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Regulation of Reactivated Contraction in Teleost Retinal Cone Models by Calcium and Cyclic Adenosine Monophosphate

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ABSTRACT We have been using lysed cell models of teleost retinal cones to examine the mechanism of contraction in nonmuscle cells. We have previously reported that dark-adapted retinas can be lysed with the detergent Brij-58 to obtain cone motile models that undergo Ca^{++} - and adenosine triphosphate (ATP)-dependent reactivated contraction. In this report we further dissect the roles of ATP and Ca^{++} in activation of contraction and force production by (a) characterizing the Ca^{++} and nucleotide requirements in more detail, (b) by analyzing the effects of inosine triphosphate (ITP) and the ATP analog $\text{ATP}\gamma\text{S}$ and (c) by testing effects of cyclic adenosine monophosphate (cAMP) on reactivated cone contraction. Exposing lysed cone models to differing free Ca^{++} concentrations produced reactivated contraction at rates proportional to the free Ca^{++} concentration between 3.16×10^{-8} and 10^{-6} M. A role for calmodulin (CaM) in this Ca^{++} regulation was suggested by the inhibition of reactivated contraction by the calmodulin inhibitors trifluoperazine and calmidazolium (R24571). The results of analysis of nucleotide requirements in lysed cone models were consistent with those of smooth muscle studies suggesting a role for myosin phosphorylation in Ca^{++} regulation of contraction. $\text{ATP}\gamma\text{S}$ and ITP are particularly interesting in that $\text{ATP}\gamma\text{S}$, on the one hand, can be used by kinases to phosphorylate proteins (e.g., myosin light chains) but resists cleavage by phosphatases or adenosine triphosphatases (ATPases), e.g., myosin ATPase. ITP, on the other hand, can be used by myosin ATPase but does not support Ca^{++} /calmodulin mediated phosphorylation of myosin light chains by myosin light chain kinase. Thus, these nucleotides provide an opportunity to distinguish between the kinase and myosin ATPase requirements for ATP. When individual nucleotides were tested with cone motile models, the nucleotide requirement was highly specific for ATP; not only ITP and $\text{ATP}\gamma\text{S}$, but also guanosine triphosphate, cytosine triphosphate, adenylyl-immidodiphosphate (AMPPNP) failed to support reactivated contraction when substituted for ATP throughout the incubation. However, if lysed cones were initially incubated with $\text{ATP}\gamma\text{S}$ and then subsequently incubated with ITP, the cones contracted to an extent that was comparable to that observed with ATP. As observed in skinned smooth muscle, adding cAMP to contraction medium strongly inhibited contraction in lysed cone models.

Recent research suggests that contraction in nonmuscle cells resembles that in muscle not only in its dependence on the actin-myosin interaction for force production but also in its regulation by calcium (18, 26, 32, 36, 38, 45). Ca^{++} regulation of contraction by a myosin-linked mechanism has been described in a variety of vertebrate smooth muscle cells (1, 2, 17, 55), as well as nonmuscle cells (3, 29, 70). Although there is controversy concerning the Ca^{++} regulatory mechanism of smooth muscle contraction (27, 67), considerable biochemi-

cal, physiological, and pharmacological evidence has accumulated in favor of the myosin phosphorylation theory (7, 64, 65). According to this theory, the actin-myosin interaction is regulated by Ca^{++} calmodulin-dependent phosphorylation of the 20,000 dalton light chain of myosin kinase (MLCK)¹

¹ *Abbreviations used in this paper:* ATPase, adenosine triphosphatase; $\text{ATP}\gamma\text{S}$, analog of adenosine triphosphate; CaM, calmodulin; ITP, inosine triphosphate; MLCK, myosin light chain kinase; TFP, trifluoperazine.

(1, 2, 17, 36, 55). When this light chain is phosphorylated, myosin can interact with actin to produce contraction. Upon removal of Ca^{++} , myosin light chains are dephosphorylated by endogenous phosphatase(s) resulting in a decrease in actomyosin adenosine triphosphatase (ATPase) activity and subsequent relaxation (17, 25, 37). Calmodulin-dependent myosin phosphorylation has also been shown to mediate actomyosin ATPase activity in several nonmuscle cells (3, 29, 70).

To investigate further the mechanism by which contraction is regulated in a nonmuscle cell, we have been examining reactivated contraction in detergent-lysed, teleost retinal cone models. *In vivo*, teleost retinal cones undergo dramatic length changes, elongating to 100 μm in the dark and contracting to 6 μm in the light. These movements are part of a coordinated rearrangement of the visual cells, which position the photoreceptors for optimal vision at different light intensities (12). We have reported previously that when dark-adapted retinas are lysed with the nonionic detergent, Brij-58, the long slender cones produce motile models that undergo Ca^{++} and adenosine triphosphate (ATP)-dependent reactivated contraction, with morphology and rate comparable to those observed *in vivo* (13, 46). We have also demonstrated that reactivated contraction of cone models is inhibited by both *N*-ethylmaleimide subfragment-1 and *N*-ethylmaleimide heavy meromyosin, thus providing evidence that cone contraction is actomyosin-mediated (46). The primary advantage of the cone motile models over other cell types used to study cell shape changes (59) is the ease with which movement can be quantified. Contraction is linear, excursions are large (20–30 μm), and responses are very reproducible. Thus, we can provide quantitative analysis of contraction for comparison with results from studies using highly purified proteins.

In this report we characterize further the nucleotide requirements and role of Ca^{++} in regulating reactivated contraction. To compare the mechanism of Ca^{++} regulation in cone contraction with previously reported studies in smooth muscle and other nonmuscle cells, we have examined the effects of CaM inhibitors, cyclic adenosine monophosphate (cAMP), and the ATP analog, ATP γ S, on reactivated contraction in cone models. The phenothiazine derivatives, such as trifluoperazine (TFP) and calmidazolium (R24571), have been shown to bind to CaM and inhibit its interaction with CaM-dependent enzymes (28, 40, 60); although not absolutely specific to CaM, these drugs have been shown to inhibit myosin phosphorylation and actomyosin ATPase activity in both smooth muscle (16, 53) and nonmuscle cells (35). cAMP has been shown to inhibit actomyosin interaction in both smooth muscle and nonmuscle cells by catalyzing the phosphorylation of MLCK with subsequent reduction in its affinity for Ca^{++} /CaM (1, 4, 7, 23). ATP γ S, when used with the nucleotides cytosine triphosphate or inosine triphosphate (ITP) provides a most useful probe for analyzing the role of myosin phosphorylation in Ca^{++} regulation. ATP γ S serves as a substrate for myosin phosphorylation by MLCK but not for myosin ATPase activity or for phosphatase activity (15, 33, 65). In contrast, ITP and cytosine triphosphate support myosin ATPase activity but do not serve as substrates for MLCK (42, 68). Thus these nucleotides can be used to distinguish between kinase and myosin ATPase requirements for ATP. We have therefore tested the effects of these various agents on reactivated cone contraction to evaluate further the postulated role for myosin phosphorylation in nonmuscle contraction.

MATERIALS AND METHODS

Animals: Experiments were carried out on green sunfish (*Lepomis cyanellus*). Most experiments were performed on fish obtained from Funez Fish Farm in Sebastopol, CA and Shadowlake Fish Ranch in Redding, CA. Because the 1982–1983 California floods made it impossible to obtain fish from our local suppliers, we were forced to obtain fish from Ohio (Fender's Fish Hatchery, Baltic, OH). Cone models from the Ohio fish produced slightly reduced excursions and rates of reactivated contraction. For this reason, and because we found minor seasonal variation in extents of contraction, all experimental manipulations in each table or figure are compared to controls from the same fish population. To minimize the effects of circadian fluctuations on cone length (41), all experiments were done at the same time of day. Fish were placed in an aerated-dark box at noon and dark-adapted for ~1.5–2 h. This procedure assures that cones are not so long and fragile that they are lost during dissection and yet provides sufficiently long cone myoids (50–60 μm) for easy measurement of length changes.

Preparation of Retinas and Cone Length Measurements: Experiments were performed under infrared illumination with an infrared converter as previously described (46). Eyes of dark-adapted fish were enucleated and the retinas were gently detached with gassed (95% O_2 /5% CO_2), Ca^{++} -free ($<10^{-8}$ M) modified Earles' Ringer solution containing 5 mM EGTA, 1 mM MgSO_4 , 24 mM sodium bicarbonate, 20 mM glucose, 3 mM HEPES, 1 mM ascorbic acid, pH 7.4. The retinas were bisected along the choroid fissure to produce four half-retinas per fish. One half-retina was placed directly into fix and served as the t_0 (initial cone length). The other three halves were subjected to the two-step lysis-incubation procedure as described previously (46). In brief, retinas were lysed for 3 min in a reactivation medium (Table I) containing 1% Brij-58 (polyoxyethylene 20 cetyl ether; Sigma Chemical Co., St. Louis, MO) and then transferred to detergent-free medium for 15 min incubation. t_0 and treated retinas were placed in 6% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, overnight, and then cut into 25–50- μm thick slices with a manual tissue chopper for examination with Nomarski optics (Fig. 1). Cone myoid length was measured with an ocular micrometer, as the distance from the outer limiting membrane to the base of the cone ellipsoid (13, 46). Extents and rates of contraction were determined by comparing experimental values to the t_0 from the same fish. In all figures, *n* refers to the number of retinas examined.

Ca^{++} and Nucleotide Studies: Ca^{++} /EGTA buffers were prepared with 10 mM EGTA according to formulae from Steinhardt et al. (57) (Table II). These calculations assume an association constant for EGTA with calcium of $10^{10.7}$. Free Ca^{++} levels above 10^{-5} M were not explored because Ca^{++} /EGTA buffering is not reliable at concentrations $>10^{-6}$ M (47).

ATP, adenosine diphosphate, AMPPNP, cytosine triphosphate, guanosine triphosphate, ITP, cAMP (Sigma Chemical Co., St. Louis, MO) and ATP γ S (Boehringer-Mannheim, Federal Republic of Germany) were prepared as aqueous stock solutions, adjusted to pH 7.0, and stored at -20°C . We did not further purify the ATP γ S from Boehringer-Mannheim. Cassidy et al. (15) found that ATP γ S was contaminated with 3% ADP, but that the commercial preparation did not behave differently from column-purified ATP γ S in experiments with skinned smooth muscle. Also ADP fails to support contraction in cone-lysed cell models, so the small contaminating ADP should not compromise ATP γ S results. Unless otherwise stated, specified Ca^{++} and nucleotide concentrations were present in both steps of the two-step reactivation procedure. In all solutions nucleotide concentrations were matched with equimolar concentrations of Mg^{++} . The Ohio fish used for the ATP γ S/ITP study exhibited slower rates of cone movement, therefore we extended the postlysis incubation to 27 min to obtain greater excursions.

Calmodulin Inhibitor Studies: TFP and R24571 were gifts from Smith, Kline & French Laboratories in Philadelphia, PA, and Janssen Pharmaceutics, Life Sciences, Beerse, Belgium, respectively. TFP, dissolved in distilled water, and R24571, dissolved in dimethyl sulfoxide (DMSO), were both stored as 5 mM stock solutions at -20°C .

For these inhibitor studies, two methods of experimental protocol were employed. In the first method, drugs were added to Ca^{++} -free Earles' Ringer (5

TABLE I
Reactivation Media

Contraction	Relaxation	Rigor
0.1 M PIPES*	0.1 M PIPES	0.1 M PIPES
10 mM EGTA	10 mM EGTA	10 mM EGTA
1 mM free MgSO_4	1 mM free MgSO_4	1 mM free MgSO_4
10^{-5} M free CaCl_2	$<10^{-8}$ M free CaCl_2	10^{-5} M free CaCl_2
4 mM MgATP	4 mM MgATP	No MgATP

* pH, 6.94.

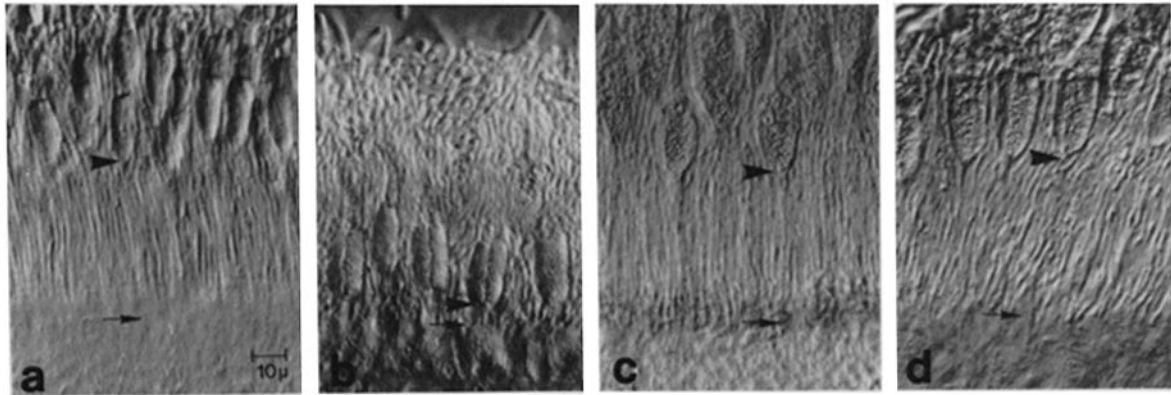


FIGURE 1 Nomarski light micrographs of chopped retinal slices of four half-retinas taken from the same fish; half retinas were fixed either immediately after dissection (a), or after 18 min in the following reactivation media: (b) contraction medium; (c) relaxation medium; and (d) rigor medium (see Table I). Cone myoid length was measured as the distance from the base of the ellipsoid (arrowheads) to the outer limiting membrane (arrows). $\times 625$.

TABLE II
Formulae for $Ca/EGTA$ Buffers

pH	Concentration				Calculated		pCa
	EGTA	MgSO ₄	CaCl ₂	free Mg ⁺⁺	free Ca ⁺⁺		
	M	M	M	M	M		
6.94	1×10^{-2}	1.33×10^{-3}	None	10^{-3}	10^{-8}	<8.0	
6.94	1×10^{-2}	1.33×10^{-3}	1.75×10^{-4}	10^{-3}	10^{-8}	8.0	
6.94	1×10^{-2}	1.32×10^{-3}	5.33×10^{-4}	10^{-3}	3.16×10^{-8}	7.5	
6.94	1×10^{-2}	1.28×10^{-3}	1.51×10^{-3}	10^{-3}	10^{-7}	7.0	
6.94	1×10^{-2}	1.21×10^{-3}	3.61×10^{-3}	10^{-3}	3.16×10^{-7}	6.5	
6.94	1×10^{-2}	1.12×10^{-3}	6.4×10^{-3}	10^{-3}	10^{-6}	6.0	
6.94	1×10^{-2}	1.02×10^{-3}	9.48×10^{-3}	10^{-3}	10^{-5}	5.0	

mM EGTA; $<10^{-8}$ M free Ca^{++}), detergent, and detergent-free contraction media, to yield final concentrations of 50, 100, and 200 μ M TFP and 10 μ M R24571. Dark-adapted retinas were preincubated in culture 15 min in Ca^{++} -free Earles' Ringer (5 mM EGTA; $<10^{-8}$ M free Ca^{++}) alone or with either drug. Retinas were then transferred to either contraction medium (10^{-5} M free Ca^{++}) alone, or with either drug for the two-step lysis-incubation procedure (18 min). DMSO (0.2%) in Earles' Ringer and in contraction medium served as a control for the R24571 studies. Drug concentrations remained constant throughout the consecutive culture and two-step procedures.

In the second method, lysed cones were incubated with the drugs for 90 min in relaxation medium before transfer to contraction medium containing the drugs. Dark-adapted retinas were lysed for 3 min in relaxation medium containing 1% Brij-58, and transferred to detergent-free relaxation media, either alone, or containing TFP or R24571 for 90 min incubation. One half-retina from each fish was fixed after the 90 min incubation to serve as a t_0 while other half-retinas were subsequently transferred to either contraction medium alone, or to contraction medium containing either TFP or R24571 for 18 min. All drug concentrations remained constant throughout the consecutive incubations.

Culture Studies in Ca^{++} -free Medium: To investigate whether external calcium is required for light-induced cone contraction in culture, dark-adapted retinas were first dissected with Ca^{++} -free Hanks' BSS containing 5 mM EGTA ($<10^{-8}$ M Ca^{++}) and then placed in either Ca^{++} -free or normal Hanks' balanced salt solution (1.3 mM $CaCl_2$; no EGTA) for 10 min in the light. As a control, some retinas were incubated in Ca^{++} -free Hanks' balanced salt solution in complete darkness. After incubation, retinas were then fixed and chopped into retinal slices for examination.

RESULTS

Effects of Free Ca^{++}

Concentration on Contraction

Fig. 2 illustrates the effects of altering free Ca^{++} levels on extents of reactivated contraction achieved by lysed cone

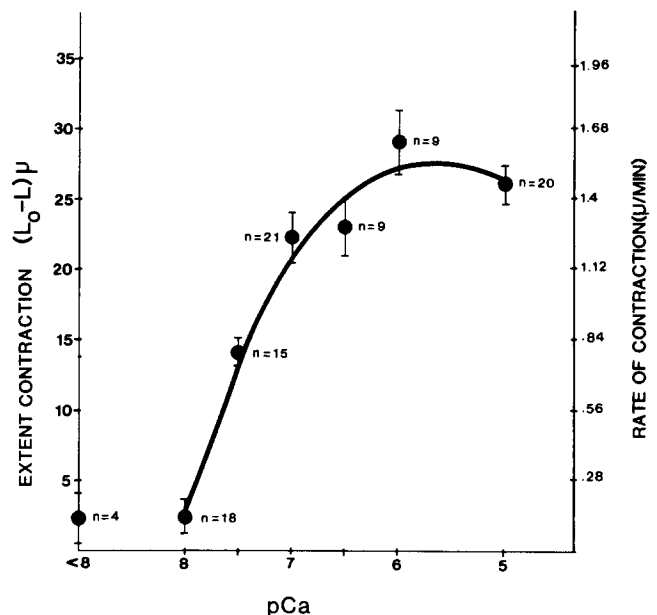


FIGURE 2 Effects of free Ca^{++} level on the extent of reactivated contraction observed after 18 min in contraction medium. Because contraction rates were linear for 18 min at two of the indicated Ca^{++} concentrations (see Fig. 3), equivalent rates of contraction have been indicated on the right axis. Bars, \pm SEM; n, number of retinas examined. Extent of contraction ($L_0 - L$) was obtained by subtracting final (reactivated) cone myoid length (L) for each retina from the initial cone myoid length (L_0) measured in directly fixed (t_0) retinas from the same fish.

models after 18 min in contraction medium containing 4 mM ATP. Reactivated contraction did not occur if free Ca^{++} was $\leq 10^{-8}$ M (pCa 8.0). Maximal extent contraction was observed at $\geq 10^{-6}$ M free Ca^{++} (pCa 6.0). At intermediate free Ca^{++} levels, cone models contracted to intermediate extents with extent of contraction being proportional to free Ca^{++} concentration (Fig. 2).

To ascertain whether the intermediate extents of contraction observed in 18 min resulted from Ca^{++} effects on the rate of cone contraction, we examined the kinetics of contraction at two free Ca^{++} concentrations (pCa 5.0 and 7.5). At both concentrations rates of contraction were linear throughout the 18-min incubation procedure, with the rate at pCa 5.0 ($1.6 \mu\text{m}/\text{min}$) approximately twice that at pCa 7.5 ($0.8 \mu\text{m}/\text{min}$) (Fig. 3). These results suggest, therefore, that changes in free Ca^{++} influence the rate rather than the ultimate extent of reactivated contraction. Thus an ordinate for rate is also provided in the dose-response curve in Fig. 2.

Transient Calcium Requirement for Reactivated Contraction

Cone models contracted similarly whether they were exposed to Ca^{++} throughout the 18-min two-step procedure or exposed just during the initial 3-min lysis step (Table III). This persistence of contraction after Ca^{++} removal indicates

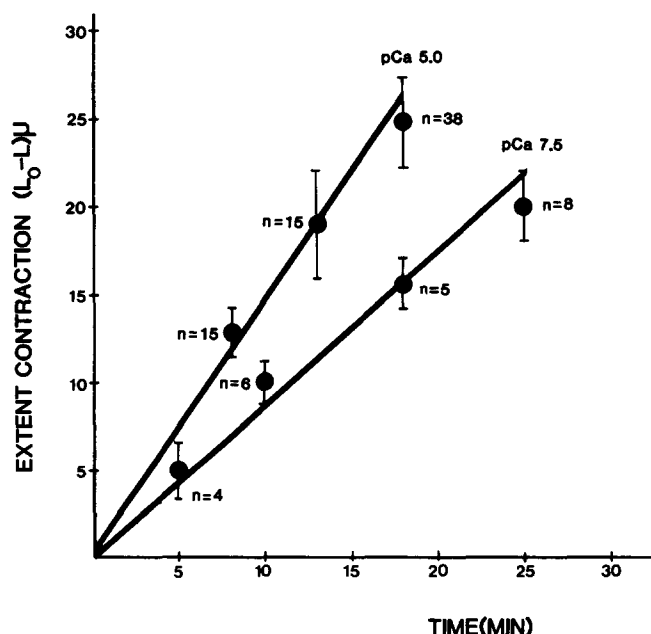


FIGURE 3 Kinetics of reactivated contraction for pCa 7.5 and 5.0. Because rates were linear for 18 min, we can express the different extents of contraction measured at 18 min and illustrated in Fig. 2 as rates. Thus the rate of contraction is proportional to the free Ca^{++} concentration. Bars, \pm SEM; n, number of retinas examined.

TABLE III
Transient Calcium Requirement

After 3 min in contraction medium (10^{-5} M Ca^{++} , 4 mM ATP) with 1% Brig-58:	Extent of contraction ($L_0 - L$) $\mu\text{m} \pm \text{SEM}$ (n)
+15 min in relaxation medium	30.5 ± 3.2 (4)
+15 min in contraction medium	27.9 ± 1.2 (10)

that in the cone models a transient exposure to Ca^{++} is sufficient to support subsequent contraction for at least 15 min.

Stability of Contractile Apparatus in Lysed Cone Models in the Absence of Ca^{++}

The contractile apparatus and Ca^{++} regulatory machinery of lysed cone models appear to be stable for at least 90 min in the absence of Ca^{++} . When cone models were lysed and incubated in relaxation medium ($<10^{-8}$ M free Ca^{++} , 10 mM EGTA) for 90 min and then transferred to contraction medium (10^{-5} M Ca^{++}), reactivated contraction was obtained that was indistinguishable from models lysed directly in contraction medium (Table IV). Apparently no necessary components are lost during the 90-min Ca^{++} -free incubation.

Effect of TFP and R24571 on Reactivated Contraction

To begin to investigate the role of CaM in mediating Ca^{++} control of contraction, we examined effects of the inhibitors TFP and R24571 on reactivated contraction in lysed cell models. Because transient Ca^{++} exposure suffices to activate full contraction for the 18-min incubation procedures (see above), it was clearly important for the drugs to be fully active before exposing the lysed cones to calcium. We used two approaches to effect this. Initially, retinas were exposed to the CaM inhibitors for a 15-min preincubation in culture before lysis. Addition of TFP to both culture and incubation steps inhibited reactivated contraction, although high concentrations (100 μM) were required for full inhibition. R24571 at 10 μM also inhibited contraction by 75%. Because the high concentration of TFP required for complete inhibition of contraction may have resulted from our allowing only 15 min for equilibration with the drug before lysis, we used a second protocol to allow maximal penetration. After lysis in relaxation medium cones were exposed to drugs for 90 min and then transferred to contraction medium containing the drug. With this procedure TFP strongly inhibited reactivated cone contraction at much lower concentrations (10^{-5} M) (Table V). R24571 also inhibited contraction under these conditions (Table V). DMSO (0.2%) did not affect reactivated contraction.

The extent of contraction observed with contraction medium controls in this series of experiments (Table V) was less than that observed in previous studies using the same procedure (Table IV). The reason for this discrepancy is not clear; it may reflect the fact that the fish were from different suppliers and the experiments were done in different seasons (summer with California fish for Tables III and IV, winter

TABLE IV
Stability of Contractile Apparatus in the Absence of Calcium

After 3 min in relaxation medium (10^{-8} M Ca^{++} , 4 mM ATP) with 1% Brig-58:	Extent of contraction ($L_0 - L_{\text{final}}$) $\mu\text{m} \pm \text{SEM}$ (n)
+15 min in relaxation medium	2.9 ± 1.7 (6)
+90 min in relaxation medium	5.5 ± 2.4 (6)
+15 min in relaxation medium	
+90 min in relaxation medium	25.2 ± 1.9 (9)
+15 min in contraction medium (10^{-5} M Ca^{++} , 4 mM ATP)	

with Ohio fish for Table V). Whatever the reason for the reduced cone excursions in controls, they do not compromise the interpretation of results in Table V inasmuch as all the reported results of experimental treatments are compared with contraction medium controls from the same fish.

Nucleotide Requirements for Reactivated Contraction

Fig. 4 is a dose-response curve illustrating the effect of varying MgATP concentration on the extent of cone contraction observed during an 18-min reactivation in 10^{-5} M Ca^{++} . Virtually no contraction was observed at 0 mM MgATP (rigor condition), whereas maximal contraction occurred at concentrations ≥ 1 mM MgATP. Cone models contracted to intermediate extents at concentrations between 0 and 1 mM MgATP. A half-maximal extent of contraction was observed at 9×10^{-5} M ATP.

The abilities of other nucleotides to support reactivated cone contraction are illustrated in Fig. 5. No significant contraction was observed when ATP was substituted with guanosine triphosphate, cytosine triphosphate, ITP, ADP, or AMPPNP in contraction medium (Fig. 5). These results are comparable with those observed in rigor medium (0 mM ATP).

Effect of ITP and ATP γ S on Reactivated Contraction

Table VI illustrates the effects of ITP and ATP γ S on reactivated contraction. In that these experiments were done using Ohio animals that produced smaller contraction excursions

in 18 min (see above and Table V), we increased the time to 30 min to allow greater excursions. ATP γ S when substituted for ATP in contraction medium did not support reactivated contraction. Earlier experiments (using the 18-min reactivation procedure) had shown that ITP substitution

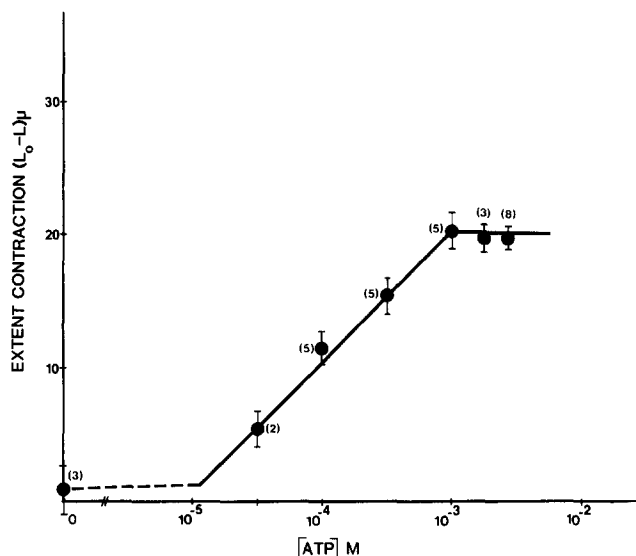


FIGURE 4 Effects of ATP concentration on reactivated cone contraction. All preparations had 1 mM free Mg in addition to the MgATP indicated. Bars, mean \pm SEM; number in parenthesis, number of retinas examined.

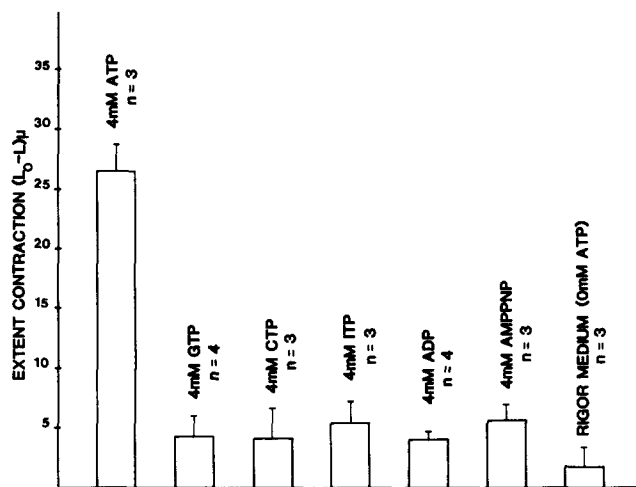


FIGURE 5 Nucleotide specificity of reactivated contraction. Substitution of ATP with 4 mM concentrations of guanosine triphosphate, cytosine triphosphate, ITP, ADP, or AMPPNP in contraction medium produced no significant contraction; this result was equivalent to that observed with rigor medium (0 mM ATP). Bar, \pm SEM; n, number of retinas examined.

TABLE V
Effect of TFP and R24571 on Reactivated Contraction

Incubation period	Extent of contraction ($L_0 - L$) $\mu\text{m} \pm \text{SEM} (n)$
90 min in relaxation medium + 18 min in contraction medium (4 mM MgATP; 10^{-5} M free Ca^{++})	14.7 ± 1.2 4
90 min in relaxation medium + 10 μM TFP + 18 min in contraction medium + 10 μM TFP	3.2 ± 1.0 2
90 min in relaxation medium + 25 μM TFP + 18 min in contraction medium + 25 μM TFP	0.55 ± 1.7 2
90 min in relaxation medium + 0.2% DMSO + 18 min in contraction medium + 0.2% DMSO	14.3 ± 1.8 4
90 min in relaxation medium + 10 μM R24571 (in DMSO) + 18 min in contraction medium + 10 μM R24571 (in DMSO)	-1.4 ± 1.8 4

Retinas were lysed for 3 min in relaxation medium ($<10^{-8}$ M free Ca^{++}) with 1% Brij-58 and then incubated.

TABLE VI
Effect of ITP and ATP γ S on Reactivated Contraction

Media for first 3-min lysis step	Media for second 27-min incubation step	Extent of contraction ($L_0 - L$) $\mu\text{m} \pm \text{SEM} (n)$
10^{-5} M Ca^{++} /4 mM ATP	10^{-5} M Ca^{++} /4 mM ATP	22.2 ± 1.5 (6)
10^{-5} M Ca^{++} /4 mM ATP	10^{-5} M Ca^{++} /—	6.4 ± 0.8 (3)
10^{-5} M Ca^{++} /4 mM ATP	—/4 mM ITP	21.9 ± 2.8 (3)
10^{-5} M Ca^{++} /4 mM ATP γ S	10^{-5} M Ca^{++} /4 mM ATP γ S	1.8 ± 0.7 (3)
10^{-5} M Ca^{++} /4 mM ATP γ S	10^{-5} M Ca^{++} /4 mM ITP	19.4 ± 1.5 (3)

Half retinas were lysed for 3 min in indicated reactivation media, then incubated for 27 min as indicated, and then fixed for cone length measurements.

for ATP also produced no contraction (see Fig. 5). Reactivated contraction did occur, however, if retinas were first exposed to ATP γ S during lysis for 3 min and then incubated with ITP for 27 min (Table VI).

We reported above that transient exposure to Ca⁺⁺ supports subsequent contraction for 15 min in the absence of Ca⁺⁺ if ATP is present throughout both steps (see above and Table IV). Therefore we reasoned that, if Ca⁺⁺ and ATP were supplied for the first step, ITP might successfully substitute for ATP in the second 15-min incubation in the absence of Ca⁺⁺. Reactivated cones behaved as we predicted. Cones did contract if retinas were first lysed in normal contraction medium (10⁻⁵ M Ca⁺⁺; 4 mM ATP) and then incubated in relaxation medium (<10⁻⁸ M Ca⁺⁺) with 4 mM ITP instead of ATP (Table VI). The extent of reactivated contraction observed with ITP in the second step (both with and without Ca⁺⁺) was indistinguishable from that observed in the control (normal contraction medium with 4 mM ATP).

Effect of cAMP on Reactivated Contraction

Addition of cAMP to contraction medium (10⁻⁵ M Ca⁺⁺, 4 mM ATP) strongly inhibited reactivated cone contraction at a concentration ≥ 10 μ M (Fig. 6).

Effect of External Calcium Removal on Cone Contraction in Cultured Retina

Ca⁺⁺ removal from the external medium did not interfere with the ability of intact cones to undergo light-induced contraction in culture. If dark-adapted retinas were cultured in the light for 10 min in the absence of Ca⁺⁺ (<10⁻⁸ M Ca⁺⁺ buffered with 5 mM EGTA), cones contracted to an extent comparable with that obtained in normal Ca⁺⁺-containing Hanks' balanced salt solution (~1.3 mM CaCl₂; no EGTA) (28.1 \pm 1.2 μ m vs. 34.3 \pm 1.2 μ m). This result suggests that extracellular Ca⁺⁺ is not required for cone contraction in culture at least for the first 10 min of contraction.

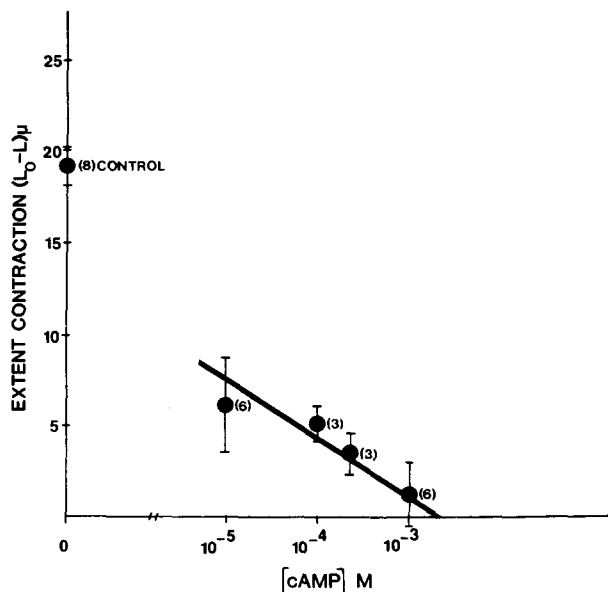


FIGURE 6 Effects of cAMP on reactivated contraction in cone models. cAMP when added to contraction medium (10⁻⁵ M Ca⁺⁺, 4 mM ATP) inhibited reactivated contraction in a dose-dependent manner. Bars, \pm SEM; n, number of retinas examined.

DISCUSSION

In this study we have investigated the role of Ca⁺⁺ in regulating cone contraction. A considerable body of work has now accumulated that suggests that in nonmuscle cells, as in smooth muscle, myosin phosphorylation plays a major role in Ca⁺⁺ regulation of the actin-myosin interaction (3, 23, 29, 50, 70). Therefore, we have specifically employed a variety of treatments designed to evaluate the possible role of myosin phosphorylation in regulating cone contraction. Unfortunately, the cones that we study comprise a minor component of the volume of the whole retinas used in our model procedures; thus we are unable to ascertain directly whether cone myosin is phosphorylated during reactivated contraction in the models. Instead, we have analyzed the responses of the models to Ca⁺⁺, CaM inhibitors, nucleotide analogs, and cAMP, so that these responses can be compared with the effects of these agents on purified preparations of smooth and nonmuscle proteins and with other lysed cell models. In all treatments we have employed, cone models responded in a manner consistent with the postulated role for myosin phosphorylation in Ca⁺⁺ regulation.

Contraction in cone models was Ca⁺⁺ dependent, with the rate of contraction proportional to a free Ca⁺⁺ concentration between 3.16 \times 10⁻⁸ and 10⁻⁶ M. This dose-response curve reported here for Ca⁺⁺ effects on the rate of reactivated cone contraction is at least an order of magnitude lower than is generally observed for reactivated contraction in skinned smooth muscle fibers or glycerinated myofibrils (26, 37). There are several possible explanations for this discrepancy. Cone myoids are small (<5 μ m diam) and not under tension, thus relatively fewer activated myosin heads may suffice to support contraction than in smooth or skeletal muscle. Also, if indeed cone contraction is activated by myosin phosphorylation, the Ca⁺⁺ curve would be highly sensitive to the relative extents of extraction of CaM, MLCK, and MLCK phosphatase after lysis.

Phosphatase would appear to be lost or relatively inactive in our models because a brief exposure of cone models to Ca⁺⁺ suffices to activate full subsequent contraction in 10 mM EGTA. This situation contrasts to that of smooth muscle where exposure to EGTA after Ca⁺⁺ produces relaxation within 5 min (15). Our results might also reflect a low cone phosphatase activity in vivo. Because cone contraction occurs under normal conditions only once a day at dawn, and subsequent elongation (relaxation) is not required until 10–14 h later at dusk, these cells would not appear to have as great a need for a highly active myosin light chain phosphatase as other cell types with physiological changes in contractility of a shorter time course. Such a suggestion is consistent with results of recent studies with several fish species indicating that very brief light flashes (milliseconds) are sufficient to induce the full extent of cone contraction in a subsequent 30–45 min of darkness (43, 62). In vivo, as in the models, once activated, cone contraction tends to continue to completion.

Other components of the Ca⁺⁺ regulatory machinery appear, in contrast, to be effectively retained in cone models, because models exposed to 10 mM EGTA for 90 min produce full contraction if Ca⁺⁺ is subsequently added. In skinned smooth muscle preparations, adding 5 μ M exogenous CaM shifts the Ca⁺⁺ curve for tension activation a full order of magnitude lower (37). Levels for endogenous CaM in this range have been estimated for brain (51) and smooth muscle (5). Inasmuch as the maximal rate of reactivated contraction

in cone models approximates that observed *in vivo*, we may well be retaining physiological levels of CaM after lysis in the models. Similar retention of CaM with the cytoskeleton in the absence of Ca⁺⁺ is observed in the intestinal brush border (34).

The retention of MLCK in our lysed cell models would not be surprising because MLCK has been shown to be associated with stress fibers in several nonmuscle cells (24) and with the membrane cytoskeleton in lymphocytes (10).

Myosin also appears to be retained in our cone models. In smooth muscle, there is considerable controversy as to the state of assembly of myosin in the relaxed and contractile states (56). Recent studies of smooth muscle and nonmuscle myosin assembly *in vitro* suggest that myosin phosphorylation may be necessary for myosin assembly as well as for myosin-actin interaction (20, 48, 49, 58). The finding of Cande et al. (14) that myosin is extracted under relaxation conditions in glycerinated smooth muscle, is consistent with this suggestion. If the suggested assembly mechanisms hold for cones, then one might expect cone myosin to be extracted during the 90-min incubation in relaxation medium. However, inasmuch as we observe maximal contraction upon addition of Ca⁺⁺, we must conclude that either cone myosin filaments did not dissociate in relaxation medium or that they failed to diffuse out of the models and thus were able to reassemble upon addition of Ca⁺⁺.

Calmodulin Inhibitors

Reactivated cone contraction is strongly inhibited by the CaM inhibitors, TFP (40) and R24571 (28, 60). Although not completely specific for CaM, these inhibitors have been widely used to study the involvement of CaM in several Ca⁺⁺-regulated physiological processes, including smooth muscle and nonmuscle motility (3, 28, 40, 60). TFP has been shown to inhibit Ca⁺⁺-activated tension and myosin light chain phosphorylation in skinned smooth muscle strips (16) without inhibiting myosin ATPase activity (21, 31, 53). Although it is clear that our CaM inhibitor results are not conclusive demonstration of CaM participation, they are nonetheless consistent with those obtained with smooth muscle and other nonmuscle studies (3, 16) and with the postulated role of CaM in activation of cone contraction.

Nucleotide Studies

The ATP dose-response curve reported here for lysed cone models indicated that 1 mM ATP was required for maximal contraction, with half-maximal produced by 10⁻⁴ M ATP. This requirement for high ATP concentrations resembles that for reactivated contraction in glycerinated smooth muscle cells (14) and is consistent with the reported *K_m* for platelet MLCK of 121 μM (6).

Results of our studies using the ATP_γS are consistent with the postulated role of myosin phosphorylation in Ca⁺⁺ regulation of cone contraction. The nucleotide requirement for the phosphorylation of the myosin light chain is highly specific for ATP and is not supported by other nucleotides (44). However, the ATP analog, ATP_γS, will serve as a substrate for MLCK, and leads to the thiophosphorylation of myosin light chains and consequent activation of myosin ATPase (15). ATP_γS does not, however, serve as substrate for myosin ATPase (15). Cassidy et al. (15) has shown that incubation of skinned smooth muscle fibers with ATP_γS in the presence of

Ca⁺⁺ led to myosin phosphorylation, but tension development (contraction) occurred only when ATP was subsequently added. In contrast, the nucleotide, ITP, does not support MLCK activity (68) but is hydrolyzed by myosin ATPase even better than ATP (42, 68). The effects of both ATP_γS and ITP on reactivated contraction in cone models are analogous to those for ATP_γS and ITP (68) or cytosine triphosphate (65) in skinned smooth muscle fibers and thus provide the strongest evidence for a role for myosin phosphorylation.

Effects of cAMP

We report here that the presence of cAMP in contraction medium (10⁻⁵ M Ca⁺⁺, 4 mM ATP) strongly inhibits reactivated cone contraction. This result is consistent with previous reports that culturing retinas with dibutyl-cAMP likewise inhibits contraction in intact cones (22), and even induces cone elongation (11). It is also consistent with the observation that retinal cAMP levels are high in the dark and fall with light onset (11). Inhibition of contraction by cAMP has also been observed in smooth muscle where it has been suggested that cAMP-dependent phosphorylations might decrease contractile activity indirectly, by lowering the concentration of Ca⁺⁺ in the cell, and/or directly, by decreasing the sensitivity of the contractile apparatus to Ca⁺⁺ (7, 8, 52, 54). In lysed cell models of cones, a direct effect on the contractile machinery is more likely than an alteration of Ca⁺⁺ levels in that free Ca⁺⁺ is regulated by 10 mM EGTA buffers. A direct effect of cAMP on MLCK has been reported both for smooth muscle (7) and nonmuscle cells (3, 4, 30). MLCK is phosphorylated by a cAMP-dependent kinase; when phosphorylated at two sites it binds Ca⁺⁺/CaM 15–20 times more weakly than unphosphorylated MLCK (19). In the cone models cAMP inhibition of contraction is 70% complete at 100 μM, and complete at 1 mM. Because the *K_m* for cAMP-dependent kinase is in the micromolar range, our graded dose-response curve at higher concentrations is perhaps surprising. However, higher cAMP concentrations may be required because we have added cAMP and Ca⁺⁺ simultaneously to the models. MLCK affinity for Ca⁺⁺/CaM is reduced only if both cAMP-dependent sites are phosphorylated, and the second site cannot be phosphorylated if Ca⁺⁺/CaM is bound to the MLCK (19). cAMP may also inhibit cone contraction directly by catalyzing phosphorylation of some protein component of the contractile machinery other than MLCK. cAMP-dependent phosphorylation of actin (2, 66), filamin (63), and severin (9, 39) have been reported in other cell types. Though we cannot rule out an indirect effect of cAMP on free Ca⁺⁺ levels in intact cones *in vivo*, our results with cone models suggest that a direct effect of cAMP on the motile machinery plays an important role in regulating cone motility.

Sources of Ca⁺⁺ in Vivo

Several observations suggest that *in vivo* the Ca⁺⁺ responsible for activating cone contraction is derived both from internal stores and from extracellular sources. We report here that intact cones in cultured retinas exhibited normal light-induced contraction in Ca⁺⁺-free culture medium (5 mM EGTA; <10⁻⁸ M free Ca⁺⁺) for at least 10 min after light onset; thus it seems clear that light onset can induce Ca⁺⁺ release from internal stores. However, cones failed to contract to the fully light-adapted position after longer incubations (30 min) in the light with Ca⁺⁺-free culture medium (1 mM

EGTA; $<10^{-8}$ M free Ca^{++}) (22). Together these results suggest that cone contraction can be initiated by Ca^{++} release from internal stores but that an external source of Ca^{++} is necessary for completion of contraction over a longer time course. A similar situation has been reported for some smooth muscles, where agonist-induced contraction could be initiated in EGTA, but for sustained contraction, extracellular Ca^{++} was required (61, 69).

Conclusions

The observations reported here strongly suggest that myosin phosphorylation plays a role in Ca^{++} regulation of cone contraction. Under a wide variety of experimental conditions, cone motile models behaved in ways consistent with those observed in skinned or glycerinated smooth muscle, where contraction has been directly correlated with myosin phosphorylation (1, 17, 33). Because the conditions required for contraction in lysed cone models were also consistent with those reported for Ca^{++} activation of myosin ATPase in actomyosin preparations from other nonmuscle cells, our results further support the previously postulated role for myosin phosphorylation in regulation of nonmuscle contractility. We are currently trying to develop motile models of isolated cones so that we may characterize myosin phosphorylation directly.

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