BINDING CHARACTERISTICS OF ^3H -DIHYDROALPRENOLOL TO β -ADRENOCEPTORS OF RAT HEART TREATED WITH NEURAMINIDASE

Takafumi NAGATOMO, Miyuki SASAKI, Hiroshi TSUCHIHASHI
and Shoichi IMAI*

Department of Pharmacology, Niigata College of Pharmacy, Niigata 950-21, Japan

*Department of Pharmacology, Niigata University School of Medicine, Niigata 951, Japan

Accepted April 2, 1983

Abstract—Binding characteristics of the β -blockers and β -agonists with the β -adrenoceptors were investigated in ^3H -dihydroalprenolol (^3H -DHA) binding to rat heart membranes treated with neuraminidase. When 60% of the total sialic acid content in the membranes was removed, reproducibility of the binding assay became much better than was attainable without neuraminidase treatment, and the maximum density of β -adrenoceptors was increased. These data suggest that the binding of the test compounds with the β -adrenergic receptors in cardiac muscle was under the influence of the sialic acid of the glycocalyx of the membrane. The ^3H -DHA binding sites in membranes treated with neuraminidase showed a strict stereo-specificity when tested with propranolol. The ranking order of inhibition of β -antagonists or agonists is: dl-propranolol > oxprenolol > alprenolol > pindolol > YM-09538 > labetalol > acebutolol > atenolol > metoprolol > sotalol > butoxamine > practolol as antagonists or l-isoproterenol > l-epinephrine > l-norepinephrine as agonists. A good correlation ($r=0.91$, $P<0.001$) was observed between the K_i values observed by the present binding assay and the pA_2 observed in the guinea-pig atria relative to the positive inotropic effect by Bieth et al. (Br. J. Pharmacol. **68**, 563–569, 1980), indicating that the present method will be useful for screening new β -adrenergic receptor antagonists or agonists.

Numerous studies have been conducted with direct radioligand binding techniques (1–3) to delineate the characteristics of cardiac autonomic receptors. In our previous report (4), it was demonstrated that treatment of the crude rat heart plasma membrane (sarcolemma) fraction with neuraminidase resulted in a better reproducibility than was attainable without neuraminidase treatment, with an increase in the maximum number of binding sites (B_{max}) for the specific β -adrenergic receptors. This implies that the removal of sialic acid from the glycocalyx of the sarcolemma induced an alteration of the environment of the receptor site, thus improving the reproducibility of binding of

the test compounds with the receptor site. The object of this study is to examine the characteristics of the binding of β -blockers with different potency and selectivity with the β -adrenoceptors in the membrane of rat heart treated with neuraminidase and to see whether the treatment with neuraminidase would bring about any change in the binding characteristics of the β -adrenoceptors.

Materials and Methods

Male Wister rats weighing between 150–250 g were killed by a blow on the head. After removal of the heart, the ventricular muscles were minced with small scissors in 250 mM sucrose, 10 mM Tris-HCl, pH 7.6,

and were then homogenized using a Polytron homogenizer, twice for 10 sec at setting 8. The homogenate was filtered through 4 layers of cheese cloth. To this suspension, 3M KCl and 250 mM sodium pyrophosphate were added to attain a final concentration of 0.3 M for KCl and 25 mM for sodium pyrophosphate, necessary to solubilize contractile and other proteins. The suspension was centrifuged at 177,000 g for 45 min, and the pellets were then treated with neuraminidase (6.2 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. The suspension treated with neuraminidase was centrifuged at 177,000 g for 45 min, and the resultant pellets were suspended in 6 ml of an incubation buffer per gram of the original tissue weight.

The cardiac β -receptor binding assay was carried out in duplicate with the method described previously (4). For ^3H -dihydroalprenolol (^3H -DHA) binding, 0.5 ml of membrane suspension (0.4–0.6 mg protein) was incubated with shaking for 20 min at 23°C with 2.5 nM ^3H -DHA and different concentrations of various β -blockers in a total volume of 1 ml of 60 mM Tris-HCl, pH 8.0, and 20 mM MgCl_2 . At the end of the incubation, the incubation medium was immediately filtered through GF/C glass filters. The filters were then washed three times with 5 ml of cold buffer. Ten ml of a Triton-toluene based scintillation fluid was added, and the radioactivity of ^3H -DHA bound to the membrane in the absence and presence of β -blockers was counted. The difference of the mean value of the total binding and the non-specific binding was taken as the specific binding. The specific binding routinely amounted to approx. 50% of the total binding. Values of the inhibition constant (K_i) for each of the β -blockers were calculated according to the following equation (5):

$$K_i = \frac{\text{IC}_{50}}{(1 + D^*/K_d^*)}$$

where IC_{50} =the concentration of non-radioactive drugs that inhibits 50% of the radioactive drug binding, K_i =inhibition constant of a non-radioactive drug for the radioactive drug binding site, D^* =free concentration of the radioactive drug (2.5 nM) in the incubation solution, and K_d^* =equilibrium dissociation constant (2.67 nM) of the radioactive drug for the binding site.

The sialic acid contents of the heart muscle treated with different concentrations of neuraminidase were measured by the method of Warren (6). One unit (1.0 U) of neuraminidase was defined as the amount of enzyme activity which could liberate 1 nmole sialic acid/min under the assay conditions. The reaction mixture (0.1 ml) was composed of 0.1 M KCl, 10 mM MgCl_2 , 20 mM Tris-maleate, pH 7.4, containing 1.0 mM of N-acetyl neuraminyl lactose and the neuraminidase (0.25 mg/ml). The homogenates were treated with the different concentrations of neuraminidase in a medium containing 100 mM KCl, 10 mM MgCl_2 , 20 mM Tris-maleate, pH 7.4, at 25°C for 30 min. Protein determination was carried out by the method of Lowry et al. (7).

^3H -Dihydroalprenolol (^3H -DHA) (49.4 Ci/mmole) was purchased from New England Nuclear Corp. Neuraminidase (Type V, purified from *Clostridium perfringens*), l-isoproterenol D-bitartrate, l-epinephrine, l-norepinephrine were purchased from the Sigma Company. l-, d- and dl-Propranolol were kindly donated by ICI Pharmaceuticals and YM09538, 5-[1-hydroxy-2-[[2-(o-methoxy phenoxy)-ethyl]-amino] ethyl]-2-methylbenzenesulfonamide hydrochloride, from Yamanouchi Pharmaceutical Co., Ltd. The following drugs were also used in this study: Atenolol, pindolol, oxprenolol, labetalol HCl, alprenolol HCl, atenolol, acebutolol HCl,

MJ 1999 (Sotalol), practolol, butoxamine HCl, metoprolol, salbutamol and dichro-isoprotenerol.

Results

The amount of the membrane protein obtained with the present method from the rat heart was 62.8 ± 10.8 mg per g wet weight without neuraminidase treatment, while it was 53.7 ± 1.7 mg/g after treatment with neuraminidase.

Figure 1 depicts the effect of neuraminidase treatment on the binding of radioligand to β -adrenoceptors in rat heart muscles. The density of the specific binding sites was increased after treatment of the membrane with neuraminidase as the result of a decrease in the non-specific binding sites. In addition, as decreases in the standard error show, the reproducibility of the binding became much

better. Whereas the coefficient of variation of the specific binding was 83.5% without neuraminidase treatment, it was only 12.1% in neuraminidase-treated preparations. As illustrated in Fig. 2, with the increased removal of sialic acid from the heart muscle brought about with higher concentrations of neuraminidase, the number of β -adrenoceptor sites was increased, reaching a plateau value when 60% of the sialic acid was removed from the heart muscles.

On the basis of these findings, preliminary experiments were performed in order to standardize the β -adrenergic receptor binding assays with the cardiac membrane preparations treated with neuraminidase. The specific binding of ^3H -DHA (2.5 nM) to the membrane fraction was linear with protein concentrations between 0.25 and 0.75 mg/incubation medium when the antagonist (dl-propranolol) was used at a saturating concentration. Total and specific bindings at 23°C were rapid, reaching steady-state within 15 min when 2.5 nM ^3H -DHA was used. Binding in the presence of $5 \mu\text{M}$ propranolol (non-specific binding) using 2.5 nM ^3H -DHA reached equilibrium in 5 min and remained constant thereafter. Figure 3 illustrates the relation between the concentration of ^3H -DHA in the incubation medium and the binding of this compound to the receptor. Scatchard analysis to determine the affinity and number of binding sites is shown in the inset. Values of the equilibrium dissociation constant (K_d) and the maximum binding capacity (B_{max}) in the heart muscle treated with neuraminidase calculated from the Scatchard plot were found to be 2.67 ± 0.5 nM and 83.5 ± 8.9 fmoles/mg protein, respectively. The value of the Hill coefficient equalled to unity.

Table 1 summarizes the K_i values (inhibition constants) for antagonists and agonists tested in displacement experiments using heart homogenates treated with neur-

Fig. 1. Effects of neuraminidase treatment on the binding of radioligand to β -adrenoceptor of rat heart muscle. The binding assay was carried out in the medium containing 2.5 nM ^3H -DHA, 60 mM Tris-HCl, pH 8.0, and 20 mM MgCl_2 in the presence or absence of $5 \mu\text{M}$ dl-propranolol at 23°C for 20 min. Numbers in parentheses are the number of experiments.

Fig. 2. Effect of neuraminidase treatment on the number of specific binding sites of β -adrenoceptor (upper plot, left ordinate) and the amount of sialic acid released from the heart muscle (lower plot, right ordinate). The conditions for neuraminidase treatment were as described in Materials and Methods. Values are the mean $\pm$ S.E.

Fig. 3. The binding of ^3H -DHA to membrane fractions of cardiac muscle treated with neuraminidase. The preparation was incubated with increasing concentrations of ^3H -DHA (0.25–10 nM) in the presence (non-specific) and the absence (total) of $5 \mu\text{M}$ dl-propranolol. The inset shows a Scatchard plot of specific ^3H -DHA binding to the membrane fraction. Each point represents the mean of 4 experiments, each conducted in duplicate.

Table 1. Comparison of K_i values determined from binding-inhibition experiments with ^3H -DHA

Compounds	K_i (nM)	n
Antagonists		
l-propranolol	1.91 ± 0.70	4
dl-propranolol	5.18 ± 0.81	4
d-propranolol	118.43 ± 38.62	4
Oxprenolol	7.10 ± 2.27	3
Alprenolol	8.72 ± 3.02	3
Pindolol	9.83 ± 3.60	3
YM-09538	51.38 ± 8.40	3
Labetalol	127.08 ± 39.90	3
Acebutolol	741.47 ± 415.55	2
Atenolol	2336.43 ± 251.38	3
Metoprolol	3118.95 ± 2743.61	3
Sotalol	3737.94 ± 302.43	3
Practolol	13378.10 ± 5855.31	5
Dichloroisoproterenol	449.89 ± 262.10	3
Butoxamine	4819.21	1
Agonists		
l-isoproterenol	777.37 ± 438.19	3
l-epinephrine	4127.06 ± 2316.32	3
l-norepinephrine	8355.72 ± 2861.01	3
Sulbutamol	972.30 ± 360.06	3

Values are the mean $\pm$ S.E. n=number of experiments.

Fig. 4. Relationship between potencies of β -adrenoceptor blockers relative to inhibition of ^3H -DHA binding to the receptor of the neuraminidase-treated rat heart and the pA_2 values as regards the inhibition of the positive inotropic effect of isoproterenol assessed by pharmacological methods.

aminidase. The binding of propranolol showed strict stereospecificity with l-propranolol, exhibiting two orders of magnitude higher affinities than d-propranolol. Among 10 β -blockers tested, dl-propranolol, oxprenolol, alprenolol and pindolol were the most potent, while sotalol and practolol were the least potent. Labetalol and YM-09538, a combined α - and β -adrenoceptor antagonist, occupied an intermediate position as ^3H -DHA binding antagonists. Relative to antagonists, agonists compounds occupied a much smaller fraction of the ^3H -DHA binding sites. The rank order of the potency was l-isoproterenol > l-epinephrine > l-norepinephrine.

Figure 4 depicts the relationship between the log (K_i) values of 9 β -blockers and the pA_2 values of the same β -blocking agents against the positive inotropic actions of isoproterenol in the guinea-pig myocardium, which were taken from the paper of Bieth et al. (8). A good correlation was found between K_i and pA_2 values, giving a straight line which can be described by the equation $y = 0.68x + 3.22$ ($r = 0.91$, $P < 0.001$).

Discussion

Treatment of the cardiac membrane fraction with neuraminidase resulted in a higher reproducibility of the binding assay than was obtainable without neuraminidase treatment. The coefficient of variation of the specific binding was 12.1% as compared with 83.5% for the non-treated preparation. As is well known, the sarcolemma of the cardiac muscle has a glycocalyx that consists mainly of glycosaminoglycans, so called acid mucopolysaccharides, external to the unit membranes (9). It may be concluded that the removal of the sialic acid or some other chemical moieties from the membranes by treatment with deoxyribonuclease or neuraminidase could produce a crucial change in the environment of the β -adrenoceptors, producing a better reproducibility with an

increase in the binding sites (4, 10). Contrary to our expectation, binding characteristics of β -adrenoceptor agonists or antagonists in the cardiac muscle membrane treated with neuraminidase expressed as mean values were not much different from those observed in the membrane without treatment with neuraminidase (8). Thus, the main advantage of using the neuraminidase-treated preparation is the much better reproducibility of the binding assay. Another advantage of the treatment with neuraminidase is the feasibility of conducting the binding assay without preparing the purified membrane fractions from cardiac muscle, thus eliminating long and tedious work and a resultant low yield of the membrane fraction.

The K_d values for the β -adrenoceptors obtained in the heart muscle treated with neuraminidase coincided with (11, 12) or were slightly lower than those (13–15) obtained in the membrane fractions not treated with this enzyme. The ^3H -DHA binding sites exhibited strict stereospecificity for binding of the representative β -adrenergic antagonist propranolol (Fig. 4). In addition, K_i values obtained by Bieth et al. (8) were identical to those of the membrane fraction treated with neuraminidase ($r = 0.98$). Thus, the order of potencies for inhibition of binding observed with various β -adrenoceptor antagonists is in agreement with those reported by other investigators (8, 16). The cardioselective β -blockers like metoprolol, acebutolol and practolol were found to have lower affinities to binding sites. Their lower affinity for binding sites was perhaps related to steric hindrance by the side chain (16) or low partition coefficients (17, 18). On the other hand, propranolol, pindolol, alprenolol and oxprenolol, all of which have higher partition coefficients, showed higher affinities to membrane receptors.

There was a good correlation between K_i

values of the various β -adrenoceptor blocking agents determined on the neuraminidase-treated membranes and the pA_2 values of these compounds observed in the atria of the different species as regards the positive inotropic effects by Bieth et al. (8). The excellent correlation provided further evidence for the identity of binding sites with the receptor. Thus, the validity of using this binding assay for the screening of new compounds reacting with β -adrenoceptors seems to be further justified.

References

- 1) Lefkowitz, R.J.: Clinical physiology of adrenergic receptor regulation. *Am. J. Physiol.* **243**, E43-E47 (1981)
- 2) Hoffman, B.B. and Lefkowitz, R.J.: Radioligand binding studies of adrenergic receptors: New insights into molecular and physiological regulation. *Annu. Rev. Pharmacol. Toxicol.* **20**, 581-608 (1981)
- 3) Watanabe, A.M., Jones, L.R., Manalan, A.S. and Besch, H.R., Jr.: Cardiac autonomic receptors. Recent concepts from radiolabeled ligand-binding studies. *Circ. Res.* **50**, 161-174 (1982)
- 4) Nagatomo, T. and Sasaki, M.: Effects of neuraminidase and deoxyribonuclease on the β -adrenergic receptors in rat heart. *Japan. J. Pharmacol.* **33**, 481-484 (1982)
- 5) Cheng, Y.-C. and Prusoff, W.H.: Relationship between the inhibition constant (K_i) and the concentration of inhibition which causes 50% inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**, 3099-3108 (1973)
- 6) Warren, L.: The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* **234**, 1971-1975 (1959)
- 7) Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275 (1951)
- 8) Bieth, N., Rouot, B., Schwartz, J. and Velly, J.: Comparison of pharmacological and binding assays for ten β -adrenoceptor blocking agents and two β -adrenoceptor agonists. *Br. J. Pharmacol.* **68**, 563-569 (1980)
- 9) Langer, G.A.: The structure and function of the myocardial cell surface. *Am. J. Physiol.* **235**, H461-H468 (1978)
- 10) Taeko, S., Daly, M.J., Anand-Srivastava, M.B. and Dhalla, N.S.: Influence of neuraminidase on rat heart sarcolemma. *J. Mol. Cell. Cardiol.* **12**, 211-217 (1980)
- 11) Harden, T.K., Wolfe, B.B. and Molinoff, P.B.: Binding of iodinated beta-adrenergic antagonists to proteins derived from rat heart. *Mol. Pharmacol.* **12**, 1-15 (1976)
- 12) Baker, S.P., Boyd, H.M. and Potter, L.T.: Distribution and function of β -adrenoceptors in different chambers of the canine heart. *Br. J. Pharmacol.* **68**, 57-63 (1980)
- 13) Williams, L.T., Lefkowitz, R.J., Watanabe, A.M., Hathaway, D.R. and Besch, H.R., Jr.: Thyroid hormone regulation of β -adrenergic receptor number. *J. Biol. Chem.* **252**, 2787-2789 (1977)
- 14) Limas, C. and Limas, C.J.: Reduced number of β -adrenergic receptors in the myocardium of spontaneously hypertensive rats. *Biochem. Biophys. Res. Commun.* **83**, 710-714 (1978)
- 15) Alexander, R.W., Williams, L.T. and Lefkowitz, R.J.: Identification of cardiac β -adrenergic receptors by (-) ^3H alprenolol binding. *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1564-1568 (1975)
- 16) Chenieux-Guicheney, P., Dausse, J.P., Meyer, P. and Schmitt, H.: Inhibition of [^3H] dihydroalprenolol binding to rat cardiac membranes by various β -blocking agents. *Br. J. Pharmacol.* **63**, 177-182 (1978)
- 17) Jack, D.B.: Is there a relationship between the physical properties and cardioselectivity of β -adrenoceptor antagonists? *Br. J. Clin. Pharmacol.* **11**, 402-406 (1981)
- 18) Deacon, C.S., Lennard, M.S., Bax, N.D.S., Woods, H.F. and Tucker, G.T.: Inhibition of oxidative drug metabolism by β -adrenoceptor antagonists is related to their lipid solubility. *Br. J. Clin. Pharmacol.* **12**, 429-431 (1981)