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Research report

Inhibitor of antagonist binding to the muscarinic receptor is elevated in Alzheimer's brain ☆

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Abstract

The 100,000 × *g* supernatant fraction of human brain contains endogenous inhibitors of antagonist binding to the muscarinic receptor. Significantly greater inhibition was observed with Alzheimer's than non-demented control supernatant fractions. Low molecular weight inhibitor was separated from larger inhibitor species by membrane dialysis (3,500 dalton cut-off). The activity of low molecular weight inhibitor was greatly increased by sulfhydryl reducing agents. While the low molecular weight inhibitor was stable to heat, acid and base for short time periods (< 20 min), it was inactivated by acid hydrolysis (50% loss after 16 h, 100% loss after 96 h). The low molecular weight inhibitor activity is elevated approximately three-fold in Alzheimer's brain. The low molecular weight inhibitor from Alzheimer's brain was found to be a non-competitive inhibitor. This is the first report of endogenous inhibitors in human brain of ligand binding to the muscarinic receptor and of increased inhibitor activity in Alzheimer's disease.

Key words: Alzheimer's; Muscarinic receptor; Endogenous inhibitor; Brain

1. Introduction

Available evidence indicates that the muscarinic cholinergic receptor (mAChR) and cholinergic transmission are crucial to memory and learning [6,8,10]. A loss of cholinergic function, including markedly decreased levels of brain choline acetyltransferase [7] and a loss of cholinergic neurons in the nucleus basalis [31], have been demonstrated in patients with Alzheimer's disease (AD), the most prevalent memory disorder [8]. Numerous clinical studies have been conducted to treat AD patients through pharmacologic manipulation of the cholinergic system [8]. Consequently, endogenous substances regulating central muscarinic cholinergic function are of considerable importance.

Several investigators have reported the presence of endogenous inhibitors of antagonist binding to the mAChR in mammals [1,4,9,15,12,24]. However, the exact nature and identity of the mammalian inhibitors remains unknown. Hu et al. [20] have recently reported that protamine causes complex allosteric modulation of the mAChR of rat heart, and Jerusalinsky et al. [21] have isolated two polypeptides from snake venom which selectively inhibit binding of antagonists to the central mAChR. We now report the presence in human brain of endogenous inhibitors of antagonist binding to the mAChR, at least one of which is a low molecular weight substance whose activity is significantly increased in AD.

2. Materials and methods

2.1. Materials

Materials used were as follows: [^{3}H]quinuclidinyl benzilate (47 Ci/mol) was from Amersham. β-Mercaptoethanol, dithiothreitol, Tris-HCl, atropine sulfate, vitamin B_{12}, acetylcholine, acetylcholinesterase (EC 3.1.1.7), *E. coli* alkaline phosphatase (EC 3.1.3.1), choline, and Sephadex G-10 and G-25 were purchased from Sigma.

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Spectra/Por 3 dialysis tubing was from Spectrum Labs, BCA protein reagent kit was from Pierce, glass fiber filter circles were from Whatman, Optifluor was from Packard and [^{3}H]pirenzipine (70 μCi/mmol) was from DuPont NEN.

2.2. Membrane muscarinic cholinergic receptor preparations

mAChR Method A. For the initial inhibitor studies, subcellular membranes from non-demented adult human frontal cortex (normal tissue by neuropathology) were prepared according to Frey et al. [14] a modification of von Hungen and Roberts [30]. All steps were performed at 4°C. Frontal cortex gray matter was homogenized with a Waring blender in 9 vols. of 0.32 M sucrose. The homogenate was centrifuged at 1000× *g* for 10 min. The resulting supernatant fraction was centrifuged at 15,000× *g* for 25 min, the pellet discarded, and the supernatant fraction was then centrifuged at 100,000× *g* for 60 min. The resulting pellet, containing microsomal membranes and accompanying muscarinic receptors, was resuspended at 5% w/v in water and allowed to lyse for 1 h at 4°C. This resuspension was centrifuged at 100,000× *g* for 60 min. The final washed pellet was then resuspended at 15% wt/vol in 50 mM Tris-HCl, pH 8.1, aliquoted, frozen in liquid nitrogen and stored at −70°C for use in the binding assays. Thawed aliquots were briefly re-homogenized in a glass/glass hand-held homogenizer prior to assay.

mAChR Method B. Membranes rich in mAChRs were prepared by a modification of the method used by Marks and Collins [23]. Gray matter from non-demented adult human frontal cortex was homogenized in 9 vols. of 50 mM Tris-HCl, pH 7.4, at 37°C, using 5 strokes of a glass/Teflon motor-driven homogenizer. The homogenate was centrifuged at 27,000× *g* for 20 min at 4°C, and the subsequent pellet resuspended in 10 vols. of cold deionized water with 5 strokes of the homogenizer. The resuspension was incubated at 37°C for 5 min, then was centrifuged as before. The resulting pellet was resuspended, incubated and centrifuged again as above. The final pellet was weighed, resuspended at 15% w/v in 50 mM Tris-HCl buffer, aliquoted in small portions, flash-frozen in liquid nitrogen and stored at −70°C for subsequent assays for protein content and binding. Before use in binding assays, the thawed membrane preparation was briefly re-homogenized with 10 strokes in a glass/glass homogenizer. A typical mAChR membrane preparation bound 300 pmol ^{3}H-QNB/g protein.

2.3. Inhibitor preparation

Brain tissue selection. We have been preparing the soluble inhibitor from human brain tissue obtained from cases of AD and non-demented controls matched for age, sex and postmortem interval between death and freezing of tissue. These cases come from our large dementia brain bank of about 1,500 cases of AD, other dementias and normal non-demented controls. On chart review, the AD cases met retrospective clinical criteria for primary degenerative dementia according to the *Diagnostic and Statistical Manual of Mental Disorders* – 3rd version: revised (American Psychiatric Association, 1987). The pathologic diagnosis of AD was based on the presence of an age-adjusted moderate-to-severe number of neuritic plaques in the neocortex similar to published criteria without evidence of other neurologic disease [22,25]. Cases were excluded if their medical histories revealed recent exposure to drugs with cholinergic central nervous system activity. Non-demented control cases were without clinical or neuropathologic evidence of neurologic or psychiatric disease. Autopsy brain tissue was frozen in liquid nitrogen and stored at −70°C between 5 and 24 h after death.

Method A. For the initial inhibitor studies, 100,000× *g* supernatant fractions were prepared from either AD or normal control frontal cortex gray matter by the same subcellular fractionation procedure as in mAChR Method A, except that the tissue was initially homogenized in 9 vols. of water and the third centrifugation was for 90 min (instead of 60 min) to insure that all insoluble membrane fragments were pelleted. The final pellet was discarded and the supernatant fraction was frozen in liquid nitrogen and stored at −70°C. For gel filtration analysis, these supernatant fractions were concentrated by lyophilization and resuspended to 1/20 the original volume in water.

Method B. Gray matter from human frontal cortex (either normal non-demented or AD by neuropathology) was homogenized in 9 vols. of 1.0 M acetic acid for 40 s at 80°C in a Waring blender, heated for 5 min in an 80°C water bath, then centrifuged at 1200× *g* for 10 min at 4°C. The resulting supernatant fraction was centrifuged at 11,000 × *g* for 20 min at 4°C. The 11,000× *g* supernatant fraction was centrifuged at 100,000× *g* for 100 min at 4°C, then the 100,000× *g* supernatant fraction was lyophilized and resuspended in water at 1/20 the original homogenate volume. The 100,000× *g* supernatant fraction was transferred to a Spectra/Por 3 dialysis (3500 dalton cut-off) membrane bag, and dialyzed against 20 vols. of water at 4°C for 24 h with gentle stirring. The resulting < 3500 dalton fraction (dialysate) was lyophilized and resuspended in water to the volume originally placed in the dialysis bag, then both the < 3500 (low molecular weight (MW) inhibitor) and > 3500 fractions (dialysis retentate) were frozen in liquid nitrogen and stored at −70°C for subsequent assays for protein content and inhibitor activity. A typical low MW inhibitor preparation contained about 2.5 mg/ml protein.

2.4. Inhibitor activity assay

Assay method A. Inhibitor activity was measured using a modification of the method of Fields et al. [13] to assess the binding of [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB), a non-specific muscarinic receptor antagonist, to the mAChR. [^{3}H]QNB binding was determined with 45 mM Tris-HCl, pH 7.4, at 37°C, 30 mM β-mercaptoethanol (β-ME), 75 μg/ml membrane (mAChR method B) and 2.5×10^{-10} M [^{3}H]QNB, with and without addition of inhibitor. To control for non-specific binding, 12.5 μM atropine sulfate (an mAChR antagonist) was added to several tubes. Subtracting non-specific binding from total binding yielded specific binding. The Tris buffer, β-ME, inhibitor fraction, mAChR preparation and enough water to make all reaction volumes equal 4 ml total, were combined in glass test tubes, mixed and preincubated in an ice bath for 30 min. Preincubation of the inhibitor with the mAChR as described resulted in more consistent inhibition. The binding reaction was initiated by adding [^{3}H]QNB, vortexing the tubes briefly, and then incubating the tubes in a 37°C water bath with moderate shaking. After 60 min the binding reaction was terminated by adding 5 ml of cold 50 mM Tris buffer, pH 7.4, to each tube and chilling the tubes in an ice bath. The tube contents and 3×5 ml rinses were filtered through Whatman GF/B glass fiber filters using a vacuum manifold. The dried filters were placed in scintillation fluor and counted in a Beckman LS-7000 scintillation counter set for tritium detection. Counting efficiency was about 45%.

Assay method B. Initial binding studies followed essentially the same assay procedure as method A, but omitted the β-ME and the preincubation on ice, and used the muscarinic receptor membrane preparation mAChR method A.

2.5. Protein assay

Protein concentrations were measured using the bicinchoninic acid (BCA) protein assay method, essentially as described by Smith et al. [28].

2.6. Alkaline phosphatase treatments

Twenty μl of low MW inhibitor preparation were incubated with 1 unit of alkaline phosphatase in 0.4 M Tris-HCl (pH 8.0) at 25°C for

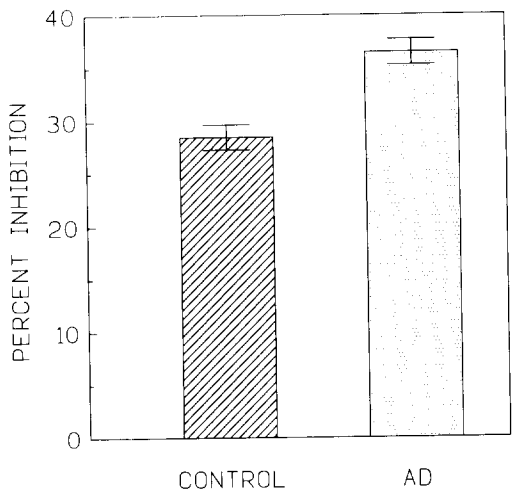

Fig. 1. Inhibition of [^{3}H]QNB binding to normal human brain mAChR by supernatant fractions from twelve matched pairs of normal and AD frontal cortex. Brains were matched for age (AD 70±6; normal 70±7) and sex (AD 8 male, 4 female; normal 9 male, 3 female). The mean age of dementia onset was 63±8 years and the mean duration of disease 8±3 years. Values shown are average percent inhibition±standard error of duplicate determinations. For Figs. 1–3, assays (Method B) were performed without β-ME using a microsomal membrane mAChR preparation (mAChR Method A) and 100,000×*g* supernatant (Inhibitor preparation method A).

60 min. Before the treated inhibitor was tested in the binding assay (Assay method A), the alkaline phosphatase was inactivated by placing the sample in a boiling water bath for 10 min, followed by an ice-water bath for 20 min. Heat-treated controls were included to account for any effect of inactivated alkaline phosphatase on the binding assay.

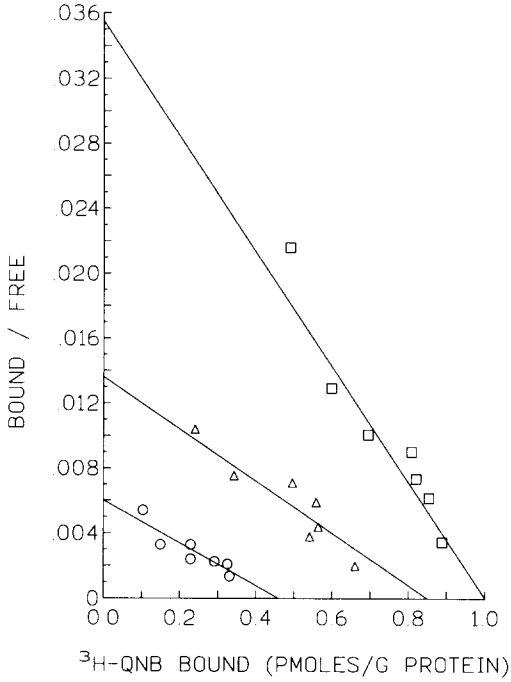

Fig. 2. Scatchard analysis of the effects of AD and normal supernatant fractions on [^{3}H]QNB binding to mAChR. Representative 100,000×*g* supernatant fractions from AD and normal frontal cortex were assayed for inhibitor activity using a microsomal membrane mAChR preparation. B_{max} values are 1.0 pmol/g protein for the control without supernatant (□), 0.85 pmol/g for the normal supernatant fraction (△), and 0.46 pmol/g for the AD supernatant fraction (○), from the Scatchard analysis [27]. See Table 1 for the means and standard errors of the B_{max} and K_d values for all 12 matched pairs.

3. Results

While examining subcellular fractions of human frontal cortex from both Alzheimer's patients and normal non-demented control cases, we noted that the 100,000×*g* supernatant fractions inhibited [^{3}H]QNB binding to the mAChR of the microsomal membrane fraction from normal brain (Fig. 1). The inhibition was dependent on the amount of supernatant fraction added to the QNB binding assay (not shown). Comparison of supernatant fractions prepared from the frontal cortex gray matter of 12 neuropathologically-confirmed AD cases and 12 non-demented controls matched for age and sex demonstrated a significantly greater inhibition ($P < 0.05$) of [^{3}H]QNB binding by AD supernatant fractions (Fig. 1).

Both the AD and control supernatant fractions significantly decreased mAChR density (B_{max}), with a greater reduction in B_{max} observed when the AD supernatant fraction was added (Fig. 2, Table 1). Further, while addition of both AD and normal supernatant fractions increased the dissociation constant (K_d), only the increase caused by the AD supernatant was statistically significant (Fig. 2, Table 1). The above differences could not be explained by patient age, sex or duration of illness. Variations in postmortem time interval (5–24 h) were not associated with any significant effects on either inhibitor activity or binding parameters (not shown). These initial studies indicated that one or more endogenous inhibitors of antagonist binding to the mAChR were present in the soluble fraction of human frontal cortex grey matter and suggested inhibitor activity was elevated in AD. In addition to inhibiting [^{3}H]QNB binding, the 100,000×*g* super-

Table 1
Effect of normal and AD brain 100,000×*g* supernatant fractions on [^{3}H]QNB binding parameters

Binding parameters	No additions	Normal	AD
K_d (pM)	39 ±6	61 ±9	111 ±25 *
B_{max} (pmol/g protein)	1.5±0.2	1.1±0.3 *	0.9± 0.2 **

The effect of supernatant fractions from 12 matched pairs (described in Fig. 1 legend) on the binding of [^{3}H]QNB to mAChR from normal human brain was studied. Binding parameters obtained in the presence of either normal or AD supernatant fractions were compared to those obtained with no addition of supernatant fractions. Significant differences are indicated by * $P < 0.05$ or ** $P < 0.005$.

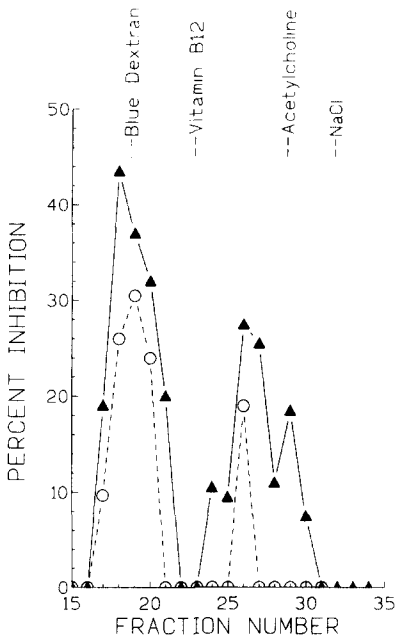

Fig. 3. Chromatography of inhibitor from AD and normal brain on Sephadex G-10. 2.5 ml vols. of the 100,000× *g* supernatant samples from normal (○) and AD (▲) frontal cortex were applied to a Sephadex G-10 column (0.8×68 cm), equilibrated with 50 mM Tris-HCl, pH 7.4, at 4°C, eluted at a rate of 6 ml/h, collected in 3.4 ml fractions and tested for inhibition of [^{3}H]QNB binding. Molecular weight standards [blue dextran (2×10^6 daltons), vitamin B_{12} (1350), acetylcholine (182) and NaCl (58)] eluted as indicated.

natant fraction from AD brain also inhibited the binding of [^{3}H]pirenzepine, an M_1 antagonist (not shown).

Chromatography of the AD and non-demented control supernatant fractions on either Sephadex G-10 (Fig. 3) or Sephadex G-25 (not shown) revealed two peaks of inhibitor activity, one in the void volume and one (the low MW inhibitor) that eluted after vitamin B_{12} (1,350 daltons). This indicated at least two inhibitory factors. Since the low MW inhibitor eluted ahead of acetylcholine (182 daltons) (Fig. 3), and its activity was not destroyed by acetylcholinesterase (not shown), it was not acetylcholine. Further, choline, the major breakdown product of acetylcholine, did not cause inhibition of QNB binding at concentrations up to 2.5 μM in the assay (not shown). This corresponds to a tissue concentration of 500 μM which is at least ten times greater than physiologic levels in rat brain [3]. Thus it is unlikely that the inhibitor is choline.

Following these preliminary studies, we focused our efforts on characterizing the low MW inhibitor (Inhibitor prep method B) and comparing its activity in AD and non-demented control brain. Also, because the microsomal membrane preparation (mAChR method A) used in the initial studies contained only a small fraction of the cellular mAChRs, all subsequent studies were conducted with a lysed, washed 27,000 × *g* membrane preparation (mAChR method B) which was much higher in mAChR density [5] (see section 2 for details). Similar inhibition was observed by AD and non-demented control supernatant fractions with either membrane preparation (not shown).

The activity of the low MW inhibitor from AD brain was not decreased by treatment with base (0.1 M NaOH for 20 min at 2°C) or acid (0.1 M HCl for 20 min at 2°C). The low MW (182–1,350 daltons) and stability of the inhibitor suggested it might be a peptide. Guidotti et al. [16] have previously reported the homogenization of rat brain tissue in 1 M acetic acid at 80°C for the isolation of an endogenous peptide. The use of acid and heat not only inactivates proteases but also denatures and precipitates many contaminating proteins. Utilizing a modification of Guidotti's procedure followed by centrifugation and dialysis of the 100,000 × *g* supernatant fraction through Spectra/Por 3 (Inhibitor preparation method B), we obtained two fractions, one > 3,500 daltons and the other < 3,500 daltons. Both fractions retained inhibitor activity. Thus the low MW inhibitor could also be separated by dialysis through Spectra/Por 3 membranes from other larger MW inhibitors.

Our research was greatly simplified by the discovery that the activity of the low MW inhibitor from either AD or non-demented control brain was greatly increased by β-ME. With small amounts of low MW inhibitor, which were insufficient to cause inhibition in the absence of β-ME, inhibitor activity was increased in a concentration-dependent manner with β-ME. Maximum inhibition was observed with 30 mM β-ME (Fig. 4). Dithiothreitol (DTT), another sulfhydryl reducing agent, similarly caused a dramatic increase in the activity of the low MW inhibitor with 0.25 mM DTT giving maximum inhibition (not shown). Neither 0.25 mM DTT nor 30 mM β-ME altered [^{3}H]QNB binding more than ±7% with our standard assay conditions. Because inclusion of 30 mM β-ME in the assay not only maximally increased the activity of the low MW inhibitor but also gave higher assay reproducibility, all subsequent assays included 30 mM β-ME.

The activity of the > 3,500 dalton inhibitory fraction from AD brain was also increased by β-ME or DTT (not shown). Analysis of the > 3,500 dalton and < 3,500 dalton fractions prepared from five Alzheimer's brains revealed that 64 ± 2% of the recovered activity was found in the low MW fraction.

Acid hydrolysis (6 M HCl, 110°C, for 16 h) of the low MW inhibitor isolated from either AD or normal brain resulted in loss of approximately 50% of inhibitor activity (Fig. 5). While most peptides are completely hydrolyzed under these conditions, those with peptide linkages between non-aromatic hydrophobic amino acids such as valine, leucine or isoleucine require longer times for complete acid hydrolysis [29]. Peptides with amino-terminal valine, leucine or isoleucine are also

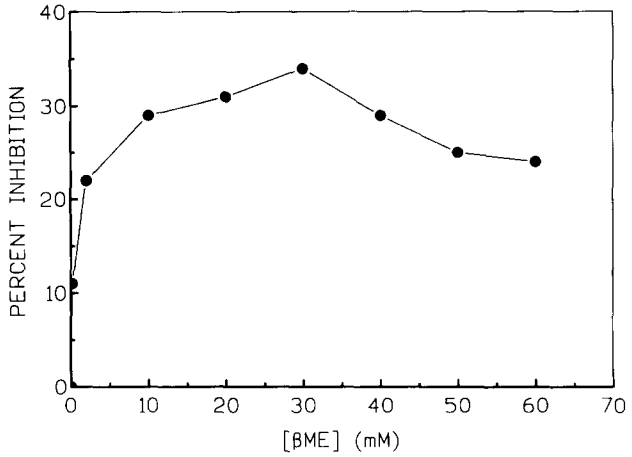

Fig. 4. Low MW inhibitor activity increased by β-ME. Low MW inhibitor from AD brain was assayed (Method A) for activity in the presence of increasing concentrations of β-ME. Binding in the absence of inhibitor was compared to binding in the presence of inhibitor at the β-ME concentrations shown in 4 separate experiments. Points shown are the averages of duplicate determinations of a representative experiment. For the subsequent experiments shown in Figs. 5–9, 30 mM β-ME was included in the assay (Method A) which utilized a lysed, washed 27,000 × g membrane mAChR preparation (mAChR Method B) and low MW inhibitor preparation (Method B).

known to be resistant to acid hydrolysis [29]. Acid hydrolysis for 96 h of low MW inhibitor prepared from three normal and three AD brains resulted in nearly complete loss of inhibitor activity for all but one of the six inhibitor preparations (Fig. 6). Treatment of the low MW inhibitor with alkaline phosphatase did not alter inhibitor activity, indicating that the inhibitor is not a phosphomonoester.

Beginning with an equal wet weight of tissue,

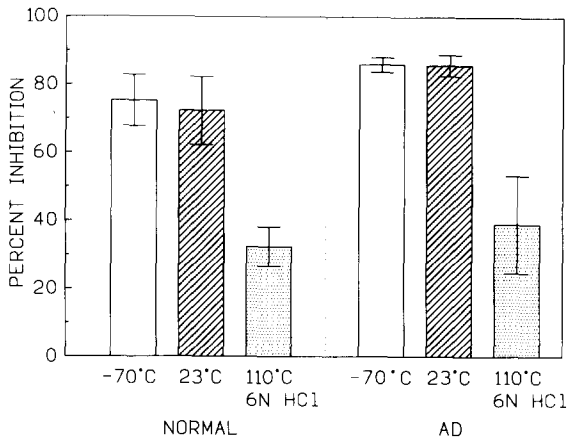

Fig. 5. 16 h acid hydrolysis of low MW inhibitor from normal and AD brain. Low MW inhibitor prepared from 4 normal and 5 AD brains was heat-sealed under N_2(g) in glass ampules containing 6 M HCl and heated to 110°C [18] in a Pierce Reacti-Block. Control ampules with inhibitor but no acid were kept at 23°C or at −70°C. After 16 h, acid hydrolyzed samples were dried under N_2(g), resuspended and neutralized before being assayed in triplicate for inhibitor activity.

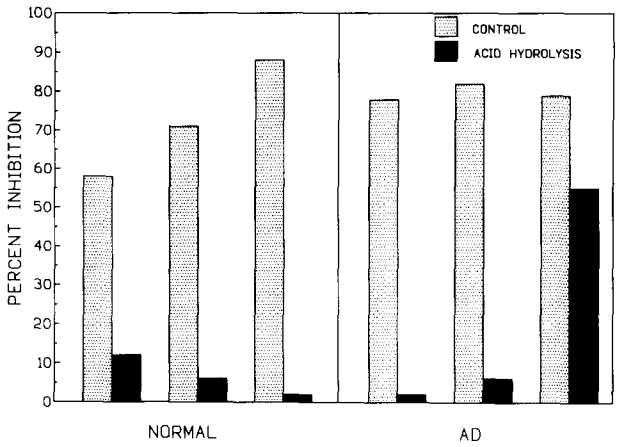

Fig. 6. 96 h acid hydrolysis of low MW inhibitor from normal and AD brain. Low MW inhibitor prepared from 3 normal and 3 AD brains were acid hydrolyzed with 6 M HCl at 110°C as described in Fig. 5. After 96 h, the ampules were opened and the contents were dried, resuspended, neutralized and assayed in triplicate for inhibitor activity. Control ampules with inhibitor but no acid were kept at −70°C.

< 3,500 dalton fractions were prepared from the frontal cortex gray matter of 5 severe AD cases (81 ± 5 years) and 4 normal cases (86 ± 5 years). In all cases, the activity of the low MW inhibitor increased with increasing concentration of inhibitor in the assay, reaching a maximum of 93–94% inhibition for the inhibitor from both the AD and normal brain tissue. (See Eadie-Hofstee analysis (Fig. 7).) A Hill plot analysis of these data (Fig. 8) revealed only slight positive cooperativity with a Hill coefficient of 1.2 for the inhibitor from both

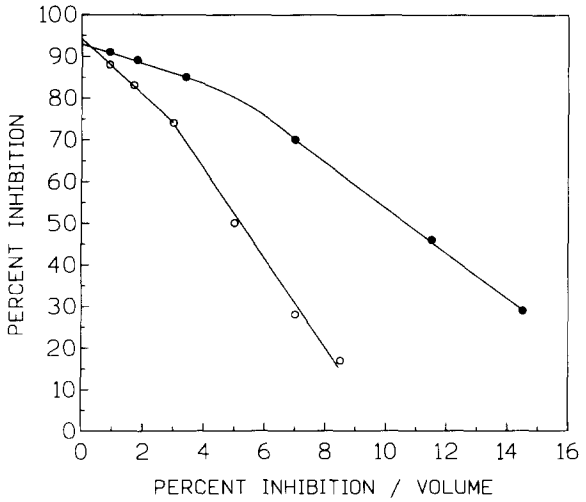

Fig. 7. Eadie-Hofstee analysis of inhibition of [^{3}H]QNB binding by normal and AD low MW inhibitors. Low MW inhibitor was prepared from 4 normal (○) and 5 AD (■) brains. The mean percent inhibition of [^{3}H]QNB binding is presented for both AD and normal low MW inhibitor preparations for each volume of inhibitor tested. AD and normal low MW inhibitors caused a maximum inhibition of 93% and 94%, respectively, from the Eadie-Hofstee analysis [11,19]. In Figs. 7 and 8, each point shown represents the mean of four or five triplicate determinations of inhibitor activity.

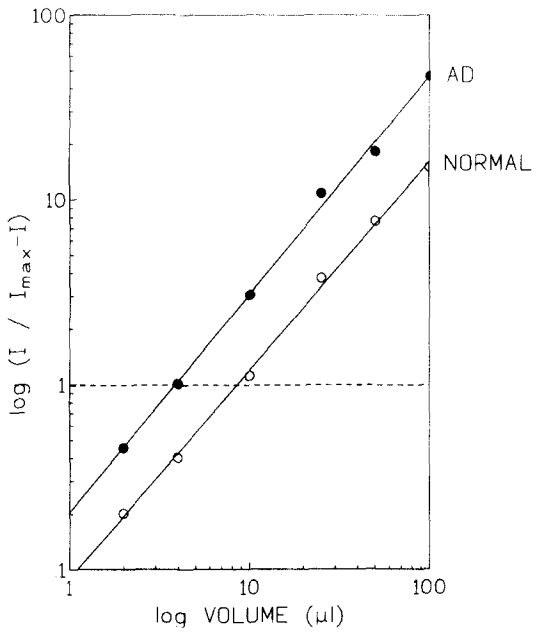

Fig. 8. Hill plot of inhibition of [^{3}H]QNB binding by the low MW inhibitor isolated from AD and normal brain. A Hill plot of the data in Fig. 7 is shown [17]. 1 μl of low MW inhibitor was derived from 1.9 mg of frontal cortex gray matter for both AD and normal brain. The volume of low MW inhibitor required to produce half-maximal inhibition of [^{3}H]QNB binding is determined from the intersection with the dashed line where $(I/I_{max} - I) = 1$. The Hill coefficient is determined from the slope of each line.

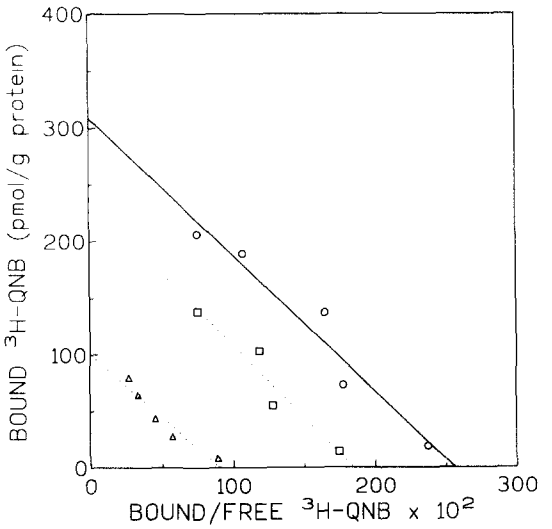

Fig. 9. Eadie-Hofstee plot of the effect of low MW inhibitor from AD brain on [^{3}H]QNB binding. Low MW inhibitor preparations from 6 AD brains were pooled. Zero (○), 2.5 μl (□) or 10 μl (△) of inhibitor were added to the assay. Assay concentrations of [^{3}H]QNB ranged between 0 and 400 pM. Analysis of the resulting data by Eadie-Hofstee plot yielded the following B_{max} values: no inhibitor = 309 pmol/g protein; 2.5 μl inhibitor = 236 pmol/g protein; 10 μl inhibitor = 100 pmol/g protein. The kinetic analysis was performed three times. In the representative analysis shown, each point is the average of duplicate determinations.

AD and normal brain. However, the volume of the < 3,500 dalton fraction from AD brain required to produce half-maximal inhibition of QNB binding, was 2.7 times less than that for normal brain ($P < 0.005$). These data suggest that low MW inhibitor activity is about three times greater in AD brain than in normal control brain on a per gram wet weight tissue basis.

Analysis of the effect of low MW inhibitor from AD brain on binding to the mAChR as a function of [^{3}H]QNB concentration indicates that the principal effect is to decrease B_{max} (Fig. 9). No significant effect on K_d was observed, suggesting that the low MW inhibitor is a non-competitive inhibitor.

4. Discussion

This is the first report of endogenous inhibitors in human brain of ligand binding to the mAChR and of increased inhibitor activity in AD. The data presented demonstrate that inhibitor activity is greatly increased by the reducing agents β-ME (Fig. 4) and DTT. It is not known whether these sulfhydryl reagents are acting on the inhibitors or on the membrane itself to alter the interaction of the inhibitor with the mAChR.

Our comparison of endogenous inhibitor activity in 100,000 × *g* supernatant fractions of AD and non-demented brain indicates that inhibitor activity is significantly increased in AD brain (Figs. 1 and 2, Table 1). This difference is even more evident in the < 3,500 dalton fraction containing the low MW inhibitor whose activity is increased approximately three-fold in AD (Fig. 8). This inhibition appears to be non-competitive as no significant effect on K_d, a measure of receptor affinity for the ligand, was observed (Fig. 9).

The characteristics of the low MW inhibitor from AD brain differ from those of several other inhibitors of ligand binding to the mAChR reported to occur in the tissues of other mammals. The earliest report of an endogenous factor interfering with antagonist binding to the mAChR was from Acton et al. [1] who described a competitive inhibitor from bovine brain with an approximate MW of 1,500. Though similar in size, the low MW inhibitor from AD brain exhibits non-competitive kinetics (Fig. 9).

Creazzo and Hartzell [4] have isolated from rat heart and brain an endogenous low MW inhibitor of the mAChR. The activity of this endogenous soluble factor was blocked by DTT. While this is opposite to the effect of DTT described here for the low MW inhibitor found in AD brain, there are a number of similarities including low molecular weight and resistance to heat and acid.

Diaz-Arrastia et al. [9] have also reported a low MW, moderately heat- and acid-stable inhibitor isolated from calf thymus which required Zn^{2+} (10–500

μM) for activity and whose activity was totally destroyed by hydrolysis with 6 M HCl at 110°C for only 6 h. As shown, even 16 h of hydrolysis with 6 M HCl only destroyed half of the activity of the low MW inhibitor from human brain (Fig. 5) and addition of 100 μM Zn^{2+} did not increase activity of the inhibitor (not shown). However, again in spite of these differences, there are a number of similarities including low MW, heat and acid stability and similar non-competitive kinetics with certain assay conditions.

Fryer and El-Fakahany [15] have also reported an endogenous factor in rat heart supernatant fractions which altered the affinity of rat heart mAChR for selective antagonists. The activity of this factor was destroyed by heat, however, clearly distinguishing it from the inhibitor activities described here for human brain. Fang et al. [12] have found a heat-stable, trypsin-sensitive, soluble factor from guinea pig ileal muscle that reduced the B_{max} for [^{3}H]QNB but did not alter K_d. However, the MW of this factor was greater than 10,000. Finally, Maslinski et al. [24] have described a soluble trypsin-sensitive factor (MW < 10,000) from rat thymocytes which reduced the B_{max} for [^{3}H]QNB but did not alter the K_d. Although this trypsin-sensitive soluble factor was inactivated by heat ($t_{1/2}$ = 12 min at 56°C), its other properties are not inconsistent with those we describe here for the low MW inhibitor from AD brain.

Peptides isolated from non-mammalian sources have also been found to alter ligand binding to the mAChR. Hu et al. [20] have reported that salmon protamine caused allosteric modulation of mAChR but did not alter B_{max}. This differs not only from our low MW inhibitor in its action but also from that reported by others [4,9,24] who also observed a decrease in B_{max}. Jerusalinsky et al. [21] have described two polypeptides from snake venom that selectively inhibited binding of [^{3}H]pirenzepine. These polypeptides, around 7,000 MW, are competitive inhibitors and thus appear to differ from the low MW inhibitor found in AD brain both in size and type of inhibition.

It is not possible to ascertain from the studies performed thus far whether or not the low MW inhibitor is a peptide. While the inactivation of the inhibitor by prolonged acid hydrolysis is consistent with a peptide having linkages between non-aromatic hydrophobic amino acids or amino terminal valine, leucine or isoleucine, our preliminary results with immobilized and soluble proteases do not allow any firm conclusions at this time. Inactivation of the low MW inhibitor by acid hydrolysis together with its elution ahead of acetylcholine on Sephadex G-10 make it extremely unlikely that the inhibitor is simply a metal ion.

Andriamampandry and Kanfer [2] have reported that a phosphomonoester elevated in AD brain inhibits choline acetyltransferase. The low MW inhibitor described here, however, is not inactivated by treatment with alkaline phosphatase, unlike the one reported by Andriamampandry and Kanfer.

Studies now in progress to further purify the low MW inhibitor from human brain and to determine its MW, composition and receptor specificity should help to identify this potentially important regulator of mAChR function. Increased activity of endogenous inhibitors of mAChR function in AD may contribute to the cholinergic deficit of this disease and interfere with therapeutic attempts to increase mAChR activity. Also, evidence for increased levels of hippocampal nerve growth factor and brain-derived neurotrophic factor mRNA following stimulation of mAChR has been presented and reviewed by da Penha Berzaghi et al. [5]. Endogenous inhibitors of mAChR function, such as those described here for human brain, may alter this regulation leading to reduced levels of brain neurotrophic factors and neurodegeneration. Further, Nitsch et al. [26] have reported that release of amyloid precursor protein derivatives is stimulated by activation of mAChR. Thus, endogenous inhibitors of antagonist binding to the mAChR may play a role in the regulation of amyloid release and deposition which contributes to the neuropathology of AD.

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