*REDUCTION AND SPECIFIC ALKYLATION OF THE RECEPTOR FOR ACETYLCHOLINE**

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Acetylcholine (ACh) causes a change in the permeability of the membrane of electrogenic cells to ions.[1] An objective central to the explanation of this phenomenon in molecular terms is the characterization of the receptor for ACh. This has been inferred to be a protein component of the cell membrane,[2, 3] possibly with multiple, cooperatively interacting binding sites.[4, 5] An approach to this objective is to label the receptor in the living cell by a specific, covalent reaction. The specificity may be obtained by directing the reaction toward the active site by combining in one reagent a group which will react covalently with amino acid side chains and a group which will reversibly bind to the active site. Methods of directing covalent reactions toward active sites have been successfully used to label and to map these sites in enzymes and in antibodies (for reviews see refs. 6 and 7). Similar methods have also been used to label the active sites of receptors for catecholamines[8–11] and for ACh.[9, 12–14]

We present here a two-step process for covalently labeling the ACh-receptor in the electroplax of *Electrophorus electricus* based on the following observations: (1) the response of the electroplax to receptor activators such as ACh and carbamylcholine is specifically inhibited by prior short exposure of the cell to dithiothreitol (DTT), a potent reducing agent;[15] (2) the inhibition is completely reversed by subsequent exposure of the cell to 5,5′-dithio-bis (2-nitrobenzoate) (DTNB), an oxidizing agent;[16] and (3) application of N-ethylmaleimide (NEM) after DTT, at a concentration otherwise without an effect, prevents the reversal by DTNB. It was concluded that the receptor contains a relatively easily reduced disulfide bond and that at least one of the sulfhydryl groups thereby formed may be alkylated by NEM.[3] In order to confer specificity for the receptor on this alkylation following reduction, we prepared and used a maleimide derivative, 4-(N-maleimido)phenyltrimethylammonium iodide (MPTA), containing a group, phenyltrimethylammonium, which is a potent depolarizer of the electroplax,[17] and presumably has considerable affinity for the active site of the ACh-receptor. We present evidence that the rate of reaction of MPTA with the reduced disulfide of the receptor is two to three orders of magnitude faster than that of NEM or of the tertiary amine analogue of MPTA, 4-(N′-maleimido)-N, N-dimethyl aniline (MDA), and, hence, that MPTA is a specific *affinity label*[6, 7] of the reduced receptor. Furthermore, the distance of the double-bond of the maleimido moiety of MPTA from the quaternary nitrogen provides an estimate of the distance of the negative subsite within the active site from the disulfide which is reduced and alkylated. That this disulfide is close to the active site is further supported by the finding that following reduction, hexamethonium, normally a competitive inhibitor of the receptor, acts as an activator, causing a depolarization. It appears likely that reduction alters the conformation and, thereby, the specificity of the active site.

Materials and Methods.—Electroplax were isolated,[18] mounted in a Lucite holder (details of which will appear elsewhere), and perfused as previously described with a modified Ringer's solution.[19] All reagents were added to the innervated side of the cell. The potential difference across the cell from the noninnervated to innervated side and that across the noninnervated membrane were recorded simultaneously. The change in the ratio of the permeability of Na to that of K may be estimated as a function of these two changes in potential difference.[19] However, for the purposes of this work, the response to an activator is taken to be the sum of the change in these two potential differences, which is just the change across the innervated membrane, 80 sec after the addition of the activator to the innervated side of the cell (as in ref. 3).

MDA was made by the procedure of Cava *et al.*[20] and isolated as bright orange crystals, mp 151–152°C. These polymerized when recrystallized but could be chromatographed on silica gel to yield the pure material. *Analysis:* Calculated for $C_{12}H_{12}N_2O_2$: C, 66.65%; H, 5.59%; N, 12.95%. Found: C, 66.66%; H, 5.91%; N, 12.86%. The infrared and nuclear magnetic resonance spectra are consistent with the assigned structure.

Methylation was accomplished by heating a methyl iodide solution of the tertiary amine in a pressure bottle for 8 hr on a steam bath. Trituration with ether and recrystallization from methanol-benzene yielded MPTA, a pale yellow powder, mp 191–193°C in 67% yield. *Analysis:* Calculated for $C_{13}H_{15}IN_2O_2$: C, 43.55%; H, 4.24%; I, 35.43%; N, 7.83%. Found: C, 43.71%; H, 4.18%; I, 35.81%; N, 7.82%. The infrared and nuclear magnetic resonance spectra confirm the structure, and by the latter the purity is estimated to be 98%.

The second-order rate constants for the reaction of L-cysteine with NEM, with MPTA, and with MDA in the same modified Ringer's solution (pH 7.05) used on the electroplax and at 25°C were determined as follows: 55 ml of deoxygenated Ringer's solution was added to a tubular cell of 10-cm light path; 0.5 ml of a solution of one of the maleimide derivatives in water was added and the absorbance (NEM at 300 mμ; MDA at 305 mμ; MPTA at 320 mμ) determined (A_0); then 0.05 ml of L-cysteine solution was added with a micropipette and the solution quickly mixed. Starting at 10 sec after mixing, the absorbance (A_t) was determined initially at 5-sec intervals and finally at 30-sec intervals for 4 min. The initial concentrations of the cysteine and the maleimide derivative were in each case equal and hence the following equation applies: $kt = c/a_0\,(a_0 - c)$, where k is the rate constant in M^{-1} sec^{-1}, t is time in sec, c is the concentration of product in M, and a_0 is the initial concentration of the reactants. For any nonequal extinction coefficients for the maleimide and the product and for the extinction coefficient for cysteine equal to zero, $c/a_0(a_0 - c) = (A_0 - A_t)/a_0(A_t - A_\infty)$. The resulting equation may be rearranged to $A_t = (A_0 - A_t)/a_0kt + A_\infty$; hence, the plot of A_t versus $(A_0 - A_t)/t$ should lie on a straight line with slope $1/a_0k$ and intercept A_∞. a_0 was 2.5×10^{-5} M for MDA and NEM and was 2.11×10^{-5} M for MPTA. The resulting points were reasonably colinear.

Results and Discussion.—The effectiveness of a maleimide derivative in alkylating the reduced disulfide of the receptor may be inferred from experiments such as the one shown in Figure 1. Following a control response to carbamylcholine, 1 mM DTT is applied for ten minutes. The next response is inhibited due to the reduction. Thereafter, a maleimide derivative, in this case 10^{-7} M MPTA, is added for a short time and then washed out with Ringer's solution. Another response is elicited which shows the inhibition due to both reduction and alkylation. DTNB is then added for ten minutes to reform those disulfides which have not been blocked by alkylation, and two more responses are elicited: the second is usually larger than the first; however, the response does not recover appreciably more thereafter. As has been shown previously, the final response in the absence of alkylation is equal to the initial one; i.e., the reduction alone is

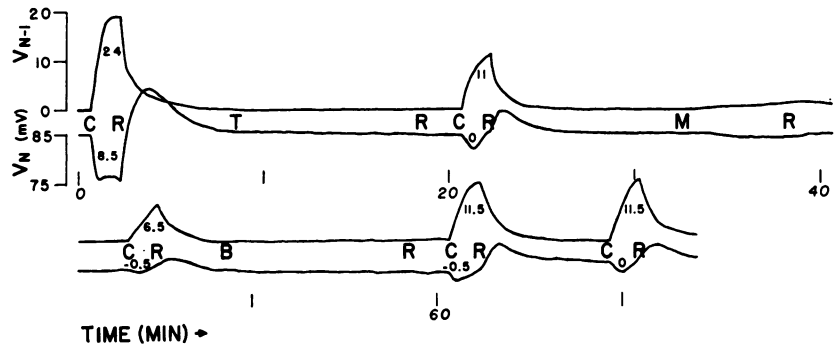

FIG. 1.—The irreversible inhibition of the reduced receptor by MPTA. The potential difference across the noninnervated membrane, V_N, and across the entire cell from the noninnervated to the innervated side, V_{N-I}, were measured simultaneously, with agar bridges in the two outside solutions and a glass microelectrode of 5–10 MΩ inside the cell. The changes in potential difference 80 sec after adding carbamylcholine are indicated. The solutions, perfused past the innervated membrane, were added as indicated on the record: *C*, 40 μM carbamylcholine chloride in *R*; *R*, modified Ringer's solution; *T*, 1 mM DTT in Tris-Ringer's solution[3] (pH 8.0); M, 10^{-7} *M* MPTA in *R*; B, 1 mM DTNB in Tris-Ringer's solution (pH 8.0).

completely reversible.[3] The initial response minus the final response is a measure of the unreversed inhibition and, hence, of the number of the receptors which have been alkylated. The initial response minus the response after the maleimide is added is a measure of the number of receptors which have been either reduced or both reduced and alkylated. The ratio of the former difference to the latter is therefore roughly equal to the fraction of the reduced disulfides which have been alkylated and has the virtue of being equal to zero for total reversibility of the inhibition and of being equal to one for total irreversibility. In the experiment shown in Figure 1, this ratio equals 0.79 (= (32.5–11.5)/(32.5–6.0)).

The ratios obtained with the three maleimides tested, multiplied by 100 and expressed as per cent irreversible inhibition, are plotted in Figure 2 versus the concentration of the maleimide multiplied by the duration of its application. During the alkylation reactions, the maleimide concentration is constant since the solution is rapidly perfused past the electroplax. The extent of alkylation is then a function of (second-order rate constant) × (concentration of maleimide) × (time of application). Therefore, for equal "per cent irreversible inhibition," a function of the extent of alkylation, the ratio of the second-order rate constants of the alkylation by two maleimide derivatives is inversely proportional to the ratio of the (concentration × time)'s required to produce the inhibition.

From the data presented in Figure 2 it may be concluded that NEM (○) and MDA (△) are approximately equally active in alkylating the reduced disulfide; MPTA (□), on the other hand, appears to react three orders of magnitude faster than the other two. At 50 per cent irreversible inhibition, the ratio of the "rate-constants" is 1660. A small part of this large difference is due to an intrinsically greater reactivity of MPTA compared with NEM and MDA, as indicated by the rate constants of the reaction with L-cysteine in the same modified Ringer's

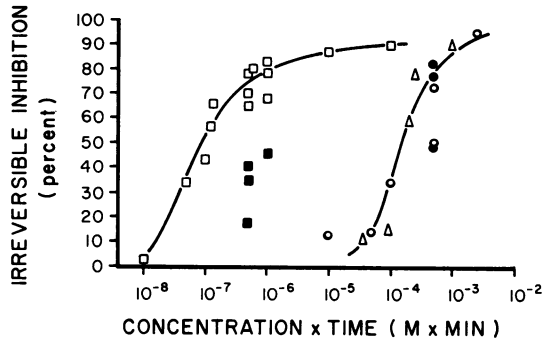

FIG. 2.—The concentration dependence of the irreversible inhibition of the reduced receptor by maleimide derivatives. The ordinate is the per cent of the total inhibition due both to reduction and alkylation which is not reversed by oxidation. The abscissa is the concentration of maleimide applied multiplied by the duration of application. MPTA, □; MDA, △; NEM, ○; MPTA in 1 mM hexamethonium, ■; NEM in 1 mM hexamethonium, ●.

solution used on the electroplax (Table 1). Taking 1660 as the ratio of the rate constant of alkylation of the receptor by MPTA to that by NEM and dividing by the ratio of the rate constants for the alkylation of cysteine, we find an *enhancement*[6] of the alkylation of the receptor by MPTA relative to NEM of 300-fold, due presumably only to the binding of the charged quaternary ammonium group to the active site of the receptor. When the differences in intrinsic reactivity are taken into account, MDA appears to be less effective than NEM in alkylating the receptor so that, in the absence of appreciable affinity for the active site, the size of a molecule may be an important factor influencing the rate of alkylation of the receptor. Taking into account the differences in intrinsic reactivity, we find that MPTA alkylates the reduced receptor 720-fold faster than MDA, its tertiary analogue. It has been reported that 10^{-4} *M* *p*-(trimethylammonium) benzenediazonium fluoborate, which, like MPTA, contains the phenyltrimethylammonium moiety, irreversibly inactivates the ACh-receptor of the electroplax and that the tertiary amine analogue has almost no effect.[5] For the purpose of labeling the receptor, the two-step process described here may be even more specific than indicated above for the alkylation step alone, since the first step, the reduction, is already selective for disulfide bonds unusually susceptible to reduction.

Supporting the hypothesis that MPTA can bind reversibly to the active site is the observation that, in the absence of prior reduction of the receptor by DTT,

TABLE 1. *Rate constants for the reaction of maleimide derivatives with cysteine and with reduced receptor.*

Derivative	Rate constant for reaction with cysteine k (M^{-1} sec^{-1})	k/k_{NEM}	Rate constant for alkylation of receptor relative to NEM	Enhancement of alkylation of receptor due to affinity
NEM	1620 ± 130*	1.0	1	1.0
MDA	3920 ± 80	2.4	1	0.4
MPTA	8850 ± 700	5.5	1660	300.

The second-order rate constants for the reaction with cysteine were determined as described in *Materials and Methods*. The mean value ± SEM is given. The relative rate constants for the alkylation of receptor were calculated from the curves in Fig. 2 and are the ratio of the (conc.) × (time) of MPTA or of MDA resulting in 50% irreversible inhibition to that of NEM. The enhancement due to affinity is the relative rate constant for alkylation of the receptor divided by the relative rate constant for alkylation of cysteine.

* An expression by Gorin *et al.*[21] relating k for this reaction to pH yields k = 1720 for pH 7.05.

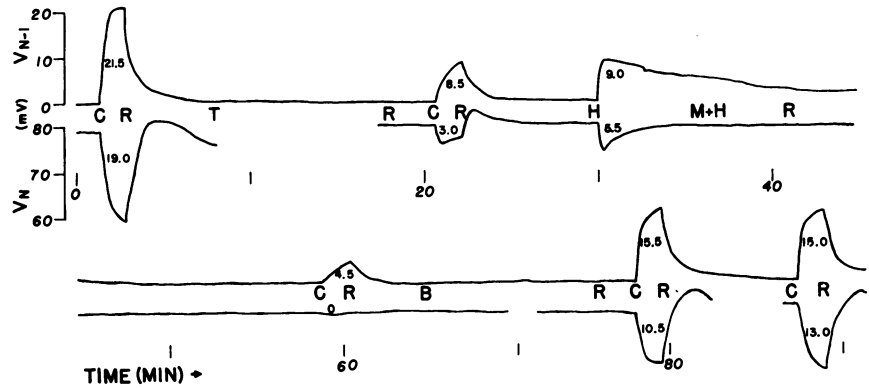

FIG. 3.—The partial protection of the reduced receptor by hexamethonium against irreversible inhibition by MPTA. The experiment was conducted as in Fig. 1. *C*, 40 μM carbamylcholine; *R*, modified Ringer's solution; *T*, 1 mM DTT (pH 8.0); *H*, 1 mM hexamethonium chloride; *H* + *M*, 10^{-7} *M* MPTA in 1 mM hexamethonium; *B*, 1 mM DTNB (pH 8.0).

MPTA acts simply as a reversible competitive inhibitor of the receptor. MPTA at 5×10^{-4} *M* (50,000-fold the concentration at which it causes 50 per cent irreversible inhibition after DTT) has no irreversible effects on the response of the electroplax without prior treatment by DTT. Moreover, the dissociation constant of the complex of MPTA with the *unreduced* receptor may be estimated by the method of Gaddum[22] to be 8×10^{-5} *M*. This result does not, however, indicate what the affinity of MPTA for the *reduced* receptor might be, since the active site in this form is undoubtedly altered (see below). This observation further supports the view that MPTA is indeed acting by alkylating a reduced disulfide, since there is no effect in the absence of prior reduction. It should be mentioned in this connection that after reduction, when MPTA reacts, it invariably causes a small response of 1–2 mv which is not reversed when the MPTA is washed out.

Hexamethonium (hexamethylene-bis(trimethylammonium chloride)) is a relatively small, readily reversible, competitive inhibitor of the ACh-receptor in the electroplax. Transmission at the vertebrate neuromuscular junction is not particularly sensitive to hexamethonium, which is generally thought of as a ganglionic blocking agent (e.g., ref. 23). However, it blocks the neurally evoked action potential of the electroplax[24] and competitively inhibits the depolarization caused by carbamylcholine with an apparent dissociation constant of 3×10^{-5} *M*.[25] The effect of hexamethonium on the reaction of the reduced receptor with MPTA provides further evidence that the reversible binding of MPTA at the active site precedes the alkylation. A typical experiment is shown in Figure 3. The procedure is the same as in Figure 1 except that after DTT, 1 mM hexamethonium is added for six minutes to allow it to equilibrate with the receptor and then 10^{-7} *M* MPTA in 1 mM hexamethonium is applied for five minutes. The irreversible block in this case is 35 per cent (compare with 79% in Fig. 1). The results of several such experiments have been plotted in Figure 2. Hexametho-

nium partially protects the receptor against alkylation by MPTA, i.e., it slows the reaction with MPTA. The alkylation by NEM, however, does not appear to be slowed by the presence of 1 mM hexamethonium (Fig. 2). It appears, therefore, that hexamethonium is protecting by specifically competing with MPTA for binding to the active site, rather than by sterically hindering the reaction at the sulfhydryl, which is probably just outside the active site (see below).

A surprising result, shown in Figure 3, is that after DTT, hexamethonium causes a depolarization i.e., is a receptor-activator. This it does only after DTT; moreover, reoxidation with DTNB causes the receptor to regain its former specificity, and hexamethonium no longer activates. A likely explanation for this phenomenon is that the reduction of a disulfide near the active site changes the conformation of this site and, thereby, its specificity. In comparison, selective reduction under nondenaturing conditions of two disulfide bonds in trypsin[26] and of one disulfide of the basic trypsin inhibitor of bovine pancreas[27] results in no loss in their activities. On the other hand, reduction of a disulfide in thioredoxin causes localized conformational change of this protein.[28] That reduction changes the specificity of the active site of the receptor is consistent with previous observations that treatment with DTT decreases the apparent affinity constant of the receptor for activators[3] and, also, decreases the apparent cooperativity between sites in the response to activators.[4]

In the MPTA molecule, the distance from the quaternary nitrogen to the ethylenic double-bond of the maleimide moiety, is more or less fixed and may be estimated using a molecular model to be approximately 8 Å. The distance from the double-bond to the periphery of the methyl groups on the quaternary nitrogen is approximately 10 Å. As a first approximation, then, 8–10 Å may be taken as the distance from the negative site within the active site to one of the sulfhydryls formed by reduction of a disulfide and, possibly, to the disulfide itself. The latter requires that the conformational change upon reduction not be too great; in fact, the residual activity of the reduced receptor as well as the reversibility by reoxidation argue for a small change. Since the over-all length of the ACh-molecule is approximately 9 Å, it is probable, given a similar orientation of MPTA and of ACh with respect to the negative subsite, that the disulfide in question lies at the periphery of the active site. Proximity of a disulfide bridge to an active site has precedents in the "histidine loop" present in "serine proteases"[29] and may yet be found in acetylcholinesterase, which, however, is not inactivated by DTT under conditions which inactivate the receptor.[30]

Summary.—A disulfide bond in the acetylcholine-receptor of the electroplax may be reversibly reduced and reoxidized. Alkylation with maleimide derivatives following reduction prevents the reoxidation. One such derivative, 4-(N-maleimido)phenyltrimethylammonium iodide (MPTA), has considerable affinity for the active site of the receptor and, by virtue of such affinity, alkylates the reduced receptor two to three orders of magnitude faster than its tertiary amine analogue or than N-ethylmaleimide (NEM), which lack such affinity. Hexamethonium, ordinarily a reversible competitive inhibitor of the receptor, is an activator of the *reduced* receptor, indicating that reduction of the disulfide has altered the specificity of the active site. Hexamethonium also competes with

MPTA for the active site since it slows the reaction of MPTA, but not of NEM, with the reduced disulfide. From a model of MPTA, it may be estimated that the disulfide which is reduced and alkylated is of the order of 10 Å from the negative subsite which binds the quaternary nitrogen group common to all activators of the receptor and to MPTA.

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