Effects of Bifemelane Hydrochloride on Various Cholinergic Markers in Cortical and Subcortical Regions of Aged Rats

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Abstract—The effects of bifemelane hydrochloride (BF) upon various cholinergic markers, muscarinic receptors, acetylcholinesterase (AChE) and choline acetyltransferase (CAT), in the aged rat brain were examined. Marked reduction of the density of muscarinic receptors (B_{max}) as well as AChE and CAT activity concomitant with aging was observed. Administration of BF in daily doses of 10 mg/kg for 4 weeks to aged rats significantly decreased the apparent dissociation constant (K_d) for QNB in muscarinic receptors in the forebrain, but did not affect the value of B_{max} . CAT activity also increased significantly compared with that of control aged rats, but administration of BF did not alter AChE activity. These results indicate that long-term treatment with BF enhances the affinity of muscarinic receptors for QNB as well as CAT activity and that BF may have therapeutic application in the treatment of CNS cholinergic dysfunctions.

Bifemelane hydrochloride (BF), designated chemically as 4-(*o*-benzyl phenoxy)-*N*-methylbutylamine hydrochloride, is a new compound which displays antianoxic, EEG-activating and memory retrieval effects in laboratory research animals (1–3). This drug is also reported to be effective in promoting the uptake of glucose into the brain (4). In addition to these pharmacological properties, BF was also found to be effective for the treatment of cerebrovascular disease in clinical trials (5).

Recently, it has been reported that BF improved scopolamine-induced amnesia and potentiated the effects of physostigmine, and the effects of BF were antagonized by atropine (6). The drug also has been shown to prevent the decreases in acetylcholine levels that ordinarily result from ischemia, hypoxia and scopolamine injection (7). BF has been used clinically in the treatment of the memory impairment symptoms observed in patients with senile dementia or cerebrovascular disease (8). However, the mechanism of the effects of BF on the central nervous system has not been fully elucidated. These observa-

tions prompted us to investigate the effects of BF on cholinergic neurons. The present study was concerned with the effects of BF on various cholinergic markers, muscarinic receptors, acetylcholinesterase (AChE) and choline acetyltransferase (CAT) in the aged rat brain.

Materials and Methods

1. Materials: ^3H -QNB ((-)-quinuclidinyl benzilate, 43 Ci/mmol) and acetyl- ^3H -coenzyme A (200 mCi/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Atropine sulfate, acetyl coenzyme A, choline bromide and physostigmine sulfate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bifemelane hydrochloride (BF) was donated by the Eisai Co., Ltd. (Tokyo, Japan). Pirenzepine hydrochloride was supplied by Boehringer Ingelheim Japan. All other chemicals were obtained from Wako Pure Chemical, Industries, Ltd., Japan.

2. Rat brain tissues: The young male Wistar rats used in the experiments weighed 200–300 g (2 months), while the aged male Wistar rats weighed 500–600 g and were all over 24

months old. The rats were kept in an environmentally controlled room (20–23°C, 50–60% humidity, illumination from 7:00 to 19:00 hours) with food and water available *ad libitum*. On the day of the assay, the rats were sacrificed by decapitation. The brains were rapidly removed from the skulls and kept in a dish of ice-cold saline for one hour at most before dissection of the three different brain regions was performed. The brain areas used were as follows: (a) forebrain, (b) temporal, parietal and occipital cortices (which were combined for all assays and are generically referred to below as cortical regions) and (c) hippocampus, globus pallidus, thalamus, nucleus basalis of Meynert, caudate nucleus, putamen and striatum (which were combined for all assays and are generically referred to below as subcortical structures). To minimize individual variations, tissues from at least 5 animals were pooled for use in each experiment. After weighing, the tissues were homogenized in 5 vols. of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$. The homogenates were centrifuged at $35,000\times g$ for 15 min, and then the pellets were resuspended in the same buffer; this procedure was repeated twice. The crude synaptic membrane fractions (P_2 fraction) obtained by this centrifugation were used as the AChE and muscarinic receptor preparations. The tissues were homogenized in 5 vols. of ice-cold 50 mM phosphate buffer (pH 7.4) containing 10 mM EDTA and 2.5% Triton X-100. These preparations were allowed to stand for 15 min at 0°C and then centrifuged at $35,000\times g$ for 20 min. The supernatants obtained by this centrifugation were used as the choline acetyltransferase (CAT) preparations.

3. Drug administration: To elucidate the *in vivo* effects of BF on muscarinic receptors, AChE and CAT activity, BF in daily doses of 10 mg/kg was orally administered to aged rats for 4 weeks. Rats receiving physiological saline orally for 4 weeks served as the controls. All rats were decapitated 24 hr after the final dose.

4. Ligand binding assay (9): Aliquots of tissue homogenate (100 μ l, 1 mg/ml protein) were incubated at 37°C for 30 min with various concentrations of 3H -QNB (0.04 nM–

0.6 nM) in a final assay volume of 1.0 ml in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$. Differences between measurements in the presence and absence of atropine (1 μ M) were used to define specific binding. Reactions were terminated by rapid filtration through glass fiber filters (Whatman GF/C) under reduced pressure, and the filters were immediately washed three times with 5-ml aliquots of ice-cold 10 mM Tris-HCl buffer (pH 7.4) containing 15 mM NaCl. After the filters were dried overnight, 8 ml of scintillation cocktail were added. Samples were quantified by liquid scintillation spectroscopy (Packard Tri-Carb) with an efficiency of 45%. Typically, counts were 95% specific for 3H -QNB binding. 3H -QNB was used a concentration of 0.04 nM for experiments involving the competitive displacement of 3H -QNB binding by atropine, pirenzepine or BF.

5. Enzyme activity: Acetylcholinesterase (AChE) activity was measured in accordance with the method of Ellman (10). The 1.0 mg/ml enzyme sample were incubated in 100 mM phosphate buffer (pH 8.0) containing 10 mM 5,5'-dithiobis-2-nitrobenzoic acid in a final volume of 3.5 ml at 37°C for 5 min. After adding 20 μ l of acetylthiocholine (75 mM), the 412 nm absorbance was measured with a Hitachi 557 spectrophotometer. Enzymic activity was expressed as nmol/min/mg of protein. The V_{max} and K_m values were obtained from Lineweaver-Burk plots corresponding to various concentrations of acetylthiocholine.

Choline acetyltransferase (CAT) activity was measured by a minor modification of the radiometric method of Fonnum (11) using 3H -acetyl coenzyme A as substrate. Aliquots of brain homogenate were treated with a solution containing 50 mM phosphate buffer (pH 7.4) and 2.5% Triton X-100 to obtain a concentration of 1 mg/ml protein for each sample. The incubation solutions contained 20 mM EDTA, 50 mM phosphate buffer (pH 7.4), 0.1 mM physostigmine, 8 mM choline bromide, 0.3 M NaCl and 0.2 mM acetyl coenzyme A. Eight-microliter samples and 20 μ l aliquots of incubation solution were combined and incubated at 37°C for 15 min. The reaction was terminated by adding cold 10 mM phosphate buffer. The reaction products

were extracted with 2.5 ml of toluene-acetonitrile containing 0.5% Na-tetraphenylborate (4:1, v/v). Samples of the extract were mixed with Triton X-100-toluene scintillation liquid, and their radioactivities were measured by liquid scintillation spectrometry. The results were calculated from dpm values and expressed as nmol of acetylcholine synthesized/min/mg of protein. The V_{max} and K_m values were obtained from Lineweaver-Burk plots corresponding to various concentrations of choline bromide.

6. Protein: Protein content was determined by the method of Lowry et al. (12) using bovine serum albumin as the standard.

7. Analysis of results: Binding data derived from the saturation experiments were analyzed by computerized linear regression analysis to estimate the apparent dissociation constant (K_d) and the density of receptors (B_{max}). The statistical significance of differences between young and aged rats or normal aged and BF-treated aged rats was calculated using Student's *t*-test.

Results

1. Effects of BF on muscarinic receptors in vitro: The effects of BF on muscarinic receptors in the forebrain of aged rats were investigated. As shown in Fig. 1, ^3H -QNB

Fig. 1. Inhibition of specific ^3H -QNB binding by BF, pirenzepine and atropine. Membrane preparations of the forebrain of aged rats were incubated with 0.04 nM ^3H -QNB and various concentrations of BF (●—●), pirenzepine (○—○) and atropine (◆—◆) in 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM MgCl_2 for 30 min at 37°C. Each point represents the mean of the specific ^3H -QNB binding values determined in three separate experiments.

Fig. 2. Lineweaver-Burk plots of the ^3H -QNB binding with and without BF. A membrane preparation of aged rat forebrain was incubated for 30 min at 37°C in the absence (○—○) or presence of 2 μM BF (●—●) or 10 μM BF (▲—▲). Values indicated are the means of duplicate determinations.

binding was strongly displaced with increasing concentrations of BF. The displacement curve was parallel to the typical curves of pirenzepine or atropine, known as antagonists of muscarinic receptors. Furthermore, BF manifested a competitive behavior with respect to muscarinic receptors, as indicated by Lineweaver-Burk double reciprocal plots (Fig. 2). BF also competed for ^3H -QNB binding sites in the other two different brain regions, i.e., cortical regions and subcortical structures (data not shown). Very similar results were obtained in the three different brain regions of young rats and BF-treated aged rats (data not shown).

2. Effects of repeated administration of BF on muscarinic receptors: Figure 3 compares the results concerning specific ^3H -QNB binding in the three different brain regions of control young and aged rats as well as those of aged rats after oral administration of BF (10 mg/kg/day) for 4 weeks. The only significant difference observed was in the forebrain of control aged rats vs. BF-treated aged rats, where ^3H -QNB specific binding in BF-treated aged rats was about 15% greater than that in the control group. In order to determine whether this increase in ^3H -QNB specific binding induced by BF was due to an increase in the density of muscarinic receptors or to an increase in the affinity of the

Fig. 3. Effect of repeated administration of BF on QNB binding in three different brain regions of aged rats. Membrane preparations were incubated for 30 min at 37°C with 0.15 nM ^3H -QNB in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM MgCl_2 . Specific binding was expressed by the difference in binding in the presence and absence of an excess amount of unlabeled atropine. The indicated data are the means and standard errors of the mean values for 5 rats. Statistical significance of differences was assessed by Student's *t*-test. * $P < 0.05$, ** $P < 0.01$. FB: forebrain, CR: cortical regions, SS: subcortical structures.

receptors for QNB, kinetic analyses of ^3H -QNB binding were performed. Figure 4 shows the results of Scatchard analyses of these data and indicates that BF increased the affinity of the receptors but did not affect the density of muscarinic receptors (B_{max}) in the forebrain of aged rats (Table 1). There was a slight age-related change in the affinity of the ligand for muscarinic receptors in the forebrain (0.123 ± 0.001 nM for young vs. 0.107 ± 0.005 nM for aged) and subcortical structures (0.087 ± 0.008 nM for young vs. 0.042 ± 0.006 nM for aged). By contrast, significant age-related differences were found with respect to the density of muscarinic receptors (B_{max}) in the three different brain regions of aged rats (Table 1).

3. Effects of repeated administration of BF

Fig. 4. Scatchard analyses of specific ^3H -QNB binding in the forebrain of aged and BF-treated aged rats. Membrane preparations were incubated with the indicated concentrations of ^3H -QNB for assessment of binding to muscarinic sites. Nonspecific binding was determined in the presence of 1 μM atropine. The abscissa represents the amount of bound radioligand, expressed as pmol/mg of protein; the ordinate represents the ratio of bound to free radioligand, expressed as pmol/mg of protein/nM. Each data point represents the mean of two experiments in which measurements were performed in triplicate. (●—●) young rats, (○—○) aged rats, (◆—◆) BF-treated aged rats.

on AChE and CAT activity: The activities of AChE and CAT are shown in Tables 2 and 3. With advancing age, V_{max} values of AChE and CAT declined in the three areas of the aged rat brains. However, no substantial differences were observed between the K_m values of these enzymes in young and aged rats. After repeated administration of BF to aged rats, AChE activity in the forebrain and cortical regions was not significantly different in the BF-treated aged rats as compared with the control aged rats. However, a moderate increase of AChE activity was noted in the subcortical structures of BF-treated aged rats. By contrast, administration of BF for 4 weeks resulted in a significant increase in V_{max} values of CAT in the three brain areas of BF-treated aged rats as compared with those of the control aged rats.

Table 1. Binding of ^3H -QNB to muscarinic ACh receptors in the brain of young, aged and BF-treated aged rats

$B_{\max}$			
	Young rats	Aged rats	BF-treated aged rats
Forebrain	2.35 $\pm$ 0.15	1.63 $\pm$ 0.03**	1.50 $\pm$ 0.09**
Cortical regions	1.82 $\pm$ 0.07	1.40 $\pm$ 0.08**	1.23 $\pm$ 0.06***
Subcortical structures	1.16 $\pm$ 0.17	0.56 $\pm$ 0.03**	0.73 $\pm$ 0.04*
K_d			
	Young rats	Aged rats	BF-treated aged rats
Forebrain	0.123 $\pm$ 0.001	0.107 $\pm$ 0.005*	0.081 $\pm$ 0.005***
Cortical regions	0.089 $\pm$ 0.007	0.112 $\pm$ 0.009	0.076 $\pm$ 0.013#
Subcortical structures	0.087 $\pm$ 0.008	0.042 $\pm$ 0.006**	0.074 $\pm$ 0.003##

Binding data are derived from saturation analysis; binding for each concentration of radioligand was measured in triplicate, and nonspecific binding was defined as binding in the presence of 1 μM atropine, also in triplicate. Results represent the maximal density of muscarinic receptors ($B_{\max}$) and apparent dissociation constant (K_d) for ^3H -QNB binding and are expressed as the mean $\pm$ S.E. of the values obtained for five rats. $B_{\max}$: pmol/mg protein, K_d : nM. * P <0.05, ** P <0.02, *** P <0.01, compared with young rats; # P <0.05, ## P <0.02, ### P <0.01, compared with aged rats. BF: Bifemelane, 10 mg/kg, p.o., 4 weeks.

Table 2. Effects of repeated administration of BF on AChE activity

	Young rats		Aged rats		BF-treated aged rats	
	$V_{\max}$	K_m	$V_{\max}$	K_m	$V_{\max}$	K_m
Forebrain	283.8 $\pm$ 10.2	83.0 $\pm$ 16.0	205.0 $\pm$ 13.0**	77.9 $\pm$ 8.6	206.5 $\pm$ 9.3**	78.2 $\pm$ 5.5
Cortical regions	140.8 $\pm$ 9.2	62.8 $\pm$ 7.8	92.2 $\pm$ 1.8**	69.3 $\pm$ 2.6	86.2 $\pm$ 6.6**	74.9 $\pm$ 5.8
Subcortical structures	188.7 $\pm$ 8.3	56.0 $\pm$ 3.3	122.5 $\pm$ 5.3**	58.5 $\pm$ 5.2	154.9 $\pm$ 7.7*#	53.9 $\pm$ 2.5

Each value represents the mean $\pm$ S.E. for five rats. $V_{\max}$ and K_m values were determined from Lineweaver-Burk double reciprocal plots of values obtained from graphic representation of kinetic data. $V_{\max}$: nmol/min/mg protein, K_m : μM . * P <0.05, ** P <0.01, compared with young rats; # P <0.05, compared with aged rats.

Table 3. Effects of repeated administration of BF on CAT activity

	Young rats		Aged rats		BF-treated aged rats	
	$V_{\max}$	K_m	$V_{\max}$	K_m	$V_{\max}$	K_m
Forebrain	1.89 $\pm$ 0.33	2.82 $\pm$ 0.15	1.58 $\pm$ 0.1	2.61 $\pm$ 0.58	2.73 $\pm$ 0.21*#	2.52 $\pm$ 0.39
Cortical regions	1.28 $\pm$ 0.03	1.77 $\pm$ 0.18	1.09 $\pm$ 0.09	2.07 $\pm$ 0.13	1.41 $\pm$ 0.10*#	1.99 $\pm$ 0.31
Subcortical structures	1.29 $\pm$ 0.18	9.21 $\pm$ 0.51	0.92 $\pm$ 0.09	8.14 $\pm$ 2.30	2.32 $\pm$ 0.39***	12.28 $\pm$ 1.90

Each value represents the mean $\pm$ S.E. for five rats. $V_{\max}$ and K_m values were determined from Lineweaver-Burk double reciprocal plots of values obtained from graphic representation of kinetic data. $V_{\max}$: nmol/min/mg protein, K_m : mM against choline bromide. * P <0.05, ** P <0.01, compared with young rats; # P <0.05, ## P <0.01, compared with aged rats.

Discussion

In general, the membrane receptors of neurotransmitters decrease concomitantly with rising neurotransmitter levels or after administration of agonists, which is generally regarded as a down regulation phenomenon. As indicated in our previous paper (13), when BF was orally administered to young rats for 4 weeks, the density of muscarinic receptors decreased significantly in the forebrain. We have reported that this decrease phenomenon of density of muscarinic receptors is not attributed to down regulation; in view of results indicating that BF possesses activating effects upon cholinergic neurons in the central nervous system and an antagonistic action with respect to the effects of atropine (6), it seems likely that BF directly alters the lipid organization or protein composition of the muscarinic receptors in the synaptosomes after long-term administration of BF. By contrast, in the present study, when aged rats were given daily injections of BF for 4 weeks, specific ^3H -QNB binding in the forebrain increased by about 15% as compared with the level for aged control rats. To determine whether or not the increase in specific ^3H -QNB binding by BF was due to an increase in the density of muscarinic receptors, kinetic analysis of QNB binding were performed. As shown in Fig. 4, BF significantly increased the affinity but did not affect the density of muscarinic receptors for QNB in the forebrain of aged rats. Thus, BF possesses a unique mechanism of action.

Biochemical studies have consistently confirmed that a loss of cholinergic function occurs during natural aging (14–17). In the present study, marked reduction of AChE and CAT activity concomitant with aging was also observed. The long-term treatments with BF increased significantly the $V_{\max}$ values, but not the K_m values of CAT for its substrates in the three regions of the brain as compared with the control group of aged rats. This indicates that BF increased the amounts of CAT, without altering its affinity for its substrates. However, in our previous paper, no effects of chronic administration of BF upon the $V_{\max}$ and K_m values of CAT were observed in young rats (13). Moreover, BF did not significantly

alter CAT activity in vitro. The selective mechanism of this increase in $V_{\max}$ values following administration of BF to aged rats remains unexplained.

Recently, changes in endogenous concentrations of neurotransmitters, alterations in the activity of synthetic or degradative enzymes and changes in the binding of ligands to receptors have been reported in senile dementia (18–21). In particular, CAT activity is generally accepted as a good indicator of cholinergic function, as well as the degree of severity of Alzheimer's disease (22). Muscarinic receptors are also known to be markedly reduced in the frontal cortex of patients with cerebrovascular dementia (23). A number of drugs and substances have been tested for possible improvement of the memory impairment symptoms observed with patients of senile dementia. Pantoyl-GABA (24) is reported to increase both the release of ACh and the quantity of CAT, without altering its affinity for its substrates. Chronic administration of dihydroergotoxine (25, 26) is also reported to enhance CAT activity and to promote recovery of muscarinic receptor binding in the temporal cortex and hippocampus in the aged rat brain. It has also been suggested that calcium hopantenate (27) increases the affinity of the GABA receptors in the cerebral cortex.

The results of the present study clearly demonstrate that BF also possesses a unique action, increasing both the affinity of muscarinic receptors for QNB and CAT activity in aged rats. Thus, all the above findings suggest that BF may have therapeutic applications in the treatment of senile dementia, including Alzheimer's disease and cerebrovascular dementia.

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