Influence of Polymeric Effectors on Binding of 3H-Dihydroalprenolol to β-Adrenergic Receptor of Rat Brain

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Abstract—The significance of anionic and cationic charges of glycocalyx, phospholipid or protein, etc. on the cell surface of the rat brain was examined for β-adrenoceptors using the radioligand binding assay method. Thus, this experiment was designed to assess the effects of polymeric effectors, DNA, heparin, polymyxin B, histone, gelatin, colominic acid and bovine serum albumin (BSA), on the affinity of β-adrenoceptors. The rat brain was used and the β-adrenoceptor binding assay was carried out using 3H-dihydroalprenolol as a radioligand. Polymyxin B, DNA and heparin significantly caused a reduction in the maximum number of β-adrenoceptors (B_max), but only small changes were observed with histone, gelatin, BSA and colominic acid. Only DNA induced a decrease in the value of the dissociation constant (K_d) of β-adrenoceptors. These results suggest that anionic or cationic charges in the environment of the receptor sites could have a crucial role in drug-receptor interaction.

As is well known, the cell surface of the mammalian tissues contains negatively charged sites. Negatively charged surface sites, phospholipids, sialic acid moieties in glycoproteins or glycolipids, and acidic mucopolysaccharide, and positively charged sites of peripheral proteins play a crucial role in the various cellular functions, cell-to-cell recognition, cell aggregation, or drug-receptor interaction, etc. (1). The cardiac muscle cells also have negatively charged sites based on a glycocalyx that is composed of two layers, the surface coat and the external lamina (2). In our previous report (3), it was demonstrated that the treatment of rat heart membrane with neuraminidase or deoxyribonuclease resulted in a good reproducibility of binding data and caused an increase in the binding sites for the β-adrenergic receptors using 3H-dihydroalprenolol as a ligand. On the other hand, these treatments were not required for the β-adrenoceptor binding assay using rat brain homogenates (H. Tsuchihashi et al., unpublished observation). These results suggested that brain membranes were simple compared to heart membranes and that the environment of the receptor site could have a crucial role in the drug-receptor interaction. Therefore, the object of this study is to examine the significance of anionic and cationic charges on the cell surface of rat brain and to assess the effects of polymeric chemicals, which are thought to bind to negative or positive charges on the surface of the cell membranes, on the affinity of β-adrenoceptors.

Materials and Methods
Materials: 3H-dihydroalprenolol (3H-DHA) (104.8 Ci/mmmole) was purchased from New England Nuclear Corp. Albumin from bovine serum (BSA), polymyxin B, colominic acid from Escherichia coli, and histone from calf thymus were purchased from Sigma Chem. Co. (St. Louis, MO, U.S.A.). The deoxyribonucleic acid (DNA) from the calf thymus was purchased from P-L Biochemicals, Inc. (Milwaukee, WI, U.S.A.). The purified gelatin was purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan). The heparin was purchased from Seikagaku Kogyo Co., Ltd.
Membrane preparation: Male Wistar rats weighing between 300–350 g were killed by a blow on the head. After the removal of the brain, the cerebellum and cerebral cortex were minced with small scissors in 250 mM sucrose, 10 mM Tris-HCl, pH 7.6, and were then homogenized using a glass homogenizer. The homogenate was filtered through 4 layers of gauze. The filtrate was centrifuged at 40,000 g for 30 min, and the resultant pellets were rinsed at one time and then homogenized with the glass homogenizer using 20 ml of 75 mM Tris-HCl, 25 mM MgCl₂, pH 8.0. The prepared membrane was stored at 4°C and used within 1 hr.

Binding assay: The β-adrenoceptor binding assay was carried out in duplicate with 3H-DHA in the presence (non-specific) and absence (total) of 100 μM dl-propranolol. For 3H-DHA binding, 0.25 ml of membrane suspension (about 0.25 mg) was incubated with shaking for 30 min at 23°C with 1.2 nM of 3H-DHA and different concentrations of various effectors in a total volume of 0.5 ml containing 60 mM Tris-HCl, 20 mM MgCl₂ (pH 8.0). The Scatchard analyses were carried out in duplicate with various concentrations of 3H-DHA in the presence and absence of 50 μg/ml of various effectors. At the end of the incubation period, the incubation medium was immediately filtered through a GF/C glass fiber filter using an improved method (H. Tsuchihashi et al., unpublished observation), which was continuously filtered, washed for 2 sec (about 1 ml of cold buffer), and dried for 30 sec. The filters were added to 5 ml of a Tl76 scintillation fluid. The difference in mean values between total and non-specific binding was taken as the specific binding. All binding assays were performed within 3 hr from the removal of the brain from the rat. Protein was determined by the method of Lowry et al. (4). Significant differences were analyzed using Student’s t-test.

Results

The yield of the membrane protein with the present method from the rat brain was 75.42±1.15 mg per g wet weight (n=16). Preliminary experiments were performed in order to standardize the β-adrenergic receptor binding assays with the membrane preparations. The specific binding of 3H-DHA (1.2 nM) to the membrane fraction was linear.

![Fig. 1. Effects of polymyxine B for β-adrenoceptor binding. The data represent the mean±S.E. of duplicate determinations from five separate experiments. Total (---), specific (---) and non-specific (---) binding. *P<0.05, ***P<0.001 vs. without polymyxin B.](image-url)
with protein concentrations below 0.75 mg per incubation when the antagonist (dl-propranolol) was used at a saturating concentration. Total, non-specific and specific bindings at 23°C were rapid, reaching steady-state within 10 min when 1.2 nM \(^3\)H-DHA was used.

The data presented in Figs. 1–4 show the

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**Fig. 2.** Effects of heparin for β-adrenoceptor binding. The data represent the mean±S.E. of duplicate determinations from five separate experiments. Total (——), specific (— —), and non-specific (— ▲ —) binding. *P<0.05, **P<0.02, ***P<0.001 vs. without heparin.

**Fig. 3.** Effects of DNA for β-adrenoceptor binding. The data represent the mean±S.E. of duplicate determinations from five separate experiments. Total (——), specific (— —), and non-specific (— ▲ —) binding. *P<0.05, **P<0.02, ***P<0.001 vs. without DNA.
effects of increasing concentrations of polymyxin B (Fig. 1), heparin (Fig. 2), DNA (Fig. 3) and histone (Fig. 4) on the \( \beta \)-adrenoceptor binding. The binding of \(^3\)H-DHA to brain membranes was inhibited by 30–40\%, 30–40\% and 20–70\% when the membranes were incubated with over 4 \( \mu g/\)mg of membrane protein of heparin, over 40 \( \mu g/mg \) of membrane protein of polymyxin B, and over 40 \( \mu g/mg \) protein of DNA, respectively. The addition of gelatin, BSA and colominic acid induced no changes of drug-receptor interaction. The addition of histone increased the amount of non-specific and total binding, but no change in specific binding was observed when the membranes were incubated with over 400 \( \mu g/mg \) membrane protein of histone.

Table 1 shows data demonstrating the influence of the addition of these effectors on \( \beta \)-adrenoceptors obtained by Scatchard analysis. Polymyxin B, DNA and heparin significantly caused the reduction (inhibition by 31\%, 67\% and 27\%, respectively) in the density of \( \beta \)-adrenoceptors (\( B_{\text{max}} \)), but only slight changes were observed with histone.

![Fig. 4. Effects of histone for \( \beta \)-adrenoceptor binding. The data represent the mean±S.E. of duplicate determinations from five separate experiments. Total (■—■), specific (○—○), and non-specific (▲—▲) binding. *P<0.05 vs. without histone.](image_url)

<table>
<thead>
<tr>
<th>Numbers of experiment</th>
<th>( K_d ) (nM)</th>
<th>( B_{\text{max}} ) (fmole/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.05±0.86</td>
<td>372.10±20.24</td>
</tr>
<tr>
<td>DNA</td>
<td>4.06±0.72**</td>
<td>158.20±27.06***</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>8.46±1.09</td>
<td>258.74±15.91***</td>
</tr>
<tr>
<td>Heparin</td>
<td>6.21±0.33</td>
<td>272.62±26.28**</td>
</tr>
<tr>
<td>Colominic acid</td>
<td>8.27±0.63</td>
<td>316.94±34.00</td>
</tr>
<tr>
<td>BSA</td>
<td>10.48±0.72</td>
<td>379.42±35.40</td>
</tr>
<tr>
<td>Histone</td>
<td>11.26±1.12</td>
<td>306.91±30.44</td>
</tr>
<tr>
<td>Gelatin</td>
<td>13.10±3.94</td>
<td>316.43±46.62</td>
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</tbody>
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*All effectors were added at a concentration of 100 \( \mu g/mg \) protein of membrane. **P<0.02 vs. control. ***P<0.001 vs. control. Numbers are given as the mean±S.E.
Fig. 5. Lineweaver-Burk plots of \( \beta \)-adrenoceptor binding using the rat brain membrane with or without addition of DNA, heparin and polymyxin B. (a) no-addition, (b) addition of heparin, (c) polymyxin B, and (d) DNA.

gelatin, BSA and colominic acid. Only DNA induced a decrease in the value of the dissociation constant \( (K_d) \) of \( \beta \)-adrenoceptors.

Figure 5 shows the Lineweaver-Burk plots of the drug-receptor interaction using rat brain membrane with or without the addition of polymeric effectors. The addition of DNA and heparin showed the uncompetitive inhibition, and the addition of polymyxin B showed noncompetitive inhibition.

Discussion

The significance of anionic and cationic charges on the cell surface for the drug-receptor interaction was examined to assess the effect of seven kinds of polymeric chemicals on the affinity of \( \beta \)-adrenoceptors. Polymyxin B, DNA and heparin significantly caused a reduction in \( B_{\text{max}} \), and only DNA induced a decrease in the value of \( K_d \). BSA, which consists of a carbohydrate-free and the neutral polypeptide chain, was used as the control for this experiment. As expected, BSA had no effect on the drug-receptor interaction.

As is well known, the cell surfaces of mammalian tissues contain negatively and positively charged sites. Negatively charged sites are composed of phospholipids, sialic acid moieties in glycoproteins or glycolipids, and acidic mucopolysaccharide, etc., and positively charged sites are composed of protein, etc. Polymyxin B is an amphiphilic peptidolipid containing positively charged 2,4-diaminobutyric acid groups (5) and thus can interact with negatively charged surface sites, and the binding requires the presence of anionic phospholipid (6, 7). The present results imply that the effect of polymyxin B on \( \beta \)-adrenoceptors is due to covering of the receptor sites through its binding to anionic phospholipid in the membranes.

Cellular fibronectin is a major cell surface glycoprotein synthesized by a variety of cell types and appears to function as an adhesive molecule, affecting a wide variety of cellular events (8–11). Its important function is to serve as a link between collagenous structures and the surface of cells. Thus, the attachment of a cell to the collagen substrate is mediated by fibronectin (12), and fibronectin binds to acidic mucopolysaccharide such as heparin (13). As shown in the above results, heparin, one of the acidic mucopolysaccharides,
caused a reduction in the number of receptor sites. Gelatin which is derived from collagen by hydrolytic action caused no change in the number of the sites and the affinity of the drug-receptor interaction. These results imply that the binding sites of heparin on the cell surface such as fibronectin also controls the drug-receptor interaction.

DNA and histone are chromosomal substances. DNA is a strongly acidic material and binds to positively charged materials such as histones. Conversely, histones are small proteins (MW 10000–20000) attached to the DNA of cell nuclei by ionic linkages. In a previous report, deoxyribonuclease treatment of rat cardiac homogenate was found to be beneficial for the isolation of sarcolemma from cardiac muscle (14) and resulted in good reproducibility in the β-adrenoceptor binding assay (3). As described above, the present results indicated that DNA caused a reduction in the number of β-adrenoceptor sites and an increase in the affinity of drug-receptor interaction, and they indicated that histones caused an increase in the number of non-specific binding sites regardless of the drug-receptor interaction. These facts suggest that the presence of DNA and/or histones interfere with the binding assay. Therefore, it is necessary to remove DNA and histones from the myocardium membrane preparation. Furthermore, these results imply that the amounts of histone and DNA in the homogenates of rat brain are much smaller than those of the heart.

In a previous report, the removal of sialic acid moieties from rat cardiac membrane resulted in good reproducibility in the β-adrenoceptor binding assay (3). Polysialic acid, colominic acid, caused no change in the number of receptor sites and the affinity of the drug-receptor interaction. These results suggest that the removal of positively charged materials such as histones may have occurred when sialic acid moieties, which are negatively charged surface sites, were removed from the cell surface by neuraminidase treatment, thus resulting in good reproducibility for the β-adrenoceptor binding assay.

As described above, it is reasonable to presume that DNA, heparin and polymyxin B attached to positively or negatively charged sites of the cell membrane by ionic linkage. It is well known that heparin interacts with cell-surface components and lysosomal enzymes (15). Addition of heparin to various acid hydrolases results in either reduction or apparent increase in enzyme activity (16). Most of the lysosomal enzymes interact with heparin in pH-dependent, reversible and electrostatic binding (15–17). DNA and polymyxin B possessed the properties of binding reversibility for basic proteins and/or cell membrane; i.e., histone and acidic moieties of the cell membrane, respectively (5–7, 18–22). Thus, these polymeric chemicals are reversible effectors for cell-surface components. The inhibition styles of the added DNA and polymyxin B were uncompetitive and non-competitive, respectively. On the other hand, the inhibition of heparin looked like the uncompetitive type (Fig. 5). However, that by heparin may be non-competitive because the addition of heparin did not show any changes in the Kₐ value as compared to control. Thus, these results imply that the positively and negatively charged sites on the cell surface of rat brain containing β-adrenoceptor molecules could have a crucial role in the drug-receptor interaction. Particularly, it is suggested that the binding of these chemicals to the positively and negatively charged sites of the β-adrenoceptor molecule caused conformational changes of the receptor molecule by allosteric interaction.

Negatively charged materials like heparin and DNA caused a reduction of the number of receptors. A possibility is that these results may be related to the binding of divalent cation to these effectors in the experimental medium. DNA and heparin strongly bind divalent cations such as Mg²⁺ and Ca²⁺. However, the evidence that the divalent cations did not affect the binding of an antagonist but affected those of an agonist (23) seem to discount this possibility.

In conclusion, the environment of the receptor site could have a crucial role in drug-receptor interaction.

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References


