observed when $f = 0$, i.e., all of the bilayer is annular.

The fraction of probe in the bulk lipid region is related to the probe partition coefficient, K, and the ratio of bulk to annular lipid area, R, by

$$f = KR/(1 + KR) \quad \text{(A4)}$$

The area of an individual annular domain in units of the area of a phospholipid is designated σ, i.e., $\sigma = a_A/a_L$. The mean number of coat proteins in a region of area a_A is $x\sigma$. According to Poisson statistics, the probability of finding an area of radius a_A which contains no coat proteins is $\exp(-x\sigma)$. Letting $a_A = \pi r_A^2$, it must be the case that the lipid at the center of the protein-free area is at least a distance r_A from the nearest coat protein and is therefore in the bulk phase. From this we have

$$R = e^{-x\sigma}/(1 - e^{-x\sigma}) = (e^{x\sigma} - 1)^{-1} \quad \text{(A5)}$$

Combining eq A5, A4, and A2 yields eq 1 of the text.

References

Asbeck, F., Beyreuther, K., Kohler, H., von Wettstein, G., & Braunitzer, G. (1969) *Hoppe-Seyler's Z. Physiol. Chem. 350*, 1047.

Berde, C. B., Hudson, B. S., Simoni, R. D., & Sklar, L. A. (1979) *J. Biol. Chem. 254*, 391.

Burstein, E. A., Vendenkina, N. S., & Ivkova, M. N. (1973) *Photochem. Photobiol. 18*, 263.

Chapman, D., Connell, B. A., Eliaz, A. W., & Perry, A. (1977) *J. Mol. Biol. 113*, 517.

Chapman, D., Gomez Fernandez, J. C., & Boni, F. M. (1979) *FEBS Lett. 98*, 211.

Curatolo, W., Verma, S. D., Sakura, J. D., Small, D. M., Shipley, G. G., & Wallach, D. F. H. (1978) *Biochemistry 17*, 1802.

Fung, B., & Stryer, L. (1978) *Biochemistry 17*, 5241.

Hagen, D. S., Weiner, J. H., & Sykes, B. D. (1978) *Biochemistry 17*, 3860.

Knippers, R., & Hoffman-Berling, H. (1966) *J. Mol. Biol. 21*, 281.

Oldfield, E., Gilmore, R., Glaser, M., Gutowsky, H. S., Hshung, J. C., Kang, S. Y., King, T. E., Meadows, M., & Rice, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4657.

Racker, E., Chein, T. F., & Kandrach, A. (1975) *FEBS Lett. 57*, 14.

Sandermann, H., Jr. (1978) *Biochim. Biophys. Acta 515*, 209.

Sklar, L. A., Hudson, B. S., & Simoni, R. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1649.

Sklar, L. A., Hudson, B. S., & Simoni, R. D. (1977a) *Biochemistry 16*, 819.

Sklar, L. A., Hudson, B. S., & Simoni, R. D. (1977b) *Biochemistry 16*, 5100.

Sklar, L. A., Miljanich, G. P., & Dratz, E. A. (1979) *Biochemistry 18*, 1707.

Tecoma, E. S., Sklar, L. A., Simoni, R. D., & Hudson, B. S. (1977) *Biochemistry 16*, 829.

van Zoelen, E. J. J., van Dijck, P. W. M., DeKruijff, B., Verkleij, A. J., & van Deenen, L. L. M. (1978) *Biochim. Biophys. Acta 514*, 9.

Wickner, W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4749.

Wickner, W. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1159.

Wickner, W. (1977) *Biochemistry 16*, 254.

Wolber, P. K., & Hudson, B. (1979) *Biophys. J. 28*, 197.

Interaction of Local Anesthetics with *Torpedo californica* Membrane-Bound Acetylcholine Receptor†

Steven G. Blanchard, Janet Elliott, and Michael A. Raftery*

ABSTRACT: The effects of local anesthetics on the rate of the agonist-induced increase in ligand affinity of membrane-bound acetylcholine receptor from *Torpedo californica* were examined. The rate of the transition in receptor affinity was determined by following the time-dependent increase in inhibition of iodinated α-bungarotoxin binding caused by 1 μM carbamylcholine. At concentrations below those that directly inhibited the binding of iodinated α-bungarotoxin, dibucaine increased the rate of the transition to a high-affinity state and tetracaine decreased this rate. The measured rate constants were 0.026 ± 0.008 s^{-1} in the presence and 0.010 ± 0.002 s^{-1} in the absence of dibucaine while tetracaine decreased the rate to 0.006 ± 0.002 s^{-1} as compared to a control value of 0.012 ± 0.003 s^{-1}. A parallel was observed between the effectiveness of a compound in increasing or decreasing the rate of the agonist-induced transition in affinity and the change in its apparent inhibition constant in the presence of carbamylcholine (increase or decrease) measured by the displacement of tritiated perhydrohistrionicotoxin. This parallel could be explained by assuming (a) that local anesthetics bound directly to the specific histrionicotoxin binding site or (b) that they bound to a different site and the observed effects were caused by conformational changes.

The release of acetylcholine at the neuromuscular junction results in a transient depolarization of the postsynaptic membrane. This response is mediated by nicotinic acetylcholine receptors (AcChR)[1] in this membrane and is considered to involve the binding of AcCh followed by a transient increase in membrane conductance. The ligand binding properties of the AcChR have been studied in vitro by using membrane fragment preparations enriched in this molecule obtained from several species of marine electric ray (Cohen et al., 1972;

† From the Church Laboratory of Chemical Biology, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125. *Received February 15, 1979.* Contribution No. 5970. Supported by U.S. Public Health Service Grant NS10294, by a grant from the Muscular Dystrophy Association of America, and by Grant RR07003 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health.

[1] Abbreviations used: AcChR, acetylcholine receptor; [^{125}I]-α-BuTx, α-bungarotoxin iodinated with ^{125}I; carb, carbamylcholine; H_{12}-HTX, perhydrohistrionicotoxin; PAA, procaine amide azide.

0006-2960/79/0418-5880$01.00/0

Duguid & Raftery, 1973; Reed et al., 1975). However, the ion translocation function of the AcChR has not been as extensively characterized in vitro. In vivo, local anesthetics block the normal response of the AcChR to the binding of agonists, and electrophysiological studies of the actions of local anesthetics on the AcChR (Ruff, 1976; Neher & Sakmann, 1976; Steinbach, 1977) have led to proposals suggesting that these compounds act by binding to the open-channel form of the AcChR, thus decreasing the ionic conductance. In addition, local anesthetics increase the rate of pharmacological desensitization (Magazanik, 1976; Magazanik & Vyskocil, 1973), thus facilitating the conversion of the receptor to a nonconducting state. Upon prolonged exposure to agonists, membrane-bound AcChR in vitro is converted to a state of high affinity for agonists (Weber et al., 1975; Weiland et al., 1976; Lee et al., 1977; Quast et al., 1978) and it has been suggested that this process is an in vitro correlate of desensitization in vivo. It may therefore be concluded that studies of the action of local anesthetics on AcChR function in vitro may provide information on the process of ion translocation.

In the experiments reported here, we detail the effects of local anesthetics on AcChR-enriched membrane fragments from *Torpedo californica* with respect to (a) inhibition of the rate of [^{125}I]-α-BuTx–AcChR complex formation, (b) changes in the rate of the agonist-induced increase in receptor affinity, measured by the inhibition of [^{125}I]-α-BuTx binding in the presence of carb, and (c) their ability to displace [^{3}H]H_{12}-HTX specifically bound to the AcChR in the membrane-associated state.

Materials and Methods

AcChR-enriched membrane fragments were prepared from *T. californica* electric organ as previously described (Duguid & Raftery, 1973; Reed et al., 1975; Elliott et al., 1979b). The concentration of [^{125}I]-α-BuTx binding sites was determined by the DEAE-cellulose disk method of Schmidt & Raftery (1973) using [^{125}I]-α-BuTx prepared as described by Blanchard et al. (1979). Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

The time course of [^{125}I]-α-BuTx–AcChR complex formation was followed by a modification of the DEAE disk method (Schmidt & Raftery, 1973). For each time point a 0.1-mL aliquot of the reaction mixture was pipetted onto a Whatman DE-81 filter paper disk, thus quenching the reaction (Blanchard et al., 1979). The disks were then washed in three changes of buffer containing 100 mM NaCl, 0.1% (v/v) Triton X-100, and 10 mM NaP_i, pH 7.4. Radioactivity was determined by using a Packard liquid scintillation spectrometer (Model 3375) or a Beckman 4000 γ counter. The rate of [^{125}I]-α-BuTx binding to the receptor was determined by using an 8–10-fold molar excess of [^{125}I]-α-BuTx over its binding sites. Under these conditions the reaction followed pseudo-first-order kinetics and the rate of [^{125}I]-α-BuTx–AcChR complex formation was taken from the slope of a plot of ln $(C_\infty - C_t)$ vs. time, where C_t was the counts per minute bound to the disk at time t and C_∞ was the counts per minute bound at completion of the reaction (typically measured 3–5 h after the start of the reaction). For any given set of [^{125}I]-α-BuTx and AcChR concentrations, the value of C_∞ was found to be the same in the presence or absence of local anesthetic (not shown). This method has been shown to give an accurate measurement of the rate of [^{125}I]-α-BuTx–AcChR complex formation (Blanchard et al., 1979).

Membrane fragment preparations were routinely tested for their ability to undergo the agonist-induced transition from a state of low to high affinity for cholinergic ligands by comparing the rate of [^{125}I]-α-BuTx binding in the presence of 1 μM carb with and without 15 min of preincubation (Lee et al., 1977). Preparations in which a significant amount of the receptor was initially in a state of high affinity were discarded. For measurement of the rate of the receptor transition from the low- to high-affinity state for agonists, membranes were incubated with the desired anesthetic for 15–20 min at room temperature followed by addition of carb (1 μM final concentration). After a given time, [^{125}I]-α-BuTx was added and the rate of toxin–receptor complex formation, k_{app}, was determined as described above. The variation of k_{app} vs. time of incubation was fit to an exponential decay of the form

$$k_{app}(t) = (k_0 - k_\infty)e^{-k't} + k_\infty \quad (1)$$

where k_0 was the rate of toxin binding in the presence of 1 μM carb without preincubation, k_∞ was the rate of toxin binding in the presence of 1 μM carb when all of the receptor was in the high-affinity state (for the purposes of these experiments, this condition was fulfilled after 15 min of preincubation), and k' was the rate of the transition in receptor affinity for the agonist. Estimates for k' were obtained from a linear least-squares fit of the data to the logarithmic form of eq 1.

The preparation of [^{3}H]H_{12}-HTX and the measurement of its binding to membranes by centrifugation assay have been previously described (Elliott & Raftery, 1977, 1979). Briefly, solutions 1.2 μM in [^{125}I]-α-BuTx binding sites (0.3 μM H_{12}-HTX sites), 0.4 μM [^{3}H]H_{12}-HTX, and the desired concentration of local anesthetic were incubated for 20 min at room temperature (40 μM unlabeled H_{12}-HTX was also included in duplicate samples for the determination of nonspecifically bound [^{3}H]H_{12}-HTX). Triplicate 10-μL samples were withdrawn for the estimation of radioactivity both before and after centrifugation in a Beckman airfuge. The amount of [^{3}H]H_{12}-HTX specifically bound was taken as the difference between that amount bound by the membranes in the absence and the presence of saturating concentrations of unlabeled H_{12}-HTX. Apparent inhibition constants, K_I, for the compounds tested were calculated by assuming competitive inhibition and utilizing values for the K_d of [^{3}H]H_{12}-HTX of 0.55 μM in the absence of carb and of 0.35 μM in the presence of carb.

Lidocaine was purchased from Pfaltz and Bauer, Inc., carb and procaine were from Sigma Chemical Co., and dibucaine and tetracaine were from ICN Pharmaceuticals. Unless otherwise indicated, the buffer used in all experiments was *Torpedo* Ringers (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.02% NaN_3 buffered by either 5 mM Tris or 20 mM Hepes, pH 7.4).

Results

In general, the local anesthetics tested inhibited [^{125}I]-α-BuTx binding only weakly, with inhibition constants in the millimolar range (Figure 1). Only dibucaine showed a significant inhibition of toxin binding at lower concentrations (K_I = 56 ± 6 μM; Figure 1). At a concentration of 5 μM, dibucaine did not significantly alter the rate of toxin binding, as predicted from its K_I. If, however, 1 μM carb was added simultaneously with the [^{125}I]-α-BuTx to membranes containing this concentration of dibucaine, the observed rate of toxin binding was slower than that observed in the presence of only carb or only dibucaine (Figure 2A). Assuming competitive inhibition of toxin binding by both of these ligands, a decreased rate of [^{125}I]-α-BuTx binding would reflect an increased affinity of the AcChR for carb or dibucaine or for

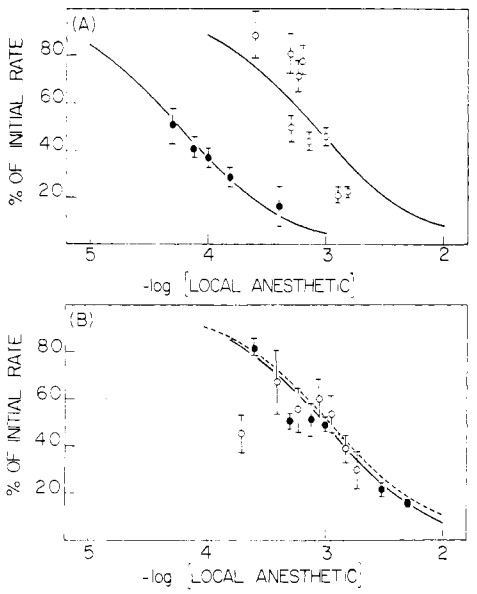

FIGURE 1: Inhibition of toxin binding to membrane-bound AcChR as a function of local anesthetic concentration. Membrane fragments 63 nM in toxin sites were incubated with local anesthetic for 20–30 min. The reaction was initiated by adding [^{125}I]-α-BuTx (final concentration 520 nM), and the kinetics of toxin binding were followed to at least 50% completion. The apparent rate constants for toxin binding in the presence of ligand, $k_{[L]}$, were determined from linear least-squares estimations of the slopes of logarithmic plots of the data as described under Materials and Methods. (A) (●) Dibucaine and (○) tetracaine; (B) (●—●) procaine and (○---○) lidocaine. The curves were drawn by using apparent inhibition constants (K_I values listed in Table I) calculated from a weighted linear least-squares fit to the equation $k_{[L]}^{-1} = k_{[L]=0}^{-1}(1 + [L]/K_I)$. These calculations assumed simple competitive inhibition of [^{125}I]-α-BuTx binding by local anesthetics.

both compounds. The experiments described below allowed discrimination between these possibilities.

Membrane fragments were incubated with carb to induce conversion of the receptor to the high-affinity state. When such membranes were diluted 40-fold into buffer containing dibucaine and toxin, the initial rate of [^{125}I]-α-BuTx–receptor complex formation was the same as that observed when the membranes were diluted into buffer containing only [^{125}I]-α-BuTx (Figure 2B). If dibucaine association with the AcChR was in rapid equilibrium (with respect to toxin binding), this demonstrated that the affinity of the receptor for dibucaine was independent of the affinity state (high or low) for agonists. It could be deduced then that dibucaine increased the affinity of the AcChR for carb.

If the high-affinity state for agonists in the presence of carb and dibucaine is the same as that state induced by carb alone, there are two possible explanations for this result. The first is that dibucaine induced the change in affinity, but its affinities for the two states did not differ appreciably. This type of behavior has been observed for both hexamethonium and *d*-tubocurarine (Quast et al., 1978). In order to test this possibility, membrane fragments were incubated with dibucaine for 2 h and the rate of toxin binding was measured after 40-fold dilution into buffer containing 1 μM carb and [^{125}I]-α-BuTx. As shown in Figure 2C, the rate did not differ from that of a control which had not been exposed to dibucaine. Thus, the local anesthetic alone did not cause conversion of the AcChR from a state of low to one of high affinity. The second possibility was therefore considered, i.e., that dibucaine modified the *rate* of the carb-induced time-dependent affinity change. The data shown in Figure 2D demonstrate that dibucaine did indeed greatly increase the rate of this transition.

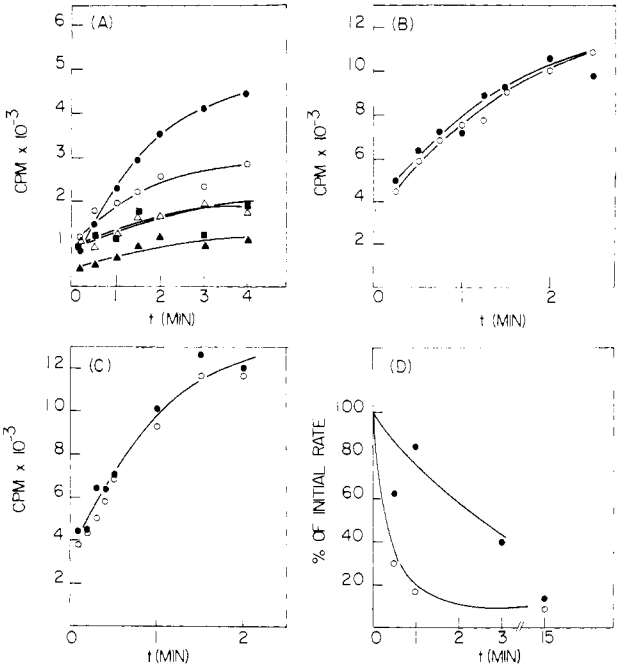

FIGURE 2: (A) Inhibition of [^{125}I]-α-BuTx binding to AcChR-enriched membranes by carb in the presence of dibucaine. The formation of [^{125}I]-α-BuTx–AcChR complex was followed as described under Materials and Methods. The concentrations were 30 nM in toxin sites, 240 nM in [^{125}I]-α-BuTx, 1 μM in carb, and 5 μM in dibucaine. (●) [^{125}I]-α-BuTx binding to membrane fragments; (○) carb and [^{125}I]-α-BuTx were added simultaneously to membrane fragments; (▲) membrane fragments were incubated with carb for 15 min before the addition of [^{125}I]-α-BuTx; (△) dibucaine, carb, and [^{125}I]-α-BuTx were added to membrane fragments simultaneously; (■) membrane fragments were incubated 15 min with dibucaine, followed by addition of carb and [^{125}I]-α-BuTx. (B) [^{125}I]-α-BuTx binding to membranes in the high-affinity state for agonists in the presence of dibucaine. Membrane fragments (2.6 μM in toxin sites) were incubated with 1 μM carb for 45 min at room temperature to convert the receptor to the high-affinity state. Aliquots were diluted 40-fold into *Torpedo* Ringers containing [^{125}I]-α-BuTx (470 nM) without (●) or with (○) 5 μM dibucaine. (C) Binding of [^{125}I]-α-BuTx to AcChR-enriched membranes following prolonged exposure to dibucaine. (○) Membrane fragments 1.6 μM in [^{125}I]-α-BuTx sites were incubated for 2 h at 4 °C with 0.83 μM dibucaine and then diluted (20-fold) into *Torpedo* Ringers containing 1 μM carb and 0.47 μM [^{125}I]-α-BuTx, and formation of toxin–receptor complex was monitored. (●) Membranes were incubated for 2 h in *Torpedo* Ringers only and then assayed as described above. (D) Increase in the rate of the carb-induced affinity change in the presence of dibucaine. AcChR-enriched membrane fragments (87 nM in [^{125}I]-α-BuTx sites) were incubated for 5 min without (●) or with (○) 1 μM dibucaine. Carb was added (final concentration, 1 μM), the membranes were incubated for the indicated times before [^{125}I]-α-BuTx (final concentration 0.51 μM) was added, and the rate of [^{125}I]-α-BuTx–AcChR complex formation was followed under pseudounimolecular conditions as described under Materials and Methods. Plots show the apparent rate of toxin binding, k_{app}, vs. time of incubation with carb. The solid lines were calculated by using apparent rate constants for the affinity transition of $k' = 0.010 \pm 0.002$ s^{-1} in the absence of dibucaine, in agreement with previously reported values (Lee et al., 1977; Quast et al., 1978), and $k' = 0.026 \pm 0.008$ s^{-1} in the presence of dibucaine. These values were obtained from weighted linear least-squares fits to the logarithmic form of eq 1.

The effects of several other compounds on the rate of the carb-induced increase in affinity were tested. In all cases the concentration of anesthetic used was well below the K_I determined by [^{125}I]-α-BuTx inhibition so that a direct inhibition of toxin binding by the compound being tested could be ruled out. The results of these studies are summarized in Table I. The local anesthetic tetracaine presented an especially interesting case. The data in Figure 3 can be interpreted as a demonstration that, at 50 μM, tetracaine *decreased* the rate

Table I: Effects of Local Anesthetics on Properties of AcChR Membrane Fragments

local anesthetic	inhibn of [^{125}I]-α-BuTx binding, K_I (μM)	displacement of [^{3}H]H_{12}-HTX specifically bound, K_I (μM)		effect on rate of carb-induced affinity change	
		−carb	+carb	effect[a]	concn tested (μM)
dibucaine	56 ± 6	80	7.5	++	1-5
tetracaine	800 ± 100	1.5	30	−	50
lidocaine	1100 ± 200	2500	600	+	150
procaine	930 ± 60	3000	3000	0	150

[a] + = increase rate; − = decrease rate; 0 = no effect.

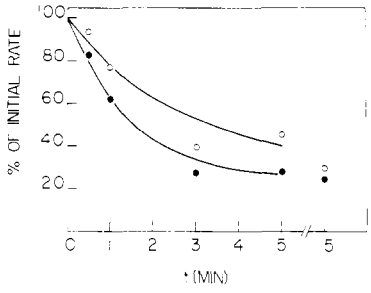

FIGURE 3: Decreased rate of the carb-induced affinity change in the presence of tetracaine. The experimental details were the same as those in Figure 2D, except that 50 μM tetracaine was used in place of dibucaine. The calculated rates for the carb-induced increase in affinity were $k' = 0.012 \pm 0.003$ s^{-1} (●) in the absence of tetracaine and $k' = 0.006 \pm 0.002$ s^{-1} (○) in the presence of 50 μM tetracaine.

of the low- to high-affinity transition caused by carb. This result was in contrast to those obtained with the other compounds which either increased or did not affect the rate of this process under similar conditions.

The local anesthetics were also found to displace [^{3}H]H_{12}-HTX specifically bound to the AcChR-enriched membrane fragments. However, only tetracaine and dibucaine were effective in this regard in the micromolar concentration range (Figure 4). In the presence of 10 μM carb, the effective concentrations at which the local anesthetics displaced [^{3}H]H_{12}-HTX were shifted from those observed in the absence of carb (Figure 4; Table I). Because this concentration of carb was sufficient to completely convert the AcChR to the high-affinity state for agonists (Quast et al., 1978), the different apparent K_I values in the presence and absence of carb presumably reflect the effectiveness with which a particular compound displaces [^{3}H]H_{12}-HTX bound to the high- and low-affinity states of the AcChR for agonist. Figure 5 shows a double-reciprocal plot of bound vs. free HTX at various tetracaine concentrations. The plot indicates that, for tetracaine, the assumption of competitive inhibition is valid.

Discussion

The local anesthetics inhibited the binding of [^{125}I]-α-BuTx only weakly, in agreement with results reported earlier by other investigators (Weber & Changeux, 1974; Weiland et al., 1977). From the results presented in Table I, it can be seen that (with the exception of tetracaine) the K_I values for direct inhibition of [^{125}I]-α-BuTx binding and for the displacement of specifically bound [^{3}H]H_{12}-HTX in the absence of carb are similar for a given anesthetic. However, it should be noted that we have previously shown (Elliott & Raftery, 1977) that HTX and α-BuTx bind to different sites. In addition, with membranes in the high-affinity state for agonist, dibucaine

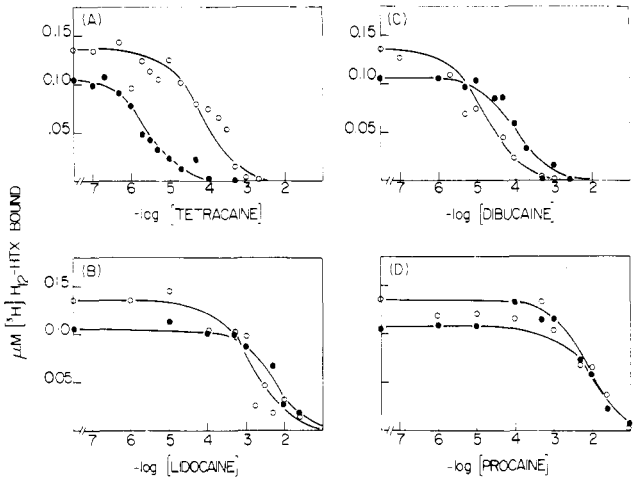

FIGURE 4: Displacement by local anesthetics of [^{3}H]H_{12}-HTX specifically bound to AcChR-containing membranes. The displacement of [^{3}H]H_{12}-HTX by increasing concentrations of local anesthetics was measured as described in the text. Displacement was measured in the absence of carb (●) and in the presence of 10 μM carb (○), which ensured conversion of the receptor to its high-affinity state for agonists. The increased amount of [^{3}H]H_{12}-HTX bound to the membranes in the presence of carb was a reflection of the somewhat smaller K_d for this compound to high-affinity membranes (0.35 μM) as compared to low-affinity membranes (0.55 μM). The curves were calculated by using the K_I values listed in Table I. (A) Tetracaine; (B) lidocaine; (C) dibucaine; (D) procaine.

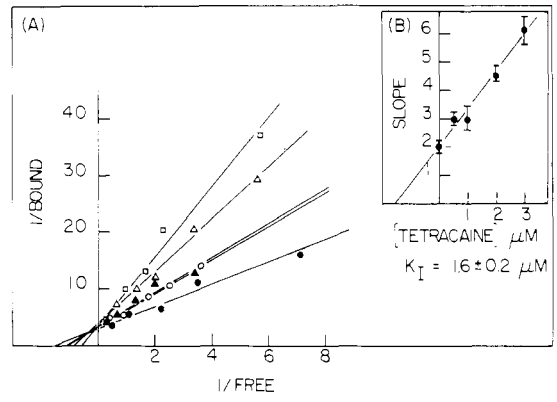

FIGURE 5: (A) Double-reciprocal plots of [^{3}H]H_{12}-HTX binding to membranes in the presence of various tetracaine concentrations. The concentration of α-BuTx sites was 1.2 μM with (●) no tetracaine present, (○) [tetracaine] = 0.5 μM, (▲) [tetracaine] = 1.0 μM, (△) [tetracaine] = 2.0 μM, and (□) [tetracaine] = 3.0 μM. (B) Replot of the slopes obtained from the double-reciprocal plots vs. tetracaine concentration to yield K_I, which equals −(x intercept).

displaced [^{3}H]H_{12}-HTX at a concentration 10-fold lower than it inhibited [^{125}I]-α-BuTx binding (compare Figure 4B and Figure 2C). If dibucaine bound to a single site both inhibited the binding of [^{125}I]-α-BuTx and caused the displacement of [^{3}H]H_{12}-HTX, then the apparent K_I values for these two effects on high-affinity membranes should be equal. It was therefore concluded that the sites of local anesthetic interaction responsible for inhibition of [^{125}I]-α-BuTx binding and the displacement of specifically bound [^{3}H]H_{12}-HTX were distinct.

Both local anesthetics (Weiland et al., 1977; Briley & Changeux, 1978) and general anesthetics (Young et al., 1978) have been found to affect the agonist-induced increase in ligand affinity of *Torpedo* membranes at concentrations below those that directly inhibit the binding of α-toxins to the AcChR. We have observed that the magnitude of this effect depends on the compound being tested; whereas the local anesthetics dibucaine and lidocaine caused an increase in the rate of the carb-induced change (Figure 2D and Table I), both procaine

(Table I) and procaine amide azide (Blanchard & Raftery, 1979) showed little effect on the rate of this transition. The most interesting case, however, was that of tetracaine (Figure 3). The rate of the carb-induced affinity change was slower in the presence of this compound than in its absence. In contrast, Weiland et al. (1977) reported that local anesthetics facilitated the conversion of the receptor to a high-affinity state. However, the data reported by these authors were obtained in the absence of calcium while the results reported here were performed in *Torpedo* Ringers buffer (containing 4 mM calcium). Since calcium has been shown to increase the rate of the affinity change for cholinergic ligands in vitro (Lee et al., 1977), the results of these two studies cannot be directly compared.

As can be seen in Table I, the effects of local anesthetics on the rate of the carb-induced increase in receptor affinity for agonists roughly paralleled the differences in their apparent affinities for the low- and high-affinity states of the receptor as reflected by $[^3H]H_{12}$-HTX displacement (in the absence and presence of 10 μM carb, respectively). A possible explanation for this parallel is that the rate of the agonist-induced transition in receptor affinity was modulated by compounds bound to the $[^3H]H_{12}$-HTX binding site with the magnitude of the effect determined by the ratio of the K_I values to the two states: with compounds such as dibucaine, that bind more tightly to the high-affinity state, causing an increased rate of transition and tetracaine, which binds more tightly to the low-affinity state, decreasing the rate of the transition. This explanation is also consistent with the observation that compounds such as HTX (Elliott & Raftery, 1977) and PAA (Blanchard & Raftery, 1979), that did not appreciably change the rate of the receptor transition, also showed little or no difference in their apparent affinities for the low- and high-affinity states.

Another possible explanation of the effects of local anesthetics on the carb-induced increase in AcChR affinity is that these compounds perturb the resting conformation of the receptor in a nonspecific manner such as disturbing the lipid annulus surrounding the receptor. Such a mechanism has been previously suggested as a possible explanation of the effects of both local (Weiland et al., 1977) and general (Young et al., 1978) anesthetics on membrane-bound AcChR from *Torpedo*, as well as of the effects of anesthetics on proteins in general (Richards et al., 1978). The fact that local anesthetics displace specifically bound $[^3H]H_{12}$-HTX does not rule out this explanation; following dissolution of AcChR-enriched membranes in the nonionic detergent Triton X-100, both the ability to undergo the agonist-mediated transition in affinity (V. Witzemann and M. Raftery, unpublished experiments) and specific $[^3H]H_{12}$-HTX binding (Elliott & Raftery, 1977) were lost. In addition, 2% (w/v) sodium cholate extracts of AcChR-enriched membranes from *T. californica* showed no specific binding of $[^3H]H_{12}$-HTX (Elliott & Raftery, 1977), but following dilution of the detergent to 0.5% (w/v), binding similar to that seen for intact membranes was recovered (Elliott & Raftery, 1979). Considering the sensitivity of the specific binding of $[^3H]H_{12}$-HTX to perturbations of the membrane environment of the AcChR, binding of local anesthetics to sites other than the specific $[^3H]H_{12}$-HTX binding site might result in displacement of specifically bound HTX by means of changes in receptor conformation. As an example, Triton X-100 displaced bound $[^3H]H_{12}$-HTX at concentrations in the range of 0.0005% (w/v), which are well below ($\sim$20-fold) those at which the membranes begin to dissolve (Raftery et al., 1973), and the displacement curve was shifted to lower concentrations in the presence of carb (not shown). Therefore, the parallel observed between the effects of local anesthetics on $[^3H]H_{12}$-HTX displacement and on the rate of the low- to high-affinity transition (for agonists) might be expected even if these effects were the result of nonspecific perturbation of the membranes by local anesthetics.

With respect to the identification of possible binding sites of local anesthetics, we have recently shown that the local anesthetic analogue PAA specifically labeled a polypeptide in *T. californica* membrane fragments of molecular weight 43 000 upon photolysis (Blanchard & Raftery, 1979). This labeling could not be prevented by H_{12}-HTX, indicating a separation of sites for these two compounds. In addition, we have recently shown (Elliott & Raftery, 1979) that $[^3H]H_{12}$-HTX binds not to the 43 000-dalton polypeptide but rather that the specific association of this toxin appears to be with the AcChR when these components are dissolved in buffers containing sodium cholate. Membrane fragments depleted in the 43 000-dalton polypeptide bound both $[^3H]H_{12}$-HTX (Elliott et al., 1979a) and a local anesthetic analogue competitive with H_{12}-HTX (Neubig et al., 1978) with the K_d and number of binding sites for both compounds being no different from those for membranes containing this component. Thus, it has been demonstrated that some compounds that are classified as local anesthetics associate with proteins in *T. californica* postsynaptic membranes which are not involved in the binding of H_{12}-HTX. As discussed above, other sites of interaction are also possible for this diverse class of compounds.

Several authors have suggested (Weber et al., 1975; Weiland et al., 1976; Lee et al., 1977; Quast et al., 1978) that the transition of the AcChR to a state having high affinity for agonists may be an in vitro correlate to the electrophysiological phenomenon of desensitization (Rang & Ritter, 1970), and local anesthetics are known to enhance the rate of desensitization in vivo (Magazanik & Vyskocil, 1973; Magazanik, 1976). Thus, the effects of local anesthetics on the rate of the in vitro affinity change reported here provide additional evidence for correlation of this process with desensitization in vivo. Inhibition of α-BuTx and H_{12}-HTX binding gives only indirect evidence as to possible local anesthetic binding sites and their relationships to the observed effects of these compounds on channel blockade and desensitization. The use of more direct techniques such as local anesthetic photoaffinity labels (Blanchard & Raftery, 1979) may prove useful in this regard.

Acknowledgments

Synthetic H_{12}-HTX and H_8-HTX were gifts of Dr. Y. Kishi. We are also grateful to Dr. S. M. J. Dunn and Dr. Paul Hartig for helpful comments, John Racs for technical assistance, Valerie Purvis for drafting the figures, and Carolyn Sprague for typing the manuscript.

References

Blanchard, S. G., & Raftery, M. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 81–85.

Blanchard, S. G., Quast, U., Reed, K., Lee, T., Schimerlik, M. I., Vandlen, R., Claudio, T., Strader, C. D., Moore, H.-P. H., & Raftery, M. A. (1979) *Biochemistry 18*, 1875–1883.

Briley, M. S., & Changeux, J.-P. (1978) *Eur. J. Biochem. 84*, 429–439.

Cohen, J. B., Weber, M., Huchet, M., & Changeux, J.-P. (1972) *FEBS Lett. 26*, 43–47.

Duguid, J. R., & Raftery, M. A. (1973) *Biochemistry 12*, 3593–3596.

Elliott, J., & Raftery, M. A. (1977) *Biochem. Biophys. Res. Commun. 77*, 1347–1353.
Elliott, J., & Raftery, M. A. (1979) *Biochemistry 18*, 1868–1874.
Elliott, J., Blanchard, S. G., Wu, W., Miller, J., Strader, C. D., Hartig, P., Racs, J., & Raftery, M. A. (1979a) *Biochem. J.* (in press).
Elliott, J., Dunn, S. M. J., Blanchard, S. G., & Raftery, M. A. (1979b) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2576–2579.
Lee, T., Witzemann, V., Schimerlik, M., & Raftery, M. A. (1977) *Arch. Biochem. Biophys. 183*, 57–63.
Magazanik, L. G. (1976) *Annu. Rev. Pharmacol. 16*, 161–175.
Magazanik, L. G., & Vyskocil, F. (1973) in *Drug Receptors* (Rang, H. P., Ed.) pp 105–119, Macmillan, London.
Neher, E., & Sakmann, B. (1976) *Nature (London) 260*, 779–802.
Neubig, R. R., Krodel, E. K., & Cohen, J. B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 690–694.
Quast, U., Schimerlik, M., Lee, T., Witzemann, V., Blanchard, S., & Raftery, M. A. (1978) *Biochemistry 17*, 2405–2414.
Raftery, M. A., Schmidt, J., Martinez-Carrion, M., Moody, T., Vandlen, R., & Duguid, J. (1973) *J. Supramol. Struct. 1*, 360–367.
Rang, H. P., & Ritter, J. M. (1970) *Mol. Pharmacol. 6*, 357–382.
Reed, K., Vandlen, R., Bode, J., Duguid, J., & Raftery, M. A. (1975) *Arch. Biochem. Biophys. 167*, 138–144.
Richards, C. D., Martin, K., Gregory, S., Keightley, C. A., Hesteth, T. R., Smith, G. A., Warren, G. B., & Metcalfe, J. C. (1978) *Nature (London) 276*, 775–779.
Ruff, R. L. (1976) *Biophys. J. 16*, 433–439.
Schmidt, J., & Raftery, M. A. (1973) *Anal. Biochem. 52*, 349–355.
Steinbach, J. H. (1977) *Biophys. J. 18*, 357–358.
Weber, M., & Changeux, J.-P. (1974) *Mol. Pharmacol. 10*, 15–34.
Weber, M., David-Pfeuty, T., & Changeux, J.-P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3443–3447.
Weiland, G., Georgia, B., Wee, V. T., Chignell, C. F., & Taylor, P. (1976) *Mol. Pharmacol. 12*, 1091–1105.
Weiland, G., Georgia, B., Lappi, S., Chignell, C. F., & Taylor, P. (1977) *J. Biol. Chem. 252*, 7648–7656.
Young, A. P., Brown, F. F., Halsey, M. J., & Sigman, D. S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4563–4567.

Protein–Lipid Interactions. High-Field Deuterium and Phosphorus Nuclear Magnetic Resonance Spectroscopic Investigation of the Cytochrome Oxidase–Phospholipid Interaction and the Effects of Cholate†

David M. Rice,‡ J. C. Hsung, Tsoo E. King, and Eric Oldfield*

ABSTRACT: Deuterium quadrupole-echo Fourier transform nuclear magnetic resonance spectra (at 34 MHz) and phosphorus-31 Fourier transform nuclear magnetic resonance spectra (at 60.7 MHz) have been obtained of 1-(6,6-dideuteriopalmitoyl)-2-oleyl-*sn*-glycero-3-phosphocholine dispersions in excess water in the absence of, and complexed with, the membrane enzyme cytochrome oxidase (cytochrome *c*:O_2 oxidoreductase, EC 1.9.3.1). Thereby, we have investigated the effects of the detergent sodium cholate, and of temperature, on protein–lipid interactions in this system. Our results strongly suggest that residual detergent in these protein–lipid complexes causes a significant disordering of hydrocarbon chain and head group organization as determined by deuterium quadrupole splittings ($\Delta\nu_Q$) and phosphorus chemical shielding anisotropies ($\Delta\sigma$). At low (~2 wt %) cholate levels, C_6 $\Delta\nu_Q$ and ^{31}P $\Delta\sigma$ values in the protein–lipid complexes (containing ~70 wt % protein) are only about 8% smaller than in pure phospholipid bilayers at the same temperature, between 20 and 35 °C, suggesting rather similar structural organization. There are, however, significant line-width increases, especially on approaching T_c in both deuterium and phosphorus spectra, indicating increased correlation times, in the region of the membrane surface. These results obtained on protein–lipid complexes containing an unsaturated phospholipid are in agreement with those of a previous study utilizing a disaturated phospholipid [Kang, S. Y., Gutowsky, H. S., Hsung, J. C., Jacobs, R., King, T. E., Rice, D., & Oldfield, E. (1979) *Biochemistry 18*, 3257–3267] but differ significantly from those of a similar study employing 1-(5,5-dideuteriopalmitoyl)-2-oleyl-*sn*-glycero-3-phosphocholine [Seelig, A., & Seelig, J. (1978) *Hoppe-Seyler's Z. Physiol. Chem. 359*, 1747–1756].

The nature of the interactions between proteins and lipids in biological membranes is a topic of considerable current interest due to the frequent observation that a variety of membrane enzymes appear to require lipids in order to express their full biological activity [e.g., Yu et al. (1975), Gennis & Jonas (1977), Tanford & Reynolds (1976), Bennett et al. (1978), and Vik & Capaldi (1977)]. The most frequent model for the nature of the protein–lipid interaction is that the lipids are somehow immobilized (Jost et al., 1973a,b, 1977; Marsh et al., 1978; Warren et al., 1974, 1975; Longmuir et al., 1977) by the rigid protein "wall" (Moore et al., 1978), a cholesterol-like effect (Hong & Hubbell, 1972; Kleemann & McConnell, 1976), and that as a result the lipid hydrocarbon chain organization becomes far more restricted, leading to increased molecular order parameters (Dahlquist et al., 1977; Marčelja, 1976; Jost & Griffith, 1978; Scott & Cherng, 1978).

† From the Department of Chemistry, University of Illinois at Urbana–Champaign, Urbana, Illinois 61801 (D.M.R. and E.O.), and the Department of Chemistry and the Laboratory for Bioenergetics, State University of New York at Albany, Albany, New York 12222 (J.C.H. and T.E.K.). *Received April 11, 1979; revised manuscript received August 15, 1979.* This work was supported by the National Institutes of Health (Grant HL-19481 to E.O. and Grants GM-16767 and HL-12576 to T.E.K.) and in part by the Alfred P. Sloan Foundation (E.O.).

* Correspondence should be addressed to this author. He is an Alfred P. Sloan Research Fellow, 1978–1980.

‡ Supported in part by a National Institutes of Health, Cell and Molecular Biology Training grant (Grant GM-07283).

0006-2960/79/0418-5885$01.00/0