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# Ligand-Induced Effects at Regions of Acetylcholine Receptor Accessible to Membrane Lipids†

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ABSTRACT: The effectiveness of fluorescence quenching of pyrene-1-sulfonyl azide, a hydrophobic probe used to photolabel acetylcholine receptor (AcChR)-rich electroplax membranes [Sator, V., Gonzalez-Ros, J. M., Calvo-Fernandez, P., & Martinez-Carrion, M. (1979) *Biochemistry 18*, 1200], is used to study the accessibility of the covalently attached fluorophore to extramembranous quenchers as a function of occupancy of cholinergic receptor binding sites. In these membranes, binding of water-soluble cholinergic ligands to specific sites at the extracellular side affects the fluorophore located in a distant topographical area of the AcChR molecule. When a neurotransmitter analogue (carbamylcholine) is present, the susceptibility of the covalently attached fluorophore to quenching with externally added nitromethane decreases in comparison with that of the same membranes in the absence of carbamylcholine. This neurotransmitter agonist effect is, however, reversible as removal of carbamylcholine by dialysis restores the quenching effectiveness to that of resting nonliganded membranes. The presence of bound $\alpha$-bungarotoxin produces an opposite effect to that of carbamylcholine and induces an increase in susceptibility to quenching agent. These results are interpreted in terms of long-range effects induced by occupancy of cholinergic sites which are detected by covalently bound fluorophore located at regions of the AcChR protein accessible through the lipid matrix of the *Torpedo* membrane. Such effects are presumably due to molecular rearrangements within the membrane-bound AcChR structure.

The role of the acetylcholine receptor (AcChR)[1] in promoting increased cation permeability upon agonist binding at the cholinergic binding sites in postsynaptic muscle membranes is well documented (Adams, 1981; Conti-Tronconi & Raftery, 1982; Martinez-Carrion et al., 1982). Nevertheless, little is known regarding molecular mechanisms for formation of the ion channel or induction by cholinergic agonists of reversible transitions of the receptor between "sensitized" (low-affinity) and "desensitized" (high-affinity) states. In this regard, conformational changes of the AcChR upon ligand binding were postulated early (Nachmansohn, 1955). Consistent with this hypothesis were reports of changes both in the intrinsic fluorescence of the receptor (Bonner et al., 1976; Barrantes, 1976) and in its increased labeling with a water-soluble probe, ethidium azide, in the presence of cholinergic ligands (Witzemann & Raftery, 1978). More recently, Kaneda et al. (1982) have reported ligand effects on the intrinsic fluorescence of purified, detergent-solubilized *Narke* (another electric fish) receptors which they attributed to conformational changes occurring within the AcChR protein.

We previously reported pyrene-1-sulfonyl azide (PySA) as a nonperturbing hydrophobic fluorescent probe useful in the identification of AcChR subunits accessible from the hydrophobic membrane matrix (Sator et al., 1979a). In this and in a subsequent study (Gonzalez-Ros et al., 1979b), the distribution of PySA photoproducts in labeled membranes was extensively characterized. It seems that in this compound the

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[1] Abbreviations: AcChR, acetylcholine receptor; PySA, pyrene-1-sulfonyl azide; $\alpha$-Bgt, $\alpha$-bungarotoxin; Tris, tris(hydroxymethyl)aminomethane.

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light-generated nitrene has a marked preference for covalent binding to hydrophobic proteins. Of the generated photoproduct population associated with the membrane, approximately 90% remain *noncovalently* associated with the lipid matrix. At least 70% of these noncovalently bound photoproducts can be readily removed by exchange with plain lipid vesicles (Gonzalez-Ros et al., 1979b). Of the remaining 10% of photoproducts found in *covalent* attachment to membrane protein components, almost all are bound to the AcChR protein. Subunit analysis has further revealed (Sator et al., 1979a) that PySA labeling results in specific tagging of the $\beta$ (50000 molecular weight) and $\gamma$ (60000 molecular weight) subunits of the AcChR protein complex. Furthermore, covalent attachment of the PySA label does not affect the binding of $\alpha$-neurotoxins or the ability of the membrane-bound AcChR to undergo agonist-induced desensitization-like phenomena such as time-dependent binding of $\alpha$-bungarotoxin ($\alpha$-Bgt) (Gonzalez-Ros et al., 1979a). This means that photolabeled membranes highly enriched in fluorophore bound at a known location on a functional AcChR protein can be obtained.

Here we report how PySA-labeled AcChR-enriched membranes can be used to monitor the ability of cholinergic ligands to induce long-range molecular events at the strategic area of the AcChR protein accessible to the hydrophobic lipid matrix.

## Materials and Methods

Frozen *Torpedo californica* electroplax was purchased from Pacific Biomarine Supply Co., and AcChR-enriched membranes in $Ca^{2+}$-free Ringer's solution were prepared as described elsewhere (Sator et al., 1979a). Specific activities of the preparations [between 2 and 3 nmol of $\alpha$-bungarotoxin ($\alpha$-Bgt) binding sites/mg of protein] were determined with a DEAE filter disk assay (Schmidt & Raftery, 1973) by using $^{125}$I-$\alpha$-Bgt (New England Nuclear). Protein concentrations were determined by the method of Lowry et al. (1951). PySA was purchased from Molecular Probes, and [$^3$H]PySA was obtained by titration by the Whilzbach method (by ICN Corp.) and purified as previously described (Sator et al., 1979a). All handling of PySA was performed in the dark, and glassware were covered with aluminum foil. AcChR-enriched membranes were labeled with PySA (Sator et al., 1979a), and the majority of noncovalently bound PySA photoproducts were removed by several exchanges with lipid vesicles prepared by sonicating total lipid extracts from AcChR-enriched membranes (Gonzalez-Ros et al., 1979b). The resulting "clean" PySA-labeled, AcChR-enriched membranes were used in all subsequent fluorescence measurements. These membranes retain most of the total amount of protein initially present as well as both the AcChR specific activity ($\alpha$-Bgt binding sites) and the ability to undergo "desensitization" by cholinergic agonists measured by the time-dependent decrease in binding of $\alpha$-Bgt (Gonzalez-Ros et al., 1979a). Samples for fluorescence determinations were prepared by diluting 100-$\mu$L aliquots of a membrane suspension (1–2 mg of protein/mL) in 5 mM Tris, pH 7.4, containing 0.5 M sucrose and 0.02% $NaN_3$, to a final volume of 1.8 mL. Samples were deaerated by bubbling with pure argon for 10 min prior to fluorescence measurements. In another procedure, a solution containing only PySA ($\sim 10^{-5}$ M) in the above-mentioned buffer was photolyzed to obtain PySA photoproducts having no covalent association to any membrane component. Completeness of photoproduct production was determined spectrally in a Cary 210 spectrophotometer as previously described (Sator et al., 1979a). The photolyzed solution was then lyophilized and the resulting residue extracted with either tetrahydrofuran or chloroform. The extracted PySA photoproducts thus obtained were incorporated into AcChR-enriched membranes in a similar fashion to nonphotolyzed PySA (Sator et al., 1979a). The final concentration of the photoproducts was approximately $10^{-4}$ M. These membrane samples were used as a control in which the effects of noncovalently associated PySA photoproducts within the AcChR-rich membrane matrix were compared to membranes containing PySA photoproducts covalently bound to the AcChR protein.

Table I: Fluorescence Lifetime Values of PySA-Labeled, AcChR-Rich Membrane Fragments in the Presence of Ligands or Membrane Perturbants

| ligand | fluorescence lifetime (ns) |
|---|---|
| none (control) | 13.7 |
| carbamylcholine ($10^{-6}$ M) | 13.6 |
| carbamylcholine ($10^{-3}$ M) | 13.7 |
| $\alpha$-bungarotoxin ($2.5 \times 10^{-7}$ M) | 13.6 |
| $\alpha$-bungarotoxin ($2.5 \times 10^{-7}$ M) and then carbamylcholine ($10^{-3}$ M) | 13.7 |
| $\alpha$-cobratoxin ($2.5 \times 10^{-7}$ M) | 13.4 |
| *d*-tubocurarine ($2.5 \times 10^{-5}$ M) | 13.4 |
| tetracaine ($10^{-6}$ M) | 13.5 |
| carbamylcholine ($10^{-3}$ M) and then tetracaine ($10^{-6}$ M) | 13.0 |
| $\alpha$-cobratoxin ($2.5 \times 10^{-7}$ M) and then tetracaine ($10^{-6}$ M) | 13.3 |
| tetracaine ($10^{-3}$ M) | 8.7 |
| carbamylcholine ($10^{-3}$ M) and then tetracaine ($10^{-3}$ M) | 8.4 |
| $\alpha$-cobratoxin ($2.5 \times 10^{-7}$ M) and then tetracaine ($10^{-3}$ M) | 8.3 |
| Triton X-100 (1%) | 8.2 |
| labeled purified AcChR (protein alone in a 0.1% Triton X-100 solution) | 8.4 |

Fluorescence lifetimes were measured at 25 °C by the cross-correlation phase method described by Spencer & Weber (1969) using an SLM 4800 series subnanosecond fluorometer with the excitation light (346 nm) modulated at 6 MHz. The average of phase lifetime values relative to a glycogen scattering solution was used for all calculations. Corning filters 4-96 and 7-59 were used in the emission pathway.

Quenching titrations of the lifetime of the fluorescence samples were performed by adding increasing aliquots from a deaerated stock solution of the quenchers into the sample cuvette.

## Results

Fluorescence lifetime values of PySA-labeled AcChR-enriched membranes in the presence of several ligands and membrane perturbants are shown in Table I. Neither addition of ligands nor low concentrations of local anesthetics led to significant changes in the fluorescent lifetime of the fluorophore. However, drastic perturbation of the membrane lipid matrix such as that which occurs upon addition of 1% Triton X-100 resulted in a substantial reduction of the fluorescence lifetime. High concentrations of the local anesthetic tetracaine (millimolar) also shortened the lifetime measurements. Because of the lack of information provided by the lifetime measurements alone, we determined the accessibility of the fluorophore to external quenchers from measurements of fluorescence lifetime values in the presence of increasing concentrations of three different quenchers: iodide anions, thallous cations, and neutral nitromethane (Figure 1). These agents have previously been shown to be effective quenchers for the singlet excited state of pyrene analogues incorporated into lipid vesicles or membranes (Sator et al., 1979b) and can

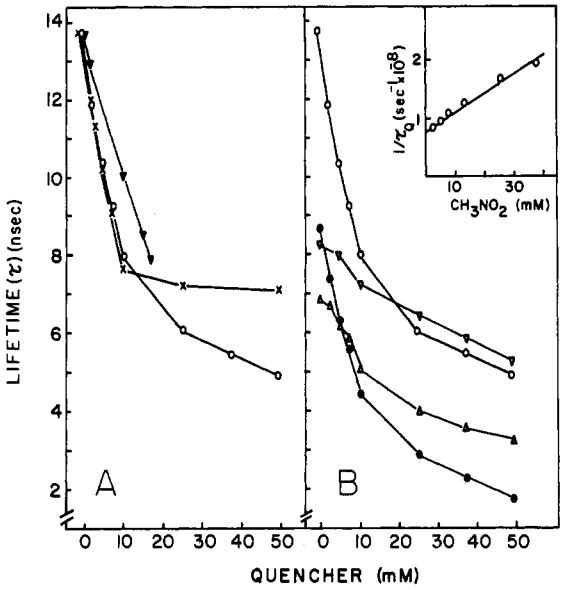


FIGURE 1: (A) Effect of increasing concentrations of externally added quenchers on the fluorescence lifetime of PySA-labeled AcChR-enriched membranes. Fluorescence lifetime quenchers were (○) nitromethane; (×) iodide anions; and (▼) thallous cations. (B) Effect of membrane perturbants on the nitromethane quenching pattern of PySA-labeled AcChR-enriched membranes: (○) "control" (no membrane perturbants added); (▽) 1% Triton X-100; (●) $10^{-3}$ M tetracaine; (△) $10^{-2}$ M procaine. Insert: Stern–Volmer plot of nitromethane quenching on control PySA-labeled AcChR-enriched membranes.

act as indexes of the accessibility of protein-bound fluorophore to water-soluble agents. Nitromethane appears to be the most adequate quencher (for PySA-labeled AcChR membranes) since it provides an efficient quenching of PySA photoproduct fluorescence over a wide range of concentrations (Figure 1A) while demonstrating a fit to Stern–Volmer plots with correlation coefficients of 0.98–0.99 (Figure 1B, insert). Quenching titration curves of PySA-labeled membranes with or without addition of different concentrations of membrane perturbants are shown in Figure 1B. All major perturbations of the hydrophobic environment of the PySA photoproducts result in alterations of the quenching pattern. In the case of Triton X-100, this result is attributable to the disruption of the intact membrane matrix during solubilization. For local anesthetics such as tetracaine or procaine, these alterations in quenching patterns could reflect quenching due to the presence of high concentrations of the local anesthetics themselves prior to addition of nitromethane as well as to physical perturbation of the membrane. These experiments do not differentiate between these alternatives. In contrast, low concentrations of local anesthetics do not produce deviation from the behavior of the "control" PySA-labeled membranes.

Figure 2 illustrates results obtained when nitromethane was added to labeled membranes exposed to cholinergic ligands prior to the addition of the quencher. The presence of carbamylcholine at concentrations above the dissociation constant for membrane-bound AcChR (higher than $10^{-6}$ M) results in a poorer accessibility of the quencher to the fluorophore, as indicated by the relatively longer fluorescence lifetime. Conversely, preincubation of AcChR-rich membranes with α-Bgt produces the opposite effect; that is, quenching by nitromethane is more effective. Further additions of carbamylcholine ($10^{-3}$ M) to the latter membranes cannot reproduce the effect of carbamylcholine alone in decreasing the accessibility of the fluorophore to the quencher, and the quenching pattern remains identical with that of samples subjected to

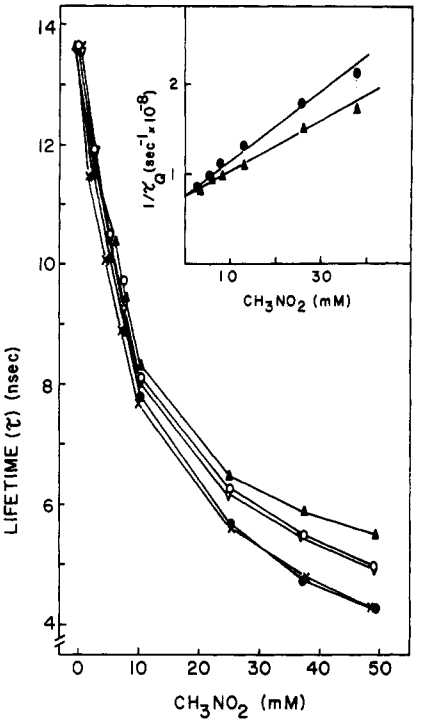


FIGURE 2: Nitromethane quenching patterns of PySA-labeled, AcChR-enriched membranes in the presence of cholinergic ligands: (▽) control (no ligands are added); (○) $10^{-6}$ M carbamylcholine; (▲) $10^{-3}$ M carbamylcholine; (●) α-bungarotoxin (2-fold molar excess to α-bungarotoxin binding sites); (×) α-bungarotoxin followed by $10^{-3}$ M carbamylcholine. Conditions for the preparation of the samples are indicated under Materials and Methods and in Table II. Insert: Stern–Volmer plots of nitromethane quenching on PySA-labeled AcChR-enriched membranes in the presence of $10^{-3}$ M carbamylcholine (▲) and α-bungarotoxin (●).

Table II: Effect of Cholinergic Ligands on the Apparent Rate Constants of Nitromethane Quenching of PySA-Labeled AcChR Membranes from *Torpedo californica*

| ligand | $k_2^{app} \times 10^{-8}$ $(M^{-1}\ s^{-1})^a$ |
|---|---|
| none (control) | 29.7 |
| carbamylcholine ($10^{-6}$ M) | 29.5 |
| carbamylcholine ($10^{-3}$ M) | 25.9 |
| α-Bgt[b] | 35.8 |
| α-Bgt and then carbamylcholine ($10^{-3}$ M) | 35.6 |

[a] Apparent second-order rate constants for the collisional quenching process were obtained from the slopes of Stern–Volmer plots of the quenching curves like those in the insert of Figure 2. [b] Samples were preincubated for 30 min with a 2-fold excess of α-Bgt over total α-Bgt binding sites.

preincubation with α-Bgt only. The effect of binding of these ligands to membrane-bound AcChR is reflected in the various values of the apparent second-order rate constant for the collisonal quenching process of PySA photoproducts fluorescence by nitromethane (Table II) as calculated from Stern–Volmer plots (Figure 2, insert).

In contrast to membranes where PySA photoproducts are covalently bound to AcChR, when the tetrahydrofuran-soluble PySA photoproducts are noncovalently incorporated into AcChR membranes, they give nitromethane quenching patterns which are unaffected by the presence of cholinergic ligands (Figure 3). In this case, the initial fluorescence lifetime values of incorporated soluble PySA photoproducts which are noncovalently associated within the AcChR-rich membranes are consistently lower (by about 3 ns) than those

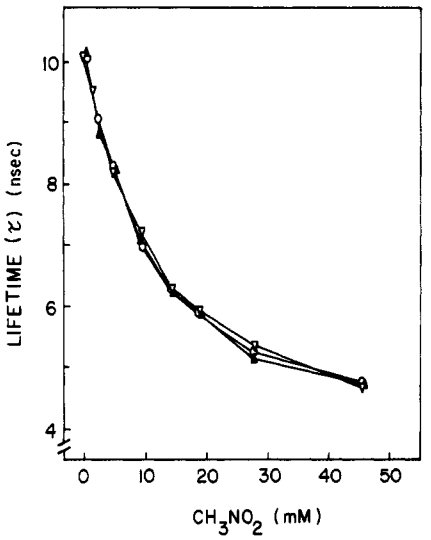


FIGURE 3: Nitromethane quenching patterns of the fluorescence emitted by noncovalently bound PySA photoproducts incorporated into AcChR membranes after complete photolysis in buffer (see Materials and Methods): (▽) control (no ligands are added); (▲) $10^{-3}$ M carbamylcholine; (○) $10^{-6}$ M carbamylcholine.

of the total PySA photoproducts generated by irradiation by PySA in AcChR-rich membranes.

## Discussion

The entry of a water-soluble quencher into the hydrophobic domain of a membrane or plain lipid vesicle is governed by properties of both the quenching agent and the membrane, including the following: (a) the charge at the membrane–water interface; (b) the charge of the quencher; (c) the separation and nature of the phospholipid polar head groups; (d) the fluidity of the membrane interior; and (e) the partition coefficient of the quenching agent between water and lipid. In order to optimize the conditions for fluorescence quenching in our system, we tested neutral compounds as well as several positively and negatively charged ions as quenchers of the fluorescence of PySA-labeled AcChR membranes.

Nitromethane, a noncharged molecule, provides the most effective quenching pattern in our PySA-labeled membranes over a wide range of quencher concentrations. This may be because the neutral character of nitromethane minimizes the possibility that a charged membrane surface could influence the accessibility of an added quencher to the hydrophobic fluorophore domains.

The quenching of PySA photoproduct fluorescence by nitromethane appears to behave as a dynamic, collisional quenching process. The conclusion is based on the definition of collisional (diffusion) quenching, whereby the quenching phenomena obeys the Stern–Volmer equation:

$$\frac{1}{\tau_Q} = \frac{1}{\tau_0} + k_2^{app}[Q]$$

where $\tau_0$ and $\tau_Q$ are respectively the fluorescence lifetime in the absence and presence of a concentration of quencher ([Q]) and $k_2^{app}$ is the apparent second-order rate constant for the quencher–fluorophore collision.

For weakly partitioning quenchers such as nitromethane, Gratzel & Thomas (1976) concluded that the apparent quenching constant is given by

$$k_2^{app} = \frac{k_2 n}{k' + k_2}$$

where $k_2$ is the true second-order rate constant and $n$ and $k'$ are the respective rates for the entry and exit of the quencher into the membrane environment. This means that alterations of the hydrophobic environment of the membrane which are able to modify the partitioning of the quencher as well as the rates of entry or exit could presumably lead to alterations in the $k_2^{app}$ values which do not necessarily imply changes in the true $k_2$ values. In our system, this is not likely to be the case for two reasons. First, we have independently shown by using freely diffusing *pyrene* within the lipid bilayer (Sator et al., 1979b) that occupany of the ligand binding sites of the receptor by pharmacologic agents does not perturb the quenching constant for nitromethane. Second, as shown in our controls, the nitromethane quenching patterns of the PySA photoproducts produced by photolysis outside of the membrane and then incorporated into the membrane lipid matrix (Figure 3) are also unaffected by the addition of ligands of the AcChR.

When the fluorophore is photolyzed within the membrane and, therefore, photoproducts covalently bound to membrane components occur, our results strongly suggest that the observed alterations in the quenching patterns detected as changes in the $k_2^{app}$ are related to events associated with the AcChR protein in the membrane rather than to an alteration of the membrane lipid matrix. We have previously shown (Sator et al., 1979a; Gonzalez-Ros et al., 1979b) that only a small population of PySA photoproducts are covalently bound to neutral membrane lipids and that the majority of fluorophore not covalently bound to AcChR protein is free in the lipid bilayer where its fluorescence properties are unresponsive to ligand addition. Also in support of the idea that observed fluorescence changes are related to alterations in protein structures is the observation that the decreased extent of quenching of the fluorophore by nitromethane upon addition of carbamylcholine can be fully prevented by prior preincubation of the AcChR with the specific antagonist $\alpha$-Bgt. Furthermore, binding of $\alpha$-Bgt alone appears to induce the opposite effect to that of carbamylcholine by increasing the quenching efficiency of the neutral quencher. This effect of bound neurotoxin appears to be irreversible (as is its specific binding to the AcChR), as subsequent additions of carbamylcholine cannot reproduce the effect of carbamylcholine alone.

Under our conditions of high quencher concentration with respect to that of covalently bound fluorophore, the apparent second-order rate constant should be indicative of the accessibility of PySA photoproducts to have a collision with the water-soluble quencher. We therefore interpret the observed changes in quencher effectiveness upon addition of cholinergic ligands as changes in the exposure (accessibility) of AcChR-bound fluorophore domains to quencher. Because nitromethane quenching of *free* PySA photoproducts incorporated into membrane controls is unaffected by the presence of either carbamylcholine or $\alpha$-Bgt, it appears that only after covalent attachment of the fluorophore to the AcChR can we detect the effect of cholinergic ligands.

As we have shown, one out of three fluorophore molecules in our "clean" labeled membranes is covalently bound to the AcChR protein (Gonzalez-Ros et al., 1979b). Therefore, the differences detected in the fluorophore accessibility upon cholinergic ligand binding reflect an overall average response of both covalently attached fluorophores and the somewhat more abundant noncovalently, lipid-associated ones. For this reason, we conclude that the effect of cholinergic ligands on rearranging the receptor protein subunits or inducing local conformational changes in the region of fluorophore attachment must be of sufficient magnitude to be detected in the

average measurement of fluorescence lifetimes of both covalent and noncovalent bound fluorophores. The full magnitude of the fluorescence response to ligand could be elicited if sufficient amounts of purified, PySA-labeled AcChR could be reconstituted into lipid vesicles lacking PySA photoproducts other than those covalently bound to the AcChR protein. Unfortunately, we have as yet been unable to obtain reconstituted AcChR-rich vesicles containing suitable amounts of hydrophobically PySA-labeled receptor subunits for use in fluorescence experiments. Until the technical problems of this approach can be overcome, we will not be able to unequivocally demonstrate that the small population of PySA-labeled neutral lipids plays no role in the fluorescence responses we detect in the presence of various cholinergic ligands. However, it seems to us unlikely that the specific fluorescence changes we report are due to alterations in the lipid matrix rather than to conformational rearrangements at the hydrophobic domain of the AcChR protein. Our interpretation regarding the magnitude of the effect of ligand binding in rearranging the AcChR protein is also consistent with other observations we have made by using the technique of differential scanning calorimetry (Farach & Martinez-Carrion, 1983) when addition of carbamylcholine significantly stabilizes the thermal transitions of proteins in membranes rich in AcChR.

Regarding the range of the effect, we emphasize the location of the PySA fluorophore, which from our previous studies we know to be attached to two subunits exposed to the hydrophobic lipid matrix ($\beta$ and $\gamma$ subunits) (Sator et al., 1979a). Since the binding sites for water-soluble cholinergic agents are located on the exocytoplasmic side of the $\alpha$ subunit of the AcChR protein (Conti-Tronconi & Raftery, 1982), it is of interest to note that the changes induced by ligand binding must be transmitted over a relatively long distance within the receptor–matrix organization. In this respect, recent structural studies (Kistler et al., 1982) of the AcChR molecule have led to postulation of the need of some conformational changes in the protein structure to account for the formation of the ion channel. Our observations with PySA-labeled membranes would suggest that the postulated rearrangement seems to be detectable at least on the $\beta$ and $\gamma$ subunits where the PySA probe is attached.

AcChR desensitization occurs within a short time scale; hence, we cannot distinguish between conformational changes related to the agonist-induced desensitization process and those due to the formation of the transient ion channel. Since continued exposure of AcChR to agonists, as in our experiments, induces desensitization, the observed fluorescence changes should reflect the environmental change of the probe when AcChR is in a desensitized state. Yet, distinction between environmental changes of the chromophore as induced by agonist densensitization and those producing ion channel formation seems feasible in the future. Fast spectroscopic and rapid mixing techniques (ligand–receptor) can be used to distinguish the rapid (milliseconds) ion channel opening from the slower (seconds) desensitization process. We are currently pursuing this approach by using the covalent fluorophore techniques described here.

**Registry No.** Carbamylcholine, 462-58-8; $\alpha$-Bgt, 11032-79-4; $\alpha$-cobratoxin, 69344-74-7; *d*-tubocurarine, 57-95-4; tetracaine, 94-24-6.

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