Non-co-ordinate development of β -adrenergic receptors and adenylate cyclase in chick heart

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We have studied the properties of β -adrenergic receptors and of their interaction with adenylate cyclase in the chick myocardium during embryogenesis. Between 4.5 and 7.5 days *in ovo* the number of receptors determined by (–)-[³H]dihydroalprenolol ([³H]DHA) binding is constant at approx. 0.36 pmol of receptor/mg of protein. By day 9 the density decreases significantly to 0.22 pmol of receptor/mg of protein. At day 12.5–13.5 the number was 0.14–0.18 pmol of receptor/mg of protein. This number did not change further up to day 16. The same results were obtained with guanosine 5'-[β , γ -imido]triphosphate (p[NH]ppG) added to the assay mixtures. There was no significant change in receptor affinity for the antagonist [³H]DHA between days 5.5 and 13. Despite the decrease in numbers of β -adrenergic receptors, there was no change in basal, p[NH]ppG-, isoprenaline- or isoprenaline-plus-p[NH]ppG-stimulated adenylate cyclase activity between days 3 and 12 of development. We conclude that β -adrenergic receptors and adenylate cyclase are not co-ordinately regulated during early embryonic development of the chick heart. Some of the β -adrenergic receptors present very early in the ontogeny of cardiac tissue appear not to be coupled to adenylate cyclase since their loss is not reflected in decreased activation of the enzyme.

Functional innervation of the heart requires the presence not only of the presynaptic neuron but also postsynaptic receptors for transmitters that transduce chemical signals into biochemical and/or electrical events in target cells. There is evidence that β -adrenergic receptors are present in myocardium in very young (day 4) chick embryos (McCarty *et al.*, 1960). These receptors are coupled to adenylate cyclase, at least in part, since β -adrenergic agonists can stimulate cyclic AMP formation in these developing hearts (Polson *et al.*, 1977; Renaud *et al.*, 1977). However, the cyclic AMP response diminishes by day 7 (Polson *et al.*, 1977) or 15 (Renaud *et al.*, 1977). This decreased response might reflect changes in the β -adrenergic receptor, in adenylate cyclase or in receptor-enzyme coupling during embryogenesis. In the present study, we tested these possibilities by characterizing the β -adrenergic receptors and adenylate cyclase throughout cardiac embryogenesis. Although the course of β -adrenergic receptor density has been studied in foetal-mouse heart, beginning at the third trimester, there is little in-

formation regarding earlier development and in particular regarding the relationship to adenylate cyclase (Chen *et al.*, 1979). The developing chick heart is particularly well suited for such studies because the gestational age can be determined precisely and because there is an extensive literature describing the time of occurrence of key developmental events that may be related to the synthesis and function of β -adrenergic receptors and adenylate cyclase. These include the development of autonomic innervation (Pappano, 1977), physiological responsiveness to catecholamines (Pappano, 1977) and ion-channel appearance (Sperelakis & Shigenobu, 1972; Galper & Catterall, 1978). Accordingly, we have established the time course of development of the β -adrenergic receptors and of their coupling to adenylate cyclase to permit these events to be related to the development of other systems.

Methods

Embryonated Leghorn chicken eggs (Flock MR56) were obtained from Spafas Inc., Norwich, CT, U.S.A. In all cases, the embryonic ages were

Abbreviations used: [³H]DHA, (–)-[³H]dihydroalprenolol; p[NH]ppG, guanosine 5'-[β , γ -imido]triphosphate.

determined by comparison with the description of Hamburger & Hamilton (1945). Hearts were quickly removed from embryos of various ages and placed in cold (4°C) buffered medium (75 mM-Tris/HCl/25 mM-MgCl₂, pH 7.4). Blood and any attached membranes were removed by vigorously drawing the hearts and buffer into a Pasteur pipette several times. The hearts to be assayed for β -adrenergic receptors were placed in fresh cold medium and homogenized with 35 strokes in a tight-fitting Dounce homogenizer. The homogenate was centrifuged at 50 000 *g* for 25 min at 4°C. The supernatant phase was discarded and the pellet was resuspended in Tris-buffered medium using the Dounce homogenizer to a final protein concentration of 1–2 mg/ml. Phase-contrast microscopy revealed no intact cells. The centrifugation and second homogenization step improved reproducibility of the β -adrenergic receptor assay over that obtained with crude homogenates, presumably because of more complete disruption of cells and more uniform dispersion of the small fragments. Hearts to be assayed for adenylate cyclase activity were homogenized in 0.25 M-sucrose/50 mM-Tris/

HCl/10 mM-MgCl₂/1 mM-dithiothreitol/1 mM-disodium EDTA, pH 7.6, and enzyme activity was determined as previously described (Neer, 1978).

β -Adrenergic receptors were assayed by using [³H]DHA (New England Nuclear; sp. radioactivity 52 Ci/mmol) as previously described (Alexander *et al.*, 1975*a,b*). Total binding was about 1% of added counts. Specific binding was defined as that displaceable by 10 μ M-(+)-propranolol and at 10 nM-[³H]DHA was 60–80% of total binding at all ages. The (–)-isomers of both the antagonist propranolol and agonist isoprenaline were more potent in competing for binding that were the (+)-isomers (Fig. 1). The potency order of displacement by agonist was: (–)-isoprenaline > (–)-noradrenaline (results not shown). These characteristics, together with the property of saturability (see Fig. 3 below), define the binding sites as β -adrenergic receptors. Data in all Figures and Tables refer to specific binding.

Protein was measured by the method of Lowry *et al.* (1951).

Results

Fig. 2 demonstrates that the number of β -adrenergic receptors per mg of protein in chick hearts is dependent on embryonic age. Thus, hearts 4.5–5.5 and 6.5–7.5 days *in ovo* have 0.36 ± 0.03 (mean $\pm$ S.E.M.) and 0.36 ± 0.04 pmol of receptor/mg

Fig. 1. Characteristics of binding of [³H]DHA to 7-day-embryonic chick hearts

[³H]DHA (approx. 10 nM) was incubated for 15 min at 37°C with 100–200 μ g of the particulate fraction. The reaction was terminated by addition of 2 ml of cold (4°C) assay medium and rapid filtration through a Whatman GF/C glass-fibre filter under reduced pressure. The assay tube was washed three times with 4 ml of the cold assay medium. The filter was dried, placed in 10 ml of Beckman General Purpose Liquid Scintillation Counting Fluid and counted at 42% efficiency in a Beckman Liquid Scintillation Counter. Symbols: ●, (–)-propranolol; ○, (+)-propranolol; ■, (–)-isoprenaline; □, (+)-isoprenaline.

Fig. 2. Effects of embryonic age on number of chick heart β -adrenergic receptors

Saturating concentrations of [³H]DHA (40–50 nM) were incubated for 15 min at 37°C with 100–200 μ g of the chick heart particulate fraction and β -adrenergic receptor assay was as described in the legend to Fig. 1; 30–40 of the youngest hearts and two to three of the oldest hearts were used for each experiment. The bars indicate $\pm$ 1 S.E.M. *P* values were determined by using Student's *t* test.

of protein respectively. By day 9 the density decreased significantly to 0.22 ± 0.02 pmol/mg of protein ($P < 0.01$). There was a further decrease by day 12.5–13.5 to 0.14 ± 0.01 pmol/mg of protein ($P < 0.01$) but no further change was observed as late as day 16–17 (0.16 ± 0.01 pmol/mg of protein). In separate experiments we found that the density of β -adrenergic receptors at days 5 and 13 was not altered by assaying in the presence of $100 \mu\text{M}$ -concentrations of the non-hydrolysable guanosine triphosphate analogue p[NH]ppG.

To determine whether the affinity as well as the density of β -adrenergic receptors might undergo changes during embryonic development, Scatchard analyses were performed of [^3H]DHA binding to the particulate fraction of hearts from 5.5- and 13-day embryos. Representative Scatchard plots for each age are shown in Fig. 3. In the case of the 5.5-day hearts, the maximum binding capacity was 0.288 pmol/mg of protein, with an equilibrium dissociation constant (K_d) of 8.4 nM. In contrast, the receptor density in the 13-day heart was 0.166 pmol/mg of protein, although the K_d (9.5 nM) was similar. The data from Scatchard analysis of saturation binding curves from 5.5-day hearts (four experiments) and 13-day hearts ($n = 3$) are summarized in Table 1. Hearts from 5.5- and 13-day embryos had a receptor density of 0.35 ± 0.06 and 0.18 ± 0.01 pmol/mg of protein respectively ($P < 0.05$). There was no difference in receptor affinity. The values for receptor number at the two ages determined from Scatchard analysis agree well with those determined by using single saturating concentrations of the ligand (Fig. 2). Thus the β -adrenergic receptors of the chick heart decrease in number but not affinity early in the course of embryogenesis.

The development of β -adrenergic-receptor-coupled adenylate cyclase was assessed in 3–6-, 8–9- and 10–12-day-embryo hearts (Table 2, group A). There was no difference in the activity of basal or isoprenaline ($10 \mu\text{M}$)-stimulated adenylate cyclase at the three ages. The apparent K_m for (–)-isoprenaline stimulation of adenylate cyclase was 700 nM in both 5- and 9-day-embryo hearts. To

exclude the possibility that receptor–enzyme coupling might be linked by low levels of guanine nucleotides, adenylate cyclase activity was measured in separate experiments in the presence of p[NH]ppG ($10 \mu\text{M}$) in 5-, 8–9- and 10-day-embryo hearts (Table 2, group B). Both p[NH]ppG and isoprenaline stimulated basal adenylate cyclase activity to a similar extent (3–4-fold) at all three ages. Stimulation of adenylate cyclase activity by the combination of (–)-isoprenaline ($10 \mu\text{M}$) and p[NH]ppG ($10 \mu\text{M}$) was greater than the sum of activity stimulated by either agent alone. There was, however, no difference in the extent of stimulation at any of the ages tested. Thus, there appears to be no appreciable change in adenylate cyclase activity with increasing embryonic age despite the marked decrease in β -adrenergic receptor density.

The change in receptor density occurs over a

Fig. 3. Scatchard analysis of [^3H]DHA saturation curves in 5.5- and 13-day-embryonic chick hearts. Representative experiments at each age are shown. The K_d was 8.4 and 9.5 nM at 5.5 (●) and 13 days (○) respectively. The line represents the best fit determined by linear regression ($r = 0.93$ and 0.99 for 5.5- and 13-day-embryo hearts respectively).

Table 1. The binding parameters resulting from Scatchard analysis of [^3H]DHA saturation curves

The binding assay was performed as described in the Methods section and in the legend to Fig. 1. Concentrations of [^3H]DHA between about 3 and 50 nM were utilized. The line of best fit for each individual experiment was determined by linear regression analysis. The maximum binding capacity (B_{max}) and K_d for the group were determined from the mean values of the individual experiments. Results are means $\pm$ S.E.M. for the numbers of observations shown in parentheses. The P value was determined by an unpaired t test. Abbreviation used: N.S., not significant.

Age (days)	B_{max} (pmol/mg of protein)	P	K_d nM	P
5.5 (4)	0.35 ± 0.06	<0.05	13.3 ± 3.0	N.S.
13 (3)	0.18 ± 0.01		10.5 ± 0.5	

Table 2. *Adenylate cyclase activity*

Assays were performed at 37°C with homogenates of chick hearts as described in the text; 2–15 hearts (depending on age) were used per experiment. (–)-Isoprenaline and p[NH]ppG were each 10 μ M. The earlier experiments (group A) did not include measurement of guanine nucleotide activation.

Age (days)	Number of experiments	Adenylate cyclase activity (pmol of cyclic AMP/ mg of protein per 10 min)			
		No addition	(–)-Isoprenaline	p[NH]ppG	p[NH]ppG and (–)-isoprenaline
Group A					
3–6	12	387 ± 63	899 ± 132		
8–9	6	379 ± 56	938 ± 154		
10–12	7	356 ± 60	943 ± 178		
Group B					
5	3	391 ± 80	1006 ± 65	1135 ± 75	3182 ± 421
8–9	3	407 ± 53	1196 ± 93	1876 ± 498	4279 ± 630
10	3	486 ± 28	1282 ± 146	1265 ± 211	3629 ± 291

relatively short time span between 7 and 12 days *in ovo*. In contrast, heart weight and total protein increase continuously over the period studied in the experiments. Preliminary experiments on whole homogenates also demonstrated a decrease in density of cardiac β -adrenergic receptors during development, suggesting that the changes in density in the crude particulate fraction from young- and old-embryonic hearts do not result from systematic differences in the ratio of plasma membrane to total protein in this fraction. Furthermore the constancy of adenylate cyclase activity (itself a plasma-membrane marker) argues against a trivial explanation of the decrease in β -adrenergic receptor density by a general increase in myocardial protein content. Other plasma-membrane markers such as muscarinic cholinergic receptors (Galper *et al.*, 1977) also maintain an essentially constant relationship with total protein in chick heart during the time β -adrenergic receptor decreases markedly. Thus, the observed changes appear to be specific for the β -adrenergic receptor.

Discussion

Although it has been shown that β -adrenergic receptors and adenylate cyclase are different molecules, the functional stoichiometry of the two is not known. Several studies of mature tissues have supported the idea that the full number of β -adrenergic receptors is needed for maximal activation of the enzyme by agonists (Brown *et al.*, 1976a,b; Lucas & Bockaert, 1977). In some of these studies decreasing the number of β -adrenergic receptors by genetic manipulation (Johnson *et al.*, 1979) or by binding of slowly reversible ligands (Maguire *et al.*, 1976) led to a decreased maximal activation of the enzyme. The number of β -adrenergic receptors

decreases during maturation of the rat erythrocyte and this is accompanied by diminished adenylate cyclase activity (Hollenberg, 1979). In other kinds of studies, Tolkovsky & Levitzki (1978) have shown that decreasing receptor number in turkey erythrocytes diminished the rate of hormone activation but not the maximal level attained.

Our studies show that early in embryogenesis the production of cardiac β -adrenergic receptors and adenylate cyclase is not co-ordinately regulated. Early in cardiac development a large number of β -adrenergic receptors are present which do not seem to be functionally linked to adenylate cyclase. Decreasing their number by approximately half during embryogenesis does not diminish the adenylate cyclase response to isoprenaline stimulation. Thus the drop in hormone-stimulated cyclic AMP levels reported to occur during cardiac development (Polson *et al.*, 1977; Renaud *et al.*, 1977) does not appear to be due to diminished adenylate cyclase responsiveness.

Our findings do not define the signal resulting in decreased cardiac β -adrenergic receptor density. There are, however, at least two developmental events temporally related to the decrease in receptor density. First, very young embryonic chick hearts are activated via tetrodotoxin-insensitive slow Na⁺ channels (Sperelakis & Shigenobu, 1972; Galper & Catterall, 1978). Tetrodotoxin-sensitive fast Na⁺ channels become active on day 5 and cation fluxes through these channels increase until day 18. Cation fluxes through the slow channels greatly increase at about day 8. Thus, major changes in membrane electrical properties occur concomitantly with the decrease in β -adrenergic receptor density.

Another major developmental event temporally correlated with decreased β -adrenergic receptor density in the chick heart is the initiation of sympathetic innervation. Although functional

sympathetic neuroeffector transmission may not occur until day 17 (Pappano, 1977), sympathetic nerves appear considerably earlier. Between day 7 and day 10, catecholamine levels increase markedly from low initial levels, presumably reflecting synthesis by neurons *in ovo* (Ignarro & Shideman, 1968a,b). Similarly, [^3H]noradrenaline uptake that is reserpine- and cocaine-sensitive, and hence presumably neuron-specific, first appears at day 5 *in ovo*, increases over the next 2 days and reaches a plateau between days 8 and 10 (Ignarro & Shideman, 1968a,b). Although fluorescent histochemical studies could not demonstrate catecholamine-containing neurons in the chick ventricle until day 16 *in ovo* (Enemar *et al.*, 1965), morphological and histological evidence suggests that ingrowth of sympathetic nerves into the chick heart occurs between days 6 and 10 *in ovo* (Romanoff, 1960). Catecholamine uptake into sympathetic nerve terminals that appears before noradrenaline concentrations increase enough to be detected by fluorescence microscopy has been reported also in the iris (Black & Mytilineous, 1976). The close correlations among adrenergic innervation, increased catecholamine content and decrease in density of β -adrenergic receptors raises the possibility that these developmental events are related. It is possible that the decrease in receptor number reflects agonist induced down-regulation (Mukherjee *et al.*, 1975) by increasing levels of noradrenaline during innervation.

There has been intense investigation of factors involved in the development, stabilization and maintenance of neuronal and neuronal-effector networks (Rees, 1978; Landmesser & Pilan, 1978). It has been postulated that retrograde effects of trophic substances produced by target tissues attract and stabilize nerve terminals and that antegrade factors (possibly including neurotransmitters) localize receptors to the synaptic area of the cell membrane and control receptor synthesis (Changeux & Danchin, 1976). The glycoprotein nerve-growth factor, which stimulates the growth of adrenergic neurons of sympathetic ganglia both *in vitro* and *in vivo*, is thought to be a postsynaptic trophic substance (Mobley *et al.*, 1979). An example of antegrade control by the neuron of postsynaptic events is the difference in distribution of nicotinic cholinergic receptors on innervated and denervated skeletal-muscle cells (Miledi, 1960). It is interesting to speculate that the decrease in density of β -adrenergic receptors in the chick heart that occurs between days 7 and 13 *in ovo* may be determined by noradrenergic neurons that are developing their neuroeffector relationship with the heart during this time.

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