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BINDING SITES ON ACETYLCHOLINESTERASE FOR REVERSIBLE LIGANDS AND PHOSPHORYLATING AGENTS

A THEORETICAL MODEL TESTED ON HALOXON AND PHOSPHOSTIGMINE

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Abstract—The reaction of acetylcholinesterase (EC 3.1.1.7; human erythrocytes) with phosphostigmine, haloxon and VX was studied, and the effect of three reversible ligands (TMA, edrophonium, coumarin) and of acetylthiocholine upon the time-dependent and time-independent (reversible) inhibition by the organophosphates was evaluated. The three ligands and acetylthiocholine decreased the second-order rate constant of phosphorylation by a factor proportional to the enzyme–ligand dissociation constant, or to both K_m and K_{ss} (Michaelis constant and the substrate-inhibition constant for acetylthiocholine) irrespective of the organophosphate. However, the time-independent inhibitions by phosphostigmine and haloxon were differently affected. Acetylthiocholine affected the time-independent inhibition by phosphostigmine by a factor proportional to K_m, and that by haloxon by a factor proportional to K_{ss}. Coumarin had no effect on the time-independent inhibition by phosphostigmine, while TMA and edrophonium displaced phosphostigmine from its complex. Coumarin displaced haloxon from its complex with the enzyme, while TMA and edrophonium had no effect. We conclude that phosphostigmine and haloxon bind reversibly to different sites on the enzyme and the experiments agree with a theoretical model that haloxon binds reversibly to a peripheral site on acetylcholinesterase, and phosphostigmine to the catalytic site.

Acetylcholinesterase has a considerable capacity for binding structurally diverse ligands, and there is substantial evidence that the enzyme has binding sites remote from the catalytic site [1–8]. Reversible binding to acetylcholinesterase has been extensively investigated, but the properties of the peripheral sites and their effect upon the catalytic site are still not understood.

In this paper we have investigated the binding sites on acetylcholinesterase by studying the effect of three competing ligands and one substrate on phosphorylation of the catalytic site. The phosphorylating agents were haloxon, phosphostigmine and VX†. The competing ligands were TMA, edrophonium and coumarin. The substrate was acetylthiocholine. The choice of the compounds was based upon previous findings that TMA and edrophonium bind to the catalytic site of the enzyme, while coumarin binds to a peripheral site [4, 7, 9, 10]. Haloxon was shown to react with acetylcholinesterase in two ways: one is phosphorylation of the catalytic site and the other reversible binding to a peripheral site [9]. Phosphostigmine also binds reversibly to the enzyme [11], but the binding site has not been investigated. VX is a phosphorylating agent for which there is no evidence of a reversible step in its reaction with acetylcholinesterase.

In the theoretical treatment we have tested a model based upon the following assumptions: (a) the catalytic site and the peripheral site are independent concerning the binding of inhibitors or substrates (i.e. the affinity of a substrate or inhibitor for any of the two sites is not affected by the occupancy of the other one), (b) the binding of inhibitors or substrates to the peripheral site prevents phosphorylation of the catalyltic site, and (c) substrate inhibition is caused by the binding of substrate to the peripheral site. This model was tested with emphasis on whether haloxon and phosphostigmine bind reversibly to the same site, and the theoretical equations are given in the Appendix.

MATERIALS AND METHODS

Enzyme. The enzyme preparation was human erythrocyte acetylcholinesterase (EC 3.1.1.7) purified by gel chromatography as described by Wright and Plummer [12] or used as erythrocyte ghosts prepared according to Dodge and co-workers [13]. Both preparations were stored in 1.0% gelatine at −20°; the enzyme activity remained unaltered during storage up to 15 days. Immediately before use the preparation was thawed and diluted in 1.0% gelatine. The final concentration during enzyme assay was about 20 μg protein/ml, and 90 μg protein/ml for the

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† Abbreviations—phosphostigmine, 3-*N*-trimethylanilinium diethylphosphate methyl sulphate; haloxon, di-2-chloroethyl 3-chloro-4-methylcoumarin-7-yl phosphate; VX, *O*-ethyl S-2(diisopropylamino)ethyl methylphosphonothioate; TMA, tetramethylammonium iodide; edrophonium, 3-hydroxy-*N*-ethyl dimethyl anilinium chloride; coumarin, 3-chloro-7-hydroxy-4-methylcoumarin.

purified enzyme and erythrocyte ghosts respectively; the final gelatine concentration was 0.1%. No difference was observed between the two enzyme preparations with respect to the studied reactions, and all results on both preparations are therefore included into this paper.

Inhibitors. The following inhibitors were used: VX, phosphostigmine (prepared by Professor A. Deljac, Laboratory of Organic Chemistry, Faculty of Science, University of Zagreb, Yugoslavia), haloxon and coumarin (Copper's Technical Bureau, Berkhamsted, Herts, U.K.), TMA (BDH Chemical Ltd., Poole, Dorset, U.K.), edrophonium (F. Hoffmann-LaRoche, Basel, Switzerland).

Stock solutions were prepared in water (phosphostigmine, TMA and edrophonium), ethanol (haloxon and coumarin) or propylene glycol (VX). Further dilutions were prepared in water, except haloxon and coumarin which were prepared in 20% (v/v) ethanol. In the reaction medium with haloxon and coumarin the final ethanol concentration was 2 or 4% (v/v). In the reaction medium with VX the final propylene glycol concentration was $\leqq$0.0005% (v/v).

Substrate. Solutions of acetylthiocholine iodide (Fluka AG, Buchs SG, Switzerland) were daily prepared in water.

Experimental conditions and measurement of enzyme activity. All experiments were carried out at 25° with 20 mM phosphate buffer at pH = 7.4.

The enzyme activity was determined by the spectrophotometric method of Ellman and co-workers [14] using DTNB (5,5'-dithiobis (2-nitrobenzoic acid)) (Fluka AG, Buchs SG, Switzerland) as the thiol reagent. Stock solutions of DTNB (10 mM) were prepared in buffer and stored at 4°; the final DTNB concentration during enzyme assay was 0.33 mM.

The assay medium for determining enzyme activity contained buffer (2.1 ml), DTNB (0.1 ml), enzyme (0.20 ml), inhibitor (0.3 ml) and substrate (0.3 ml). Control samples contained water (0.3 ml) instead of the inhibitor. The enzyme activity was determined by measuring the increase of absorbance at 412 nm in 1.0 cm cells at 15 sec intervals up to 2 min. The first reading was done 15 sec after the addition of the last reactant (substrate or enzyme). The increase in absorbance was linear in all control and inhibited samples except in those containing phosphostigmine concentrations $\geqq$60 μM. In these samples the plot log (ΔA/15 sec) vs time of assay was linear, and the enzyme activity was calculated by extrapolating the line to initial time.

Corrections for spontaneous substrate hydrolysis were done for concentrations $\geqq$1.0 mM acetylthiocholine.

Time-independent inhibition (reversible inhibition). The enzyme was added to the assay medium containing buffer, DTNB, inhibitor and substrate. The activity was determined from the increase in absorbance as described above.

Time-dependent inhibition (progressive inhibition). The inhibitor (haloxon, VX or phosphostigmine) was added to the assay medium containing buffer, DTNB and enzyme. After a given time of inhibition the substrate was added, and the activity determined from the increase in absorbance as described above.

Effect of competing agents (TMA, coumarin, edrophonium) on progressive inhibition. The inhibitor (haloxon, VX or phosphostigmine) was added to the assay medium containing buffer, DTNB, enzyme and competing agent. After a given time of inhibition the substrate was added, and the activity determined from the increase in absorbance as described above.

Effect of substrate on progressive inhibition. The enzyme was added to the assay medium containing buffer, DTNB, substrate and inhibitor (haloxon, VX or phosphostigmine), and the increase in absorbance measured up to 16 min. The first reading was done 15 sec after the addition of the enzyme, and subsequent readings every 15 sec or 1.0 min. A plot of log (ΔA per interval) vs time of assay was linear. From the slope of the line the first-order rate constant of inhibition was calculated.

Effect of ethanol. Haloxon and coumarin are poorly soluble in water. The reaction medium therefore had to contian 2 or 4% ethanol, as stated in the tables. These concentrations increased the enzyme-inhibitor dissociation constants and decreased the second order rate constants of inhibition. The same was the case for the effect of 2% ethanol on reactions of phosphostigmine, VX, TMA and edrophonium. The effect of ethanol was not studied in detail, because for a given compound the same kinetic equations were valid irrespective of ethanol. In the theoretical calculations only constants derived at the same ethanol concentration were compared with each other.

Calculation of constants. All constants derived for the reaction of the enzyme with inhibitors and the substrate were calculated by linear regression analysis.

RESULTS AND DISCUSSION

Time-independent inhibition. The effect of acetylthiocholine was studied upon time-independent inhibition of acetylcholinesterase by haloxon, phosphostigmine, TMA, edrophonium and coumarin. At each substrate concentration the inhibition was determined with 2–3 different inhibitor concentrations, and the degree of inhibition expressed as the ratio $v \cdot I/(v_0 - v)$, where v_0 and v are the enzyme activities at a given substrate concentration (S) in the absence and in the presence of the inhibitor (I). For all compounds the degree of inhibition was a linear function of the substrate concentration (cf. Fig. 1 for inhibition by phosphostigmine and haloxon) and the enzyme-inhibitor dissociation constants K_{inh} were calculated from equations (1) or (2) (Table 1):

$$\frac{v \cdot I}{v_0 - v} = K_{inh} + \frac{K_{inh}}{K_m} S \quad (1)$$

$$\frac{v \cdot I}{v_0 - v} = K_{inh} + \frac{K_{inh}}{K_{ss}} S \quad (2)$$

The Michaelis constant K_m and the substrate-inhibition constant K_{ss} were calculated from the same equations (Table 1).

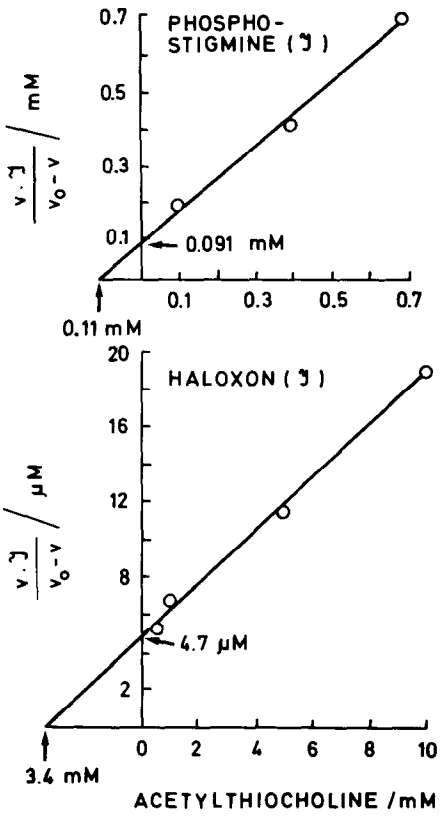

Fig. 1. The effect of substrate on the time-independent inhibition of acetylcholinesterase by phosphostigmine and haloxon. Each point is the mean value of 5–10 measurements with 2–3 different inhibitor concentrations. The reaction with haloxon was measured in 2% ethanol.

Both constants were also derived independently from pS-curves in the absence of inhibitors; K_m from plot of S/v_0 vs S, K_{ss} from plot of $1/v_0$ vs S (Table 1).

Equation (1) should hold for pure competitive inhibition occurring in the enzyme active site [15], while equation (2) was derived [9, 16] for competitive inhibition with excess substrate, which in turn was interpreted to occur at a peripheral allosteric site. These equations can therefore be used to distinguish between two types of reversible binding.

Table 1 shows that the five inhibitors fall into two categories. Phosphostigmine, TMA and edrophonium compete with considerably lower substrate concentrations than haloxon and coumarin, and this is quantitatively expressed by the intercept of the inhibition lines with the abscissa (cf. Fig. 1). For the first three compounds these values are between 0.11 and 0.27 mM, i.e. significantly lower than the corresponding values for haloxon and coumarin 3.4–4.0 mM (Table 1). Because of this difference reversible inhibition by phosphostigmine could only be measured at substrate concentrations up to 1.0 mM, while for haloxon it was measurable up to 10 mM acetylthiocholine (Fig. 1).

These results indicate two different types of binding. Reversible inhibition by phosphostigmine, TMA and edrophonium conforms approximately with equation (1) while equation (2) seems more applicable to reversible inhibition by haloxon and coumarin. However the K_m constant derived independently from pS-curves was lower, and the K_{ss} constant higher, than the corresponding values derived from equations (1) and (2) (Table 1).

Table 1. The time-independent inhibition of acetylcholinesterase

Inhibitor	Ethanol (%)	K_{inh} (μM)	K_m or K_{ss} (mM)
Phosphostigmine	0	91	0.11
TMA	0	1280	0.11
	2	2900	0.27
Edrophonium	0	0.44	0.23
	2	0.62	0.26
Haloxon	2	4.7	3.4
Coumarin	2	51	4.0
	4	61	2.9
Substrate	Ethanol (%)	*K_m (mM)	*K_{ss} (mM)
Acetylthiocholine	0	0.046	12
	2	0.10	18

Constants were derived from equations (1) and (2), except those indicated with an asterisk which were derived from pS-curves in the absence of inhibitors.

Time-dependent inhibition. Progressive inhibition of acetylcholinesterase by VX, phosphostigmine and haloxon (AB) was studied over a time period up to 10 min using 4–6 different inhibitor concentrations. At any given inhibitor concentration the decrease in enzyme activity was a first-order reaction wherefrom the first-order rate constants k were calculated.

Inhibition by VX proceeded according to scheme 1 (EH and EA are the free and phosphorylated enzyme resp.) and the second-order rate constant k_a was calculated from equation (3).

$$EH + AB \xrightarrow{k_a} EA + BH \qquad \text{Scheme 1}$$

$$k = k_a \cdot AB \qquad (3)$$

Inhibition by phosphostigmine and haloxon showed characteristics already described [9, 11]: with increasing inhibitor concentrations the first-order rate constants k approached a saturation value indicating the presence of an enzyme-inhibitor complex. Two reaction schemes are applicable to these results:

$$EH + AB \underset{k_{-1}}{\overset{k_{-1}}{\rightleftharpoons}} EHAB \xrightarrow{k_{+2}} EA + BH \qquad \text{Scheme 2}$$

$$\underbrace{\qquad\qquad\qquad\qquad}_{k_a}$$

$$k = \frac{k_{+2} \cdot AB}{K_a + AB} \qquad (4)$$

$$K_a = \frac{k_{-1} + k_{+2}}{k_{+1}} \qquad (5)$$

$$k_{+2}/K_a = k_a \qquad (6)$$

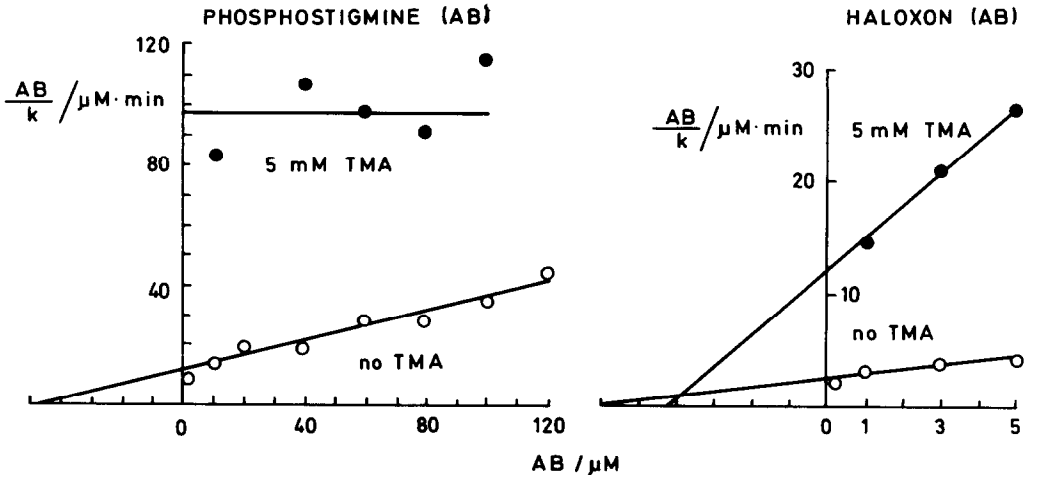

Fig. 2. The effect of TMA on the time-dependent inhibition of acetylcholinesterase by phosphostigmine and haloxon. The k is the first-order rate constant of inhibition, and each k is the mean value obtained in 3–4 experiments. The reaction with haloxon was measured in 2% ethanol.

$$\begin{array}{l} EH + AB \xrightarrow{k_a} EA + BH \quad \text{Scheme 3} \\ + \\ AB \\ K_i \downarrow\uparrow \\ EHAB \end{array}$$

$$k = \frac{k_a \cdot K_i \cdot AB}{K_i + AB} \qquad (7)$$

EHAB in scheme 2 stands for the reversible complex in the enzyme active site, while EHAB in scheme 3 stands for a reversible complex formed at a peripheral site. Scheme 3 was derived earlier for the inhibition of acetylcholinesterase by haloxon [9, 16].

From these experiments it could not be concluded which of the two schemes holds for a given phosphorylating agent, because equations (4) and (7) are kinetically undistinguishable. By applying either equation we have calculated the rate constants k_a and the enzyme-inhibitor dissociation constants K_a or K_i. The calculation was done from a linear transformation of the above equations as stated in the Appendix (equations A1 and A2) and as shown in Figs. 2 and 3. The obtained constants are given in Tables 2 and 3.

In order to distinguish between schemes 2 and 3 the progressive inhibition was studied in the presence of TMA, edrophonium, coumarin and acetylthiocholine, and the rate constants and dissociation constants in the presence of these compounds were evaluated as described in the Appendix.

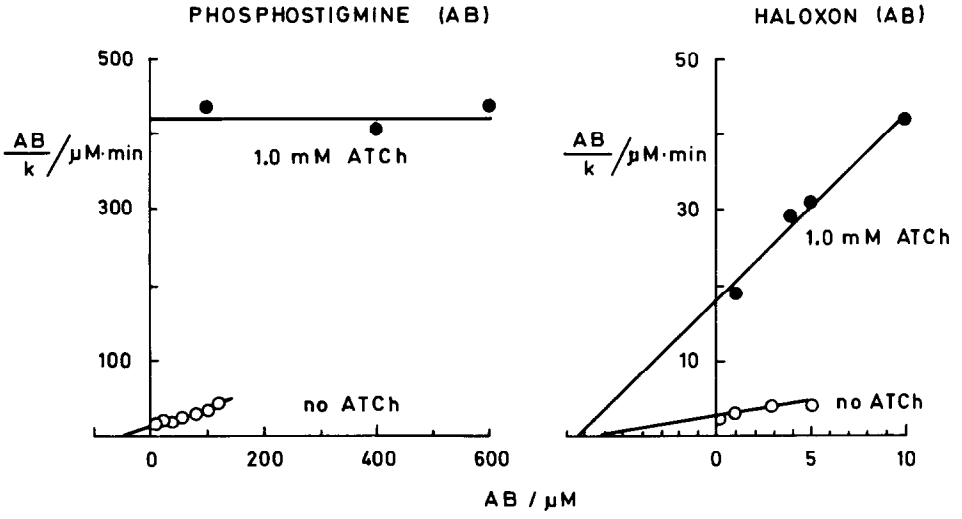

Fig. 3. The effect of substrate on the time-dependent inhibition of acetylcholinesterase by phosphostigmine and haloxon. The k is the first-order rate constant of inhibition, and each k is the mean value obtained in 3–4 experiments. The reaction with haloxon was measured in 2% ethanol.

Table 2. The time-dependent inhibition of acetylcholinesterase by VX, phosphostigmine and haloxon

Phosphorylating agent	Reversible ligand or substrate	Ethanol (%)	$k_a \times 10^{-4}$ (moles^{-1}L min^{-1}) Measured	$k'_a \times 10^{-4}$ (moles^{-1}L min^{-1}) Measured	$k'_a \times 10^{-4}$ (moles^{-1}L min^{-1}) Calculated
VX	None	0	3100	—	—
	TMA, 1.25 mM	0	—	1440	1600
	5.0 mM	0	—	380	640
	ATCh, 0.40 mM	0	—	330	310
	1.0 mM	0	—	150	130
	None	2	1600	—	—
	Coumarin, 30 μM	2	—	750	990
	60 μM	2	—	470	720
Phosphostigmine	None	0	9.0	—	—
	TMA, 2.5 mM	0	—	1.5	3.1
	5.0 mM	0	—	0.96	1.8
	Edroph. 0.5 μM	0	—	1.5	4.2
	1.0 μM	0	—	1.0	2.7
	ATCh 0.40 mM	0	—	1.1	0.90
	1.0 mM	0	—	0.24	0.37
	None	2	3.7	—	—
	Coumarin 30μM	2	—	1.6	2.3
	60 μM	2	—	1.0	1.7
Haloxon	None	2	44	—	—
	TMA 2.5 mM	2	—	12	24
	5.0 mM	2	—	8.3	16
	Edroph. 0.20 μM	2	—	8.4	33
	0.40 μM	2	—	5.0	27
	ATCh 0.50 mM	2	—	11	7.1
	1.0 mM	2	—	5.7	3.8
	None	4	29	—	—
	Coumarin 30 μM	4	—	12	19
	60 μM	4	—	4.7	15

The k_a and k'_a are second-order rate constants of inhibition in the absence and presence of reversible ligands or substrate (ATCh). The k'_a were calculated from equations (A7) and (A12).

The dissociation constants K_a and K_i were affected in the following way (Table 3). In the presence of TMA or edrophonium no reversible complex with phosphostigmine was observed ($K'_a \gg K_a$) (cf. Fig. 2), while in the presence of coumarin no reversible complex with haloxon was observed ($K'_i \gg K_i$). TMA and edrophonium had no effect on the complex with haloxon ($K'_i = K_i$) (cf. Fig. 2), and coumarin had no effect on the phosphostigmine complex ($K'_a = K_a$). Acetylthiocholine increased significantly the dissociation constant for phosphostigmine, but had little or no effect on haloxon (cf. Fig. 3).

If the criteria outlined in the Appendix are applied, it follows that phosphostigmine and haloxon must bind to different sites on the enzyme. TMA and edrophonium affected the binding of phosphostigmine in agreement with equations (A3) and (A8), coumarin in agreement with equation (A4), and acetylthiocholine with equations (A10) and (A13). For inhibition by haloxon the following equations were valid: equation (A5) for the effect of TMA and edrophonium, equations (A6) and (A9) for coumarin, and equations (A11) and (A14) for acetylthiocholine. We have therefore concluded that phosphostigmine reacts with the enzyme according to scheme 2 and haloxon according to scheme 3. The respective dissociation constants were denoted as K_a and K_i for phosphostigmine and haloxon resp.

According to the theoretical model the second-order rate constants of inhibition should be equally affected by ligands irrespective of the binding sites for AB and I [equations (A7) and (A12)]. A comparison between the measured and calculated k'_a values (Table 2) confirmed this prediction. The calculated k'_a tended to be higher than the measured k'_a. We have no explanation for this trend, which was not restricted to any pair of the tested compounds, and was even observed for VX, which reacts with acetylcholinesterase according to the simple scheme 1. This disagreement was not related to the presence of ethanol, because it was also seen in reactions without ethanol (cf. Table 2).

CONCLUSION

The binding sites on acetylcholinesterase were studied with organophosphorus compounds and three non-phosphorylating inhibitors. The emphasis was on whether haloxon and phosphostigmine bind reversibly to the catalytic site or peripheral site on the enzyme.

From the effect of substrate on the time-indepen-

Table 3. The enzyme-inhibitor dissociation constants derived from the time-dependent inhibition of acetylcholinesterase by phosphostigmine and haloxon in the absence (K_a and K_i) and presence (K_a' and K_i') of reversible ligands or substrate (ATCh)

Phosphorylating agent	Reversible ligand or substrate	Ethanol (%)	Dissociation constants (μM)		
Phosphostigmine			K_a		K_a'
			Measured	Calculated	Measured
	None	0	46	—	—
	TMA, 2.5 mM	0	—	—	*
	5.0 mM	0	—	—	*
	Edrophonium				
	0.5 mM	0	—	—	*
	1.0 mM	0	—	—	*
	ATCh, 0.4 mM	0	—	31	304
	1.0 mM	0	—	—	*
	None	2	122	—	—
	Coumarin, 30 μM	2	—	—	129
	60 μM	2	—	—	105
Haloxon			K_i		K_i'
			Measured	Calculated	Measured
	None	2	5.9	—	—
	TMA, 2.5 mM	2	—	—	4.1
	5.0 mM	2	—	—	4.2
	Edrophonium				
	0.2 μM	2	—	—	6.1
	0.4 μM	2	—	—	5.5
	ATCh, 0.5 mM	2	—	3.3	3.4
	1.0 mM	2	—	6.6	7.0
	None	4	8.3	—	—
	Coumarin, 30 μM	4	—	—	*
	60 μM	4	—	—	*

K_a was calculated from equation (A13) inserting $K_a' = 304$ μM and $K_m = 46$ μM. K_i was calculated from equation (A14) inserting $K_{ss} = 18$ mM and $K_i' = 3.3$ or 6.6 μM.

* Reversible complex not detectable ($K_a' \gg K_a$ or $K_i' \ll K_i$)

dent inhibition it was possible to classify the inhibitors into two groups (Table 1). Phosphostigmine, TMA and edrophonium competed with acetylthiocholine at low substrate concentrations (K_m from inhibition = 0.11–0.27 mM), and haloxon and coumarin at high substrate concentrations (K_{ss} from inhibition = 3.4 – 4.0 mM) (Table 1). We have interpreted this effect as competition between phosphostigmine and substrate at the catalytic site and between haloxon and substrate at a peripheral, substrate-inhibition, site.

The time-dependent inhibition of acetylcholinesterase by phosphostigmine and haloxon revealed a reversible step in the kinetics of the reaction where from the enzyme-inhibitor dissociation constants were calculated, K_a for phosphostigmine and K_i for haloxon. The values of these constants agreed well with the dissociation constants obtained from the time-independent inhibition K_{inh} (Tables 1 and 3) showing that in both reactions the same complex was being determined.

The effect of TMA, edrophonium, coumarin and acetylthiocholine on progressive inhibition confirmed the difference between phosphostigmine and haloxon, and the kinetics was consistent with phosphostigmine reacting according to scheme 2 and haloxon according to scheme 3.

The theoretical equations used for the calculation of dissociation constants and rate constants were based on the assumptions (see Introduction and Appendix) which almost certainly oversimplify the properties of binding sites on the enzyme. However, the models describe satisfactorily the reaction of acetylcholinesterase with haloxon and phosphostigmine, and the theoretical equations seem generally applicable for a qualitative differentiation between reactions described in schemes 2 and 3.

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APPENDIX: THEORETICAL EQUATIONS AND EVALUATION OF CONSTANTS

Rate constants and dissociation constants in schemes 2 and 3 were calculated from a linear transformation of equations (4) and (7), respectively.

AB binds to catalytic site

$$\frac{AB}{k} = \frac{K_a}{k_{+2}} + \frac{AB}{k_{+2}} \tag{A1}$$

AB binds to peripheral site

$$\frac{AB}{k} = \frac{1}{k_a} + \frac{AB}{k_a K_i} \tag{A2}$$

When phosphorylation of the enzyme by AB proceeded in the presence of a ligand (I) four equations were considered:

AB and I *bind to catalytic site*

$$\frac{AB}{k} = \frac{K_a}{k_{+2}}\left(1 + \frac{I}{K_{inh}}\right) + \frac{AB}{k_{+2}} \tag{A3}$$

AB binds to catalytic and I *to peripheral site*

$$\frac{AB}{k} = \frac{K_a}{k_{+2}}\left(1 + \frac{I}{K_{inh}}\right) + \frac{AB}{k_{+2}}\left(1 + \frac{I}{K_{inh}}\right) \tag{A4}$$

AB binds to peripheral and I *to catalytic site*

$$\frac{AB}{k} = \frac{1}{k_a}\left(1 + \frac{I}{K_{inh}}\right) + \frac{AB}{k_a K_i}\left(1 + \frac{I}{K_{inh}}\right) \tag{A5}$$

AB and I *bind to peripheral site*

$$\frac{AB}{k} = \frac{1}{k_a}\left(1 + \frac{I}{K_{inh}}\right) + \frac{AB}{k_a K_i} \tag{A6}$$

Equations (A3) and (A4) apply to scheme 2, and (A5) and (A6) to scheme 3. Equation (A3) is from Iverson (ref. 8 above). Equations (A4), (A5) and (A6) were derived here from the following assumptions: the enzyme has two binding sites for AB and I (the catalytic and the peripheral sites), these sites are independent as far as binding is concerned, but when the peripheral site is occupied by either AB or I the catalytic site cannot be phosphorylated (for more details cf. ref. 16 above).

It follows from equations (A3) to (A6) that the second-order rate constant of phosphorylation will decrease by the same factor irrespective of the binding sites for AB and I:

$$k_a' = k_a / \left(1 + \frac{I}{K_{inh}}\right) \tag{A7}$$

The same equation is valid when phosphorylation follows scheme 1 in the presence of reversible ligands (I). This equation was used earlier for inhibition by organophosphorus compounds in the presence of tetraalkylammonium ions [17, 18].

The dissociation constants for AB will be unaffected by I, when AB and I bind to different sites (equations (A4) and (A5) (cf. Fig. 2 for the effect of TMA on haloxon), but will increase by the same factor when AB and I bind to the same site [equations (A3) and (A6)]:

$$K_a' = K_a\left(1 + \frac{I}{K_{inh}}\right) \tag{A8}$$

$$K_i' = K_i\left(1 + \frac{I}{K_{inh}}\right) \tag{A9}$$

Equations (A8) and (A9) represent the intercepts on the abscissa in Fig. 2. If the enzyme affinity for AB becomes apparently very small, i.e. K_a' and K_i' very large, no reversible complex with AB will be detectable in the presence of I, and the inhibition line will be parallel with the abscissa (cf. Fig. 2 for the effect of TMA on phosphostigmine).

When the phosphorylation of the enzyme proceeded in the presence of substrate two equations were considered

AB binds to catalytic site and S *to both sites*

$$\frac{AB}{k} = \frac{K_a}{k_{+2}}\left(1 + \frac{S}{K_m}\right)\left(1 + \frac{S}{K_{ss}}\right) + \frac{AB}{k_{+2}}\left(1 + \frac{S}{K_{ss}}\right) \tag{A10}$$

AB binds to peripheral site and S *to both sites*

$$\frac{AB}{k} = \frac{1}{k_a}\left(1 + \frac{S}{K_m}\right)\left(1 + \frac{S}{K_{ss}}\right) + \frac{AB}{k_a K_i}\left(1 + \frac{S}{K_m}\right) \tag{A11}$$

Equation (A10) applies to scheme 2, and equation (A11) to scheme 3. It follows from these equations that the second-order rate constant of phosphorylation will decrease by the same factor irrespective of the binding site for AB:

$$k_a' = k_a / \left[\left(1 + \frac{S}{K_m}\right)\left(1 + \frac{S}{K_{ss}}\right)\right] \tag{A12}$$

while K_a and K_i will be affected by S according to:

$$K_a' = K_a\left(1 + \frac{S}{K_m}\right) \tag{A13}$$

$$K_i' = K_i\left(1 + \frac{S}{K_{ss}}\right) \tag{A14}$$

Equations (A13) and (A14) represent intercepts on the abscissa in Fig. 3, and it follows from the equations that the effect of substrate on K_i will be much less pronounced than the effect of substrate on K_a (compare haloxon and phosphostigmine in Fig. 3).