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RATE, FORCE AND CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE RESPONSES TO (−)-ADRENALINE IN NEONATAL RAT HEART TISSUE

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1 (−)-Adrenaline sensitivity in 1 to 20 day-old rat heart tissue was investigated as rate, force and cyclic adenosine 3′,5′-monophosphate (cyclic AMP) production responses together with sensitivity to (±)-propranolol blockade.

2 Resting performances were measured and responsiveness to (−)-adrenaline then determined as ED_{50} values and maximal responses together with sensitivity to propranolol (pA_2 values).

3 Resting force, corrected for sample size, did not change with age, whereas resting atrial rate doubled between 1 and 20 days.

4 ED_{50} concentrations in atria were constant with age, but decreased in ventricles. Cocaine (10^{-5} M) and other drugs did not consistently affect ED_{50} values.

5 Maximal responses were not age-dependent in right atria, but increased in left atria. In 2 to 5 day-old hearts there was no inotropic response to adrenaline and the very small maximal response in ventricles rose 5 to 7 fold by 20 days.

6 Propranolol sensitivity increased slightly (2 to 4 times) with age in all tissue from a pA_2 value of 7.5 at 2 days to 8.2 at 20 days.

7 Control cyclic AMP was higher in 2 day than in 20 day-old tissue and in atria than ventricular strips or hearts. In 2 and 20 day-old atria, hearts and ventricles, force increases with different adrenaline concentrations correlated linearly with the log of the increase in cyclic AMP. Such a correlation was not seen in 2 day hearts and ventricles for cyclic AMP rose without corresponding force increases.

8 Results suggest slight maturational changes in atrial β-receptors. In 1 to 5 day-old ventricles, normal ED_{50} concentrations and good cyclic AMP response in the presence of a much reduced force response may indicate receptor-response transduction inefficiency, assuming a β_1-receptor occupation and cyclic AMP production response mechanism for inotropism with adrenaline.

Introduction

Studies on β_1-adrenoceptors for rate and force responses are usually performed on adult cardiac tissue. Cultured cells have been used as a simple system for correlating response with receptor occupation, but in cultured rat heart cells radioactive adrenaline accumulation is not readily related to response (Walker, 1978). Furthermore, beating responses to adrenaline appear earlier than propranolol sensitivity in such cultures (Walker, 1977). Such anomalies prompted the examination of source tissue (neonatal ventricles) for responsiveness to adrenaline and it appeared that hearts from rats aged 1 to 5 days did not have a force response to adrenaline (Walker & Au, 1975). This lack of adrenaline response in tissue used to produce adrenaline-responsive cultures seemed worth investigating. In addition, Mirkin (1972) suggested that an ontogenic approach is useful in investigating adrenergic mechanisms.

In the rat, sympathetic innervation is relatively sparse at birth (Iversen, De Champlain, Glowinski & Axelrod, 1967; Atwood & Kirshner, 1968) and its control of heart rate is not fully developed for the next 20 days (Wekstein, 1965). Force responses in new born (1 and 2 weeks old) rat atria are supersensitive to noradrenaline, but not isoprenaline, while electrical field stimulation responses develop during the first three weeks of life (Standen, 1978). However, adrenergic innervation is delayed in ventricles (De Champlain, Olson, Malmfors & Sachs, 1970). Rate responses to isoprenaline in right atria, and force responses in left, are maximum at birth (Standen, 1968), suggesting mature β-adrenoceptors. Adenylate cyclase

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is present in foetal tissue and full activity is found in heart (Martin, Levey & Levey, 1973) and brain (Schmidt, Palmer, Dettbam & Robson, 1970) at birth.

In view of the above, we investigated rate, force and cyclic adenosine 3',5'-monophosphate (cyclic AMP) responses to (−)-adrenaline in neonatal rat hearts, atria and ventricular strips in the absence and presence of drugs influencing adrenaline distribution.

Methods

General

Experiments were on isolated cardiac tissue from Wistar rats; tissues were bathed in a Krebs-Henseleit solution containing (mM): Na^+ 143, K^+ 5.9, HCO_3^- 25, $H_2PO_4^+$ 1.2, Ca^{2+} 2.5, Mg^{2+} 1.3, Cl^- 128, SO_4^{2} 1.3, disodium edetate (EDTA) 0.1 and glucose 11, bubbled with 5% CO_2 in O_2 at 31°C.

A special breeding colony was used to give a single animal from each litter as a source of tissue for a particular age. Randomly chosen animals were sometimes used, but experiments showed between-animal variance to be greater than that between litters. Rat ages were accurate to within 12 h.

Hearts from decapitated animals were used for different preparations. These were:

(1) Whole hearts perfused at an aortic root pressure of 75 mmHg (Langendorff) with force recorded by isometric strain gauge (Statham/Grass) attached to the apex. Hearts could be paced via atrial platinum electrodes stimulated supramaximally at 1.5 to 5 V, 1 to 2 ms and 180 beats/min.

(2) Atria (right and left) were removed from flushed and cleaned hearts. The ventricular borders of atria were connected by cotton thread to a force transducer and the other border was hooked onto punctate platinum electrodes suitable for electrical stimulation.

(3) Ventricular strips were cut as tissue triangles (0.5 to 1.0 cm base and 1.0 cm height) from right ventricles and hooked on twin platinum electrodes for supramaximal stimulation at 3 to 8 V, 1 to 4 ms pulse width and a frequency of 180 beats/min.

Resting tensions

Resting tension-developed tension curves were obtained for each preparation and adrenaline responses recorded at a resting tension which was 50% of that required for maximum developed tension.

Responsiveness to adrenaline was determined by equilibrium cumulative dose-response techniques and propranolol sensitivity as pA_2 values computed from log(dose-ratio − 1)/concentration plots (after Schild). Propranolol blockade reached a maximum within 10 min (half-onset times of 2 to 3 min in all tissues) and so propranolol additions were made 10 min before re-obtaining adrenaline dose-response curves and measurement of dose-ratios.

In some experiments, attempts were made to nullify possible perturbing effects of $uptake_1$ and $uptake_2$, α-adrenoceptors, etc. by obtaining dose-response data in the presence of cocaine (10^{-4} M) or the following combination of drugs (as used by Patil, Patel & Krell, 1971): cocaine 10^{-5} M, 17-β-oestradiol 5×10^{-5} M, phentolamine 10^{-5} M and thujaplicin 10^{-4} M (all as bases).

Data analysis

Mean dose-response curves were obtained by interpolating concentrations needed to produce various percentile responses from individual dose-response curves, for this method gives the best estimate of dose-response curves and ED_{50} values (Ariens, 1964). Curves were only obtained after responses to a test dose became constant. The mean curve for each tissue was computed from data obtained on at least five different preparations.

Propranolol sensitivity was determined with 3 to 4 concentrations in 2 to 6 different preparations with pA_2 values extrapolated from lines of best fit assuming a unity slope.

Cyclic AMP determinations

All cyclic AMP assays were with Amersham-Searle kits (TRK 432). Whole hearts were freeze-clamped on the perfusion apparatus by means of a modified Wollenberger clamp (stainless steel ball and socket). Frozen tissue was weighed before being powdered between the ball and socket. Atria and ventricles, prepared as indicated previously, were incubated within a stainless steel mesh holder. Adrenaline was added for 40 s, after 30 min incubation in control Krebs solution, before the tissue was rapidly frozen in liquid nitrogen, weighed and powdered. Control experiments showed that cyclic AMP levels reached a maximum with 40 s incubation in adrenaline.

Powdered tissue was homogenized in 5% cold trichloracetic acid and treated as described previously (Collins & Sutter, 1975) for cyclic AMP assay.

Materials

(−)-Adrenaline hydrochloride was prepared from stock before use; stock solutions (at 4°C) consisted of 10^{-2} M in 5 mM ascorbic acid and 0.1 mM EDTA. Other drugs were (±)-propranolol (Ayerst), β-thujaplicin (Koch-Light), cocaine (BDH), 17-β-oestradiol (Calbiochem) and phentolamine (Ciba).

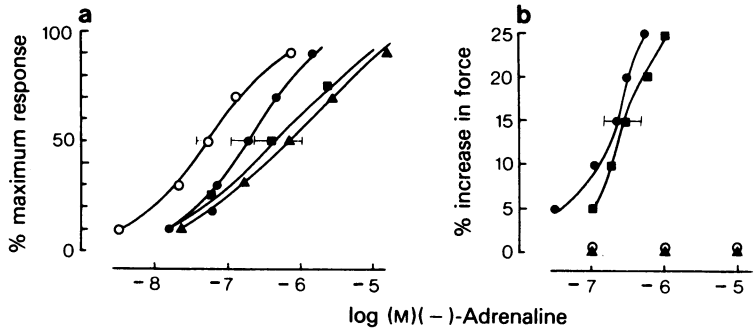

Figure 1 Dose-response curves for (−)-adrenaline in perfused neonatal rat hearts of 2 to 20 days post-natal age. (a) Rate in unpaced hearts; (b) force in paced hearts. Neonatal hearts were perfused with Krebs solution by the Langendorff technique at 31°C or 37°C for 2 day-olds. Adrenaline was added cumulatively and doses producing different percentile responses found by interpolation from individual dose-response curves (Ariens, 1964). Each curve is the mean from 5 to 6 hearts; representative s.e. means are shown for ED_{50} values. In (a) hearts beat spontaneously and responses are expressed as percentage of maximum response; in (b) stimulation was at 180 beats/min and responses are expressed as percentage increase of control. In (a): (■) 2 day-old at 37°C; (▲) 2 day-old; (○) 5 day-old; (●) 20 day-old. In (b): (▲) 2 day-old; (○) 5 day-old; (■) 10 day-old and (●) 20 day-old.

Results

In initial experiments on whole hearts, inotropic responses changed with age, while chronotropic responses did not. Chronotropic dose-response curves were similar for different ages and temperatures (31° and 37°C) as is shown in Figure 1a, where ED_{50} values vary from 5×10^{-8} M to 6×10^{-7} M in a non age-dependent manner. Inotropic responses were age-dependent (Figure 1b) in hearts, paced to beat at 180 beats/min, for young (2 and 5 day-old) hearts showed no inotropic responses, whereas 14 and 20 day-old hearts gave noticeable responses. In view of these initial findings, we then examined responses to adrenaline in the different heart chambers of 1 to 20 day-old rats.

Adrenaline responsiveness was expressed in conventional terms of affinity (ED_{50}) and intrinsic activity (maximum), with allowance for control (absence of adrenaline) performance of tissue. To make this allowance, we noted resting beating rates, and obtained full resting tension-developed tension curves; such curves did not change appreciably from tissue to tissue. Typical right atria values were 0.2 g resting tension for 50% of maximum developed tension and 0.8 g for maximum in 1 day tissue against 0.4 g and 1.0 g in 20 day-old. Similar observations gave Table 1 from which it is apparent that weight-corrected control force, did not change markedly with age, although a tendency for greater force development with age was seen in left atria and a rise in beating rate in right atria. In view of the above findings, adrenaline responses were expressed as percentage change from resting values.

With the above normalisation, affinity and intrinsic activity were thus expressed in terms of ED_{50} and maxima (Figures 2 and 3). ED_{50} concentrations did not fall with age in any tissue, even in ventricles where maxima values (Figure 3) were very small in 1 to 5 day-old tissue. The ED_{50} for right atrial force increased (decreased affinity) with age from 7×10^{-8} M at day 1 to 3×10^{-7} M at day 20, while that for rate did not change. The right ventricular force ED_{50} rose from 1×10^{-7} M to 6×10^{-7} M over days 1 to 20, whereas that for left atrial force fell from 4.5×10^{-7} M to 1×10^{-7} M over the same period.

Uptake systems and α-adrenoceptors possibly distort affinity estimates, and so responses were also

Table 1 Resting (control) values in cardiac tissues from rats of different ages

Tissue age (days)	*Force (mg/mg wet wt.)* *RVF*	*RAF*	*LAF*	*Rate (beats/min)* *RARa*
2	20 ± 6	14 ± 3	13 ± 2	115 ± 20
5	19 ± 3	13 ± 4	16 ± 4	115 ± 20
10	16 ± 4	16 ± 4	20 ± 5	212 ± 8

R, L, A, V, F and Ra refer to right, left, atria, ventricular strip, force and rate respectively. All values are mean ± s.e. mean from 5 to 7 preparations and were obtained after at least 30 min equilibration in Krebs solution at 31°C. Left atria were stimulated at 180 beats/min. Force values (corrected for sample weight) were obtained at a diastolic tension 50% of that required to produce maximum developed tension.

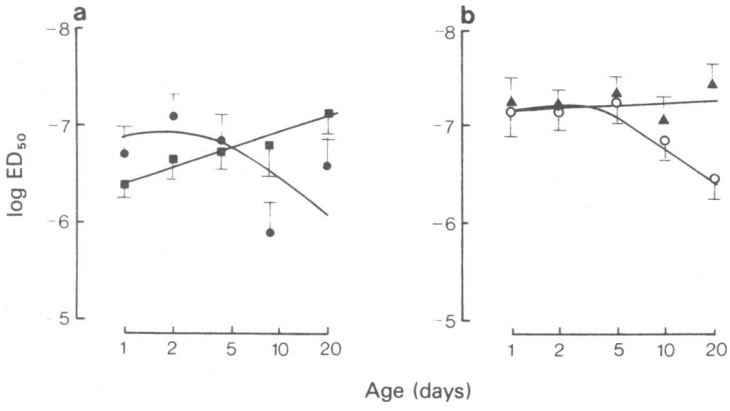

Figure 2 The effect of age on ED_{50} concentrations for different chambers of neonatal rat hearts. (a) Left atrial and right ventricular strip force. (b) Right atrial force and rate. With each tissue, ED_{50} values were obtained as in Figure 1 and mean ED_{50}s (as log M concentrations) are plotted against tissue age in days. Mean curves for tissues of different ages, but the same type, were always found to be parallel. Note logarithmic scale for age. In (a): (●) right ventricular strip force and (■) left atrial force. In (b): (▲) right atrial rate and (○) right atrial force. Vertical lines show s.e. mean.

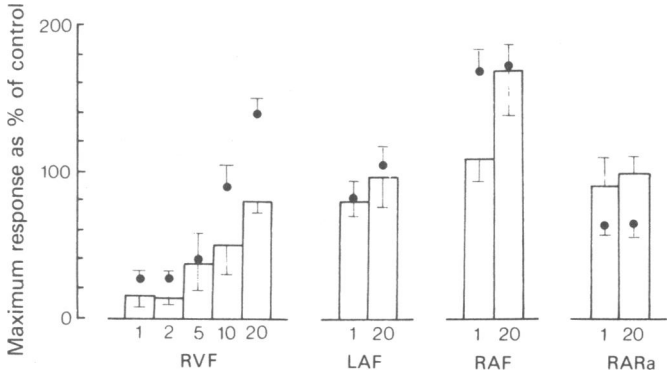

Figure 3 Maximum responses to adrenaline in different chambers from neonatal rat hearts in the absence and presence of cocaine 10^{-5} M. Each column indicates the mean (vertical lines show s.e. mean) of maximum responses (as percentage increase of control) obtained with adrenaline in 5 to 7 preparations. Means (●) with s.e. mean obtained in 10^{-5} M cocaine. R, L, A, V, F and Ra refer to right, left, atrial, ventricular strip, force and rate, respectively, while age of tissue is given beneath each column.

obtained in tissues treated with cocaine alone, or with cocaine plus the other drugs indicated in Methods. Estimates of ED_{50} in normal Krebs solution were not statistically different from those obtained in cocaine or cocaine plus the other blocking drugs (Table 2).

While examination of ED_{50} values did not show adrenaline affinity to change with age, considerable changes with age were seen in ventricular maximal responses. Figure 3 shows that maximal responses with, or without, cocaine increased only minimally with age in atria; the apparent change in maxima (from 110% of control to 170%) for right atrial force response at day 1 (Figure 3c) was probably a statistical artefact. In data not shown, the mean of right atrial maximal force for days 1, 2, 5, 10 and 20 was 140% in control tissue and 120% in cocaine. In ventricular strips however (Figure 3a), a marked increase in maximum responses occurred with age. Maximum responses in 1 and 2 day-old ventricles were 18 and 17% of control, respectively, and increased to 80% by

20 days *post partum*. In ventricles, unlike other tissues, cocaine consistently potentiated maximum responses to adrenaline. Ventricles therefore show a marked maturation of maximum response to adrenaline with age.

Propranolol sensitivity

The β-adrenoceptor in neonatal heart tissue was also investigated in terms of sensitivity to propranolol blockade of adrenaline responses. Initial experiments showed propranolol blockade to develop in a saturating fashion with time, such that the half-time to maximum blockade was 1 to 2 min and maximum blockade occurred within 10 min. For the data shown, linear regression analysis was used for pA_2 values assuming unity slopes. Not all tissues were examined but values showed a small but consistent increase with age (Table 3). Left atrial force pA_2 rose from 7.59 at 2 days to 8.01 at 20 days; a 2.6 fold increase. Increases for right atrial force and rate were 2.9 and 1.3 fold respectively.

Propranolol sensitivity in 2-day-old ventricle strips was not measured owing to the limited adrenaline force response; with a maximum increase of tension of 18%, it was difficult to obtain estimates of dose-ratios.

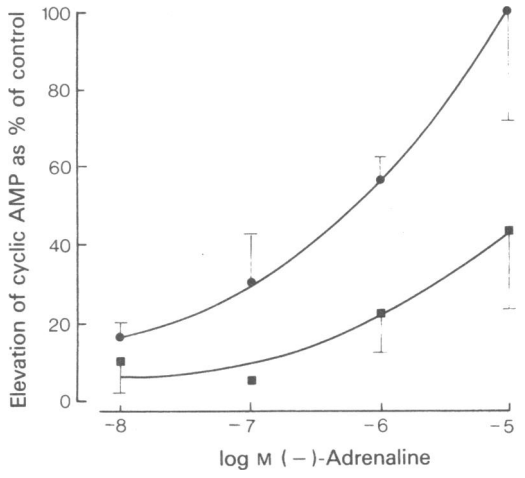

Figure 4 Dose-cyclic AMP response curves to adrenaline in 2 and 20 day-old hearts. Points are means of elevation (as percentage increase of control) induced in 2 (●) and 20 day (■) hearts by the concentration of (−)-adrenaline shown on abscissa scale. Each mean is from 5 to 7 hearts; vertical lines show s.e. mean. Resting cyclic AMP levels are given in the text as are methods.

Cyclic AMP responses

Another index of β-adrenoceptor activation is cyclic AMP production with various concentrations of adrenaline. Dose-response curves for cyclic AMP production in whole hearts perfused with adrenaline are shown in Figure 4 where cyclic AMP production was not maximum even at 10^{-5} M adrenaline and slopes were low. This contrasts with rate and force dose-response curves where maxima occurred below 10^{-5} M and where slopes were such that 50 to 60% of total response occurred in a decade of concentration.

Figure 4 also shows that 2 day-old hearts produced more cyclic AMP, at each adrenaline concentration, than did 20 day-old hearts, despite higher control resting levels of cyclic AMP in the younger tissue (0.82 ± 0.08 pmol/mg wet tissue at 2 day versus 0.51 ± 0.07 at 20 day). It was assumed that cyclic

Table 2 ED_{50} concentrations for adrenaline in control and treated cardiac tissue from 1 and 20 day-old rats

Treatment	Age (days)	ED_{50} values (−log M ± s.e. mean) RARa	RAF	LAF	RVF
Control	1	7.2 ± 0.3	7.2 ± 0.2	6.4 ± 0.4	6.7 ± 0.3
Control	20	7.0 ± 0.2	6.5 ± 0.4	7.2 ± 0.3	6.6 ± 0.3
Cocaine	1	6.9 ± 0.2	7.1 ± 0.2	6.9 ± 0.3	6.9 ± 0.2
Cocaine	20	7.4 ± 0.2	7.1 ± 0.1	7.1 ± 0.1	6.6 ± 0.2
Combination	1				
Combinations	20	7.5 ± 0.2	7.5 ± 0.3	7.5 ± 0.3	

R, L, A, V, F and Ra refer to right, left, atria, ventricular strip, force and rate, respectively. All values are mean ± s.e. mean. Responses were obtained in control Krebs solution at 31°C and in Krebs containing cocaine 10^{-5} M or a combination of cocaine, phentolamine, β-thujaplicin and 17-β-oestradiol (see Methods). Dose-response curves were obtained before and after 20 min exposure to the drugs and ED_{50} concentrations obtained from normalised curves. The number of preparations (n) = 5–7.

AMP levels in whole hearts reflected the levels in ventricles because of their greater contribution to the mass of the heart. However, control cyclic AMP levels in bathed atria were higher than those in ventricles (0.66 for 2 day-old atria versus 0.47 for right and 0.45 for left ventricle strips). Atria were more responsive to adrenaline stimulation, with 10^{-5} M adrenaline causing a 450% increase in cyclic AMP levels in 2-day-old atria and a 150% increase in 20 day-old atria. The corresponding right ventricle figures were 50% for 2 day-olds and 60% for 20 day-olds, which were approximately those for left ventricle strips (2 day-old only).

If cyclic AMP is simply the second messenger for cardiac β_1-adrenoceptor-mediated responses, cyclic AMP production in adrenaline-stimulated tissue should be consistently related to response (inotropism). To test this, we plotted inotropic response against the log of the appropriate cyclic AMP production for various adrenaline concentrations (Figure 5). In Figure 5a the relationship between cyclic AMP and inotropism is shown for 2 and 20 day-old atria and in both cases a near linear relationship was found with the older tissue producing more force for cyclic AMP elevations. Corresponding data for 2 and 20 day-old whole hearts and ventricles is shown in Figure 5b. In 20 day-old hearts and ventricles the relationship between force and log cyclic AMP was linear, but the slope was less than for atria (10% versus 20% increase in force for a doubling of cyclic AMP content), although displaced to the left. For younger 2 day-old ventricle tissue (whole hearts, right and left ventricles) no such relationship was found for there were immeasurable force increases with marked elevations of cyclic AMP.

Discussion

Pappano (1977) has reviewed the ontogenesis of cardiac autonomic transmission; such knowledge is important for understanding maturation processes and for giving insight into receptor mechanisms (Mirkin, 1972). In the rat, sympathetic innervation appears before birth (Adolph, 1971; Wildenthal & Wakeland, 1973; Robkin, Shepard & Dyer, 1976) but sympathetic tone does not reach adult levels for 20 days *post partum* (Wekstein, 1965). Standen (1978) has investigated neonatal rat atria for responsiveness to noradrenaline, isoprenaline, tyramine and electrical stimulation. Responses to electrical stimulation and tyramine were consistent with developing adrenergic innervation for the first four weeks of growth. In the first two weeks *post-partum*, left atria inotropic responses were supersensitive to noradrenaline, indicating a relative lack of the uptake$_1$ process while there was no evidence for β-receptor maturation; ventricles were not investigated. However, *in vivo*, Seidler & Slotkin (1979) found rate responses to isoprenaline to be lower in young rats (4 to 16 day-old) than in adults. It seems that β-adrenoceptors for rate appear early in foetal life for adrenaline-sensitive adenylate cyclase has been reported for rat foetuses 60 to 20 days post conception (Clark, Beatty & Allan, 1973). Our findings for atria are thus in general accord with previously reported data.

Apart from reports on effectiveness of propranolol, little is known about sensitivity changes with age. The small change in propranolol sensitivity we found may reflect some maturation of a propranolol binding site not exactly identical to the adrenaline recognition site, a situation reminiscent of cultured heart cells where propranolol sensitivity develops slowly compared with adrenaline rate-response development (Walker, 1977).

Reduced adrenaline responses in ventricles and whole heart were not expected from atrial findings and the knowledge that cells cultured from ventricles are sensitive to adrenaline (Walker, 1977). The discrepancy between no force responses in the whole heart and the small response in right ventricle strips was probably due to the methods used, for greater precision was possible when recording from ventricular strips. The interesting finding was normal ED_{50} values in young ventricles but very much reduced maxima, indicating unchanged affinity but reduced intrinsic activity (receptor-response transduction or total number of activable receptors).

While cyclic AMP production is held to be the transduction link between β-adrenoceptor activation

Table 3 Propranolol sensitivity (pA_2 and Schild plot slopes) in 2 and 20 day-old neonatal rat heart tissue

Tissue	*Age (days)*	n	*Slope*	pA_2
RAF	20	7	0.7 ± 0.2	8.46 ± 0.13
RARa	20	13	1.0 ± 0.1	8.12 ± 0.13
LAF	20	5	1.0 ± 0.1	8.01 ± 0.06
RVF	20	12	0.9 ± 0.1	8.53 ± 0.07
RAF	2	14	0.9 ± 0.1	8.00 ± 0.02
RARa	2	12	1.1 ± 0.1	8.00 ± 0.02
LAF	2	11	1.0 ± 0.2	7.59 ± 0.04

Abbreviations as before. Full cumulative adrenaline dose-response curves were obtained in 2 to 5 atria with 10^{-8} to 10^{-6} M (±)-propranolol. Values are mean ± s.e. mean. (Dose ratio − 1) against log propranolol concentration plots give slopes not statistically significantly different from unity. All 20 day-old tissue pA_2 values were statistically ($P < 0.01$) different from the values in 2 day-old tissue.

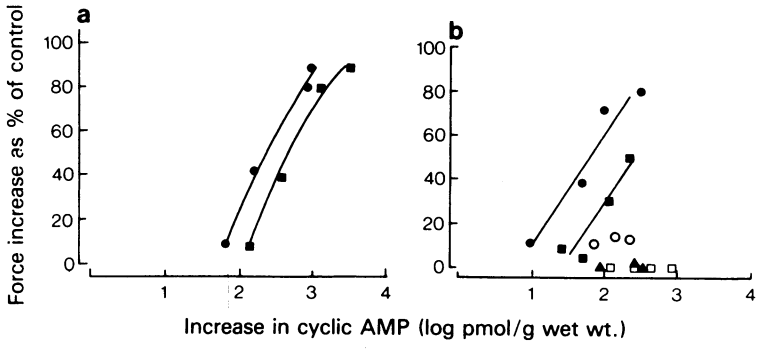

Figure 5 Relationship between cyclic AMP elevation and maximal force increase due to different adrenaline concentrations. (a) In 2 and 20 day-old atria. (b) In 2 and 20 day-old whole hearts, right and left ventricular strips. Atria, whole hearts and ventricular strips were incubated in adrenaline as indicated in text for estimation of cyclic AMP elevation. Maximum positive inotropic responses (expressed as percentage increase of control) were found as indicated for Figures 1 and 3. Maximum response as percentage of control shown on the ordinate scale while elevation of cyclic AMP content is given as log pmol/g wet tissue wt. on the abscissa scale. Each point represents the mean of values obtained from 4 to 7 preparations. In (a): (■) 2 day-old atria and (●) 20 day-old atria. In (b): (■) 20 day-old whole hearts; (▲) 2 day-old whole hearts; (●) 20 day-old right ventricular strips, (○) 2 day-old right ventricular strips and (□) 2 day-old left ventricular strips. The lines shown are assumed lines of best fit.

and response, the system has still to be fully elucidated. Adenylate cyclase activity is present early in foetal life (Clark *et al.*, 1973) and fluoride-stimulated enzyme is found in many rat foetal tissues (Schmidt *et al.*, 1970; Hommes & Baere, 1971) and so it is not surprising that we found all neonatal cardiac tissue to produce cyclic AMP on exposure to adrenaline. In response to adrenaline, 2 day-old tissue produced more cyclic AMP than older tissue (20 day-old) although concentrations were also slightly higher in the younger tissue. Thus, the β-adrenoceptor, defined by cyclic AMP response, was developed in all heart tissue, whereas the force response in early (1 to 5 day-old) ventricles was uncommon.

The exact relationship between cyclic AMP and response has still to be elucidated but the linear relationships in Figure 5 varied only slightly between tissues, except for 1 to 5 day-old ventricles. A relative inefficiency of the cyclic AMP link in embryonic chick heart has already been described (Polson, Goldberg & Shideman, 1977).

Our data are open to a number of non-mutually exclusive explanations. In the simplest model of one β_1-adrenoceptor irrevocably linked through cyclic AMP production to response, our results indicate a lack of cyclic AMP receptors in early (0 to 5 day-old) ventricles. Alternative explanations require more than one pool of cyclic AMP and/or more than one β-adrenoceptor.

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