Ontogenetic changes in adenylate cyclase, cyclic AMP phosphodiesterase and calmodulin in chick ventricular myocardium

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The activities of cyclic AMP phosphodiesterase (3',5'-cyclic nucleotide 5'-nucleotidohydrolase, EC 3.1.4.17) and adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] and calmodulin content during development of chick ventricular myocardium were determined. The specific activity of cyclic AMP phosphodiesterase was relatively low in early embryos, increased during embryogenesis by about 4-fold to reach highest values just before hatching, and then decreased by approx. 30% within 1 week after hatching. In contrast, adenylate cyclase did not change during embryonic development, but increased by approx. 50% within 1 week after hatching. Calmodulin content remained constant at 9 µg/g wet wt. during embryonic development and decreased to 6 µg/g wet wt. by 1 week after hatching. DEAE-Sephacel chromatography of chick ventricular supernatant revealed a single major form of cyclic nucleotide phosphodiesterase activity in early embryonic (9-day E) and hatched (6-day H) chicks. This enzyme form was eluted at approx. 0.27 M-sodium acetate, hydrolysed both cyclic AMP and cyclic GMP, and was sensitive to stimulation by Ca²⁺-calmodulin, with an apparent *K_m* for calmodulin of approx. 1 nM. In contrast, ventricular supernatant from late-embryonic (18-day E) chicks contained two forms of phosphodiesterase separable on DEAE-Sephacel: the same form as that seen at other ages, plus a cyclic AMP-specific form which was eluted at approx. 0.65 M-sodium acetate and was insensitive to stimulation by Ca²⁺-calmodulin. The ontogenetic changes in cyclic AMP phosphodiesterase activity in chick ventricular myocardium are consistent with reported ontogenetic changes in the steady-state contents of cyclic AMP in this tissue and suggest that this enzyme may be responsible for the changes that occur in this nucleotide during development of chick myocardium.

INTRODUCTION

During embryonic development of chick hearts, studies on β -adrenergic-receptor formation, sympathetic innervation and tissue cyclic AMP content have been performed. Positive chronotropic effects of adrenaline have been observed as early as day 2 after fertilization, and some investigators have observed positive inotropic effects of catecholamines on day 4 after fertilization, accompanied by β -adrenergic-receptor-mediated increases in cyclic AMP content (reviewed by Pappano, 1981). Several reports on ontogenetic changes in cyclic AMP contents in chick hearts have shown the values to be highest in early-embryonic hearts and to decline steadily to reach lowest values just before hatching (McLean *et al.*, 1975; Hosey & Green, 1977; Renaud *et al.*, 1978; Lipshultz *et al.*, 1981). Despite this marked decline in cyclic AMP content during chick heart ontogenesis, except for one preliminary report (Hosey & Green, 1977), no detailed studies have been done on regulation of the enzymes that control cyclic AMP contents in this species. In the present paper, we examine the changes in the activities of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] and cyclic AMP phosphodiesterase (3',5'-cyclic nucleotide 5'-nucleotidohydrolase, EC 3.1.4.17) and calmodulin that

occur during development of chick hearts, coupled to ontogenetic changes in cyclic AMP content. Preliminary accounts of some of this work have appeared previously (Andrenyak *et al.*, 1981; Smith & Pappano, 1983).

MATERIALS AND METHODS

Materials

Cyclic [2,8-³H]AMP (32.3 Ci/mmol) and [α -³²P]ATP (29.4 Ci/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A. Cyclic [³H]AMP was purified by chromatography on Dowex 1-X8 (200–400 mesh) and stored at –20 °C in acidic 50% (v/v) ethanol before use. Snake (*Ophiophagus hannah*) venom, cyclic AMP, ATP, phosphocreatine, creatine kinase, dithiothreitol, bovine serum albumin and neutral alumina were purchased from Sigma, St. Louis, MO, U.S.A. Dowex 1-X8 was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. All other chemicals were of commercial analytical-grade quality.

Tissue preparations

Fertilized eggs (White Leghorn, from SPAFAS, Norwich, CT, U.S.A.) were kept at 37.5 °C in a humidified incubator until the embryos reached the desired state of development. Chicks hatched on day 21

Abbreviation used: p[NH]ppG, guanosine 5'-[γ -imido]triphosphate.

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of incubation (Hamburger–Hamilton Stage 46). The animals were decapitated, and the hearts rapidly excised, and rinsed in modified Tyrode's solution containing (mM): K^+ , 5.4; Na^+ , 149; Ca^{2+} , 1.8; Mg^{2+} , 1.0; Cl^- , 148; HCO_3^- , 11.9; $H_2PO_4^-$, 0.4; and dextrose, 5.5. After rinsing, the ventricles were separated from the atria. For preparations less than 10 days after fertilization, both presumptive ventricles were used. For preparations 10 days and more after fertilization, only the right ventricle was used. For assay of adenylate cyclase activity, ventricular tissue was homogenized (4 °C) at 50 mg wet wt./ml in 10 mM-Tris/HCl (pH 7.8)/1 mM-dithiothreitol/0.2 mM- $MgCl_2$ in a Dual homogenizer. For assay of cyclic AMP phosphodiesterase activity, ventricular tissue was homogenized (4 °C) at 10 mg wet wt./ml in 40 mM-Tris/HCl (pH 7.5)/0.25 M-sucrose in a Potter–Elvehjem homogenizer, except for preparation of supernatants before DEAE-Sephacel chromatography, where tissue was homogenized at 40 mg wet wt./ml and centrifuged at 133 000 g_{max} for 45 min.

Assay of adenylate cyclase activity

This was done by the method of Saloman (1979). Reaction mixtures (0.1 ml) contained 25 mM-Tris/HCl (pH 7.8), 3 mM- $MgCl_2$, 1 mM-dithiothreitol, 1 mM-cyclic AMP, 0.3 mM-ATP, 1×10^6 – 3×10^6 c.p.m. of [α - ^{32}P]ATP, 0.17 mg of phosphocreatine, 7.2 units of creatine kinase, and 1 mg of ventricular tissue. Reactions were initiated by addition of tissue homogenate and incubated for 10 min at 30 °C. Reaction linearity was verified for at least 20 min under these conditions. Reactions were stopped by the addition of 0.1 ml of a solution of 2% SDS, 1.4 mM-cyclic AMP and 4 mM-ATP. Tracer cyclic [3H]AMP (5000 c.p.m.) was added to monitor recovery, and cyclic [^{32}P]AMP product formed (approx. 0.05–0.1% of the substrate) was isolated by sequential chromatography on Dowex 50 and alumina columns.

Assay of cyclic AMP phosphodiesterase activity

Phosphodiesterase activity was determined by the two-step radioisotope procedure of Thompson *et al.* (1979a), by using chromatography on Dowex 1-X8 to separate products. Reaction mixtures (0.4 ml) contained 40 mM-Tris/HCl (pH 8.0), 1.25 mM-2-mercaptoethanol, 10 mM- $MgCl_2$, cyclic [3H]AMP (100 000 c.p.m.), unlabelled cyclic AMP as indicated, 50 μ g of bovine serum albumin, tissue homogenate, and either EGTA or $CaCl_2$, as indicated. Further details of the application of this assay system to the avian heart have been described previously (Biegon *et al.*, 1980).

Determination of calmodulin

A rat brain calmodulin-dependent cyclic nucleotide phosphodiesterase was prepared, and dose/response curves for activation of the enzyme by purified bovine brain calmodulin were prepared as described previously (Volpi *et al.*, 1981). For determination of calmodulin, samples of ventricular homogenates were heated at 100 °C for 3 min in 0.1 ml of 40 mM-Tris/HCl (pH 8.0). Denatured protein was not removed, but rather, rat brain phosphodiesterase and other components necessary for its assay were added directly to the heated sample, and the degree of stimulation of the enzyme was determined. The amount of calmodulin in the sample was determined from the equivalent amount of purified

bovine brain calmodulin (Epstein *et al.*, 1982) necessary to produce the same degree of enzyme stimulation.

Protein determination

Proteins were determined on 5% -trichloroacetic acid precipitates by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

RESULTS

Cyclic AMP content during chick heart development

Chicks hatch 21 days after fertilization. Ontogenetic changes in the cyclic AMP content of chick hearts have been investigated extensively in several laboratories (McLean *et al.*, 1975; Hosey & Green, 1977; Renaud *et al.*, 1978; Lipshultz *et al.*, 1981). These studies show cyclic AMP content to be highest at the early-embryo stage, and to decrease steadily, by 4-fold or more, reaching lowest values at the late-embryo stage. This pattern of a sharp decrease in cyclic AMP content during embryonic development is observed whether the cyclic AMP content is calculated on the basis of the amount of protein (McLean *et al.*, 1975; Hosey & Green, 1977; Renaud *et al.*, 1978) or the amount of DNA (Lipshultz *et al.*, 1981). A representative example of the change in cyclic AMP content, taken from McLean *et al.* (1975), is shown in Fig. 1 (top panel); the cyclic AMP content decreases by approx. 4-fold from the early-embryonic (5E) to the late-embryonic (18E) age. After hatching, cyclic AMP content remains about the same as that at the late-embryo state, when expressed either on the basis of protein (McLean *et al.*, 1975) or on the basis of DNA (Lipshultz *et al.*, 1981), but a small (50%) increase is observed by 1 week after hatching when cyclic AMP content is expressed on the basis of wet weight (Biegon & Pappano, 1980).

Adenylate cyclase and cyclic AMP phosphodiesterase activities during chick heart development

Protein content and basal activities of adenylate cyclase and cyclic AMP phosphodiesterase were examined in whole crude homogenates of chick ventricular myocardium during embryogenesis and up to 1 week after hatching (Fig. 1). Protein content increased by 45%, from a low value of 9.05% of the wet weight measured in 8-day embryos, to a high value of 13.14% measured in 1-week-hatched chicks. The specific activity of adenylate cyclase in embryonic ventricles was relatively constant, from a value of 16.4 ± 1.1 pmol/min per mg of protein in 10–11-day embryos to 14.9 ± 1.1 pmol/min per mg in 17–18-day embryos; then, by 1 week after hatching, it increased by 50% to 22.4 ± 1.5 pmol/min per mg.

The sensitivity of adenylate cyclase to stimulation by p[NH]ppG and isoprenaline has been examined as a function of developmental age (Smith & Pappano, 1985). p[NH]ppG stimulates adenylate cyclase activity at all ages, although the degree of stimulation at the early embryonic age is greater than that at later ages. Isoprenaline stimulation of adenylate cyclase activity is also greatest at the early-embryonic age. The net effect of this is that the activity of adenylate cyclase at the 10–11E age is about equal to that at the 5–6H age, when

Fig. 1. Cyclic AMP content, protein content, and adenylate cyclase and cyclic AMP phosphodiesterase activities in developing chick ventricular myocardium

The values for cyclic AMP content are taken from McLean *et al.* (1975). Protein content and adenylate cyclase and cyclic AMP phosphodiesterase activities were determined as described in the Materials and methods section. Phosphodiesterase activity was measured at 1 μ M- and 100 μ M-cyclic AMP as indicated, in the presence of 0.1 mM- CaCl_2 . The numbers in parentheses represent the numbers of experiments performed, and data are plotted as means $\pm$ S.E.M. All adenylate cyclase assays were done in triplicate, and all phosphodiesterase assays in duplicate. In some cases the data from chicks in close proximity of age were averaged together, and the brackets on the abscissa represent the range of ages used for the determination of phosphodiesterase activity at that stage of development. Adenylate cyclase activities were determined in 10–11-day-embryonic, 17–18-day-embryonic and 7-day-hatched chicks. 'H' on the abscissa represents the time of hatching; 'E' refers to embryonic age, or days *in ovo*.

determined in the presence of either p[NH]ppG or isoprenaline. However, the late-embryo age (17–19E) is extremely sub-sensitive to isoprenaline stimulation; there is almost a complete lack of stimulation of adenylate cyclase by 10 μ M-isoprenaline at this age. Hence,

Table 1. Ca^{2+} -calmodulin stimulation of cyclic AMP phosphodiesterase activity in homogenates of chick ventricle as a function of developmental age

Results represent means of duplicate determinations. Basal activity was determined by inclusion of 1 mM-EGTA. Where indicated, Ca^{2+} and calmodulin (+ Ca^{2+} -CaM) were added, as 0.2 mM- CaCl_2 and 20 nM purified bovine brain calmodulin. The numbers in parentheses represent the percentage increase in activity over basal.

Chick age	Condition	Phosphodiesterase activity (nmol/min per mg of protein)	
		With 1 μ M-cyclic AMP	With 100 μ M-cyclic AMP
9E	Basal	0.359	1.96
	+ Ca^{2+} -CaM	0.683 (190)	3.58 (183)
18E	Basal	0.594	2.34
	+ Ca^{2+} -CaM	0.714 (120)	2.77 (118)
6H	Basal	0.298	1.97
	+ Ca^{2+} -CaM	0.967 (324)	3.42 (174)

adenylate cyclase activity at the late embryonic age is severalfold lower than at other ages when determined in the presence of isoprenaline.

Cyclic AMP phosphodiesterase activity was assayed at high (100 μ M) and low (1 μ M) substrate concentrations and showed nearly identical developmental patterns when assayed at these two substrate concentrations (Fig. 1). The specific activity was lowest at the early-embryonic stage (0.53 ± 0.04 nmol/min per mg of protein, measured at 100 μ M-cyclic AMP, in 6-day embryos), steadily increased 4-fold, to 2.17 ± 0.08 nmol/min per mg in 17–18-day embryos, and then decreased by 30%, to 1.53 ± 0.11 nmol/min per mg, by 1 week after hatching.

Ca^{2+} -sensitivity of cyclic nucleotide phosphodiesterase

One form of phosphodiesterase in mammalian cardiac tissue has been shown to be sensitive to activation by Ca^{2+} -calmodulin (Ho *et al.*, 1976; Appleman & Terasaki, 1975), and in liver of developing chicks the Ca^{2+} -sensitivity of phosphodiesterase was shown to be strongly dependent on the developmental state (Tanigawa *et al.*, 1976); hence we examined the Ca^{2+} -sensitivity of phosphodiesterase in chick heart by comparing the activities in 0.1 mM- CaCl_2 with those in 1 mM-EGTA at different developmental ages from 6E to 8H. We found that, when assayed at 100 μ M-cyclic AMP, the activities were consistently higher in CaCl_2 than in EGTA. The activities ranged from 17 to 30% lower in EGTA, with a mean depression by EGTA of $22.4 \pm 2.3\%$. The decrease in activity by EGTA did not appear to depend on the developmental stage of the heart, and was apparent throughout embryogenesis as well as after hatching. This is in contrast with chick liver phosphodiesterase, which showed strong (78%) EGTA inhibition at the early-embryo stage, much less (14%) at hatching, and no EGTA inhibition in the adult (Tanigawa *et al.*, 1976).

We also examined the sensitivity of phosphodiesterase in chick heart homogenates to stimulation by exogenously added calmodulin (Table 1). Phosphodiesterase in

Table 2. Calmodulin content of developing chick heart

Heart ventricular tissue was homogenized in 40 mM-Tris/HCl (pH 8.0)/0.25 M-sucrose at 20 mg/ml wet wt., and calmodulin content was determined as described in the Materials and methods section. All assays were performed in duplicate. E refers to embryonic age (days *in ovo*); H refers to hatched age (days after hatching). Results are presented as the mean $\pm$ S.E.M. for the numbers of chicks in parentheses: *significantly different from 11–13E and 17–18E ($P < 0.05$).

Chick age	Calmodulin content ($\mu\text{g/g}$ wet wt.)
8E (3)	9.66 ± 4.74
11–13E (6)	8.27 ± 1.12
17–18E (5)	9.14 ± 1.85
5–6H (4)	$5.97 \pm 0.92^*$

chicks hearts was sensitive to stimulation by calmodulin at all ages, although the degree of stimulation in early embryos (9E) and hatched chicks (6H) was about 2-fold, which is considerably greater than that in late embryos (18E), which only show a stimulation of about 20%.

Calmodulin contents in chick ventricular myocardium

Since the Ca^{2+} -sensitivity of phosphodiesterase appears to be mediated through calmodulin (Klee *et al.*, 1980), the amount of calmodulin in chick ventricular myocardium was also determined at different developmental ages (Table 2). The amount of calmodulin appeared constant, at approx. $9 \mu\text{g/g}$ wet wt., during embryogenesis and then decreased by 33%, to $6 \mu\text{g/g}$ wet wt., by 5–6 days after hatching. On the basis of an average intracellular water content of 54 ml/100 g wet wt. in embryonic-chick ventricles (McDonald & DeHaan, 1973), these amounts of calmodulin correspond to cellular concentrations of about $1.0 \mu\text{M}$ in embryonic-chick heart and $0.7 \mu\text{M}$ in 5–6-day-neonatal heart. For comparison, calmodulin contents were also determined in the pectoral muscles of 6-day-hatched chicks; these were $10.9 \pm 1.6 \mu\text{g/g}$ wet wt. (S.E.M., $n = 3$), approx. 80% greater than corresponding values in the heart of an animal of equivalent age when expressed in terms of tissue content.

DEAE-Sephacel chromatography of cyclic nucleotide phosphodiesterase in chick ventricular supernatant

Cyclic nucleotide phosphodiesterase activities in chick ventricular supernatants from early-embryonic (9E), late-embryonic (18E) and 6-day-hatched (6H) chicks were compared by DEAE-Sephacel chromatography (Fig. 2). The 9E and 6H ventricular supernatants each showed a single major peak of cyclic nucleotide phosphodiesterase activity eluted at approx. 0.27 M-sodium acetate. This form hydrolysed $1 \mu\text{M}$ -cyclic AMP and $1 \mu\text{M}$ -cyclic GMP with about equal activity, and the hydrolysis of both of these nucleotides was stimulated severalfold by Ca^{2+} -calmodulin. Similarly, the supernatant from a 6-month-old adult-chicken ventricular preparation also showed a single major form of cyclic nucleotide phosphodiesterase, similar in properties to

that seen at these two ages (Andrenyak, 1984). By contrast with these other ages, DEAE-Sephacel chromatography of ventricular supernatant from the late-embryonic (18E) chick revealed two distinct peaks of cyclic AMP phosphodiesterase activity (Fig. 2). One form, eluted at approx. 0.25 M-sodium acetate, appeared to be very similar to the form of the enzyme seen at the other ages, but the other form, eluted at approx. 0.65 M-sodium acetate, was specific for cyclic AMP at $1 \mu\text{M}$ substrate, and was not stimulated by Ca^{2+} -calmodulin. This enzyme form is seen only at the late embryonic age. The properties of these enzyme forms have been studied in detail (Andrenyak, 1984). Of particular note is that both the Ca^{2+} -calmodulin-sensitive and -insensitive enzyme forms display high affinity for cyclic AMP as a substrate. In the presence of Ca^{2+} -calmodulin, the calmodulin-sensitive phosphodiesterases from all ages (early embryonic, late embryonic, neonatal and adult) had K_m values for both cyclic AMP and cyclic GMP substrates of approx. $1\text{--}2 \mu\text{M}$. The calmodulin-insensitive enzyme form from late-embryonic chicks also displayed high affinity, with a K_m for cyclic AMP of $1.4 \mu\text{M}$.

Calmodulin stimulation of the Ca^{2+} -calmodulin-sensitive phosphodiesterase isolated by DEAE-Sephacel chromatography

Homogenates from late-embryonic chick hearts showed much less stimulation by calmodulin than did those from early-embryonic or hatched chicks (Table 1). This could be due to differential sensitivity of the Ca^{2+} -calmodulin-sensitive phosphodiesterase forms from different ages to stimulation by Ca^{2+} -calmodulin. However, when Ca^{2+} -calmodulin-sensitive forms, isolated by DEAE-Sephacel chromatography, from late-embryonic (18E) and hatched (10H) chicks were compared for their ability to be stimulated by Ca^{2+} -calmodulin, they were found not to differ. Enzyme forms isolated from both ages were maximally stimulated 4–5-fold by calmodulin, and both enzyme forms displayed an apparent K_D for calmodulin of approx. 1.0 nM . These data indicate that the sub-sensitivity of late-embryonic chick heart homogenates to stimulation by calmodulin is not due to an intrinsic difference in the calmodulin-sensitive phosphodiesterase of this age. The sub-sensitivity may result instead from the selective appearance of an appreciable amount of calmodulin-insensitive phosphodiesterase activity at this age. The lower stimulation in homogenates may also result, in part, from the presence of particulate phosphodiesterase activity, present throughout development, which comprises approx. 32% of the total activity and is insensitive to stimulation by calmodulin (Andrenyak & Epstein, 1984).

DISCUSSION

It is clear from studies in several laboratories that the cellular content of cyclic AMP changes dramatically during embryogenesis of chick ventricular myocardium. Cyclic AMP begins at a high concentration, and steadily decreases to reach lowest values before hatching (McLean *et al.*, 1975; Hosey & Green, 1977; Renaud *et al.*, 1978; Lipshultz *et al.*, 1981). In studying the enzymes that regulate cyclic AMP concentrations, we find little correlation in adenylate cyclase activities with cyclic

Fig. 2. DEAE-Sepharose chromatography of chick ventricular supernatant

DEAE-Sepharose chromatography was performed as described by Thompson *et al.* (1979b), with slight modification. Chick ventricular supernatant was loaded by ascending flow to a column (4.0 cm $\times$ 1.6 cm) of DEAE-Sepharose equilibrated in 5 mM-Mes (pH 6.5)/30% (v/v) ethylene glycol/1 mM-dithiothreitol/5% (w/v) glucose/10 mM-sodium acetate/1 mM-EGTA/10 mM-NaF/10 μ M-tosyl-l-lysylchloromethane (DEAE Running Buffer). After loading, the column was washed with 75 ml of the DEAE Running Buffer and eluted with an 80 ml linear (0.01–1 M) sodium acetate (NaAc; ○ in top line) gradient in DEAE Running Buffer. The flow rate was 0.5 ml/min, and 1 ml fractions were collected. Phosphodiesterase activity was determined at 1 μ M-cyclic AMP and 1 μ M-cyclic GMP in the presence (○) and absence (●) of 0.2 mM-CaCl₂ and 20 mM-calmodulin. Panels represent ventricular supernatant from (a) 9-day embryonic chick (9E), (b) 18-day embryonic chick (18E), (c) 6-day-hatched chick (6H). Recoveries of activities from the columns, given as basal and calmodulin-stimulated activities respectively, were: 9E, 53% and 87%; 18E, 68% and 87%; 6H, 63% and 76%.

AMP content during embryogenesis, but we do find changes in phosphodiesterase activity that appear to correlate with changes in cellular cyclic AMP content. Although cyclic AMP content falls during embryogenesis, basal adenylate cyclase activity changes very little during embryogenesis and increases by 50% after hatching. Even when adenylate cyclase activity is measured in the presence of a saturating concentration of guanine nucleotide (p[NH]ppG), which may more closely approximate to conditions in the intact cell, the activity of the enzyme is the same in the 9–10-day-embryonic and 5–6-day-hatched ages (Smith & Pappano, 1985), whereas cyclic AMP content is severalfold lower at the hatched age. In contrast with adenylate cyclase, cyclic AMP phosphodiesterase activity begins low and steadily increases, to reach highest values before hatching. This is reciprocal to the observed changes in cyclic AMP content. From these observations, we suggest that phosphodiesterase may function to regulate cellular cyclic AMP content during chick heart embryogenesis.

In addition to the developmental regulation of ventricular cyclic AMP content, the accumulation of cyclic AMP in response to hormone stimulation is also developmentally regulated. Polson *et al.* (1977) and Renaud *et al.* (1978) both showed differences in responsiveness of embryonic-chick hearts to isoprenaline-induced increases in cyclic AMP at different embryonic ages. Similarly, Hosey & Green (1977) showed that 10 μ M-isoprenaline produced a 4.4-fold increase in cyclic AMP in ventricles from 8-day-embryonic chicks, but produced no increase in cyclic AMP in newborn chicks. In the presence of the phosphodiesterase inhibitor, RO 20-1724, however, isoprenaline increased the cyclic AMP content of 8-day-embryonic ventricles by 17.4-fold, and that of newborn chicks by 40-fold. From these observations, Hosey & Green (1977) suggested that the decreased sensitivity of the newborn chicks to isoprenaline may be due to a change in the metabolism of cyclic AMP by phosphodiesterase. Our data are consistent with this interpretation, since we find regulation of both the activity and the apparent expression of cyclic AMP phosphodiesterase forms during chick heart development.

In addition to the developmental regulation of the accumulation of cyclic AMP in response to hormone stimulation, a 10-fold sub-sensitivity to isoprenaline stimulation of the force of contraction was observed in 18-day-embryonic-chick ventricles, as compared with earlier embryos and hatched chicks (Biegon *et al.*, 1980; Higgins & Pappano, 1981). This sub-sensitivity may result in part from an alteration in the hormone-responsive adenylate cyclase at that age, as evidenced by a lack of isoprenaline stimulation of adenylate cyclase activity in ventricular homogenates of 18-day embryos (Smith & Pappano, 1985). It could also result from the elevated cyclic AMP phosphodiesterase and the appearance of a new phosphodiesterase form which we detect at that age, which could limit the accumulation of cyclic AMP in response to isoprenaline. Such a mechanism of increased phosphodiesterase activity accounting for sub-sensitivity has been proposed for the sub-sensitivity to isoprenaline increase of cyclic AMP in mononuclear leucocytes isolated from individuals with atopic disorders (Grewe *et al.*, 1982).

Cyclic AMP concentrations appear to be regulated by

Ca^{2+} in cardiac tissue. This view is supported by observations of a reciprocal relationship between external Ca^{2+} concentration and cellular cyclic AMP content in mammalian cardiac tissue (Namm *et al.*, 1968; Endoh *et al.*, 1976; Harary *et al.*, 1976; Dobson *et al.*, 1976). Since the phosphodiesterase in heart was found to be Ca^{2+} -sensitive, and since the Ca^{2+} -sensitivity of phosphodiesterase is due to activation by a Ca^{2+} -calmodulin complex (Klee *et al.*, 1980), we measured the amounts of calmodulin during development. We found no change in calmodulin during embryogenesis, and a 33% decrease after hatching. It has been reported that calmodulin is a constitutively synthesized protein, which undergoes little change in amount during development (Smoake *et al.*, 1974; Means & Dedman, 1980), and our results are consistent with this. Hence, the Ca^{2+} regulation of cyclic AMP contents in cardiac tissue, if it occurs through the Ca^{2+} -sensitive phosphodiesterase, probably results from transient changes in the steady-state concentration of Ca^{2+} , rather than through developmental regulation of calmodulin.

Hosey & Green (1977) determined the activities of adenylate cyclase and phosphodiesterase in chick ventricular myocardium in 7–9-day embryos and newborn chicks. They found that adenylate cyclase activity was about 20% lower, and phosphodiesterase activity about 40–50% lower, in newborn chicks as compared with 7–9-day embryos. In contrast, we found adenylate cyclase to be about 50% higher in hatched chicks and phosphodiesterase activity to be about the same, as compared with this age of embryo. The reason for this discrepancy in the observed changes in these enzyme activities is not readily apparent, but could result from slight methodological differences. Nevertheless, from all of their data, Hosey & Green (1977) concluded that phosphodiesterase activity was important in determining the accumulation of cyclic AMP in response to isoprenaline, and similarly, from our data, we suggest that phosphodiesterase may be important in regulating the changes in cyclic AMP concentrations that occur during chick heart development, and possibly also responsible in part for the sub-sensitivity of the 18-day-embryonic hearts to isoprenaline stimulation.

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