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SURFACE INTERACTION OF [^{3}H]NOREPINEPHRINE WITH CULTURED CHICK EMBRYO MYOCARDIAL CELLS

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SUMMARY

Cultured chick embryo cardiac myoblasts specifically bind [^{3}H]norepinephrine. The binding is rapid and reversible. Bound [^{3}H]norepinephrine, dissociated by 1 M HCl, can be rebound to fresh cells. β-Adrenergic catecholamines were most potent in displacing [^{3}H]norepinephrine from the cellular binding sites. The binding reaction did not show stereospecificity. α-Adrenergic amines were much less potent. Propranolol, but not phentolamine, competed for the sites. Approximately $2.5 \cdot 10^6$ specific binding sites are present per myocardial cell. The sites appear to be present predominantly at the cell surface in that norepinephrine linked to agarose beads competes for the sites. Similarly, the sites were degraded by either trypsin or trypsin bound to agarose. Two different binding constants, $K = 2 \cdot 10^6$ and $1 \cdot 10^5$, were observed. Proteolytic enzymes decreased binding whereas certain phospholipases led to an increase in specific binding. Divalent cations at concentrations > 1 mM diminished binding as did chelating agents.

INTRODUCTION

The actions of catecholamines on a variety of tissues, including the heart, appear to be mediated via binding to specific β-adrenergic receptors and activation of the enzyme adenyl cyclase [1]. Recently, studies from this and other laboratories have described characteristics of binding of [^{3}H]norepinephrine or [^{3}H]epinephrine to membrane fragments from heart [2–6], liver [7–9], erythrocytes [10,11], and splenic capsule [12] which have many of the properties to be expected of binding to physiologic adrenergic receptor binding sites. The in vitro binding was generally reversible and of high affinity. The affinity of catecholamines for the binding sites generally reflected their in vivo biologic effects or their capacity to activate the enzyme adenylate cyclase present in the same membrane fractions.

In order to study binding of catecholamines to myocardial cells in a more intact form we have investigated the properties of the interaction of [^{3}H]norepinephrine with cultured chick embryo cardiac myoblasts [13]. The advantages of such a system are: (1) the cells used for binding studies are morphologically intact and can be quantitated easily; (2) the pharmacologic characteristics of catecholamine

action on these cells (increased rate of beating) have been previously well characterized and provide data for comparison [14]; and (3) the cells are reasonably homogeneous and free of symphathetic nerves and blood vessels.

MATERIALS

(±)-[7-^{3}H]Norepinephrine, 10–15 Ci/mmole was from New England Nuclear Company. (±)-Isoproterenol bitartrate and (+)- and (−)-norepinephrine bitartrate were obtained from Sterling Winthrop. (−)-Epinephrine bitartrate, dopamine hydrochloride (dihydroxyphenethylamine), (±)-dihydroxyphenylalanine, and all enzymes were from Sigma. Agarose–trypsin, 73.8 mg/g dry weight, was obtained from Worthington Biochemicals. (±)-Propranolol was a gift from Ayerst; phentolamine was from Ciba. Norepinephrine was covalently linked to agarose via a 30-Å side arm by previously published methods [4].

METHODS

Chick embryo myoblasts were isolated from pooled 12–13-day chick embryo hearts. After trimming to remove connective tissue, the cells were minced and dissociated with trypsin by a modification [15] of the method of DeHaan [16]. Cells were grown in 150-mm petri dishes in F-12 media (Grand Island Biological Company) supplemented with 5% heat-inactivated fetal calf serum under 95% air and 5% CO_2. After three days of growth, the cells were harvested by gentle scraping and suspended in 0.25 M sucrose containing 20 mM K^+ phosphate buffer, pH 7.4. Cells were washed once and finally suspended in 0.25 M sucrose phosphate buffer. Photomicrographs of the cells are shown in Fig. 1.

Binding was studied by incubating [^{3}H]norepinephrine at concentrations of $3 \cdot 10^{-9}$–$5 \cdot 10^{-9}$ M with aliquots of myocardial cells ($5 \cdot 10^5$–$7.5 \cdot 10^5$ cells/ml) for 30–60 min at 37° C. [^{3}H]Norepinephrine bound to the cells was quantitated by rapid

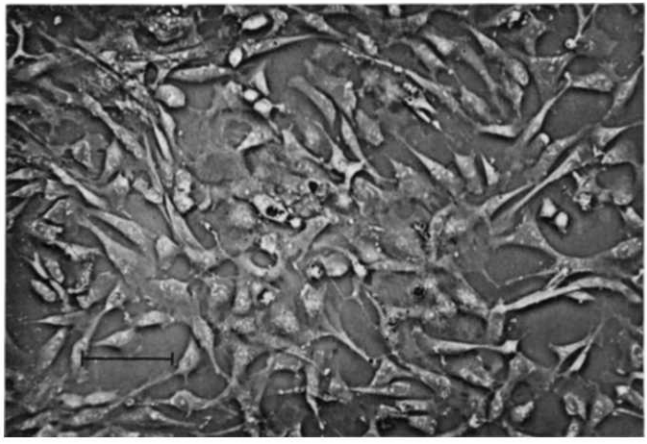

Fig. 1. Photomicrograph of chick embryo myoblasts used for binding studies. Bar represents 100 μm.

Millipore filtration of each incubation mixture followed by a 20-ml buffer wash. The filters (Millipore Company, 0.45-μm pore size) were then dissolved in scintillation fluid [17] and counted in a Packard liquid scintillation spectrometer. Under these conditions, less than 0.5% of the filtered [^{3}H]norepinephrine was found on the Millipore filters when either no cells were included in the incubation or when an excess (10^{-4} M) of unlabeled norepinephrine was included in the incubation to block binding of [^{3}H]norepinephrine to specific sites on the cells. Such controls were performed in each experiment and were subtracted from each experimental value to determine the amount of [^{3}H]norepinephrine specifically bound to the cells. In the absence of added drugs, the amount of [^{3}H]norepinephrine specifically bound to the cells was generally 8–10 times the nonspecific binding. In figures and tables "control binding" refers to the amount of [^{3}H]norepinephrine bound in the absence of added compounds or treatments. This was generally in the range of 2.2–2.8 pmoles/mg protein.

The surface area of the myocardial cells was estimated in the following manner. Cells were photographed against a 10000-μm^2 grid with 400 × magnification. Typical myocardial cells were then traced using a fine, uniform quality paper and the tracings cut out and weighed. The weights of the cutout traced cells were then compared with the weight of a similar cutout tracing of the 10000-μm^2 grid. The surface area of the myocardial cells was taken as a fraction of the weight of the cell tracings to that of the standard grid and multiplied by two. This procedure probably underestimates the true surface area in that the cell is assumed to have only two sides. Partial refoldings of the cell membrane which might increase the surface area are also ignored in these estimates.

RESULTS

Binding of [^{3}H]norepinephrine to the myocardial cells was directly proportional to the number of cells present (Fig. 2). As is shown in Fig. 3, equilibrium binding was reached within 15 min at 37 °C. The process was reversible as indicated by the very rapid dissociation of bound [^{3}H]norepinephrine when 10^{-4} M labeled norepinephrine was added to the incubation (Fig. 4). Approximately 75% of the bound [^{3}H]norepinephrine was rapidly displaced, followed by very little further dissociation during the time period studied. One hundred percent of the bound [^{3}H]norepinephrine could be dissociated after equilibrium binding by addition of 1 M HCl. The norepinephrine displaced by HCl could be rebound to fresh myocardial cells. There was no alteration in the binding properties of the dissociated norepinephrine as compared to native [^{3}H]norepinephrine.

Fig. 5 shows the results obtained when increasing amounts of [^{3}H]norepinephrine were incubated with cells. The maximum amount bound was 66000 ± 8000 dpm/ml of incubation mixture, which corresponds to 0.03 μCi [^{3}H]norepinephrine bound. Since the specific activity of the [^{3}H]norepinephrine was 13.7 Ci/mmole, 0.03 μCi is equivalent to $2.2 \cdot 10^{-12}$ moles [^{3}H]norepinephrine bound or, multiplying by $6.023 \cdot 10^{23}$, $1.33 \cdot 10^{12}$ molecules bound. Since the average number of cells in these experiments was 543000 per ml incubation, approximately $2.5 \cdot 10^6$ molecules were bound per cell. If it can be assumed that one molecule combines with one binding site, then the number of available binding sites per cell is $2.5 \cdot 10^6$.

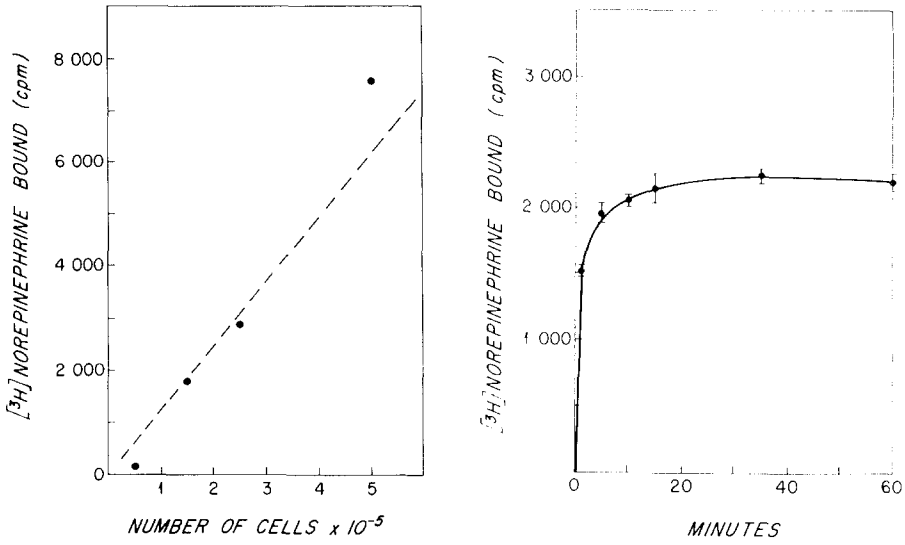

Fig. 2. Relation of binding to cell density. Incubation conditions were as described under Methods. Each point is the mean of duplicate determinations.

Fig. 3. Time course of binding of [^{3}H]norepinephrine to cultured myocardial cells. Each point represents mean ± S.E. of triplicate determinations.

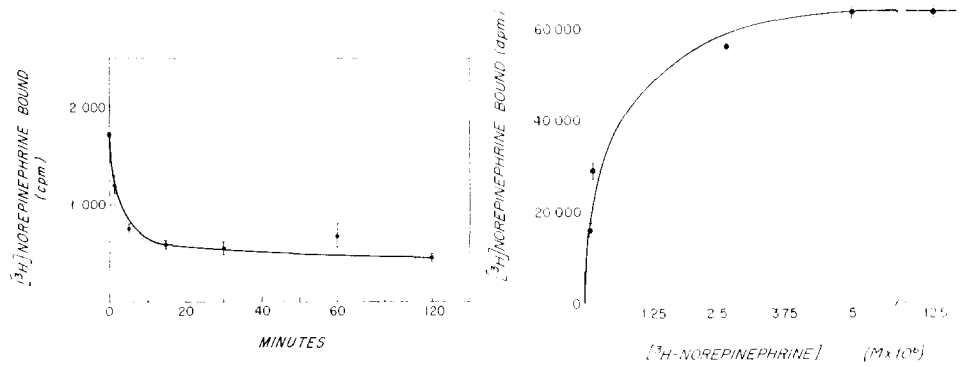

Fig. 4. Time course of dissociation of [^{3}H] norepinephrine from cultured myocardial cells. Incubations were carried out at 37 °C for 30 min. At this time, 10^{-4} M unlabeled norepinephrine was added to the incubations (marked 0 time) and the amount of [^{3}H]norepinephrine bound was determined serially. Each point is the mean ± S.E. of three determinations.

Fig. 5. Dependence of [^{3}H]norepinephrine binding on concentration of [^{3}H]norepinephrine. For each concentration of [^{3}H]norepinephrine tested, [^{3}H]norepinephrine specifically bound to the cells was determined by millipore filtration as described under Methods. Control experiments were performed in which each incubation was performed in the presence of 10^{-4} M unlabeled norepinephrine to block all the specific binding sites. In the presence of the unlabeled norepinephrine, a small constant percent of the added cpm (approx. 0.5% or less) was found on the Millipore filters. This value was subtracted from each experimental value to determine the amount of [^{3}H]norepinephrine actually specifically bound at each concentration. Each point is the mean ± S.E. of four determinations.

Fig. 6 shows a Scatchard plot [18], the data for which were obtained by adding increasing amounts of unlabeled norepinephrine to incubations containing a fixed amount of [^{3}H]norepinephrine. Two distinct binding affinities are defined: a higher one corresponding to a K of $2 \cdot 10^6$–$3 \cdot 10^6$ and a lower one with a K of about $1 \cdot 10^5$. With different batches of cells, the K of the high order sites varied from $2 \cdot 10^6$ to $6 \cdot 10^6$ and the K of the low order site from $1 \cdot 10^5$–$7 \cdot 10^5$. The total number of sites per cell obtained from the Scatchard plot was generally several fold (about 6 ×) higher than that calculated by saturation experiments with [^{3}H]norepinephrine alone.

Unlabeled catecholamines and structurally related drugs competed with [^{3}H]-norepinephrine for occupancy of the sites. The specificity of the binding site was defined by testing a variety of drugs for their ability to compete for the [^{3}H]norepinephrine binding sites. β-Adrenergic agonists, such as isoproterenol, epinephrine and norepinephrine, were most active (Fig. 7) followed by dopamine and dihydroxy-

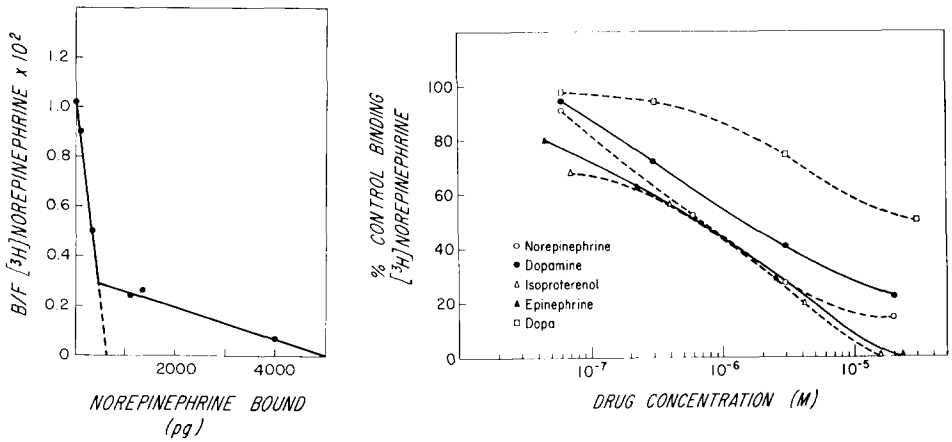

Fig. 6. Scatchard plot for binding of [^{3}H]norepinephrine to cultured myocardial cells. Each point is the mean of duplicates.

Fig. 7. Inhibition of [^{3}H]norepinephrine binding to myocardial cells by β-adrenergic drugs. Labeled norepinephrine and unlabeled drugs were added together and reactions started by addition of membranes. Each point is the mean of four determinations: ○, norepinephrine; △, isoproterenol; ●, dopamine, ▲, epinephrine; □, dihydroxyphenylalanine.

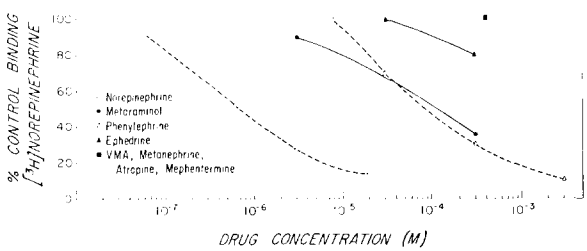

Fig. 8. Inhibition of [^{3}H]norepinephrine binding to myocardial cells by α-adrenergic drugs and other compounds. Labeled norepinephrine and unlabeled drugs were added together and reactions started by addition of membranes. Each point is the mean of three determinations: ○, norepinephrine; ●, metaraminol; △, phenylephrine; ▲, ephedrine; □, vanillylmandelic acid, normetanephrine, atropine, mephentermine, methoxamine, glucagon.

phenylalanine. α-Adrenergic amines and other compounds whose biologic activity is normally due to release of endogenous catecholamines from catechol storage sites were much less active (Fig. 8). A number of metabolites including vanillylmandelic acid and normetanephrine, as well as the cholinergic blocking drug atropine, had no effect on binding at concentrations up to $3 \cdot 10^{-4}$ M.

The β-adrenergic antagonist, propranolol, caused a 40% inhibition of [^{3}H]norepinephrine binding when present at 10^{-4} M, whereas the α-antagonist, phentolamine, had no effect. Binding of catecholamines did not exhibit stereospecificity: thus, as depicted in Fig. 9, the (+) and (−) isomers of norepinephrine competed equally well for binding sites.

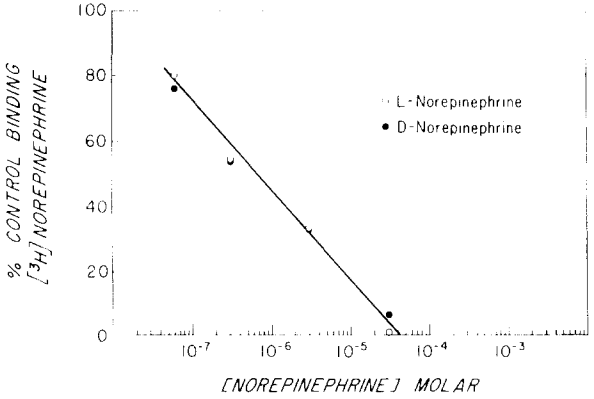

Fig. 9. Inhibition of [^{3}H]norepinephrine binding to myocardial cells by (+) and (−) isomers of norepinephrine. Labeled norepinephrine and unlabeled drugs were added together and reactions started by addition of membranes. Each point is the mean of four determinations: ○, (−)-norepinephrine, ●, (+)-norepinephrine.

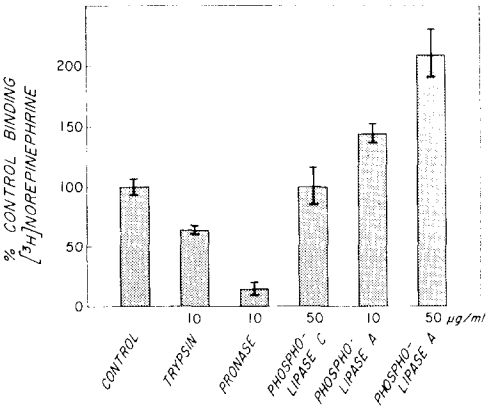

Fig. 10. Inhibition of [^{3}H]norepinephrine binding to myocardial cells by various enzymes. 1-ml aliquots of cells were preincubated for 30 min at 37 C with one of the enzymes at the indicated concentration. Controls were preincubated in a similar fashion without added enzymes. After the preincubation, [^{3}H]norepinephrine was added and the incubation continued for an additional 60 min. Each value represents the mean ± S.E. of four determinations.

The effects of preincubation of the cells with a variety of enzymes on subsequent binding of [^{3}H]norepinephrine is shown in Fig. 10. Proteolytic enzymes, such as trypsin and pronase, inhibited binding. Sulfhydryl reagents, such as *p*-chloromercuribenzoate (PCMB), markedly inhibited binding. PCMB at 1 mM caused an 83%

TABLE I

EFFECTS OF DIVALENT CATIONS AND CHELATORS ON [^{3}H]NOREPINEPHRINE BINDING

"Control" binding was 2.5 ± 0.2 pmoles [^{3}H]norepinephrine bound per mg protein. Each value is the mean ± S.E. of four determinations.

Addition	Concentration (mM)	Percent control binding
Ca^{2+}	0.01	86 ± 4
	0.10	88 ± 4
	1.00	36 ± 3
	10.00	0
Mg^{2+}	0.01	98 ± 5
	0.10	93 ± 4
	1.00	68 ± 4
	10.00	22 ± 2
EDTA	0.1	48.3 ± 4
	1.0	10.6 ± 2
EGTA	0.1	58.7 ± 1
	1.0	23.2 ± 1

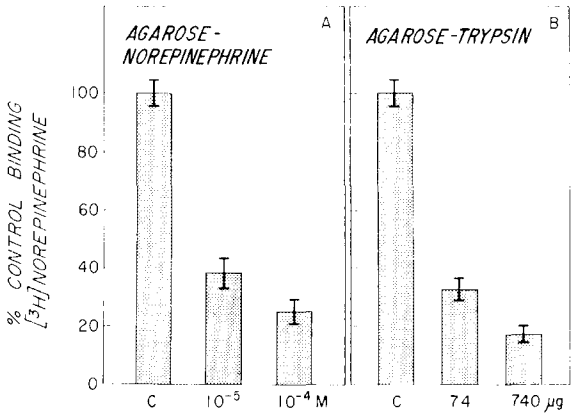

Fig. 11. Inhibition of [^{3}H]norepinephrine binding to myocardial cells by (a) agarose–norepinephrine and (b) agarose–trypsin. In each case, the beads were extensively washed prior to use, then diluted to give the appropriate norepinephrine or trypsin concentrations. [^{3}H]Norepinephrine and agarose derivatives were added simultaneously and the reaction started by addition of membranes. The concentrations in the figure refer to the concentrations of resin-linked norepinephrine or trypsin actually present in the incubations. Agarose–norepinephrine contained approximately 5 mM norepinephrine (settled gel), the agarose–trypsin contained 73.8 mg trypsin per g dry gel. Each value represents the mean ± S.E. of four determinations.

inhibition of binding. Divalent cations also inhibited binding as did the chelating agents, EDTA or EGTA (Table I), suggesting that trace amounts of some divalent cation(s) are necessary for the binding reaction.

To test the hypothesis that the binding sites under study were localized to the cell membrane, [^{3}H]norepinephrine binding was studied in the presence of norepinephrine covalently linked to agarose which assures that only surface interactions can take place. The agarose–norepinephrine inhibited binding (Fig. 11a) by competition with [^{3}H]norepinephrine for membrane sites. In a similar fashion, trypsin bound covalently to agarose beads also inhibited binding presumably because of its proteolytic activity at the cell surface (Fig. 11b). These experiments suggest that the adrenergic binding sites studied are located predominantly on the cell membrane. In control experiments, unsubstituted agarose did not inhibit binding. Stability of the agarose trypsin or agarose–norepinephrine complexes was determined by incubating the complexes with buffer or cells and testing the supernatants for inhibition of binding after removal of the agarose by filtration. The lack of inhibition found indicated that no free norepinephrine or trypsin was released under the incubation conditions.

DISCUSSION

The characteristics of binding of [^{3}H]norepinephrine to cultured myocardial cells are strikingly similar to those previously reported for binding to subcellular membrane fragments from cardiac and other tissues [2–12]. In particular, the following characteristics of the binding observed here have been reported previously for binding of [^{3}H]norepinephrine or [^{3}H]epinephrine to subcellular membrane fragments: (1) Reversible binding which does not alter the biologic activity of the bound amine [4]. (2) Two kinetic orders of sites with binding constants differing by about one order of magnitude [4, 9, 12]. (3) Similar specificity of binding with beta agonists most potent and α-active amines and metabolites inert [2–4, 10, 11]. (4) Inhibition of binding by propranolol but only at concentrations well above those known to cause blockade of β-adrenergic effects in other systems [2–5, 8–12]. (5) Lack of stereospecificity of binding [4, 8, 11]. (6) Inhibition of binding by proteolytic enzymes and sulfhydryl reagents [4, 9]. (7) Inhibition of binding by chelating agents suggesting the possible involvement of trace amounts of divalent cations in the binding process [4]. These may be involved in forming a bridge between the two ring-hydroxyl groups and some portion of the binding site [19].

In addition to demonstrating a striking similarity to the norepinephrine binding sites studied in subcellular particles, the current binding studies with intact myocardial cells provide several new pieces of information. First, the studies with agarose norepinephrine and agarose–trypsin seem clearly to localize the binding sites studied to the cell surface, i.e. the cell membrane. Of interest in this regard are two recent reports demonstrating the biologic activity of insolubilized catecholamine derivatives. Venter et al. [20] reported that epinephrine covalently bound to glass beads was biologically active in increasing the rate of beating of isolated chick embryo myocardial cells (similar to those used in the current studies) as well as the whole heart preparations. Blecher et al. [21] reported that agarose–norepinephrine conjugates were active in causing lipolysis in intact fat cells. Of interest was our observation

(see Figs 6 and 11) that agarose–norepinephrine was only about 10% as active as native norepinephrine in inhibiting the binding of [^{3}H]norepinephrine. This is presumably due to steric factors which prevent full access of all the immobilized norepinephrine molecules to the cell surface.

The biologic significance of the binding observed in these studies cannot be definitely stated at this time. Certain points, however, are clear. The binding is not related to neuronal uptake [22] or nerve storage vesicle uptake [23], as these components are not present in the cell cultures. These neuronal uptake processes have totally different specificities as well. Similarly, this binding process is not the so-called uptake 2 process [22] which operates only at much higher norepinephrine concentrations and which also has a different specificity (normetanephrine, the most potent inhibitor) [22]. Similarly, the binding is clearly unrelated to the catecholamine-metabolizing enzymes since bound [^{3}H]norepinephrine can be dissociated and shown to retain biologic activity. Further, the specificities are different. For example, catechol *O*-methyl transferase has equal affinity for all catechols and a K_M several orders of magnitude higher than that demonstrated here [24]. Monoamine oxidase is clearly not involved since catechol compounds lacking an amino group can bind.

The data are quite consistent with pharmacologic studies performed with similar myocardial cells. Thus, Ertel et al. [14] found that catecholamines stimulated the rate of beating of chick embryo myocardial cells with specificity very similar to that reported here. Norepinephrine gave half maximum stimulation at a concentration of about 40 ng/ml, identical to the concentration giving half maximum displacement of binding in our experiments. In addition, phenylephrine had chronotropic effects only when 50–100 times higher concentrations were used. This finding is also in close agreement with the present results. It has also been demonstrated by others that the rate of beating of intact early chick and rat embryonic hearts can be increased by β-agonists [25]. Thus, the β-receptors are known to be present very early in development.

Two findings in the present study, although in agreement with results previously published for binding studies with subcellular membranes [4], are at variance, however, with what might have been expected for binding of [^{3}H]norepinephrine to physiologic beta adrenergic receptor sites. The first is the very high concentrations of the β-adrenergic blocking drug propranolol, which were needed to block binding, as compared to the very low concentrations of the drug which will block the chronotropic effects of catecholamines on these cells.

A second unexpected feature is the lack of stereospecificity of the binding reaction. (−)-forms of catecholamines are generally 5–20 times more active than (+)-forms [26], although the actual ratio of potencies vary very widely from one species to another, and data on this point have not been published for isolated myocardial cells grown in tissue culture. This finding is similar to the documented lack of stereospecificity previously found for binding of [^{3}H]norepinephrine to myocardial membranes and [^{3}H]epinephrine to purified liver plasma membranes as well as [^{3}H]isoproterenol binding to erythrocyte membranes [4,8,11]. The significance of this finding is not clear at present. The catecholamines presumably interact with β-adrenergic receptor sites and adenyl cyclase in vivo at several different points of contact [27]. Likely sites are the ring hydroxyls, the β-carbon OH and the N terminus. It is possible that in these in vitro studies major contact is made at only one or two of

these potential contact points so that stereospecificity at the β-carbon is less important.

This interpretation is similar to the suggestion of Bilzekian and Aurbach [11] that activation of adenyl cyclase by β-active catecholamines may require binding at two distinct sites — one specific for the catechol nucleus, the other for the ethanolamine portion of the molecule. The binding reflected in these and similar studies would be primarily to the catechol specific site. Adrenergic blockers like propranolol might interact at the ethanolamine site and hence be relatively weak inhibitors of this in vitro binding. The finding of Bilzekian and Aurbach that non-physiologically active catechol compounds inhibit isoproterenol-stimulated cyclase in erythrocytes is consonant with this suggestion [11].

An additional new finding with this system is the data permitting calculations of the number of binding sites per cell. When this was experimentally determined by adding increasing amounts of [^{3}H]norepinephrine to cells, a figure of approximately $2.5 \cdot 10^6$ sites per cell was obtained. However, when total binding sites per cell is calculated from the intercept of the Scatchard plot (Fig. 6), a number several fold higher is obtained. The exact significance of this discrepancy is not entirely clear. The number of sites reflected by the Scatchard plot represents the total of all binding sites of both low and high order. Perhaps the experiments with [^{3}H]norepinephrine reflect interaction primarily with the high order sites. Another consideration is that the intercept on the Scatchard plot represents an extrapolation from that portion of the curve which is most inaccurate since the percent binding is the lowest. On the other hand, the saturation curve with [^{3}H]norepinephrine should be most accurate at the higher concentrations of [^{3}H]norepinephrine since the absolute number of cpm bound is highest. From photomicrographs of these chick embryo myocardial cells, the size of a typical cell has been estimated (see Methods). The surface area of such a cell is approx. 4,474 μm^2. $2.5 \cdot 10^6$ sites/cell would then correspond to approx. 560 sites/μm^2 surface area if all the sites were on the cell surface. If we assume there are about $3 \cdot 10^8$ muscle cells/g [28], there would then be approx. $7.25 \cdot 10^{14}$ sites/g of cells or approx. 1.2 nmole sites/g.

It is of interest that this estimate of $7.25 \cdot 10^{14}$ sites/g is remarkably close to several estimates of the number of adrenergic receptors actually present in various tissues. Thus Clark estimated 10^{14} epinephrine receptors per g of tissue [29], Lewis and Miller found $1.7 \cdot 10^{13}$ adrenergic receptors per g of seminal vesicle smooth muscle by labeling with [^{3}H]phenoxybenzamine [28], and May et al. [30] found $1.15 \cdot 10^{12}$ sites/mg protein in the aorta by labeling with *N,N*-[^{3}H]dimethyl-2-bromo-2-phenylethylamine [30]. In view of the number of assumptions involved in these calculations and the markedly different techniques used, the agreement is striking.

As with studies of membrane fragments, two orders of binding sites were found with association constants differing by about one order of magnitude. Two or more distinct orders of binding sites have also been found for interaction of insulin [31] and adrenocorticotropin [32] with plasma membranes as well as for the binding of cholinergic ligands to cholinergic binding sites [33]. It is not presently clear whether both types of sites are physiologically significant or what the relation of the two sites to each other is.

The finding that the binding site is a protein with crucial SH groups is similar to findings in cardiac and liver membranes [4,8]. The apparent increase in binding

after exposure of the cells to phospholipase A is quite similar to the enhanced binding of [^{125}I]insulin by fat cells after phosphooipase C or A digestion recently reported by Cuatrecasas [34]. Studies are currently in progress to determine if the properties of these newly exposed sites differ in any way from those normally available.

The observation that chelating agents have marked inhibitory effects on the binding is consistent with the suggestion of Belleau [19] that the two catechol ring OH groups may link to some component of the receptor via formation of a bridge through a divalent cation.

The ability of norepinephrine and trypsin covalently linked to agarose to interact with the sites strongly suggests that they are located on the outer surface of the cell. In view of current concepts which localize adenyl cyclase to cell membranes and the close relationship of β-adrenergic receptors and adenyl cyclase, this seems entirely reasonable. It will be noted that the agarose–norepinephrine was only about 10% as active as equimolar amounts of unaltered norepinephrine. This is presumably due to the steric constraints imposed by linkage of the N terminus of the molecule to the gel matrix [35].

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