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Cardiac β-adrenoceptor binding characteristics with age following adrenal demedullation

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1 The role of adrenal medullary catecholamines in the regulation of cardiac function becomes more important when adrenergic neural influences in the heart are decreased. Since adrenergic nervous input to the heart decreases with age, it would be expected that catecholamine influence on cardiac neuroeffector junction would increase.

2 Fischer-344 rats of 6-, 12- and 24-months were adrenal demedullated or sham-operated and the animals were killed at the end of two weeks. β-Adrenoceptors were studied in the membrane preparations from the ventricles of rat hearts. [^{125}I]-iodopindolol was used as the radioligand. The density of β-receptors (B_{max}), dissociation constant (K_D) and the ratio of cardiac β-adrenoceptor subtypes were studied. The relative percentages of β-receptor subtypes were determined by use of ICI 89,406 (β_1-selective antagonist) and ICI 118,551 (β_2-selective antagonist).

3 In 24-month-old animals which were adrenal demedullated, hydrocortisone replacement was employed for one week; the animals were killed one week later.

4 The data indicate that there was a diminution of the B_{max} following adrenal demedullation at all ages but that the ratios of β_1:β_2-adrenoceptors remain the same as in the controls (67:33). The effect of adrenal medullary catecholamines on cardiac β-receptor binding characteristics did not seem to be influenced by age.

Introduction

The importance of the adrenal medulla in maintaining homeostasis has been amply demonstrated (e.g., Axelrod & Reisine, 1984). Its role in cardiac regulation becomes more important when adrenergic neural influences in the heart are decreased (Mueller *et al.*, 1969; deChamplain & Nadeau, 1971; Gauthier *et al.*, 1972). It has been shown that when adrenal catecholamines are elevated and gain access to the heart, subsensitivity of cardiac β-receptors results (Torda *et al.*, 1981). Since adrenergic nervous input to the heart decreases with age (Daly *et al.*, 1988), it would be expected that the influence of adrenal medullary catecholamines on adrenergic neurochemical transmission at the cardiac neuroeffector junction would increase and cause downregulation of β-receptors.

It has been shown that adrenomedullary activity, as measured by tyrosine hydroxylase and dopamine β-hydroxylase activity, increases with age (Kvetnansky *et al.*, 1978; Banerji *et al.*, 1984), suggesting an increased availability of catecholamines to act on the heart. Furthermore, it has been demonstrated that although secretion rates of adrenaline and noradrenaline from the adrenal gland under resting conditions varied widely, they increased significantly in old rats (Ito *et al.*, 1986). In addition, Ito *et al.* (1986) found that sympathetic efferent nerves innervating the adrenal medulla showed increased neural activity with age in resting conditions. This increase in neural activity paralleled the increased catecholamine secretion from the medulla. Basal and stimulated levels of adrenaline in the circulation of man do not appear to change with age (Barnes *et al.*, 1982; Pfeifer *et al.*, 1983). However, Avakian *et al.* (1984) have shown that arterial plasma adrenaline concentration in older rats is significantly elevated both in basal conditions and in response to cold stress, suggesting enhanced adrenal medullary activity with advanced age.

To explore whether the effect of adrenal medullary catecholamines on postjunctional β-adrenoceptors is influenced by age, binding characteristics of these receptors were determined after the adrenal medulla had been removed surgically from young and old Fischer-344 male rats. β-Adrenoceptor density, affinity and ratio of β_1- to β_2-adrenoceptors were studied in the ventricles of rat hearts by use of radioligand methodology.

Methods

Animals

Fischer-344 (F-344) male rats at 3 ages (6-, 12- and 24-months old) were used in the experiments. These rats were maintained under barrier conditions at Harlan Laboratories, Inc. (Indianopolis, Indiana, U.S.A.). At our institution, rats were housed under barrier conditions in standard filtered cages, two per cage, in a temperature regulated environment (21 ± 1°C), on a 12 h light/dark cycle. The animals were fed *ad libitum* on a pasteurized rodent diet (19–26% protein, 4–6% fat) and on autoclaved water adjusted to pH 3. Maximum life span for F-344 rats maintained under these conditions is approximately 35 months, with a mortality rate of about 50% at 29 months (Coleman *et al.*, 1977).

Adrenal demedullation

Bilateral adrenal demedullation were carried out with sodium pentobarbitone (50 mg kg^{-1}, i.p.) as the anaesthetic agent.

The surgery was performed by making an incision bilaterally below the last rib on the dorsum near the centre of the back of the animal. After locating the adrenal gland, an incision was made through the cortex to permit expression of the medulla when the gland is gently squeezed. For the sham-operated animals the same procedure was followed up to the point of incision of the cortex.

After adrenal demedullation, to minimize postsurgical difficulties related to infection and dehydration, rats were kept in separate cages and were administered 10 ml of 5% dextrose in 0.9% saline, subcutaneously, daily for one week. Penicillin G, 50,000 u per rat per day, i.m., was administered for one week. With this procedure the survival rate was extremely good (95%), except in 24-month-old animals (five of seven died within 72 h). It was hypothesized that in older animals the

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adrenal cortex required several days to resume secretory activity after surgery. During this interval the older animals succumbed. To improve the survival rate in older animals, hydrocortisone (A-hydrocort) was administered intramuscularly $12\,mg\,kg^{-1}$ for 3 days, $6\,mg\,kg^{-1}$ for 2 days; and $2\,mg\,kg^{-1}$ for 2 days. The animals were killed one week later by decapitation. At this time blood samples were collected from the site of decapitation to determine corticosterone levels to ascertain whether the adrenal cortex became operational after surgery. Plasma samples were sent to Endocrine Sciences Laboratories for radioimmunoassay analysis. The survival rate was 75% in 24-month-old animals treated with hydrocortisone.

To determine whether hydrocortisone replacement therapy affects the binding characteristics of β-receptors, hydrocortisone was administered to sham-operated 24-month-old animals, in the same schedule as in the 24-month-old demedullated animals, and receptor binding characteristics measured at the same relative postoperative time.

Preparation of tissue

After decapitation the hearts were removed quickly and chilled in ice-cold buffer (Tris-Isosaline pH 7.5). The ventricles were dissected free of the atria, fat and major vessels. Crude membrane preparations were prepared from the ventricles as described by Tumer *et al.* (1987). The protein content of membrane preparations was approximately 30–60 μg per assay. Membrane preparations were stored at −70°C.

Total β-adrenoceptor binding assay

Assay of β-adrenoceptor binding was carried out on washed crude membrane preparations of heart tissue employing a modification of the radioligand binding method described by Hedberg *et al.* (1985). [^{125}I]-iodopindolol (IPIN) (2200 $Ci\,mmol^{-1}$) was used at 9 concentrations to determine the affinity and the total number of β-adrenoceptors. The total assay volume was 250 μl, which contained IPIN at concentrations ranging from 20 pM to 250 pM, 100 μM GTP, 100 μM (−)-isoprenaline, 100 μl tissue preparation and 50 μl of BSA/ascorbic acid solution (0.0002% bovine serum albumin/0.284 mM ascorbic acid). The assay volume was placed in disposable polypropylene tubes and set in an ice waterbath. Aliquots of the solution were incubated at 37°C for 25 min. The reaction was stopped by addition of 8 ml of 10 mM cold Tris-isosaline and rapidly filtered through glass fibre filters (Schleicher and Schuell No. 30) on a Brandel Cell Harvester followed by two washes using 8 ml of the same buffer. The filter bound activity was determined in a Beckman Gamma Counter at a counting efficiency of 75%. Specific binding was defined as the difference between binding of IPIN in the absence and presence of 100 μM (−)-isoprenaline. The density of binding sites (B_{max}) and their affinity (K_D) for IPIN were determined by linear regression analysis of saturation isotherm data, linearly transformed according to the method of Scatchard (1949). A Scatchard analysis was performed with a computer programme which was developed at the Department of Pharmacology, University of Pennsylvania, U.S.A.

Protein content was determined according to the Bradford Coumassie Brilliant Blue Method (1976) with reagents prepared by Biorad (Rockville Centre, N.Y., U.S.A.).

Assay of β-adrenoceptor subtypes

To quantitate β-adrenoceptor subtypes (β_1- and β_2-), the membrane preparation (200 μl) from the ventricles was incubated for 25 min at 37°C in disposable polypropylene tubes (Sarstedt, Inc.: Princeton, NJ, U.S.A.) in an assay mixture (0.5 ml) containing 100 μM isoprenaline, 100 μM GTP and 110 pM IPIN (2.2 $\mu Ci\,\mu mol^{-1}$). To determine the relative fractions of β_1- and β_2-adrenoceptor, selective compounds ICI 89,406 (β_1-selective antagonist) and ICI 118,551 (β_2-selective antagonist) were used in 23 concentrations ranging from 25 pM–40 μM. After the incubation period, the reaction was stopped with 10 ml of 10 mM Tris-isosaline. The samples were filtered rapidly under reduced pressure, using a Brandel cell harvester and glass fibre filter (Schliecher and Schuell No. 30). The filters were washed with 10 ml of wash buffer. The washed filter bound activity was measured in a Beckman gamma counter with 75% efficiency.

Inhibition of specific binding of IPIN by ICI 89,406 and ICI 118,551 was analysed according to Hofstee (1952) by using the specific analytical procedure, FITSITES which is a computer-aided, nonlinear, least-square regression curve-fitting techniques (McGonigle & Perry, 1985). For this purpose the PROPHET system, BBN Lab. Inc. Cambridge, MA, was used at the Department of Pharmacology, University of Pennsylvania, U.S.A.

The method of Cheng & Prusoff (1973) was used to calculate K_D values for the inhibition of specific binding of IPIN by ICI 89,406 and ICI 118,551.

Statistical analysis

Differences in means were compared statistically by two factor analysis of variance (ANOVA), i.e. age (6, 12 and 24 months) and group (adrenal demedullated vs sham-operated). A difference was considered to be statistically significant when the P value was less than 0.05. When ANOVA indicated significant differences, the Tukey post-hoc procedure and simple effects t tests were applied to determine which specific age groups differed from one another.

For comparing the differences in corticosterone levels a 3 (age) by 2 (sham vs demedullations) ANOVA was performed. Simple effect t tests were used to determine differences between the age groups at each of the conditions (sham-operated vs adrenal demedullation).

Radioligands and reagents

The following agents were employed: [^{125}I]-iodopindolol (New England Nuclear), ICI 89,406 ICI 89,406 (1-(2-(cynophenoxy)-3-β-(3-phenylureido)ethylamino-2-propanol) and ICI 118,551 ICI 118,551 (erythro-(±)-1-(7-methylindan)-4-yloxy)-3-isopropylaminobutan-2-ol) (ICI America Wilmington Delaware), A-hydrocort (Abbott). The chemicals used in the

Results

Assay of total number of β-adrenoceptors was carried out on crude membrane preparations of heart tissue following sham-operation or adrenal demedullation. Figure 1 depicts representative Scatchard plots obtained from 6-month-old heart assays were purchased from Sigma.

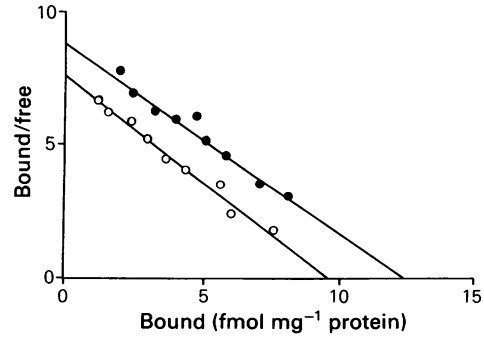

Figure 1 [^{125}I]-iodopindolol (IPIN) binding to ventricular membrane preparation obtained from the heart of 6-month-old male F-344 rats (representative plot). Control (●) and adrenal demedullated (○). Scatchard analysis of IPIN binding: receptor densities (B_{max}) are 12.5 and 9.7 fmol mg^{-1} protein; the dissociation constants (K_D) equal; 0.234 and 0.186 nM, and the correlation coefficient are $r = -0.957$ and -0.965 for sham-operated and adrenal demedullated animals, respectively.

Table 1 Effect of adrenal demedullation on binding characteristics of cardiac β-adrenoceptors as a function of age

	6 months	*12 months*	*24 months*
Control (sham-operated)			
B_{max} (fmol mg^{-1})	13.0 ± 2.0	12.6 ± 1.8	10.9 ± 1.3 11.0 ± 2.9†§
K_D (nM)	0.219 ± 0.07	0.189 ± 0.06	0.237 ± 0.04 0.239 ± 0.07†§
Adrenal demedullated			
B_{max} (fmol mg^{-1})	10.7 ± 0.8*	10.7 ± 1.2*	9.2 ± 1.5*§
K_D (nM)	0.209 ± 0.05	0.164 ± 0.05	0.176 ± 0.03§

Values represent means ± s.d. (n = 8).
* Significantly different from the controls (ANOVA, $P < 0.05$).
§ These values were determined one week after hydrocortisone replacement was terminated.
† Sham-operated group (n = 4) treated with hydrocortisone (see Methods section).

preparations. Table 1 summarizes the effect of adrenal demedullation on cardiac β-adrenoceptor binding characteristics as a function of age. There was a significant diminution in receptor density (B_{max}) but not in dissociation constant (K_D) in preparations from 6-, 12-, and 24-month-old adrenal demedullated animals compared to controls (sham-operated). The B_{max} values were 13 ± 2.0; 12.6 ± 1.8 and 10.9 ± 1.3 fmol mg^{-1} protein for 6-, 12- and 24-month-old sham animals respectively, and 10.7 ± 0.8; 10.7 ± 1.2; 9.2 ± 1.5 fmol mg^{-1} protein for 6-, 12- and 24-month-old adrenal demedullated animals respectively. There was a statistically significant decline in B_{max} after adrenal demedullation at all ages ($P < 0.05$). However, in neither the sham-operated, nor the adrenal demedullated groups did the B_{max} change with age ($P > 0.05$) (Table 1).

Dissociation constants (K_D) were 0.219 ± 0.07; 0.189 ± 0.06; 0.237 ± 0.04 nM for 6-, 12- and 24-month-old sham-operated animals respectively and 0.209 ± 0.05; 0.164 ± 0.05; 0.176 ± 0.03 nM for 6-, 12- and 24-month-old adrenal demedullated animals respectively ($P > 0.05$). To determine if the results depended on the length of time after adrenal demedullation, receptor density and K_D were examined in four animals 3 and 4 weeks after adrenal demedullation; the results appeared to be similar to those observed 2 weeks after adrenal demedullation (data not shown).

The relative ratios of cardiac β_1- to β_2-adrenoceptors determined by use of ICI 89,406 (β_1-selective antagonist) and ICI 118,551 (β_2-selective antagonist) were approximately 67:33 in ventricular preparations from 6- and 24-month-old animals. There were no statistically significant differences in the ratios between the sham-operated and adrenal demedullated animals regardless of age ($P > 0.05$). Figure 2 (a and b) represents typical plots for inhibition of specific IPIN binding by ICI 89,406 and ICI 118,551, respectively. Displacement curves of antagonists competing for antagonist ligand are shallow.

Table 2 Corticosterone levels in plasma (μg dl^{-1})†

6 months:	Sham-operated	(n = 5):	80.6 ± 13.1
	Demedullated	(n = 6):	51.2 ± 11.9
12 months:	Sham-operated	(n = 4):	63.3 ± 23.8
	Demedullated	(n = 4):	42.8 ± 19.5
24 months:	Sham-operated	(n = 5):	49.4 ± 20.8
	Demedullated	(n = 2):	21.0§
	Demedullated	(n = 6):	45.5 ± 17.4*

Values represent mean ± s.d.
§ No hydrocortisone treatment after surgery.
* After hydrocortisone replacement.
† All samples were taken 2 weeks after sham operation or demedullation.

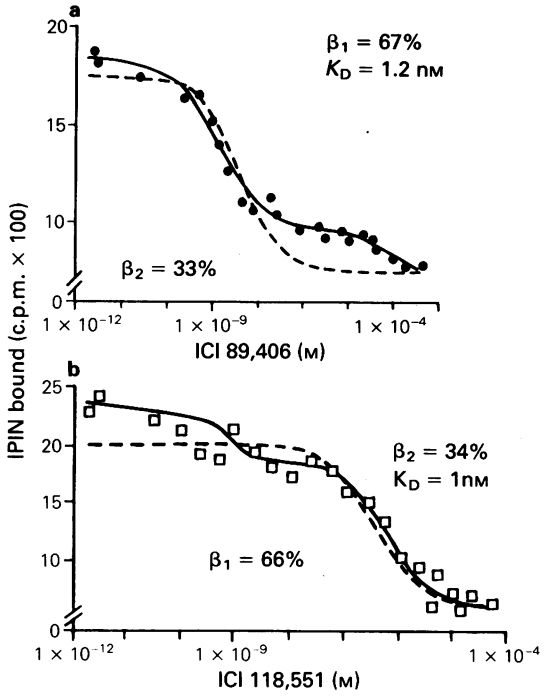

Figure 2 Representative plots for inhibition of binding of [^{125}I]-iodopindolol (IPIN) by ICI 89,406 (β_1-antagonist) (a) and for inhibition of IPIN binding by ICI 118,551 (β_2-antagonist) (b) in ventricles from the hearts of 6-month-old F-344 rats. Continuous line represents the proportions of β_1- and β_2-adrenoceptors derived from nonlinear regression analysis assuming two coexisting receptor sites with drug interaction. The dashed line represents the computer assisted best fit of the data for a single class of receptor. K_D values were calculated according to the method of Cheng & Prusoff (1973). IC_{50} = the concentration of ICI 89,406 or ICI 118,551 inhibiting the specific binding of IPIN by 50% i.e. 10^{-9} M for β_1-antagonist and 5×10^{-7} M for β_2-antagonist.

Plasma levels of corticosterone were decreased after demedullation in 6- and 12-month-old animals (Table 2). In 6-month-old shams, corticosterone levels were 80.6 ± 13.1 while in demedullated animals they were 51.2 ± 11.9 μg dl^{-1} ($P < 0.05$). In 12-month-old animals plasma corticosterone levels were 63.3 ± 2.38 and 42.8 ± 19.5 μg dl^{-1} for sham-operated and demedullated animals respectively ($P < 0.05$). In 24-month-old sham-operated animals, the corticosterone levels were significantly lower than those in 6-month-old animals. After hydrocorticosterone replacement therapy, the corticosterone levels in demedullated animals were similar ($P > 0.05$) to the sham-operated controls i.e. 49.4 ± 2.08 compared to 45.5 ± 17.4 μg dl^{-1}.

It appears that hydrocortisone replacement therapy did not influence receptor binding characteristics. Two animals (24 months old) survived without hydrocortisone replacement; the B_{max} (10.0 fmol mg^{-1}) and K_D (0.204 nM) values determined for these animals were similar to the group treated with hydrocortisone (Table 1). Furthermore, in the 24-month-old sham-operated group (n = 4) given hydrocortisone, the B_{max} and K_D were similar to those of the sham-operated group (24 months old) which had not been administered hydrocortisone (Table 1). It also appears that the decrease in corticosterone levels with age did not influence the binding characteristics of the β-receptors, since there was no significant changes in B_{max} or K_D with age (Table 1) in either the sham-operated or demedullated animals.

Discussion

In 6-, 12- and 24-month-old animals the removal of the adrenal medulla appears to result in a decrease in B_{max} (receptor density) of the ventricles. A reciprocal relationship exists between adrenergic activity in the heart and in the

adrenal medulla, that is, when adrenergic nervous activity in the heart decreases, adrenal medulla activity increases and *vice versa* (Gauthier *et al.*, 1972). The absence of adrenal medullary activity following removal of the adrenal medulla might result in an increase in adrenergic nervous activity in the heart; as a result a greater amount of the transmitter would be presented to the adrenoceptors in the heart and this could result in downregulation (reduced number of receptors).

The findings of the present study confirm previous data from this laboratory and others (Guarnieri *et al.*, 1980; Abrass *et al.*, 1982; Tumer *et al.*, 1987), that β-receptor density and dissociation constant do not change with age in the rat heart nor does the ratio of β-adrenoceptor subtypes i.e. β_1 to β_2 (Tumer *et al.*, 1989). Similar observations were made in the present study.

The present investigation indicates that when adrenal medullary secretion is removed the number of β-receptors in the heart decreases. This was unexpected as there is evidence that adrenal medullary secretions increase with age (Kvetnansky *et al.*, 1978; Banerji *et al.*, 1984), hypothetically resulting in a downregulation of β-receptors in the heart. On this basis demedullation would be expected to result in upregulation, which was not seen. The fact that adrenergic innervation in the heart was intact may have masked any alterations resulting from demedullation, i.e. when the adrenal medullary activity is reduced adrenergic activity in the heart increases. Another possibility to explain downregulation of receptors after demedullation is that sufficient time after removal of the adrenal medulla did not elapse to allow for upregulation to occur. The experiments were conducted 2–4 weeks after demedullation.

Basal circulatory levels of catecholamines in the rat with age have been variously described (Roberts & Tumer, 1987). In the Fischer male rat, Chieuh *et al.* (1980) found that basal levels increase with age. In the present study, it appears that such an increase in circulatory catecholamines did not influence β-adrenoceptor binding characteristics, since there was no significant difference in B_{max} or K_D regardless of age. In man, a plasma catecholamine increase with age has been frequently observed (for review, Roberts & Tumer, 1987). It is not known whether such increases in circulatory catecholamines in man effect the binding characteristics of β-adrenoceptors in the heart.

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