RECONSTITUTION OF A PARTIALLY PURIFIED ENDPLATE ACETYLCHOLINE RECEPTOR PREPARATION IN LIPID BILAYER MEMBRANES*

G. Kemp[1], J.O. Dolly[2], E.A. Barnard[2], and C.E. Wenner[1]

[1]New York State Department of Health, Roswell Park Memorial Institute, Buffalo, New York 14203 and [2]Department of Biochemistry, State University of New York at Buffalo, Buffalo, New York 14214

Received August 1, 1973

SUMMARY - Incorporation of a fraction isolated from rat diaphragm muscle that contained the specific endplate cholinergic receptor into phospholipid bilayer membranes resulted in the production of an acetylcholine-stimulated conductance increment of large magnitude. The acetylcholine-stimulated conductance shows several characteristics of the in vivo post-synaptic excitable system.

The binding of ACh** at the post-synaptic membrane of the neuromuscular junction results in an increase in membrane ion permeability and a partial depolarization of that membrane. A number of workers (for review see ref.1) have solubilized and substantially purified, from fish electric organs, an ACh receptor protein involved in this system. We report here initial studies on the reconstitution in vitro of this excitable system, by the incorporation into a phospholipid bilayer membrane of a receptor-containing fraction isolated from vertebrate skeletal muscle. Such a system should permit identification of the components necessary for the post-synaptic response to ACh.

De Robertis and coworkers (2) first attempted such reconstitution, using a proteolipid obtained by chloroform-methanol extraction of eel electroplax and subsequent chromatography on Sephadex LH-20. Transient increments of current were obtained when ACh was applied directly on the membrane at a concentration of 10^{-3}M or greater, from a distance of a few millimeters in an unstirred or slightly stirred bathing solution. However, 0.05 ACh in the stirred bathing solution was required to change irreversibly the conductance. This concentration is several orders of magnitude greater than would be expected from electrophysiological data. Other cholinergic ligands that altered the conductance were

*Supported by USPHS grant Nos. GM-11754 and CA-13784, and post-doctoral fellowship (to J.O.D.) of Muscular Dystrophy Association of America

**ACh - Acetylcholine

used in correspondingly high concentrations, and antagonists such as dimethyl-d-tubocurarine also gave conductance increments when applied alone.

Jain et al. (3) have reported large conductance changes when a commercial preparation of eel electroplax acetylcholinesterase was added to the medium bathing bilayer membranes, but only after subsequent addition of ACh. Although not a reconstitution, perhaps the most convincing in vitro study yet of the ACh receptor is the work of Kasai and Changeux (4,5), who measured the permeability to various tracer ions of vesicles formed by membrane fragments from eel electroplax. Sensitivity of a variety of pharmacological agents known to act upon the receptor in vivo was quantitatively retained.

These previous studies have used eel electroplax as the source of excitable material. In our work, ACh receptor preparation from vertebrate skeletal muscle is used, since the post-synaptic system there is well characterized in situ.

Experimental

The partial purification of the muscle endplate ACh receptor (fraction with apparent molecular weight about 550,000) from rat diaphragm has been described previously (6). Bilayer membranes were formed across an aperture of Teflon or glass one mm in diameter, according to the technique first described by Mueller et al. (7). A voltage gradient was generated and capacitance monitored by devices designed by Dr. D. Wobschall. Membranes were formed from egg lecithin (Supelco, Inc., Bellefonte, Pennsylvania), and were dissolved in either hexadecance or in hexadecane-tetradecane (1:1). The dependence of capacitance upon hydrocarbon solvent reported by Haydon et al. (8) was also observed with our apparatus. Current vs. voltage plots obeyed Ohm's law up to $\pm$ 100 mV a polarizing potential of 10 or 20 mV was used. The receptor preparation contained 0.6% Triton X-100; this was finally diluted by a factor of 0.3 -2 x 10^3. All bathing solutions were in 2mM Tris-HCl, pH 7.0, and contained 0.1 M KCl, or NaCl when stated. The temperature of the bathing solution was 27°C. The receptor was added to the well-stirred bathing solution after the membrane was formed and

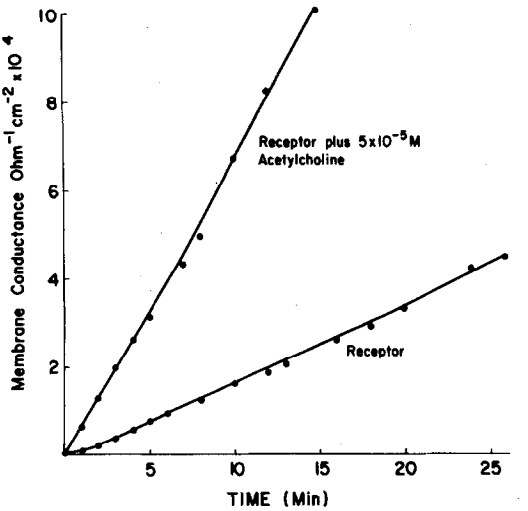

Fig. 1. Stimulation of Conductance by Receptor Preparation. Bathing solutions (both sides of the thinned membrane) contained 0.1 M KCl and, for the upper curve, ACh. The receptor preparation was added (4.5 μg/ml-final protein conc.) to one side, at zero time.

thinned, and the rate of conductance increase was then monitored. Numerous preparations have been made using both normal and denervated (14 days after denervation) diaphragm preparations (6). These preparations varied considerably in their ability to introduce conductance; the basis for this variability in preparations is currently being investigated. Nevertheless, the qualitative properties of the activity as described in this paper appeared consistent from preparation to preparation. The activity in assays of any given preparation was reproducible, and was little changed after storage frozen for several weeks.

Results and Discussion

Fig. 1 shows the increase in membrane conductance produced by the addition of the receptor preparation (4.5 μg/ml final protein concentration) to the stirred bathing solution. The conductance does not appear to reach an equilibrium value for at least 30-40 min., the lifetime of the longest-lived membranes obtained with our lipid preparations. When the bathing solutions initially contained 5 x 10^{-5} M ACh, the rate of conductance increase was accelerated approximately four-fold (Fig. 1). In addition, when the receptor and ACh were added to opposite sides of the membrane prior to thinning, the membrane (after thinning) again

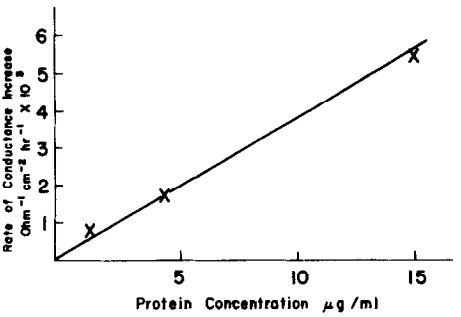

Fig. 2. Stimulation of Conductance in Relation to Concentration of a Receptor Preparation Added.

showed an approximately four-fold stimulation of the conductance increase; but when the receptor and ACh were added to the same side of the membrane, no such stimulation of conductance was observed. When 5 x 10^{-5}M ACh was added to a thinned membrane on the side opposite to the receptor, there was a rapid rise in conductance following the addition, and a subsequent four-fold increase in the rate of conductance increase. Again, the stimulation of conductance by ACh was not observed when the ACh was added to the same side of the membrane as the receptor.

Fig. 2 demonstrates that the rise in conductance produced by the receptor is proprotional to the amount of receptor material added. This first-order dependence of conductance on concentration is in contrast to the results of Parisi et al. (2) and Jain et al. (3) who found (with eel electroplax preparations) a fourth-order dependence of conductance on concentration. The two most active denervated muscle preparations were 30-50 times more active (per µg protein) than the normal diaphragm preparations (but otherwise similar in behavior), interesting in view of the 20 fold increase in receptor concentration found in such denervated muscles (6).

The stimulation by ACh of the induced conductance was prevented by two specific blocking agents for the ACh receptor, d-tubocurarine and α-bungarotoxin (Table I). In the absence of ACh, these agents had little or no blocking effect; they certainly gave no conductance increment. The antagonism provides evidence that the ACh receptor in the preparation is involved in the activity measured.

Table I. Effects of ACh and Cholinergic Inhibitors upon the Induced Conductance.

Medium	ACh(5×10^{-5}M) Addition	Relative Rate of Conductance Increase*
(1) 0.1 M KCl	None	1.0
	Both sides	3.8
	Receptor side	1.0
	Opposite side	3.8
+ 6×10^{-6}M α-bungarotoxin	None	1.2
+ 6×10^{-6}M α-bungarotoxin	Both sides	0.7
+ 9×10^{-5}M α-tubocurarine	None	0.8
+ 9×10^{-5}M α-tubocurarine	Both sides	0.9
(2) 0.1 M NaCl	None	0.3
	Both sides	0.8

*Rate measured over the first 15 min. after addition of receptor (see Fig. 1) and expressed relative to the rate with some receptor preparation and concentration in 0.1 M KCl alone. All active receptor preparations appeared to give the same set of relative values (within experimental error limits).

It was also observed that the activity was quite labile. When stored at 4°C, the preparation gradually lost its capacity to induce conductance, approximately 75% being lost over a five-day period: one sample stored at room temperature for 24 hr. became totally inactive during that period. The protein fraction containing the non-specific α-bungarotoxin-binding component of muscle (peak III of ref. 6) was tested in parallel experiments; it showed little or no ability to increase membrane conductance, even at a protein concentration of 15 μg/ml, and gave no response to ACh. It is not yet known whether another component must be introduced with the receptor molecule for the membrane conductance rise to be achieved. An observation was made that the receptor α-bungarotoxin binding capacity remained intact when the overall activity here spontaneously disappeared, which suggests such a requirement.

The magnitude of the conductance that can be generated by this receptor preparation is remarkable; in Fig. 1 the conductance in the presence of ACh has reached 10^{-3} ohm^{-1} cm^{-2} in 15 min. Based upon the extent of ^{3}H-labeled α-bungarotoxin binding (6) to samples of the preparations (assuming one α-bungarotoxin molecule bound per receptor) the receptor concentration was estimated to be of the

order of 10^{-10}-10^{-11} M at the protein concentration presented. Malonyl-gramicidin (9), chosen for comparison because it is an ionophoric agent which also shows first-order conductance kinetics, would give a similar stimulation of conductance at a concentration of approximately 10^{-11} M.

Preliminary investigation of the ion specificity of the conductance increase (Table I) indicated an approximate 3:1 selectivity of K^+ over Na^+, both in the presence and absence of ACh.

It is not unreasonable to expect that the ACh receptor system, when placed in a membrane, would (if it is reasonably intact) give rise to an increased membrane conductance in response to addition of ACh, as occurs *in vivo*. However, it has been shown (10,11) that several enzymes, including acetylcholinesterase, when placed in a membrane system and allowed to combine with their substrates, can give rise to membrane conductance changes. We believe that the properties of the present reconstitution system, as described above, distinguish it from the latter, relatively non-specific, type. We can note that Del Castillo *et al*, (10) who found that trypsin, chymotrypsin, or lactic dehydrogenase in the bathing solution at 30 μg/ml gave rise to membrane conductance changes upon addition of their substrates, observed the same effects in unthinned membranes, and concluded that the phenomemon was due to some change in membrane state in the unthinned regions of the membrane resulting in the formation of conducting channels. In contrast, the conductance changes reported in this communication do not occur in unthinned membranes. Leuzinger and Schneider (11) repeated the experiments of Del Castillo *et al*. using membranes incubated with acetylcholinesterase and subsequently exposed to ACh, but no criteria of membrane thinning were reported. An increase in conductance from 10^{-8} ohm^{-1} cm^{-2} to 10^{-6} ohm^{-1} cm^{-2} was produced by adding ACh to membranes treated with 1 μg/ml acetylcholinesterase (11). Our preparation contained no detectable (6) trace of this enzyme, and the conductance increment obtained is up to four orders of magnitude greater. Larger changes than those of Leuzinger and Schneider were demonstrated recently (3) upon ACh addition to bilayer membranes in the presence of a commercial electroplax acetylcholinesterase preparation. It is not known whether this activity was due to the

enzyme or to possible contamination by the ACh receptor system, particularly in the light of the reversal of the effect by both an acetylcholinesterase inhibitor, neostigmine, and receptor blocking agents d-tubocurarine and α-bungarotoxin.

The reconstitution with the endplate-specific components does not reproduce well the resting and ACh-excited states of the post-synaptic membrane, in that the conductance here in the absence of ACh is so great such that only a further 4-fold increase is possible with ACh. Possible explanations are that control of the resting state in vivo is due to some other component, or some feature of the natural membrane or mode of insertion in it of the conductance-modulating component, that is lacking in the present simplified system.

Acknowledgments: We thank Mr. T. Chiu for preparation of the α-bungarotoxin, Dr. E.X. Alburquerque for kindly denervating rats, and Dr. D. Papahadjopoulos for frequent and invaluable discussions. J.O. Dolly is a post-doctoral fellow of the Muscular Dystrophy Associations of America.

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