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[^{3}H]NOREPINEPHRINE BINDING AND LIPOLYSIS BY ISOLATED FAT CELLS

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SUMMARY

[^{3}H]norepinephrine was shown to bind to specific sites on isolated fat cells. A Scatchard plot of norepinephrine binding showed two apparent K_a of $1.9 \cdot 10^6$ and $1.2 \cdot 10^5$ LM^{-1}. $1.4 \cdot 10^{-4}$ M Norepinephrine covalently-linked to agarose beads reduced [^{3}H]norepinephrine binding by over 50 %. Several structurally related drugs were compared as inhibitors of [^{3}H]norepinephrine binding and as stimulators of lipolysis in preparations of similarly prepared cells. Dose-response curves for norepinephrine, epinephrine and isoproterenol showed the affinities for binding inhibition and for stimulation of lipolysis to be in the same range of $6 \cdot 10^{-7}$–$2 \cdot 10^{-6}$ M. Dopamine and dopa were potent inhibitors of [^{3}H]norepinephrine binding at $8.5 \cdot 10^{-7}$ M and $2.0 \cdot 10^{-6}$ M respectively, but did not stimulate lipolysis even at 10^{-4} M. Propranolol, a β-adrenergic antagonist, had no effect on [^{3}H]norepinephrine binding at 10^{-4} M but completely inhibited catecholamine-stimulated lipolysis at 10^{-5} M. Phentolamine, an α-adrenergic antagonist, did not inhibit binding or catecholamine-stimulated lipolysis at 10^{-4} M. Ephedrine, metaraminol, phenylephrine and normetanephrine were also ineffective both as [^{3}H]norepinephrine binding inhibitors and as stimulators of lipolysis. The results suggested the catechol ring of catecholamines is more important than the ethanolamine side chain as a requirement for binding, while both an intact catechol moiety and ethanolamine function appear necessary for physiological effect.

INTRODUCTION

The in vitro binding of radiolabeled catecholamines has been used to study the interaction of adrenergic agonists with several tissues [1]. The use of membrane fractions for many of these studies has precluded a correlation of binding kinetics with the kinetics of a well-characterized physiological response. Such correlation is an important criterion for the ultimate definition of biological receptors [2].

A well-known response of white adipose tissue to catecholamines is stimulation of lipolysis. This has been shown in vitro in whole fat pads [3], in isolated cells [4] and in tissue homogenates [5]. The effect of catecholamines on lipolysis appears to involve interaction with beta-adrenergic receptors [6] and subsequent mediation by cyclic 3′5′ adenosine monophosphate [3]. In this study we have utilized isolated fat cells to directly demonstrate a set of specific sites which bind [^{3}H]norepinephrine and related compounds. The results are compared to other data recently reported [7]. In addition we have compared the kinetics of [^{3}H]norepinephrine binding to the kinetics of the lipolytic response in similar cell preparations.

METHODS

Isolation of cells

Sprague-Dawley male rats (200–350 g) were sacrificed by a blow to the head. Epididymal fat pads were removed and rinsed in calcium-free Krebs–Ringer's phosphate buffer, pH 7.4. The pads were dissected free of major blood vessels, rinsed in the Krebs buffer, and minced finely with a scissors. Fat cells were isolated by the method of Rodbell [4] as follows. All steps were carried out at room temperature and using siliconized or plastic vessels. Fat cells were dispersed by incubating the minced adipose tissue in a 25 ml polyethylene bottle containing 10 mg collagenase (Worthington, Type I) and 3 ml Krebs buffer for each gram of tissue. After 1h of continuous vigorous shaking at 37 °C, the resulting suspension was filtered through two layers of cheesecloth followed by one volume of Krebs buffer. The suspension was centrifuged at $50 \times g$ for 1 min. Stroma and vascular debris sedimented and were aspirated and discarded along with the suspending medium, leaving the fat cells, which float as a compact layer. The cells were then washed four times by flotation at $50 \times g$ for 1 min in 0.25 M sucrose containing 1 mM $MgCl_2$ and 5 mM sodium phosphate buffer, pH 7.4. If cells were to be used for binding studies, a fifth and final wash was carried out in the sucrose–Mg^{2+}–phosphate buffer. For cells to be used in lipolysis experiments, the fifth wash was done in the aforementioned Krebs buffer.

Binding of [^{3}H]norepinephrine to isolated cells

The packed cell layer was suspended in sucrose–Mg^{2+}–phosphate buffer. The tendency of the cells to float hindered uniform pipetting. This was overcome by continuous gentle swirling. Cell numbers were reproducibly estimated by quickly transferring a drop of the continuously swirled suspension to a hemocytometer, gently and rapidly lowering a coverslip, and counting intact cells in the usual manner. Binding studies were carried out at 37 °C by incubating $1.5–2.5 \cdot 10^5$ cells with $2 \cdot 10^{-8}$ M l-[7-^{3}H(*N*)]norepinephrine (6.4 Ci/mM, New England Nuclear) in a 1-ml vol. After 45 min of incubation, bound and unbound [^{3}H]norepinephrine were separated by rapid Millipore filtration and assayed by scintillation counting [8]. Two sets of controls for non-specific binding were included in each experiment. Incubations were carried out without cells to determine the amount of [^{3}H]norepinephrine non-specifically bound to the Millipore filter. Cells were also incubated with the usual amount of [^{3}H]norepinephrine plus an excess of unlabeled norepinephrine ($3 \cdot 10^{-4}$ M) in order to determine the amount of non-displaceable [^{3}H]norepinephrine bound

to the cells. The radioactivity in the controls was subtracted from values obtained in experimental incubations. Specific binding of [^{3}H]norepinephrine under these conditions was 8–10 times the amount non-specifically bound.

If bovine serum albumin (Sigma, Fraction V) was included in the binding assay the absolute amount of [^{3}H]norepinephrine bound was reduced. This effect was dependent on albumin concentration suggesting the concentration of free [^{3}H]norepinephrine may have been lowered by non-specific binding to albumin. This would not be expected to affect the dose-response kinetics of competition by unlabeled drugs, since the absolute amount of [^{3}H]norepinephrine bound to cells is a linear function of free [^{3}H]norepinephrine at these low concentrations (see Results). Therefore to maximize the sensitivity of the binding assay, albumin was not included in the incubations.

Lipolysis experiments

The packed cell layer was not disturbed following the final wash in Krebs buffer. Using a plastic tipped Eppendorf pipette, 0.1 ml aliquots of packed cells ($\approx 4 \cdot 10^5$ cells, or $\approx$250 μg total cell protein) were transferred to 0.9 ml Krebs buffer containing bovine serum albumin (f.c. 4 %). The albumin had been defatted by the method of Chen [9]. The cells were pre-incubated at 37 °C for 10 min and 0.05 ml drug was added to each tube at timed intervals. After 60 min the incubation was terminated by adding 5 ml of a lipid extraction mixture [10]. Free fatty acids were extracted by the method of Dole et al. [11] as modified by Trout et al. [10].

When a potential blocking drug was tested, it was added before the preincubation. The blocking effect was compared to a control tube containing only the stimulating drug (10^{-5} M epinephrine). In every experiment, each variable was included in duplicate tubes. Two controls were included in each experiment, one containing no drug addition and the other containing only 10^{-5} M epinephrine.

Free fatty acids were titrated with dilute NaOH by the method of Dole [11] to an endpoint of 9.8 on an automatic titrator (Rainin Instruments) and compared to a palmitic acid standard. Protein was determined by the method of Lowry et al. [12].

RESULTS

Assay conditions

In the presence of $2 \cdot 10^{-8}$ M [^{3}H]norepinephrine, binding was linear with increasing cell number up $\cdot \approx 3 \cdot 10^5$ cells/ml (Fig. 1). In a typical experiment using $1.5–2.5 \cdot 10^5$ cells/ml, the amount bound was 0.41 pmoles ($\pm$0.036, S.E.M.) per 10^5 cells. Binding at 30 min was typically 90 % of the maximum seen after 45 min. With other conditions held constant, binding was linear with increasing amounts of [^{3}H] norepinephrine in the range tested ($5 \cdot 10^{-9}$ to $3 \cdot 10^{-8}$ M).

A Scatchard analysis [13] of the data for competition of [^{3}H]norepinephrine binding by unlabeled norepinephrine in the range 10^{-8}–10^{-6} M yielded an estimate of $k_{assoc} = 1.9 \cdot 10^6$ l mole^{-1} (Fig. 2). For higher concentrations of norepinephrine (10^{-6}–10^{-5} M), Fig. 2 suggests a possible second association constant of $1.2 \cdot 10^5$ l mole^{-1}. These values for k are similar to those reported by Jarett et al. [7]. Further experiments will be necessary to explain the apparent second order of sites. From Fig. 2 the amount bound to the high affinity sites is about 12 pmoles/10^5 cells. This

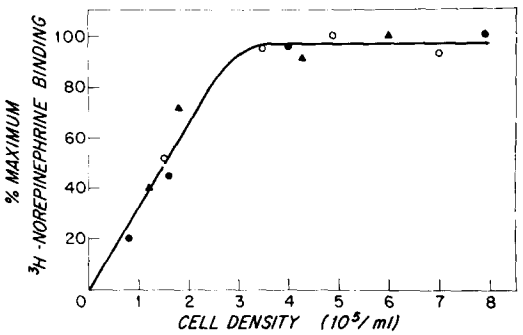


Fig. 1. The effect of increasing cell density on binding of [^{3}H]norepinephrine to isolated fat cells. Increasing numbers of cells (abscissa) were incubated at 37 °C for 45 min with a fixed concentration ($2 \cdot 10^{-8}$ M) of [^{3}H]norepinephrine as described in the text. For each experiment, the maximum cpm bound at high cell density was defined as maximum binding. Cpm bound at other cell densities were expressed as percent maximum (ordinate) to facilitate comparison. Symbols ●, ○, ▲, are three separate experiments. Each point is the mean of duplicate determinations.

estimate of binding capacity is high compared to similar estimates for other hormones [2]. While this question needs further clarification, there is no a priori reason to expect similar numbers of sites for different hormones, especially since catecholamines are relatively small molecules that may enter the cell and possibly interact at intracellular sites. In fact, catecholamine binding to subcellular fractions has been demonstrated in vitro [7].

Under the conditions described in "Methods" for lipolysis, the release of free fatty acids in the presence of 10^{-5} M epinephrine was linear with increasing cell number. The lipolytic response was measurable at 20 min and continued linearly with time up to 45 min; a further small increase was seen if the incubation was continued to 90 min (Fig. 3). The typical lipolytic response observed after 60 min with 10^{-5} M epinephrine was 0.6–0.8 μEq free fatty acid/$4 \cdot 10^5$ cells. The dose-response curve for free fatty acid release in the presence of various concentrations of

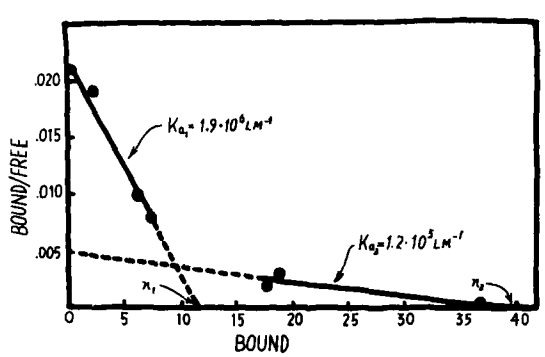


Fig. 2. Scatchard analysis of binding of l-norepinephrine to adipocytes. 1.5–2.5 · 10^5 cells/ml were incubated with 10^{-7}–$6 \cdot 10^{-5}$ M l-norepinephrine in the presence of labeled hormone as described in the text. The ordinate (Bound/Free) has units of ml/10^5 cells, the abscissa, pmoles/10^5 cells. The affinity constants were estimated from the slopes and the maximum pmoles bound per 10^5 cells from n, the abscissa intercept. Each point is the mean of 4–12 determinations from 2–6 experiments.

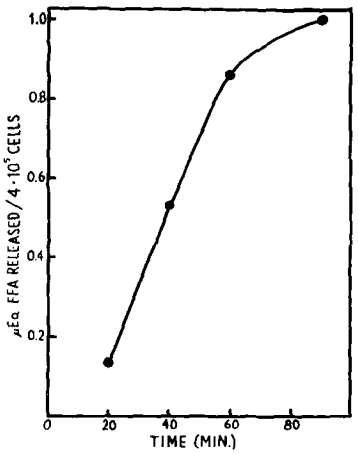


Fig. 3. The release of free fatty acids as a function of time in the presence of epinephrine. About $4 \cdot 10^5$ isolated adipocytes were incubated at 37 °C in Krebs-Ringers phosphate buffer, pH 7.4 containing 4 % albumin and 10^{-5} M l-epinephrine. Free fatty acids were quantitated as described in the text. A zero time control was below the sensitivity of the assay, and is not included in the Figure.

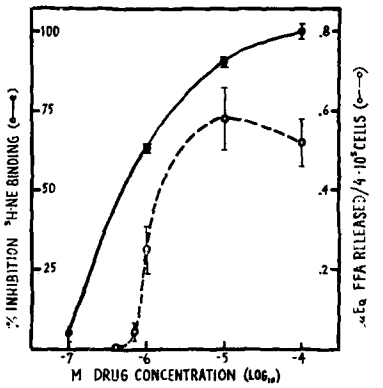


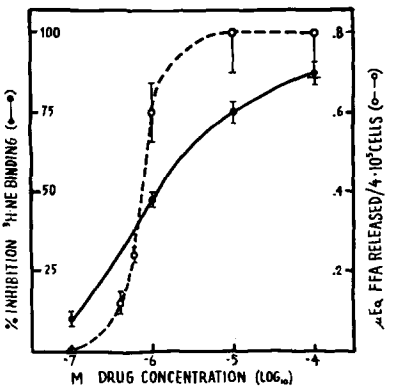
Fig. 4. A comparison of [^{3}H]norepinephrine binding inhibition and free fatty acid release at various concentrations of l-norepinephrine. For binding experiments isolated cells were incubated with $2 \cdot 10^{-8}$ M [^{3}H]norepinephrine and varying concentrations of l-norepinephrine as described in the text. Percent inhibition [^{3}H]norepinephrine binding was calculated by comparing cpm bound at each concentration to a control containing no competing drug. Using similarly prepared cells, free fatty acid release was also measured at various concentrations of l-norepinephrine with other conditions described in the text. Each point is the mean ± S.E.M. of 4–6 determinations from two to three separate experiments.

norepinephrine (see Fig. 4) was virtually identical to those reported by others under similar conditions [14].

If lipolysis was carried out in the sucrose–Mg^{2+}–phosphate buffer used for the binding experiments (but with 4 % albumin added) total free fatty acid release was reduced by an average of 55 % compared to a control of Krebs buffer. This reduction is probably due to the absence of K^+; others have reported a similar reduction in lipolysis if K^+ is omitted from the incubation [15, 16]. If 5 mM KCl was included in the sucrose–Mg^{2+}–phosphate buffer, free fatty acid release was restored to the level seen in Krebs buffer. KCl (1 or 5 mM) was not necessary for, and had no effect on, [^{3}H]norepinephrine binding in sucrose–Mg^{2+}–phosphate buffer (data not shown).

Adrenergic agonists

The affinities for binding and for stimulation of lipolysis agreed closely for norepinephrine, epinephrine and isoproterenol (Figs. 4, 5, 6). These have both an intact catechol ring and a hydroxyl group on the beta carbon of the ethanolamine moiety. These typical β-adrenergic agonists gave 50 % displacement of [^{3}H]norepinephrine binding in the range of $6 \cdot 10^{-7}$ to $2 \cdot 10^{-6}$ M. A similar range of $6 \cdot 10^{-7}$–$1 \cdot 10^{-6}$ M was observed for 50 % maximum lipolytic response. More exact estimates of affinities for binding and physiological effect are given in Table I along with similar estimates for other compounds tested.



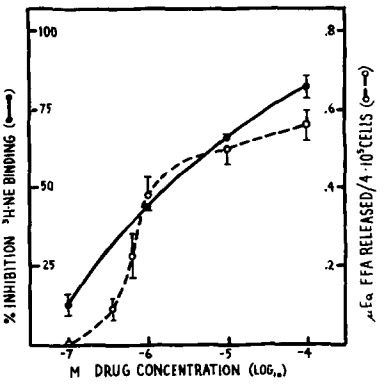


Fig. 5. A comparison of [^{3}H]norepinephrine binding inhibition and free fatty acid release at various concentrations of l-epinephrine. Binding experiments were carried out as described in Fig. 4, except that l-epinephrine was the competing drug. Using similarly prepared cells, free fatty acid release was measured at various concentrations of l-epinephrine with other conditions as described in the text.

Fig. 6. A comparison of [^{3}H]norepinephrine binding inhibition and free fatty acid release at various concentrations of l-isoproterenol. Conditions for binding and lipolysis are the same as in Fig. 6, except that isoproterenol was used in place of norepinephrine.

Compounds like dopamine and dopa, which have the usual catechol structure but lack the beta-carbon hydroxyl, were potent inhibitors of [^{3}H]norepinephrine binding (Fig. 7) but had no measurable effect on lipolysis even at 10^{-4} M (Table I).

TABLE I

Comparison of relative potencies of several compounds for inhibition of [^{3}H]norepinephrine binding and for effect on lipolysis. For each compound, the concentration required to inhibit [^{3}H]norepinephrine binding by 50 % (Column I) was estimated from the dose–response curves for binding competition (Figs. 4–9). The concentration necessary to cause 50 % of the maximum lipolytic effect (Column II) was estimated from similar dose–response curves of free fatty acid release (Figs. 4–6, 9). The incubation conditions for binding and lipolysis are described in Methods.

Compound Tested	Concentration (M) required for 50 % maximum effect	
	I. Binding	II. Lipolysis
Norepinephrine	$6.3 \cdot 10^{-7}$	$1.1 \cdot 10^{-6}$
Epinephrine	$1.1 \cdot 10^{-6}$	$7.4 \cdot 10^{-7}$
Isoproterenol	$1.6 \cdot 10^{-6}$	$6.6 \cdot 10^{-7}$
Dopamine	$8.5 \cdot 10^{-7}$	no effect at 10^{-4}
Dopa	$2.0 \cdot 10^{-6}$	no effect at 10^{-4}
Metaraminol	$5.6 \cdot 10^{-6}$	no effect at 10^{-4}
Phenylephrine	$1.1 \cdot 10^{-5}$	no effect at 10^{-4}
Ephedrine	no effect at 10^{-4}	no effect at 10^{-4}
Phentolamine	no effect at 10^{-4}	no effect at 10^{-4}*
Propranolol	no effect at 10^{-4}	$5.2 \cdot 10^{-7}$*
Normetanephrine	**	no effect at 10^{-4}

* tested for inhibition in presence of 10^{-5} M epinephrine

** at 10^{-4} M, binding of ^{3}H-norepinephrine was enhanced by 17 % (see Fig. 9)

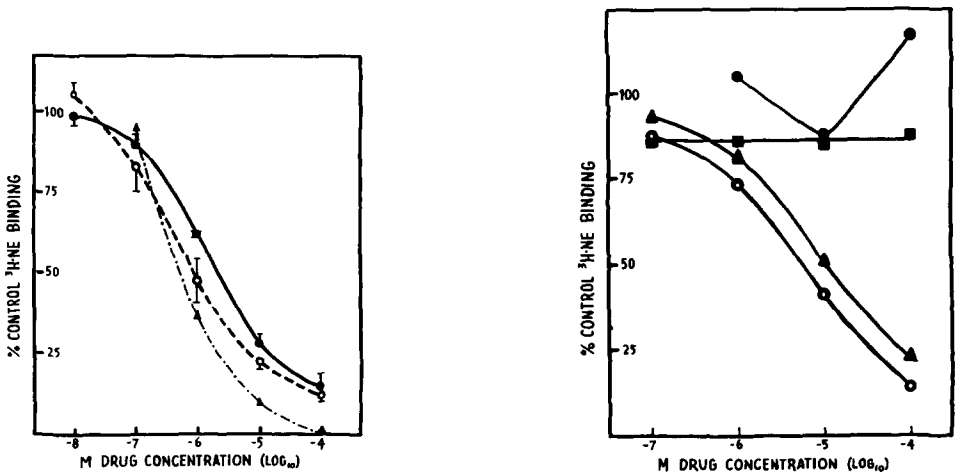


Fig. 7. Inhibition of [^{3}H]norepinephrine binding to fat cells by unlabeled dopa (●) and dopamine (○). Norepinephrine (▲) is also shown for comparison. Conditions of the incubations were as described in Fig. 5 for binding except that dopa or dopamine replaced norepinephrine as the competing drug.

Fig. 8. Inhibition of [^{3}H]norepinephrine binding to fat cells by unlabeled analogs. Conditions of the incubation were as described in Fig. 5 for binding, except that metaraminol (○), phenylephrine (▲), ephedrine (■), or normetanephrine (●) were used instead of norepinephrine as the competing drug. Each point is the mean of 4–6 determinations from 2–6 experiments. For simplicity, S.E.M.'s are not included but were usually $\pm 4\%$.

Phenylephrine, an alpha-adrenergic agonist lacking the catechol ring hydroxyl in the 4 position, but having a beta-carbon hydroxyl, was not effective either as an inhibitor of binding or as a stimulator of lipolysis at 10^{-4} M. Metaraminol and ephedrine, indirectly active compounds, and normetanephrine, an *o*-methylated metabolite, were also tested. These compounds are structurally similar to phenylephrine in that the catechol ring is altered, but the usual beta-carbon hydroxyl group is present on the ethanolamine moiety. These drugs did not stimulate lipolysis (Table I), nor did they compete effectively for [^{3}H]norepinephrine binding sites (Fig. 8). In fact, normetanephrine at high concentrations (10^{-4} M) enhanced binding by an average of 17%; similar results have been noted for the effect of metanephrine on [^{3}H]epinephrine binding [7].

Adrenergic antagonists

Phentolamine, an alpha antagonist, did not compete with binding nor affect lipolysis at 10^{-4} M. Propranolol, a beta adrenergic antagonist lacking a catechol ring, did not compete for binding sites at 10^{-4} M but completely inhibited catecholamine-stimulated lipolysis at much lower concentrations (10^{-5} M) (Fig. 9).

Localization of binding sites

To determine whether [^{3}H]norepinephrine-binding occurred at the cell surface, experiments were carried out with norepinephrine covalently linked to agarose beads [8, 17]. At concentrations of $\approx 1.4 \cdot 10^{-4}$ M agarose-bound norepinephrine (the only concentration tested) [^{3}H]norepinephrine binding was more than 50% inhibited

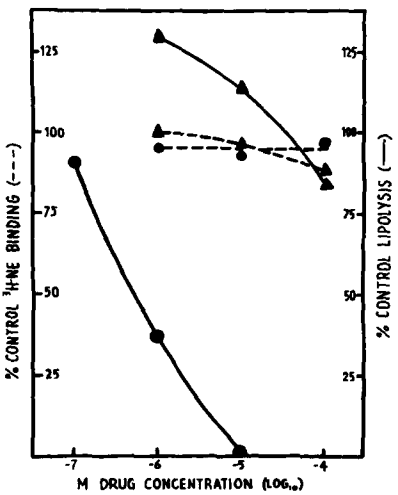


Fig. 9. Inhibition of [^{3}H]norepinephrine binding and lipolysis by adrenergic antagonists. Propranolol (●) and phentolamine (▲) were tested for effectiveness as inhibitors of [^{3}H]norepinephrine binding (- - -) to isolated fat cells; details of the binding assay were the same as in Fig. 5, with details given in Methods. The two antagonists were also tested for effectiveness as inhibitors of free fatty acid release in the presence of 10^{-5} M epinephrine (—). The details of the lipolysis assay are given in Methods. Each point is the mean of 4–6 determinations from 2–6 experiments.

over a control incubation containing unsubstituted agarose. Others have reported that agarose-bound norepinephrine prepared by similar methods stimulates lipolysis demonstrating that this agent is physiologically active [18]. In another study [8], it has been shown that under similar incubation conditions, norepinephrine does not dissociate from the agarose–norepinephrine complex. Examination of the cells incubated with the agarose–norepinephrine complex using phase contrast microscopy showed clearly that agarose–norepinephrine was excluded from the cells by virtue of size and that the complex was associated with the fat cell surface (Fig. 10). The observed inhibition of binding by agarose–norepinephrine presumably reflects surface interaction. Others have recently reported [^{3}H]epinephrine binding to subcellular fractions such as mitochondria and microsomes as well as to plasma membranes [7]. As noted above, free catecholamines are relatively small molecules that might gain access to intracellular sites. Since only about 50 % of [^{3}H]norepinephrine binding was inhibited by $\approx 10^{-4}$ M agarose–norepinephrine, it is possible that the uninhibited [^{3}H]norepinephrine binding was intracellular. The higher concentrations of agarose–norepinephrine ($1.4 \cdot 10^{-4}$ M) versus free norepinephrine ($2.7 \cdot 10^{-6}$ M) required for equivalent inhibition is thus somewhat unexpected, but is probably due to steric restrictions imposed by the agarose–norepinephrine complex. In any case, this result strongly suggests that at least some norepinephrine binding sites are on the cell surface.

DISCUSSION

The classic beta adrenergic agonists norepinephrine, epinephrine, and isoproterenol were effective both as stimulators of lipolysis and inhibitors of [^{3}H]norepi-

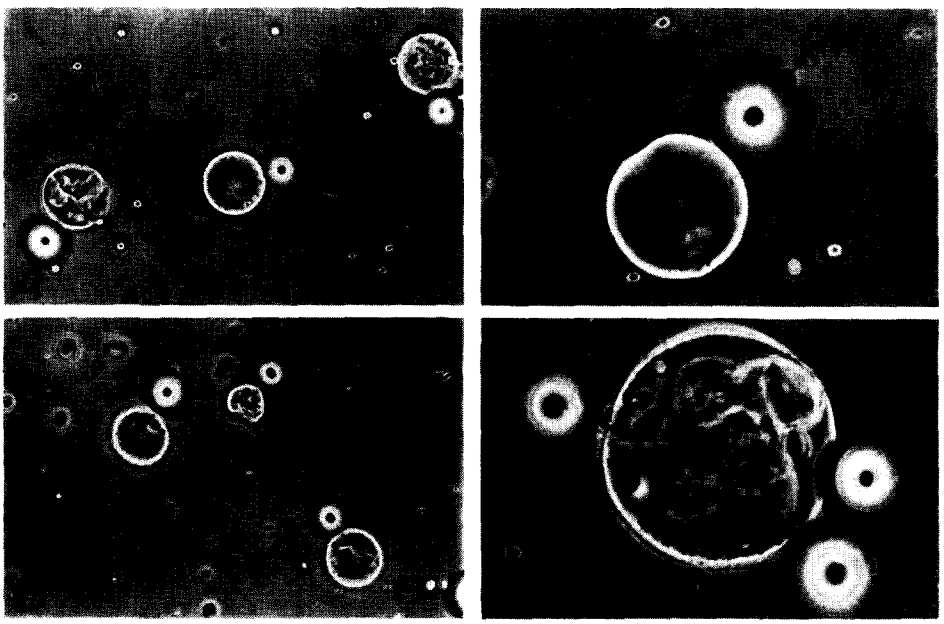


Fig. 10. Phase contrast microscopy of isolated fat cells associated with agarose-norepinephrine. Fat cell nuclei (N) and cell membranes (M) are indicated by arrows. A and B show several cells individually associated with beads (larger spheres). C and D are enlarged views. In D, three cells are associated with the same bead. The bar represents 100 μ. Nonsubstituted agarose did not associate with isolated fat cells.

nephrine binding. The concentrations of each of these agents required to reduce binding by 50 % agreed closely with the concentration required to give 50 % of the maximum lipolytic response in similar preparations of intact cells. Jarett et al., also found agreement in comparing the kinetics of [^{3}H]epinephrine binding to adipocyte membranes with the kinetics of lipolysis [7]. This fulfills an important criterion for the eventual definition of meaningful biological receptors.

The data indicated that drugs with an intact catechol group were more potent inhibitors of binding than those with altered catechol OH groups. The integrity of the ethanolamine portion of the catecholamine molecule appeared less important as a requirement for binding. Similar observations have been made in two recent studies of catecholamine binding to adipocyte membranes [2, 7] as well as in the other systems, e.g. chick embryo myoblasts [8] and membrane fractions from rat liver [19], turkey erythrocytes [20, 21] and dog heart [22, 23].

Propranolol was an ineffective competitor of [^{3}H]norepinephrine binding. Under conditions similar to those in this study, higher concentrations of propranolol ($> 10^{-4}$ M) did inhibit [^{3}H]norepinephrine binding to isolated chick myocardial cells and adipocyte and myocardial membranes, as well as [^{3}H]isoproterenol binding to erythrocyte membranes [7, 8, 21, 22]. Propranolol is structurally dissimilar to catecholamines with respect to the catechol moiety, but the ethanolamine group closely resembles that of the potent beta-agonists. The importance of the catechol ring as a requirement for binding is again underscored.

Both an intact catechol ring and a beta carbon OH appeared to be required for stimulation of lipolysis. Thus, dopamine and dopa, both of which lack the beta-carbon OH were ineffective at 10^{-4} M even though the characteristic catechol moiety was present. Analogs with the opposite characteristics, i.e. lacking the complete catechol ring but having a beta-carbon OH and an otherwise typical ethanolamine side chain were also ineffective in stimulating lipolysis.

On the other hand, propranolol was shown to be an effective inhibitor of catecholamine-stimulated lipolysis. The binding inhibition data suggest that this blocking effect of propranolol is not due to competition with norepinephrine for binding sites with a predominant specificity for the catechol portion of the catecholamine molecule. Drugs like propranolol might be envisaged as interfering with the interaction of the ethanolamine moiety of the beta-agonist molecule at a separate portion of the receptor site [21, 23]. Full biological activity of beta-adrenergic agonists appears to require both the "catechol" and "ethanolamine" functions. An antagonist drug could conceivably work by interfering with agonist binding at either the catechol or ethanolamine portions of the molecule, as has been suggested by Bilezikian and Aurbach [20]. Binding at the catechol-specific portion of the sites would thus still be intact, at a time when propranolol had abolished the biological activity of the agonist by interfering with the "ethanolamine" interaction. If this interpretation is correct, one might expect compounds like dopa and dopamine to compete with catecholamines for the catechol-specific portion of the site and thus potentially act as antagonists. This prediction can be evaluated from data in at least two independent reports: in turkey erythrocytes membranes, catechol compounds apparently did inhibit catecholamine-stimulated adenyl cyclase activity [20]. However, others reported that catechol compounds did not inhibit catecholamine-stimulated adenyl cyclase activity in turkey-erythrocytes membranes nor glycerol release in fat cells [3]. In the interest of resolving this question, we intend to carry out further studies in our cell preparations in order to test a series of drugs lacking either the catechol or ethanolamine moiety of typical catecholamines for the ability to inhibit lipolysis and adenyl cyclase activity in the presence of epinephrine.

Recently it has been suggested that [^{3}H]norepinephrine binding might represent interaction with catechol-*o*-methyl transferase [2] (EC 2.1.1.6.). However, a comparison of subcellular localization of catechol-*o*-methyl transferase activity and [^{3}H]catecholamine binding in adipocytes shows that cathechol-*o*-methyl transferase activity is clearly localized in the plasma membrane fraction [24] while catecholamine binding is distributed uniformly in mitochondria, microsomes and plasma membranes [7]. Further study is needed to clarify the biological significance of binding to subcellular fractions, but the comparison shows that catechol-*o*-methyl transferase interaction and catecholamine binding are not simply related. A similar comparison has been made for liver. In this tissue catecholamine binding is localized in plasma membranes [19] but catechol-*o*-methyl transferase activity is localized primarily in a $78\,000 \times g$ supernatant fraction [25]. Other points that would seem to caution the interpretation of [^{3}H]norepinephrine binding as interaction with catechol-*o*-methyl transferase have been presented elsewhere [26].

The physiological significance of the [^{3}H]norepinephrine binding sites is not clear at the present stage of investigation. On the one hand, the binding characteristics agree in some respects with the expected behavior of beta adrenergic receptors: Potent

agonists such as norepinephrine, epinephrine, and isoproterenol are also potent inhibitors of [^{3}H]norepinephrine binding. The affinities for binding and physiological effect (lipolysis) agree closely. Indirectly active compounds, metabolites, as well as alpha adrenergic agonists and antagonists are ineffective both as inhibitors of [^{3}H] norepinephrine binding and as stimulators of lipolysis. On the other hand, certain observations appear to be inconsistent with the pharmacological characteristics of beta adrenergic interaction: Dopa and dopamine did not stimulate lipolysis but were potent inhibitors of binding. Propranolol was an effective lipolytic inhibitor, but had no effect on binding.

In any case, these binding sites differ in their specificity from all other known catecholamine binding sites [26] and are apparently not related to catechol-*o*-methyl transferase. The data show the sites clearly bind catecholamines over precisely the concentration range where their effects are exerted and hence may be involved in some way in regulating physiological response.

Much work remains to be done before biological adrenergic receptors can be unequivocally defined by classic binding techniques. The use of a homogeneous population of cells is advantageous for the study of catecholamine binding sites. Fat cells should continue to be especially useful, since their behavior is well characterized and easily quantitated at several levels of cellular organization.

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