An analysis of contractile proteins in developing chick heart by SDS polyacrylamide gel electrophoresis and electron microscopy

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SUMMARY

Chick heart development was studied using transmission electron microscopy and SDS-polyacrylamide gel electrophoresis in combination with densitometry. Myosin heavy chain, α-actinin, actin and tropomyosin accumulations were analysed in developing hearts from preheartbeat stage 9 (Hamburger-Hamilton staging series) through 2 days after hatching. At the preheartbeat stage, electron microscopy revealed a significant number of thin filaments scattered throughout the cytoplasm of the myoblasts; however, very few thick filaments were seen. There was no obvious association between the two filament types. SDS-polyacrylamide tube gels of heart muscle homogenates demonstrated the presence of all five proteins in hearts at the preheartbeat stage. Further analyses of the proteins by gel densitometry indicated that both actin and myosin accumulated rapidly during heart development while α-actinin and tropomyosin levels remained relatively static. Our results show that detectable quantities of myosin heavy chain, α-actinin, actin and tropomyosin accumulate in myocardial cells prior to the appearance of myofibrils and initiation of the contractile function.

INTRODUCTION

The now classical description of the myofibril as an interdigitating hexagonal array of thick and thin filaments (Huxley & Hanson, 1954) and the subsequent localization of the various proteins by biochemical and immunohistochemical methods has provided a means, though limited, by which the electron microscopist can identify and follow the aggregation and organization of these contractile proteins into the adult sarcomere structure (ref. reviews by Hermann, Heywood & Marchok, 1970; Fischman, 1970). Nevertheless, considerable disagreement exists regarding the temporal relationships of the various filaments and filament aggregates at both the ultrastructural (Allen & Pepe, 1965; Fischman, 1967, 1970; Hay, 1963; Heuson-Stiennon, 1965) and biochemical levels (Heywood & Rich, 1968; Masaki & Yoshizaki, 1972; Potter & Hermann, 1970; Roy, Sreter & Sarkar, 1979).

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Certain biochemical events associated with protein synthesis and assembly have been described in some detail. Of the several myofibrillar proteins, myosin and actin have been the most extensively monitored in synchronous populations of differentiating skeletal muscle cells (Emerson & Beckner, 1975; Emerson, 1977). These investigations have demonstrated that synthesis of both myosin and actin increase dramatically during and shortly after myoblast fusion in skeletal muscle cell cultures. In more recent studies, Devlin & Emerson (1978) showed that the synthesis of myosin heavy chain, two myosin light chains, two subunits of tropomin, and two subunits of tropomyosin are initiated simultaneously at the time of myoblast fusion. Synthesis of only one myofibrillar protein, α-actin, is detected in cultures of dividing myoblasts. Isotope dilution studies (Allen, Stromer, Goll & Robson, 1978, 1979) also show an increase in tropomyosin during and after the initiation of muscle cell fusion.

The bulk of biochemical studies on contractile proteins in developing muscle have concentrated on skeletal muscle cells in culture. To date, no comparable studies have been made on developing cardiac muscle tissue. Before we can begin to understand myofibrillogenesis in cardiac muscle, it will be necessary to know the relative quantities of proteins present at different stages of development. In skeletal muscle, myofibrillar protein synthesis is highly coincident with myotube formation. Since developing cardiac muscle cells do not fuse and since myofibrillogenesis has not been examined in detail in developing heart, we undertook the present study to correlate the ultrastructural features of myofibril formation with the accumulation patterns of the major myofibrillar proteins. Myosin, actin, tropomyosin and α-actinin were chosen because they represent the primary components of the thick filament, thin filament, calcium regulatory complex and Z-line respectively.

Our results show that all four proteins are present in the heart tube at the preheartbeat stage even though morphologically, no organized myofibrils are seen. With further development, a marked increase in the accumulation of actin and myosin are noted, corresponding to the increase in number and organization of thin and thick filaments. However, the levels of accumulation of α-actinin and tropomyosin remain relatively constant throughout development.

**MATERIALS AND METHODS**

**Procurement of tissues**

Fertilized White Leghorn eggs were incubated at 38 °C for 26 h to 21 days. The Hamburger-Hamilton staging system for chick embryos was used. Hearts from embryos ranging from Hamburger-Hamilton stage 9 (26 h of incubation) through stage 44 (18 days of incubation) and 2-day-old chicks were investigated.

**Contractile protein standards**

Contractile proteins extracted and purified by published methods were used
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as standards in the electrophoresis experiments. These included: 1) chicken skeletal muscle myosin (Richards, Chung, Menzel & Olcott, 1967); 2) chicken skeletal muscle actin (Spudich & Watt, 1971); 3) porcine skeletal muscle α-actinin (Goll, Suzuki, Temple & Holmes, 1972); 4) chicken heart tropomyosin (Eisenberg & Kielly, 1974; Bailey, 1948); 5) chicken skeletal muscle tropomyosin (Eisenberg & Kielly, 1974; Bailey, 1948); and 6) chicken skeletal muscle myofibrils (Etlinger, Zak & Fischman, 1976).

Electron microscopy

Embryonic hearts were fixed by perfusion with 2% formaldehyde, 2.5% glutaraldehyde and 0.1% Picric acid buffered to pH 7.3 with 0.15 M-phosphate buffer (modified from Ito & Karnovsky, 1968). Drawn glass capillary tubing was used for perfusion of the stage-9 and stage-15 embryos, while a syringe with a small gauge needle was used in the older embryos. After perfusion fixation, small pieces of the myocardium were then immersed in the fixative for an additional 2 h, postfixed in 1% OsO4, embedded in Epon and routinely processed for transmission electron microscopy. Thin sections were mounted on copper grids, double stained with lead citrate and uranyl acetate and viewed on Phillips 200 or Hitachi HU-11D electron microscopes at accelerating voltages of 60 or 75 kV.

SDS-polyacrylamide gel electrophoresis

Embryonic and adult heart tissues were dissected in quantity and prepared for analysis using the stacking gel method of Laemmli (1970). Tissue samples included: a) embryonic hearts from Hamburger-Hamilton stage 9 (26–30 h of incubation), stage 15 (51–56 h of incubation), stage 28–30 (6 days of incubation), stage 38 (12 days of incubation) and stage 44 (18 days of incubation); b) ventricular and atrial heart regions of newly hatched 2-day-old chicks; c) adult chicken myofibrils from cardiac and skeletal muscle prepared by the method of Etlinger et al. (1976); d) purified protein standards; and e) non-muscle control using brain from 12-day embryo and 2-day-old chicks.

Particular care was taken in the dissection of the newly fused heart tubes of stage-9 embryos. The heart tube, including the prospective atria, was very carefully removed by a cut just cephalad to the conus region followed by cuts caudal to the sinus venosus. A hair loop was used to remove any non-myocardial tissue. About 175 hearts were required for each tube gel.

After dissection, all tissues were homogenized at 0°C in 0.1 M-Tris–HCl buffer pH 7.4 to which the proteolytic inhibitor phenyl methyl sulphonyl fluoride was added to a final concentration of 1.0 mM. The samples were assayed for protein concentration (BIORAD method) and suspended in a 10% (w/v) glycerol, 5% (v/v) beta-mercaptoethanol, 2.3% (w/v) SDS and 0.0625 M-Tris–HCl, pH 6.8 and heated at 100°C for 5 min.

Samples with 35–50 μl of protein were layered on the tops of 10 cm × 5 mm tube gels containing 10% acrylamide and electrophoresed at 1.5 milliamper./gel.
To ensure linearity between O.D. and protein quantity, the same samples were run at two or three different concentrations. Optimal linearity of samples with the best resolution was achieved when 42 μg of protein was loaded. Co-electrophoresis with actin, myosin, α-actinin and tropomyosin standards as well as mixtures of standards and unknowns were used to identify the various protein bands in the unknowns. The latter was found to be the more effective method of identifying the protein bands in tube gels. When the gels were scanned, the enlarged peak (due to addition of the specific purified protein standard) could be easily determined. The gels were stained routinely with 0.05% Coomassie brilliant blue R250. Graphic traces were made using a Gilford Spectrophotometer Model 250 (Gilford Instruments, Oberlin, Ohio) equipped with a gel-scanner attachment at an absorbance wavelength of 550 nm. From the gel scans, ratios of each of the contractile proteins to total protein and to actin were determined by use of a tracing device linked to a Numonics digitizer interfaced with a HP9815S programmable calculator.

RESULTS

Electron microscopy

At stage 9, after the fusion of its paired primordia, the heart is a nearly straight tube. Electron micrographs (Fig. 1) reveal that the cytoplasm of cardiac myoblasts contain free ribosomes, glycogen and lipid droplets. Thin (6 nm) filaments are often seen though thick (15 nm) filaments are rare.

Spontaneous contractions begin in the chick heart at stage 10 (nine to ten somites) on the right side of the ventricle. By stage 15, the heart is beating rhythmically and electron microscopy reveals a marked increase in the cytoplasmic complexity of developing myocardial cells (Fig. 2). Numerous myofibrils at various stages of assembly are scattered throughout the cytoplasm; although the highly differentiated myofibrils with distinct A bands, I bands and Z-lines tend to be located in the peripheries of the cells. Granular endoplasmic reticulum, free ribosomes and Golgi complexes are apparent and adjacent cell membranes are interspersed with desmosomes and other regions of electron density.

Fig. 1. Transmission electron micrograph of a stage-9 embryo (seven somites). Intercellular spaces (is) sometimes separate adjacent myoblasts. The granular appearance of the cytoplasm is due to the abundance of free ribosomes and glycogen. Mitochondria (m), rough endoplasmic reticulum, and lipid droplets (li) often are seen. At this preheartbeat stage, no formed sarcomeres are visible, although thin (6 nm) filaments (arrowheads) are scattered throughout the cytoplasm. ×4000; Scale bar = 2 μm.

Fig. 2. Transmission electron micrograph of stage-15 embryonic myocardium. The heart is beating rhythmically by this time. Myofibrils at various stages of assembly are seen in the cytoplasm; some complete with Z-lines (Z). Desmosomes (d) and fascia adherentes (fa) and other regions of increased electron density are often present at adjacent cell membranes. ×16 000; Scale bar = 1 μm.
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Figs 1 & 2
Table 1. Per cent of myosin heavy chain, alpha-actinin, actin and tropomyosin in the developing chick heart

<table>
<thead>
<tr>
<th>Age</th>
<th>Tissue</th>
<th>Percent of total protein</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Myosin</td>
</tr>
<tr>
<td>Stage 9</td>
<td>Fused heart-tubes</td>
<td>1.84 ± 0.5</td>
</tr>
<tr>
<td>Stage 15</td>
<td>Whole heart</td>
<td>3.34 ± 0.8</td>
</tr>
<tr>
<td>6 days</td>
<td>Embryo ventricle</td>
<td>7.03 ± 0.7</td>
</tr>
<tr>
<td>72 days</td>
<td>Embryo ventricle</td>
<td>9.4 ± 1.0</td>
</tr>
<tr>
<td>18 days</td>
<td>Embryo ventricle</td>
<td>10.32 ± 0.9</td>
</tr>
<tr>
<td>2 days post-hatches</td>
<td>Newly-hatched chick ventricle</td>
<td>16.92 ± 0.8</td>
</tr>
</tbody>
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In this table, values for percent total protein are expressed as the mean ± standard deviation. Five SDS-electrophoresis gels of embryonic heart tissues were analysed for each developmental stage except stage 9 where three were evaluated. Statistical comparisons were made using the Student’s t-test.

After six days of incubation, myofibrils several sarcomeres in length align parallel to each other, with the Z-lines in register (Fig. 3). As myofibrillogenesis progresses, the number, size, and complexity of the myofibrils increase such that at 18 days of incubation, the cytoplasm of the myocardial cells is filled with organized myofibrils (Fig. 4).

SDS polyacrylamide gel electrophoresis

All heart tissues examined contain proteins which co-migrate with myosin heavy chain (200 000 daltons), actin (45 000 daltons), α-actinin (100 000 daltons), and tropomyosin (34 000 daltons) standards on SDS polyacrylamide gels (Fig. 5). Densitometric scans of the gels (Fig. 6) are analysed to determine the percentage of each specific contractile protein relative to the total protein content in the heart. A summary of findings is given on Table 1 and Fig. 7.

Fig. 3. Transmission electron micrograph of 6-day embryonic ventricular myocardium. Myofibrils are more numerous at this stage and some are in parallel arrays, with their Z-lines (Z) in register. ×8000; Scale bar = 2 μm.

Fig. 4. Transmission electron micrograph of 18-day embryonic ventricular myocardium. The number of myofibrils has increased significantly and now occupy most of the sarcoplasm, with clear regions mostly at the ends of the nucleus (n). The myocardium contains fully developed myofibrils complete with distinct A-bands, I-bands, Z-lines, and M-lines. ×11 000; Scale bar = 1 μm.
Fig. 5. SDS polyacrylamide gels of heart homogenates and protein standards include: (a) preheartbeat stage-9 hearts; (b) stage-15 hearts; (c) 6-day ventricles; (d) 12-day ventricles; (e) 18-day ventricles; (f) ventricle from 2-day-old chick; (g) adult chicken ventricular myofibrils; (h) tropomyosin standard; (i) actin standard; (j) α-actinin standard; (k) myosin heavy chain standard. The specific proteins that were examined include: M, myosin heavy chain; α-A, α-actinin; A, actin; T, tropomyosin.
Fig. 6. Our estimation of the approximate percentage of total protein represented by myosin heavy chain (M), α-actinin (α-A), actin (A) and tropomyosin (T) is based on the gel scans. Total protein content is represented by the area under the entire gel scan, determined by a tracing device linked to a programmable computer to integrate the area covered. Peak areas are defined by perpendicular lines dropped to the baseline.

Myosin heavy chain comprises 1.84% of the total protein in stage-9 hearts (preheartbeat). A steady increase in percent accumulation of this protein continues throughout development. By 18 days, myosin HC accumulation has increased five-fold to represent 10.32% of the total protein. At two days after hatching, there is a sharp rise in myosin content to 16.78% of total protein.

Similarly, actin (45,000 daltons) represents 8.53% of the total protein in stage-9 hearts. This actin component showed steady and consistent increases in accumulation with development. At 2 days post-hatching, actin comprises about 16.94% of the total protein in the chick heart.

As expected, α-actinin (1.14%) and tropomyosin (1.0%) accumulations at stage 9 are smaller than either actin or myosin. Although both α-actinin and tropomyosin showed relatively small increases numerically, their accumulation doubled between stage 9 and 2 days after hatching.

Analyses of the gel scans for non-muscle control tissue (brain) indicated that even as late as 2 days posthatching, the actin and myosin contents still appeared
DISCUSSION

Our results show significant increases in actin and myosin accumulation throughout development while \(\alpha\)-actinin and tropomyosin levels remain relatively constant. SDS polyacrylamide gels of the precontractile heart indicate the presence of the four proteins although electron microscopy reveals only a few thick and mostly thin filaments scattered in the cytoplasm. No thick–thin filament arrangement or Z-lines are seen. In general, the appearance of the sarcomere is taken as a direct measure of macromolecular assembly. However, at the preheartbeat stage, no direct morphological correlates can be made. Obviously, the mere presence of these proteins is not sufficient for the formation of the sarcomere apparatus. Both quantitative and qualitative changes may be needed before these proteins can eventually assemble into an identifiable myofibril. For example, a critical concentration may be needed before polymerization of these individual proteins can occur. Alternatively, an inhibitor to be slightly less than the levels in precontractile hearts; furthermore, no relative increases in the contractile proteins were noted in the brain tissue with advancing embryonic development.
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To polymerization for actin could be present at this time. Since there are defined stoichiometric relationships among the proteins in the myofibril (Potter, 1974), one expects that its assembly might depend upon changes in relative quantities of the protein components.

Of the proteins investigated, actin represents the most abundant myofibrillar protein throughout development. Although our one-dimensional gel analyses of actin do not distinguish between muscle and non-muscle actin, our two-dimensional gel analyses of the same contractile proteins in the chick heart show the presence of both α-actin, a muscle-specific actin isozyme, and β-actin, a 'non-muscle' type actin isozyme in equivalent amounts (Woodroofe, Lim-Spiker & Lemanski, 1980). This finding was also confirmed by Wiens & Spooner (1981). α-Actin becomes the dominant actin form as development progresses.

In skeletal muscle cell cultures, several reports indicate a rapid burst in protein synthesis related to myoblast fusion. Myosin synthesis has been reported to occur either during cell fusion (Devlin & Emerson, 1978; Emerson & Beckner, 1975; Young, Goll & Stromer, 1975; Paterson & Strohman, 1972), or shortly after cell fusion (Coleman & Coleman, 1972). Actin synthesis also increases rapidly in fused cells (Paterson, Roberts & Yaffe, 1974; Rubinstein, Chi & Holtzer, 1974; Rubinstein et al. 1976; Garrels, 1979a,b). Similar relationships exist for α-actinin synthesis (Allen et al. 1979) and tropomyosin synthesis (Allen et al. 1978; Allen et al. 1979; Carmon, Neuman & Yaffe, 1978; Garrels, 1979a,b) which also reflect the same abrupt change during the final phase of skeletal muscle differentiation. Actin and myosin in the heart do not accumulate in rapid 'bursts' but rather show steady increases in protein accumulation throughout development.

Since significant quantities of the myofibrillar proteins have already accumulated in the heart cells prior to sarcomere formation, it appears that only small increases in quantities of the proteins from the preheartbeat stage 9 to the newly postheartbeat stage 15 are necessary for myofibril formation to occur. Application of the Student's t-test to the data (Table 1) reveals that between stage 9 and stage 15 only myosin (P < 0.01) and actin (P < 0.05) show statistically significant increases and even these increases are very small. This is not surprising since heart function is precocious in vertebrate embryos. The interrelated events to cytodifferentiation and establishment of myocardial architecture occur very rapidly to produce a functional organ early in embryonic life. In less than 40 h of incubation, precardiac mesoderm has undergone re-organization, from a sheet of undifferentiated cells to a tubular heart whose cells exhibit a high degree of specialization. Thus, it appears that contractile proteins must accumulate in myocardial cells in significant quantities well in advance of a heartbeat.

It is obvious that one-dimensional SDS-polyacrylamide gel electrophoresis does not discriminate between muscle and non-muscle forms of these proteins. We believe, however, that it is the muscle-specific forms of these proteins which contribute to the increase in myofibrillar organization and complexity of
developing cardiac muscle since the contractile protein accumulation in the non-muscle tissue (brain), even at the most advanced stage, are less than in preheartbeat heart tubes. Furthermore, non-muscle tissue does not show significant increases in contractile proteins during development. Similar results have been reported in non-muscle tissues in the axolotl (Ambystoma mexicanum) system (Lemanski, 1976; Lemanski, Mooseker, Peachey & Iyengar, 1976).

In conclusion, our results show that significant quantities of myosin heavy chain, α-actinin, actin and tropomyosin are present in preheartbeat cardiomyocytes prior to the appearance of myofibrils. Increased protein accumulation during heart development is reflected in a corresponding increase in myofibrillar organization and complexity.

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