Epinephrine Binding to the Catecholamine Receptor and Activation
of the Adenylate Cyclase in Erythrocyte Membranes
(hormone receptor/β-adrenergic receptor/cyclic AMP/turkey)

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ABSTRACT Turkey erythrocyte membranes showed specific binding of [3H]epinephrine. The concentration of hormone required for half-maximal binding (30 µM) was the same as that required for half-maximal activation of the adenylate cyclase located in the same membrane preparation. The binding reaction at 37°C reached completion during the first minute of incubation, which agrees well with the known rapidity of the biological response to catecholamines. Specific binding was abolished by heating the membranes 1 min at 100°C. Chromatography of the bound [3H]epinephrine, after its extraction from the membranes, indicated that the hormone had retained its chemical structure. Epinephrine binding was inhibited by the β-adrenergic blocking agent propranolol, which also inhibited the activation of adenylate cyclase by the hormone. The specificity of phenethylamine derivatives in displacing [3H]epinephrine from the binding sites showed that a typical catecholamine receptor was responsible for the binding. Displacement of the bound hormone by analogs lacking the catechol group was more extensive at 37°C than at 0°C. Some of the analogs that displaced epinephrine from the binding site caused only a feeble activation of the adenylate cyclase, but were able to inhibit the activation of the enzyme by epinephrine. Thus, binding to a catecholamine receptor on a membrane preparation is an essential, but insufficient, condition to elicit a response.

Knowledge about cell receptors for hormones and neurotransmitters has mostly been obtained indirectly by study of the responses of tissues to the active agents. To date, none of the hormone receptors residing in cell membranes has been defined in molecular terms. However, recent information has been obtained about the specific binding of ACTH (1), glucagon (2), acetylcholine (3, 4), and insulin (5) to membrane preparations containing the respective receptors for these hormones. In the work with ACTH by Lefkowitz et al. (1), and particularly in the extensive studies with glucagon by Rodbell et al. (2), convincing evidence was obtained that binding indeed occurred at the true, functional receptor, i.e., the preparation retained the hormone-responsive adenylate cyclase.

It seemed to us of particular importance to study the binding of the catecholamines, epinephrine and norepinephrine, to the receptor for the following reasons. The catecholamine receptors are ubiquitous, being present in almost every cell type of a mammal. The catecholamines act not only as hormonal hormones, but also as neurotransmitters in the brain and in other nerve cells. There are at least two types of receptors for catecholamines, α- and β-, defined on the basis of responses in the intact cell; the nature of the difference between these two receptors at the molecular level is unknown. The β-receptor activates, and appears to be part of, the adenylate cyclase system (6). Its mechanism of action can, therefore, be studied in a cell-free membrane preparation. The relative specificity of the catecholamine receptor with respect to different hormone derivatives varies widely from tissue to tissue for unknown reasons (7). An almost inexhaustible collection of catecholamine derivatives is available to test the function of each chemical group in the molecule in the binding reaction and in the activation that results from such binding.

There are major obstacles that render the study of catecholamine binding to its receptor quite difficult. Hormones of this group have a relatively low affinity for the receptor, and readily dissociate from it during washing (8, 9). They undergo spontaneous oxidation at alkaline pH, are taken up for storage at nerve endings, and are readily metabolized by most tissues (10). There have been, therefore, few attempts to study attachment of the hormone to the receptor. Binding of labeled epinephrine to liver plasma membranes has been reported (11). The pH optimum for binding was 10.5, and the bound radioactivity was not released by 5% trichloroacetic acid. No evidence was presented to show that the radioactivity bound was epinephrine. Very recently, binding of norepinephrine to heart microsomes was reported to have the specificity of a β-receptor (12). However, there was no information on whether the fraction studied contained the hormone-responsive adenylate cyclase, or how much of the bound radioactivity was unchanged norepinephrine. Previous work on heart microsomes by other investigators had demonstrated that much of the bound material after incubation with [3H]norepinephrine was composed of products of the monoamine oxidase (EC 1.4.3.4) reaction and other reactions (13).

For the present study, turkey erythrocyte membranes were chosen since Øye and Sutherland showed an epinephrine-responsive adenylate cyclase in this system (14). The cellular purity, the ease of preparation of cell membranes, the absence of nerve endings that take up epinephrine, and the probable paucity of the usually potent enzymes that metabolize epinephrine appeared to offer great advantages for the erythrocyte system in the study of the catecholamine receptor. This communication reports on the specificity of the receptor for the binding of sympathomimetic amines and activation of the adenylate cyclase.

MATERIALS AND METHODS
[3H]Epinephrine and [3H]ATP were products of New England Nuclear Corp. The various catecholamines and phenethyl-
amines were purchased from Sigma Chemical Co. and K and K(U.S.A.) and from Biotec (Sweden).

Turkey erythrocyte membranes (ghosts) were prepared according to Øye and Sutherland (14). Adenylate cyclase assays were performed in triplicate by the procedure of Krishna and Brodie (15), with minor modifications (9). There was only a trace of enzyme activity in the absence of epinephrine or fluoride. Activation by the hormone was measured as pmoles of cAMP formed when epinephrine was present in the reaction mixture. Activation by a hormone analog was measured similarly, by substituting it for the hormone. Some hormone analogs, when present in the reaction mixture, inhibited the activation caused by epinephrine. This inhibition is expressed as percent decrease of the activation relative to that caused by epinephrine alone. Binding of [3H]epinephrine to the erythrocyte membranes was determined by a method developed for measurement of [3H]cAMP binding (16). Unless otherwise stated, the assays contained erythrocyte membranes, 0.3-2.5 mg of protein, 20 mM of glycylglycine (pH 7.5), 1-10 μM [3H]epinephrine (106 cpnm), with or without further addition of hormone analogs, in a final volume of 1 ml. Incubation was for 10 min at 0°C or 5 min at 37°C. Centrifugation and the determination of bound and free radioactivity in the pellet were described (16). The pellet was usually rinsed once with 20 mM of glycylglycine (pH 7.5) before assay.

Inhibition of binding of [3H]epinephrine by various unlabeled analogs was taken as a relative measure of the binding of these analogs to the receptor. It is expressed as percent decrease of the [3H]epinephrine bound in presence of an analog relative to that bound in its absence.

RESULTS

Incubation of turkey erythrocyte membranes with [3H]epinephrine led to rapid binding of the hormone. The process at 37°C appeared complete in less than 1 min (Fig. 1). At 0°C, 5 min were required for maximal binding. When binding was studied as a function of hormone concentration, it was found that saturation was reached at about 50 μM epinephrine (Fig. 2). Half-maximal binding was at 30 μM (from Fig. 2 and additional experiments). The $K_m$ of the adenylate cyclase for epinephrine in the membrane preparation was measured from a Lineweaver–Burk plot of the data and was also found to be 30 μM. It is possible that at saturating epinephrine concentrations, binding also occurs at sites other than...
of various were used to criteria bound indeed (b) receptor. of sensitivity and fluoride 66 and on the pH 6.8, the therefore, experiments that were performed at 1–10 μM of epinephrine. In order to define the nature of the binding, and in view of the relative instability of epinephrine under oxygen in cell-free extracts, we analyzed the bound radioactive material. About 99% of the radioactivity was readily eluted from the membranes by 0.1 N acetic acid. When the eluted radioactivity was analyzed by thin layer chromatography in two solvents, only one peak, which corresponded in its mobility to epinephrine, was observed (Fig. 3). Some radioactive contaminants present in the stock of original [3H]epinephrine were not bound to the membranes. The recovery of radioactivity on the chromatograms was 90–100%; therefore, the hormone structure remained intact during the binding reaction.

When the membrane preparation was heated 1 min at 98°C at pH 6.8, the binding was completely inactivated. The dependence of activity on pH varied somewhat with the buffer and the temperature. The binding activity did not change much in the pH range from 6.8 to 8.5, but dropped to almost zero at pH 6.0. A test at pH higher than 8.5 is probably not meaningful, because of the instability of epinephrine (10). 0.2 mM β-Hydroxymethylbenzeneacetic had no inhibitory effect on epinephrine binding, but inhibited the adenylylate cyclase 66 and 43% when the enzyme was activated by 10 mM of fluoride and 0.1 mM of epinephrine, respectively. Thus, the sensitivity of adenylylate cyclase to sulfhydryl reagents (9, 14, 18) is probably inherent in the catalytic unit and not in the hormone receptor.

It seemed crucial to establish whether the hormone was indeed bound to a functional catecholamine receptor. Two criteria were used to examine this problem: (a) Specificity of various hormone analogs as inhibitors of [3H]epinephrine binding; (b) Specificity of the hormone analogs as activators of adenylylate cyclase, and also as inhibitors of enzyme activation by epinephrine. We assumed that the effect of a hormone analog was a measure of its specific binding to the receptor. Table 1 shows the specificity of binding at 0°C, in comparison with adenylylate cyclase activation. It is evident that only the true catecholamines, those with a 3,4-dihydroxybenzene ring and an amino group attached to the α-carbon of the side chain, possess a high affinity for the binding site. Absence of one of the 3,4-dihydroxyl groups, as in phenylephrine, or its methylation, as in metanephrine, resulted in a drastic loss of affinity for the binding site. Presence of a carboxyl group on the α-carbon, as in dihydroxyphenylalanine (DOPA), also caused a steep decline in affinity. These findings are in excellent agreement with the well-known specificity of the catecholamine receptors in animal systems (7, 10). No less important was the finding that the specificity of activation of the adenylylate cyclase by hormone analogs was similar to the specificity of binding. However, dopamine, which showed a high binding affinity, demonstrated a relatively low adenylylate cyclase activation, confirming the importance of the β-hydroxyl for the activation process (7).

Some additional observations in Table 1 are noteworthy. Interference with binding caused by the carboxyl group in DOPA is partially overcome either by esterification, as in DOPA methyl ester, or by addition of a β-hydroxy, as in DOPS (see Table 1). Thus, the β-hydroxy can have a contributory function in the binding process that was not evident from comparison of dopamine with epinephrine and norepinephrine. While DOPA methyl ester, DOPS, and serotonin at relatively high concentrations showed some affinity for the binding site, these compounds activated the adenylylate cyclase poorly or not at all (Table 1). It is, of course, possible that a compound binds to the receptor that controls adenylylate
### Table 2. Inhibition by hormone analogs of the adenylate cyclase response to epinephrine, and the effect of temperature on binding to the receptor

<table>
<thead>
<tr>
<th>Hormone analog</th>
<th>Inhibition of binding of [3H]epinephrine, %</th>
<th>Inhibition of the activation of adenylate cyclase by epinephrine, (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0°C</td>
<td>37°C</td>
</tr>
<tr>
<td>Ephedrine</td>
<td>100</td>
<td>20</td>
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<tr>
<td>Phenylephrine</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>Metanephrine</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>DOPA</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>DOPA methylester</td>
<td>100</td>
<td>70</td>
</tr>
<tr>
<td>DOPS</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>Dopamine</td>
<td>30</td>
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<tr>
<td>Serotonin</td>
<td>100</td>
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<tr>
<td>Propranolol</td>
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<tr>
<td>Propranolol</td>
<td>10</td>
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<tr>
<td>Phentolamine</td>
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<td>Phentolamine</td>
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</table>

Epinephrine concentration was 10 μM throughout. The enzyme activity at 10 μM was about 25% lower than that shown in Table 1 at 0.1 mM. All other conditions were as in Table 1. In assays containing only epinephrine, the binding activity at 37°C was either the same as at 0°C or 30% lower, depending on the membrane batch.

cyclase activity, but fails to induce the change required for enzyme activation. In this case, the compound may compete with a true activator for the receptor, and may thus inhibit adenylate cyclase activation. Table 2 demonstrates that a number of compounds inhibit the activation of adenylate cyclase caused by epinephrine. Since adenylate cyclase was measured at 37°C, inhibition of [3H]epinephrine binding was tested at 37°C, as well as at 0°C. Inhibition of binding at 37°C is roughly equivalent to the inhibition of the epinephrine-activated adenylate cyclase in the case of epinephrine, phenylephrine, metanephrine, and serotonin. Dopamine and DOPA by themselves stimulate the adenylate cyclase to some extent (Table 1) and are, therefore, not expected to inhibit the enzyme drastically in the presence of epinephrine. DOPA methyl ester inhibited adenylate cyclase activation by epinephrine, but its effect on binding seemed to be more powerful (Table 2).

Propranolol, a specific β-receptor-blocking agent, caused complete inhibition of the adenylate cyclase when only part of the binding was blocked. Phentolamine, an α-receptor blocking agent, did not inhibit binding when tested at 0.02 mM. At this concentration, it specifically and completely inhibited the α-receptor in rat parotid gland (19). The effect of phentolamine at the higher concentration is probably nonspecific. It is also evident from Table 2 that several compounds inhibit epinephrine more effectively at 37°C than at 0°C. This characteristic appears to be typical of those derivatives that do not possess the catechol structure, the top three compounds in Table 2.

**DISCUSSION**

We used several criteria to ascertain that the binding measured indeed occurs between the hormone and the biologically relevant catecholamine receptor. The bound radioactivity was extracted and analyzed to establish that it was unchanged epinephrine. The specificity of binding of hormone analogs and derivatives was examined to establish whether it fits the gross biological specificity of the catecholamine receptor, as found in other systems (7, 10). Finally, the specificity of hormone binding and of adenylate cyclase activation were compared with each other in the same membrane preparation. The findings support the conclusion that the binding sites measured and characterized indeed belong to a typical catecholamine receptor. The specificity pattern of the binding reaction was quite different from that of the known enzymes involved in the synthesis and breakdown of epinephrine (10), and it is therefore unlikely that these enzymes could have contributed significantly to the binding.

In comparison of the effect of a hormone analog on the binding reaction with its effect on adenylate cyclase activation, it must be remembered that the two processes are measured under different assay conditions. It is, therefore, surprising that the data for binding and enzyme activation show, in most cases, a better fit than can be expected on the basis of the above qualifications. However, the β-adrenergic blocking agent, propranolol, seemed to inhibit the adenylate cyclase activation more extensively than it inhibited the binding of the hormone. One possible explanation is that when propranolol occupies only part of the receptor sites, it prevents activation of the adenylate cyclase at neighboring sites that are still occupied by epinephrine.

Half-maximal binding occurred at 30 μM epinephrine, which was equivalent to the Kₘ for activation of the adenylate cyclase in the erythrocyte membrane. This value is somewhat higher than the Kₘ reported for the fat cell (20) and parotid gland (9) enzymes. It must be emphasized that many of the physiologically important adenylate cyclases, such as those of heart (8), liver (8), and brain (21), become saturated with the catecholamine only at a concentration of 0.1 mM or higher. Therefore, in this respect also, there is no essential difference between the turkey erythrocyte and other catecholamine-responsive systems. It seems reasonable to expect that the findings on the catecholamine receptor of the turkey erythrocyte membrane will be relevant to the action of this receptor in other tissues.

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