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Effects of anticholinesterases on catalysis and induced conformational change of the peripheral anionic site of murine acetylcholinesterase

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ABSTRACT

Conventional insecticides targeting acetylcholinesterase (AChE) typically show high mammalian toxicities and because there is resistance to these compounds in many insect species, alternatives to established AChE inhibitors used for pest control are needed. Here we used a fluorescence method to monitor interactions between various AChE inhibitors and the AChE peripheral anionic site, which is a novel target for new insecticides acting on this enzyme. The assay uses thioflavin-T as a probe, which binds to the peripheral anionic site of AChE and yields an increase in fluorescent signal. Three types of AChE inhibitors were studied: catalytic site inhibitors (carbamate insecticides, edrophonium, and benzylpiperidine), peripheral site inhibitors (tubocurarine, ethidium bromide, and propidium iodide), and bivalent inhibitors (donepezil, BW284C51, and a series of *bis*(*n*)-tacrines). All were screened on murine AChE to compare and contrast changes of peripheral site conformation in the TFT assay with catalytic inhibition. All the inhibitors reduced thioflavin-T fluorescence in a concentration-dependent manner with potencies (IC_{50}) ranging from 8 nM for *bis*(6)-tacrine to 159 μM for benzylpiperidine. Potencies in the fluorescence assay were correlated well with their potencies for enzyme inhibition (R^2 = 0.884). Efficacies for reducing thioflavin-T fluorescence ranged from 23–36% for catalytic site inhibitors and tubocurarine to near 100% for ethidium bromide and propidium iodide. Maximal efficacies could be reconciled with known mechanisms of interaction of the inhibitors with AChE. When extended to pest species, we anticipate these findings will assist in the discovery and development of novel, selective bivalent insecticides acting on AChE.

1. Introduction

Acetylcholinesterase (EC3.1.1.7, AChE) is a fast acting enzyme located at the synaptic cleft to hydrolyze the neurotransmitter, ACh, and is present in many organisms, including mammals and insects [1]. The inhibition of AChE results in the accumulation of ACh, and leads to hyperexcitation, convulsions, and death. Due to its critical function in the nervous system, AChE is the target for many insecticides, such as organophosphates and carbamates [2]. However, mammalian toxicities (both acute and chronic) and the development of resistance are major disadvantages of the use of these chemicals in agriculture and vector borne disease control [3–7]. Accordingly, there is a need to discover and develop alternatives to conventional AChE inhibitors.

Previous X-ray crystallographic studies found that AChE has a catalytic pocket, connected with a 20-Å, deep and narrow gorge [8–10]. In most species, there are two important ligand binding sites at each end of this gorge: the catalytic site, containing a catalytic triad (Ser^{203}-Glu^{334}-His^{447} as numbered for *Mus musculus* AChE [9]), is located at the bottom of the gorge, and the peripheral anionic site, which consists principally of one negatively charged residue (Asp^{74}) and multiple aromatic amino acid residues (Tyr^{72}, Tyr^{124}, Trp^{286}, and Tyr^{341} [9]), is at the entrance of the gorge [8,11,12]. It has been reported that the binding of ligands to either site modulates the conformation or the activity of the other site [13–16]. The interaction between these two ligand binding sites can be determined by using a fluorescent probe, TFT (Fig. 1), which binds to the peripheral anionic site of AChE, and increases its fluorescence over that of free TFT in solution [17]. According to Stsiapura et al. [18], TFT has a nonplanar conformation in the ground

Abbreviations: AChE, acetylcholinesterase; ATC, hacetylthiocholine; DTNB5, 5'-dithiobis-(2-nitrobenzoic acid); IC_{50}, inhibitory concentration needed to inhibit 50% of the enzyme activity; mAchE, murine acetylcholinesterase; TcAChE, Torpedo californica acetylcholinesterase; TFT, thioflavin-T.

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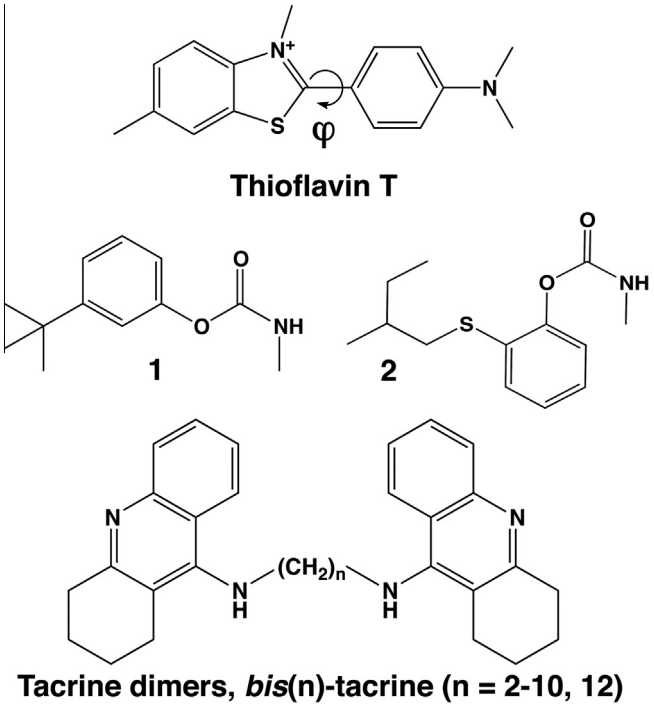

Fig. 1. Structures of compounds referred to in the text. Thioflavin T is shown, along with the axis of rotation related to fluorescence. Other structures are experimental carbamate insecticides (**1**–**2**) and *bis*-tacrine dimers.

state with a torsion angle between the benzothiazole and the dimethylaminobenzene rings of around 37°. When excited by light, the TFT molecule is twisted, the torsion angle increases to about 90° if the dye is in a low viscosity microenvironment, and yields no fluorescent signal. If the TFT molecule is located in a viscous microenvironment, such as bound to AChE, the transition of the TFT molecule from the excited state to the unexcited state will be suppressed, which increases fluorescence [18]. When ligands bind to AChE, a drop in TFT fluorescence may occur either by inhibiting binding of TFT in the peripheral site, or indirectly by reducing rotational rigidity of bound TFT [17,19].

We used this fluorescent assay to study the interaction between the peripheral anionic site and the catalytic site of murine AChE (mAChE) in the present of various AChE inhibitors, including catalytic site inhibitors, peripheral site inhibitors, and bivalent inhibitors binding to both sites. The effects of different AChE inhibitors on mAChE were screened by using the fluorescent assay as well as enzyme activity assay to compare and contrast changes of peripheral site conformation and catalytic ability in response to AChE inhibitors.

2. Materials methods

2.1. Enzyme and chemicals

mAChE was expressed in cell lines and purified by affinity and size exclusion chromatography, as described by Ekstrom et al. [20]. The mAChE was aliquoted into 1 mL samples, and stored at −80 °C. Immediately prior to assay, a vial with frozen mAChE was thawed and kept on ice before use.

Inhibitors of AChE in this study were selected from various categories, including peripheral site inhibitors, catalytic site inhibitors, and bivalent inhibitors that occupy both sites. The peripheral site inhibitors used here were propidium iodide, ethidium bromide, and *d*-tubocurarine. The catalytic site inhibitors were edrophonium, tacrine, benzylpiperidine, propoxur, bendiocarb, pirimicarb, aldicarb, two experimental carbamates [21], 3-*tert*-butylphenyl methylcarbamate (**1**), and 2-(2-methylbutylthio)phenyl methylcarbamate (**2**) (Fig. 1). The bivalent inhibitors were donepezil (E2020) and BW284c51. Another group of AChE ligands is the tacrine dimers, which are two tacrine monomers separated by 2–12 methylene units, and labeled as *bis*(*n*)-tacrine, where *n* equals the number of carbon atoms in the alkyl chain (Fig. 1). The tacrine dimers are bivalent AChE inhibitors [22,23].

Propidium iodide, ethidium bromide, *d*-tubocurarine, edrophonium, tacrine, propoxur, bendiocarb, pirimicarb, aldicarb, donepezil, BW284c51, and TFT were all purchased from Sigma–Aldrich (St. Louis, MO, USA). Compounds **1** and **2** were prepared with methods identical or equivalent to those described previously [21], as were the *bis*(*n*)-tacrines [22].

All the candidate inhibitors except ethidium bromide and tubocurarine, which were dissolved in assay buffer, were dissolved in DMSO to make original stocks. In both fluorescence assay and enzyme activity assay with these AChE inhibitors, the final concentration of DMSO in assays was maintained as 0.1%. TFT was dissolved in methanol, and diluted with assay buffer to a methanol concentration of 0.1%.

2.2. Enzyme fluorescence assay

The fluorescence assay used was modified from established procedures [16,17,24], and was performed in black Costar 96-well plates (Corning, Tewksbury, MA, USA). The fluorescence was monitored by a SyntaxMax plate reader (BioTek, Winooski, VT, USA) in 20 mM sodium phosphate with 0.02% Triton X-100, pH 7.4, at 28 °C. Fluorescence was monitored using 450 and 490 nm wavelengths for excitation and emission, respectively, with excitation and emission slits of 10 nm (excitation) and 20 nm (emission). In order to determine the fluorescent signal resulting from the binding of TFT to the peripheral anionic site of mAChE, TFT auto-fluorescence, enzyme auto-fluorescence, buffer auto-fluorescence, AChE inhibitors' auto-fluorescence, and AChE inhibitors fluorescence with TFT as well as mAChE were all subtracted from TFT fluorescence with enzyme. Non-specific binding of TFT was also subtracted from the total binding fluorescence to obtain specific binding of TFT, by co-incubating 10 μM donepezil with mAChE and TFT for 1 h [19,25]. Unless otherwise indicated, 30 μL of cell lysate derived mAChE and 20 μM of TFT were used per reaction.

Equilibrium binding studies of TFT was performed by incubating serial concentrations of TFT from 0–10 μM with mAChE. In other experiments, inhibitory dose–response curves for candidate inhibitors were determined by incubating mAChE with at least six concentrations of inhibitor for 1 h at room temperature prior to adding 20 μM of TFT.

2.3. Enzyme inhibition assay

Inhibition of mAChE by candidate inhibitors was determined by using the Ellman assay in a 96-well plate format [26]. The mAChE samples were thawed and diluted 100-fold with 0.1 M sodium phosphate buffer, pH 7.4 before use. The dilution of mAChE (30 μL) was then preincubated with at least six concentrations of inhibitors for 1 h at room temperature prior to adding 300 μM DTNB and 400 μM ATCh, which were both dissolved in 0.1 M sodium phosphate buffer, pH 7.4. The kinetic reading of absorbance at 405 nm was started immediately after adding DTNB and ATCh with a Dynex Triad multimode plate reader (Dynex Technologies, Chantilly, VA, USA). The experiment was repeated in triplicate with different enzyme dilutions to obtain means and SEMs of IC_{50} values for selected inhibitors.