Effects of Bunitrolol on Adrenergic and Serotonergic Receptors

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Abstract—To assess the importance of anti-adrenergic and anti-serotonergic activities of bunitrolol for its efficacy as an antihypertensive and antianginal agent, effects of this substance on the binding of adrenergic and serotonergic agents to the respective receptors of the rat brain, rat heart, dog brain, and/or dog aorta were examined using the radioligand binding assay methods. In addition, the pA2 values of bunitrolol as an antagonist against the positive chronotropic and inotropic actions (β1-adrenoceptor) of isoproterenol were also determined by pharmacological methods using the isolated guinea pig atria. To assess the specificity, pA2 values were also obtained in the isolated trachea (β2-adrenoceptor) using isoproterenol as an agonist and in the isolated aorta from the guinea pig and the rat using phenylephrine as an agonist (α1-adrenoceptor). A strong inhibition by bunitrolol of 3H-dihydroalprenolol (3H-DHA) binding to β-adrenoceptors was observed, while the inhibition of 3H-prazosin binding to α1-adrenoceptors, 3H-serotonin binding to 5HT1-receptors, 3H-p-aminoclonidine binding to α2-adrenoceptors, and 3H-ketanserin binding to 5HT2-receptors were found to be very weak. The rank order of antagonistic potencies of bunitrolol against the adrenergic receptors as assessed with pA2 values were β1 > β2 > α1. From these two different types of experiments, it is clear that the antihypertensive and antianginal effects of bunitrolol are mainly due to its β-blocking actions, with the α1-blocking action of this drug playing a minor role.

Bunitrolol has been clinically used because of its antihypertensive and antianginal efficacy. As is well-known, bunitrolol has a very potent β-adrenergic blocking action (1–3). However, it was reported that, in addition, bunitrolol possessed a weak α1-blocking action (4). To assess the relative importance of these two actions for clinical efficacy, in the present study, we attempted to determine the antagonistic effects of bunitrolol on α- and β-adrenergic receptors with radioligand binding methods as well as by pharmacological assessment. Furthermore, the effect of bunitrolol on serotonergic receptors was examined, as serotonin is also important as a regulator of the blood pressure in the body (5–9).

Materials and Methods

Materials: 3H-DHA (90 Ci/mmol), 3H-prazosin 80.9 Ci/mmol, 3H-p-aminoclonidine (42.2 Ci/mmol), 3H-5-hydroxytryptamine creatine sulfate (3H-serotonin; 24.3 Ci/mmol), and 3H-ketanserin (95Ci/mmol) were purchased from New England Nuclear Co., Ltd. Bunitrolol, O-[2-hydroxy-3-(tert.-butylamino)propoxy]benzonitrile HCl, was kindly donated by Nippon Boehringer Ingelheim Co., Ltd.

Animals: In these experiments, male Wistar rats weighing 200–350 g, mongrel dogs of either sex weighing 10–25 kg, and male Hartley guinea pigs weighing 350–550 g were used.
Preparation of the membrane-enriched fraction: The membrane-enriched fractions from the rat brain and heart used for $^3$H-DHA binding were prepared by the methods described previously (10–12). The membrane-enriched fractions from the rat brain and dog aorta used for $^3$H-prazosin binding were prepared by the methods described previously (13). The membrane-enriched fractions from the dog brain used for $^3$H-DHA binding, those from the brain used for $^3$H-prazosin binding, and those from the dog and rat brain used for $^3$H-$\alpha$-aminoclonidine binding were prepared by the following methods. After removal, the brain was immediately frozen in liquid nitrogen and stored at $-80^\circ$C until used. The brain tissue was defrosted at room temperature and minced with small scissors in 10 vol. of 0.25 M sucrose, 10 mM Tris-HCl buffer (pH 7.4). The suspension was homogenized using a glass homogenizer and filtered through 4 layers of gauze. The filtrate was centrifuged at 40,000 g for 30 min. The pellet obtained was homogenized using a glass homogenizer in an incubation buffer for the radioligand binding assay described below. The membrane-enriched fraction from the dog brain used for $^3$H-serotonin and $^3$H-ketanserin binding was prepared by the following method: The brain defrosted at room temperature was minced with small scissors and homogenized with a glass homogenizer in 10 vol. of 0.25 M sucrose, 1 mM MgCl$_2$, 5 mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 18,000 g for 15 min. The pellet was suspended in 10 vol. of 60 mM Tris-HCl buffer and then incubated for 10 min at 37°C. The suspension was centrifuged at 40,000 g for 30 min. Both pellets from the dog brain and aorta were homogenized using a glass homogenizer.

Binding assays: $\beta$-Adrenoceptor binding assays using the rat brain and heart treated with neuraminidase were carried out by the methods described previously (10–12). In the case of dog brain, the membrane suspension (0.25 mg of protein) was incubated with constant shaking for 30 min at 23°C with 1.0 nM $^3$H-DHA in a total volume of 0.5 ml containing 60 mM Tris-HCl, 20 mM MgCl$_2$ buffer (pH 7.4), and the indicated concentration of unlabelled drugs. $\alpha_1$-Adrenoceptor binding assay using the rat brain and the dog aorta was carried out by the methods described previously (13). The membrane suspensions (0.25 mg of protein) from dog brain were incubated for 60 min at 23°C with 0.2 nM $^3$H-prazosin in a total volume of 1 ml containing 15 mM Tris-HCl, 5 mM MgCl$_2$ buffer (pH 7.4). In the case of $\alpha_2$-adrenoceptor binding, the membrane suspension (0.25 mg of protein) from the rat and dog brain was incubated for 60 min at 23°C with 0.6 nM $^3$H-$\alpha$-aminoclonidine in a total volume of 1 ml containing 15 mM Tris-HCl, 5 mM MgCl$_2$ buffer (pH 7.4). Binding of $^3$H-serotonin (1.0 nM) to the dog brain (0.5 mg of protein) was determined at 37°C for 30 min by incubating with 30 mM Tris-HCl, 5 mM MgCl$_2$, 2 mM CaCl$_2$, 2.85 mM ascorbic acid, 4 $\mu$M pargyline buffer (pH 7.4) in a final volume of 1 ml of the reaction mixture. $^3$H-Ketanserin binding to the membrane fraction (0.25 mg of protein) from the brain and the aorta was determined at 37°C for 30 min in an incubation medium containing 50 mM Tris-HCl, 2.85 mM ascorbic acid, 5 $\mu$M pargyline buffer (pH 7.4). Reactions of all the binding assays were started by adding the membrane suspension. After incubation, the reaction was terminated by a rapid filtration under a vacuum through GF/C glass fiber filters using an Automatic Cell Harvester Labomash (LM-101, Labo Science) (10). Five ml of scintillation fluid (Scintisol EX-H, Dojindo Laboratories) was added to the filters, and the radioactivity was counted using a scintillation counter (Aloka LSC-700). The
specific binding of $^3$H-DHA and $^3$H-prazosin to receptors was defined by the previously described methods (10-13). The specific binding of $^3$H-p-aminoclonidine, $^3$H-serotonin, and $^3$H-ketanserin to receptors in the brain or aorta was defined as the amount of radioligand bound in the absence of competing drug minus the amount in the presence of 0.1 mM I-norepinephrine ($^3$H-p-aminoclonidine binding), 0.1 mM serotonin ($^3$H-serotonin binding), 0.1 mM methysergide ($^3$H-ketanserin binding to the brain), and 10 $\mu$M cinacalcin ($^3$H-ketanserin binding to the aorta). Protein was determined using the method of Lowry et al. (14).

Kinetic analysis: All kinetics analyses were carried out on a NEC PC-9801F computer system that performs iterative non-linear regression, which is based on the theory of Munson and Rodbard (15). The goodness of fit was evaluated on a model having only one receptor subtype and a model having the two receptor subtypes by a Scatchard analysis as described previously (16). In the displacement analysis, the goodness of fit was evaluated on a general model for the interactions of one ligand with one class of receptor site (17). The values of inhibition constants ($K_i$) were calculated by the methods previously described (17) and expressed as $pK_i$ values in this report. In order to quantify the mode of saturation, Hill numbers were determined by Hill plots (18).

Pharmacological observations: $\beta$-Blocking actions were determined as described previously (12, 19), and $\alpha_1$-blocking actions were studied as described previously (13, 20). The right and left atria of the guinea pig were used for the assessment of the antagonistic potencies against the positive chronotropic and inotropic actions of isoproterenol ($\beta_1$-effect). The rate of the spontaneous contraction of the right atria was recorded on a thermo-stylist oscillograph (Watanabe Mark V) to test the positive chronotropic actions. The left atria stimulated electrically by a squarewave pulse stimulator (Nihon Kohden MSE-40) at the frequency of 1 Hz with voltages 30% above the threshold were used to evaluate the inotropic effects. Their contractile tension was recorded on a thermostylist oscillograph (Watanabe Mark V) with a strain gauge transducer (Toyo-Baldwin T7-30-240) and a carrier amplifier (San-ei 1829). Isolated tracheal smooth muscle preparations of the guinea pig were used to assess the antagonistic effects towards the $\beta_2$-adrenoceptor. Isoproterenol was used as an agonist. Seven tracheal rings removed from guinea pigs were sutured together and mounted vertically in a 5 ml organ bath. The contractile tension of the preparation was recorded on a potentiometric recorder (Watanabe SR6204). Drugs were administered in a cumulative fashion, and $pA_2$ values of each of the chemicals were calculated using the equation described previously (12, 20). The bathing solution used was Krebs-Henseleit solution (containing in mM: NaCl, 118; KCl, 4.7; CaCl$_2$, 2.5; NaHCO$_3$, 25.0; MgSO$_4$, 1.2; KH$_2$PO$_4$, 1.2; glucose, 12) which was aerated with a mixture of 95% O$_2$ and 5% CO$_2$. The temperature of the solution was maintained at 32±0.30°C. Isolated preparations of the rat and guinea pig aorta were used to assess the antagonistic effects towards the $\alpha_1$-adrenoceptor. The descending aorta was cut into rings of approximately 2 mm width, and the preparations were mounted in a 10 ml organ bath. The contractile tension of these preparations was recorded on a potentiometric recorder (Watanabe SR6204) using a strain gauge transducer (Toyo-Baldwin T7-30-240) and a carrier amplifier (San-ei 1829). The bathing solution used was the Krebs-Henseleit solution as described above which was aerated with a mixture of 95% O$_2$ and 5% CO$_2$. The temperature of the solution was maintained at 37±1°C.

Results

Table 1 summarizes the dissociation constants ($K_d$) and the capacity of binding sites ($B_{max}$) of the membrane preparations of the various tissues for adrenergic and serotonin radioligands. All Scatchard plots were of uniphasic character, and the Hill coefficients were equal to unity.

Table 2 summarizes the $pK_i$ values of bunitrolol derived from the displacement experiments. Bunitrolol exhibited a higher affinity to $\beta$-adrenoceptors than dl-propranolol and it had negligibly lower af-
Table 1. Binding affinities (K<sub>a</sub>) and capacities (B<sub>max</sub>) of various tissues for adrenergic and serotonergic radioligands

| Radioligands       | Receptor | Tissues       | K<sub>a</sub> (nM) | B<sub>max</sub> (fmoles/mg protein) | B<sub>max</sub> (fmoles/g tissue) | Hill Coefficient |
|--------------------|----------|---------------|---------------------|-------------------------------------|----------------------------------|------------------|
| <sup>3</sup>H-DHA  | <i>β</i>  | Rat heart (3)<sup>a)</sup> | 2.67±0.53           | 83.50± 8.89                        | 4596.29± 415.95                 | 0.97±0.03        |
|                    |          | Rat brain (6)<sup>b)</sup> | 1.05±0.09           | 69.55± 7.72                        | 5125.73± 478.56                 | 1.00±0.07        |
|                    |          | Dog brain (5)  | 1.12±0.17           | 23.49± 3.20                        | 1689.94± 260.44                 | 1.01±0.03        |
| <sup>3</sup>H-Prazosin | <i>α-1</i> | Rat brain (3)<sup>c)</sup> | 0.16±0.04           | 28.76± 8.62                        | 2286.26± 713.38                 | 1.01±0.08        |
|                    |          | Dog brain (3)  | 0.36±0.07           | 33.77± 6.39                        | 2092.67± 362.46                 | 0.99±0.02        |
|                    |          | Dog aorta (7)<sup>c)</sup> | 0.19±0.07           | 85.39±12.85                       | 289.06± 70.21                   | 0.93±0.09        |
| <sup>3</sup>H-p-Aminoclonidine | <i>α-2</i> | Rat brain (3) | 0.35±0.07           | 58.96±21.86                       | 4054.34±1497.36                 | 0.99±0.03        |
|                    |          | Dog brain (3)  | 0.37±0.02           | 60.03±3.42                        | 4045.91±304.27                  | 1.00±0.02        |
| <sup>3</sup>H-Serotonin | 5HT<sub>1</sub> | Dog brain (4) | 2.29±0.50           | 136.32±18.46                      | 12375.04±1466.46                | 0.92±0.04        |
| <sup>3</sup>H-Ketanserin | 5HT<sub>2</sub> | Dog brain (6) | 0.61±0.05           | 35.23±5.52                        | 2831.42±169.71                  | 0.98±0.01        |
|                    |          | Dog aorta (3)  | 1.20±0.29           | 36.80±3.68                        | 138.16±41.84                    | 0.99±0.04        |

Values are the means±S.E. Numbers in parentheses represent the numbers of experiments. The values marked as <sup>a)</sup>, <sup>b)</sup> and <sup>c)</sup> were obtained from the reports described previously: <sup>a)</sup> Ref. 11, <sup>b)</sup> Ref. 10, <sup>c)</sup> Ref. 13.
### Table 2. pKi values of bunitrolol for adrenergic and serotonergic receptors of various tissues

| Radioligands     | Receptors | Tissues   | Drugs               | pKi       |
|------------------|-----------|-----------|---------------------|-----------|
| $^3$H-DHA        | $\beta$   | Rat brain | dl-Propranolol (8)  | 7.68±0.14 |
|                  |           |           | Bunitrolol (3)      | 7.86±0.11 |
|                  |           | Rat heart | dl-Propranolol (12) | 8.30±0.06 |
|                  |           |           | Bunitrolol (3)      | 8.89±0.42 |
|                  |           | Dog brain | dl-Propranolol (4)  | 7.54±0.31 |
|                  |           |           | Bunitrolol (5)      | 8.45±0.42 |
| $^3$H-Prazosin   | $\alpha$-1| Rat brain | Prazosin (9)        | 9.45±0.24 |
|                  |           |           | Clonidine (7)       | 5.97±0.21 |
|                  |           |           | Bunitrolol (3)      | 4.93±0.39 |
|                  |           | Dog brain | Prazosin (7)        | 8.92±0.30 |
|                  |           |           | Clonidine (3)       | 4.97±0.63 |
|                  |           |           | Bunitrolol (4)      | 4.90±0.22 |
|                  |           | Dog aorta | Prazosin (7)        | 10.33±0.33|
|                  |           |           | Clonidine (7)       | 5.96±0.20 |
|                  |           |           | Bunitrolol (3)      | 5.28±0.10 |
| $^3$H-p-Aminoclonidine | $\alpha$-2| Rat brain | Clonidine (3)       | 8.78±0.09 |
|                  |           |           | Prazosin (3)        | 4.78±0.02 |
|                  |           |           | Bunitrolol (3)      | 4.36±0.04 |
|                  |           | Dog brain | Clonidine (4)       | 8.88±0.10 |
|                  |           |           | Prazosin (4)        | 4.73±0.12 |
|                  |           |           | Bunitrolol (3)      | 4.30±0.35 |
| $^3$H-Serotonin  | 5HT$_1$   | Dog brain | Serotonin (9)       | 8.04±0.26 |
|                  |           |           | Ketanserin (5)      | 5.20±0.29 |
|                  |           |           | Bunitrolol (3)      | 4.61±0.12 |
| $^3$H-Ketanserin | 5HT$_2$   | Dog brain | Ketanserin (14)     | 8.67±0.16 |
|                  |           |           | Cinancerin (5)      | 8.27±0.30 |
|                  |           |           | Bunitrolol (4)      | 4.33±0.34 |
|                  |           | Dog aorta | Ketanserin (7)      | 8.47±0.38 |
|                  |           |           | Cinancerin (6)      | 7.40±0.50 |
|                  |           |           | Bunitrolol (7)      | 5.74±0.68 |

Values are the means±S.E. Numbers in parentheses represent the numbers of experiments.

### Table 3. Comparison of adrenoceptor blocking activities of bunitrolol, dl-propranolol and prazosin

| Preparation       | Receptor | Animal   | Agonist      | Antagonist   | pA$_2$ value |
|-------------------|----------|----------|--------------|--------------|--------------|
| Right atrial strip| $\beta$-1| Guinea pig| Isoproterenol| dl-Propranolol (5) | 8.49±0.02 |
|                   |          |          |              | Bunitrolol (6) | 9.02±0.15 |
| Left atrial strip | $\beta$-1| Guinea pig| Isoproterenol| dl-Propranolol (5) | 8.39±0.04 |
|                   |          |          |              | Bunitrolol (5) | 9.06±0.03 |
| Trachea           | $\beta$-2| Guinea pig| Isoproterenol| dl-Propranolol (6) | 8.37±0.12 |
|                   |          |          |              | Bunitrolol (5) | 8.71±0.10 |
| Aorta             | $\alpha$-1| Guinea pig| Phenylephrine| Prazosin (5) | 8.30±0.06 |
|                   |          |          |              | Bunitrolol (5) | 5.04±0.04 |
|                   |          |          |              | dl-Propranolol (5) | 5.29±0.09 |
| Rat               |          |          | Phenylephrine| Prazosin (5) | 10.80±0.19 |
|                   |          |          |              | Bunitrolol (5) | 6.72±0.06 |
|                   |          |          |              | dl-Propranolol (5) | 5.52±0.07 |

Values are the means±S.E. Numbers in parentheses represent the numbers of experiments.
finities for $a_1$, $a_2$, 5HT$_1$- and 5HT$_2$-receptors than prazosin, clonidine, serotonin and ketanserin, respectively.

Table 3 and Fig. 1 show the results of the pharmacological assessment of antagonistic effects of bunitrolol against adrenergic receptors. The rank order of antagonistic potency of bunitrolol was: $\beta_1 > \beta_2 > a_1$.

**Discussion**

In the present study, it was found by Scatchard analyses that $K_d$ values of the binding of a radioligand to a receptor were much the same in different tissues, while the $B_{\text{max}}$ values (fmoles/g tissues) were different depending on the tissues. For example, the $B_{\text{max}}$ values expressed as fmoles/g tissue of the binding of $^3$H-prazosin, $^3$H-p-amino- clonidine and $^3$H-ketanserin were lower in the aorta than in the brain. This is probably due to the lower protein yields ascribable to the technical difficulties inherent in the preparation of membranes. As regards the $B_{\text{max}}$, there was also a species difference. For example, the numbers of $\beta$-adrenoceptors were greater in the rat brain than in the dog.
In our previous work, affinities of \( \beta \)-antagonists to receptors as assessed by \( K_i \) values were lower in the brain than in the heart; especially marked was the lower affinity of S-596 towards \( \beta \)-receptor in the brain as compared with that in the heart (10). In the present study, the \( pK_i \) values of bunitrolol obtained in the rat brain for \( ^3 \)H-DHA binding were lower than those in the rat heart. Thus, the blocking activities of chemicals against the same type of receptors are not necessarily the same in different preparations.

Based on pharmacological studies, bunitrolol is admittedly a \( \beta \)-blocker with a weak \( \alpha_1 \)-blocking action (1-4). This was confirmed in the present study, in which the antagonistic potencies were assessed both by the binding assay method and by pharmacological observations. We have already reported that \( \beta \)- and \( \alpha_1 \)-blocking potencies of compounds as assessed from \( K_i \) or \( IC_{50} \) values obtained from the radioligand binding assays correlate well with those assessed with \( pA_2 \) values obtained by pharmacological methods (13, 15). The result obtained in the present study with these two methods is another example of a good correlation between \( pA_2 \) and \( K_i \).

The antihypertensive effects of \( \beta \)-adrenergic blocking drugs are well documented in man. Recently, several \( \beta \)-adrenergic blocking drugs with an \( \alpha_1 \)-adrenergic blocking effect have been developed. In the case of labetalol, a prototype \( \beta \)-blocker with \( \alpha_1 \)-blocking activities, an acute hypotensive effect was observed, indicating that not only the \( \beta \)-blocking effect but also the \( \alpha_1 \)-blocking effect may be responsible for the antihypertensive effects of this compound (21). However, in the case of bunitrolol, the \( \alpha_1 \)-blocking activities may not be important in the antihypertensive effect of the compound, for the \( \alpha_1 \)-blocking activities were found in the present study to be extremely weak as compared with those of labetalol.

The present study clearly showed the existence of \( 5HT_1 \)- and \( 5HT_2 \)-receptors in the dog brain and \( 5HT_2 \)-receptors in the dog aorta. Central serotonergic neurons were shown to influence the blood pressure (8). Furthermore, the hypertension produced by serotonin has been shown to be mediated through \( 5HT_2 \)-receptors in the peripheral blood vessels (5-7). Serotonin receptors have been demonstrated in the various tissues, and highly significant correlations were found between the concentration of the compound necessary for half-maximal contraction of vessels (ED50) and the binding affinity for binding sites (8). Ketanserin has been developed as an antihypertensive agent because this drug was found to antagonize the vascular effect of serotonin via \( 5HT_2 \)-receptor in vivo (9). However, the possible contribution of the serotonergic blocking effects of bunitrolol to its antihypertensive effects was excluded in the present study; practically no inhibition of \( ^3 \)H-serotonin and \( ^3 \)H-ketanserin binding was observed with bunitrolol.

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