SYNTHESIS OF FLUORESCENT *ACYL*-CHOLINES WITH AGONISTIC PROPERTIES: PHARMACOLOGICAL ACTIVITY ON *ELECTROPHORUS* ELECTROPLAQUE AND INTERACTION IN VITRO WITH *TORPEDO* RECEPTOR-RICH MEMBRANE FRAGMENTS

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1. Introduction

The physiological post-synaptic receptor of acetylcholine is nowadays a well identified protein which can be selectively labelled, purified and studied in its membrane environment [1–3]. However, progress in the understanding of the transient change of permeability triggered by acetylcholine brings upon the difficulty to correlate the actual binding of acetylcholine to its receptor site and the subsequent opening of the associated ionophores in the time scale of synaptic transmission, i.e. in the millisecond range.

Several fluorescent cholinergic ligands have already been used to study the binding properties of the nicotinic receptor protein from fish electric organ in its membrane-bound [4–6], and purified [7] forms and to monitor its conformational transitions [4,5,8]. These compounds behave in vivo on *Electrophorus* electroplaque or at the neuromuscular junction either as competitive antagonists [6,7] local anesthetics [8] or mixed effectors with both an agonistic character and a noncompetitive blocking activity [4,5]. It was therefore of importance to develop fluorescent ligands which, without ambiguity, would exhibit an agonistic character in vivo.

A series of compounds has been synthetized which, like DNS-chol [9,4,5] possess a fluorescent dansyl residue and a quaternary nitrogen. In addition, hydrocarbon chains with varying lengths (1 to 5 carbon atoms) have been inserted between the dansyl group and the acylcholine moiety. Those [1-(5-dimethylamino naphthalene)sulfonamido] *n*-alkanoic acid β-(*N*-trimethylammonium bromide)ethyl esters (C_nDAChol) (see fig.3 for formula) show all the absorption and fluorescence properties of the dansyl chromophore. On the isolated electroplaque from *Electrophorus*, the first two members of the series (C_1 and C_2 DAChol) behave primarily as non competitive blocking agents. However the C_3, C_4 and C_5 derivatives applied in bath cause a significant depolarization of the electroplaque and both their potency and apparent affinity increase with the length of their hydrocarbon chain. Preliminary ^{1}H n.m.r. experiments do not reveal any preferential conformation of the probe in solution which might explain the differences in pharmacological activity noticed among the various compounds synthesized. In vitro experiments carried out with the receptor-rich membrane fragments from *T. marmorata* and C_5 DAChol show that, within a wide range of concentrations, this fluorescent agonist

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may be conveniently used to selectively label the cholinergic receptor site.

2. Materials and methods

2.1. *Synthesis of dansyl-acylcholines: $C_nDAChol$*

Glycine, β-alanine, γ,δ,ω-aminoacids, dansyl-chloride, bromoethanol, trimethylamine, dicyclohexylcarbodiimide (DCC) were from Fluka. All the *N*-carbobenzoxy-amino acids (*Z*-amino acids) were prepared by standard methods [10].

2.1.1. *N*-carbobenzoxyaminoacids β-bromoethyl ester

A solution of DCC (0.01 mol) in 30 ml of dry tetrahydrofuran was added dropwise at 0°C under nitrogen to a mixture of the appropriate Z-amino acid (0.01 mol), β-bromoethanol (0.01 mol) and 2 ml of pyridine in 30 ml of tetrahydrofuran. The reaction mixture was stirred for 24 h at 20°C. The formed dicyclohexylurea was filtered and the excess of DCC was precipitated by addition of 1 ml of acetic acid. The solution, evaporated to dryness in vacuo was dissolved in 50 ml of benzene and washed successively with HCl 1 N, H_2O, $NaHCO_3$, H_2O and dried over Na_2SO_4. The filtrate was evaporated to dryness and gave a residual oil (yield 70–80%). The purity of the compounds obtained in this step was checked by thin layer chromatography on silicagel using benzene–ether 50/50 mixture and n.m.r. spectroscopy.

2.1.2. Amino acid β-bromoethyl ester hydrobromides

The Z-amino acid β-bromoethyl esters ($8 \cdot 10^{-3}$ mol) were dissolved in glacial acetic acid (10 ml) and a stream of dry hydrogen bromide was bubbled through the solution for 15 min. The excess of HBr was eliminated by a stream of dry nitrogen. The solution was evaporated to dryness and the syrup used without purification for the next step.

2.1.3. (5-dimethylaminonaphthalene-1 sulfonyl) amino acid β-bromoethyl ester

Under nitrogen, a mixture of dansyl chloride ($6.2\ 10^{-3}$ mol) and triethylamine ($6.2 \cdot 10^{-3}$ mol) in 30 ml of dry dichloromethane was added slowly at 0°C to a stirred solution of any of the above hydrobromides ($6.2 \cdot 10^{-3}$ mol) and triethylamine ($6.2 \cdot 10^{-3}$ mol) in 30 ml of anhydrous methanol. After 24 h, the solution was evaporated to dryness in vacuo and the yellow oily residue purified by chromatography on a silicagel column with benzene–ether 50/50 as the elution agent (yield: 50–60%).

2.1.4. [1-(5-dimethylaminonaphthalene) sulfonamido] *n*-alkanoic acid β-(*N*-trimethylammonium bromide)ethyl esters ($C_nDAChol$).

An excess of a cold solution of trimethylamine in benzene (30%) was added to a solution of an above bromo ester ($1.27 \cdot 10^{-3}$ mol) in 5 ml of dry acetone and the mixture was heated in a sealed tube at 70°C for 24 h. The crystalline hydrobromide which separated was filtered and the solution evaporated in vacuo to give a precipitate. The solid was recrystallized from methanol–acetone 50/50 (yield 40–50%).

All the intermediates and the expected $C_nDAChol$ derivatives give centesimal analysis in accordance with theory and their structure was verified by n.m.r.

The different steps of the synthesis of $C_nDAChol$ may be summarized as follows:

$$Z\text{-}NH(CH_2)_n\text{-}COOH \xrightarrow[DCC \quad THF]{HOCH_2CH_2Br}$$

$$Z\text{-}NH(CH_2)_nCOOCH_2CH_2Br \xrightarrow[CH_3COOH]{HBr}$$

$$Br^-H_3{}^+N(CH_2)_nCOOCH_2CH_2Br$$

$$\xrightarrow[2\ Et_3N]{\text{Dansyl chloride}}$$

$$DNS\text{-}NH(CH_2)_nCOOCH_2CH_2Br \xrightarrow[\text{Acetone}]{N(CH_3)_3}$$

$$DNS(CH_2)_nCOOCH_2CH_2N^+(CH_3)_3Br^-$$

n = 1 to 5; Z = $C_6H_5CH_2$-O-CO-

2.2. *Electrophysiology*

The pharmacological activity of the $C_nDAChol$ was studied in vivo on the isolated electroplaque from *Electrophorus electricus* [11]. Given concentrations of ligand were applied in bath and membrane potentials recorded according to Higman et al. [12].

2.3. *Preparation of receptor-rich membrane fragments from* Torpedo marmorata *electric organ.*

The membrane fragments were purified by centrifugation in sucrose gradients following the method of Cohen et al. [13] with minor modifications. The purified fragments were concentrated by centrifugation and resuspended in a solution of 10^{-4} M paramethyl sulfonyl fluoride (a protease inhibitor [14]) and 0.02% NaN_3 in distilled water.

The concentration of cholinergic receptor sites was estimated by the method of Weber and Changeux [15] following the binding of [^{3}H]α-toxin from *Naja nigricollis.*

Proteins were assayed by the method of Lowry et al. [16]. The specific activity of the membrane preparations used ranged from 1100 to 1500 nmol of [^{3}H]α-toxin binding site per g of protein.

2.4. *[^{3}H]acetylcholine displacement experiment*

Membrane fragments ($6{\cdot}10^{-8}$ M [^{3}H]α-toxin binding sites, 54 μg protein per ml) in *Torpedo* physiological saline solution (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$ and 5 mM sodium phosphate, pH 7.0) were first incubated for 60 min with $5{\cdot}10^{-5}$ M Tetram, an acetylcholinesterase inhibitor [17]. Then tritiated acetylcholine bromide (250 Ci/mol, Amersham) was introduced to a final concentration of $6{\cdot}10^{-8}$ M. This solution was divided into 2 ml fractions, to which given concentrations of C_5 DAChol ranging from 0 to $5\ 10^{-5}$ M, were added. Membranes were pelleted by a 120 min 20 000 rev/min centrifugation in a JA21 rotor of a J21 Beckman centrifuge (~ 45 000 *g*). 400 μl aliquots of the supernatants were counted with 3.5 ml Bray for 4 min. in the ^{3}H-channel of an Intertechnique scintillation counter. Corresponding aliquots of the samples were taken before centrifugation and counted as well. The radioactivity of the supernatants after centrifugation gave the concentrations of free acetylcholine. The difference between the total radioactivity of the suspension and that of the supernatants, after centrifugation, was taken as estimates of acetylcholine bound to the membrane fragments. No quenching effects due to varying concentrations of C_5 DAChol were observed.

2.5. *Spectroscopic measurements*

The C_nDAChol were dissolved in *Torpedo* physiological solution and the absorption and fluorescence spectra recorded respectively with an Unicam SP800 spectrophotometer and a Jobin-Yvon spectrofluorimeter. The PMR spectra were recorded in DMSO d_6 and D_2O buffer on a Bruker WH 90 spectrometer in the Fourier transform mode, at a probe temperature of 30 ± 1°C, with tetramethylsilane (TMS) as external reference.

The interaction of the C_nDAChol with *Torpedo* membrane fragments was followed in *Torpedo* physiological saline solution with a FICA differential recording spectrofluorimeter. The quartz fluorescence cells containing the suspension supplemented with $5{\cdot}10^{-5}$ M Tetram were thermostated at 20°C during the experiment.

3. Results

3.1. *Spectral properties of C_nDAChol in aqueous solution*

The absorption and fluorescence emission spectra of all the C_nDAChol compounds studied appear identical (fig.1). The absorption spectra show the same absorption maxima at 216, 246 and 328 nm. Upon illumination at 328 nm, maximal fluorescence emission is observed at 560 nm. In agreement with previous findings [4–6], the spectral characteristics of the fluorescence spectrum change with solvent polarity: for instance transfer from water to ethanol causes an approx. 100 fold increase in maximal fluorescence intensity accompanied by an approx. 50 nm shift to the blue; also a shoulder appears around 450 nm.

3.2. *^{1}H n.m.r. spectroscopy of C_nDAChol*

The n.m.r. spectrum of C_5 DAChol is shown in fig.2. No significant difference was noticed between the C_nDAChol compounds studied in DMSO d_6 and D_2O buffer pH 7.0 at the same concentration. Only a slight variation of the C_nDAChol spectrum with concentration was observed indicating little, if any, tendency to intermolecular interactions in aqueous solution [18]. Moreover, at low concentrations of the compound (5×10^{-4} M), the chemical shift of the trimethylammonium group does not change when the length of the chain increases. This indicates that, despite differences in length, the several C_nDAChol

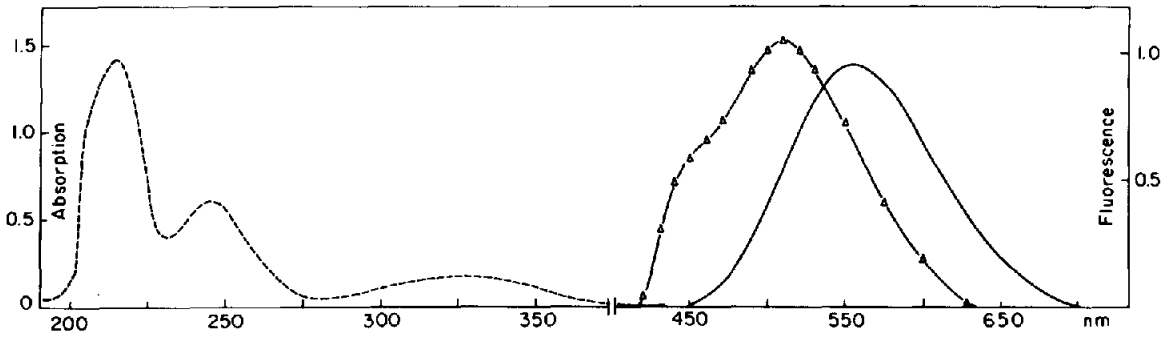

Fig.1. Spectral properties of C_5DAChol. (– – –) Absorption spectrum of $4 \cdot 10^{-5}$ M C_5DAChol in *Torpedo* physiological solution. $\epsilon_{216} = 3.55 \cdot 10^4$, $\epsilon_{246} = 1.55 \cdot 10^4$, $\epsilon_{328} = 0.55 \cdot 10^4$ M^{-1} cm^{-1}. (————) Fluorescence spectrum of 10^{-5} M C_5DAChol in *Torpedo* physiological solution (λ_{ex} = 328 nm). (△ – △) Fluorescence spectrum of 10^{-7} M C_5DAChol in ethanol (λ_{ex} = 328 nm).

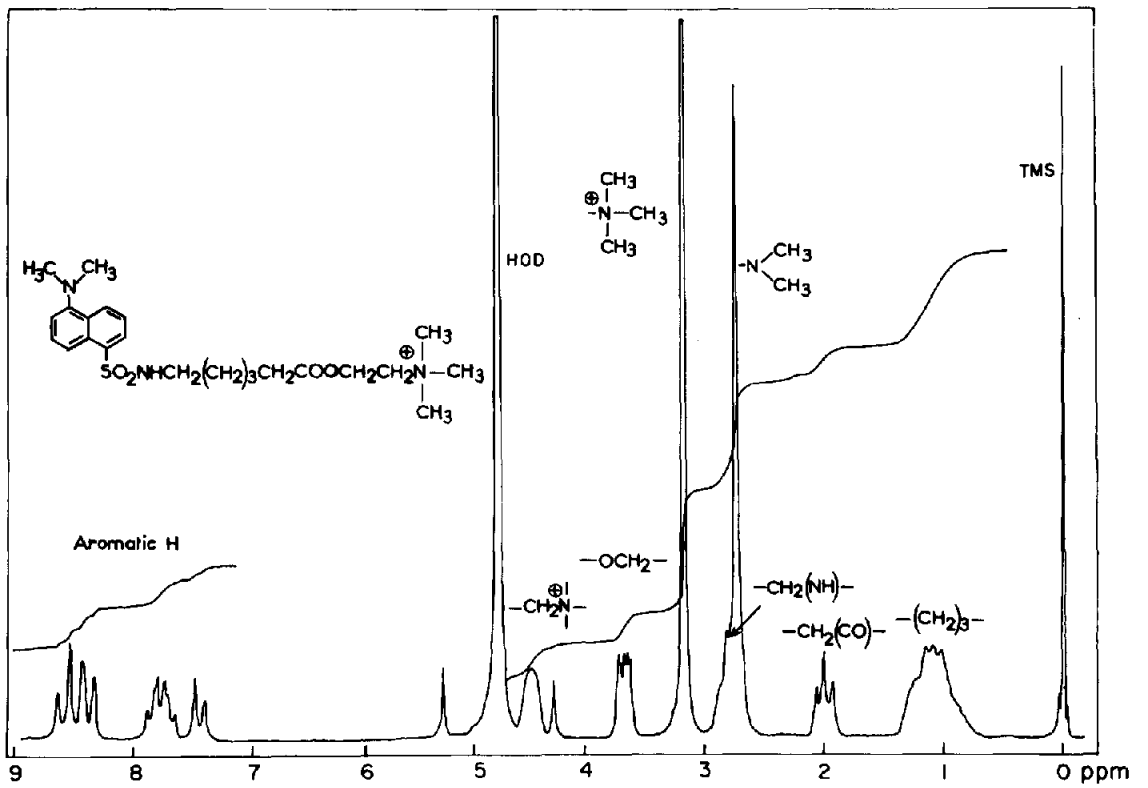

Fig.2. ^{1}H n.m.r. spectrum of 10^{-2} M C_5DAChol in a *Torpedo* physiological solution made in D_2O.

compounds exhibit similar conformations. In particular, they do not show any tendency to intramolecular folding upon the aromatic moiety when the number of carbon atoms in the intermediary segment increases.

3.3. *Pharmacological activity in vivo of C_nDAChol on* Electrophorus electricus *electroplaque*

Bath application of C_1 or C_2 DAChol, at concentrations ranging from 10^{-7} to 10^{-4} M does not cause any significant change of membrane potential. However, both of them block reversibly the response to 3×10^{-5} M carbamylcholine in a non-competitive manner. On the other hand, C_3, C_4 or C_5 DAChol behave, at low concentrations, as agonists (fig.3). The amplitude of the maximal depolarization recorded increases with n and reaches 15 mV (the third of the maximal response to carbamylcholine) for n = 5 and the apparent dissociation constant (the concentration of effector giving half of the maximal response) decreases when n increases. For instance, K_{app} = 2.5×10^{-6} M and 7×10^{-7} M for C_4 and C_5 DAChol respectively and with all of them, the dose–response curve has a bell shape: at high concentrations of the compounds, the amplitude of the response decreases and becomes negligible. In this range of concentration, however, the C_nDAChol block reversibly the response to carbamylcholine and behave as non-competitive antagonists.

Since C_5-DAChol turned out to be the most potent agonist, it was most extensively studied in the following biochemical experiments.

3.4. *Effect of C_5DAChol on the binding in vitro of [^{3}H]acetylcholine to* Torpedo *receptor-rich membrane fragments.*

The binding of [^{3}H]acetylcholine was followed by centrifugation (see Materials and methods) under conditions where acetylcholine binds almost exclusively to the cholinergic receptor site (complete displacement by *N. nigricollis* α-toxin). Fig.4 shows that, when approx. 65% of the receptor sites were occupied by acetylcholine, C_5DAChol displaces quantitatively acetylcholine bound to the membrane fragments. At

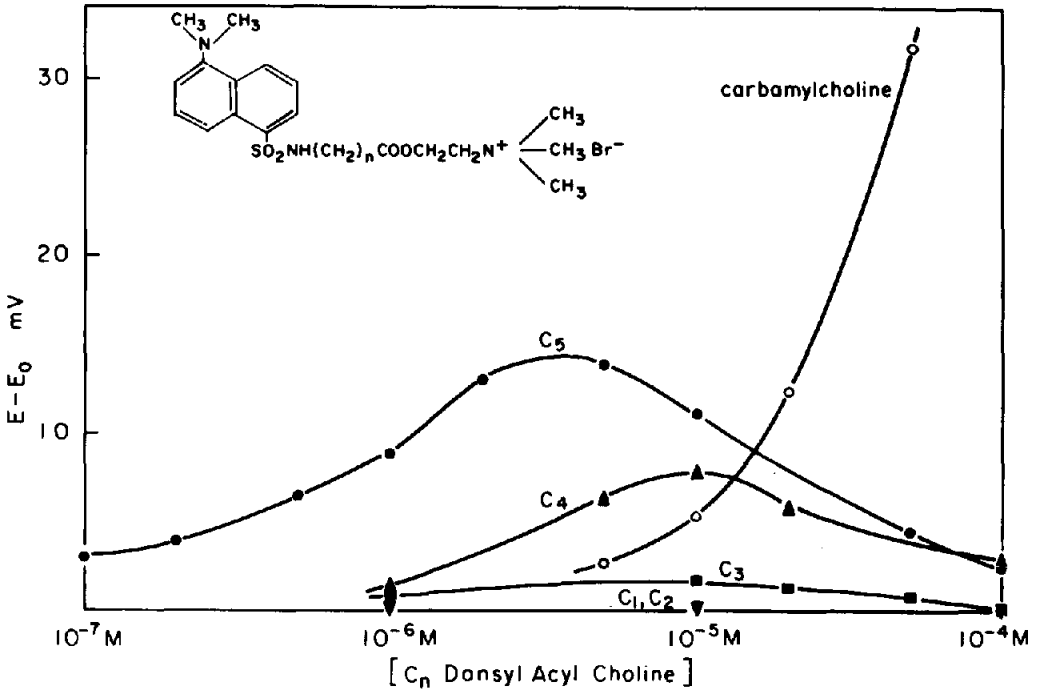

Fig.3. Pharmacological response of the isolated electroplaque from *Electrophorus electricus* to C_nDAChol. E = membrane potential; E_0 = resting potential (– 80 mV); the amplitude of the maximal response to carbamylcholine ($E - E_0$) was approx. 50 mV.

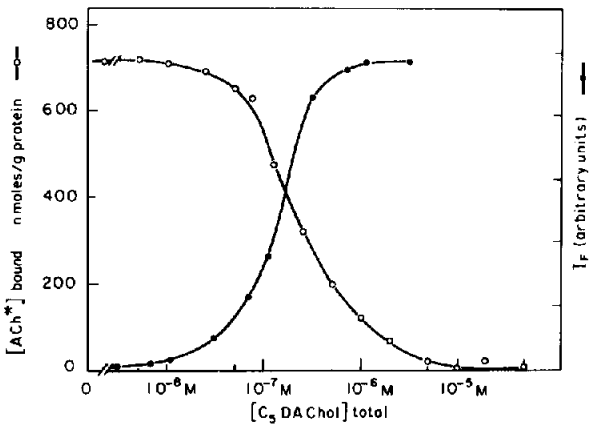

Fig.4. Binding of C_5DAChol to *Torpedo* receptor-rich membrane fragments as followed either by displacement of [^{3}H]acetylcholine or by fluorescence spectroscopy. (○ – ○) Displacement of [^{3}H]acetylcholine (see Materials and methods). (· – ·) Fluorescence experiment. The sample and reference cells contained 2.5 ml of *Torpedo* physiological solution supplemented with membrane fragments (2×10^{-7} M in *N. nigricollis* α-toxin sites, 130 μg protein per ml) and 10^{-4} M Tetram. A saturating concentration of acetylcholine bromide (10^{-4} M) was added to the reference cell; the indicated concentration of C_5DAChol was then introduced in both cells. λ_{ex} = 290 nm (energy transfer), λ_{em} = 557 nm (maximum emission wavelength).

saturating levels of C_5DAChol negligible amounts of [^{3}H]acetylcholine remain associated to the membrane fragment. Assuming a mutually exclusive binding of C_5DAChol and acetylcholine and equal numbers of acetylcholine and [^{3}H]α-toxin binding sites, one estimates from the displacement curve, a dissociation constant of 4.5×10^{-8} M for C_5DAChol (and of 1.2×10^{-8} M [5] for acetylcholine).

3.5. *The interaction in vitro of C_5DAChol with* Torpedo *receptor-rich membrane fragments followed by fluorescence spectroscopy*

In a first series of experiments the fluorescence emission of C_5DAChol was followed by direct excitation in one of the bands where it maximally absorbs (λ_{ex} = 325 nm). Addition of membrane fragments (2×10^{-7} M final concentration in [^{3}H]α-toxin sites) to a 1 μM solution of C_5DAChol in *Torpedo* physiological solution causes a significant increase in fluorescence intensity between 400 and 560 nm (about 30% at 500 nm, the intrinsic protein fluorescence being subtracted). The subsequent addition of *N. nigricollis* α-toxin or carbamylcholine to the mixture further modifies the fluorescence spectrum: at wavelengths larger than 490 nm, as expected, the signal intensity decreases (by about 20% at 560 nm). C_5DAChol, therefore, interacts with the membrane fragments at a site sensitive to specific nicotinic ligands. Under these conditions a significant enhancement of the fluorescence at wavelengths shorter than 490 nm was observed (about 80% at 460 nm).

In order to record fluorescence signals more exclusively related to C_5DAChol bound to the membrane fragments, excitation by energy transfer from membrane proteins (λ_{ex} = 290 nm) was used instead of direct excitation.

Under excitation at 290 nm the emission of C_5DAChol free in solution is minimum. However, an overlap between protein fluorescence emission and C_5DAChol excitation spectra indeed exists. The measurements were therefore performed by differential spectroscopy under the following conditions: both reference and sample cells contained the suspension of membrane fragments (2×10^{-7} M in [^{3}H]α-toxin sites) in physiological saline solution and C_5DAChol was added to the sample cell. Fig.5 shows the emission spectrum recorded in the presence of 1 μM C_5DAChol. A comparison with the emission spectrum of free C_5DAChol reveals that the wavelength of the C_5DAChol emission maximum is shifted by about 10 nm to the blue. In order to test the specificity of the fluorescence emission signal, 8 μM α-toxin from *N. nigricollis* was added to both cells: a marked decrease of fluorescence intensity took place without significant change of the wavelength of maximal emission. The same observation was obtained with another typical nicotinic antagonist: flaxedil. On the other hand, when an agonist, such as carbamylcholine, was used instead of an antagonist, the fluorescence emission decreased as well but a marked shift to the blue, of about 55 nm, was noticed. This observation confirms and further amplifies previous findings with DNS-chol and the same membrane fragments [4,5]

As already suggested in the case of DNS-chol [4,5] the simplest interpretation of the data is that C_5DAChol binds to, at least, two categories of sites: the cholinergic receptor site and secondary sites, distinct from the previous ones, where C_5DAChol fluorescence emission differs when the receptor site

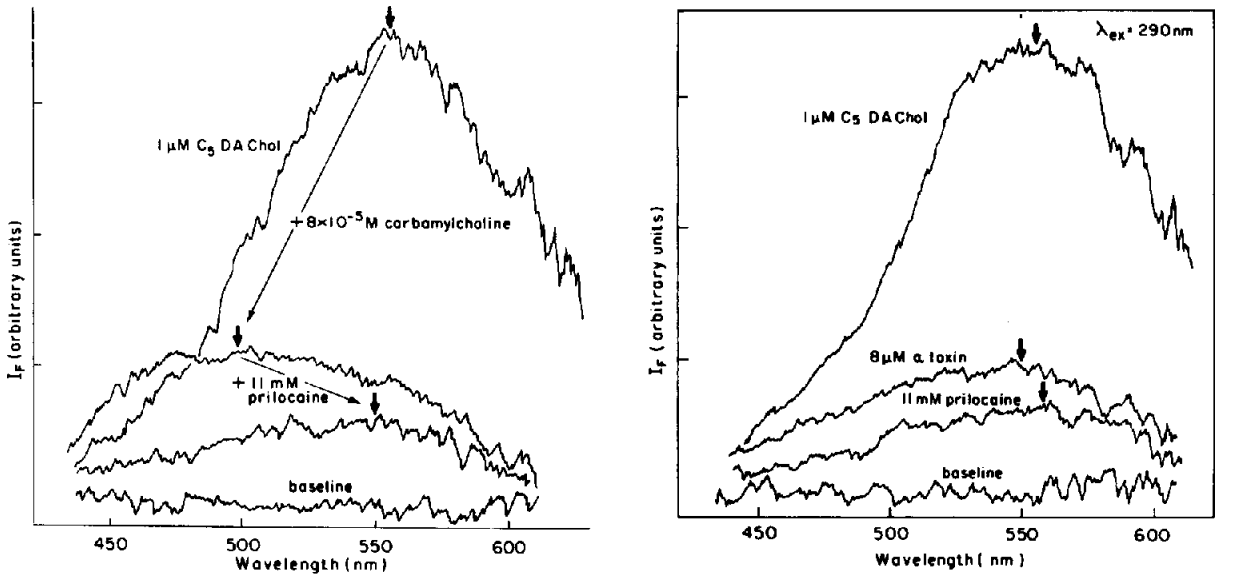

Fig.5. Differential fluorescence emission spectra of C_5DAChol in the presence of *Torpedo* receptor-rich membrane fragments. Effect of cholinergic ligands. Membrane fragments ($2 \cdot 10^{-7}$ M *N. nigricollis* α-toxin binding sites, 180 μg protein per ml) in *Torpedo* physiological solution were present in sample and reference cells (base line). C_5DAChol was added to sample cell and spectrum recorded. In (A), the indicated concentration of carbamylcholine and then of prilocaine was introduced in both cells; for (B), α-toxin followed by prilocaine, to both cells. The arrows indicate the emission maxima.

is occupied by an agonist or an antagonist [4,5]. To test the hypothesis [5] that these secondary sites are, in some manner, related to the local anesthetic binding site, prilocaine (1–11 mM) was added after the suspension was supplemented with carbamylcholine or α-toxin. A further decrease of fluorescence intensity took place and, again, as found with DNS-chol [5] prilocaine reversed the blue shift observed in the presence of agonists.

Qualitatively, C_5DAChol behaves in a manner similar to DNS-chol. Important quantitative differences, however, were noticed. First, the blue shift caused by the agonists is significantly larger than that found with DNS-chol. Moreover, conditions can be found under which the fluorescence signal associated with the binding of C_5DAChol to the receptor site can be separated almost completely from that associated with the secondary sites and free C_5DAChol: measurements of the decrease of fluorescence intensity which accompanies the displacement of C_5DAChol from the cholinergic receptor site by either α-toxin or agonists indicate that at low concentrations ($< 6 \cdot 10^{-7}$ M) of C_5DAChol the fluorescence signal which is not associated with ACh binding sites represents less than 15% of the total signal.

Moreover the incidence, of these remaining 15% can be diminished using the following experimental set up. Both sample and reference cells contained membrane fragments plus C_5DAChol and an excess of acetylcholine was added to the reference cell. The difference signal was then studied as a function of C_5DAChol concentration (fig.4). Assuming that the fluorescence quantum yield of C_5DAChol bound to the ACh receptor site is independant of the average occupancy of the sites and that the number of α-toxin and ACh binding sites are equal, one can estimate a dissociation constant for C_5DAChol of $4.5 \cdot 10^{-8}$ M.

Interestingly, this value is very close to that found by direct displacement of [^{3}H]acetylcholine bound at equilibrium to the membrane-bound receptor.

At concentrations of C_5DAChol larger than 10^{-6} M, the acetylcholine binding sites are already saturated and the contribution to the fluorescence signal from

C_5DAChol bound to the secondary sites and from free C_5DAChol becomes significant. Yet, for technical reasons the dissociation constants of C_5DAChol from these secundary sites have not been measured.

4. Conclusion

Electrophysiological and biochemical observations confirm that the C_nDAChol compounds, like DNS-chol, behave both as agonistic and non-competitive blocking agents. However, increasing the length of the chain between the choline moiety and the dansyl residue markedly enhances the agonistic character of the molecule as manifested by both the amplitude of the maximal response and the apparent dissociation constant. Preliminary ^{1}H n.m.r. experiments, however, do not reveal differences of conformation which might simply explain the phenomenon.

Fluorescence studies done by energy transfer with the C_5 member of the series show that this compound, like DNS-chol, binds to *Torpedo* membrane fragments at the level of two distinct population of sites: the acetylcholine receptor site and secondary sites related to the local anesthetic binding site. However, at variance with what was found with DNS-chol and probably because of the preferential and high affinity of C_5DAChol to the cholinergic receptor site, conditions can be defined where the fluorescence signal reflects, almost exclusively, the occupancy of the receptor site by C_5DAChol. This compound may therefore become an useful probe to follow, by rapid kinetic techniques, the binding of an agonist to the membrane bound cholinergic receptor protein and to analyse the conformational changes it triggers.

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