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Effects of huperzine A on acetylcholinesterase isoforms in vitro: comparison with tacrine, donepezil, rivastigmine and physostigmine

Qin Zhao, Xi Can Tang*

State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, People's Republic of China

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Abstract

Five inhibitors of acetylcholinesterase, huperzine A, donepezil, tacrine, rivastigmine and physostigmine, were compared with regard to their effects on different molecular forms of acetylcholinesterase in cerebral cortex, hippocampus, and striatum from the rat brain. In general, huperzine A preferentially inhibited tetrameric acetylcholinesterase (G4 form), while tacrine and rivastigmine preferentially inhibited monomeric acetylcholinesterase (G1 form). Donepezil showed pronounced selectivity for G1 acetylcholinesterase in striatum and hippocampus, but not in cortex. Physostigmine showed no form-selectivity in any brain region. In cortex, the most potent inhibitors of G4 acetylcholinesterase were huperzine A (K_i 7×10^{-9} M) and donepezil (K_i 4×10^{-9} M). The potent inhibitors of cortical G1 acetylcholinesterase were donepezil (K_i 3.5×10^{-9} M) and tacrine (K_i 2.3×10^{-8} M). In hippocampus, huperzine A and physostigmine were the most potent inhibitors of G4 acetylcholinesterase, while donepezil and tacrine were most potent against G1 acetylcholinesterase. In striatum, huperzine A and donepezil were the most potent against G4 acetylcholinesterase, while again donepezil was the most potent against G1. Although the inhibition constants (K_i) of these acetylcholinesterase inhibitors differed significantly from region to region, the nature of the inhibition did not vary. These results suggest that the use of acetylcholinesterase inhibitors in treatment of Alzheimer's disease must consider both form-specific and region-specific characteristics of acetylcholinesterase inhibition.

Keywords: Acetylcholinesterase isoform; Huperzine A; Tacrine; Donepezil; Rivastigmine; Physostigmine; Alzheimer's disease; (Rat)

1. Introduction

Alzheimer's disease is the most common age-related neurodegenerative disease with many cognitive and neuropsychiatric manifestations that result in progressive disability and eventual incapacitation. A decrease of acetylcholine in the brain of patients with Alzheimer's disease appears to be a critical element in producing dementia (Dekosky and Scheff, 1990; Terry and Masliah, 1991). This finding has led to research on ways to enhance the dwindling amount of cholinergic neurotransmitter (Candy et al., 1983). One approach is to inactivate acetylcholinesterase, the enzyme that cleaves synaptic acetylcholine and terminates neuronal signaling. Acetylcholinesterase inhibitors increase the availability of acetylcholine in central cholinergic synapses and are the most promising currently available drugs for the treatment of Alzheimer's disease (Giacobini, 2000). Acetylcholinesterase inhibitors approved by the U.S. Food and Drug Administration for the symptomatic treatment of patients with mild to moderate Alzheimer's disease include tacrine, donepezil and rivastigmine. Huperzine A, a novel *Lycopodium* alkaloid discovered from the Chinese folk medicine *Huperzia serrata*, has been found to inhibit acetylcholinesterase selectively (Tang et al., 1999) and is well tolerated, properties that may be especially suitable for Alzheimer's disease treatment.

Acetylcholinesterase exists in multiple molecular forms that can be distinguished by their subunit associations and hydrodynamic properties (Brimijoin, 1983; Massoulie and Bon, 1982). Because of their differing sedimentation coefficients, these multiple molecular forms can be readily separated by ultracentrifugation on sucrose density gradients (Saez-Valero et al., 1993). In mammalian brain, the bulk of acetylcholinesterase occurs as a tetrameric, G4 form (10S) together with much smaller amounts of a monomeric, G1 (4S) form (Bon et al., 1979; Grassi et al., 1982). There is

* Corresponding author. Tel.: +86-21-6431-1833x405; fax: +86-21-6437-0296.

E-mail address: xctang@mail.shcnc.ac.cn (X.C. Tang).

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evidence that acetylcholinesterase inhibitors, including those of clinical significance, do not inhibit all forms equally well. In particular, although physostigmine inhibits G4 and G1 forms with similar potency, heptylphysostigmine and the newer analog, rivastigmine, have been reported to exhibit marked preference for G1 acetylcholinesterase (Ogane et al., 1992; Polinsky, 1998). This phenomenon of form-specific selectivity complicates the interpretation of experiments with acetylcholinesterase inhibitors but also opens the door to discovery of therapeutic agents directed towards the most appropriate targets.

The theory behind much current Alzheimer's disease treatment revolves around the likelihood that the associated dementia reflects dysfunctional cholinergic signaling in the cerebral cortex and hippocampus (Bartus et al., 1982; Coyle et al., 1983). These acetylcholinesterase-rich areas of the brain are prominent sites of neuropathology in Alzheimer's disease and should be prime targets for acetylcholinesterase inhibitor therapy. However, cholinergic interneurons in the striatum are an even richer source of acetylcholinesterase and would also be affected strongly by such enzyme inhibitors (Kawaguchi et al., 1995). To date, there have been no extensive studies to determine the relative preferences of clinically relevant acetylcholinesterase inhibitors for different molecular forms of acetylcholinesterase from different regions of brain. Here we report observations on the selectivity of five acetylcholinesterase inhibitors for G4 and G1 acetylcholinesterase forms from cortex, hippocampus and striatum of the rat. These findings may be useful in future work on functional effects of acetylcholinesterase inhibitor in brain.

2. Materials and methods

2.1. Acetylcholinesterase inhibitors

Natural (−)-huperzine A (a colourless powder with purity of >98%), isolated from *H. serrata* (Thunb) Trev, was provided by the Department of Phytochemistry in this institute. Donepezil and rivastigmine (both colourless powder with purity of >98%) were prepared by the Department of Synthetic Chemistry in this institute; tacrine and physostigmine were purchased from Sigma (St. Louis, MO, USA).

2.2. In vitro assay of brain acetylcholinesterase

Adult Sprague–Dawley male rats, weighing 200 ± 35 g, were supplied by Shanghai Experimental Animals Center, Chinese Academy of Sciences. Rats were decapitated, the brain was rapidly dissected on ice into cortex, hippocampus and striatum, and then weighed and homogenized in five volumes of cold 75 mM sodium phosphate buffer, pH 7.4. Homogenates were centrifuged at 13 000 × *g* for 30 min at 4 °C; supernatants used as acetylcholinesterase sources were divided into aliquots and stored at −20 °C. Acetylcholinesterase activity was measured by the principle of the Ellman method (Ellman et al., 1961). Enzyme samples in 10 mM phosphate buffer, pH 7.5, were incubated 150 s at 37 °C with 0.3 mM acetylthiocholine iodide in the presence of 50 μM tetraisopropyl pyrophosphoramide, a selective inhibitor of butyrylcholinesterase, and 5,5′-dithiobis-(2-nitrobenzoic acid), for color development (all chemicals from Sigma). Production of the yellow anion of 5-thio-2-nitrobenzoic acid was measured with a SPECTRAmax 96-well plate reader (Molecular Devices, Sunnyvale, CA, USA) at 412 nm. Protein concentration was measured with the Coomassie brilliant blue protein-binding method (Bradford, 1976) using bovine serum albumin as standard. Acetylcholinesterase activity was expressed in international units (1 IU = 1 μmol acetylthiocholine hydrolyzed per second) per mg protein.

2.3. Separation of acetylcholinesterase molecular forms

The molecular forms of acetylcholinesterase extracted from cortex, hippocampus and striatum were separated by gel-exclusion chromatography followed by ultracentrifugation on sucrose gradients as previously described according to their different molecular weights and sedimentation coefficients (Zhao and Tang, 2002; Ogane et al, 1992). For gel-exclusion chromatography, a HiPrep™ 16/60 Sephacryl™ S-200 HR column in an FPLC system (Amersham Pharmacia Biotech, Sweden) was equilibrated with sodium phosphate buffer (pH 7.2, 50 mM) containing 5 mM edetic acid. Each of acetylcholinesterase supernatant extracted from brain areas was thawed at 4 °C and filtered with a 0.2-μm syringe filter. Filtrate was loaded on the column and the absorbed protein was eluted with the same buffer as above (0.5 ml/min). Fractions of 1.5 ml were collected and assayed for acetylcholinesterase activity. Acetylcholinesterase activity assay of aliquots from all the fractions collected revealed two major activity peaks. Protein markers such as myosin (200 kDa), calmodulin-binding protein (130 kDa), rabbit phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), rabbit actin (43 kDa) and bovine carbonic anhydrase (31 kDa) were loaded on the HiPrep™ 16/60 Sepharcryl™ S-200 HR column and eluted with same conditions as above with the aim of elucidating the molecular weight of different acetylcholinesterase peaks. A linear relationship of log molecular weight against elution volume was obtained with the protein markers used. Upon plotting the elution profile of the separated samples against this linear graph, the rapidly eluting high-molecular weight peak was designated to G4-enriched fraction for it was coincidental with the previous documented G4 molecular weight (Massoulie and Bon, 1982), while the slowly eluting low-molecular weight peak was G1-enriched fraction. The G4-riched and G1-riched fractions were layered respectively onto 5–25% (w/v) sucrose gradients in sodium phosphate buffer (50 mM, pH 7.2) containing edetic acid (5 mM). Ultracentrifugation was

performed at 160 000 × *g* for 21 h, 4 °C in a Beckman SW 41 Ti rotor. Catalase from beef liver (11.3S) and bovine serum albumin (4.3S) were used as internal sedimentation markers. Fractions were collected from the bottom of each tube and assayed for acetylcholinesterase activity. Acetylcholinesterase activity assay revealed one activity peak for G4-riched or G1-riched fraction. The corresponding sedimentation coefficients were 10S and 4S, which assigned to G_4 and G_1 acetylcholinesterase forms (Bon et al., 1979; Grassi et al., 1982). As a rule, the high-molecular weight Sephacryl eluate sedimented as a single 10S peak, designated as G4 form, while the low-molecular weight eluate sedimented as a single 4S peak, designated as G1 form.

2.4. Determination of inhibition constant, K_i

K_i values were derived from IC_{50} values with the aid of the Cheng equation (Cheng and Prusoff, 1973), using concurrent estimates of substrate K_m from double-reciprocal Lineweaver–Burk plots. For IC_{50} determination, acetylcholinesterase inhibitors in various concentrations (25 nM to 25 mM for huperzine A, tacrine, donepezil and physostigmine, and 25 μM to 0.25 M for rivastigmine) were incubated at 37 °C with enzyme (G4 or G1, 20 μl) in 10 mM phosphate buffer, pH 7.5 (30 μl) in the presence of acetylthiocholine (0.3 mM). IC_{50} values were determined graphically from log concentration–inhibition curves between 20% and 80% of initial activity. All assays were performed in triplicate for three independent experiments. Each determination was repeated three times, and the values obtained for the measured parameter were averaged.

2.5. Determination of inhibition type

To determine the type of enzyme inhibition, triplicate 20-μl enzyme samples (G4 or G1) were incubated at 37 °C with acetylthiocholine in the above described buffer (30 μl) in the presence or absence of acetylcholinesterase inhibitor (10 and 1 μM for huperzine A, tacrine, donepezil and physostigmine; 10 and 1 mM for rivastigmine). Inhibition type was determined graphically from double-reciprocal Lineweaver–Burk plots of data from three independent experiments.

2.6. Statistical analysis

Statistical analysis was carried out by ANOVA when appropriate, using SPSS software (Chicago, IL, USA).

3. Results

3.1. Effects of acetylcholinesterase inhibitors on G4 and G1 forms

Multiple molecular forms of acetylcholinesterase were separated from cortex, hippocampus and striatum by gel-exclusion chromatography and sucrose density gradient centrifugation. Pooled fractions of isolated G4 and G1 forms were used to assess the effects of acetylcholinesterase inhibitors. Fig. 1A shows inhibition curves of five acetylcholinesterase inhibitors for the G4 and G1 forms from cerebral cortex. Although these raw data do not perfectly reflect the K_i values (see below), it is obvious that huperzine A inhibited the G4 form more potently than the G1 form under these experimental conditions. Tacrine and donepezil showed much less selectivity, however, and physostigmine none at all, while rivastigmine inhibited G1 more potently than G4. Effects of the five acetylcholinesterase inhibitors in hippocampus and striatum were similar but not identical to those in cortex (Fig. 1B and C). The main differences between cortex and these two tissues were (1) a less marked preference of huperzine A for G4 acetylcholinesterase, and (2) a strong preference of tacrine and donepezil for G1 acetylcholinesterase.

3.2. Inhibition constants (K_i) with G4 and G1 forms

Selectivity of the acetylcholinesterase inhibitors for the different molecular forms of acetylcholinesterase was clearly shown by different inhibition constants (K_i) (Table 1). In the cortex, huperzine A, tacrine and rivastigmine, but not donepezil or physostigmine, exhibited significant differences in K_i between G4 and G1 forms, while in hippocampus and striatum, only physostigmine failed to show form-based selectivity (Table 1). Huperzine A and donepezil both inhibited cortex G4 acetylcholinesterase with high potency, while tacrine, physostigmine and rivastigmine showed lower potency for this form. In fact, huperzine A had much higher specificity than the other four inhibitors for G4 acetylcholinesterase in cortex (K_i G4/K_i G1: 200), while rivastigmine was at the opposite extreme in its specificity for cortical G1 acetylcholinesterase (K_i G4/K_i G1: 0.01). Donepezil and tacrine, also potent inhibitors of G1 acetylcholinesterase, were not especially selective in cortex.

It seems worthwhile to rank the acetylcholinesterase inhibitors in terms of their K_i values for G4 acetylcholinesterase in different brain regions. In the cortex, the most potent inhibitors, by a factor of 10, were huperzine A and donepezil; in the hippocampus and striatum, they were physostigmine and huperzine A. A similar look at K_i values for inhibition of G1 acetylcholinesterase shows that in all three sampled brain regions, donepezil was most potent against this form, with tacrine close behind.

3.3. Inhibitory nature of acetylcholinesterase inhibitors with acetylcholinesterase molecular forms

The nature of the acetylcholinesterase inhibition was investigated to determine whether all five acetylcholinesterase inhibitors were acting in the same basic manner (competitive, noncompetitive, mixed). We also investigated whether the inhibitors acted differently with different forms

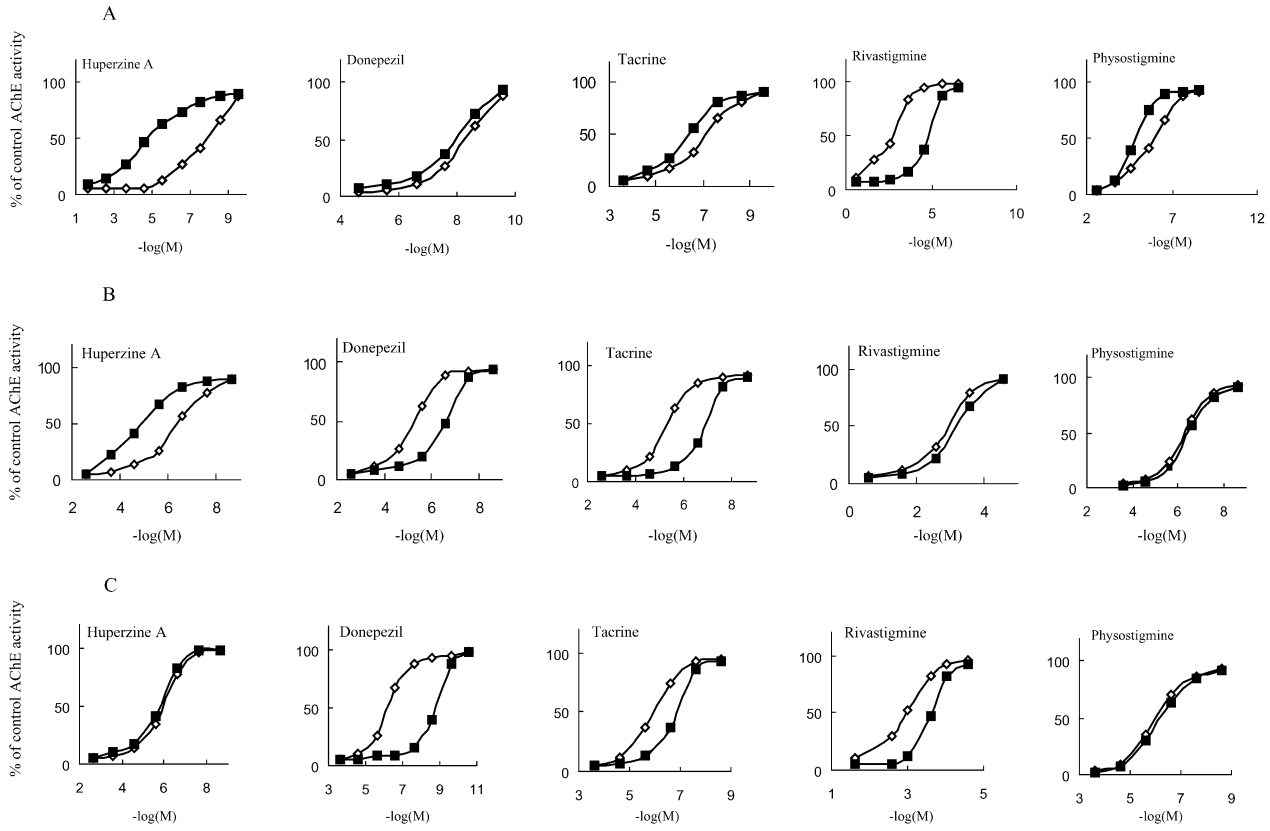

Fig. 1. Effects of huperzine A, donepezil, tacrine, rivastigmine and physostigmine on the G4 (–◇–) and G1 (–■–) forms in the cortex (A), hippocampus (B) and striatum (C). Acetylcholinesterase inhibitors with various concentrations were incubated at 37 °C with enzyme (G4 or G1) in 10 mM phosphate buffer, pH 7.5, in the presence of acetylthiocholine as substrate. All assays were performed in triplicate for three separate experiments. Values are expressed as percent of control acetylcholinesterase (G4 or G1) activity against concentration of acetylcholinesterase inhibitors.

of the enzyme in different brain regions. For this purpose, we examined double-reciprocal plots of velocities measured at eight different concentrations of acetylthiocholine substrate and three different concentrations of inhibitor (including zero). With G4 acetylcholinesterase from cortex, the linear regressions with a single point of intersection in the fourth quadrant indicate a mixed-competitive inhibition by huperzine A and physostigmine (Fig. 2A). The plots with donepezil, tacrine and rivastigmine on the other hand, with consistent intersections on the *x*-axis, indicate noncompetitive inhibition (Fig. 2A). The same pattern of inhibition types was obtained when these five acetylcholinesterase

Table 1
Inhibition constant (K_i) of five acetylcholinesterase inhibitors on acetylcholinesterase G4 and G1 form of cortex, hippocampus and striatum

		K_i (M)		
		Cortex	Hippocampus	Striatum
Huperzine A	G4	$7.0 \pm 3.5 \times 10^{-9}$**	$5.0 \pm 0.6 \times 10^{-7}$*	$1.1 \pm 0.1 \times 10^{-7}$**
	G1	$3.5 \pm 1.5 \times 10^{-7}$	$8.4 \pm 0.9 \times 10^{-7}$	$6.0 \pm 6.0 \times 10^{-7}$
Donepezil	G4	$4.0 \pm 1.5 \times 10^{-9}$	$5.6 \pm 1.4 \times 10^{-6}$	$5.2 \pm 0.9 \times 10^{-7}$
	G1	$3.5 \pm 1.2 \times 10^{-9}$	$2.9 \pm 3.5 \times 10^{-9}$**	$1.4 \pm 0.4 \times 10^{-10}$**
Tacrine	G4	$6.0 \pm 1.4 \times 10^{-8}$	$5.4 \pm 4.3 \times 10^{-6}$	$1.7 \pm 0.5 \times 10^{-6}$
	G1	$2.3 \pm 0.3 \times 10^{-8}$*	$3.2 \pm 2.0 \times 10^{-9}$**	$1.9 \pm 0.2 \times 10^{-8}$**
Rivastigmine	G4	$1.5 \pm 1.4 \times 10^{-3}$	$3.5 \pm 0.5 \times 10^{-4}$	$1.0 \pm 4.0 \times 10^{-4}$
	G1	$1.6 \pm 2.1 \times 10^{-5}$**	$3.1 \pm 0.8 \times 10^{-5}$**	$1.5 \pm 1.1 \times 10^{-5}$*
Physostigmine	G4	$9.7 \pm 1.9 \times 10^{-7}$	$3.3 \pm 0.3 \times 10^{-8}$	$6.6 \pm 1.1 \times 10^{-8}$
	G1	$5.8 \pm 1.2 \times 10^{-7}$	$3.0 \pm 2.2 \times 10^{-8}$	$3.6 \pm 1.2 \times 10^{-8}$

Data represent mean ± S.D. All assays were performed in triplicate for three independent experiments.
K_i values were derived from IC_{50} values with the aid of the Cheng equation, using concurrent estimates of substrate K_m from double-reciprocal Lineweaver–Burk plots. IC_{50} values were determined graphically from log concentration–inhibition curve from 20% to 80% of initial activity.
*$p < 0.05$, significant difference between G4 and G1 forms.
**$p < 0.01$, significant difference between G4 and G1 forms.

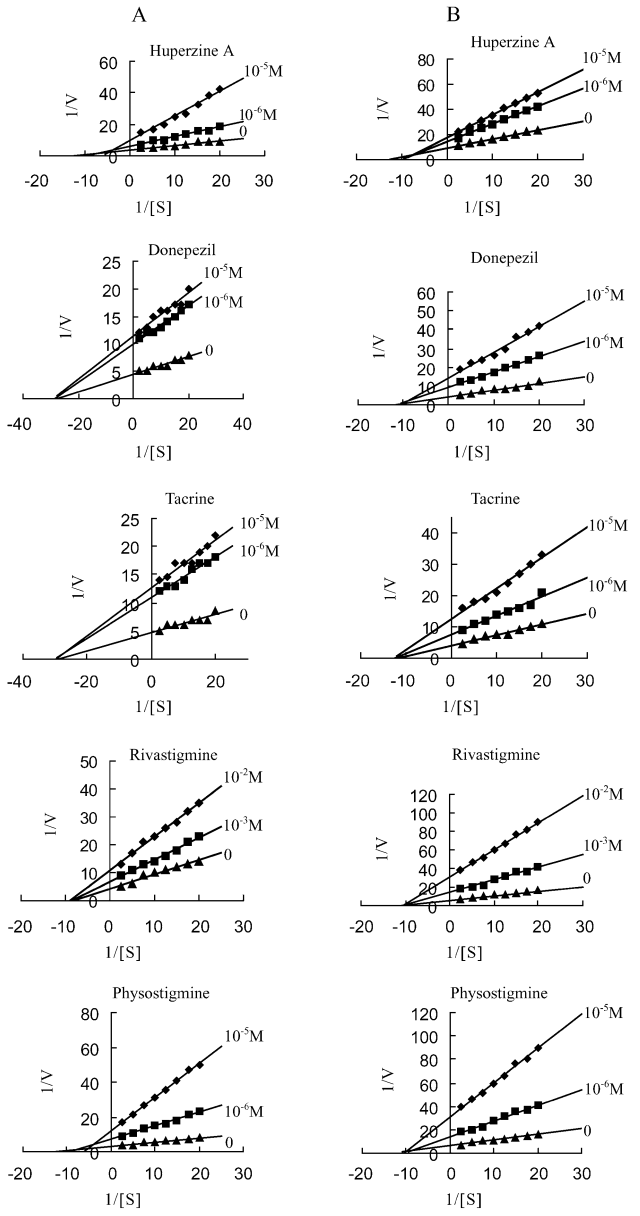

Fig. 2. Double-reciprocal plots for inhibition of cortex G4 (A) and G1 (B) by huperzine A, donepezil, tacrine, rivastigmine and physostigmine. The graphical plots were conducted at eight different concentrations of the substrate acetylthiocholine and at two different concentrations of the inhibitors or without acetylcholinesterase inhibitors. Three independent experiments were carried out in triplicate.

inhibitors were tested against G1 acetylcholinesterase of cortex (Fig. 2B), or against G4 and G1 acetylcholinesterase from hippocampus and striatum. In other words, it was the inhibitor that determined the nature of inhibition, not the molecular form of acetylcholinesterase or its tissue source.

4. Discussion

Cholinesterase inhibitors are the only pharmacological agents proved effective for the treatment of Alzheimer's disease (Giacobini, 2000). In this study, we found some acetylcholinesterase inhibitors that affected different acetylcholinesterase isoforms to differing extents, and sometimes behaved differently with the same isoform from different brain areas. Although such findings must be confirmed by more extensive studies with highly purified enzyme preparations, our striking and consistent results require explanation. A pressing question is how this type of selectivity can arise, since the active sites for all of the molecular forms are apparently identical and are derived from a single gene by alternative C-terminal splicing (Vigny et al., 1978; Taylor et al., 1986; Taylor and Radic, 1994). Thus, the differential inhibition of acetylcholinesterase molecular forms is difficult to ascribe to intrinsic conformational differences in the active site gorge, such as those that distinguish acetyl- and butyrylcholinesterase (Saxena et al., 1999). To date, the growing literature on the X-ray crystal structure of acetylcholinesterase and its complexes with various acetylcholinesterase inhibitors (Silman and Sussman, 2000) lacks information to explain catalytic differences among the molecular forms. On the other hand, the different globular forms are known to have different degrees of hydrophobicity (Taylor et al., 1986), and the 'aromatic patch' of residues in acetylcholinesterase's active center allows for versatile interactions with inhibitors (Ariel et al., 1998; Radic and Taylor, 2001). Furthermore, the microenvironment of the active center may play a role in determining the selectivity of substrate and inhibitors for acetylcholinesterase isoforms. For example, posttranslational modifications of the enzyme surface, perhaps tissue-specific, might affect inhibitor interactions with acetylcholinesterase monomers and tetramers, especially near the peripheral site. It is also possible that the local abundance and nature of tissue lipids may affect catalytic function. Although environmental effects of this sort might be considered ''extraneous'', they could be critically important in determining responses to acetylcholinesterase inhibitors in vivo, in a therapeutic setting.

The G4 form of acetylcholinesterase is the major form in most regions within the mammalian brain (Skau and Shipley, 1999). Approximately 60–90% of this enzymatic form is ectocellular (Das et al., 2001). Ectocellular G4 acetylcholinesterase is the major form for metabolizing acetylcholine (Taylor et al., 1981), and this form is selectively depleted in Alzheimer's disease (Siek et al., 1990). This information suggests that G4 acetylcholinesterase is the physiologically relevant form at cholinergic synapses, and its inhibition would be expected to prolong the action of acetylcholine. By contrast, G1 acetylcholinesterase occurs primarily in the neural cytoplasm, where its inhibition would be unlikely to affect synaptic physiology (Skau and Shipley, 1999). Consequently, it is not surprising that potent inhibitors of G4 acetylcholinesterase in cortex and hippocampus, such as huperzine A, have proven effective for symptomatic treatment of Alzheimer's disease.

Alzheimer's disease is in part characterized by a loss of cholinergic neurons in the basal forebrain that project to

the cerebral cortex and hippocampus (Gottwald and Rozanski, 1999). The cognitive deficits associated with Alzheimer's disease are also thought be primarily related to defects of cholinergic neurotransmission in cerebral cortex and hippocampus (Serena et al., 2001). Potent inhibitors of G4 acetylcholinesterase in the cortex and hippocampus would be expected to be effective for treating such deficits. Huperzine A was found to have higher specificity for cortex and hippocampus G4 form than other acetylcholinesterase inhibitors in this study. This finding corresponds well with previous reports that huperzine A is able to enhance cognition in several different animal species (Tang et al., 1999). In fact, clinical trials have demonstrated that huperzine A induces significant improvements in memory of aged subjects and patients with Alzheimer's disease (Xu et al., 1995). Thus, the inhibitory potency of acetylcholinesterase inhibitors towards different acetylcholinesterase forms may predict their potential in vivo activity.

In the present studies, we have shown that huperzine A is G4-selective in multiple brain areas, while rivastigmine is G1-selective, and donepezil varies from region to region between G4- and G1-selectivity. Although the clinical relevance of these pharmacological characteristics remains to be explored, form-specific and region-specific relative potency of acetylcholinesterase-inhibition are factors that may influence tolerability profiles. Information regarding the differential inhibitory sensitivity among acetylcholinesterase molecular forms in particular brain areas deserves to be considering in developing acetylcholinesterase inhibitors for treatment of Alzheimer's disease.

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