Phosducin and PP33 Are *in vivo* Targets of PKA and Type 1 or 2A Phosphatases, Regulators of Cell Elongation in Teleost Rod Inner-Outer Segments

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Teleost rod photoreceptors elongate in the light and shorten in darkness. We are investigating the role of cAMP-dependent protein kinase (PKA), phosphatases and target phosphoproteins in the regulation of photoreceptor cell shape. Preparations of rod fragments, consisting of the motile inner segment with attached photosensory outer segment (RIS-ROS), undergo light-stimulated elongation in culture. The PKA-selective inhibitor, H89, enhanced RIS-ROS elongation in both light and darkness, suggesting that elongation is associated with dephosphorylation of PKA substrates. Okadaic acid and calyculin A, inhibitors of type 1 and 2A phosphatases, blocked light-dependent and light-independent elongation with relative potencies suggesting that elongation requires dephosphorylation by type 1 phosphatase in light and type 2A phosphatase in darkness. To identify targets of PKA and phosphatases, RIS-ROS were isolated from retinas prelabeled with ^{32}P -orthophosphate, and then incubated in the presence of kinase inhibitors or phosphatase inhibitors. Two phosphoproteins, PP33 and PP35, were phosphorylated by PKA and dephosphorylated by type 1 or 2A phosphatases in light- and dark-cultured RIS-ROS. PP35 (but not PP33) was immunoprecipitated by an antibody to phosducin, a PKA-regulated modulator of phototransduction (Lee et al., 1992); PP35 was also phosphorylated *in vitro* by a Ca^{2+} -calmodulin-activated kinase. PP33 further differed from PP35 in its phosphopeptide maps and phosphorylation by PKC. We conclude that RIS-ROS elongation is correlated with the dephosphorylation of PKA substrates by type 1 or 2A phosphatases. Candidate mediator proteins include PP35, a fish phosducin homolog, and PP33, a newly described photoreceptor phosphoprotein.

[Key words: rods, photoreceptor, phosphorylation, PKA, phosphatase, phosducin, cell motility, H89, okadaic acid, calyculin A]

Received Mar. 6, 1995; revised May 31, 1995; accepted June 1, 1995.

We thank Dr. R. Lee for purified phosducin and Dr. W. Fletcher for PKA catalytic subunit and PKI, and for their helpful discussions. We are grateful to Dr. T. Shinohara for the phosducin antibody and Dr. B. Fung for the transducin and phosphodiesterase antibodies. We also thank Ms. Debbie Tran for help with Figure 1. This work was supported by NEI Grant #03575 to Beth Burnside.

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Light triggers a fall in cyclic nucleotide levels within vertebrate photoreceptors. The light-induced decrease in cGMP is mediated by increased activity of rod outer segment cGMP-phosphodiesterase, leading to closure of cGMP-gated channels and membrane hyperpolarization (Yau, 1994). A light-induced fall in cAMP was measured in the photoreceptor layer (Orr et al., 1976; Cohen, 1982) where the highest densities of cAMP-binding proteins were detected in inner segments (Caretta and Saibil, 1989). cAMP has been implicated in the regulation of several light-regulated retinal processes, although its exact role is not well understood. Examples include outer segment renewal (Besharse et al., 1982; Eckmiller and Burnside, 1983), melatonin biosynthesis (Iuvone and Besharse, 1986), modulation of cGMP-gated channels (Furman and Tanaka, 1989), and photoreceptor shape changes in lower vertebrates (Besharse et al., 1982; Burnside et al., 1982; O'Connor and Burnside, 1982). In addition, light-induced dephosphorylation at the PKA phosphorylation site of phosducin, a $G_{\beta\gamma}$ -binding protein (Lee et al., 1984, 1987, 1990a), has been implicated in light adaptation (Lee et al., 1992; Yoshida et al., 1994).

To expand our understanding of light- and cAMP-mediated events in rods, and the role of cAMP in regulating neuronal cell shape, we have been studying the motility of teleost rod inner segments with attached outer segments (RIS-ROS) in cultured preparations. RIS-ROS motility is consistent with rod motility observed on intact retinas (O'Connor and Burnside, 1981, 1982): elongation is actin-dependent (Pagh-Roehl et al., 1992a), stimulated by light (Nagle and Burnside, 1984), and inhibited by cyclic nucleotide analogs (Liepe and Burnside, 1993a). Light-induced RIS-ROS elongation was also blocked by an inhibitor of type 1 and type 2A protein phosphatases while elongation was stimulated in darkness by protein kinase inhibitors (Liepe and Burnside, 1993a). Phosphorylation by PKA but not cGMP-dependent protein kinase was detectable in RIS-ROS (Pagh-Roehl et al., 1993). Therefore, we proposed that RIS-ROS motility is regulated by the relative phosphorylation of one or more PKA substrates with dark favoring their phosphorylation and light favoring their dephosphorylation by type 1 or 2A phosphatases (Liepe and Burnside, 1993a).

In this study, we further tested the hypothesis that PKA and type 1 or 2A phosphatases play a role in the pathway mediating rod cell elongation. We used a PKA-selective inhibitor, two distinct phosphatase inhibitors, and *in vivo* phosphorylation patterns to determine whether the phosphorylation state of a specific subset of phosphoproteins varied consistent with pharmacological manipulations affecting RIS-ROS elongation; such phosphopro-

teins would be candidate regulators of rod motility. We report that only two phosphoproteins, PP33 and PP35, were regulated appropriately by light, PKA and phosphatases. PP35 is the fish homolog of phosducin while PP33 is a distinct photoreceptor protein not previously described. Our results suggest that proteins which regulate RIS-ROS motility undergo phosphorylation by PKA and dephosphorylation by phosphatase 2A in darkness, while maintaining a relatively high level of phosphorylation. In the light, these proteins are dephosphorylated by phosphatase 1 to produce a reduced level of phosphorylation which is correlated with cell elongation.

Materials and Methods

Experimental animals. Green sunfish, *Lepomis cyanellus*, were obtained from Fender Fish Hatchery (Baltik, OH) and kept in indoor aquaria on a daily cycle of 14 hr of light and 10 hr of darkness. Fish were 4.5–6 inches in length.

Cell culture and physiology. Fish were dark-adapted from 2–3 hr to facilitate enrichment of photoreceptor suspensions with rod fragments, and dissections were carried out under infrared illumination using an image convertor as discussed previously (Pagh-Roehl et al., 1993). To prepare suspensions of RIS-ROS, isolated retinas were shaken in 0.75–1.0 ml of a HEPES-based Earle's Ringer containing 5 mM taurine (HERT; Pagh-Roehl et al., 1993). Cells from both retinas of one fish were pooled; 90 μ l of the RIS-ROS suspension were added to the well of a 96-well microtiter plate containing 10 μ l of a 10 $\times$ dose of drug or buffer control which contained a final concentration of 1.0–1.4% DMSO. Specimens were cultured for 45 min in the dark or with incandescent white light (6.5 lux), then fixed with a final concentration of 2% glutaraldehyde. Myoid lengths were measured using a 40 $\times$ phase objective and ocular micrometer as described previously (Pagh-Roehl et al., 1992b).

Protein kinase and phosphatase inhibitors were stored at -20°C as concentrated stock solutions. H89 (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA) was prepared as a 10 mM stock in 100% DMSO and diluted with 10% or 14% DMSO to give the appropriate final concentrations in 1.4% DMSO. Okadaic acid (Gibco BRL, Gaithersburg, MD) and calyculin A (LC Laboratories, Woburn, MA) were stored at 500 μ M in 10% DMSO.

In situ phosphorylation. Retinas from five fish were isolated in the dark and cultured for up to 45 min in 0.5 ml of HERT. Retinas were then transferred to 24 well plates, with two to three retinas per well containing 0.5 ml of phosphate-free HERT and 1 mCi/ml of carrier-free ^{32}P orthophosphate (Amersham, Arlington Heights, IL). Isolated retinas were cultured in the dark for another 45 min on an orbital shaker. In some experiments, 0.25 mM IBMX was added for the final 15 min of labelling. After labelling, retinas were rinsed twice in HERT, then vortexed at low speed for 10 sec in 1 ml of HERT per retina to generate RIS-ROS.

RIS-ROS suspensions obtained from 10 retinas were filtered through 100 μ m nylon mesh (Tetko, Elmsford, NY) and pooled; 900 μ l samples were added to Eppendorf tubes containing 100 μ l of a 10 $\times$ concentration of H89, okadaic acid, calyculin A, IBMX, or HERT containing the drug carrier DMSO. RIS-ROS were further cultured for 10 min in the light or dark. Reactions were stopped with a final concentration of 10% trichloroacetic acid (TCA) and samples were immediately frozen. TCA pellets were washed twice with cold acetone and resuspended in 25–50 μ l of SDS sample buffer (containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol in Tris-HCl, pH 6.8). The resuspended pellet was further incubated at room temperature for 30 min with intermittent vortexing and neutralization of the pH with ammonium fumes to facilitate resolubilization of protein.

In vitro phosphorylation. RIS-ROS suspensions, generated by shaking retinas individually in HERT, then filtered, were pelleted at 1000 $\times$ g for 10 min and resuspended in 100 μ l per retina of a hypotonic lysis buffer containing protease inhibitors (Pagh-Roehl et al., 1992a). For experiments investigating cAMP-dependent phosphorylation, the lysis buffer consisted of 50 mM Tris-HCl, pH 7.2, and 10 mM EGTA. For experiments investigating calcium- and phospholipid-dependent phosphorylation, the lysis buffer consisted of 50 mM Tris-HCl, pH 7.2, and 1 mM EGTA, with the appropriate calcium concentrations obtained by adding CaCl_2 as determined by the method of Steinhart et al. (1977).

The resuspended lysate was divided and 0.5–1.0 mg/ml of RIS-ROS protein were added to Eppendorf tubes containing 50 μ M ATP, 2–5 μ Ci ^{32}P ATP (3000 Ci/mmol; Amersham), 10 mM MgCl_2 plus protein kinase

activators and inhibitors. To demonstrate PKA activity, 10 μ M 8-bromo-adenosine 3':5'-cyclic monophosphate (8Br-cAMP; Boehringer Mannheim, Indianapolis, IN), 1 mM isobutylmethylxanthine (IBMX; Sigma, St. Louis, MO) and 50 mM of the PKA inhibitor PKI (kindly provided by Dr. W. Fletcher, Jerry L. Pettis VA Hospital, Loma Linda, CA) were added to the homogenate. To stimulate Ca^{2+} /CAM kinase activity, purified exogenous bovine brain calmodulin (Sigma) was added at a final concentration of 50 μ g/ml. 1-oleoyl-2-acetyl-glycerol (OAG) at 50 μ M and phosphatidylserine (PS) at 30 μ g/ml (both from Sigma) were added to stimulate PKC. OAG in DMSO and PS in hexane were first dried under a stream of dry nitrogen, then resuspended in 10% Triton X-100 and vortexed twice (Kitano et al., 1986). To evaluate the action of phosphatases *in vitro*, RIS-ROS proteins were phosphorylated with 100 nM of a purified PKA catalytic subunit (with a specific activity of 5–10 μ mol of phosphate transferred/min/ μ g protein; provided by Dr. W. Fletcher) for up to 90 min in the presence or absence of 1 μ M microcystin-LR (GIBCO BRL). Reactions were stopped with a third volume of SDS sample buffer containing 3 $\times$ concentrations of SDS, 2-mercaptoethanol, and glycerol for PKC and Ca^{2+} /CAM kinase assays, or with 10% TCA for PKA and phosphatase assays.

Immunoprecipitation. RIS-ROS homogenates from six fish were phosphorylated *in vitro* in the dark with ^{32}P ATP using 100 nM of the catalytic subunit of PKA (provided by Dr. W. Fletcher). The reaction was stopped with 20 mM of EDTA and cooled immediately on ice. All subsequent steps were performed at 4°C in the light. Homogenates were centrifuged at 8000 $\times$ g for 10 min. Supernatants were recovered and incubated on a shaker with a 1/100 dilution of an immune serum to amino acids 6 through 35 of mammalian phosducin, VS-3132, or the corresponding preimmune serum (kindly provided by Dr. T. Shinohara, NIH, Bethesda, MD). After 1 hr, a 1/50 dilution of prewashed Pansorbin (Calbiochem, San Diego, CA) or Protein A Sepharose (Sigma) was added and incubated for another hour. Immune complexes were pelleted at 8000 $\times$ g for 5 min, washed three times with Tris-buffered saline, resuspended in SDS sample buffer, and heated to 90°C for 5 min to release bound protein. The supernatant from the first centrifugation of the immune complex was precipitated with 10% TCA, washed twice with acetone, and resuspended in SDS sample buffer.

Cleveland digests. RIS-ROS homogenates and a purified bovine phosducin-transducin₈ complex (kindly provided by Dr. R. Lee, VA Medical Center, Sepulveda, CA) were phosphorylated *in vitro* using 100 nM of the catalytic subunit of PKA and ^{32}P ATP. After electrophoresis, gels were briefly stained and destained for approximately 30 min to minimize acid hydrolysis of proteins. Phosducin, PP33 and PP35 were localized by autoradiography and excised from the dried gel. After rehydration in water, gel pieces were equilibrated in sample buffer containing 1 mM EDTA, then applied to the lanes of a 1.5 mm thick, 15% SDS gel. The gel pieces were overlaid with a protease solution in sample buffer containing 10% glycerol (Cleveland et al., 1977). Proteases tested were 100 ng of V8 protease (Sigma) and 500 ng of TLCK-treated α -chymotrypsin (Worthington Biochemical Corporation, Freehold, NJ). Electrophoresis was performed essentially as described by Cleveland et al. (1977) except that the electrophoresis run was continuous.

Gel electrophoresis and autoradiography. Samples were electrophoresed on 15% or 5–22% SDS polyacrylamide gels of 1 mm thickness or 5–22%, 0.5 mm thick minigels and exposed for autoradiography as described previously (Pagh-Roehl et al., 1992b, 1993). Quantitation of phosphorylation was performed using the volume integration function of a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Since background increases nonlinearly with increasing molecular weight (Pagh-Roehl et al., 1993), background was subtracted adjacent to each band of interest using a box equal to the band area.

Immunoblotting. After electrophoresis on minigels, samples were electroblotted onto Immobilon (Millipore, Medford, MA) as described previously (Pagh-Roehl et al., 1993). Immunoblotting was performed using a 1/250 dilution of a transducin₈ polyclonal antibody, LAP636B and a 1/500 dilution of a rod phosphodiesterase polyclonal antibody, MOE (kindly provided by Dr. B. Fung, Jules Stein Institute, UCLA), or a 1/10,000 dilution of the phosducin anti-peptide antiserum, VS31/32. Labeled bands were visualized using an alkaline phosphatase labeled secondary antibody (Pagh-Roehl et al., 1993). Immunoblots were computer-scanned and labeled bands were quantitated using NIH Imaging.

Results

Physiological and biochemical studies were carried out using preparations of RIS-ROS isolated from the retinas of dark-adapted

Figure 1. Phase light micrographs showing RIS-ROS cultured in the dark or light in the presence of the phosphatase inhibitors okadaic acid and calyculin A, or the PKA-selective inhibitor H89. RIS-ROS were isolated from dark-adapted green sunfish under infrared illumination. RIS-ROS preparations used for phosphorylation and physiology are shown in (A). RIS-ROS were either fixed immediately after isolation (B) or cultured for 45 min in the light (C–E) or dark (F–I). Light samples were in control Ringer (C), 1 μ M okadaic acid (D) or 100 nM calyculin A (E). Dark cultures were in control Ringer (F), 10 μ M H89 (G), 10 μ M H89 and 1 μ M okadaic acid (H), or 10 μ M H89 and 100 nM calyculin A (I). *M*, Myoid; *e*, ellipsoid; *IS*, inner segment; *OS*, outer segment.

green sunfish (Fig. 1A). When cultured in the light, RIS-ROS are stimulated to elongate (Fig. 1B,C). PKA substrates were previously implicated in RIS-ROS elongation because elongation was blocked by the phosphodiesterase inhibitor IBMX, high concentrations of cyclic nucleotide analogs, or the phosphatase inhibitor okadaic acid (Liepe and Burnside, 1993a). Furthermore, the relative effectiveness of the kinase inhibitors H7 and H8 in stimulating RIS-ROS elongation were shown to be more consistent with an effect on PKA than PKC (Liepe and Burnside, 1993a).

However, subsequently we found that IBMX or cyclic nucleotide analogs did not consistently inhibit elongation (K. Pagh-Roehl, unpublished observations). When effective, these agents, which elevate intracellular cyclic nucleotide levels, could inhibit motility by creating ionic imbalances since opening of the outer segment channels by cGMP is further enhanced by cAMP (Furman and Tanaka, 1989) and only 1% of the channels are open under normal conditions (Yau, 1994). Therefore, we extended our physiological characterization of the roles of PKA and phosphatases in RIS-ROS elongation, to characterize phosphatase inhibitors in more detail and to examine the effects of H89, a new, more specific inhibitor of PKA than H7 and H8 (Hidaka et al., 1991).

Effects of phosphatase inhibitors on light-dependent RIS-ROS elongation

Light-induced RIS-ROS elongation was inhibited in a dose-dependent manner by both okadaic acid and calyculin A (Fig. 1B,E, 2A), two membrane permeant inhibitors which are most potent against type 1 and type 2A serine/threonine phosphatases (Ishihara et al., 1989; Cohen et al., 1990). In control samples cultured in the light for 45 min, RIS-ROS myoids elongated 10–16 μ m relative to time zero (compare B and C in Fig. 1), whereas in parallel cultures maintained in the dark, myoids elongated only 5–7 μ m (Figs. 1F, 3); thus, control motility is consistent with that previously reported (Pagh-Roehl et al., 1992b; Liepe and Burnside, 1993a). Light-induced elongation was most effectively blocked by calyculin A, with half-maximal inhibition occurring at approximately 100 nM (Fig. 2A). Okadaic acid also inhibited light-induced RIS-ROS elongation, as reported previously (Liepe and Burnside, 1993a), but required approximately 10-fold higher concentrations than calyculin A for half-maximal inhibition. Higher concentrations of both inhibitors (1 μ M for calyculin A and 10 μ M for okadaic acid) blocked elongation completely (Fig. 2A). In the presence of either phosphatase inhibitor, RIS-ROS morphology was normal; the triangular shape of the ellipsoid portion of the inner segment was maintained although the myoids failed to elongate (Fig. 1D,E). Since okadaic acid and calyculin A differ structurally from one another (Cohen et al., 1990), it seems likely that inhibition of RIS-ROS elongation was due to effects on phosphatase activity.

Effects of phosphatase inhibitors on light-independent elongation

The PKA-selective protein kinase inhibitor H89 stimulated elongation of dark-cultured RIS-ROS to myoid lengths attained in the light (Fig. 1, compare B and C to F and G). Stimulation of RIS-ROS elongation in the dark was half-maximal at 2.5 μ M and peaked at approximately 10 μ M (Fig. 3). Effective concentrations are similar to those with a physiological effect reported in other whole cell studies (Chijiwa et al., 1990). H89 also enhanced light-induced elongation at 10 μ M (Fig. 3). Above 10 μ M, H89 appeared to be toxic, causing retraction of myoids and rounding of ellipsoids into highly refractile structures. For phosphorylation studies, we therefore used H89 at 10 μ M.

Okadaic acid and calyculin A both inhibited light-independent RIS-ROS elongation induced by H89 in the dark (Fig. 2B). Treated RIS-ROS had somewhat rounded ellipsoids with short myoids emerging from the ellipsoid at abnormal angles (Fig. 1H,I). Calyculin A was equally effective at inhibiting light- and H89-induced elongation, with half-maximal inhibition at 100 nM (Fig. 2B,C). Okadaic acid inhibition of H89-induced elongation was approximately twice as effective as calyculin A, with half-

Figure 2. Inhibition of light-dependent (A) and light-independent elongation induced by H89 in the dark (B) by the phosphatase inhibitors okadaic acid (OKA) and calyculin A (Cal A). RIS-ROS were isolated from dark-adapted fish under infrared illumination and cultured for 45 min in the dark or light in control Ringer (C) or with the indicated concentrations of calyculin A (□) or okadaic acid (●). All dark samples contained 10 μM H89. Data are expressed as mean ± SEM for six fish. In C, means for calyculin A and okadaic acid treatments in the light and dark are directly compared.

Figure 3. Stimulation of RIS-ROS elongation by the PKA-selective kinase inhibitor H89 in dark and light cultures. RIS-ROS were isolated from dark-adapted fish and cultured for 45 min in the presence of control Ringer (C) or concentrations of H89 as indicated. Data are expressed as the mean ± SEM for four fish.

maximal inhibition at 50 nM (Fig. 2B). Okadaic acid was therefore 50-fold more effective at inhibiting H89-induced elongation than it was at inhibiting light-induced elongation (Fig. 2C). This suggests that different phosphatase activities are required for elongation in the light and in darkness.

RIS-ROS motility in preparations used for analysis of protein phosphorylation

For technical reasons, procedures typically used to prepare RIS-ROS were modified for phosphorylation studies, including retinal culture and RIS-ROS isolation procedures (see Materials and Methods). In addition, rods were exposed to Cerenkov radiation during *in situ* labeling, which may have partially light-adapted cells (Biernbaum and Bownds, 1991). Therefore, we evaluated the effects of light, H89, and okadaic acid on RIS-ROS motility in preparations used for phosphorylation studies to insure that the regulation of RIS-ROS motility was not compromised.

Although spontaneous dark elongation was enhanced and sensitivity to H89 was reduced, RIS-ROS nonetheless maintained their responsiveness to light and okadaic acid. RIS-ROS cultured in the light elongated 8.3 μm (±0.5 μm). Okadaic acid reduced elongation to 3.1 μm (±0.2 μm). Elongation in the dark was 5.9 μm (±1.2 μm), a greater fraction of the light elongation than observed in the physiological studies described in preceding sections. Thus, stimulatory effects of H89 over this high background were not significant (6.5 μm ± 1.5 μm). As in the light, elongation in the dark was reduced by okadaic acid to 1.5 μm (±0.2 μm) in the absence of H89 and to 2.5 μm (±0.5 μm) in the presence of H89. These results suggest that light/dark differences may be underrepresented in RIS-ROS preparations used for phosphorylation studies.

Phosphorylation of RIS-ROS proteins in situ

Sixteen phosphoproteins, ranging in molecular weight between 10 and 200 kDa, were detected in dark-adapted RIS-ROS isolated from cultured retinas that were labeled *in situ* with ³²P-orthophosphate for 45–60 min. RIS-ROS preparations are shown by Coomassie blue staining in Figure 4A and by autoradiography in Figure 4, B and C. Major phosphoproteins migrated at 145, 100, 67, 50, 42, 35, 33 (Fig. 4B, lane 1) and 19 kDa (not shown). Nine minor phosphoproteins and a major high molecular weight

Figure 4. Effects of okadaic acid and calyculin A on phosphorylation of RIS-ROS proteins in the dark and light. RIS-ROS were isolated after ^{32}P -labeling of dark-cultured retinas. After dark- or light-culture of RIS-ROS for an additional 10 min in the presence of phosphatase inhibitors, proteins were precipitated and electrophoresed on 15% SDS gels. Coomassie blue stained SDS gel of isolated RIS-ROS (lane 1) and protein standards (lane 2) are shown in A. Autoradiograms in B and C show phosphorylation patterns after RIS-ROS were incubated in the dark (B, lanes 1–3, C, lanes 1–2) or light (B, lanes 4–6, C, lanes 3–4) in the presence of 50 nM okadaic acid (OKA; B, lanes 2, 5), 1 μM okadaic acid (B, lanes 3, 6) or 100 nM calyculin A (C, lanes 2, 4).

Figure 5. Effect of light (*A*) and okadaic acid in the light (*B*) on phosphorylation of prominent RIS-ROS phosphoproteins. RIS-ROS were isolated from 32 P-labeled retinas in the dark and incubated for 10 min in the dark or in the light in the absence (*A*) or presence (*B*) of 1 μ M okadaic acid. Phosphorylation in SDS gels was quantified using a phosphorimager. Data are expressed as a percent of the ratio of radiolabel in light- versus dark-cultured samples (*A*) and okadaic acid-treated versus light controls (*B*) $\pm$ the SEM for three experiments in *A* and two or five experiments as indicated in *B*.

phosphoprotein at 190 kDa were detectable with longer or shorter exposures, respectively. Quantitation by phosphorimager was performed on phosphoproteins at 100, 67, 50, 42, 35, and 33 kDa, and for most experiments, the minor phosphoproteins were analyzed for changes by visual inspection.

Comparison of phosphoproteins in dark- and light-adapted RIS-ROS in situ

Several proteins decreased in phosphorylation after RIS-ROS were exposed to light for 10 min, a treatment which bleaches approximately 30% of rhodopsin and promotes maximal elongation of RIS-ROS (Liepe and Burnside, 1993b). Phosphorylation decreases were not obvious in the autoradiograms because the background was increased in the light samples (compare lanes 1 and 4 of Fig. 4*B* and lanes 1 and 3 of 4*C*). However, when background was subtracted using a phosphorimager (see Materials and Methods for details on background subtraction), a 20–25% reduction in phosphate incorporation was detectable in proteins at 33, 35, and 50 kDa (Fig. 5*A*). While some phos-

phoproteins seemed unchanged in the light, PP67 decreased in phosphorylation by more than 60% and PP100 increased by 20%. Opsin was the major light-stimulated phosphoprotein (Fig. 4*B*, lanes 4–6 and Fig. 4*C*, lanes 3 and 4), which obscured quantitation of PP42.

In a previous study of mammalian phosducin, a light-induced decrease in phosphorylation was only detectable when retinas were precultured in IBMX in the dark, to elevate basal phosphorylation levels (Lee et al., 1984). In our experiments, culturing retinas in the presence of IBMX did not alter the effects of light on phosphorylation patterns (data not shown).

Effects of phosphatase inhibitors on dark- and light-adapted RIS-ROS in situ

Incubating RIS-ROS in the light with either okadaic acid or calyculin A consistently increased the phosphorylation of two phosphoproteins, PP33 and PP35 (Fig. 4*B*, compare lanes 5 and 6 with 4, and in 4*C*, lane 4 with 3). After treatment of RIS-ROS for 10 min with 1 μ M okadaic acid, an increase in phosphory-

Table 1. Effects of okadaic acid and calyculin A on phosphorylation of RIS-ROS proteins in the dark and light

kDa	Dark (% of control)			Light (% of control)		
	OKA (50 nM)	OKA (1 μ M)	Cal A (100 nM)	OKA (50 nM)	OKA (1 μ M)	Cal A (100 nM)
33	128	165	123	116	146	155
35	202	321	203	148	304	344
50	119	116	124	101	97	83
67	62	82	80	148	108	80
100	85	68	130	100	105	77

RIS-ROS were isolated from 32 P-labeled retinas in the dark, and cultured for 10 min in the dark or light in control Ringer or in the presence of 50 nM or 1 μ M okadaic acid (OKA) or 100 nM calyculin A (Cal A). Protein was precipitated with TCA and electrophoresed on 15% SDS gels. Radiolabeled bands were quantitated using a phosphorimager. Numbers are expressed as a percentage of the treated versus the control for one experiment.

lation over the light control averaged 155% for PP33 and 218% for PP35 (Fig. 5B). Other phosphoproteins measured showed no increases after subtraction of the background, which is elevated after okadaic acid treatment, giving the impression that all phosphoproteins are somewhat more heavily phosphorylated (as in Fig. 4B; compare lanes 2 and 3 with 1; see Materials and Methods for details on background subtraction). The increase in phosphorylation of PP33 and PP35 was dose dependent, with 50 nM okadaic acid causing less increase than 1 μ M (Table 1). After treatment of RIS-ROS for 10 min in the light with 100 nM calyculin A, PP33 and PP35 were the only RIS-ROS phosphoproteins increased in phosphorylation (Table 1).

Incubating dark-cultured RIS-ROS with either okadaic acid or calyculin A also increased phosphorylation of PP33 and PP35 (compare lanes 1 with 2 and 3 in Fig. 4B and lane 1 with 2 in Fig. 4C). Phosphorylation levels were increased to a greater extent in the dark than observed with incubation in the light. In particular, phosphorylation of PP33 and PP35 was higher in the dark than in the light (Table 1; e.g., PP35 showed a threefold vs twofold elevation in phosphorylation in the dark and light, respectively). Inhibitor treatment in the dark increased phosphorylation of PP50 124% relative to dark controls but had no effect on phosphorylation of PP50 in the light (Table 1), suggesting that this phosphoprotein is phosphorylated by a kinase which is active in the dark but not in the light, while kinases phosphorylating PP33 and PP35 are active in both darkness and light.

Identification of PKA substrates *in situ*

When RIS-ROS were incubated in the dark with the phosphodiesterase inhibitor IBMX, highest increases in phosphorylation were observed for PP67, 35, 33, and 17 in each of two experiments (Fig. 6A lane 1 vs 2). Since IBMX treatments elevate cAMP levels in RIS-ROS (B. Liepe and B. Burnside, unpublished observations), PKA activity is also likely to increase correspondingly. Thus, phosphoproteins with increased phosphorylation levels after IBMX treatment are candidate PKA substrates. In one experiment in which quantitation was performed, IBMX increased PP67 phosphorylation nearly threefold, while PP35 and PP33 increased 60% and 40%, respectively. After subtraction of the higher backgrounds obtained in the IBMX-treated samples, other phosphoproteins were either unaffected by IBMX incubations (e.g., PP50) or decreased in phosphorylation (PP42), suggesting that they are not PKA substrates. Additional phosphoproteins (at approximately 19 and 38 kDa) showing an in-

crease in phosphorylation were not observed in all preparations; they are probably contaminants of the RIS-ROS preparation.

Treatment of dark-adapted RIS-ROS with the PKA inhibitor H89 for 10 min resulted in a decrease in phosphorylation of PP67 and PP33 ($n = 4$; Fig. 6B; compare lanes 1 and 2), and, in some experiments (not shown), it also reduced basal phosphorylation levels of PP35. In one experiment in which phosphorylation was quantitated, PP67 decreased by 56% and PP33 decreased by 60%. Since basal phosphorylation levels of PP35 were usually very low in dark-adapted RIS-ROS, effects by H89 were therefore undetectable. However, when phosphorylation levels of PP35 were increased after dephosphorylation was inhibited by 1 μ M okadaic acid, it was then possible to detect an H89-induced reduction in PP35 phosphorylation (Fig. 6B, lanes 4 and 6). This suggested that PKA phosphorylated PP35 under basal conditions in the dark, but that the PKA phosphorylation site of PP35 turned over rapidly in isolated RIS-ROS. In contrast, H89 reduced phosphorylation of PP33 when phosphorylation levels were elevated by both 50 nM and 1 μ M okadaic acid (compare lanes 3 and 5, 4 and 6 in Fig. 6B). Effects of H89 on phosphorylation of PP67, PP35, and PP33 were observed in light-adapted RIS-ROS as well (results not shown), suggesting that PKA substrates are phosphorylated by PKA in both light and darkness.

H89 also increased the phosphorylation of phosphoproteins such as PP42 ($n = 3$). However, it is not likely that these phosphoproteins are directly involved in elongation since they were not significantly affected by okadaic acid, which blocked cell elongation stimulated by H89 as well as by light.

Phosphorylation of PP33 and PP35 by PKA *in vitro*

To obtain further evidence that phosphoproteins affected *in situ* by IBMX and H89 treatments are indeed PKA substrates, *in vitro* experiments were performed under conditions known to directly stimulate or inhibit PKA. When RIS-ROS homogenates were treated with 8BrcAMP in the presence of 32 P γ ATP, five phosphoproteins at 67, 35, 33, 25 and 17 kDa showed an increase in phosphorylation (Fig. 7; compare lanes 1 and 3; Pagh-Roehl et al., 1993), further indicating that the corresponding phosphoproteins labeled *in situ* are PKA substrates. PP25 has been previously shown not to be present in Percoll-purified RIS-ROS (Pagh-Roehl et al., 1993), thus coming from some unidentified retinal contaminant of the RIS-ROS preparations; it was therefore not considered in our phosphorylation studies. PKI, a specific inhibitor of PKA (Walsh and Glass, 1991), antagonized the cAMP-stimulated phosphorylation almost completely (Fig. 7, lane 4), also indicating that these proteins are phosphorylated by PKA. Furthermore, basal phosphorylation of PP33 was inhibited by PKI (Fig. 7, lane 2) as observed *in situ* with H89 (Fig. 6B, lane 2). These cAMP-stimulated phosphoproteins also increased in phosphorylation in the presence of purified catalytic subunit of PKA (Pagh-Roehl et al., 1993).

Dephosphorylation of PP33 and PP35 by type 1 or 2A phosphatases *in vitro*

We further examined whether type 1 and 2A phosphatases dephosphorylate PP33 and PP35 in *in vitro* phosphorylation experiments. In RIS-ROS homogenates labeled in the presence of 32 P γ ATP by exogenous PKA catalytic subunit, phosphorylation of PP33 and PP35 peaked by 10–15 min (presumably due to the depletion of ATP), then decreased in the absence of phosphatase inhibitors (Fig. 8A), indicating that PKA phosphorylation sites

Figure 6. Identification of PKA substrates in RIS-ROS using the phosphodiesterase inhibitor IBMX (**A**) and the PKA-selective kinase inhibitor H89 (**B**). RIS-ROS were isolated from ^{32}P -labeled retinas in the dark and cultured for an additional 10 min in the dark. After TCA precipitation of proteins, and electrophoresis on 15% SDS gels, ^{32}P -labeled phosphoproteins were visualized by autoradiography. **A**, 1 mM IBMX was added in lane 2. Phosphoproteins at 67, 35, 33, and 17 kDa showing increased phosphorylation are indicated. **B**, RIS-ROS were incubated in the presence of 10 μM H89 (lanes 2, 5, 6) and okadaic acid at 50 nM (OKA +) or 1 μM (OKA++) in lanes 3, 5 and 4, 6, respectively. Phosphoproteins at 67, 35, 33 kDa showing decreased phosphorylation by H89 are indicated.

are highly susceptible to dephosphorylation *in vitro*. At 10–15 min of incubation, PP35 appeared more phosphorylated than PP33 but subsequently was more rapidly dephosphorylated (lanes 30, 60, and 90 min). Observations that PP35 is particularly susceptible to phosphatase action *in vitro* corroborates observations made about sensitivities to phosphatase inhibitors within intact RIS-ROS.

We examined whether the dephosphorylation of PP33 and PP35 could be inhibited by microcystin-LR (Fig. 8B), a specific inhibitor of type 1 and 2A phosphatases (MacKintosh et al., 1990). Microcystin-LR was chosen for these *in vitro* studies because it inhibits these phosphatases at lower concentrations than calyculin A and okadaic acid (MacKintosh et al., 1990); the latter two inhibitors were used for whole cell studies, on the other hand, because they are lipophilic, unlike microcystin and therefore membrane-permeable (Cohen et al., 1990). As shown in Figure 8B, dephosphorylation was inhibited by 1 μM microcystin-LR, suggesting that PP33 and 35 are dephosphorylated *in vitro* by type 1 or type 2A phosphatases, in agreement with phosphatase inhibitor studies done *in situ* (Figs. 4, 5).

Further characterization of PP33 and PP35

Since the activity of protein phosphatases type 1 or 2A enhances myoid elongation in cultured RIS-ROS (see also Liepe and Burnside, 1993a), and results in the dephosphorylation of two prominent PKA substrates, PP33 and PP35, these two phosphoproteins could be mediators of rod motility and were therefore further characterized. Previously, we identified PP33 and PP35 as PKA substrates by *in vitro* phosphorylation (Pagh-Roehl et al., 1993); we proposed that PP33 and PP35 are phosducin-related proteins because both PP33 and PP35 migrate as acidic proteins on 2-D gels, like mammalian phosducin (Lee et al., 1984), and they comigrate on 1-D gels with two phosducin-immunoreactive bands found to be present in teleost RIS-ROS (Pagh-Roehl et al., 1993). In this study, we analyzed the relationship of PP33 and PP35 to mammalian phosducin in more detail.

Immunoprecipitation of PP35 with a phosducin antibody

We investigated whether PP33 and PP35 could be immunoprecipitated by an antibody against mammalian phosducin. The an-

Figure 7. PKA substrates in RIS-ROS identified by *in vitro* phosphorylation. RIS-ROS were isolated from the retinas of dark-adapted fish, homogenized in Tris-EGTA buffer and incubated for 10 min with $^{32}\text{P}\gamma\text{ATP}$ in the presence of 10 mM 8BrcAMP (lanes 3 and 4) to stimulate endogenous PKA activity and 50 nM PKI (lanes 2 and 4) to inhibit PKA activity. Precipitated proteins were electrophoresed on 5–22% minigels and subjected to autoradiography. Phosphoproteins showing increased phosphorylation with 8BrcAMP and decreased phosphorylation with PKI are indicated.

tibody tested was made against an N-terminal sequence consisting of amino acids 6–35 (Dr. T. Shinohara, personal communication). To identify PP33 and PP35 *in vitro*, exogenous PKA and $^{32}\text{P}\gamma\text{ATP}$ were incubated with RIS-ROS homogenates. After the reaction was terminated with EDTA, the membranes were pelleted and the supernatant, containing most of the total PP33 and PP35, was mixed initially with preimmune or immune serum, then with Pansorbin or Sepharose–Protein A to pellet immunoglobulin and bound antigen. PP35 appeared in the pelleted fraction of the immune serum samples only (compare lane 1 and 2 in Fig. 9) while PP33 was faintly present in pellets of both immune and preimmune sera. After pelleting of the immune serum, most of the PP35 was removed from the remaining supernatant fraction (Fig. 9; compare lanes 3 and 4), while PP33 abundance remained constant in the immune and preimmune supernatants.

Since mammalian phosducin readily forms a complex with transducin $\beta\gamma$ (Lee et al., 1987), we also tested whether the β -transducin subunit coimmunoprecipitated with PP35. In Western blots of the immunoprecipitates probed with an anti-trans-

Figure 8. Sensitivity of PP33 and PP35 to phosphatase action *in vitro*. RIS-ROS were isolated from retinas of dark-adapted fish, homogenized in Tris-EGTA buffer, then incubated for the indicated times with $^{32}\text{P}\gamma\text{ATP}$ and 100 nM of the catalytic subunit of purified PKA. **A**, Autoradiograph from a 5–22% minigel showing the time course in minutes of phosphorylation of PP33 and PP35 by exogenous PKA. **B**, Phosphorimager quantitation of total PP33 and PP35 radiolabel after phosphorylation by PKA in the presence or absence of 1 μM microcystin and electrophoresis on 15% gels. Data represent one experiment.

ducin β antibody, we found that transducin β was reduced by 75% from the supernatant of the immune serum as compared with the amount in the samples incubated with preimmune serum. In contrast, using an antibody to rod phosphodiesterase, no differences were detected between the supernatants of samples treated with the preimmune versus the immune serum. The phosphodiesterase also was not immunoprecipitated by the phosducin antibody, indicating that coimmunoprecipitation of PP35 and transducin β is specific. Therefore, our results suggest that RIS-ROS PP35, like mammalian phosducin, interacts with transducin $\beta\gamma$.

Cleavage digests of purified phosducin, PP35, and PP33

To further evaluate the relatedness of PP33, PP35, and mammalian phosducin, Cleveland phosphopeptide maps of the three phosphoproteins were compared after *in vitro* phosphorylation using exogenous PKA. Autoradiographs of the phosphorylated

Figure 9. Immunoprecipitation of teleost PP35 by a phosducin anti-peptide antibody. RIS-ROS homogenates were phosphorylated in the dark with $^{32}\text{P}\gamma\text{ATP}$ and exogenous PKA catalytic subunit. The reaction was stopped by EDTA at 4°C , and supernatants recovered after centrifugation at $8000 \times g$ for 5 min. RIS-ROS supernatants were incubated for 1 hr with a 1/100 dilution of VS-31/32 phosducin immune serum (*I*; lanes 1 and 3) or preimmune serum (*PI*; lanes 2 and 4), incubated further with a 1/4 dilution of Pansorbin. The $8000 \times g$ pellet was washed three times with Tris-buffered saline, then resuspended in SDS sample buffer and heated at 90°C for 5 min (lanes 1 and 2). Supernatants (lanes 3 and 4) were TCA-precipitated and resuspended in SDS sample buffer.

proteins showed that PP35 and phosducin comigrated with one another (Fig. 10A). Although mammalian phosducin is reported to migrate on SDS gels with a molecular weight of 33 kDa, we routinely calibrated its migration relative to markers as 35 kDa in our gel system. PP33, PP35, and phosducin were excised from the gel and Cleveland digestion was performed on the gel pieces. Distinctly different phosphopeptide patterns for PP33 and PP35 were obtained with V8 protease ($n = 3$; Fig. 10B, lanes 2 and 4) although a major peptide of similar molecular weight was produced with α -chymotrypsin (Fig. 10B, lanes 1 and 3). In contrast, phosducin and PP35 had similar phosphopeptides produced by V8 protease (compare lanes 4 with 6; the arrow in Fig. 10B points to the lowest molecular weight phosphopeptide shared by PP35 and phosducin but not by PP33). No similarities were observed between the phosphopeptide patterns of PP33 and phosducin. Phosphopeptide maps with V8 protease are consistent with phosducin and PP35 being related proteins, both differing from PP33. Small differences in phosphopeptide patterns

between PP35 and phosducin may be due to differences in the amounts of proteins digested, or to posttranslational modifications.

Phosphorylation of PP33 and PP35 by PKC and Ca^{2+} /CAM kinase *in vitro*

Because phosducin has three consensus sequences for PKC (Lee et al., 1990) and 2 for Ca^{2+} /CAM kinase (see Lee et al., 1990b, and Groshan et al., 1993, for phosducin sequences and Pearson and Kemp, 1991, for protein kinase consensus sequences), we tested the ability of these kinases to phosphorylate PP33 and PP35 *in vitro*. In RIS-ROS homogenates incubated in the presence of $^{32}\text{P}\gamma\text{ATP}$, addition of 5 μM calcium enhanced phosphorylation of PP35 (Fig. 11, compare lanes 1 and 2). Phosphorylation of PP35 was further enhanced with the addition of exogenous calmodulin (Fig. 11, lane 4), while in the absence of calcium, exogenous calmodulin had no effect on phosphorylation relative to the controls (data not shown). In the presence of calcium, addition of two PKC activators, phosphatidylserine (PS) and 1-oleoyl-2-acetyl-glycerol (OAG), stimulated phosphorylation of PP33 (Fig. 11, lane 3). These results indicate that in RIS-ROS homogenates *in vitro*, PP33 is phosphorylated by PKC while PP35 is phosphorylated by a Ca^{2+} /CAM kinase, further suggesting that these are distinct phosphoproteins.

Discussion

We have used a preparation of motile rod inner-outer segments (RIS-ROS) to characterize the regulation of photoreceptor shape changes. Physiological studies indicate that light-induced RIS-ROS elongation requires dephosphorylation of PKA substrates by type 1 phosphatase (Fig. 12). In darkness, PKA substrates are phosphorylated but remain in a constant state of turnover with dephosphorylation mediated by type 2A phosphatase (Fig. 12); therefore, inhibiting PKA would lead to dephosphorylation and cell elongation. Biochemical studies indicate that the phosphorylation state of two PKA substrates varied consistently with the inhibition of PKA and phosphatase activity: a fish phosducin homolog and a phosphoprotein not previously described in photoreceptors. These phosphoproteins may play a role in regulating RIS-ROS elongation.

Analysis of the phosphorylation of RIS-ROS proteins *in situ* showed that only PP33 and PP35 were phosphorylated by PKA, dephosphorylated by type 1 or type 2A phosphatases in dark- and light-adapted RIS-ROS, and dephosphorylated in response to light. PP33 and PP35, previously identified as PKA substrates by phosphorylation *in vitro*, copurified with RIS-ROS after their separation from retinal contaminants (Pagh-Roehl et al., 1993). A third PKA substrate, PP25, did not copurify with RIS-ROS (Pagh-Roehl et al., 1993). We also showed here that a fourth PKA substrate, PP67, was not dephosphorylated by type 1 or 2A phosphatases in intact RIS-ROS. PP50, which was dephosphorylated by type 1 or 2A phosphatases in darkness, was generally unaffected by inhibitors of these phosphatases in the light, unlike PP33 and PP35. We cannot rule out the possibility that the light-induced fall in phosphorylation of RIS-ROS proteins is due in part to ATP reduction during light exposure, as shown to occur in frog RIS-ROS by Biernbaum and Bownds (1985). High light exposures used in this study bleach approximately 30% of opsin, signaling maximal RIS-ROS elongation (Liepe and Burnside, 1993b). The phosphorylation patterns of PP33 and PP35 are nevertheless consistent in every other respect with their being mediators of a pathway signaling rod cell elongation.

Figure 10. Phosphorylation of mammalian phosducin, teleost PP35 and PP33 by PKA catalytic subunit (**A**) and comparison of Cleveland phosphopeptide maps (**B**). Bovine phosducin, purified in a complex with transducin_{βγ}, and RIS-ROS homogenates were phosphorylated with ³²PγATP and PKA catalytic subunit. Following electrophoresis on 15% gels and autoradiography, phosducin, PP33, and PP35 were located (**A**) and excised from the gel. Gel pieces were rehydrated and electrophoresed in the presence of 100 ng of V8 protease (**B**, lanes 2, 4, and 6) or 500 ng of α-chymotrypsin (**B**, lanes 1, 3, and 5). The arrowhead indicates a prominent phosphopeptide produced with V8 protease from PP35 and phosducin (lanes 4 and 6) but not from PP33 (lane 2).

Observations suggesting that PP35 is the fish homolog of phosducin are based on characteristics of phosducin from mammalian ROS (Lolley et al., 1992). Like mammalian phosducin (Lee et al., 1984), PP35 is an acidic, PKA substrate (Pagh-Roehl et al., 1993). A phosducin antibody immunoprecipitated all of PP35. As expected of mammalian phosducin, which forms a 1:1 complex with transducin_{βγ} (Lee et al., 1987), teleost transducin_{βγ} was immunoprecipitated together with PP35 by the phosducin antibody. Furthermore, a mammalian phosducin standard and PP35 comigrated on 1-D gels. Finally, phosphopeptide maps of mammalian phosducin and PP35 were similar, consistent with a homology between the two proteins.

Several new observations about rod phosducin were made in this study. *In vitro* phosphorylation of PP35 was enhanced by calcium and exogenous calmodulin, suggesting that fish phosducin is phosphorylated by Ca²⁺/CAM kinase. Although phos-

ducin contains consensus sequences for Ca²⁺/CAM kinase phosphorylation (Lee et al., 1990b; Pearson and Kemp, 1991), *in vitro* phosphorylation studies using mammalian ROS have failed to show a calcium-activated phosphorylation of phosducin (Newton and Williams, 1993). This can be explained by the absence of Ca²⁺/CAM kinase activity in ROS (Binder et al., 1989). In teleost RIS-ROS, phosducin was immunolocalized primarily to the inner segment, with only minor staining of ROS, in the axonemal region (V. Yee and K. Pagh-Roehl, unpublished observations). Thus, RIS-ROS phosducin is likely to be primarily from RIS where it may be exposed to Ca²⁺/CAM kinase and other kinases not present in ROS. This could produce the multiple isoforms of teleost phosducin observed in RIS-ROS (Pagh-Roehl et al., 1993).

A second new observation is that the phosphorylation state of teleost phosducin appears to be dynamic. Since phosphatase inhibitors increased phosducin phosphorylation more than any other phosphoprotein within RIS-ROS, PP35 must be highly sensitive to phosphatase activity. The rapid reduction of H89-sensitive phosphorylation in RIS-ROS further suggests that the PKA phosphorylation turns over rapidly *in vivo*. Sensitivity of phosducin to dephosphorylation *in vitro* has been noted in this and other studies (Hawes et al., 1994; Yoshida et al., 1994).

PP33, the other PKA substrate dephosphorylated by type 1 or 2A phosphatases in RIS-ROS, appears to be distinct from PP35 and mammalian phosducin. In a previous *in vitro* phosphorylation study, we proposed that PP33 might be a phosducin homologue because it comigrated with a second phosducin immunoreactive band present in RIS-ROS, and it had an acidic pI similar to PP35 and mammalian phosducin (Pagh-Roehl et al., 1993). However, studies reported here suggest that PP33 is different from phosducin and PP35: it is not immunoprecipitated by a phosducin antibody; it generates distinct V8 protease digestion patterns; and it is phosphorylated by PKC, not by Ca²⁺/CAM kinase. However, PP33 may be a phosducin-related protein. Multiple phosducin-like proteins have been reported

Figure 11. Effect of calcium, calmodulin, phosphatidylserine and 1-oleoyl-2-acetylgllycerol on phosphorylation of PP33 and PP35 *in vitro*. RIS-ROS were isolated from dark-adapted fish, homogenized in Tris-EGTA buffer and incubated for 10 min with ³²PγATP in the absence (lane 1) or presence of 5 μM calcium (lanes 2–4). Lane 4 contained 50 μg/ml exogenous calmodulin (CaM). Lane 3 contained 30 μg/ml phosphatidylserine (PS) and 50 μM 1-oleoyl-2-acetylgllycerol (OAG). Samples were diluted with 3× SDS sample buffer and electrophoresed on 5–22% SDS gels.

Dark: **Light:**

Figure 12. Model depicting the effect of darkness and light on the phosphorylation state of specific PKA substrates. Phosphorylation by PKA is favored over dephosphorylation by phosphatase type 2A (PP2A) in the dark. Phosphorylation of PKA substrates is associated with shortening of rods. In the light, dephosphorylation of PKA substrates by phosphatase type 1 (PP1) is favored over phosphorylation by PKA. Dephosphorylation of PKA substrates is associated with rod cell elongation. P, Phosphate.

(Kuo et al., 1993) and predicted from gene products (Abe et al., 1993). Furthermore, phosducin does have PKC consensus sequences (Lee et al., 1990b). Splicing variations at the two splicing sites within the N-terminal part of phosducin (Abe et al., 1993) could account for the absence of Ca^{2+} /CAM kinase phosphorylation sites at serine 54 and 73, and failure to immunoprecipitate PP33 by a phosducin antibody directed towards epitopes at the N-terminus. However, sufficient portions of the N-terminus may remain to allow recognition of a 33 kDa band by the phosducin antibody on immunoblots, as reported previously (Pagh-Roehl et al., 1993). Even though phosducin is reported to be sensitive to proteases (Lee et al., 1987), it is unlikely that PP33 is a breakdown product of PP35. PP33 was not more abundant in RIS-ROS homogenates incubated in the absence of protease inhibitors (K. Pagh-Roehl, unpublished observations). Furthermore, removal of Ca^{2+} /CAM kinase phosphorylation sites by proteases would result in a protein lower in molecular weight than PP33.

Evidence that RIS-ROS elongation entails dephosphorylation of mediator proteins by different phosphatases in light and darkness is based on the availability of membrane permeant phosphatase inhibitors with differing sensitivities toward type 2A phosphatases. Type 1 and 2A phosphatases are inhibited by approximately the same concentration of calyculin A while okadaic acid distinguishes between the two phosphatases, inhibiting type 2A at 50–100-fold lower concentrations (Cohen et al., 1990). We found that 50-fold higher concentrations of okadaic acid were required to block light-induced elongation than to block H89-induced elongation in darkness. However, no differences were detected in the amount of calyculin A required to block elongation suggesting that massive changes in total phosphatase or PKA activity are unlikely to have occurred. Rather, the requirement for 50-fold higher okadaic acid concentrations to block light-induced elongation is more likely due to its weaker action against type 1 phosphatase. In further support of this,

calyculin A, which is a more potent blocker of type 1 phosphatase than okadaic acid (Ishikawa et al., 1989), is more effective than okadaic acid at blocking light-induced elongation. In several other studies, greater sensitivity of an intracellular process to calyculin A relative to okadaic acid correlates with type 1 phosphatase activity (e.g., Eriksson et al., 1992; Downey et al., 1993). The light-specific requirement for type 1 phosphatase activity in producing RIS-ROS elongation may reflect a change in location of phosphatase or substrate (Shenolikar, 1994). In this regard, it is of interest that phosducin is reported to translocate from outer to inner segments in the light (Whelan and McGinnis, 1988).

Results from this and a previous study (Liepe and Burnside, 1993a) suggest that PKA substrates are dephosphorylated both during light-dependent and light-independent elongation (Fig. 12). Light and H89-induced elongation were not additive, indicating that they are operating through the same pathway. Although H89 could inhibit other protein kinases at the micromolar concentrations required to compete with intracellular ATP (Hidaka et al., 1991), *in situ* phosphorylation experiments showed that treatment of RIS-ROS with H89 reduced phosphorylation only of substrates identified as PKA substrates by other approaches. H89 may stimulate elongation in the dark by inhibiting sustained activity of PKA, and thus promoting the dephosphorylation of PKA target sites. Finally, PP33 and PP35 are both PKA substrates and the only phosphoproteins showing a rapid response to type 1 or 2A phosphatase activity in both darkness and light.

In conclusion, we have identified two phosphoproteins that may play a role in the light-regulated PKA pathway mediating elongation in teleost rods. Phosducin, a candidate regulator of rod motility, has direct effects on G-protein activity (Lee et al., 1992; Yoshida et al., 1994). In rod outer segments, phosducin thus modulates cGMP-phosphodiesterase activity (Lee et al., 1992; Yoshida et al., 1994). In inner segments, where the ma-

jority of rod phosducin is located (Lee et al., 1988), phosducin is expected to modulate adenylate cyclase and phospholipase C- β (see Yoshida et al., 1994). Through these pathways, phosducin may exert effects on the actin cytoskeleton, which mediates rod cell elongation (O'Connor and Burnside, 1982; Pagh-Roehl et al., 1992a). Further study will be required to understand the exact role of phosducin in inner segment physiology and to characterize PP33, a newly described rod phosphoprotein.

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