REVERSIBLE DISAGGREGATION OF MYOFILAMENTS IN VERTEBRATE SMOOTH MUSCLE

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ABSTRACT

Strips of taenia coli from guinea pigs were incubated under isometric conditions in Krebs-Ringer bicarbonate saline (MKR) containing various concentrations of Ca^{+2} and/or Mg^{+2}. Spontaneous or chemically induced contractile activity was abolished within 15 min of exposure to MKR containing Ca^{+2} at concentrations below 10^{-6} M; contractile activity was restored by reincubation in normal MKR after 1–2 h. Exposure of taenia coli to MKR containing Ca^{+2} at concentrations below 10^{-6} M for 1 h or more led to loss of thick and thin myofilaments from the sarcoplasm as observed with the electron microscope. Except for the loss of these two filament types, the cells contained all other structural features observed in preparations incubated in MKR containing Ca^{+2} at its normal level (1.3×10^{-3} M). The loss of thick and thin myofilaments in strips exposed to a Ca^{+2} concentration below 10^{-6} M was reversed by reincubation for 30 min in MKR containing normal Ca^{+2} levels. The observed loss of thick and thin myofilaments in response to low Ca^{+2} is interpreted as resulting from the disaggregation of some or all of the molecular components of these two filament types.

The importance of Ca^{+2} in the contractility of smooth muscle is underscored by abnormal changes in membrane permeability (1), membrane depolarization (2), and loss of excitation-contraction coupling (3) when Ca^{+2} is experimentally removed or decreased in concentration in the bathing medium. In addition to these fundamental roles, Ca^{+2} can exert an effect on the interaction and aggregation of various isolated contractile proteins (4–6) and "native" myofilaments (7, 8). The present experiments were performed to determine if the structure of the myofilaments within intact vertebrate smooth muscle fibers is dependent on Ca^{+2}. The observations suggest that the thick and thin myofilaments can reversibly disaggregate and aggregate within intact muscle fibers in response to changes in the extracellular Ca^{+2} concentration.

MATERIALS AND METHODS

Paired strips of taenia coli were dissected from guinea pigs previously killed by a blow on the head. Each muscle strip of the pair was mounted in an organ bath at 37°C as previously described (9) and force was monitored isometrically with a transducer (model FT03, Grass Instrument Co., Quincy, Mass.). Only pairs of muscle strips showing spontaneous contractile activity similar to that in Fig. 1 were used in these experiments. After 60 min of incubation in mammalian Krebs-Ringer bicarbonate saline (MKR) the organ bath was drained and substituted by MKR containing diminished levels of Ca^{+2} and/or Mg^{+2}. The reduction in divalent cation concentration was accomplished by addition of either ethylenediaminetetraacetic acid (EDTA) (Sigma Chemical Co., St. Louis, Mo.), diethylenetriamine pentaacetic acid (DTPA) (Sigma Chemical Co.), or ethyleneglycol *N*, *N'*-tetraacetic acid (EGTA) (Sigma Chem-

ical Co.) to the normal MKR. The stock solution of each chelator was adjusted to pH 7.4 in all experiments. Preparations were fixed after either 15, 30, 60, 120, or 180 min in MKR with diminished concentrations of divalent cations by the injection of glutaraldehyde into the organ bath to a final concentration of 2.5%. Strips of taenia coli fixed after exposure to normal MKR for either 75, 90, 120, 180, or 240 min served as controls for this part of the study. The contractile response to electrical or chemical stimulation was tested at frequent intervals during the experiment. Acetylcholine was injected into the bath to a final concentration of 1 μg/ml and after the maximal response was observed, the bath was rinsed and refilled with solution identical to that in the bath before chemical stimulation. The response to electrical stimulation was tested at frequent intervals during the experiment by passing a brief train of square wave impulses (100 V, 1 ms duration, 10 pulses/s) between the hooks at either end of the preparation. In normal MKR this pattern of electrical stimulation resulted in a reproducible contraction similar to that observed in response to K^+ (9).

In order to check for the reversibility of the effects of MKR containing lowered concentrations of divalent cations, some preparations were incubated in normal MKR after a 60 min period of incubation with reduced Ca^{+2} and/or Mg^{+}.[2] The preparations were fixed between 30 and 90 min after reexposure to normal MKR. Strips of taenia coli fixed in the organ bath were removed therefrom after 10–15 min and fixation continued in the same solution at 4°C for 24 h. The strips were then prepared for electron microscopy (10). The concentration of Ca^{+2} and Mg^{+2} in MKR after addition of a chelating agent was calculated as described by Pull et al. (11). The calculation was performed utilizing a value of 7.4 for the pH of MKR and stability constants for the various divalent cation-chelator complexes at 25°C (12, 13). An unavoidable but small error is thus introduced since the experiments were performed at 37°C but neither stability constants at 37°C nor thermodynamic data required for making temperature corrections of the constants were available for all of the metal-chelate complexes. Thermodynamic data (12) required for the calculation of stability constants for the EDTA-divalent cation complexes at 37°C were, however, available. Calculation of the constants at 37°C indicated that the Ca^{+2} and Mg^{+2} concentrations determined using stability constants at 25°C underestimate the Ca^{+2} and Mg^{+2} concentration in the present experiments at 37°C by no more than 15%.

RESULTS

A typical record showing changes in spontaneous contractile activity upon reduction in the concentration of Ca^{+2} and Mg^{+2} from 1.2×10^{-3} M and 1.3×10^{-3} M, respectively, in normal MKR to 1.6×10^{-8} M and 1.6×10^{-6} M is shown in Fig. 1. As was typical of all preparations studied, within 3 min of the decrease in Ca^{+2} and Mg^{+2} concentrations, spontaneous contractile activity was abolished. The contractile response to a test dose of acetylcholine (1 μg/ml) or brief electrical stimulation was always abolished in less than 15 min. The effect of reexposure of the same taenia coli strip to normal MKR is also shown in Fig. 1. As was typical of all preparations studied, normal spontaneous contractile activity returned. The mean time interval between reexposure to normal MKR and resumption of spontaneous contractile activity was 86 ± 9 (SE) min. The contractile response to acetylcholine or electrical stimulation returned with a similar time course.

A profile of a fiber from a muscle strip that was incubated in normal MKR for 60 min is shown in Figs. 2 and 9 *a*. The thin myofilaments are found in lattice-like arrays between the abundant and widely distributed thick myofilaments. The 100-Å diameter filaments predominate around areas containing dense bodies. The numbers and pattern of distribution of the myofilments shown in Figs. 2 and 9 *a* are characteristic of the preparations incubated in MKR for intervals ranging between 1 and 4 h.

A typical cross section of a fiber from a muscle strip incubated for 60 min in MKR with lowered Ca^{+2} and Mg^{+2} levels (5 mM EDTA) is shown in Figs. 3 and 9 *b*. Thick myofilaments are not

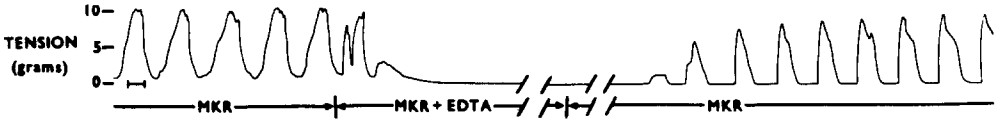

FIGURE 1 Polygraph record of the contractile activity of a taenia coli preparation. During interval denoted by *MKR + EDTA*, preparation was incubated in MKR containing 5 mM EDTA. Break in record during this interval was 50 min. Break in record within the last period was 85 min. Time mark at lower left is 1 min interval.

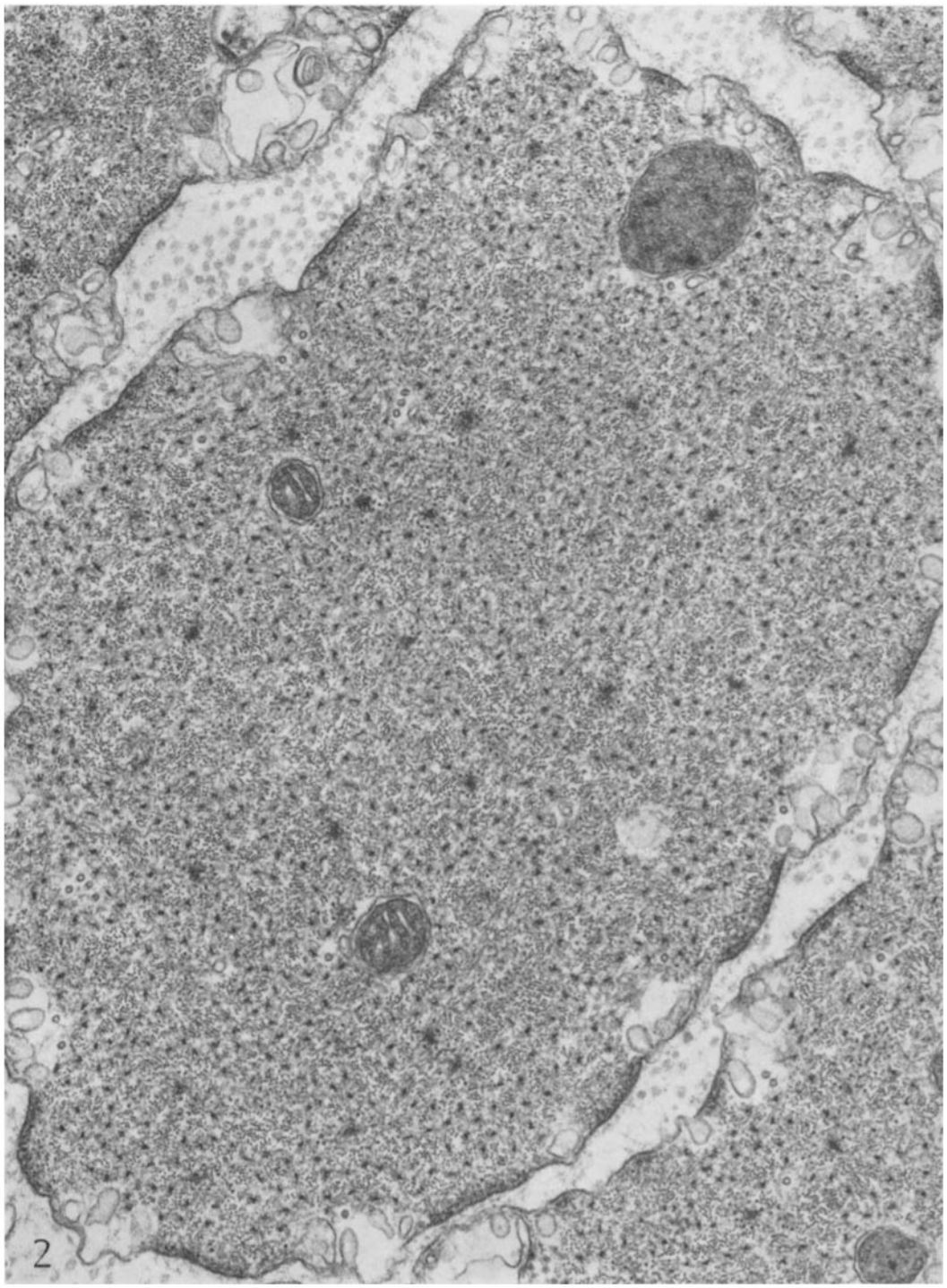

FIGURE 2 Cross-sectional profile of a fiber from a muscle strip incubated in normal MKR for 60 min. Thick and thin myofilaments are abundant. × 40,000.

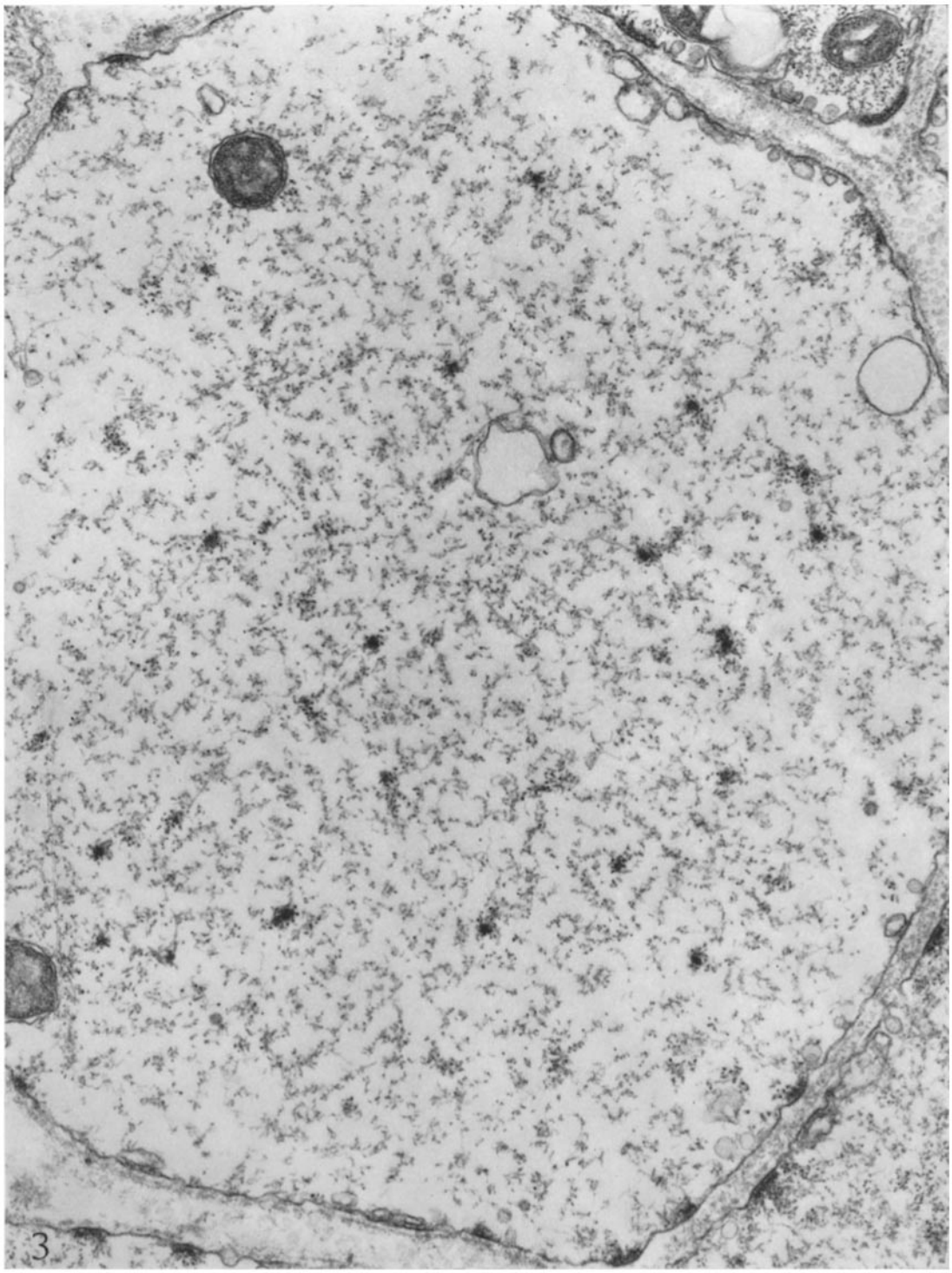

FIGURE 3 Cross-sectional profile of a fiber from a muscle strip incubated in MKR with lowered levels of Ca^{+2}/Mg^{+2} (5 mM EDTA) for 60 min. Thick myofilaments are absent. Thin myofilaments are much reduced in number. The principal components remaining are 100-Å filaments and dense bodies. Micropinocytotic vesicles at the cell membrane are reduced in size. × 40,000.

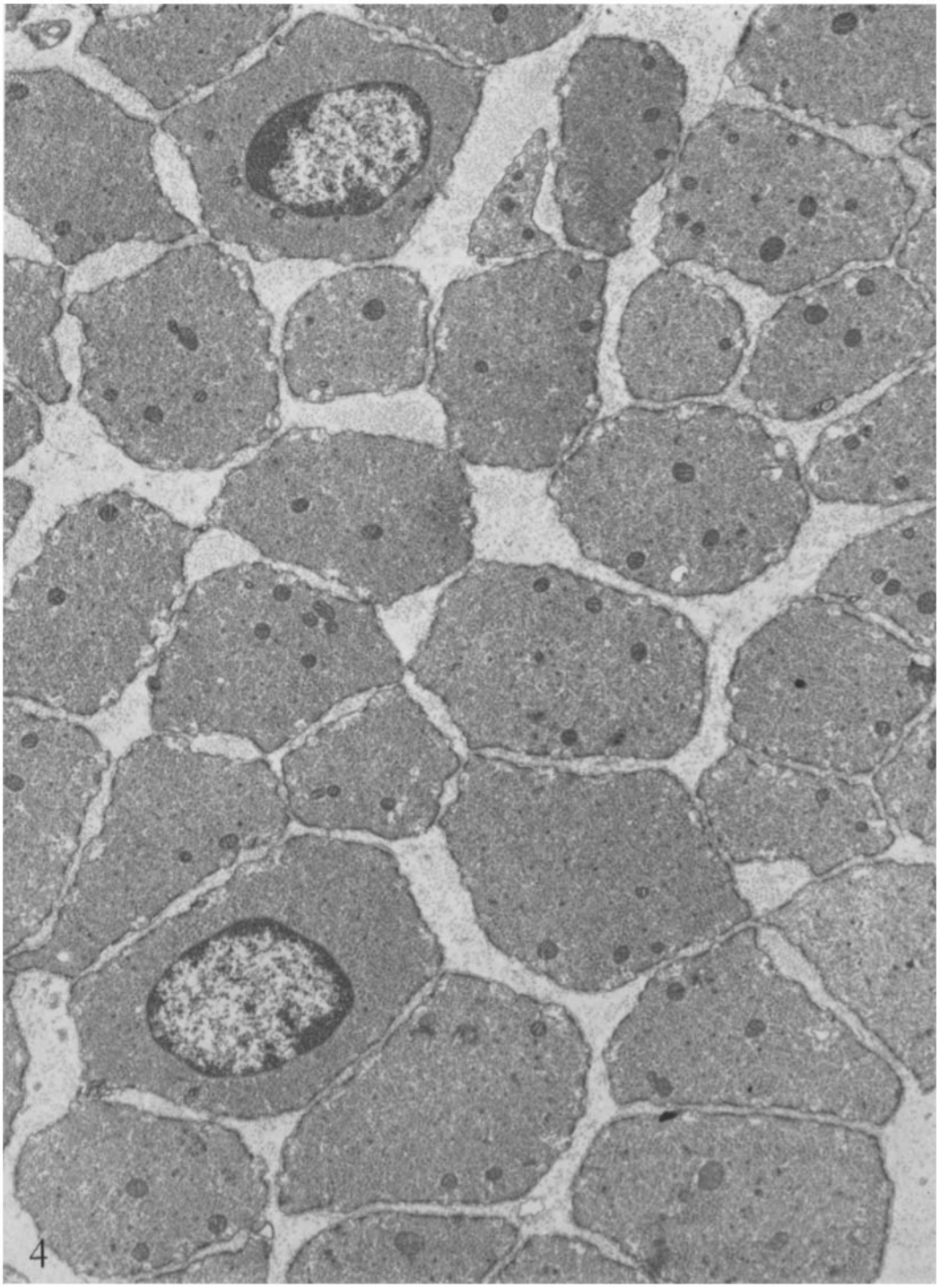

FIGURE 4 Group of fiber profiles from a muscle strip incubated in normal MKR. The sarcoplasm is comparatively dense (see Fig. 5). × 10,000.

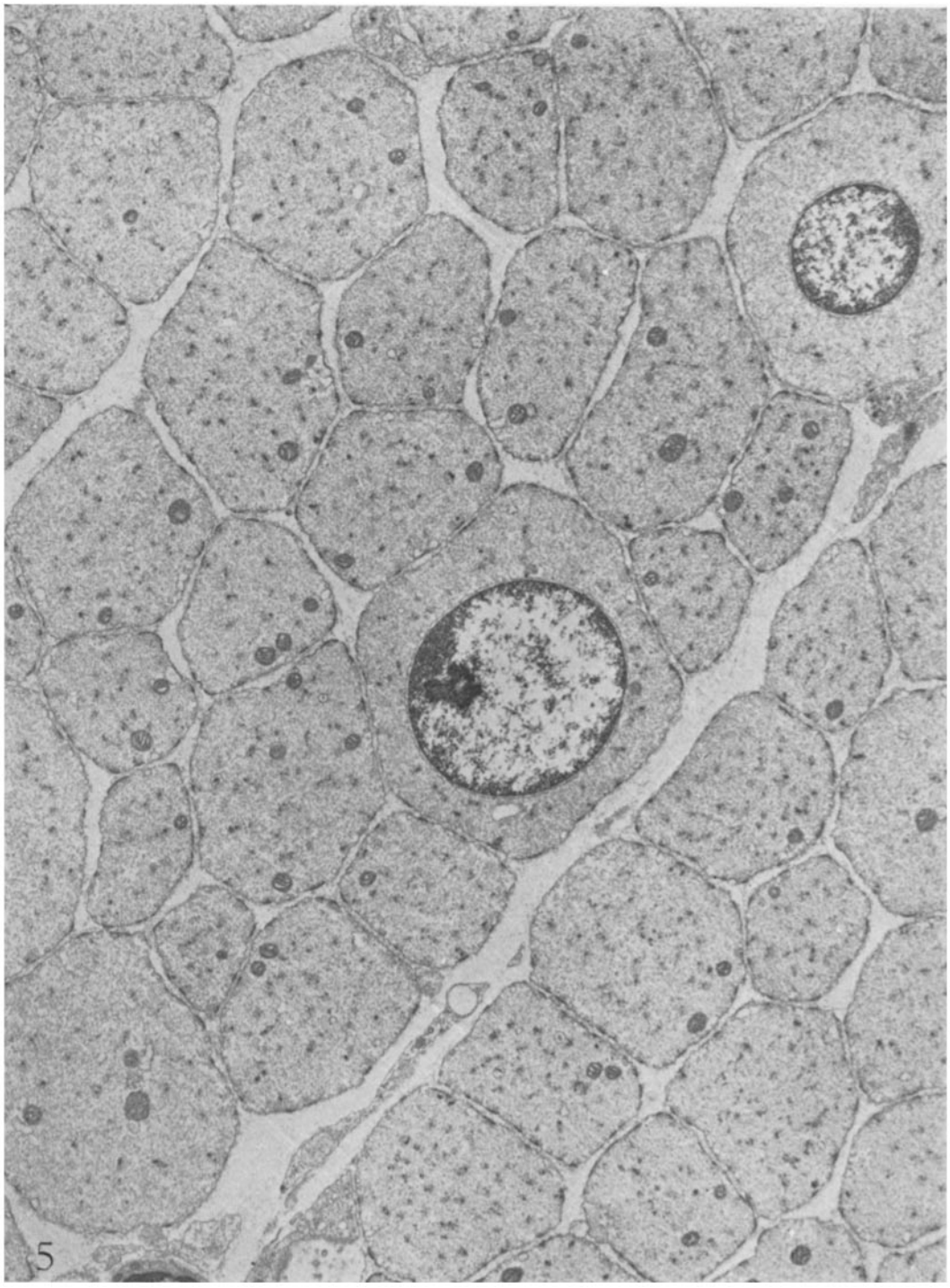

FIGURE 5 Group of fiber profiles from a muscle strip incubated in MKR with lowered levels of Ca^{+2}/Mg^{+2} (5 mM EDTA) for 60 min. The sarcoplasm in the fiber profiles is uniform and less dense than those in Fig. 4. × 10,000.

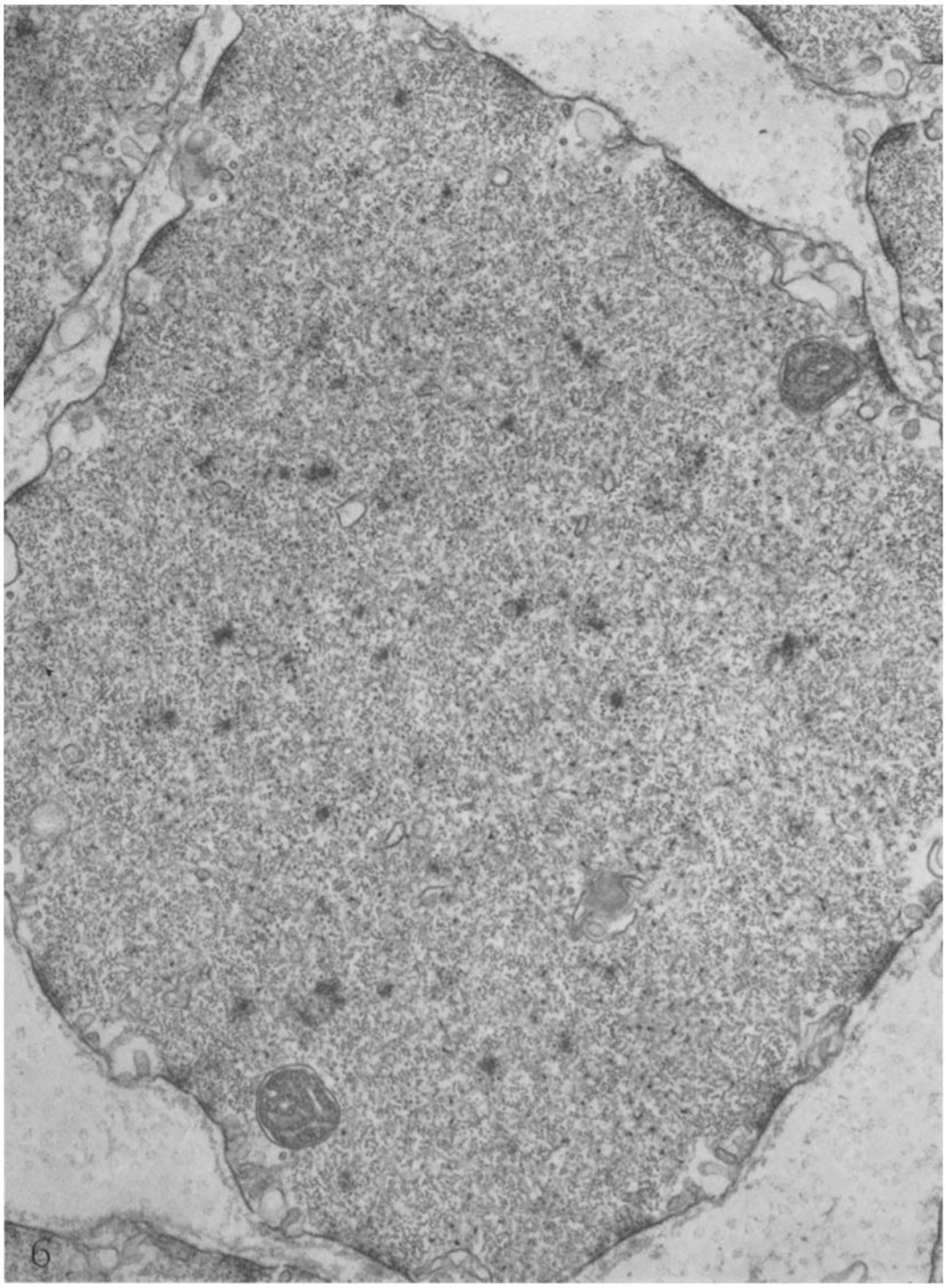

FIGURE 6 Cross-sectional profile of a fiber from a muscle strip incubated at reduced Ca^{+2}/Mg^{+2} levels for only 30 min. Thin myofilaments are plentiful, but thick myofilaments are much reduced in number and diameter. × 40,000.

demonstrable, and thin myofilaments are very much reduced in number. The 100-Å diameter filaments and dense bodies are the only major components remaining. This condition of incubation is also related to a decrease in the size of the micropinocytotic vesicles of the cell membrane. Cross sections from preparations exposed to MKR with EDTA for intervals as long as 3 h were identical to the profile shown in Figs. 3 and 9 *b*.

The absence of most of the myofilaments in cross sections of fibers treated with EDTA makes their sarcoplasm less electron-opaque as compared to fibers in normal MKR. This difference in density and the uniformity in the appearance of the sarcoplasm among fibers in either type of preparation is illustrated in Figs. 4 and 5.

After shorter periods of incubation in MKR (30 min) with reduced Ca^{+2} and Mg^{+2}, the thin filaments are still numerous in cross sections of fibers, but thick filaments, if present at all, are much reduced in number (Figs. 6 and 9 *c*). Exposure of taenia coli to low Ca^{+2}/Mg^{+2} for shorter periods (10–15 min) had no demonstrable effect on ultrastructure in all but the outermost fibers in the strips examined. Ultrastructural changes similar to those shown in Figs. 3, 5, and 9 *b* were obtained after 1 h when 5 mM DTPA or EGTA was substituted for 5 mM EDTA as the chelating agent. EGTA, unlike the other chelating agents, led to marked dispersion of nuclear chromatin and crenulation of the cytoplasmic membranes. Although the three different chelating agents produce essentially the same changes in the filamentous complement of smooth muscle, (i.e. loss of thick and thin myofilaments) the extent of reduction in Ca^{+2} and Mg^{+2} concentrations in these experiments is not equivalent. Table I summarizes the ultrastructural observations and the corresponding extracellular concentrations of Ca^{+2} and Mg^{+2}. Loss of thick and thin myofilaments is correlated solely with a reduction in extracellular Ca^{+2} concentration. The threshold for the ultrastructural changes associated with reduction in extracellular Ca^{+2} is probably below 10^{-6} M, because when taenia coli were incubated in MKR containing 3.0×10^{-6} M Ca^{+2} and 1.3×10^{-3} M Mg^{+2} for up to 3 h, no ultrastructural changes were noted.

Figs. 7 and 9 *d* show a smooth muscle fiber from a strip of taenia coli which was incubated for 1 h in normal MKR subsequent to incubation in MKR with reduced Ca^{+2} (1.6×10^{-8} M). Both thick and thin filaments in lattice-like arrays are clearly evident. This reversibility was found for virtually all the fibers in a strip (Fig. 8). Similar structural details were noted in preparations incubated in normal MKR for shorter periods (30 min). Hence, normal ultrastructural relationships appear to return before spontaneous or electrically or chemically inducible contractile responses can be detected.

DISCUSSION

These experiments indicate that loss of demonstrable thick and thin myofilaments in smooth muscle can be induced by lowering the level of extracellular Ca^{+2} to below 10^{-6} M. Decreasing the extracellular Ca^{+2} concentration reportedly leads to a prompt increase in the efflux of Ca^{+2} from smooth muscle (14, 15) and a consequent decrease in the levels of tissue Ca^{+2} (15). However, the loss of demonstrable myofilaments may not be a direct consequence of decreased Ca^{+2} concentration per se, since changes in extracellular Ca^{+2} also lead to changes in permeability of the smooth muscle cell membrane (3, 16) with consequent changes in the intracellular levels of numerous ionic species (2, 17, 18). Furthermore, as a con-

TABLE I

The Effect of Ca^{+2} and Mg^{+2} Concentration on Thick and Thin Myofilaments

Medium	Calculated extracellular concentration:		Ultrastructure	
	Ca^{+2}	Mg^{+2}	Thick filaments	Thin filaments
	M	*M*		
MKR	1.2×10^{-3}	1.3×10^{-3}	Present	Present
MKR + 5 mM EDTA	1.6×10^{-8}	1.6×10^{-6}	Absent	Absent
MKR + 5 mM DTPA	6.0×10^{-8}	3.0×10^{-6}	Absent	Absent
MKR + 5 mM EGTA	5.0×10^{-8}	1.3×10^{-3}	Absent	Absent
MKR + 5 mM EDTA + 3.9 mM $MgSO_4$	3.0×10^{-6}	1.3×10^{-3}	Present	Present

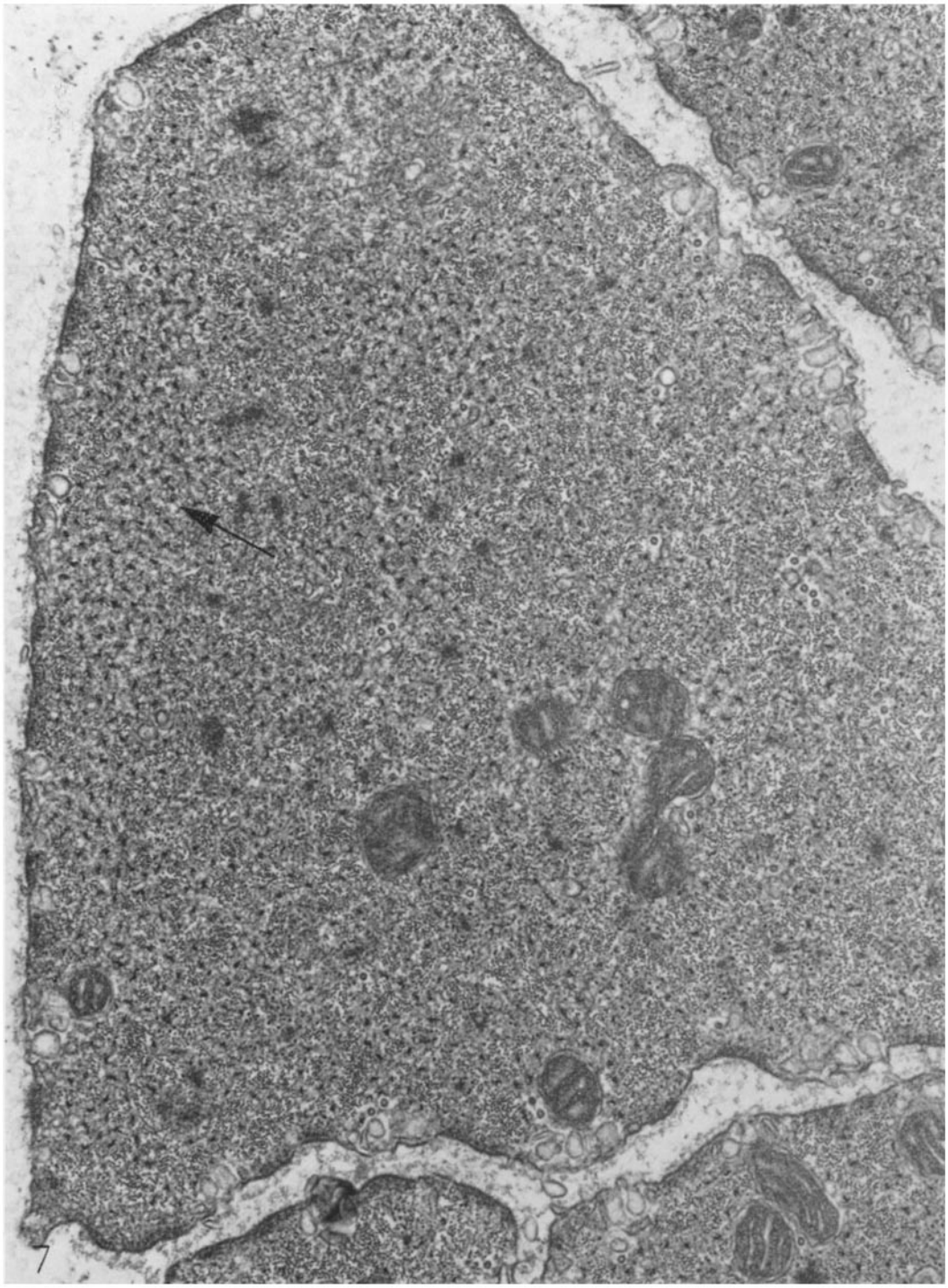

FIGURE 7 Cross-sectional profile of a fiber from a muscle strip first incubated at reduced Ca^{+2}/Mg^{+2} levels for 60 min, then returned to normal MKR for 60 min. Thin myofilaments are numerous and present in lattice-like arrays. Thick myofilaments are abundant and occasionally aggregate into bundles containing no intervening thin myofilaments (arrow). The micropinocytotic vesicles are approximately the same size as those at the cell membrane of fibers from strips in normal MKR. × 40,000.

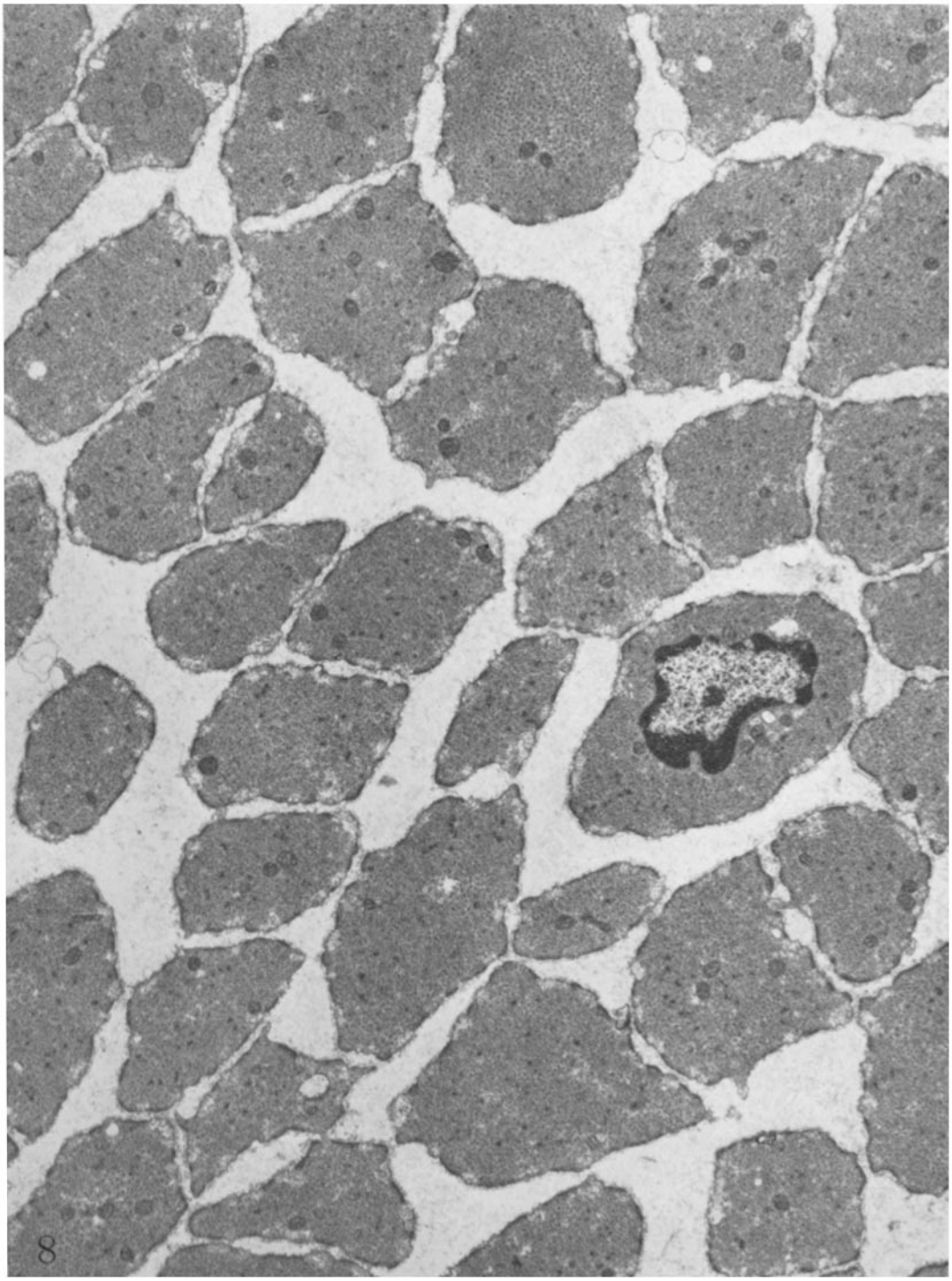

FIGURE 8 Group of fiber profiles from a muscle strip treated as in Fig. 7. The sarcoplasm of the fibers is uniformly dense, reflecting the abundance of myofilaments (compare with Figs. 4 and 5). × 10,000.

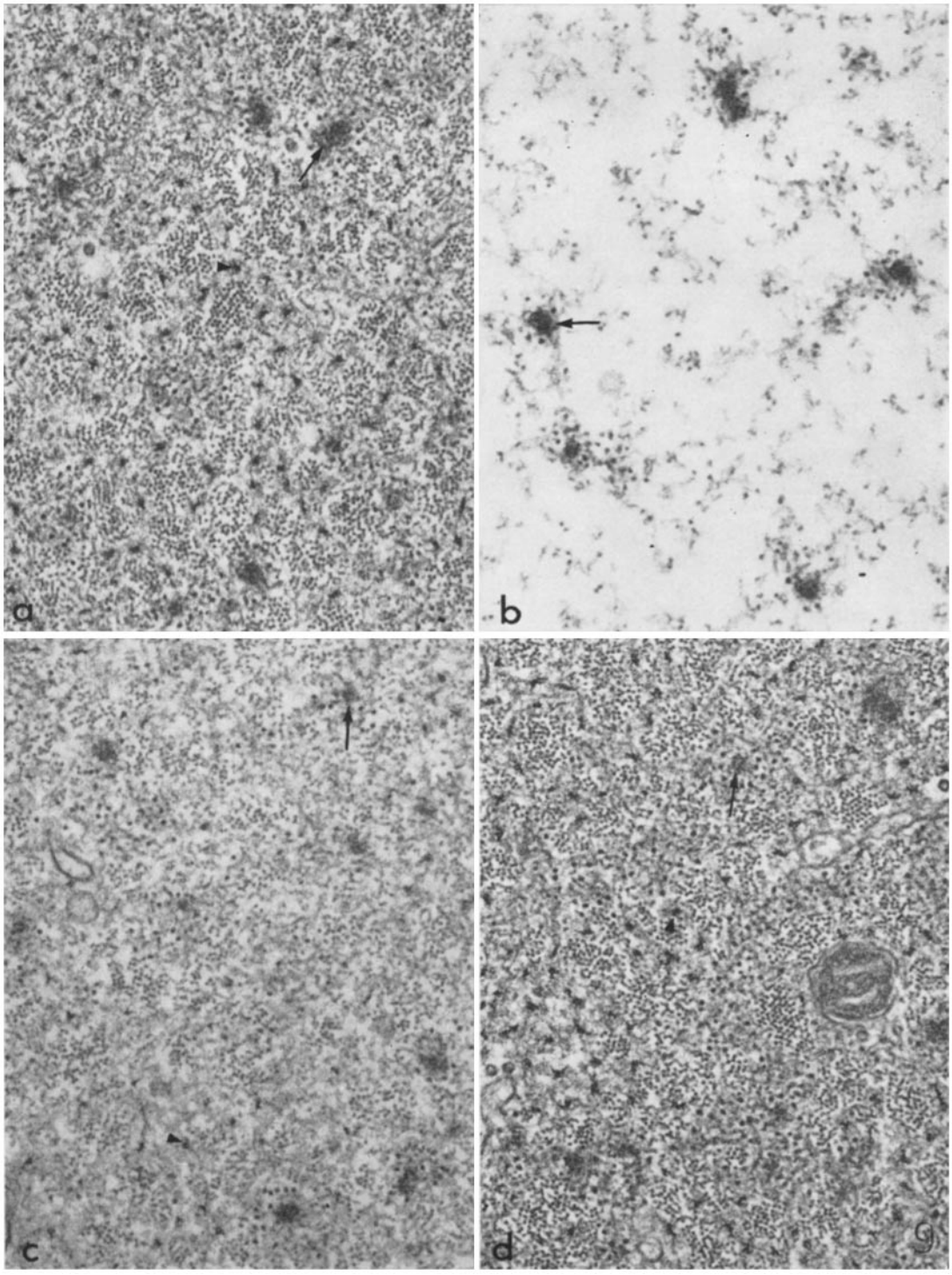

FIGURE 9 Areas of smooth muscle fibers in cross section showing the types of myofilament profiles found with various treatments. Arrows point to dense bodies with associated 100-Å filaments. Arrowheads point to thick myofilaments. The thin filaments are the smallest profiles and when present they lie in lattice-like arrays or bundles between the thick myofilaments and dense bodies. (*a*) Area from fiber in normal MKR as in Fig. 2. (*b*) Area from fiber in EDTA for 1 h as in Fig. 3. (c) Area from fiber in EDTA for 30 min as in Fig. 6. (*d*) Area from fiber in EDTA for 1 h and subsequently reincubated in normal MKR as in Fig. 7. × 90,000.

sequence of decreasing extracellular Ca^{+2} levels, changes in smooth muscle cellular metabolism might also be expected, since numerous metabolic pathways are known to be influenced by the concentration of Ca^{+2} and other ions. It is, therefore, difficult to precisely pinpoint what intracellular change or changes are the cause of the loss of thick and thin myofilaments.

The apparent loss of thick and thin myofilaments observed in response to decreased extracellular Ca^{+2} may be due to either (*a*) a lack of staining or (*b*) a gradual disaggregation of some or all of the molecular species which comprise the thick and thin myofilaments. The first possibility seems unlikely, because there is no alteration in the staining properties of intracellular structures as evidenced by the normally stained 100-Å filaments, mitochondria, plasma membranes, nuclei, and rough endoplasmic reticulum in the presence of low Ca^{+2} concentrations. A more likely explanation of the observed ultrastructural changes is that filament "loss" reflects the gradual disaggregation of molecules which comprise the thick and thin myofilaments. This interpretation is consistent with the observation of Shoenberg (7) that aggregation of the protein myosin into filaments requires the presence of Ca^{+2} in addition to Mg^{+2} and adenosine 5-triphosphate. Purified *f*-actin from striated muscle is not, however, disaggregated by low Ca^{+2} concentration (19). Apparent disaggregation of the thin filaments may, therefore, reflect an effect of low Ca^{+2} on one or more of the nonactin proteins which comprise the thin filament (20). Similarly, the apparent disaggregation of the thick myofilaments may result from the effect of low Ca^{+2} on a nonmyosin component of the filament. Further consideration of the mechanisms responsible for thick and thin filament loss in the present studies will require more detailed information regarding both intracellular changes evoked by low extracellular Ca^{+2} and the chemistry and structure of thick and thin myofilaments of smooth muscle.

The inability to consistently demonstrate thick and/or thin myofilament arrays in early studies of smooth muscle (21–23) may be related to the effects of low Ca^{+2} in the present study. Thick myofilaments typical of those formed from myosin were usually not found in smooth muscle fixed with osmium tetroxide. Sporadically, they were present, suggesting that the fixation process was generally insufficient to preserve the apparently labile myosin-containing filaments. The similarity in appearance of smooth muscle fixed in osmium tetroxide to that in which the extracellular Ca^{+2} has been removed by chelation, suggests the possibility that in the early studies, sufficient Ca^{+2} may have been lost to lead to the disaggregation of numerous thick and thin myofilaments.

The present observations add further support to previous studies (10, 24–26) indicating the presence of a third class of filaments, 100 Å in diameter within smooth muscle. The observed stability of 100-Å filaments in the presence of decreased extracellular Ca^{+2} indicates that these myofilaments have different properties from the thick and thin myofilaments of smooth muscle, further supporting the contention that they represent a separate class of filaments.

These studies may also have a bearing on past attempts to differentiate between different Ca^{+2} pools involved in excitation-contraction coupling. A common mode of approach to this problem has been to reduce extracellular Ca^{+2} levels and to determine at what Ca^{+2} level the response to different agonists are lost (2, 27, 28). The present results suggest that care must be exercised in interpreting the loss or decrease in responsiveness to an agonist in a Ca^{+2}-free medium as being due to depletion of a Ca^{+2} pool involved in excitation-contraction coupling. In order for this interpretation to be valid in view of the present study, it must be shown that the contractile apparatus is still intact and functional. If disaggregation of subunits comprising the thick and thin filaments takes place, it seems unlikely that net force would result from activation of the actin-myosin system. Upon diminution of extracllular Ca^{+2} levels in taenia coli, the loss of contractile responses to acetylcholine or electrical stimulation occurred considerably before any loss of filaments was noted; upon returning Ca^{+2} to the medium, filaments were noted before contractile responses could be detected. Thus, in this preparation filament structure appeared to be less sensitive to decreased extracellular Ca^{+2} levels than the process of excitation-contraction coupling, thus validating conclusions drawn from the effects of Ca^{+2} removal regarding the Ca^{+2} requirement of excitation-contraction coupling. It remains to be seen if decreased extracellular Ca^{+2} has similar effects on the myofilaments of other smooth muscle and what role, if any, these changes play in the loss of responsiveness to contractile stimuli in the presence of low extracellular Ca^{+2} concentration.

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