Effects of Deoxyribonuclease I and Neuraminidase Treatments on the Specific Binding of $^3$H-Prazosin and $^3$H-Quinuclidinyl Benzilate ($^3$H-QNB) to $\alpha$-Adrenergic and Muscarinic Receptors in Rat Myocardial Membranes

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Abstract—The effects of neuraminidase and deoxyribonuclease I (DNase) treatments on the specific binding of $^3$H-prazosin and $^3$H-quinuclidinyl benzilate ($^3$H-QNB) to $\alpha$-adrenoceptors and muscarinic cholinergic receptors in the membrane of the rat myocardium was examined, and the dissociation constant ($K_d$) and the maximum number of binding sites ($B_{max}$) was analyzed using the method of Scatchard analysis. Although no changes in $K_d$ values were observed when DNA or sialic acid was removed from cardiac muscles by treatments with DNase or neuraminidase, the $B_{max}$ values of $\alpha$-adrenergic and muscarinic receptors were markedly decreased after treatment with DNase, while neuraminidase treatment induced an increase in the $B_{max}$ values of the $\alpha$-adrenoceptors. The possibility that these results provide the basis for elucidation of the characteristics of these receptors in the rat myocardium was discussed.

Our previous reports showed that neuraminidase or deoxyribonuclease I (DNase) treatments resulted in an improvement in the reproducibility of radioligand binding assay for $\beta$-adrenoceptors in myocardial membranes (1) and that the membrane preparations treated with neuraminidase could be used for assessment of newly synthesized chemicals as $\beta$-blockers (2, 3). In view of the findings that $\alpha$-adrenoceptors exist in the myocardium subserving positive inotropic effects through mechanisms other than those associated with the activation of the adrenoceptors (4–6) and because it is important to have precise knowledge of the different binding characteristics of these two receptors in the myocardium, the present study was undertaken to clarify whether the DNase or neuraminidase treatment would affect the $\alpha$-adrenoceptors in the cardiac muscle membranes just as it did in the $\beta$-adrenoceptors. For comparison, the effects of the treatment with these enzymes on the muscarinic cholinergic receptor sites reported to be in the myocardium (7–10) were also studied.

Hearts were excised from male Wistar rats weighing 200–300 g stunned with a blow on the head. Subsequent steps were carried out at 4°C. The heart muscles were blotted, weighed and minced with small scissors in a small volume (ca. g tissue/3 ml) of 0.25 M sucrose, 10 mM Tris-HCl buffer, pH 7.6.

The minced hearts were suspended in 10 volumes of the same buffer and homogenized in a Polytron homogenizer using 2×10 sec bursts separated by 1 min cooling periods. The homogenates were then filtered through 4 layers of gauze and centrifuged at 40,000 g for 45 min. A portion of the pellet was then treated with neuraminidase or DNase as described previously (1). In brief, the pellets were treated with neuraminidase (0.31 unit/mg protein) in a medium containing 100 mM KCl, 10 mM MgCl$_2$, 20 mM Tris-maleate, pH
7.4, at 25°C for 30 min or with DNase I (60 Kunitz units/mg protein) in a medium containing 250 mM sucrose, 10 mM Tris-HCl, pH 7.6, at 37°C for 60 min. The suspension treated with these enzymes was centrifuged at 40,000 g for 45 min, and the pellets were homogenized again with a glass homogenizer using 5 strokes and then finally resuspended in the incubation buffer (100 mM Tris-HCl, 20 mM MgCl₂, pH 8.0). The remaining portion of the pellet was incubated in the same manner in a medium without the enzymes, centrifuged at 40,000 g for 45 min, and suspended in the incubation buffer just described (non-treated preparation).

The assays of the cardiac α-adrenergic and muscarinic receptors were performed in duplicate with ³H-prazosin and ³H-quinuclidinyl benzilate (³H-QNB) as ligands in the presence (non-specific) and absence (total) of 0.1 mM phentolamine or atropine sulfate, respectively. For the assays of these two receptors, 0.5 ml of membrane suspension (0.4–0.6 mg protein) and different concentrations of each radioligand were incubated with shaking for 30 min at 23°C in a total volume of 1 ml containing 50 mM Tris-HCl, 20 mM MgCl₂, pH 8.0. At the end of the incubation period, the incubation medium was immediately filtered through a GF/C glass fiber filter with a modified automatic cell harvester (LM-101, Labo Science, Tokyo, Japan). The filters were washed with approximately 1 ml of incubation buffer and put into vials containing 5 ml of a Triton-toluene based scintillation fluid. The radioactivity of ³H-prazosin and ³H-QNB bound to the membrane was counted. The maximum numbers of α-adrenergic and muscarinic receptor binding sites (Bₘ₅ₐₓ) and dissociation constant (Kᵦ) were determined by the method of Scatchard (1949).

DNA and sialic acid were determined by methods of Croft and Lubran (11) and Warren (12), respectively. Protein was assayed by the method of Lowry et al. (13).

³H-Prazosin (23 Ci/m mole) and ³H-QNB (33.2 Ci/m mole) were purchased from the New England Nuclear Co., Ltd. Neuraminidase (type D) and DNase (DN-100) were purchased from the Sigma Chemical Company.

Membrane protein yield and the concentrations of DNA and sialic acid of the preparations incubated in two types of incubation buffers described previously without DNase I or neuraminidase (non-treated preparation) were not different from each other. As shown in Table 1, membrane protein yields per gram wet weight of the cardiac muscle preparations treated with either DNase I or neuraminidase were lower than that of the nontreated control (71.44±4.06 mg protein/g tissues), being 51.72±2.98 (DNase-treated, P<0.02) and 60.57±3.82 (neuraminidase treated) mg protein/g tissues, respectively. DNase and neuraminidase treatment also induced a significant decrease in DNA or sialic acid contents (Table 1).

The ratio of the specific binding to the total binding of ³H-prazosin usually amounted to approximately 63% and that of ³H-QNB, 69%. As shown in Table 2, Kᵦ values of the α-adrenergic and muscarinic receptors of the muscle membrane treated with DNase or neuraminidase were not statistically different from those of the non-treated control. However, a marked reduction of Bₘ₅ₐₓ values of α-adrenergic and muscarinic receptors was found in the myocardial membrane treated with DNase, while the neuraminidase treatment induced an increase in the values

|                | Protein yield (mg protein/g) | DNA (µg/mg protein) | Sialic acid (µg/mg protein) |
|----------------|-----------------------------|---------------------|-----------------------------|
| No treatment  (9) | 71.44±4.06                 | 50.31±2.86          | 2.70±0.19                   |
| DNase (4)      | 51.72±2.98**               | 6.24±1.76***        | 2.61±0.36                   |
| Neuraminidase (5) | 60.57±3.82                | 47.72±2.32          | 1.62±0.22***                |

Values are means±S.E. Numbers in parentheses represent the numbers of experiments. Significant differences: **P<0.02, ***P<0.01.
of B$_{\text{max}}$ of the $\alpha$-adrenoceptors. The values of the Hill coefficient of $\alpha$-adrenergic and muscarinic receptors of the nontreated preparations were 1.02 and 0.94, respectively.

It was found that neuraminidase treatment could increase the number of $\alpha$-adrenoceptors in the cardiac muscle membrane, just as it did with respect to $\beta$-adrenoceptors. Thus, the removal of sialic acid from membranes of the myocardium showed almost the same effects on the $\alpha$- and $\beta$-adrenoceptors, indicating that the sialic acid moieties existing in the vicinity of $\alpha$- and $\beta$-adrenoceptors and/or in both adrenoceptors themselves may not play an important role in the different binding characteristics of these two types of adrenoceptors.

DNA is a chromosomal substance and an acidic chemical. In addition, DNA also induces the aggregation of the plasma membrane during the preparation of the sarcolemmal fraction from the myocardium. Thus, it is now accepted that DNase treatment is useful for the purification of the sarcolemmal fraction prepared from the myocardium (14, 15). The preparation of the crude membrane fraction for the radioligand binding assay used here is one step in the preparations of the sarcolemmal fraction using DNase from myocardium as described above. In the present study, the chemical materials including the DNA in the homogenate fraction removed with DNase must have been important in determining the differences in the characteristics of $\alpha$- and $\beta$-adrenoceptors, because treatment of cardiac muscle with this enzyme induced the decrease of the $\alpha$-adrenoceptors density in contrast to changes in the $\beta$-adrenoceptors described previously in our report (1). Chuang et al. (16) also reported that when the erythrocyte membrane prepared from bullfrog was treated with DNase, $^3$H-DHA binding to the membrane fraction did not change. It is now accepted that $\alpha$-1 adrenergic agonists produce myocardial inotropic responses independent of the $\beta$-adrenergic mechanism (4). Thus, the results presented in this paper may support the idea that these two types of adrenoceptors have different binding characteristics or functions.

The treatment of the membrane fraction of the myocardium with neuraminidase clearly induced the decrease in the number of muscarinic receptors in agreement with the reports of Wei and Sulakhe (7). As Venter and Fraser (17) suggested the possibility that $\alpha$-adrenoceptor might be structurally related to the muscarinic receptors, it is of interest to note that neuraminidase treatment induced diverse effects on the $\alpha$-adrenergic and muscarinic receptors.

In conclusion, the above findings indicate that the treatment of the membrane preparation with DNase or neuraminidase is useful for the elucidation of the important binding characteristics of the adrenoceptors and muscarinic receptors in the myocardium.

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