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Carbamylcholine and Acetylcholine-Sensitive, Cation-Selective Ionophore as Part of the Purified Acetylcholine Receptor

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Summary. Black lipid membranes were formed with oxidized cholesterol in the presence of either the acetylcholine receptor, purified from the electric organ of the electric ray *Torpedo californica* or its tryptic digest. In both cases, conductance of cations increased and was dependent on the concentration of the receptor protein. Conductance of Ca^{++} was dependent on its concentration, but addition of carbamylcholine gave no reproducible or consistent effects. Only in the case of the tryptic digest of the acetylcholine receptor did carbamylcholine and acetylcholine consistently induce monovalent cation selective conductance ($P_{Na,K}: P_{Cl}=4.4$). The induced monovalent cationic conductance due to carbamylcholine (10 μM) varied from 10- to over 100-fold. Curare (10 μM) prevented the action of carbamylcholine.

Na-dodecyl sulfate gel electrophoresis of the acetylcholine receptor, before and after tryptic digestion, indicated that this mild enzyme treatment hydrolyzed the receptor molecule subunits. Nevertheless, the receptor molecule retained its full binding of [*acetyl*-^{3}H]acetylcholine; and analytical gel electrophoresis indicated that it remained intact possibly through hydrogen, hydrophobic and disulfide bonding.

It is generally accepted that binding of acetylcholine (ACh) to its receptor in the postsynaptic membrane induces specific ion fluxes across that membrane. But, it is not known whether or not a cation selective ionophore[1] is an integral part of the ACh-receptor molecule.[2] Strong evidence for the identity of a pure molecule as the ACh-receptor is its successful reconstitution, by incorporating part or all of it, with or without

1 Any material that causes an increase of conductance of lipid membranes is considered to be an ionophore. Although the word means "ion carrier", it is used in the general sense without implication as to whether it is a channel or a carrier.

2 The purified noncatalytic protein that binds ACh, α-bungarotoxin and cholinergic activators and inhibitors with high affinities is defined as the ACh-receptor molecule.

associated molecules, in a bimolecular lipid membrane, and causing specific ion fluxes in response to application of cholinergic activators and the inhibition of such responses by antagonists such as occurs *in vivo*. Although relatively pure preparations of ACh-receptors have become available during the past two years (Karlsson, Heilbronn & Widlund, 1972; Eldefrawi & Eldefrawi, 1973*a*; Klett, Fulpius, Cooper, Smith, Reich & Possani, 1973; Schmidt & Raftery, 1973; Chang, 1974; Lindstrom & Patrick, 1974; Meunier, Sealock, Olsen & Changeux, 1974), none of these characterized receptors has been reconstituted until possibly very recently. This failure may have been due to the masking or loss of ionophores during purification of the ACh-binding macromolecules. Impure ACh-receptors (Kemp, Dolly, Barnard & Wenner, 1973) or proteins of unspecified purity (Goodall, Bradley, Saccomani and Romine, 1974; Jain, 1974; Romine, Goodall, Peterson & Bradley, 1974), possibly containing ACh-receptors, were incorporated in bimolecular lipid membranes and increases in conductance to Na^+, K^+ or Cl^- were observed in the presence of activators. Also, proteins of membranes enriched in ACh-receptors were reconstituted into microsacs, and an increase in [$^{22}Na^+$]efflux was observed in the presence of carbamylcholine (carb) (Hazelbauer & Changeux, 1974). Very recently pure *Torpedo* ACh-receptors were incorporated into vesicles made from *Torpedo* phospholipids and the presence of carb or ACh accelerated [^{22}Na]efflux of some ACh-receptor preparations (Michaelson & Raftery, 1974; Michaelson, Vandlen, Bode, Moody, Schmidt & Raftery, 1974).

We have available a purified ACh-receptor from the electric organ of the electric ray *Torpedo californica* with the high specific binding per mg protein of 10 nmoles of either ACh or α-bungarotoxin (Edelstein, Beyer, Eldefrawi & Eldefrawi, 1975). This compares with specific binding of 2–4 (Karlin & Cowburn, 1973), 6.5 (Chang, 1974; Meunier *et al.*, 1974), 6.6 (Klett *et al.*, 1973) or 4.1 (Lindstrom & Patrick, 1974) nmoles per mg protein for ACh-receptors purified from the electric eel *Electrophorus electricus* and 6 (Schmidt & Raftery, 1973) or 10 (Michaelson & Raftery, 1974) nmoles per mg protein for the *Torpedo* sp. ACh-receptor. This ACh-receptor has also been characterized immunologically and physiologically (Eldefrawi & Eldefrawi, 1975; Sanders, Schleifer, Eldefrawi, Norcross & Cobb, 1975).

The aim of the present study was to determine whether this highly purified ACh-receptor contained cation-selective ionophores as part of its structure. Utilizing the same techniques that were successful in the isolation of the Na^+-ionophore from ($Na^+ + K^+$)-ATPase (Shamoo &

Albers, 1973; Shamoo, 1974; Shamoo & Myers, 1974) and the Ca^{++}-ionophore from ($Ca^{++} + Mg^{++}$)-ATPase (Shamoo & MacLennan, 1974), cation specific ionophores were discovered and the ACh-receptor was successfully reconstituted in bimolecular lipid membranes.

Materials and Methods

Purification of the ACh-Receptor

The ACh-receptor was purified by affinity adsorption on a Sepharose 4B gel to which the α-neurotoxin from the venom of the cobra *Naja naja siamensis* was attached as we previously reported (Eldefrawi & Eldefrawi, 1973*a*). Desorption of the receptor was accomplished by stirring the gel for 16 hr at 23 °C in 1 M carb (without Triton). Removal of carb was by seven consecutive dialyses of the receptor solution containing 2.5 mg protein against 2.5 liters of 5 mM Na_2HPO_4. Protein concentration was determined by the method of Lowry, Rosebrough, Farr and Randall (1951). Electric organs from live *T. californica* (from Pacific Biomarine, Venice, Calif.) were used fresh or frozen at −16 °C for less than one month. Also, solutions used in purification were first deaerated and the removal of carb by extensive dialysis was performed under nitrogen. As a result, the maximum number of ACh-binding sites in the purified preparation was raised from 7.8 to 10 nmoles/mg protein (based on protein values obtained by the Lowry *et al.* method), and the number of free SH groups was raised from 2.4 to 20 nmoles/mg protein (Eldefrawi, Eldefrawi & Wilson, 1975). Another modification was the use of a solution of 0.2 M NaCl, 1 mM Na ethylenediaminetetraacetate and 5 mM Na_2HPO_4 during purification instead of Krebs original Ringer's phosphate, which contained 0.67 mM Ca^{++}. This reduced the Ca^{++}, that was tightly bound to the purified ACh-receptor, from 1 172 to 150 nmoles/mg protein, as assayed by atomic absorption (Eldefrawi, Eldefrawi, Penfield & O'Brien, 1975).

Binding of [*acetyl*-^{3}H]acetylcholine (sp. act. 49.5 Ci/mole-New England Nuclear) to the ACh-receptor was studied by equilibrium dialysis in Krebs original Ringer's phosphate at 4 °C for 16 hr as previously described (Eldefrawi, Eldefrawi & O'Brien, 1971). The dialysis tubing (Union Carbide) was pretreated to eliminate contaminants (McPhie, 1971).

To inhibit the activity of ACh-esterase present (1 active site per 20,000 ACh-receptor sites), the receptor preparation was incubated with 10^{-5} M Tetram, (*O,O*-diethyl *S*-diethylaminoethyl phosphorothiolate) for 30 min at 23 °C and 10^{-5} M Tetram was added to the dialysis bath. At such concentrations, Tetram did not affect ACh-binding by the ACh-receptor (Eldefrawi, Britten & Eldefrawi, 1971; Eldefrawi, Britten & O'Brien, 1971).

Tryptic Digestion of the ACh-Receptor

The purified ACh-receptor was incubated for 1 hr at 23 °C with trypsin (bovine, type X1, DCC treated, crystallized—from Sigma) at a weight ratio of 50 receptor: 1 trypsin. Trypsin inhibitor (from soybean, type 1-S from Sigma) was then added at a weight ratio of 2 inhibitor: 1 trypsin. The ACh-receptor was subjected to disc gel electrophoresis. For incorporation in the BLM, the tryptic-digested (TD) ACh-receptor was incubated with Sephadex CM-50 for 20 min (CMC), then filtered on a glass filter and the filtrate used.

Disc Gel Electrophoresis

The analytical method of Davis (1963) was used with only running gel of 7% acrylamide-0.18% bisacrylamide gel (pH 8.9) as previously described (Eldefrawi & Eldefrawi, 1973*a*).

Coomassie brilliant blue (0.25%) was used for staining and 7% acetic acid for destaining. For sodium dodecyl sulfate-(SDS-) gel electrophoresis, the ACh-receptor was lyophilized, dissolved, incubated at 37 °C for 2 hr in 2% SDS and 2% β-mercaptoethanol, boiled for 2 min and the procedure of Weber and Osborn (1969) followed with 7.5% polyacrylamide and 0.2% bisacrylamide (1X cross-linking) gel (7.5 cm long).

Conductance Measurements

The circuitry was as previously described (Shamoo, 1974) with a digital Keithly Electrometer Model No. 616 as ammeter/voltmeter. The electrometer was connected directly either in series (ammeter) or in parallel (voltmeter) with the bimolecular lipid membrane. The electrometer output was connected simultaneously to a two-channel recorder and an X-Y recorder. The voltage source was a function generator IEC F52A. Materials to be assayed were placed in both compartments, separated by the black lipid membrane (BLM). The membrane was formed after the addition of the designated bathing solution and the receptor protein. Oxidized cholesterol was prepared by the method of Tien, Carbone and Davidowicz (1966). The membrane diameter was 1 mm, and the solution volume for each of the Teflon cup and the outside compartment was 5 ml. The conductance (G) was expressed in mho for the area of the membrane (1 mm diameter). The standard electrolyte solution contained 3 mM chloride salts of various cations and 5 mM histidine (pH 7.3) at room temperature.

Results

Properties of Intact Acetylcholine Receptor on Lipid Bilayer Conductance

In the first group of experiments, the ACh-receptor was added to both compartments before the formation of the BLM, and the sensitivity of the bilayer conductance to ACh-receptor in the presence of Ca^{++} was studied. Fig. 1 is representative of six experiments on conductance of black lipid membrane against time in the presence of various amounts of ACh-receptor. The experiment was performed on the same day with the same bathing fluid (10 mM $CaCl_2$ + 5 mM histidine, pH 7.3), and the same batch of receptor. Each curve was obtained on a different membrane. All conductances were average conductances. There was a variability in the time of onset of the increase in conductance and in the maximal conductance either before the membrane broke (arrow ↑) or reached quasi-steady state or steady state. Nevertheless, it is clear that as the concentration of ACh-receptor increased, the rate of increase in conductance and the maximal conductance increased. The bilayer conductance was very sensitive to ACh-receptor concentration in the presence of Ca^{++}. Curve C represents $\sim 2.3 \times 10^{-11}$ M concentration of the receptor. The addition of carb or ACh at various concentrations (0.1–100 μM) gave no reproducible or consistent effects. Six experiments were conducted

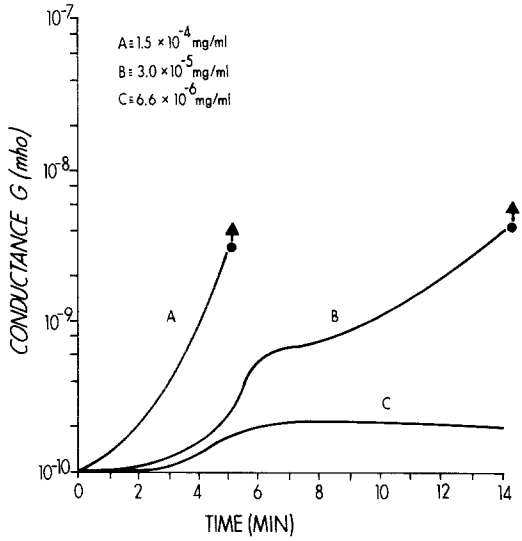

Fig. 1. The time response of black lipid membrane (BLM) conductance in the presence of 10 mM $CaCl_2$, 5 mM histidine, pH 7.3. The ACh-receptor is present in both compartments before the formation of the bilayer at the indicated concentration for *A*, *B* and *C*. The conductance of the bilayer is continuously monitored under the condition of triangular waveform of ± 75 mV maximum voltage clamp and at frequency of 0.015 Hz. The arrow indicates breaking of the membrane. Data are representative of six experiments

to relate conductance to Ca^{++} concentration (Fig. 2). The conductance increased markedly at Ca^{++} concentrations above 0.5 mM. In measurements of diffusion potential, it was found that there was no selectivity between Ca^{++} and Cl^-. Calcium (up to 50 mM) alone or Triton (at the maximal concentration present in the purified receptor preparation) had no effect on lipid bilayer conductance in the absence of receptor.

When Na^+ was the cation present in the bathing fluid, the bilayer conductance was also sensitive to ACh-receptor (Fig. 3). Five experiments were made on the conductance of BLM against time in the presence of various dosages of ACh-receptor. It was found that the concentration of ACh-receptor required to cause an equal increase in BLM conductance in the presence of NaCl was 20- to 100-fold greater than that in the presence of $CaCl_2$. Sometimes carb increased Na^+ conductance as in *C*, and sometimes it did not, so that there was neither reproducible nor specific effect of carb on conductance in the presence of the receptor.

After failing to induce, with carb, reproducible conductance increases in BLM in the presence of NaCl, we exposed the ACh-receptor to tryptic digestion which did not reduce its binding of ACh, hoping for a better

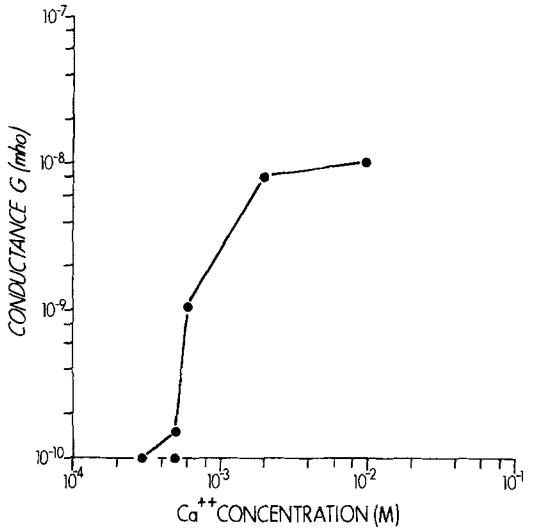

Fig. 2. The relationship between BLM conductance and $CaCl_2$ concentration. The BLM conductance is measured in the presence of 5 mM histidine, pH 7.3, and also in the presence of a concentration of 0.14 μg/ml ACh-receptor in both compartments. The concentration of $CaCl_2$ is increased by the addition of small volumes of highly concentrated $CaCl_2$(1 M) to both sides of the membrane bathing fluid. The conductance reaches a steady state within 2–4 min after each salt addition. Data are representative of six experiments

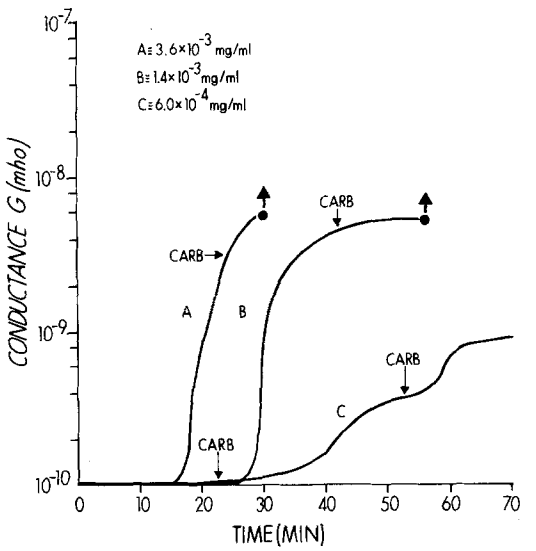

Fig. 3. The time response of BLM conductance in the presence of 0.1 M NaCl, 5 mM histidine, pH 7.3. The ACh-receptor is present in both compartments before the formation of the bilayer at the indicated concentration for *A*, *B* and *C*. The arrow next to carb indicates its addition at 10 μM final concentration. Data are representative of five experiments

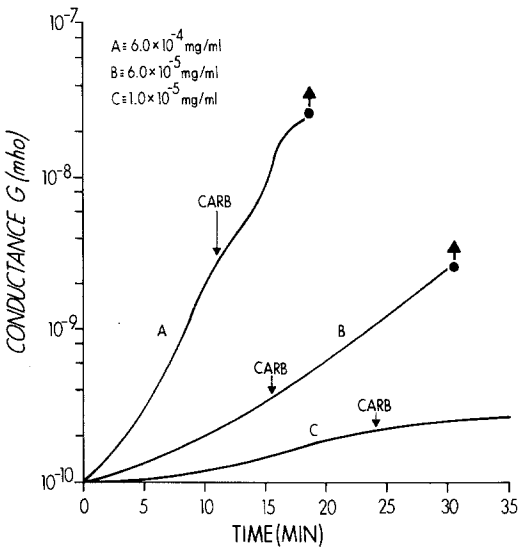

Fig. 4. The time response of BLM conductance in the presence of 3 mM $CaCl_2$, 5 mM histidine, pH 7.3. The TD-CMC-treated ACh-receptor is present in both compartments before the formation of the bilayer at the indicated concentrations for *A*, *B* and *C*. The final concentration of carb is 10 μM. Data are representative of six experiments

exposure of the ionophoric site in the bilayer system with the maintenance of its cholinergic sensitivity.

Fig. 4 is representative of six experiments on conductance of lipid bilayer against time in the presence of 3 mM $CaCl_2 + 5$ mM histidine, pH 7.3. There was no qualitative difference found when using 10 mM $CaCl_2$ instead of 3 mM $CaCl_2$. The ACh-receptor, digested by trypsin and exposed to carboxymethyl cellulose (CMC), as described under Materials and Methods, was present in both compartments at various concentrations (*A*, *B* and *C*). The addition of carb (0.1–10 μM) had no reproducible effect on the bilayer conductance. Tryptic digestion caused little or no change in the properties of the receptor on bilayer conductance in the presence of Ca^{++}. In measurements of diffusion potential, it was found that there was no selectivity between Ca^{++} and Cl^-.

Fig. 5 is representative of four experiments on conductance of lipid bilayer against time for each of the indicated ionic conditions. The bathing fluid in both compartments, in addition to the presence of 3 mM chloride salt of either Ca^{++}, Mg^{++}, Ba^{++}, Sr^{++}, Zn^{++}, or Ni^{++}, contained 0.3 μg/ml of the TD-CMC-treated ACh-receptor. Whatever the cause of the increased conductance due to the addition of the digested TD-CMC-treated ACh-receptor, the latter had a preference to Ca^{++}, Mg^{++}, and Ba^{++} over Sr^{++}, Zn^{++} or Ni^{++}.

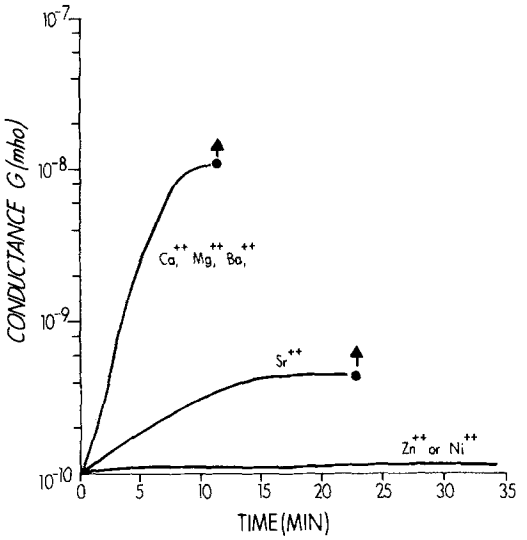

Fig. 5. The time response of BLM conductance in the presence of 3 mM chloride salt of the divalent cation, 5 mM histidine, pH 7.3. The TD-CMC-treated ACh-receptor is present in both compartments before the formation of the bilayer at a concentration of 0.3 μg/ml. Data are representative of four experiments

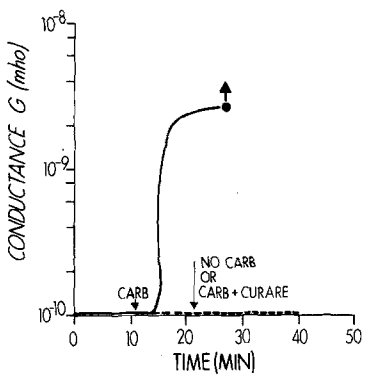

Fig. 6. The time response of BLM conductance in the presence of 0.1 M NaCl, 5 mM histidine, pH 7.3. The TD-CMC-treated ACh-receptor is present in both compartments before the formation of the bilayer at a concentration of 0.8 μg/ml. The concentration of carb is 10 μM; and curare is 10 μM. Data are representative of five experiments

Fig. 6 is representative of five experiments on lipid bilayer conductance against time. The membrane was formed while the bathing solution contained 0.1 M NaCl, 5 mM histidine, pH 7.3 and the TD-CMC-treated ACh-receptor at only 0.8 μg/ml so that Na^+ conductance was at a steady

state. In every experiment, the addition of carb caused a dramatic increase in conductance. If curare was present with salt, receptor, and then carb was added after the formation of the bilayer, no increase in conductance was observed.

If carb was present with the TD-CMC-treated ACh-receptor before the formation of the bilayer, further addition of carb produced no change in conductance. If curare was added after BLM formation in the presence of TD-CMC-receptor and NaCl, no effect on conductance was observed.

If the TD-CMC-treated ACh-receptor was present at a high concentration above 4 μg/ml in the bathing medium before the addition of carb, we observed an increase in conductance without carb addition. The concentrations of TD-CMC-treated ACh-receptor, required to induce an increase in conductance without carb, varied within 10-fold from batch to batch of ACh-receptor. However, it was somewhat constant for the same batch. Moreover, for each batch the type of experiment just described was repeated several times to locate the TD-CMC-treated ACh-receptor dosage, which caused no increase in conductance by itself. Once that was established in five experiments, we then proceeded to add carb and observe the increased conductance. The amount of conductance change due to carb varied from a 10-fold increase to over 100-fold increase.

Within a given batch of ACh-receptor the following were some means and ranges where conductance increased in the presence of NaCl:

a) The minimum concentration of TD-CMC-receptor causing a spontaneous increase in conductance of a BLM in the presence of NaCl was 5.6 ± 1.5 μg/ml for $n=5$ and the range was from 4–7 μg/ml.

b) The concentration used giving an increase in conductance only with the addition of carb was 0.7 ± 0.3 μg/ml for $n=5$ and the range was 0.3–1.0 μg/ml.

c) After the formation of BLM for 5 to 20 min, carb was added. BLM conductance formed under identical conditions as those described for carb experiments and were stable for at least 1 hour and sometimes overnight afterwards.

Preliminary results indicate that the carb-induced conductance is not reversed by later addition of curare. We feel that the rigidity of the oxidized cholesterol bilayer may not allow a conformational change induced by curare binding, to take place. Other lipids are being tested.

When experiments were repeated with 0.1 M KCl instead of NaCl similar data was obtained as in Fig. 6. There was no selectivity difference between Na^+ and K^+. However, in experiments, measuring diffusion potential after carb addition, it was found that P_{Na}/P_{Cl} was 4.4, where

P stands for permeability. This indicates that the selectivity between cations and anions was present only when the conductance increased in response to carb.

When the ACh-receptor was left at room temperature for two days, causing it to lose capacity to bind ACh, no ionophoric properties could be detected whether with Ca^{++} or Na^{+} and with or without carb.

Effects of Tryptic Digestion on the ACh-Receptor

The binding of [*acetyl*-^{3}H]acetylcholine to the purified ACh-receptor, before and after tryptic digestion, was studied by equilibrium dialysis. The mild tryptic digestion (1 hr with 50 receptor: 1 trypsin, w/w) did not affect the ACh binding at concentrations ranging from 5 nM to 5 μM of ACh. As previously reported (Eldefrawi & Eldefrawi, 1973*a, b*) there was high and low affinity binding of ACh, with positive cooperativity at the low concentrations. However, when the weight ratio of receptor to trypsin was reduced from 50 to 10 and incubation was extended from 1 to 2 hr, there was a 10% reduction of binding at 1 μM ACh. The reduction was up to 27% after 24 hr incubation with trypsin at 23 °C.

To determine the effect of tryptic digestion on the molecular weight of the ACh-receptor, disc gel electrophoresis was performed. The receptor protein appeared as a single band that migrated very little into the gel, and the mild tryptic digestion did not change the pattern except for a 5% reduction in that protein band (Fig. 7). If tryptic digestion was for 4 hr, the same single band appeared, but its concentration was reduced by about 15%. However, the pattern obtained by analytical SDS-gel electrophoresis was greatly changed (Fig. 8). Before digestion, mainly four subunits were obtained as previously reported for the *T. marmorata* receptor (Carroll, Eldefrawi & Edelstein, 1973) with the major one having a molecular weight of 43,000 and most of the others being larger. After digestion, six subunits appeared and almost all the peptides had a molecular weight smaller than 43,000, the major ones being about 32,000 and 14,000. In both cases, there was a peak of large molecular weight close to the start, possibly SDS micelles with protein and salt.

Before incorporation in the BLM, the TD-ACh-receptor was incubated for 20 min with Sephadex CM 50 ion exchange gel, then the filtrate used. After incubation with Sephadex CM 50, $93 \pm 4\%$ of the protein was recovered in the filtrate. On the other hand, incubation with DEAE-Sephadex A-50, resulted in recovery of only $5\% \pm 2\%$ of the receptor protein.

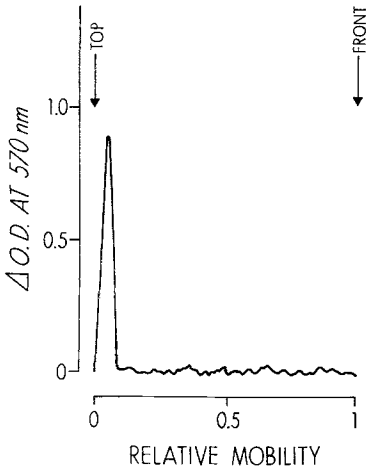

Fig. 7. Scan of gel electrophoresis of the ACh-receptor after 1 hr incubation with trypsin (50:1, w/w). The scan is identical to that of the purified ACh-receptor before incubation except that the TD-ACh-receptor contains 5% less protein than the untreated receptor. Gel stained with Coomassie Brilliant Blue. Experiment run in triplicate

Discussion

Conductance increases in BLM have been observed upon addition of substrates to the bathing solution containing their respective enzymes (trypsin, chymotrypsin, lactic dehydrogenase and ACh-esterase) (Del Castillo, Rodriguez & Romero, 1967; Leuzinger & Schneider, 1972). Such changes contrast with the selective ionophoric activities recently observed, such as Na^+-dependent and Ca^{++}-dependent and selective ionophores from $(Na^+ + K^+)$-ATPase and $(Ca^{++} + Mg^{++})$-ATPase, respectively (Shamoo & Albers, 1973; Shamoo, 1974; Shamoo & MacLennan, 1974; Shamoo & Myers, 1974). Increases in BLM conductances were reported by several researchers using impure receptor preparations or preparations suspected to contain the receptor protein (Kemp *et al.*, 1973; Jain, 1974; Goodall *et al.*, 1974; Romine *et al.*, 1974). In some of these studies transient increases in conductance were observed either in the presence of the protein alone (Romine *et al.*, 1974) or in the presence of cholinergic ligands as well (Parisi, Reader & DeRobertis, 1972). In the present study, we observed transient fluctuations in conductance, but they were not reproducible phonomena. We report on conductance changes under steady-state conditions. The observed increase in BLM conductance of Na^+ and K^+ when the membrane is formed in the presence of the TD-CMC-treated receptor is triggered by carb or ACh and is inhibited by

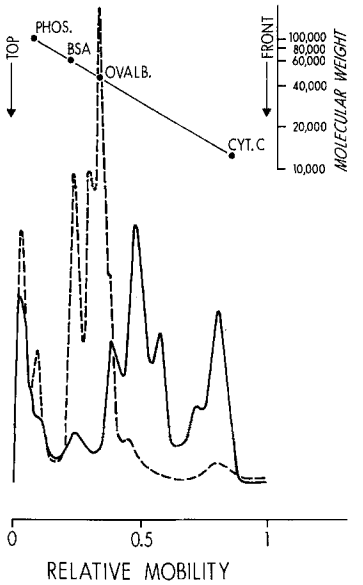

Fig. 8. Scan of SDS-gel electrophoresis of the purified ACh-receptor. Straight line receptor after incubation with trypsin (50:1 w/w) for 1 hr; broken line-untreated receptor. Standards are run on a separate gel. Phos, phosphorylase *a* (94,000); BSA, bovine serum albumin (66,000); Ovalb, ovalbumin (43,000); Cyt. *c*, cytochrome (11,700). Experiment run in triplicate

curare (Fig. 6). It is not transient, which is to be expected under our experimental condition, where the concentration of activator is constant during the experiments. This activator-induced conductance increase is monovalent cation selective ($P_{Na,K}$: P_{Cl}=4.4), which is consistent with the *in vivo* responses of the receptor to activators (Ruiz-Manresa & Grundfest, 1971). Similar cation selectivity was observed in BLM with a contaminant of an ACh-esterase preparation, suspected to be the ACh-receptor (Jain, 1974). The magnitude of the maximum presently observed activator-induced conductance increase of over 100-fold is much higher than the 10-fold reported using purified ACh-receptor in vesicles (Michaelson & Raftery, 1974).

The ACh-receptor preparation used in this study appears as a single molecular species as judged by isoelectrofocusing and gel filtration (Eldefrawi, Eldefrawi & Wilson, 1975), and by sedimentation equilibrium measurements, with absorption optics and a scanner-computer system, in presence of 0.1% Triton (Edelstein *et al.*, 1975). It has a molecular weight of 330,000 in the presence of 0.1% Triton, as determined by sedimentation equilibrium but some molecules aggregate to 660,000 as a result of the removal of most of the Triton. This ACh-receptor preparation contains two

impurities: ACh-esterase [one active site for every 20,000 ACh-receptor sites and 0.0007% Triton X-100 equal to 0.113 mg/mg receptor protein, determined by use of the tritiated detergent (Edelstein *et al.*, 1975)]. Three other reconstituted protein systems that show ACh-receptor-like activity contain little (Hazelbauer & Changeux, 1974; Michaelson & Raftery, 1974) or no detergent (Jain, 1974). On the other hand, removal of Triton from one protein preparation rapidly denatures it as judged by bilayer activity (Romine *et al.*, 1974). The receptor protein is acidic [pI = 4.8 (Eldefrawi & Eldefrawi, 1973*a*)] with about 22 mole % of aspartic and glutamic acids, and it contains 2.4 mole % tryptophan, which impart characteristic fluorescence on the molecule (Eldefrawi, Eldefrawi & Wilson, 1975).

In the present study, we observe increases in BLM conductances of monovalent and divalent cations, when the membrane is formed in the presence of the receptor protein (Figs. 1–3). The magnitude of these conductances is directly related to the concentration of the receptor protein, but the effect of activators on conductance is not reproducible. Conductance increases become monovalent cation selective, and consistently sensitive to the effect of activators when the receptor protein is pretreated with trypsin before use. However, it may be that such a treatment is not necessary, since an ACh-receptor, from the same species and of the same purity, was successfully reconstituted into vesicles from *Torpedo* phospholipids, and the presence of ACh caused an acceleration of $[^{22}Na^+]$-efflux which was inhibited by the presence of α-bungarotoxin (Michaelson & Raftery, 1974). In those experiments, cation selectivity was not demonstrated and not all receptor preparations yielded chemically excitable vesicles. It is known that trypsin preferentially catalyzes the hydrolysis of peptide bonds between the carboxy group of arginine or lysine and the amino group of another amino acid. It also acts as an esterase and amidase. Exposure of the purified ACh-receptor to a low concentration of trypsin for 1 hr results in hydrolysis of the receptor subunits into smaller ones (Fig. 8). However, the molecule retains its capacity to bind ACh and apparently remains intact (Fig. 7), with its subunits attached possibly through disulfide, hydrogen and hydrophobic bonds. The following observations may explain why the ACh-receptor molecule remains intact after trypsin treatment. The receptor is found to be unusually resistant to treatment with guanidine hydrochloride (Carroll *et al.*, 1973). About 82% of all cysteic acid sulfur of this purified ACh-receptor forms disulfide bonds (Eldefrawi, Eldefrawi & Wilson, 1975). These observations suggest that the receptor exists in a highly folded conformation, which is supported

by a recent study of the far ultraviolet circular dichroism spectrum of a *Torpedo nobiliana* ACh-receptor (Moore, Holladay, Puett & Brady, 1974). Therefore, the subunits of the trypsin-treated ACh-receptor may remain attached by disulfide, hydrogen and hydrophobic bonds.

Based on the loss of 5–7% of the receptor protein in gel electrophoresis (Fig. 7) after its exposure to trypsin and CMC, we may suggest that trypsin separates 5–7% of the receptor as positively charged subunits. Such a small reduction in molecular weight cannot be detected by this gel electrophoresis. More extended exposure to trypsin seems to increase the loss of protein and reduces ACh-binding. It is possible that the new side chains, exposed by trypsin digestion, permit the incorporation of the receptor in BLM in a functional conformation that allows interaction between the active site and the ionophoric activities. The importance of the correct conformation is emphasized also by the failure to induce monovalent cationic conductance in BLM when the membrane is formed in the presence of carb and TD-CMC-treated ACh-receptor.

An interesting observation is the large increase in BLM conductance in the presence of Ca^{++} and either ACh-receptor or TD-CMC-treated ACh-receptor (Figs. 1, 2, 4, 5). This increase is nonselective between cations and anions. Therefore, it may be that the receptor in the presence of Ca^{++} interacts strongly with the lipid bilayer to produce large holes, not within, but surrounding the receptor molecule. This increase in conductance is probably not related to the cholinergic activator induced cation selective conductance. It is consistent with the fact that the conductance increase does not require the presence of a cholinergic activator (Fig. 4). Thus, the conductance increase may indicate a chemical event occurring due to interaction of Ca^{++}-receptor with lipids. What has been observed in conductance with Ca^{++} and the receptor is similar to the recent report that a mitochondrial glycoprotein, which has a very high affinity for Ca^{++}, causes a dramatic nonselective increase in conductance (Prestipino, Ceccarelli, Conti & Carafoli, 1974). It is possible that the glycoprotein and the ACh-receptor do not easily become associated with the bilayer unless Ca^{++} is present. The importance of Ca^{++} is underscored by the need for it along with Mg^{++} to form sealed vesicles, that retain [$^{22}Na^{+}$], which are made of solubilized proteins from membrane fragments rich in ACh-receptors and supplanted with crude lipids extracted from native membranes (Hazelbauer & Changeux, 1974). Another role for Ca^{++} in membranes is as a modulater of selectivity among monovalent cations as was found in the transport of alkali metals by the isolated mid-gut of the American silkworm (Zerahn, 1971).

The purified receptor protein was found to have a high Ca^{++} content as measured by atomic absorption (Eldefrawi *et al.*, 1975). When purified in the presence of 1 mM ethylenediamine tetraacetate, its Ca^{++} content is 0.15 μmoles/mg protein, but if purified in Ringer's (containing 0.67 mM Ca^{++}) its final content of Ca^{++} rises to 1.17 μmoles/mg protein. This Ca^{++} is tightly bound. The increased conductance of BLM in the presence of Ca^{++} and receptor may be related to this special relationship between Ca^{++} and receptor, and suggests a physiological role for Ca^{++}. Models have been proposed that emphasize a regulatory role for membrane bound Ca^{++} and a close relationship between it and the ACh-receptor (Chang & Triggle, 1973; Neumann, Nachmansohn & Katchalsky, 1973).

The data presented demonstrate the presence of monovalent cation selective ionophores as part of the ACh-receptor molecule. Further studies are needed to elucidate the role of Ca^{++}, to reduce the variabilities observed, to test different synthetic lipids and to study the dose response relationship for various agonists. It is also important to speed up the response which still takes minutes instead of seconds, as *in vivo*, to develop. We hope to see transient response if we can reduce the concentration of ACh rapidly such as by having ACh-esterase present. In addition, studies will be required to determine whether the whole receptor molecule acts as the ionic channel or whether a small part is the ionophore; and if the latter is the case, to isolate it.

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