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A Fraction of the Ventricular Myocardium That Has the Specificity of the Cardiac Beta-Adrenergic Receptor

(norepinephrine binding/displacement)

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ABSTRACT **The 78,000 × *g* microsomal fraction of canine ventricular myocardium effected a 20-fold concentration of [^{3}H]norepinephrine from a 10^{-9} M solution. The [^{3}H]norepinephrine bound was displaced by physiologic concentrations of the beta-adrenergic catecholamines isoproterenol, epinephrine, and norepinephrine, in that order, which is the order of effectiveness of their actions on the force and rate of cardiac contraction. Alpha-adrenergic compounds did not displace [^{3}H]norepinephrine until concentrations four orders of magnitude greater were reached. The beta-adrenergic blocker propranolol displaced at 10^{-6} M, whereas the alpha-adrenergic blocker phentolamine was inactive. Metabolites of the catecholamines did not compete for this binding site. It is concluded that, on the basis of specificity and affinity of binding, these microsomal particles are likely to contain the beta-adrenergic receptor.**

For over 20 years the concept of specific alpha and beta receptors for catecholamines has differentiated apparently separable physiologic and pharmacologic actions of norepinephrine, epinephrine, and related compounds (1). Direct studies of tissue uptake and binding of norepinephrine, however, have been complicated by a number of discrete and apparently very different binding mechanisms for adrenergic agents. In particular, cardiac uptake of specific catecholamines is poorly correlated with the effects of these agents on the rate and force of cardiac action (2). Uptake is a complex process, which may be separated into neural uptake (2), neural vesicle (storage granule) uptake (3), and receptor uptake. Extensive studies have defined the specificities of the neural (2) and neural vesicle (4, 5) uptakes of [^{3}H]norepinephrine but neither of these specificities correlate with cardiac effects. Binding of labeled norepinephrine to the beta-adrenergic receptor has not been previously demonstrated. We describe here the characteristics of binding of [^{3}H]-norepinephrine to microsomal particles from canine ventricular myocardium. The binding is blocked by catecholamines in direct proportion to their beta-adrenergic potency on cardiac action.

MATERIALS AND METHODS

[7-^{3}H]D,L-Norepinephrine, 9–10 Ci/mmol, was obtained from New England Nuclear Corp., L-norepinephrine bitartrate, isoproterenol hydrochloride, and phenylephrine hydrochloride were from Winthrop Pharmaceuticals; L-epinephrine hydrochloride was from Parke-Davis, DL-propranolol hydrochloride from Ayerst, phentolamine mesylate from CIBA, metaraminol bitartrate and α-methyl dihydroxyphenylalanine (α-methyl Dopa) from Merck Sharpe and Dohme, ephedrine sulfate from Lilly, methoxamine hydrochloride from Burroughs-Wellcome Co., and mephentermine sulfate from Wyeth. Dihydroxyphenylethylamine (Dopamine), D,L-dihydroxyphenylalanine (DOPA), dihydroxymandelic acid, metanephrine, normetanephrine, vanillylmandelic acid, homovanillic acid, and β-phenethylamine were from Sigma. Millipore filters (0.45-μm pore size, 25 mm diameter) and Millipore filter holders were from the Millipore Corp. Other chemicals were reagent grade of the highest purity obtainable from commercial sources.

Preparation of ventricular microsomes

Hearts were removed from mongrel dogs (15–30 kg) immediately after exsanguination and were placed in ice-cold 0.25 M sucrose–0.005 M Tris·HCl (pH 7.4)–0.001 M $MgCl_2$ (sucrose buffer). After the atria and great vessels had been removed, the ventricles (40–80 g) were weighed, washed thoroughly, and homogenized in 3 volumes of the sucrose buffer. This was accomplished by mincing the tissue with scissors, blending in an "Osterizer" blender at high speed for 30–45 sec, and finally homogenizing with a motor-driven Teflon-tipped pestle. All procedures were carried out at 0°C. The tissue homogenates thus obtained were centrifuged in a No. 30 rotor at 30,000 rpm (78,000 × *g* average force) for 5 min (including time of acceleration) in a model L3-50 Beckman Spinco preparative ultracentrifuge to remove coarse tissue fragments. Supernatants were recentrifuged at 30,000 rpm for 1 hr. The reddish microsomal pellet thus obtained was resuspended in one-third to one-half the original homogenate volume and either used directly or frozen at −20°C. Microsomes prepared in this way contained 4–7 mg of particles per ml and their binding activity was stable to freezing for several weeks.

Binding experiments

1 ml of microsomal particles was incubated with [^{3}H]D,L-norepinephrine 5×10^{-9} M (50,000 cpm/ml), generally for 2 hr at 37°C. Compounds to be tested for effects on binding were included in the incubation mixtures at the stated concentrations. Binding to the microsomal particles was assessed by counting the particles either after centrifugation at 78,000 × *g* for 1 hr or after Millipore filtration with Millipore filters (0.45-μm pore size) in a Packard liquid scintillation counter.

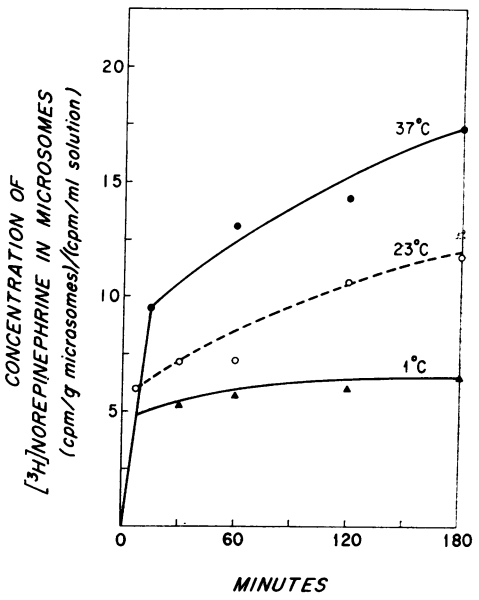


FIG. 1. Binding of [^{3}H]norepinephrine to cardiac microsomes. For conditions of incubation, see *Methods*. Each point is the mean of duplicates.

RESULTS

Binding of [^{3}H]norepinephrine to sites in the microsomal particles was a rapid, temperature-dependent process (Fig. 1). In the experiments reported, the concentration of [^{3}H]-norepinephrine in the particles was computed as (dpm/g of particles)/(dpm/ml of solution). In the absence of inhibitors this quotient was generally 20 after 2 hr at 37°C. In more recent experiments, dilution and further washing of the particles has led to a 20-fold increase in the particle/solution ratio of counts, so that at 2 hr, the concentration of [^{3}H]-norepinephrine in the particles was 400–500 times that in the medium.

Several compounds were tested for their ability to inhibit [^{3}H]norepinephrine binding. Agents with direct beta-adrenergic activity on the heart were most effective (Fig. 2,*A* and *B*). In the range of 10^{-6}–10^{-9} M isoproterenol was most potent, with threshold about 10^{-9} M and 50% inhibition at 1.8×10^{-7} M. Epinephrine and norepinephrine were somewhat less potent. Dopamine and DOPA were significantly less effective.

In contrast to the potency of the direct-acting beta-adrenergic agents, compounds whose beta-adrenergic action is largely due to norepinephrine release and alpha-adrenergic compounds did not compete effectively until much higher concentrations were reached (Fig. 3). Metaraminol displaced

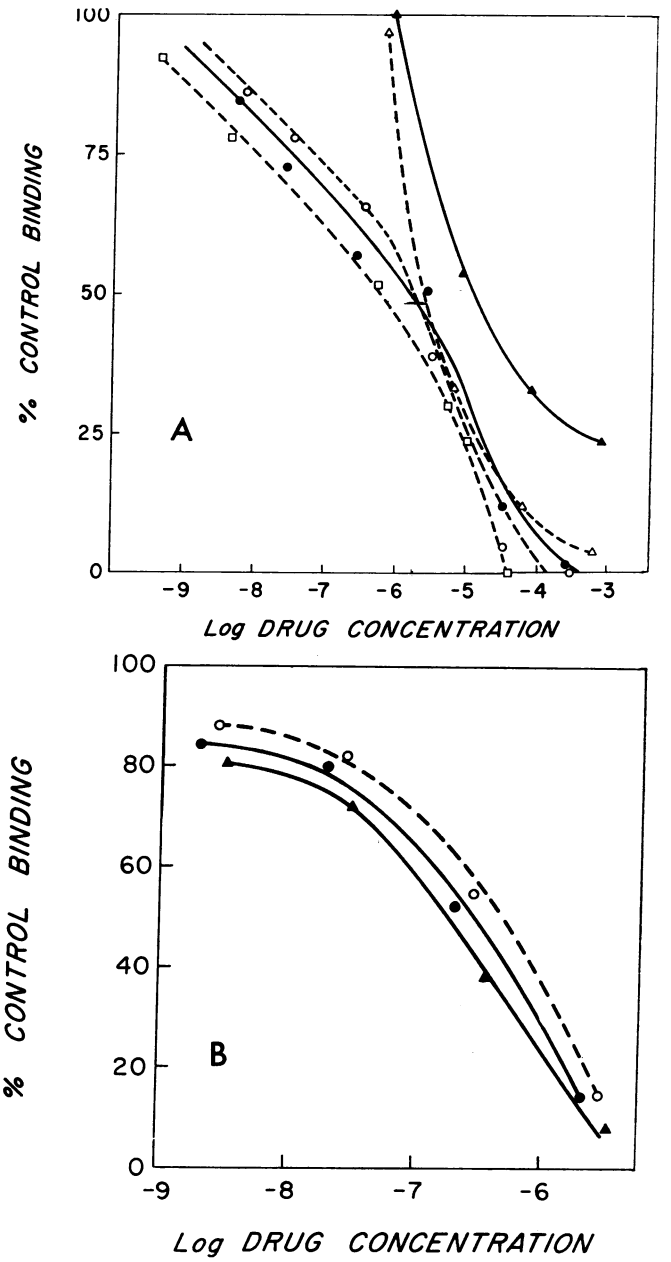


FIG. 2*A*. Inhibition of [^{3}H]norepinephrine binding to cardiac microsomes by beta-adrenergic agents: ○, norepinephrine, ● epinephrine, △ Dopamine, ▲ DOPA, □ isoproterenol. Each point is the mean of duplicates. In *B*, particles were diluted 1:20 with sucrose buffer before use (final concentration of particles, 250 μg/ml). ▲ isoproterenol, ● epinephrine, ○ norepinephrine. Each point is the mean of three determinations.

TABLE 1. *Inhibition of [^{3}H]norepinephrine binding to cardiac microsomes by various compounds*

Compound	50% inhibition	Threshold for inhibition
Dihydroxymandelic Acid	1×10^{-5} M	2×10^{-7} M
α-Methyl DOPA	5×10^{-6} M	1×10^{-7} M
Metanephrine	No inhibition at 4×10^{-4} M	
Normetanephrine	No inhibition at 4×10^{-4} M	
4-OH-3-methoxymandelic acid	No inhibition at 4×10^{-4} M	
4-OH-3-methoxyphenylacetic acid (homovanillic acid)	No inhibition at 4×10^{-4} M	
Phenethylamine	No inhibition at 4×10^{-3} M	

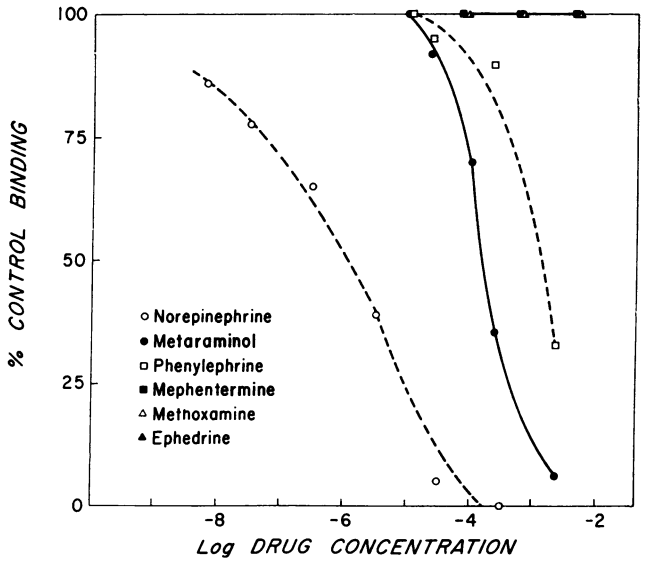


FIG. 3. Inhibition of [^{3}H]norepinephrine binding to cardiac microsomes by alpha-adrenergic agents and indirectly-active beta-adrenergic agents.

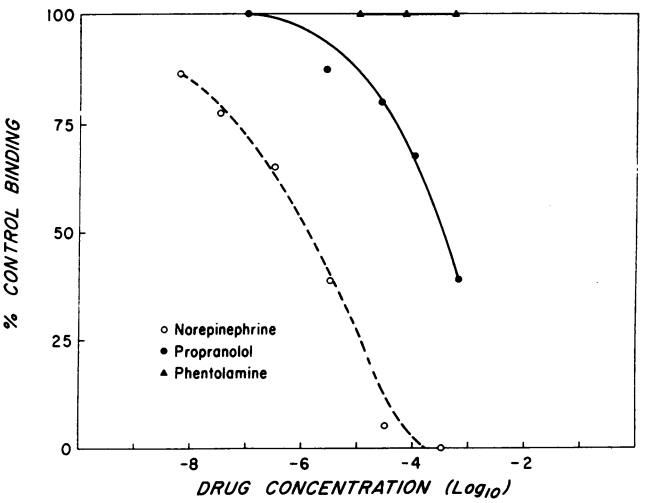


FIG. 4. Inhibition of [^{3}H]norepinephrine binding to cardiac microsomes by adrenergic blocking agents. Each point is the mean of duplicates.

significantly only at concentrations above 10^{-4} M. Phenylephrine was even less potent, with 50% inhibition occurring only at 1.5×10^{-3} M. The alpha-adrenergic agent methoxamine and the indirectly beta-active agents mephentermine and ephedrine did not displace at any concentration tested up to 5×10^{-3} M.

The beta-blocking agent propranolol inhibited binding, whereas the alpha-blocker phentolamine was not active at concentrations below 10^{-3} M (Fig. 4).

Compounds possessing the dihydroxyphenyl configuration competed for receptors. Thus, both dihydroxymandelic acid and α-methyl DOPA inhibited binding in an intermediate concentration range (Table 1). 3-*O*-Methylated metabolites such as normetanephrine, metanephrine, vanillylmandelic acid, and homovanillic acid were inactive (Table 1). β-Phenethylamine, lacking both ring hydroxyl groups and the β-hydroxyl, was inactive even at 4×10^{-2} M.

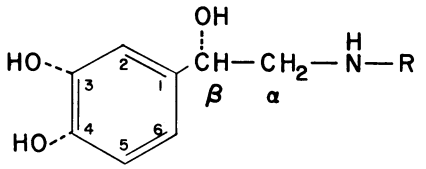
DISCUSSION

We have demonstrated binding of catecholamines and related compounds to a cardiac microsomal fraction that seems to have a binding specificity identical to that of the beta-adrenergic receptor. The order of cardiac potency of beta-adrenergic agents exactly parallels their potency in displacing [^{3}H]norepinephrine from the microsomal binding sites: isoproterenol > epinephrine > norepinephrine > dopamine > DOPA (Table 2) (6).

Displacement was detectable in the threshold range of drug concentrations which produce pharmacologic effects. Moreover, 50% inhibition of binding occurred at concentrations of catecholamines ($1–5 \times 10^{-7}$ M) that correspond closely to those resulting in one-half maximal effects in a

TABLE 2. *Order of potency of adrenergic agents*

Inotropic and chronotropic effects on the heart (6)	Blocking norepinephrine binding to cardiac microsomes	Blocking uptake of [^{3}H]norepinephrine by whole heart (2)	Blocking uptake of [^{3}H]norepinephrine by splenic nerve granules (4)	Blocking uptake of [^{14}C]epinephrine by adrenal medullary chromaffin granules (5)
Isoproterenol	Isoproterenol	Metaraminol	Phenethylamine	Phenethylamine
Epinephrine	Epinephrine	Dopamine	Metaraminol	Epinephrine
Norepinephrine	Norepinephrine	Norepinephrine	Dopamine	Norepinephrine
Dopamine	Dopamine	Epinephrine		Phenylephrine
DOPA	DOPA	Mephentermine	Ephedrine	Isoproterenol
Indirectly active or not active	*Not active at concentrations below 10^{-4} M*	Phenethylamine	Mephentermine	Ephedrine
Ephedrine	Metaraminol	Ephedrine		
Mephentermine	Ephedrine	Phenylephrine		
Methoxamine	Phenylephrine	Isoproterenol		
Phenethylamine	Mephentermine	DOPA		
Indirectly active or weakly beta active	Methoxamine	Methoxamine		
Metaraminol	Phenethylamine			
Phenylephrine				



Action (NE = norepinephrine)	Substituents: Required for activity	Substituents: Not required, but enhance activity	Substituents: Decrease activity
β-Adrenergic inotropy and chronotropy (6)	3—OH, 4—OH	β—OH, R=CH_3, $CH(CH_3)_2$	—
Inhibition of NE binding to cardiac microsomes	3—OH, 4—OH	β—OH, R=CH_3, $CH(CH_3)_2$	—
Inhibition of NE uptake by neural vesicles (4,5)	None	β—OH	R=CH_3, $CH(CH_3)_2$
Inhibition of NE uptake by whole heart (2)	None	3—OH, 4—OH	β—OH, R=CH_3, $CH(CH_3)_2$

FIG. 5. Structure–activity relationships for inhibition of [^{3}H]norepinephrine binding to cardiac microsomes by adrenergic agents.

number of other *in vitro* systems (Table 3). Alpha-adrenergic agents and indirectly active beta-adrenergic agents effected threshold displacement of [^{3}H]norepinephrine only at concentrations five orders of magnitude higher, or not at all. The beta-adrenergic blocker propranolol inhibited binding, whereas the alpha blocker phentolamine did not. Metabolites of catecholamines did not interact appreciably with these binding sites.

Various biological mechanisms for uptake and binding of norepinephrine have been demonstrated. The specificity of these binding processes differ markedly, however, from that of the beta-adrenergic receptor (Table 2). Thus the uptake of [^{3}H]norepinephrine into adrenergic nerve tissue in organs such as the heart is most potently blocked by metaraminol and dopamine, with the potent beta-agonist isoproterenol almost inactive (2). A second uptake process in such tissues is most effectively blocked by 3-*O*-methylated metabolites such as metanephrine (2). Binding of norepinephrine in isolated storage granules from bovine splenic nerves and of epinephrine in adrenal medullary granules has also been extensively studied (4, 5). Again a markedly different specificity was found, with phenethylamine among the most potent inhibitors and isoproterenol less effective. Further, uptake in these storage particles is apparently less specific than that of the beta-receptor, and is blocked equally well by a variety of alpha- and beta-blocking agents (7, 8).

The structure–activity relationships for interaction of catecholamines with beta-receptors as determined in this system may be considered with reference to Fig. 5. The ring 3- and 4-hydroxyl groups are required for binding. The β-hydroxyl group, though not required, enhances binding. Substitution on the amino group increases binding. These are the precise structural requirements of the beta-adrenergic receptor as determined by pharmacologic studies (9). Structure–activity considerations for the other norepinephrine binding processes are summarized for contrast in Fig. 5.

Binding of [^{3}H]epinephrine to liver membranes has been studied, though at concentrations of catecholamines far outside the physiologic range (10).

In vitro binding of [^{3}H]norepinephrine to cardiac microsomal particles has been previously reported from several laboratories (11–13), though the specificity of binding has not been examined. It was shown that the *in vitro* uptake of [^{3}H]norepinephrine by cardiac microsomes differed markedly from that of the adrenergic storage granules: cardiac microsomal uptake was insensitive to reserpine, which potently blocked nerve granule uptake (11, 13); cardiac microsomal uptake was also insensitive to ATP, which stimulated nerve

TABLE 3. *Physiological effects of catecholamines on in vitro cardiac preparations*

	One-half maximal concentration		Reference
Inhibition of [^{3}H]norepinephrine binding to cardiac microsomes	Norepinephrine Epinephrine Isoproterenol	5×10^{-7} M 3×10^{-7} M 1.8×10^{-7} M	This work
Change in tension in isometrically contracting cat right ventricular papillary muscle	Norepinephrine	$2–3 \times 10^{-7}$ M	Epstein *et al.* (14)
Increase in relative contractile force and cyclic AMP generation in isolated perfused working rat hearts	Epinephrine	1.4×10^{-7} M	Robison *et al.* (15)
Increase in cyclic AMP generation in broken-cell adenylate cyclase preparations from canine heart	Norepinephrine Epinephrine Isoproterenol	3×10^{-6} M 1×10^{-6} M 9×10^{-7} M	Murad *et al.* (16)

granule uptake (11, 13); after sucrose gradient centrifugation of cardiac microsomes, the fractions that bound [^{3}H]norepinephrine most avidly differed from those containing the norepinephrine storage depots (13).

These findings support the conclusion suggested by the specificity of the *in vitro* cardiac microsomal binding of [^{3}H]norepinephrine, namely, that this represents the physiologic beta-adrenergic receptor.

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