MOLECULAR PHARMACOLOGY, 6, 41–45 (1970)

Kinetic Effects of Leptocurares and Pachycurares on the Methanesulfonylation of Acetylcholinesterase

A Correlation with Pharmacodynamic Properties

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(Received September 2, 1969)

SUMMARY

It was shown previously that the alkyltrimethylammonium ion series of cholinergic and anticholinergic drugs not only fail to protect acetylcholinesterase (AChE) against irreversible esterification by methanesulfonyl fluoride of its active serine hydroxyl, but actually accelerate the reaction, thus showing that the alkyl chains do not bind on the catalytic esteratic sites themselves (*exo* orientation). Acceleration has been explained in terms of conformational perturbations, and maximum potency in this regard was exhibited by the *n*-hexyl member, followed by a sharp reversal of the acceleration trend at the *n*-heptyl member. Parallel trends and shifts in potencies at the receptor level are well documented. Using the same experimental techniques, it has now been found that leptocurares (polymethoniums of the decamethonium series, including succinylcholine) also behave as accelerators of the methanesulfonylation reaction, peak activity being observed with decamethonium. Succinylcholine was 4 times more active than the latter in stimulating the methanesulfonylation reaction. The trend in stimulating potencies closely parallels the relative blocking potencies at the motor end plate level. In marked contrast, *d*-tubocurarine and gallamine *protect* AChE against methanesulfonyl fluoride by a mechanism which does not obey the laws of competitive kinetics. The application of conventional assay techniques had previously led to the conclusion that pachycurares are competitive inhibitors of the *cationic* substrate ACh at both the enzyme and myoneural junction levels. However, evidence that the inhibition is rather of the partially competitive type has recently been reported. Since the methanesulfonyl fluoride molecule, unlike ACh, carries no charge, it can hardly compete directly with pachycurares for charged binding sites. Hence, the observed partially competitive relationship must be the result of pachycurare-induced change transmitted to the esteratic center from outer anionic sites. These contrasting properties of leptocurares and pachycurares as modifiers of conformation find an exact parallel in the divergence of their mechanisms of blockade at the myoneural junction level. These observations furnish new insight into the interaction topographies underlying the ligand-induced conformational changes.

The rate of irreversible inhibition of acetylcholinesterase by methanesulfonyl fluoride is markedly sensitive to the effects of quaternary ion binding on the anionic centers of the enzyme (1, 2). For instance, sulfonylation of the esteratic serine hydroxyl is accelerated about 6-fold by the tetra-

This investigation was supported by Research Grant 2001-2 from the Defence Research Board of Canada.

TABLE 1

Effects of lepto- and pachycurares on the velocity of irreversible methanesulfonylation of erythrocyte acetylcholinesterase at 25° and pH 7.4 in 0.1 M NaCl and 0.02 M $MgCl_2$

The relative velocities of the methanesulfonylation reaction with AChE were estimated by a slight modification of the method of Kitz and Wilson (1, 2). At various time intervals, 0.25-ml portions of incubation solution were withdrawn and then immediately assayed for residual AChE activity against ACh at a concentration of 3.4×10^{-4} M in salt solution having a final volume of 3 ml at enzyme concentrations of 10 or 14 units/ml or a final volume of 25 ml at enzyme concentrations of 80 units/ml. For control incubations, only methanesulfonyl fluoride was omitted. The rate of disappearance of AChE activity is proportional to the velocity of the methanesulfonylation reaction with the esteratic center (1–3). Duplicates were run in all cases. As expected, semilogarithmic plots of percentage of residual AChE activity against time were all linear [pseudo-first-order kinetics (1, 2)]. In the last column, the acceleration (or inhibition) values (A) represent the ratios of the slopes of the initial velocity of AChE inactivation by methanesulfonyl fluoride at two different concentrations of each modifier. For all practical purposes, the individual A values were independent of modifier concentration in the range of concentrations given in the second column. Since duplicates were run for each modifier concentration, the A values represent averages of four determinations, which permitted estimation of the standard errors.

Effector salt	Effector salt concentration	Methanesulfonyl fluoride concentration	AChE concentration	Acceleration of reaction, A (avg ± SE)
	M	M	*units/ml*	
Tetramethonium	92.10×10^{-3}	13.9×10^{-6}	10	1.80×0.1
	11.40×10^{-3}	20.6×10^{-7}	80	
Pentamethonium	30.30×10^{-3}	20.6×10^{-7}	80	1.50 ± 0.1
	34.60×10^{-3}	20.6×10^{-7}	80	
Hexamethonium	10.40×10^{-2}	13.9×10^{-6}	10	1.30 ± 0.2
	64.60×10^{-4}	20.6×10^{-7}	80	
Heptamethonium	91.00×10^{-5}	61.8×10^{-7}	80	4.20 ± 0.1
	19.20×10^{-5}	61.8×10^{-7}	80	
Octamethonium	62.50×10^{-5}	20.6×10^{-7}	80	6.30 ± 0.1
	15.30×10^{-4}	20.6×10^{-7}	80	
Nonamethonium	17.30×10^{-5}	20.6×10^{-7}	80	8.90 ± 0.05
	23.50×10^{-5}	20.6×10^{-7}	80	
Decamethonium	5.08×10^{-5}	6.2×10^{-7}	80	9.30 ± 0.1
	9.40×10^{-5}	20.6×10^{-7}	80	
Undecamethonium	2.01×10^{-5}	61.8×10^{-7}	80	9.10 ± 0.05
	3.11×10^{-5}	61.8×10^{-7}	80	
Dodecamethonium	9.50×10^{-6}	61.8×10^{-7}	80	8.70 ± 0.1
	13.30×10^{-6}	61.8×10^{-7}	80	
Succinylcholine	5.00×10^{-3}	30.9×10^{-6}	80	42.00 ± 2
	9.00×10^{-3}	30.9×10^{-6}		
d-Tubocurarine	2.22×10^{-3}	11.3×10^{-6}	14	0.45 ± 0.05
	5.44×10^{-3}	11.3×10^{-6}	14	
Gallamine	8.00×10^{-3}	11.3×10^{-6}	14	0.60 ± 0.05
	2.00×10^{-3}	11.3×10^{-6}	14	

methylammonium ion, an effect which is suggestive of effector-induced changes of conformation in the enzyme (3, 4). Accordingly, in the absence of complicating factors, rate variations in the methanesulfonylation reaction may give some measure of *effector potencies as inducers of conformational changes*. This may constitute one of the key physical parameters possibly allowing meaningful comparisons with pharmacological potencies, since it is conceivable that quaternary salts induce analogous changes of conformation in the membrane receptors (4). In this respect, comparisons of affinity

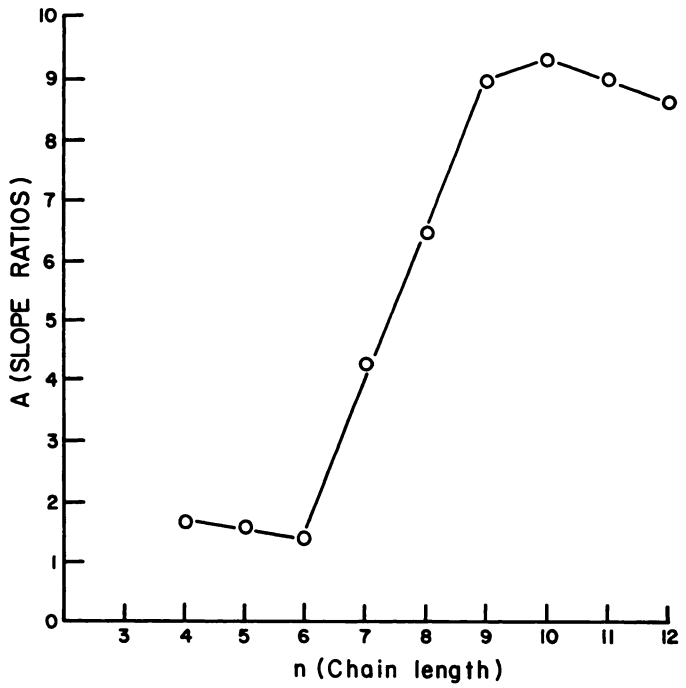

FIG. 1. *Plot of the ratio of the slopes (A) of the initial velocities of irreversible methanesulfonylation of AChE against n, the number of carbon atoms separating the two charges in polymethonium leptocurares*
Experimental conditions are defined in Table 1 (see the text also).

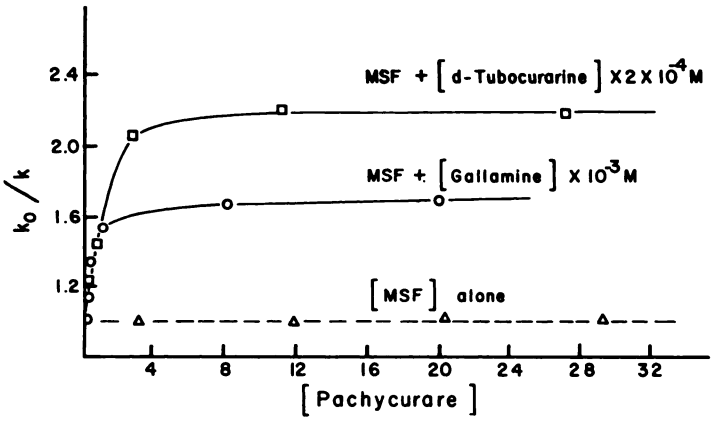

FIG. 2. *Plots of the rate constant ratios (k_0/k) for irreversible methanesulfonylation of AChE against the concentrations of d-tubocurarine and gallamine*
Experimental conditions are defined in Table 1, except that a range of concentrations for the reagents was used. The curves quickly reach a plateau (do not extrapolate to 1), a mechanism of inhibition at variance with the laws of competitive kinetics. MSF, methanesulfonyl fluoride.

constants are of very limited value, since they bear no direct relation to the nature and degree of conformational change (5).

Using the assay methods of Kitz and Wilson (1, 2), we have recently discovered (6) that all members of the *n*-alkyltrimethylammonium ion series of drugs (up to *n*-dodecyl) not only fail to protect AChE[1]

[1] The abbreviations used are: AChE, acetylcholinesterase; ACh, acetylcholine.

against methanesulfonyl fluoride, but also actually accelerate the reaction, thus establishing that all the alkyl chains of the effectors, regardless of length, assume an orientation on the enzyme surface which permits free access of methanesulfonyl fluoride to the esteratic sites. Moreover, the alkyltrimethylammonium ions are competitive inhibitors of ACh hydrolysis (7), thus showing that they share with ACh only the cation-binding site. Hence, the alkyl chains must be *exo*-oriented relative to the methanesulfonyl fluoride site of reaction[2] (see Fig. 3). Equally significant was the observation (6) that acceleration is dependent on chain length, reaching a sharp peak at hexyltrimethylammonium (about 8.5 times the control velocity), followed by a sharp reversal of the trend as the chain is further elongated. Relative *potencies* at the receptor level (9) follow an almost parallel pattern, which suggests that the conformational response of AChE to alkyltrimethylammonium ions may be not unlike that of the receptors.[3]

In order to test the generality of this approach to the problem of the conformational response of AChE and ACh receptors to quaternary drugs, we have now examined the effects of leptocurares and pachycurares (10) on the rate of the methanesulfonylation reaction with AChE. The ligands tested included polymethoniums (tetra- to dodecamethylene), succinylcholine, *d*-tubocurarine, and gallamine. The initial velocities of ACh hydrolysis were all evaluated at 25° and pH 7.4 (CO_2-free nitrogen atmosphere) by the pH-stat technique previously described in detail (11). Salt solutions 0.1 M in NaCl and 0.02 M in $MgCl_2$ were used throughout. Control incubations from which only methanesulfonyl fluoride was omitted were run in parallel, so that the inhibitory effects of the quaternary salts on ACh hydrolysis in the assay medium were automatically compensated for. The effector concentrations were saturating with respect to enzyme, as estimated from their binding constants (12). As expected, no reaction between the effectors alone and methanesulfonyl fluoride was observable.

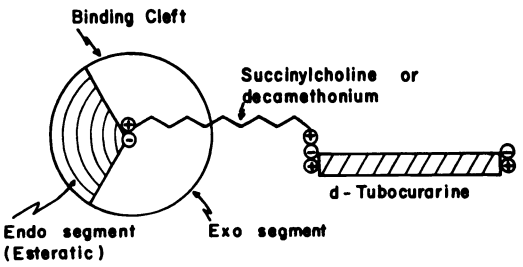

FIG. 3. *Schematic representation of the alignment of leptocurares and pachycurares on the AChE surface*

The main chain of alkyltrimethylammoniums and leptocurares projects away from the esteratic center where methanesulfonyl fluoride reacts. Pachycurares are shown to occupy outer sites, one of which would be identical with a leptocurare-binding site (see the text).

The ratio of the slopes of the initial velocities of methanesulfonylation gave *A*, the acceleration or inhibition values. The data summarized in Table 1 and Figs. 1 and 2 suggest the following conclusions.

1. Like their monoquaternary counterparts (6), the homologous leptocurares of the polymethonium series must form *exo* complexes with AChE (Fig. 3), since they all fail to protect against attack of the esteratic site by methanesulfonyl fluoride (Table 1). Equally striking is the fact that peak acceleration occurs at decamethonium (Fig. 1), which is also the best agonist of the series on the motor end plate receptors (10, 13). Hence, the monoalkyl quaternaries and polymethonium leptocurares cause parallel responses in both AChE (acceleration) and the motor end plate membrane [depolarization (14a)]. Moreover, succinylcholine is about 4 times more effective than decamethonium in accelerating the methanesulfonylation reaction (Table 1) and is also about 4 times more active as a leptocurare (14). An optimum fit of these two

[2] These findings may be regarded as constituting an experimental demonstration of the existence of accessory receptor areas for competitive blocking agents, as proposed by Ariëns (8).

[3] Pentyltrimethylammonium has generally been observed to surpass hexyltrimethylammonium in agonistic potency (9), but the differences are relatively small. On the other hand, heptyltrimethylammonium is a partial agonist (9), and it is precisely at this chain length that a sharp transition in the trend of accelerations is observed (6).

ligands on AChE would require the presence of an auxiliary anionic site (15) outside the ACh-binding cleft[4] (Fig. 3).

2. In marked contrast, the pachycurares *d*-tubocurarine and gallamine *protect* AChE against methanesulfonyl fluoride (Table 1), and the observed inhibition does not follow the classical laws of competitive kinetics, but rather resembles feedback inhibition of regulatory enzymes[5] (Fig. 2) (21, 22). On that basis, pachycurares may preferentially bind on *outer* anionic sites (Fig. 3) and induce a change which is unlike that produced by leptocurares.[5] At the end plate receptor level, leptocurares and pachycurares also induce dissimilar changes (14): whereas the former block by depolarization, the latter block without it. In Fig. 3, leptocurares and pachycurares are shown to share one auxiliary anionic site, in agreement with other data[4] and with the observation that pachycurares readily "reverse" inhibition of *ACh hydrolysis* by leptocurares (16).

The above results show that the kinetic or *conformational response* of AChE to these quaternary drugs follows trends that are strongly reminiscent of the receptor response to the same drugs. It seems possible that parallel interaction topographies and specificities may be involved at the enzyme and receptor levels, a problem which is currently under investigation.

[4] Labeling experiments with 2-^{14}C-*N*, *N*-dimethyl-2-phenylaziridinium chloride, an anionic site-directed inhibitor (10), have confirmed this conclusion (B. Belleau, V. DiTullio, and M. DiTullio, in preparation).

[5] It should be noted that, unlike ACh, methanesulfonyl fluoride carries no charge and thus can hardly compete directly with pachycurares for charged sites. Hence, the lack of a competitive relationship between methanesulfonyl fluoride and pachycurares shows that their respective sites of interaction are probably of the nonoverlapping type. Significantly, pachycurare inhibition of AChE hydrolysis of the charged *substrate* ACh is also of the partially competitive type (16), a result at variance with previous conclusions (15, 17, 18). Similarly, it has long been accepted on the basis of conventional assay techniques (19) that a purely competitive relationship for pachycurares and ACh exists at the myoneural junction. However, close examination of more recent data (20) reveals that pachycurares cause nonparallel shifts of the dose-response curves for ACh acting on leech muscle, thus also pointing to a partially competitive type of antagonism at the receptor level.

REFERENCES

1. R. Kitz and I. B. Wilson, *J. Biol. Chem.* **237**, 3245 (1962).
2. R. Kitz and I. B. Wilson, *J. Biol. Chem.* **238**, 745 (1963).
3. I. B. Wilson, *Ann. N. Y. Acad. Sci.* **144**, 664 (1967).
4. B. Belleau, *Advan. Drug Res.* **2**, 89 (1965).
5. B. Belleau, *Ann. N. Y. Acad. Sci.* **144**, 705 (1967).
6. B. Belleau, *in* "Physico-Chemical Aspects of Drug Actions" (E. J. Ariëns, ed.), p. 207. Pergamon Press, Oxford, 1968.
7. B. Belleau, H. Tani and F. Lie, *J. Amer. Chem. Soc.* **87**, 2283 (1965).
8. E. J. Ariëns and A. M. Simonis, *Ann. N. Y. Acad. Sci.* **144**, 842 (1967).
9. J. M. van Rossum and E. J. Ariëns, *Arch. Int. Pharmacodyn. Ther.* **118**, 418 (1959).
10. D. Bovet, *in* "Curare and Curare-Like Agents" (D. Bovet, F. Bovet-Nitti and G. B. Marini-Bettolo, eds.), p. 252. Elsevier, Amsterdam, 1969.
11. B. Belleau and H. Tani, *Mol. Pharmacol.* **2**, 411 (1966).
12. F. Bergmann and R. Segal, *Biochem. J.* **58**, 692 (1954).
13. R. B. Barlow and H. R. Ing, *Brit. J. Pharmacol. Chemother.* **3**, 298 (1948).
14. (a) S. Thesleff, *Acta Physiol. Scand.* **34**, 218 (1955); (b) J. del Castillo and B. Katz, *Proc. Roy. Soc. (London)* **146**, 339 (1956–57).
15. J. P. Long, *in* "Handbuch der experimentellen Pharmakologie" (G. B. Koelle, ed.), p. 398. Springer-Verlag, Berlin, 1963.
16. J.-P. Changeux, *Mol. Pharmacol.* **2**, 369 (1966).
17. F. Bergmann, I. W. Wilson and D. Nachmansohn, *Biochim. Biophys. Acta* **6**, 217 (1950).
18. J. A. Cohen and R. A. Oosterbaan, *in* "Handbuch der experimentellen Pharmakologie" (G. B. Koelle, ed.), p. 300. Springer-Verlag, Berlin, 1963.
19. D. H. Jenkinson, *J. Physiol. (London)* **152**, 309 (1960).
20. W. Flacke and T. S. Yeoh, *Brit. J. Pharmacol. Chemother.* **33**, 154 (1968).
21. J. C. Gerhart and A. B. Pardee, *Fed. Proc.* **23**, 727 (1964).
22. C. A. Woolfolk and E. R. Stadtman *Biochem. Biophys. Res. Commun.* **17**, 313 (1964).