products of incubation using anti-PIPKIIα immunoprecipitates as a source of enzyme and 80 μM synthetic dipalmitoyl PI-3-P, PI-4-P, or PI-5-P as substrates. (B) HPLC analysis of the PIP kinase reaction products. Panel I shows the elution of [^{32}P]gPI-3,4-P_2 and [^{32}P]gPI-3,5-P_2 from deacylated PI-3-P phosphorylation products. Panel II shows the elution of [^{32}P]gPI-4,5-P_2 from deacylated PI-4-P phosphorylation products. Panel III shows the elution of [^{32}P]-gPI-4,5-P_2 from deacylated PI-5-P phosphorylation products. The elution profiles of the standards [^{3}H]gPI-4-P and [^{3}H]gPI-4,5-P_2 are shown by the dashed lines.

PIPKIIα as an enzyme source and synthetic dipalmitoyl-PI, PI-3-P, PI-4-P, or PI-5-P as substrates. The products of the enzyme assays were separated by TLC, identified by autoradiography, and quantified by scintillation counting. As shown in Figure 3A, PI-5-P was the preferred substrate for PIPKIIα in bovine ROS, followed by PI-4-P and PI-3-P. The phosphorylation products of PI-4-P and PI-5-P migrated at the position of PI-4,5-P_2 on TLC plates. The phosphorylation product of PI-3-P migrated as a PI bisphosphate, but more slowly than PI-4,5-P_2. The relative amounts of PIP_2 formed

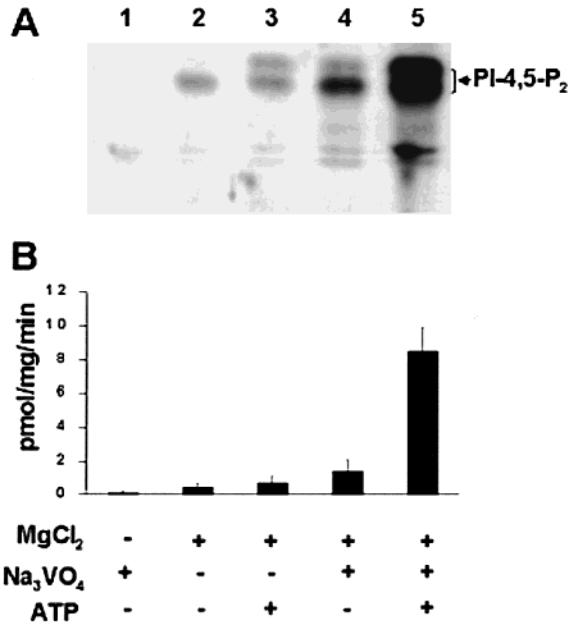

FIGURE 4: Effect of tyrosine phosphorylation on PIP kinase activity in ROS. (A) TLC autoradiogram of products of ROS preincubated as indicated and assayed for PIP kinase activity using 80 μM dipalmitoyl PI-5-P as substrate. (B) PIP kinase activities of five independent ROS preparations. The difference between lane 5 and the other lanes is significant at $P < 0.001$.

by ROS PIPKIIα were 13:2:1 for PI-5-P, PI-4-P, and PI-3-P, respectively, at PIP a concentration of 80 μM. When PI was used as a substrate, no phosphorylated lipid products were found in anti-PIPKIIα immunoprecipitates from ROS (data not shown). The possibility that the phosphorylation of PI-3-P and PI-4-P was due to contamination with other PI kinases in the immunoprecipitates was eliminated because the immunoprecipitates could not phosphorylate PI to PI-5-P (*40*) and the control immunoprecipitates (absence of primary antibody) did not show any enzymatic activity toward PI-4-P. Therefore, the PIP kinase activity in the immunoprecipitates toward PI-3-P and PI-4-P is due to PIPKIIα.

The phosphorylated products of PI-3-P, PI-4-P, and PI-5-P produced by anti-PIPKIIα immunoprecipitates were identified by HPLC (Figure 3B). The deacylated products from the kinase reactions using PI-3-P as a substrate separated into two peaks by HPLC and migrated at the positions of PI-3,4-P_2 and PI-3,5-P_2 (Figure 3B I) (*41*), which indicated that PI-3-P can be phosphorylated on both the D-4 and D-5 positions. One small unidentified peak from the phosphorylated PI-3-P products eluted at about 22 min. HPLC analysis showed that phosphorylated products of the PI-4-P and PI-5-P were PI-4,5-P_2 (Figure 3B, panels II and III). No labeled PI-3,4,5-P_3 was found in any of these incubations.

Effect of Tyrosine Phosphorylation on PIP Kinase Activity in Bovine ROS. While characterizing the enzymatic activity of PI 3-kinase, we discovered that PIP kinase activity was stimulated by tyrosine phosphorylation. Bovine ROS were incubated in a buffer with or without $MgCl_2$, ATP, and/or Na_3VO_4 (a protein tyrosine phosphatase inhibitor), using 80 μM PIP-5-P as substrate, to examine the effects of tyrosine phosphorylation on PIP kinase activity (Figure 4). In the absence of ATP and $MgCl_2$, PIP kinase activity was very

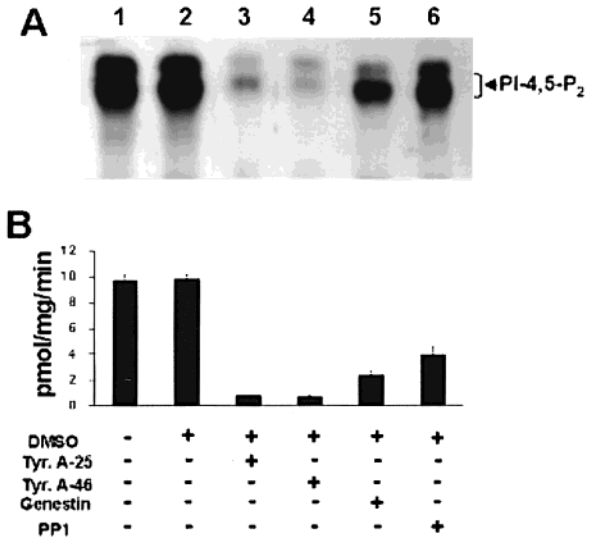

FIGURE 5: Effect of tyrosine kinase inhibitors on PIP kinase activity in ROS. (A) TLC autoradiogram of products of ROS preincubated as indicated and then incubated under tyrosine phosphorylation conditions (Figure 4, lane 5) in the presence of tyrosine kinase inhibitors. PIP kinase assays were conducted using 80 μM dipalmitoyl PI-5-P as substrate. (B) PIP kinase activities of five independent ROS preparations. The difference between lanes 1 or 2 and the other lanes is significant at $P < 0.001$.

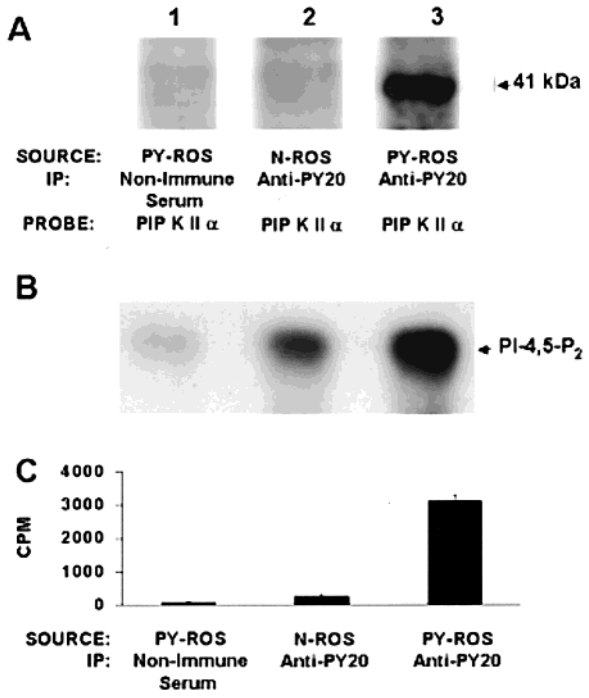

FIGURE 6: Identification of PIPKIIα in anti-PY20 immunoprecipitates. PY-ROS (300 μg) and N-ROS (300 μg) were immunoprecipitated with anti-PY20. (A) Western blots of 41 kDa proteins immunoprecipitated with anti-PY20 or nonimmune serum and probed with anti-PIPKIIα. (B) TLC autoradiogram of PI-4,5-P_2 formed by incubation of the immunoprecipitated proteins, using 80 μM dipalmitoyl-PIP-5-P as substrate. (C) PIP kinase activities of three independent ROS preparations. The difference between N-ROS and PY-ROS is significant at $P < 0.001$.

low (lane 1). The addition of $MgCl_2$ alone (lane 2), or with either ATP (lane 3) or Na_3VO_4 alone (lane 4), caused a small increase in PIP kinase activity. The most dramatic increase in PIP kinase activity was found in the ROS incubated in the presence of Na_3VO_4, ATP, and $MgCl_2$ (lane 5), a condition known to stimulate tyrosine phosphorylation in ROS (*32*). PIP kinase activity in PY-ROS (Figure 4B, lane 5) was 6 times greater than that of ROS incubated with $MgCl_2$ and Na_3VO_4, but without ATP (lane 4), and 60 times greater than that of ROS incubated with only Na_3VO_4 (lane 1). These values are from five independent ROS preparations, and the differences are significant at $P < 0.001$.

Several tyrosine kinase inhibitors were tested for their effect on PIP kinase activity in bovine ROS (Figure 5A). Two positive controls, lane 1 without 1% DMSO and lane 2 with 1% DMSO, did not show any difference in their kinase activity. Tyrphostin A-25 (lane 3) and tyrphostin A-46 (lane 4) almost abolished the kinase activity at a concentration of 80 μM, whereas genestin (lane 5) was less effective at the same concentration. PP1 (lane 6) inhibited about 50% of the kinase activity at a concentration of 10 μM. The difference between lanes 1/2 and the other lanes is significant at $P < 0.001$ (Figure 5B).

PIPKIIα in Anti-PY20 Immunoprecipitates from PY-ROS. Anti-phosphotyrosine antibody (anti-PY20) was used to immunoprecipitate tyrosine phosphorylated proteins from PY-ROS and N-ROS. Immunoblot analysis of anti-PY20 immunoprecipitates probed with anti-PIPKIIα showed that the 41 kDa protein previously identified as PIPKIIα in ROS was present in anti-PY20 immunoprecipitates from PY-ROS (Figure 6A, lane 3) but not from N-ROS (lane 2). The control immunoprecipitates from PY-ROS probed with anti-PIPKIIα did not show a positive immunoreaction of the 41 kDa protein in the western blots (lane 1). PIP kinase assays using synthetic dipalmitoyl-PI-5-P as substrate showed that the activity in anti-PY20 immunoprecipitates from PY-ROS (Figure 6B and 6C, lane 3) was about 13 times higher than that from N-ROS (lane 2). The control immunoprecipitates from PY-ROS using nonimmune mouse IgG contained only negligible PIP kinase activity (lane 1). The presence of PIPKIIα in anti-PY20 immunoprecipitates from PY-ROS and the higher PIP kinase activity present in anti-PY20 immunoprecipitates from PY-ROS compared with N-ROS (Figure 6C) suggests that the activity of PIPKIIα is controlled by tyrosine phosphorylation in ROS.

Effect of Tyrosine Kinase Inhibitors on PIP Kinase Activity in Anti-PY20 Immunoprecipitates. To further probe the role of tyrosine phosphorylation in PIP kinase activity in bovine ROS, ROS were preincubated briefly with or without tyrosine kinase inhibitors, followed by incubation under conditions used to generate PY-ROS (*32*). The resulting ROS were immunoprecipitated with anti-PY20 or nonimmune mouse IgG, and the immunoprecipitates were used as an enzyme source to conduct the PIP kinase assays using PI-5-P as substrate (Figure 7). Consistent with the PIP kinase assays on ROS (Figure 5), tyrphostin A-25 and tyrphostin A-46 had the most significant effect on the kinase activity in anti-PY20 immunoprecipitates (Figure 7, lanes 3 and 4) as compared with the control values (lane 1). These two tyrosine kinase inhibitors virtually abolished the kinase activity in the immunoprecipitates at a concentration of 80 μM. Genestin (80 μM, lane 5) and PP1 (10 μM, lane 6) also inhibited PIP kinase activity. The normal mouse IgG (lane 2) immunoprecipitates had negligible PIP kinase activity. The inhibition of PIP kinase activity by tyrosine kinase inhibitors in anti-PY20 immunoprecipitates further confirms that PIP kinase

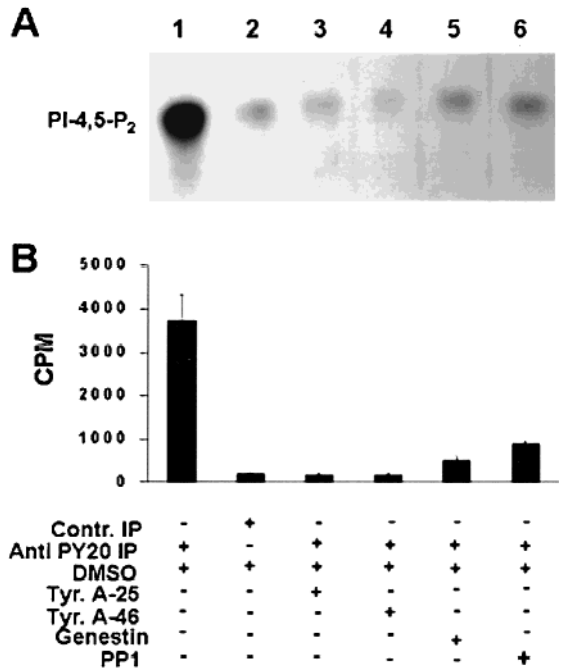

FIGURE 7: Effect of tyrosine kinase inhibitors on PIP kinase activity in anti-PY20 immunoprecipitates. ROS were treated as described for Figure 5, except that the samples were immunoprecipitated prior to PIP kinase assay. (A) TLC autoradiogram of PI-4,5-P_2 products. (B) PIP kinase assays of four independent ROS preparations. The difference between lane 1 and the other lanes is significant at $P < 0.001$.

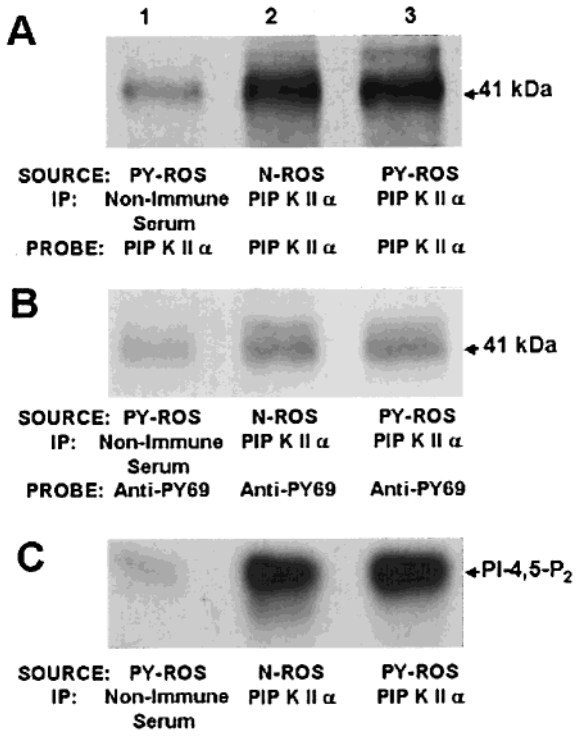

FIGURE 8: Tyrosine phosphorylation of PIPKIIα. PY-ROS and N-ROS were immunoprecipitated with or without anti-PIPKIIα, and the resulting immunoprecipitates were probed with anti-PIPKIIα (panel A) or anti-PY69 (panel B) or used to measure PIP kinase activity (panel C). The integrated densities of PIPKIIα immunoreactions (panel A, lanes 2 and 3) and the kinase activities (panel C, lanes 2 and 3) are not significantly different.

activity in ROS may be controlled by tyrosine phosphorylation.

Anti-PIPKIIα Immunoprecipitates from PY-ROS and N-ROS. To examine whether the PIPKIIα activity stimulated by tyrosine phosphorylation was due to phosphorylation of the enzyme, anti-PIPKIIα antibody was used to immunoprecipitate PIP kinase from PY-ROS and N-ROS. Western blot analysis of the immunoprecipitates probed with anti-PIPKIIα showed an immunoreactive 41 kDa band in both PY-ROS and N-ROS (Figure 8A, lanes 2 and 3). A much weaker 41 kDa immunoreaction band was seen in the immunoblots that were probed with anti-phosphotyrosine antibody (anti-PY69) (Figure 8B, lanes 2 and 3), and there were no obvious differences between PY-ROS and N-ROS. Using anti-PIPKIIα immunoprecipitates from ROS as substrate, and c-Src or anti-type I insulin-like growth factor receptor immunoprecipitates from ROS as sources of tyrosine kinase, incubation with [γ-^{32}P]ATP in the tyrosine phosphorylation buffer showed no obvious phosphorylation of the 41 kDa protein under conditions where other proteins were labeled (data not shown). These results provide no evidence that PIPKIIα is tyrosine phosphorylated in ROS.

When anti-PIPKIIα immunoprecipitates from PY-ROS and N-ROS were incubated with PI-5-P, there was no significant difference in PIP kinase activity between PY-ROS and N-ROS (Figure 8C).

Immunocytochemical Localization of PIPKIIα in Rat and Bovine Retinas. The cellular localization of PIPKIIα in rat and bovine retinas was determined using the same anti-PIPKIIα antibody used for immunoprecipitations and western blots. Unstained frozen sections of bovine retinas (Figure 9A−C) show primary localization of immunoreactivity in the outer segments, with small amounts of reactivity in other retinal layers (C), which was not present when primary antibody was not used (A) or was pretreated with blocking peptide (B). Toluidine blue stained sections of frozen rat retinas showed an identical picture, with most of the reaction product in ROS (F). Omission of primary antibody (D) or blocking with the immunogenic peptide (E) resulted in an absence of reaction product in the retina.

DISCUSSION

The classic pathway for PI-4,5-P_2 synthesis is the successive actions of PI 4-kinase and PI-4-P 5-kinase. A new pathway for PI-4,5-P_2 formation has recently been established by the discovery of the presence of PI-5-P in mammalian cells (*15*) and defining PIPKII as a predominantly PI-5-P 4-kinase (*15*). Previous studies from our laboratory using radiolabeled inositol have shown that the enzymes for PI-4,5-P_2 synthesis are present in bovine ROS (*27*). In the current study, we used several approaches to establish that the PIPK in these membranes is type II. In agreement with Rameh et al. (*15*), we found that the preferred substrate for ROS PIPKIIα is PI-5-P; however, the enzyme could also use PI-3-P and PI-4-P as substrates, as shown by others (*16*, *42*). Furthermore, incubation with heparin blocked PI-4,5-P_2 formation, while phosphatidic acid had no effect and spermine only a modest effect. This behavior is consistent with the properties of PIPKII, since all three stimulate PIPKI activity, especially phosphatidic acid (*19*). Western blots with an antibody to PIPKIIα establish the presence of this isoform in bovine ROS. Immunocytochemistry in both bovine and rat retinas also demonstrates its presence in ROS. The paucity of immunoreaction in other retinal layers (Figure 9) suggests

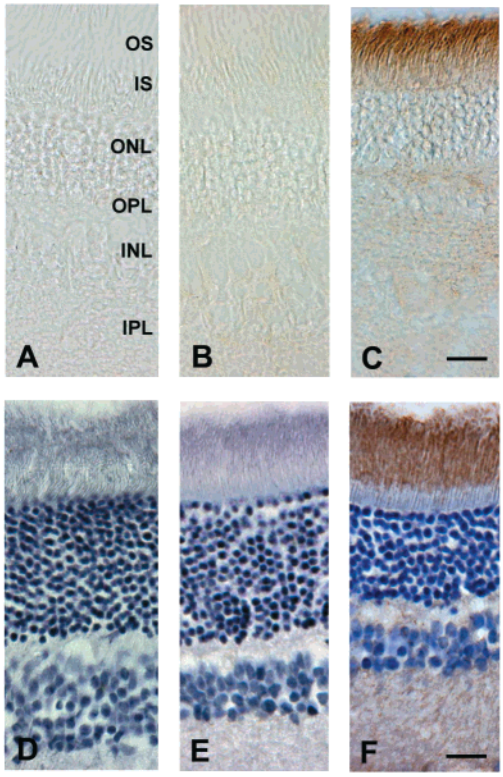

FIGURE 9: Bovine (A−C) and rat (D−F) retina frozen sections (14 μm) from light-adapted eyes subjected to immunocytochemistry. The bovine sections (A−C) were not stained; the rat sections (D−F) were stained with toluidine blue. (A, D) Incubation with nonimmune goat IgG; (B, E) incubation with anti-PIPKIIα/blocking peptide mixture; (C, F) incubation with anti-PIPKIIα. The bar length is 20 μm. The retinal cell layers are as follows: OS, photoreceptor outer segments; IS, photoreceptor inner segments; ONL, outer nuclear layer; OPL, outer plexiform cell layer; INL, inner nuclear layer; IPL, inner plexiform layer.

that PIPKIIα may be expressed solely in photoreceptor cells. Whether this enzyme is present in cone photoreceptor cells cannot be determined from the light micrographs, due to the small number of cones in the retinas of cattle and rats and the difficulty in distinguishing them morphologically.

The dramatic blocking effect of heparin on PIPK activity in ROS (Figure 1C) supports our argument that PIPKIIα is the major isoform in these membranes. If any of the PI-4,5-P_2 formed from endogenous substrate were due to the activity of PIPKI, some labeled PI-4,5-P_2 should have been synthesized in the presence of heparin and more following the addition of PA. We cannot rule out the possibility that PIPKI is present in ROS as a soluble enzyme, since cytosolic proteins would not be present in our preparation. Jones et al. (*43*) recently reported that PIPKI, but not PIPKII, could be recruited from the cytosol to Golgi in the presence of ADP-ribosylation factor 1.

Previous reports (*20*, *44*−*46*) have shown that the isoforms of PIPKII are ∼47−53 kDa proteins. The PIP kinase we identified on Western blots with the anti-PIPKIIα antibody had an apparent molecular mass of 41 kDa. In renaturation experiments, only the gel slice containing this 41 kDa protein had regenerable PIPK activity. Also, the 41 kDa protein was immunoprecipitated by the anti-PIPKIIα antibody and was enzymatically active, preferentially converting PI-5-P to PI-4,5-P_2. The reason for the difference in apparent molecular masses is not obvious. The smaller ROS protein may be a splice variant that is recognized by the polyclonal antibody raised against a peptide sequence in the enzyme.

The mechanism of activation of PIP kinases is not well understood. Jones et al. (*43*) showed that the recruitment of PIPKI, but not PIPKII, to Golgi membranes resulted in increased formation of PI-4,5-P_2. Itoh et al. (*47*) recently reported that all three isoforms of PIPKI have protein kinase activity and that the lipid kinase activity may be regulated by autophosphorylation on serine and threonine residues. Itoh et al. (*46*) have previously shown that PIPKIIγ is phosphorylated on serine residues following mitogen stimulation. Subcellular fractionation of the γ-isoform showed that it was located predominantly in the smooth and rough endoplasmic reticulum and not in the cytosol. Hinchliffe et al. (*48*) reported that PIPkin C (PIPKII) was dephosphorylated in platelets following thrombin stimulation, which resulted in a 2−3-fold increase in enzyme activity. They later showed that PIPKII could be phosphorylated on serine-304 (*49*). In none of these studies was evidence presented for tyrosine phosphorylation of PIPKII or activation of enzyme activity through tyrosine phosphorylation of other proteins. However, Castellino and Chao (*22*) found that PIPKII co-immunoprecipitated with the activated EGF receptor in A431 and PC12-615 cells and with members of the ErbB tyrosine kinase family in activated MCF7 cells. In both instances, tyrosine phosphorylation of the EGF and ErbB receptors was necessary for these receptors to be immunoprecipitated by the anti-PIPKII antibody.

We present evidence here that tyrosine phosphorylation is involved in regulation of PIPKIIα activity in bovine ROS. Several lines of evidence support this conclusion. First, preincubation with ROS under conditions known to stimulate endogenous tyrosine kinases led to a stimulation of PI-4,5-P_2 formation from endogenous and exogenous substrates. These reactions were carried out in washed ROS membranes free of cytosolic proteins and thus represent activation of the enzyme rather than recruitment of a cytosolic protein to the membrane. Second, preincubation of ROS membranes with several tyrosine kinase inhibitors reduced PIPK activity significantly. Again, these incubations were carried out with washed ROS membranes free of cytosolic proteins. Third, immunoprecipitation of PY-ROS and N-ROS with an anti-PY antibody resulted in recovery of PIPK activity in the PY-ROS immunoprecipitates. The 41 kDa protein shown on renaturation experiments to have PIPK activity was recognized by the anti-PIPKIIα antibody in the PY-ROS immunoprecipitates but not in the N-ROS immunoprecipitates. Fourth, preincubation of ROS with tyrosine kinase inhibitors prior to immunoprecipitation with anti-PY antibodies greatly reduced the PIPK activity in all of the immunoprecipitates, except in the control that did not contain tyrosine kinase inhibitors. These results support our conclusion that tyrosine phosphorylation by an endogenous, membrane-bound tyrosine kinase in bovine ROS leads to the activation of PIPKIIα in these membranes.

Although activated by tyrosine phosphorylation of some protein, we find no evidence that PIPKIIα is tyrosine phosphorylated. Rather, our results to date suggest the contrary. Immunoprecipitation of N-ROS and PY-ROS with the anti-PIPKIIα antibody resulted in the recovery of PIPKIIα in both immunoprecipitates, and western blots probed with anti-PY antibody showed no difference in

phosphorylation of the 41 kDa protein in N-ROS and PY-ROS (Figure 8). Furthermore, PIPK activity was about equal in both immunoprecipitates. There are several possible explanations of these results. First, absence of anti-PY immunoreactivity could result from the anti-PIPKIIα antibody not recognizing or not immunoprecipitating PIPKIIα that is tyrosine phosphorylated. If significant amounts of nonphosphorylated 41 kDa protein were present in these membranes and the amount of anti-PIPKIIα antibody was not saturating, the results presented in Figure 8 could be obtained. Also, immunoprecipitation with anti-PIPKIIα may have dissociated nonphosphorylated PIPKIIα from a tyrosine phosphorylated adapter protein, resulting in equal PIPK activities in N-ROS and PY-ROS immunoprecipitates. It is interesting that, in the experiments of Castellino and Chao (*22*), the EGF and ErbB receptors were tyrosine phosphorylated, but no mention is made of the phosphorylation state of PIPKII.

The mechanism whereby the PIPKIIα activity is mediated by tyrosine phosphorylation remains to be determined. PIPKIIα contains a proline-rich region (amino acids 307−329) highly homologous to the sequence of the SH3-binding domain (*50*). It is possible that PIPKIIα directly interacts with tyrosine kinases or a tyrosine-phosphorylated protein that contains an SH3 domain (*51*, *52*) or with SH2 adapter proteins, such as Grb2 (*53*) that will connect the enzyme to protein tyrosine kinase or tyrosine phosphorylated protein. Thus, the modulation of PIPKIIα activity might directly involve tyrosine phosphorylation of the enzyme, which we doubt, or it might be indirect, by an as yet unidentified tyrosine phosphorylated protein that binds the enzyme.

In summary, our results show that PIPKIIα is present in bovine and rat ROS membranes and that its activity in vitro in bovine ROS is stimulated by tyrosine phosphorylation. Although we cannot rule out completely the possibility that PIPKIIα is itself tyrosine phosphorylated, the most likely explanation is that tyrosine phosphorylation of other ROS proteins, which has been shown to occur in vivo (*31*) and in vitro (*32*), leads to the activation of PIPKIIα in these membranes.

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