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DISTRIBUTION AND FUNCTION OF β-ADRENOCEPTORS IN DIFFERENT CHAMBERS OF THE CANINE HEART

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1 An improved binding assay involving (−)-[^{3}H]-dihydroalprenolol (DHA) and KCl-washed cardiac membranes was developed to study β-adrenoceptors in the canine heart quantitatively.

2 Receptor numbers varied from 3.8 to 7.1 pmol/g fresh tissue, showing a steady increase from left atrium → right atrium → right ventricle → interventricular septum → left ventricle. With one minor exception, the same pattern was found for adenylate cyclase activity and Na^+, K^+-activated ATPase activity.

3 The binding of DHA was inhibited in the expected manner by β-adrenoceptor agonists and antagonists, and was stereospecific, in confirmation of previous studies. Dissociation constants determined from Scatchard analyses included DHA: 2.5 nM; (−)adrenaline: 230 nM; (−)noradrenaline: 1167 nM. Kinetic analyses of the binding of DHA yielded a K_D of about 4 nM.

4 The distribution of β-receptors is closely related to that of blood flow and the arrival plus retention of a circulating catecholamine, but is markedly different from that of endogenous noradrenaline, and thus adrenergic nerve terminals. Most receptors thus appear not at synapses but diffusely localized where they can react with circulating adrenaline.

5 Evidence is discussed that β-receptors at synapses respond primarily to neural noradrenaline, less to circulating adrenaline, and hardly at all to circulating noradrenaline; responses mediate increased cardiac output during exercise. In contrast most cardiac β-receptors appear to respond only to adrenaline, and to be used, except at times of severe circulatory stress, during psychological stress.

Introduction

It is well known that the heart responds to environmental stress with an increased rate and force of contraction. While moment-to-moment changes in force appear largely dependent upon fibre length, major increases in rate, force and output normally depend upon adrenergic stimulation of the heart (Rushmer, 1976). Both noradrenaline, released from nerves within the heart, and adrenaline, released from the adrenal glands, are known to be involved, and both act on β-adrenoceptors. In this study we have used a quantitative ligand-binding assay to demonstrate that β-receptors are distributed in different chambers of the canine heart in proportion to blood flow and the arrival of circulating catecholamines rather than to adrenergic innervation. These results are discussed in relation to Rushmer's (biological) conclusion in 1976 that "... it is now generally recognized that circulating neurohormones are probably of little significance in normal cardiovascular control."

Methods

Mongrel dogs of either sex weighing 15 to 20 kg were anaesthetized with intravenous pentobarbitone and their hearts were removed and placed in ice-cold 0.9% w/v NaCl solution (saline). The atria, ventricles and interventricular septum were separated from each other, leaving the interatrial septum with the left atrium; excess fat and the valves were discarded. The tissues were homogenized with 9 volumes of 10 mM Tris-HCl buffer, pH 8, utilizing a Waring blender at top speed for 30 to 45 s for the ventricles and interventricular septum, and a Polytron blender equipped with a P10 ST generator operating at setting 6 out of 10, for 30 to 45 s for the atria. The whole homogenates were poured through fine nylon tea sieves to remove large connective tissue fragments.

β-Adrenoceptors were measured by assessment of the binding of DHA to membranes. Most previous studies of cardiac β-receptors have been made on microsomal membrane preparations (Alexander, Wil-

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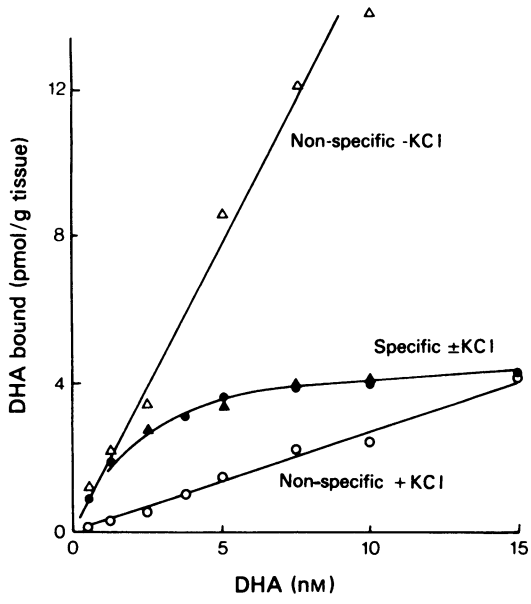

Figure 1 Specific (●) and non-specific (○) binding of dihydroalprenolol (DHA) to KCl-washed particles from the ventricles of one dog heart, and specific (▲) and non-specific (△) binding of DHA to membranes prepared in buffer alone from the ventricles of a second heart. Standard assay conditions were used except for the concentrations of DHA indicated.

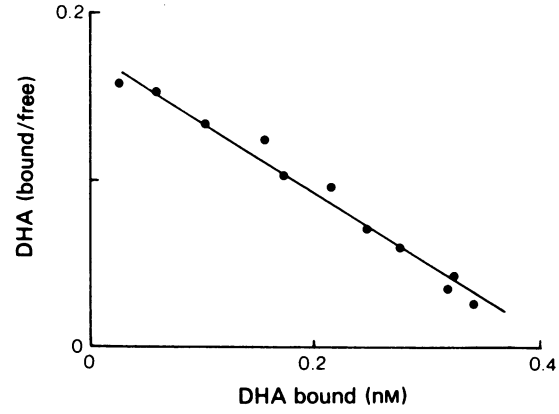

Figure 2 Scatchard analysis of the specific binding of dihydroalprenolol (DHA) to KCl-washed membranes; the data are taken from Figure 1.

liams & Lefkowitz, 1975; Krawietz, Poppert, Erdmann, Glassman, Struck & Konrad, 1976; Harden, Wolfe & Molinoff, 1976; Williams, Lefkowitz, Watanabe, Hathaway & Besch, 1977), but these membranes have, in our hands, only 5 to 20% of the total binding sites of the tissue. Homogenates and buffer-washed membranes collected by ultracentrifugation have all the sites, but are difficult to use for routine assays because of high non-specific ligand binding. To minimize non-specific binding (see Figure 1) each homogenate was diluted with an equal volume of ice-cold 1 M KCl and left on ice for 10 min to facilitate the dissolution of contractile and other proteins. Membranes were then collected for assay by centrifugation at 48,000 g_{max} for 10 min. (Further centrifugation yielded very small amounts of particulate material having only a few percent of the specific binding sites of the first sediment.) Pellets were gently resuspended with the Polytron blender in the original volume of 10 mM Tris buffer, resedimented, and finally resuspended as before for assays. Membranes from 2 to 8 mg of tissue were incubated in triplicate with 0.6 to 15 nM DHA in 50 mM sodium N-2-hydroxyethylpiperazine-N'-2-ethanesulphonate (HEPES) buffer, pH 8.0, containing 4 mM $MgCl_2$, with and without 10 μM (±)-alprenolol, in a final volume of 150 μl, for 20 min at 25°C. At the end of each incubation, 4 ml of ice-cold 25 mM HEPES buffer, pH 8.0, containing 4 mM $MgCl_2$, was added to each tube, and the fluid was filtered under reduced pressure through a Whatman GF/C glass fibre filter. Each tube and filter was then rinsed with two additional 4 ml aliquots of buffered $MgCl_2$, the total time between dilution of the incubation medium and the end of the last rinse being 8 to 12 s. Filters were placed in scintillation phials, dried in an oven at 40 to 60°C, covered with 5 ml of a toluene/Triton X-100-based scintillation fluid, and counted. Specific DHA binding is defined as the difference between total binding in the absence of alprenolol, and the nonspecific binding (to particles and filters) found in the presence of 10 μM alprenolol.

During binding assays involving additional drugs, the incubation media contained 0.1% ascorbic acid as an antoxidant, and 20 μM pargyline to inhibit monoamine oxidase. Neither addition was found to have any effect on the dissociation constant for DHA binding (K_D) or the maximum number of binding sites (B_{max}) determined by Scatchard analyses.

With the membrane preparations used in this study the specific binding of DHA was proportional to the concentration of membrane protein used between 0.05 and 0.7 mg per incubation tube and glass fibre filter. Larger amounts of particulate material caused a decrease in the rate of washing of filters, and were therefore avoided. Specific binding increased from pH 5.5 to pH 7.0, after which a plateau was reached and maintained through pH 9.5.

Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as the standard.

Adenylate cyclase activity was determined as follows. Samples of homogenates from 2 to 8 mg of tissue were incubated in triplicate with 1.6 mM α-^{32}P-

ATP, 5 mM $MgCl_2$, 30 mM KCl, 1 mM EGTA, 10 mM theophylline, 0.01% bovine serum albumin, 50 mM Tris-HCl buffer, pH 7.6, [^{3}H]-cyclic AMP as an internal standard, 3 mM phosphoenolpyruvate, 0.154 mg/ml pyruvate kinase, 0.1 mm isoprenaline and 0.1 mM GppNHp, in a final volume of 0.1 ml, for 10 min at 37°C. The product, cyclic AM^{32}P, was collected after passage through a column of neutral alumina (Ramachandran, 1971) and counted. Enzyme activity from all chambers was shown to be linear with respect to protein and time for at least 10 min. Since isoprenaline causes variable activation of enzyme activity beyond that seen with GppNHp alone, this assay must be regarded as a measure of total cyclase rather than of cyclase specifically associable with β-adrenoceptors.

Na^+, K^+-activated ATPase (ATPase) was quantified with a ouabain-binding assay (Gelbart & Goldman, 1977). Samples of homogenates from 1 to 3 mg of tissue were incubated in triplicate with 50 nM [^{3}H]-ouabain for 2 h at 37°C with and without 5 mM ATP, and membranes were then collected and washed on glass fibre filters, as previously described. Specifically-bound ouabain was calculated as the difference in binding between samples with and without ATP, the latter being only a few percent of the former. We have established for canine atrial and ventricular tissue that there is only one population of saturable binding sites for ouabain in the range 0.1 nM to 100 nM, with a K_D of 6 to 7 nM, and that 0.005 to 0.05% deoxycholate does not enhance binding (unpublished observations). The latter result indicates that ouabain has access to all available binding sites.

Drugs

(−)[2,3-^{3}H-propyl]-dihydroalprenolol (DHA, 32–48 Ci/mmol), [^{3}H-general]-ouabain (14 Ci/mmol), and [^{3}H-general]-adenosine 3′,5′-cyclic phosphate (cyclic AMP; 36 Ci/mmol), were purchased from New England Nuclear Corporation; α-^{32}P adenosine triphosphate (ATP) and 5′-guanyl-imidodiphosphate (GppNHp) from ICN Pharmaceuticals, Inc.; crystalline rabbit muscle pyruvate kinase from Boehringer-Mannheim; and the (−)-isomers of isoprenaline, noradrenaline and adrenaline from Sigma Chemical Corp.

The following drugs were provided by pharmaceutical companies: (−)- and (+)-alprenolol (Hässle); (±)-atenolol (ICI U.S. Inc.); (±)-tolamolol (Pfizer); (±)-labetalol, (±)-AH35/25, and (±)-34/74 (Allen and Hanburys Research Ltd.); (+)-isomers of isoprena-

Table 1 Distribution of various substances in different parts of canine hearts

	Values per g of fresh tissue				
	Left atrium	*Right atrium*	*Right ventricle*	*Inter-ventricular septum*	*Left ventricle*
β-Adrenoceptors	3.3, 4.4, 3.7	4.6, 4.1, 5.0	5.3, 6.4, 4.6	5.7, 4.6, 5.3	8.4, 6.7, 6.1
(pmol DHA bound)	(3.8)*	(4.6)	(5.3)	(5.3)	(7.1)
Adenylate cyclase activity	343, 339, 440	291, 524, 582	885, 943, 1173	932, 1312, 1173	1336, 1421, 1428
(pmol cyclic AMP/s)	(374)	(466)	(1000)	(1140)	(1395)
Na^+, K^+-activated ATPase	228, 179, 186	142, 140	446, 474	465, 458, 473	522, 510, 538
(pmol ouabain bound)	(198)	(141)	(450)	(465)	(523)
Blood flow†					
(% i.v. dose of ^{86}Rb retained after 20 s)	0.037	0.038	0.043	0.063	0.064
Delivery of [^{3}H]-noradrenaline‡					
(pmol retained 15 min after trace i.v. dose)	53	61	76	110	122
Noradrenaline content§	8,400	14,000	4,400	5,200	3,900
(pmol)	9,200	11,500	3,600	3,300	3,300
	5,300	5,900	1,800	—	1,500
	8,300–14,000	12,000–22,000	4,700–6,500	5,300	3,600–5,900

* Mean values from this study are in parentheses. These were presented at a symposium of the American Physiological Society in Hollywood, Florida, October, 1977.
† Levy & de Oliveira (1961).
‡ Potter, Cooper, Willman & Wolfe (1965).
§ The four sets of data are from Potter *et al.* (1965); Angelakos (1965); Spurgeon, Priola, Montoya, Weiss & Alter (1974), and Harvey (1978).

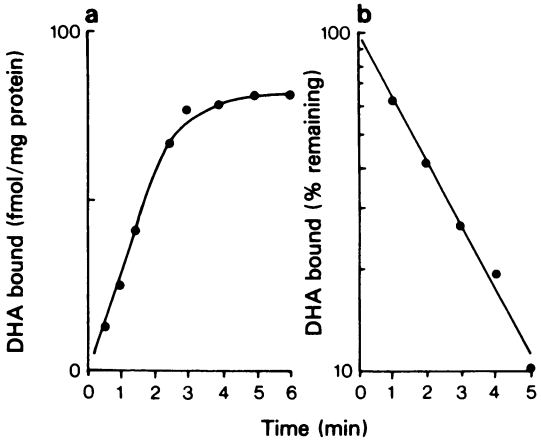

Figure 3 Association (a) and dissociation (b) of dihydroalprenolol (DHA) at 25°C. For (a), standard assay conditions were used, except for the times of incubation indicated. For (b) 20 μl of (±)-propranolol was added after standard incubations to bring the final concentration of 10 μM; free and bound DHA were then determined at the further times indicated.

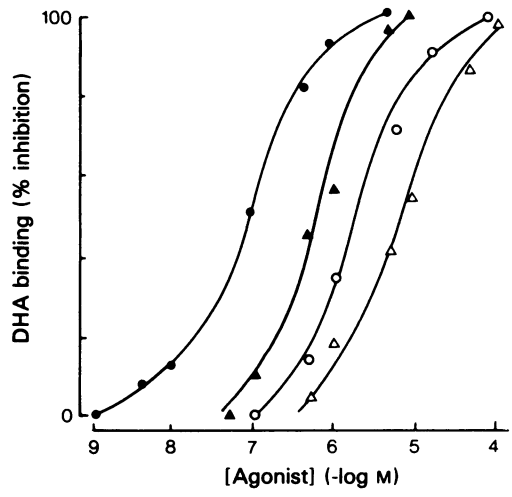

Figure 4 Inhibitory effects of (−)-isoprenaline (●), (−)-adrenaline (▲), (−)-noradrenaline (○) and (+)-isoprenaline (△) on the binding of dihydroalprenolol (DHA) at equilibrium to β-receptors in the dog heart.

line, noradrenaline and adrenaline (Sterling-Winthrop); propranolol and practolol (Ayerst); phentolamine (Ciba-Geigy); and pargyline (Abbott).

Results

Figure 1 shows saturation curves for specific DHA binding sites in preparations of membranes from two canine left ventricles; the values are typical of more than 20 experiments. The use of hypertonic KCl during membrane preparation reduced nonspecific, nonsaturable binding six fold, but had no effect on specific, saturable binding. Analysis of the equilibrium data in this figure by the method of Scatchard (Figure 2) indicated a single type of binding site with a K_D of approx. 2.5 nM and a calculated B_{max} of 5.8 pmol for the membranes recovered from 1 g of tissue. Similar analyses of the binding of DHA to KCl-washed membranes from all cardiac chambers yielded comparable K_D values (between 2 and 4 nM) and the saturation levels given in Table 1.

Analyses of the association and dissociation of DHA with and from KCl-washed membranes from canine left ventricles also provided evidence for a single type of binding site (Figure 3). The calculated rate of association was approx. 0.095 nM^{-1} min^{-1}, and that for dissociation was about 0.395 min^{-1}. The dissociation constant calculated from these data is approx. 4, in accord with the equilibrium data. Since the half-time for dissociation was about 2 min, and dissociation of DHA is slower at 4° than 25°C (Williams, Jarrett & Lefkowitz, 1976), it may be concluded that little DHA is lost from β-receptors during dilution and filter-washing procedures at 4°C lasting less than 15 s.

In confirmation of earlier studies with cardiac microsomes (Alexander *et al.*, 1975; Krawietz *et al.*, 1976; Harden *et al.*, 1976), the equilibrium binding of DHA to unfractionated KCl-washed membranes was inhibited by various drugs as expected for sites having the properties of β-adrenoceptors. As shown in Figure 4, a potency order of (−)-isoprenaline > (−)-adrenaline > (−)-noradrenaline > (+)-isoprenaline was found, indicative of binding of DHA to sites having the pharmacological specificity of β-receptors (Ahlquist, 1948). For these three compounds and for alprenolol, (−)-isomers were much more potent in competing for DHA binding sites than their (+)-isomers, with alprenolol showing the greatest (262 fold) difference (Table 2). Alprenolol was the most potent inhibitor tested, showing a calculated K_D value, 2.8 nM, in excellent agreement with the K_D value for DHA determined by Scatchard analyses. The calculated K_D value for noradrenaline (1167 nM) was significantly higher than that for adrenaline (233 nM); corresponding values for rat heart microsomes, determined with the ligand ^{125}I-hydroxybenzylpindolol, have been reported as 2500 and 50 nM, respectively (Harden *et al.*, 1976).

The distribution of β-receptors in different cardiac chambers is given in Table 1. For comparison the table also shows: (1) the distribution of the enzyme, adenylate cyclase, which is activated following cate-

Table 2 Inhibition of the binding of dihydroalprenolol (DHA) β-adrenoceptors by various drugs

Drug	*Observed* I_{50} (nM)	*Calculated* K_D (nM)
(−)-Alprenolol	8.5	2.8
(+)-Alprenolol	2,200	733
(+)-Propranolol	18	6
(+)-Labetolol	60	20
(+)-Tolamolol	88	29
(−)-Isoprenaline	90	30
(+)-Isoprenaline	8,000	2,666
(−)-Adrenaline	700	233
(+)-Adrenaline	86,000	29,000
(±)-Atenolol	800	267
(−)-Noradrenaline	3,500	1,167
(+)-Noradrenaline	110,000	37,000
(±)-Practolol	5,000	1,667
(±)-AH 34/74	6,200	2,067
(±)-AH 35/25	16,000	5,330
Phentolamine	over 500,000	
Pargyline	over 500,000	

I_{50} values represent the drug concentration required to reduce specific binding of DHA by 50%, and were estimated from the data in, or like that shown in Figure 4. K_D values were calculated according to the equation:

$$K_D(\text{drug}) = \frac{IC_{50}}{1 + \frac{[\text{DHA}]}{K_D \text{ for DHA}}}.$$

cholamine-receptor interactions; (2) levels of endogenous noradrenaline, as the best available measure of the distribution of noradrenergic nerve terminals; (3) values for the regional distribution of blood flow and the retention of trace amounts of circulating catecholamines in nerves, as measures of the regional delivery of circulating catecholamines to β-receptors; and (4) the distribution of Na^+, K^+-activated ATPase, whose function is unrelated to that of catecholamines, β-receptors and cyclase activation. To facilitate comparisons, all values except noradrenaline content are compared on a percentage basis in Figure 5.

It is apparent that there is an excellent correlation between the regional distribution of β-receptors, blood flow and the retention of a trace dose of a circulating catecholamine in all parts of the dog heart. Each of these parameters, and also adenylate cyclase activity and Na^+, K^+-ATPase (excepting left > right atrium) increased with progression from the left atrium → right atrium → right ventricle → interventricular septum → left ventricle. In contrast, the distribution of endogenous noradrenaline is 2 to 4 fold greater in the atria than ventricles, with an increasing progression from the left ventricle → right ventricle → septum → left atrium → right atrium (Table 1). The ratio of β-receptors to endogenous noradrenaline con-

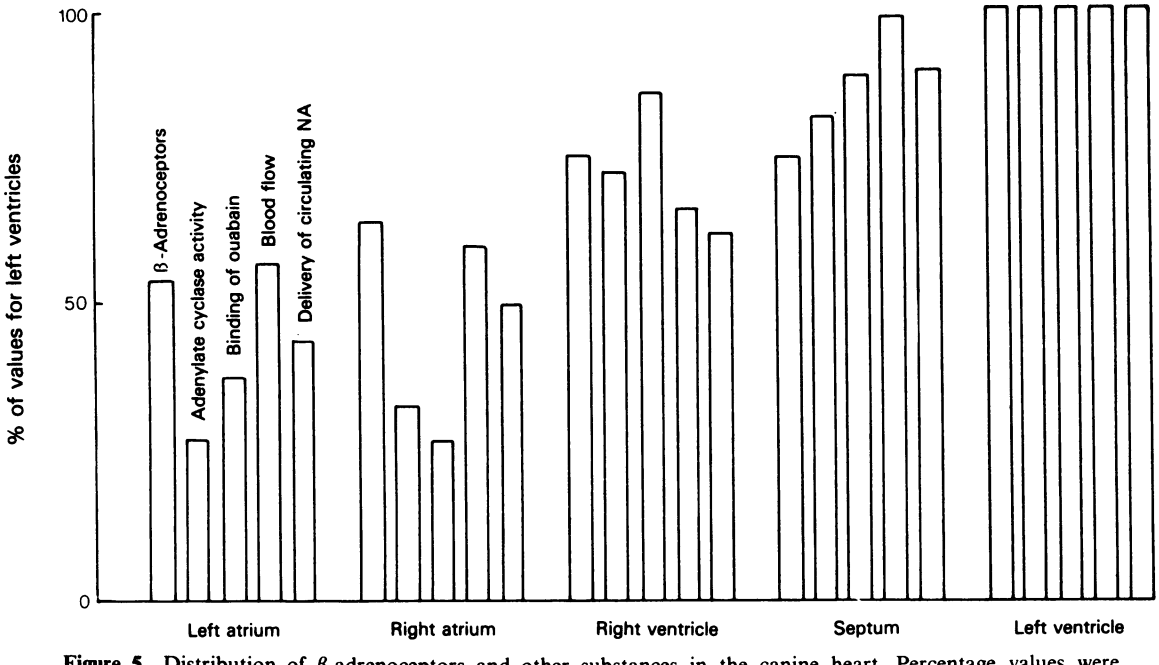

Figure 5 Distribution of β-adrenoceptors and other substances in the canine heart. Percentage values were calculated from Table 1. Relative values for endogenous noradrenaline (NA) content are not included for the sake of clarity; various values for the right atrium of the dog heart average 3.7 fold higher than those for the left ventricle (Table 1). Corresponding ratios for the cat, rabbit, guinea-pig and rat are 1.4, 2.0, 3.2 and 3.7, respectively (Muscholl, 1959).

tent was 5.6 fold higher in the minimally-innervated left ventricle than the maximally-innervated right atrium. The corresponding value for receptors/cyclase activity was 10.6 fold.

Discussion

Since the regional binding of DHA in the canine heart does not parallel levels of endogenous noradrenaline (and thus adrenergic nerve terminals), it appears that factors other than innervation are primarily responsible for the distribution of those receptors. Their distribution is closely related to that of blood flow, and the arrival plus retention of a circulating catecholamine. Thus most of the receptors appear not to be at synapses, but to be diffusely localized where they can react with circulating adrenaline. It remains a moot question what factors do control placement of these receptors; presumably both general cellular factors and the degree of activation of receptors are involved. There is evidence that β-responsiveness appears before innervation (Pappano, 1977) and remains on cultured cardiocytes (Hill-Smith & Purves, 1978). It also appears that the number of β-receptors in normal, growing hearts is proportional to the surface area of myocardial cells (Baker & Potter, 1979).

Four comments may be made about the β-receptors which are at synapses. First, since major increases in cardiac output are generally accomplished by large changes in heart rate rather than stroke volume, and since large increases in rate depend primarily upon the effects of adrenergic nerve stimulation (on the sinus node, the rate of impulse conduction and the rate of tension development), it may be concluded that the relatively few β-receptors near nerves can account for near-maximum changes in performance. Second, stimulation of the heart via its nerves accounts for the major increases in cardiac rate, force and output seen during exercise (Rushmer, 1976). Even severe exercise in dogs (Ohokuzi, 1966) and man (Callingham, 1977) is not accompanied by much secretion of adrenaline. When Donald, Ferguson & Milburn (1968) studied a dose of propranolol in racing greyhounds that blocked all but 4% of peak cardiac responses to isoprenaline, but left 26% of increases which followed supramaximal stimulation of the right stellate ganglion, they found negligible change in racing times. During more prolonged heavy exercise, high doses of propranolol in man, which should effectively block most effects of circulating adrenaline, reduce cardiac performance only about 20% (Epstein, Robinson, Kahler & Braunwald, 1965). Thus only a minority of the β-receptors in the heart, those at synapses, appear important for exercise. Third, even when there are large amounts of circulating adrenaline, this catecholamine probably has much less effect on synaptic receptors than noradrenaline from nerves. Perhaps the best illustration is the fact that racing greyhounds with adrenergically denervated hearts achieve only 60% of normal increases in heart rate during peak excitement before a race (Donald *et al.*, 1968), despite abrupt and major secretion of adrenaline, considerable supersensitivity of the sinus node to catecholamines (Donald & Shepherd, 1965), and dependence of the myocardium on circulating adrenaline for peak (unchanged) race performance (Donald *et al.*, 1968). Finally the effects of circulating noradrenaline on synaptic β-receptors are probably insignificant. The levels found in animals and man during rest, exercise or psychological stress, 1 to 10 nM (Callingham, 1975), are simply too low to exert much effect on receptors with a K_D of 1167 to 2500 nM for this catecholamine.

From the foregoing, it is obvious that the majority of the β-receptors in the heart must respond to circulating adrenaline but not noradrenaline, at times unrelated to exercise *per se*. Plasma levels of adrenaline rise towards the K_D value for this catecholamine at cardiac β-receptors (50 to 230 nM) in two general sets of conditions: life-threatening circumstances including severe haemorrhage, hypoxia, hypoglycaemia, acidosis and the discharge of phaeochromocytomas; and circumstances involving psychological stress (Callingham, 1975). In each case adrenaline presumably acts on all β-receptors, and its effects will be relatively greater on the rate and degree of tension development than on heart rate, compared to the effects of noradrenaline acting only near nerves. The large increases in heart rate which occur concomitantly are probably due largely to neural activity. For practical purposes, it thus appears that most cardiac β-receptors in modern, socialized man are used during such activities as dealing with the normal problems of business life, taking an intelligence exam and watching an arousing film, all of which are accompanied by considerable secretion of adrenaline (Levi, 1967).

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