REACTIVITY TO ACETYLCHOLINE DEVELOPED BY ARTIFICIAL MEMBRANES CONTAINING A PROTEIN FROM ELECTROPHORUS ELECTRICUS: THE EFFECT OF URANYL IONS.

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SUMMARY

A special hydrophobic protein fraction from the electroplax of Electrophorus electricus, when incorporated into artifical lipid membranes, induces a reactivity to acetylcholine. Uranyl ions increase 10-20 times the conductivity of such membranes, produce discrete current jumps, and strongly potentiate the effect of acetylcholine. The response to acetylcholine was studied in media containing KCl,NaCl,Choline-Cl and Na-Propionate in the presence or absence of uranyl ions. Control membranes made of lipids or containing a non-cholinergic protein from the same tissue showed no reactivity to acetylcholine and had only a slight increase in conductance at very high concentrations of uranyl ions.

INTRODUCTION

A special hydrophobic protein fraction (i.e.: proteolipid) from the electroplax of Electrophorus electricus binding acetylcholine was isolated (1) and incorporated into artificial lipidic membranes. Such membranes became sensitive to the application of acetylcholine and "reacted" with a rapid and transient increase in conductance (2). Specific effects were also found with other nicotinic agents (3). Controls involving the use of other hydrophobic proteins from the electroplax or from different tissues gave negative results. Other investigators have shown that the surface charge of lipidic membranes can be modified by the adsorption of divalent cations, such as uranyl ions (4) and that this effect can be used as an ionic probe (5) in such membranes. The main purpose of the present investigation has been to study the effect of uranyl ions on the resistance of artificial membranes containing the cholinergic protein fraction from

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Electrophorus electricus and to obtain further information about the response to acetylcholine. In this way it was hoped to obtain a deeper insight into the ionic mechanisms underlying these phenomena.

EXPERIMENTAL

The membrane forming solution (MFS) of control membranes contained 10 mg/ml synthetic cholesterol (Sigma Chemical Co.,St.Louis,Mo.,99+%) and dipalmitoyl-DL-α-lecithin (Sigma Chemical Co., St.Louis,Mo., Standard for chromatography) dissolved in chloroform-methanol-tetradecane (1.0:0.8:0.6). The experimental membranes contained in addition up to 80 μg/ml of hydrophobic protein from the electroplax of Electrophorus electricus. Only protein fractions 1 and 3, the latter having binding capacity for cholinergic drugs were used after the separation of the five protein peaks by column chromatography in Sephadex LH-20 (Pharmacia Fine Chemicals,Inc.,Uppsala,Sweden)(1).

The membranes were formed across a 1 mm diameter hole in an horizontal Teflon septum and the membrane current was recorded as previously described (2,3). The pharmacological agents were added in 50 μl aliquots with capillary tubes (3). All experiments were carried out at a temperature of 22°C . The bath solution contained 100 mM NaCl and 1 mM Tris-Cl buffer (pH 7.0), unless otherwise specified.

Control membranes had a basal conductance of about 1.5×10^{-6} mho. cm^{-2} which increased up to 5×10^{-6} mho.cm^{-2} by the addition of the protein (3). Because uranyl ions have been reported (4) to reduce the conductance of phospholipid membranes containing a cation selective carrier, the action of this divalent cation was investigated. Control membranes and membranes containing the protein from peak 1 showed no significant change in conductance when formed in the presence of uranyl acetate 10^{-6} M (Fig. 1A and B) while those containing peak 3 showed a definite increase (Fig. 1C).

Fluorescence was determined on a Perkin Elmer MPF-3 fluorometer standardized with a fluorescent standard (p-terphenyl gel, $\sim 3 \times 10^{-5}$ M) at an excitation wavelength of 390 nm, an emission wavelength of 470 nm, and a slit width such that a 1.35×10^{-6} M solution of quinine sulfate in 0.1 N sulfuric acid gave one relative fluorescence unit.

RESULTS

This method was capable of detecting small concentrations of arginine splitting proteases: trypsin, thrombin, and pepsin (1). Chymotrypsin, on the other hand, was totally inactive. This is to be expected as chymotrypsin preferentially hydrolyzes aromatic amino acid peptides (1).

Trypsin was detectable at concentrations as low as 5 ng/ml at 20 minutes. The rate of hydrolysis was proportional to enzyme concentration over a thousand-fold range (Figure 1). After twenty minutes the rate of hydrolysis decreased (data not shown). This assay, therefore, did not prove useful for incubation times longer than twenty minutes with trypsin.

Thrombin was capable of hydrolysing the DNPP at a concentration as low as 8.3 NIH units/ml at 40 minutes. The rate of hydrolysis was proportional to enzyme concentration over at least a five-fold range (Figure 2). After 40 minutes the rate of hydrolysis decreased (data not shown). Both thrombin and trypsin were inhibited by 4.5×10^{-3} M n-acetylimidazole (Table I).

Amino acid analysis of the protamine sulfate used in these studies is shown in Table II and compared to that previously reported (2).

DISCUSSION

Protamine, with its amino terminal group blocked with DNFB, offers an advantage as a substrate for trypsin-like enzymes. It did not yield any fluorescence with fluorescamine and was stable for months at room temperature.

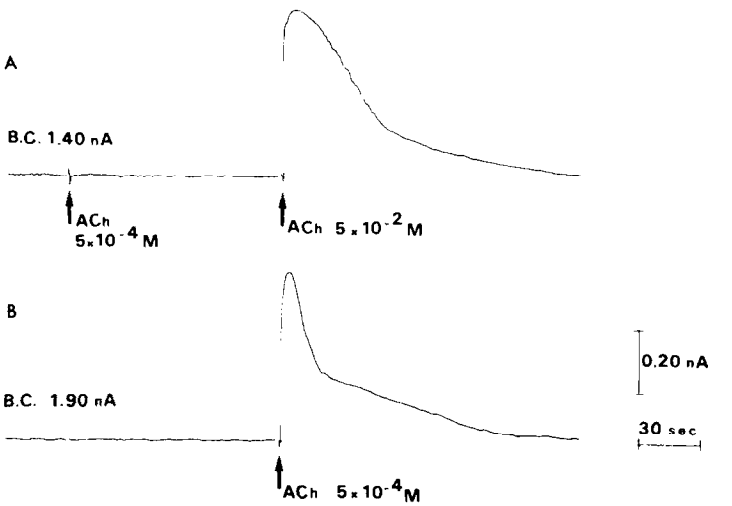

FIGURE 2 Response of membranes containing peak 3 to acetylcholine chloride (ACh). A) in the presence of NaCl 100 mM and Tris-Cl buffer 1 mM. B) in the presence of NaCl 100 mM, Tris-Cl buffer 1 mM and 10^{-6} M UO_2^{++} . Voltage: 100 mV.

Control membranes and those containing the protein from peak 1 showed no change in conductance with uranyl acetate up to 5 x 10^{-5} M in all the media tested. On the contrary, in those membranes containing peak 3 , at concentrations as low as 5 x 10^{-6} M the uranyl induced a dramatic increase in conductance(Fig. 3C). The membrane conductance increases up to 10^{-4} mho.cm^{-2} . This increase in conductance takes about ten minutes to reach an equilibrium, and after that sometimes it may slowly decrease. With 5 x 10^{-4} M uranyl acetate a slight and slow increase in conductance(up to two-fold) was observed in control and peak 1 membranes (Fig. 3), while in those containing peak 3 the increase was rapid and of a much higher magnitude (10-20 fold). In some cases the increase in conductance induced by uranyl ions in the presence of peak 3 may be in discrete steps of about 6 x 10^{-10} mho and the record shows a "staircase" appearance (Fig. 3D).

The response to ACh-Cl (5 x 10^{-2} M in the pipette) given by membranes containing peak 3 was studied in media containing NaCl,

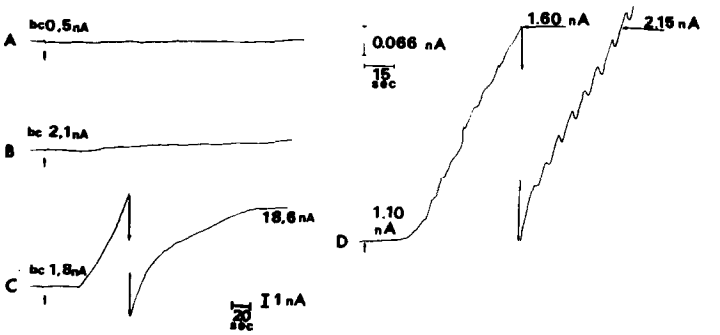

FIGURE 3 Records of DC current across artificial lipid membranes. A) Control membrane; B) Membrane containing peak 1 ; C) Membrane containing peak 3. The arrows show the addition of UO_2^{++} to the lower chamber (negative side). Voltage: 100 mV.
D) "Staircase" increase in membrane current, in the presence of UO_2^{++} 10^{-5} M in the lower chamber. Voltage: 50 mV.

KCl, Choline-Cl and Na-Propionate (100 mM in each case) and with the addition of uranyl ions to the lower chamber (negative side). In all cases the response obtained at the equilibrium level was proportional to the increase in conductance induced by uranyl ions. These low resistance membranes did not show any change in conductance after the injection of NaCl, Choline-Cl or KCl (Table I). On the other hand, control membranes or those containing peak 1 did not react towards ACh-Cl, even in the presence of 5×10^{-4} M uranyl acetate in the lower chamber.

An observation which may be of interest in relation to the ionophoric function of the cholinergic protein is that after storage for a few days it generally looses its capacity to decrease the membrane resistance and simultaneously the reactivity to uranyl ions and ACh-Cl is greatly reduced or completely lost.

DISCUSSION

It has been postulated that polyvalent cations, such as UO_2^{++} may bind to phospholipids and by this mechanism could change the surface charge of the membrane (6). A positive surface charge decreases the

TABLE I

Effect of uranyl ions on the conductance of artificial membranes containing the peak 3 protein fraction

Media	Basal current nA		Transient increase induced by ACh (5×10^{-2} M in the pipette) nA	
	Before uranyl treatment	After uranyl treatment	Before uranyl treatment	After uranyl treatment
NaCl	2.00	11.00	0.20	3.80
KCl	1.60	8.00	0.60	3.00
Choline-Cl	1.40	6.30	0.25	1.50
Na-Propionate	2.20	16.60	0.40	4.00

concentration of positive ions and increases that of negative ones ir the membrane thus increasing the conductance for negative species and depressing it for positive ones (5). Furthermore it has been reported that uranyl ions reduce the conductance of artificial membranes containing cation selective carriers (4). Because of the low permeability of control lipidic membranes this surface charge effect could be difficult to detect (7).

The effect observed with uranyl ions on the membranes containing peak 3 could be due to a large and specific increase in anionic translocation because of the adsorption of UO_2^{++} to some special phospholipid present in the peak 3 fraction. However it could also be due to a special binding to the protein moiety resulting in the formation of some type of anionic channel. Because of their high electron density, the adsorption of uranyl ions to the membranes containing peak 3 could be detected under the electron microscope and the results obtained will be published elsewhere (8).

We have previously reported on the observation of current jumps in artificial membranes containing the peak 3 fraction (3,9). In general such jumps had a conductance of 3×10^{-10} mho. In some of the experiments with uranyl ions the increase in conductance showed a "staircase" shape, each step having about 6×10^{-10} mho. It is

intriguing the fact that the small fluctuations observed, either in control conditions or in the presence of uranyl ions are not regularly observed and their appearance can not be related to a particular experimental condition. Recently conductance jumps of about 2.2×10^{-10} mho have been reported with crude extracts of electroplax incorporated into artificial membranes (10). Their magnitude and frequency is reminiscent of the fluctuations previously observed by us with the cholinergic protein of the electroplax (3,9). Discrete conductance jumps, having about the same amplitude (i.e.: 1×10^{-10} mho) have been found by Katz and Miledi (11,12) studying the effect of ACh on the membrane potential noise of the myoneural junction and a similar conductance value is ascribed to single Na^+ channels in the axon membrane (13). The possible relationship between our findings in reconstituted lipid-proteolipid membranes and those observed in chemical and electrically excitable membranes is a matter of conjecture.

One of the most interesting effects of uranyl ions on the membranes is the induction of a 100 fold increase in sensitivity towards ACh. It has been previously suggested that the protein fraction contained in peak 3 represents the cholinergic receptor of the electroplax (14) and this concept has been recently supported by the finding that it binds α-bungarotoxin in the relation of 1 mole to 37,000 g (15).

In a chemical receptor two main functions are recognized, i.e. the specific recognition site and the ionophore for the translocation of ions. The ideal goal would be the reconstitution of both functions in an artificial membrane. The system we have described has a specific reaction to ACh, however differences with the physiological situation are encountered. They refer mainly to the high concentra-

tions of acetylcholine needed to activate the receptor, the different time base of the response and the possible ionic mechanisms involved.

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