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Effects of Local Anesthetics and Calcium on the Interaction of Cholinergic Ligands with the Nicotinic Receptor Protein from *Torpedo marmorata*

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SUMMARY

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Studies are presented of the interaction in a physiological ionic environment of aromatic amine local anesthetics (prilocaine, lidocaine, and dimethisoquin) and Ca^{++} with receptor-rich membrane fragments isolated from *Torpedo* electric organ. The environmentally sensitive fluorophore 1-(5-dimethylaminonaphthalene-1-sulfonamido)ethane 2-trimethylammonium iodide (DNS-chol) interacts with two classes of sites in the membrane fragments: the cholinergic receptor site and secondary sites characterized by probe emission properties (λ_{max}) sensitive to the pharmacological nature (agonist or antagonist) of the cholinergic ligand bound to the receptor site. Fluorescence studies show that the local anesthetics cause an *increase* of affinity of the membrane-bound receptor for DNS-chol and for cholinergic ligands, both agonists and antagonists. The increase of affinity is not associated with a change of DNS-chol emission properties. At the same concentrations at which the anesthetics control receptor affinity, they also affect the fluorescence of DNS-chol bound to the secondary sites: the presence of a local anesthetic causes a loss of the DNS-chol spectral properties characteristic of the binding of agonists to the receptor site. Local anesthetics also control the binding of [^{3}H]acetylcholine to the membrane-bound receptor. In the absence of prilocaine the acetylcholine binding curve is slightly sigmoid (Hill coefficient, n_H = 1.4, half-saturation at 10 nM free acetylcholine). In the presence of 3 mM prilocaine there is a decrease of cooperativity and an increase of affinity (n_H = 1.0, half-saturation at 6 nM free acetylcholine). The concentrations at which the local anesthetics act on the membrane fragments are those at which they block the permeability response of *Electrophorus* electroplax upon addition to the bath of the agonist carbamylcholine. Fluorescence and radioactive ligand assays demonstrate that Ca^{++} also causes an *increase* of receptor affinity for cholinergic ligands, but in a manner significantly different from that observed with local anesthetics. Solubilization of membrane fragments by detergent leads to changes in the binding properties of the receptor protein. On the membrane fragments the binding data for each agonist can be analyzed in terms of a homogeneous population of sites, while after solubilization heterogeneity of the binding con-

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stants appears. Prilocaine or Ca^{++} no longer affects the binding of acetylcholine to the solubilized receptor protein. The observed effects of local anesthetics and Ca^{++} on the affinity of the cholinergic receptor are related to the phenomenon of receptor desensitization.

INTRODUCTION

Several classes of pharmacological agents are known to regulate the cationic permeability of the postsynaptic membrane from fish electroplax (1). The agonists, which include the physiological transmitter acetylcholine, cause an increase of permeability to Na^+ and K^+ ions both *in vivo*, on the monocellular electroplax, and *in vitro*, on excitable microsacs prepared from *Electrophorus* electric organ (2). The antagonists, such as *d*-tubocurarine or gallamine, block the effect of the agonists by increasing the apparent dissociation constant for the agonist with little (3) or no (4) change of the maximal response (competitive effect). The local anesthetics, on the other hand, block the increase of permeability (5) caused by the agonists, largely by reducing the maximal response with little effect on the apparent dissociation constant (noncompetitive effect). Finally, Bartels and Nachmansohn (6, 7) have studied a group of compounds with structures intermediate between those of the agonists and the local anesthetics, some of which, typified by benzoylcholine, show a dual action on *Electrophorus* electroplax and behave both as agonists and as noncompetitive blocking agents.

To analyze further the mechanism by which these various pharmacological agents control the permeability of the postsynaptic membrane to alkali cations, we have used the electric organ of *Torpedo marmorata* instead of that from *Electrophorus*. With *Torpedo* no monocellular electroplax preparation has yet been developed, but the high density of innervation of the electroplax and its high content of cholinergic receptor makes the electric organ from this fish a better source of biochemical material (8). In the membrane fragments from *Torpedo* we have used, the cholinergic receptor constitutes about 30% of the proteins (9). These fragments originate most likely from the areas of the cytoplasmic membrane which underlie the nerve terminals (10, 11). With this membrane preparation the structural properties of the membrane-bound receptor can be investigated by electron microscopy (12, 13) and by X-ray diffraction (14). Conditions have been defined under which *Torpedo* microsacs preserve the ability to respond *in vitro* to cholinergic agonists by an increase of permeability to $^{22}Na^+$ (15).

Furthermore, the specific binding of acetylcholine and decamethonium (16) to the cholinergic receptor site present in these membrane fragments has been characterized. This binding can be blocked by cholinergic antagonists, by *Naja* α-toxins (see ref. 17), and also by high concentrations of local anesthetics. However, the anesthetic concentrations necessary to prevent acetylcholine binding are much higher than those at which they prevent the agonist-induced depolarization of the eel electroplax. It was concluded that the local anesthetics block the response to cholinergic agonists by binding to sites (local anesthetic binding sites) topographically distinct from the cholinergic receptor site (18).

Thanks to the introduction of a cholinergic fluorophore sensitive to environment, 1-(5-dimethylaminonaphthalene-1-sulfonamido)-ethane 2-trimethylammonium iodide (19), it then became possible, with the receptor-rich membrane fragments from *Torpedo*, to relate the physiologically important response to the agonists to a characteristic change of membrane structure (20). In these experiments, and in several of those presented in this paper, the interaction of DNS-chol[2] with

[2] Abbreviations used are: DNS-chol, 1-(5-dimethylaminonaphthalene-1-sulfonamido)ethane 2-trimethylammonium iodide [the same compound was used previously (20, 21) but was incorrectly identified as a propane derivative]; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; SDS, sodium dodecyl sulfate; Tetram, *O*,*O*'-diethyl *S*-(β-diethylamino)ethyl phosphorothiolate; SKF 525-A, diethylamino ester of diphenylpropylacetic acid; gallamine, gallamine triethiodide (Flaxedil).

the membrane fragments was followed by energy transfer from the membrane proteins (λ_{ex} = 287 nm). Analysis of the effect of cholinergic ligands and *Naja* α-toxin on the fluorescence of DNS-chol emitted under these conditions led to the conclusion that DNS-chol binds to at least two classes of membrane sites: the cholinergic receptor site and "secondary" sites located on or in the vicinity of the receptor protein.

At micromolar concentrations of DNS-chol and cholinergic receptor sites, the fluorescence decreases by 70% in the presence of cholinergic ligands and α-toxin. This fluorescence decrease was attributed to the displacement of DNS-chol from the cholinergic receptor site. The spectroscopic dissociation constant of DNS-chol calculated from the data (K_{fluo} = 20 μM) agrees with the dissociation constant determined indirectly via the binding of radioactive ligands. The fluorescence of DNS-chol associated with its secondary sites represents only a small fraction of the total fluorescence detected by energy transfer and is observed under conditions in which DNS-chol is displaced from the receptor site, i.e., in the presence of an excess of cholinergic ligands or *Naja* α-toxin. When the cholinergic ligand is an antagonist or *Naja* α-toxin, maximum emission takes place at 537 $\pm$ 3 nm; in the presence of an excess of agonist the maximum emission shifts toward the blue by about 15 nm. On the basis of this result, it was concluded that upon binding of cholinergic agonists some change of membrane structure takes place which is reversed by the antagonists and hence is associated with the physiological response of the membrane.

We report here studies on the effect of local anesthetics and calcium ions on receptor-rich membrane fragments as sensed by the fluorescent properties of DNS-chol and the binding of radioactive cholinergic ligands. In a first series of experiments done with the isolated electroplax from *Electrophorus*, we show that DNS-chol has a dual pharmacological action and behaves like benzoylcholine, both as an agonist and as a noncompetitive blocking agent or local anesthetic. Since DNS-chol binds *in vitro* both to the cholinergic receptor site and to secondary sites, we investigated the possibility that these secondary sites were responsible for the noncompetitive blocking action of DNS-chol on the electroplax; in other words, that they were related to the local anesthetic binding site.

We have found that local anesthetics do decrease the fluorescence intensity of DNS-chol associated with the secondary sites. Moreover, we show an apparent paradoxical effect of several local anesthetics on the binding properties of the cholinergic receptor site present in the same membrane fragments: all of them cause a marked increase of affinity of the receptor site for DNS-chol and for the cholinergic agonists and antagonists tested. This effect takes place at concentrations of local anesthetics which are just those necessary to block the agonist-induced depolarization of *Electrophorus* electroplax. Detailed studies of the binding of [^{3}H]acetylcholine show that one of them, prilocaine, changes the shape of the binding curve from sigmoid to hyperbolic. Calcium ions also enhance the affinity of the membrane-bound receptor for cholinergic ligands, but via a different mechanism. After solubilization of the membrane fragments by detergents, local anesthetics and calcium ions no longer control the binding properties of the cholinergic receptor site. A preliminary report of this work has been published (21).

MATERIALS AND METHODS

Preparation of Receptor-Rich Membrane Fragments

The isolation by sucrose density centrifugation of membrane fragments rich in cholinergic receptor has been described (9). The binding of [^{3}H]α-toxin from *Naja nigricollis* (22) was used to estimate the concentration of cholinergic receptor sites (16). Proteins were assayed by the method of Lowry *et al.* (23) with bovine serum albumin as standard. Sixty grams of fresh electric tissue from *Torpedo marmorata* yield about 6 mg of proteins in fragments that band at 38% (w/v) sucrose. These fragments contain 1–2 μmoles of [^{3}H]α-toxin binding sites per gram of protein.

As described previously (20), an additional

centrifugation-resuspension procedure was used to transfer the membranes from concentrated sucrose to a suitable ionic environment, which for most spectroscopic experiments was *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. When the effect of divalent cations on the binding properties of the membrane-bound cholinergic receptor was studied, the membrane fragments were resuspended in a saline solution free of divalent cations: 250 mM NaCl, 5 mM KCl, 0.1 mM EGTA, and 5 mM sodium phosphate, pH 7.0 (Ca^{++}- and Mg^{++}-free buffer).

In order to characterize the polypeptide composition of the purest membrane fragments available, polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed with fragments prepared by the following method. First 20 ml of the low-speed supernatant fraction were layered on 5 ml of 35% sucrose (w/v) and centrifuged at 105,000 × *g* for 90 min at 4°. The pellet (0.9 μmole of toxin sites per gram of protein) was resuspended in double-distilled water with a Potter Teflon-glass homogenizer. The resuspension (0.5 mg of protein per milliliter) was then placed on a linear sucrose gradient, centrifuged, and collected as described by Cohen *et al.* (9). Maximal toxin binding activity was found at 38% (w/v) sucrose.

SDS-polyacrylamide gel electrophoresis of these fragments demonstrated that their polypeptide composition is relatively simple. Figure 1 shows a gel scan of a membrane sample possessing high specific activity (3.5 μmoles of α-toxin binding sites per gram of protein). Above mol wt 40,000 there are five major bands, with the predominant band at 44,000. For preparations of lower specific activity (1 μmole/g of protein) the same major bands are observed, but the relative dominance of the 44,000 band is reduced.[3]

[3] Note added in proof: This 44,000 band is the only one labeled by the affinity reagent 4-(N-maleimido)-benzyltri[^{3}H]methyl ammonium iodide developed by Karlin and Cowburn (45). This labeling is prevented by preincubation of the membranes fragments with *Naja* α-toxin (A. Sobel, unpublished results).

Solubilization of Receptor-Rich Membrane Fragments

Solubilization of the membrane fragments can be achieved by the addition of anionic (sodium cholate) or neutral (Emulphogene BC-720 or Triton X-100) detergents. The memdrane fragments in 38% (w/v) sucrose were diluted in an equal volume of double-distilled water and centrifuged at 80,000 × *g*

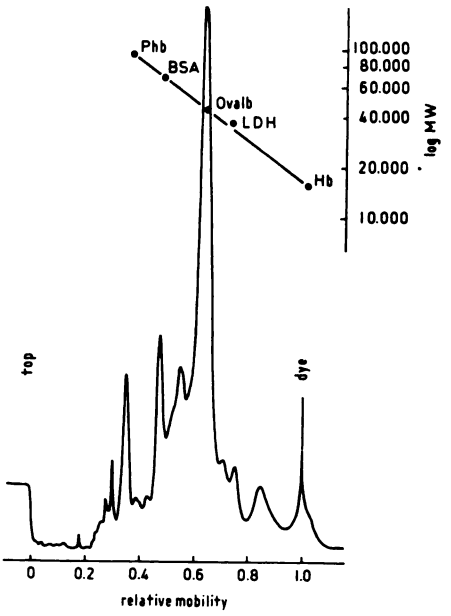

FIG. 1. *Polyacrylamide gel electrophoresis on sodium dodecyl sulfate of highly purified preparation of receptor-rich membrane fragments from Torpedo*

The suspension of membrane fragments contained 3.5 μmoles of *Naja* α-toxin sites per gram of protein (see MATERIALS AND METHODS). Polyacrylamide gel electrophoresis was performed according to Davies and Stark (24) with 0.1 M sodium borate buffer containing 0.1% sodium dodecyl sulfate adjusted to pH 8.5 with glacial acetic acid. A membrane sample (25 μg of protein) was boiled for 2 min in 2% sodium dodecyl sulfate–0.2% 2-mercaptoethanol. After migration (8 mamp/gel) the gels were shaken overnight in 25% 2-propanol–10% acetic acid, stained with Coomassie blue, and destained with 30% methanol 7.5% acetic acid. Standards were run on a separate gel. Ph b, rabbit muscle glycogen phosphorylase *b*; BSA, bovine serum albumin; Ovalb, ovalbumin; LDH, lactate dehydrogenase; Hb, hemoglobin.

for 75 or 90 min. The supernatant fluid was discarded, and the pellet was resuspended in a small volume of medium to a protein concentration of 2–6 mg/ml. Concentrated sodium cholate (50% w/v) was then added to a final concentration of 1–4% (w/v), with a mass ratio between 5 and 10 mg of cholate per milligram of protein. After incubation at room temperature for 5–30 min, the suspensions were centrifuged at 100,000 × *g* for 90 min. Solubilized receptor is defined operationally as the [^{3}H]α-toxin binding sites remaining in the supernatant fraction. For the solubilized receptor, [^{3}H]α-toxin binding was assayed by the procedure of Olsen *et al.* (25), and protein by the method of Lowry *et al.* (23) with bovine serum albumin in the presence of detergent as a standard. The yield upon solubilization by sodium cholate was 30–100% for the α-toxin binding sites and 80–100% for protein. No systematic difference in yield was observed when solubilization was achieved in various media (distilled water, *Torpedo* physiological saline solution with or without divalent cations, or 0.5 M NaCl–0.1 M Tris-HCl, pH 7.4).

Fluorescence Experiments

All fluorescence spectra were measured at 21° using a FICA differential recording spectrofluorometer under conditions described previously (20). A Hamamatsu photomultiplier, model R372 UH, was used to detect signal emission, and no corrections were made for the wavelength variation of the photomultiplier sensitivity. All spectra presented and all determinations of the wavelength of maximum emission were obtained by differential spectroscopy. Both cells contained the receptor preparation (0.3 ml), and DNS-chol was added to the sample cell. Other reagents were added to both sample and reference cells.

Two fluorescence assays were used to study the interaction of DNS-chol with the receptor preparations. The apparent affinity of DNS-chol for the acetylcholine receptor site under various environmental conditions was determined from the dependence of fluorescence on DNS-chol concentration in the absence and presence of high concentrations of a high-affinity cholinergic ligand (wavelength of excitation, λ_{ex} = 287 nm; wavelength of emission, λ_{em} = 550 nm). For DNS-chol concentrations up to 50 μM in the presence of 1 mM carbamylcholine or 5 μM *Naja* α-toxin, all cholinergic receptor sites are occupied by the latter ligands. The difference of DNS-chol fluorescence intensity observed in their presence and absence is a measure of the fluorescence of DNS-chol bound to the receptor site (20).

These experiments were analyzed by a double-reciprocal plot of the total DNS-chol concentration and the magnitude of the fluorescence signal sensitive to the cholinergic ligands. The abscissa intercept in such a plot is $(-1/K_{fluo})$, where K_{fluo} is a "spectroscopic" dissociation constant (20), and the ordinate intercept measures the maximum signal when all sites are occupied by DNS-chol.

A second parameter, R, was used in the course of these studies. It is the ratio of the light intensity, I_{287}, observed at 550 nm for excitation at 287 nm (protein excitation) to the intensity, I_{330}, observed for excitation at 330 nm (DNS-chol excitation): $R = I_{287}/I_{330}$. The absolute value of R must be an empirical function of the spectrometer, but for a particular spectrometric configuration relative changes of R observed in the presence of different perturbants (solvent, proteins) must reflect a change in the wavelength structure of the excitation spectrum. For DNS-chol in physiological saline, R = 0.43 ± 0.03. The same value was observed in the presence of up to 1 mg/ml of serum albumin or up to 5% detergent (sodium cholate, Emulphogene BC-720). Values greater than R = 0.43 were used as a measure of the preferential excitation of DNS-chol by energy transfer.

A fluorescence assay (λ_{ex} = 287 nm, λ_{em} = 550 nm) was used to determine the apparent affinity of the receptor for nonfluorescent cholinergic ligands relative to that of DNS-chol. For a fixed concentration of DNS-chol (usually 7.2 μM), increasing concentrations of a second ligand were added to the receptor preparations until maximum DNS-chol fluorescence decrease was observed. C_{50}, the ligand concentration reducing DNS-chol fluorescence by 50% of its total decrease, was used to characterize the affinity of the ligand for the receptor (20). For these ex-

periments concentrated stock solutions of ligands were used so that the initial volume (0.3 ml) was not increased by more than 5% over the titration curve.

Binding of Radioactive Ligands

Binding at equilibrium of [^3H]acetylcholine and [^3H]decamethonium to membrane fragments. This was measured by the ultracentrifugation method described by Weber and Changeux (16). The membrane suspension was always diluted in *Torpedo* physiological saline solution before the addition of ligand.

When the effect of local anesthetics was studied, the diluted suspension was incubated for at least 30 min with the desired concentration of anesthetic, before the addition of ligand. For divalent cations the membrane suspension was diluted first in Ca^{++}- and Mg^{++}-free buffer and then supplemented with the desired concentration of $CaCl_2$ and $MgCl_2$.

When [^{3}H]acetylcholine was used as a ligand, the diluted membrane suspension was first incubated for 30 min with 0.1 mM Tetram, a potent cholinesterase inhibitor (26). It was verified that under these conditions inhibition of [^{3}H]acetylcholine binding occurs only above 0.2 mM Tetram. After the incubation with Tetram [^{3}H]acetylcholine was added to the mixture. The total concentration of [^{3}H]acetylcholine was varied between 1.5 and 60 nM by changing the concentration of labeled ligand without isotopic dilution. Since the solutions of [^{3}H]acetylcholine were made in ethanol, the membrane suspensions always contained 0.16% ethanol. It was ascertained that up to 1% (v/v) ethanol has no significant effect on the binding of [^{3}H]acetylcholine.

The membrane suspension was equilibrated for 10–30 min with the radioactive ligand. The radioactivities of the media before and after centrifugation at 100,000 $\times$ g for 90 min were counted on 400-μl samples in 10 ml of Multisol (Intertechnique) or Unisolve (Koch-Light Laboratories) in an Intertechnique scintillation counter. The efficiency of counting was 40 $\pm$ 3%.

Initial rate of [^3H]α-toxin binding to membrane fragments. This was measured by filtration on Millipore filters, as described by Weber and Changeux (16). The concentration of [^{3}H]α-toxin in the media was 0.6 nM, and that of [^{3}H]α-toxin binding sites was 1.2 nM. Under these conditions the amount of toxin bound was linear with time for at least 8 min.

Binding of [^3H]acetylcholine to solubilized receptor protein. We used the equilibrium dialysis technique described by Gilbert and Müller-Hill (27). Samples of solubilized receptor protein (250–300 μl) were first incubated for 30 min in the presence of 0.1 mM Tetram and placed in small dialysis bags. The bags were dialyzed for 18 hr at 4° on a rocking shaker against 20 ml of buffer supplemented with 1% (w/v) sodium cholate, 0.01 mM Tetram, and the desired concentration of [^{3}H]acetylcholine. The buffer used was either *Torpedo* physiological saline solution or Ca^{++} and Mg^{++}-free buffer supplemented with the desired concentration of $CaCl_2$. Local anesthetics were added both inside and outside the bags.

After dialysis, radioactivities were counted on 50–75-μl samples in 10 ml of Unisolve. Protein concentration inside the bag was measured by the method of Lowry *et al.* (23), using bovine serum albumin as standard. No significant dilution was observed during dialysis. In these experiments little (<10%) or no receptor inactivation occurred during dialysis, as measured by [^{3}H]α-toxin binding in control experiments.

Chemical Products

The iodide salt of DNS-chol was prepared as described by Weber *et al.* (19). Cholinergic ligands and local anesthetics obtained commercially were used without further purification. The hydrochloride salts of prilocaine, lidocaine, and dimethisoquin were gifts of Laboratoire Roger Bellon, Neuilly, France. The purified α-toxin of *Naja nigricollis* was a gift of Dr. P. Boquet, and the tritiated [^{3}H]α-toxin, a gift of Drs. A. Menez, J. L. Morgat, and P. Fromageot.

Choice of Local Anesthetics

Since we wished to study by fluorescence techniques the effect of local anesthetics on the interaction between DNS-chol and the receptor-rich membrane fragments, anesthetics that do not have undesirable optical

properties of their own had to be found. Ideal compounds would have negligible absorption and fluorescence above 270 nm. The spectral characteristics of six local anesthetics were surveyed (Table 1). Prilocaine and lidocaine have very little absorption and no detectable fluorescence above 270 nm. The other four compounds have absorption bands above 280 nm and are fluorescent. Figure 2 shows the effects of different local anesthetics on free DNS-chol fluorescence. The quenching caused by procaine, tetracaine, dimethisoquin, and prilocaine reflects their extinction coefficients at 287 nm. Thus, although lidocaine can be studied at concentrations up to 20 mM, above 3 mM prilocaine optical density effects become noticeable. Similarly, 0.1 mM dimethisoquin and 0.02 mM procaine or tetracaine are the upper concentrations at which they are "transparent."

It is apparent (18, 28, 29) that the physiologically active concentrations of procaine and tetracaine exceed 0.02 mM. However, for lidocaine, prilocaine, and dimethisoquin, physiologically important concentrations are accessible without expected interference with spectroscopic measurements.

Structural formulae of DNS-chol and the three local anesthetics to be used in this study are presented in Fig. 3. Lidocaine and dimethisoquin are tertiary amines, the former with an amide linkage and the latter with an ether linkage to the aromatic portion. Prilocaine is a secondary amine with an amide linkage.

Choice of Detergents

It is known that a variety of neutral and anionic detergents can be used to solubilize cholinergic receptor from electric tissue in forms that retain the capacity to bind *Naja* α-toxins and cholinergic ligands (30). For fluorescence studies with the solubilized cholinergic receptor, aromatic detergents such as Triton are unacceptable because of their intense intrinsic fluorescence. Cholate and Emulphogene BC-720 do not possess chromophores interfering with fluorescence studies for excitation above 280 nm. However, the fluorescence properties of DNS-chol are affected by the presence of detergents (Fig. 4). DNS-chol quantum yield increases with increasing detergent concentrations, and the increase of quantum yield is accomapnied by a blue shift of the emission maximum. At 1% sodium cholate the fluorescence intensity increases by about a factor of 3 and the emission maximum shifts from 553 to 540 nm.

Although the detergents cause a small shift of excitation maximum toward the red (not shown), the basic structure of the excitation spectrum is not changed. For emission at 550 nm, $R = I_{287}/I_{330}$ remains 0.43 ± 0.03 for sodium cholate or Emulphogene up to 5%.

TABLE 1

Spectral characteristics of local anesthetics in physiological saline solution

Anesthetic	Absorption			Fluorescence	
	λ_{max}	ϵ_{max}[a]	ϵ_{287}	λ_{max}[b]	Relative intensity[b]
	nm	$M^{-1}\ cm^{-1}$	$M^{-1}\ cm^{-1}$	*nm*	
Prilocaine	230	5,600	$\leqq 100$		0
Lidocaine	270	310	$\leqq 6$		0
Dimethisoquin	270 (320)	8,000 (4,000)	3,200	360	1.0
Dibucaine	328	4,300	3,000	420	0.7
Tetracaine	310	23,000	15,000	355	4.0
Procaine	290	17,000	17,000	355	0.3

[a] Extinction coefficient of major absorption bands at $\lambda \geqq 250$ nm, except for prilocaine, for which there is only a short wavelength shoulder at 230 nm.

[b] Uncorrected for emission photomultiplier wavelength sensitivity.

RESULTS

Dual Pharmacological Action of DNS-chol on Electrophorus Electroplax

All our fluorescence and binding studies utilized membrane fragments from *Torpedo*. However, no monocellular electroplax preparation has yet been developed with this fish. The pharmacological action *in vivo* of DNS-chol was therefore studied with *Electrophorus* electroplax by following steady-state membrane potentials according to Higman *et al.* (4). Bath application of 10–30 μM DNS-chol to the innervated face of the electroplax causes only a small depolarization (less than 5 mV); at higher concentrations the depolarization becomes negligible. Study of the combined action of low concentrations of DNS-chol and of the agonist carbamylcholine shows that DNS-chol has a dual action (Fig. 5). In the presence of carbamylcholine at concentrations lower than 30 μM, DNS-chol *potentiates* by as much as 200% the depolarization caused by this agonist. At higher concentrations of carbamylcholine DNS-chol *decreases* the response to carbamylcholine in a noncompetitive manner. A similar dual action has also been reported for another aromatic cholinergic agent, benzoylcholine (6).

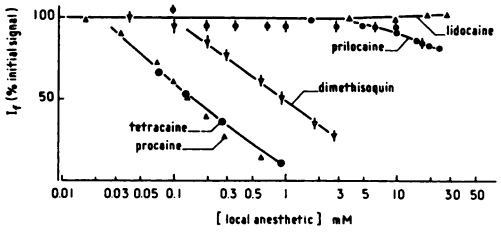

FIG. 2. *Effect of local anesthetics on DNS-chol fluorescence intensity in physiological saline solution*

Excitation, 287 nm; emission, 550 nm. Physiological saline solution was present in both the sample and reference cells. DNS-chol (7.2 μM) was added to the sample cell, and local anesthetics to both cells. The decrease of DNS-chol fluorescence intensity was not accompanied by any change in wavelength of maximum emission (λ_{max} = 553 nm) for prilocaine concentrations up to 20 mM or for tetracaine concentrations to 70 μM. The DNS-chol excitation spectrum is distorted because of the optical density of the concentrated anesthetic solutions. I_f = relative fluorescence intensity.

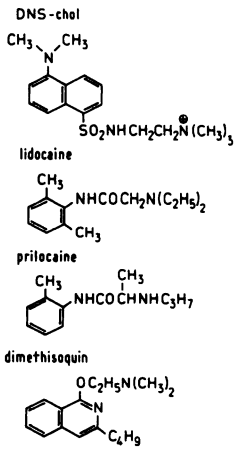

FIG. 3. *Structures of compounds used*

For comparison we show in Fig. 6 the effects of two typical local anesthetics used in this work, lidocaine and prilocaine. Both behave as noncompetitive blocking agents. No potentiation of the response takes place at low concentrations of carbamylcholine, but the reduction of the depolarization by a given concentration of anesthetic appears somewhat larger at low than at high concentrations of agonist. For instance, 0.2 mM lidocaine reduces by only 33% the response caused by 100 μM carbamylcholine, but by 66% that given by 20 μM carbamylcholine. As a first approximation, we characterized the pharmacological action of the local anesthetics by the concentration which reduces by 50% the depolarization caused by 50 μM carbamylcholine. For both lidocaine and prilocaine that concentration is 0.2 mM; for dimethisoquin it is 2 μM (18).

Because of its dual action, the apparent affinities of DNS-chol as an agonist and as a noncompetitive blocking agent cannot be rigorously estimated. However, DNS-chol potentiates carbamylcholine action when present at 10 μM and reduces by 50% the depolarization caused by 100 μM carbamylcholine at around 40 μM. As a noncompetitive blocking agent DNS-chol appears as potent as tetracaine (5), or 10 times as potent as lidocaine or prilocaine.

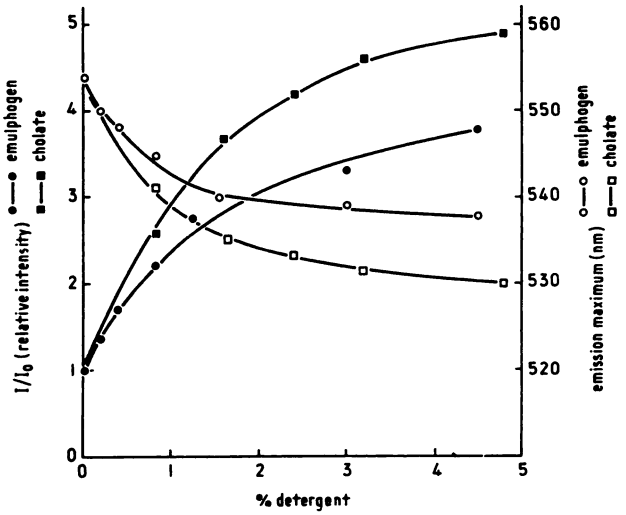

FIG. 4. *Emission properties of DNS-chol (7.2 μM) in physiological saline solution containing detergent*
Cholate concentrations are weight per volume; Emulphogene BC-720 concentrations are volume for volume. The intensities are expressed as the ratio of peak heights observed in the presence and absence of detergent. λ_{ex} = 340 nm.

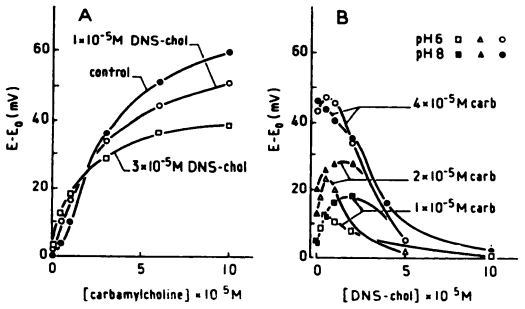

FIG. 5. *Pharmacological action of* DNS-*chol in-vivo*

A. Effect of DNS-chol on response of *Electrophorus* isolated electroplax to increased concentrations of carbamylcholine. The ordinate shows steady-state membrane depolarization recorded in the presence of a given concentration of carbamylcholine; E_0, resting potential (−75 mV). The bath solution was *Electrophorus* Ringer's solution (160 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 1.5 mM sodium phosphate, pH 7.0).

B. Effect of increasing DNS-chol concentration at pH 6 and 8 on steady-state membrane depolarization caused by fixed concentrations of carbamylcholine (*carb*). The bath solutions were *Electrophorus* Ringer's solution adjusted to pH 6 with 5 mM sodium phosphate, or to pH 8 with 2 mM Tris.

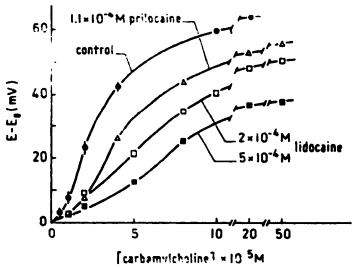

FIG. 6. *Effect of prilocaine and lidocaine on response of Electrophorus isolated electroplax to carbamylcholine*

The bath solution was *Electrophorus* Ringer's medium, pH 7.0.

The electroplax response to DNS-chol depends upon pH (Fig. 5). At pH 6 DNS-chol potentiates the response to carbamylcholine only up to 5 μM, while at pH 8 the same effect occurs up to 20 μM. Under these conditions the apparent dissociation constant of carbamylcholine does not change significantly (31). Hence increasing the pH reduces the blocking effect of DNS-chol, as it does for typical local anesthetics (32).

In this last case the effect of pH appears directly related to the ionization of the teritiary amine. However, the pK_a of the

tertiary amine of DNS-chol is probably close to 3 (44), while that of the typical local anesthetics lies usually near 8 (29). The effect of pH on the pharmacological action of DNS-chol might therefore be accounted for either by ionization of the sulfonamido nitrogen or by a change in the binding properties of the membrane sites.

Our previous spectroscopic studies (20) done *in vitro* with purified *Torpedo* membrane fragments showed that DNS-chol binds to at least two distinct classes of membrane sites: the cholinergic receptor site and secondary sites sensitive to the agonistic character of the cholinergic ligands bound by the cholinergic receptor site. Here we demonstrate that DNS-chol behaves on *Electrophorus* electroplax both as an agonist and as a noncompetitive blocking agent. To test the possibility that these two pharmacological actions are related to the two classes of sites demonstrated *in vitro*, further studies on the effect of local anesthetics on the binding properties of *Torpedo* receptor-rich membrane fragments were undertaken.

Effect of Local Anesthetics on Interaction of DNS-chol with Receptor-Rich Membrane Fragments Followed by Fluorescence Spectroscopy

Effect on DNS-chol interaction with secondary sites (in the presence of an excess of cholinergic ligands). Figure 7 shows a series of emission spectra of DNS-chol in the presence of membrane fragments, cholinergic ligands, and prilocaine. As already discussed (20), the decrease of DNS-chol fluorescence intensity caused by the addition of carbamylcholine (Fig. 7A) or *Naja* α-toxin (Fig. 7B) corresponds to the displacement of DNS-chol from the cholinergic receptor site. The wavelength of maximum emission of the spectrum recorded at high levels of a cholinergic ligand depends upon its pharmacological action as an agonist (λ_{max} = 525 nm) or as an antagonist (λ_{max} = 540 nm). The observed spectral shift was attributed to a change in the emission properties and/or the binding properties of DNS-chol interacting with a secondary class of sites present in the receptor-rich membrane fragments.

In order to examine the relationship between this secondary class of sites and the binding sites for local anesthetics, prilocaine was then added in the presence of excess carbamylcholine or *Naja* α-toxin. A further decrease of fluorescence intensity occurs. It is more pronounced in the presence of carbamylcholine than in the presence of α-toxin, but before the addition of prilocaine the residual intensity is larger with carbamylcholine. Above 1 mM prilocaine the signal intensities are the same in the presence of

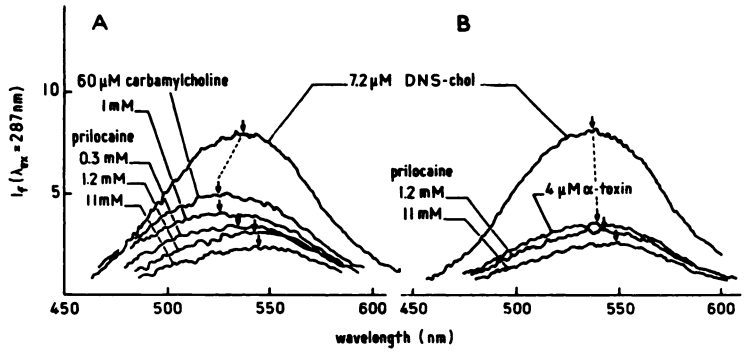

FIG. 7. *Differential fluorescence emission spectra of suspensions of Torpedo receptor-rich membrane fragments containing DNS-chol, cholinergic ligands, and prilocaine*

Membrane suspensions (0.4 µM *Naja* α-toxin sites, 0.4 g of protein per liter) in physiological saline were present in the sample and reference cells, and DNS-chol (7.2 µM) in the sample cell. The ordinate is relative fluorescence with excitation at 287 nm. The spectrum was initially recorded in the presence of DNS-chol (7.2 µM). Then, in A, carbamylcholine chloride was added to 60 µM and then 1 mM. Increasing concentrations of prilocaine were added to sample and reference cells as indicated. For B, *Naja* α-toxin (4 µM) was added to sample and reference cells, followed by prilocaine.

either ligand. The intensity decrease is accompanied by a clear shift of λ_{max} toward 553 nm, the emission maximum of DNS-chol in aqueous solution.

In Fig. 8A we present the variation of λ_{max} with prilocaine concentration under the experimental conditions of Fig. 7. In the absence of membrane fragments no change of λ_{max} occurs up to 15 mM prilocaine. Hence there is no direct interaction between prilocaine and DNS-chol. On the other hand, with the membrane suspension, λ_{max} varies with prilocaine concentration, and in a more pronounced manner in the presence of carbamylcholine than of α-toxin. Addition of 0.5 mM prilocaine to the membrane suspension containing carbamylcholine results in a shift of λ_{max} from 525 to 540 nm, the value

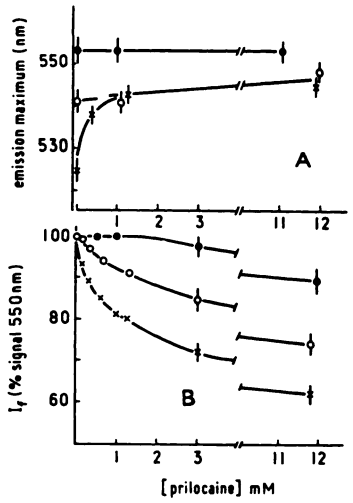

FIG. 8. *Effect of prilocaine on wavelength of maximum emission (A) and intensity of fluorescence (B) of DNS-chol when membrane suspension contained 1 mM carbamylcholine chloride* (×——×) *or 4 μM Naja α-toxin* (○——○)

Excitation at 287 nm; DNS-chol, 7.2 μM; conditions as in Fig. 7. In B the ordinate is the percentage of intensity prior to addition of prilocaine (i.e., in the presence of carbamylcholine or *Naja* α-toxin). Results are significant within ±3%. The difference between intensities in the presence of carbamylcholine and toxin for prilocaine concentrations greater than 3 mM reflects the different initial values; the observed intensities are the same in the two cases (see Fig. 7). Included are values for a control experiment with 7.2 μM DNS-chol in physiological saline (●——●).

observed initially in the presence of α-toxin. At higher prilocaine concentration λ_{max} shifts further toward the red in the presence of either ligand.

In Fig. 8B are compared the changes of fluorescence intensities which accompany the shifts in λ_{max} shown in Figs. 7 and 8A. In a control experiment done in the absence of membrane fragments, we first show that DNS-chol emission does not change up to 2 mM prilocaine. Above 2 mM prilocaine the decreased fluorescence intensity reflects the absorption of the incident light due to the significant optical density of the local anesthetic solution. Within the range of concentrations where this effect is absent, prilocaine decreases DNS-chol fluorescence intensity in the presence of membrane fragments and carbamylcholine or *Naja* α-toxin. The maximal decrease appears relatively smaller in the presence of α-toxin than in the presence of carbamylcholine. The concentrations of prilocaine which give half the effect caused by 2 mM prilocaine are 0.4 mM in the presence of carbamylcholine and 0.8 mM in the presence of α-toxin. These concentrations lie close to those which reduce by 50% the maximal response of *Electrophorus* electroplax to carbamylcholine, K_{app} = 0.2 mM. The effect of prilocaine on DNS-chol bound to its second class of sites appears significant under the same conditions as those of its pharmacological activity as a local anesthetic.

Analysis of the spectra recorded above 2 mM prilocaine indicates that even under these conditions DNS-chol still interacts with the membrane fragments. The spectral parameter $R = I_{287}/I_{330}$ (see definition in MATERIALS AND METHODS) decreases from 0.68 in the presence of 4 μM *Naja* α-toxin to 0.58 at 3 mM prilocaine. At 11 mM prilocaine R = 0.49, while for free DNS-chol in the absence of membrane fragments R = 0.41 at that concentration. Even at 11 mM prilocaine DNS-chol appears to be excited preferentially by energy transfer and, hence, to interact with the membrane fragments.

Results similar to those just described have been observed (not shown) with other cholinergic ligands and another local anesthetic. Decamethonium, an agonist, mimics

carbamylcholine; gallamine, an antagonist, behaves similarly to the *Naja* α-toxin. Also over the same range of concentrations as prilocaine, the anesthetic lidocaine causes similar shifts of λ_{max} and decreases of fluorescence intensity. Whether one considers the DNS-chol emission wavelength or intensity, in the presence of 3 mM prilocaine or lidocaine, the fluorescence of DNS-chol from the secondary sites is no longer sensitive to the pharmacological nature of the cholinergic ligand bound to the receptor.

These experiments were performed at a single DNS-chol concentration and at concentrations of cholinergic ligands which saturate the cholinergic receptor site. They show that the fluorescent properties of DNS-chol bound to the secondary class of sites is sensitive to the presence of local anesthetics at physiological concentrations; they do not distinguish, however, between a change of quantum yield associated with a modification of the environment of DNS-chol bound to the secondary sites and a displacement of DNS-chol from these sites.

Effect on DNS-chol interaction with cholinergic receptor site. We have previously shown that at sufficiently low DNS-chol concentrations excitation by energy transfer leads to a fluorescence emission of DNS-chol resulting primarily from molecules bound to the cholinergic receptor site (20). While typical cholinergic ligands decrease this fluorescence emission as a result of DNS-chol displacement from the receptor site, local anesthetics show the opposite effect. Increasing the concentration of prilocaine, dimethisoquin, and lidocaine (Fig. 9) causes an increase of DNS-chol fluorescence, followed by a more or less pronounced decrease of fluorescence intensity at higher concentrations. These three compounds are active within different ranges of concentrations and cause different maximal fluorescence increases. The maximal effects produced by dimethisoquin, prilocaine, and lidocaine are 18% at 5 μM, 26% at 4 mM, and 10% at 2 mM, respectively. Included for comparison in Fig. 9 are the effects of two cholinergic ligands, carbamylcholine and atropine. These two compounds cause only a decrease of DNS-chol fluorescence. Even at concentrations 10 times lower than those

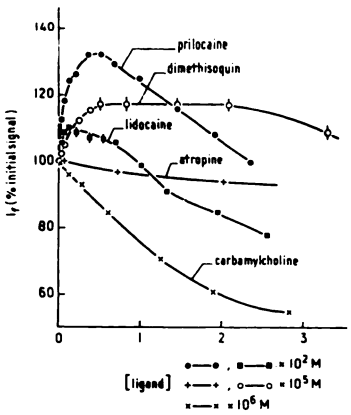

FIG. 9. *Concentration dependence of effect of local anesthetics on fluorescence of DNS-chol (7.2 μM) in the presence of suspension of receptor-rich membrane fragments (1.2 μM α-toxin sites, 0.8 g of protein per liter) in physiological saline solution*

λ_{ex} = 287 nm; λ_{em} = 550 nm. The intensities were determined by differential spectroscopy and represent probe fluorescence only. Intensities are expressed as a percentage of intensity observed prior to the addition of local anesthetic or cholinergic effectors. Three local anesthetics (prilocaine, dimethisoquin, and lidocaine) caused an increase of fluorescence while a potent (carbamylcholine) and a weak (atropine) nicotinic ligand caused only a decrease of intensity. Although not indicated, both atropine and carbamylcholine caused a maximal fluorescence decrease of 65%, and 0.5 ± 0.1 mM atropine or 1.3 ± 0.1 μM carbamylcholine was necessary to cause half that maximal decrease.

shown in Fig. 9, no evidence exists for an increase of fluorescence. Similar results have been observed (20) for other cholinergic ligands, both potent, such as acetylcholine, and weak, such as tetraethylammonium or choline.

The enhancement of DNS-chol fluorescence caused by local anesthetics was analyzed in detail in the case of prilocaine. In order to relate the observed effect to a possible change of affinity of the receptor site for DNS-chol, the concentration of DNS-chol was varied from 0.3 to 50 μM in both the presence and absence of *Naja* α-toxin (Fig. 10). Prilocaine causes an increase of fluorescence in the absence of α-toxin and a decrease in its presence. The experimental conditions were such that the differences in

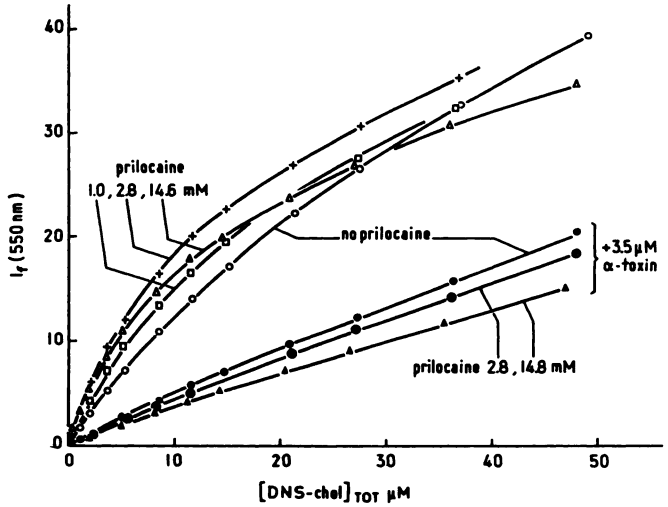

FIG. 10. *Effect of prilocaine on fluorescence of DNS-chol interacting with suspension of receptor-rich membrane fragments*

The sample and reference cells contained membrane suspension (0.85 μM *Naja* α-toxin sites, 0.5 g of protein per liter) in physiological saline. The indicated concentration of prilocaine and/or α-toxin were present in both cells prior to the addition of DNS-chol (TOT = total) to the sample cell. λ_{ex} = 287 nm; λ_{em} = 550 nm. The α-toxin concentration was such that all receptor sites were occupied by α-toxin under these conditions. In the absence of α-toxin the DNS-chol emission maximum was not sensitive to prilocaine: λ_{max} = 537 ± 3 nm for prilocaine concentrations up to 14.8 mM.

intensity measured in the absence and presence of toxin could be assigned to DNS-chol bound to the cholinergic receptor site (20). Figure 11 shows a double-reciprocal plot of this difference as a function of the total DNS-chol concentration at various prilocaine concentrations. All plots are linear from 2 to 50 μM DNS-chol. In the presence of prilocaine the slope of the straight line decreases with little change of the ordinate intercept. At 14.6 mM prilocaine a small decrease of the maximal fluorescence signal takes place, presumably as a consequence of a significant absorption of the prilocaine solution. The spectroscopic dissociation constant, K_{fluo}, determined from the abscissa intercept, decreases from 27 μM in the absence of prilocaine to 9 μM at 2.8 mM or 14.6 mM prilocaine. The apparent affinity of DNS-chol for the cholinergic receptor site therefore increases up to 3-fold in the presence of anesthetic. The concentration of prilocaine which gives a half-maximal increase of apparent affinity is 0.3 mM (Fig. 12), again a value close to its apparent dissociation constant (K_{app} = 0.2 mM) measured *in vivo* on *Electrophorus* electroplax.

Effects of Local Anesthetics on Interaction of Several Cholinergic Ligands with Receptor-Rich Membrane Fragments

Fluorescence assay with DNS-chol. At a fixed concentration of DNS-chol the decrease of its fluorescence caused by increasing concentrations of cholinergic ligands reflects the binding of these ligands to the receptor site. We have used this indirect method to study the effect of local anesthetics on the binding of cholinergic agonists and antagonists to the receptor.

For cholinergic ligands (acetylcholine, *Naja* α-toxin) whose dissociation constants ($K_d \leqq$ 10 nM) are much smaller than the DNS-chol dissociation constant and the concentration of receptor sites, the DNS-chol displacement curve becomes linear. The end point of the displacement curve determines the number of binding sites of the high-affinity ligand (20). Prilocaine concentrations up to 10 mM do not change the end point of the curves obtained with *Naja* α-toxin and acetylcholine. Thus prilocaine does not change the number of binding sites for either ligand.

For cholinergic effectors possessing a lower affinity ($K_d \geqq 1$ μM) the fluorescence assay becomes convenient for determining the parameters of their interaction with the receptor site. The fluorescence intensity varies in a hyperbolic manner with the concentration of effector added. Since the dissociation constant of DNS-chol for the receptor is 20 μM at the concentration (5 μM) of free DNS-chol used for the assay, the ligand concentration, C_{50}, which produces a half-maximal decrease of DNS-chol fluorescence is an approximate measure of the dissociation constant, K_d, of the ligand for the receptor (20). Figure 13 shows such a displacement experiment in the case of an agonist, decamethonium. In the absence of prilocaine C_{50} is 1.5 μM, a value close to the dissociation constant of decamethonium measured directly in the absence of DNS-chol (16). When the concentration of prilocaine increases, C_{50} *decreases* to 1.0 μM at 3.3 mM prilocaine. In Fig. 14B C_{50} for decamethonium is plotted as a function of prilocaine concentration. The concentration of prilocaine which gives a half-maximal decrease of C_{50} is again 0.3 mM.

Similar results have been observed (Table 2) for other cholinergic ligands. Prilocaine decreases the C_{50} both for agonists such as carbamylcholine and for antagonists like gallamine and *d*-tubocurarine. The decreases of C_{50} never exceed a factor of 2, and with tetraethylammonium no effect was observed.

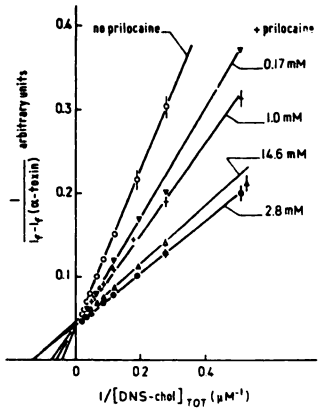

FIG. 11. *Effect of prilocaine on fluorescence of DNS-chol bound to cholinergic receptor site in receptor-rich membrane fragments*

The data are taken from the experiment of Fig. 10. The DNS-chol fluorescence associated with the acetylcholine binding site is the difference between intensities observed in the absence and presence of saturating quantities of *Naja* α-toxin. The ordinate is the reciprocal of that intensity; the abscissa is the reciprocal of total DNS-chol concentration.

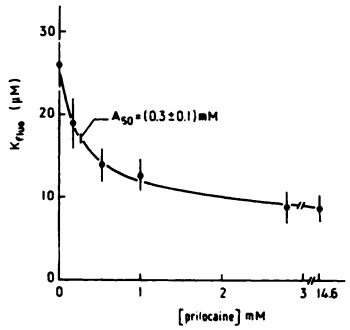

FIG. 12. *Effect of prilocaine on spectroscopic dissociation constant, K_{fluo}, of DNS-chol for cholinergic receptor site*

K_{fluo} was determined from the intercept with the abscissa in Fig. 11. A_{50} is the concentration of prilocaine that causes 50% of the observed total decrease of K_{fluo}.

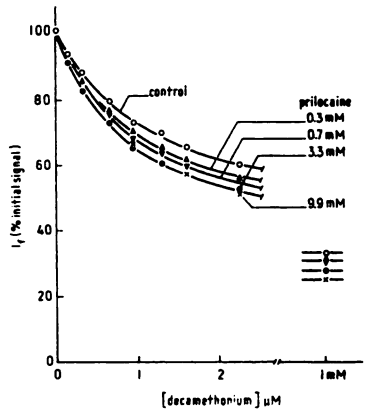

FIG. 13. *Effect of decamethonium on fluorescence of DNS-chol in receptor-rich membrane suspension (0.65 μM Naja α-toxin sites, 0.55 g of protein per liter) in the presence of different concentrations of prilocaine*

Membrane suspension in physiological saline; 7.2 μM DNS-chol. λ_{ex} = 287 nm; λ_{em} = 550 nm. Membranes were first incubated with the indicated prilocaine concentrations prior to the addition of decamethonium chloride. The ordinate is the ratio of the signal intensity observed in the presence of decamethonium to that observed initially in the presence of prilocaine only.

Another local anesthetic, lidocaine, causes a decrease of C_{50} for cholinergic ligands.

All the experimental data obtained by the fluorescence assay indicate that local anesthetics within a given range of concentration cause a decrease of C_{50} and, hence, an increase of affinity of the cholinergic receptor site for its specific ligands. It should be noted that the observed decrease of C_{50} is smaller than the increase of ligand affinity, because the effect of local anesthetics on the receptor site is an increase of affinities both for DNS-chol (which would tend to increase the observed C_{50}) and for the cholinergic ligand (which would tend to decrease C_{50}).

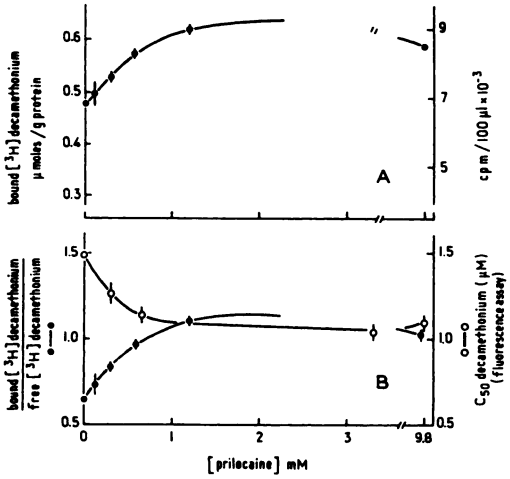

FIG. 14. *Effect of prilocaine on the binding of decamethonium to the cholinergic receptor site.*

A. Effect of prilocaine on binding of [^{3}H]-decamethonium chloride to membrane fragments. Membrane fragments [0.8 μM *Naja* α-toxin sites, 0.5 g of protein per liter in physiological saline solution, also containing 15% (w/v) sucrose] were incubated with [^{3}H]decamethonium chloride (400 Ci/mole, Radiochemical Centre), 0.54 μM. The suspensions were then incubated for 30 min in the presence of prilocaine. The free decamethonium in equilibrium with the membrane fragments was determined from the supernatant radioactivity after ultracentrifugation at 140,000 × *g* for 60 min at 15°. Bound decamethonium was determined from the difference between total and free decamethonium. At least 95% of the bound decamethonium was displaced by 2 μM α-toxin.

B. Comparison of effects of prilocaine on displacement of DNS-chol by decamethonium (fluorescence assay) and on binding of [^{3}H]-decamethonium (ultracentrifugation). O——O, concentration of decamethonium required to cause 50% of the maximal decrease of DNS-chol fluorescence (from Fig. 13); ●——●, ratio of bound to free [^{3}H]decamethonium (experiment of Fig. 14A).

Binding of [^{3}H]decamethonium. The binding of [^{3}H]decamethonium to the membrane fragments was followed by ultracentrifugation. The experimental conditions were such that all bound decamethonium could be displaced by stoichiometric amounts of α-toxin and, therefore, was specifically associated with the cholinergic receptor site (16). Fig. 14A shows the effect of prilocaine on [^{3}H]-decamethonium binding when the total decamethonium concentration was fixed at 0.5 μM. Under these conditions the fraction of receptor sites occupied by decamethonium was initially 20%. The addition of prilocaine up to 2 mM increases the amount of decamethonium bound to the membrane fragments.

TABLE 2

Effect of prilocaine on interaction of cholinergic ligands with cholinergic (nicotinic) receptor present in Torpedo membrane fragments

A fluorescence assay was used to determine the affinities of the cholinergic ligands (see the text). C_{50}^0 indicates the concentration of cholinergic ligand necessary to cause a half-maximal decrease of DNS-chol (7.2 μM) fluorescence in the absence, and C_{50}, in the presence, of prilocaine.

Ligand	C_{50}^0	C_{50} (0.7 mM prilocaine)/C_{50}^0	C_{50} (3.3 mM prilocaine)/C_{50}^0
	M		
Carbamylcholine	2.2×10^{-6}	0.75[a, b]	
Decamethonium	1.5×10^{-6}	0.75[a, b]	0.7[a]
Gallamine	9.0×10^{-6}	0.6[a]	0.5[c]
d-Tubocurarine	0.7×10^{-6}	0.5[b]	
Tetraethyl-ammonium	0.3×10^{-3}		1.0[c]

[a] The membrane suspension in physiological saline contained 0.6 μM α-toxin sites (0.7 g of protein per liter).

[b] The membrane suspension in physiological saline contained 0.3 μM α-toxin sites (0.6 g of protein per liter).

[c] The membrane suspension in physiological saline contained 0.7 μM α-toxin sites (0.5 g of protein per liter).

At 10 mM prilocaine this amount begins to diminish. The dissociation constant of decamethonium bound to the receptor site estimated from these data is 0.9 ± 0.2 μM in in the absence of prilocaine and 0.5 ± 0.1 μM at 1.2 mM prilocaine. In Fig. 14B the ratio of bound to free decamethonium for various concentrations of prilocaine is compared with the C_{50} value determined by the fluorescence assay at the same prilocaine concentration. In both cases 0.3–0.4 mM prilocaine caused about 50% of the maximal effect observed.

Because of the rather low affinity of decamethonium for the receptor site, the enhancement of affinity caused by local anesthetics was studied in greater detail in the case of acetylcholine, whose binding can be followed with rather high precision.

Binding of [^3H]acetylcholine. Since the purified membrane fragments from *Torpedo* contain non-negligible amounts of acetylcholinesterase, binding of [^{3}H]acetylcholine was always measured at 20° in the presence of 0.1 mM Tetram (see MATERIALS AND METHODS), a potent acetylcholinesterase inhibitor, which at that concentration does not interfere with the binding of [^{3}H]acetylcholine or [^{3}H]decamethonium to the cholinergic receptor site (see also 26).

As reported previously (16), when the binding of acetylcholine to different membrane suspensions prepared according to Cohen *et al.* (9) was studied, certain variations were noticed. Some preparations bound acetylcholine in a cooperative manner (Hill coefficient, n_H = 1.3–1.4), whereas others did not (n_H = 1.0). Also we observed small variations of the concentration ($F_{1/2}$) of free acetylcholine giving half-saturation of the receptor sites (Table 3).

For the three preparations studied, prilocaine causes a shift of the acetylcholine binding curve toward lower concentration (Figs. 15–17; Table 3). The concentration of acetylcholine needed for half-saturation ($F_{1/2}$) decreases by 40–65% without a change in the number of acetylcholine binding sites. Although $F_{1/2}$ varied from one preparation to another in the absence of prilocaine, in its presence $F_{1/2}$ reached the same minimal value (6.1 nM) with all membrane preparations tested. The concentration of prilocaine giving half maximal decrease of $F_{1/2}$ is, as in the previous experiments, close to 0.4 mM.

In the presence of prilocaine the Hill coefficient of the binding curve for acetylcholine decreases (Figs. 15 and 16). Prilocaine both increases the affinity for acetylcholine and changes the shape of the binding curve from sigmoid to hyperbolic. Again the same minimal value of n_H (1.05) was found with the preparations exhibiting cooperativity.

Since the effect of prilocaine presents striking analogies with the well-documented case of allosteric activators of typical regulatory proteins (33), it was analyzed in similar terms. A plot of the binding data in which, for each point of the binding curve, a phenomenological dissociation constant, K_d, is calculated on the basis of a bimolecular reaction with a homogeneous population of sites is shown in Fig. 18. In the absence of prilocaine K_d decreases markedly with in-

TABLE 3

Effect of prilocaine on binding of [^3H]acetylcholine to three different membrane suspensions

The temperature indicated is that at which acetylcholine binding was measured. $F_{1/2}$ = concentration of acetylcholine needed for half-saturation; n_H = Hill coefficient.

Preparation	[^{3}H]α-Toxin binding sites	[^{3}H]-Acetylcholine binding sites	Temperature	No prilocaine		0.3 mM prilocaine		3 mM prilocaine	
				$F_{1/2}$	n_H	$F_{1/2}$	n_H	$F_{1/2}$	n_H
	μmoles/g protein			*nM*		*nM*		*nM*	
A	2.0	1.8	20°	10.3	1.47			6.0	1.05
B	1.8	2.0	20°	14.8	1.16	11.2	1.10	6.2	1.05
C	0.44	0.40	4°	17.0	1.0	8.4	1.0	6.0	1.0

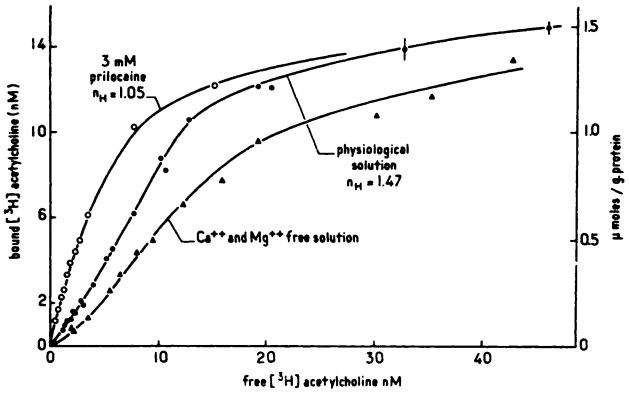

FIG. 15. *Binding of [^{3}H]acetylcholine to membrane fragments: effects of prilocaine and divalent cation*; ●——●, *Torpedo* membrane fragments (preparation A of Table 3; 1.0 μM *Naja* α-toxin binding sitess 0.5 g of protein per liter) were diluted 50-fold in physiological saline solution supplemented with 0.1 mM Tetram. ○——○, dilution in the same medium plus 3 mM prilocaine; ▲——▲, dilution in Ca^{++}- and Mg^{++}-free buffer supplemented with 0.1 mM Tetram. In each case extrapolation of a double-reciprocal plot of the data gave a value of 17.5 nM for the total number of [^{3}H]acetylcholine binding sites. The concentration of [^{3}H]α-toxin binding sites was 20 nM.

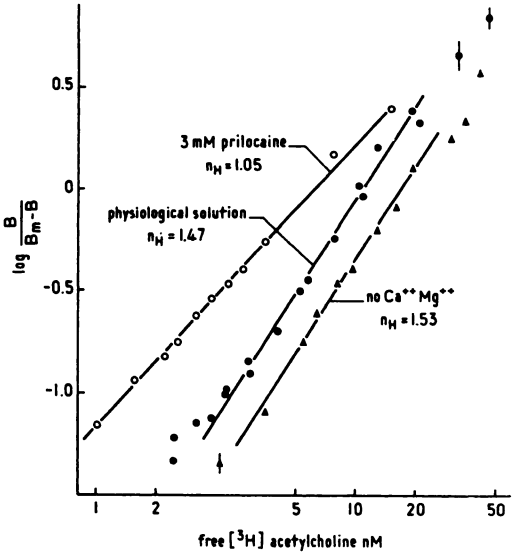

FIG. 16. *Binding of [^{3}H]acetylcholine to membrane fragments: Hill plot*

B is the concentration of bound acetylcholine; B_m, the total concentration of its binding sites determined by extrapolation of a double reciprocal plot. The data are from Fig. 15. The straight lines are a least-squares fit of the data.

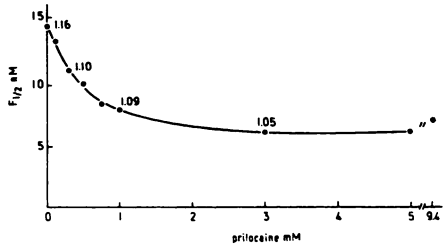

FIG. 17. *Effect of prilocaine on position and shape of binding curve of [^{3}H]acetylcholine to receptor-rich membrane fragments*

Membrane preparation B of Table 3 (0.8 μM *Naja* α-toxin binding sites; 0.45 g of protein per liter) was diluted 80-fold in physiological saline solution supplemented with 0.1 mM Tetram and the indicated concentrations of prilocaine. $F_{1/2}$ is the concentration of free [^{3}H]acetylcholine giving half-saturation of the binding sites. Also indicated is the Hill index, n_H, at 0, 0.3, 1, and 3 mM prilocaine.

creasing acetylcholine concentration. Positive cooperative interaction for acetylcholine binding takes place. In the presence of 3 mM prilocaine K_d no longer varies with acetylcholine concentration: at all concentrations of acetylcholine tested, K_d has the same value as that found in the absence of prilocaine at saturating levels of acetylcholine. In other words, the same high affinity of the cholinergic receptor site appears to be reached in the presence of either prilocaine or high concentrations of acetylcholine.

For still unknown reasons, some membrane preparations do not exhibit coopera-

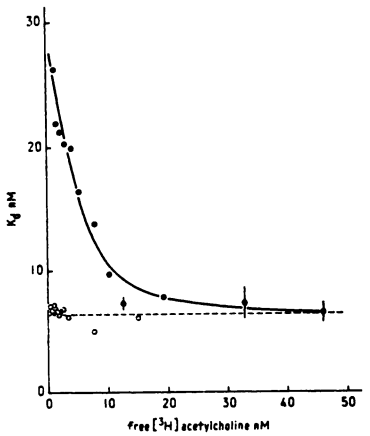

FIG. 18. *Variation of K_d as a function of free [^{3}H]acetylcholine*

Data are from Fig. 15. K_d was calculated by the formula $K_d = (B_m - B)/B \times F$, where B (or F) is the concentration of bound (or free) [^{3}H]-acetylcholine, and B_m is the number of binding; acetylcholine, and B_m is the number of binding sites. ●——●, *Torpedo* physiological saline; O- - -O, *Torpedo* physiological saline supplemented with 3 mM prilocaine.

tive acetylcholine binding. With such preparations, however, prilocaine still causes a marked increase of affinity for acetylcholine (Table 3). Although a strong correlation exists between the loss of cooperative interaction and the enhancement of acetylcholine affinity, this finding suggests that the two properties can be uncoupled, presumably as a consequence of an alteration of membrane structure.

To test the reversibility of the effect of prilocaine on the binding of [^{3}H]acetylcholine, a membrane suspension was first incubated for 30 min with 3 mM prilocaine in Ca^{++}- and Mg^{++}-free buffer. The suspension was then diluted in the same medium without prilocaine, giving a final local anesthetic concentration of 0.2 mM. After 1 hr the binding of [^{3}H]acetylcholine was measured. Under these conditions the binding curve is exactly superimposable on that obtained when 0.2 mM prilocaine was added directly to the suspension ($F_{½}$ = 18 nM, n_H = 1.31), and clearly differs from that obtained with 3 mM prilocaine ($F_{½}$ = 9 nM, n_H = 1.15). The effects of prilocaine on the affinity for acetylcholine and on its cooperative binding are thus entirely reversible.

To ascertain that the observed enhancement of acetylcholine affinity was associated with the pharmacological action of prilocaine as a local anesthetic, the effect of another local anesthetic, dimethisoquin, was studied. We also tested SKF 525-A, a compound known to increase the rate of desensitization of the motor endplate to acetylcholine (34). When applied to *Electrophorus* electroplax, SKF 525-A blocks the response to carbamylcholine in a noncompetitive manner, half-reduction of the maximal response to carbamylcholine taking place at 1 μM SKF 525-A. In agreement with the observation of Franklin and Potter (35), this compound decreases the initial rate of [^{3}H]α-toxin binding to *Torpedo* membrane fragments by 40% at 1 mM, a concentration 1000 times larger than that at which it blocks the physiological response. By these criteria SKF 525-A behaves as a local anesthetic (18).

As shown in Fig. 19, prilocaine, dimethisoquin, and SKF 525-A increase the binding of acetylcholine to almost the same extent in the range of concentration where they

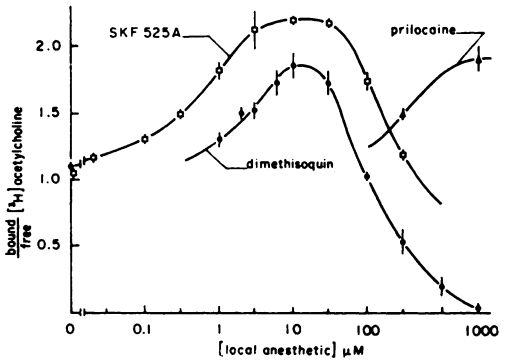

FIG. 19. *Effects of various local anesthetics on binding of [^{3}H]acetylcholine to receptor-rich membrane fragments*

A membrane suspension (0.3 μM *Naja* α-toxin binding sites; 0.6 g of protein per liter) was diluted 15-fold in physiological saline solution supplemented with 0.1 mM Tetram and the indicated concentrations of local anesthetics. Binding was measured in the presence of 20 nM total [^{3}H]-acetylcholine. In the absence of local anesthetic the concentration of bound ligand was 11 ± 1 nM.

block the response of *Electrophorus* electroplax to carbamylcholine. At much higher concentrations (50–1000 times their apparent dissociation constants *in vivo*) a decrease of acetylcholine binding takes place. That decrease parallels the decrease of the initial rate of [^{3}H]α-toxin binding to the same membrane fragments. The effects occurring at high concentrations have been interpreted on the basis of a direct interaction of the local anesthetics with the cholinergic receptor site (18).

We also examined the effect of DNS-chol on the binding of acetylcholine to the membrane-bound receptor. No increase of acetylcholine binding was observed for DNS-chol concentrations lower than those causing a decrease of the amount of acetylcholine bound. This result was not unexpected, since spectroscopic studies have shown (20) that DNS-chol binds with greater affinity to the receptor site than to its secondary sites.

Naja [^{3}*H*]*α-toxin binding.* Weber and Changeux (18) showed that local anesthetics do not increase the initial rate of [^{3}H]α-toxin binding at those concentrations where they strengthen the association of acetylcholine and decamethonium with the receptor site. They further demonstrated that, by following the decrease of the initial rate of [^{3}H]-α-toxin binding as a function of cholinergic ligand concentration, one can estimate the dissociation constant of these ligands for the cholinergic receptor site. This method might therefore be used to monitor the changes of binding properties of the receptor site for cholinergic ligands in rather indirect but quite reliable fashion.

Figure 20 confirms that 3 mM prilocaine has little effect on the initial rate of [^{3}H]α-toxin binding in the absence of effectors but shifts the protection curve by decamethonium toward lower concentrations, as expected for an increased affinity of the receptor site for this agonist. Figure 20 also shows that this is the case for an antagonist, *d*-tubocurarine. Prilocaine also increases the apparent affinity of the cholinergic receptor site for another antagonist, gallamine (not shown).

Effect of Ca^{++} on Interaction of Cholinergic Ligands and Local Anesthetics with Receptor-Rich Membrane Fragments

With many pharmacological preparations, local anesthetics interfere with events requiring Ca^{++} (for review see ref. 28). On *Electrophorus* electroplax Ca^{++} (but not Mg^{++}) potentiates the response to carbamylcholine (36). We thus investigated the effect of Ca^{++} on the binding properties of the receptor protein in relation to the effect of local anesthetics. Both fluorescence and direct binding methods were used.

Fluorescence assay with DNS-chol. The membrane fragments taken from the sucrose gradient were resuspended in a buffered saline solution free of divalent cations (250 mM NaCl, 1 mM EGTA, and 5 mM sodium phosphate, pH 6.8), and the effects of Ca^{++} or prilocaine on the fluorescence properties of DNS-chol interacting with the membrane

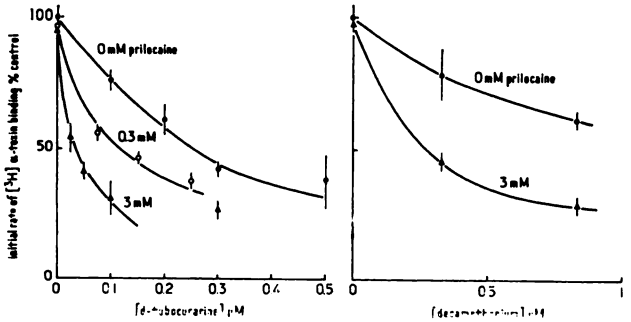

FIG. 20. *Initial rate of Naja* [^{3}*H*]*α-toxin binding to Torpedo membrane fragments: effect of prilocaine on protection by cholinergic ligands*

Data are expressed as a percentage of the initial rate measured in the absence of both prilocaine and cholinergic ligands.

fragments were studied in this environment (Fig. 21A). A double-reciprocal plot of the fluorescence intensities measured at various DNS-chol concentrations shows that Ca^{++}, like prilocaine, *increases* the apparent affinity of DNS-chol for the receptor site (Fig. 21B) without changing the extrapolated maximal fluorescence signal. In Table 4 are listed the K_{fluo} values determined by this method.

In the presence of an excess (1 mM) of carbamylcholine, a condition under which DNS-chol fluorescence results from its interaction with the secondary sites, Ca^{++}, like local anesthetics, cause a *decrease* of fluorescence intensity.

As for the local anesthetics, the increased affinity of DNS-chol for the cholinergic receptor site is not accompanied by any change of the wavelength of maximum emission of DNS-chol (λ_{max} = 537 ± 3 nm). However, unlike the anesthetics, the decrease of fluorescence of DNS-chol bound to the secondary sites is not accompanied by a change in the wavelength of maximum emission when the receptor sites are occupied by carbamylcholine. The blue shift of DNS-chol emission from the secondary sites caused by agonists exists in the absence or presence of Ca^{++} (λ_{max} = 523 ± 3 nm). Similarly, the absence or presence of Ca^{++} does not change λ_{max} when all receptor sites are occupied by gallamine (λ_{max} = 532 ± 3 nm). With respect to both classes of sites, saturating levels of Ca^{++} appear less potent than high prilocaine concentrations. For instance, 5 mM Ca^{++} gives about half the effect of 5 mM prilocaine at the level of both classes of sites. Moreover, the effects of prilocaine and Ca^{++} on the receptor site are not strictly additive. Addition of 5 mM prilocaine in the presence of 5 mM Ca^{++} increases the effect observed with Ca^{++} alone, but up to the same level as prilocaine in the absence of Ca^{++}. Addition of 5 mM Ca^{++} to a membrane suspension which already contains 5 mM prilocaine has only a slight effect on DNS-chol interaction with its two classes of sites.

Figure 22 shows the concentration dependence of the effect of Ca^{++} on DNS-chol fluorescence in both the presence and absence of prilocaine. The fluorescence increase associated with DNS-chol bound to the re-

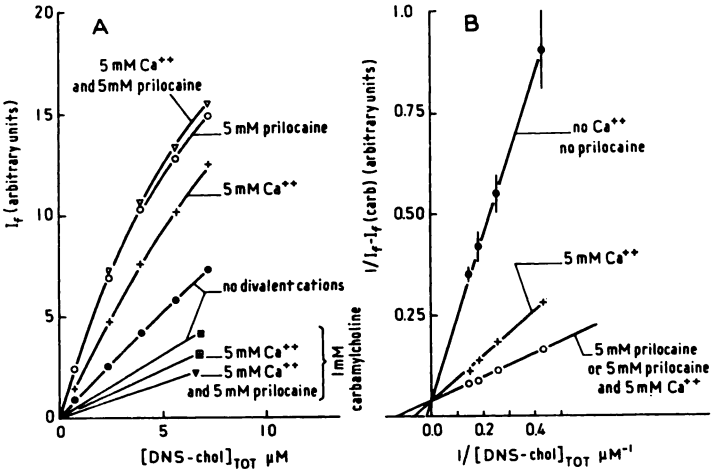

FIG. 21. *Effect of Ca^{++} and prilocaine on fluorescence of DNS-chol bound to receptor-rich membrane fragments*

The suspension contained 1.0 μM *Naja* α-toxin sites and 0.8 g of protein per liter in 250 mM NaCl, 1 mM EGTA, and 5 mM sodium phosphate, pH 6.8. The indicated concentrations of Ca^{++}, prilocaine, and carbamylcholine were present prior to the addition of DNS-chol. λ_{ex} = 287 nm; λ_{em} = 550 nm. The presence of 1 mM carbamylcholine ensured that all receptor sites would be occupied by carbamylcholine under the conditions of these experiments. A. Direct plot of the data. B. Double-reciprocal plot. The reciprocal of the fluorescence decrease caused by 1 mM carbamylcholine is plotted against the reciprocal of the total DNS-chol concentration.

ceptor site reaches a maximum around 3 mM Ca^{++} (curve *A1*). As found for prilocaine, the concentration dependence of the effect of Ca^{++} monitored at the level of the secondary class of DNS-chol sites (in the presence of carbamylcholine) follows closely that observed at the level of the receptor site (curve *B1*). Adding prilocaine to the same membrane suspension, but in the absence of Ca^{++}, gives the expected increase of DNS-chol fluorescence at the level of the receptor site (curve *A2*), while adding prilocaine in the presence of 5 mM Ca^{++} gives a further increase of fluorescence which is only slightly larger (15%) than that observed in the presence of prilocaine alone. The variation of this last effect as a function of prilocaine concentration is almost the same as that observed in the absence of Ca^{++}.

Addition of Ca^{++} in the presence of saturating levels of prilocaine (curve *A3*) gives a slight further increase of DNS-chol fluorescence. This effect takes place in the same range of Ca^{++} concentration as the major effect of Ca^{++} observed in the absence of prilocaine. In other words, although both Ca^{++} and prilocaine enhance DNS-chol binding to the receptor site, there is no evidence of an effect of prilocaine on Ca^{++} binding and, conversely, of Ca^{++} on prilocaine binding.

The observed effect of Ca^{++} on the receptor site is not limited to DNS-chol binding. Ca^{++} also increases the affinity of the receptor site for carbamylcholine as measured by the DNS-chol fluorescence assay (Table 4).

Binding of [^3H]acetylcholine. The binding data obtained with [^{3}H]acetylcholine in the presence of Tetram confirm and further extend those obtained by fluorescence spectroscopy. The binding curves of acetylcholine in the presence of Ca^{++}- and Mg^{++}-free buffer and in the presence of normal physiological saline solution are compared in Fig. 15. The number of acetylcholine molecules bound at saturation does not change, but half-saturation occurs at a higher concentration of free acetylcholine in the absence of divalent cations ($F_{1/2}$ = 17 nM) than in their presence ($F_{1/2}$ = 10 nM). On the other hand, the effect on the shape of the binding curve is rather small: n_H = 1.53 in Ca^{++}- and Mg^{++}-free buffer and 1.47 in physiological solution.

The concentration dependence of the Ca^{++} effect was followed at a fixed total con-

TABLE 4

Comparison of effects of Ca^{++} and prilocaine on binding of DNS-chol and carbamylcholine to membrane-bound cholinergic receptor

Ca^{++}	Prilocaine	DNS-chol K_{fluo}[a]	Carbamylcholine C_{50}[b]
mM	*mM*	*μM*	*μM*
0	0	33 ± 3	3.0 ± 0.5
5	0	18 ± 3	1.5 ± 0.1
0	5	7.5 ± 0.3	0.95 ± 0.1
5	5	7.4 ± 0.3	1.1 ± 0.1
0	15		0.9 ± 0.1
5	15		0.7 ± 0.1

[a] K_{fluo} = spectroscopic dissociation constant.

[b] C_{50} is the concentration of cholinergic ligand necessary to cause a half-maximal decrease of DNS-chol (7.2 μM) fluorescence. The membrane suspension is described in the legend to Fig. 21.

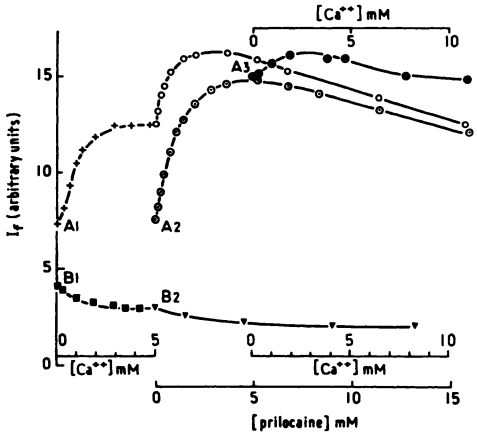

FIG. 22. *Concentration dependence of effect of Ca^{++} or prilocaine on fluorescence of DNS-chol (7.2 μM) in suspension of receptor-rich membrane fragments in buffered saline solution free of divalent cations*

The suspension was the same as that of Fig. 21. Curve *A1*, Ca^{++} added up to 5 mM (+——+), then prilocaine to 15 mM (O——O); *A2*, in the absence of Ca^{++} prilocaine was added up to 15 mM; *A3* in the presence of 4.8 mM prilocaine Ca^{++} was added up to 10 mM; *B1*, in the presence of 1 mM carbamylcholine Ca^{++} was added up to 5 mM; *B2*, then prilocaine to 12 mM. In the presence of 1 mM carbamylcholine, 14 mM prilocaine was associated with the same DNS-chol fluorescence intensity in the presence and absence of 5 mM Ca^{++}.

centration of acetylcholine. Ca^{++} increases acetylcholine binding, which reaches a plateau at 5 mM Ca^{++}, half of the maximum effect taking place at 1 mM Ca^{++} (Fig. 23). The maximal enhancement of acetylcholine affinity is also smaller than that reached with local anesthetics, and the effects of Ca^{++} and prilocaine on acetylcholine binding are not strictly additive: addition of 5 mM Ca^{++} produces little or no enhancement of acetylcholine affinity in the presence of 3 mM prilocaine. Varying Mg^{++} concentration in the absence of Ca^{++} has no significant effect on acetylcholine binding. In addition, 5 mM Mg^{++} does not modify the potentiating effects on acetylcholine binding of 5 mM Ca^{++} or 3 mM prilocaine (not shown). In agreement with the pharmacological experiments *in vivo* Ca^{++} cannot be replaced by Mg^{++}.

Consequences of Solubilization on Interaction of Cholinergic Ligands, Local Anesthetics, and Ca^{++} with Receptor Protein

Eldefrawi *et al.* (37) and Franklin and Potter (35) reported that solubilization of *Torpedo* receptor protein by mild detergents modifies the binding properties of the cholinergic receptor site. It was therefore of importance to determine whether local anesthetics and Ca^{++} ions still control the affinity of the cholinergic receptor in the soluble form.

Fluorescence assay with DNS-chol. Treatment of *Torpedo* membrane fragments with sodium cholate under the conditions given in MATERIALS AND METHODS yields a preparation of receptor protein which does not sediment after centrifugation at 100,000 × *g* for 1 hr and which binds 1–3 μmoles of *Naja* [^{3}H]α-toxin per gram of protein. The concentration of accessible sites is large enough (micromolar) to follow the interaction of DNS-chol with the solubilized receptor protein by fluorescence spectroscopy. In the presence of 7 μM DNS-chol 1 mM carbamylcholine causes a significant (20%)

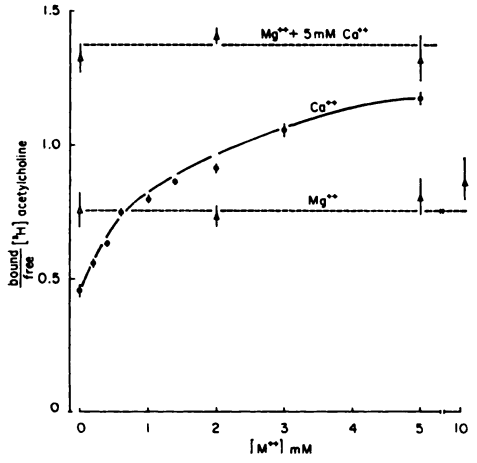

FIG. 23. *Effects of Ca^{++} and Mg^{++} on binding of [^{3}H]acetylcholine to receptor-rich membrane fragments*

A membrane suspension (0.4 μM *Naja* α-toxin sites; 0.6 g of protein per liter) was diluted in Ca^{++}- and Mg^{++}-free buffer supplemented with the indicated amounts of divalent cation. The final concentration of α-toxin sites was 20 nM. ●, 25 nM total [^{3}H]acetylcholine; △, 12 nM total [^{3}H]-acetylcholine.

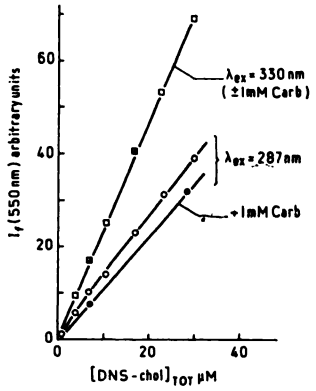

FIG. 24. *Effect of carbamylcholine on DNS-chol fluorescence in detergent-solubilized preparation of receptor-rich membrane fragments*

Solubilization was accomplished by adding sodium cholate (to 0.7%, w/v) to a suspension of receptor-rich membrane fragments in physiological saline. After 20 min of stirring at 20°, the solution was centrifuged at 95,000 × *g* for 60 min, and the supernatant solution (1.2 μM α-toxin sites; 0.35 g of protein per liter) was used for the fluorescence experiments. The solubilized receptor solution was added to both the sample and reference cells, and DNS-chol, to the sample cell only. Excitation at 287 nm: ●——●, intensities observed when carbamylcholine chloride was present in both cells prior to the addition of DNS-chol. Excitation at 330 nm: ◪, intensities observed in both the presence and absence of 1 mM carbamylcholine (Carb); □, in the absence only.

decrease of fluorescence intensity only for excitation at 287 nm (not at 330 nm) (Fig. 24). The interaction of DNS-chol with the receptor protein in solution can therefore be followed by energy transfer.

$R = I_{287}/I_{330}$ decreases from 0.59 at 7 μM DNS-chol in the absence of carbamylcholine to 0.44 ± 0.03 in the presence of 1 mM carbamylcholine (Fig. 24). For $\lambda_{ex} = 287$ nm, DNS-chol emission has $\lambda_{max} = 533$ nm prior to the addition of cholinergic ligands or when all receptor sites are occupied by an agonist (carbamylcholine) or *Naja* α-toxin. For $\lambda_{ex} = 330$ nm, DNS-chol emission has $\lambda_{max} = 543$ nm. For free DNS-chol in the presence of 0.7% sodium cholate in physiological saline solution, $R = 0.44$ and $\lambda_{max} = 542$ nm for $\lambda_{ex} = 287$ nm or 330 nm. Hence with this preparation there is no evidence for the existence of secondary sites for DNS-chol characterized by preferential excitation by energy transfer from proteins. There is no evidence for secondary sites whose spectral properties distinguish between cholinergic receptor sites occupied by agonists or antagonists. However, the origin of the different emission maxima for 287 and 330 nm excitation is not clear.

The fraction of DNS-chol fluorescence sensitive to carbamylcholine, however, is too small to permit accurate determination of a dissociation constant under the present experimental conditions. A double-reciprocal analysis of the available fluorescence data shows that K_{fluo} for DNS-chol must be larger than 20 μM. In any case, the affinity of the receptor protein in solution for DNS-chol is certainly not higher than that found for the membrane-bound receptor.

As expected, the *Naja* α-toxin and a variety of cholinergic ligands cause the same maximum decrease of fluorescence intensity as carbamylcholine (Fig. 25). As with the membrane-bound receptor, the α-toxin, because of its high affinity, gives a linear titration curve. With the other ligands the displacement curves are no longer fitted by a hyperbola and appear rather complex. Their shape indicates that the solubilized receptor possesses multiple affinities for ligands such as acetylcholine or carbamylcholine. Starting from membrane fragments characterized by a single affinity for each ligand (16), solubilization creates a heterogeneity of the affinities for a single ligand.

Despite this complexity, we have again characterized each cholinergic ligand by its concentration, C_{50}, producing a half-maximal decrease of DNS-chol fluorescence. Although this parameter does not have a simple physical meaning, certain results are clear. Under the solubilization conditions of Fig. 25 the C_{50} values for all agonists tested are considerably higher than the values found for the membrane-bound receptor: 100-fold

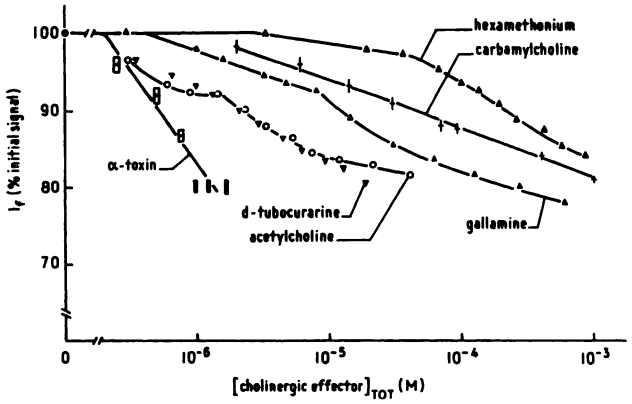

FIG. 25. *Concentration dependence of effect of cholinergic ligands on fluorescence of DNS-chol (7.2 μM) interacting with detergent-solubilized preparation of receptor-rich membrane fragments*

The intensities determined at 550 nm are expressed as percentages of the intensity observed in the absence of cholinergic ligands: $\lambda_{ex} = 287$ nm. The receptor solubilization was performed as described in Fig. 24. The acetylcholine experiment was performed in the presence of 0.1 mM Tetram.

for acetylcholine, 50-fold for carbamylcholine, and 10-fold for decamethonium.

The C_{50} values measured for the antagonists tubocurarine, gallamine, and hexamethonium increased only slightly (Table 5). However, other solubilization conditions (see legend for Fig. 26A) resulted in a solution characterized by lower C_{50} values for agonists: C_{50} = 1.7 μM for decamethonium and 4.5 μM for carbamylcholine (see Fig. 27). In order to clarify these results, we measured the binding of [^{3}H]acetylcholine by equilibrium dialysis.

Binding of [^{3}H]acetylcholine. After solubilization of receptor-rich membrane fragments with sodium cholate the binding curve of [^{3}H]acetylcholine measured by equilibrium dialysis in the presence of Tetram reveals, in the range of acetylcholine concentration explored (0.01–6 μM), a clear-cut heterogeneity of binding constants. As observed in the fluorescence experiments, the shape and position of the curves obtained vary with the condition of solubilization. When the solubilization is done with 1–4% (w/v) cholate and 5–10 mg of cholate per milligram of protein, the data can be interpreted in terms of binding to two classes of sites. Depending upon the solubilization, from 30 to 50% of them bind acetylcholine with a dissociation constant K_d = 0.04–0.07 μM, and the rest with a K_d close to 0.8 μM. Binding of acetylcholine to both classes of sites is completely blocked by prior incubation of the extracts with *N. nigricollis* α-toxin, and the total number of [^{3}H]acetylcholine sites is in the same range as that of [^{3}H]α-toxin. In agreement with the DNS-chol fluorescence assay, one preparation characterized by low C_{50} values for agonists contained a large fraction of sites binding acetylcholine with high affinity (50% with K_d = 0.04 μM).

Effect of prilocaine and Ca^{++}. Prilocaine between 0.01 and 3 mM has no significant effect on the binding of [^{3}H]acetylcholine to the cholate-solubilized receptor (Fig. 26A) in either its high-affinity (revealed at 0.02 μM free acetylcholine) or low-affinity (revealed at 1.1 μM free acetylcholine) forms. In the presence of 10 mM prilocaine a slight decrease of bound acetylcholine was noticed, as was true for the membrane-bound receptor. Also, Ca^{++} (0.2–5 mM) has no effect (Fig. 26B) on the binding of acetylcholine to the solubilized receptor protein initially in Ca^{++}- and Mg^{++}-free buffer and 1% sodium cholate.

Florescence studies show that prilocaine (0.01–2 mM) causes no increase of the fluo-

TABLE 5

Interaction of cholinergic ligands with Torpedo receptor present in membrane fragments and after solubilization with sodium cholate

Ligand	Membrane fragments		Solubilized[c]	C_{50} sol/C_{50} mem
	K_p (protection against [^{3}H]α-toxin binding)[a]	C_{50} (DNS-chol fluorescence assay)[b]	C_{50} (DNS-chol florescence assay)	
	μM	μM		
Acetylcholine	0.8×10^{-2}	—[d]	3 ± 0.5	≧100
Carbamylcholine	0.5	1.5	50 ± 20	25–50
Decamethonium	0.7	1.5	10 ± 2	10
d-Tubocurarine	0.2	1.0	3.5 ± 0.5	4
Gallamine	8.0	12.0	22 ± 8	3
Hexamethonium	40	80	260 ± 50	4

[a] data from Weber and Changeux (16).

[b] data from Cohen and Changeux (20).

[c] for the solubilized receptor, the conditions were those of Fig. 24.

[d] Affinity too great to be estimated by fluorescence assay. DNS-chol displacement curve is linear with added acetylcholine.

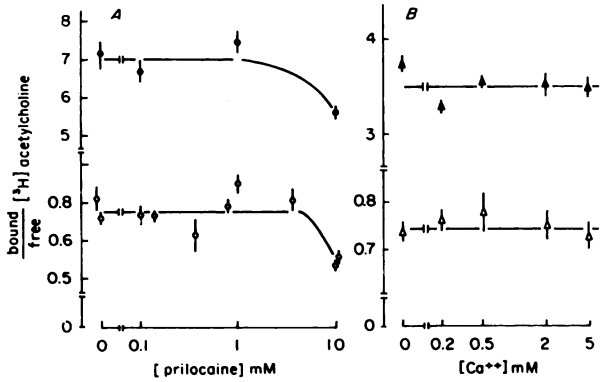

FIG. 26. *Binding of [³H]acetylcholine to detergent-solubilized preparations of receptor-rich membrane fragments*

A. Effect of prilocaine. A membrane suspension (10 μM *Naja* α-toxin sites; 6 g of protein per liter) in physiological saline solution was dissolved with 3.3% (w/v) sodium cholate. After centrifugation at 100,000× *g* for 1 hr, about 50% of the α-toxin sites remained in the supernatant solution, which then was diluted with physiological saline solution to a final cholate concentration of 0.9% (w/v) (1.1 μM α-toxin sites). Equilibrium dialysis performed as described in MATERIALS AND METHODS revealed in the absence of prilocaine at least two classes of binding sites for [³H]acetylcholine (K_d = 0.04 μM, B_{max} = 0.44 μM; K_d = 0.8 μM, B_{max} = 0.3–0.5 μM). Free [³H]acetylcholine: ○, 1.1 μM; ●, 0.02 μM.

B. Effect of Ca^{++}. A membrane suspension (1.9 μM α-toxin sites; 1.6 *g* of protein per liter) in Ca^{++}- and Mg^{++}-free buffer was dissolved with 1% (w/v) sodium cholate (6.4 mg of cholate per milligram of protein). The recovery of α-toxin sites was 85–100% of the starting material. Equilibrium dialysis in the absence of Ca^{++} revealed high- (K_d = 0.086 μM; B_{max} = 0.4 μM) and low- (K_d = 0.8 μM; B_{max} = 0.8 μM) affinity sites. Free [³H]acetylcholine: △, 0.8 μM, ▲; 0.03 μM.

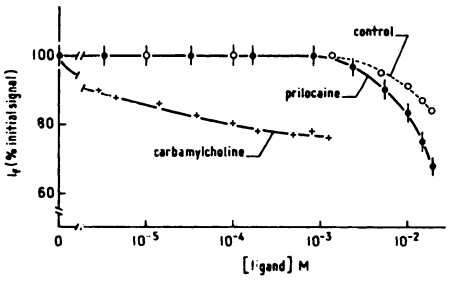

FIG. 27. *Comparison of effects of prilocaine and carbamylcholine on intensity of fluorescence of DNS-chol (7.2 μM) interacting with sodium cholate-solubilized receptor*

Solubilization was performed as described for Fig. 26A. The control experiment was conducted by adding prilocaine to 7.2 μM DNS-chol in physiological saline solution in the absence of receptor protein.

rescence of DNS-chol interacting with the cholate-solubilized receptor (Fig. 27) and, hence, no change of the receptor affinity for DNS-chol. The only effect of prilocaine is to cause a decrease of DNS-chol fluorescence at prilocaine concentrations above 2 mM, and a large fraction of that decrease is due to the optical density of the prilocaine solution. Also, Ca^{++} (0.1–5 mM) has no effect (no more than 2%) on the fluorescence intensity of DNS-chol (2–7 μM) interacting with the cholate-solubilized receptor described in Fig. 26A.

We also used fluorescence techniques to study the effect of prilocaine on the receptor-rich membrane fragments solubilized by 2% Emulphogene BC-720. In that case the solubilized receptor is characterized by high affinity for acetylcholine (80% of sites with K_d = 0.02 μM)[4] and by C_{50} = 0.3 μM for carbamylcholine. Upon addition of prilocaine (0.01–2 mM) no increase of DNS-chol (7.2 μM) fluorescence intensity was observed. Prilocaine likewise does not increase the affinity for DNS-chol of the purified receptor protein from *Electrophorus*, although in that case the soluble protein exhibits much higher affinity for the agonists than does the membrane-bound form (38).

[4] H. Sugiyama, personal communication.

DISCUSSION

Previous spectroscopic studies (20), using receptor-rich membrane fragments purified from *Torpedo* electric organ revealed two major classes of sites for a cholinergic fluorescent ligand, DNS-chol: the cholinergic receptor site and secondary sites where DNS-chol indicates a reversible change of membrane structure associated with the binding of agonists to the cholinergic receptor site.

DNS-chol shows a dual pharmacological action on the isolated *Electrophorus* electroplax. At low concentrations of carbamylcholine it potentiates the response and therefore acts as a receptor agonist. Why the effect of DNS-chol is not simply additive with that of carbamylcholine is not understood; a similar potentiation has been noticed with *Electrophorus* electroplax between two agonists, carbamylcholine and decamethonium (39). At high concentrations of carbamylcholine the maximal response decreases in the presence of DNS-chol. This effect resembles that of a local anesthetic on the electroplax response to agonist, and 30 μM DNS-chol is as potent as 0.2 mM lidocaine or prilocaine.

While binding of DNS-chol to the cholinergic receptor site has been demonstrated with *Torpedo* membrane fragments, the relationship is not as clear between the secondary sites studied by fluorescence techniques and the sites of action of DNS-chol as a local anesthetic. First, no quantitative pharmacological data are yet available with *Torpedo* electroplax or microsacs, and so we always refer here to the action of anesthetics on *Electrophorus* electroplax. Local anesthetics decrease the fluorescence of DNS-chol bound to the secondary sites *in vitro* and abolish the spectroscopic distinction caused by the binding of agonist or antagonist at the receptor site. This effect takes place at anesthetic concentrations in the range of those which block the response of *Electrophorus* electroplax. However, further studies are needed to understand the mechanism by which local anesthetics modify the spectral properties of DNS-chol bound to the secondary sites.

An extensive study of the effect of local anesthetics on the binding of fluorescent and radioactive ligands to the cholinergic receptor site present in *Torpedo* membrane fragments has shown that these compounds *control* the affinity of the membrane-bound receptor at concentrations at which they are physiologically active (Table 6). All the anesthetics tested cause an increase of affinity without change in the number of sites. The increase is the same for the different anesthetics studied, although they are effective at different concentrations. It is also the same for a given anesthetic and different cholinergic ligands: acetylcholine, a small polar agonist (K_d = 10 nM), or the aromatic DNS-chol (K_d = 30 μM). The local anesthetics increase the affinity for both cholinergic antagonists and agonists. In-

TABLE 6

Comparison between physiological effects of various local anesthetics and their effects on binding of cholinergic ligands to membrane-bound cholinergic receptor of Torpedo

I_{50} is the concentration of local anesthetic which decreases by half the depolarization of the eel electroplax by bath application of the agonist. A_{50} (spec) is the concentration of anesthetic causing half the maximal observed increase of DNS-chol fluorescence (from Fig. 9). A_{50} (binding) is the anesthetic concentration causing half the maximal increase of binding of [^{3}H]acetylcholine (from Fig. 19).

Anesthetic	*In vivo:* membrane potential of isolated electroplax, I_{50}	*In vitro:* effect on binding of DNS-chol and acetylcholine to membrane-bound receptor (*Torpedo*)	
		A_{50} (spec)	A_{50} (binding)
	M	*M*	*M*
SKF 525-A	1×10^{-6} [a]		1×10^{-6}
Dimethisoquin	2×10^{-6} [b]	1.5×10^{-6}	2×10^{-6}
Prilocaine	2×10^{-4} [a] 8×10^{-5} [b]	4×10^{-4}	3×10^{-4}
Lidocaine	2×10^{-4} [a]	2×10^{-4}	

[a] Data from this work. The response was followed in the presence of 50 μM carbamylcholine.

[b] Data from Weber and Changeux (18). The response was followed in the presence of 2 μM decamethonium.

direct methods have been used to show the effect with antagonists, and it cannot be decided yet whether the increase of affinity is of exactly the same magnitude with antagonists as with agonists. When one uses DNS-chol to probe the properties of the receptor site, the spectroscopic data show that anesthetics cause no change in the maximum fluorescence signal (quantum yield) or in the wavelength of maximum DNS-chol emission. The control of affinity occurs without change of the receptor site environment as sensed by the spectral properties of DNS-chol.

With most of the membrane preparations tested, the binding curve of acetylcholine is S-shaped, with a Hill coefficient of 1.4. In the presence of local anesthetics the shape of the binding curve for acetylcholine is converted from sigmoid to hyperbolic. This conversion of shape, which accompanies the increase of affinity, is reminiscent of a characteristic property of regulatory enzymes (33). The local anesthetics would play a role similar to that of allosteric activators such as valine, in the case of biosynthetic L-threonine deaminase (40), or ATP, in the case of aspartate transcarbamylase (41). However, the relationship between the increase of affinity and the uncoupling of cooperative interactions is not as clear with the cholinergic receptor as it is with the regulatory enzymes: in membrane preparations which bind acetylcholine without significant cooperative effect, prilocaine still causes an increase of affinity for acetylcholine.

Calcium ions cause, as do the local anesthetics, an increase of affinity of the membrane-bound *Torpedo* receptor for cholinergic ligands. However, these two classes of compounds appear to act in a rather different manner. With 3 mM prilocaine the same affinity is reached whether or not calcium is present in the medium, but this is not true for the effect of calcium in the presence or absence of local anesthetics. Ca^{++} also causes an increase of affinity for acetylcholine without significant change of cooperativity. Furthermore, the spectroscopic experiments indicate that calcium, like local anesthetics, causes a decrease of DNS-chol fluorescence from the secondary sites. However, this decrease is smaller than that caused by the anesthetics and is *not* accompanied by a change in the wavelength of maximum emission.

Solubilization of *Torpedo* receptor-rich membrane fragments by cholate is accompanied by a marked change of the binding properties of the cholinergic receptor site. On the membrane fragments a single (high) affinity constant is present for each ligand studied; a marked heterogeneity of binding constants appear after solubilization (37), and the affinities for the agonists decrease more significantly than for the antagonists. Furthermore, no evidence has been found that prilocaine or calcium can increase the affinity of the solubilized receptor in either its high- or low-affinity form. The control of receptor affinity by these ligands appears characteristic of the membrane-bound form of the receptor protein.

Finally, we would like to discuss the possible relationship between the effect observed *in vitro* on the binding properties of the membrane-bound receptor and the control of membrane pern eability. At first glance there is a paradox: both local anesthetics and Ca^{++} cause an increase of receptor affinity. Local anesthetics, however, ultimately prevent the permeability change caused by the agonists, while calcium does not. We have already mentioned several experimental results which suggest that local anesthetics and Ca^{++} increase the affinity of the receptor site via different sites and most likely by different mechanisms. Characterization of these differences might lead to identification of the structures involved in the coupling between receptor and ionophore.

Several electrophysiological results, however, emphasize a parallel between the action of calcium and local anesthetics. Upon iontophoretic application of agonists at the frog neuromuscular junction, the membrane conductance, after a rapid increase, decreases rapidly. Desensitization takes place. Magazanik and Vyskočil (34) have shown that both Ca^{++} and local anesthetics increase the rate of desensitization. SKF 525-A, the most effective compound in enhancing desensitization, behaves in our system *in vivo* and *in*

vitro as a local anesthetic. One of the models proposed by Katz and Theshleff (42) and Magazanik and Vyskočil (34) to explain desensitization postulates a third structural state of the receptor, in addition to the "resting" and "active" states. Such a state would have an affinity for agonists higher than that of the "active" state. It is therefore possible that the high-affinity state of the receptor protein observed in the presence of local anesthetics corresponds to a "desensitized" form of the receptor.

An important question concerns the physiological significance of the affinities for acetylcholine and other cholinergic ligands of the membrane-bound form of the receptor in our preparations of membrane fragments *in vitro*. Since the affinity of the receptor can be controlled by its membrane environment and since changes by a factor of 100 take place upon detergent solubilization, one cannot be certain that the high-affinity form of the receptor is that of the receptor in its native synaptic environment. In fact, a simple calculation tends to suggest the opposite. Knowing the dissociation constant of acetylcholine for the high-affinity state (10 nM) and assuming that the rate of association of acetylcholine with the receptor site is bimolecular and limited by diffusion ($k \sim 10^9$ M^{-1} sec^{-1}) one can calculate a unimolecular rate of dissociation of the acetylcholine-receptor complex of about 10 sec^{-1}. In other words, the average time for which a single acetylcholine molecule would be bound to the receptor site would be on the order of 0.1 sec. Even if the association rate constant is 10 times higher, the expected time of receptor occupation (0.01 sec) would exceed the duration of the postsynaptic potential [about 0.005 sec (43)]. Although such a situation is not impossible, it is difficult to imagine why acetylcholine would be bound to the receptor for as much as 25 times the duration of the conductance change. Comparison of the kinetics of binding and of the change of conductance upon iontophoretic application of a fluorescent agonist to the live cell might help to clarify this situation.

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