Role of β -adrenoceptor-adenylate cyclase system in the developmental decrease in sensitivity to isoprenaline in foetal and neonatal rat heart

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1 The inotropic and chronotropic sensitivity to noradrenaline and isoprenaline (Iso) of foetal and neonatal rat heart decreases as the heart becomes sympathetically innervated. In the present study, we have examined adenylylase (AC) activation and β -adrenoceptor binding to determine whether a developmental decrease in sensitivity was demonstrable in the β -receptor-AC system of atrial and ventricular membranes from the 15 day foetus and 1 day and 7 day neonates.

2 While the maximum activation of AC by Iso increased with age, the sensitivity expressed in terms of pD_2 values decreased from the 15th foetal day to the first day after birth in the atria, and from the first day to the 7th day after birth in the ventricle.

3 In contrast, activation of AC by forskolin was almost identical at all ages both in atria and ventricle.

4 The maximum equilibrium binding of [³H]-dihydroalprenolol decreased with age, the dissociation constant being about the same at all ages in both the atria and ventricle.

5 In conclusion, we have demonstrated a developmental decrease in the sensitivity of AC to Iso in myocardial membrane fractions consistent with the developmental decrease in chronotropic and inotropic sensitivity to β -adrenoceptor agonists. Although a reduction in β -adrenoceptor number partly accounts for the decrease in sensitivity, some other factors such as decreased coupling to AC may largely be responsible.

Introduction

It is known that nerves exert a trophic influence upon muscle to regulate its responsiveness to neurotransmitters and exogenously applied agonists. This phenomenon has been most extensively investigated in skeletal and smooth muscle. However, relatively little is known regarding the trophic influence of autonomic nerves on cardiac muscle. There are several reports including those from our own laboratory, which suggest that sympathectomy of the rat heart results in post-junctional supersensitivity to agonists (Nomura *et al.*, 1980; Ishii *et al.*, 1982; 1985; Goto *et al.*, 1985). In organ culture conditions, in the absence of sympathetic innervation, rat atria acquire a high sensitivity similar to that after denervation, which can be prevented by adding the neurotransmitter (noradrenaline) to the culture medium (Tanaka *et al.*, 1988a). These results support the hypothesis that sympathetic nerves exert a trophic influence upon cardiac muscle to maintain its sensitivity at a normal level.

With respect to changes in the chronotropic and inotropic sensitivity of cardiac muscle to noradrenaline (NA) and isoprenaline (Iso) during development, we have recently shown that the β -adrenoceptor sensitivity is high in the foetus followed by a ten fold decrease, which occurs in the late foetal period at the sinus node and during the first postnatal week in the ventricle (Shigenobu *et al.*, 1988; Tanaka *et al.*, 1988a,b). The time course of this decrease correlates well with the development of sympathetic innervation in each region (Shigenobu *et al.*, 1988; Tanaka *et al.*, 1988a,b), which also suggests that myocardial sensitivity is influenced by sympathetic innervation.

As for the molecular basis of the developmental decrease in sensitivity, we have suggested the contribution of some

changes in the β -receptor-adenylate cyclase (AC) system based on pharmacological considerations (Shigenobu *et al.*, 1988; Tanaka *et al.*, 1988a), but no direct measurement has been made. Therefore, in the present study, we examined β -adrenoceptor binding and the activation of adenylylase in foetal and neonatal rat myocardial membranes to determine whether the developmental decrease in sensitivity is demonstrable at the level of adenosine 3':5'-cyclic monophosphate (cyclic AMP) production and whether it is accompanied by changes in receptor number and/or affinity. We used atrial and ventricular membranes from the 15 day foetus, 1 day neonate and 7 day neonate, and compared the time course of the changes with the developmental decrease in chronotropic and inotropic sensitivity.

Methods

Pregnant Wistar strain rats were anaesthetized with ether and foetuses were removed on the 15th day of gestation (birth usually occurs on the 21st day of gestation). The atria and ventricle were obtained from these 15-day foetuses, and one day and 7-day old neonates and they were used either for AC measurements or for binding assays.

AC measurements were performed according to methods previously reported (Ishikawa *et al.*, 1988). Tissues were homogenized with a glass-Teflon homogenizer in 20 mM Tris/HCl buffer (pH 7.4) containing 0.25 M sucrose. The homogenate was centrifuged at 100,000 *g* for 1 h, and the pellet resuspended in the buffer was used for the measurements. Membrane fractions equivalent to 30–40 μ g protein were incubated at 37°C for 10 min in 300 μ l of the reaction mixture. The reaction mixture consisted of (mM): Tris/HCl 40 (pH 7.4), KCl 10, MgCl₂ 5, EGTA 1, ATP 1, theophylline 10, creatine phosphate 5, and GTP 1 μ M, creatine kinase 75 μ g ml⁻¹ and bovine

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serum albumin 1 mg ml⁻¹. Various concentrations of Iso or forskolin were added. Reactions were terminated by boiling for 3 min. Cyclic AMP thus formed was assayed by means of a radioimmunoassay kit (Yamasa Shouyu). The increase in AC activity produced by each concentration of drug was calculated. The pD₂ values were obtained by proportional allotment between the two concentrations which produce nearly a half-maximal response, as described previously (Tanaka & Shigenobu, 1989). pD₂ is defined as the negative logarithm of the molar concentration of a drug required to produce a half maximum response. Therefore, pD₂ is an indicator of sensitivity to a drug and a higher pD₂ value reflects a higher sensitivity.

Binding assays were performed according to the methods previously described (Abe *et al.*, 1988; Koike *et al.*, 1988). The isolated atria and ventricles were minced and homogenized with a polytron homogenizer (Kinematica; setting 11, 5 s × 5 times) in 10 ml ice-cold sucrose buffer. The homogenate was first centrifuged at 3000 *g* for 10 min. The pellet was resuspended in 10 ml of sucrose buffer and recentrifuged under the same conditions. The supernatants from the two centrifugations were mixed well and further centrifuged at 10,000 *g* for 15 min. The resulting supernatant was centrifuged at 100,000 *g* for 60 min. The final pellet was resuspended in ice-cold Tris/HCl buffer (pH 7.4), and used for the assays: 200 µl of the final suspension equivalent to 15–20 µg protein was incubated with various concentrations of [³H]-dihydroalprenolol ([³H]-DHA) at 37°C for 30 min. The incubation was terminated by filtration through Whatman GF/F glass filters, which were rinsed twice with 3 ml ice-cold Tris/HCl buffer. The filters were dried and radioactivities were counted with a liquid scintillation counter (Aloka, LSC-700). The amount of [³H]-DHA bound in the absence and presence of 10 µM propranolol was regarded as total and non-specific binding, respectively. The maximum binding capacity (*B*_{max}) and the equilibrium dissociation constant (*K*_d) were calculated from Scatchard analysis of the saturation data. Regression lines were drawn by the least square method.

Data were expressed as the mean ± s.e.mean of three independent observations each performed in duplicate. Statistical analyses were carried out by Bartlett's analysis of variance followed by Tukey's test for multiple comparisons (Grimm, 1973).

Results

Stimulation of adenylate cyclase by isoprenaline

Both in the atrium and in the ventricle, there were no significant changes in the basal AC activity during development (Table 1). Iso, at concentrations from 10⁻⁹ M to 10⁻⁵ M, stimulated in a concentration-dependent fashion the AC activity of

Figure 1 Dose-response curves for the effect of isoprenaline (Iso) on adenylate cyclase activity of atrial (a) and ventricular (b) membranes from the foetus (●), 1-day old neonate (○) and 7-day old neonate (■). Abscissa scale: concentration of Iso (–log M); ordinate scale: cyclic AMP formed per min per mg protein (pmol). Each point represents the mean (with s.e.mean shown by vertical bars) from 3 experiments performed in duplicate.

both atrial and ventricular membranes of all ages. The maximum response to Iso increased during the first postnatal week both in the atrium and in the ventricle (Figure 1). However, cyclic AMP production induced by 10⁻⁸ M Iso (around this concentration, chronotropic or inotropic response were examined) was greater in foetal atria and foetal and 1-day old ventricle. In fact, when pD₂ values for cyclic AMP production were calculated to determine the sensitivity changes, they decreased significantly from the foetal period to

Table 1 Activation of adenylate cyclase (AC) by isoprenaline

	Atria			Ventricle		
	Foetus	1-day-old	7-day-old	Foetus	1-day-old	7-day-old
Basal activity	8.9 ± 1.2	11.8 ± 1.1	12.9 ± 1.3	9.6 ± 1.2	11.6 ± 0.9	12.8 ± 1.9
Maximum increase by Iso	8.2 ± 1.6	7.5 ± 0.7	13.4 ± 1.6*	8.6 ± 1.7	9.8 ± 2.1	16.7 ± 1.6*
Increase by 10 ⁻⁸ M Iso	4.5 ± 0.4	2.9 ± 0.5*	2.3 ± 0.4*	4.6 ± 1.4	5.2 ± 1.2	0.8 ± 0.6*
pD ₂ value for Iso (AC)	8.18 ± 0.17	7.60 ± 0.18*	7.23 ± 0.15*	8.05 ± 0.19	7.93 ± 0.18	6.81 ± 0.14*
pD ₂ value for Iso (mechanical)	9.48 ± 0.13	8.52 ± 0.08*	8.54 ± 0.05*	8.80 ± 0.04	8.72 ± 0.09	7.54 ± 0.06*

Activities are expressed as pmol of cyclic AMP min⁻¹ mg⁻¹ protein. Values are the mean ± s.e.mean from three different experiments in duplicate. pD₂ values for the mechanical (chronotropic and inotropic) responses to Iso were obtained in our previous studies (Shigenobu *et al.*, 1988).

* Indicates statistically significant difference (*P* < 0.01) from the corresponding value in the foetus.

Figure 2 Dose-response curves for the effect of forskolin on adenylyl cyclase activity of atrial (a) and ventricular (b) membranes from the foetus (●), 1-day old neonate (○) and 7-day old neonate (■). Abcissa scale: concentration of forskolin ($-\log M$); ordinate scale: cyclic AMP formed per min per mg protein (pmol). Each point represents the mean (with s.e.mean shown by vertical bars) from 3 experiments performed in duplicate.

the first day after birth in the atrium, and from the first day to the 7th day after birth in the ventricle (Table 1). These decreases in sensitivity were paralleled by decreases in pD_2 values for the chronotropic and inotropic responses to Iso obtained in our previous study (Table 1).

Stimulation of adenylyl cyclase by forskolin

Forskolin, which is known to activate directly the catalytic subunit of AC (Seamon & Daly, 1983), stimulated the AC

Figure 3 Saturation curves of specific [3H]-dihydroalprenolol binding to atrial (a) and ventricular (b) membranes from the foetus (●), 1-day old neonate (○) and 7-day old neonate (■). Each point represents the mean (with s.e.mean shown by vertical bars) from 3 experiments performed in duplicate. Scatchard plots of the data are shown in the insets.

activity in both atrial and ventricular membranes in a concentration-dependent manner and the dose-response curve was identical at all ages both in the atrium and in the ventricle (Figure 2). The increase in cyclic AMP production was much greater than that produced by Iso in all preparations.

Table 2 Summary of binding parameters

	Atria			Ventricle		
	Foetus	1-day-old	7-day-old	Foetus	1-day-old	7-day-old
B_{max} (fmol mg^{-1} protein)	217 ± 21	183 ± 11	$156 \pm 8^*$	197 ± 18	176 ± 10	$127 \pm 7^*$
K_d (nM)	0.94 ± 0.13	0.90 ± 0.08	0.79 ± 0.18	1.08 ± 0.08	0.97 ± 0.07	0.86 ± 0.12

β -Adrenoceptor number (B_{max}) and apparent dissociation constant (K_d) were determined by linear regression analysis of Scatchard plots in Figure 3. Values indicate the mean $\pm$ s.e.mean from 3 different experiments in duplicate.

* Indicates statistically significant difference ($P < 0.01$) from the corresponding value in the foetus.

[³H]-dihydroalprenolol binding

Specific [³H]-DHA binding to both atrial and ventricular membranes was saturable and of high affinity. The Scatchard plots of the data were linear in all preparations indicating the existence of a single binding site (Figure 3). The B_{max} for [³H]-DHA decreased gradually with a similar developmental time course in the atrium and in the ventricle, i.e. the B_{max} value at 1 week after birth was 2/3 of that in the foetus both in the atrium and in the ventricle (Table 2). The dissociation constant was almost identical in all membranes (Table 2).

Discussion

The present study has demonstrated that a β -adrenoceptor agonist, Iso, was able to stimulate the AC system as early as the 15th day of gestation. This observation and our previous results (Shigenobu *et al.*, 1988) indicate that β -adrenoceptor agonists are able to activate AC as well as to produce chronotropic and inotropic responses as early as the 15th day of gestation, which is before the onset of sympathetic innervation (Gomez, 1958; Ishii *et al.*, 1982; Shigenobu *et al.*, 1988). It has been reported that, in the chick ventricle, Iso is able to activate AC and produce a positive inotropic effect in the 11-day old embryo while sympathetic innervation begins on the 16th embryonic day (Higgins & Pappano, 1981; Smith & Pappano, 1985). Therefore, it seems to be a general phenomenon that postjunctional cells are responsive to adrenoceptor agonists before innervation is established (Pappano 1977).

In cardiac muscle, it has been postulated that autonomic innervation may exert a trophic influence upon muscle properties (Shigenobu, 1983). With regard to the inotropic and chronotropic sensitivity (pD_2 values) of the rat myocardium during development, there seems to be general agreement that sensitivity to NA is high at foetal or neonatal stages followed by decrease with age (Mackenzie & Standen, 1980; Ishii *et al.*, 1982; Shigenobu, 1983; Shigenobu *et al.*, 1988; Tanaka *et al.*, 1988a,b). Although it has been suggested that these changes were caused by prejunctional mechanisms (Mackenzie & Standen, 1980), we have observed in the right atria and in the ventricle that sensitivity to Iso decreased in parallel to the sensitivity to NA during development (Shigenobu *et al.*, 1988; Tanaka *et al.*, 1988a,b), and concluded that the changes were mainly postjunctional in nature. This decrease in sensitivity correlates well with the increase in sympathetic innervation in each region, as determined by examining responsiveness to tyramine (Shigenobu *et al.*, 1988; Tanaka *et al.*, 1988a,b), NA content (Iversen *et al.*, 1967; Mirkin, 1972), intensity of the fluorescence of catecholamine containing fibres (De Champlain *et al.*, 1970; Lipp & Rudolph, 1972), capacity for NA uptake and retention (Iversen *et al.*, 1967) and by morphological observations (Gomez, 1958).

Under organ culture conditions, in the absence of neuronal influence, the sensitivity of foetal and neonatal atria to NA and Iso is maintained at a high level, similar to that observed in the foetal atria before innervation. Addition of the neurotransmitter (NA) to the culture medium results in normal sensitivity similar to that observed in intact atria with sympathetic innervation (Tanaka *et al.*, 1988a). Furthermore, there are several reports including those from our laboratory that sympathectomy of the rat heart results in postjunctional supersensitivity to agonists (Nomura *et al.*, 1980; Ishii *et al.*, 1982; 1985; Goto *et al.*, 1985). All these results support the hypothesis that sympathetic nerves exert trophic influences upon cardiac muscle to maintain its sensitivity at normal level.

Although the correlation between the sensitivity to agonists and sympathetic innervation has been demonstrated by many investigators, the cellular basis for these sensitivity changes has not been systematically investigated. It has been reported that sympathetic denervation of rat heart by 6-

hydroxydopamine results in an increase in β -receptor number with no change in affinity (Nomura *et al.*, 1980), and that reserpine treatment induces an increase in β -adrenoceptor number (Latifpour & McNeil, 1984) as well as an increase in the maximum activation of AC by adrenaline (Cros & McNeil, 1987) in guinea-pig atrium.

We have already reported that the developmental decrease in sensitivity can be demonstrated in the presence of a phosphodiesterase inhibitor, isobutyl-methyl xanthine, and that the sensitivity to forskolin and dibutyryl cyclic AMP remains unchanged during development (Shigenobu *et al.*, 1988). These results suggest that the decrease in sensitivity is unlikely to be produced by an increase in phosphodiesterase activity or some changes in the process distal to cyclic AMP. It is possible that some change in the process leading to cyclic AMP production is the major cause of the decrease in sensitivity. In the present study, we measured the activation of AC by Iso and directly demonstrated a developmental decrease in sensitivity at the level of cyclic AMP production (Figure 1, Table 1). The decrease occurred before birth in the atria, and after birth in the ventricle, which is in good correlation with the decrease in chronotropic and inotropic sensitivity (Table 1). Therefore, the developmental decrease in chronotropic and inotropic sensitivity may be explained, at least in part, by the decrease in the sensitivity of the β -receptor-AC system.

The concentration-range of Iso that activated AC was much higher than that required to produce chronotropic and inotropic responses (Figure 1, Table 1), a phenomenon which has been documented by many investigators (Venter, 1979; Scholz, 1980; Porzig, 1982; Ishikawa *et al.*, 1988). Although the attenuation of the sensitivity of AC activity during the procedure of homogenization cannot be totally excluded (Porzig, 1982), it may be explained by considering 'spare receptors', namely, only partial activation of AC is sufficient to elicit mechanical responses (Venter, 1979). Therefore, the amount of cyclic AMP produced by threshold concentrations of Iso may be important in producing mechanical responses. Taking the above into account, the important observation made in the present study may be that there was a correlation between the increase in cyclic AMP production by 10^{-8} M Iso (which is in the concentration-range to produce chronotropic and inotropic responses) and the developmental decrease in sensitivity (Table 1).

In contrast to these changes in the responsiveness of AC to Iso, no developmental changes were observed in the responsiveness to forskolin (Figure 2). This is in agreement with our previous results which show that the inotropic and chronotropic sensitivity to forskolin remains unchanged during development (Shigenobu *et al.*, 1988; Tanaka *et al.*, 1988a). Therefore, it is most likely that the decrease in sensitivity is produced by some changes in the β -receptor and/or its coupling to AC. The degree of AC activation by forskolin was much greater than that by Iso in all preparations, which may be due to its direct action on the catalytic subunit of AC.

Both in the atrium and in the ventricle, the B_{max} of [³H]-DHA binding decreased with age while the K_d values remained unchanged. This is in agreement with a previous report on ventricular membranes which showed a higher B_{max} of [³H]-DHA binding in the 1-day old neonate compared to the adult (Whitsett & Beckerman, 1981). The present authors have also reported a developmental decrease in the B_{max} values for a partial β -receptor agonist [³H]-befunolol in foetal and neonatal myocardium (Koike *et al.*, 1988). It is well known that the larger the number of receptors in a tissue, the larger the pD_2 value of the agonist that should be obtained (Kenakin, 1984). Therefore, the decrease in β -receptor number may partly account for the decrease in sensitivity. However, the magnitude of the decrease in receptor number seems to be rather small when compared with the tenfold decrease in sensitivity (Table 1, 2). Moreover, the time course of the developmental decrease in receptor number and that of the decrease in cyclic AMP production were not completely parallel. Therefore, it is likely that some other factors such as

decreased coupling to AC are also involved. Similar decreases in the sensitivity to Iso and changes in the β -receptor-AC system have been reported in the chick embryo ventricle before hatching (Higgins & Pappano, 1981; Smith & Pappano, 1985). As the subsensitivity was transient and was not prevented by reserpine administration, factors other than sympathetic innervation seem to be the cause of subsensitivity in the case of the chick embryo (Higgins & Pappano, 1981).

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(Received August 15, 1989)

Revised November 30, 1989

Accepted December 24, 1989)