N-Ethylmaleimide-modified Subfragment-1 and Heavy Meromyosin Inhibit Reactivated Contraction in Motile Models of Retinal Cones

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ABSTRACT The mechanism of contraction in motile models of teleost retinal cones has been examined by using *N*-ethylmaleimide (NEM)-modified myosin fragments (NEM-S-1 and NEM-heavy meromyosin [HMM]) to prevent access of native myosin to actin filaments during reactivation of contraction. In the diurnal light/dark cycle, retinal cones of green sunfish (*Lepomis cyanellus*) and bluegill (*Lepomis macrochirus*) exhibit length changes of >90 μm. The motile myoid region of the cone contracts from 100 μm in the dark to 6 μm in the light. Motile models for cone contraction have been obtained by lysis of dark-adapted retinas with the non-ionic detergent, Brij-58. These cone motile models undergo Ca^{++}-and ATP-dependent reactivated contraction, with morphology and rate comparable to those observed in vivo (Burnside, B., B. Smith, M. Nagata, and K. Porrello, 1982, *J. Cell Biol.*, 92:198–206). The cone myoids contain longitudinally oriented actin filaments which bind myosin subfragment-1 (S-1) to form characteristic "arrowhead" complexes which dissociate in the presence of MgATP (Burnside, B., 1978, *J. Cell Biol.*, 78:227–246). Modification of S-1 or HMM with the sulfhydryl reagent, NEM, produces new species, NEM-S-1 or NEM-HMM, which still bind actin but which fail to detach in the presence of MgATP (Meeusen, R. L., and W. Z. Cande, 1979, *J. Cell Biol.*, 82:57–65). We have used NEM-S-1 and NEM-HMM to test whether cone contraction depends on an actomyosin force-generating system. We find that reactivated contraction of cone models is inhibited by NEM-S-1 and NEM-HMM but not by the unmodified species, S-1 and HMM. Thus, reactivated cone contraction exhibits NEM-S-1 and NEM-HMM sensitivity as well as Ca^{++}- and ATP-dependence. These observations are consistent with an actomyosin-mediated mechanism for force production during cone contraction.

Actomyosin contractile systems play a central role in a variety of cellular processes such as cytokinesis, phagocytosis, and cell locomotion (8, 10). The contractile proteins of nonmuscle cells are similar to those found in muscle; however, their polymers appear to be less highly ordered, less stable, and vary in composition among different cell types (8, 21). The major components of these systems are actin and myosin. Although the interactions of actin and myosin are well characterized in muscle (12), the mechanisms of force production and transmission for nonmuscle cell contraction remain poorly understood.

To further characterize the structure and physiology of contraction in a nonmuscle cell, we have been examining cone contraction in the teleost retina. Teleost retinal cones elongate in darkness and contract in light. These movements are part of a coordinated morphological rearrangement of the photoreceptors and pigment granules of the retinal pigment epithelium, which serve to position the photoreceptors and pigment optimally for vision in bright or dim light (1, 2). Cone contraction is mediated by the necklike myoid region which contains longitudinally oriented actin filaments (as identified by subfragment 1 [S-1] binding) and thick (myosinlike) filaments (5).

To study the mechanism of contraction in teleost cones, we have developed detergent-lysed motile models (6). Because cone contraction is uniaxial and exhibits a large excursion, it is more easily quantified in cone models than in reported models from other cell types (3, 7, 11, 19, 25). Long dark-adapted cones, lysed with the nonionic detergent, Brij-58, yield

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cone motile models which undergo Ca^{++}- and ATP-dependent reactivated contraction, with morphology and rates comparable to those observed in vivo (6).

In this study we have used *N*-ethylmaleimide–subfragment 1 (NEM–S-1) and NEM-heavy meromyosin (HMM) to prevent access of native myosin to actin filaments in reactivated cone models. Enzymatic digestion of rabbit skeletal muscle myosin with trypsin or papain yields either HMM or S-1, respectively (15, 16). Since both of these fragments contain the myosin binding sites, they bind tightly to actin filaments to form characteristic arrowhead complexes. S-1 and HMM therefore have been useful tools in studying microfilament-based systems where they serve to identify filaments as actin, as well as define filament polarity (13). Modification of HMM or S-1 with the sulfhydryl reagent, NEM, yields a product (NEM-HMM or NEM–S-1) which still binds to actin filaments but is not removed by the presence of MgATP (17). Thus, NEM-HMM or NEM–S-1 can act in situ to prevent access of native myosin to actin filaments and consequently block actomyosin-mediated force production for contraction. Accordingly, NEM–S-1 and NEM-HMM have therefore been used as probes for investigating actomyosin-mediated functions in various cell types (17, 18, 27). NEM-HMM blocks contraction in glycerinated muscle myofibrils and inhibits cytokinesis when injected into amphibian eggs (18), while NEM–S-1 inhibits cytokinesis in permeabilized PTK_1 cells (7). This action appears to be specific for actomyosin systems, since these NEM-fragments do not interfere with the in vitro polymerization of microtubules or the beating of demembranated cilia (17).

We report here that NEM-modified S-1 and HMM block reactivated contraction in teleost retinal cone models. This finding further reinforces the argument that cone contraction is actomyosin-mediated and thus provides a useful model for physiological and structural studies of the mechanism of force production in nonmuscle cells.

MATERIALS AND METHODS

Animals: All experiments were carried out on retinas from dark-adapted green sunfish (*Lepomis cyanellus*) or bluegill (*Lepomis macrochirus*). Studies in the lab primarily focus on green sunfish; the closely related bluegill was used for some studies because of temporary difficulties in obtaining green sunfish. The behavior of cones from the two fish is indistinguishable. Fish were obtained from Funez Fish Farm (Sebastopol, CA) and maintained either in outdoor ponds or in laboratory aquaria. Indoor fish were kept on a light cycle to mimic that of outdoors.

Effects of circadian fluctuations on cone length were avoided by performing the experiments at the same time of day (14). Fish were placed in an aerated dark box at 11 AM and experiments begun at approximately 4 PM. Dark-observed at night (14). The advantage of this procedure is that it avoids the loss of very long fragile cones during dissection, yet still provides cones with sufficiently long myoids (40–60 μm) for easy measurement of length changes.

Preparation of Retinas: Experiments on bluegill were carried out under dim red light while those on green sunfish were done under infrared illumination with a Find-R-Scope infrared converter (FJW Industries, Mt. Prospect, IL). Retinas were gently detached with oxygenated Ca^{++}-free Hanks' balanced salt solution containing 5 mM EGTA ($<10^{-8}$ M free Ca^{++}) and then bisected along the choroid fissure, thus producing four half-retinas per fish. One half-retina was placed directly into fix (t_0) to provide initial cone length measurements. The other three halves were incubated for 3 min in Linbro tissue culture trays with 6-mm wells containing contraction media (Table I) with 1% Brij-58 (polyoxethylene 20 cetyl ether; Sigma Chemical Co., St. Louis, MO), a non-ionic detergent. The retinas were then transferred to detergent-free contraction medium with either no addition, S-1 (or HMM), or NEM–S-1 (or NEM-HMM), and incubated for 15 min. They were then fixed and either prepared for electron microscopy (see below) or prepared as retinal slices. For the latter, treated and t_0 retinas were placed in 6% glutaraldehyde with 0.1 M phosphate buffer, pH 7.0, overnight, and then cut into 25–50 μm thick slices with a manual tissue-chopper as previously described (6).

TABLE I

Reactivation Media

Contraction	Relaxation	Rigor
0.1 M PIPES, pH 6.94	0.1 M PIPES, pH 6.94	0.1 M PIPES, pH 6.94
5 mM EGTA	5 mM EGTA	5 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 Mg-ATP	4 mM Mg-ATP	No Mg-ATP

The free $[Ca^{++}]$ used are based on formulae for calcium/EGTA buffers from Steinhardt et al. (24); this calculation assumes an association constant for EGTA with calcium of $10^{10.7}$.

TABLE II

Formulae for Ca/EGTA Buffers Concentration, M

pH	EGTA	$MgSO_4$	$CaCl_2$	Calculated free Mg^{++}	Calculated free Ca^{++}
6.94	5×10^{-3}	1.16×10^{-3}	None	10^{-3}	10^{-8}
6.94	5×10^{-3}	1.01×10^{-3}	4.74×10^{-3}	10^{-3}	10^{-5}

Electron Microscopy: For ultrastructural examination, retinas were placed in fix containing 1% glutaraldehyde (TAAB) in 0.1 *M* phosphate buffer, pH 7.0, with 0.2% tannic acid and 1.0 mM $MgCl_2$, for 1–1.5 h at room temperature. The retinas were then cut into blocks and postfixed in cold 1% OsO_4 in 0.1 M phosphate buffer, pH 6.0, for 45 min. After dehydration in graded ethanols, the retinas were embedded in Epon 812 and sectioned parallel to the long axis of the cones. Thin sections were stained with uranyl acetate and lead citrate and viewed with a JEOL 100S electron microscope. Thick (1-μm) sections were used for cone length measurements.

NEM-modified S-1 and HMM Studies: NEM-S-1 and NEM-HMM were prepared from rabbit skeletal muscle myosin as described elsewhere (17). Some preparations were lyophilized for storage and others were stored in 50% glycerol. Preparations stored by both procedures were effective in blocking contraction in cone motile models; however, glycerol-stored NEM–S-1 produced better arrowheads in morphological decoration studies. For the bluegill studies, lyophilized S-1, NEM–S-1, HMM, and NEM-HMM were resuspended in detergent-free contraction medium (Table I) to yield final concentrations of 2–4 mg/ml. Meeusen and Cande (17) found that newly prepared NEM-HMM and NEM–S-1 retained substantial Ca^{++}- ATPase activity, thus suggesting that some HMM and S-1 had not reacted with NEM. Since, after lyophilization and storage, only approximately one-third of this Ca^{++}-ATPase activity remained, we suspected that denaturation had occurred during storage and consequently we increased the concentration of both NEM–S-1 and S-1 from 4 to 12 mg/ml for inhibition tests with green sunfish cone models. 6 mg/ml glycerin-stored NEM–S-1 was just as effective at inhibiting cone contraction as 12 mg/ml lyophilized protein. NEM–S-1 and NEM-HMM were exhaustively dialyzed to remove unreacted NEM. We are confident that unreacted NEM did not contribute to inhibition of contraction since heat-denatured NEM-HMM and NEM-bovine serum albumin (BSA) produced no inhibition of contraction in glycerinated myofibrils (17).

S-1 Decoration: S-1 for the actin filament decoration of green sunfish was a gift from Dr. Roger Cooke (University of California, San Francisco). S-1 stored in 75% glycerol was added to detergent-free rigor medium (Table I) to a final protein concentration of 5 mg/ml (7.5% glycerol). Dark-adapted retinas were lysed in rigor medium containing 1% Brij-58 for 3 min and then transferred to detergent-free rigor plus S-1 medium for 15-min incubation. The retinas were then placed in fix and prepared for electron microscopy. As a control, one retina was incubated in S-1 with 4 mM MgATP present.

NEM–S-1 Decoration: It has been our experience that NEM-HMM and NEM–S-1 deteriorate more rapidly during prolonged storage than unmodified HMM or S-1. This problem has been the experience of others as well and it is advisable to use freshly prepared modified proteins promptly. To obtain satisfactory decoration, we used NEM–S-1 that was freshly prepared and stored in 50% glycerol (at −70°C). This NEM-S-1 preparation was added to both detergent-free contraction and rigor media to a final protein concentration of 6 mg/ml (with 25% glycerol), and these solutions were used in the standard reactivation protocol. Retinas were lysed in either contraction or rigor medium containing 1% Brij-58 for 3 min and then transferred to detergent-free contraction or rigor medium with NEM–S-1 for 15-min incubation. The retinas were then placed in fix and prepared for electron microscopy.

Measurement of Cone Lengths: Retinal slices were examined at × 40 with Nomarski optics (see Fig. 2). Plastic 1-μm sections were examined with bright field. Cone myoid length was measured with an ocular micrometer, as the distance from the ellipsoid base to the outer limiting membrane (OLM). Twenty representative cones were measured from each retina. In all figures, *n* refers to numbers of retinas examined. Extents and rates of contraction were determined by comparing experimental values to t_0's for the same fish. The significance of differences between mean cone lengths was statistically tested by analysis of variance. Light micrographs served as permanent records.

RESULTS

Effects of NEM-S-1 and NEM-HMM on Reactivated Cone Contraction

BLUEGILL STUDIES: Initial experiments were performed on bluegill using dim red light for dissection. When dark-adapted bluegill retinas were lysed and incubated in contraction medium containing 2–4 mg/ml of NEM-S-1, reactivated cone contraction was inhibited 84%. However, lysed cones incubated in contraction medium alone or with S-1 proceeded to contract to similar extents at rates similar to those observed in vivo for green sunfish (6). The extent of reactivated contraction was slightly greater in contraction medium alone (33.5 ± 2.2 μm) than it was when S-1 was present (28.9 ± 5.4 μm). Lysed cones incubated in NEM-S-1 exhibited myoid lengths comparable to those observed in directly fixed (t_0) retinas.

Parallel experiments were carried out using HMM and NEM-HMM in place of S-1 and NEM-S-1 respectively, to compare their relative inhibitory effects on reactivated cone contraction. As with S-1, normal HMM had little effect on reactivated contraction, while NEM-HMM inhibited contraction by 45%. NEM-HMM inhibition, however, was less effective than that observed with NEM-S-1, at approximately the same concentrations (45% vs. 84%).

GREEN SUNFISH STUDIES: All subsequent experiments were performed on green sunfish when they again became available, since this species has been used for previous motile model studies in this lab. To avoid all possibility that dim red light might trigger cone contraction and thus compromise t_0 cone lengths, we carried out all subsequent dissections under infrared illumination.

Fig. 1 illustrates effects of various media on the extent of reactivated cone contraction in green sunfish. When dark-adapted retinas were subjected to the two-step lysis-incubation procedure in contraction medium, cones exhibited reactivated contraction (Fig. 1), at a rate comparable to light-induced contraction in vivo. No contraction was observed if free calcium concentrations were $<10^{-8}$ M (relaxation medium; Table I) or if MgATP was deleted (rigor medium; Table I) (Fig. 1). Characterization of the two-step reactivation procedure and comparison of model contraction rates to those in vivo have been described in detail in a previous paper (6).

Adding S-1 to contraction medium had no effect on reactivated cone contraction (Figs. 1 and 2 *b* and *c*). However, adding NEM-S-1 not only completely inhibited contraction but actually produced slight cone elongation over t_0 values (Fig. 2 *a* and *d*)—resulting in a negative value for extent contraction (Fig. 1). Similar cone elongation was produced by rigor medium with S-1 (Fig. 1). Higher concentrations of NEM-S-1 were used for green sunfish (12 mg/ml) than for bluegill (2–4 mg/ml) experiments; thus it is not surprising that the effectiveness of NEM-S-1 inhibition is greater in the green sunfish experiments.

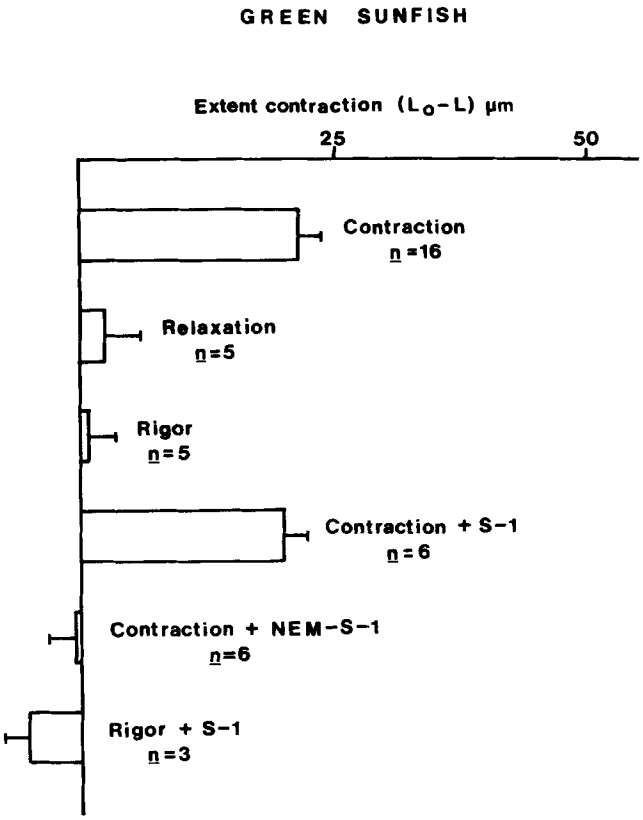

FIGURE 1 Histogram illustrating extent of reactivated cone contraction in green sunfish obtained with two-step lysis-incubation procedure. Effects of contraction, relaxation, and rigor media (see Table I), of contraction medium with added S-1 and NEM-S-1 and rigor medium with added S-1 and NEM-S-1, are indicated. Bar represents standard error. Extent of contraction (L_o-L) was obtained by subtracting final (reactivated) cone myoid length (L) for each retina from the initial cone myoid length (L_o) measured in directly fixed (t_0) retinas from the same fish.

Ultrastructure of Cone Models

A more detailed examination of cone model ultrastructure has been described in a previous paper (6). Treatment with our two-step lysis-incubation procedure produced extensive extraction of the otherwise dense cone-myoid cytoplasmic matrix, leaving the cytoskeletal elements well preserved (Figs. 3 and 4). Thin (actin) filaments were clearly visible in sections cut tangential to the cone myoid plasma membrane (Fig. 3). Most visible thin filaments were closely associated with the plasma membrane. Microtubules and intermediate filaments were also present in the myoids of lysed cone models (Fig. 3).

The ellipsoids of cones in directly fixed retinas contain prominent bundles of 50–100 thin filaments which originate in the microvillus-like calyceal processes at the base of the outer segment and course down the periphery of the ellipsoid just beneath the plasma membrane (Fig. 4 *a*). The center of the ellipsoid is occupied by densely packed mitochondria (Fig. 4 *a*).

In Brij-extracted cones, the ellipsoid mitochondria became vesiculated and severely extracted (Fig. 4 *b* and *c*). However, the plasma membrane was rarely disrupted or fragmented; in most cones, plasma membranes appeared to be intact (Fig. 4 *b* and *c*) (6). (The disruption of cone plasma membranes in Fig. 4 *e* and *f* was observed only in NEM-S-1 preparations containing high concentrations of glycerol.) The bundles of thin filaments in the ellipsoid were not disrupted or splayed by

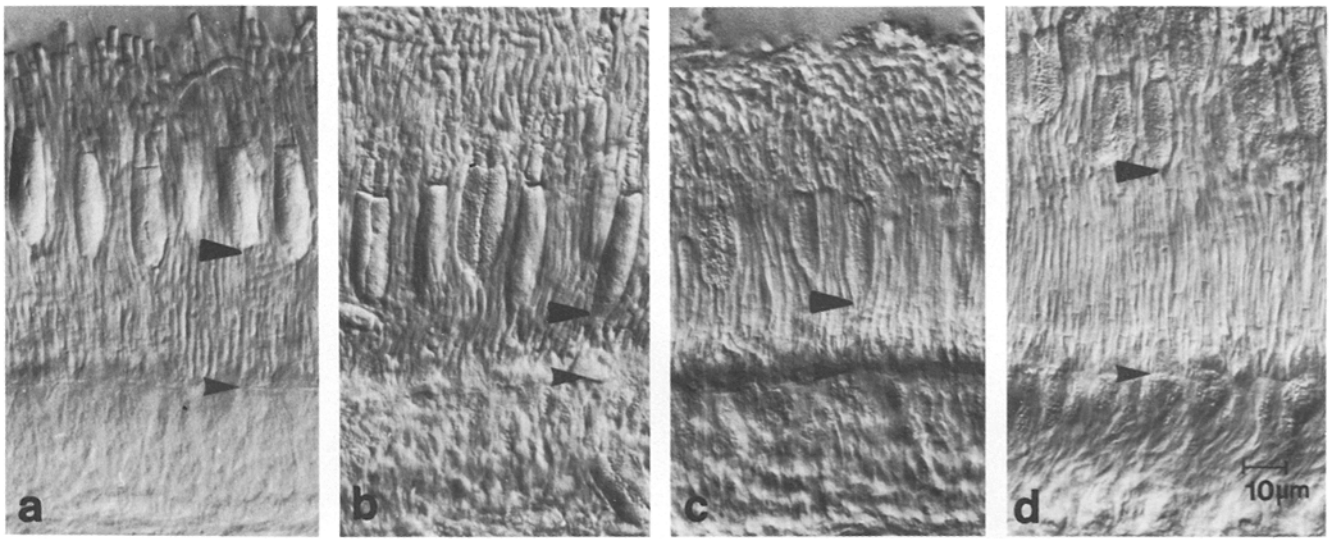

FIGURE 2 Nomarski light micrographs of four retinal slices from a single green sunfish illustrating results shown in Fig. 1. Slices from four half-retinas from the same fish were fixed (*a*) immediately after dissection (t_0), and (*b*, *c*, and *d*) after 18 min of two-step lysis-incubation procedure: (*b*) in contraction medium alone; (*c*) in contraction medium + S-1; and (*d*) in contraction medium + NEM-S-1. Cone myoid length was measured from the base of the ellipsoid (large arrowhead) to the outer limiting membrane (small arrowhead). × 625.

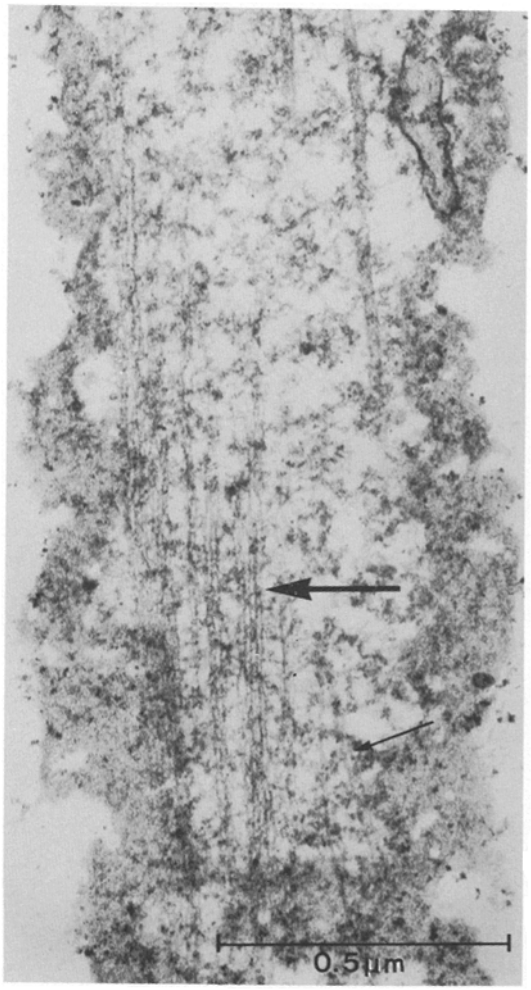

FIGURE 3 Electron micrograph of a green sunfish cone myoid after two-step procedure in rigor medium. The myoid is cut slightly tangential to the long axis. Actin filaments (large arrow) are prominent and abundant close to the plasma membrane but rare in the center of the myoid. Intermediate filaments (small arrow) and microtubules are also present. × 75,000.

detergent treatment (Fig. 4*b*). If detergent-lysed cone models were incubated with unmodified S-1 in rigor medium (no ATP), actin filaments were sparsely decorated with characteristic arrowhead complexes, though apparently not all actin binding sites were occupied (Fig. 4*c* and *d*). Under these conditions the ellipsoid actin bundles were often splayed into individually decorated filaments (Fig. 4*d*). Thus, it seems clear that S-1 does cross the plasma membrane in detergent-treated models, though not so freely as occurs after glycerin extraction of retinas (5). Actin filaments did not decorate in the detergent-treated models if ATP (4 mM) was included with the unmodified S-1 (Fig. 4*b*).

When detergent-lysed cone models were incubated with NEM–S-1 in either contraction (Fig. 4*e*) or rigor (Fig. 4*f*) medium, actin filaments appeared decorated with occasional recognizable arrowheads, though decoration was clearly inferior to that obtained with unmodified S-1. Nonetheless, NEM–S-1 decorated filaments were distinguishable from undecorated filaments in S-1 plus ATP-treated preparations (compare Fig. 4*e* and *f* to *b*). Thus, NEM–S-1 appears to be crossing the plasma membrane in detergent-treated cone models, though binding to actin is not so extensive as observed with unmodified S-1. Clearly, it is not necessary for the NEM–S-1 to saturate all actin binding sites for successful inhibition of contraction. Reactivated contraction was fully blocked in the NEM–S-1-treated retinas shown in Fig. 4*e* in spite of the sparse arrowhead formation.

DISCUSSION

We have previously shown that detergent-lysed teleost retinal cones undergo Ca^{++}- and ATP-dependent reactivated contraction, with morphology and rate comparable to that observed in vivo (6). We report here that reactivated cone contraction is inhibited by NEM–S-1 and NEM-HMM. At concentrations of 12 mg/ml, NEM–S-1 totally blocks reactivated contraction (Fig. 1), whereas at 2–4 mg/ml cone contraction is only partially inhibited. Inhibition by NEM-HMM is less effective than that by NEM–S-1 at similar concentrations. This observed difference in inhibitory effectiveness might result because S-1

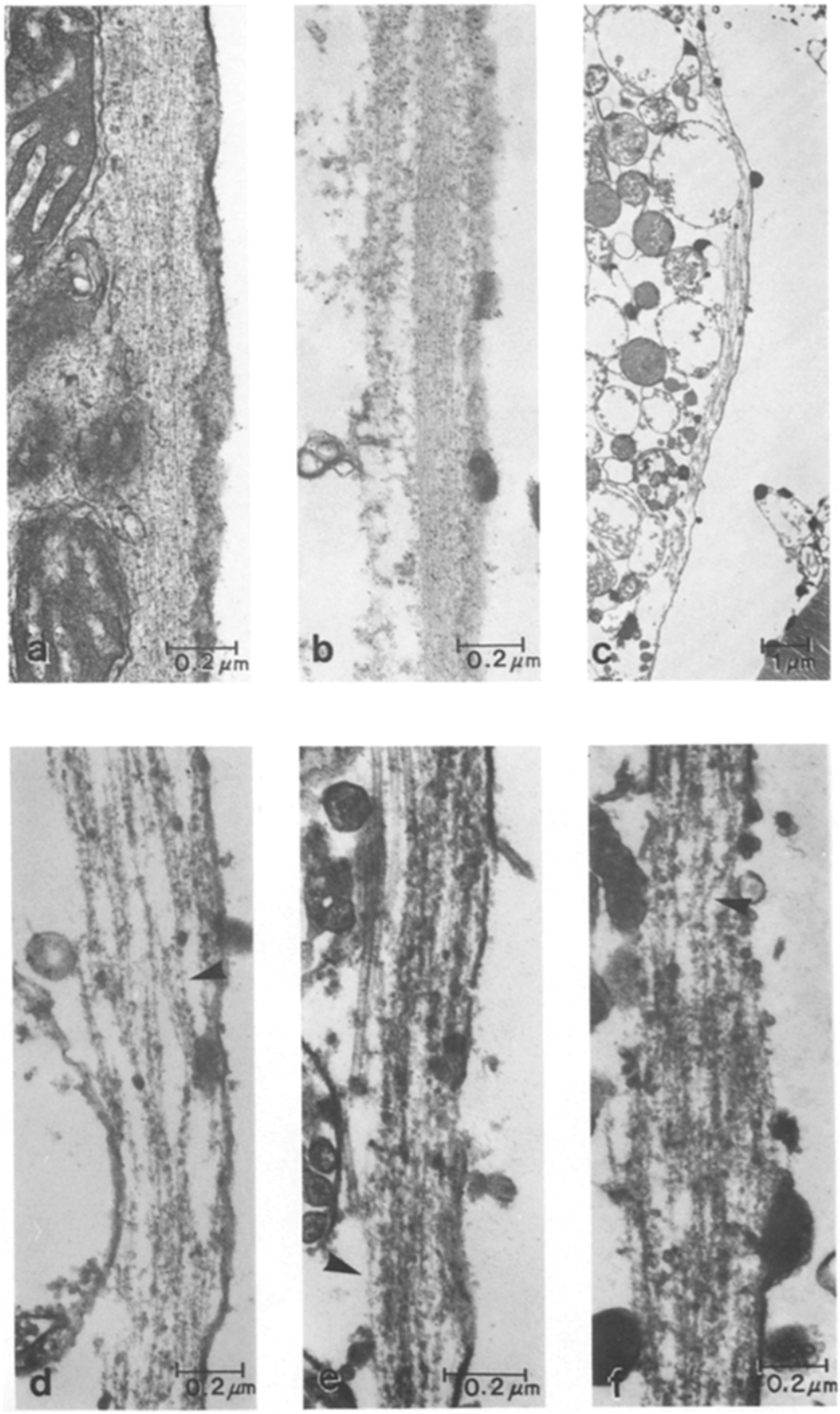

FIGURE 4 Electron micrographs of green sunfish cone ellipsoids from retinas fixed (*a*) directly after dissection (t_0); or after 18 min in two-step reactivation procedure with (*b*) contraction medium + S-1 (with ATP); (*c* and *d*) rigor medium + S-1 (no ATP); (*e*) contraction medium + NEM-S-1 (with ATP); (*f*) rigor medium + NEM-S-1 (no ATP). Bundles of actin filaments which lie just beneath the plasma membrane (*a*) are not decorated in S-1 + ATP (*b*) but are decorated by S-1 in the absence of ATP (*c* and *d*). In *d*, arrowheads are visible (arrows) though actin binding sites are clearly not saturated. After incubation with NEM-S-1 (with or without ATP), (*e* and *f*), some arrowheads are visible (arrows) but decoration is more sparse than that obtained with unmodified S-1 (compare *e*, *f*, and *d*). Nonetheless, NEM—S-1-decorated filaments (*e* and *f*) are clearly distinguishable from undecorated filaments in S-1 + ATP (*b*). (*a* and *b*) × 50,000. (*c*) × 7500. (*d*) × 62,500. (*e* and *f*) × 50,000.

(115,000 daltons) is significantly smaller than HMM (340,000 daltons) (15,16) and thus may diffuse more efficiently into the models through their detergent-permeabilized plasma membranes. Nonetheless, both NEM-S-1 and NEM-HMM significantly inhibit reactivated cone contraction.

We have demonstrated here, and previously (4, 5), that cone thin filaments bind with S-1 to form arrowhead complexes (decoration), thus characterizing them as actin. We also report here that NEM-modified S-1 binds to actin filaments in situ in the models, in both the presence and absence of 4 mM Mg-ATP. Although actin filament decoration obtained with NEM-S-1, in both rigor and contraction media, is not so distinct nor

so uniform as that observed with S-1 binding, cone actin filaments decorated with NEM–S-1 are clearly distinguishable from undecorated actin filaments in cones incubated with S-1 plus ATP. Similar reduced decoration has been observed in negatively stained preparations of actin filaments with NEM-HMM as compared with unmodified HMM (9, 17). It seems likely that chemical modification of S-1 or HMM with NEM reduces the proportion of the protein which has actin-binding activity. Though actin filament decoration by NEM–S-1 is sparse and irregular, it nonetheless appears to be sufficient to produce inhibition of contraction.

It has been suggested that NEM–S-1 and NEM-HMM inhibit actomyosin contraction by binding irreversibly to native actin filaments and thereby inhibiting their interaction with native myosin (17, 22, 23). Although NEM does not abolish the ability of HMM and S-1 to bind actin filaments, it does abolish MgATP-induced dissociation of the fragments from actin filaments (17, 22, 23). Irreversible binding of NEM-modified myosin fragments to only a few sites on actin filaments could effectively obstruct the access of native myosin to the myosin-binding sites on actin filaments and, thus, block force production in actomyosin systems.

NEM–S-1 and NEM-HMM inhibition of reactivated contraction in cone models reinforces previous arguments that teleost retinal cone contraction depends upon an actomyosin-mediated mechanism (4, 5, 26). Cone myoids contain thin (actin) filaments and thick (myosinlike) filaments oriented parallel to the axis of contraction (5). Thus their distribution is appropriate for a sliding-filament mechanism of contraction (5). Passive shortening resulting from depolymerization of myoid microtubules can be ruled out because colchicine, high pressure, and low temperature disrupt myoid microtubules but do not produce contraction in long dark-adapted cones (4, 26). The persistence of microtubules during contraction in our Brij-extracted cone models demonstrates that microtubule disassembly is not required for shortening. The calcium and ATP requirements for reactivated contraction in teleost cone models are comparable to those observed in glycerinated myofibrils and other contractile models of nonmuscle cells (3, 10, 19). Furthermore, ocular injections of the cytochalasins (B and D) completely block light-induced cone contraction in vivo (4), thus indicating that cone shortening is comparable to the numerous previously studied examples of nonmuscle contraction which exhibit cytochalasin-sensitivity (8). For all these reasons, we believe that teleost retinal cone models provide powerful tools for studying the mechanism and regulation of actomyosin contraction in nonmuscle cells.

The authors wish to express their appreciation to Kent McDonald and Barbara Nagle for critical reading of the manuscript.

This study was supported by National Science Foundation grant PCM 8011792.

Received for publication 14 June 1982, and in revised form 13 September 1982.

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