Effects of Extracellular Ca^{++}, K^+, and Na^+ on Cone and Retinal Pigment Epithelium Retinomotor Movements in Isolated Teleost Retinas

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ABSTRACT We have examined the effects of changes in extracellular ionic composition on cone and retinal pigment epithelium (RPE) retinomotor movements in cultured isolated teleost retinas. In vivo, the myoid portion of teleost cones contracts in the light and elongates in the dark; RPE pigment disperses in the light and aggregates in the dark. In vitro, cones of dark-adapted (DA) retinas cultured in constant darkness contracted spontaneously to their light-adapted (LA) positions if the culture medium contained $\geq 10^{-3}$ M Ca_o^{++}. DA cones retained their long DA positions in a medium containing $\leq 10^{-6}$ M Ca_o^{++}. Low $[Ca^{++}]_o$ (10^{-5}–10^{-7} M) also permitted darkness to induce cone elongation and RPE pigment aggregation. Light produced cone contraction even in the absence of Ca_o^{++}, but the extent of contraction was reduced if $[Ca^{++}]_o$ was $<10^{-3}$ M. Thus, full contraction appeared to require the presence of external Ca^{++}. High $[K^+]_o$ (≥ 27 mM) inhibited both light-induced and light-independent Ca^{++}-induced cone contraction. However, low $[Na^+]_o$ (3.5 mM) in the presence of $\leq 10^{-6}$ M Ca_o^{++} did not mimic light onset by inducing cone contraction in the dark. High $[K^+]_o$ also promoted dark-adaptive cone and RPE movements in LA retinas cultured in the light. All results obtained in high $[K^+]_o$ were similar to those observed when DA or LA retinas were exposed to treatments that elevate cytoplasmic cyclic 3′,5′-adenosine monophosphate (cAMP) content.

INTRODUCTION

Teleost retinas respond to variations in light intensity by elongation and contraction of the myoid region of photoreceptor inner segments and by rearranging the distribution of pigment granules within retinal pigment epithelium (RPE) (Walls, 1942; Burnside and Nagle, 1983). In darkness, rod myoids are short, cone myoids are long, and pigment granules are aggregated toward the basal end of RPE cells; in light, rods elongate, cones contract, and RPE pigment disperses (Fig. 1). Regulation of these retinomotor movements is thought to involve both cAMP and calcium since treatments that elevate cAMP induce dark-

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adaptive movements (Burnside et al., 1982*a*; Burnside and Basinger, 1983) and Ca^{++} is required for contraction in detergent-lysed cone models (Burnside et al., 1982*b*).

We decided to examine the effects of changing extracellular ionic composition on retinomotor position more closely when we discovered that a low level of free extracellular calcium ($[Ca^{++}]_o$) was required for dark-adapted (DA) cones to maintain their normal dark position (i.e., long myoids) during culture of isolated retinas in constant darkness. In previous experiments, culturing isolated fish retinas in constant dark in a normal Ringer solution containing 1.8 mM Ca^{++} induced cones to contract spontaneously to the short light-adapted (LA) state (Burnside and Basinger, 1983). Thus, it seemed possible that low $[Ca^{++}]_o$ preserved some condition necessary for maintenance of long cone myoids in DA isolated retinas in vitro. We therefore undertook more detailed dose-response studies with a range of EGTA-buffered Ca_o^{++} levels to further clarify the effects of external Ca^{++} concentration on retinomotor position in dark, light, and dark/light transitions. The effects of calcium ionophore A23187 and agents that reportedly influence Ca^{++} fluxes were also examined.

In addition, we undertook experiments designed to study the influence of changes in extracellular $[K^+]$ and $[Na^+]$ upon retinomotor movements. Since cones are known to hyperpolarize in response to light (Baylor and Fuortes, 1970), it seemed possible that changes in membrane potential might play a role in the regulation of these movements by light. To investigate this theory, we sought to alter cone membrane potential experimentally by changing $[K^+]_o$ or $[Na^+]_o$. High $[K^+]_o$ has been reported to depolarize rods and cones in the dark and prevent light-induced hyperpolarization (Cervetto, 1973; Capovilla et al., 1980); low $[Na^+]_o$, on the other hand, has been reported to hyperpolarize rods and cones in the dark (Cervetto, 1973; Capovilla et al., 1981). We found that high $[K^+]_o$ blocked light-induced cone contraction, but low $[Na^+]_o$ in the presence of low $[Ca^{++}]_o$ failed to mimic light onset by inducing cone contraction in the dark.

Both low $[Ca^{++}]_o$ and high $[K^+]_o$ favored DA retinomotor positions. Since low $[Ca^{++}]_o$ activates adenylate cyclase activity in rabbit photoreceptors (DeVries et al., 1982) and high $[K^+]_o$ enhances cAMP content in a variety of retinas (Wassenaar and Korf, 1976; Watling et al., 1980; Cohen, 1982), it seemed possible that altering extracellular Ca^{++} and K^+ levels might be affecting retinomotor movements by influencing intracellular cAMP concentration. Previous work in this laboratory (Burnside et al., 1982*a*) has shown that treatments that elevate intracellular cAMP levels elicit dark-adaptive movements. Therefore, we compared the effects of changes in $[Ca^{++}]_o$ and $[K^+]_o$ to the effects of 3-isobutyl-1-methylxanthine (IBMX) and/or dibutyryl-cAMP (dbcAMP) addition. We found that low $[Ca^{++}]_o$, high $[K^+]_o$, and agents that elevate cytoplasmic [cAMP] all produced similar results, i.e., they favored DA retinomotor positions.

MATERIALS AND METHODS

Preparation and Procedure

Green sunfish, *Lepomis cyanellus*, were entrained to a 12-h light/12-h dark cycle for at least 3 wk before use. All experiments were performed at the same time in this cycle, viz., at

light-to-dark transition, so that the influence of circadian rhythm on retinomotor position (Levinson and Burnside, 1981) would be equivalent for all experiments. For dark adaptation, fish were placed in complete darkness for 1–2 h before an experiment. This period of time was found to produce cone myoid lengths in the range of 35–60 μm. All manipulations on DA fish and retinas were conducted under infrared illumination of wavelength >880 nm (Wratten filter 87A; Eastman Kodak Co., Rochester, NY) with the aid of an image converter (FJW Industries, Mt. Prospect, IL).

Fish were killed, their eyes were enucleated and hemisected, and the retinas (with attached RPE in the case of LA fish) were removed by gently pipetting a stream of $<10^{-8}$ M Ca^{++} Ringer (1 mM EGTA with no Ca^{++} addition) between retina and choroid. After severance of the optic nerve, isolated retinas with or without RPE were bisected along the choroid fissure, thus creating four similar preparations from each fish.

Cultures were carried out by modification of the procedure described in Burnside and Basinger (1983). Each retinal section was placed vitreous down in a well of a Falcon multiwell dish (Falcon Labware, Oxnard, CA) containing 0.5 ml medium at a given free [Ca^{++}]. Then 0.3 ml medium was removed from the well to ensure favorable gaseous exchange. The dish was placed in an enclosed chamber (either clear or opaque, depending on the desired light condition) gassed with 95% O_2/5% CO_2. The chamber was situated on a rotating platform, and retinas were incubated for the specified time (30–60 min) at 22°C in either total darkness or under a white light providing an intensity of 200 fc (model 214 light meter; General Electric, Cleveland, OH), which is the equivalent of 8.85×10^{14} photons/s·cm^2 at 546 nm. Following culture, retinal halves were fixed in 6% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0.

Materials and Solutions

The basic Ringer solution used in the experiments described here was a modified Earle's buffered salt solution containing (all concentrations are mM): 116.4 NaCl, 5.4 KCl, 1.0 NaH_2PO_4, 25.5 glucose, 24.0 $NaHCO_3$, 3.0 HEPES, 1.0 ascorbic acid, and 1.0 EGTA. $MgSO_4$ and $CaCl_2$ were added to obtain 1 mM free [Mg^{++}] and a specified free [Ca^{++}] according to Steinhardt et al. (1977). Because of the variability in reported values of association constants of EGTA and divalent cations, the concentrations of EGTA, $MgSO_4$, and $CaCl_2$ added to the medium to yield the desired reagent levels are shown in Table I. Although the Ca^{++}-buffering effectiveness of 1 mM EGTA is reduced at levels of free [Ca^{++}] $\geq 10^{-5}$ M, EGTA was included in all media so that results could be directly compared. Medium was freshly prepared before each experiment from a 10× stock solution (Gibco Laboratories, Grand Island, NY) containing the requisite amounts of NaCl, KCl, and NaH_2PO_4, as well as 5.5 mM glucose upon dilution. Other Ringer components were added to this. EGTA was added from a 0.1-M stock solution adjusted to pH 7.40 with NaOH. The final solution was equilibrated with 95% O_2/5% CO_2 and pH was adjusted to 7.40.

Calcium ionophore A23187 (Calbiochem-Behring Corp., San Diego, CA) solutions were prepared immediately before use from a concentrated (19.1 mM) stock of A23187 in 100% dimethylsulfoxide (DMSO). Light exposure of ionophore was minimized. The ability of our A23187 solution to enhance membrane permeability to Ca^{++} was tested by application to a suspension of sea urchin (*Lytechinus pictus*) eggs in artificial seawater. At a final concentration of 30 μM A23187 in 1% DMSO, fertilization membranes were observed to elevate within 30 s, a reaction induced by increased $[Ca^{++}]_i$ (Steinhardt and Epel, 1974). In experiments with DA retinas, ionophore addition was carried out under infrared illumination by pipetting 2 μl of a 3-mM solution in 100% DMSO into the culture dish well containing the retina in 200 μl of a given [Ca^{++}] Ringer solution. Thus, the final

concentration of A23187 was 30 μM and that of DMSO was 1% (vol/vol) for experimental retinas; control retinas were exposed to 1% DMSO alone delivered in the same manner.

In experiments with high K^+ medium, additional KCl was added directly to the above Ringer or as an equimolar replacement for NaCl. In experiments with low Na^+ Ringer, the chloride and bicarbonate salts of choline were substituted on an equimolar basis for NaCl and $NaHCO_3$.

The calcium antagonists verapamil, dantrolene sodium, and cinnarizine were gifts of Jean Ehinger at Knoll Pharmaceutical Co., (Whippany, NJ). Frank Ebetino at Norwich-Eaton Pharmaceuticals (Norwich, NY), and Howard Grimwood at Janssen Pharmaceutica (Piscataway, NJ), respectively. Each of these compounds was dissolved directly in the appropriate Ringer solution to produce the desired concentration and was included in both the dissecting and culture media of experimental retinas. Control retinas were exposed to normal Ringer.

TABLE I

Divalent Cation Buffer Composition of Ringer Solution

[EGTA]	Added [$MgSO_4$]	Added [$CaCl_2$]	Calculated free [Mg^{++}]	Calculated free [Ca^{++}]
mM	*mM*	*mM*	*mM*	*M*
1.0	1.08	—	1.0	$<10^{-8}$
1.0	1.07	0.13	1.0	10^{-8}
1.0	1.03	0.60	1.0	10^{-7}
1.0	1.01	0.94	1.0	10^{-6}
1.0	1.00	1.00	1.0	10^{-5}
1.0	1.00	1.10	1.0	10^{-4}
1.0	1.00	2.00	1.0	10^{-3}

Calculated free Mg^{++} and Ca^{++} concentrations are based on Steinhardt et al. (1977), assuming an association constant for EGTA and Ca^{++} of $10^{10.7}$; pH = 7.40.

Quantitation of Retinomotor Position

After fixation, retinomotor positions of cones and RPE were quantified as previously described (Burnside et al., 1982*a*; Burnside and Basinger, 1983). Morphological measurements were made on ~20-μm-thick slices of each retina using a light microscope equipped with an ocular micrometer and Nomarski interference contrast optics. Since rod positions were not reliably discernible by this technique, only cone and RPE retinomotor positions were examined. Cone myoid length, the distance between the outer limiting membrane (OLM) and the base of a cone ellipsoid, was determined for 20 representative cells from each half-retina. 10 measurements of pigment dispersion in RPE were made for each retinal piece. This value, termed the pigment index, is the quotient resulting from dividing the distance between Bruch's membrane and the most vitreal extent of RPE by the distance from Bruch's membrane to the OLM (see Fig. 1). Data are presented as mean ± standard error of the mean, where *n* refers to the number of retinal halves examined. The significance of the difference between control and experimental group means was ascertained by a two-tailed Student's *t* test.

RESULTS

Effects of Changing $[Ca^{++}]_o$ on Dark-adapted Retinas Cultured in Darkness

Dose-response studies were conducted by culturing isolated DA retinas in constant darkness in EGTA-buffered media containing differing free calcium con-

centrations. Fig. 2*a–d* shows light micrographs of such DA retinas after a 30-min culture in the dark. At free external calcium levels of $\leq 10^{-6}$ M, retinal cones of DA fish remained in their normal DA positions during dark culture (Figs. 2*a–c* and 3). However, in $[Ca^{++}]_o$ of 10^{-3} M, full cone myoid contraction occurred even in the absence of light (Figs. 2*d* and 3). Similar results in 10^{-3} M Ca_o^{++} were reported by Burnside and Basinger (1983). Extracellular Ca^{++} concentrations of 10^{-4}–10^{-5} M resulted in intermediate extents of contraction (Fig. 3).

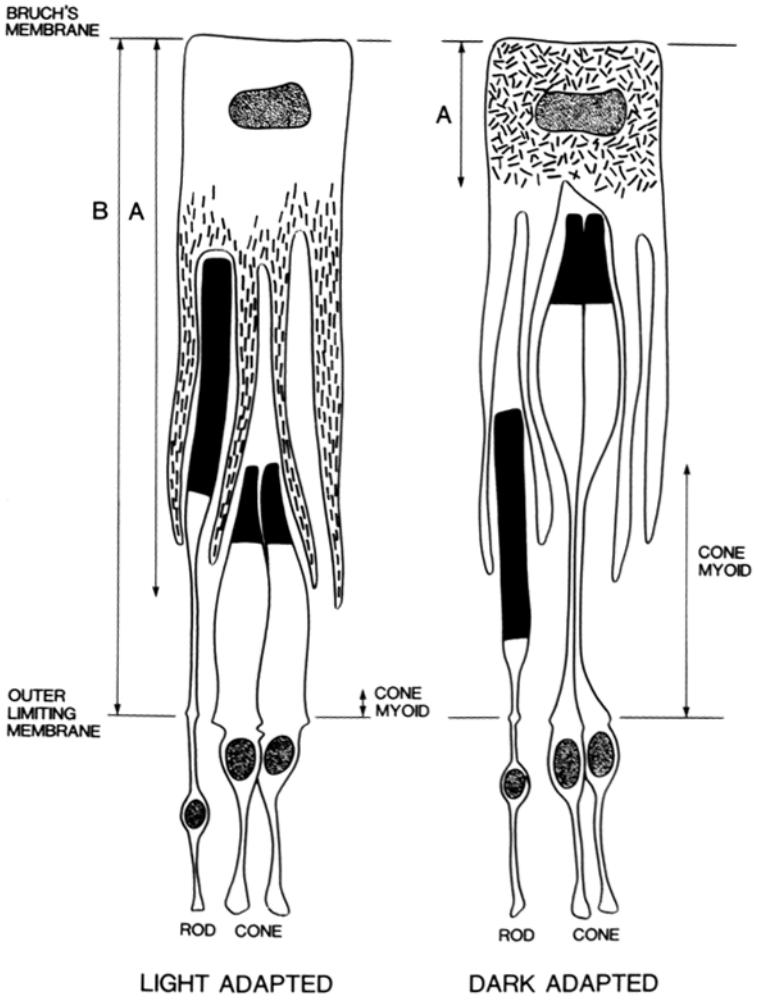
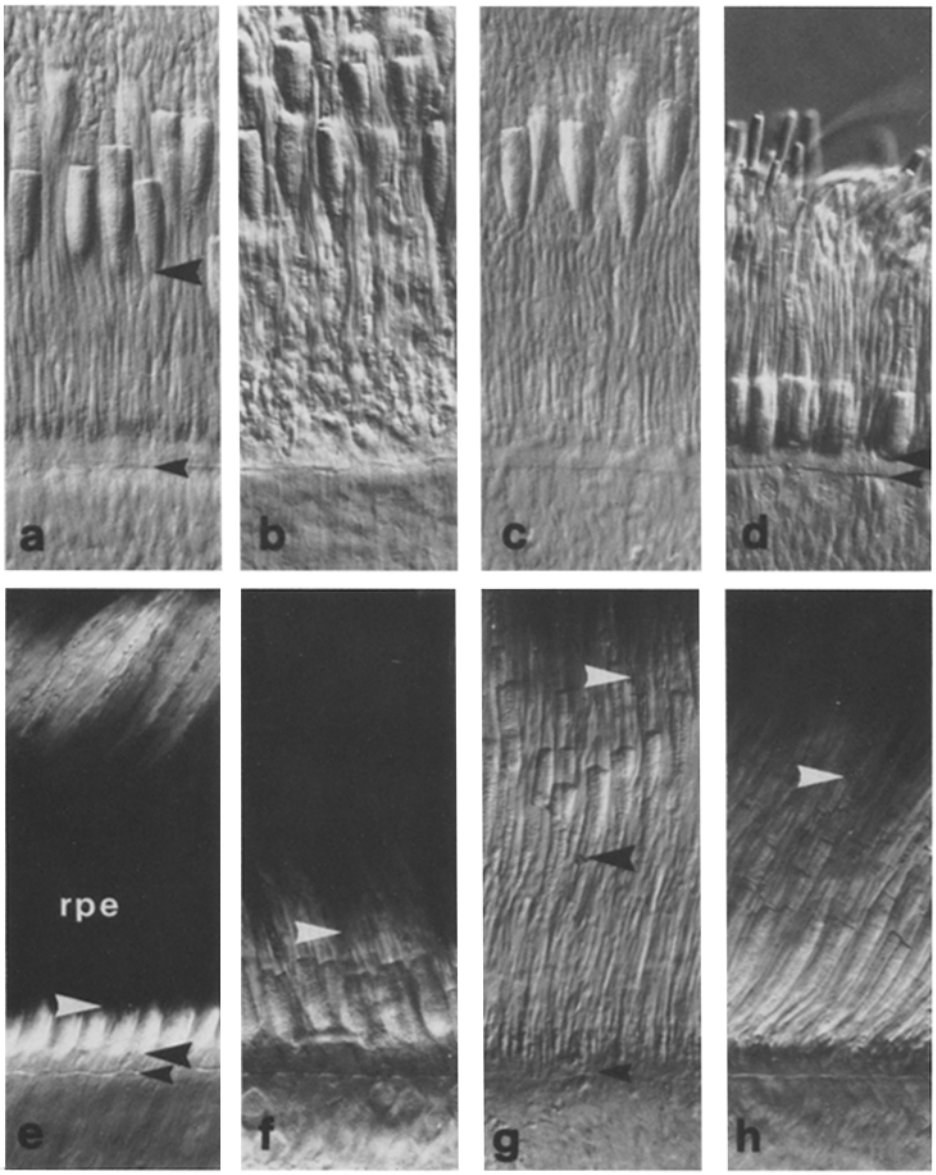


FIGURE 1. Schematic diagram of rod, cone, and RPE retinomotor positions in dark- and light-adapted retinas. Cone myoids are long in the dark and short in the light. Pigment index, calculated as distance *A* divided by distance *B*, is small in the dark when pigment is aggregated and large in the light when pigment is dispersed.

Ca^{++} Ionophore

We knew from other work performed in this lab on detergent-lysed cell models that cone contraction could be activated by elevating the free Ca^{++} level bathing the contractile apparatus to $\geq 10^{-7}$ M (Porrello and Burnside, 1982). Therefore, we tested the effect of calcium ionophore A23187 on intact retinas cultured in darkness with various concentrations of free Ca^{++} in the Ringer solution to see if ionophore would shift the $[Ca^{++}]_o$ dose-response curve toward that observed



in lysed-cell models. Addition of 30 μM A23187 in 1% DMSO to the incubation media during dark culture resulted in cone contraction at $[Ca^{++}]_o$ approximately two orders of magnitude lower than in the absence of ionophore (Fig. 3). In 1% DMSO alone, cone positions did not differ from those found in normal Ringer at a given $[Ca^{++}]_o$. Thus, the ionophore dose-response curve was similar to that seen in both detergent-lysed cell models of teleost cones (Porrello and Burnside, 1984) and detergent-lysed smooth muscle (Gordon, 1978). This finding implies that changing $[Ca^{++}]_o$ influences cone retinomotor position at least in part by altering $[Ca^{++}]_i$.

We were surprised that 10^{-3} M extracellular Ca^{++} induced light-adaptive cone movements in vitro in the absence of light since such levels are generally assumed to be present in vivo and are employed in most physiological studies of isolated retinas. An immediate concern was the possibility that cone plasma membranes were leaky under our culture conditions. However, our dose-response curves with and without ionophore indicate that under our culture conditions, cones successfully maintained a calcium gradient across their plasma membranes. We further demonstrated photoreceptor plasma membrane integrity by incubating whole isolated retinas in 50 μM didansylcysteine (DDC), a fluorescent compound used by Yoshikami et al. (1974) to assess the relative permeability of cell membranes in isolated retinas. They found that in a preparation with intact plasma membranes, only cone outer segments were fluorescent; rod outer segments and all photoreceptor inner segments did not absorb sufficient dye to fluoresce. In our preparations, addition of DDC to isolated DA retinas immediately after dissection or after 30 min of dark or light culture in 10^{-3} M Ca^{++}-Ringer resulted in fluorescence being observed only in cone outer segments; no fluorescence was visible in rod outer segments or any photoreceptor inner segments. However, when isolated retinas were exposed to a hypotonic medium, all photoreceptor inner and outer segments became fluorescent. Thus, the integrity of cone inner segment membranes was retained unless the cells were subjected to osmotic shock. Taken together, our A23187 and DDC experiments

FIGURE 2. (*opposite*) Light micrographs of 20-μm-thick retinal slices obtained with Nomarski interference contrast optics. The outer limiting membrane and the base of a cone ellipsoid are indicated by small and large black arrows, respectively. Cone myoid length corresponds to the distance between these arrows. The apical most pigment granules of the RPE are indicated by white arrows. 500×. (*a–d*) Retinas without RPE were removed from DA fish and fixed immediately after dissection (*a*) or after a 30-min culture in the dark with $[Ca^{++}]_o = 10^{-8}$ (*b*), 10^{-6} (*c*), or 10^{-3} M (*d*). Extracellular $[Ca^{++}] \leq 10^{-6}$ M maintained long DA cone myoids; in 10^{-3} M Ca_o^{++}, cones contracted to their short LA positions. (*e–h*) Retinas with attached RPE were removed from LA fish and fixed immediately after dissection (*e*) or after a 60-min culture in the dark with $[Ca^{++}]_o = 10^{-8}$ (*f*), 10^{-6} (*g*), or 10^{-4} M (*h*). Cultures of LA retinas differed from those of DA retinas in that RPE remained attached to LA retinas and LA RPE-retinas were cultured for a longer time since cone elongation occurs at a slower rate than contraction (Burnside et al., 1982*b*). Dark-adaptive retinomotor movements (cone elongation and RPE pigment aggregation) were maximal at $[Ca^{++}]_o = 10^{-6}$ M.

suggest that cone inner segment plasma membranes remained intact and capable of maintaining a Ca^{++} gradient under our culture conditions. However, we cannot at this time exclude the possibility that Ca^{++} extrusion was partially compromised.

Effects of Changing $[Ca^{++}]_o$ on DA Retinas Cultured in Light

The extent of cone contraction was also analyzed after culture of DA retinas in varying $[Ca^{++}]_o$ in the light. We found that, after 30 min, cones contracted to

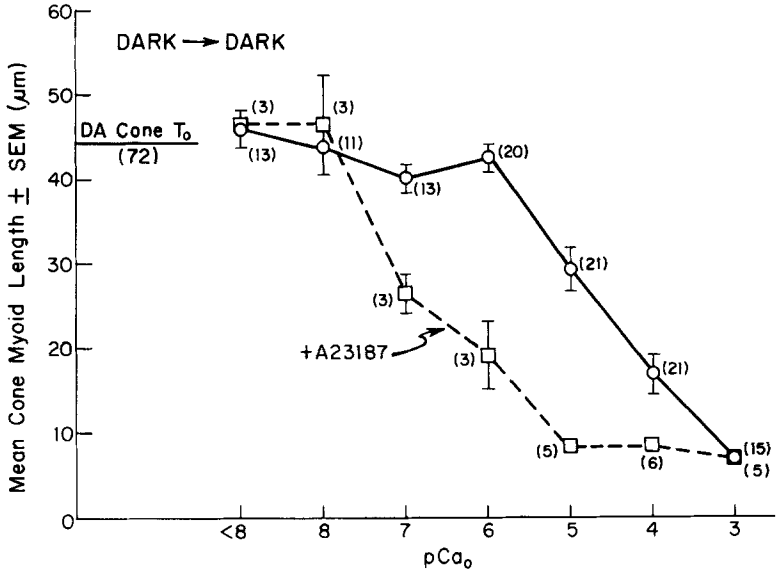


FIGURE 3. Effect of extracellular Ca^{++} concentration on cone myoid length during culture of isolated DA retinas in constant dark. Retinas were removed from DA fish under infrared illumination and cultured in a bicarbonate/CO_2-buffered medium, pH 7.40, containing 1 mM EGTA and a specified free Ca^{++} concentration for 30 min in total darkness (circles connected by solid line). Some retinas were cultured in the presence of 30 μM Ca^{++} ionophore A23187 in 1% DMSO (squares connected by dotted line). In 1% DMSO alone, cone position did not differ from that found in normal Ringer at a given $[Ca^{++}]_o$. Without ionophore, cones remained in their DA positions at $[Ca^{++}]_o \leq 10^{-6}$ M; at 10^{-3} M Ca_o^{++}, cones contracted to their LA positions. When A23187 was added, cones contracted maximally at $[Ca^{++}]_o \geq 10^{-5}$ M.

their fully light-adapted positions only if 10^{-3} M Ca^{++} was present in the medium (Fig. 4). At lower concentrations of Ca_o^{++}, the extent of contraction was limited. Even in the absence of extracellular Ca^{++} ($<10^{-8}$ M), cones contracted to 49% of the maximal extent observed in 10^{-3} M Ca_o^{++}.

We were concerned that exposure to a low $[Ca^{++}]_o$ solution inhibited light-induced cone contraction by compromising the contractile machinery or some other essential cellular process. To examine this possibility, we preincubated DA isolated retinas in either $<10^{-8}$ or 10^{-6} M Ca_o^{++} and then transferred them to

10^{-3} M Ca_o^{++} to determine if cones could still contract normally. After 15 min of exposure to a solution having $<10^{-8}$ M Ca_o^{++}, cone contraction was diminished when retinas were subsequently exposed to 10^{-3} M Ca_o^{++} in either dark or light (Table II). This finding implies that prolonged absence of Ca^{++} from the external medium ($<10^{-8}$ M Ca_o^{++} in 1 mM EGTA) is harmful to some element(s) necessary for cone contraction, and that the presence of 10^{-3} M Ca_o^{++} and light thereafter cannot completely compensate for the impairment resulting from Ca^{++} deprivation. On the other hand, a prolonged exposure to 10^{-6} M Ca_o^{++} did not interfere with subsequent light-induced cone contraction in 10^{-3} M Ca_o^{++} (Table II).

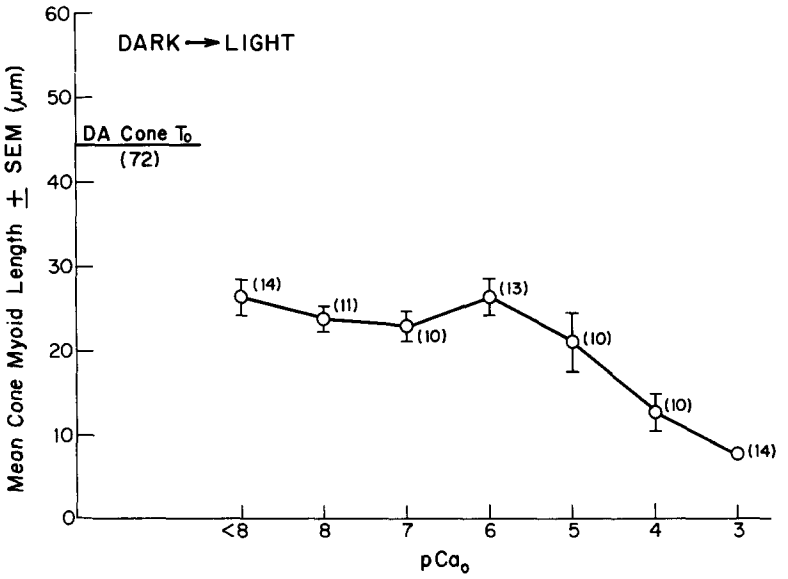


FIGURE 4. Effect of extracellular Ca^{++} concentration on cone myoid length during culture of isolated DA retinas in the light. Dissection and culture conditions were the same as in Fig. 3 except that, during culture, isolated retinas were illuminated with a white light providing an intensity of $\sim 8.8 \times 10^{14}$ photons/s·cm^2. Cones contracted to their LA positions in the presence of 10^{-3} M Ca_o^{++}; at $[Ca^{++}]_o \leq 10^{-6}$ M, the extent of contraction was approximately half that observed in 10^{-3} M Ca_o^{++}.

Ca^{++} Antagonists

Since 10^{-6} M Ca_o^{++} did not compromise later light-induced cone contraction in 10^{-3} M Ca_o^{++}, the incomplete contraction induced by light in 10^{-6} M Ca_o^{++} (Fig. 4) implies a requirement for extracellular Ca^{++} in the contractile process. To investigate this possibility further, we attempted to inhibit the movement of Ca^{++} across cone plasma membranes by incubating isolated retinas in the Ca^{++} antagonists verapamil, Co^{++}, Cd^{++}, La^{+++}, cinnarizine, or dantrolene. These agents block Ca^{++} channels in excitable membranes (Rosenberger and Triggle, 1978; Desmedt and Hainaut, 1979; Godfraind, 1982), and micromolar quantities of D-600 (a verapamil derivative), Co^{++}, and Cd^{++} block Ca^{++}-dependent regenerative potentials in rods (Fain et al., 1980). Only verapamil influenced the extent of

TABLE II

Effect of Preincubation in Low $[Ca^{++}]_o$ Upon Cone Myoid Lengths in Culture of Isolated DA Retinas in Dark or Light

Preincubation conditions			Culture conditions			Cone myoid length $\bar{x} \pm$ SEM (n)
pCa_o	Dark/light	Time	pCa_o	Dark/light	Time	
		min			*min*	*μm*
						T_o = 43.9±3.5 (10)
	None (control)		3	Dark	30	7.0±0.3 (15)
<8	Dark	15	3	Dark	30	17.2±0.8 (2)*
<8	Dark	15	3	Light	30	18.2±3.6 (2)*
	None (control)		3	Light	30	7.5±0.5 (14)
6	Dark	30	3	Light	30	7.7±1.2 (4)

Cone myoid lengths of experimental retinas were recorded after preincubation in the dark with the indicated $[Ca^{++}]_o$ and subsequent culture in either dark or light in 10^{-3} M Ca_o^{++}. Control retinas were not preincubated. The T_o retinas were fixed immediately upon dissection.

* Significantly different from control ($P < 0.001$).

TABLE III

Effect of Calcium Antagonists Upon Cone Myoid Lengths in Culture of Isolated DA Retinas in Dark or Light

Treatment	Dark/light	Cone myoid length $\bar{x} \pm$ SEM (n)	
		$pCa_o < 8$	$pCa_o = 3$
		μm	*μm*
		T_o = 44.5±0.8 (72)	—
1 mM EGTA-Ringer (control)	Dark	45.9±2.1 (13)	7.0±0.3 (15)
50 μM verapamil	Dark	—	10.4±1.3 (8)*
50 μM $CoCl_2$	Dark	—	6.8±0.4 (4)
50 μM $CdCl_2$	Dark	—	6.0±0.5 (3)
		T_o = 46.6±2.0 (4)‡	
1 mM EGTA-Ringer (control)	Dark	—	16.5±2.0 (6)‡
50 μM cinnarizine	Dark	—	16.5±1.5 (6)‡
1 mM Na acetate	Dark	—	20.8±1.5 (3)‡
1 mM La acetate	Dark	—	17.1±1.8 (3)‡
1 mM EGTA-Ringer (control)	Light	26.3±2.2 (14)	7.5±0.5 (14)
50 μM verapamil	Light	25.8±2.0 (3)	5.5±0.4 (3)
30 μM dantrolene	Light	23.5±3.8 (4)	7.2±0.8 (4)

Cone myoid lengths were recorded after 30 min of dark or light culture with the indicated conditions. The T_o retinas were fixed immediately upon dissection.

* Significantly different from control ($P < 0.01$).

‡ During experiments with cinnarizine and La^{+++}, cones of control retinas did not contract to fully LA positions in 10^{-3} M Ca_o^{++}. Therefore, these experimental data should be compared with the separate T_o and 1 mM EGTA-Ringer (control) data included.

cone contraction, and its effect was small (9% inhibition) and limited to light-independent Ca^{++}-induced cone contraction (Table III). Thus, it seems possible that most of the Ca^{++} influx relevant to cone contraction occurs via carrier-mediated facilitated diffusion, e.g., by a Na^{+}-Ca^{++} antiporter, rather than through a channel (see Borle, 1981; DiPolo and Beauge, 1983). Our results with Ca^{++} antagonists may also suggest that the effects of altering $[Ca^{++}]_o$ on cone retinomotor position are at least partly expressed through an indirect route, e.g., by changing the activity of a membrane-bound Ca^{++}-sensitive enzyme (Robinson et al., 1980), by modifying membrane potential (Yau et al., 1981), or by influencing cytoplasmic Ca^{++} buffering capacity (Greenblatt, 1983).

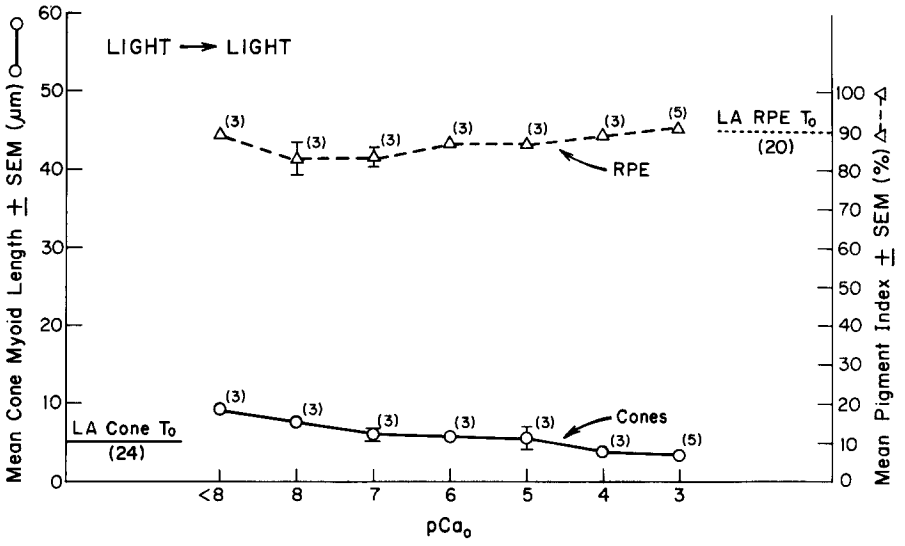


FIGURE 5. Effect of extracellular Ca^{++} concentration on cone myoid length and RPE pigment distribution during culture of isolated LA retinas with attached RPE in constant light. Retinas with adherent RPE were removed from LA fish and cultured in the light for 60 min. Retinomotor positions of both cones and RPE remained close to their original (T_o) values regardless of $[Ca^{++}]_o$.

Effects of Changing $[Ca^{++}]_o$ on LA Retinas Cultured in Light

In LA animals, RPE was firmly attached to the retina so that RPE was removed with the retina during dissection, and the normal RPE-retina association was maintained during culture. Thus, technical considerations dictated that the conditions of these experiments did not exactly match those described above for culture of DA retinas since RPE spontaneously detached during dissection of the latter and was therefore not present during culture. However, the maintenance of RPE-retina association in LA tissue allowed us to assess retinomotor positions of both cones and RPE.

Dose-response studies with varying $[Ca^{++}]_o$ were performed on isolated LA RPE-retinas cultured in continuous light. We found little change in either cone

position or RPE pigment distribution regardless of $[Ca^{++}]_o$; both cones and RPE remained close to their original LA (T_o) positions (Fig. 5).

Effects of Changing $[Ca^{++}]_o$ on LA Retinas Cultured in Darkness

When LA RPE-retinas were cultured in the dark with varying $[Ca^{++}]_o$, maximal cone myoid elongation and RPE pigment aggregation occurred between 10^{-5} and 10^{-7} M Ca_o^{++} (Figs. 2*e–h* and 6). At concentrations of Ca_o^{++} above or below this range, cones and RPE pigment migrated toward their DA positions but only to intermediate extents. Thus, while 10^{-5}–10^{-7} M Ca_o^{++} permitted darkness to induce dark-adaptive cone and RPE retinomotor movements, $[Ca^{++}]_o \leq 10^{-8}$ M

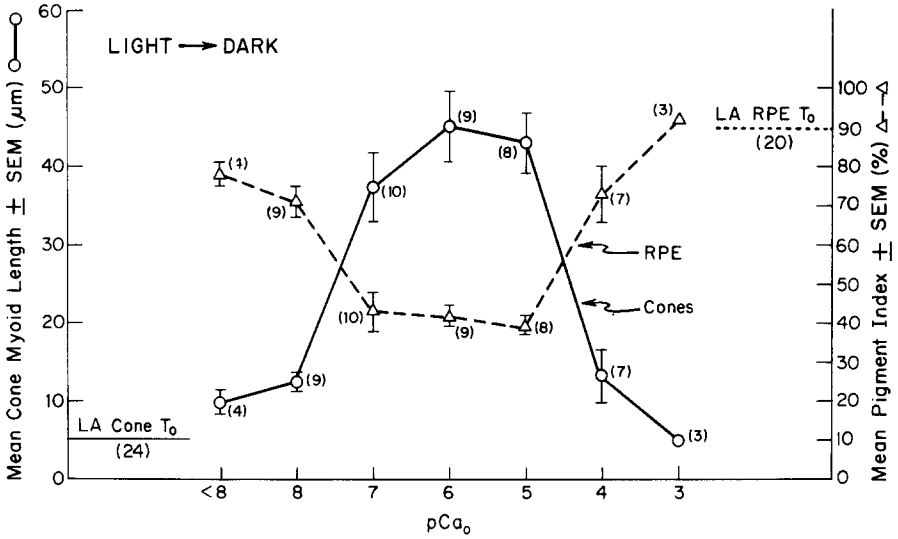


FIGURE 6. Effect of extracellular Ca^{++} concentration on cone myoid length and RPE pigment distribution during culture of isolated LA RPE-retinas in the dark. After removal from LA animals, retinas with adherent RPE were cultured in total darkness for 60 min. Maximal dark-adaptive retinomotor movements (cone myoid elongation and RPE pigment aggregation) occurred between 10^{-5} and 10^{-7} M Ca_o^{++}. At concentrations of Ca_o^{++} above or below this range, extents of cone elongation and RPE pigment migration were greatly reduced.

inhibited these movements. Since $[Ca^{++}]_o < 10^{-8}$ M also compromised subsequent light-induced cone contraction in 10^{-3} M Ca_o^{++} (Table II), it seems likely that prolonged exposure to very low $[Ca^{++}]_o$ ($\leq 10^{-8}$ M) disrupts a cell function essential to both cone contraction and elongation.

Effects of High $[K^+]_o$ and Low $[Na^+]_o$

Since light onset is accompanied by hyperpolarization of cone plasma membranes (Baylor and Fuortes, 1970) as well as cone myoid contraction, we sought to investigate whether hyperpolarization might be a prerequisite for cone contraction. Therefore, we attempted to block both light-induced and light-independent

Ca^{++}-induced cone contraction by increasing extracellular $[K^+]$. High $[K^+]_o$ depolarizes rods and cones in the dark and prevents light-induced hyperpolarization (Cervetto, 1973; Capovilla et al., 1980). It should therefore eliminate the influence of light-evoked membrane potential change on cone retinomotor movement. We found that light-induced cone contraction was blocked by a 10-fold increase in $[K^+]_o$ (i.e., by 54 mM K_o^+) in media with either $< 10^{-8}$ or 10^{-3} M Ca_o^{++} (Table IV). In addition to blocking light-induced cone contraction, high $[K^+]_o$ also prevented the induction of cone contraction by high ($\geq 10^{-3}$ M) $[Ca^{++}]_o$ in darkness (Fig. 7). These results suggest that K^+-induced depolarization might prevent the occurrence of light- and Ca^{++}-elicited events that lead to contraction. Our observations are thus consistent with the idea that light-stimulated hyperpolarization might be a necessary step in a sequence of reactions culminating in cone myoid contraction.

To further test this possibility, we sought to impose an artificial hyperpolarization in the absence of light to see if this change in membrane potential could

TABLE IV

Effect of High $[K^+]_o$ and (dbcAMP + IBMX) Upon Cone Myoid Lengths in Light Culture of Isolated DA Retinas

	Cone myoid length $\bar{x} \pm$ SEM (n)	
	$pCa_o < 8$	$pCa_o = 3$
	μm	μm
	$T_o = 47.6 \pm 1.7$ (18)	—
1 mM EGTA-Ringer (control)	28.7±4.7 (4)	8.0±0.6 (7)
54 mM K_o^+ replacement of Na_o^+	46.8±3.5 (4)*	45.7±1.2 (4)*
54 mM K_o^+ addition	—	45.4±4.1 (6)*
3 mM dbcAMP + 5 mM IBMX	46.8±1.8 (4)*	50.0±4.3 (4)*

Cone myoid lengths were recorded after 30 min of light culture with the indicated conditions. The T_o retinas were fixed immediately upon dissection.

* Significantly different from control ($P < 0.001$).

mimic the effect of light on cone contraction. To induce membrane hyperpolarization, $[Na^+]_o$ was lowered to 3.5 mM (1.0 mM in NaH_2PO_4 and 2.5 mM caused by EGTA addition) and the remainder was replaced with choline salts. A decrease in $[Na^+]_o$ with choline substitution has been shown to hyperpolarize rod and cone membrane potential in the dark (Cervetto, 1973; Capovilla et al., 1981). If such a voltage change served as the requisite signal for cone contraction, then one might expect hyperpolarization in the dark to cause contraction of the cones in DA retinas. Because cones contracted spontaneously (i.e., independent of light) when $[Ca^{++}]_o$ was $>10^{-6}$ M, these experiments were conducted at $[Ca^{++}]_o \leq 10^{-6}$ M. We found that the cones of DA retinas subjected to low $[Na^+]_o$ treatment during dark culture remained in their DA positions at both $<10^{-8}$ and 10^{-6} M Ca_o^{++} (Table V). Thus, low $[Na^+]_o$ treatment did not mimic the effect of light on cone contraction. This result suggests that under these conditions, low $[Na^+]_o$ either does not induce sufficient hyperpolarization or that hyperpolarization alone is not sufficient to cause cone contraction.

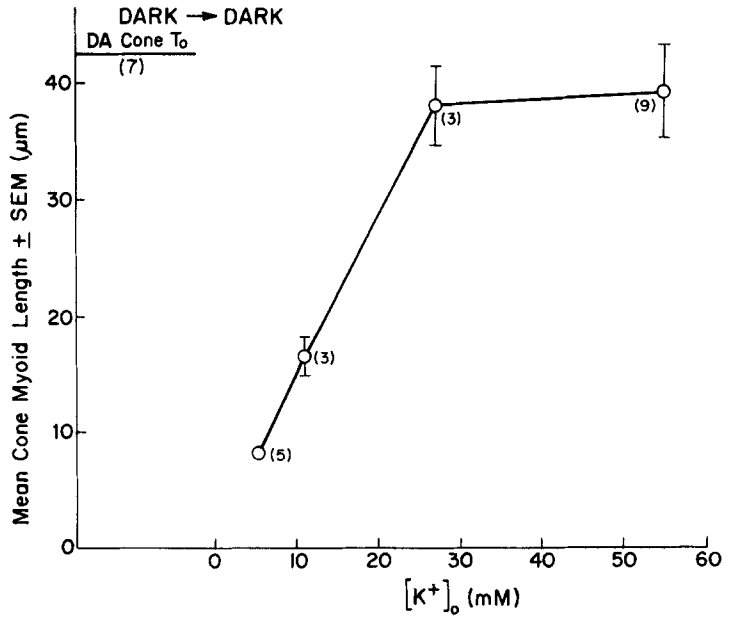


FIGURE 7. Maintenance of DA cone retinomotor positions by high $[K^+]_o$ in isolated DA retinas cultured in constant dark. Retinas were removed from DA fish and cultured for 30 min in the dark in a medium containing 10^{-3} M Ca^{++} and a specified concentration of K^+. Light-independent Ca^{++}-induced cone contraction was counteracted by elevated $[K^+]_o$ in a dose-dependent fashion. At 27–54 mM K_o^+, cones remained in their DA positions even in the presence of 10^{-3} M Ca_o^{++}.

Effects of Altering Intracellular cAMP Levels

Elsewhere it has been shown that increasing extracellular $[K^+]$ can substantially enhance cAMP levels in intact retinas (Wassenaar and Korf, 1976; Watling et al., 1980; Cohen, 1982). Since we have previously shown that treatments which elevate cAMP levels induce dark-adaptive retinomotor movements (cone elongation and RPE pigment aggregation) in LA retinas (Burnside et al., 1982*a*;

TABLE V

Effect of Low $[Na^+]_o$ Upon Cone Myoid Lengths in Dark Culture of Isolated DA Retinas

	Cone myoid length $\bar{x}$ ± SEM (n)	
	$pCa_o < 8$	$pCa_o = 6$
	μm	μm
	T_o = 49.2±1.8 (9)	—
1 mM EGTA-Ringer (control)	47.6±2.8 (4)	49.8±3.0 (3)
140.4 mM choline replacement of Na_o^+	43.0±1.6 (9)	45.1±2.0 (6)

Cone myoid lengths were recorded after 30 min of constant dark culture with the indicated conditions. The T_o retinas were fixed immediately upon dissection. Experimental means were not significantly different from control means ($P < 0.10$).

Burnside and Basinger, 1983), we tested to see if such treatment with IBMX/dbcAMP could also inhibit light-stimulated cone contraction. Cone contraction was blocked at both $<10^{-8}$ and 10^{-3} M Ca_o^{++} when DA retinas were cultured in light in the presence of 3 mM dbcAMP and 5 mM IBMX (Table IV). Thus, incubation in either IBMX/dbcAMP or high $[K^+]_o$ can block light-induced cone retinomotor movement.

In addition, IBMX also prevented light-independent Ca^{++}-induced cone contraction (Fig. 8). In media containing normal $[K^+]_o$ (5.4 mM) and $\geq 10^{-3}$ M Ca_o^{++} without IBMX, cones spontaneously contracted to their LA positions in the absence of light. This contraction was blocked by either high $[K^+]_o$ (Fig. 7) or

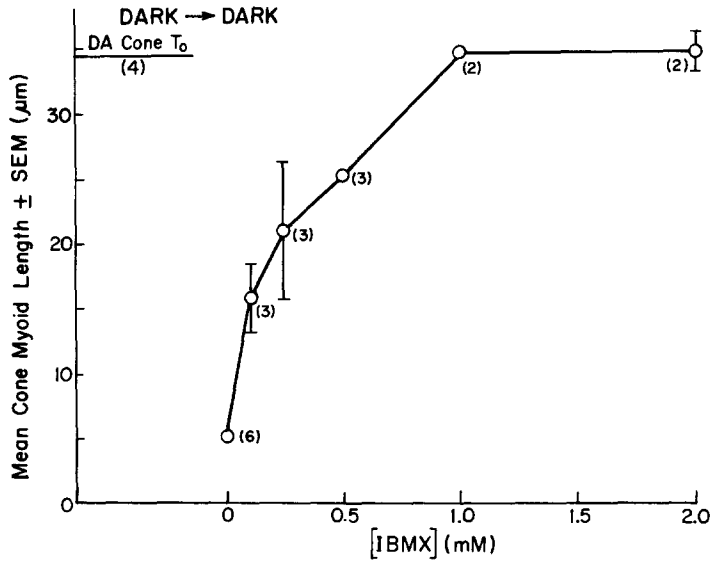


FIGURE 8. Maintenance of DA cone retinomotor positions by IBMX in isolated DA retinas cultured in constant dark. Retinas were removed from DA fish and cultured for 60 min in the dark in a medium containing 1.8 mM Ca^{++} and a specified concentration of IBMX. Light-independent Ca^{++}-induced cone contraction was inhibited by IBMX in a dose-dependent manner. At 1–2 mM IBMX, cones remained in their DA positions even in the presence of 1.8 mM Ca_o^{++}.

IBMX (Fig. 8) in a dose-dependent fashion. Effects of increasing $[K^+]_o$ or [IBMX] thus parallelled the results obtained by lowering $[Ca^{++}]_o$ (Fig. 3). All of these treatments worked in a concentration-dependent manner to retain long cone myoids during dark culture of DA retinas; $[Ca^{++}]_o \leq 10^{-6}$ M, [IBMX] ≥ 1 mM, and $[K^+]_o \geq 27$ mM were equally effective.

The above observations suggested that high $[K^+]_o$ and IBMX/dbcAMP application might block cone contraction by a common mechanism, e.g., by modifying cytoplasmic cAMP levels. If this suggestion is valid, one might expect elevated $[K^+]_o$ to induce dark-adaptive movements in LA retinas cultured in light in a manner analogous to IBMX/dbcAMP. To test this possibility, we compared the effects of incubating LA RPE-retinas in continuous light with high $[K^+]_o$ to the

effects of IBMX/dbcAMP treatment. At 10^{-3} M Ca_o^{++}, high $[K^+]_o$ produced partial dark-adaptive retinomotor movements in both cones and RPE; at 10^{-6} M Ca_o^{++}, high $[K^+]_o$ induced pronounced cone elongation and RPE pigment aggregation (Fig. 9). These results resembled those obtained by incubating LA RPE-

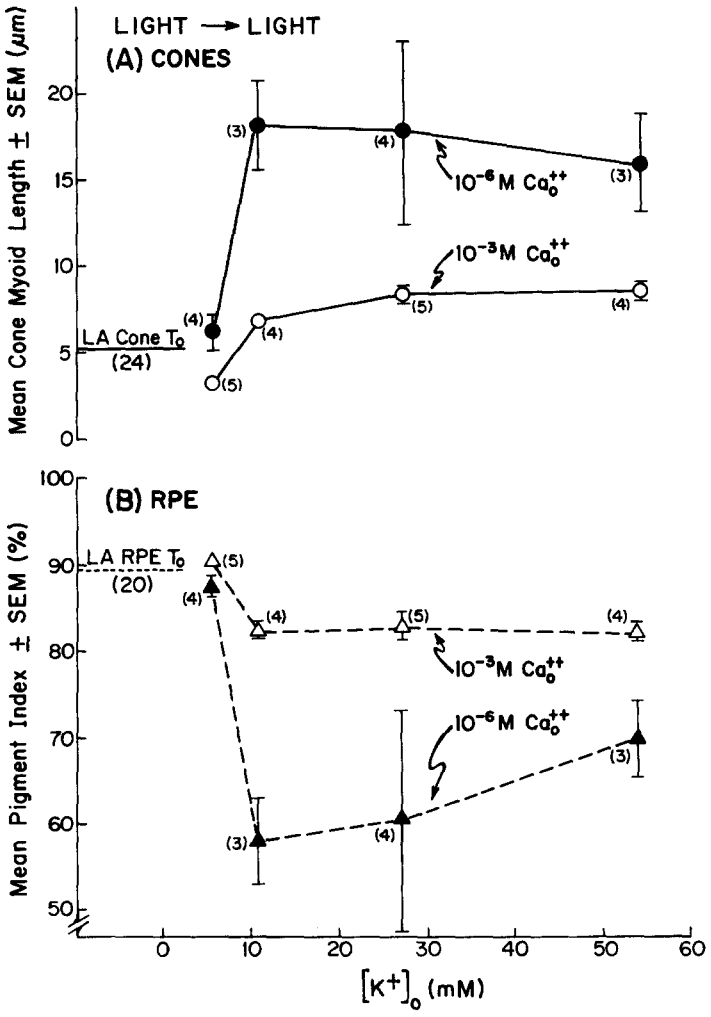


FIGURE 9. Promotion of dark-adaptive cone (*A*) and RPE (*B*) retinomotor movements by high $[K^+]_o$ in isolated LA RPE-retinas cultured in constant light. Retinas with adherent RPE were removed from LA fish and cultured for 60 min in the light in a Ringer solution containing either 10^{-3} M (open circles and triangles) or 10^{-6} M (solid circles and triangles) Ca_o^{++} and a specified concentration of K^+. Cone myoid elongation and RPE pigment aggregation were partially induced by high $[K^+]_o$ in the presence of 10^{-3} M Ca_o^{++}. When $[Ca^{++}]_o$ was reduced to 10^{-6} M, the extents of dark-adaptive cone and RPE movements induced by high $[K^+]_o$ were increased.

retinas in a medium containing normal $[K^+]_o$ (5.4 mM), 1.8 mM Ca_o^{++}, and added IBMX/dbcAMP (Fig. 10). Our data thus support the notion that high $[K^+]_o$ and high $[cAMP]_i$ may work through a common mechanism.

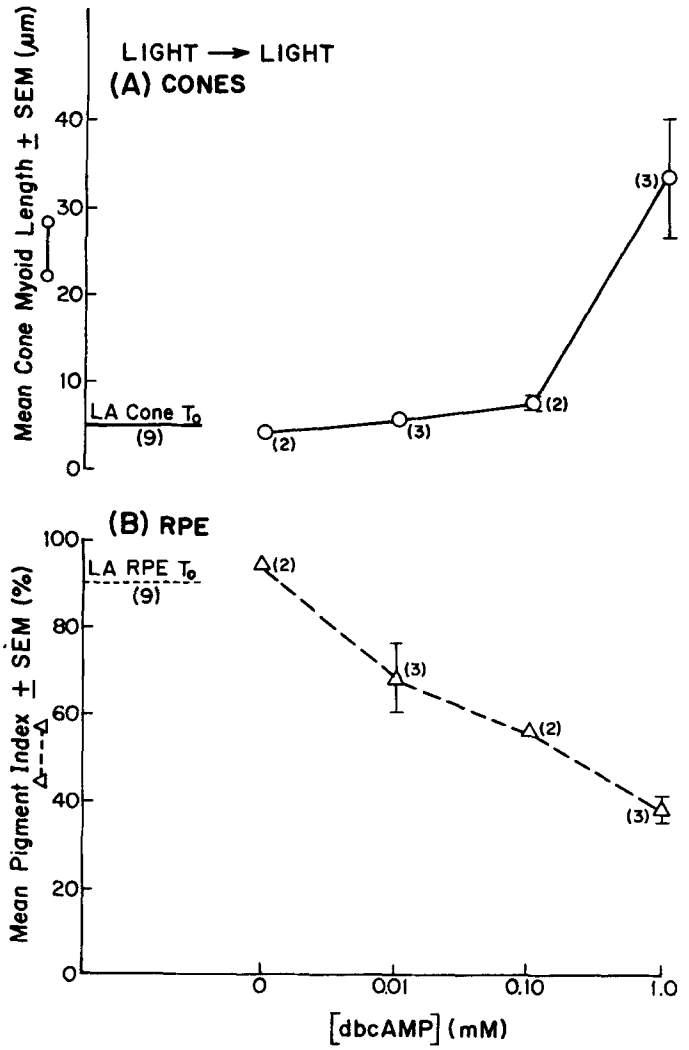


FIGURE 10. Promotion of dark-adaptive cone (*A*) and RPE (*B*) retinomotor movements by dbcAMP in isolated LA RPE-retinas cultured in constant light. Retinas with adherent RPE were removed from LA fish and cultured for 60 min in the light in a Ringer solution containing 1.8 mM Ca^{++}, 5.4 mM K^+, 0.1 mM IBMX, and a specified concentration of dbcAMP. IBMX alone at this concentration had no effect on either cone or RPE retinomotor position. Dark-adaptive cone RPE retinomotor movements were elicited by dbcAMP in a concentration-dependent manner. RPE pigment migration was sensitive to lower [dbcAMP] than was cone elongation.

DISCUSSION

Our results indicate that changes in ionic composition of the extracellular medium had significant effects on cone and RPE retinomotor movements in isolated teleost retinas. Low $[Ca^{++}]_o$ and high $[K^+]_o$ favored dark-adaptive retinomotor movements. Low $[Ca^{++}]_o$ maintained DA cone positions in DA retinas cultured in constant darkness and permitted dark-induced cone and RPE movements in LA retinas cultured in darkness. High $[K^+]_o$ prevented either Ca^{++}- or light-induced cone contraction and induced dark-adaptive cone and RPE movements in the light. All of these effects of high $[K^+]_o$ on cone length were reproduced by treatments that elevate cytoplasmic cAMP content.

Effects of Ca^{++} and cAMP on Cone Retinomotor Movements

In our experiments, high $[Ca^{++}]_o$ induced light-adaptive cone contraction regardless of dark/light condition. Treatments that increase intracellular cAMP concentration induced dark-adaptive cone elongation in the light and prevented activation of cone contraction by either high $[Ca^{++}]_o$ or light. Thus, high $[Ca^{++}]_o$ appears to be associated with cone contraction, while high $[cAMP]_i$ appears to be required for cone elongation. In other studies in our laboratory using lysed-cell models of *Lepomis cyanellus* cones, we have found that reactivated cone contraction requires both increased free $[Ca^{++}]$ and decreased [cAMP]; conversely, reactivated cone elongation requires both low free $[Ca^{++}]$ and high [cAMP] (Porrello and Burnside, 1984; Gilson et al., 1983). Therefore, cone movement in either direction appears to be associated with antagonistic changes in intracellular Ca^{++} and cAMP levels. The results reported here for effects of $[Ca^{++}]_o$ and treatments that elevate $[cAMP]_i$ are thus in accordance with data obtained from lysed-cell models of teleost cones. In addition, our findings are consistent with evidence indicating that cAMP inhibits contraction and induces relaxation in smooth muscle (Webb and Bohr, 1981). In these cells, it has been suggested that cAMP may either act directly on the contractile machinery to reduce its sensitivity to Ca^{++} or act indirectly by activating mechanisms that lower $[Ca^{++}]_i$ (Mueller and van Breemen, 1979; Conti and Adelstein, 1981). Cyclic AMP has been found to similarly influence both the contractile apparatus and $[Ca^{++}]_i$ in nonmuscle cells (Hathaway et al., 1981; Feinstein et al., 1983). Therefore, it seems likely that cAMP inhibits contraction in intact cones by interfering with actin-myosin interaction and/or by decreasing free $[Ca^{++}]_i$.

Relationship Between $[Ca^{++}]_o$ and $[cAMP]_i$

Several observations suggest that alterations of $[Ca^{++}]_o$ might be influencing cytoplasmic cAMP levels in our cultured retinas. In our studies, $\geq 10^{-3}$ M Ca_o^{++} induced cone contraction even in the absence of light; however, adding IBMX completely blocked this Ca^{++}-induced contraction. Thus, it seems likely that during culture in $\geq 10^{-3}$ M Ca_o^{++} without IBMX, cAMP levels declined, perhaps because of Ca^{++} activation of cone myoid phosphodiesterase and/or Ca^{++} inhibition of adenylate cyclase (Piascik et al., 1980). It is also possible that some factor controlling endogenous cAMP or Ca^{++} concentration was lost upon removal of retinas from their in vivo environment. Exposure of DA retinas to

$\geq 10^{-3}$ M Ca_o^{++} in the dark has previously been reported to reduce the cAMP content in isolated mouse retinas (Cohen et al., 1978) and the cGMP content in isolated frog retinas (Woodruff and Fain, 1982). Such a decline in the cAMP level in cone myoids of our DA cultured retinas could account for light-independent Ca^{++}-induced cone contraction. We were unable to test this directly by measuring cAMP concentrations in these experiments since measurements of either whole retina (e.g., Farber et al., 1981) or photoreceptor inner segment layer (e.g., Orr et al., 1976) [cAMP] would be dominated by the contribution of the vastly more numerous rods.

Extracellular Ca^{++} Levels In Vivo

A possible implication of the effects of $[Ca^{++}]_o$ reported here is that extracellular Ca^{++} levels in intact retinas in vivo may differ in dark and light. Low $[Ca^{++}]_o$ was required to maintain DA cone myoid positions, while 10^{-3} M Ca_o^{++} was required in order for light to induce full cone myoid contraction. These results suggest that $[Ca^{++}]_o$ in the dark may be less than $[Ca^{++}]_o$ in the light. This idea is consistent with previously reported physiological experiments with isolated retinas where low $[Ca^{++}]_o$ in the dark was required to observe light-induced increases in $[Ca^{++}]_o$ (Gold and Korenbrot, 1980; Yoshikami et al., 1980), rapid light-induced decreases in $[cGMP]_i$ (Kilbride, 1980), and light-induced decreases in phtoreceptor Na^+ permeability (Woodruff et al., 1982).

Ca^{++} Release from Internal Stores in Cones

Our results suggest that light induces a release of Ca^{++} from internal stores. Even in a medium containing $<10^{-8}$ M Ca^{++} and 1 mM EGTA, DA retinal cones contracted partially upon stimulation by light. In 30 min, cones contracted to ~50% of the extent seen in 10^{-3} M Ca_o^{++}. Porrello and Burnside (1984) have shown that cones contract at normal rates (~1.5 μm/min) for the first 10 min after light onset under these conditions. Thus, cones appear to move normally for a short time and then stall at an intermediate length. Our result here is similar to that observed in barnacle muscle fibers: when these fibers were bathed in caffeine to release internal Ca^{++} stores, the peak tension in $<10^{-8}$ M Ca_o^{++} was approximately half that in 1.5 mM Ca_o^{++} (Caputo and DiPolo, 1978). In smooth muscle, agonist-induced contraction consists of two phases: a short-lived phasic contraction that does not require external Ca^{++}, and a longer tonic contraction that does require external Ca^{++} (Watkins and Davidson, 1980; van Breemen et al., 1982). Thus, in smooth muscle, the proper stimulus can initiate contraction by evoking Ca^{++} release from an intracellular store, but sustained contraction requires the presence of extracellular Ca^{++}. Our results suggest that a similar mechanism obtains in retinal cones, i.e., that light can elicit an increase in $[Ca^{++}]_i$ from internal stores sufficient to activate cone myoid contraction, but that the full extent of contraction requires the presence of extracellular Ca^{++}.

Effects of Ca^{++} on RPE Pigment Distribution

In addition to permitting dark-induced cone elongation, low $[Ca^{++}]_o$ was necessary for dark-induced aggregation of RPE granules. Burnside et al. (1983) have

shown that pigment granule dispersion is actin dependent and have suggested that light onset induces RPE pigment dispersion by increasing intracellular Ca^{++} concentration. The low $[Ca^{++}]_o$ requirement for pigment aggregation reported here is consistent with this hypothesis. Calcium effects on pigment movements similar to those found here have been reported in fish melanophores (Ishibashi, 1957; Novales and Novales, 1965), fly retinas (Kirschfeld and Vogt, 1980), and crayfish retinas (Frixione and Arechiga, 1981). Thus, our results are in agreement with previous work suggesting that low $[Ca^{++}]$ is required for dark-induced pigment aggregation.

Effects of High $[K^+]_o$ and Low $[Na^+]_o$

Prevention of light-stimulated cone contraction by high $[K^+]_o$ suggests that light-induced hyperpolarization might be a requirement for cone contraction. However, exposing cones to low $[Na^+]_o$ in order to induce hyperpolarization in the dark failed to elicit light-adaptive cone retinomotor movement. Because these low $[Na^+]_o$ experiments had to be performed in low $[Ca^{++}]_o$ conditions to prevent spontaneous contraction, it is conceivable that our low $[Na^+]_o$ treatment may not have adequately mimicked the hyperpolarizing effect of light. In the presence of low $[Ca^{++}]_o$, photoresponses can still be elicited from rods after Na_o^+ removal (Yau et al., 1981; Bastian and Fain, 1982; Oakley and Pinto, 1983). Our results are consistent with these findings and suggest that low $[Na^+]_o$ in the presence of low $[Ca^{++}]_o$ does not fully reproduce the effect of light on cone retinomotor movement.

It is also possible that light and high $[K^+]_o$ influence cone retinomotor movement through a voltage-independent mechanism. This is the case in smooth muscle, where changes in membrane potential and contractile tension can be experimentally separated (Casteels and Droogmans, 1982). High $[K^+]_o$ could interrupt light-stimulated cone contraction by mechanisms that may or may not be voltage dependent. High $[K^+]_o$ not only influences membrane potential but also enhances cAMP content in brain slices (Daly, 1977) and in isolated retinas from various species (Wassenaar and Korf, 1976; Watling et al., 1980; Cohen, 1982). Since adding IBMX/dbcAMP produced cone movements similar to those seen in high $[K^+]_o$, it seems probable that cAMP is a crucial effector of both treatments.

Conclusions

Manipulation of the external ionic environment surrounding isolated teleost retinas dramatically influenced cone and RPE retinomotor movements. High $[Ca^{++}]_o$ favored light-adaptive movement regardless of dark/light condition. Low $[Ca^{++}]_o$ or high $[K^+]_o$ favored dark-adaptive movement (cone elongation and RPE pigment aggregation) and maintenance of dark-adapted positions. The effects of low $[Ca^{++}]_o$ and high $[K^+]_o$ were similar to the effects of treatments that elevate intracellular cAMP concentration. The results of these experiments on intact cells in whole, isolated retinas and of experiments on lysed-cell models reported elsewhere (Gilson et al., 1983; Porrello and Burnside, 1984) are consistent with the suggestion that in cone myoids light onset is accompanied by

high $[Ca^{++}]_i$ and low $[cAMP]_i$, whereas onset of darkness is accompanied by low $[Ca^{++}]_i$ and high $[cAMP]_i$. Thus, regulation of retinomotor movements by light appears to involve a complex and antagonistic relationship between Ca^{++} and cAMP.

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