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Calcium and Cyclic Nucleotide Dependent Regulatory Mechanisms during Development of Chick Embryo Skeletal Muscle†

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ABSTRACT: Late prenatal and early postnatal development of skeletal muscle regulatory systems was studied in chick embryos from 7 days before to 7 days after hatching. The following protein concentrations or enzyme activities were measured daily in pectoralis and in leg muscle extracts: parvalbumin, calmodulin (the heat-stable ubiquitous calcium-dependent regulator), calcium–calmodulin-dependent myosin light-chain kinase, cyclic adenosine 3′,5′-monophosphate (cAMP) dependent and independent protein kinases, and the heat-stable protein kinase inhibitor. The appearance of adult contractile properties did not correlate with variations in cAMP-dependent protein kinases or the protein kinase inhibitor, which are already present at day −7 and continue to fluctuate around the same level. Muscle development is accompanied by a decrease of cAMP-independent protein kinase activity, which becomes minimal at days +1 to +3, and of the calmodulin content after day +3. These changes may be ascribed to the decrease in the percentage of proliferating cells. By contrast, the synthesis of calcium-modulated myosin light-chain kinase and parvalbumin occurs around the time of hatching, together with that of the sarcoplasmic reticulum Ca^{2+}–Mg^{2+}–ATPase [Martonosi, A., Roufa, D., Boland, R., Reyes, E., & Tillack, T. W. (1977) *J. Biol. Chem. 252*, 318–332]. Myosin light-chain kinase activity, initially low, increases rapidly from day −2 to reach a maximum at day +3 to +4. Similarly, parvalbumin, measured by a sensitive radioimmunoassay, is almost absent from all types of muscle until day −2. Active synthesis first begins in leg muscles and then in pectoralis muscle several days later (day +4) and at a much lower rate. cAMP-dependent protein kinase (and its inhibitor) and calmodulin, the ubiquitous regulatory proteins which mediate the effects of cAMP and Ca^{2+} ions, respectively, are synthesized early in embryonic development. Fast muscle differentiation, which involves the switch off of slow-twitch muscle myosin and the withdrawal of multiple innervation [Gauthier, G. F., Lowey, S., & Hobbs, A. W. (1978) *Nature (London) 274*, 25–29], is more closely correlated with the late synthesis of the elements of the calcium cycle, namely, the sarcoplasmic reticulum Ca^{2+} pump, the calcium-dependent myosin light-chain kinase, and the soluble relaxing factor, parvalbumin.

Regulation of physiological or metabolic processes in response to an external hormonal or neural stimulus is achieved through two main intracellular second messengers, calcium ions (Rasmussen & Goodman, 1977) and cyclic nucleotides (Robison et al., 1971). Skeletal muscle is a good example of a tissue in which the two systems not only coexist but also control each other in a concerted manner (Fischer et al., 1976). For instance, stimulation of glycogenolysis by catecholamines involves the sequential stimulation of the membrane-bound adenylate cyclase, activation by cAMP[1] of cAMP-dependent protein kinases (Hofmann et al., 1975), activation of glycogen phosphorylase kinase by phosphorylation, and finally activation of glycogen phosphorylase by the same covalent modification. However, glycogen phosphorylase kinase has an absolute requirement for Ca^{2+} ions probably via its Ca^{2+} binding subunit,

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[1] Abbreviations used: PKI, protein kinase inhibitor; R and C, the regulatory and catalytic subunits of cAMP-dependent protein kinase, respectively; cAMP, cyclic adenosine 3′,5′-monophosphate; Pa, parvalbumin; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; SR, sarcoplasmic reticulum.

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identified as calmodulin (Cohen et al., 1978).[2] Calcium released by the sarcoplasmic reticulum will similarly turn on other enzymes controlled by calmodulin, namely, cyclic nucleotide phosphodiesterases (Lin et al., 1974a) and myosin light-chain kinase (Dabrowska et al., 1978; Yazawa et al., 1978), that may perhaps provide a thick filament control of muscle contraction (Morimoto & Harrington, 1974; Lehman, 1978), in addition to the well-known troponin switch on the thin filament (Potter & Gergely, 1975).

The role of myosin light-chain phosphorylation in skeletal muscle is, however, much less clearly understood than in smooth muscle (Dabrowska et al., 1978; Sherry et al., 1978), although there are indications that it may significantly affect the affinity of the regulatory light chain for Ca^{2+} (Alexis & Gratzer, 1978).

Muscle relaxation occurs when Ca^{2+} ions are rapidly scavenged by parvalbumin, acting as a soluble relaxing factor in fast twitch muscle (Pechere et al., 1977; Haiech et al., 1979), from which they can be pumped back into SR vesicles (Blum et al., 1977). Adult contractile properties are acquired in the late prenatal and early postnatal period through modification of the type of innervation and switch off of the slow myosin synthesis that is widespread during early development (Gauthier et al., 1978). Also, synthesis of the sarcoplasmic reticulum Ca^{2+}-Mg^{2+}-ATPase has been shown to begin during the last week of embryonic development and to proceed rapidly during the first 9 days after hatching (Martonosi et al., 1977). Considerable information is available about the coordinate synthesis of contractile proteins (myosin, actin, troponin, and tropomyosin) that is first detected at the time of myoblast fusion (Devlin & Emerson, 1978) and about the synthesis of soluble enzymes, such as phosphorylase, which appears shortly before hatching and increases in the following week (Hauschka, 1968). By contrast, little is known about the development of the regulatory systems in the chick embryonic muscle. It was therefore felt that a study of several proteins involved in calcium or cyclic nucleotide dependent regulations could provide a better insight into the development of regulatory mechanisms in muscle than measurements carried out on a single enzyme.

Chick embryo skeletal muscles were studied from 7 days before to 7 days after hatching. Breast and leg muscles were analyzed separately since, in the adult bird, they have been reported to differ in their parvalbumin content (Blum et al., 1977) and thus in their calcium regulation. The following protein concentrations or enzyme activities in the cytosol were measured each day: (a) parvalbumin, i.e., the single major chick muscle parvalbumin (Blum et al., 1977) [in contrast to the parvalbumin-like material also reported to be present in chicken muscle (Heizmann et al., 1977)], for which a highly sensitive radioimmunoassay was developed; (b) calmodulin, measured by the activation of calmodulin-deficient myosin light-chain kinase; (c) calmodulin-dependent myosin light-chain kinase; (d) cAMP-dependent and -independent protein kinases (using histone as substrate); and (e) the heat-stable protein kinase inhibitor (Walsh et al., 1971; Demaille et al., 1977) specific for the catalytic subunit of cAMP-dependent protein kinase (Demaille et al., 1978).

Materials and Methods

^{32}P and ^{125}I were from CEA, Saclay. [γ-^{32}P]ATP, ~780 mCi/mmol, was prepared by the procedure of Glynn & Chappell (1964). Whatman P 81 phosphocellulose paper was obtained from Reeve-Angel; the histone IIA mixture and cAMP were from Sigma. TPCK-trypsin (278 units/mg) and soybean trypsin inhibitor (1 mg inhibits 1.58 mg of trypsin) were from Worthington.

The catalytic subunit of bovine heart cAMP-dependent protein kinase type II was prepared and stored as previously described (Demaille et al., 1977; Peters et al., 1977). The protein kinase inhibitor was prepared from skeletal muscle as in Demaille et al. (1977) and used without prior separation of the isoinhibitors (Ferraz et al., 1979). The concentration of the C subunit was determined by using the absorption coefficient $A_{280nm}^{1\%,1cm} = 14.9$ (Peters et al., 1977), and that of PKI was determined by amino acid analysis. Calmodulin-dependent myosin light-chain kinase was prepared from bovine heart according to Walsh et al. (1979).

Myosin light chains were prepared from rabbit skeletal muscle myosin and from bovine cardiac myosin according to Perrie & Perry (1970) by ethanol precipitation from 5 M guanidine hydrochloride. The whole light-chain fraction was used as substrate for myosin light-chain kinase; it contained sufficient contaminating calmodulin for maximal activation, as checked with purified calmodulin-free enzyme. The light chain susceptible to phosphorylation was partially purified from the bovine cardiac whole light-chain fraction and freed from contaminating calmodulin essentially according to Frearson & Perry (1975).

Calmodulin was prepared from bovine brain by a modification of the method of Teo et al. (1973), as described by Walsh & Stevens (1978).

Chicken parvalbumin was prepared by the procedure previously described (Blum et al., 1977; Pechere et al., 1971) from leg muscles only, since breast muscle was reported to contain no parvalbumin (Blum et al., 1977). Antisera exhibiting high titer and high avidity were obtained by repeated injections of a parvalbumin emulsion in Freund's complete adjuvant to New Zealand white female rabbits. Intramuscular injections were performed initially in the four legs (1 mg of protein in each) and then in the front legs after 3 weeks and in the hind legs 3 weeks later. Two more booster injections were made at 3-week intervals. Total bleeding was carried out through the carotid artery. The titer of the antisera was checked by using several dilutions of antiserum and of antigen by double diffusion on Ouchterlony plates.

Preparation of Skeletal Muscle Extracts from Chick Embryos. For each day of development, breast and leg muscles from one chick embryo were dissected separately, weighed, and homogenized at 4 °C with a Potter homogenizer in 2.5 volumes (v/w) of 15 mM 2-mercaptoethanol and 4 mM EDTA, pH 7.0. The homogenate was centrifuged at 7000*g* for 30 min, and the supernatant was filtered through glass wool and stored between assays at −70 °C. Samples from the same homogenates were used for each set of assays.

Myosin light-chain kinase was assayed at 30 °C during 30 min in 1 mM dithiothreitol, 13 mM magnesium acetate, 2 mM [γ-^{32}P]ATP (50–100 cpm/pmol) and 50 mM Tris buffer, pH 7.6, containing 15 mg/mL whole light-chain fraction in a final volume of 80 μL. The reaction was stopped by pipetting 50 μL of the incubation mixture on Whatman 3MM filter papers (23 mm ϕ), which were washed in 10% trichloroacetic acid (Corbin & Reimann, 1974) before counting in toluene scintillant.

The Ca^{2+}-dependent activity was given by the difference obtained between assays performed in the presence of 0.1 mM Ca^{2+} vs. 3 mM EGTA under otherwise identical conditions.

[2] The name calmodulin has been proposed for the calcium-dependent regulator (CDR) or modulator protein or calcium-dependent activator of cyclic nucleotide phosphodiesterase.

Optimal results were obtained with 10-μL aliquots of the extracts. Addition of exogenous calmodulin was unnecessary since both the whole light-chain fraction used as substrate and the muscle extract contained enough of the regulatory protein.

cAMP-dependent protein kinase was measured enzymatically rather than by its cyclic nucleotide binding capacity, in view of the existence of another adenine nucleotide binding protein (Ueland & Døskeland, 1978). Assays were performed in 70 μL of 0.3 mM dithiothreitol, 4.3 mM magnesium acetate, 0.24 mM [γ-^{32}P]ATP, 7 μM cAMP, and 12 mM potassium phosphate, pH 7.0, containing 4.3 mg/mL histone IIA mixture at 30 °C. After 30 min, [^{32}P]histone was separated from excess [γ-^{32}P]ATP by pipetting 50 μL on Whatman P 81 phosphocellulose paper; the papers were washed according to Witt & Roskoski (1975) and counted in toluene scintillant. The activity of the catalytic subunit of cAMP-dependent protein kinase was given by the difference obtained between two otherwise identical assays performed in the absence and presence of 16 units of PKI (i.e., 168 nM PKI or 1.5 μg/mL), respectively (Demaille et al., 1977). Since the specific activity of the C subunit increases with dilution to reach a plateau (Peters et al., 1977; Hofmann et al., 1977), serial twofold dilutions of 20 μL of extract (1:1 to 1:128) were performed. Optimal results were obtained when 5-μL aliquots of the extracts were assayed. With this amount of extract, the error introduced by the presence of endogenous PKI was lower than 10% and was neglected. Phosphate incorporation observed in the presence of excess PKI provided a measure of cAMP-independent histone kinases.

Protein kinase inhibitor (PKI) was only measured on leg muscle extracts because of the limited amount of breast muscle. To 1 mL of the extract was added 1 mL of water, and the sample was heated for 3 min at 90 °C, immediately cooled on ice, and centrifuged for 10 min at 3000 rpm in a clinical centrifuge. The supernatant (1.6 mL) was made 0.2 M in ammonium bicarbonate. To one-half of the supernatant (PKI test) was added soybean trypsin inhibitor (100 μg) before trypsin (25 μg), and the sample was lyophilized. The other half of the supernatant (PKI blank) was incubated at 37 °C for 30 min with trypsin before addition of soybean trypsin inhibitor. Serial twofold dilutions of PKI tests and blanks (1:2 to 1:128) were assayed in parallel in the presence of 10 nM C subunit as previously described (Demaille et al., 1977). For each sample, the PKI blank was taken as 100% activity. In this range of dilutions, inhibition decreased from ca. 90 to 5%. The amount of extract giving 50% inhibition contained 1 unit of PKI, corresponding to 6.46 ng of inhibitor (Demaille et al., 1977) or 0.734 pmol, assuming M_r 8800 (Ferraz et al., 1979).

Calmodulin was assayed by its ability to stimulate purified bovine cardiac muscle myosin light-chain kinase. An aliquot of each extract, made 5 mM in $CaCl_2$, was heated at 90 °C for 4 min, cooled immediately on ice, and centrifuged for 10 min at 3000 rpm in a clinical centrifuge. The supernatant solutions were diluted fivefold with 10 mM ammonium acetate and 5 μM $CaCl_2$ and dialyzed against this buffer. The dialyzed solutions were lyophilized and redissolved in a volume of 10 mM ammonium acetate and 5 μM $CaCl_2$ equal to that of the original extract. Aliquots of the resultant solutions were incubated for 20 min at 30 °C in 25 mM Tris-HCl, pH 7.6, 8 mM magnesium acetate, 1.6 mM dithiothreitol, 2 mM [γ-^{32}P]ATP (50–100 cpm/pmol), and 0.2 mM $CaCl_2$ containing 5 mg/mL purified light chain of bovine cardiac myosin and a constant level (26.4 pmol of ^{32}P transferred per min) of myosin light-chain kinase in a final volume of 70 μL. The reaction was quenched by pipetting 60 μL of the incubation mixture onto Whatman 3MM filter papers (23 mm ϕ), which were then washed in cold 10% followed by 5% (w/v) trichloroacetic acid before counting in toluene scintillant. Since coprecipitation at the heat step may result in calmodulin losses, levels of calmodulin reported herein represent minimal values.

Radioimmunoassay of Chicken Parvalbumin. Iodination of chicken parvalbumin (6 μg) was carried out by the procedure of Hunter & Greenwood (1962). To the protein dissolved in 15 μL of 0.25 M potassium phosphate, pH 7.5, were added sequentially 2 mCi of carrier-free Na^{125}I (in 10 μL), 50 μg of chloramine T (in 10 μL of 0.25 M phosphate buffer, pH 7.5), 120 μg of sodium metabisulfite, and 200 μg of KI (each in 10 μL of 0.05 M phosphate buffer, pH 7.5), and after 2 min 5 mg of bovine serum albumin (in 1 mL of 0.05 M phosphate buffer, pH 7.5) was added. Labeled parvalbumin was freed from excess unreacted ^{125}I by gel filtration on a 10-mL Sephadex G-50 superfine column in 0.05 M phosphate buffer, pH 7.5. Its specific activity was 105 μCi/μg, corresponding to 0.66 iodine atom introduced per molecule, presumably on the single tyrosyl residue. The diluent used throughout the assay was 0.15 M NaCl, 0.02% sodium azide, and 10 mM phosphate buffer, pH 7.4, containing 2% (v/v) normal human serum. By use of 70 fmol of [^{125}I]parvalbumin as tracer (100000 cpm), 32% binding was obtained with a final antiserum dilution of 1:50000, i.e., a range of sensitivity adequate for this study.

The assay was carried out in triplicate in a final volume of 0.5 mL by using disposable transparent polystyrene tubes (11 × 55 mm). The unknown or standard (50 μL) was added with 350 μL of diluent, followed by the antiserum (50 μL) and, finally, the tracer (50 μL). The tubes were incubated at 30 °C overnight. Separation of free and bound parvalbumin was achieved simply by addition of 0.5 mL of saturated ammonium sulfate solution, since parvalbumins do not precipitate at neutral pH below 70% saturation. After 15 min, the tubes were centrifuged for 15 min at 3000 rpm at 4 °C and the supernatants were discarded. The pellets were resuspended in 1 mL of half-saturated ammonium sulfate solution, spun down as above, and counted in a Beckman γ counter for 2 min.

Standard curves were prepared from triplicate assays of suitable dilutions of a stock parvalbumin solution of known concentration as determined by amino acid analysis. Nonspecific binding measured in the absence of antiserum and in the presence of antiserum containing an excess of unlabeled parvalbumin (50 pmol) amounted to ~8500 cpm, which was subtracted from all other figures.

Radioactivity bound in the absence of unlabeled protein was 40500 cpm (32% binding). Triplicate assays of muscle extracts were carried out on 50-μL samples subjected to serial twofold dilutions from 1:5 to 1:10240. Data were treated on a minicomputer (Olivetti P 6060) according to the equation

$$\log b/(b_0 - b) = -\log [\mathrm{Pa}] + Y$$

where Y is a constant and b_0 and b are the radioactivities bound in the absence and in the presence of unlabeled parvalbumin, respectively.

For the assay of parvalbumin samples, only dilutions where activities fell within the linear portion of the standard curve (50–1000 fmol) were taken into account, and the data were plotted as described above. The unknown concentration of parvalbumin, $[\mathrm{Pa}]_u$, was calculated from the intercept (Y') with the y axis according to the equation

$$Y' = Y - \log [\mathrm{Pa}]_u$$

in which Y is the intercept for the standard curve.

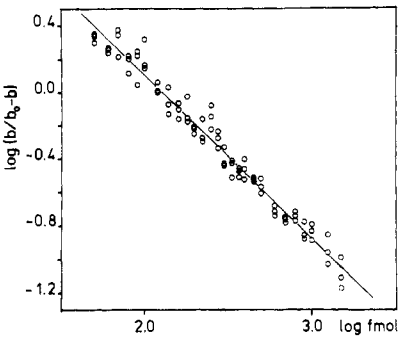

FIGURE 1: Standard curve for the radioimmunoassay of chicken parvalbumin. Triplicate samples were processed as described under Materials and Methods. The best linear fit to the experimental points was obtained through linear regression by the least-squares method using an Olivetti P 6060 minicomputer. Data below 50 fmol and above 2 pmol are not illustrated since they yield different slopes. Quantitation of embryonic muscle parvalbumin was made only in the range illustrated here (50–1000 fmol).

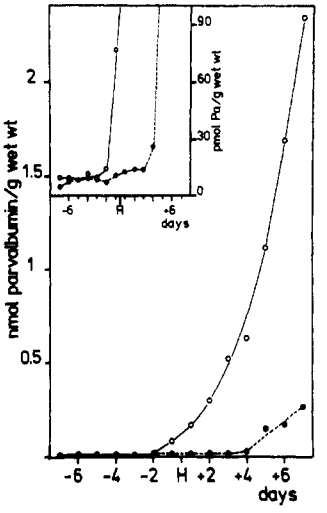

FIGURE 2: Parvalbumin levels in chick embryo leg (○) and breast (●) muscles, determined by the radioimmunoassay described under Materials and Methods. Inset: the ordinate has been enlarged by a factor of 10 to better illustrate the beginning of synthesis; H = hatching. The precision of the assay is 0.1%.

Results

Parvalbumin. By use of the sensitive radioimmunoassay described under Materials and Methods, parvalbumin gave a perfectly linear response between 50 and 1 pmol of antigen, as shown in Figure 1. The experimental slope between these points is −0.98, very close to the theoretical value of −1 expected from the equation with a correlation coefficient of 0.99. From the standard deviation, the precision of the assay is 0.08%. Below 50 fmol, the absolute value of the experimental slope is lower (−0.23). The sensitivity of the assay is determined by the minimal amount of unlabeled antigen, $[Pa]_0$, for which the scatter of the experimental points is lower than the absolute slope value. This corresponded to ≤5 fmol unlabeled.

Parvalbumin was found to be present at very low levels (≤10 pmol/g wet wt) until 2 days before hatching (Figure 2). Synthesis of parvalbumin in hind leg muscles begins at day −2 to −1, when it proceeds very rapidly, reaching 2.33 nmol/g wet wt at day +7. In contrast, parvalbumin synthesis in breast muscle is essentially nonexistent before day +4 to +5 and then proceeds at a much lower rate, reaching a level of only 0.26 nmol/g wet wt at day +7.

Calmodulin Levels in Embryonic Muscle. During studies on the calmodulin-dependent myosin light-chain kinase of bovine cardiac muscle, it became apparent that this system

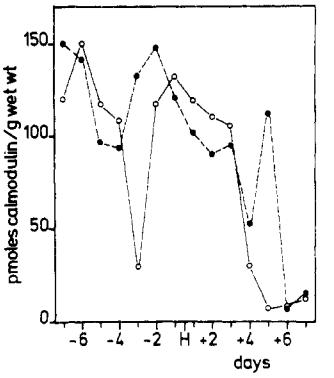

FIGURE 3: Levels of calmodulin in extracts of chick hind leg (○) and pectoralis (●) muscles at different stages of embryonic development. Aliquots of suitable dilutions of the extracts were treated and assayed in the presence of 0.2 mM $CaCl_2$ as described under Materials and Methods, H = hatching. As the extent of coprecipitation at the heat step could not be determined, the precision of the assay was not evaluated.

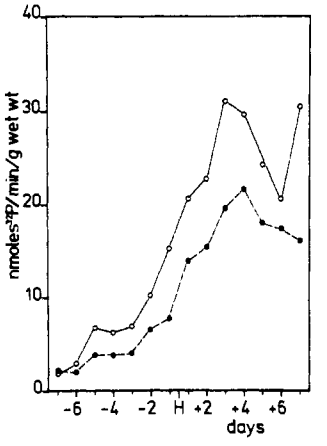

FIGURE 4: Determination of Ca^{2+}-dependent myosin light-chain kinase activity in chick embryo leg (○) and breast (●) muscles (see Materials and Methods); H = hatching.

provides a significantly more sensitive and convenient assay for calmodulin than the classical method relying on the activation of phosphodiesterase (Lin et al., 1974b). The procedure is particularly useful for microassays since the activity of the kinase is totally dependent on Ca^{2+} ions and the affinity of the enzyme for calmodulin is high ($K_{d(app)}$ = 1.3 nM) (Walsh et al., 1979). Half-maximal stimulation is obtained with 91 fmol or 1.5 ng of calmodulin per assay.[3]

By use of this procedure, concentrations of calmodulin in the embryonic muscle extracts were determined as illustrated in Figure 3. In hind leg muscles, the initially high level of calmodulin (up to 151 pmol/g wet wt at day −6) gradually decreases to reach a minimum of 30 pmol/g at day −3 before coming back to 133 pmol/g at day −1. Its level then decreases, gradually at first and then precipitously after day +3 to reach low values at days +5 to +7 (7–12 pmol/g).

Similar fluctuations were observed in breast muscle, with a high initial level followed by a minimum at days −5 to −4 (94 pmol/g), a maximum at day −2 (148 pmol/g), and then a steady decrease only interrupted by a peak at day +5, before reaching the low levels observed at days +6 and +7 (7–15 pmol/g).

Calmodulin-Dependent Myosin Light-Chain Kinase. A marked increase in the Ca^{2+}-dependent myosin light-chain kinase activity occurs from day −2 before hatching to a max-

[3] The standard curve has been submitted to reviewers for examination and will be sent to interested readers upon request.

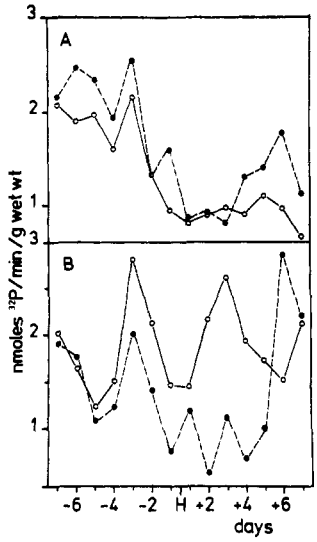

FIGURE 5: Evolution of protein kinase activities in chick embryo leg (○) and breast (●) muscles at different stages of embryonic development. (A) cAMP-independent protein kinase activity measured with histone as substrate in the presence of an excess of cAMP and PKI, as described in Materials and Methods. (B) cAMP-dependent protein kinase activity obtained in the presence of an excess of cAMP but in the absence of PKI and from which the independent activity (*A*) was subtracted (see Materials and Methods). H = hatching.

imum attained at day +3 and +4 for hind leg and breast muscles, respectively, as shown in Figure 4. This late synthesis appears to be rather characteristic, since differences in activity between the two muscle types are minor. A basal level of light-chain kinase activity observed in the presence of 4 mM EGTA (not illustrated) was subtracted from the figures obtained in the presence of Ca^{2+}. It was rather invariant, displaying no peak of activity and only a slow decrease from ~10 nmol min^{-1} g^{-1} to a minimum reached within 3 days after hatching.

Cyclic Nucleotide Dependent and Independent Protein Kinases. As shown in Figure 5A, the cAMP-independent protein kinases (i.e., measured with histone as substrate in the presence of an excess of PKI) exhibit from 7 to 3 days before hatching an activity as high as that observed for the cAMP-dependent kinases, with a little peak appearing at day −3, for both types of enzymes. From there on the activity in both muscles decreases steadily to reach a minimum [<1 nmol min^{-1} (g wet wt)$^{-1}$] after hatching (day +1 to +3). The minimum is maintained in leg muscle but appears to increase to ~60% of the −7 to −3 day value in breast muscle.

In contrast to the cyclic nucleotide independent kinases, cAMP-dependent kinase activities, illustrated in Figure 5B, exhibit marked cyclic variations around a mean value of ~2 nmol min^{-1} (g wet wt)$^{-1}$. In hind leg muscles, three minima are observed at day −5, −1 to +1, and +6, separated by two peaks of activity at day −3 and +3. Activities in breast muscle extract appear to be somewhat lower, especially from day +2 to +5 when the second activity peak is missing.

Protein kinase inhibitor levels, measured only in hind leg muscles, are rather stable, averaging between 30 and 40 pmol/g wet wt except for a deep trough appearing 2 days after hatching, followed by a slow recovery (Figure 6). The determination of PKI was rendered significantly more accurate by comparison with blanks in which PKI was destroyed by tryptic digestion. Nonspecific inhibition observed in these blanks was as high as 85% in the first dilution (corresponding to 200 μL of heat-treated muscle extract) and became almost negligible from the third dilution on (corresponding to 50 μL

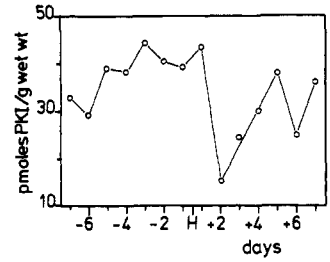

FIGURE 6: Evolution of the level of protein kinase inhibitor in chick embryo leg muscle determined as described under Materials and Methods. H = hatching. As the extent of coprecipitation at the heat step could not be determined, the precision of the assay was not evaluated.

of heat-treated extract). Figures were not corrected for possible losses during the heat step and are in the same range as those reported for adult rabbit skeletal muscle [20–60 units/g wet wt as compared to 35 units/g wet wt in rabbit skeletal muscle, also uncorrected for losses at the heat step (Demaille et al., 1977)]. PKI is thus already present at day −7 at levels comparable to those observed in adult skeletal muscle.

Discussion

While this report is primarily concerned with the development of regulatory mechanisms in chick embryonic skeletal muscle, it also provides new techniques for the accurate and sensitive quantitation of calmodulin, parvalbumin, and PKI. The single-stage procedure followed to quantitate calmodulin by the activation of calmodulin-deficient myosin light-chain kinase is ≥5 times more sensitive than the widely used two-step phosphodiesterase activation assay of Lin et al. (1974b). A further advantage lies in the fact that purified myosin light-chain kinase appears to be inactive in the absence of Ca^{2+}, whereas calmodulin-deficient phosphodiesterase exhibits a significant basal level of activity even in the absence of Ca^{2+} ions. A possible limitation of the quantitation procedure could result from coprecipitation of calmodulin at the heat step, though there is no indication of that.

Parvalbumin determination by radioimmunoassay is not only highly sensitive but is also extremely specific, since the parvalbumin-like protein described by Heizmann et al. (1977) does not cross-react with parvalbumin and the cross-reacting material of M_r ~60 000 (Blum et al., 1977) is not likely to be detected at the high dilutions at which the radioimmunoassay is carried out. This provides evidence that the breast muscle parvalbumin is identical with the leg muscle protein which was used to elicit antibodies.

The target proteins of the two intracellular second messengers cAMP and Ca^{2+}, namely cAMP-dependent protein kinase (and its inhibitor) and calmodulin, appear before the third week of embryonic development, at a time when active synthesis of contractile proteins has already occurred but the elements of the fast-twitch calcium cycle are not yet present.

There is no obvious explanation for the cyclic variations in the cAMP-dependent protein kinase level or for the difference observed between leg muscle and pectoralis muscle, in which the cAMP-dependent kinase peak at day +3 is absent. Similarly, there is no obvious reason for the deep trough in PKI content observed at day +2. Since the proportion of the assayed proteins or enzymes that remained bound to or trapped in the 7000*g* pellet during the preparation of the muscle extracts could not be determined, part of the observed fluctuations might be due to variable binding to the sediment fraction.

cAMP-dependent kinase activities were not corrected for the amount of endogenous PKI, which in the sample used for kinase activity (5 μL) would amount to 0.04–0.12 PKI unit.

PKI levels do not vary significantly between day −5 to +1 after hatching and thus could not affect the kinase activity peak observed at day −3 (Figure 5B). By contrast, the marked drop in PKI at day +2 (Figure 6) probably must contribute to the full expression of the second kinase activity peak, even though the PKI trough occurs 1 day earlier than the maximum of kinase activity.

Even though cAMP-independent kinases were measured with histone as substrate, whereas most of them display a higher affinity for acidic substrates such as casein or phosvitin (Hathaway & Traugh, 1979), the decrease in activity from high initial levels to a minimum attained around hatching is obvious. Since some of these enzymes seem to be involved in the control of protein synthesis through phosphorylation of the initiation factors (Ranu & London, 1976; Datta et al., 1977; Trachsel et al., 1978; Hathaway & Traugh, 1979), the decrease in the level of cAMP-independent kinases may perhaps be associated with the marked drop in the percentage of proliferating cells (from 23.8% to 2.5% from 8 days before to 8 days after hatching) reported by Marchok & Herrmann (1967).

Since calmodulin plays a major role in mediating the disassembly of tubulin triggered by Ca^{2+} (Marcum et al., 1978) and has been found in the mitotic apparatus of eucaryotic cells (Welsh et al., 1978), the decrease in cell proliferation may perhaps be correlated to the drop in calmodulin level following hatching.

Embryonic rabbit tissues (lung, heart, brain, and liver) have also been shown to synthesize calmodulin as early as the 14th to the 15th day of gestation (Singer et al., 1978). Furthermore, cAMP phosphodiesterase appears to be predominantly calmodulin dependent in embryonic liver, whereas little or no Ca^{2+}–calmodulin-dependent phosphodiesterase was said to be present in adult liver (Tanigawa et al., 1976). Embryonic muscle thus appears to be more closely related to nonmuscle cells, in which calmodulin is involved in cell division and in the control of stress fiber contractility (Dedman et al., 1978) and cAMP phosphodiesterase activity. Muscle differentiation is essentially marked by the late synthesis of the regulatory proteins that trigger contraction and relaxation of fast-twitch muscle through a calcium cycle. The synthesis of the SR Ca^{2+} pump begins only during the third week of embryonic development, and the rate of synthesis is maximal during the first week after hatching (Martonosi et al., 1977). At approximately the same time, the Ca^{2+}–calmodulin-dependent myosin light-chain kinase and parvalbumin, which allows fast relaxation (Haiech et al., 1979), are also synthesized. Therefore, simultaneously with the switch off of slow myosin synthesis and the withdrawal of multiple innervation (Gauthier et al., 1978), the regulatory features of fast-twitch muscles are established. Parvalbumins are indeed found only in fast-twitch muscles (Blum et al., 1977), and myosin light-chain kinase activity is substantially higher in fast muscle than in red or cardiac muscles (Frearson & Perry, 1975; Walsh et al., 1979). This is also true for the sarcoplasmic reticulum Ca^{2+}–Mg^{2+}–ATPase.

An interesting particular case is provided by the chicken pectoralis muscle, in which parvalbumin synthesis is delayed and proceeds at a much lower rate, as if mechanical activity or stretch played a major role in initiating the synthesis of the elements of the calcium cycle.

Muscle development appears to proceed through two major coordinate syntheses. At the time of myoblast fusion, all contractile proteins are synthesized coordinately (Devlin & Emerson, 1978) and a "slow myosin" cell type is produced that contains calmodulin and is capable of active cell proliferation. The transition to fast-twitch fibers occurs around hatching through the coordinate synthesis of fast myosin, the SR Ca^{2+} pump, parvalbumin, and myosin light-chain kinase.

References

Alexis, M. N., & Gratzer, W. B. (1978) *Biochemistry 17*, 2319–2325.

Blum, H. E., Lehky, P., Kohler, L., Stein, E., & Fischer, E. H. (1977) *J. Biol. Chem. 252*, 2834–2838.

Cohen, P., Burchell, A., Foulkes, J. G., Cohen, P. T. W., Vanaman, T. C., & Nairn, A. C. (1978) *FEBS Lett. 92*, 287–293.

Corbin, J. D., & Reimann, E. M. (1974) *Methods Enzymol. 38*, 287–290.

Dabrowska, R., Sherry, J. M. F., Aromatorio, D. K., & Hartshorne, D. J. (1978) *Biochemistry 17*, 253–258.

Datta, A., De Haro, C., Sierra, J. M., & Ochoa, S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3326–3329.

Dedman, J. R., Welsh, M. J., & Means, A. R. (1978) *J. Biol. Chem. 253*, 7515–7521.

Demaille, J. G., Peters, K. A., & Fischer, E. H. (1977) *Biochemistry 16*, 3080–3086.

Demaille, J. G., Peters, K. A., Strandjord, T. P., & Fischer, E. H. (1978) *FEBS Lett. 86*, 113–116.

Devlin, R. B., & Emerson, C. P., Jr. (1978) *Cell 13*, 599–611.

Ferraz, C., Demaille, J. G., & Fischer, E. H. (1979) *Biochimie 61*, 645–651.

Fischer, E. H., Becker, J. U., Blum, H. E., Byers, B., Heizmann, C., Kerrick, G. W., Lehky, P., Malencik, D. A., & Pocinwong, S. (1976) in *Molecular Basis of Motility* (Heilmeyer, L., et al., Eds.) pp 137–158, Springer-Verlag, West Berlin.

Frearson, N., & Perry, S. V. (1975) *Biochem. J. 151*, 99–107.

Gauthier, G. F., Lowey, S., & Hobbs, A. W. (1978) *Nature (London) 274*, 25–29.

Glynn, I. M., & Chappell, J. B. (1964) *Biochem. J. 90*, 147–149.

Haiech, J., Derancourt, J., Pechere, J. F., & Demaille, J. G. (1979) *Biochemistry 18*, 2752–2758.

Hathaway, G. M., & Traugh, J. A. (1979) *J. Biol. Chem. 254*, 762–768.

Hauschka, S. D. (1968) *Results Probl. Cell Differ. 1*, 37–57.

Heizmann, C. W., Hauptle, M.-T., & Eppenberger, H. M. (1977) *Eur. J. Biochem. 80*, 433–441.

Hofmann, F., Beavo, J. A., Bechtel, P. J., & Krebs, E. G. (1975) *J. Biol. Chem. 250*, 7795–7801.

Hofmann, F., Bechtel, P. J., & Krebs, E. G. (1977) *J. Biol. Chem. 252*, 1441–1447.

Hunter, W. M., & Greenwood, F. C. (1962) *Nature (London) 194*, 495–496.

Lehman, W. (1978) *Nature (London) 274*, 80–81.

Lin, Y. M., Liu, Y. P., & Cheung, W. Y. (1974a) *J. Biol. Chem. 249*, 4943–4954.

Lin, Y. M., Liu, Y. P., & Cheung, W. Y. (1974b) *Methods Enzymol. 38*, 262–273.

Marchok, A. C., & Herrmann, H. (1967) *Dev. Biol. 15*, 129–155.

Marcum, J. M., Dedman, J. R., Brinkley, B. R., & Means, A. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3771–3775.

Martonosi, A., Roufa, D., Boland, R., Reyes, E., & Tillack, T. W. (1977) *J. Biol. Chem. 252*, 318–332.

Morimoto, D., & Harrington, W. F. (1974) *J. Mol. Biol. 88*, 693–709.

Pechere, J. F., Demaille, J., & Capony, J. P. (1971) *Biochim. Biophys. Acta 236*, 391–408.

Pechere, J. F., Derancourt, J., & Haiech, J. (1977) *FEBS Lett. 75*, 111–114.
Perrie, W. T., & Perry, S. V. (1970) *Biochem. J. 119*, 31–38.
Peters, K. A., Demaille, J. G., & Fischer, E. H. (1977) *Biochemistry 16*, 5691–5697.
Potter, J. D., & Gergely, J. (1975) *J. Biol. Chem. 250*, 4628–4633.
Ranu, R. S., & London, I. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4349–4353.
Rasmussen, H., & Goodman, D. B. P. (1977) *Physiol. Rev. 57*, 421–509.
Robison, G. A., Butcher, R. W., & Sutherland, E. W. (1971) *Cyclic AMP*, Academic Press, New York.
Sherry, J. M. F., Gorecka, A., Aksoy, M. O., Dabrowska, R., & Hartshorne, D. J. (1978) *Biochemistry 17*, 4411–4418.
Singer, A. L., Dunn, A., & Appleman, M. M. (1978) *Arch. Biochem. Biophys. 187*, 406–413.
Tanigawa, Y., Shimoyama, M., Tai, J., Fujii, K., & Ueda, I. (1976) *Biochem. Biophys. Res. Commun. 73*, 19–24.
Teo, T. S., Wang, T. H., & Wang, J. H. (1973) *J. Biol. Chem. 248*, 588–595.
Trachsel, H., Ranu, R. S., & London, I. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3654–3658.
Ueland, P. M., & Døskeland, S. O. (1978) *J. Biol. Chem. 253*, 1667–1676.
Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer, E. H., & Krebs, E. G. (1971) *J. Biol. Chem. 246*, 1977–1985.
Walsh, M., & Stevens, F. C. (1978) *Can. J. Biochem. 56*, 420–429.
Walsh, M., Vallet, B., & Demaille, J. G. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 2952a.
Welsh, M. J., Dedman, J. R., Brinkley, B. R., & Means, A. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1867–1871.
Witt, J. J., & Roskoski, R., Jr. (1975) *Anal. Biochem. 66*, 253–258.
Yazawa, M., Kuwayama, H., & Yagi, K. (1978) *J. Biochem. (Tokyo) 84*, 1253–1258.

Regulatory Properties of Single-Headed Fragments of Scallop Myosin†

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ABSTRACT: Calcium control was studied in single-headed myosin and subfragment-1 (S1) preparations obtained by papain digestion of scallop myosin. Single-headed myosin, containing light chains in stoichiometric amounts, was calcium regulated; in contrast, the actin-activated Mg–ATPase of all S1 species lacked calcium sensitivity. Both regulatory and essential light chains were retained by S1 and single-headed myosin preparations provided divalent cations were present during papain digestion, although a peptide amounting to 10% of the mass was removed from regulatory light chains. The modified regulatory light chain retained its ability to confer calcium binding and restore calcium sensitivity to the ATPase of desensitized myofibrils. Regulatory light chains protected the essential light chains from fragmentation by papain. S1 bound regulatory light chains with a uniformly high affinity and appeared to consist of a single species. The results demonstrate that head to head interactions are not obligatory for calcium control, although they may occur in the intact myosin molecule, and suggest a role for the subfragment-2 region in calcium regulation of myosin.

Contractile activity in all muscles is regulated by intracellular levels of calcium ion [cf. Ebashi & Endo (1968), Weber & Murray (1973), and Szent-Györgyi (1975)]. Upon stimulation, calcium is released into the sarcoplasm of the muscle and is bound by regulatory proteins on the myofilaments. Binding of calcium allows the two types of myofilaments to interact with each other to produce contractile force. The regulatory proteins are located on either or both types of filaments. In many invertebrates, notably mollusca, the regulation is associated with myosin, while in vertebrate striated muscles the regulatory proteins are located on the actin containing thin filaments (Kendrick-Jones et al., 1970; Lehman & Szent-Györgyi, 1975). Actin-linked regulation is mediated through the troponin complex and tropomyosin which prevent interaction of actin and myosin at low levels of calcium (Ebashi & Ebashi, 1964). The myosin molecule is an enzyme composed of two high molecular weight subunits (M_r 200 000) and two pairs of low molecular weight subunits (M_r 16 000–30 000) (Lowey & Risby, 1971; Weeds & Lowey, 1971). In molluscan systems, notably scallops, one pair of low molecular weight subunits (regulatory light chains) has been shown to be directly involved in regulation while the role of the other pair in regulation, if any, remains obscure (Szent-Györgyi et al., 1973; Kendrick-Jones et al., 1976).

One mole of regulatory light chain (R-LC)[1] can be removed from myosin of scallop by treatment with 10 mM EDTA at 0 °C, resulting in complete loss of regulation with concomitant loss of 1 mol of calcium binding sites per myosin (Szent-Györgyi et al., 1973). The actin-activated ATPase activity

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[1] Abbreviations used: S1, myosin subfragment-1; R-LC, regulatory light chain; SH-LC, essential light chain; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; IAA, iodoacetic acid; DEAE, diethylaminoethyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris, tris(hydroxymethyl)aminomethane.

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