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Studies on the Chemical Properties of the Acetylcholine Receptor Site of the Frog Neuromuscular Junction

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Summary. The effect of a water-soluble carbodiimide has been used to study the nature of the presumed anionic part of the acetylcholine (ACh) receptor at the frog neuromuscular junction. The ACh sensitivity has been measured by the moving fluid electrode method and by recording end plate potentials with microelectrodes. The carbodiimide blocked ACh sensitivity without marked effect on the membrane resistance or potential difference. The conditions of reversibility of the block and the results obtained with phospholipids suggest that a carboxyl group is important in the combination of ACh with the receptor.

Presumably, the receptor site for the cation acetylcholine (ACh) contains an anionic group. This assumes the receptor-ACh combination to be ionic. Evidence concerning the chemical nature of this presumed anionic site is incomplete. The changes of sensitivity of the frog neuromuscular junction to carbamylcholine (which behaves similarly to ACh) in the presence of UO_2^{++} led Liu and Nastuck (1966) to suggest that phosphate groups are involved. Sokoll and Thesleff (1968) have reported similar findings with ACh. Other evidence concerning the nature of the site is available from pH studies, but the published results appear to conflict. Del Castillo, Nelson and Sanchez (1962) concluded that the ACh effect on the end plate membrane of frog muscle is the same at pH 7 and pH 4, if allowance is made for the pH effect on the membrane resistance. However, Sokoll and Thesleff (1968) found the ACh sensitivity in denervated rat muscles to be reduced by about 90% at pH 5.2.

Thus, the pH data must be interpreted with care. Furthermore, the UO_2^{++} data, although suggestive, are far from definitive. For these reasons, another approach to the problem has been taken. The effects on the ACh depolarization of the frog neuromuscular junction of a chemical treatment specific for phosphate and carboxylate groups are reported here.

Methods

Paired sartorius muscles from the frog *Rana pipiens* were used. The depolarization produced by ACh was measured by the moving electrode method of Fatt (1950). The paired muscles were mounted vertically in a bath which contained Ringer or appropriate test solutions. At the top, the tendon ends were tied to separate hooks; the pelvic ends were fixed to hooks below. Agar-KCl electrodes made contact by cotton wicks with both tendon ends, and another electrode was in the bath below the pelvic end. The fluid was drained from the chamber at a uniform rate; the potential difference recorded between the electrode on the tendon and that in the bath below the muscle is related to whatever potential exists between the tendon end of the muscle and a point on the muscle at the level of the bath solution. The potential along the muscle was recorded with a penwriter. The motor end plates are depolarized by ACh, and since the end plates tend to be distributed in two bands (Eccles, Katz & Kuffler, 1941; Fatt, 1950), the potentials recorded in ACh usually show two peaks. The magnitude of the depolarization depends on the ACh concentration (Fatt, 1950), as well as the membrane resistance and the resting membrane potential (del Castillo *et al.*, 1962).

In the usual procedure, after the muscles were in position, recordings of the potential were made in Ringer solution at 10-min intervals until the recorded potential changes were reproducible. Then the bath solution was changed to Ringer with 5.5×10^{-5} M ACh. After 30 sec, the bath was emptied and the potentials recorded. The next bath solution contained 1.1×10^{-4} M ACh, and the same procedure was repeated. Then potentials were similarly recorded in 2.75×10^{-4}, 5.5×10^{-4} and 1.1×10^{-3} M ACh.

The time interval of 30 sec was selected because ACh depolarization appeared to be almost at a maximum at this time (Fig. 1), and yet the interval was short enough to minimize desensitization effects (Thesleff, 1955). The depolarization appeared to decrease slowly with time after about 1 min, presumably due to desensitization.

After the highest ACh concentration was applied, the muscles were bathed in Ringer for at least 30 min before a second test or chemical treatment. A plot of depolarization as a function of ACh concentration for three ACh test sequences spaced at 30-min intervals is presented in Fig. 2. In some cases, following the 30-min wash, one of each pair of muscles was treated as noted, and the other was used as a control.

The experiments were performed at room temperature (21 °C).

Modification of Carboxyl Groups

The procedure used was based on that described by Hoare and Koshland (1962). The water-soluble carbodiimide [1-ethyl-3 (3-dimethylaminopropyl) carbodiimide HCl] was obtained from Ott Chemical Co. (Muskegon, Mich.). Glycine methyl ester was purchased from Mann Research Lab. (New York, N.Y.).

Experimental muscles were treated for 5 min with Ringer solution containing 10 or 100 mM carbodiimide with or without 50 mM glycine methyl ester. The pH was set at 4.75 with dilute HCl. The pH was measured before and after the treatment. Control muscles were treated for the same time period in Ringer solution at pH 4.75 made hyperosmotic with sucrose.

To learn more about the reactive sites for the carbodiimide molecule, the kinetics of its reaction with three different phospholipids were studied. Carbodiimide and glycine methyl ester were dissolved in 10 ml of dioxane at 25 °C. The pH was adjusted to 4.75 with 0.5 mM HCl, and then phosphatidyl choline, phosphatidyl ethanolamine or phosphatidyl serine was added. Samples were removed at 2, 5, 10, 15 and 30 min, evaporated

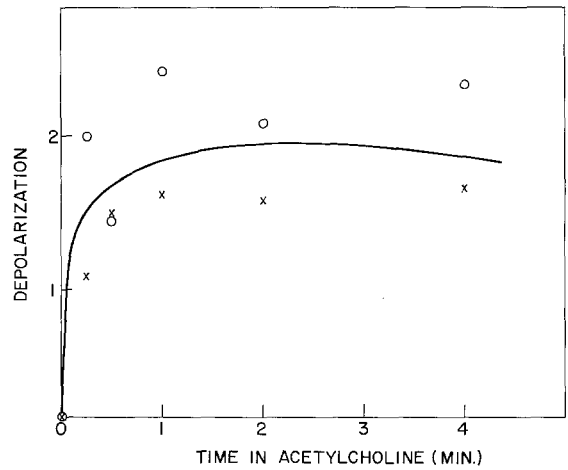


Fig. 1. Effect of duration of exposure to 5.5×10^{-4} M ACh on magnitude of depolarization in two muscles denoted by ∘ and ×. Muscles were washed for 20 min between trials. The sequence of trial periods used was: 15 sec, 1 min, 4 min, 2 min, 30 sec and 90 sec

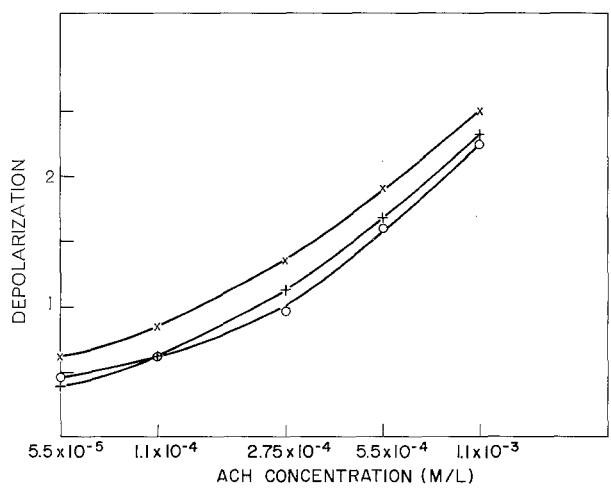


Fig. 2. Effect of time and desensitization on ACh concentration-membrane depolarization relation. Abscissal scale is logarithmic. Three runs were made at intervals of about 30 min. First run, ×; second run, +; third run, ∘

to dryness under nitrogen and separated by two-dimensional, thin layer silica chromatography. The amount of lipid in each spot was quantitated by determination of the inorganic phosphorus.

Electrophysiological Experiments

End plate transmission was blocked by addition of Mg and reduction of Ca (del Castillo & Katz, 1954). Under these conditions, a microelectrode inserted into a muscle at an end plate records end plate potentials (e.p.p.) of several-millivolts amplitude which

are about 10 % of the normal size. The microelectrode was connected to an amplifier designed by John P. Hervey of Rockefeller University. The input circuit includes a bridge so that the resistance of the muscle membrane may be measured following the technique described by Purple and Dodge (1966). The accuracy of the measurement in these experiments was somewhat limited since, as used, the technique measures either the resistance of the microelectrode (5 or more MΩ) or that of the microelectrode plus muscle membrane (the latter is of the order of several tenths of a MΩ).

Results

Carbodiimide Treatment

After 5 min in 10 mM carbodiimide and 50 mM glycine methyl ester, the sensitivity of ACh was reduced by about 50 %. Treatment for 10 min in the same solution completely abolished the ACh response (Fig. 3). The effects of the carbodiimide addition in the presence of glycine methyl ester were not reversed by 30-min exposure to frog Ringer made to pH 4.1 by addition of HCl.

Muscles treated with carbodiimide alone show a similar loss of sensitivity to ACh. However, subsequent to treatment of these muscles with frog Ringer at pH 4.1 for 5 min, the ACh sensitivity was largely restored (Fig. 4).

Electrophysiological Studies

Possible explanations for the loss of ACh sensitivity subsequent to carbodiimide treatment include depolarization of the muscle membrane or a marked decrease in membrane resistance. Either or both of these would produce a marked reduction in the magnitude of the ACh depolarization.

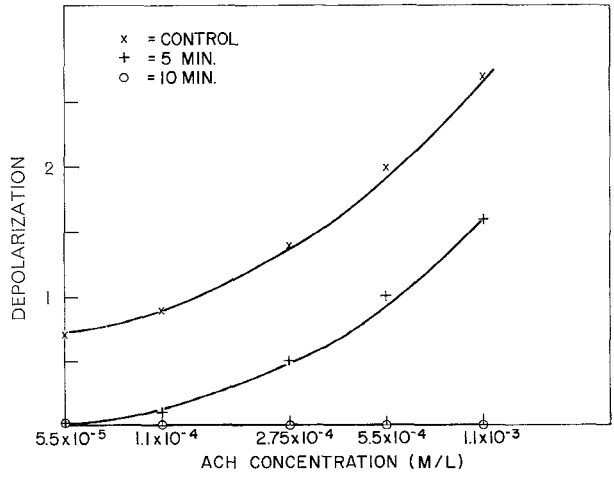


Fig. 3. Effect of time on carbodiimide effect on ACh depolarization. The bath for the treatment contained 10 mM carbodiimide. Abscissal scale is logarithmic

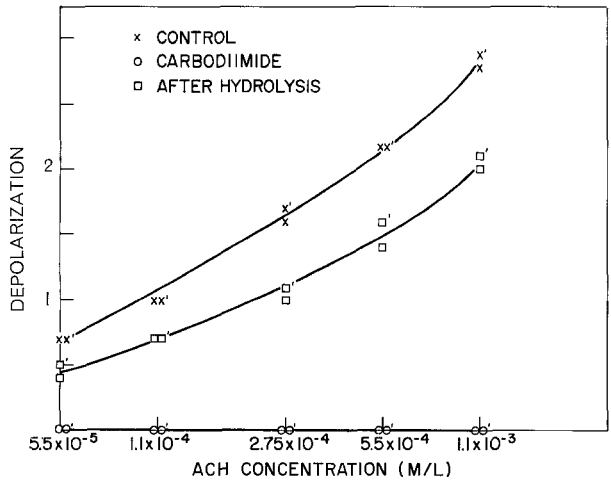


Fig. 4. Effect of hydrolysis (5 min at pH 4.1) on carbodiimide desensitization. No glycine methyl ester present. Results for two muscles are shown, and data for one are marked with primes. Abscissal scale is logarithmic

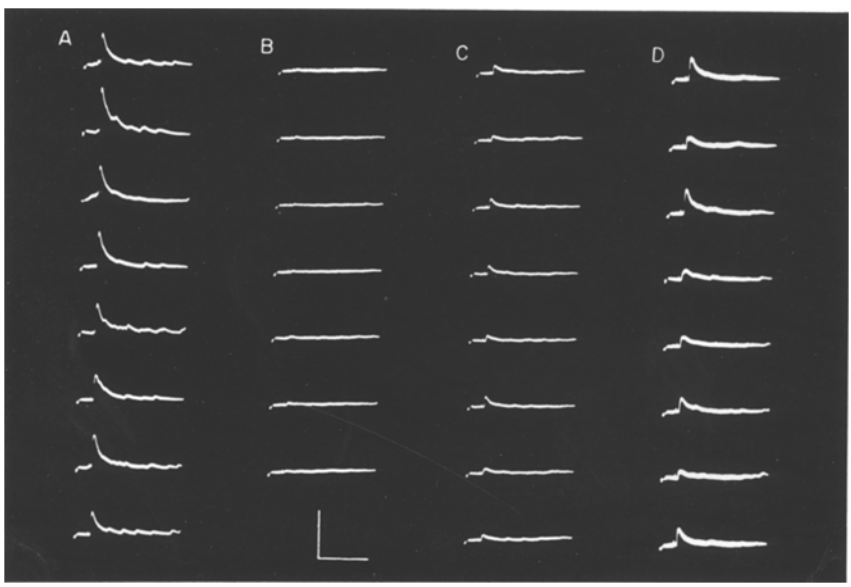


Fig. 5A – D. Effect of carbodiimide (10 mM) treatment on end plate potentials, and its partial reversal by hydrolysis. (A) before treatment; (B) 3 min after carbodiimide; (C) 12 min in Ringer (pH = 4) after washout of carbodiimide; (D) 65 min in same solution as in (C). Scale: 5 mV and 50 msec

Records of e.p.p. recorded in the presence of increased Mg and reduced Ca by a microelectrode in a muscle fiber at the end plate are shown in Fig. 5A. If the Ringer bath was replaced with a solution identical except

for the addition of 10 mM carbodiimide and adjusted to pH 4.2, the e.p.p. disappeared within minutes (Fig. 5B). It was difficult to follow the exact membrane potential under these conditions, but any changes were less than 10 mV. A small depolarization in acid pH has been reported by Hutter and Warner (1967). Furthermore, the membrane resistance was increased by an average of 127% in four experiments (range: 7 to 325%). A twofold resistance under a similar change to acid pH was reported by del Castillo *et al.* (1962) and is presumably due to a decrease in membrane chloride conductance (Hutter & Warner, 1967). Subsequent to replacement of the bath with an acid solution (pH 4.2) but without carbodiimide, the e.p.p. returned (Fig. 5C & D).

Phospholipid Reaction Studies

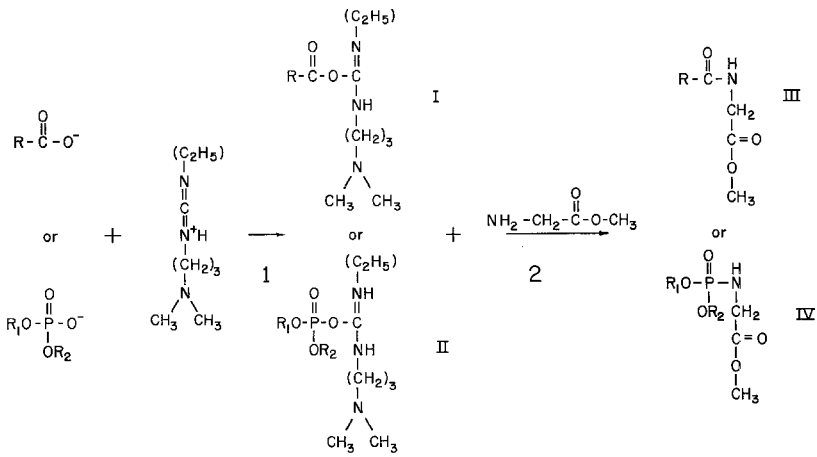
The thin layer chromatography plates containing samples taken from the reaction mixtures including carbodiimide, glycine methyl ester and either phosphatidyl choline or phosphatidyl ethanolamine showed three spots: the unreacted lipid and the two areas previously identified as the reactants, i.e., carbodiimide and glycine methyl ester. Analysis of inorganic phosphorus showed that these two areas contained $6.3 \pm 0.8\%$ of the phosphatidyl choline and $10.8 \pm 0.9\%$ (15 determinations, SE) of the phosphatidyl ethanolamine. However, the phosphatidyl serine plate contained an additional spot near the unreacted lipid which contained $45.4 \pm 2.5\%$ of the total lipid. The area occupied by carbodiimide and glycine methyl ester also contained $8.6 \pm 2.3\%$ of the phosphatidyl serine. All reactions seemed to be complete by the end of 5 min.

Discussion

The evidence that the receptor is in fact a protein has been reviewed by Nachmansohn (1968). The work presented here assumes that the ACh-receptor combination is ionic. The data presented are consistent with this assumption. The behavior of the combination between ACh and the enzyme acetylcholinesterase has been discussed by O'Brien (1970), and he cites evidence suggesting that this combination is not ionic. However, evidence of the type cited is not available for the ACh-receptor reaction.

The water-soluble carbodiimide reagent used would be expected to exist as a protonated amine in the pH range employed. Because of this charge, it would not be expected to penetrate the muscle membrane readily, and, therefore, its action would be restricted to the membrane surface. From its known chemical properties, the carbodiimide would be expected to react

with carboxylic groups (Hoare & Koshland, 1962) of sialic acid residues, phosphatidyl serine and of membrane proteins, and with phosphodiester groups (Khorana, 1953) of membrane phospholipids according to Reaction tion 1:



The reaction products I and II are unstable and are usually hydrolyzed by water, which leads to regeneration of the original groups. Compound I may also rearrange, however, to a stable acylurea derivative. In the presence of an excess of glycine methyl ester, reaction products III and IV are formed according to Reaction 2. Compound III is stable but the phosphoramidate compound IV is acid labile. Prolonged exposure of compound IV to acid leads to hydrolysis and to regeneration of the original phosphodiester group. The results of the phospholipid reactions noted above are consistent with the stability of compound III and the lability of compound IV.

The results obtained with muscles treated with the carbodiimide reagent may be rationalized on the basis of the above chemical considerations. In the absence of glycine methyl ester in the reaction medium, the carbodiimide reacts with carboxyl and phosphodiester groups to form compounds I and II and thus reduces the sensitivity of treated muscles to ACh. After washing of the reacted muscles with the fresh acid buffer, regeneration of the original groups occurs leading to restoration of the ACh sensitivity. In the presence of an excess of glycine methyl ester, however, compounds III and IV are formed which would also inhibit the ACh sensitivity of the treated muscles. Exchange of the medium and a 30-min exposure of the treated muscles to pH 4.1 did not restore the ACh sensitivity. This would imply that compound III was formed leading to an irreversible inhibition of the ACh sensitivity. This result would implicate carboxyl groups of sialic acid, of

membrane protein or of phosphatidyl serine as being essential for the ACh binding site. However, two uncertainties still remain. Hydrolysis of compound IV at pH 4.1 may be slow, or formation of compound III may have caused structural alteration of the muscle membrane, thus indirectly changing the structure of the ACh binding site. With these reservations, one may tentatively conclude, however, that carboxyl groups may be an essential part of an anionic site for ACh binding. It should be noted that Karlin and Winnik (1968) have recently shown that reduction of a disulfide bond in the electroplax of *Electrophorus electricus* blocks the ACh depolarization, suggesting that this bond is close to the active site.

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