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Interaction of merocyanine 540 with nicotinic acetylcholine receptor membranes from *Discopyge tschudii* electric organ

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Abstract

Interactions between merocyanine 540 (MC540) and nicotinic acetylcholine receptor (AChR) have been studied by visible absorption spectroscopy using native receptor-rich membranes from *Discopyge tschudii* electric tissue and liposomes obtained by aqueous dispersion of endogenous lipids extracted from the same tissue. The fact that merocyanine partitions into the membrane when this is in the liquid-crystalline state, exhibiting a characteristic peak at 567 nm, was exploited to obtain quantitative information about the physical state of the AChR-rich membrane. Spectra of MC540 revealed that this molecule was preferentially incorporated into AChR-rich membranes, with an affinity ($K_d$app 30 μM) 10-fold higher than that in liposomes ($K_d$app 290 μM). Changes were observed in the equilibrium dissociation constant of MC540 at different temperatures: the two-fold higher affinity at 8°C than at 23°C can be rationalized in terms of a higher value of the overall dimerization constant (K_{dim}) at the lower temperature. The local anaesthetic benzocaine competed for MC540 binding sites with higher potency in AChR-rich native membranes than in liposomes made with endogenous lipids. This competition was found to be AChR concentration-dependent, whereas in liposomes the displacement was constant at different lipid/MC540 molar ratios. Titration experiments yielded an apparent dissociation constant for benzocaine of 0.6 mM and 0.7 mM for liposomes and AChR-rich membranes, respectively. The possible location of the benzocaine binding site is deduced from the competition experiments to be at the lipid annulus surrounding the nicotinic AChR protein.

Key words: Cholinergic receptor; Local anaesthetic; Lipid annulus; Torpedinidae; Cyanine dye

1. Introduction

Merocyanine 540 (MC540) is a cyanine dye that changes its optical properties, such as absorption and emission, upon binding to lipid bilayer membranes [1–4], a variety of biomembranes from different origin [1,3,5] and surfactant micelles [6]. Such spectroscopic changes have been ascribed principally to the physical state of the lipids in the membrane and to the electrical potential at the interface or across the membrane [1,7]. For this reason, MC540 has been widely used to measure cell membrane potentials in different excitable tissues [8–11].

The mechanism by which MC540 responds to changes in the membrane has been the subject of several investigations, and plausible models have emerged [2,12–15]. In essence, these hypotheses postulate that MC540 is present in the membrane in a monomer-dimer equilibrium, with differing optical properties for each species. When the membrane is in the fluid state, MC540 exhibits a peak at 570 nm; in the presence of membranes in the gel state, the spectrum is comparable to that of MC540 in water without lipids, i.e., it shows peaks at 500 and 530 nm. Hence, the increase in the 570 nm peak with respect to that at 500 nm can be related to the gel–fluid transition of the membrane. The absorption at 570 nm is ascribed to the presence of monomer in the membrane in equilibrium to monomers and dimers in the adjacent aqueous solution. In addition, MC540 exhibits a mechanism of intramembranous dimerization coupled to a rate-limiting reorientation of the dye in the membrane [2].

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The nicotinic acetylcholine receptor (AChR) is the paradigm rapid ligand-gated neurotransmitter receptor. It can be obtained in native membrane-bound form from the electric tissue of various Torpediniformes species such as the South American ray *Discopyge tschudii*. The natural neurotransmitter acetylcholine binds to two α-subunits and controls the opening of the channel that is a constituent part of this protein. Competitive antagonists also bind to the same sites and inhibit nicotinic AChR function. A third class of ligands is constituted by an heterogeneous collection of noncompetitive antagonists which are postulated to bind with high affinity to the lumen of the channel, and with less affinity to other sites, possibly at the lipid/receptor protein interface [16]. The noncompetitive antagonists provoke the inhibition of ion-flux by two different mechanisms: sterically, by blocking of the ion channel, and secondly by enhancing the rate of the nicotinic AChR desensitization process (reviewed in [17]. The latter is an inherent property of the nicotinic AChR protein: prolonged exposure to agonists leads the receptor protein to a closed-channel state.

Local anaesthetics are a particular class of noncompetitive antagonists that presumably bind to the hydrophobic surface of the nicotinic AChR. Among these drugs benzocaine has been relatively well studied. It displays a dissociation constant of 114 μM, as measured by the patch-clamp technique [18]. We have recently determined that the spin-labelled analogue of benzocaine is a drug of high affinity for the nicotinic AChR [19] relative to spin-labelled phosphatidylcholine, a lipid of low specificity [20], as determined by electron spin resonance (ESR) spectroscopy. We have also shown by ESR and fluorescence spectroscopy that benzocaine competes with spin-labelled lipids such as cholestane, a cholesterol analogue [21], and phosphatidylinositol and stearic acid [22].

In this work we have studied the interaction of MC540 with nicotinic AChR native membranes from *D. tschudii* electric organ, and compared its properties with those in liposomes made from total lipids from the same tissue. We have also studied the effect of thermal perturbation on the absorbance of MC540 in nicotinic AChR native membranes. Finally, we have analyzed the interaction of MC540 with nicotinic AChR-rich and lipid membranes in the presence of benzocaine, and obtained the apparent dissociation constant of this ligand.

2. Materials and methods

Materials

Discopyge tschudii specimens were obtained from the South Atlantic coast at Necochea (Buenos Aires, Argentina). DEAE-cellulose sheets (DE-81) were obtained from Whatman (Clifton, NJ). Merocyanine 540, α-bungarotoxin, benzocaine, leupeptin, chymostatin, antipain, and chloramine T were obtained from Sigma (St. Louis, MO). $Na^{125}I$ and Sephadex G-25 were purchased from New England Nuclear (Waltham, MA) and Pharmacia (Uppsala, Sweden), respectively. All other reagents were of the highest purity available.

Methods

Purification of nicotinic AChR native membranes. Electric fish were killed by pithing and the electric organs were dissected, frozen in liquid nitrogen, and stored at −70°C. The preparation of nicotinic AChR-containing membranes followed the method of Dwyer [23], with some modifications. Briefly, the tissue (about 300 g) was thawed, diced, and homogenized with 1 vol. of 10 mM Na-phosphate buffer (pH 7.4), containing 0.4 M NaCl, 5 mM EDTA, 5 mM EGTA, 3 mM phenylmethanesulfonyl fluoride, 5 mM iodoacetamide, and 5 μg/ml of each of the following proteinase inhibitors: leupeptin, chymostatin and antipain. The tissue was homogenized in a Virtis for four 15 s intervals. The homogenate was centrifuged in a JA-14 rotor at 4600 rpm for 10 min at 4°C. The supernatant was filtered through cheesecloth and pelleted by centrifugation in a JA-17 rotor at 16000 rpm for 60 min. The pellets were resuspended in 20 mM Na-phosphate, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.02% NaN_3 (pH 7.4), and sucrose was added to give a final concentration of 30% (w/w). Aliquots (7 ml) of the suspension were layered onto a discontinuous gradient of 8 ml 50% sucrose (w/w), 9 ml 39% sucrose (w/w), and 8 ml 35% sucrose (w/w), each prepared in the same buffer. The gradient was overloaded with 2 ml buffer and centrifuged for 60 min at 45000 rpm in a 55.2 Ti rotor. Bands at the interface between 50–39% and 39–35%, were collected separately, diluted with 1 vol. of bidistilled water, homogenized and pelleted by centrifugation at 17000 rpm for 1 h in a JA-17 rotor. To store membranes at −70°C, pellets were resuspended in 20 mM Na-phosphate buffer (pH 7.4), containing 0.25 M sucrose and 1 mM EDTA.

In order to study MC540-nicotinic AChR membrane interactions, AChR native membranes were washed and resuspended in 10 mM TRIS-HCl buffer (pH 7.4), at the desired concentrations.

Toxin binding experiments. α-Bungarotoxin was iodinated by the chloramine T method of Hunter and Greenwood [24], and purified on a Sephadex G-25 column. Specific activity of α-[^{125}I]bungarotoxin was 2–4 Ci/mmol. The specific activity of the nicotinic AChR membranes was assayed using the α-[^{125}I]bungarotoxin/DE-81 ion-exchange filter paper method [25]. Typically, values of 1600–2000 pmol α-[^{125}I]bungarotoxin/mg protein were obtained. Protein was determined according to the method of Lowry et al. [26].

Preparation of nicotinic AChR-free liposomes. Total lipid extracts were made from *D. tschudii* electric tissue according to Bligh and Dyer [27]. Organic solvents were evaporated under nitrogen and dried total lipid content was determined by weighing. Total phospholipids were quantified by phosphorus analysis [28], and total cholesterol (free + esterified) was determined enzymatically (Wiener Laboratories, Rosario, Argentina). Total lipid content was about 1 mg/mg protein and the phospholipid/cholesterol molar ratio was approximately 1.3.

Liposomes were prepared as follows: a chloroform/methanol solution of total lipids was evaporated under nitrogen and the dry film was resuspended in 10 mM Tris-HCl buffer (pH 7.4), by sonication for 10 min.

Titration experiments. Both liposomes and nicotinic AChR native membranes (2.2–2.5 ml final volume), at the desired lipid concentrations, were titrated with increasing aliquots of MC540 from a stock solution (1 mg/ml). In parallel, MC540 solutions were titrated with increasing aliquots of stock suspensions of each membrane preparation. Spectra of MC540 were obtained between 400–600 nm in a Hitachi model 100–60 U.V.-visible spectrophotometer at 8°C and 23°C.

In the presence of nicotinic AChR-rich membranes, the spectrum of merocyanine is composed of a peak at 570 nm, another one at 530 nm and a third one at 500 nm. The peak at 570 nm corresponds to dye monomers in the membrane, the peak at 530 nm corresponds to dimers in the membrane and monomer in solution, and the peak at 500 nm corresponds to the dimer in water. As the contribution from each species at 530 nm cannot be resolved, it may be assumed under the conditions in which the membrane is in excess with respect to the dye that the presence of monomer and dimer in solution is negligible. This is supported by the absence of the peak at 500 nm.

The equilibrium between monomer and dimer in the membrane is given by the relative magnitude of the peaks at 570 nm and 530 nm. Under conditions in which turbidity is low the absorbance is equal to the optical density. Thus,

$$[OD]_{570} = A_{570} = E_M[MC_M] + E_D[MC_D]$$

and,

$$[OD]_{530} = A_{530} = E_m[MC_m] + E_D[MC_D]$$

where E_M is the extinction coefficient of the monomer in the membrane, E_D the extinction coefficient of the dimer in the membrane and E_m the extinction coefficient of the monomer in the solution. $[MC_M]$, $[MC_D]$ and $[MC_m]$ are the corresponding concentrations. Considering that the optical density at 570 nm is mainly due to monomers in the membrane and that $[MC_m]$ is very low, the difference between $A_{(570)}$ and $A_{(530)}$ is the difference between monomer and dimer in the membrane.

Data from the MC540 spectra were plotted as the double reciprocal of the Langmuir adsorption isotherm as:

$$1/\Delta A = (K_d/\Delta A_{max}[MC540_{total}]) + 1/\Delta A_{max} \quad (1)$$

where ΔA is the difference in absorption values between 567 nm and 532 nm for different merocyanine concentrations ($[MC540_{total}]$), and K_d is the apparent dissociation constant of the membrane-MC540 system. ΔA_{max} is the maximum difference in absorption obtained at saturation for a given lipid concentration.

Displacement experiments. At the desired total lipid/MC540 molar ratio for each type of membrane, MC540 was titrated with increasing concentrations of the local anaesthetic benzocaine using a stock solution in absolute ethanol (1 mg/ml). In parallel, nicotinic AChR

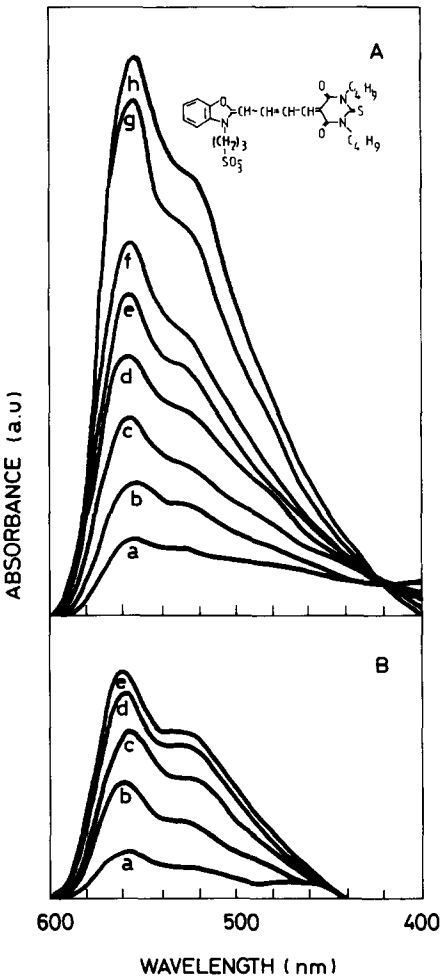

Fig. 1. Merocyanine 540 titration in: (A) liposomes prepared by aqueous dispersion of total lipids extracted from *D. tschudii* electric tissue. Inset: molecular structure of merocyanine 540. (B) Spectra corresponding to native nicotinic AChR membranes prepared from the same electric tissue. MC540 concentrations (in μM) were: (a) 3.6, (b) 7.1, (c) 10.7, (d) 14.2, (e) 17.7, (f) 21.2, (g) 24.7 and (h) 28.2.

native membranes were incubated for 30 min with 0.1 mM benzocaine and titrated with MC540. The observed absorption values were corrected for dilution in all experiments.

3. Results

Interaction of merocyanine 540 with nicotinic AChR membranes and liposomes

Absorption spectra of MC540 in the presence of liposomes prepared from total lipids from *Discopyge tschudii* electric tissue are shown in Fig. 1A. There is no evidence of isosbestic points between 450 and 600 nm, which would denote the equilibration between dye in solution and membrane-bound dimer and equilibration between membrane-bound monomer and dimer. The spectra exhibit two well-defined maxima at 532 nm and 567 nm at the highest dye concentration, since under these conditions MC540 is present both in solution and in the membrane. The absorption peak at 567 nm can be seen to increase more than the one at 532 nm between 3.58 μM and 28.2 μM dye (Fig. 1A).

Spectra corresponding to the titration of nicotinic AChR-rich membranes with MC540 are shown in Fig. 1B. The spectra are essentially similar to those obtained with receptor-free liposomes prepared from the membrane lipids (Fig. 1A). Quantitative differences between the two can be obtained by means of double-reciprocal plots (Fig. 2A) according to Eq. (1) (see Materials and methods).

The slopes of the straight lines in this figure are proportional to the $K_d/\Delta A_{max}$ values, and the intercepts yield the reciprocal of ΔA_{max} values. The figures of K_d calculated for both types of membranes at 23° and 8° are given in Table 1.

Table 1
Dissociation constant (K_d) of MC540 for liposomes prepared with endogenous *Discopyge* lipids and for nicotinic AChR-rich membranes

Membrane	Temperature (°C)	K_d	A_{max}	r
Liposomes	23	298.4	2.09	0.999
AChR-rich	23	29.8	0.28	0.991
AChR-rich	8	14.9	0.07	0.973

K_d values were obtained according to Eq. (1) from Fig. 2A and 4A respectively. r is the correlation coefficient.

Titration of MC540 with both membrane preparations was performed in parallel (Fig. 2B). The absorption difference (ΔA, $A_{567}-A_{532}$), which is directly proportional to the affinity of MC540 for the membrane, increases faster for the nicotinic AChR-rich preparation than for the lipid vesicles, in good agreement with the results of Fig. 2A and Table 1.

The equilibrium between monomer and dimer in the membrane can be estimated from the optical density at 567 nm (Table 2).

At total lipid/MC540 molar ratios higher than 10, it can be assumed that almost all the probe molecules are membrane-bound, either as monomers (MC_M) or dimers (MC_D) [2]. The equilibrium between these two membrane-bound species is given by:

$$2\,MC_M \rightleftharpoons MC_D \quad (2)$$

for which the equilibrium constant, K_{eq}, is defined by:

$$K_{eq} = [MC_M]^2/[MC_D] \quad (3)$$

The conservation condition [2] dictates:

$$[MC540_{total}] = [MC_M] + 2\,[MC_D] \quad (4)$$

where $[MC540_{total}]$ is the total merocyanine concentration, and $[MC_M]$ and $[MC_D]$ are the monomer and

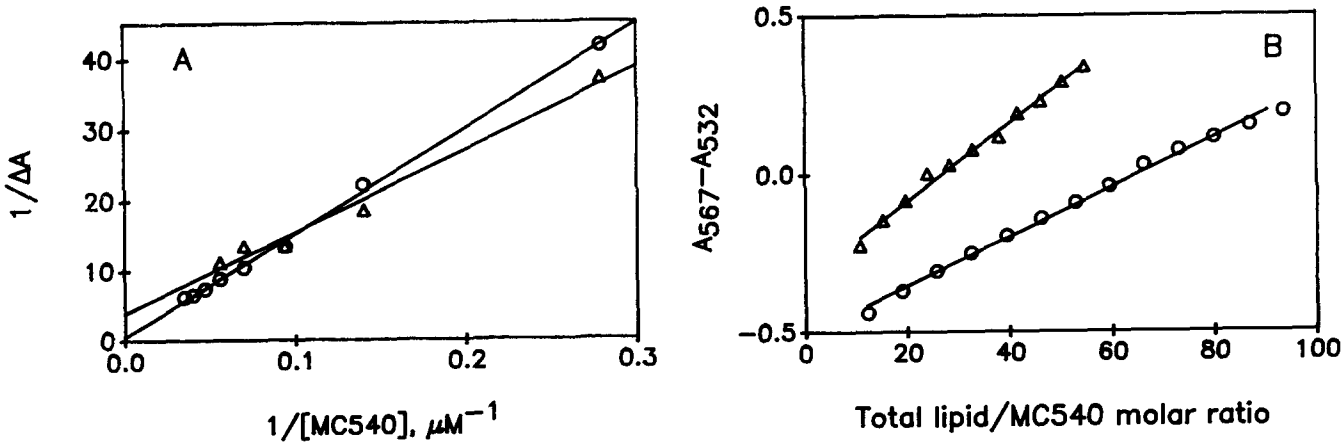

Fig. 2. (A) Double reciprocal of the Langmuir adsorption isotherm (see Eq. 1) for MC540 in liposomes (o) ($r = 0.999$) and nicotinic AChR native membranes (triangles) ($r = 0.991$). (B) Difference absorption ($A_{567}-A_{532}$) as a function of total lipid/MC540 molar ratio for liposomes (○) and nicotinic AChR native membranes (triangles).

Table 2
Monomer-dimer equilibrium constant for MC540 in liposomes and nicotinic AChR-rich membranes

[MC540] (μM)	Total lipid/ MC540 (molar ratio)	OD_{567} ($\times 10^3$ M^{-1})	$[MC_M]$ (μM)	$[MC_D]$ (μM)	K_{eq} (μM)
Liposomes					
3.58	114	0.144	2.19	1.39	3.5
7.13	57	0.228	4.18	2.95	5.9
10.68	38	0.302	6.15	4.53	8.4
14.21	29	0.379	8.11	6.10	10.8
17.73	23	0.446	10.04	7.69	13.1
21.23	19	0.518	11.98	9.25	15.5
24.72	16	0.590	13.90	10.82	17.9
28.20	14	0.650	15.79	12.41	20.1
Nicotinic AChR membranes					
3.58	73	0.103	2.07	1.51	2.8
7.13	37	0.156	3.97	3.16	5.0
10.68	24	0.254	6.00	4.68	7.7
14.21	18	0.283	7.82	6.39	9.6
17.73	15	0.402	9.91	7.82	12.6

OD_{567} is the measured optical density of MC540, at a given membrane lipid concentration. $[MC_M]$ and $[MC_D]$ are the concentration of monomeric and dimeric species of MC540, which were calculated from Eqs. (3) and (4), by assuming molar extinction coefficients of $\epsilon_M = 170000\ M^{-1}$ and $\epsilon_D = 6000\ M^{-1}$, respectively [14]. K_{eq} are the monomer-dimer equilibrium constants, determined according to Eq. (3).

dimer concentrations in the membrane, respectively. Such concentrations can be calculated from the following equation:

$$OD_{567} = E_M[MC_M] + E_D[MC_D] \quad (5)$$

where OD_{567} are the measured optical density values at 567 nm, and E_M and E_D are the molar extinction coefficients of the membrane-bound MC540 monomer and dimer, respectively. Equilibrium constants (K_{eq}) can be calculated according to Eq. (3) from the parameters given in Table 2. The plot of K_{eq} vs. the total lipid/MC540 molar ratio for both membrane preparations indicates that the equilibrium constant given by

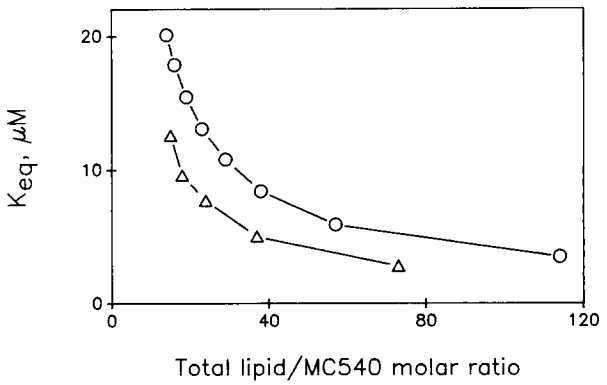

Fig. 3. Relationship between monomer-dimer equilibrium constant (K_{eq}) and total lipid/MC540 molar ratio for liposomes (○) and nicotinic AChR native membranes (triangles).

Eq. (3) decreases in parallel with the ratio in both cases (Fig. 3). Furthermore, the K_{eq} values are lower for nicotinic AChR-containing membranes than for the lipid vesicles.

Effect of temperature on MC540 dimerization

In order to evaluate plausible reaction mechanisms, nicotinic AChR native membranes were titrated with MC540 at 8°C. The temperature-dependent MC540 titration curves plotted according to Eq. (1) are shown in Fig. 4A. The K_d values obtained at the low temperature (8°C) indicate that the affinity of MC540 for nicotinic AChR native membranes was 2-fold higher than that at 23°C (Table 1).

It has previously been shown that the overall dimerization process consists of two steps [2,15]: the reorientation of MC540 monomers in the membrane from a perpendicular (M_{per}) to a parallel (M_{par}) orientation with respect to the plane of the membrane, and the dimerization process proper. The product of the second step, the dimers (D_2), are normally oriented parallel to the plane of the membrane. Thus, the overall

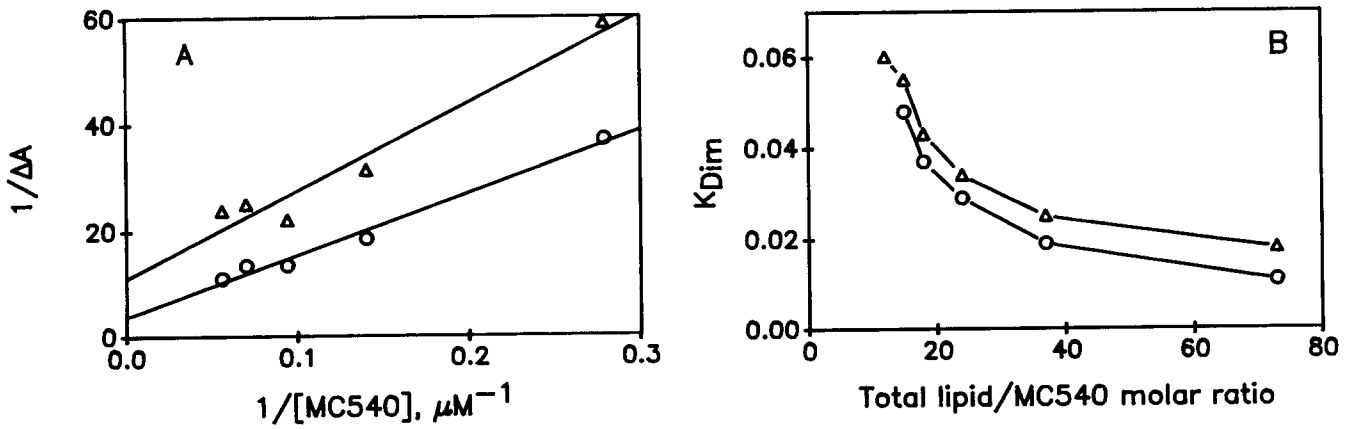

Fig. 4. (A) Effect of temperature on MC540-nicotinic AChR native membrane binding. Values for the titration at 8°C (triangles) and 23°C (○) are shown. (B) Temperature effect on the overall MC540 dimerization constant (K_{dim}).

dimerization process can be spatially represented by the following equation:

$$2M_{per} \rightleftharpoons 2M_{par} \rightleftharpoons D_2 \quad (6)$$

Under the present experimental conditions, at high total lipid/MC540 molar ratio, where the concentration of MC540 in solution is negligible, it is possible to define M_{per}, M_{par} and D_2 as dimensionless concentrations of merocyanine in the membrane related to $[MC_M]$ and $[MC_D]$ in the following manner [2]:

$$[M_{par}] + [M_{per}] = [MC_M]/[\text{Total lipid}] \quad (7)$$

and

$$[D_2] = [MC_D]/[\text{Total lipid}] \quad (8)$$

The overall dimerization constant, K_{dim}, can be related to K_{eq}, and becomes dimensionless:

$$K_{dim} = ([M_{par}] + [M_{per}])^2/[D_2] = K_{eq}/[\text{Total lipid}] \quad (9)$$

In order to demonstrate the relationship between temperature and the overall dimerization process, we calculated the K_{dim} values according to Eq. (9) and plotted it against the total lipid/MC540 molar ratio (Fig. 4B). From this it becomes apparent that the K_{dim} values are higher at low temperatures than at high temperatures throughout the titration, and that such differences are more pronounced at higher total lipid/MC540 molar ratios. At low total lipid/MC540 molar ratios (i.e., at concentrations of MC540 between 10.7 and 17.7 μM) the K_{dim} values change by about 14%. At high total lipid/MC540 molar ratios (between 3.6 and 7.1 μM MC540) the K_{dim} values change by more than 25%.

Effect of benzocaine on the binding of MC540 to membranes

We also studied the changes in the spectra of MC540 induced by the local anaesthetic benzocaine. The intensity of the absorption maxima at 567 nm and 532 nm of MC540 decreased, whereas the absorption maximum at about 500 nm increased (data not shown). In order to quantify this effect, competition experiments were performed using a fixed lipid/MC540 and varying concentrations of the local anaesthetic. From the data we were able to calculate the concentration of benzocaine inhibiting the specific binding of MC540 by 50% (IC_{50}) (Table 3).

Considering a pseudo-Hill coefficient equal to 1, these values can be converted into an inhibitory constant, which is theoretically equivalent to an equilibrium dissociation constant:

$$K_d\text{app} = IC_{50}/(1 + [\text{MC540}]/K_d) \quad (10)$$

where $K_d$app is the apparent dissociation constant for benzocaine, and [MC540] and K_d are the concentration and dissociation constants for merocyanine, respectively. The resulting $K_d$app values are also shown in Table 3 for both types of membrane.

Table 3
Displacement of MC540 from membranes by benzocaine

Membrane	Total lipid/MC540 (molar ratio)	IC_{50} (mM)	$K_d$app (mM)	[AChR] (μM)	r
Liposomes	35	0.80	0.73	–	0.999
	106	0.80	0.75	–	0.997
AChR membranes	29	0.99	0.59	0.95	0.994
	42	0.82	0.51	1.27	0.999
	100	0.68	0.41	2.20	0.993

$K_d$app, apparent dissociation constant for benzocaine, determined according to Eq. (10). IC_{50}, concentration of benzocaine giving half-maximal inhibition of MC540-membrane binding, determined graphically from plotting similar to Fig. 4A. [AChR], concentration of nicotinic acetylcholine receptor. r, correlation coefficient of non-linear regression fit.

Ethanol, the vehicle used for benzocaine addition, is a drug exhibiting, albeit at higher concentrations than those used here, general anaesthetic-like pharmacological properties on the nicotinic AChR [29,30]. In order to demonstrate that benzocaine molecules block specifically the binding of MC540 to nicotinic AChR membranes, and that ethanol does not interfere with this effect, titrations were carried out on nicotinic AChR membranes preincubated with ethanol without benzocaine for 30 min at room temperature and, in

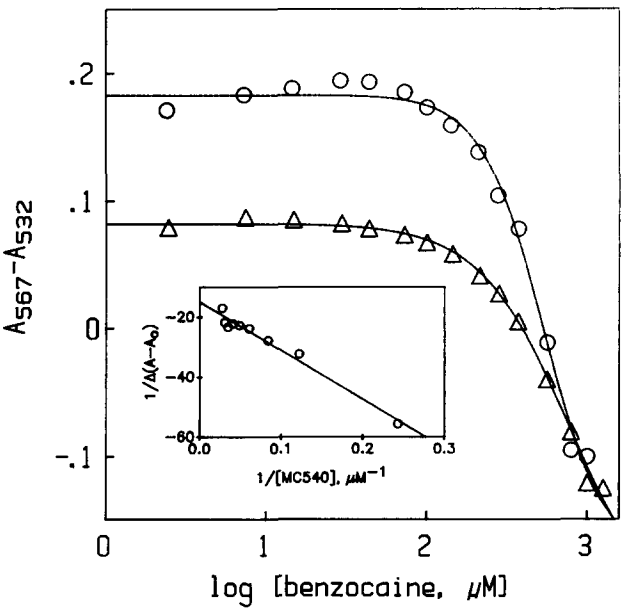

Fig. 5. Displacement by benzocaine of MC540 from liposomes (○) and nicotinic AChR native membranes (triangles). Inset: double reciprocal of the Langmuir adsorption isotherm for 0.1 mM benzocaine-pretreated nicotinic AChR membranes relative to control. A corresponds to the difference ($A_{567}-A_{532}$) of the samples treated with benzocaine and A_0 to the same difference for control samples without benzocaine.

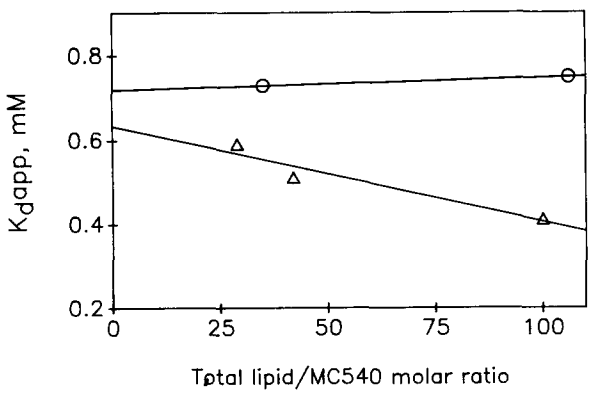

Fig. 6. Apparent dissociation constant ($K_d$app) of benzocaine as a function of total lipid/MC540 molar ratio for liposomes (○) and nicotinic AChR native membranes (triangles) at 23°C.

parallel, nicotinic AChR membrane samples preincubated without benzocaine were also titrated with MC540 at 23° (Fig. 5).

The resulting difference absorption spectra should reflect exclusively the effect of benzocaine on MC540-nicotinic AChR binding. From the inset in Fig. 5 it is apparent that the dissociation constant for MC540 in benzocaine-treated nicotinic AChR membranes is 11 μM higher than in control, non-treated membranes.

The apparent benzocaine dissociation constant for each membrane type was plotted as a function of total lipid/MC540 molar ratios (Fig. 6). From these it is evident that the $K_d$app in liposomes does not change at either low or high total lipid/MC540 molar ratios, whereas in the case of nicotinic AChR membranes at high total lipid/nicotinic AChR ratios the $K_d$app decreases.

4. Discussion

Possible location of the MC540 binding site in nicotinic AChR-rich membranes

The measurement of the steady-state MC540 absorption changes associated with the interaction of this dye with nicotinic AChR membranes and liposomes (Fig. 1) enabled us to determine firstly that MC540 has a 10-fold higher affinity for nicotinic AChR-rich than for pure lipid membranes (i.e., membranes without the receptor protein (cf. Table 1)). In addition, the apparent dissociation constant of merocyanine in liposomes obtained from *D. tschudii* total lipid extracts was 298.4 μM (Table 1), i.e., much higher than that reported for liposomes prepared from soybean lecithin (10 μM; [3]). This considerable difference could be ascribed to membrane lipid composition. The lipid composition of *D. tschudii* electric organ and nicotinic AChR membranes prepared therefrom has been previously reported by our laboratory [31]. A high proportion of zwitterionic phospholipids such as phosphatidylcholine (PC) (36.3%) and phosphatidylethanolamine (32.4%) are characteristically observed. Anionic phospholipids such as phosphatidylserine (PS), phosphatidate and phosphoinositides are also present in proportions of 15.2%, 2.0% and 2.2%, respectively. Thus, liposomes prepared from this source have a net negative charge. Since MC540 also exhibits a net negative charge (see molecular structure in inset to Fig. 1A), the repulsion between the two in this system might determine a lower affinity in comparison with liposomes prepared from PC's.

The fact that MC540 shows a 10-fold higher affinity for nicotinic AChR native membranes than for pure lipid liposomes (Table 1) is possibly due to the presence of the protein moiety. In other native biological membranes, such as those of mitochondria, apparent K_d values of 5.0–7.2 μM have been reported for MC540 [3,5]. These values are, again, lower than those found in AChR native membranes, and suggest that differences in K_d values may arise from specific differences in the composition of both lipid and protein in nicotinic AChR membranes.

In the case of the nicotinic AChR α-subunit from *Torpedo californica* two positively charged residues, Arg-429 and His-408, have been purported to be present at the membrane-water interface (the lipid polar headgroup region) [32]. These residues provide suitable sites for attachment of the negatively charged group of a photoactivable analogue of PS to a specific nicotinic AChR transmembrane region [32]. If this were the case, the electrostatic barrier to MC540 partition into pure lipids could decrease due to the partial neutralization of the negatively charged lipids by positively charged amino acids at relevant sites in nicotinic AChR transmembrane segments.

Dimerization of MC540 in nicotinic AChR membranes

The studies of Dragsten and Webb [15] and Verkman and Frosch [2] have demonstrated two kinetic processes applicable to MC540: (i) the partitioning of the dye into the membrane, in a diffusion-limited manner, with delays of about 100 ms; and (ii) a faster process of coupling between the reorientation (0.5–1 ms) and the dimerization (< 6 μs) of the dye in the membrane, namely the overall dimerization process. The rate-limiting step corresponds to the reorientation of monomers, normally oriented both parallel to and perpendicular to the plane of the membrane, and dimers, usually oriented parallel to the plane of the membrane. In this model, MC540 penetrates in the water-hydrocarbon interface and it is assumed that the whole molecule must be reoriented prior to dimerization [2,15].

From the titration parameters and equilibrium data given in Table 2 it is possible to deduce a higher

monomer-dimer equilibrium constant for liposomes than for nicotinic AChR native membranes at all MC540/lipid ratios, with higher K_{eq} values for low total lipid/MC540 molar ratios. That is, the equilibrium is displaced towards the dimers in the presence of nicotinic AChR protein in the membrane. This could be a consequence of a decrease in lipid mobility in the presence of protein, making the membrane a poorer solvent for the partition of the monomers. The rigidity of the lipid phase can be increased in the fluid-to-gel transition, or alternatively by insertion of proteins in the membrane bilayer. In the presence of a rigid phase, the equilibrium is displaced to dimers because monomer is excluded from the fluid phase. In this case, the association of merocyanine molecules would take place in the plane of the membrane as a consequence of the reorientation of the monomer. In other words, the presence of a more rigid environment due to the protein-lipid interface would favour the hydrophobic interaction of the merocyanine molecules among themselves rather than with the lipid phase. The lipid annulus around the nicotinic AChR protein [33] (see reviews in [34,35]) is likely to be the locus of such interaction.

Temperature effects on the interaction of MC540 with nicotinic AChR membranes and refinement of the possible location of MC540 and benzocaine binding sites

It is known that the fraction of membrane lipids in the fluid state increases at higher temperatures, changing the physicochemical properties of the membrane. Conversely, the displacement of the equilibrium of MC540 to dimers in the membrane phase would be favoured by decreases in temperature. Since the overall dimerization process of MC540 is temperature-dependent [2], the 2-fold higher affinity of MC540 at 8°C than at 23°C (Fig. 4 and Table 1) might correspond either to a temperature-induced change in MC540 absorption or to a true monomer-dimer association-dissociation process.

The overall dimerization constant (K_{dim}) in nicotinic AChR membranes was also sensitive to temperature, and depended on the total lipid/dye ratio (Fig. 4B). The overall dimerization process is more pronounced at lower temperatures and is indicative of an enthalpic driven process for the MC540 reorientation step followed by dimerization, as suggested by the observations of Verkman and Frosch [2,15]. It seems reasonable to assume that the occurrence of MC540 dimers is therefore responsible for the higher affinity of the dye for nicotinic AChR membranes at lower temperatures (Fig. 4A and Table 1). It is also probable that dimers are oriented parallel to the plane of the membrane, and deeply embedded in the bilayer, such that an equilibrium between MC540 dimers in water and in the membrane is established, leading to a higher affinity of the dye for AChR membranes.

One can also distinguish the existence of two temperature regimes in the ligand-membrane interaction: a 'low temperature regime' exhibiting a saturation behaviour that could be interpreted as a consequence of a saturating partition of MC540 in the lipid annulus, and a 'high temperature regime' (Table 1), for which the ratio remains constant, at a relatively high value. This could be interpreted in terms of an increased number of lipid shells in the annulus surrounding the nicotinic AChR, with lipids in the liquid-crystalline state. There would be a sort of 'recruitment' effect, whereby a second (or more) shells in the annulus become more fluid at higher temperatures and allow more MC540 to be partitioned into the nicotinic AChR membrane. As expected, the less mobile lipid shell becomes more conspicuous at low temperatures [33].

Since both membranes and MC540 exhibit net negative charges, in order to minimize the Coulombic repulsion between the tail carrying the sulfonate group of the dye and the membrane, MC540 must be oriented outwards from the latter. Negatively charged sialic acid residues in the carbohydrate moiety of the five nicotinic AChR chains are possibly too distant (protruding into the extracellular space) from the polar head region-aqueous interface to pose any barrier to the MC540 molecule partitioning into the membrane itself. Thus, the driving force for the binding would arise from the interaction of the two tetramethylene tails of MC540 with the hydrophobic portion of the membrane [36].

Although it is possible that insertion into or adsorption onto the membrane bilayer of *any* membrane protein could displace the monomer-dimer equilibrium of merocyanine by induction of a rigid phase, and that the effect could be different for different membrane proteins, it appears that in the case of the nicotinic AChR the perturbation induced in the membrane and detected by MC540 exhibits pharmacological specificity. Thus, the results obtained by displacement of MC540 by benzocaine indicate that this local anaesthetic can compete for MC540-membrane binding sites. The data show concurrence with previous results from our laboratory using ESR spectroscopy and native nicotinic AChR membranes from *Torpedo marmorata* [19]. Benzocaine binding sites also appear to overlap, at least partially, with those for spin-labelled cholestane, a cholesterol analogue [21], and phospholipid and fatty acid spin labels [22]. Thus, a variety of ligands seem to have in common with MC540 the ability to compete for a site(s), presumably hydrophobic in nature, on the surface of the nicotinic AChR protein [34,35]. Interestingly, the binding of MC540 to gel-phase membranes is affected by the amount of cholesterol (between 5 and 35% cholesterol/phospholipid ratio

[38]). Taking into account the putative location of MC540 dimers in the bilayer [37], it is possible to deduce the magnitude of one dimension of the benzocaine (and MC540) binding sites: they must reside close to the region where the hydrophobic portion of the acyl chains meet the interfacial polar head group of the phospholipids.

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6. References

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