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Tyrosine phosphorylation is involved in phosphatidylinositol 3-kinase activation in bovine rod outer segments

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Purpose: We have previously shown that phosphatidylinositol 3-kinase (PI 3-kinase) activity is present in bovine rod outer segments (ROS). The present study was undertaken to investigate the mechanism of PI 3-kinase activation in these membranes.

Methods: Tyrosine-phosphorylated ROS (PY-ROS) were obtained by incubating ROS with ATP, $MgCl_2$, and orthovanadate (Na_3VO_4), a tyrosine phosphatase inhibitor. Non-phosphorylated ROS (N-ROS) were obtained by incubating ROS under the same conditions, but without ATP and orthovanadate. Both were subjected to immunoprecipitation using antibodies against the regulatory p85 (anti-p85) subunit of PI 3-kinase, the catalytic p110 (anti-p110) subunit of PI 3-kinase, or phosphotyrosine (anti-PY). The immunoprecipitates (IPs) were assayed for PI 3-kinase activity. Enzyme assay products were separated by thin-layer chromatography (TLC), deacylated, and identified by high performance liquid chromatography (HPLC).

Results: PI 3-kinase activity in anti-p85 and p110α IPs was significantly higher in PY-ROS than in N-ROS. No enzyme activity was recovered in anti-p110β IPs. PI 3-kinase activity in anti-PY IPs from PY-ROS was six-fold greater than those from N-ROS. Immunoblot analysis showed that the amount of p85 in PY IPs from PY-ROS was significantly higher than those from N-ROS. However, tyrosine phosphorylation of p85 and p110α was not observed in anti-p85 and anti-p110α IPs that were probed with anti-PY.

Conclusions: This study indicates that the p85/p110α complex of PI 3-kinase is present in ROS and tyrosine phosphorylation is involved in the regulation of its activity.

Phosphatidylinositol 3-kinase (PI 3-kinase) activation is known to be essential for a number of cellular functions, including membrane trafficking [1], cytoskeletal rearrangement [2,3], migration [4], mitogenesis [5], differentiation [6], and protection from apoptosis [7]. Several different classes of PI 3-kinase have been identified based on their structure, lipid substrate preference, and probable mode of activation [8]. The best-characterized class of PI 3-kinase is the p85/p110 family, which consists of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit. The class I_A catalytic subunits, which exist in three isoforms (α, β, and δ) [9-11], phosphorylate the D-3 position of the inositol ring of phosphoinositides to produce four phosphorylated inositol lipids, PI-3-P, PI-3,4-P_2, PI-3,5-P_2, and PI 3,4,5-P_3 [11]. The p85 regulatory subunit contains one SH3 and two SH2 domains [12,13] that can function as adapters to link PI 3-kinase to tyrosine phosphorylated proteins. The other classes of PI 3-kinase, which do not interact with p85, include PI 3-kinases regulated by G-proteins [14], PI 3-kinases containing a C2-domain [15,16], and PI 3-kinase analogues of yeast vps34p [17].

The p85/p110 family of PI 3-kinase has been shown to be a common element of many signaling pathways involving tyrosine kinases (see reviews [11] and [18]). A variety of stimuli can trigger distinct and specific biological responses in different cell types. We have previously reported that bovine photoreceptor rod outer segments (ROS) contain a p85/p110 enzyme complex [19]. The question of how PI 3-kinase activity is regulated in photoreceptors remains to be addressed. In this report, we investigated the possible involvement of tyrosine phosphorylation in PI 3-kinase activation in bovine ROS.

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METHODS

Materials: Polyclonal antisera to the p85 regulatory subunit (Cat. No. 06-195) and the p110α catalytic subunit (Cat. No. 06-567) of PI 3-kinase were from Upstate Biotechnology, Inc (Lake Placid, NY). Polyclonal anti-p110α (H 201) and anti-p110β (H-239) were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-PY20 was from Transduction Laboratories (Lexington, KY). [γ-^{32}P]ATP and [^{3}H] inositol-1,3,4,5-P_4 were from New England Nuclear (Boston, MA). Deacylated [^{3}H]PI-4,5-P_2 (gPI-4,5-P_2) was from American Radiolabeled Chemicals (St. Louis, MO). Electrophoresis reagents, nitrocellulose sheets, and alkaline phosphatase-conjugated goat anti-rabbit IgG were from Bio-Rad (Hercules, CA). HRP-conjugated donkey anti-rabbit IgG and sheep anti-mouse IgG, enhanced chemiluminescence (ECL) reagents, and Hyperfilm ECL were from Amersham Life Science (Arlington, IL). BCA reagents were from Pierce (Rockford, IL). PI-

4,5-P_2, protein A-Sepharose, silica gel thin-layer chromatographic (TLC) plates, and other chemicals were from Sigma (St. Louis, MO).

Preparation of ROS: Fresh bovine eyes were obtained at a local abattoir and dissected on ice. ROS were prepared on a continuous sucrose gradient (25%-50%) as previously described [20]. For fractionation of ROS, the ROS pellet was resuspended in 65% sucrose solution, loaded onto a second 25-50% continuous sucrose gradient, and centrifuged at 80,000 x g for 2 h at 4 °C. One ml fractions were withdrawn and collected from the bottom of the centrifuge tube by using a 16 G needle. Protein determination was performed with BCA reagents following the manufacturer's instructions.

Tyrosine phosphorylation of ROS: Tyrosine-phosphorylated ROS (PY-ROS) were prepared by incubating ROS with 50 mM Tris-HCl (pH 7.4), 1.5 mM ATP, 1 mM EGTA, 2 mM $MgCl_2$, and 0.2 mM Na_3VO_4 for 15 min at 37 °C [21]. Non-phosphorylated ROS (N-ROS) were prepared by incubating ROS with 50 mM Tris-HCl (pH 7.4), 1 mM EGTA, and 2 mM $MgCl_2$.

Immunoprecipitation: PY-ROS and N-ROS were solubilized in 500 µl of lysis buffer [1% Triton X-100, 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM PMSF, 0.2 mM Na_3VO_4, 10 µg/ml leupeptin, and 1 µg/ml aprotinin] and clarified by centrifugation at 20,000 x g for 20 min at 4 °C. The solubilized ROS were incubated with either anti-p85 (1:300), anti-p110α (10 µg/ml), anti-p110β (10 µg/ml), or anti-PY20 (10 µg/ml) antibodies for 4 h at 4 °C. Forty µl of protein A-Sepharose was subsequently added and the incubation continued for an additional 2 h at 4 °C. As a control, solubilized ROS were similarly treated using either normal rabbit serum or goat anti-mouse IgG. The immunoprecipitates (IPs) were washed twice with Buffer 1 [15.7 mM NaH_2PO_4 (pH 7.4), 1.47 mM KH_2PO_4, 137 mM NaCl, 2.68 mM KCl, 0.2 mM Na_3VO_4, and 1% Triton X-100], twice with Buffer 2 [0.1 M Tris-HCl (pH 7.5), 0.5 M LiCl, and 0.2 mM Na_3VO_4], and twice with Buffer 3 [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, and 0.2 mM Na_3VO_4]. The final IPs were resuspended in 30 µl of Buffer 3 for enzyme assays or 30 µl of SDS-PAGE sample buffer for SDS-PAGE and western blot analysis.

SDS-PAGE and western blot analysis: Samples were resolved by 8% or 10% SDS-PAGE and stained with Coomassie blue or transferred onto nitrocellulose membranes. Nitrocellulose sheets were blocked with 5% bovine serum albumin (BSA) overnight at 4 °C and incubated with either anti-p85 antibody (0.25 µg/ml), anti-p110α (H-201, 1 µg/ml), anti-p110β (1 µg/ml), or anti-PY20 (5 µg/ml) antibodies for 3 h at room temperature. Following primary antibody incubations, immunoblots were incubated either with alkaline phosphatase-conjugated anti-rabbit IgG and developed using alkaline phosphatase substrates or with HRP-linked secondary antibodies and developed by ECL. For quantitation, western blot membranes were scanned using DeskScan II (Hewlett Packard, Palo Alto, CA) and optical density of the major band (p85) was quantitated by ONE-Dscan (Scanalytics, Bellerica, MA).

PI 3-kinase assay: Enzyme assays were carried out as described by Kaplan et al. [22], with minor modifications. Briefly, assays were performed directly on anti-p85, anti-p110α, anti-p110β, or anti-PY IPs in 50 µl of the reaction mixture containing 0.2 mg/ml PI-4,5-P_2, 50 µM ATP, 0.2 µCi [γ-^{32}P]ATP, 5 mM $MgCl_2$, and 10 mM HEPES buffer (pH 7.5).

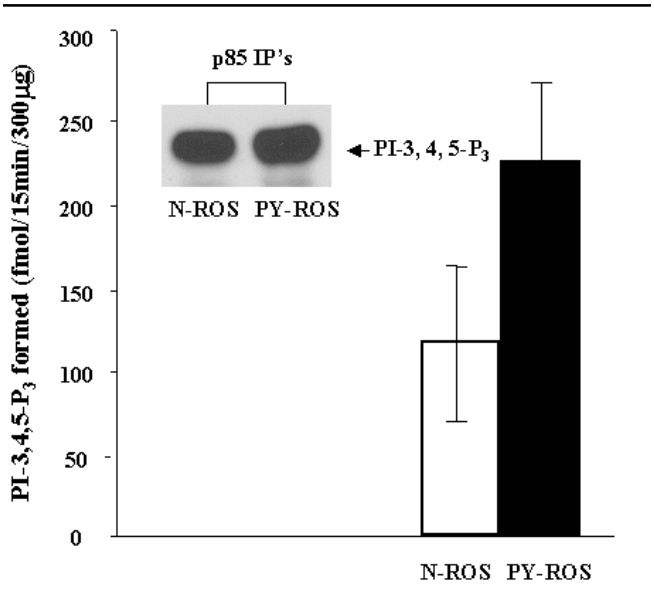

Figure 1. PI 3-kinase activity in anti-p85 immunoprecipitates from PY-ROS and N-ROS. Anti-p85 immunoprecipitates from PY-ROS and N-ROS (300 µg protein each) were assayed for PI 3-kinase activity with [γ^{32}P]ATP using PI-4,5-P_2 as a substrate. The [^{32}P]-labeled phospholipids were separated by TLC and visualized by autoradiography (inset). PI-3,4,5-P_3 spots were scraped and counted for radioactivity. Values are mean and standard deviation for five independent ROS preparations ($p<0.003$).

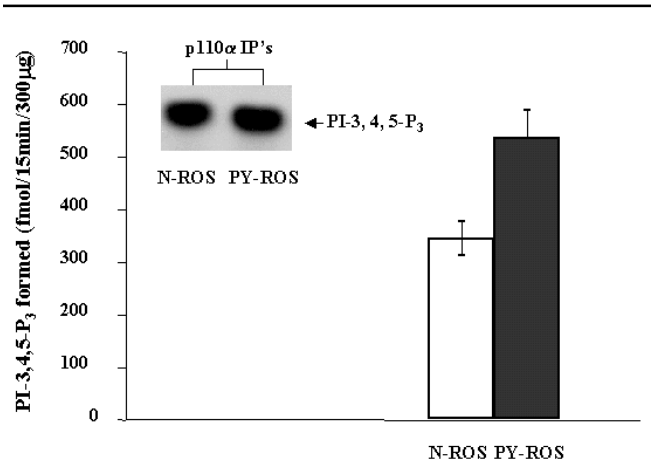

Figure 2. PI 3-kinase activity in anti-p110α immunoprecipitates from PY-ROS and N-ROS. Anti-p110α immunoprecipitates from PY-ROS and N-ROS (300 µg protein each) were assayed for PI 3-kinase activity with [γ-^{32}P]ATP using PI-4,5-P_2 as a substrate. The [^{32}P] labeled phospholipids were separated by TLC and visualized by autoradiography (inset). PI-3,4,5-P_3 spots were scraped and counted for radioactivity. Values are mean and standard deviation for four independent ROS preparations ($p<0.02$).

The reactions were carried out for 15 min at room temperature and stopped by the addition of 100 µl of 1 N HCl and 200 µl of chloroform/methanol (1/1, V/V). Lipids were extracted and resolved on oxalate-coated TLC plates (silica gel 60) with a solvent system of 1-propanol/2 M acetic acid (65/35, V/V). The oxalate-coated TLC plates were prepared by placing in 1% (W/V) potassium oxalate in 50% methanol (V/V) and baked in an oven at 100 °C for 1 h prior to use. TLC plates were exposed to X-ray film overnight at -70 °C and radioactive lipids were scraped and quantified by liquid scintillation counting.

HPLC analysis of PI 3-kinase products: Phospholipids were deacylated with methylamine reagent as described by Auger et al. [23]. Briefly, the [^{32}P]-labeled lipids from PI 3-kinase assays were incubated with 1.8 ml of a methylamine reagent (methylamine/methanol/n-butanol, 42.8/45.7/11.4, by vol) for 50 min at 53 °C. The sample was dried under vacuum, resuspended in 2 ml H_2O, and dried again. The dried products were then dissolved in 2 ml H_2O and extracted twice with 2 ml of n-butanol/light petroleum ether/ethyl formate (20/4/1, by vol). The aqueous phase containing deacylated products was dried under vacuum and dissolved in 0.5 ml of 10 mM $(NH_4)_2HPO_4$ (pH 3.8). HPLC analysis was performed on a Partsphere 5 Sax anion exchange column (Whatman, Clifton, NJ). Separation was achieved by pumping water for 10 min followed by a linear gradient of $(NH_4)_2HPO_4$ (pH 3.8) from 0.0 to 1.0 M in 100 min at 1 ml/min. The radioactivity of the eluate from the HPLC column was monitored by an on-line Flow-one A250 radioisotope detector (Radiomatic Instruments, Tampa, FL).

Data analysis: Data from PI 3-kinase assays were analyzed using paired Student's t-test. P-values less than 0.05 were considered statistically significant.

RESULTS

Effect of tyrosine phosphorylation on PI 3-kinase activity in ROS: To examine the effect of tyrosine phosphorylation on PI 3-kinase activity, tyrosine-phosphorylated PY-ROS and N-ROS were solubilized, immunoprecipitated with anti-p85, and assayed for PI 3-kinase activity using PI-4,5-P_2 as substrate. As shown in Figure 1, PI 3-kinase activity in anti-p85 IPs from PY-ROS was two-fold greater than those from N-ROS ($p<0.003$, n = 5 independent ROS preparations). Western blots of p85 immunoprecipitates probed with anti-p85 antibody did not show any differences in the amounts of p85 between N-ROS and PY-ROS (data not shown).

Since p85 is a regulatory subunit of PI 3-kinase, the presence of PI 3-kinase activity in anti-p85 IPs indicates the association of a p110 catalytic subunit. To determine which p110 subunit is present in ROS and responsible for the increase of PI 3-kinase activity, p110α and p110β immunoprecipitates were obtained from PY-ROS and N-ROS and assayed for PI 3-kinase activity using PI-4,5-P_2 as substrate. As shown in Figure 2, PI 3-kinase activity in anti-p110αIPs from PY-ROS was higher than those from N-ROS ($p<0.02$, n=4). PI 3-kinase activity was not recovered in anti-p110β IPs from PY-ROS (data not shown).

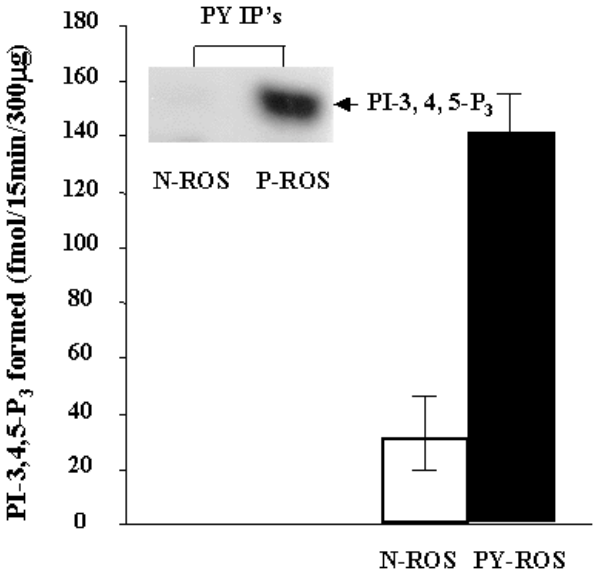

Figure 3. PI 3-kinase activity in anti-PY immunoprecipitates from PY-ROS and N-ROS. Anti-PY immunoprecipitates from PY-ROS and N-ROS (300 µg protein each) were assayed for PI 3-kinase activity with [γ-^{32}P]ATP using PI-4,5-P_2 as a substrate. The [^{32}P]-labeled phospholipids were separated on TLC and visualized by autoradiography (inset). PI-3,4,5-P_3 spots were scraped and counted for radioactivity. Values are mean and standard deviation for five independent ROS preparations ($p<0.0002$).

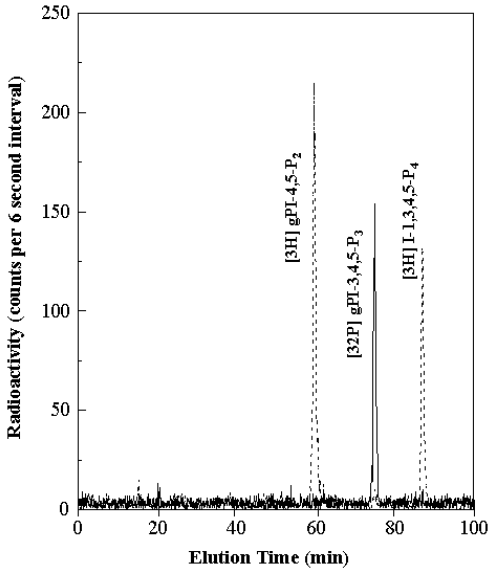

Figure 4. HPLC analysis of deacylated [^{32}P]PIP_3 from anti-PY immunoprecipitates of PY-ROS. The immunoprecipitates were assayed for PI 3-kinase activity with [γ-^{32}P]ATP using PI-4,5-P_2 as a substrate. The [^{32}P]-labeled phospholipids were separated on TLC, deacylated, and analyzed by HPLC with flow-through scintillation counting. Dotted lines represent the elution profile of the standards gPI-4,5-P_2 and I-1,3,4,5-P_4.

PI 3-kinase activity recovered in anti-PY immunoprecipitates from PY-ROS: Anti-PY IPs from PY-ROS and N-ROS (300 µg of each) were assayed for PI 3-kinase activity using PI-4,5-P_2 as substrate (Figure 3). Activity in the IPs from PY-ROS was six-fold greater than those from N-ROS ($p<0.0002$, n=5 independent ROS preparations). To further confirm that [^{32}P]PIP_3 produced in the anti-PY IPs was PI-3,4,5-P_3 rather than some other phospholipid, [^{32}P]PIP_3 was deacylated with methylamine reagent and the product [^{32}P]gPIP_3 was mixed with standard [^{3}H]gPI-4,5-P_2 and [^{3}H] I-1,3,4,5-P_4 and analyzed by HPLC. [^{32}P]gPIP_3 was eluted at 74.1 min, which was before [^{3}H]-I 1,3,4,5-P_4 and after [^{3}H]gPI-4,5-P_2 (Figure 4). This elution time and pattern is consistent with that reported for gPI-3,4,5-P_3 by other investigators [23].

p85 protein associated with anti-PY immunoprecipitates: Anti-PY IPs from PY-ROS and N-ROS were subjected to western blot analysis using anti-p85 to determine if the higher PI 3-kinase activity observed in anti-PY IPs from PY-ROS is due to increased amounts of p85. The optical density of p85 was quantitated to determine the relative amounts of p85 in each sample. Anti-PY IPs from PY-ROS contained about three times the amount of p85 compared to anti-PY IPs from N-ROS (Figure 5A; $p<0.017$, n=4). However, tyrosine phosphorylation of p85 was not observed on western blots of anti-p85 IPs from PY-ROS or N-ROS probed with anti-PY (Figure 5B). Anti-p110α IPs from PY-ROS and N-ROS were also subjected to western blot analysis using anti-PY to determine if p110α was tyrosine phosphorylated. Tyrosine phosphorylation of p110α was not observed (data not shown).

Identification of p85/p110α PI 3-kinase in ROS: To further confirm that the p85/p110α complex of PI 3-kinase is present in ROS and not due to contamination from other cell types, the second of successive continuous sucrose gradients used to purify ROS was fractionated and subjected to SDS-PAGE and immunoblot analysis using anti-p85 and anti-p110α. As shown in Figure 6A, gradient fractions 12 through 17, which represent the ROS collected during normal preparation, contained the highest amounts of rhodopsin. Immunoblot analysis shows that gradient fractions 12 through 17 similarly contained the highest amounts of p85 (Figure 6B) and p110α (Figure 6C).

DISCUSSION

We have previously shown that bovine ROS contain PI 3-kinase activity that is increased in membranes prepared from light-adapted retinas [19]. The p85 regulatory and p110 catalytic subunits were identified on immunoblots of ROS and PI 3-kinase activity was recovered in anti-p85 immunoprecipitates. To investigate which p110 isoform is present in bovine ROS, ROS were subjected to western blot analysis using anti-p110α and anti-p110β antibodies. A distinct 110 kDa band was observed in ROS using anti-p110α, but multiple proteins of various molecular weights were recognized by anti-p110β. Anti-p110α and anti-p110β IPs from PY-ROS and N-ROS were assayed for PI 3-kinase activity using PI-4,5-P_2 as substrate. We found that anti-p110α IPs, but not anti-p110β IPs, contained PI 3-kinase activity.

To ensure that the presence of PI 3-kinase in ROS was not due to contamination from other retinal cells, ROS were purified on a second continuous sucrose gradient that was subsequently fractionated into 1 ml aliquots, which were subjected

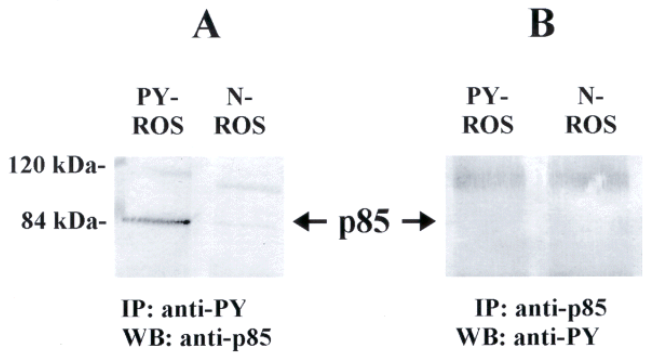

Figure 5. Detection of the p85 subunit of PI 3-kinase in anti-PY and anti-p85 immunoprecipitates. Equal amounts of PY-ROS and N-ROS (300 µg each) were solubilized and immunoprecipitated with anti-PY or anti-p85. The immunoprecipitates (IP) were subjected to western blot (WB) analysis using anti-PY (Panel **A**) or anti-p85 (Panel **B**). Molecular weight markers in kilodaltons are indicated on the left.

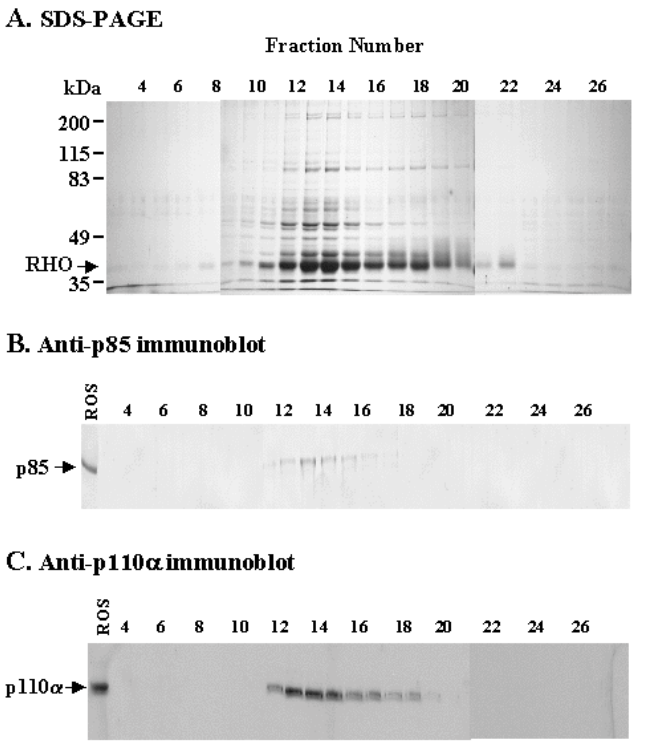

Figure 6. Identification of PI 3-kinase in ROS. ROS and ROS gradient fractions 4 through 27 were subjected to 8% SDS-PAGE and stained with Coomassie blue (Panel **A**). Rhodopsin (RHO) is indicated by arrow. The samples were also subjected to western blot analysis using anti-p85 (Panel **B**) and anti-p110α(Panel **C**). Immunoreaction products of p85 and p110α are indicated.

to western blot analysis using anti-p85 and anti-p110α. The fractions that contained the highest amounts of rhodopsin, a rod photoreceptor cell-specific protein, also contained the highest levels of p85 and p110α, thus confirming that the p85/p110α complex is present in ROS and that it is of the Class I type.

Protein tyrosine phosphorylation is one of the ways by which cells regulate the activity of PI 3-kinase [22,24-28]. Tyrosine phosphorylation of the p85 regulatory subunit has been shown to occur in several cell types in response to various stimuli such as platelet-derived growth factor [29] and insulin [30]. However, in most cases, PI 3-kinase is recruited to signaling complexes involving receptor or non-receptor tyrosine kinase(s) without apparent tyrosine phosphorylation of the p85 subunit [9,13,31]. To determine if tyrosine phosphorylation is involved in activation of PI 3-kinase in ROS, we carried out a series of experiments under conditions that promote tyrosine phosphorylation. We found that PI 3-kinase activity from p85 and p110α IPs was about two-fold higher in PY-ROS than in N-ROS. Since p85 is a regulatory subunit of PI 3-kinase and lacks kinase activity, the presence of enzyme activity in p85 IPs indicates that p85 is associated with a p110 catalytic subunit. Since neither of the PI 3-kinase proteins appears to be phosphorylated, the increase in catalytic activity in the IPs from the PY-ROS may be due to a tyrosine-phosphorylated protein that was brought down by either p85 or p110 antibodies, as part of a complex that controls PI 3-kinase activity in these membranes.

We investigated the effect of tyrosine phosphorylation on PI 3-kinase activity by assaying anti-PY IPs from PY-ROS and N-ROS. PI 3-kinase activity in anti-PY IPs from PY-ROS was six-fold greater than that from N-ROS, suggesting that either the p85 or p110α subunit of PI 3-kinase becomes tyrosine phosphorylated or p85 binds to other tyrosine phosphorylated protein(s) in ROS. To address this question, anti-p85 IPs and anti-p110α IPs from PY-ROS or N-ROS were subjected to western blot analysis using antibodies to phosphotyrosine. p85 and p110α were not detected by anti-PY in these IPs, indicating that p85 and p110α were not tyrosine phosphorylated. However, western blot analysis using anti-p85 showed that significantly higher amounts of p85 were present in anti-PY IPs from PY-ROS than in those from N-ROS (Figure 5). These results suggest that the p85 regulatory subunit is most likely bound to and co-immunoprecipitates with a tyrosine-phosphorylated protein in ROS.

The identity of the tyrosine kinase(s) or adapter protein(s) in ROS responsible for the activation of PI 3-kinase has not been established. However, Ghalayini et al. [32] recently reported that light stimulates tyrosine phosphorylation of several ROS protein in vivo in rats. Furthermore, Bell et al. [21] have shown that at least ten endogenous proteins in bovine ROS are tyrosine-phosphorylated in vitro under the same conditions used in our studies. We hypothesize that a tyrosine-phosphorylated protein interacts with the SH2 domain of the p85 subunit, resulting in the activation of PI 3-kinase. The question of which protein may be responsible for the regulation of PI 3-kinase in ROS still needs to be addressed.

It has been reported that PI 3-kinase products (PI-3,4,5-P_3 and PI-3,4 P_2) serve as second messengers in the activation of several potential downstream kinases, including the novel protein kinase C (PKC) isoforms δ, ε, and η [33], the $p70^{s6k}$ ribosomal subunit [34], and the serine/threonine kinase, Akt [35,36]. Akt possesses a PI-3,4 P_2/PI-3,4,5-P_3 binding domain and has been implicated in the prevention of apoptosis [7,37,38]. Further investigation of the PI 3-kinase regulatory mechanism and its functions in ROS should provide a better understanding into the role of this enzyme in photoreceptors.

ACKNOWLEDGEMENTS

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