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IN VITRO PROTECTION OF ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE BY TETRAHYDROAMINOACRIDINE

COMPARISON WITH PHYSOSTIGMINE

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Abstract—The protective action of 1,2,3,4-tetrahydro-9-aminoacridine (THA) against the long-lasting inactivation of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) brought about by diisopropylfluorophosphate (DFP) and physostigmine, as well as by neostigmine in the case of AChE only, was evaluated by a dilution technique using *Electrophorus electricus* AChE and horse serum BuChE as target enzymes. In parallel experiments, the ability of physostigmine itself to protect these enzymes from DFP was evaluated and compared with that of THA. THA pretreatment was seen to prevent in a dose-dependent manner the inhibition of both AChE and BuChE. However, it was appreciably more potent towards AChE than towards BuChE. THA mean EC_{50} values for protecting AChE against 10, 40 and 100 μM DFP were 0.04, 0.16 and 0.45 μM, respectively; against 1 μM physostigmine the value was 1.8 μM and against 1.2 μM neostigmine it was 3.0 μM. The THA mean EC_{50} value for protecting BuChE against 3 μM physostigmine was 0.55 μM and the values for protecting against 3, 10 and 40 μM DFP were 1.5, 3 and >10 μM, respectively. The protective action of THA was time independent: recovery of the maximal enzymic activity was immediate upon dilution. Unlike THA, the protective action of physostigmine developed progressively after dilution and was maximal within 3–4 (AChE) or 6–8 hr (BuChE). Under our experimental conditions, 0.3 μM physostigmine protected approximately 70% of AChE from 40 μM DFP and 5 μM physostigmine protected 9 and 47% of BuChE from 40 and 3 μM DFP, respectively. The results of this work suggest that THA exerts its protective action by shielding the active site of AChE and BuChE from the attack of the inactivating agents on account of its higher enzymic affinity, whereas the protective action of physostigmine against DFP takes advantage also of the carbamylation of the enzyme. These results are in line with the hypothesis that protection of AChE is the primary mechanism responsible for the antidotal action of THA against organophosphorus poisoning.

1,2,3,4-Tetrahydro-9-aminoacridine (THA†) is a polycyclic primary amine endowed with potent inhibitory activity towards both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) [1–4]. Interest in this drug arises mainly from reports indicating that it is effective in improving the clinical condition of certain patients with Alzheimer's disease [5].

In line with a previous observation [6], we have reported recently that THA exerts a protective action against acute diisopropylfluorophosphate (DFP) poisoning in mice [7]. These findings point to an analogy with some carbamate anti-ChE agents such as physostigmine and pyridostigmine which, at low doses and in the presence of atropine therapy, may also protect laboratory animals from organophosphorus poisoning (see Refs 8 and 9 for reviews). As for carbamates [10], the primary mechanism of THA's antidotal action seems to be represented by the ability of this drug to protect AChE from irreversible inactivation caused by organophosphorus agents [7, 11–13]. However, this point is far from being fully established. In fact THA has also been reported to block ion channels [14], to interact with muscarinic receptors [4], to attenuate NMDA receptor-mediated neurotoxicity [15], to block high-affinity choline uptake [16] and to slow the rate of aging of sarin-inhibited AChE [17]. Each of these actions may in theory be responsible for, or at least contribute to, the antidotal effect of the drug. Besides, recent findings indicate that the *in vivo* protective effects of carbamates may also be unrelated to AChE protection [18].

To acquire more information on this point, in the present investigation we have evaluated directly the ability of THA to protect both AChE and BuChE from inactivation by DFP and carbamates using purified preparations of the two enzymes. The action of THA was compared also with that of other purely

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† Abbreviations: ChE, cholinesterase(s); AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; THA, 1,2,3,4-tetrahydro-9-aminoacridine; DFP, diisopropylfluorophosphate; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); ATCh, acetylthiocholine; BuTCh, butyrylthiocholine; BW284C51, 1,5-bis-(4-allyldimethylammoniumphenyl)-pentane-3-one.

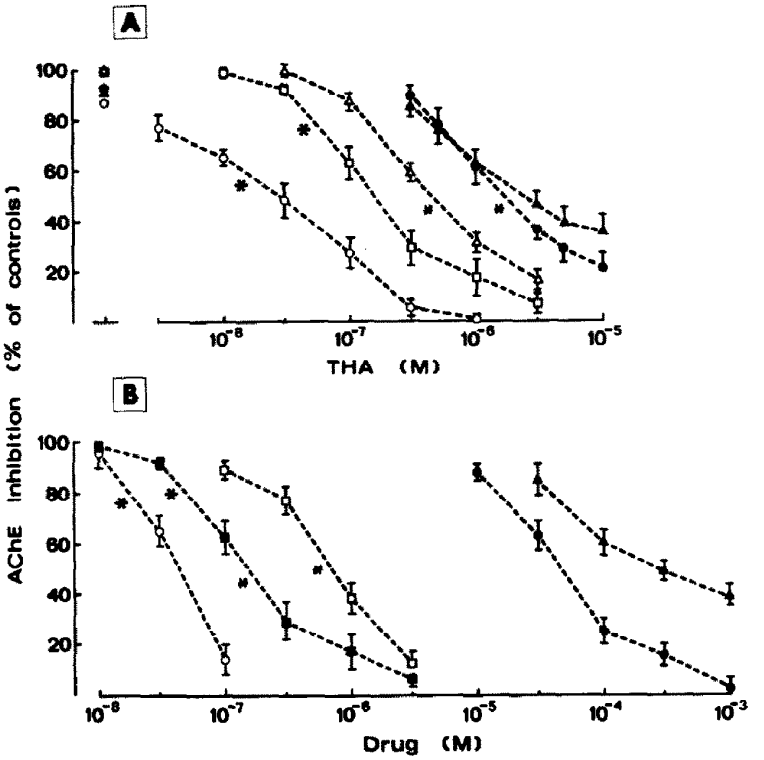

Fig. 1. (A) Protection of AChE from 10 μM (○), 40 μM (□), 100 μM (△) DFP, 1 μM physostigmine (●) and 1.2 μM neostigmine (▲) by increasing concentrations of THA. The values on the left-hand side of the graph denote enzymic inhibition in the absence of THA pretreatment. (B) Protection of AChE from 40 μM DFP by the drugs: BW284C51 (○), THA (■), edrophonium (□), dextrorphan (●) and ranitidine (▲). Aliquots (2.2 U) of electric eel AChE premixed with scalar concentrations of the test compounds were incubated at 25° for 15 min with a fixed concentration of DFP, physostigmine or neostigmine. The samples were then diluted 1000 times with 0.25 mM DTNB solution and assayed immediately for enzymic activity as described in Materials and Methods. The values are the means ± SEM of 3–4 separate experiments performed in duplicate. The EC_{50} values of the curves sharing the same symbols (*, #) differ from each other at $P < 0.05$ level (F test).

reversible AChE inhibitors and with that of physostigmine itself.

MATERIALS AND METHODS

Materials. AChE (EC 3.1.1.7) from *Electrophorus electricus*, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCh) and butyrylthiocholine iodide (BuTCh) were purchased from Boehringer Mannheim GmbH. Horse serum BuChE (EC 3.1.1.8), THA hydrochloride, physostigmine sulfate, neostigmine bromide, edrophonium chloride and 1,5-bis-(4-allyldimethylammonium-phenyl)pentane-3-one dibromide (BW284C51) were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). DFP was from Fluka AG (Buchs, Switzerland): a stock solution of DFP was prepared in propylene glycol and stored at 4°. Ranitidine was a gift from Glaxo SpA (Verona, Italy). Dextrorphan tartrate was kindly supplied by Dr H. Gutman, F. Hoffmann-La Roche Co. (Basel, Switzerland).

Measurement of ChE inhibition. This was carried out according to the photometric method of Ellman *et al.* [19]. Fifty-microliter aliquots of AChE from *E. electricus* or BuChE from horse serum were made up to 2.85 mL with 0.25 mM DTNB in 50 mM sodium phosphate buffer, pH 7.2, and mixed with scalar concentrations of THA or buffer alone at 25°. Enzymic hydrolysis was started immediately by the addition of 0.5 mM ATCh (AChE assays) or 1 mM BuTCh (BuChE assays). The total volume of the assay was 3 mL. The variations in optical absorbance (412 nm, 25°) were measured for 1 min. All concentration values refer to final concentrations in the activity assay.

Measurement of ChE protection. Fixed, highly inhibitory concentrations of the inactivating agents (DFP, physostigmine or neostigmine) or buffer alone (controls), were added to aliquots of eel AChE (2.2 U) or horse serum BuChE (14.4 U) previously mixed with scalar concentrations of THA, or of a different protective agent or buffer alone (controls), in a total volume of 100 μL and incubated for 15 min at 25°. Then 10-μL fractions of the incubates were diluted rapidly 1000 (AChE) or 5000 (BuChE) times with 0.25 mM DTNB and assayed immediately for

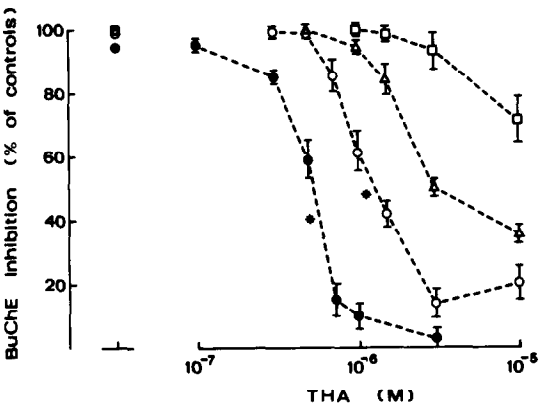

Fig. 2. Protection of BuChE from DFP and physostigmine by increasing concentrations of THA. Aliquots of horse serum BuChE (14.4 U) mixed with scalar concentrations of THA were incubated at 25° for 15 min with 3 μM (○), 10 μM (△) and 40 μM (□) DFP and 3 μM physostigmine (●), and with buffer alone (controls). All samples were then rapidly diluted 5000 times with 0.25 mM DTNB solution and assayed for enzymic activity as described in Materials and Methods. The values on the left-hand side of the graph denote BuChE inhibition in the absence of THA pretreatment. The values are the means ± SEM of 3–4 separate experiments performed in duplicate. The EC_{50} values of the curves sharing the symbol (*) differ from each other at the $P < 0.05$ level (F test).

AChE or BuChE activity in the presence of 0.5 mM ATCh or 1 mM BuTCh, respectively. The dilution of the samples and the measurement of reaction velocity were accomplished in less than 2 min. In the experiments designed to evaluate the effects of the addition times of THA on ChE protection, the sequence of the addition of drugs was varied. However, also in these experiments, the 15-min incubation was started from the moment of the addition of the inactivating agent (DFP or physostigmine). The drug concentration values in these experiments refer to final concentrations in the incubation mixtures, before dilution of the samples, while DTNB and substrate concentrations are those present in the activity assay.

In the experiments designed to evaluate the protective effects of physostigmine, this drug was preincubated with the enzyme for 15 min before addition of DFP. Besides, in these experiments, the recovery of enzymic activity was evaluated at different times within 4–6 hr of dilution. The other steps of the procedure were as for THA.

The protective effect is described in the results as a decrease in enzymic inhibition consequent to THA pretreatment. The per cent enzymic inhibition was calculated from enzyme blanks assayed during the procedure.

RESULTS

THA is a strong inhibitor of ChE. In our experiments the THA IC_{50} values for eel AChE and horse serum BuChE were 31 ± 3 and 8.2 ± 0.8 nM, respectively. To evaluate its protective action towards these enzymes, therefore, THA had to be removed as completely as possible from the incubation medium before measuring residual enzymic activity. In our experiments this was achieved by extensively diluting the ChE preparations which had been pre-incubated with THA and inactivators, before assaying ChE activity. To keep to a minimum the spontaneous activity recovery of carbamate-inhibited enzymes, the whole procedure was accomplished as rapidly as possible (1–2 min). It was assumed that inhibition by DFP and the carbamates physostigmine and neostigmine would not be significantly affected by the operation owing to the basically irreversible character of the inhibitory effects of these drugs [20], while that by THA should be greatly reduced or abolished altogether by extensive dilution on account of its reversibility [21].

Figure 1 shows the results obtained in the experiments with eel AChE. In this case the enzymic incubates were diluted 1000 times and assayed immediately for AChE activity. Under these conditions, THA up to the concentration 1 μM caused no enzymic inhibition in the absence of irreversible inhibitors. Inhibition caused by 3 and 10 μM THA blanks averaged 4 and 15% of controls, respectively. Therefore, when THA was used at these or higher concentrations, a small fraction of enzymic activity was maintained, inhibited by THA itself in spite of the dilution. The plots in panel A of Fig. 1 show that THA effectively protected AChE from all the inactivators tested, that is 10, 40 and 100 μM DFP, 1 μM physostigmine, and 1.2 μM neostigmine. In the absence of THA, these compounds inhibited AChE by an average of 87, 100, 100, 93 and 92%, respectively. THA protective action was dose dependent and showed a pattern of progress which resembled that (not shown) of inhibition. THA was markedly more effective against DFP (EC_{50} = 0.04 ± 0.006 μM, 10 μM DFP; 0.16 ± 0.04 μM, 40 μM DFP; 0.45 ± 0.1 μM, 100 μM DFP) than against physostigmine (EC_{50} = 1.8 ± 0.4 μM, 1 μM physostigmine) or neostigmine (EC_{50} = 3.0 ± 0.6 μM, 1.2 μM neostigmine).

Panel B of Fig. 1 shows the results obtained in these experiments using other reversible AChE inhibitors as protective agents and 40 μM DFP as inactivator. To enable a direct comparison between these drugs and THA, the curve of the latter drug has also been included in Panel B of Fig. 1. The bis-anilinium derivative, BW284C51, which is the potent and selective inhibitor of AChE [22], was the most effective among the compounds tested with an EC_{50} value of 0.04 ± 0.008 μM. Edrophonium was less potent than THA as a protective agent but its EC_{50} value was still in the sub-micromolar range (0.7 ± 0.15 μM). Dextrorphan, which is a weak inhibitor of AChE [23], and ranitidine, which is an uncompetitive inhibitor of the same enzyme [24], protected AChE at relatively high concentrations only (EC_{50} of 45 ± 9 and 300 ± 50 μM, respectively).

The results obtained using horse serum BuChE are shown in Fig. 2. In this case the enzymic mixtures were diluted 5000 times on account of the particularly high inhibitory potency of THA towards this enzyme. Under these conditions, THA up to 3 μM did not

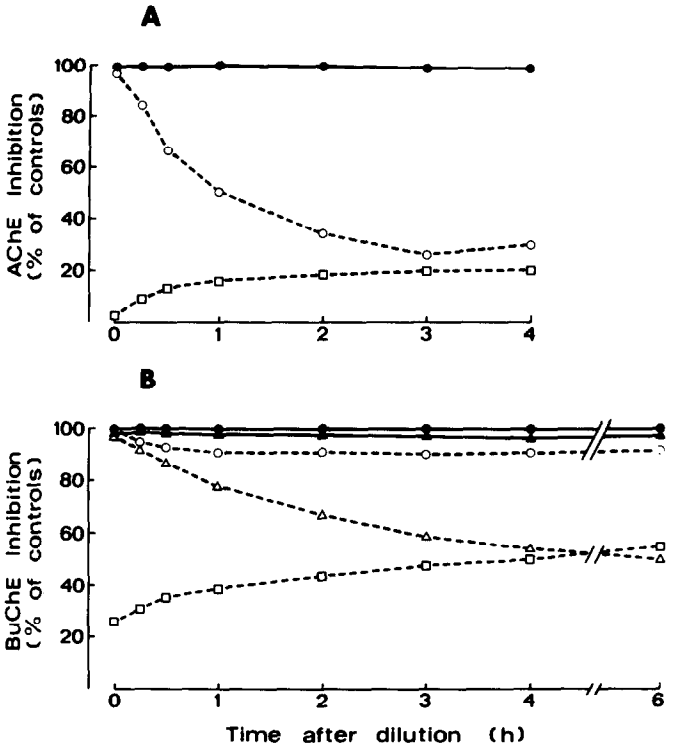

Fig. 3. Protection of AChE (A) and BuChE (B) from DFP by pretreatment with physostigmine and THA: time-course of enzymic activity recovery following extensive dilution. (A) Aliquots of electric eel AChE (2.2 U) pretreated (15 min at 25°) with buffer alone (●), 0.3 μM physostigmine (○) and 3 μM THA (□) were incubated for an additional 15 min with 40 μM DFP prior to being rapidly diluted 1000 times. (B) Aliquots of horse serum BuChE (14.4 U) pretreated (15 min at 25°) with buffer alone (●, ▲), 5 μM physostigmine (○, △) and 5 μM THA (□) were incubated for an additional 15 min with 3 μM (▲, △, □) or 40 μM (●, ○) DFP prior to being diluted 5000 times. All samples were then assayed for enzymic activity at different times after dilution. AChE and BuChE controls were assayed along with the appropriate test samples. The points in the graph are the means of two separate experiments in duplicate which gave very similar results.

inhibit BuChE when diluted, while 10 μM THA caused a 10% inhibition of control BuChE. EC_{50} values for the protection of this enzyme from 3 μM physostigmine, 3, 10 and 40 μM DFP were 0.55 ± 0.1, 1.5 ± 0.2, 3 ± 0.4 and >10 μM, respectively. The mean per cent inhibition of BuChE by 3 μM physostigmine, 3, 10 and 40 μM DFP in the absence of THA pretreatment was 95, 99, 100 and 100%, respectively. Unlike AChE, therefore, THA was more effective in protecting BuChE from physostigmine than from DFP. BW284C51, edrophonium and dextrorphan failed to protect BuChE from 40 μM DFP to any appreciable extent.

The recovery of AChE and BuChE activity, following dilution, was diminished markedly when THA was added to the enzyme after DFP or physostigmine (data not shown).

To measure the protective effect of physostigmine against AChE and BuChE phosphorylation by DFP, a slightly different experimental protocol was used. ChE inhibition by physostigmine is time dependent; accordingly, the regeneration of free active enzyme upon dilution is a time-consuming process [20]. In the experiments in which physostigmine was used as a protective agent, therefore, the recovery of enzymic activity was examined at different times after dilution to allow full hydrolysis of the carbamyl enzyme. The results of Fig. 3 (panel A) show that pretreatment of eel AChE with 0.3 μM physostigmine protected approximately 70% of the enzyme from DFP inactivation. The enzyme, however, recovered its activity only 3–4 hr after dilution. This was the maximal protective effect we could obtain from physostigmine under our experimental conditions. The recovery of maximal activity was immediate in the case of pretreatment with 3 μM THA. In this case, however, there was a subsequent increase in enzymic inhibition which led to AChE values similar to those obtained with physostigmine. The results obtained with BuChE (panel B in Fig. 3) closely paralleled those with AChE. In this case, however, DFP was also used at 3 μM since both physostigmine and THA (data not shown) provided very poor protection of BuChE from 40 μM DFP. As observed with AChE, the recovery of enzymic activity in THA-pretreated samples was immediate after dilution. In the subsequent time intervals, however, the decrease in BuChE activity was very marked in

Table 1. Effect of preincubation time on the protective effects of physostigmine and THA against AChE inhibition by DFP

Preincubation time (min)	AChE inhibition (% of controls)	
	Physostigmine	THA
0	60	33
1	47	37
2	44	38
5	35	36
15	30	36
30	27	35
60	28	38

Aliquots of electric eel AChE were preincubated with 0.3 μM physostigmine or 0.3 μM THA for the indicated times prior to the addition of 40 μM DFP. The samples were then incubated for an additional 15 min, diluted 1000 times and assayed for enzymic activity 3 hr (physostigmine samples) or immediately (THA samples) after dilution. In the absence of physostigmine or THA, 40 μM DFP caused a mean 99.5% inhibition of AChE.

Values are the means of two separate experiments in duplicate which gave very similar results.

these samples and led within 5–6 hr to inhibition values which were slightly higher than those observed with 5 μM physostigmine. Separate experiments have shown that this effect is probably due to DFP which, although diluted up to 0.6 nM, maintains a considerable inhibitory activity towards BuChE.

The protective effect of physostigmine was seen to be time dependent. The results in Table 1 show that in physostigmine-pretreated samples, AChE inhibition decreased from 60 to 27% of the controls when the pretreatment varied from 0–30 min. In contrast, THA's action was scarcely affected by the duration of its contact with the enzyme. In any case, this operation did not ameliorate the protective efficacy of the drug.

DISCUSSION

The data reported in this work indicate that THA can effectively protect AChE and BuChE from inactivation by DFP and physostigmine and also, in the case of AChE, by neostigmine. In agreement with previous findings, THA appears to be particularly effective in protecting AChE from DFP [7, 11–13]. THA was more effective in this action than the tertiary amines eseroline [25] and meptazinol [26]. THA also protects AChE against physostigmine and neostigmine. This action, however, requires markedly higher THA concentrations than those necessary to protect against DFP. In agreement with this finding, Marquis [13] failed to detect the protective action of 0.1 μM THA against physostigmine. THA's action is only preventive. The addition of the drug to the enzyme after DFP or physostigmine invariably results in a marked loss of protective efficacy.

Unlike physostigmine and carbamates in general, THA lacks a reactive acylating group and cannot steadily block ChE [20]. Its protective action, therefore, is likely to take place at the formation of the reversible complex between inhibitor and enzyme. A simple scheme describing the reactions studied is:

$$E + I \underset{}{\overset{K_d}{\rightleftarrows}} E.I \overset{k_2}{\rightarrow} EI \overset{k_3}{\rightarrow} E + Met$$

$$+$$

$$THA$$

$$\Updownarrow K_i$$

$$E.THA$$

in which E = free enzyme, I = inactivating agent (DFP or carbamate), E.I = enzyme–inactivating agent complex, EI = phosphorylated or carbamylated enzyme, E.THA = enzyme–THA complex and Met = inhibitor metabolites. Assuming that under our experimental conditions EI deacylation proceeds negligibly and that $k_3 \ll k_2$ [27], it can be derived from this reaction scheme that the first-order overall rate constant for the reaction of E with I (k_{obs}) is:

$$k_{obs} = \frac{k_2[I]}{[I] + K_d} \frac{1}{1 + [THA]/K_i}$$

According to this hypothesis, THA might prevent the attack of an irreversible inhibitor by competing with it at the active site of the enzyme. THA would in general be favored in this action by its higher affinity for ChE in comparison with the blocking agent. In fact, the K_i for the reversible interaction between THA and eel AChE is reported to be 8.5 nM [12], while the K_d for DFP, physostigmine and neostigmine inhibition of the same enzyme are 1100, 17 and 14 μM, respectively [28]. Analogously, THA's K_i for BuChE is considerably lower than that of physostigmine or DFP (5.2 nM vs 12.7 and 0.9 μM, respectively.* Also, the EC_{50} values for protection of AChE by the other reversible agents tested in this work or reported previously [25, 26] correlate well with the respective K_i values ($r = 0.998$). The efficacy of THA as a protective agent against inhibition by DFP or carbamate depends not only on the ratio K_i/K_d but also on I and k_2. For instance, the much lower efficacy of THA towards DFP inhibition of BuChE than of AChE can be explained on the basis of the higher overall inhibitory potency of DFP for the former than for the latter enzyme [20]. Unlike THA, protection by carbamates is likely to take place at not only the formation of the reversible enzyme–inhibitor complex, but also at the carbamylation of the enzyme [10]. This latter step may explain the time dependence of physostigmine protective action.

The remarkable efficacy of THA in protecting AChE from agents which interact with the catalytic site of the enzyme and the parallelism with the protective curves of competitive anti-ChE agents, such as edrophonium and BW284C51, suggest strongly that THA itself binds to an area closely adjacent to the catalytic site rather than to a peripheral allosteric site [2, 3, 13]. The findings that

* Galli A and Mori F, unpublished observation.

THA: (1) prevents [^{14}C]DFP binding to AChE [12], (2) binds to a site distinct from that of propidium [12] and gallamine [28] and (3) inhibits AChE in a mixed competitive–noncompetitive way [2, 29, 30] are in agreement with our point.

Recently, we have reported that THA doses in the range of 2.5–7.5 mg/kg protect mice from acute intoxication by DFP [7]. Although it is difficult to generalize about the *in vivo* situation from the *in vitro* one, it appears from our results that such doses of the drugs are high enough to protect AChE. Also, the weak protective effect of THA against physostigmine correlates with the observation that THA is ineffective as an antidote against physostigmine poisoning in the mouse.* The results of this work can also provide an explanation for the finding that THA [7] and other reversible agents such as eseroline [25] and meptazinol [26], unlike physostigmine and carbamates in general [8, 9], may exert a prophylactic action against ChE poisoning in the absence also of atropine co-administration. In fact, the prompt reversibility of the interaction of THA and related drugs with AChE is likely to favor the availability of free active enzyme in the period of time which is probably the most critical for survival, that is that immediately following poisoning. In the case of carbamates, we have shown that AChE, on the contrary, remains inhibited for a considerable time after poisoning by both the protective agent and poison, making necessary supportive therapy with anticholinergics.

On the whole, our findings appear in line with the concept that the primary mechanism of the antidotal action of THA against DFP poisoning, like that of physostigmine, resides in the ability of these drugs to prevent the irreversible inactivation of AChE. On the other hand, the protection of BuChE does not seem to play an important role in the mechanism of action of these drugs.

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