TOPICS IN MOLECULAR PHARMACOLOGY VOLUME 1

A. S. V. BURGEN
AND
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EDITORS

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Preface

In recent years, there have been considerable advances in our knowledge of the details of small molecule-macromolecule interactions in general, as well as in the characterisation of receptor molecules themselves. These advances have brought us substantially closer to the goal of a detailed understanding of drug, hormone and neurotransmitter action at a truly molecular level.

There is, of course, still a long way to go. Further progress will depend upon the integration of many different kinds of information -- from physical chemistry to physiology. We feel that the development of molecular pharmacology would be helped by publication of a series of reviews of relevant advances in these areas.

It is our intention that the series should deal both with the fundamental general principles governing drug-receptor interactions and their biological consequences and with analysis of specific receptor systems. In this first volume, the articles are primarily concerned with analysis of specific systems, although in each case some general principles can clearly be seen to be emerging. In some cases, such as for example hormone-activated adenyl cyclase or the sodium channel, we are concerned with the structural and functional organisation of the receptor system. In others, particularly those where the receptor is a soluble enzyme, we are concerned with the atomic details of the drug-receptor interaction. The common feature, in this and in future volumes, is a concern to push as far as possible toward the molecular level, to understand drug action in terms of the three-dimensional structure and dynamics of the drug-receptor system.

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Kinetics of ligand binding to the nicotinic acetylcholine receptor and acetylcholinesterase

Alfred MAELICKE

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Abbreviations: dansyl-*n*-chol and NBD-*n*-chol represent dansyl- and NBD-labelled aminoacylcholines with their bridge of methylene groups consisting of *n* members; see structures 1 and 2 in Fig. 1.

A.S.V. Burgen & G.C.K. Roberts (eds.) Topics in Molecular Pharmacology

1. Introduction

Synapses are biological devices designed for rapid transmission of information between adjacent cells. A representative example is the neuromuscular junction which can transmit chemical impulses with frequencies of up to 50 per second. Its fast response to repetitive stimuli depends both on rapid coupling of binding and primary response and on rapid removal of transmitter from the cleft between excitatory events. Whilst the latter, i.e. binding and turnover of acetylcholine by acetylcholinesterase has been studied thoroughly, little is known about the way in which binding of transmitter and cellular response are linked. This is because only the receptor and not the associated ion channel (ionophore) is presently accessible to biochemical studies. As a result, ligand recognition by the receptor and response are presently studied by completely different techniques, the former being a domain of biochemistry, the latter of electrophysiology.

Although the time resolution of electrophysiological techniques permits certain inferences about the kinetics of excitation at cholinergic synapses (Colquhoun, 1979), a full understanding of the underlying chemical events will have to await the independent determination of all rate constants involved. At present the following reaction steps are accessible to quantitative studies: (i) interaction of acetylcholine and analogs with acetylcholinesterase; (ii) interaction of cholinergic ligands with the acetylcholine receptor in purified and membrane-bound form; (iii) the rates of conformational changes of the receptor protein; (iv) the rates of opening and closing of the receptor controlled ion channel; and (v) the rates of secondary responses. Because all these reaction steps have to fit into the time frame of a few milliseconds, they have to be considered together. Kinetic studies may help to answer the following questions: (1) How does binding and recognition of transmitter molecules lead to changes in membrane permeability; how are these processes coupled? (2) What is the nature and what are the rates of the chemical and physical reactions responsible for transmitter recognition and induction of the primary response? (3) Which steps in transmitter binding and recognition are rate-limiting for the onset of the permeability change? Are they subject to regulation or modification? On a more general level, answers to some or all of these questions will help our understanding of the basic principles of intercellular communication, of the functional properties of integral membrane proteins and of the effect of the membrane and its constituents on these.

In this chapter I describe briefly the current status of biochemical studies of the kinetics of cholinergic excitation. Since these events mostly occur in the subsecond time range, rapid kinetic techniques are required for their studies. These must be based on changes in the spectroscopic properties of the reactants and I therefore begin with an overview of the molecular probes that can be applied.

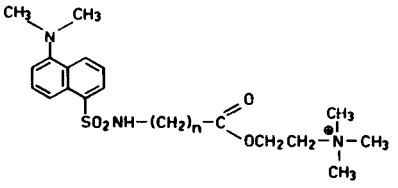
2. *Molecular probes for the study of receptor mechanisms*

Useful molecular probes have to be sensitive and should introduce as little perturbation as possible. Sensitivity is best achieved by fluorescent probes because their signals are linearly proportional to the intensity of the applied light source. Since the natural transmitter is non-fluorescent under normal conditions, the intrinsic fluorescence of the receptor protein appears to be the most direct probe. Unfortunately, the receptors from *Electrophorus* and *Torpedo* have very weak intrinsic fluorescence, and the *Torpedo* receptor contributes only a few percent of the total fluorescence of purified membrane fragments (Barrantes, 1978). Furthermore, the maximal changes in intrinsic fluorescence of the receptor protein in the presence of cholinergic ligands are only of the order of 5–10% (Cohen and Changeux, 1973; Barrantes, 1978).

These disadvantages can be overcome by covalently attaching a fluorescent probe to the receptor protein. Well suited for these purposes is a disulfide bond in the vicinity of a ligand binding site at the receptor (Karlin, 1969) which, after reduction, can be affinity alkylated (Weill et al., 1974). In this way Dunn et al. (1980) have attached the fluorescent probe 5-(iodoacetamido)salicylic acid (IAS) to the membrane-bound receptor and have studied the changes in fluorescence of the probe induced by non-fluorescent cholinergic ligands.

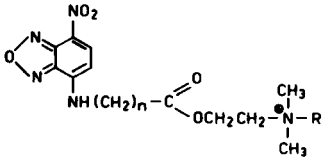
Various types of extrinsic fluorescent probes have been applied to receptor-ligand studies. These are meaningful only if (i) they have unambiguous pharmacology, and (ii) their interaction with the receptor is accompanied by specific and large changes in their spectroscopic properties. Only few of the compounds that have been applied so far meet these requirements. The best studied and most satisfactory examples are the dansyl-acylcholines (Waksman et al., 1976, 1980) and the NBD *n*-acylcholines synthesized in my laboratory (Jürss et al., 1979a, 1981). Both (Fig. 1) are analogs of acetylcholine in which the fluorescent moiety is linked via a bridge of methylene groups in the choline moiety. For example, the C_6 member of the dansyl-acylcholine acts as a depolarizing effector (agonist) on the isolated electroplaque from *Electrophorus electricus*, producing a half-maximal response at 10^{-6} M. The corresponding dose-response curve is sigmoid and can be characterized by a Hill coefficient of 2.0. At concentrations above 5 μM or after longer periods of incubation, dansyl-6-chol shows pharmacological desensitization when studied by $^{22}Na^+$ efflux from excitable microsacs from *Torpedo marmorata* (Heidmann and Changeux, 1979a). Of the NBD *n*-acylcholines, NBD-5-chol has been studied in most detail by physiological methods. It induces isometric contractions of rat diaphragm, depolarizes frog muscle fibers and has a pronounced desensitizing action. 0.4 μM NBD-5-chol produces the same response as 30 μM carbamylcholine or 1 μM acetylcholine as determined by measuring the endplate current of voltage clamped *Rana pipiens* Sartorius muscles. The mean open time of the NBD-5-chol activated channel at 17°C and −76 mV holding potential is 2.6 ± 0.2 ms and is almost identical to the decay time constant of miniature endplate currents (2.85 ms) recorded in the same preparation (Jürss et al., 1979a). All trimethylammonium derivatives synthesized so far (n = 4–7) act as agonists of acetylcholine at the neuromuscular junction whilst the *N,N*-dimethyl-*N*-propylammonium derivative is a competitive antagonist at low concentration with additional weak depolarizing effects at higher concentration (Jürss et al., 1981; Prinz and Maelicke, 1981). Hence,

(1)



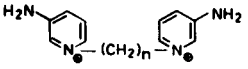
n = 1 - 7, 10

(2)



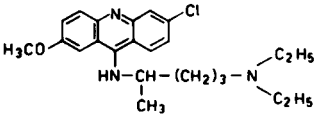
n = 4 - 7
R = CH_3, n-C_3H_7, CH_3 *

(3)



n = 6, 10

(4)



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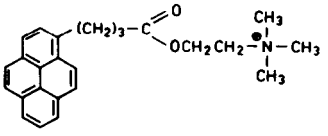

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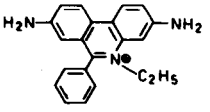

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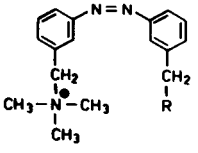
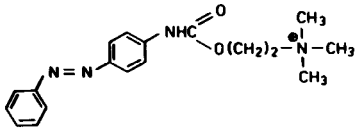


R = $\overset{\oplus}{N}(CH_3)_3$, Br

(8)



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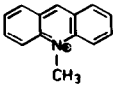

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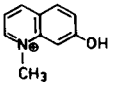

the structure-function relationships of NBD *n*-acylcholines agree with those previously observed with representative cholinergic ligands (Beers and Reich, 1970).

Another physiologically well characterized fluorescent probe is 1-pyrenebutyrylcholine which partially acts as curare-like antagonist (Barrantes et al., 1975). Since it has rather complicated pharmacologic and considerable non-cholinergic effects, it has not been applied to detailed biochemical studies. Bisquaternary ammonium compounds such as bis-3-(aminopyridinium)-1,10-decane (DAP) (Fig. 1) were originally designed for the study of acetylcholinesterase (Mooser et al., 1972). Both, the hexane and the decane derivative act as partial blockers at the frog neuromuscular junction (Maelicke, unpublished). They have been applied to binding studies with the purified acetylcholine receptor (Martinez-Carrion and Raftery, 1973; Maelicke et al., 1977a).

A probe which does not interact directly with the ligand recognition site but which does probably bind to the receptor protein is the fluorescent antimalarial drug and local anaesthetic quinacrine. Quinacrine does not significantly alter the resting potential of the eel electroplaque but blocks the depolarization caused by 30 μM carbamylcholine, 50% inhibition taking place at about 10 μM. Quinacrine reversibly decreases the response to carbamylcholine with only little effect on the concentration for half-maximal response (Grünhagen and Changeux, 1976a, b). A probe of rather indirect and little defined action is the mutagenic and intercalating drug ethidium (Quast et al., 1978).

Another approach is to link an independent monitoring reaction with an event affected by the receptor in the course of cholinergic excitation. Such probes are the chelating agents murexide and arsenazo III both of which can monitor the amount of Ca^{2+} released by the receptor upon interaction with acetylcholine (Eldefrawi et al., 1975; Neumann and Chang, 1976). Similarly Rb^{2+} has been used to mimick the effects of Ca^{2+} on ligand binding (Rübsamen et al., 1978).

Besides these fluorescent molecular probes there exist other compounds which after manageable synthetic modifications might have the potential of excellent monitoring ligands. To these belong the cholinergic analogs of azobenzene introduced by Bartels et al. (1971) (Fig. 1). These compounds exist in two photoisomerizable conformations, the *trans*-isomer acting as an agonist and the *cis*-isomer being inactive at the neuromuscular junction. These and related probes have been applied in concentration jump and voltage jump experiments to study the ion channel characteristics of the eel electroplaque (Nass et al., 1978; Lester et al., 1980; Lester, 1980) and, hence, are well characterized electrophysiologically. After attachment of a fluorescent moiety, for example NBD, they should be uniquely suited to time-resolved studies of agonist binding and the onset of the response.

Fig. 1. Structural formulae of fluorescent probes used in the study of receptor-ligand and esterase-ligand interactions. (1) Dansyl-*n*-chol, [1-(5-dimethylaminonaphthalene)sulfonamido)-*n*-alkanoic acid β-(*N*-trimethylammonium)ethyl ester; (2) NBD-*n*-chol, *N*-7-(4-nitrobenzo-2-oxa-1,3-diazole)-ω-aminoalkanoic acid β-(*N*-trimethylammonium)ethyl ester; (3) DAP, bis(3-aminopyridinium)-1,*n*-alkane; (4) Quinacrine, 3-chloro-7-methoxy-9-(1-methyl-4-diethylaminobutylamino)acridine; (5) 1-Pyrenebutyrylcholine; (6) Ethidium, 3,8-diamino-5-ethyl-6-phenylphenanthridium; (7) Bis-Q, 3,3-bis[α-(trimethylammonium)methyl]azobenzene; (8) QBr *p*-phenylazophenyl carbamicacid β-(*N*-trimethylammonium)ethyl ester; (9) *N*-Methylacridinium; (10) 1-Methyl-7-hydroxyquinolinium.

A completely different time range of receptor function can be explored by means of fluorescent neurotoxins. These have several orders of magnitude slower dissociation rates than the low molecular weight ligands, act as competitive antagonists of the curare type and can be used to monitor relatively slow changes in receptor properties (Anderson and Cohen, 1974; Ravdin and Axelrod, 1977; Kang and Maelicke, 1980). Their physiological properties are defined by the specificity and potency of their blocking action and by their toxicity at the neuromuscular junction.

3. Molecular probes for the study of esterase mechanisms

The structural requirements for molecular probes of acetylcholinesterase are hardly less stringent than for the receptor. With the noteworthy exception of polypeptide neurotoxins all ligands of the acetylcholine receptor are also ligands of the esterase. The ligands of the receptor can be subdivided into agonists and antagonists (competitive blockers), those of the esterase into substrates and competitive inhibitors. This situation indicates two-step mechanisms for both receptor activation and substrate hydrolysis. There is evidence that the initial binding reaction (and hence the structure of the ligand binding site) is rather similar for both receptor and esterase. The overall mechanism of enzymatic acetylcholine hydrolysis has been established by steady-state kinetic studies (Rosenberry, 1975) and the remaining questions largely relate to the rates of association of the natural substrate acetylcholine and the properties of some intermediates. To answer these questions compounds with essentially identical properties to those of acetylcholine are required. It is therefore remarkable that until recently only enzyme inhibitors (DAP, *N*-methylacridinium, 1-methyl-7-hydroxyquinolinium, Fig. 1) have been applied in fluorescence kinetic studies of the enzyme. Of these *N*-methylacridinium and DAP (Mooser et al., 1972; Mooser and Sigman, 1974; Taylor and Lappi, 1975) were shown to have one binding site per active site of the enzyme, whilst this has not been established for 1-methyl-7-hydroxyquinolinium (Rosenberry and Bernhard, 1971; Rosenberry and Neumann, 1977). The NBD *n*-acylcholines (Jürss et al., 1979a; Prinz et al., 1980a,b; Jürss et al., 1981) competitively inhibit enzymatic hydrolysis of acetylthiocholine ($K_i \approx 5 \cdot 10^{-8}$ M) and are also poor substrates of the enzyme. Their turnover numbers increase with increasing chain length but they are hydrolyzed by the enzyme several orders of magnitude more slowly than acetylcholine itself.

4. Fluorescence binding studies with the acetylcholine receptor

Binding and kinetic studies critically depend on the pharmacological specificity of the molecular probes applied and the properties of the observed changes in fluorescence. Furthermore, binding studies are often a prerequisite for the kinetic analysis of a reaction sequence. These properties are therefore summarized and discussed here before considering the specifically kinetic applications.

4.1 Labelled acetylcholine receptor

Dunn et al. (1980) have used 5-(iodoacetamido)salicylic acid (IAS) to label sulfhydryl groups of receptor enriched membrane fragments from *Torpedo californica*. In addition to considerable non-specific labeling, some IAS was covalently attached to one or two sulfhydryl groups in the vicinity of a ligand binding site. The fluorescence of these IAS molecules is enhanced upon interaction of the membrane fragments with cholinergic ligands while the fluorescence of the non-specific sites appears to be unaffected. Using the fluorescence enhancement as a means of monitoring binding of carbamylcholine to the membrane fragments a K_D value of $1.7 \cdot 10^{-7}$ M was determined. This value corresponds to the high affinity state of the receptor which according to Moore and Raftery (1979) and contrary to the findings of Dunn et al. (1980) is converted to a low affinity state by treatment with dithiothreitol and iodoacetamide alkylation of the sulfhydryl groups.

4.2 Dansylderivatives of choline and aminoacylcholines

Dansylcholine (Weber et al., 1971) was first studied by Cohen and Changeux (1973) as a ligand of the acetylcholine receptor in aqueous solution. Interaction with receptor-rich membrane fragments causes both spectral shifts and a strong enhancement of the fluorescence of the drug. These are partially reversed in the presence of other cholinergic ligands including α-neurotoxin indicating two classes of sites, one cholinergic, the other "secondary". An apparent K_D value for binding to the cholinergic sites of the order of $2 \cdot 10^{-5}$ M was determined by the effect of dansylcholine on the initial rates of α-toxin binding to the receptor, and by equilibrium competition binding studies with acetylcholine and receptor. The fluorescence characteristics of dansylcholine bound to the secondary sites are influenced by the nature of the ligand bound to the cholinergic sites. Binding to the secondary sites is competitive with the local anaesthetic prilocaine; the relative stoichiometry of cholinergic and secondary sites, however, was not established. Because dansylcholine acts on the electroplax as partial agonist and mixed blocker, studies with this compound are of limited value and were thus discontinued.

More promising ligands are the dansyl derivatives of aminoacylcholines, dansyl-*n*-chol (Fig. 1; Waksman et al., 1976, 1980). Of these the C_1 and C_2 members act as noncompetitive blocking agents while the C_3 and longer chain members act as agonists in addition to their noncompetitive effects. The noncompetitive effects are due to the naphthalene ring system of the fluorescent moiety as shown by the full agonist properties of methylsulfonamidoacylcholines (Waksman et al., 1980). The concentrations for half-maximal response (K_{app}) of dansylamidoacylcholines as determined with *Electrophorus* electroplaques decrease with increasing chain length. K_{app} for dansyl-5-chol is $8 \cdot 10^{-7}$ M while binding of this compound to the cholinergic sites of receptor enriched membrane fragments from *Torpedo* is characterized by an equilibrium dissociation constant, K_D, of $5 \cdot 10^{-8}$ M. Binding can be monitored by the changes in fluorescence of the drugs, i.e. the strong increase in intensity and the shift of the fluorescence spectrum. With dansyl-5-chol 85% of the fluorescence effect is reversed upon addition of representative cholinergic ligands, the remaining changes in fluorescence being due to binding of dansyl-5-chol to other than cholinergic sites (Waksman et al., 1976).

Dansyl-6-chol shows similar spectral effects upon interaction with receptor-rich membrane fragments from *Torpedo marmorata* as described for the C_5 derivative. Its fluorescence effects are apparently more specific for interaction with the cholinergic sites at the receptor. However, there remains a fluorescence effect which is not competitive with cholinergic ligands but is largely reversed by prilocaine. The two classes of sites differ markedly in their maxima of fluorescence emission (557 nm when bound to cholinergic sites, 475 nm when bound to secondary sites). Hence, by selecting the appropriate spectral range, the interaction of dansyl-6-chol with cholinergic and secondary sites at the receptor can be measured separately. Interestingly, α-neurotoxin appears to block binding of dansyl-6-chol to both classes of sites. By differential energy transfer studies (λ_{EX} = 290 nm, λ_{EM} = 557 nm) the affinity of binding ($K_D \approx 10^{-7}$ M) to the cholinergic sites and the ratio of ligand to toxin binding sites (1 : 1) was determined. The effect on the initial rates of toxin–receptor interaction indicates a K_D value of $1 \cdot 10^{-8}$ M for binding to the cholinergic sites while the concentration for half-maximal response (K_{app}) is 10^{-6} M.

4.3 NBD derivatives of aminoacylcholines

In search of less bulky and less hydrophobic fluorophors we have applied NBD chloride (Haugland, 1968) since 1975 in the synthesis of analogs of choline and acylcholines. Of these only the larger NBD *n*-acylcholines (Jürss et al., 1979a) were pharmacologically advantageous compounds. They are full agonists of acetylcholine at the neuromuscular junction and closely mimick the natural transmitter in the kinetics of receptor channel gating. The excitation maximum of NBD *n*-acylcholines (484 nm) is close to a major emission line of argon lasers; the fluorophor is very resistant to photobleaching. The fluorescence quantum yield is approximately 0.02. Binding of NBD *n*-acylcholines to membrane-bound or to purified receptors results in large and specific changes of the drug's fluorescence. Practically total quenching of fluorescence is observed with purified receptor from *E. electricus*. The effect is specific for the fluorescent choline ester in that neither NBD-ethylamine nor NBD-aminoalkanoic acids produce any quenching of fluorescence when incubated with the receptor under identical conditions. When explored over a wide range of receptor and ligand concentrations the fluorescence changes show the existence of two classes of binding site of different affinities for NBD *n*-acylcholines at the receptor. Both classes of site fulfill the criteria for specific cholinergic sites; binding is competitively inhibited by other cholinergic ligands (α-neurotoxins, acetylcholine, decamethonium, tubocurarine). There are equal amounts of high affinity and low affinity sites. NBD 5-chol and its *N*-*n*-propyl derivative were employed as probes to study the binding of typical non-fluorescent cholinergic ligands to the purified solubilized receptor (Prinz and Maelicke, 1981). These studies established the following characteristics of the binding sites. All cholinergic ligands compete for the same number of binding sites. There exists one class of binding sites for antagonists at the receptor but apparently two classes of binding site for agonists. The equal number of high affinity and low affinity binding sites for agonists suggests an arrangement of sites in pairs. The data are consistent with the idea that all the agonist binding sites are initially identical. Agonist binding to one site of a pair then causes a decrease in affinity of the second site for agonists. Accord-

ingly, binding of agonists to pairs of interacting sites is sequential, the negative cooperativity between them leading to the appearance of two classes of sites. The following binding constants were obtained: acetylcholine, $K_D(1) = 1 \cdot 10^{-7}$ M, $K_D(2) = 2 \cdot 10^{-6}$ M, carbamylcholine, $K_D(1) = 4 \cdot 10^{-6}$ M, $K_D(2) = 6 \cdot 10^{-5}$ M; decamethonium, $K_D(1) = 1 \cdot 10^{-8}$ M, $K_D(2) = 6 \cdot 10^{-7}$ M; tubocurarine, $K_D = 2 \cdot 10^{-7}$ M; gallamine, $K_D = 1 \cdot 10^{-7}$ M; hexamethonium, $K_D = 2 \cdot 10^{-5}$ M. The particular advantage of fluorescence binding studies with NBD acylcholines is the absence of any unspecific change in fluorescence or any remaining fluorescence after binding that could introduce ambiguity in the analysis of data. With purified receptor from *Torpedo marmorata* the existence of two separate classes of agonist binding site is indicated by their different fluorescence characteristics: binding to one class of sites results in an enhancement, binding to the other in a quenching of the drug's fluorescence. These findings indicate considerable structural differences at the position where the fluorescent moiety binds close to the ligand binding sites of the receptors from *Electrophorus* and *Torpedo*. Such differences must also exist between receptor-rich membrane preparations and purified receptor: interaction of NBD *n*-acylcholines with membrane preparations from both *Electrophorus* and *Torpedo* results in a quenching of fluorescence; the effect, however, is considerably smaller than observed with the purified receptor from *Electrophorus*. The K_D values for interaction of NBD 5-chol with the receptor protein from *Electrophorus* are K_D (1) = $3 \cdot 10^{-9}$ M and K_D (2) = $2 \cdot 10^{-7}$ M.

4.4 Bisquaternary ammonium compounds and pyrene derivatives

The fluorescence (λ_{EX} = 300 nm, λ_{EM} = 400 nm) of bis-(3-amino-pyridinium)-1,10-decane (DAP) (Fig. 1) is completely quenched upon interaction of the drug with the purified receptor from *Torpedo californica*. The binding can be described by a single equilibrium dissociation constant, with a value at pH 7.4 and 24°C of the order of $1 \cdot 10^{-7}$ M. The affinity of DAP strongly depends on the pH and is decreased in a competitive fashion by monovalent (Na^+, K^+) and divalent (Ca^{2+}, Mg^{2+}, Sr^{2+}) cations (Martinez-Carrion and Raftery, 1973). More detailed studies with a whole series of fluorescent bis-methonium compounds (n = 4–12) show that the quenching effect is almost completely specifically cholinergic, that the (single) affinity of binding increases with increasing number of methylene groups, and that interaction with the receptor occurs via both ends of the bifunctional probes. DAP is not displaced completely from receptor by all cholinergic ligands. Non-fluorescent bis-methonium compounds displace all the DAP in a single step from the receptor protein, while tubocurarine does so only in a stepwise fashion and carbamylcholine and acetylcholine only partially. Hence, DAP and the latter ligands might bind at separate sites, and the existence of ternary complexes is indicated. Binding of DAP to *Torpedo* membrane fragments is biphasic with the high affinity K_D value being of the order of $1 \cdot 10^{-8}$ M. A considerable amount of non-specific binding is also observed (Bode et al., 1979). With the purified receptor from *E. electricus* we observed for both the decane and hexane derivative complete quenching of fluorescence in the bound state and a single affinity of binding. From competition binding studies with ^{3}H-toxin we obtained K_i values of $3 \cdot 10^{-9}$ M and $6 \cdot 10^{-9}$ M for the fluorescent hexane and decane derivative, respectively (Maelicke et al., 1977a). Besides

their complicated pharmacology all DAP compounds have the disadvantage of low fluorescence quantum yield and marked photolability.

With respect to spectroscopic properties, the pyrene derivative of butyrylcholine (Barrantes et al., 1975) is certainly of advantage. It has a high quantum yield of fluorescence, a long half-life of the excited state (92 ns) and is much more resistant to photobleaching than DAP. However, its affinity to hydrophobic sites strongly overshadows its rather weak cholinergic properties and, consequently, it does not possess the selectivity required for a useful active site probe. No quantitative binding data have been published with the probe.

4.5 Fluorescent local anaesthetics

The fluorescent antimalarial drug quinacrine has some structural and functional analogies to local anaesthetics (Grünhagen and Changeux, 1976a). Interaction with membrane fragments from *Torpedo marmorata* causes an increase in fluorescence which is further augmented in presence of carbamylcholine, acetylcholine, decamethonium, hexamethonium and gallamine but not α-toxin. Prilocaine and quotane, two typical local anaesthetics each partially, and to different extents, reverse the fluorescence increase observed upon incubation of membrane fragments with quinacrine. That the reversal of fluorescence is not complete has been interpreted as being due to a direct effect of the two local anaesthetics on quinacrine and its fluorescence properties. Quinacrine does not bind to a fixed number of sites at the membrane fragments but partitions between aqueous and particulate phase. Carbamylcholine does not seem to affect this phase equilibrium but apparently increases the quantum yield of quinacrine molecules bound to membrane fragments. These binding properties show that quinacrine has general effects on membranes and thereby *indirectly* influences cholinergic sites. The latter effect can be used to measure apparent dissociation constants for cholinergic ligands. Their values are similar to those obtained by other methods (Weber and Changeux, 1974).

The mutagenic and intercalating dye ethidium is known to have direct effects on phospholipid membranes, partial blocking and local anaesthetic action. Its interaction with enriched membrane fragments from *Torpedo californica* causes both a strong enhancement of fluorescence and a shift of the emission to longer wavelengths (Schimerlik et al., 1979). Fluorescence enhancement for binding to sites competitive with cholinergic ligands is 2–3 times that for binding to secondary sites. Ethidium binds to only one class of cholinergic sites with a K_D value of the order of $3 \cdot 10^{-6}$ M. Incubation with cholinergic agonists or antagonists causes a further increase in fluorescence. This is due to an increase in quantum yield of the bound dye and appears to reflect a conformational change of the receptor protein. Competition binding studies with representative cholinergic ligands yield the following inhibition constants: carbamylcholine, $5 \cdot 10^{-8}$ M; decamethonium, $1 \cdot 10^{-7}$ M; nicotine, $4 \cdot 10^{-7}$ M; d-tubocurarine, $4 \cdot 10^{-8}$ M; gallamine, $1 \cdot 10^{-6}$ M; and hexamethonium, $2 \cdot 10^{-5}$ M. The binding of some local anaesthetics enhances the fluorescence of ethidium–receptor mixtures at low concentration and quenches the fluorescence at higher concentrations; other local anaesthetics quench the fluorescence of ethidium at all concentrations investigated. Binding of ethidium to secondary sites is non-saturable.