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Protease inhibitors and indoleamines selectively inhibit cholinesterases in the histopathologic structures of Alzheimer disease

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ABSTRACT **Neurofibrillary tangles and amyloid plaques express acetylcholinesterase and butyrylcholinesterase activity in Alzheimer disease. We previously reported that traditional acetylcholinesterase inhibitors such as BW284C51, tacrine, and physostigmine were more potent inhibitors of the acetylcholinesterase in normal axons and cell bodies than of the acetylcholinesterase in plaques and tangles. We now report that the reverse pattern is seen with indoleamines (such as serotonin and its precursor 5-hydroxytryptophan), carboxypeptidase inhibitor, and the nonspecific protease inhibitor bacitracin. These substances are more potent inhibitors of the cholinesterases in plaques and tangles than of those in normal axons and cell bodies. These results show that the enzymatic properties of plaque and tangle-associated cholinesterases diverge from those of normal axons and cell bodies. The selective susceptibility to bacitracin and carboxypeptidase inhibitor indicates that the catalytic sites of plaque and tangle-bound cholinesterases are more closely associated with peptidase or protease-like properties than the catalytic sites of cholinesterases in normal axons and cell bodies. This shift in enzymatic affinity may lead to the abnormal protein processing that is thought to play a major role in the pathogenesis of Alzheimer disease. The availability of pharmacological and dietary means for altering brain indoleamines raises therapeutic possibilities for inhibiting the abnormal cholinesterase activity associated with Alzheimer disease.**

Neurofibrillary tangles and amyloid plaques are the major histopathologic hallmarks of Alzheimer disease (AD). Light and electron microscopic studies have demonstrated that these structures contain histochemically and immunohistochemically definable acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) and that these AD-related cholinesterases (ADChEs) have different histochemical properties from the cholinesterases associated with intact neuronal cell bodies and axons (1–6). Specifically, the ADChEs are more resistant to traditional cholinesterase (ChE) inhibitors (7, 8), require more substrate for inhibition (9), and are histochemically more intensely reactive at a lower pH than the AChE of normal cells and fibers (4).

We have searched for modifiers of ChE function that may act preferentially on the ADChEs. One set of modifiers that we have investigated is the indoleamine derivatives, including 5-hydroxytryptamine (serotonin; 5-HT) and 5-hydroxytryptophan (5-HTP; a serotonin precursor). This is based on evidence that these substances can inhibit an acetylcholine hydrolyzing esterase from several mammalian sources (10–14) and on information that there is a aryl-acylamidase activity associated with purified ChEs that is inhibited by indoleamines (15–17). We have also examined protease inhibitors as AChE may have a protease function or be closely associated with one (18, 19).

We report that 0.05–1 mM concentrations of indoleamines, carboxypeptidase inhibitor (CPI), and the nonspecific peptidase inhibitor bacitracin (but not tyrosine, tyramine, glycine, or pepstatin A) inhibit the *in situ* ChE activity of plaques and tangles but not of normal perikarya and axons. These results have implications for understanding the nature of the AD-ChEs and for designing potential therapeutic strategies.

MATERIALS AND METHODS

The observations in this report are based on four brains with the clinical and pathological criteria of AD (67, 82, 88, and 88 years old), two control brains with no clinical or pathological evidence for AD (55 and 71 years old), and one brain with no clinical history of dementia but with pathological changes of AD (99 years old). Interval from death to autopsy was <24 hr. Coronal slabs 1–2 cm thick were cut and fixed by immersion in 0.1 M phosphate buffer (pH 7.4) with 4% paraformaldehyde for 24 hr at 4°C. The tissue was then taken through a 10–40% sucrose gradient in 0.1 M phosphate buffer (pH 7.4). Slabs were frozen on dry ice and cut by microtome into 40-μm thin sections. For diagnostic evaluation, sections were stained with thioflavin S and examined for histofluorescence for confirmation of AD. Cresyl violet staining was used for identification of cytoarchitectonic boundaries. Mounted tissue sections were preincubated with various concentrations of inhibitors for 1 hr at 25°C. Visualization of the ChE reaction product was then achieved by tissue incubation for 2–5 hr in diluted (10%) Karnovsky–Roots solution at pH 6.8 followed by metal ion-diaminobenzidine intensification. The technique used for ChE histochemistry has been described in detail (4, 7). It is based on our modification of the methods of Tago *et al.* (20) and Hanker *et al.* (21). Acetylthiocholine was used as the substrate for the AChE reaction, and butyrylthiocholine was used for the BChE reaction, each at 366 μM.

RESULTS

In AD, the AChE and BChE histochemical reaction products (as defined with the respective use of acetylthiocholine and butyrylthiocholine as the substrates) are found in the vast majority of plaques and tangles. No equivalent staining is seen in control brains. The AChE reaction product is also seen in intact cortical cell bodies and axons of the control and AD brains.

Traditional ChE inhibitors such as physostigmine, tacrine (tetrahydroaminoacridine), and BW284C51 (a selective inhibitor of AChE) totally abolish AChE activity in normal cell bodies and axons but not that in plaques or tangles at

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Abbreviations: AD, Alzheimer disease; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ADChE, AD-related cholinesterase; 5-HT, 5-hydroxytryptamine (serotonin); 5-HTP, 5-hydroxytryptophan; CPI, carboxypeptidase inhibitor.

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Table 1. Effect of traditional ChE inhibitors, protease inhibitors, and amines upon axonal, perikaryal, plaque, and tangle ChEs

Compound	Conc., mM	AChE in intact axons and perikarya	Plaque and tangle AChE	Plaque and tangle BChE
Baseline enzyme activity		+++	+++	+++
Physostigmine	0.00001	−	+++	++
	0.1	−	−	−
Tacrine	0.00001	−	+++	++
	0.1	−	−	−
CPI (potato)	0.05	+++	+	+
	0.1	+++	−	−
Bacitracin	0.05	+++	+	−
	0.1	+++	+	−
	0.5	+++	+	−
	1	+++	−	−
5-HTP	0.05	+++	++	++
	0.1	+++	+	+
	0.5	+++	−	+
	1	+++	−	−
5-HT	0.05	+++	+	+++
	0.1	+++	−	+++
	0.5	++*	−	−
	1	++*	−	−
L-Tryptophan	0.05	+++	+++	+++
	0.1	+++	+++	+++
	0.5	+++	+++	+
	1	+++	++	−
Tryptamine	0.05	+++	+++	+++
	0.1	+++	+++	+++
	0.5	+++	+++	++
	1	+++	++	+
Tyrosine	1	+++	+++	+++
Tyramine	1	+++	+++	+++
Glycine	1	+++	+++	+++
Pepstatin	0.1	+++	+++	+++

All comparisons are based on matching tissue sections from the same cytoarchitectonic area and cortical layer in the seven brains described in the text. The examination included the hippocampus, entorhinal cortex, inferotemporal cortex, insula, medial frontal cortex, and cingulate gyrus. Only results that were consistently obtained in all specimens investigated for a given variable are included. +++, Intense baseline staining; ++, moderate decrease from baseline intensity; +, severe decrease from baseline intensity; −, total inhibition of staining.

*At these concentrations of 5-HT, a patchy precipitate formed over the sections. In the areas that were free of these precipitates, the AChE staining in intact cell bodies and axons appeared preserved or only very slightly decreased.

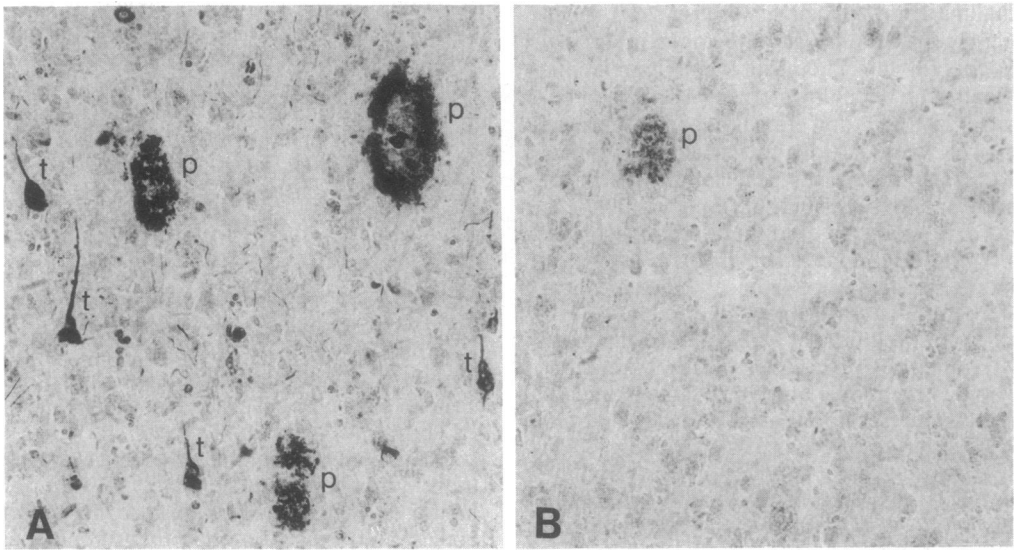

FIG. 1. L-Tryptophan inhibits BChE in plaques and tangles in layer 5 of ventral parainsular cortex. (*A*) In control sections, plaques (p) and tangles (t) yield an intense BChE reaction as indicated by the presence of the black reaction product. (*B*) In an adjacent section from the same parainsular region, the presence of 0.5 mM L-tryptophan in the incubation medium causes a severe inhibition of the BChE reaction. Tangle staining is completely abolished and only a few plaques (p) continue to show some staining but at a much reduced intensity. Tissue is from an 82-year-old patient with AD. (×140.)

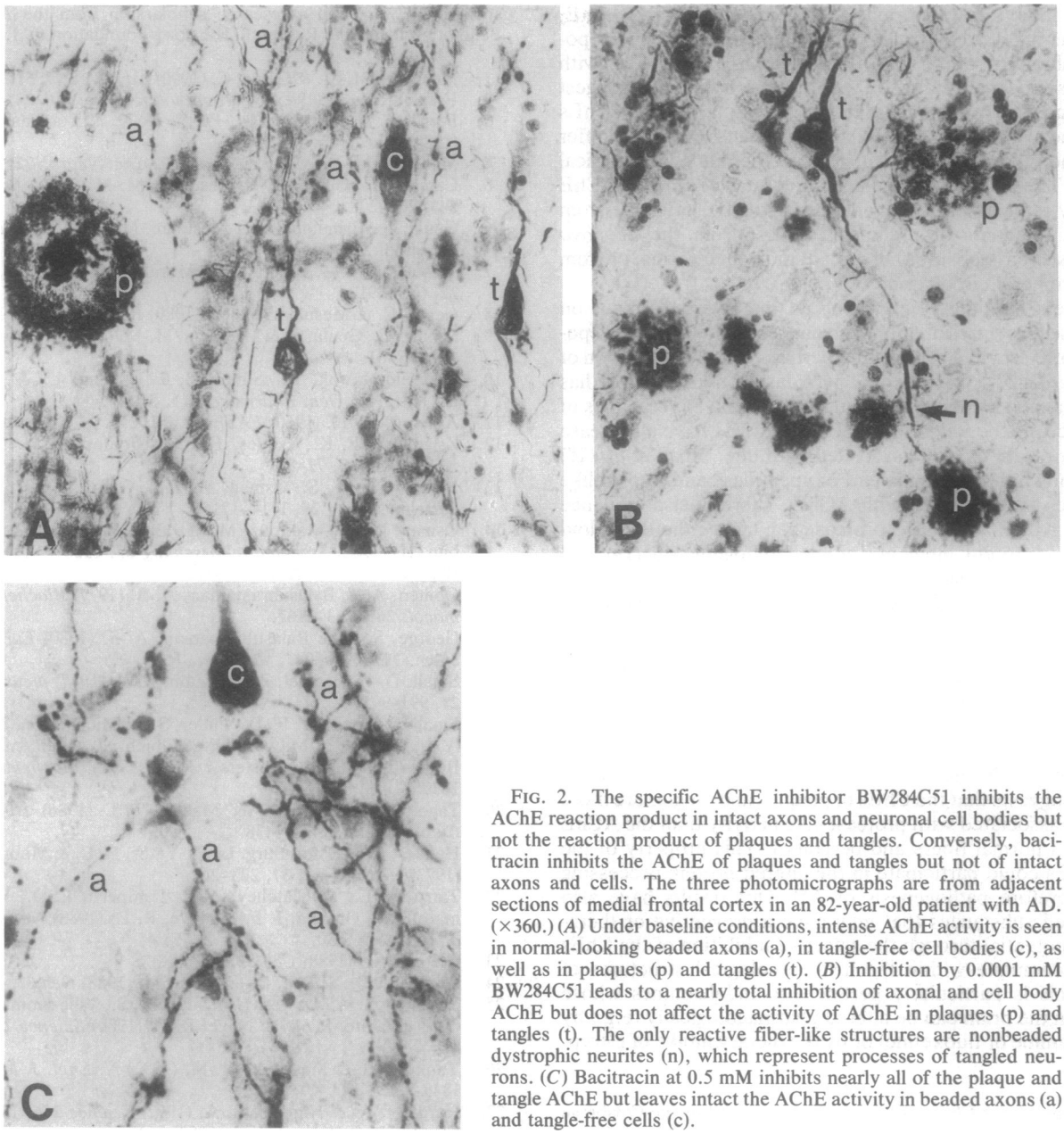

FIG. 2. The specific AChE inhibitor BW284C51 inhibits the AChE reaction product in intact axons and neuronal cell bodies but not the reaction product of plaques and tangles. Conversely, bacitracin inhibits the AChE of plaques and tangles but not of intact axons and cells. The three photomicrographs are from adjacent sections of medial frontal cortex in an 82-year-old patient with AD. (×360.) (*A*) Under baseline conditions, intense AChE activity is seen in normal-looking beaded axons (a), in tangle-free cell bodies (c), as well as in plaques (p) and tangles (t). (*B*) Inhibition by 0.0001 mM BW284C51 leads to a nearly total inhibition of axonal and cell body AChE but does not affect the activity of AChE in plaques (p) and tangles (t). The only reactive fiber-like structures are nonbeaded dystrophic neurites (n), which represent processes of tangled neurons. (*C*) Bacitracin at 0.5 mM inhibits nearly all of the plaque and tangle AChE but leaves intact the AChE activity in beaded axons (a) and tangle-free cells (c).

0.0001–0.00001 mM. At 0.1 mM, the reaction in the histopathologic structures of AD is also abolished. At this higher concentration, the selective BChE inhibitor tetraisopropylphosphoramide has no effect on the AChE reaction but abolishes the BChE reaction in the plaques and tangles. There is thus enzymatic specificity to the AChE and BChE reactions, and traditional inhibitors are more effective in inhibiting the AChE of axons and neuronal perikarya than the ChEs of the histopathological structures of AD.

The converse pattern is seen with indoleamines and certain protease inhibitors (Table 1). *In situ* enzymatic activity of the ADChE is partially inhibited by 0.05 mM 5-HTP or 5-HT and completely inhibited at 0.5–1 mM. At these concentrations of 5-HTP and 5-HT there is no substantial interference with the AChE activity of normal cell bodies and axons in AD or control brains.

Other indoleamines such as tryptophan and tryptamine also display preferential inhibition of the ADChEs but at higher concentrations than 5-HTP and 5-HT (Fig. 1). The AChE and BChE in plaques and tangles are equally affected by indoleamines except for 5-HT, which is a more potent inhibitor of the AChE. The ADChE inhibition affects the tangles and related dystrophic neurites before the plaques. Glycine, tyramine, and tyrosine at concentration of up to 1 mM have no effect on the visualization of AChE or BChE in any tissue component.

Protease inhibitors were also examined. CPI (from potato) begins to inhibit histochemically detectable ADChE at 0.01 mM and completely inhibits it at 0.1 mM, without preventing the visualization of AChE in normal axons and cell bodies. The general protease inhibitor bacitracin has a similar effect (Fig. 2). In contrast, the acid protease inhibitor pepstatin A does not prevent visualization of AChE or BChE in any tissue component at 0.1 mM concentration.

More than 500 tissue sections from the cingulate cortex, entorhinal area, hippocampal complex, insula, inferotemporal cortex, and medial frontal cortex were examined in the course of these studies and the results noted above were seen in all of the areas and all specimens that were examined.

DISCUSSION

We investigated the *in situ* inhibition of ChEs in AD and control brains. We found that traditional anti-ChE substances such as BW284C51, tacrine, and physostigmine are more

potent inhibitors of normal axonal and perikaryal AChE, whereas indoleamines and protease inhibitors are more potent inhibitors of the AChE and BChE associated with plaques and tangles. These experiments provide the strongest evidence yet available that the enzymatic properties of ChEs detected within the histopathologic structures of AD differ from those normally found in neuronal perikarya and cortical fibers. The source of the plaque and tangle-associated ChE activity remains poorly understood. It could originate from altered remnants of premorbid ChEs, from the *de novo* synthesis (or deposition) of variant molecular forms, or from the peripheral circulation.

Current hypotheses regarding AD pathogenesis focus on protease/protease inhibitor imbalances that cause the deposition of β-amyloid protein and perhaps also the formation of tangles (22–27). The potential peptidase activity of ChEs has been claimed by some and denied by others. Several lines of evidence can be taken to suggest that ChEs have a protease activity or are closely associated with one. Small *et al.* (18) have shown that there are carboxypeptidase and trypsin-like activities that copurify with AChE. There is also evidence that an AChE-associated protease can cleave the β-amyloid precursor protein, albeit at a nonamyloidogenic site (19), and that a 5-HT-inhibitable aryl-acylamidase activity is associated with purified AChE and BChE (16, 17). The presence of the aryl-acylamidase activity and the esterase activity could give the ChEs the potential to act as proteases since these two activities may be used for protein bond cleavage (15).

Whether proteolytic activity is a property of some forms of the AChE and BChE gene products or of copurifying substances remains to be determined (28–30). The selective inhibition of the ADChEs by 5-HTP, 5-HT, CPI, and bacitracin suggests that the ChEs of plaques and tangles are more closely associated with protease-like activity than the AChE found in normal fibers and perikarya. This property of the ADChEs could participate in the altered protein processing and therefore pathogenesis of AD. Our results have also identified substances that may have a preventive or therapeutic potential through the selective inhibition of the ChEs that accumulate within the histopathological structures of AD. This is particularly pertinent to indoleamines whose levels in the brain can be altered pharmacologically (e.g., by fenfluramine or fluoxetine) or by the dietary intake of L-tryptophan.

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