INCORPORATION OF EEL ELECTROPLAX ACETYLCHOLINESTERASE INTO BLACK LIPID MEMBRANES. A POSSIBLE MODEL FOR THE CHOLINERGIC RECEPTOR.*

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Summary: Addition of low concentrations of acetylcholine or carbamylcholine to solutions bathing a black lipid membrane into which electroplax acetylcholinesterase has been incorporated elicits a dramatic increase in the membrane conductance. This change is prevented or reversed by addition of neostigmine or atropine to the system. The magnitude of the conductance increase of the acetylcholinesterase-treated membrane is proportional to the fourth power of the carbamylcholine concentration and, at constant carbamylcholine concentration, to the fourth power of the enzyme concentration in the medium.

INTRODUCTION

Since Mueller, Rudin, Tien, and Westcott first reported the preparation of black lipid membranes (BLM) (1), these structures have been extensively employed as models for biological membranes (2). One of the most important properties of biological membranes for which simpler models have been sought is the coupling of a chemical reaction to a transmembrane transport process. This property bears on a number of important membrane phenomena, including the functioning of biological receptors. In efforts to identify and characterize the cholinergic receptor, incorporation of protein-containing fractions into BLM have yielded partially successful models for this receptor in three cases (3-5). We now wish to report that incorporation of a partially purified acetylcholinesterase from eel electroplax into BLM formed from oxidized cholesterol yields a preparation possessing several of the system properties of the cholinergic receptor.

METHODS AND MATERIALS

The procedure for the formation of BLM and the apparatus employed for measurement of the properties of these membranes have been previously described (6). The

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BLM employed in this study were prepared from oxidized cholesterol obtained by a minor modification of the procedure described earlier (7); specifically a 4 percent solution of cholesterol (99 + percent pure, Sigma Chemical Co.) in a 1:1 mixture of decane and tetradecane was refluxed for twenty hours with vigorous stirring. The resulting preparation yielded a stable BLM. Eel electroplax acetylcholinesterase, Type VI, (acetylcholine acetylhydrolase, EC 3.1.1.7) was obtained from the Sigma Chemical Company, who prepared the enzyme according the procedure of Lawler (8). This preparation had a specific activity of 300 units per mg protein. Results reported herein vary in detail with different acetylcholinesterase preparations. All other reagents employed were of the best grade available commercially.

RESULTS AND DISCUSSION

BLM were prepared at 33-36° in bathing solutions containing 1 M KCl and 10 mM Tris hydrochloride, pH 7.6. To one side (the cis side) of the membrane was added a freshly prepared solution of eel electroplax acetylcholinesterase at a final concentration of 4 μg/ml and the protein was permitted to interact with the membrane for a period of 6 to 10 minutes. Throughout, the cis compartment was maintained clamped at 70 mV positive with respect to the other compartment, trans, which was maintained at ground potential. As shown in Figure 1, the enzyme preparation by itself did not cause a change in the conductance properties of the BLM. However, the subsequent addition of dilute solutions of acetylcholine or carbamylcholine to either the cis or trans side of the membrane causes a marked increase in membrane conductance, reflected in increased transmembrane current, I_m, at the constant applied potential (Fig. 1). No alteration in BLM conductance was elicited by acetylcholine or carbamylcholine in the absence of enzyme, nor by either of these substances in the presence of heat-inactivated enzyme, nor by choline in the presence of the enzyme, nor by acetylcholine in cases in which the BLM has been permitted to interact with either bovine serum albumin or cytochrome c. Hence, the change in membrane conductance appears to require specifically active acetylcholinesterase and one of its substrates. As also shown in Figure 1, addition of neostigmine, an inhibitor of acetylcholinesterase, following that of acetyl- or

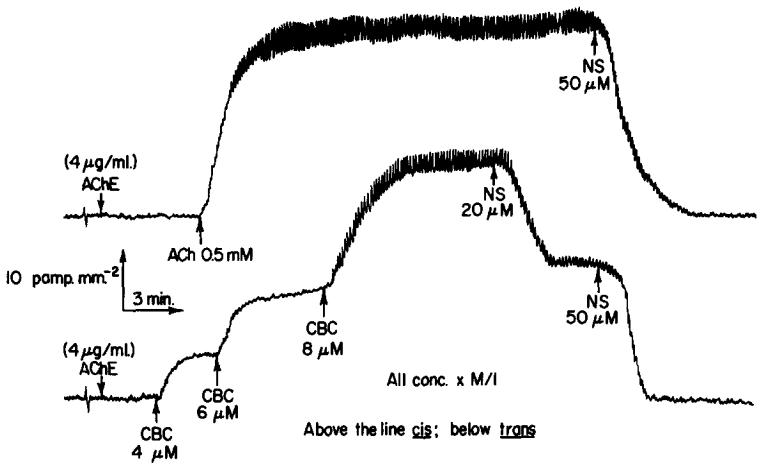

Figure 1. Plots of transmembrane current for a black lipid membrane (clamped at 70 mV potential) as a function of time. Current is measured as pico-amps per square mm of membrane area. Abbreviations employed are acetylcholinesterase, AChE; acetylcholine, ACh; carbamylcholine, CBC; and neostigmine, NS. Those substances written above the line of the trace were added to the same side of the BLM as the enzyme, the cis side; those written below were added to the opposite, trans, side. Note that the noise levels increase with increasing concentration of acetyl- and carbamylcholine; the magnitude of the noise level is accurately represented in this figure but the frequency is not. Actual oscilloscope traces indicate that the lifetime of the noise pulses is less than 10 msec and that their amplitude is less than 10^{-9} mho. These traces can be compared with plots of resting potential vs. time for the isolated electroplax; H. B. Higman, et al., Biochim. Biophys. Acta, 75, 187 (1963).

carbamylcholine to either side of the BLM causes the membrane conductance to revert to that value obtained in the absence of substrates. Moreover, 10 μM α-bungarotoxin or 20 μM d-tubocurarine added subsequent to addition of enzyme, on the trans side, but prior to addition of substrate prevents the development of increased membrane conductance when carbamylcholine is subsequently added to that compartment containing the inhibitor. However, a subsequent addition of carbamylcholine to the opposite compartment (cis) bathing the same membrane does elicit increased membrane conductance.

These results are most easily accommodated by a model in which one or more protein molecules of the acetylcholinesterase preparation form a membrane-associated aggregate in which a change of state is induced by its substrates leading to the formation of ion-conducting channels. The cholinergic receptor is generally

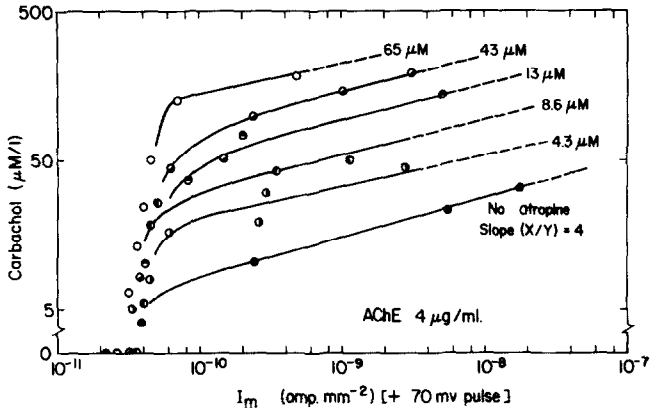

Figure 2. Double logarithmic plots of transmembrane current, I_m, as a function of the concentration of carbamylcholine (carbachol) at several concentrations of atropine for a BLM exposed to a concentration of acetylcholinesterase, AChE, of 4 μg/ml. All measurements were made with a 70 mV potential clamped across the BLM.

considered to function via a mechanism of this type (9-11). Observations of a ligand-dependent change in BLM conductance have previously been observed in systems modified by crystalline acetylcholinesterase (4) and by a proteolipid fraction (5), both isolated from the eel electroplax.

In order to further characterize this system, the dependence of conductance changes, as measured by I_m at a constant impressed voltage of 70 mV, on the concentration of both substrate and enzyme was investigated. In Figure 2, double logarithmic plots of I_m as a function of the concentration of carbamylcholine in the presence of various concentrations of atropine are shown. Above a certain minimal concentration of carbamylcholine, a linear relationship between current flow and concentration is observed; current flow is proportional to the fourth power of carbamylcholine concentration. The concentration of carbamylcholine required to achieve a given value of I_m is a linear function of atropine concentration. This concentration-dependence of the effect elicited by carbamylcholine resembles the agonist-antagonist relationships for the cholinergic receptor which have been extensively observed in pharmacological studies (12). These observations refine the model previously developed in that they suggest that opening of an ion-conducting channel requires activation by four molecules of acetyl-

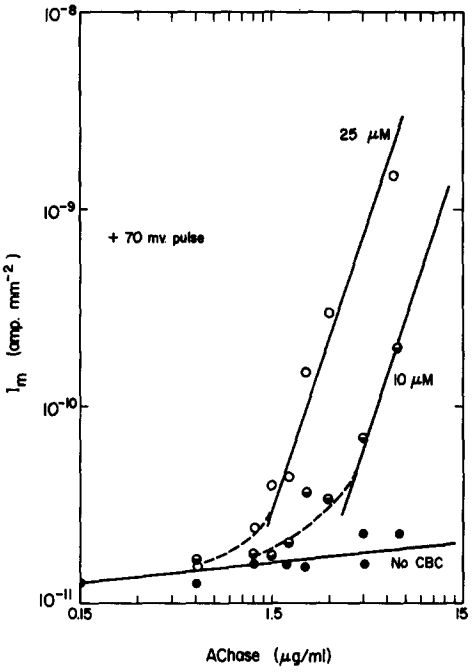

Figure 3. Double logarithmic plots of transmembrane current, I_m, as a function of the concentration of acetylcholinesterase, AChE, at the indicated concentrations of carbamylcholine. For all measurements, a potential of 70 mV was clamped across the BLM.

cholinesterase substrate and that deactivation requires several molecules, approximately six, of atropine.

In Figure 3, double logarithmic plots of I_m at carbamylcholine concentrations of 10 and 25 μM as a function of concentration of enzyme are shown. Above a minimal level the current flow increases with the fourth power of protein concentration.

These observations are consistent with models in which several molecules of acetylcholinesterase or other protein in the preparation or both aggregate in the BLM to form a potential ion-conducting channel. The first possibility is favored by the observation that related results have been obtained with crystalline acetylcholinesterase (4). On the other hand, in the light of the existing controversy concerning the nature of the cholinergic receptor (see, for example, ref. 9; M. Raftery, personal communication), the alternative possibilities which invoke

other molecules as partially or completely responsible for the receptor-like action cannot be discounted.

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