Increased K^+ Inhibits Spontaneous Contractions and Reduces Myosin Accumulation in Cultured Chick Myotubes

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ABSTRACT Increasing the K^+ from 5.4 mM to 12 mM in the culture medium of developing chick myotubes causes an immediate cessation of spontaneous contractions and leads to an inhibition of myosin accumulation. The synthesis of myosin continues at the same rate in 12 mM K^+ as in 5.4 mM K^+ as measured by [^{3}H]leucine incorporation into myosin corrected for differences in pool specific activity. Total protein synthesis and total protein accumulation are unaffected by growth in 12 mM K^+. In addition, growth in 12 mM K^+ did not alter the type of myosin heavy-chain isoform expression nor did it alter the pattern of myosin light-chain synthesis. However, the rate of myosin turnover increased threefold in cultures grown in 12 mM K^+ compared to cultures grown in 5.4 mM K^+, while total protein turnover was only marginally increased. We conclude that suppressed electrical or contractile activity of myotubes leads to an increased rate of myofibrillar protein turnover and that spontaneous mechanical and or electrical activity is required for continued myotube maturation in culture.

Recent muscle cell culture studies relating membrane activity or actual contraction to muscle fiber protein accumulation have emphasized the control of acetylcholinesterase and acetylcholine receptor synthesis and placement in the muscle fiber membrane (27, 28, 30, 31). In addition, there is an extensive literature dealing with in vivo experiments relating neural influences to the control of muscle fiber type and the control of muscle-specific enzymes including myosin isopeptides (16). There has not been as extensive an interest in a cell culture approach relating muscle fiber membrane activity to the synthesis and accumulation of myofibrillar peptides. The few cell culture reports available do, however, establish that membrane agents such as tetrodotoxin will cause muscle fiber atrophy related to an increase in myosin turnover (19, 21). It has also been shown that direct electrical stimulation of muscle fiber cultures will cause an increase in myosin accumulation (29). In the present study we report that muscle fibers in vitro, when treated with K^+ concentrations known to cause membrane depolarization, fail to accumulate myosin heavy chains (MHC's) even though MHC synthesis is normal.

Chick myotubes developing in vitro undergo a period of rapid accumulation of myosin as well as other muscle-specific proteins shortly after cell fusion (3, 5, 12, 13). Normally, by 5–6 d in culture, myotubes exhibit cross striations and spontaneous contraction (11, 17). Concomitant with the development of mechanical activity in myotubes is the development of electrical properties of myotube membranes (8, 10). Newly formed myotubes exhibit a resting potential of −15 mV which increases to −60 mV as the myotube matures (8, 14). Spontaneous contractions are observed only in myotubes exhibiting resting potentials more negative than −50 mV (8). Thus the membrane potential appears to be a limiting factor in the maturation of excitation-contraction mechanisms.

Earlier work has demonstrated a relationship between the mechanical activity of myotubes and the accumulation of muscle protein (10, 19–21). In chick myotubes cultured in the presence of 1 μM tetrodotoxin (TTX), early development proceeds normally, but by 5–6 d, when normal cultures are exhibiting spontaneous contraction, TTX cultures exhibit little or no mechanical activity and contain only 50% the myosin of control cultures (21). The reduced level of myosin accumulation is primarily due to an increased rate of myosin turnover in TTX-treated cultures (19, 21). When TTX is applied to spontaneously contracting myotubes, mechanical activity ceases immediately and, after a lag period of several hours, newly synthesized myosin begins to turn over more rapidly (21). From these data we postulated that abolition of mechanical activity in myotubes leads to instability of myosin. Although the only demonstrated direct effect of 1 μM TTX is to block fast Na^+ channels in excitable membranes (2), it remains possible that TTX has an added direct effect on myosin turnover. We have therefore sought a more physiological method

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for reducing membrane and mechanical activity in these muscle fibers.

Previous workers have shown that increasing K^+ in the growth medium of rat and mouse myogenic cultures resulted in a decrease in the resting potential of the myotube membrane and reversibly stopped the twitching of spontaneously active fibers (9, 18).

In this report we demonstrate that a relatively small increase in K^+ from 5.4 mM to 12 mM blocks spontaneous contractions in chick myotubes and leads to the same inhibition of myosin accumulation seen in TTX-relaxed cultures. Furthermore, the reduction in myosin accumulation is mediated by the same mechanism observed in TTX-relaxed cultures: an increased rate of myosin turnover. These data suggest therefore that protein turnover is a general mechanism for regulating myosin content in myotubes under a variety of physiological conditions and that turnover regulation is somehow coupled either to muscle electrical activity or to actual contraction, or to both.

MATERIALS AND METHODS

Cell Cultures

Primary cultures of 12-d embryonic muscle cells were prepared as previously described (21), except that preplating was always used to obtain a higher ratio of myoblasts to fibroblasts. In addition, cytosine arabinoside was always used at a final concentration of 5×10^{-6} M to inhibit fibroblast growth.

Myosin Heavy-Chain Accumulation

Myosin heavy-chain analysis was carried out by dissolving total culture material in SDS electrophoresis buffer and identifying the MHC band after electrophoresis on 5% SDS polyacrylamide cylindrical gels (19). Before electrophoresis, myosin heavy chains and other peptides in the extract were stained with drimarene brilliant blue according to the method of Bosshard and Datyner (1). As a standard, purified myosin was prestained in this manner, subjected to electrophoresis, and the gels were scanned at 591 nm. A linear response through the MHC band was obtained in a range of 0.3 to 8.0 μg when these amounts of myosin were applied to the standard gels. All results reported here involved the use of gel scans in the linear part of the standard curve. Normally, when contents of a single 100-mm culture dish at day 7 are dissolved in 200 μl of electrophoresis buffer, a sample of 35 μl applied to the gel would be sufficient to contain 1 to 8 μg of protein within the MHC band. Total protein was determined by the Coomassie Brilliant Blue, G-250 dye binding assay (Bio-Rad Laboratories, Oxnard, CA). On 5% SDS gels, as used here, the MHC band separates from minor high molecular weight contaminants and we have determined that over 95% of the counts associated with this band are precipitated by purified antibody to chicken myosin.

Amino Acid Pool Studies and Synthesis of Myosin and Total Protein

Amino acid pools were measured routinely using the isotope dilution method of Rubin and Goldstein (15). We report here the L-leucine specific activity measured for day-6 cultures in both normal and high potassium medium. The cultures were labeled with [^{3}H]leucine (60 Ci/mmol, Amersham Corp., Arlington Heights, IL) (10 μC/dish) in leucine-free MEM for 1 h. Pool equilibration was measured over the ensuing 60 min by analysis of replicate cultures. Leucine specific activity was determined at several time points during the 60 min. The average pool specific activity was determined by integrating the areas under the equilibration curves (Fig. 2).

MHC synthesis was measured by pulsing cultures with [^{3}H]leucine as described for pool studies. Culture plates were rinsed with minimal essential medium (MEM) containing excess unlabeled leucine. Material was dissolved from the plates directly into electrophoresis buffer containing SDS. Dissolved material was subjected to electorphoresis as described above and MHC bands were identified and excised from the gels with a razor blade. The gel slices were incubated for 90 min in scintillation vials with 0.7 ml of NCS (Amersham Corp.). After incubation, 10 ml of toluene, containing 4 gm/liter Omnifluor (New England Nuclear, Boston, MA) was added and the samples were allowed to sit overnight at room temperature. Radioactivity was determined in a Beckman LS-7000 scintillation counter (Beckman Instruments, Inc., Fullerton, CA) with ~25% counting efficiency for ^{3}H. Synthesis rates were determined using the leucine pool specific activities in dpm/pMol and the synthesis rates were then normalized to DNA values yielding results as pMol/minute per microgram DNA.

Total protein synthesis rates were determined from aliquots of the cultures dissolved in electrophoresis buffer. The aliquoted material was precipitated with cold 5% trichloroacetic acid (TCA). The precipitates were collected on glass fiber filters (GF/C, Whatman), washed with 5% TCA, followed by a methanol wash, and air dried overnight or for 30 min in a 60°C oven. The filters were incubated with 0.7 ml of NCS (Amersham Corp.) and processed for scintillation counting as described above. Total protein synthesis rates were then calculated as above for MHC.

Myosin and Total Protein Degradation

MHC degradation rates were measured after a 1-h pulse with [^{3}H]leucine as described in the pool studies. Cultures were then refed with their respective media. At various times thereafter the cells were dissolved in electrophoresis buffer. Electrophoresis was then performed as described above. MHC bands were identified and excised from the gels, and the radioactivity associated with the MHC bands was again determined as described above. Degradation rates for total protein were determined in the same manner using washed TCA precipitates of aliquots from the initial cell material dissolved in electrophoresis buffer.

Statistical analysis of turnover data includes calculation of the linear regression of the data using a computer program. The program calculates the slope of the linear regression line, the $t_{1/2}$ for the loss of radioactivity, and the degree of association between the data and the calculated line which is called the correlation coefficient.

DNA Determination

A modification of the fluorometric assay of Hinegardner (22) was used to determine DNA content. An aliquot equal to one-half of a 100-mm culture was precipitated with 2 vol of 10% TCA. A standard curve was made by precipitating calf thymus DNA (0.5–20 μg) in the presence of 100 μg of BSA. Precipitates were collected on GF/C filters (Whatman), washed with 7.5% TCA, washed twice with 10 ml of 1 N HCL, and finally washed with 10 ml of ethanol. Filters were allowed to air-dry overnight at room temperature or for 30 min in a 60°C oven in scintillation vials. A fresh solution of 2 M 3,5-diaminobenzoic acid (Aldrich Chemical Co., Milwaukee, WI, gold label) was prepared and 0.25 ml was added to each vial. The vials were capped and incubated at 60°C for 40 min. After heating, 5 ml of 1 N HCL was added to each vial. After allowing glass fiber particles to settle, the solution was transferred to glass tubes, excited at 410 nm, and read at 510 nm in a Turner model 110 fluorometer (Turner Designs, Mountain View, CA).

Myosin Heavy-Chain Peptide Maps

Limited proteolytic maps of MHC were obtained by the procedure of Cleveland et al. (24). 6-d control and K^+-treated (from day 2) cultures were labeled for 3 h with [^{35}S]methionine (100 μC/dish; > 1000 Ci/mM, Amersham). Myosin was extracted from the cultures according to Patterson and Strohman (13). Myosin was analyzed on 10% SDS slab gels according to Laemmli (25). MHC bands were cut from the gels after staining for 30 min with Coomassie Blue. The gel pieces were cut to ~4 mm and equilibrated with sample buffer (0.124 M Tris pH 6.8, 1 mM EDTA, 0.1% SDS) for 30 min and then frozen until used. The gel slices were placed into sample wells of a 15% SDS slab gel with a 3% stacking gel. The material was then overlaid with 10 μl of the proteolytic enzyme solution as described in the legend of Fig. 5. Gel electrophoresis was carried out for 40 min at 20 mA/gel. Current was then turned off and the gels were allowed to incubate with the enzyme at room temperature for 30 min. Electrophoresis was then carried out until the tracking dye (added with the proteases) was 1 cm from the bottom of the slab. The slabs were subsequently impregnated with DMSO-PPO as described by Bonner and Laskey (26) and fluorograms were prepared.

Two-dimensional SDS PAGE

[^{35}S]methionine-labeled myosin was prepared as described above and analyzed as described by O'Farrell (23). Fast and slow myosins were added to each sample to determine the mobility of fast and slow light chains in each gel. The pH range of ampholine (LKB Instruments, Inc., Rockville, MD) used was 90% of pH 5–8 and 10% of pH 3.5–10 mixture. 12.5% polyacrylamide (1/33 Bis) was used for the second dimension. Gels were stained with 0.024% Coomassie Brilliant Blue, R-250 in 40% methanol, 10% acetic acid and destained in 10% methanol and 15% acetic acid.

Osmolarity of 12 mM Potassium Media

In the following studies control myogenic cultures were grown in MEM with Earle's salts which contains 5.4 mM KCL and is buffered by 26.2 mM $NaHCO_3$

(6). To prepare 12 mM K^+ MEM, 6.6 mM $KHCO_3$ and 19.6 mM $NaHCO_3$ were used to buffer the culture medium, thus keeping the osmolarity of the medium unchanged. Additionally all culture media contained 10% horse serum and 2% embryo extract (3) which may increase the actual K^+ slightly from those values.

RESULTS

Morphological and Movement Changes Induced by Elevated Extracellular Potassium

When 12 mM K^+ medium is added to spontaneously contracting myogenic cultures, while observing them under the phase contrast microscope, one sees an immediate cessation of mechanical activity. This is not simply due to the medium change, because changing to fresh 5.4 mM K^+ medium has no inhibitory effect on contractions. In addition, no initial contracture was observed under these conditions as has been observed when the cultures are placed in isotonic KCl (130 mM) (4). Myotube cultures have been maintained in 12 mM K^+ for up to 30 d and at no time were contractions or any other movement ever observed. This effect is reversible. The time required for reversal depended on the length of time the cultures remained at the elevated [K^+]. Cultures in 12 mM K^+ for 1 wk and refed with 5.4 mM K^+ medium resumed spontaneous contractions within 48 h.

We have also grown cells from day 1 in culture in 12 mM K^+ and have observed completely normal patterns of cell fusion and of early maturation of myotubes as reflected in spreading of the myotubes and of overall growth as reflected in total protein accumulation (Fig. 3 *B*). Myotubes growing in 12 mM K^+, however, never exhibit cross striations (Fig. 1) when viewed in the phase microscope although a number of longitudinally oriented structures are apparent. This appearance persists for as long as we are able to maintain these cells.

Myosin Accumulation and Synthesis in Normal and in K^+-elevated Cells

To assess the effects of elevated external K^+ on protein synthesis and accumulation we measured first the impact on [^{3}H]leucine uptake into the intracellular leucine pool. In these experiments cells were grown in normal and in elevated potassium for 6 d. At this time muscle fibers were beginning to take on the appearance shown in Fig. 1. These cultures were then pulse-labeled for 1 h with [^{3}H]leucine, as indicated in Fig. 2, and the specific activity of the intracellular leucine pool was determined as described in Materials and Methods. The time required for equilibration of the pool with external leucine was somewhat shorter for control cells but at the end of the 1-h pulse both control and experimental cultures were showing approximately similar leucine specific activities. The average leucine pool specific activity during the entire 1-h pulse was then measured by integrating the areas under the respective curves. In normal potassium the value was 18.75 cpm/pMol and in high potassium the value was 15.93 cpm/pMol.

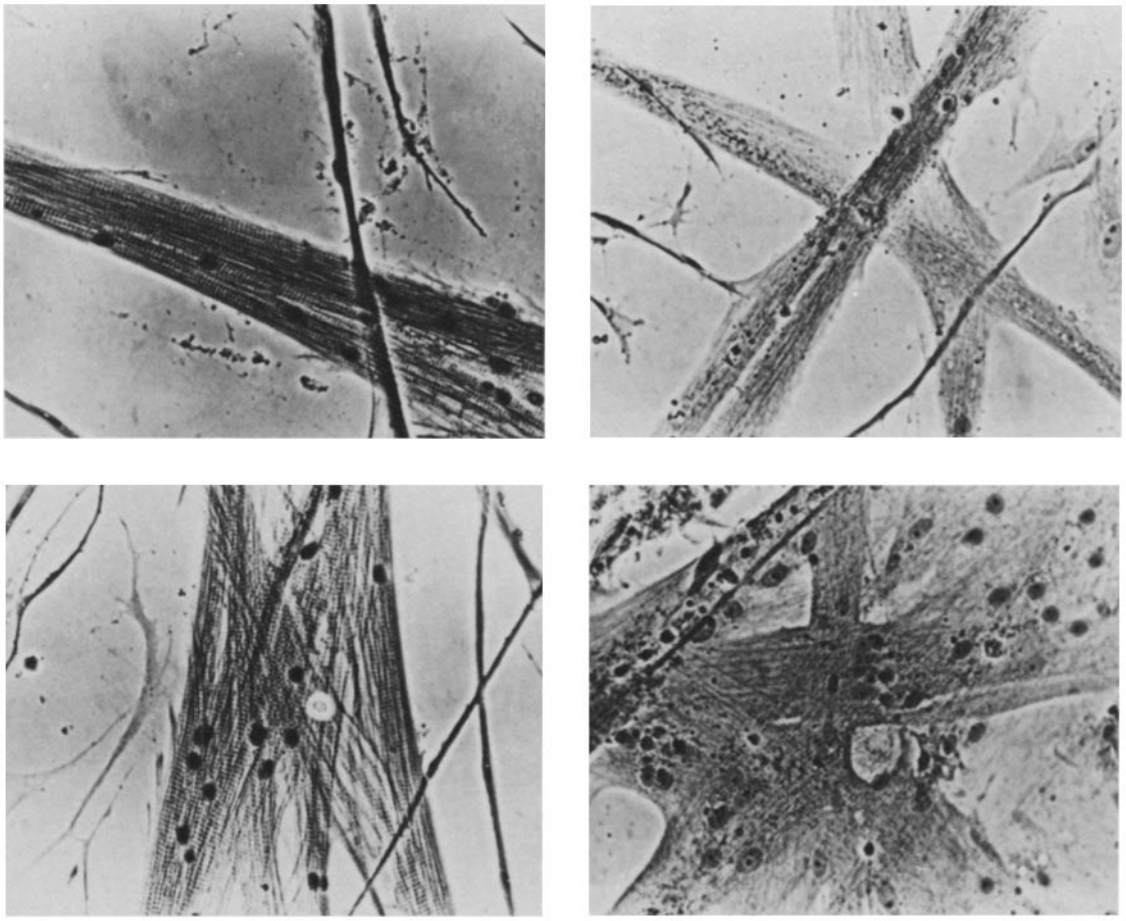

FIGURE 1 Myotubes grown in 12 mM K^+ lack phase-visible cross striations. Cultures were prepared as described in Materials and Methods. On day 2 they were fed with either normal (5.4 mM K^+, left) medium or with 12 mM K^+ (right) medium. Feeding was done in the respective media on days 4 and 7. Shown on the left are representative phase-contrast photos of cells grown in normal medium and, on the right, cells grown in 12 mM K^+ for a period of 10 d. Bar, 100 μm.

We then determined, using the above pool specific activity figures for leucine, the leucine incorporated into total protein and into myosin in the two different cultures. All incorporation data are corrected for DNA content and, since cytosine arabinoside was used to kill fibroblasts, the results yield true synthesis rates for muscle fibers in the respective cultures. These results are given in Table I. It is clear that in high potassium, which prevents spontaneous contractions and the formation of phase-apparent cross striations, there is no detectable change either in total protein synthesis or in the synthesis of myosin heavy chains when compared to control cultures within the same experiment (same cell plating).

When we now measure the extent of myosin heavy-chain accumulation in normal and in high potassium the results are strikingly different. Muscle fibers in 12 mM K^+ cease accumulating myosin heavy chains sometime between day 5 and day 6 (Fig. 3 *A*), while the accumulation of total protein was slightly depressed by day 7 (Fig. 3 *B*) and was actually elevated on days 5 and 6. In these experiments, all cultures were fed at 40 h of culture time with either normal or high potassium medium. Accumulation was measured for each time point in triplicate and analyzed for protein, myosin heavy chain, and DNA as explained in Materials and Methods.

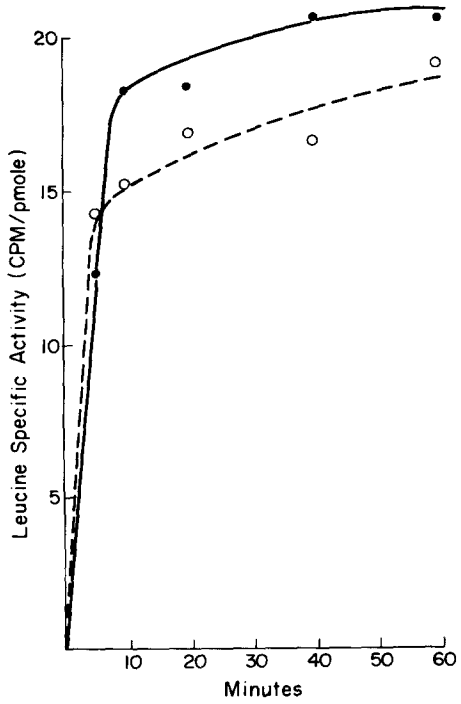

FIGURE 2 Uptake of [^{3}H]leucine into the intracellular leucine pool. Cultures grown for 6 d in 5.4 mM K^+ (--●--●--) and 12 mM K^+ (--O--O--) were labeled for 1 h with [^{3}H]leucine (5 μC/ml). At the indicated times replicate cultures were harvested and the specific activity of the intracellular leucine pool was determined as described in Materials and Methods.

TABLE I

Rate of Synthesis of Myosin and Total Protein in Myogenic Cultures Grown in 5.4 mM K^+ Medium and in 12 mM K^+ Medium

Medium	Pool specific activity	Leucine incorporated into total protein	Leucine incorporated into myosin
	cpm/pMol	*pMol/min/μg DNA*	*pMol/min/ μg DNA*
5.4 mM K^+	18.75	27.29 ± 1.83	1.06 ± .07
12 mM K^+	15.93	25.37 ± 2.42	1.02 ± .04

Myogenic cultures were prepared as described in the legend in Fig. 1. On day 6, cultures were labeled with [^{3}H]leucine (5 μCi/ml in 5.4 mM K^+ leucine-free MEM or 12 mM K^+ leucine-free MEM. The specific activity of the leucine pool was determined at 10-min intervals over a 1-h pulse by the isotope dilution method previously described (15, 19). The average pool specific activity in 5.4 mM K^+ medium and 12 mM K^+ medium during the pulse was determined by integrating the area under the equilibration curves. [^{3}H]leucine incorporated into total protein and myosin was determined as described in Materials and Methods and normalized to DNA content. The data reported are from four replicate determinations.

Myosin Heavy-Chain and Total Protein Degradation Rates in High K^+

At day 6, MHC accumulation in high K^+ had ceased even though MHC synthesis was normal. We measured, beginning exactly at this time, the rates of degradation of MHC and of total protein. Cultures were pulse-labeled with [^{3}H]leucine for 1 h on day 6 as described in Fig. 2. Cultures were then rinsed

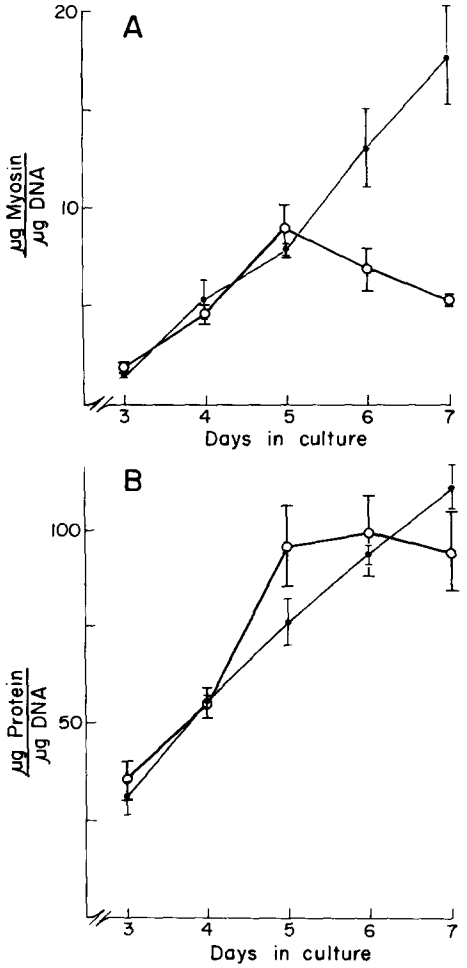

FIGURE 3 Accumulation of myosin and total protein in myogenic cultures grown in 5.4 mM K^+ medium and 12 mM K^+ medium. Primary cultures of 12-d embryonic muscle cells were prepared as described above. At 40 h, cultures were refed with either 5.4 mM K^+ or 12 mM K^+ medium, both containing 5 × 10^{-6} M cytosine arabinoside to inhibit fibroblast growth. On successive days total extracts were prepared in triplicate and analyzed for protein, DNA, and myosin content as described in Materials and Methods. (*A*) Myosin content of myogenic cultures normalized to DNA from day 3 through day 7 in 5.4 mM K^+ medium (--●--●--) and in 12 mM medium (--O--O--). (*B*) Total protein accumulation normalized to DNA in 5.4 mM K^+ medium (--●--●--) and 12 mM K^+ medium (--O--O--). Data are from one of five replicate experiments.

in MEM containing excess cold leucine and were refed with either normal or high potassium medium. At the times indicated, cultures were collected in triplicate and the radioactivity remaining in MHC was determined as described in Materials and Methods and normalized to DNA content in the respective cultures. Degradation rates for MHC were measured for the 50-h period after the [^{3}H]leucine pulse and the results are given in Fig. 4*A*. The actual turnover times ($t_{1/2}$) were estimated by assuming linear decay kinetics and calculating the linear regression of the data points (see Materials and Methods). As indicated, MHC in normal cultures has a $t_{1/2}$ of ~65 h when measured over a 2-d period, while MHC in high potassium exhibits a much reduced stability and a $t_{1/2}$ of ~23 h.

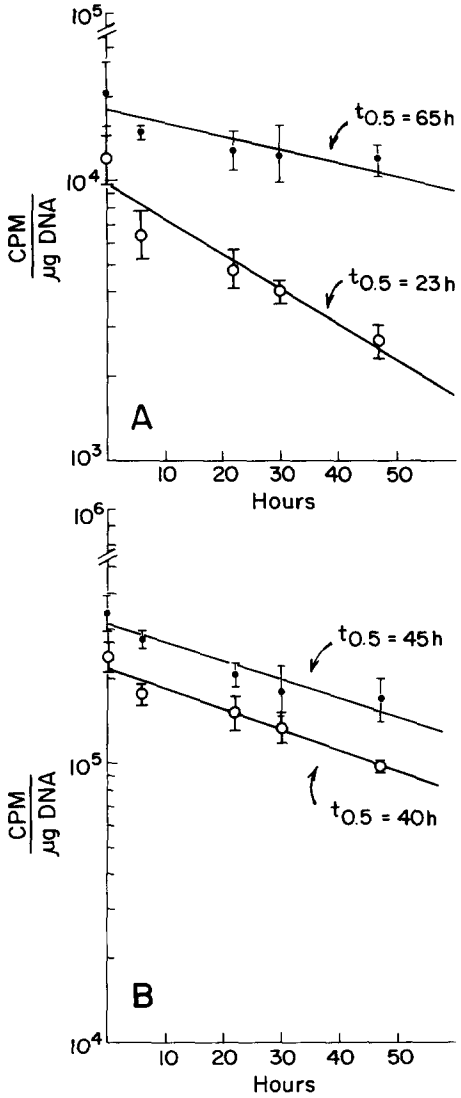

FIGURE 4 Turnover of myosin and total protein in myogenic cultures grown in 5.4 mM K^+ medium and in 12 mM K^+ medium. Myogenic cultures were prepared as described in the legend to Fig. 3. On day 6, cultures were pulse labeled for 1 h with [^{3}H]leucine (50 µC/ml) in leucine-free MEM and then refed with their respective media. At the designated time, samples were collected in triplicate and the radioactivity remaining in the myosin heavy-chain band was determined, as previously described, and normalized to DNA. $t_{1/2}$ was determined by assuming linear decay kinetics and calculating the linear regression of the data. (*A*) Myosin turnover in 5.4 mM K^+ (--●--●--) and 12 mM K^+ medium (--O--O--). (*B*) Total protein turnover in 5.4 mM K^+ (--●--●--) and 12 mM K^+ medium (--O--O--). Data are from one of three replicate experiments.

When, on the identical cultures, we measure the rate of degradation of total protein (Fig. 4*B*) it is also clear that this value is only marginally affected in high potassium compared with normal cultures. The $t_{1/2}$ for high potassium cultures was 40 h. It appears that the increased rate of myosin heavy-chain degradation is not associated with any general increase in protein degradation.

Growth in High Potassium Does Not Alter Myosin Isozyme Patterns

It is possible that chronic relaxation induced by high K^+ causes some switching in the synthesis of myosin isozymes. Under normal conditions avian and rat embryo muscle cultures synthesize an embryo-specific MHC (32, 33) and predominantly myosin light chains characteristic of fast muscle, fast light-chains 1 and 2 (FLC_1, FLC_2) (34). We therefore measured the myosin isozymes present in normal and in high K^+ cultures. The results are given in Figs. 5 and 6. First, it is apparent that the MHC synthesized in high K^+ yields a peptide map indistinguishable from the map of MHC from normal cultures. The maps are radioautographs of MHC synthesized in the presence of [^{35}S]methionine so they represent newly synthesized MHC made during the time of the pulse and therefore during the same time when MHC turnover in high K^+-cultures is elevated. We have determined by mixing experiments that we can detect a 10% contamination of embryonic MHC with adult specific MHC (unpublished data). We may conclude therefore that,

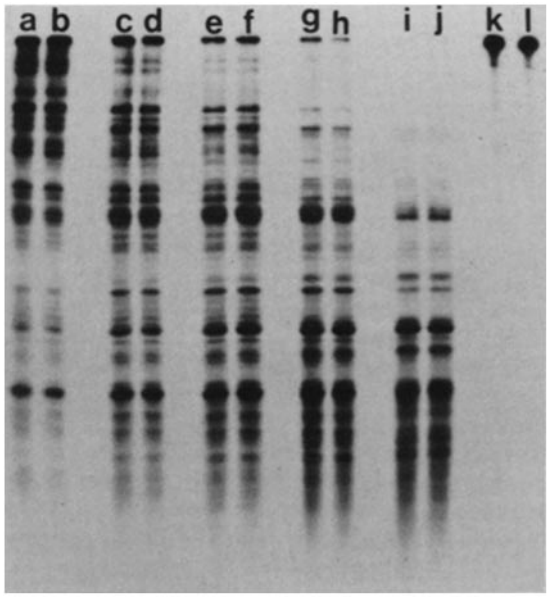

FIGURE 5 Peptide maps of myosin heavy chain from cultures grown in 5.4 mM K^+ medium and from cultures grown in 12 mM K^+ medium. [^{35}S]methionine-labeled myosin was prepared and myosin heavy-chain peptide maps were generated as described in Materials and Methods. Lanes *a*, *c*, *e*, *g*, and *i* are peptide maps of myosin heavy chain from cultures grown in 5.4 mM K^+ and lanes *b*, *d*, *f*, *h*, and *j* are myosin heavy-chain peptide maps from cultures grown in 12 mM K^+ medium. The amount of protease added to each sample is as follows: lanes *a* and *b*, 10 ng of *Staphylococcus aureus* V8 protease; lanes *c* and *d*, 25 ng of *S. aureus* V8 protease; lanes *e* and *f*, 50 ng of *S. aureus* V8 protease; lanes *g* and *h*, 100 ng of *S. aureus* V8 protease; lanes *i* and *j*, 250 ng of *S. aureus* V8 protease; lanes *k* and *l* have no protease added and represent myosin heavy chain from cultures grown in 5.4 mM K^+ and from cultures grown in 12 mM K^+, respectively.

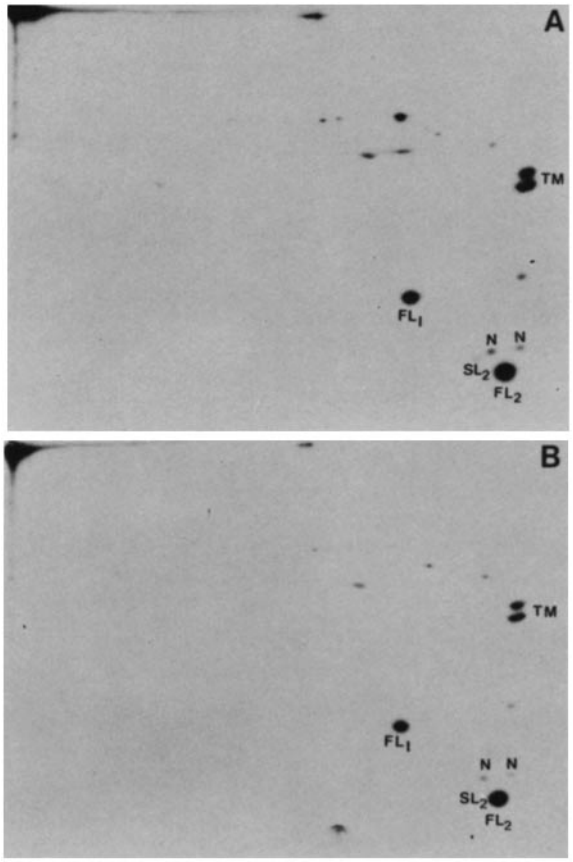

FIGURE 6 Myosin light chains synthesized in cultures grown in 5.4 mM K^+ medium and from cultures grown in 12 mM K^+ medium. [^{35}S]methionine-labeled myosin was analyzed by two-dimensional gel electrophoresis as described in Materials and Methods. (*A*) Myosin from cultures grown in 5.4 mM K^+ medium; (*B*) Myosin from cultures grown in 12 mM K^+ medium. The following proteins are indicated on the gel: FL_1-fast light chain 1, FL_2-fast light chain 2, SL_2-slow light chain 2, *TM*-tropomyosin, α and β, *N*-nonmuscle myosin light chains.

with a 10% experimental error, we are not able to detect any change in the MHC isozyme type being synthesized in high K^+ (Fig. 5).

Second, it is also apparent that myosin light-chain patterns are not altered by growth in high K^+. In both control and experimental myosin extracts the light chains are almost exclusively FLC_1 and FLC_2 with a minor synthesis of slow light chain 2 (SLC_2). We never observe either FLC_3 or SLC_1 in these cultures under normal conditions (Fig. 6). The spots labeled *N* in the figure correspond to components not exclusively associated with muscle. The *N* components are found for example in fibroblast cultures and they are peptides synthesized by muscle as well as by nonmuscle cells.

DISCUSSION

When the extracellular potassium concentration of mature chick myogenic cell cultures is increased to 12 mM, there follows: (*a*) immediate cessation of spontaneous contractions in myotubes and myofibers, (*b*) eventual failure by day 6–7 of myotubes to develop cross striation (phase-contrast observation), (*c*) eventual failure by day 5–6 to further accumulate myosin heavy chains, and (*d*) a marked increase by day 5–6 of the rate of degradation of MHC (Figs. 1, 3, and 4 above). The known direct effect of 12 mM potassium is to decrease the resting potential of myotube membranes (7).

The nature of the coupling between potassium depolarization of myotube membranes and increased MHC degradation is presently unknown. It may be direct, or it may occur as a result of loss of contraction coupled to the absence of spontaneous action potentials, or both. Clearly, the myotubes in high potassium have only 30% of the MHC of control (Fig. 3*A*) but this inhibition is not part of a general reduction in total protein accumulation. Fig. 3*B* demonstrates that total protein in 12 mM K^+ is quite similar to control values, with even a suggestion of some hypertrophy in high potassium cultures between days 4 and 6. By day 7 the total protein content in high potassium cultures is 87% of control values. Since myosin makes up about 15% of the protein of day-7 myotubes this reduction in total protein can be accounted for mostly by the reduced myosin content. In addition, as shown in Fig. 1, 7-d cultures appear quite healthy in high potassium except that they lack the clear cross-striated pattern of control cells.

The reduction of myosin accumulation in 12 mM K^+ is not due to a reduced rate of myosin synthesis. Myosin synthesis was determined by measuring [^{3}H]leucine incorporation into myosin during a 1-h pulse. It was noted that the elevated K^+ in the labeling medium reduced the uptake of [^{3}H]leucine into the cell during the pulse. Since increasing the K^+ has been shown to alter the resting potential of the myotube (see discussion below) it is not surprising that the transport properties of the cell membrane may be altered. Therefore, to compare the incorporation of [^{3}H]leucine into myosin under the different labeling conditions it was necessary to measure the leucine pool specific activity during the pulse. As shown in Table 1, when [^{3}H]leucine incorporation is corrected for differences in the pool specific activity, incorporation of [^{3}H]leucine into myosin and total protein were similar in 5.4 and 12 mM K^+ media.

Our results indicate that an increased rate of myosin turnover is primarily responsible for the reduction in myosin accumulation in myogenic cultures grown in 12 mM K^+ medium. As mentioned above (Fig. 3*A*), cultures grown in 12 mM K^+ medium for 7 d contain 33% the amount of myosin and the turnover data (Fig. 4*A*) show these same cultures to exhibit approximately three times the rate of myosin turnover as cultures grown in 5.4 mM K^+ medium. In an earlier report (19, 21) we showed that tetrodotoxin, which blocks action potentials and spontaneous contraction in cultured myotubes, will cause failure of myosin accumulation. The loss of myosin from TTX-treated cultures was also associated with a three- to fivefold increase in MHC degradation. The TTX experiments and the experiments in this report are comparable in many ways and there are some differences that require discussion. The increase in turnover of MHC is roughly the same in both cases although the $t_{1/2}$ values do differ in the control of the two series. The actual $t_{1/2}$ values for MHC vary from experiment to experiment and are most probably affected by differences in serum batches, embryo extract, and cell density. Within any given experimental series, however, the MHC halflife was always three- to fivefold greater in controls than in the respective TTX-treated or high K^+-treated culture. It appears therefore that inhibition of spontaneous contractions by either TTX or high K^+ reduces myosin accumulation by the same mechanism, i.e., by increased degradation. We have begun to measure the half-life for other myofibrillar peptides under TTX and high K^+ treatment and it appears that all of them exhibit similar behavior in treated cells compared with controls.

In the experiments reported here increased MHC turnover is correlated with a reduction in muscle-fiber resting potential. Spontaneous contractions do not usually occur until day 5 in culture because the resting potential of the myotube must reach −50 mV before an action potential can be elicited (8). The resting potential of a mature myotube is reduced to −40 mV when placed in 10.8 mM K^+ medium (7). Thus the resting potential of myotubes grown in 12 mM K^+ medium will never reach the requisite level to elicit an action potential. This is presumably the reason why cells placed or grown in 12 mM K^+ medium never exhibit spontaneous contractions. The myosin content of young, noncontractile myotubes (days 3–5) is not affected by either high potassium or TTX (19) and this is consistent with the fact that neither TTX-sensitive sodium channels nor membrane potentials necessary for spontaneous contraction arise in myotubes until later in culture development.

The question now remains: "What is the causal relationship between lack of contraction and/or electrical activity, on the one hand, and the increase in rate of MHC degradation on the other?" We have eliminated both decreased synthesis of myosin heavy chain and a general increase in proteolytic activity as potential biochemical mechanisms that can account for the overall decrease in MHC accumulation. Another possible explanation for the observed increased rate of MHC degradation would be that under depolarizing conditions the MHC that is produced is modified so that it becomes unstable or that myosin light-chain pattern is altered so that an "inappropriate" pool of light and heavy chains is produced. These modified myosin peptides would then be subjected to an increased probability of attack by steady-state proteolytic activity. But the myosin heavy-chain polypeptide is not modified, at least as far as we are able to tell by peptide map analysis (Fig. 5), nor is the myosin light-chain pattern affected (Fig. 6). Tentatively therefore we must also eliminate changes in myosin isozymes being synthesized as an explanation for the observed increase in MHC degradation.

We are left with the hypothesis that membrane agents such as TTX or high K^+ somehow have effects that are coupled to defective assembly of new myofibrils. Treated cells do not become cross striated (Fig. 1) for example, suggesting that complete assembly of sarcomere structure is defective. In addition, there is good evidence already published that assembly of myofibrils in embryonic muscle takes place just under the muscle plasma membrane (35, 36, 37). Could it be that disrupting the normal membrane potential of these fibers somehow interferes with a membrane-linked assembly process? Such disruption would lead to enlarged pools of free myofibrillar peptides that would then be relatively more exposed to endogenous muscle proteolytic activity as discussed above and would show the increased degradation rates that we measured. At the same time, at least in the short run, lack of assembly of myofibrils would not necessarily show any accelerated degradation of total protein or any significant reduction in accumulation of total protein. This hypothesis may be further tested now by the two separate relaxing strategies (TTX and high potassium) and may provide a basis for enhancing our understanding of the relationship between muscle activity and the stability of muscle structure.

Note Added in Proof: It has recently come to our attention that B. Friedman and J. A. Powell (1981, *Dev. Biol.*, 83:399–404) used changes in external K^+ to alter degradation rates of myosin heavy chain in cultured dysgenic mouse muscle. When dysgenic fibers are activated by lowering external K^+ it was shown that MHC degradation increased.

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