Title

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Journal

The Journal of General Physiology, 102(1)

ISSN

0022-1295

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Publication Date

1993-07-01

DOI

10.1085/jgp.102.1.75

Peer reviewed

Cyclic Nucleotide Regulation of Teleost Rod Photoreceptor Inner Segment Length

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ABSTRACT Retinal rod photoreceptors of teleost fish elongate in the light and shorten in the dark. Rod cell elongation and shortening are both mediated by actin-dependent mechanisms that occur in the inner segment myoid and ellipsoid. The intracellular signaling pathways by which light and dark regulate the actin cytoskeleton in the inner segment are unknown. To investigate the intracellular signals that regulate teleost rod motility, we have been using mechanically isolated rod inner/outer segments (RIS-ROS) obtained from the retinas of green sunfish, *Lepomis cyanellus*. In culture, RIS-ROS retain the ability to elongate in response to light; myoids elongate 15–20 μm in length during 45 min of light culture. A pharmacological approach was taken to investigate the role of cyclic nucleotides, cyclic nucleotide–dependent kinases, and protein phosphatases in the regulation of RIS-ROS motility. Millimolar concentrations of cAMP and cGMP analogues were both found to inhibit light-induced myoid elongation and two cyclic nucleotide analogues, SpCAMPS and 8BrcGMP, promoted myoid shortening after RIS-ROS had elongated in response to light. The cyclic nucleotide–dependent kinase inhibitor, H8, mimicked light by promoting myoid elongation in the dark. The effects of H8 were dose dependent, with maximal elongation occurring at concentrations of ~ 100 μM. Similar to the effects of cyclic nucleotide analogues, the phosphatase inhibitor, okadaic acid (0.1–10 μM), inhibited light-induced elongation and promoted shortening. The results presented here suggest that RIS-ROS motility is regulated by protein phosphorylation: phosphorylation in the dark by cyclic nucleotide–dependent protein kinases promotes rod shortening, while dephosphorylation in the light promotes rod elongation.

INTRODUCTION

Rod photoreceptors in the retinas of teleost fish undergo changes in cell length in response to changing ambient lighting conditions. In darkness rods shorten and in light rods elongate (for review see Burnside and Nagle, 1983; Burnside and Dearry, 1986). These changes in rod cell length are mediated by actin-dependent elements housed within the ellipsoid and myoid of the inner segment, and it is the myoid that

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J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/93/07/0075/24 $2.00
Volume 102 July 1993 75–98

undergoes the most dramatic changes in length (O'Connor and Burnside, 1981, 1982; Pagh-Roehl, Brandenburger, Wang, and Burnside, 1992). Thus, light and dark modulation of rod movement entails differential regulation of the actin cytoskeleton in the rod inner segment.

As a first step toward characterizing the intracellular signaling pathways by which light and dark trigger actin-dependent motility in teleost rods, we were particularly interested in examining the possible roles of cyclic nucleotides. Cyclic GMP has been shown to be the primary intracellular second messenger of phototransduction, mediating the effect of light absorption on membrane potential (Fesenko, Kolesnikov, and Lyubarsky, 1985; Yau and Nakatani, 1985). Therefore, we investigated whether cGMP might also link light absorption to rod movement and thus play multiple roles in the light regulation of rod physiological processes. In all species so far examined, including green sunfish, both retinal cGMP and cAMP levels have been reported to be higher in the dark-adapted retina than in the light-adapted retina (Orr, Lowry, Cohen, and Ferrendelli, 1976; Ferrendelli, DeVries, Cohen, and Lowry, 1980; Burnside, Evans, Fletcher, and Chader, 1982). Furthermore, cAMP analogues and agents known to elevate intracellular levels of cAMP have previously been shown to promote dark-adaptive retinomotor movements in teleost rods, cones, and retinal pigment epithelium (RPE) (for review see Dearry and Burnside, 1986*a*). For example, dibutyryl cAMP with the phosphodiesterase inhibitor IBMX promotes rod shortening in light-cultured isolated retinas (O'Connor and Burnside, 1982) and inhibits light-induced elongation in isolated rod inner/outer segments (RIS-ROS; Nagle and Burnside, 1984). In addition, both cAMP and cGMP have been implicated in the regulation of actin-based motility mechanisms in a variety of other cell types (Cheek and Burgoyne, 1987; Nishimura and van Breemen, 1989; Waldmann and Walter, 1989; Goldman and Abramson, 1990).

Previous studies have shown that isolated rod fragments (RIS-ROS) retain their ability to elongate in response to light (Dearry and Burnside, 1986*b*; Pagh-Roehl et al., 1992). Since other retinal cell types are absent in these preparations, we can use them to examine the direct effects of various experimental treatments on rod motility. In this study we have used these isolated rod fragments to test whether various agents known to affect cyclic nucleotide metabolism and/or protein phosphorylation would either mimic or block the effects of light on actin-dependent rod myoid elongation. We report that treatments that activate cyclic nucleotide–dependent kinases or inhibit phosphoprotein phosphatases inhibit light-induced elongation and promote rod shortening, thus mimicking the effect of darkness in vivo. In contrast, treatments that inhibit cyclic nucleotide–dependent protein kinases promote RIS-ROS elongation in the dark, thus mimicking the effect of light.

MATERIALS AND METHODS

Preparations and Procedures

Green sunfish, *Lepomis cyanellus*, were obtained from Chico Game Fish Farm (Chico, CA) or from Fender Fish Farms (Baltic, OH). Fish were maintained either in outdoor ponds under ambient lighting conditions or in indoor aquaria on a fixed light/dark cycle of 14 h light and 10 h dark.

All experiments were conducted in the late morning or early afternoon to minimize any possible circadian effects, although in green sunfish maintained in constant darkness there is no detectable circadian influence on the retinomotor position of rods (Burnside and Ackland, 1984). Fish were dark-adapted in aerated tanks for at least 1.5 h. Unless otherwise specified, all procedures carried out using dark-adapted fish were conducted under infrared illumination (Wratten filter 87B or Safelight filter #11; Eastman Kodak Co., Rochester, NY) with the aid of an image converter (FJW Industries, Elgin, IL). Fish were killed by spinal section and brain pithing. Retinas were removed from posterior eyecups by gently pipetting a stream of Ringer between the retina and RPE and cutting the optic nerve. Photoreceptor inner/outer segments were isolated by shaking the retina with forceps in oxygenated Ringer. The resulting suspension was composed of ~ 95% RIS-ROS, <5% detached rod outer segments, and <1% each of cone inner/outer segments, red blood cells, and RPE. Immediately after isolation an aliquot of the RIS-ROS suspension was fixed in darkness for each fish to determine the initial mean RIS-ROS myoid length (T_0). RIS-ROS samples were precultured in the dark for 15 min and then cultured for 45 min unless otherwise indicated. Mean myoid lengths after the dark preculture time were not significantly different from mean myoid lengths at T_0 as determined by Student's t test analysis ($P < 0.051$, $n = 9$, Statworks program). All cultured samples were maintained in plexiglass chambers on a rotating shaker (~ 50 rpm) housed in light-tight, ventilated, black boxes with or without a light source as described below. Cell cultures were maintained at room temperature and gassed with 95% O_2/5% CO_2. Cell viability at the end of a 45–60-min culture in normal Ringer was >95%, as demonstrated by the exclusion of didansyl cystine (DDC). Immediately after isolation or following culture, RIS-ROS were fixed with 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0.

White light stimulation was provided by a 6-W tungsten light source held ~ 15 cm from the cultures. The light from this source was projected through an acetate diffusion screen onto the plexiglass surface of the culture chamber. Irradiance was determined with a radiometer (model # 1700; International Light, Inc., Newburyport, MA) in conjunction with a silicon photodiode (model # SED038; International Light, Inc.). The irradiance provided by this light source was ~ 3×10^{13} photons per cm^2 per s at 524 nm as measured by placing the photodiode in the average position occupied by the RIS-ROS cultures in the culture chamber.

Materials and Solutions

RIS-ROS suspensions were cultured in a modified Earle's buffered salt solution. The control Ringer contained 116.4 mM NaCl, 5.4 mM KCl, 1.0 mM Na_2HPO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 25.5 mM glucose, 24 mM $NaHCO_3$, 3 mM HEPES, 1 mM ascorbic acid, and 5 mM taurine. RIS-ROS motility in the light or in the dark in the presence of 5 mM taurine was not different from motility observed in parallel cultures maintained in the absence of taurine (data not shown). The final pH was adjusted to 7.2 after equilibration with 95% O_2/5% CO_2.

cAMP and cGMP were obtained from Boehringer Mannheim Corp. (Indianapolis, IN). Adenosine, sodium nitroprusside (SNP), dibutyryl cGMP (dbcGMP), and IBMX were purchased from Sigma Chemical Co. (St. Louis, MO). Dibutyryl cAMP (dbcAMP), 8-bromo-cGMP (8BrcGMP), and 8-bromo-cAMP (8BrcAMP) were obtained from either Sigma Chemical Co. or Boehringer Mannheim Corp. The cAMP diastereomers, Rp- and Sp-adenosine-3',5'-cyclic phosphorothioate (RpCAMPS and SpCAMPS) were generously donated by Biolog Life Science Institute (Bremen, Germany). Forskolin, H8 (*N*-(2-[methylamino-ethyl]-5-isoquinoline-sulfonamide), and H7 (1-(5-isoquinolinesulfonyl)-2-methyl piperazine dihydrochloride)) were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). Rhodamine-phalloidin was obtained from Molecular Probes, Inc. (Eugene, OR). Okadaic acid was generously donated from GIBCO BRL (Gaithersburg, MD). With the exception of forskolin and okadaic acid, all agents were dissolved in Ringer at 10 times the desired concentration and diluted 1:10 into the

RIS-ROS suspension. Forskolin and okadaic acid were dissolved in 100 and 10% DMSO, respectively, and diluted into the RIS-ROS suspensions such that the final concentration of DMSO was ≤1%. DMSO at 1% had no observable effect on light-induced RIS-ROS elongation or on the slight elongation that occurs in darkness. Cell viability at the end of the culture period in Ringer plus cyclic nucleotide treatments (2 mM dbcAMP + 0.25 mM IBMX, 2 mM dbcGMP + 0.25 mM IBMX, 2 mM 8BrcAMP, 2 mM 8BrcGMP, or 2 mM IBMX) was >89% as demonstrated by the exclusion of DDC.

Rhodamine-Phalloidin Labeling of F-Actin

Rhodamine-phalloidin labeling of F-actin in RIS-ROS was accompanied as described by Pagh-Roehl et al. (1992) with the exception that the rhodamine-phalloidin was diluted 1:20 with PBS containing 1% BSA and 0.1% sodium azide. Measurements of phalloidin staining in RIS-ROS myoids and in calycal processes were performed with the aid of an ocular micrometer under fluorescence microscopy.

Quantitation of RIS-ROS Motility

After fixation, myoid lengths were measured under 40× phase optics with the aid of an ocular micrometer. For each sample, 25 RIS-ROS were selected for measurement by randomly moving the microscope stage and measuring those RIS-ROS found under the ocular micrometer. Changes in myoid length were determined for each fish by subtracting the average T_o length from the average length at the specified culture time. The mean change in myoid length was then calculated for the fish used in each experiment. In each figure, light and dark controls were cultured in parallel with experimental samples obtained from the same fish. Therefore, the number of control and experimental samples may vary from figure to figure. Percent inhibition of light-induced elongation was calculated using the controls that were cultured in parallel with the experimental samples. Data in graphs and tables are presented as mean change in myoid length ± SEM. Unless otherwise specified, *N* refers to the number of fish used to obtain each mean.

RESULTS

Light Stimulates RIS-ROS Elongation

Light stimulates myoid elongation in cultured green sunfish RIS-ROS (Figs. 1 and 2; cf. Dearry and Burnside, 1986*b*; Pagh-Roehl et al., 1992). After 60 min in culture, elongation is approximately twofold greater in RIS-ROS exposed to light than in RIS-ROS maintained in darkness. Immediately after isolation from dark-adapted fish, mean RIS-ROS myoid length is typically 7–9 μm. After 45 min of culture, mean RIS-ROS myoid length has increased by 16–20 μm in the light, but by only 5–10 μm in the dark. Elongation in the light reaches a plateau in 45–60 min. The average rate of RIS-ROS elongation for the first 45 min is 0.3–0.5 μm/min in the light and 0.1–0.2 μm/min in the dark (Fig. 1). The rate of RIS-ROS elongation in the light is similar to the rate of rod elongation observed in vivo for another fish species (cf. Burnside and Nagle, 1983) and to the rate of RIS-ROS elongation observed using time-lapse photography of living preparations (Tran, P., K. Pagh-Roehl, and B. Burnside, unpublished observations). RIS-ROS elongation in the light appears to mimic light-evoked rod motility in vivo. However, in darkness RIS-ROS elongate slightly, whereas green sunfish rods remain short in vivo, suggesting that some

signal(s) from the retina may be required to maintain rod myoids at their short dark-adaptive lengths (see Discussion). In this article we will refer to light-induced RIS-ROS elongation as the augmentation in the extent of elongation observed after 45 min in the light as compared with 45 min in the dark (see Fig. 1).

cAMP and cGMP Analogues Inhibit Light-induced RIS-ROS Elongation

Membrane-permeable analogues of both cAMP and cGMP (Braumann and Jastorff, 1985; Beebe and Corbin, 1986) inhibited light-induced RIS-ROS elongation (Fig. 2) in a dose-dependent fashion (Fig. 3, *A* and *B*). Maximal effects were observed at

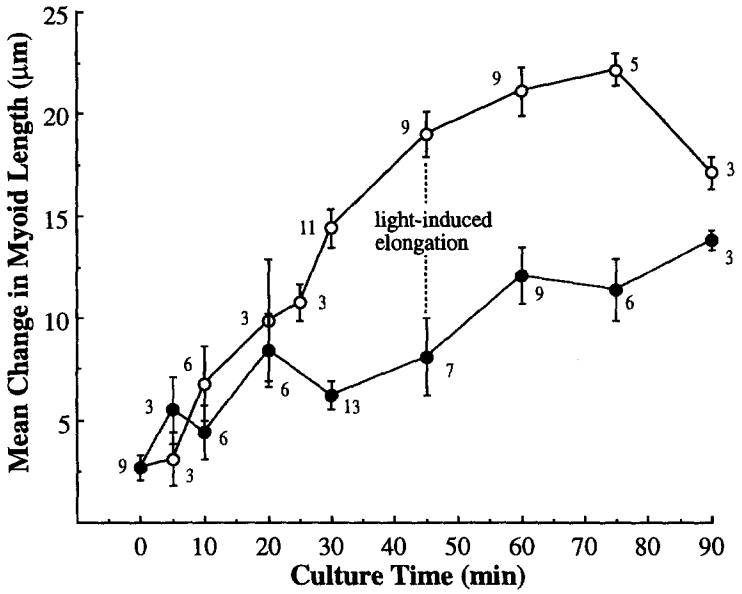

FIGURE 1. Time course of teleost RIS-ROS elongation in light and dark culture. RIS-ROS were isolated from dark-adapted fish under infrared illumination, precultured in the dark for 15 min, and then cultured in the light (○) or dark (●) for the times indicated. Light-induced myoid elongation is indicated as the difference in myoid lengths between light and dark cultures at 45 min. Time zero indicates the end of the 15-min preculture period. Data are expressed as mean ± SEM, with numbers of fish indicated for each point.

millimolar concentrations. The dose–inhibition curves of RIS-ROS elongation for both cAMP (Fig. 3 *A*) and cGMP (Fig. 3 *B*) analogues were similar. The most effective inhibition of light-induced RIS-ROS elongation was observed with the dibutyryl analogues of both cAMP and cGMP in the presence of 0.25 mM IBMX, and with the cAMP analogue SpCAMPS. Each of these treatments completely suppressed light-induced RIS-ROS elongation. At 0.25 mM, IBMX alone had no effect on light-induced RIS-ROS elongation, although it was inhibitory at higher concentrations (Fig. 3 *C*). At 2 mM the 8-bromo analogues of cAMP and cGMP each inhibited light-induced RIS-ROS elongation by 80%. In the absence of IBMX, the dibutyryl

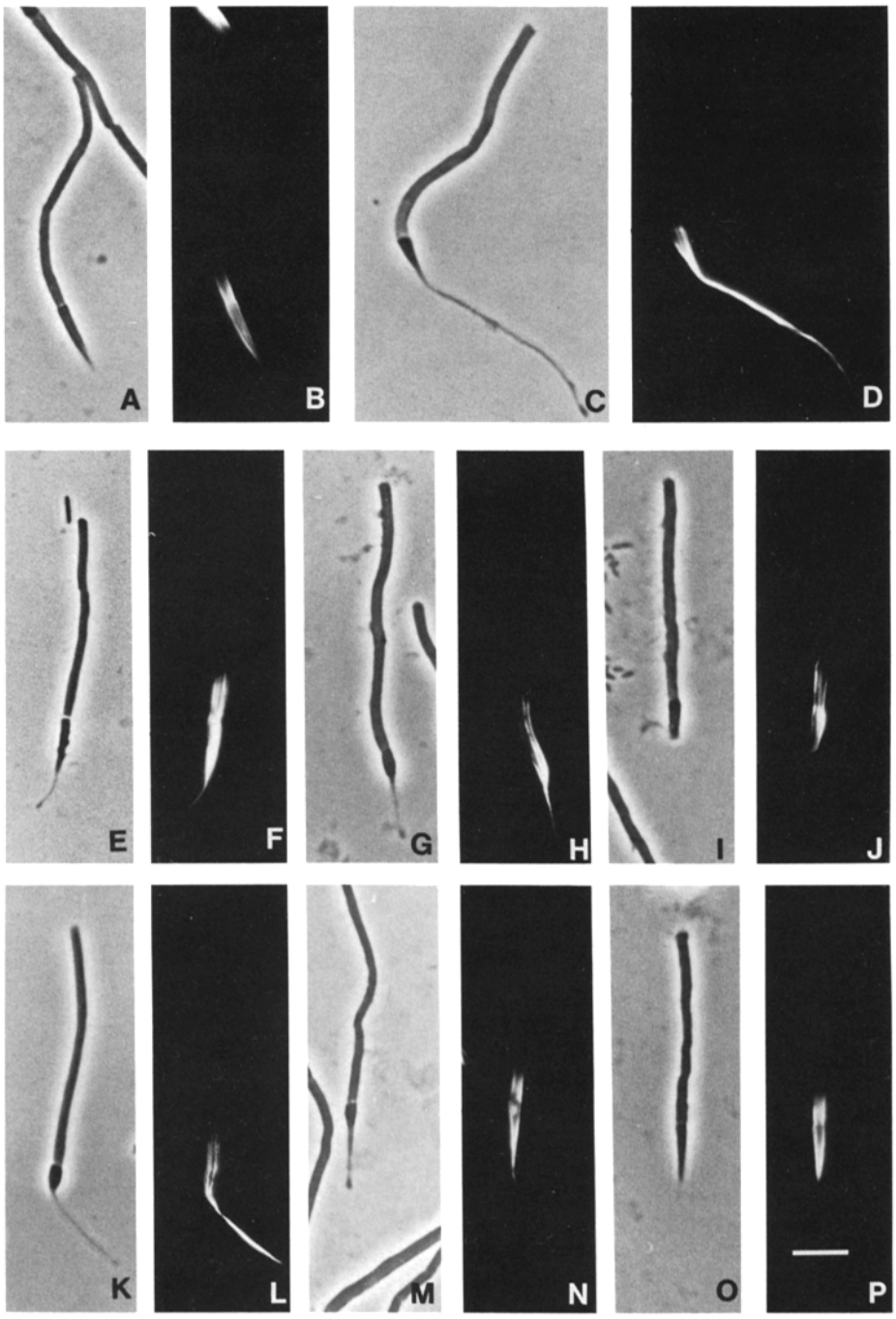

FIGURE 2. Effects of cyclic nucleotide analogues or agents known to elevate intracellular levels of cyclic nucleotides on RIS-ROS morphology. Phase (*A, C, E, G, I, K, M, O*) and fluorescence (*B, D, F, H, J, L, N, P*) micrographs of rhodamine-phalloidin-labeled RIS-ROS fixed immediately after isolation (*A, B*); after 45 min light culture (*C, D*); after 45 min dark culture (*E, F*); and after 45 min light culture with 2 mM dbcGMP + 0.25 mM IBMX (*G, H*); 2 mM 8BrcGMP (*I, J*); 1 mM IBMX (*K, L*); 2 mM dbcAMP + 0.25 mM IBMX (*M, N*); 2 mM 8BrcAMP (*O, P*). Except for preparations fixed immediately after isolation, all samples were precultured in the dark for 15 min ± cyclic nucleotide treatments. Scale bar = 10 μm.

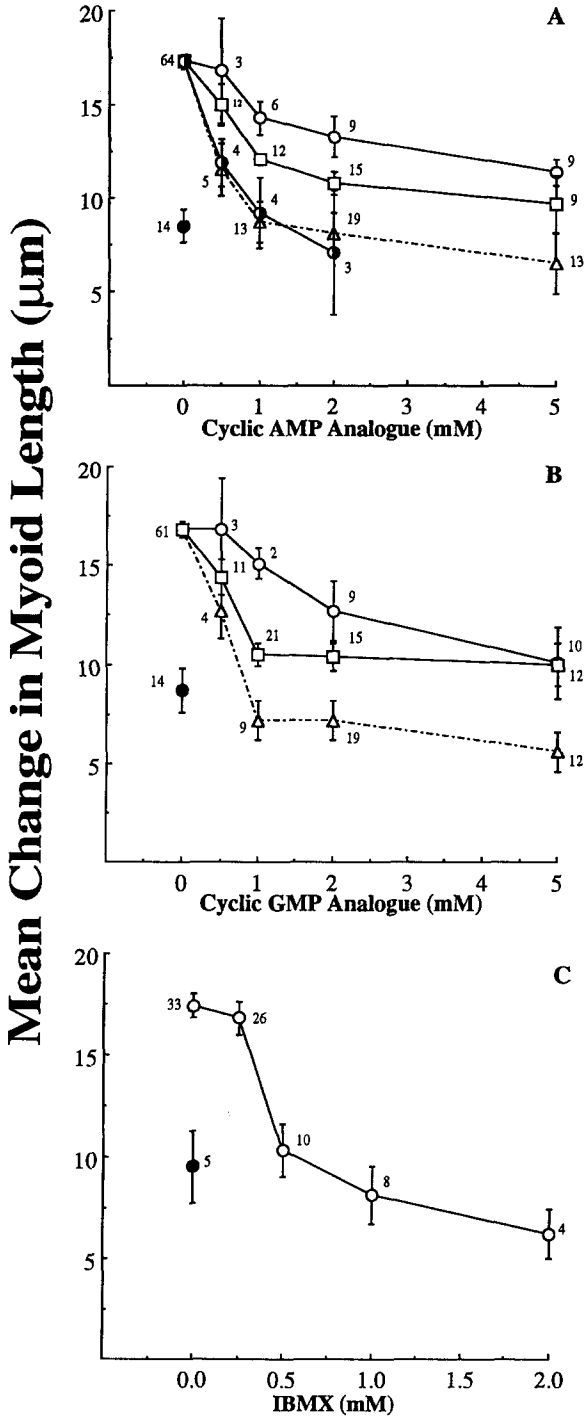

FIGURE 3. cAMP and cGMP analogues and IBMX produce dose-dependent inhibition of light-induced elongation in cultured RIS-ROS (*A*) RIS-ROS were isolated from dark-adapted fish under infrared illumination, precultured in the dark for 15 min with the indicated concentrations of db-cAMP (○), 8BrcAMP (□), db-cAMP + 0.25 mM IBMX (△), or SpCAMPS (◑), and then cultured with the indicated concentrations of the cAMP analogues in the light for 45 min. (*B*) RIS-ROS were precultured in the dark for 15 min with the indicated concentrations of db-cGMP (○), 8BrcGMP (□), or dbcGMP + 0.25 mM IBMX (△), and then cultured with the indicated concentrations of the cGMP analogues in the light for 45 min. (*C*) RIS-ROS were precultured in the dark for 15 min with the indicated concentrations of IBMX (○), and then cultured with or without IBMX in the light for 45 min. Dark controls (●) were cultured in darkness in control Ringer for 60 min. Data are expressed as mean ± SEM, with numbers of fish indicated for each point.

analogues of cAMP and cGMP were the least effective: at 2 mM they each produced only a 50% inhibition of light-induced RIS-ROS elongation. Light-induced RIS-ROS elongation was not inhibited by 2 mM adenosine, 2 mM dbcCMP with 0.25 mM IBMX, or nonderivatized cAMP or cGMP (Table I). RpCAMPS, a cAMP-dependent kinase antagonist, seemed to act like a cAMP agonist and also inhibited light-induced RIS-ROS elongation (data not shown).

Light-induced RIS-ROS elongation was also inhibited by a variety of agents known to elevate intracellular levels of cAMP and/or cGMP. Millimolar concentrations of the phosphodiesterase inhibitor IBMX completely blocked light-induced RIS-ROS elongation (Fig. 3*C*). Light-induced RIS-ROS elongation was also inhibited with activa-

TABLE I

Effects of Cyclic Nucleotides and Agents That Elevate Intracellular Levels of Cyclic Nucleotides

Treatment	Mean change in myoid length	No. of fish
	μm ± SEM	
Light control	17.3 ± 0.4	60
Dark control	7.0 ± 0.6	34
2 mM dbcGMP/0.25 mM IBMX	7.2 ± 1.0	19
2 mM 8BrcGMP	10.4 ± 0.7	15
2 mM dbcGMP	12.7 ± 1.5	9
5 mM cGMP	18.6 ± 0.7	6
1 μM SNP	7.8 ± 1.4	4
100 μM SNP	7.2 ± 1.2	9
2 mM dbcAMP/0.25 mM IBMX	8.1 ± 1.1	19
2 mM 8BrcAMP	10.6 ± 0.6	15
2 mM dbcAMP	13.3 ± 1.1	9
5 mM cAMP	14.2 ± 0.4	3
1 μM forskolin	17.2 ± 0.6	9
100 μM forskolin	9.4 ± 1.5	6
2 mM adenosine	16.0 ± 0.8	3
2 mM dbcCMP/0.25 mM IBMX	15.8 ± 0.6	6

Effects of cyclic nucleotide analogues and agents that elevate intracellular levels of cAMP and/or cGMP on light-induced RIS-ROS elongation. RIS-ROS were isolated and cultured as described in Fig. 3.

tion of either guanylate cyclase by 1 μM SNP or adenylate cyclase by 100 μM forskolin (Table I). Bath-applied SNP has been shown to elevate cGMP levels in a number of tissues, including retina (Berkelmans, Schipper, Hudson, Steinbusch, and de Vente, 1989; for review see Waldman and Murad, 1987), and forskolin has been shown to elevate cAMP levels in a variety of cell types and tissues, including green sunfish retina (cf. Dearry and Burnside, 1985).

Together these observations indicate that artificial elevation of intracellular cAMP and/or cGMP inhibits light-induced RIS-ROS elongation and leads to maintenance of short myoid lengths even after light culture. This finding is consistent with a regulatory model in which darkness is associated with elevation of intracellular cyclic

nucleotide levels and/or increased protein phosphorylation by cyclic nucleotide–dependent protein kinases, while light is associated with a fall in intracellular cyclic nucleotides levels and/or protein dephosphorylation.

SpCAMPS and 8BrcGMP Promote RIS-ROS Shortening

Since treatments that elevate cAMP or cGMP inhibited light-induced RIS-ROS elongation, we also tested whether similar treatments could induce shortening in previously light-cultured and elongated RIS-ROS. For these studies RIS-ROS were induced to elongate by preculturing in the light for 30 min (T_{30}) and then cultured with or without cyclic nucleotide analogues and/or IBMX in darkness for an

TABLE II

Induction of RIS-ROS Shortening by Cyclic Nucleotide Analogues

Treatment	Mean change in myoid length	Change in myoid length from $T = 30$	No. of fish
	$\mu m \pm SEM$	μm	
Light 30′	14.5 ± 0.5	0	11
Continuous dark 75′	11.0 ± 0.9	NA	11
All samples below cultured 30 min in light and then 45 min dark ± the indicated analogues			
30′ light + 45′ dark control	19.9 ± 0.8	5.4	11
2 mM dbcAMP/0.25 mM IBMX	17.9 ± 2.0	3.4	4
2 mM dbcGMP/0.25 mM IBMX	18.0 ± 2.6	3.5	4
2 mM dbcAMP	17.1 ± 4.4	2.6	3
2 mM dbcGMP	18.9 ± 4.2	4.4	3
2 mM IBMX	17.4 ± 2.5	2.9	4
1 mM SpCAMPS	16.9 ± 1.9	2.4	3
2 mM 8BrcAMP	13.9 ± 3.7	−0.6	4
2 mM 8BrcGMP	8.9 ± 2.8	−5.6	4
2 mM SpCAMPS	7.7 ± 2.0	−6.8	11

Capacity of cAMP and cGMP analogues to induce myoid shortening in RIS-ROS previously elongated in light culture. RIS-ROS were isolated and cultured as described in Fig. 4. Negative values in the third column indicate myoid shortening from myoid lengths achieved after 30 min of light culture. Dark controls were cultured for 75 min in the dark with no exposure to light; thus, comparison to samples after 30 min light culture is not applicable (NA).

additional 45 min. Dark controls were cultured in continuous darkness for 75 min. Two cyclic nucleotide analogues, SpCAMPS (2 mM) and 8BrcGMP (2 mM), were effective in promoting RIS-ROS shortening (Table II; Figs. 4 and 5). No detectable cell shortening was observed with the other analogues tested, though 2 mM 8BrcAMP did block the further elongation observed in control cultures (Table II). Darkness alone was not effective in inducing RIS-ROS shortening after light exposure (Table II; Figs. 4, 7 *A*, and 8 *B*). In fact, RIS-ROS precultured in the light and then cultured in darkness continued to elongate. Similar observations have been reported for cones in vivo, in which brief exposure to light promotes and maintains cones in light-adapted positions even after 40 min in darkness (Muntz and Richard, 1982). The maximal rate of rod myoid shortening in SpCAMPS or 8BrcGMP was 0.3–0.4

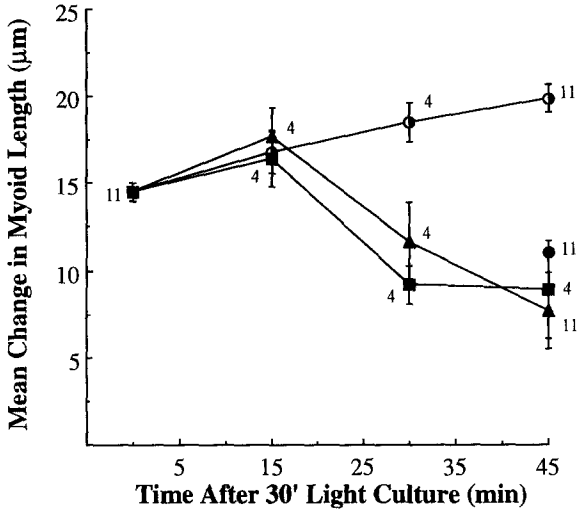

FIGURE 4. SpCAMPS and 8BrcGMP induce myoid shortening in RIS-ROS previously elongated in light culture. RIS-ROS were isolated from dark-adapted fish under infrared illumination, precultured in the dark for 15 min, and then transferred to the light for 30 min. At the end of the light culture period SpCAMPS (▲) and 8BrcGMP (■) were added to experimental cultures at a final concentration of 2 mM. All light-cultured samples, experimentals and controls (◑), were then transferred back to darkness and cultured for the times indicated. Dark controls (●) were cultured in control Ringer in the dark for 75 min. Data are expressed as mean ± SEM, with numbers of fish indicated for each point.

μm/min, a rate approximately one-third that observed for dark-induced rod shortening in vivo in another fish (cf. Burnside and Nagle, 1983).

The 8-bromo analogues were more effective than the dibutyryl analogues ± IBMX in triggering rod cell shortening (SpCAMPS = 8BrcGMP > 8BrcAMP > > dibutyryl analogues ± IBMX). This ranking contrasts with the order of effectiveness of these analogues in blocking light-induced RIS-ROS elongation (in that case the dibutyryl analogues ± IBMX were more effective than the 8-bromo analogues; see Table I; Fig. 3). These observations suggest that inhibition of light-induced elongation and activation of rod cell shortening may differ slightly in their intracellular signaling pathways. Alternatively, previously light-cultured RIS-ROS may metabolize cyclic nucleotides differently than RIS-ROS cultured only in the dark.

These observations show that cultured RIS-ROS can be induced either to elongate or shorten in culture by exposure to light or specific cyclic nucleotide analogues, respectively. The ability of cyclic nucleotide analogues to induce rod myoid shortening suggests that this shortening requires an elevation in intracellular levels of cyclic nucleotides and subsequent activation of cyclic nucleotide–dependent kinases and/or

FIGURE 5. (*opposite*) Phase micrographs of RIS-ROS elongation induced by H8 (see Fig. 6) and of RIS-ROS shortening induced by SpCAMPS (see Fig. 4) or okadaic acid (see Fig. 8). RIS-ROS elongation is shown in the upper panel. RIS-ROS were fixed immediately after isolation (*A*), or cultured in the dark for 60 min in control Ringer (i.e., 15 min preculture + 45 min culture; *B*) or in 100 μM H8 for the 15-min preculture and 45-min culture periods (*C*), Light controls were precultured in the dark for 15 min and then cultured for 45 min in the light in control Ringer (*D*). RIS-ROS shortening is shown in the lower panel. Before the application of either SpCAMPS or okadaic acid, RIS-ROS were isolated from dark-adapted fish under infrared illumination, precultured in the dark for 15 min, then transferred to the light for 30 min (*E*), and then cultured in the dark for 45 min in control Ringer (*F*), in 10 μM okadaic acid (*G*), or in 2 mM SpCAMPS (*H*). Scale bar = 10 μm.

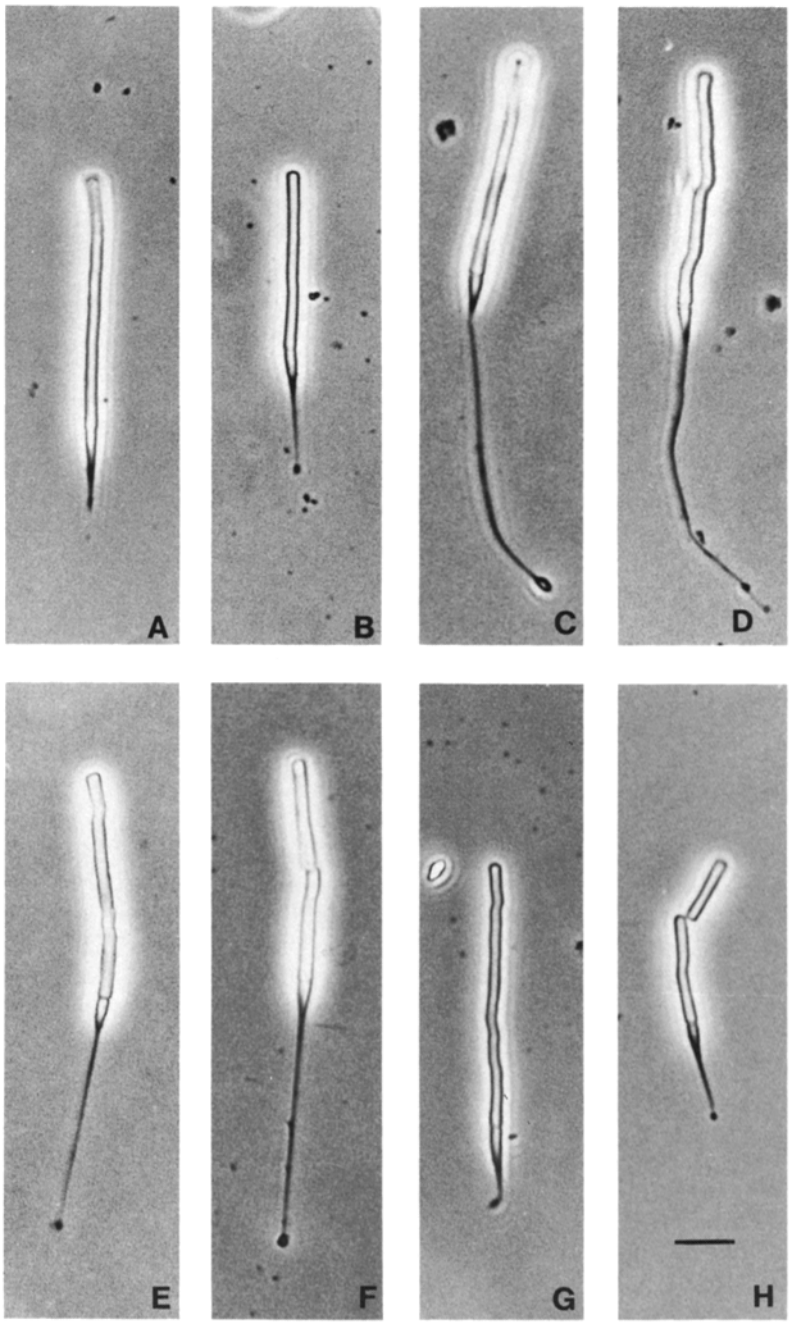

FIGURE 5.

inhibition of protein phosphatases to enhance the extent of phosphorylation of cyclic nucleotide–dependent phosphoproteins.

H8 and H7 Activate RIS-ROS Elongation in the Absence of Light

To test whether kinase activity is required for the maintenance of short RIS-ROS lengths we investigated whether kinase inhibition would promote RIS-ROS elongation in the absence of a light stimulus. In this study we used the membrane-permeant kinase inhibitors, H8 and H7 (Hidaka, Inagaki, Kawamoto, and Sasaki, 1984). In vitro, H8 is reported to strongly inhibit cyclic nucleotide–dependent kinases (K_i = 1.2 μM for cAMP-dependent kinase and 0.48 μM for cGMP-dependent kinase) and only weakly inhibit protein kinase C (PKC; K_i = 15 μM). H7 is often used as a relatively selective inhibitor of PKC; however, it is also reported to inhibit cAMP-dependent kinase (K_i = 3.0 μM) at least as effectively as PKC in vitro (K_i = 6.0 μM; Hidaka et al., 1984).

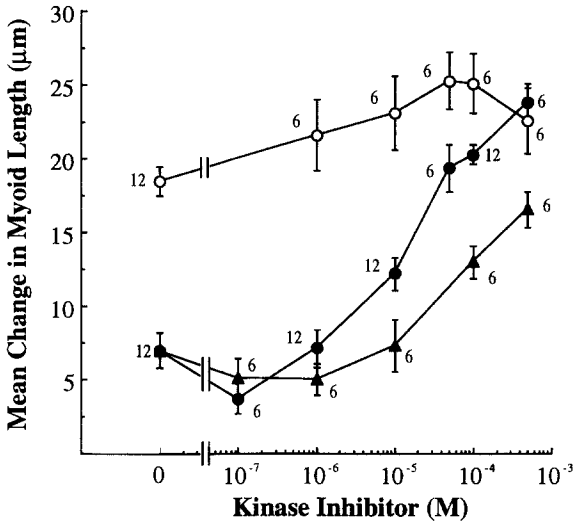

FIGURE 6. The kinase inhibitors H8 and H7 induce dose-dependent RIS-ROS elongation in the dark. RIS-ROS were isolated from dark-adapted fish under infrared illumination, precultured in the dark for 15 min with the indicated concentrations of the kinase inhibitors, and then cultured 45 min in the continual presence of H8 in light (○), H8 in the dark (●), or H7 in the dark (▲). Data are expressed as mean ± SEM, each point from six fish.

H8 and H7 induced elongation of RIS-ROS myoids in darkness (Fig. 5 *B*) in a dose-dependent manner (Fig. 6). The maximal elongation in the dark, evoked by ~100 μM H8, was equal to or greater than that produced by light in parallel cultures maintained in control Ringer (Fig. 6). Half-maximal activation of elongation, as observed under control conditions in the light, was mimicked by ~10 μM H8 and by 100 μM H7 (Fig. 6). At 100 μM, H8 also significantly enhanced the mean elongation produced by light ($P < 0.008$, N = 12; Student's t test; Figs. 6 and 7 *B*). Though higher than published K_i values for H8 based on in vitro studies, the H8 and H7 concentrations required for half-maximal stimulation of rod elongation compare favorably with other studies of nonpermeabilized cells. For example, half-maximal inhibition of cAMP-activated gene expression in cultured rat hepatocytes was achieved by H8 at a concentration of 40 μM (Buchler, Walter, Jastorff, and

Lohmann, 1988). The higher concentrations required to modulate cellular functions in intact cells are probably a result of incomplete cell penetration. Indeed, H8 uptake into intact cells was reported to have an apparent K_m of 38 μM (Hidaka et al., 1984).

The ability of both H8 and H7 to induce RIS-ROS elongation in the absence of light indicates that inhibition of kinases alone is sufficient to activate RIS-ROS elongation. The greater sensitivity of the elongation mechanisms to H8, relative to H7, suggests that inhibition of cyclic nucleotide–dependent kinases is more effective at influencing RIS-ROS motility than inhibition of PKC. These observations suggest further that continuous activity of cyclic nucleotide–dependent kinases is required for maintenance of short myoids in the dark, and that light activation of rod elongation entails a reduction and/or reversal of cyclic nucleotide–mediated phosphorylation events.

H8 Reverses RIS-ROS Shortening due to SpCAMPS and Antagonizes the Inhibitory Effects of 8BrcGMP and SpCAMPS on Light-induced RIS-ROS Elongation

Since the effectiveness of H8 in triggering RIS-ROS elongation suggested a role for kinases in regulating rod motility, we wished to examine more closely the role of kinases in mediating the abilities of cyclic nucleotide analogues to promote RIS-ROS shortening and to inhibit light-induced RIS-ROS elongation. We therefore tested whether H8 could block either the stimulatory effects of SpCAMPS on RIS-ROS shortening or the inhibitory effects of SpCAMPS and 8BrcGMP on light-induced RIS-ROS elongation.

To test whether H8 could inhibit SpCAMPS-induced RIS-ROS shortening, RIS-ROS were precultured in the light for 30 min to induce them to elongate, then cultured with SpCAMPS for 30 min in darkness to initiate SpCAMPS-induced shortening, and then cultured for another 30 min in the presence of both SpCAMPS and H8. H8 not only reversed SpCAMPS-induced RIS-ROS shortening but also induced the RIS-ROS to elongate to maximum lengths, comparable to light-cultured controls (Fig. 7 *A*). This observation suggests that SpCAMPS-induced shortening is mediated by kinases and also indicates that RIS-ROS can be induced to cycle through a round of elongation, shortening, and elongation again when sequentially exposed to light, SpCAMPS, and SpCAMPS plus H8.

In the presence of H8, RIS-ROS cultured with either 8BrcGMP or SpCAMPS elongated in the light, indicating that H8 antagonized the inhibitory effects of both a cAMP and a cGMP analogue on light-induced RIS-ROS elongation (Fig. 7 *B*). The extent of RIS-ROS elongation in the presence of either analogue with H8 was equal to or greater than the extent of elongation observed in cultures maintained in the light in control Ringer.

Together, these observations strongly suggest that the induction of RIS-ROS shortening by SpCAMPS and the inhibition of light-induced RIS-ROS elongation by 8BrcGMP and SpCAMPS are mediated by protein kinases. The reversibility of the SpCAMPS-induced RIS-ROS shortening by H8 indicates that the myoid shortening produced by SpCAMPS is a specific physiological response rather than merely myoid collapse as a result of general toxicity. Likewise, the ability of H8 to antagonize the inhibitory effects of 8BrcGMP and SpCAMPS on light-induced RIS-ROS elongation

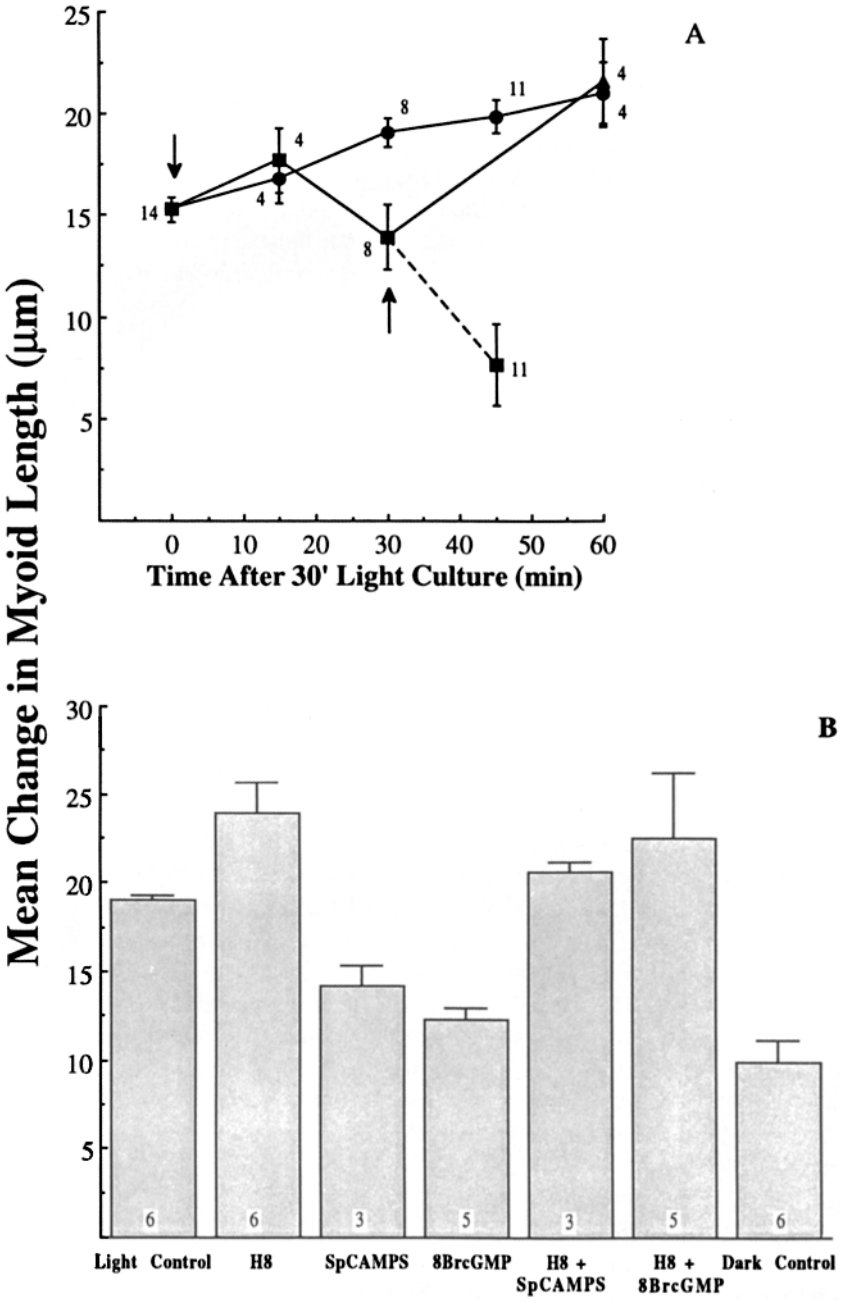
A
Mean Change in Myoid Length (μm)
Time After 30' Light Culture (min)
B
Light Control
H8
SpCAMPS
8BrcGMP
H8 + SpCAMPS
H8 + 8BrcGMP
Dark Control

FIGURE 7.

also indicates that the cyclic nucleotide–dependent inhibition of elongation is not a result of general toxicity.

The Phosphatase Inhibitor, Okadaic Acid, Inhibits Light-induced RIS-ROS Elongation and Induces RIS-ROS Shortening

The observation that H8 can activate RIS-ROS elongation in the dark suggests that kinase inhibition is sufficient to induce elongation and that light-induced elongation might entail a similar process. For example, light onset may lead to a decrease in the activity of cyclic nucleotide–dependent kinases and/or an increase in the dephosphorylation of mediator proteins. To test whether protein dephosphorylation is required for light-induced RIS-ROS elongation, we examined the ability of the membrane-permeable phosphatase inhibitor, okadaic acid, to inhibit this response (for review see Hardie, 1990). We found that okadaic acid inhibited not only light-induced RIS-ROS elongation but also the more modest elongation that occurs in dark culture (Fig. 8*A*). Inhibition was dose dependent with maximal effects at 1–5 μM. These observations suggest that protein dephosphorylation is required for light activation of RIS-ROS elongation and that dephosphorylation contributes to the modest elongation that occurs in dark-cultured RIS-ROS. In addition, we found that okadaic acid (at 5–10 μM) induced myoid shortening in RIS-ROS previously elongated by light culture (Fig. 8 *B*). This observation suggests that continuous phosphatase activity is required to promote and maintain elongated RIS-ROS myoids. These findings are consistent with those above for H8, suggesting that enhancement of the extent of cyclic nucleotide–dependent phosphorylation is correlated with inducing dark-adaptive rod shortening and with maintenance of short myoids in the dark.

Cyclic Nucleotide Treatments Do Not Disrupt the Actin Cytoskeleton

Since cAMP and cGMP have been shown in many cell types to influence cytoskeletal dynamics, we were concerned that artificial increases in cAMP and cGMP might inhibit light-induced RIS-ROS elongation by disrupting the actin cytoskeleton. In some cell types, cyclic nucleotide treatments have been shown to completely dismantle the actin cytoskeleton (Lamb, Fernandez, Conti, Adelstein, Glass, Welch, and

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FIGURE 7. (*opposite*) H8 reverses the SpCAMPS-induced shortening and antagonizes the inhibitory effects of SpCAMPS and 8BrcGMP on light-induced RIS-ROS elongation. (*A*) RIS-ROS were isolated from dark-adapted fish under infrared illumination, precultured in the dark for 15 min, and cultured in the light for 30 min to induce myoid elongation. At this time SpCAMPS (2 mM, ■) was added to the experimental cultures (*down arrow*). Samples were then transferred to darkness and cultured for the times indicated. After 30 min of the dark culture period, H8 (100 μM, ▲) was added to four of the cultures containing SpCAMPS (*up arrow*) and all samples were cultured in darkness as indicated. Control samples were cultured for 30 min in the light and then for varying times in the dark are designated by the filled circles. (*B*) RIS-ROS were isolated from dark-adapted fish under infrared illumination, precultured in the dark for 15 min with or without SpCAMPS (2 mM) or 8BrcGMP (2 mM) with or without H8 (100 μM), and then cultured in the light for 45 min. Dark controls were cultured in darkness in control Ringer for 60 min. Data are expressed as mean ± SEM, with numbers of fish indicated for each point.

Feramisco, 1988; Roger, Richaert, Huez, Authelet, Hofmann, and Damont, 1988; Goldman and Abramson, 1990). Using fluorescently labeled phalloidin, which binds to filamentous actin, we compared the actin cytoskeleton of RIS-ROS cultured in control Ringer with that of RIS-ROS cultured with cyclic nucleotide analogues and/or IBMX (Fig. 2). Previous studies have shown that in teleost rods and in RIS-ROS,

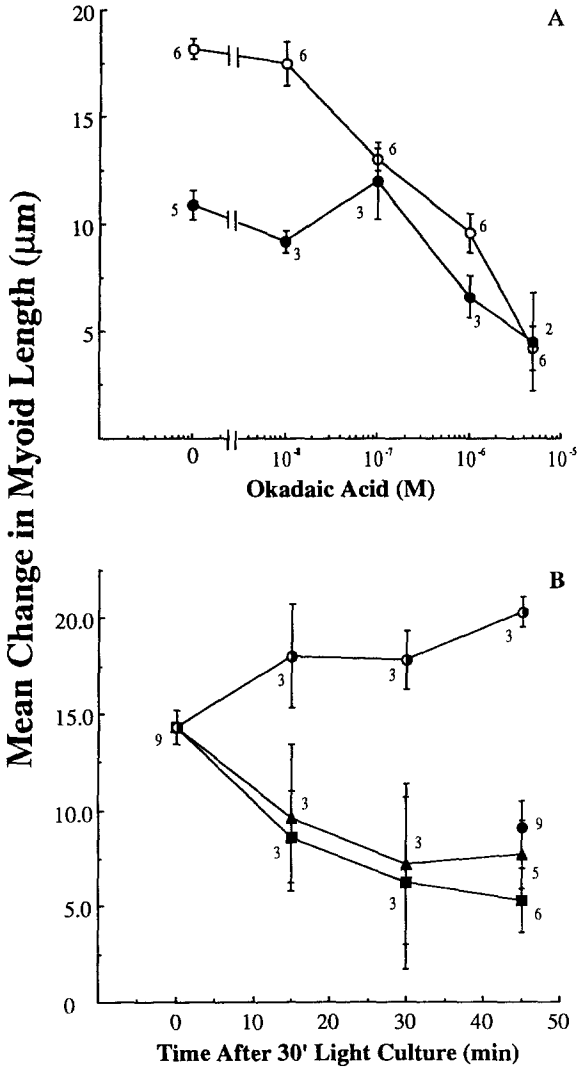

FIGURE 8. Effects of okadaic acid on RIS-ROS motility. (*A*) Okadaic acid inhibits light-induced RIS-ROS elongation in a dose-dependent manner. RIS-ROS were isolated from dark-adapted fish under infrared illumination, precultured in the dark for 15 min with or without okadaic acid, and then cultured 45 min in light (○) or dark (●) in the presence of the indicated concentrations of okadaic acid. (*B*) Okadaic acid induces myoid shortening in RIS-ROS previously elongated in light culture. RIS-ROS were isolated as described in *A*, precultured in the dark for 15 min, and then transferred to the light for 30 min. At the end of the light culture period, experimental cultures were adjusted to have 5 μM (▲) or 10 μM (■) okadaic acid. All light-cultured samples, experimentals and controls, were then transferred back to darkness and cultured for the times indicated. Dark controls (●) were cultured in control Ringer in the dark for 75 min. Light controls (◑) were cultured 30 min in the light and for the indicated times in darkness. Data are expressed as mean ± SEM, with numbers of fish indicated for each point.

parallel bundles of actin filaments form the core of calycal processes, continue proximally along the perimeter of the ellipsoid, and extend throughout the core of the myoid (O'Connor and Burnside, 1981; Pagh-Roehl et al., 1992). This characteristic pattern is maintained in RIS-ROS preparations treated with agents that artificially elevate cAMP and/or cGMP levels (Fig. 2). Furthermore, Pagh-Roehl et al.

(1992) have shown that in RIS-ROS there is an inverse relationship between calycal process length and myoid length. Dark-adapted RIS-ROS with short myoids have long calycal processes; in contrast, light-cultured RIS-ROS with long myoids have short calycal processes. This inverse relationship is also observed in RIS-ROS cultured under conditions expected to elevate cyclic nucleotide levels; measurements of myoids and calycal processes in control and cyclic nucleotide analogue treatments confirmed that this inverse relationship was retained in all treatments (data not shown). These results show that an artificial elevation of cAMP or cGMP does not inhibit elongation by the disruption of the actin cytoskeleton (at least at the level detectable by rhodamine-phalloidin staining), nor are the cyclic nucleotide treatments so disruptive as to eliminate the inverse relationship between myoid length and calycal process length found under control conditions.

DISCUSSION

All the observations presented in this paper suggest an important role for cyclic nucleotide–dependent protein phosphorylation and dephosphorylation in the regulation of rod motility. Our findings suggest that one or more mediator proteins are more highly phosphorylated in the dark than in the light, and that dephosphorylation is required for light-induced rod elongation. Observations supporting this conclusion include the inhibition of light-induced RIS-ROS elongation by cAMP and cGMP analogues, induction of RIS-ROS shortening by SpCAMPS and 8BrcGMP, induction of RIS-ROS elongation in the dark by the kinase inhibitors H8 and H7, and inhibition of light-induced RIS-ROS elongation and induction of RIS-ROS shortening by the phosphatase inhibitor, okadaic acid.

The observations that kinase inhibitors can induce elongation and that phosphatase inhibitors can induce shortening strongly suggest that both kinases and phosphatases are active at all times in the rod inner segment and that the extent of myoid elongation is determined by relative levels of protein phosphorylation in light and in darkness. The observation that kinase inhibitors can promote RIS-ROS elongation in the dark suggests that kinase activity and hence ongoing protein phosphorylation is required to maintain short myoids in the dark-adapted rod; this observation also implies that phosphatases are continuously active in the dark since kinase inactivation alone is sufficient to induce myoid elongation. Similarly, the observation that okadaic acid can promote myoid shortening suggests that phosphatase activity, hence ongoing protein dephosphorylation, is required to maintain long myoids in the light-adapted rod. In turn, this observation implies that kinases are also continuously active in the light, since blocking protein dephosphorylation alone is sufficient to induce RIS-ROS shortening. Furthermore, the observations that light-induced RIS-ROS elongation can be inhibited either by cyclic nucleotide analogues or by okadaic acid suggests that light, too, triggers rod myoid elongation by favoring protein dephosphorylation either by enhancing phosphatase activity, decreasing kinase activity, or both. Thus, phosphatases would be expected to be more active relative to kinases in the light with decreased levels of protein phosphorylation, while kinases would be expected to be more active relative to phosphatases in the dark with increased levels of protein phosphorylation. The enhancement of RIS-ROS elongation in the light by H8, as well as the block of RIS-ROS elongation in the dark by

okadaic acid, is consistent with the hypothesis that both kinases and phosphatases are active under all lighting conditions. Accordingly, during dark culture or after light exposure, RIS-ROS can be induced to elongate or shorten by inhibiting kinases or phosphatases, respectively.

The effects of kinase and phosphatase inhibitors suggest that cyclic nucleotide analogues influence RIS-ROS motility through subsequent protein phosphorylation and not through direct activation of cyclic nucleotide–dependent channels or other components of the phototransduction cascade. Strong support for this interpretation is provided by the ability of H8 to block both the inhibitory effects of cyclic nucleotide analogues on light-induced elongation and to reverse the effects of SpCAMPS on RIS-ROS shortening. Since kinase and phosphatase inhibitors affect motility in the absence of exogenous cyclic nucleotides, the ability of these inhibitors to modulate RIS-ROS motility suggests that the effects of the cyclic nucleotide treatments are unlikely to be mediated through activation of the light-sensitive channel and subsequent increases in cytosolic Ca^{2+} (cf. Ratto, Payne, Owen, and Tsien, 1988). The ability of the kinase inhibitors, H8 and H7, to induce RIS-ROS elongation in darkness indicates that elongation can occur in the absence of light absorption by rhodopsin and the subsequent biochemical cascade that triggers membrane hyperpolarization. Furthermore, we have recently found that the intensity thresholds and strength–duration characteristics of the light stimulus required to activate RIS-ROS elongation differ from those required to activate membrane hyperpolarization (Liepe and Burnside, 1993). Light activation of RIS-ROS elongation requires light intensities that are approximately six orders of magnitude higher than those reported to activate light-induced hyperpolarizing currents in frog RIS-ROS (Biernbaum and Bownds, 1985) and the photoreceptive mechanisms that mediate elongation integrate quanta over light pulse durations that are three to four orders of magnitude longer than the integration time reported for membrane hyperpolarization. These results suggest that the intracellular signals that govern motility diverge from those that modulate membrane hyperpolarization. However, from our observations we cannot rule out other indirect effects on intracellular levels of Ca^{2+} that might be mediated by cyclic nucleotide–dependent phosphorylation (for review see Rasmussen, Kelley, and Douglas, 1990; Rasmussen and Rasmussen, 1990).

Observations from other studies on vertebrate rods are consistent with a role for cyclic nucleotide–dependent phosphorylation in rod motility. Evidence from several labs strongly suggests that cAMP-dependent kinase (PKA) is present in rod outer segments (ROS) and in ROS with adjoining ellipsoids (Binder, Brewer, and Bownds, 1989; Hamm, 1990; Lee, Brown, and Lolley, 1990). Although the presence of cGMP-dependent kinase (PKG) in ROS is more controversial, one report, based on photoaffinity labeling and molecular weight analysis, suggests the presence of PKG in ROS (Thompson and Khorana, 1990). There is also evidence for PKA-dependent phosphorylation of specific rod proteins in darkness: in frog ROS, the low molecular weight proteins, components I and II, and in bovine ROS, the 33-kD protein, phosducin (Hamm, 1990; Lee et al., 1990). Consistent with a regulatory role for phosphatases in the modulation of rod motility, a type 2A phosphatase has been detected in ROS (Palczewski, McDowell, Jakes, Ingebritsen, and Hargrave, 1989). This phosphatase is inhibited by okadaic acid and has been reported to play a role in

the dephosphorylation of rhodopsin after light stimulation has promoted the phosphorylation of the photopigment (Palczewski et al., 1989). Also consistent with the apparent requirement for light-induced protein dephosphorylation, both components I and II (Polans, Hermolin, and Bownds, 1979; Bownds and Brewer, 1986; Hamm, 1990) and phosducin (Lee, Brown, and Lolley, 1984; Lee et al., 1990) dephosphorylate upon light stimulation. Finally, in vertebrate rods, both cAMP and cGMP levels are reported to decrease during illumination (Orr et al., 1976; Ferrendelli et al., 1980). Illumination triggers decreases in cGMP throughout the entire rod, and decreases in cAMP levels in the nuclear and axonal regions of the photoreceptor (Orr et al., 1976).

Several observations, including those detecting the presence of PKA in rod photoreceptors, suggest that PKA is a major player in the regulation of rod motility, rather than PKC or PKG. Other studies in our lab have shown that stimulation of PKC with phorbol esters or 1-oleoyl-2-acetyl-glycerol does not inhibit light-induced RIS-ROS elongation at the light levels used in these studies (Hammond, E., K. Pagh-Roehl, and B. Burnside, unpublished observations). Furthermore, recent observations have shown that illumination of vertebrate rods activates phospholipase C, which generates diacylglycerol, leading to the subsequent activation of PKC (Ghalayini and Anderson, 1984; Das, Yoshioka, Samuelson, and Shichi, 1986; Choe, Ghalayini, and Anderson, 1990). Therefore, since an activation of PKC is correlated with light onset, it is unlikely to be the kinase that is inhibited in the light activation of rod elongation. Although H8 has been repeatedly reported to inhibit biological functions mediated through activation of PKA (Buchler et al., 1988; Taouis, Sheldon, Hill, and Duff, 1991), it has not yet been reported that H8 inhibits cellular activities mediated through PKG in vivo or in isolated cell systems. Furthermore, whereas PKA activity in ROS has been directly demonstrated, PKG activity has not been revealed (Binder et al., 1989; Hamm, 1990; Lee et al., 1990). However, in this report we have not rigorously tested the influence on RIS-ROS motility of PKC- or Ca^{2+} calmodulin-induced phosphorylation events. Therefore, we cannot completely rule out the possibility that PKC or Ca^{2+}-calmodulin activation and/or inhibition might also play a role in the regulation of RIS-ROS motility.

From our observations it is not possible to ascertain whether cAMP, cGMP, or both cyclic nucleotides drive PKA activity in the teleost rod inner segment. cGMP has been reported to activate PKA, though at higher concentrations than cAMP (cf. Walter and Greengard, 1981). In vertebrate rods, cGMP levels may be sufficiently high to activate PKA, as has been previously suggested (Farber, Brown, and Lolley, 1979; Hamm, 1990). In ROS, cGMP levels are ~10-fold higher than cAMP (Fletcher and Chader, 1976). The subcellular location of both cAMP and cGMP binding sites are correlated with cellular compartments that appear to participate in rod motility (Caretta and Saibil, 1989). cAMP binding sites are found within the rod inner segment; although cGMP binding sites are located primarily at the plasma membrane in the outer segment, binding sites to cGMP have also been detected in the proximal outer segment at positions correlated with the calycal processes (Caretta and Saibil, 1989).

In this study we have used RIS-ROS as a tool for investigating the intracellular mechanisms regulating rod motility. Though RIS-ROS motility mimics that of rods in vivo in many attributes, there are some differences that should be noted. First, in

vivo, rods remain short in darkness, while isolated RIS-ROS elongate slowly in dark culture. Thus dark-adapted RIS-ROS appear to have been altered in some way that tends to favor light-adapted retinomotor movement, though light can still enhance elongation dramatically. Second, darkness induces rod shortening in vivo but fails to induce shortening in RIS-ROS. Both this observation and the tendency of RIS-ROS to elongate in the dark may reflect the requirement for some signal from the retina to maintain rods in their fully dark-adaptive retinomotor positions. Third, the effects of cyclic nucleotides on RIS-ROS shortening reported here differ in some details from those previously reported for rod shortening in the isolated intact retina. All membrane-permeable cAMP analogues, but not cGMP analogues, promoted rod shortening in the isolated *Tilapia* retina (O'Connor and Burnside, 1982). In contrast, SpCAMPS and 8BrcGMP were effective in promoting shortening in RIS-ROS, but the other cyclic nucleotide analogues had no effect. SpCAMPS and 8BrcGMP may have been more effective than other analogues in RIS-ROS because they are more resistant to hydrolysis by endogenous phosphodiesterase and more effective in stimulating kinase activity (Braumann, Erneux, Petridis, Stohner, and Jastorff, 1986; for review see Meyer and Miller, 1974; Richter-Landsberg and Jastorff, 1985). The mechanisms allowing cGMP analogues to modulate RIS-ROS motility, but not rod motility, may be due to species differences in cyclic nucleotide specificity requirements in the regulation of motility, since these studies were performed on different fish species.

The tendency of RIS-ROS to elongate in darkness, the failure of darkness to induce shortening in light precultured RIS-ROS, and the requirement for the highly phosphodiesterase-insensitive analogue SpCAMPS to induce RIS-ROS shortening are all consistent with the possibility that endogenous phosphodiesterase activity is increased in RIS-ROS as compared with rods in situ, thus shifting RIS-ROS slightly toward light-adaptive regulatory dynamics. This suggestion is also consistent with the observation that IBMX in the presence of either dbcAMP or dbcGMP can abolish the tendency of RIS-ROS to elongate in the dark (Liepe, B., and B. Burnside, unpublished observations). Indeed, in the isolated rabbit retina, basal phosphodiesterase activity has been shown to decrease cyclic nucleotide levels during dark culture (Goldberg, Ames, Gander, and Walseth, 1983). Whether phosphodiesterase activity is in fact enhanced in RIS-ROS cannot be determined from our studies, nor can we identify possible mechanisms for producing the enhancement if it indeed occurs. However, since RIS-ROS still exhibit light-induced elongation, and since we can produce either elongation or shortening in RIS-ROS by pharmacological manipulation of protein phosphorylation dynamics, we feel confident that the regulatory mechanisms elucidated in our RIS-ROS studies do play important roles in rod motility in vivo.

The observations presented in this report are consistent with a regulatory model in which rod motility is dictated by the degree of phosphorylation of a cytoskeletal mediator protein or proteins. In this model, protein phosphorylation is modulated by the relative activities of both PKA and phosphatases, such that the extent of phosphorylation of the mediator protein(s) is higher in the dark than in the light. Our results suggest that both activation of rod shortening and maintenance of short myoids are correlated with increased levels of cyclic nucleotide–dependent phosphorylation in rod mediator proteins. In contrast, both light activation of rod elongation

and maintenance of long myoids requires decreased levels of cyclic nucleotide–dependent phosphorylation. This postulated dephosphorylation of cytoskeletal mediator proteins with light onset may be a consequence of a light-induced decrease in cyclic nucleotides, light-induced inhibition of PKA, and/or light-induced activation of phosphatases. Ongoing investigations into the effects of light on RIS-ROS kinase and phosphatase activities and identification of cyclic nucleotide–regulated RIS-ROS phosphoproteins should increase our understanding of rod motility.

We thank Dr. Kathy Pagh-Roehl, Dana García, Homero Rey, Ernest Han, and Dr. Charlene Stone for critical feedback on the manuscript. We are indebted to Ronald Yeh and Eunice Wang for technical assistance.

Original version received 3 April 1992 and accepted version received 25 March 1993.

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