MOLECULAR PHARMACOLOGY, 10, 35–40

Binding of *Naja nigricollis* [^{3}H]α-Toxin to Membrane Fragments from *Electrophorus* and *Torpedo* Electric Organs

III. Effects of Local Anaesthetics on the Binding of the Tritiated α-Neurotoxin

MICHEL WEBER AND JEAN-PIERRE CHANGEUX

Neurobiologie Moléculaire, Institut Pasteur, Paris XV^e, France

(Received June 11, 1973)

SUMMARY

WEBER, MICHEL, AND CHANGEUX, JEAN-PIERRE: Binding of *Naja nigricollis* [^{3}H]α-toxin to membrane fragments from *Electrophorus* and *Torpedo* electric organs. III. Effects of local anaesthetics on the binding of the tritiated α-neurotoxin. *Mol. Pharmacol.* **10,** 35–40 (1974).

Local anaesthetics block noncompetitively the depolarisation of *Electrophorus* electroplax by cholinergic agonists applied in the bathing medium. The most potent local anaesthetics tested are dimethisoquin (apparent dissociation constant I_{50} = 2 μM) and dibucaine (I_{50} = 5 μM). In addition, local anaesthetics decrease the initial rate of the binding of a tritiated α-neurotoxin from *Naja nigricollis* to membrane fragments purified from *Electrophorus* and *Torpedo* electric tissues. They also inhibit competitively the binding of [^{3}H]acetylcholine to the cholinergic receptor site from *Torpedo*. As in the case of cholinergic effectors, the concentrations of local anaesthetics which decrease by half the initial rate of [^{3}H]α-toxin binding are very similar to their dissociation constants for the receptor site determined by inhibition of [^{3}H]acetylcholine binding. However, these dissociation constants are one to two orders of magnitude larger than their apparent dissociation constants, I_{50}, measured with the isolated electroplax. It is concluded that local anaesthetics bind to the cholinergic receptor site with a low affinity, but that they inhibit the depolarisation of the electroplax by binding at different sites situated on or near the cholinergic receptor protein.

INTRODUCTION

The main pharmacological effect of local anaesthetics is to block the propagation of action potential, but they also interfere with the response to cholinergic agonists of a variety of subsynaptic membranes. As shown by Podleski and Bartels (1) and Bartels (2) with *Electrophorus* electroplax and by Kasai and Changeux (3) with excitable microsacs, the inhibition by several of them (procaine and tetracaine in particular) strikingly differs from the "competitive" antagonism observed between typical cholinergic ligands. The fractional inhibition of the response to cholinergic agonists by these local anaesthetics does not significantly vary with the concentration of agonist. In other words, the maximal response decreases, but

This work was supported by grants from the National Institutes of Health, United States Public Health Service, the Centre National de la Recherche Scientifique, the Délégation Générale à la Recherche Scientifique et Technique, the Fondation pour la Recherche Médicale Française, the Collège de France, and the Commissariat à l'Energie Atomique.

the concentration of agonist which gives a half-maximal response does not change. This phenomenon thus resembles the strictly noncompetitive inhibition described with enzymes.

In order to obtain some information on the site of action of local anaesthetics in the excitable membrane and in particular to determine whether these compounds interact directly with the cholinergic receptor protein, a study of the effect of local anaesthetics on [^{3}H]α-toxin and cholinergic ligand binding to *Electrophorus* and *Torpedo* membrane fragments was undertaken. The results strongly suggest that the local anaesthetics block the response to cholinergic agonists by binding at the level of the receptor protein at a site distinct from the binding site of cholinergic agonists.

MATERIALS AND METHODS

Procedures were the same as described in the preceding papers of this series (4, 5). All experiments were done at pH 7.0.

Sources of chemicals. Procaine hydrochloride was purchased from Sigma, and tetracaine hydrochloride and dibucaine hydrochloride, from K & K Laboratories. Prilocaine hydrochloride and dimethisoquin [1-(β-dimethylaminoethoxy)3-*n*-butyliso-quinoline hydrochloride] were a gift from the Laboratoire Roger Bellon (France).

RESULTS

Effects of local anaesthetics on Electrophorus electroplax in vivo. The early results of Podleski and Bartels (1) and Bartels (2) were confirmed for procaine and tetracaine and extended to three other local anaesthetics: prilocaine, dibucaine, and dimethisoquin. They all noncompetitively inhibited the depolarisation of the electroplax by decamethonium or carbamylcholine applied in the bathing medium. The concentrations (I_{50}) of these anaesthetics which decrease by one-half the response to the agonists are listed in Table 1. The most potent were dimethisoquin (I_{50} = 2 μM) and dibucaine (I_{50} = 5 μM).

Effects of local anaesthetics on initial rate and equilibrium of [^{3}H]α-toxin binding to Electrophorus membrane fragments. As in the case of cholinergic agonists and antagonists, we attempted to measure protection constants, K_p, by following the decrease in initial rate of [^{3}H]α-toxin binding as a function of local anaesthetic concentration. The membrane fragments were initially incubated for 10 min with the designated concentration of

TABLE 1

Comparison between physiological effects of various local anaesthetics and their protective effects against [^{3}H]α-toxin binding to Electrophorus membrane fragments

K_p is the concentration of local anaesthetics which decreases by half the initial rate of [^{3}H]α-toxin binding. I_{50} is the concentration which decreases by half the depolarisation of the eel electroplax by bath application of the agonist as indicated in parentheses, or the permeability change of membrane fragments to $^{22}Na^+$ produced by the agonists. The response was followed in the presence of: 3×10^{-5} M carbamylcholine (a), 10^{-4} M carbamylcholine (b), 4×10^{-4} M carbamylcholine (c), 2×10^{-6} M decamethonium (d), 4×10^{-6} M decamethonium (e).

Anaesthetic	*In vitro*		*In vivo*	
	Protection against [^{3}H]α-toxin binding (K_p)	Efflux of $^{22}Na^+$ from microsacs at 22°	Membrane potential of isolated electroplax (I_{50})	K_p/I_{50}
	M	*M*	*M*	
Tetracaine	1.7×10^{-4}	2.0×10^{-5}(b)	4×10^{-5}(b) 2.0×10^{-5}(e)	8.5
Procaine	1.1×10^{-3}	6×10^{-4}(c)	1.3×10^{-4}(a) 1.3×10^{-4}(d)	8.5
Dibucaine	1.3×10^{-4}		5.0×10^{-6}(d)	26
Dimethisoquin	4.5×10^{-5}		2.2×10^{-6}(d)	20
Prilocaine	4.5×10^{-3}		8×10^{-5}(d)	56

anaesthetic, and the reaction was started by adding the toxin.

Figure 1 shows the protection curves obtained with a series of five local anaesthetics. As with cholinergic agonists and antagonists, the initial rate tended toward zero at high concentrations of effector. However, for instance with procaine and tetracaine, the protection curves significantly deviated from a hyperbola. As the concentration increased, the initial rate decreased much more slowly than expected from a rectangular hyperbola. Nevertheless, we still regarded as a valid empirical K_p the concentration of anaesthetic which reduced by half the initial rate of [^{3}H]α-toxin binding. The K_p values of the five local anaesthetics tested were always larger than 500 μM, and therefore were systematically higher than those measured with the cholinergic agonists and antagonists tested (Table 1).

Similar results were obtained at equilibrium. Very high concentrations of local anaesthetics were required to displace bound [^{3}H]α-toxin. In the presence of about 0.1 nM concentrations of both [^{3}H]α-toxin and binding sites, little or no effect was seen up to 10 mM prilocaine; with dimethisoquin (the most potent local anaesthetic we have tested) a 0.1 mM concentration decreased by only 20% the amount of [^{3}H]α-toxin bound, and about 1 mM would be necessary to decrease it by half (see Fig. 13 of ref. 5). By comparison, 0.1 mM decamethonium (which acts *in vivo* at the same concentrations as dimethisoquin) decreased [^{3}H]α-toxin binding 90% under the same conditions.

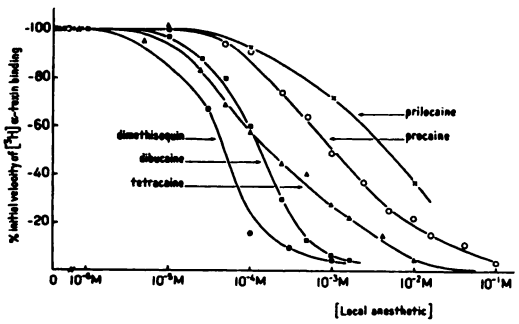

FIG. 1. *Effect of local anaesthetics on initial rate of [^{3}H]α-toxin binding to Electrophorus membrane fragments*

Membrane fragments were diluted in Ringer's solution and incubated for 10 min with the desired concentration of local anaesthetic. The reaction was started by adding [^{3}H]α-toxin. The concentrations of [^{3}H]α-toxin and binding sites varied between 1 and 5 nM.

Effect of local anaesthetics on direct binding of [^{3}H]acetylcholine to Torpedo membrane fragments. Additional information on the affinity of local anaesthetics for the cholinergic receptor site was gained by studying the displacement of [^{3}H]acetylcholine bound to *Torpedo* membrane fragments. The experimental conditions used were such that binding of [^{3}H]acetylcholine occurred almost ex-

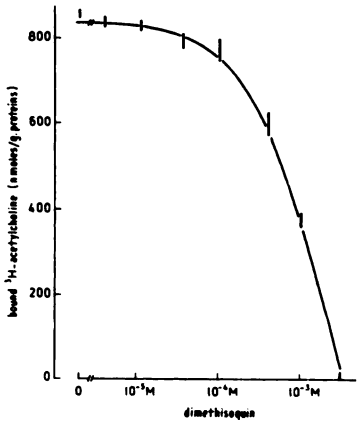

FIG. 2. *Inhibition by dimethisoquin of [^{3}H]-acetylcholine binding to Torpedo membrane fragments*

Torpedo membrane fragments were incubated for about 30 min with the indicated concentration of dimethisoquin in *Torpedo* Ringer's solution. Binding of [^{3}H]acetylcholine in the presence of 0.1 mM *O,O*-diethyl *S*-(β-diethylamino)ethyl phosphorothiolate was then measured as described previously (5). The total concentration of [^{3}H]-acetylcholine was 55 nM, and that of [^{3}H]acetylcholine binding sites was 114 nM (1730 nmoles/g of protein). The dissociation constant K_i for dimethosiquin was calculated from the formula

$$\frac{A_0}{A_0 + K_D} = 2\frac{A_{50}}{A_{50} + K_D(1 + I_{50}/K_i)}$$

where I_{50} is the concentration of dimethisoquin which reduces by half the amount of [^{3}H]acetylcholine bound, A_0 is the concentration of free [^{3}H]acetylcholine in the absence of dimethisoquin, and A_{50} is that in the presence of I_{50} dimethisoquin. K_D is the dissociation constant for acetylcholine (8.0 nM).

clusively at the level of the cholinergic receptor site. High concentrations of the local anaesthetics tested (procaine, tetracaine, and dimethisoquin) completely inhibited this binding (Fig. 2).

Figure 3 shows that tetracaine displaces [^{3}H]acetylcholine in a competitive manner. The K_D for tetracaine determined from this plot is 1.4 ± 0.5 mM. The protection constant measured with the same membrane preparation is K_p = 5 ± 1 mM. Thus there is close agreement between the equilibrium dissociation constant of tetracaine estimated by protection against [^{3}H]α-toxin binding (K_p) and that measured by following cholinergic ligand binding. However, the agreement is not as exact as observed with typical cholinergic agonists and antagonists. With the three local anaesthetics studied the K_D seems always 2–4-fold larger than the K_p (Table 2). The reasons for this minor but significant difference are not known.

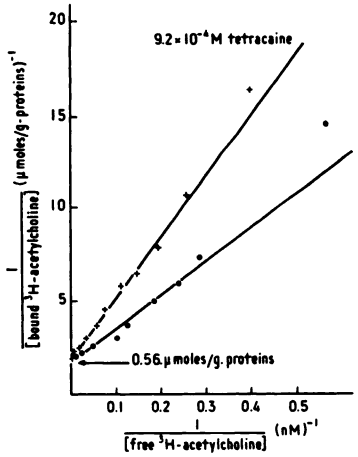

FIG. 3. *Competitive displacement by tetracaine of [^{3}H]acetylcholine from Torpedo membrane fragments (double-reciprocal plot)*

Torpedo membrane fragments were incubated for 30 min with 0.92 mM tetracaine in *Torpedo* Ringer's solution, and the binding of [^{3}H]acetylcholine was measured. The solid lines represent a least-squares fit of the data, and indicate a total number of binding sites of 73 nM (598 nmoles/g of protein) in the absence of tetracaine and 67 nM (550 nmoles/g of protein) in the presence of 0.92 mM tetracaine. The calculated dissociation constants for acetylcholine and tetracaine are 10.7 nM and 1.38 mM, respectively, ●——●, no tetracaine; +——+, 0.92 mM tetracaine.

Comparison between data from Electrophorus in vivo and in vitro. In Fig. 4 are plotted the protection curves measured *in vitro* with procaine and tetracaine and the dose-response curves recorded either with the isolated electroplax or with excitable microsacs by following the noncompetitive inhibition of the response to carbamylcholine

TABLE 2

Comparison of protection constants K_p and dissociation constants K_D of local anaesthetics for Torpedo membrane fragments

The results of two series of independent experiments performed on different preparations of membrane fragments are given for K_p values.

Anaesthetic	K_p against [^{3}H]α-toxin binding	K_D for displacement of [^{3}H]acetylcholine binding
	mM	*mM*
Procaine	0.95 ± 0.3	5 ± 1
	1.5 ± 0.3	
Tetracaine	0.5 ± 0.1	1.4 ± 0.5
	0.62 ± 0.26	
Dimethisoquin	0.01	0.058 ± 0.01
	0.018 ± 0.004	

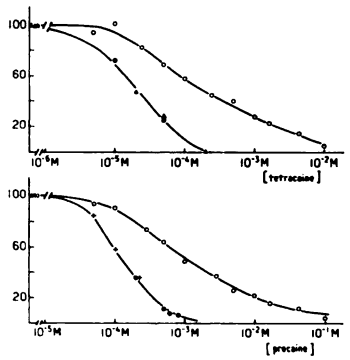

FIG. 4. *Comparison of effects of two local anaesthetics on initial rate of [^{3}H]α-toxin binding to excitable membrane fragments from Electrophorus and on response of electroplax to cholinergic agonists*

O——O, initial rate of [^{3}H]α-toxin binding (same data as for Fig. 1); ●——●, noncompetitive blockade of depolarisation of isolated electroplax by bath-applied 30 μM carbamylcholine (for procaine) or 4 μM decamethonium (for tetracaine); +——+, 2 μM decamethonium, ▲——▲, noncompetitive blockade of permeability response of excitable microsacs to 200 μM carbamylcholine. The data are from Kasai and Changeux (3).

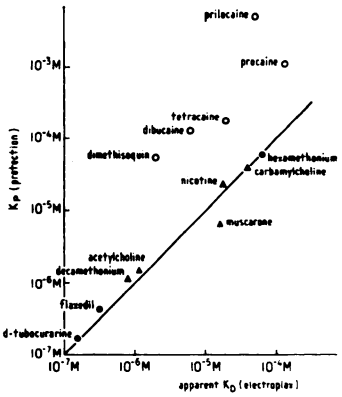

FIG. 5. *Comparison of protection constants (K_p) measured in vitro by following [³H]α-toxin binding and of apparent dissociation constants measured in vivo with isolated electroplax*

The abscissa represents the apparent dissociation constants for the cholinergic agonists and antagonists and I_{50} values for the local anaesthetics, as in Table 1.

or decamethonium. It is clear that the dose-response curves do not agree with the protection curves. The response is reduced 50% at 100 μM procaine and 20 μM tetracaine, while 50% reduction of the initial rate of [³H]α-toxin binding is seen at 1 mM procaine and 200 μM tetracaine. In both cases the protection curves are shifted towards the high concentrations of anaesthetic.

The same phenomenon is observed with all local anaesthetics tested: the protection constants K_p are one to two orders of magnitude larger than the concentrations (I_{50}) which decrease by half the physiological response of the electroplax (Table 1).

One of the most striking examples is dimethisoquin: 10 μM dimethisoquin blocks 88% of the depolarisation of the electroplax by 2 μM decamethonium, but decreases by only 10% the initial rate of [³H]α-toxin binding to *Electrophorus* microsacs.

The correlation between "protection constants" and "apparent dissociation constants" found with typical cholinergic effectors is thus not observed with local anaesthetics (Fig. 5).

DISCUSSION

Local anaesthetics constitute a chemically heterogeneous group of compounds which, in general, contain tertiary amines and hydrophobic residues, and block the propagation of the action potential (6). They also reduce, in a noncompetitive manner, the effects of cholinergic agonists on vertebrate neuromuscular junctions (7–9) and on *Electrophorus* electroplax (1, 2). Finally, some of them also modify the permeability of lipid bilayers and, in a more general manner, interact *in vitro* with lipids (10).

We have been concerned here with the effect of local anaesthetics on the response of the electroplax membrane to cholinergic agonists. First, we confirmed the results of Podleski and Bartels (1), Bartels (2), Bartels and Nachmansohn (11), and Kasai and Changeux (3), obtained with procaine and tetracaine and *Electrophorus* electroplax or microsacs, and extended them to three additional local anaesthetics: dimethisoquin, prilocaine, and dibucaine. All the local anaesthetics tested block the response to cholinergic agonists in a strictly noncompetitive manner. The apparent dissociation constants I_{50} found are also consistent with those reported previously with both *Electrophorus* electroplax and frog neuromuscular junction (7, 9).

In order to gain additional information about the noncompetitive inhibition by local anaesthetics, we studied their effect *in vitro* on the binding of either [³H]α-toxin or [³H]-acetylcholine to the cholinergic receptor site. With procaine, tetracaine, dimethisoquin and *Torpedo* membrane fragments, we found that the equilibrium dissociation constants for the cholinergic receptor site, measured by following the displacement of [³H]acetylcholine, are close although systematically larger than the constants measured by protection against [³H]α-toxin binding. Interestingly, the protection and dissociation constants were always one to two orders of magnitude larger than the corresponding "apparent" dissociation constants estimated on the electroplax. The five local anaesthetics tested bind to the cholinergic receptor site at concentrations much larger than those at which they block the response to cholinergic agonists.

The simplest interpretation of these results is that, although they bind to the cholinergic receptor site, the local anaes-

thetics do not block the response to cholinergic agonists by their interaction with the cholinergic receptor site. In agreement with Steinbach (8), the sites for their pharmacological action are therefore distinct from the cholinergic receptor site but nevertheless are located on or near the receptor protein. For all the compounds tested, the affinity for the "local anaesthetic receptor site" was higher than that for the cholinergic receptor site. It is possible that with different compounds the reverse situation occurs. This might be the case with benzoylcholine, for instance, which acts both as a partial agonist and as a noncompetitive blocking agent (2, 3). Binding of benzoylcholine to the cholinergic receptor site would then trigger a response while its binding to the "local anaesthetic receptor site" would block this effect.

The blocking effect of local anaesthetics on the response to agonists may be interpreted in various ways: for instance, they may bind to the ionophore and directly block cation transport (see ref. 9), or they may interfere with the coupling mechanism between receptor site and ionophore, either by blocking the conformational transition of the cholinergic protomer or by modifying its close environment, such as its interaction with membrane lipids.

The first hypothesis might be tested with excitable microsacs by studying an eventual competition with permeant cations. Comparative studies of the specific binding of local anaesthetics and of the conformational transitions of the receptor protein in the biological membrane, in detergent solution or integrated in lipid bilayers, should lead to unequivocal tests of the second hypothesis. If it happens to be the correct one, local anaesthetics might become adequate probes for monitoring the conformational transitions of the receptor protein.[1]

[1] Note added in proof: The validity of this prediction is supported by recent findings with a fluorescent cholinergic ligand and *Torpedo* membrane fragments (Cohen, J. B. and Changeux, J.-P., 1973, C. R. Hebd, Acad. Sc. 277, Série D, 603–605; Cohen J. B. and Changeux, J.-P., 1973, Biochemistry in press; Cohen, J. B., Weber, M. & Changeux, J.-P., in preparation).

Nothing can be said yet concerning the relation of the effects we see at the level of the cholinergic receptor protein and the pharmacological action of the local anaesthetics on the action potential.

Finally, it is worth emphasizing that snake venom α-toxin has enabled us to distinguish unambiguously the site of action of local anaesthetics from the cholinergic receptor site, although anaesthetics and cholinergic ligands often present striking similarities in structure. This is additional evidence for the particulary high selectivity of binding of these toxins to the nicotinic receptor site.

ACKNOWLEDGMENTS

We thank Professor P. Boquet for purification and a generous gift of pure α-toxin; Drs. A. Menez, J. L. Morgat and P. Fromageot for its tritiation; Professor P. G. Waser for the gift of muscarone; and the Laboratoire Roger Bellon for the gift of dimethisoquin and prilocaine. We thank Drs. R. L. Baldwin, H. Buc, J. B. Cohen, G. L. Hazelbauer, H. Lester, J. C. Meunier, R. W. Olsen, and R. Sealock for helpful criticism and suggestions and aid in the preparation of the manuscript. We thank Dr. J. Patrick for the privileged communication of a manuscript in publication.

REFERENCES

1. Podleski, T. R. & Bartels, E. (1963) *Biochim. Biophys. Acta* **75**, 387–396.
2. Bartels, E. (1965) *Biochim. Biophys. Acta*, **109**, 194–203.
3. Kasai, M. & Changeux, J.-P. (1971) *J. Membr. Biol.*, **6**, 1–23, 24–57, 58–80.
4. Weber, M. & Changeux, J.-P. (1974) *Mol. Pharmacol.*, **10**, 1–14.
5. Weber, M. & Changeux, J.-P. (1974) *Mol. Pharmacol.*, **10**, 15–34.
6. Ritchie, J. M. & Greengard, P. (1966) *Annu. Rev. Pharmacol.*, **6**, 405–430.
7. Maeno, T. (1966) *J. Physiol. (Lond)*, **183**, 592–606.
8. Steinbach, A. B., (1968) *J. Gen. Physiol.*, **52**, 144–161, 162–180.
9. Deguchi, T. & Narahashi, T. (1971) *J. Pharmacol. Exp. Ther.*, **176**, 423–433.
10. Papahadjopoulos, D. (1972) *Biochim. Biophys. Acta*, **265**, 169–186.
11. Bartels, E. & Nachmansohn, D. (1965) *Biochem. Z.* **342**, 359–374.