E_{II}-containing term in eq 19; at that point, the maximum lag time, determined solely by the E_I concentration, will be reached:

$$LT = \frac{2}{k_{-a}\left[n + \frac{1}{A} - \frac{1}{A}(1 + 2nA)^{1/2}\right]} \ln\left[1 + \frac{1 + nA - (1 + 2nA)^{1/2}}{2(1 + 2nA)^{1/2}}(1 - F_0)\right] \quad (20)$$

It should be emphasized that in eq 19 the lag time always remains dependent on the E_I concentration even if E_{II} is very small (i.e., excess E_I). This has also been found experimentally (see Figure 4) and was explained qualitatively under Discussion.

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Independent Activation of the Acetylcholine Receptor from *Torpedo californica* at Two Sites†

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ABSTRACT: Membrane vesicles enriched in acetylcholine receptor were prepared from the electroplax tissue of *Torpedo californica*. The receptor was reduced with dithiothreitol to expose a sulfhydryl group near the ligand binding site and then treated in one of the following ways: (1) affinity alkylated with bromoacetylcholine, a receptor activator, (2) affinity alkylated with maleimidobenzyltrimethylammonium, a receptor inhibitor, or (3) reoxidized to the native state with dithiobis(2-nitrobenzoate). The affinity labels blocked half of the binding sites for α-bungarotoxin. The toxin sites not protected by the affinity labels were protected by carbamylcholine based on studies of toxin binding kinetics. The functional response of native and affinity-alkylated receptors was measured by a sodium ion flux procedure. In the absence of added cholinergic activators, only slow ion flux was observed. In the presence of carbamylcholine, a receptor activator, both native and modified membranes showed the increased sodium flux associated with functional receptors. The concentration of carbamylcholine required for a 50% maximal response was higher in the affinity-labeled membranes. Preincubation of the membranes with carbamylcholine blocked the increased ion flux, indicating that desensitization could be induced. The results provide evidence for the existence of two functional sites on the acetylcholine receptor. Each site corresponds to a bungarotoxin binding site and can be independently activated and desensitized.

The nicotinic acetylcholine receptor (AcChR)[1] from electroplax tissue can be affinity alkylated by 4-maleimidobenzyltrimethylammonium (MBTA) and by bromoacetylcholine (BAC), following reduction of the receptor to expose a reactive sulfhydryl group near the active site (Karlin et al., 1975; Damle et al., 1978; Moore & Raftery, 1979a). Following alkylation, MBTA acts as an irreversible inhibitor of AcChR activation in intact electroplax cells from *Electrophorus electricus* (Karlin, 1969); in contrast, BAC acts as an irreversible activator, leading to prolonged depolarization (Silman & Karlin, 1969). Both affinity labels react specifically with the 40 000 M_r subunit of AcChR from *E. electricus* and *Torpedo californica*, and this 40 000 M_r subunit (the α chain) is presumed to contain the ligand binding site(s) (Karlin et al., 1975; Damle et al., 1978). Snake α-neurotoxins, which act as nearly irreversible inhibitors of AcChR activation, also bind to the α chain [for reviews, see Heidmann & Changeux (1978) and Barrantes (1979)]. However, there are two α-neurotoxin binding sites for each affinity-labeling site, and analysis of $NaDodSO_4$–polyacrylamide gel electrophoresis patterns indicates that there are at least two α subunits for each receptor monomer complex of M_r 250 000 (Karlin et al., 1975; Reynolds & Karlin, 1978). Since the binding of BAC and MBTA is mutually exclusive (Damle et al., 1978), it appears that one and only one of the two binding sites can be affinity labeled. Using a different affinity label, *p*-(trimethylammonium)benzenediazonium fluoroborate, Weiland et al. (1979) recently found a 1:1 ratio of toxin to affinity-labeled sites.

For membrane-bound AcChR, kinetic and equilibrium binding studies using toxins have not yet revealed differences between the two toxin sites (Blanchard et al., 1979). In detergent solution, however, kinetic heterogeneity has been ob-

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[1] Abbreviations used: AcChR, acetylcholine receptor; MBTA, 4-maleimidobenzyltrimethylammonium; BAC, bromoacetylcholine; AcCh, acetylcholine; Carb, carbamylcholine; VDB, vesicle dilution buffer; DTNB, 5,5′-dithiobis(2-nitrobenzoate); DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; [^{125}I]-α-BgTx, iodinated α-bungarotoxin.

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served and has been interpreted to represent allosteric interactions between the two sites (Bulger et al., 1977). In a recent study in this laboratory, in which a spin-labeled derivative of *Naja naja siamensis* α-neurotoxin was used, no differences between the two sites were observed either in *Torpedo* membranes or in detergent solutions of purified *Torpedo* AcChR (Ellena & McNamee, 1980).

It is not known if both toxin binding sites represent functional binding sites for the natural ligand, acetylcholine (AcCh). There has been considerable controversy over the number of AcCh binding sites (one or two) and the relationship between them [for a review, see Heidmann & Changeux (1978)].

We report and discuss here the results of experiments designed to measure the functional properties of affinity-labeled membrane vesicles from *T. californica*. Specifically, we show that BAC- and MBTA-labeled membranes respond to carbamylcholine (Carb), an AcChR activator, with a large increase in $^{22}Na^+$ permeability. Increased cation permeability of the postsynaptic membrane is the primary functional consequence of acetylcholine binding to AcChR, and purified *Torpedo* membranes have provided an excellent in vitro system for characterizing many aspects of AcChR structure and function (Heidmann & Changeux, 1978; Barrantes, 1979). We conclude that there exist two ligand binding sites for AcChR activators and that each site can be independently activated and desensitized.

Materials and Methods

Preparation of Torpedo Membranes. Liquid nitrogen frozen electric tissue (100 g) from *T. californica* was thawed and then kept at 0–4 °C. Homogenization buffer (200 mL) was added (10 mM $CaCl_2$, 1 mM EDTA, and 0.1 mM PMSF, pH 7.0), and the mixture was homogenized by using a Brinkmann Polytron (2 times for 2 min at 6–7 setting). The homogenate was centrifuged for 10 min at 5000*g*. The supernatant was filtered through four layers of cheesecloth and the pellet was discarded. The filtered supernatant was layered onto 25 mL of 37.5% (w/v) sucrose and centrifuged for 2 h at 95000*g* in a Beckman Type 35 rotor. The soft pellets were suspended in vesicle dilution buffer (VDB: 250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 0.02% NaN_3, and 10 mM Tris-HCl, pH 7.6) and centrifuged for 45 min at 140000*g*. The pellets were resuspended in VDB and used immediately or stored in liquid nitrogen.

Modification of Receptors. To 5 mL of membranes (~5 mg of protein per mL) was added 50 μL of 100 mM dithiothreitol in VDB, and reduction was allowed to proceed for 30 min at room temperature. The membranes were separated into three parts, and each was diluted with 50 mL of VDB and then centrifuged for 1 h at 150000*g*. Pellets were resuspended in 2.5 mL of VDB containing 10^{-4} M eserine to inhibit acetylcholinesterase. Part 1 was treated with 25 μL of 10 mM DTNB (final concentration of 0.1 mM DTNB), part 2 was treated with 25 μL of 10 mM MBTA, and part 3 was treated with 25 μL of 10 mM BAC. After 15 min at room temperature, each sample was diluted with 50 mL of VDB and centrifuged for 1 h at 150000*g*. Pellets were resuspended in less than 1 mL of VDB and stored in liquid nitrogen. During the final resuspension of the membrane vesicles, only a small volume of VDB was used in order to keep the protein concentration high, since the high concentration was necessary for the $^{22}Na^+$ influx procedure.

$^{22}Na^+$ Flux Assays. (1) Influx. To 30 μL of membranes at 0 °C (~5 mg of protein per mL) was added a mixture of 5 μL of 0.25 mCi/mL $^{22}NaCl$ (New England Nuclear; carrier free) and 10 μL of VDB (with and without Carb). After 1 min of influx at 0 °C, the samples were diluted with 1 mL of VDB and immediately filtered on Millipore filters (Type HA, 0.45 μm) and washed 3 times with 2.5 mL of VDB. Filters were counted in 10 mL of PCS (Amersham) in a liquid scintillation counter.

(2) Efflux. Torpedo membranes were equilibrated overnight with $^{22}Na^+$, and efflux was measured after dilution as described previously (Andreasen et al., 1979). When BAC was used as the activating ligand in the dilution buffer, 10^{-4} M eserine was included in all solutions. Concentrated BAC solutions in water were prepared and added to the dilution buffer immediately before efflux in order to minimize hydrolysis. BAC was synthesized by reaction of choline with bromoacetyl bromide (Damle et al., 1978). Both tritiated and unlabeled MBTA were prepared by the method of Karlin (1977).

Receptor Binding Assays. Three types of assays were used to measure the ligand binding properties of the receptor. The equilibrium binding of toxin was measured by the DE-81 filter disk method as described by Hamilton et al. (1979) except that iodinated α-bungarotoxin was used instead of tritiated *N. naja siamensis* toxin. The iodinated toxin was prepared and the *rate* of toxin binding was measured exactly as described previously (Andreasen et al., 1979). Tritiated MBTA labeling was measured by the affinity alkylation assay (Karlin, 1977). Protein determinations were done by the Lowry method (Lowry et al., 1951).

Results

Torpedo Membrane Vesicles. Acetylcholine receptor enriched membrane fragments prepared from the electric organ of *T. californica* are known to retain the ligand binding and ion permeability control properties expected of the AcChR (Heidmann & Changeux, 1978; Andreasen et al., 1979). For the membranes used here, 100 g of tissue gave 30 mg of membrane protein with a specific activity for iodinated α-bungarotoxin ([^{125}I]-α-BgTx) binding of 700–1000 pmol/mg.

In the absence of cholinergic activators, the influx of added $^{22}Na^+$ into the vesicles proceeded at an easily measured rate, and the ions reached equilibrium over a period of several hours (Figure 1). This influx represented passive diffusion and exchange into a heterogeneous population of vesicles and was qualitatively analogous to the passive efflux of $^{22}Na^+$ from preloaded vesicles following dilution (Andreasen & McNamee, 1977). Addition of 6×10^{-4} M Carb to the influx mixture at time 0 resulted in a significant increase in the rate of $^{22}Na^+$ influx within 10 s, the shortest influx time feasible in these studies (Figure 1). A comparably fast stimulation of ion flux was observed in efflux studies (Andreasen & McNamee, 1977). The Carb-induced increase in $^{22}Na^+$ permeability was characteristic of functional acetylcholine receptors and was completely blocked by preincubation of the receptors with 20 μM *N. naja siamensis* α-neurotoxin or with 100 μM Carb for 10 min. Inhibition of the Carb response by Carb preincubation is known as desensitization and is accompanied by a shift in the binding affinity of AcChR for agonist from a "low" to a "high" state (Weiland & Taylor, 1979; Andreasen et al., 1979; Quast et al., 1978; Weber & Changeux, 1974).

Affinity Labeling of Membrane Vesicles. Membrane vesicles were reduced with 1 mM dithiothreitol (DTT) at pH 7.6 to expose the sulfhydryl group necessary for covalent attachment of the affinity labels at the active site (Karlin et al., 1975). In the original affinity-labeling studies, a pH of 8 was used for reduction [see Karlin et al. (1975)], but we have found (see below) that the lower pH is adequate and eliminates the

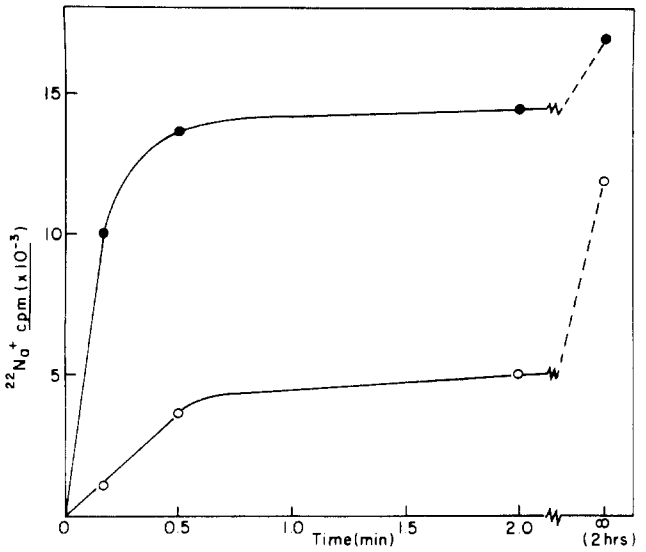

FIGURE 1: Effect of Carb on $^{22}Na^+$ influx into native AcChR membrane vesicles from *T. californica*. Vesicles were allowed to influx for varied amounts of time in the presence (●) and absence (○) of 6×10^{-4} M Carb.

Table I: Binding Properties of Modified *Torpedo* Membranes[a]

treatment	sp act. (pmol bound/mg of protein)	
	[^{125}I]-α-BgTx	[^{3}H]MBTA
none	693	369
DTT-DTNB	600	407
DTT-MBTA	356	42
DTT-BAC	344	0

[a] The equilibrium binding of [^{125}I]-α-BgTx and specific reaction with [^{3}H]MBTA were measured by procedures referenced under Materials and Methods.

need for subsequent pH adjustments. After removing the DTT by dilution and centrifugation, the membranes were alkylated either with 0.1 mM BAC or with 0.1 mM MBTA. As a control, an aliquot of the reduced membranes was reoxidized with 0.1 mM DTNB. It has been shown that DTNB reverses the effects of DTT reduction and prevents affinity labeling (Silman & Karlin, 1969). After 15 min at room temperature, excess reagent was removed by dilution and centrifugation, and the membranes were analyzed for binding and ion permeability properties.

Binding Properties of the Modified Membranes. The effectiveness of the affinity-labeling procedures was tested in three ways. The number of binding sites available to toxin was measured by allowing excess iodinated α-bungarotoxin to equilibrate with the membranes in detergent solution. As expected, the DTNB-treated membranes contained approximately the same number of binding sites as the original membranes (Table I). The MBTA- and BAC-modified membranes each contained half the number of binding sites, consistent with the occupation of half of the toxin sites by the affinity label (Table I). The native and DTNB membranes had a specific activity for tritiated MBTA labeling that was half the specific activity of toxin binding, and the BAC- and MBTA-labeled membranes specifically bound no additional MBTA (Table I). These results confirm the expectation that the affinity alkylating agents specifically block half of the available toxin binding sites. In independent binding studies, DTT alone had no effect on the number of toxin binding sites.

The *initial rate* of iodinated α-bungarotoxin binding was measured in the absence of detergent. The BAC- and

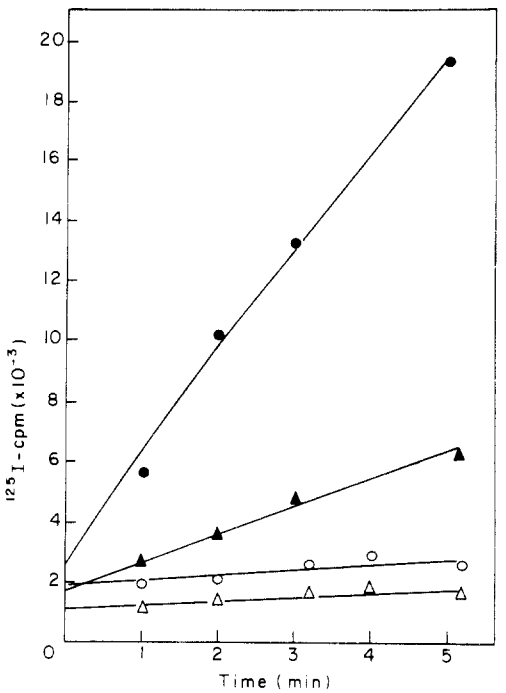

FIGURE 2: Initial rate of [^{125}I]-α-BgTx binding to *Torpedo* membranes treated with DTT–DTNB (●, ○) or BAC (▲, △). Rates were measured in the absence of Carb (solid symbols) or in the presence of 5 μM Carb after 10-min preincubation with 5 μM Carb (open symbols). Each incubation mixture contained ~3 pmol of total toxin sites and 10 pmol of [^{125}I]-α-BgTx in 700 μL of incubation buffer [see Andreasen et al. (1979) for assay details].

MBTA-labeled membranes each bound toxin at a slower rate than the native and DTNB membranes. DTT alone had no effect on the initial rate of toxin binding. The effects of Carb on the initial rates of toxin binding were also measured. Coincubation of membranes with 5 μM Carb in addition to the toxin resulted in a reduced rate of toxin binding for both the native and modified membranes (25–50% reduction). Preincubation of the membranes with the same concentration of Carb for 10 min before addition of toxin resulted in a much greater inhibition of the initial rate of toxin binding, consistent with the shift of the AcChR into a state with a higher affinity for Carb (Weiland et al., 1979; Quast et al., 1978). Typical results for DTNB- and BAC-modified membranes are shown in Figure 2.

Ion Permeability Properties of the Modified Membranes. Based on the influx curves in Figure 1, a simple influx assay procedure was chosen for measuring Carb-stimulated increases in $^{22}Na^+$ permeability. Vesicles were allowed to influx $^{22}Na^+$ for 1 min at 0 °C in the presence or absence of 10^{-3} M Carb. The difference in trapped $^{22}Na^+$ counts per minute was taken as an empirical but quantitative measure of the functional response.

In the absence of Carb, the DTNB-, MBTA-, and BAC-modified membranes behaved exactly like native membranes and showed only the slow passive diffusion. One striking implication of this result was that the BAC-modified membranes were not covalently activated at the time the influx assay was carried out due to possible desensitization of the receptor.

In the presence of 10^{-3} M Carb, the BAC- and MBTA-modified membranes gave the same maximum Carb response as the DTT–DTNB-modified membranes. In independent assays, the DTT–DTNB-modified membranes showed exactly the same influx and efflux properties as native membranes (Figure 3). The responses were dose dependent and were

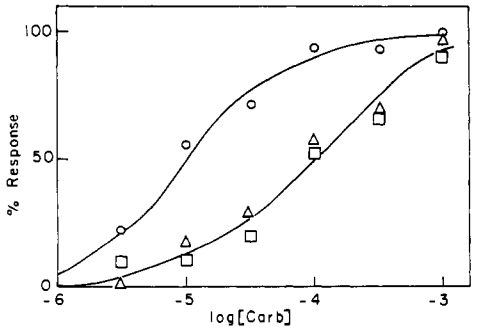

FIGURE 3: Effect of Carb concentration on $^{22}Na^+$ influx into DTNB-modified (○), MBTA-modified (□), and BAC-modified (△) AcChR membrane vesicles from *T. californica*. Vesicles were allowed to influx for 1 min after the addition of the $^{22}Na^+$–Carb solution. Each point is the mean of duplicate samples. 100% response is the difference in $^{22}Na^+$ cpm in the presence and absence of 10^{-3} M Carb for the DTT–DTNB membranes. In all cases the protein concentration was 10 mg/mL.

completely blocked by preincubation for 10 min with 20 μM *N. naja siamensis* toxin or with 100 μM Carb. The concentration of Carb that gave a 50% response was higher for the MBTA- and BAC-labeled membranes (2×10^{-4} M, Figure 3) than for the DTNB-treated membranes (2×10^{-5} M). Native membranes typically gave a 50% response at 2×10^{-5} M Carb (data not shown). The shift to higher Carb concentrations for the BAC- and MBTA-labeled membranes was similar to the shift observed simply after reduction with DTT (R. Lukas and M. McNamee, unpublished experiments). As shown in Figure 3, the DTT effect was reversed by DTNB. However, treatment of membranes with 0.1 mM DTNB *after* affinity labeling with BAC did not shift the 50% Carb response back to 2×10^{-5} M. The DTT–BAC–DTNB membranes still required 2×10^{-4} M Carb for a 50% response.

BAC Activation of Receptor. The results thus far indicate that the toxin binding site *not* involved in the affinity labeling of AcChR can be activated and desensitized (see Discussion). An underlying assumption is that the affinity-labeled site is also a functional site. The permeability response of native and DTT-reduced *Torpedo* membranes to various concentrations of BAC was measured in order to provide evidence for the functional integrity of the affinity labeled site. For native membranes, BAC behaved exactly like Carb in efflux experiments and gave a 50% response at $\sim 2 \times 10^{-5}$ M. Preincubation with 50 μM BAC for 10 min blocked the permeability response to BAC. After reduction, the BAC gave a 50% response at 2×10^{-6} M. Thus, BAC appeared to be a more efficient activator after reduction. By contrast, Carb was less efficient at activating a response following reduction, giving a 50% response at 2×10^{-4} M (R. Lukas and M. McNamee, unpublished experiments). Independent studies have shown that reduction decreases the binding affinity of AcChR for Carb (Schiebler et al., 1977; Moore & Raftery, 1979b). The increased sensitivity to low BAC concentrations probably results from the covalent attachment of the BAC, and thus the affinity-labeled site is undoubtedly an activatable site.

Discussion

The results provide direct evidence for the existence of two independent functional sites on the acetylcholine receptor from *T. californica*. The two sites bind snake α-neurotoxins and are indistinguishable by kinetic analysis of toxin binding (Blanchard et al., 1979). However, the two sites differ in their susceptibility to the affinity alkylating agents, bromoacetylcholine (BAC) and maleimidobenzyltrimethylammonium (MBTA). One and only one of the two toxin sites can be affinity alkylated by BAC or MBTA after prior reduction of the AcChR with dithiothreitol (Damle & Karlin, 1978; Damle et al., 1978). We have exploited the half-of-the-sites reactivity of the affinity labels and have measured the ion permeability control functions associated with each toxin site.

In these experiments, the stimulation of $^{22}Na^+$ flux across AcChR-rich membrane vesicles by AcChR activators was used to provide a convenient, quantitative, in vitro assay for AcChR functional activity. For experiments involving extensive chemical modifications prior to the flux assays, the influx procedure described here proved to be useful since $^{22}Na^+$ flux assays could be initiated immediately after all excess reagents were removed. For experiments designed to measure responses of native or DTT-reduced membranes to a variety of activators or inhibitors, the commonly used efflux procedure was much more convenient (Andreasen & McNamee, 1977) since the same stock solution of $^{22}Na^+$-loaded vesicles could be used. Following reduction of the *Torpedo* membranes with DTT, BAC greatly increased the efflux rate of $^{22}Na^+$ from preloaded vesicles within 10 s. The concentration of BAC in the dilution medium that gave a 50% response (2×10^{-6} M) was 100 times lower than the concentration of Carb necessary for a 50% response and 10 times lower than the BAC concentration necessary for a 50% response from unreduced receptors. We attribute this difference to the ability of BAC to react covalently with one site, thereby increasing the effective activator concentration. Thus, the site affinity labeled by BAC is a functional permeability control site. Preincubation of membranes with BAC completely blocked the increased permeability upon dilution, indicating that the covalently bound BAC could desensitize the site to which it was bound. Unlike the situation with reversibly binding ligands, the desensitization induced by covalently attached BAC was not reversible. After removal of all excess BAC, the membranes showed only the slow passive influx of $^{22}Na^+$ typical of membranes in the absence of activators, and thus the attached BAC maintained the AcChR in the high-affinity, desensitized state. MBTA is either a reversible inhibitor of AcChR (before reduction) or an irreversible inhibitor (after reduction) and thus had no direct effects on $^{22}Na^+$ influx or efflux in the absence of added activators.

Both MBTA- and BAC-labeled membranes retained the ability to respond to Carb, an AcChR activator, with a large increase in $^{22}Na^+$ influx. The activation was blocked by *N. naja siamensis* α-neurotoxin and must have been occurring at a second site since toxin could not bind to the affinity-labeled sites. That is, the added Carb was not competing with the covalent affinity label for the same site. The second site could be desensitized by prior incubation of the membranes with Carb. Thus, each site could be independently activated and desensitized.

The affinity-labeled membranes gave as large a Carb response as the native membranes, but the concentration of Carb required for a 50% response (2×10^{-4} M) was 10-fold higher for affinity-labeled membranes than for native membranes or for membranes reduced with DTT and then reoxidized with DTNB (2×10^{-5} M). The size of the response and the 50% value do not provide detailed information about the binding affinity or the *rate* of influx of $^{22}Na^+$. The time scale of these experiments (seconds to minutes) is much longer than the time for the permeability response in vivo (milliseconds). Therefore, the measured responses here are time-averaged responses from presumably heterogeneous populations of membranes. The

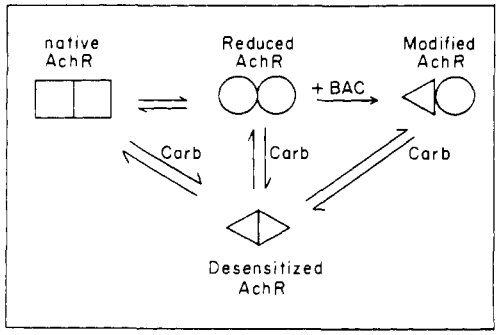

FIGURE 4: Schematic model of the different functional states of the acetylcholine receptor (see the text).

shift in apparent Carb response was the same as the shift observed after reduction with DTT alone, and the DTT effects can be explained by decreased binding affinity for Carb (R. Lukas and M. McNamee, unpublished experiments; Schiebler et al., 1977). The DTT effects, however, are completely reversed by reoxidation with DTNB. In the case of affinity-alkylated membranes, however, DTNB did *not* shift the 50% Carb response back to 2×10^{-5} M. Thus, the second site either has an inherently different Carb sensitivity than the affinity-labeled site or the effects of DTT and affinity alkylation induce an irreversible change in the second site.

The functional responses measured in *Torpedo* membranes appear to differ from the physiological responses measured in intact *Electrophorus* electroplax cells (Karlin, 1969; Silman & Karlin, 1969). For example, BAC permanently activated electroplax cells after alkylation whereas the *Torpedo* membranes were rapidly desensitized. Also, MBTA appeared to irreversibly block the Carb response in *Electrophorus* whereas here a full response could be elicited. Although the *Electrophorus* cells can be desensitized (Lester et al., 1975), the concentrations of Carb required are higher than those needed for *Torpedo* desensitization in vitro. The failure to observe desensitization in *Electrophorus* electroplax cells by covalently attached BAC is consistent with the greater resistance of *Electrophorus* to desensitization. With respect to MBTA inhibition, the test pulses of Carb used in the *Electrophorus* studies might not have been sufficient to activate the second site, especially if the *Electrophorus* AcChR shows the same shift in Carb response as the *Torpedo* AcChR. Evidence for this is provided by the observation that DTT alone reduced the Carb response of electroplax cells by over 80% under the test conditions reported (Karlin, 1969). It is also possible that the two sites are more closely coupled in intact *Electrophorus* electroplax.

The model in Figure 4 effectively summarizes the results of these experiments. We propose that there are two sites that can be independently activated or desensitized. Reduction with DTT alters the response of the membranes presumably by changing the affinity for Carb (Schiebler et al., 1977; Moore & Raftery, 1979b; R. Lukas and M. McNamee, unpublished experiments). In the absence of any evidence to the contrary, we have assumed that the two sites initially are *functionally* identical. Both the native and reduced receptors can be activated and desensitized. This latter observation (that reduced AcChR can be desensitized) is contrary to the *conclusions* reached by another group (Moore & Raftery, 1979b), but their data and our data (R. Lukas and M. McNamee, unpublished experiments) are compatible since it simply requires a higher concentration of Carb to desensitize reduced AcChR. Affinity alkylation changes one of the two sites but does not directly affect the functional properties of the second site.

Our results predict that there should be *two* small ligand binding sites per AcChR and that the ratio of toxin binding to small ligand binding should be 1:1. We cannot yet provide an explanation for the half-of-the-sites reactivity of the affinity labels. If the two α subunits on the AcChR are identical, then differences in subunit contacts could explain the different reactivities. It will be interesting to determine if there are other differences between the two sites. For example, hexamethonium is a classical antagonist of the unreduced AcChR but becomes an activator of reduced AcChR (Karlin & Winnik, 1968). Possibly the activation is occurring at only one of the two sites.

The new results reported here must be accommodated in any detailed models of AcChR function. For example, if one of the other subunits of AcChR is the ionophore, then each binding subunit could be independently coupled to its activation. Desensitization could then reflect an uncoupling phenomenon. It is also possible that each binding site has its own associated ion channel. If the assumption is made that the Carb response measured in these flux assays is proportional to the number of activated channels, then the results here favor the former model in which either of the two binding subunits can be independently coupled to the same channel. In previous studies, partial saturation of the binding sites with α-neurotoxin decreased the maximum response (Andreasen & McNamee, 1977). If the toxin sites are identical, then 50% saturation with toxin should result in a population of receptors in which both binding sites are blocked. Specifically, 25% of the AcChR would have no bound toxin, 50% would have one toxin, and 25% would have two bound toxins. Thus, there could be a 25% decrease in the number of activatable channels and a corresponding decrease in the maximum response. We are now initiating a more detailed study of flux rates and Carb responses as a function of toxin saturation. It is clear that a more detailed understanding of channel structure is required.

Acknowledgments

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Influence of Calcium on Phosphatidylglycerol. Two Separate Lamellar Structures†

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ABSTRACT: The influence of calcium on the structure of *rac*-1,2-ditetradecylglycerol-3-phosphoglycerol is investigated by differential scanning calorimetry and by X-ray diffraction. It is shown that 1 M $CaCl_2$ (pH 4.6) induces two separate lamellar phases in the same sample at 20 °C. These two phases can be clearly distinguished by their X-ray diffraction patterns. The type of phase observed depends on the pretreatment of the sample. At high temperature (90 °C), when the hydrocarbon chains are in the disordered state, the small angle reflections are in the ratio $1:1/\sqrt{3}:1/2$ and thus indicate the presence of a hexagonal phase.

It is well-known that phospholipids in aqueous systems exhibit a phase transition from an ordered structure to a more disordered state. The phase transition temperature T_t[1] does not only depend on the particular chemical structure of each phospholipid but can also be influenced by parameters of the aqueous phase. Several authors have shown that the H^+, Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations can influence T_t (Träuble & Eibl, 1974; Tocanne et al., 1974; Verkleij et al., 1974; Ververgaert et al., 1975; Jacobson & Papahadjopoulos, 1975; Galla & Sackmann, 1975; MacDonald et al., 1976). The influence of calcium on the structure of phospholipids is of particular interest, because calcium plays a central role in many biological processes.

The influence of calcium on the phase transition temperature of negatively charged phospholipids, such as phosphatidic acid, phosphatidylglycerol, phosphatidylserine, or cardiolipin, is particularly pronounced. A number of studies have reported a large increase in T_t ($\Delta T_t > 20$ °C) when using a high calcium concentration (Tocanne et al., 1974; Verkleij et al., 1974; Van Dijck et al., 1978; Portis et al., 1979). In the case of phosphatidylserine and phosphatidic acid, a phase transition could not even be detected below 70 °C after the addition of a high calcium concentration (Jacobson & Papahadjopoulos, 1975; Newton et al., 1978; Van Dijck et al., 1978). According to a recent study, the phase transition temperature of phosphatidylserine in the presence of Ca^{2+} is above 100 °C (Portis et al., 1979).

The increase in T_t induced by Ca^{2+} can be associated with a structural rearrangement of the phospholipid molecules. It was shown by X-ray diffraction that a low and a high Ca^{2+} concentration can induce two different lamellar structures in phosphatidylserine. On the addition of excess Ca^{2+}, the structure of phosphatidylserine is transformed into a crystalline-like packing of the hydrocarbon chains. This transformation was found to be exothermic (Papahadjopoulos et al., 1978; Portis et al., 1979).

Apart from inducing particular lamellar structures, Ca^{2+} is also known to induce structures with hexagonal symmetry. These so-called "hexagonal phases" have been reported in the case of cardiolipin after the addition of Ca^{2+} (Deamer et al., 1970; Rand & Sengupta, 1972; Cullis et al., 1978) and for phosphatidic acid after the addition of Mg^{2+} (Papahadjopoulos et al., 1976). At a very early stage specific models for phases with hexagonal symmetry had already been suggested (Marsden & McBain, 1948; Luzzati & Husson, 1962; Luzzati, 1968). In the present paper the term "hexagonal phase" is used solely to indicate the appearance of small angle diffraction lines in the ratio $1:1/\sqrt{3}:1/2$ and does not imply a particular molecular arrangement of the phospholipid molecules.

In the present study the influence of a high Ca^{2+} concentration on the structure of DTPG was investigated by differential scanning calorimetry and by X-ray diffraction. The ether analogue of dimyristoylphosphatidylglycerol was chosen

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[1] Abbreviations used: T_t, phase transition temperature; DTPG, *rac*-1,2-ditetradecylglycerol-3-phosphoglycerol; T_1 and T_2, main transition temperatures of phase A and B, respectively; TLC, thin-layer chromatography.

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