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Reexamination of Carbodiimide as a Possible Affinity Label for the Acetylcholine Receptor at the Frog Neuromuscular Junction

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Summary. The effects of a water-soluble carbodiimide were examined at the frog neuromuscular junction. Acetylcholine sensitivity was measured using a fluid electrode technique and intracellular recording of miniature end-plate potentials. The carbodiimide blocked synaptic sensitivity by a reversible, curare-like action. Irreversible blockade was also observed, probably due to covalent binding. The conditions of reaction and irreversibility suggest that several different residues may be attacked. The inability of cholinergic antagonists to protect the receptor from attack indicates that nonspecific sites, and not the acetylcholine binding site, are involved.

Advances have been made in recent years in isolating and analyzing the acetylcholine receptor-membrane complex. Further knowledge, however, is needed concerning the chemical properties of the receptor *in situ*. Many researchers have assumed that the combination of acetylcholine with the receptor is ionic, but the identity of the anionic binding site remains unknown. Liu and Nastuk (1966) and Sokoll and Thesleff (1968) suggested that membrane-bound phosphate groups are involved. Edwards *et al.* (1970), on the basis of studies with the chemical reagent carbodiimide, claimed that the carboxyl function was a more likely candidate. They did not demonstrate, however, that the carbodiimide actually attacked a group located near or at the binding site for acetylcholine. We therefore set out to reexamine the problem and, in particular, to investigate the interaction of carbodiimide and cholinergic drugs. We conclude from our observations that carbodiimide probably is not a specific label for the acetylcholine receptor.

Materials and Methods

Preparations and Solutions

Experiments were performed on isolated sartorius or semitendinosous muscle from the frog *Rana ridibunda*. The normal Ringer's bathing solution contained (mM) NaCl,

116; KCl, 2.5; $CaCl_2$, 0.5; $MgCl_2$, 2.5; MES [2-(N-morpholino)ethane sulfonic acid] buffer, 3 mM, pH adjusted to 7.4 by NaOH and HCl. When the bathing solution included more than 20 mM of drug, NaCl in equiosmolar amounts was deleted. Solutions containing EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl, Ott Chemical Co.], GME [glycine methyl ester HCl] and Girard's reagent T [carbazoyl methyl trimethyl ammonium Cl] were made up fresh before each experiment, as required.

Intracellular Recording

Miniature end-plate potentials (m.e.p.p.'s) were recorded using conventional electrophysiological techniques (Landau & Nachshen, 1975). Average m.e.p.p. amplitude was determined from measurements of 40–60 m.e.p.p.'s after normalizing them with respect to the initial synaptic driving force (resting potential − 15 mV)

Extracellular Recording

The sensitivity of the end-plates of isolated muscles was tested by use of the fluid electrode technique described by Fatt (1950) as modified by Lindstrom, Singer and Lennox (1972). Muscles, usually pairs from the same frog, were suspended in calibrated plastic cylinders of less than 10 cc volume. Glass tubes filled with 4% agar in normal Ringer's, and containing chlorided silver wires, served as electrodes. The pelvic electrode contacted an endplate free region of the muscle through a cotton thread, and potential difference between the muscle at the level of the meniscus of liquid and the pelvic electrode was measured. This was done over the entire length of the muscle by lowering the meniscus at a constant rate. An initial recording obtained with normal Ringer's served as the baseline of the muscle response. Agonists, either carbachol or decamethonium chloride, were then added to Ringer's, and the muscle was exposed to this solution for a predetermined time − 8 min for the sartorius muscle, 5 min for the semitendinosous. Potential along the muscle was then measured as before. The maximal potential amplitude after subtracting the base line response served as an index of synaptic sensitivity. In general, our experience with the muscles, as regarding magnitude of response to agonists, washout times, apparent K_m of the agonists and stability of response was similar to that of Lindstrom, Singer and Lennox (1972). Potential difference was recorded via an NF-1 Bioelectric electrometer, and displayed on a 5103N Tektronix dual storage oscilloscope screen. Permanent records were obtained by Feeding oscilloscope output to a Grass 79C polygraph DC driver amplifier and pen recorder unit.

Results

Control Experiments

Muscle response to carbachol or decamethonium did not deteriorate in extracellular experiments after periods of more than seven hr, although most experiments were completed in half that time. Exposure of the preparation to Ringer's at low pH, 5.4, for more than two hr did not affect the test response. Ten mmoles of GME decreased the m.e.p.p. amplitude considerably, but this effect was reversed after vigorous wash-

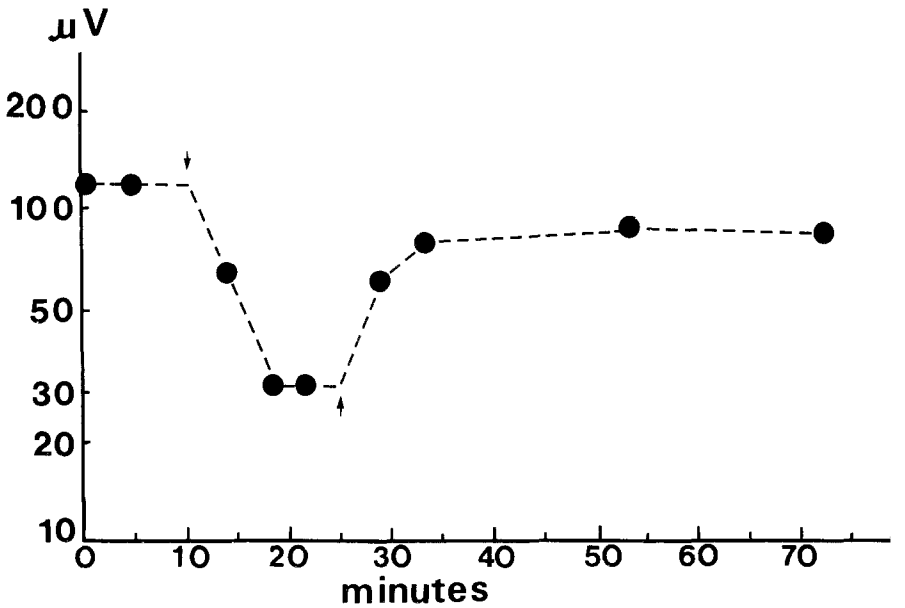

Fig. 1. Effect of 0.5 mM EDC on average m.e.p.p. amplitude. Arrows indicate wash-in and wash-out of normal Ringer's with EDC. Noise level in this experiment was 30 μV

ing with normal Ringer's. In some experiments the muscle was exposed to 100 mM of hexamethonium chloride for 1 hr. After three hours of washing, the test response recovered to $91 \pm 4\%$ (mean and SE, $n = 3$) of its control value. After an additional hour of washing, recovery was $98 \pm 4\%$ (mean and SE, $n = 3$) complete.

EDC has a curare-like effect. After only 5 min of exposure to normal Ringer's containing 0.5 mM EDC, m.e.p.p. amplitude fell to the noise level (Fig. 1). Extracellular experiments showed that EDC was about as effective as hexamethonium in blocking synaptic response to carbachol. Both drugs reduced the muscle response by half, in a concentration range of 10^{-4} to 5×10^{-5} M. Fig. 2 shows that EDC acts as a competitive antagonist of carbachol. In the presence of low concentrations of agonist a test dose of EDC reduced synaptic response to 25% of its control amplitude. When the concentration of agonist reached saturating levels, the test dose of EDC was far less effective, and blocked synaptic response by only about 50%, this indicates that EDC can compete with carbachol for a common binding site, the acetylcholine receptor. Curare-like action of EDC could be completely reversed by vigorous washing with normal

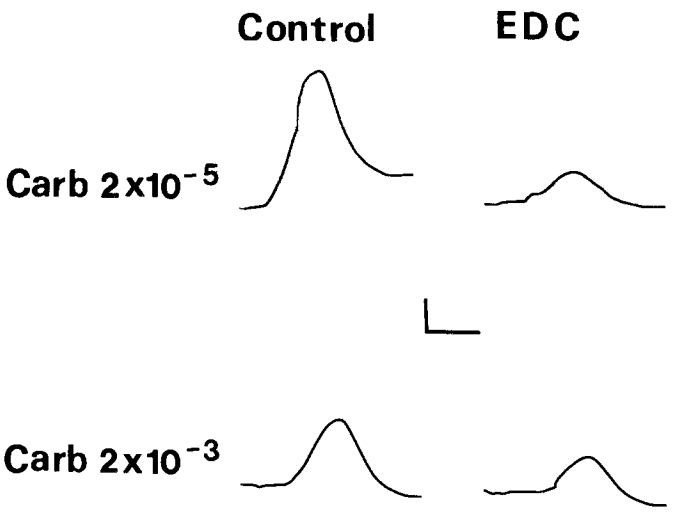

Fig. 2. Reduction of EDC's curare-like effects at a high concentration of carbachol. Upper row shows the effect of 10^{-4} M EDC on the potential along the muscle, evoked by 2×10^{-5} M carbachol after 5 min. Bottom row shows the effect of 10^{-4} M EDC on the muscle in response to 2×10^{-3} M carbachol also after 5 min. Calibration: upper row, 1 mV vertical, 5 sec horizontal; lower row, 4 mV, 5 sec. Rate of lowering of meniscus was calibrated in all experiments so that 1 sec was equivalent to 1 mm along the muscle.

Ringer's solution, provided that exposure to the carbodiimide was of short duration.

EDC acts irreversibly on muscle membrane. When the muscle was exposed to carbodiimide for periods longer than 15 min an additional effect was observed, as well as the curariform action. This consisted of a long-lasting depression of synaptic sensitivity. Fig. 1 shows that after an initial rapid recovery of receptor sensitivity the m.e.p.p. amplitude remained depressed for more than 1 hr. Presumably this depression is due to chemical binding of EDC to the muscle. After 1 hr exposure to Ringer's at pH 5.4 containing EDC, 1.5 mM, 1 hr of vigorous washing restored the test response to only $62 \pm 4\%$ of its control value (mean and SE, $n = 3$). Continued washing for one more hour increased the synaptic response only by a further 15%. This was true for muscles tested both with carbachol or decamethonium.

Nucleophiles do not increase the effectiveness of EDC. The presence of a nucleophile can modify carbodiimide's reaction, and may lead to the formation of more stable products in some systems (Means & Feeney,

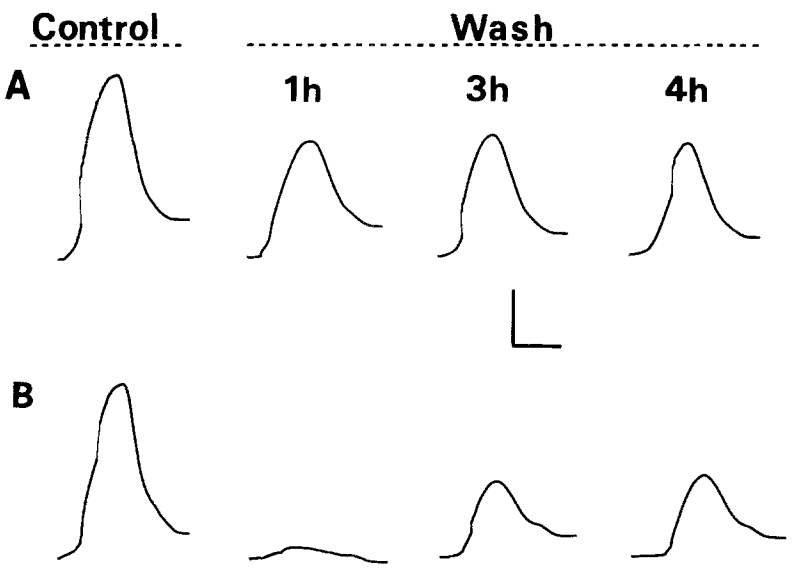

Fig. 3. Blockage of the acetylcholine receptor response in the presence of hexamethonium. Upper row: effect on muscle response of 1.5 mM EDC and 15 mM GME after 1 hr treatment. Bottom row: same as above, but 100 mM hexamethonium chloride was added to the reaction mixture. Calibration: 3 mV vertical, 2 sec horizontal

1971). We did not find this true at the neuromuscular junction. Edwards *et al.* (1970) reported that the acetylcholine response was completely blocked by bathing solution containing 10 mmoles EDC and 50 mmoles GME at pH 4.75 after 10 min. While this was observed by us, unlike Edwards *et al.* we found that vigorous washing for 30 min partially restored synaptic response. A further hour of washing with normal Ringer's completely restored muscle sensitivity. We believe that this discrepancy is due to a more effective washing of the muscle on our part.

In five muscles exposed to Ringer's (pH 5.4) along with 1.5 mM EDC and 15 mM GME for one hour, the test response after an hour of washing with normal Ringer's was $58 \pm 5\%$ (mean and SE) of control potential amplitude. After an additional hour of washing, the test response increased by a further 16%. This result does not differ significantly from that obtained when muscles were exposed to carbodiimide alone. Experiments with EDC and the nucleophile Girard's reagent T gave similar results.

We tested whether changes in pH had any effect on the reaction of carbodiimide. Lowering the pH of the bathing solution which contained EDC to 4.75, or raising it to 7.4 had no effect. Neither did

lowering the acidity of the Ringer's used to wash out the EDC from pH 7.4 to pH 5.4 have any effect on synaptic response.

EDC is not a site-specific reagent. Although EDC competes with carbachol at the acetylcholine receptor, its long-term effects do not seem to be mediated by attack of the acetylcholine binding site. In three experiments hexamethonium at a concentration of 100 mmoles was added to the reaction Ringer's along with EDC and GME. After more than 4 hr of vigorous washing, the test response was not significantly different from that of muscles treated without hexamethonium (Fig. 3). Thus, the presence of a saturating dose of hexamethonium did not afford the acetylcholine receptor a significant degree of protection from attack by carbodiimide.

Discussion

EDC irreversibly blocks the electrical response of skeletal muscle to carbachol and decamethonium, and thus appears to bind covalently to the postsynaptic membrane (Edwards *et al.*, 1970). What chemical residues are involved in generating this block? Carboxyl and phosphate groups react with carbodiimides to yield unstable intermediates. In the presence of an excess of nucleophile these are converted to stable compounds. Since no difference was found in the reaction with or without nucleophiles these reaction pathways are unlikely. Carboxyl intermediates may also be converted to stable acylureas in the absence of nucleophile, and this reaction cannot be excluded by our findings. Reactions could also occur with thiol, phenol or amine residues, since these form stable products with EDC alone. Contrary to the conclusions of Edwards *et al.* (1970), we cannot find evidence to indicate that EDC acts on muscle primarily by modifying carboxyl groups at the acetylcholine receptor.

EDC is a water soluble carbodiimide bearing a charged tertiary ammonium group in the pH range used in our experiments. Thus, it is not surprising that it has significant curare-like properties. Under suitable conditions, moreover, it irreversibly blocks the response of the acetylcholine receptor. However, a major problem in the use of site-directed reagents is that nonspecific labelling may occur at considerable rates outside the active site, in this case the acetylcholine binding site. Acceptance of EDC as an affinity label rests upon the demonstration that agonists or antagonists of acetylcholine can block attack by this reagent. This was not found.

Is the ratio of hexamethonium to EDC large enough for protection of the receptor site to be effective? A comparison of the effects of EDC with those bromoacetylcholamine (BACA) indicates that *more* than adequate concentrations of hexamethonium were used. BACA is a highly reactive reagent at the acetylcholine receptor (Ben-Haim, Landau & Silman, 1973). Because of its reactivity it begins to block the acetylcholine receptor response in frog skeletal muscle pretreated with dithiothretol, at low concentrations after short exposures. However, both one hour's exposure to 1.5 mM EDC and 20 min exposure to 6×10^{-7} M BACA reduce acetylcholine receptor sensitivity by about half. Thus, these two concentration-time doses (Karlin, 1969) are about equivalent. Whereas one mmole of hexamethonium suffices to protect the receptor from attack by BACA, a 100-fold greater concentration of hexamethonium does not modify the effects of EDC.

We conclude that EDC is not useful as an affinity reagent for the acetylcholine receptor. Although it has curare-like properties, it reacts with unidentified residues in the postsynaptic membrane, and cannot be used, at present, to label the anionic binding site of the acetylcholine receptor.

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