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Binding Characteristics of [^{3}H]Dihydroalprenolol to β-Adrenergic Receptors of Rat Brain: Comparison with Those of Rat Heart Treated with Neuraminidase

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Binding characteristics of the β-antagonists and agonists with the β-adrenoceptors were investigated in [^{3}H]dihydroalprenolol ([^{3}H]DHA) binding to rat brain membranes and the results were compared with those for rat heart membranes treated with neuraminidase. In this study, an improved automatic cell harvester LM-101 (Labo Science, Tokyo) was also used for filtration and washing of GF/C glass fiber filters. The use of this instrument allowed a large number of tubes to be assayed. The ranking order of inhibition of β-antagonists or agonists was: pindolol > alprenolol > *dl*-propranolol > labetalol > YM-09538 > oxprenolol > K-351 > S-596 > N-696 > dichloroisoproterenol > metoprolol > acebutolol > sotalol > butoxamine > atenolol > practolol as antagonists or *l*-isoproterenolol > *l*-epinephrine = *l*-norepinephrine > salbutamol as agonists. Although lower IC_{50} (concentration of drug which inhibits [^{3}H]DHA binding by 50%) values in the heart than in the brain were observed, a good correlation ($r=0.86$, $p<0.001$) was found between IC_{50} values in the binding assay with rat brain membranes and with rat heart membranes treated with neuraminidase. Thus, the radioligand binding assay method using rat brain can be useful for assessment of the relative potencies of newly synthesized chemicals as β-blockers.

Keywords——β-adrenoceptor; brain; heart; convenient binding assay; automatic cell harvester

Our previous studies have been directed towards the characterization of the β-adrenoceptor in the heart muscle.[1)] As shown in those papers, the treatment of the cardiac membrane fraction with neuraminidase resulted in a higher reproducibility of the binding assay than was obtainable without neuraminidase treatment, and there was good correlation between K_i (inhibition constant) values of the various β-adrenoceptor blocking agents determined on the neuraminidase-treated membranes and the pA_2 values on these compounds obtained from pharmacological experiments.[1)] These results imply that the neuraminidase treatment is necessary to determine the density (B_{max}) and affinity (K_d) of binding sites in the rat myocardium using the radioligand binding assay and that the environment of the receptor site could play a crucial role in the drug–receptor interaction. It is well known that β-adrenergic receptors also exist in most regions of the rat central nervous system.[2)] Thus, the object of the present study was to examine the characteristics of the binding of β-antagonists with the β-adrenoceptors in the membrane of rat brain, and to compare directly the characteristics of β-adrenoceptors in the brain and in the heart.

In addition, a preliminary experiment was carried out to improve the vacuum filtration process of the ligand binding assay using the automatic cell harvester already reported by Harris and Barsuhn.[3)]

Experimental

Chemicals described in previous reports[1a,b)] were also used in this work. The following abbreviations are used: YM-09538, 5-{1-hydroxy-2-{[2-(*o*-methoxyphenoxy)ethyl]amino}ethyl}-2-methylbenzenesulfonamide hydrochloride;

S-596, *dl*-2-(3-*tert*-butylamino-2-hydroxypropylthio)-4-(5-carbamoyl-2-thienyl)thiazole hydrochloride; K-351, 3,4-dihydro-8-(2-hydroxy-3-isopropylaminopropoxy)-3-nitroxy-2*H*-1-benzopyran; N-696, 4-(3-(*tert*-butylamino)-2-hydroxypropoxy)-*N*-methylisocarbostyril hydrochloride.

[^{3}H]Dihydroalprenolol ([^{3}H]DHA) (90 Ci/mmol) was purchased from New England Nuclear Corp. Male Wistar rats weighing between 250—350 g were killed by a blow on the head. After removal of the brain, the cerebral cortex was minced with small scissors in 10 mM Tris–HCl buffer, pH 7.6, containing 250 mM sucrose and then homogenized using a glass homogenizer (five strokes). The homogenate was filtered through 4 layers of gauze. The filtrate was centrifuged at 40000 *g* for 30 min, and the resultant pellets were rinsed at once, then homogenized with a glass homogenizer using 20 ml of 75 mM Tris–HCl buffer, pH 8.0, containing 25 mM $MgCl_2$. The prepared membrane fraction was stored at 4 °C and used within 24 h. The β-adrenoceptor binding assay was carried out in duplicate with [^{3}H]DHA in the presence (non-specific) and absence (total) of 100 μM *dl*-propranolol. For [^{3}H]DHA binding, 0.25 ml of membrane suspension containing 0.25 mg of membrane protein was incubated with shaking for 30 min at 23 °C with 1.2 nM [^{3}H]DHA and different concentrations of various β-blockers in a total volume of 0.5 ml containing 60 mM Tris–HCl and 20 mM $MgCl_2$ (pH 8.0). At the end of the incubation period, the incubation medium was immediately filtered through a GF/C glass fiber filter using the improved automatic cell harvester LM-101 (Labo Science, Tokyo). The filters were washed with continuous flow for 2 s, air-dried for 30 s, and added to 5 ml of a Tt76 scintillation fluid (counting efficiency 66%). The automatic cell harvester could continuously filter, wash, and dry twelve tubes at once. The difference in mean values between total and non-specific binding was taken as the specific binding. Specific binding routinely represented approximately 80% of the total binding. The protein was determined by the method of Lowry *et al.*[4]

Results

The yield of the membrane protein obtained by the present method from the rat brain was 77.54 ± 2.35 mg per g wet weight ($n = 35$). Preliminary experiments were performed in order to standardize the β-adrenergic receptor binding assays with the membrane preparation. The specific binding of [^{3}H]DHA (1.2 nM) to the membrane fraction was linear with respect to protein concentration below 0.75 mg per incubation when the antagonist (*dl*-propranolol) was used at a saturating concentration. Total and specific bindings at 23 °C were rapid, reaching the steady state within 10 min when 1.2 nM [^{3}H]DHA was used. Binding in the presence of

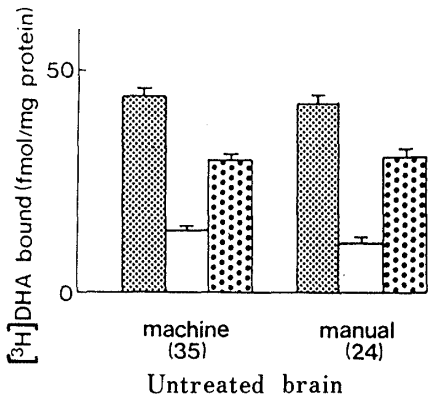

Fig. 1. Comparison of the Automatic Cell Harvester Method (Machine) and Our Conventional Method (Manual) for Binding Assay

Rat brain membranes were incubated with 1.2 nM [^{3}H]DHA for 30 min at 23 °C. The values given in parenthesis represent the numbers of experiments. ▨, total; □, non-specific; ⊞, specific. The figure shows mean values ± S.E.

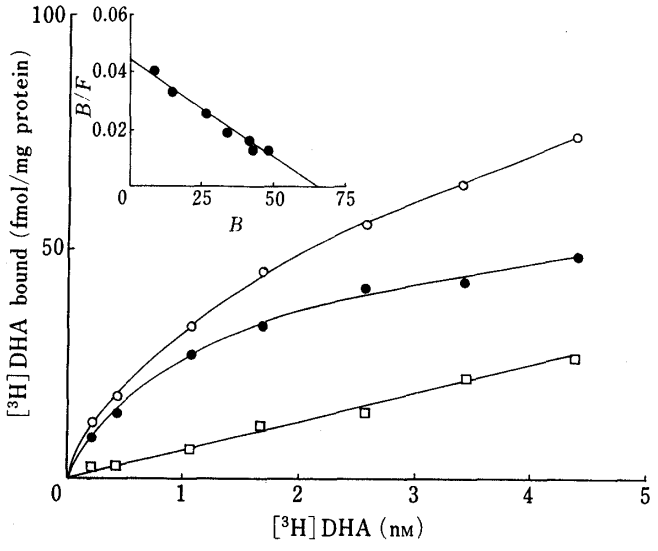

Fig. 2. The Binding of [^{3}H]DHA to Membrane Fractions of Rat Brain

The inset shows a Scatchard plot of specific [^{3}H]DHA binding to the membrane fraction, *i.e.*, the value of [^{3}H]DHA bound (fmol) divided by free ligand (fmol) was plotted as the ordinate, and the binding number of [^{3}H]DHA bound (fmol/mg protein) as the abscissa. Each point represents the mean of 6 experiments, each conducted in duplicate. The points show total (—○—), specific (—●—), and non-specific (—□—) binding.

TABLE I. IC_{50} Values of β-Antagonists and Agonists

No.[a]	Compound	Brain IC_{50} (nM)	Number of experiments	Heart[b] IC_{50} (nM)	Number of experiments
	Antagonists				
1	Pindolol	19.57±7.94	(4)	19.03±6.97	(3)
2	*dl*-Propranolol	98.44±52.10	(5)	10.02±1.57	(4)
3	Alprenolol	101.09±89.06	(3)	16.88±5.85	(3)
4	Oxprenolol	131.65±21.42	(3)	13.75±4.39	(3)
5	Labetalol	316.00±127.87	(5)	246.03±77.25	(3)
6	YM-09538	839.35±346.75	(5)	99.47±16.26	(3)
7	K-351	1296.21±982.87	(4)	38.42±11.62	(4)
8	S-596	2491.67±889.10	(6)	2.94±1.70	(3)
9	N-696	7792.67±3756.85	(4)	309.51±80.92	(4)
10	Dichloroisoproterenol	19056.44±11217.10	(4)	870.99±507.43	(3)
11	Acebutolol	23382.29±21150.53	(3)	1435.49±804.50	(2)
12	Metoprolol	35731.95±25402.05	(4)	6038.29±5311.63	(3)
13	Sotalol	48790.64±23109.58	(4)	7236.65±585.50	(3)
14	Butoxamine	96753.49±32404.82	(3)	9330.00	(1)
15	Practolol	139749.48±50599.70	(4)	25900.00±11335.88	(5)
16	Atenolol	386395.15±226992.18	(3)	4523.33±486.67	(3)
	Agonists				
17	*l*-Isoproterenol	17310.67±9243.48	(4)	1504.99±848.34	(3)
18	*l*-Epinephrine	34249.31±11812.50	(3)	7989.99±4484.40	(3)
19	*l*-Norepinephrine	39355.70±13809.98	(5)	16176.67±5538.92	(3)
20	Salbutamol	224119.40±73942.10	(6)	1882.37±697.08	(3)

Each value in parenthesis is the number of experiments. Data are the mean values±S.E. *a*) Numbers refer to the points in Fig. 3. *b*) The values in heart were obtained from the reports published by Nagatomo *et al.*[1a,b]

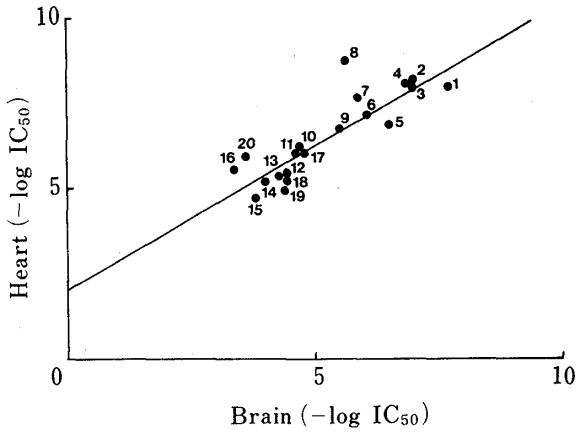

Fig. 3. Relationship between Potencies of β-Adrenoceptor-Acting Drugs Relative to Inhibition of [^{3}H]DHA Binding to the Receptor of the Rat Brain and of the Neuraminidase-Treated Rat Heart

Numbers refer to individual drugs in Table I.

100 μM *dl*-propranolol (non-specific binding) using 1.2 nM [^{3}H]DHA reached equilibrium in 10 min and remained constant thereafter. The results of comparison between the present binding assay method using the automatic cell harvester and our conventional method with rat brain membrane are shown in Fig. 1. Although no significant difference between the two results was observed, the use of the improved assay allowed a larger number of tubes to be assayed. Therefore, the improved automatic cell harvester method was used in the present study.

Figure 2 illustrates the relation between the concentration of [^{3}H]DHA in the incubation medium and the binding of the compound to the receptor. The Scatchard analysis to determine the affinity (K_d) and number of binding sites (B_{max}) is shown in the inset of the same

figure. Values of the equilibrium dissociation constant (K_d) and the maximum binding sites (B_{max}) in the brain calculated from the Scatchard plot were found to be 1.05 ± 0.09 nM ($n=6$) and 69.55 ± 7.71 fmol/mg protein ($n=6$), respectively. The value of the Hill coefficient was 1.00 ± 0.07 ($n=6$).

Table I summarizes the IC_{50} (concentration of drug which inhibits [^{3}H]DHA binding by 50%) values for antagonists and agonists tested in displacement experiments. Among 16 β-blockers tested, pindolol, alprenolol, and *dl*-propranolol were the most potent, while practolol, atenolol, and butoxamine were the least potent. The potencies of selective β-blocker compounds, metoprolol, acebutolol, atenolol, practolol, and butoxamine were weak. Labetalol and YM-09538 (combined α- and β-adrenoceptor antagonists) and N-696, S-596, and K-351 (newly synthesized antagonists) showed intermediate potency. Compared to antagonists, agonist compounds had low potency. The rank order of the potency was *l*-isoproterenol > *l*-epinephrine = *l*-norepinephrine > salbutamol.

Figure 3 shows the relationship between the $-\log IC_{50}$ values of 16 β-antagonists and 4 β-agonists from the rat brain membrane and those obtained from the rat heart membrane treated with neuraminidase.[1b] A good correlation was found with a linear relationship which can be described by the equation $y=0.83x+1.96$ ($r=0.86$, $p<0.001$). However, IC_{50} of S-596 exceeded the confidence limit ($p<0.05$) of the method of contour ellipse.[5] IC_{50} values of these compounds obtained from rat heart were smaller than those from brain.

Discussion

The use of the automatic cell harvester for the β-adrenoceptor binding assay allowed a large number of tubes to be assayed as reported by Harris and Barsuhn.[3] By this assay, the values of K_d and B_{max} for β-adrenoceptor in the brain homogenate calculated from a Scatchard analysis were found to be 1.05 ± 0.09 nM and 69.55 ± 7.71 fmol/mg protein, respectively. The K_d value coincided with one reported value[2b] and was slightly higher than another.[2c] The B_{max} value was lower than one reported value[2b] and was higher than another.[2c] It is clear that the use of the automatic cell harvester is advantageous, allowing large numbers of tubes to be assayed simultaneously.

In the present study, IC_{50} values of various β-blockers between brain and heart were compared by using the radioligand binding assay. Our previous papers have shown that the radioligand binding assay method using [^{3}H]DHA for β-adrenoceptors in the rat heart required prior neuraminidase treatment of the heart preparation.[1a] A long time was required for the enzyme treatment, and in addition, assessment of β-blocking action from the radioligand binding assay using the heart muscle may not be reliable because the membrane structure may be in part changed by the removal of sialic acid from the membranes of heart muscles. Thus, it is desirable to determine whether the results obtained from the membrane fraction of rat brain, which was not treated with the neuraminidase, are consistent with IC_{50} values of heart muscles or with pA_2 values obtained from pharmacological observations.

There was a good correlation between IC_{50} values of 20 β-adrenergic agonists or antagonists tested here for the rat brain membrane and the rat heart membrane treated with neuraminidase ($r=0.86$). However, the value of S-596 among all the drugs tested in the present paper was off the line, and the affinity of S-596 for β-receptor in the brain was much lower than that in the heart. This difference may be based on its structure. As the basic structure, β-antagonists possess either an arylethanolamine or an aryloxyethanolamine, whereas S-596 possesses a unique structure in which the aryl nucleus is replaced by a heterocyclic nucleus with an *N*-substituted thiopropranolamine moiety. This structure may result in the higher affinity of S-596 for β-adrenoceptors in the heart than for those in the brain.

On the other hand, differences of affinities of β-blockers for β-adrenoceptors between brain membranes and heart membranes treated with neuraminidase were observed, as shown in Table I. Minneman *et al.* and U'Prichard *et al.* also found higher affinities in rat heart without neuraminidase treatment than in brain.[2a,c] The main reasons why the difference of affinity between heart and brain tissues appeared may be the different membrane compositions and/or different ratios of β-1 and β-2 adrenoceptor components in the two tissues. We cannot, however, rule out the possibility that the removal of sialic acid from the cell membrane of heart muscle is the reason. In fact, we found that the neuraminidase treatment induced an increase of affinity of [^{3}H]DHA to β-adrenoceptors in rat brain without any change in the density of β-adrenoceptors (unpublished data).

It is of interest to note that a good correlation ($r=0.86$) was found between IC_{50} values of β-blockers in brain and pA_2 values obtained by pharmacological observation using guinea pig atria, as described in our previous report.[1b] In addition, a good correlation ($r=0.96$) was also found between IC_{50} values obtained from the brain and the potencies of inhibition of adenylate cyclase activity determined by U'Prichard *et al.*[2c] and Mukherjee *et al.*[6] Similar correlations were also observed in heart muscles, based on radioligand binding assays. It is well known that the ability to interact with adenylate cyclase is important, because an increase of adenylate cyclase activity in the cardiac muscle or other tissues regulates the intensity of β-adrenoceptor activation, indicating that enhancement of the pharmacological effects in tissues can be induced. Thus, IC_{50} values obtained with the brain tissue by the radioligand binding method can be useful for assessment of the relative potencies of newly synthesized drugs as β-blockers.

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