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Structural Requirements for Agonist and Noncompetitive Blocking Action of Acylcholine Derivatives on *Electrophorus electricus* Electroplaque

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SUMMARY

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Several series of acyl analogs of acetylcholine (including fluorescent ones) were synthesized and their pharmacological properties investigated on *Electrophorus electricus* electroplaque. Their action as agonist, noncompetitive blocker, or mixed agonist–noncompetitive blocker depends both on the nature of the N-terminal substituent and on the length of the alkyl chain. The C_1, C_2, and C_3 members of the dansylamidoacylcholine (C_nDAChol) and naphthylsulfonamidoacylcholine (C_nNAChol) series behave as noncompetitive blocking agents, while the C_4, C_5, and C_7 analogs exhibit a significant agonist character in addition to their noncompetitive effect. This difference might result from the folding of the ammonium group on top of the aromatic ring which would prevent access of the C_1 to C_3 members of the series to the agonist binding site. The C_{10}DAChol and NAChol are powerful noncompetitive blockers and, at variance with other members of the series, form micelles in aqueous solution. Replacement of the N-terminal aromatic ring by a polar alkyl group (as in methylsulfonamidoacylcholine) abolishes the noncompetitive blocking properties and yields a series of potent full agonists. The apparent affinity of the agonists thus obtained increases with the length of the polymethylene spacer. The partial agonistic character of some of the compounds synthesized is interpreted as resulting from their dual pharmacological action as agonists and noncompetitive blockers binding to two distinct classes of membrane sites.

INTRODUCTION

Three major classes of pharmacological agents are known to regulate the permeability of the postsynaptic membrane of a nicotinic synapse such as the neuromuscular junction or the electroplaque synapse (1). The agonists, typified by the physiological neurotransmitter, acetylcholine, cause a fast increase of permeability to Na^+ and K^+ ions. This "activation" process is responsible for the transmission of the nerve impulse through the synapse. Upon prolonged exposure to the same compounds the amplitude of the permeability response slowly decreases. A "desensitization" of the membrane to the agonist takes place (2, 3). These two reactions can be accounted for by the selective binding of agonists to discrete and interconvertible conformations of the acetylcholine receptor protein (1–3). The competitive antagonists, like curare or flaxedil, block the response elicited by the agonists by decreasing the apparent affinity without significantly altering the amplitude of the maximal response. They are postulated to stabilize the resting conformation of the receptor protein when they bind to the same or a closely located area of the agonist binding site. The third class of agents active on the nicotinic synapse is rather heterogeneous. It groups compounds, referred to as noncompetitive blocking agents, which decrease the amplitude of the permeability response to the agonists without significantly modifying their apparent affinity. They are, for instance, the aminated local anesthetics, procaine, tetracaine, dimethisoquin, and their derivatives, which result from the combination of a dialkylaminoethyl chain, corresponding to the

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$(CH_3)_3N^+—CH_2—CH_2$ sequence of AcCh,[1] linked to an aromatic ring by an ester, amide, or ether bridge. Furthermore, these compounds accelerate the reaction of "desensitization" (4) and cause *in vitro* an increase of the AcChR affinity for the agonists (1). Thus these compounds behave as allosteric effectors (5) of the AcChR site. All these results favor the assumption that the interaction of the local anesthetics and, in a more general manner, the noncompetitive blocking agents takes place at a membrane site different from the acetylcholine receptor site (6–10, 22). Recent electrophysiological evidence suggests that these compounds could interact directly with the opened ionic channel by a steric inhibition of the translocation of ions through the pore, although an "allosteric" effect of these ligands is, of course, not excluded by this kind of experiment (11–14, 31). Thus the LA[1] site might be either the AcCh ionic channel itself or a nearby related structure.

In order to analyze the binding of acetylcholine to the receptor and correlate the occupancy of the receptor site to the opening of the associated ionophore, a fluorescent analog of AcCh, which retains all the agonistic properties of the neurotransmitter, was synthesized. In a preliminary communication (15) we reported the syntheses of some C_n dansylamidoacylcholine (C_nDAChol) and the electrophysiological studies on the isolated electroplaque from *Electrophorus electricus*. At low concentration the C_5-DAChol exhibits only agonist properties and behaves as an effective fluorescent probe of the AcChR, allowing the study of the allosteric interactions between AcChR and LA sites by fast kinetic experiments (16, 17). However, the agonistic action of these C_nDAChol increases as a function of the length of the hydrocarbon chain with, in addition, a noncompetitive blocking effect in the high concentration range.

In order to explore which structural elements of molecules are responsible for the agonist and noncompetitive blocking activities, we have studied the role of the aromatic residue bound to the acylcholine part by a comparison of the pharmacological activities on the isolated electroplaque from *Electrophorus electricus* of a series of C_nDAChol (n = 1–5, 7, 10) and some of their naphthyl analogs (C_nNAChol) with those of a series of methylsulfonamidoacylcholine (C_nMAChol) (n = 1–5, 7, 10) in which the aromatic ring has been replaced by a methyl group.

The importance of both the length of the alkyl chain and the polarity of the N-terminal group were studied with the help of hexanoylcholine (HChol), nonanoylcholine (NChol), dodecanoylcholine (DChol), 11-acetylamino-undecanoylcholine (AAUChol), and 11-(β-hydroxyethylamino)-undecanoylcholine (HEAUChol).

Finally, NMR[1] studies were performed in order to distinguish between eventual preferential conformations which might be related to the biological activities.

In later papers, direct binding studies carried out with these compounds and *Torpedo* AcChR-rich membrane fragments will be presented.

[1] Abbreviations used: AcCh, acetylcholine; AcChR, acetylcholine receptor; LA, local anesthetic; Dns-Chol, 2-(5-dimethylaminonaphthalene-1-sulfonamido) ethane 1-trimethylammonium iodide; C_nDAChol, *n*-(5-dimethylaminonaphthalene-1-sulfonamido)-alkanoic acid β-(*N*-trimethylammonium bromide) ethyl ester; C_nNAChol, *n*-(1-naphthalenesulfonamido)-alkanoic acid β-(*N*-trimethylammonium bromide) ethyl ester; C_nMAChol, *n*-(methylsulfonamido)-alkanoic acid β-(*N*-trimethylammonium) ethyl ester; HChol, hexanoylcholine; NChol, nonanoylcholine; DChol, dodecanoylcholine; AAUChol, 11-acetylamino-undecanoylcholine; HEAUChol, 11-(β-hydroxyethylamino)-undecanoylcholine; tetram, *O,O'*-diethyl-*S*-(β-diethylamino) ethylphosphorothiolate; tlc, thin-layer chromatography; Bu, butyl; Et, ethyl; DMSO, dimethylsulfoxide; NMR: nuclear magnetic resonance.

MATERIALS AND METHODS

Structure of studied acylcholine derivatives. All the compounds studied are acylcholine derivatives. They have the general formula $R—(CH_2)_n—COO—CH_2—CH_2—\overset{\oplus}{N}(CH_3)_3,Br^{\ominus}$ and are listed in Table 1.

Synthesis. Hexanoic, nonanoic, and dodecanoic acids as well as the amino acids used (general formula $H_2N—(CH_2)_n—COOH$) were purchased from Aldrich. Dansyl chloride, thionyl chloride, mesyl chloride, acetyl chloride, 2-bromoethanol, triethylamine, and trimethylamine were purchased from Merck.

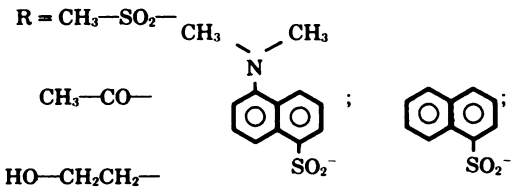
The synthesis of the compounds was carried out according to the following scheme:

$$H_2N—(CH_2)_n—COOH + HO—CH_2—CH_2—Br \xrightarrow{SOCl_2}$$

$$HCl, H_2N—(CH_2)_n—CO_2—CH_2—CH_2—Br \xrightarrow[2Et_3N]{R—Cl}$$

$$R—NH—(CH_2)_n—CO_2—CH_2—CH_2—Br \xrightarrow{(CH_3)_3N}$$

$$R—NH—(CH_2)_n—CO_2—CH_2—CH_2—\overset{\oplus}{N}(CH_3)_3,\ \overset{\ominus}{Br}$$



HChol, NChol, and DChol were obtained by the same procedure, except that the appropriate alkanoic acid was used instead of an aminoacid. Esterification by 2-bromoethanol was carried out on the unprotected amino acid. This modification of our previous method (15) renders the synthesis much easier and gives high yields of the desired ester.

The physical constants of the compounds are reported elsewhere (18). The general method is illustrated by the synthesis of C_7DAChol.

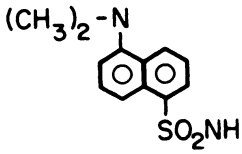
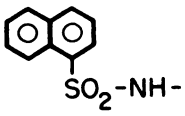
8-Aminooctanoic acid β-bromoethyl ester, hydrochloride. Thionyl chloride (11.3 mM, 0.5 ml) is added dropwise at 0°C to a mixture of 1 g of 8-aminooctanoic acid (6.28 mM) in 5 ml of 2-bromoethanol (69.22 mM). The reaction is stirred for 1 h at 0°C and, after return at room temperature, treated at 60°C for 1 h. The resulting clear solution is evaporated to dryness giving an oily residue which was precipitated by addition of dry diethyl ether. The solid is filtered, dried, and recrystallized from chloroform/diethyl ether 50/50 (yield 98%), F = 90–92°C.

The purity of all the compounds obtained in this step

TABLE 1

List of the studied compounds

$R-(CH_2)_n-CO_2-CH_2-CH_2-\overset{\oplus}{N}(CH_3)_3, Br^{\ominus}$

R	Numbers (n) of carbons	Abbreviation[a]
	1, 2, 3, 4, 5, 7, 10	C_nDAChol
	2, 5, 10	C_nNAChol
CH_3-SO_2-NH-	2, 3, 4, 5, 7, 10	C_nMAChol
CH_3-	4, 7, 10	HChol, $n = 4$ NChol, $n = 7$ DChol, $n = 10$
$CH_3-CO-NH-$	10	AAUChol
$HO-CH_2-CH_2-NH-$	10	HEAUChol

[a] AChol is the abbreviation of the group: $-COO-CH_2-CH_2-\overset{\oplus}{N}-(CH_3)_3, Br^{\ominus}$.

are checked by thin-layer chromatography (tlc) on silica gel glass plates using BuOH/AcOH/H_2O (4/1/1).

8-(5-Dimethylaminonaphthalene-1-sulfonamido)-octanoic acid β-bromoethyl ester. A solution of 1 g (3.3 mM) of the former compound, 0.891 g of dansyl chloride (3.3 mM), and 0.92 ml of triethylamine (6.6 mM) in a mixture of methylene chloride and dimethylsulfoxide (4/1) is kept stirring for 1 h. The organic solution is washed three times with water and dried over sodium sulfate. The filtrate is evaporated to dryness and gives a residual oil (yield 85%) corresponding to a pure product.

The purity is checked by tlc on silica gel glass plates using benzene/diethyl ether, 1/1. The structure was verified by NMR spectroscopy.

8-(5-Dimethylaminonaphthalene-1-sulfonamido)-octanoic acid β-(N-trimethylammonium bromide) ethyl ester. An excess (50-fold) of cold pure trimethylamine is added to a solution of 8-(5-dimethylaminonaphthalene-1-sulfonamido)-octanoic acid β-bromoethyl ester in a dry benzene/acetone mixture (1/1). The reaction is stirred for 24 h under nitrogen. The resulting precipitate is washed with diethyl ether and dried under a high vacuum (yield 45%). The amorphous powder is kept under nitrogen atmosphere. The purity of the compound is checked by tlc on silica gel using a BuOH/AcOH/H_2O (4/1/1) mixture. The structure was verified by NMR spectroscopy.

Electrophysiology. The pharmacological activity of each compound was studied *in vivo* on the isolated electroplaque from *Electrophorus electricus* as described (19) in the presence of 10^{-5} M tetram, a potent acetylcholinesterase inhibitor. Given concentrations of ligand were applied in the bath and membrane potentials recorded according to (20).

NMR studies. ^1H NMR spectra were recorded at 270 MHz on a Bruker WH 270 spectrometer operating in the Fourier transform mode and locked to the deuterium resonance of solvent D_2O. Probe temperature were regulated at ± 1°C by a Bruker BST 100/700 controller and chemical shifts, δ, were measured from an external reference made up of a capillary filled with a solution of tetramethylsilane in CCl_4. The chemical shifts, δ (in ppm), are reliable to ± 0.01 ppm. The intensity of HOD solvent resonance at 5×10^{-4} M concentration is reduced using standard homonuclear gated decoupling. Solutions were made in D_2O buffer, pH 7, using *Torpedo* physiological saline solution (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM sodium phosphate).

RESULTS

Pharmacological activity of C_nDAChol and C_nNAChol on Electrophorus electricus electroplaque.

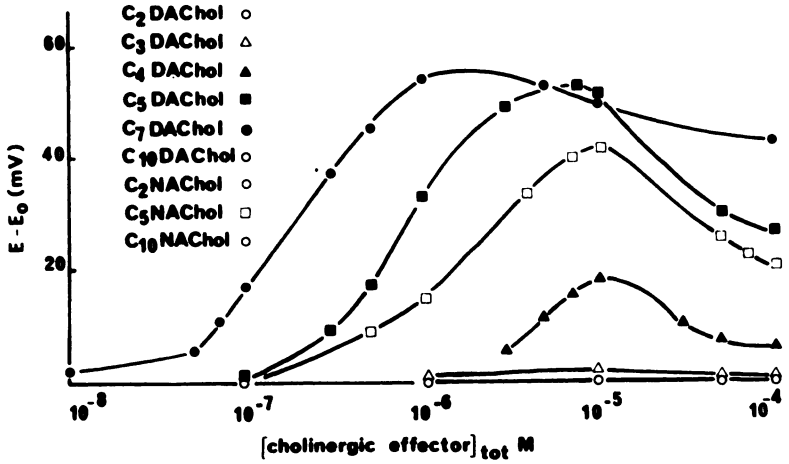


FIG. 1. *Pharmacological action of C_nDAChol and C_nNAChol on Electrophorus electricus electroplaque*

The ordinate shows steady-state membrane potential (E) in the presence of a given concentration of effector; E_0 is the resting potential (−75 ± 5 mV). The bath solution was *Electrophorus* Ringer's solution: 160 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 1.5 mM sodium phosphate, pH 7. The values of the apparent dissociation constant are listed in Table 2.

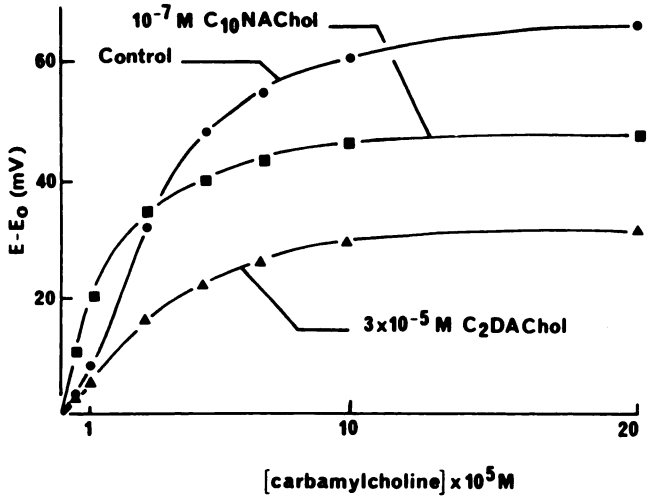


FIG. 2. *Pharmacological action of C_{10}DAChol and C_{10}NAChol*
Effect of C_2DAChol and C_{10}NAChol derivatives on response of *Electrophorus* isolated electroplaque to increased concentration of carbamylcholine.

As reported in a preliminary communication (15), bath application (from 10^{-7} to 10^{-4} M) of the C_2DAChol and C_3DAChol on the innervated face of *Electrophorus electricus* electroplaque did not cause any significant change of membrane potential (Fig. 1). However, they block reversibly the response to 2.5×10^{-5} M carbamylcholine in a noncompetitive manner as illustrated in Fig. 2 for the C_2DAChol. The pharmacological action of the naphthyl derivative C_2NAChol did not significantly differ from its dansyl analog C_2DAChol (Table 2).

As shown in Fig. 1, C_3, C_4, C_5, and C_7 DAChol behave as agonists. The amplitude of the maximal response increased with the length of the hydrocarbon chain (C_3 to C_5). Moreover, the dose-response curves were bell-shaped. These curves were characterized, in the low concentration range, by the concentration of agonist giving half of the maximal response (K_{app}) (Table 2). In the high concentration range, the amplitude of the response decreased and the curves were also characterized by the concentration giving a response equal to half of the maximal response (I_{app}). Furthermore, K_{app} decreased steadily with the length of the hydrocarbon chain whereas I_{app} did not vary significantly. In addition, at high concentration, these derivatives block reversibly the response to carbamylcholine in a noncompetitive manner.

A similar bell-shaped dose-response curve was found for the naphthyl analog C_5NAChol (Fig. 1).

Exposure of the electroplaque to C_{10}DAChol and C_{10}NAChol did not cause any major changes of the membrane potential (Fig. 1). However in the presence of 10^{-7} M C_{10}DAChol the response caused by 2×10^{-4} M carbamylcholine was reduced by 50%. C_{10}NAChol exhibited similar pharmacological properties but in a higher concentration range (Fig. 2). A potentiation of the response occurred in the presence of 10^{-7} M C_{10}NAChol and the response caused by 2×10^{-4} M carbamylcholine was reduced by 50% in the presence of 7×10^{-7} M C_{10}NAChol.

These I_{app}'s can be compared to those obtained in similar experiments with lidocaine and prilocaine (2×10^{-4} M) or dimethisoquin (2×10^{-6} M) (21, 22); C_{10}DAChol and C_{10}NAChol are, respectively, 20 and 3 times as potent as dimethisoquin.

Pharmacological properties of C_nMAChol. All the compounds of this series in which the aromatic ring is replaced by a methyl group behave as agonists within the concentration range explored (10^{-8} to 10^{-4} M). However, as illustrated in Fig. 3, the K_{app} was found to decrease when the length of the hydrocarbon chain between the methylsulfonamido group and the acylcholine moiety increased. The variation of the K_{app} values in the C_nMAChol series can be compared to that observed in the C_nDAChol series where similar changes were observed for C_4, C_5, and C_7 derivatives.

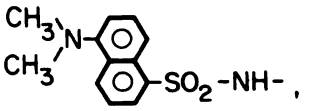
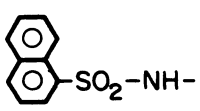
For each C_nMAChol studied, the maximal response amplitude recorded was, in general, 50 mV (except for C_2MAChol, but in this particular case a complete dose-

TABLE 2

Pharmacological properties of the studied acylcholine derivatives

ΔE_{max} is the maximal value of the recorded response *in vivo*. K_{app} is the concentration of effector giving 50% of the maximal response. IC_{50} is the concentration of noncompetitive blocking agent reducing by 50% the response caused by the 2×10^{-4} M carbamylcholine. The results are the means of several experiments performed on different *Electrophorus* electroplaques.

$R-(CH_2)_n-CO_2-CH_2-CH_2-CH_2-\overset{\oplus}{N}(CH_3)_3$

							CH_3-SO_2-NH-		CH_3-			*$CH_3-CO-NH-$ †$HO-CH_2-CH_2-NH$	
	ΔE_{max} (× mV ±5)	K_{app} (× M ±0.5)	I_{app} (× M ±0.5)	ΔE_{max} (× mV ±5)	K_{app} (× M ±0.5)	I_{app} (× M ±0.5)	ΔE_{max} (× mV ±5)	K_{app} (× M ±0.5)	ΔE_{max} (× mV ±5)	K_{app} (× M ±0.5)	I_{app} (× M ±0.5)	ΔE_{max} (× mV ±5)	K_{app} (× M ±0.5)
C_2	0	—	3×10^{-5}	0	—	5×10^{-5}	(35)	3.4×10^{-4}					
C_3	[a]	5×10^{-6}	—				52.5	3×10^{-5}					
C_4	18	4.3×10^{-6}	—				57.5	1.4×10^{-5}	62	2×10^{-6}	—		
C_5	53	8×10^{-7}	—	42	1.7×10^{-6}	—	61	4×10^{-6}					
C_7	55	2×10^{-7}	—				59	5.4×10^{-7}	37	1.1×10^{-6}	—		
C_{10}	0	—	10^{-7}	0	—	7×10^{-7}	62.5	2.2×10^{-7}	0	—	7×10^{-7}	*57 †57	*3.5×10^{-7} †9×10^{-8}

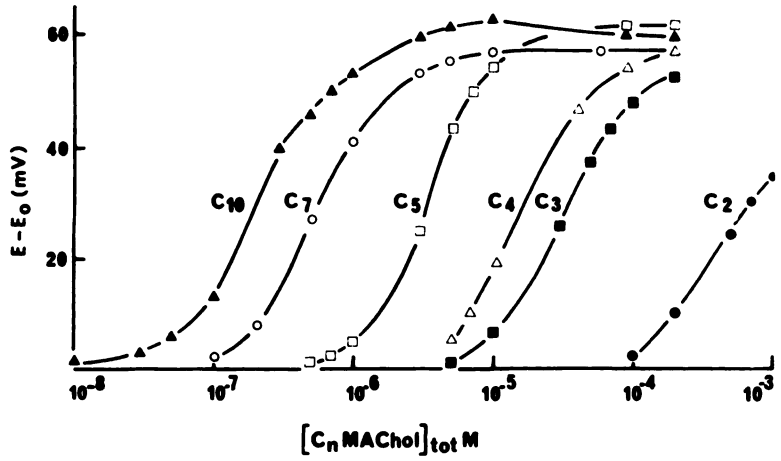


FIG. 3. *Pharmacological response of C_nMAChol*

Effect of C_nMAChol compounds on isolated electroplaque from *Electrophorus electricus*. The response is plotted as a function of C_nMAChol concentrations.

response curve could not be established). In contrast to the bell-shaped, dose–response curves of DAChol and NAChol compounds, the amplitude of the response elicited by the MAChol drugs never decreased in the high concentration range (above 2×10^{-5} M).

In Fig. 4, the cologarithm of K_{app} was plotted as a function of methylene number of the aliphatic chain in the C_nMAChol series. The value of the slope was 0.70 for C_2, C_3, C_4, and C_5 members of the series but decreased for the two last members.

Pharmacological activity of HChol, NChol, DChol, AAUChol, and HEAUChol. Three long-chain acylcholines have been studied: hexanoylcholine (HChol), nonanoylcholine (NChol), and dodecanoylcholine (DChol). Each one behaves in a different manner. Throughout a wide concentration range, HChol behaved as an agonist. The amplitude of the maximal depolarization reached 60 mV; K_{app} was 2×10^{-6} M (see Fig. 5 and Table 2).

On the other hand, NChol had a dual pharmacological action. As with the C_nDAChol, NChol behaved as an agonist at low concentrations; however, the amplitude of the depolarization caused by NChol reached a maximum at about 10^{-5} M and at higher concentrations decreased and became negligible. K_{app} was 1.1×10^{-6} M (see Fig. 5).

DChol did not cause any significant change of the membrane potential but the response to 2×10^{-4} M carbamylcholine was reduced by 50% in the presence of 7×10^{-7} M DChol without effect on the K_{app} for carbamylcholine. This compound behaved like a typical noncompetitive blocker.

Finally, two closely related compounds have been tested on the isolated electroplaque from *Electrophorus electricus*. The first, 11-acetylamino-undecanoylcholine (AAUChol), behaves as a full agonist as does the second, 11-(β-hydroxyethylamino)-undecanoylcholine (HEAUChol) (see Fig. 5). The K_{app}'s for AAUChol and HEAUChol were 3.5×10^{-7} and 9×10^{-8} M, respectively.

Conformational studies of C_nDAChol by ^1H NMR spectroscopy. Because of the flexibility of their side chains, the C_nDAChol compounds should exist as a mixture of interconverting conformational isomers. However, a folding of the polar head of these compounds on the top of the aromatic ring could take place in aqueous solution because of their high hydrophobicity. Such a preferential arrangement between acetylcholine and various aromatic rings in water has been recently reported (23). To test this possibility, it was necessary to check first an eventual occurrence of intermolecular associations. ^1H NMR spectra were recorded at various concentrations of C_nDAChol (n = 1–5) (10^{-2}, 10^{-3}, 5×10^{-4} M) dissolved in the buffer used for pharmacological investigations but with D_2O instead of H_2O. As no appreciable effect of concentration could be detected on the resonance position, it can be safely assumed that no intermolecular associations take place between C_nDAChol molecules (n = 1–5) in aqueous solution. This conclusion is further supported by the observation that the linewidths are similar when the spectra are recorded either from aqueous, or DMSO-d_6, a solvent known to disrupt intermolecular stacking between aromatic molecules (24).

In the case of C_7DAChol, however, a slight broadening

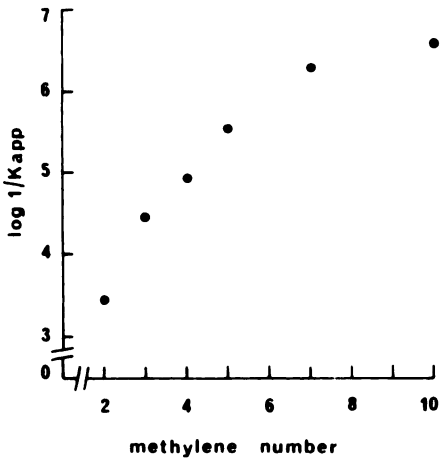


FIG. 4. *Relation between the dissociation constant of C_nMAChol with the length of the hydrocarbon chain*

Log $1/K_{app}$ is plotted as a function of methylene number separating the mesyl rest from the acylcholine moiety. The dissociation constant (K_{app}) is the concentration of cholinergic effector giving half of the maximal response.

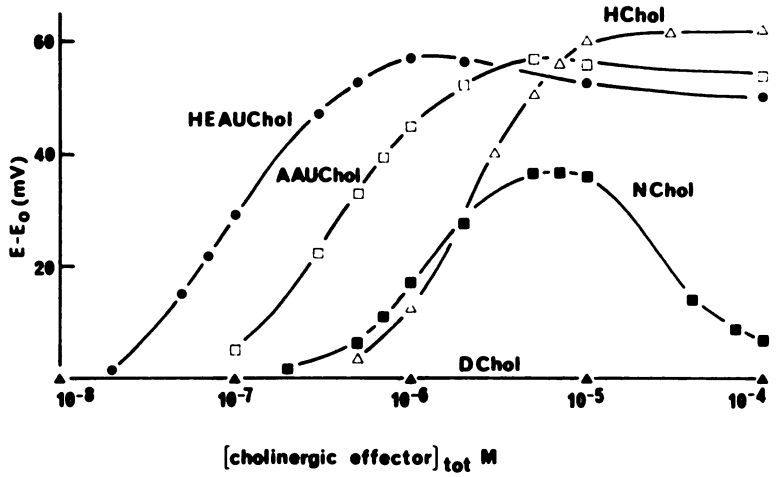


FIG. 5. *Pharmacological activity of several cholinergic effectors*

Dose-response curve of *Electrophorus electricus* electroplaque to several effectors. E_0 is the resting potential and E the steady-state potential at any given concentration of effector. HChol, hexanoylcholine; NChol, nonanoylcholine; HEAUChol, 11-(β-hydroxyethylamino)-undecanoylcholine; AAUChol, 11-acetylamino-undecanoylcholine.

of the signals was observed at high concentration (10^{-2} M) and could be related to a decrease in the degrees of freedom of the molecule caused by intermolecular associations. At variance with the results described for C_nDAChol (n = 1-5), the linewidths of all the proton signals of both C_{10}DAChol and C_{10}NAChol markedly increased when the compound was dissolved in water instead of DMSO, even at concentrations as low as 5×10^{-4} M (Fig. 6). This indicates a motional hindrance similar to that found in unsonicated aqueous solutions of lipids such as phosphatidylcholines (25).

A difference in the linewidth of ^{1}H signals of the aromatic part as well as the methylene bound to the sulfonamido group and that of the acylcholine moiety was also noticed. The degrees of freedom of the aromatic ring were therefore markedly reduced, while the polar head group of the molecule appeared comparatively less affected.

The temperature dependence of the linewidth (Fig. 7) revealed a phase transition around 40°C which corresponds to the formation of micelles (25) of C_{10}DAChol, which may exist below this temperature. A similar behavior could not be detected with C_{10}MAChol or DChol, although both compounds have the same chain length as C_{10}DAChol.

The variation of the ^{1}H chemical shifts (δ) of the aromatic and polar moieties of C_nDAChol is also worth noting (Table 3). For C_{10}DAChol and C_{10}NAChol, the ^{1}H chemical shifts of the choline part are similar to those of C_{10}MAChol (Table 2), indicating that no inter or intra molecular interactions exist between the dansyl group and the polar head. But when compared with those of the other members of the series, the aromatic protons of C_{10}DAChol appeared strongly shielded. This observation together with the differences between linewidths of the ^{1}H signals of the polar head and of the aromatic ring suggest the formation of micelles resulting from the stacking of the naphthalene rings.

In the C_nDAChol (1-5, 7) series, the protons of the choline moiety were significantly shielded for $n < 4$ when compared to their positions in the analog C_nMAChol (not shown here). These results suggest that in the population of C_nDAChol molecules, with n = 1-3, a significant fraction exists as conformers in which the polar head is folded upon the plane of the aromatic ring leading to almost globular conformations. On the contrary, the similarity between the chemical shifts of the protons of the choline moiety in C_nDAChol ($n > 4$) and the homologous C_nMAChol suggests an important degree of flexibility of the hydrocarbon chains in these derivatives.

DISCUSSION

In the series of C_nDAChol or C_nNAChol, neither the C_1, C_2, or C_3DAChol nor the C_2NAChol presented a significant agonist action. Analysis of their conformation by NMR spectroscopy indicates a strong tendency of the cationic polar head to fold upon the aromatic ring and it is possible that such highly hydrophobic globular molecules no longer exhibit a significant affinity for the "active" conformation of the acetylcholine receptor site. On the other hand, the increase of the distance between the naphthalene ring and the choline moiety and the consequent increase in the degree of freedom of the chain lead to three compounds, C_4, C_5, and C_7DAChol, which behave at low concentrations (from 10^{-7} to 10^{-5} M) as agonists with a regular decrease of their K_{app} as a function of the length of the polymethylene spacer. At higher concentration (10^{-4} M), however, these DAChol derivatives present, again, a noncompetitive blocking action. This effect is not found with corresponding C_nMAChol compounds, which behave exclusively as agonists within a wide range of concentration (from 10^{-8} to 10^{-4} M). As for the C_4 to C_7DAChol, the K_{app} of the C_nMAChol varies as a function of the chain length but, as shown in Fig. 4, in a nonlinear manner. The regular decrease of the apparent dissociation constant for the C_2 to C_5MAChol might possibly result from the interaction of their methylene subunit with some hydrophobic pocket present in the acetylcholine receptor site (26). Accordingly, the net decrease in the water-hydrocarbon contact during the binding pro-

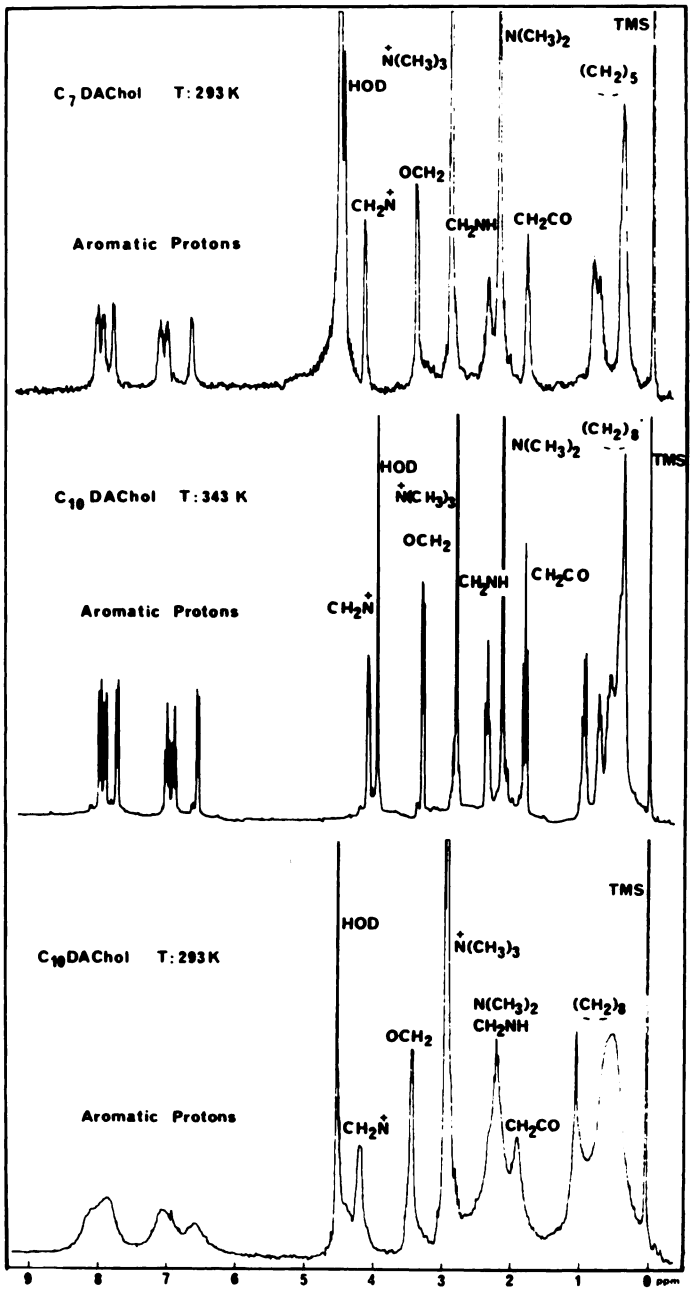


FIG. 6. *NMR spectra of C_7DAChol and C_{10}DAChol at 10^{-2} M in D_2O buffer, pH 7, at different temperatures*

cess would cause a favorable entropy change. When n becomes larger than 5, the apparent dissociation constant of the components of the series decrease more slowly when n increases. This might result from a larger loss in the degree of freedom for the longest alkyl chain when they bind to the receptor.

The substitution of a CH_3SO_2NH- group for an acetyl or hydroxyethylamino group, as in AAUChol and HEAUChol, demonstrates the role of the polar tail in maintaining a strict agonist activity. The two compounds behave exclusively as agonists whereas for a similar C_{10} alkyl chain but with a hydrophobic tail (i.e., made up of a methyl or an aromatic group) only noncompetititive blocking properties were detected (as exemplified with DChol or C_{10}DAChol and C_{10}NAChol).

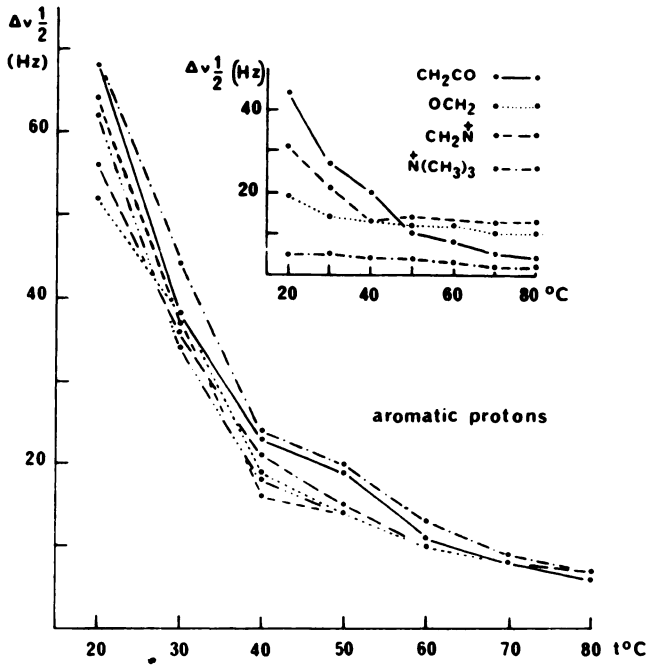


FIG. 7. *Variation in the linewidth of C_{10}DAChol protons as a function of the temperature*

This observation illustrates that the structural features required for a compound to bind to the LA site where it acts as a noncompetitive blocker are strikingly different from those required for its interaction with the active conformation of the acetylcholine receptor site. In a general manner, the increase of the hydrophobic character of the molecule enhances its apparent affinity as noncompetitive blocker. Although the presence of an aromatic ring is not absolutely required for a noncompetitive blocking action, all the compounds which contain an aromatic moiety, whatever their size, behave as blockers.

The C_{10}DAChol and the C_{10}NAChol behave almost exclusively as noncompetitive blocking agents, a rather unexpected property since their C_7 analogs are potent agonists. The noncompetitive blocking properties of these derivatives may be due to another mechanism, an interpretation reinforced by the small I_{app}'s as compared to the classical local anesthetics. Indeed, as shown by NMR, these two compounds, unlike the precedent members of the series, exhibit a strong-tendency to aggregate in aqueous solution.

Another striking feature of the dose–response curves

TABLE 3

Chemical shifts (ppm) of the protons in the choline moiety of several cholinergic effectors at 5×10^{-4} M in D_2O buffer, pH 7, at 21°C

	OCH_2	$\overset{\oplus}{CH_2}N$	$\overset{\oplus}{N}(CH_3)_3$
C_1DAChol	3.05	3.90	2.75
C_2DAChol	3.22	3.90	2.80
C_3DAChol	3.30	4.10	2.85
C_5DAChol	3.41	4.20	2.89
C_7DAChol	3.44	4.19	2.94
C_{10}DAChol	3.43	4.20	2.92
C_{10}MAChol	3.44	4.25	2.91
DChol	3.48	4.28	2.96

of the C_nDAChol or C_nNAChol concerns the concentration dependence of the agonist vs noncompetitive action of these compounds. In the low concentration range their apparent affinity as agonist varies with the length of the polymethylene spacer, whereas the decreased activity observed in the high concentration range follows nearly the same pattern for all the compounds tested and appears therefore insensitive to the length of the spacer. This shows how the same compounds may exhibit dual action on a given system as long as the interaction with the two classes of sites involved takes place in different domains of concentration. As a result the compound becomes a "partial" agonist. It is possible, but not yet shown, that the "partial" agonist character observed with several neuroleptics (27), morphinic substances (28), and a wide variety of pharmacological agents results from such dual interaction of the same compound for two distinct but strongly coupled classes of sites (29, 30).

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