Biochimica et Biophysica Acta, 352 (1974) 192–201

BBA 76645

THE RESPONSE OF ARTIFICIAL LIPID MEMBRANES CONTAINING A CHOLINERGIC HYDROPHOBIC PROTEIN FROM *ELECTROPHORUS* ELECTROPLAX

T. A. READER and E. DE ROBERTIS

Instituto de Biologia Celular, Facultad de Medicina, Paraguay 2155, Buenos Aires (Argentina)

(Received September 11th, 1973)
(Revised manuscript received December 27th, 1973)

SUMMARY

1. A cholinergic hydrophobic protein from the electroplax of *Electrophorus electricus* was incorporated into artificial lipid membranes and the d.c. responses to various nicotinic agonists and antagonists were investigated. Differences in amplitude and in the characteristics of the transient response to some of the cholinergic agents were observed.

2. The response to the agonists was antagonized by the nicotinic blocking agents d-tubocurarine, mecamylamine and two derivatives of terephtalic acid (PK-97 and PK-119). The specificity of the cholinergic response was also supported by a variety of control experiments.

3. The action of disulfide and sulfhydryl reagents on the cholinergic response was investigated. 1,4-Dithiothreitol slightly reduced the response. *N*-Ethylmaleimide did not change the rising phase of the response, but the conductance remained sustained at a high level. Treatment with 1,4-dithiothreitol followed by *N*-ethylmaleimide blocked the cholinergic response.

4. The results observed are interpreted according to a model in which there is a coordination between the binding and the ionophoric response in the cholinergic receptor.

INTRODUCTION

Physiological studies on single electroplax of *Electrophorus electricus* have demonstrated that the activation produced by cholinergic agonists is blocked by the combined treatment with 1,4-dithiothreitol, which reduces disulfide bonds, and *N*-ethylmaleimide, an alkylating agent of the newly formed sulfhydryl groups [1, 2]. These findings suggested that the acetylcholine-activated permeability system of the electroplax may be of protein nature. Furthermore, in electroplax membranes isolated by subcellular fractionation, the action of these drugs resulted in a considerable decrease in the binding of cholinergic drugs [3]. Recent work from this laboratory suggested that this type of research could be pursued to the macromolecular level.

In fact, a special hydrophobic protein fraction (i.e. proteolipid) was extracted from the electric tissue of *Electrophorus electricus* [4, 5] and from electroplax membranes [3]. This protein showed high affinity binding for nicotinic cholinergic agents, including α-bungarotoxin [6]. When this fraction was incorporated into artificial lipid membranes these became "reactive" toward acetylcholine [7–9]. Since the cholinergic protein always contained a certain amount of lipids, treatment with the above-mentioned disulfide and sulfhydryl reagents could decide if the chemical reactivity was due to the protein or to the lipid moiety.

In the present article a more detailed analysis of the cholinergic response of artificial membranes has been carried out with the use of various nicotinic agonists and antagonists. In addition it will be demonstrated that disulfide and sulfhydryl groups are probably related to the translocation of ions induced by acetylcholine and carbamylcholine in artificial lipid membranes containing the cholinergic receptor protein from *E. electricus*.

MATERIALS AND METHODS

Artificial lipid membranes were made with a membrane forming solution of chloroform–methanol–tetradecane (1.0 : 0.8 : 0.4, by vol.). Type-a membranes contained 10 mg of synthetic dipalmitoyl-DL-α-lecithin per ml and 10 mg synthetic cholesterol per ml. Type-b membranes contained only 20 mg cholesterol per ml and Type-c membranes 10 mg cholesterol per ml and 10 mg total phospholipids per ml, from bovine cerebral cortex extracted according to Folch et al. [10]. The lipid–proteolipid membranes contained, in addition, 50–80 μg of two hydrophobic protein fractions per ml separated from the electroplax of *E. electricus*. One of them (i.e. Peak 1) has no binding capacity for acetylcholine and the other (i.e. Peak 3) binds nicotinic agents with high affinity [4, 5]. Additional controls were provided by an adrenergic hydrophobic protein from the spleen [11], an adrenergic protein from heart [12] and a γ-aminobutyric acid binding protein from shrimp muscle [13]. In all cases the hydrophobic proteins were added directly into the membrane-forming solution.

The membranes were made across a 1-mm diameter hole in an horizontal Teflon septum separating two chambers containing a solution of 100 mM NaCl and 1 mM Tris–HCl buffer (pH 7.0). The pattern of membrane formation was checked with a stereomicroscope. A voltage difference across the membrane was maintained constant by a d.c. source and it was measured, via AgCl/Cl electrodes with a Keithley d.c. Voltmeter 200 B (Keithley Instruments, Cleveland, Ohio). The current was determined with a Keithley 150 A microammeter and recorded with a Heath EUW servo recorder (Heath Co., Benton Harbor, Mich.). In some experiments voltage and current were displayed on the screen of a storage oscilloscope Tektronix 5103N (Tektronix, Inc., Beaverton, Oregon). The pharmacological agents were added with capillary tubes (Kimax 34500, size 1.5–2.0 mm × 100 mm) in 50–100-μl aliquots at a distance of 2–3 mm from the membrane or added to the bathing solution as far as possible from the membrane under stirring with an electrically driven small helix of Teflon. In all cases the drugs were applied to the positive side of the membrane, kept at a constant voltage of 100 mV. All experiments were performed at 22 °C (for further technical details see refs 8 and 9).

All the salts and drugs employed were of analytical grade or of the purest grade available. The following drugs were from Sigma Chemical Co., St. Louis, Mo.: cholesterol (synthetic, 99%), dipalmitoyl-DL-α-lecithin (chromatographic grade), acetylcholine chloride (vials), carbamylcholine chloride, acetylthiocholine chloride, butyrylcholine chloride, phosphorylcholine chloride, mecamylamine chloride, 1,4-dithiothreitol and *N*-ethylmaleimide. Tetradecane (olefin free, pure) was from Koch–Light Laboratories, Calnbrook, Bucks, England and tetraethylammonium chloride from the British Drug Houses, Ltd, Poole, England. Bromoacetylcholine bromide was kindly supplied by Dr A. Karlin and the agents PK-97 and PK-119 were a generous gift from Professor M. J. Michelson from Leningrad.

RESULTS

Cholinergic response of artificial lipid membranes

Table I summarizes the experiments in which the reactivity of artificial membranes of Type-a (see Materials and Methods) to different cholinergic agents was studied. Not only the nicotinic agonists listed but also the antagonists d-tubocurarine and mecamylamine produced a transient change in conductance in the membranes containing the cholinergic protein of Peak 3. However, these two antagonists were able to block the further response to another injection of cholinergic agonists. In Table I it may be observed that control membranes and those containing the non-cholinergic protein (i.e. Peak 1) did not react to the cholinergic drugs. Results were also negative in those membranes that contained the adrenergic protein from the spleen capsule [11], the adrenergic protein from heart [12] or the protein binding γ-aminobutyric acid extracted from shrimp muscle [13]. Other controls were done with Type-b and Type-c membranes and in all cases the results were negative, provided that Peak 3 protein from electroplax was absent. Negative results were also obtained in control, Peak 1 and Peak 3 containing membranes with injections of saline solution, NaCl, KCl, sodium acetate, sucrose, and choline chloride. All these agents were

TABLE I

REACTIVITY OF ARTIFICIAL LIPID MEMBRANES TO CHOLINERGIC AGENTS

Drug used	Concentration of the drug in the pipette (M)	Type of membranes		
		Control	Peak 1	Peak 3
Acetylcholine chloride	$5 \cdot 10^{-2}$	–	–	+
Carbamylcholine chloride	$5 \cdot 10^{-2}$	–	–	+
Butyrylcholine chloride	$5 \cdot 10^{-2}$	–	–	+
Phosphorylcholine chloride	$5 \cdot 10^{-2}$	–	–	+
Acetylthiocholine chloride	$5 \cdot 10^{-2}$	–	–	+
Bromoacetylcholine bromide	$5 \cdot 10^{-2}$	–	–	+
d-Tubocurarine chloride*	10^{-3}	–	–	+
Mecamylamine chloride*	10^{-3}	–	–	+

* d-Tubocurarine chloride and mecamylamine chloride produce blockade to cholinergic agents, after a first response.

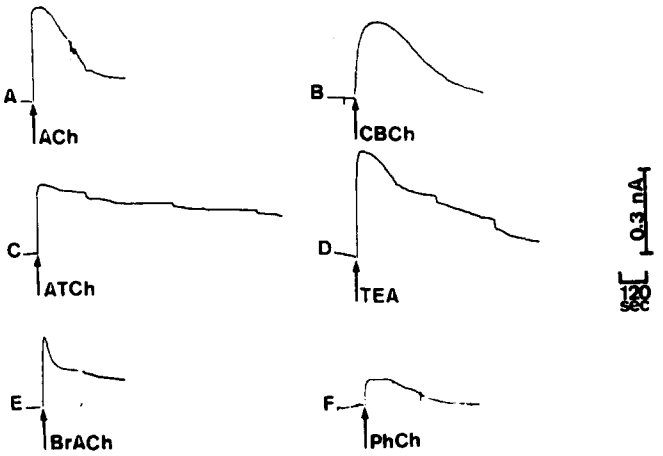


Fig. 1. Records of membrane current as a function of time. All experiments were done with Type-a membranes, containing synthetic lecithin and the cholinergic protein of Peak 3. BC, base line current. Voltage clamp: 100 mV. (A) BC: 1.30 nA. Response to acetylcholine chloride (ACh). (B) BC: 1.40 nA. Response to carbamylcholine chloride (CBCh). (C) BC: 1.20 nA. Response to acetylthiocholine chloride (ATCh). (D) BC: 1.20 nA. Response to tetraethylammonium chloride (TEA). (E) BC: 1.00 nA. Response to bromoacetylcholine bromide (BrACh). (F) BC: 1.15 nA. Response to phosphorylcholine chloride (PhCh). All drugs were injected upon the membrane in 100-μl aliquots at a concentration of $5 \cdot 10^{-2}$ M in the pipette.

applied at a concentration of 10^{-1} M in the pipette, dissolved in saline solution, making a total ionic strength of $2 \cdot 10^{-1}$ M in the pipette. Additional control experiments were performed with solutions of greater ionic strength ($3 \cdot 10^{-1}$ M of NaCl, sucrose and Tris–HCl) and the results were also negative. To avoid any pH effects all the solutions and agents tested were used at a pH of 7.0 ± 0.1. The cholinergic drugs were injected at a concentration of $5 \cdot 10^{-2}$ M; however, as previously published, responses could be obtained even with an effective concentration of 10^{-3} M in the pipette [8]. These agents were also dissolved in the saline solution, making a total ionic strength of $1.5 \cdot 10^{-1}$ M or $1.01 \cdot 10^{-1}$ M, respectively.

Fig. 1 shows typical records obtained with different cholinergic agents in membranes containing the cholinergic protein of Peak 3. Since the applied voltage remains constant throughout the experiment, an increase in conductance of the membrane is reflected in the d.c. effect. This change in conductance appears with the different cholinergic drugs employed, but the amplitude and the characteristics of the ascending and descending phases vary. For example, the response to acetylcholine increases more rapidly than that produced by carbamylcholine. With acetylthiocholine the d.c. current remains at a high level and returns very slowly to the base line. It is also of interest that the response to tetraethylammonium, a nicotinic blocking agent at autonomic ganglia, has a higher amplitude than to acetylcholine. Tetraethylammonium has been reported to exert only weak depolarization effects in the end-plates of the frog sartorius muscle [14]. The response to phosphorylcholine is rather low. The responses obtained with acetylcholine and carbamylcholine could be repeated several times with renewed injections of the drug (Fig. 2D). Both d-tubocurarine and mecamylamine, when injected upon the membranes containing the protein of Peak 3

induce a response; but afterwards they block a second response to an agonist (Fig. 2). If these blocking agents are added slowly to the bath under stirring, after a steady state is obtained, no transient d.c. is observed but the response to the injection of acetylcholine or carbamylcholine is blocked. A dose of 50 μl of 10^{-3} M d-tubocurarine is able to block the effect of $5 \cdot 10^{-2}$ M acetylcholine; since in the bath there is about a hundred times dilution the final concentration of d-tubocurarine is probably less than 10^{-5} M (Fig. 2A). Tetraethylammonium chloride also induces a conductance change. If after this response d-tubocurarine is injected the response to d-tubocurarine is smaller in amplitude than usually. A further injection of tetraethylammonium (Fig. 2C) does not produce any change in conductance.

The effect of two nicotinic blocking agents derived from terephtalic acid were also tested. As shown in the formula PK-97 has two trimethylammonium end-groups and PK-119 only one.

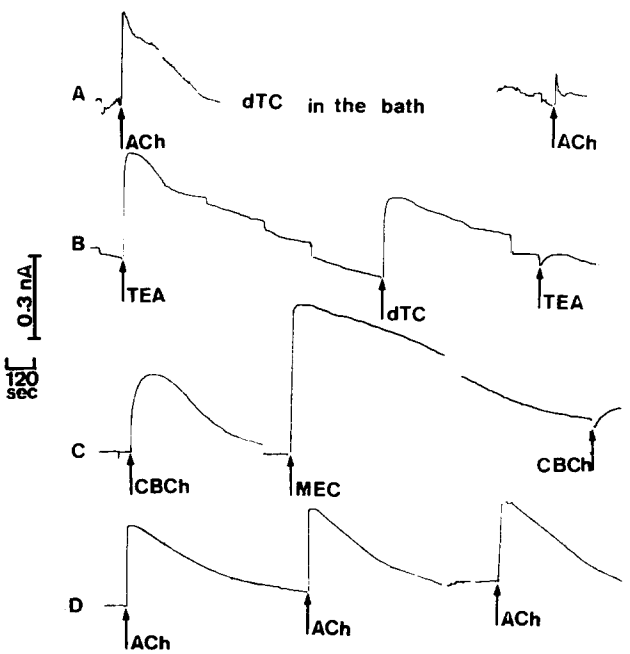


Fig. 2. Records of membrane current as a function of time showing the effect of blocking agents on the cholinergic response. (A) BC: 1.10 nA. After a first response to acetylcholine chloride (ACh) the membrane was treated with d-tubocurarine chloride (10^{-5} M) in the bath. Observe that the subsequent injection of the agonist does not elicit a response. (B) BC: 1.20 nA. In this membrane a response was obtained with tetraethylammonium chloride (TEA) and after a small response to d-tubocurarine chloride (dTC) no further response was obtained. (C) BC: 0.95 nA. The application of mecamylamine chloride (MEC) produces a blockade to the application of carbamylcholine chloride (CBCh). (D) BC: 1.60 nA. In this membrane, used as a control, several responses can be obtained with successive injections of acetylcholine chloride (ACh). All reagents when injected upon the membrane were applied in 50-μl aliquots at a concentration of $5 \cdot 10^{-2}$ M in the pipette for acetylcholine chloride, carbamylcholine chloride and tetraethylammonium chloride, and 10^{-3} M in the pipette for mecamylamine chloride and d-tubocurarine chloride.

PK-97 $(CH_3)_3-\overset{+}{N}-(CH_2)_4-O-C(=O)-C_6H_4-C(=O)-O-(CH_2)_4-\overset{+}{N}-(CH_3)_3$

PK-119 $(CH_3)_3-\overset{+}{N}-(CH_2)_4-O-C(=O)-C_6H_4-C(=O)-O-(CH_2)_3-CH_3$

On the intact animal, PK-97 is many times more effective in blocking the myoneural junction than PK-119 [15]. Both drugs when added to the bath in a concentration of 10^{-3} M in the pipette were able to block the further injection of acetylcholine or carbamylcholine. PK-97 had a more powerful blocking effect than PK-119 (Fig. 3). When these two agents were injected upon the membranes PK-97 produced a fast transient increase in conductance, but the base line very rapidly returned to its original level. This effect was not observed with PK-119.

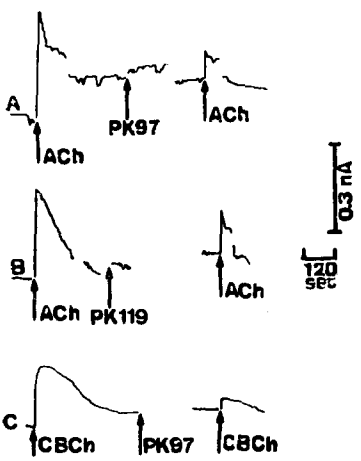


Fig. 3. Records of the response to acetylcholine chloride (ACh) and to carbamylcholine chloride (CBCh) before and after treatment with PK-97 and PK-119 (10^{-5} M in the bath). (A) BC: 2.20 nA. (B) BC: 1.60 nA. (C) BC: 1.60 nA. The cholinergic agonists were applied upon the membrane in 50-μl aliquots at a concentration of $5 \cdot 10^{-2}$ M in the pipette.

Action of disulfide and sulfhydryl reagents on the cholinergic response

Artificial membranes containing the cholinergic protein from *Electrophorus* (i.e. Peak 3) were treated with 1,4-dithiothreitol or *N*-ethylmaleimide, or with both agents, in that sequence, at a final concentration of $2 \cdot 10^{-3}$ M. 1,4-Dithiothreitol did not change the basal current, while *N*-ethylmaleimide induced a transient increase in conductance which disappeared in 1–2 min and the current returned to the initial d.c. value. After 20 min of the application of 1,4-dithiothreitol the responses to acetylcholine and carbamylcholine were slightly reduced in amplitude but the rise time and the decay of the transient remained unchanged (Fig. 4). A similar treatment with *N*-ethylmaleimide alone did not change the rising phase of the transient response produced by the cholinergic agonists. The most striking result was that the conductance remained sustained at a high level without returning to the basal line. In experiments

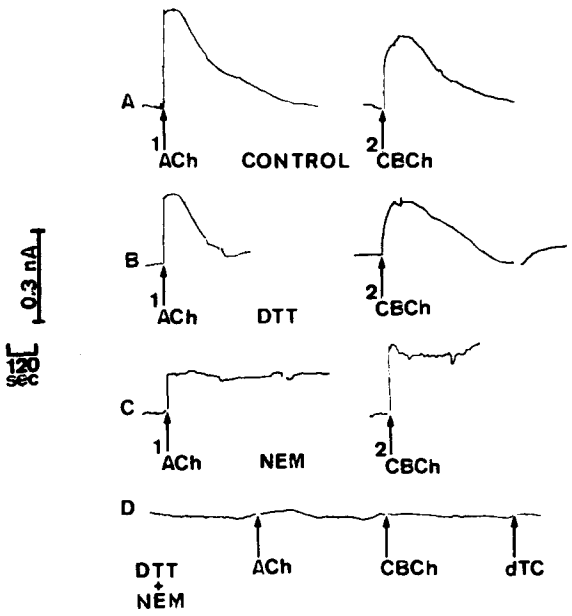


Fig. 4. Records of the response to cholinergic agents in membranes treated with disulfide and sulfhydryl group reagents. (A) Without treatment. (1) Response to acetylcholine chloride (ACh). BC: 1.10 nA. (2) Response to carbamylcholine chloride (CBCh). BC: 0.75 nA. (B) After treating the membranes with $2 \cdot 10^{-3}$ M 1,4-dithiothreitol in the bath for 20 min. (1) Response to acetylcholine chloride (ACh). BC: 1.20 nA. (2) Response to carbamylcholine chloride (CBCh). BC: 0.90 nA. (C) After treating the membranes with $2 \cdot 10^{-3}$ M *N*-ethylmaleimide in the bath for 20 min. (1) Response to acetylcholine chloride (ACh). BC: 2.40 nA. (2) Response to carbamylcholine chloride (CBCh). BC: 1.60 nA. (D) After treating the membranes with $2 \cdot 10^{-3}$ M 1,4-dithiothreitol and after 20 min with *N*-ethylmaleimide for another 20 min. BC: 1.40 nA. No responses are obtained either with acetylcholine chloride (ACh), carbamylcholine chloride (CBCh) or d-tubocurarine chloride (dTC). In all the experiments shown in this figure acetylcholine chloride, d-tubocurarine chloride and carbamylcholine chloride were injected upon the membrane in 100-μl aliquots at a concentration of $5 \cdot 10^{-2}$ M in the pipette.

in which stirring was introduced in the upper chamber to accelerate diffusion this descending phase was much shortened in the untreated membranes reacting to acetylcholine but not in the membranes treated with *N*-ethylmaleimide. It is also interesting that in order to act 1,4-dithiothreitol and *N*-ethylmaleimide should remain in contact with the membrane for at least 10 min; before that time the normal response to the cholinergic agonists is observed. The successive treatment with 1,4-dithiothreitol and *N*-ethylmaleimide (20 min each) produced a complete blockade of the cholinergic response. This inhibition of the response to acetylcholine or to carbamylcholine was studied as a function of the time of exposure to the drugs and the blockade started to be observed after 10 min.

DISCUSSION

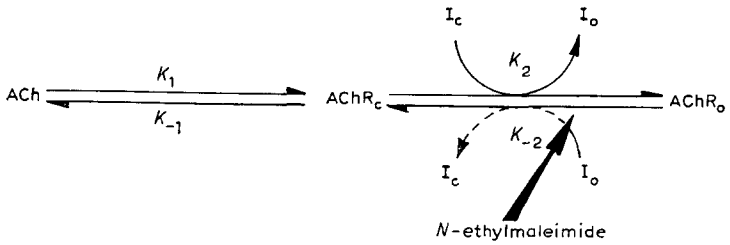
Present experiments on artificial lipid membranes containing the cholinergic hydrophobic protein (Peak 3) from the electroplax of *E. electricus* permit to gain

further insight into the mechanism of the cholinergic-induced response. Numerous control experiments support the specificity of this response. The reported variations in conductance are only induced by cholinergic agents and not by the addition of saline solutions having similar or even greater ionic strength. It is also demonstrated that these effects are not due to pH changes. Several nicotinic agents, agonists and antagonists, such as d-tubocurarine and mecamylamine, produce transient conductance changes in membranes containing the cholinergic protein, but not in membranes having other proteins from *E. electricus* (i.e. Peak 1) or from other tissues. The fact that d-tubocurarine, mecamylamine, PK-97 and PK-119 are able to block the responses elicited by acetylcholine and carbamylcholine also demonstrates the specificity of the response.

The concentration of the agonists and of the antagonists applied to the membrane seem rather high in comparison to the ones used on biological membranes. However, it is necessary to consider that the lipid matrix in which the receptor protein is embedded is very different than that of the biological membrane; in this there is also a high concentration of proteins. This difference is reflected in the high electrical resistance of the artificial membranes [8] which in our case is of the order of $5 \cdot 10^5\ \Omega \cdot \mathrm{cm}^2$. In recent experiments [9] we have observed, that in the presence of uranyl ions, the ionophoric response of the membrane toward acetylcholine may be increased by a factor of one hundred. In this case large conductance transients are obtained with concentrations of $5 \cdot 10^{-4}$ M acetylcholine in the capillary tube, which corresponds to a final concentration in the bath of $5 \cdot 10^{-6}$ M. It is interesting to recall that within the synaptic vesicle a concentration of acetylcholine of 10^{-1} M has been postulated [16] and that in physiological conditions the concentration in the synaptic gap may reach 10^{-6}–10^{-7} M [17].

An interesting observation has been the different rise time and decay of the transient response obtained with the various cholinergic agonists. This is of particular interest in view of the fact that there is no acetylcholinesterase in the system. The slower rise of the transient with carbamylcholine is specially remarkable (Fig. 1), and may be compared with the slower depolarization induced by this agent in the living electroplax [1].

Exposure of the membranes to agents that reduce disulfide bonds (i.e. 1,4-dithiothreitol) partially inhibits the cholinergic response. This is completely abolished if the newly formed sulfhydryl groups are permanently alkylated with *N*-ethylmaleimide. These findings are also comparable with the blockade previously observed in the living electroplax [1, 2]. 1,4-Dithiothreitol is a reagent that reduces disulfide bonds, with a minimum formation of mixed disulfides [18], and partially inhibits the depolarization of the electroplax membrane induced by cholinergic agonists [1, 2]. The effect of 1,4-dithiothreitol is made irreversible if *N*-ethylmaleimide is introduced in the system to alkylate the sulfhydryl groups [1, 2]. In our system the treatment with 1,4-dithiothreitol, followed by *N*-ethylmaleimide, leads not only to the complete blockade of the response to the agonist but also to the inhibition of the transient response produced by d-tubocurarine (Fig. 4). The fact that *N*-ethylmaleimide alone blocks the descending phase of the transient without affecting the ascending one, seems of particular interest in relation to the model of the cholinergic receptor postulated by De Robertis [19]. We could consider the effect of a cholinergic agonist as having the following interactions:



The first part of the diagram ($AChR_c$) corresponds to the interaction of acetylcholine with the binding site, being the ionophore in the closed condition. The second part corresponds to the opening of the ionophore in the acetylcholine–receptor complex ($AChR_o$). Albuquerque et al. [20] have recently demonstrated that histrionicotoxin, an active agent isolated from the colombian arrow-poison frog *Dendrobates histrionicus* [21] is able to interact with the receptor ionophore of the myoneural junction maintaining it in the closed condition. We think that *N*-ethylmaleimide may interact with the receptor ionophore maintaining it in a more permanent open position. Support to this hypothesis is provided by the observation that *N*-ethylmaleimide inhibits the repolarization of the living electroplax after carbamylcholine [1]. On the other hand, De Robertis and Fiszer de Plazas [3] have demonstrated that both *N*-ethylmaleimide and 1,4-dithiothreitol alone did not interfere with the binding of acetylcholine to the electroplax membranes while the combined action of 1,4-dithiothreitol and *N*-ethylmaleimide greatly reduced the binding. The blocking action of these two drugs used in combination upon the acetylcholine response is consistent with the idea put forward by Karlin and co-workers [1, 2] that disulfide groups are essential in the function of the cholinergic receptor. Furthermore Del Castillo (personal communication) has suggested that the process of activation of the cholinergic receptor implies a change in the free sulfhydryl groups of the receptor protein. In fact, activation can be induced with heavy metals or reagents such as *N*-ethylmaleimide or organic mercurials reacting monovalently with sulfhydryl groups (ref. 22 and Del Castillo, J., personal communication).

The results obtained in artificial lipid membranes containing the cholinergic protein from *Electrophorus* in general resemble those obtained in the single-cell preparation of the electroplax [1, 2], and show the same specificity of the response to different cholinergic nicotinic agents. These findings also suggest that it is the protein and not the lipid moiety of the isolated cholinergic proteolipid the one responsible for the binding and the ionophoric properties of the receptor when this is reconstituted in the artificial membrane.

ACKNOWLEDGEMENTS

We acknowledge Professor M. J. Michelson for providing us with the drugs PK-97 and PK-119 and Dr Karlin for supplying us with bromoacetylcholine bromide.

This work was supported by Grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) Unidad Neurobiológica, Legajo 1824 G/71, Argentina and from National Institutes of Health (2 ROI NS 06953-07 NEUA) U.S.A.

REFERENCES

1 Karlin, A. and Bartels, E. (1966) Biochim. Biophys. Acta 126, 525
2 Karlin, A. (1969) J. Gen. Physiol. 54, 245S
3 De Robertis, E. and Fiszer de Plazas, S. (1970) Biochim. Biophys. Acta 219, 388
4 La Torre, J. L., Lunt, G. S. and De Robertis, E. (1970) Proc. Natl. Acad. Sci. U.S. 65, 716
5 De Robertis, E., Lunt, G. S. and La Torre, J. L. (1971) Mol. Pharmacol. 7, 97
6 Fiszer de Plazas, S. and De Robertis, E. (1972) Biochim. Biophys. Acta 274, 258
7 Parisi, M., Rivas, E. and De Robertis, E. (1971) Science 172, 56
8 Parisi, M., Reader, T. A. and De Robertis, E. (1972) J. Gen. Physiol. 60, 454
9 Reader, T. A., Parisi, M. and De Robertis, E. (1973) Biochem. Biophys. Res. Commun. 53, 10
10 Folch, J., Lees, M. and Sloane Stanley, G. H. (1957) J. Biol. Chem. 226, 497
11 Fiszer de Plazas, S. and De Robertis, E. (1972) Biochim. Biophys. Acta 266, 246
12 Ochoa, E., Llorente de Carlin, M. C. and De Robertis, E. (1972) Eur. J. Pharmacol. 18, 367
13 Fiszer de Plazas, S. and De Robertis, E. (1973) FEBS Lett. 33, 45
14 Sobrino, J. A. and Del Castillo, J. (1972) Int. J. Neurosci. 3, 251
15 Michelson, M. J. (1969) Proc. IVth Int. Congr. Pharmacol. 4, 103
16 Macintosh, F. C. (1959) J. Biochim. Physiol. 37, 343
17 Del Castillo, J. and Katz, B. (1955) J. Physiol. London 128, 157
18 Cleland, W. W. (1964) Biochemistry 3, 480
19 De Robertis, E. (1971) Science 171, 963
20 Albuquerque, E. X., Barnard, E. A., Chiu, T. H., Jlapa, A. J., Oliver, J., Dolly, J., Jansson, S.-E., Daly, J. and Witkop, B. (1973) Proc. Natl. Acad. Sci. U.S. 70, 949
21 Daly, J. W., Karle, I., Myers, C. W., Tokuyama, T., Waters, J. A. and Witkop, B. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1870
22 Del Castillo, J., Escobar, I. and Guijon, E. (1971) Int. J. Neurosci. 1, 199