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Articles

Time-Dependent Binding of Paramagnetic and Fluorescent Hydrophobic Ions to the Acetylcholine Receptor from *Torpedo*†

Scott C. Hartsel, Cynthia R. Moore, Douglas E. Raines, and David S. Cafiso*
Department of Chemistry and Biophysics Program, University of Virginia, Charlottesville, Virginia 22901
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ABSTRACT: In receptor-rich vesicles isolated from *Torpedo*, paramagnetic or fluorescent phosphonium ions bind to both the acetylcholine receptor (AcChR) and the receptor membrane. When added to receptor vesicles, two to three phosphoniums undergo a slow time-dependent binding to the AcChR. The presence of agonist increases the rate but not the extent of binding of the alkylphosphonium nitroxides. Approximately one phosphonium per receptor can be displaced by the addition of saturating concentrations of the high-affinity histrionicotoxin derivative isodihydrohistrionicotoxin or by the addition of phencyclidine or quinacrine mustard. In addition, preincubation of the receptor with these channel blockers prevents approximately one phosphonium from binding to the receptor. When a series of alkyltriphenylphosphonium ions was studied, it was found that the rate of phosphonium binding to the receptor decreased with increasing probe hydrophobicity. This appears to be a function of the partitioning of the probe between membrane and aqueous phases. The phosphonium ions used here promote desensitization of the receptor, as judged by the binding rate of the fluorescent agonist NBDA-C_5-acylcholine or α-bungarotoxin. Preincubation of the receptor with isodihydrohistrionicotoxin virtually eliminates the phosphonium-mediated desensitization. The rates of the phosphonium-mediated desensitization also appear to be dependent upon the phase partitioning of the probe. These results strongly suggest that the binding sites for the phosphonium ion (and the high-affinity histrionicotoxin blocking site) are accessible only through the aqueous phase. The phosphonium binding and agonist-induced transitions observed here are not observed with a negative hydrophobic ion probe, or a negative surface amphiphile, indicating that modifications in membrane electrostatics do not contribute to the observed changes. These spin-labeled and fluorescent phosphonium derivatives provide a new set of probes to study channel blocking sites on the acetylcholine receptor.

Among the intrinsic ion channels present in excitable membranes, the chemically gated nicotinic acetylcholine receptor (AcChR)[1] represents a unique opportunity to examine both the structural and the electrical properties of a biological ion channel. The electric organs of certain eels and rays provide an abundant source of the AcChR, and this has greatly facilitated the biochemical and physical characterization of this important membrane protein [see Changeux et al. (1984) for a review]. In this receptor channel, there are a number of functionally and structurally distinct sites. In addition to the primary agonist binding site (for acetylcholine), there appear to be both high- and low-affinity sites for noncompetitive channel blockers (Heidmann et al., 1983; Oswald et al., 1983) and an additional regulatory binding site for agonist (Takeyasu et al., 1983, 1986). Compounds that bind to the high-affinity channel blocking site are particularly intriguing,

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* Address correspondence to this author.

[1] Abbreviations: AcChR, nicotinic acetylcholine receptor ion channel complex; PCP, phencyclidine; HTX, histrionicotoxin; isoHTX, isodihydrohistrionicotoxin; EbTX, erabutoxin b; Carb, carbamylcholine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; TEMPOL, 4-hydroxy-TEMPO; NBDA, (7-nitro-2,1,3-benzoxadiazol-4-yl)-amino; $TPMP^+$, triphenylmethylphosphonium; $TPDP^+$, triphenyldodecylphosphonium; EPR, electron paramagnetic resonance.

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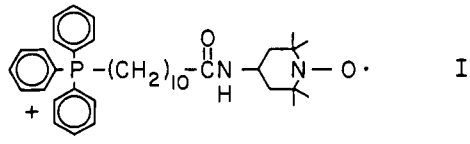
since they interact with regions of the receptor complex that regulate gating and ion flow through the channel. These compounds are generally cations containing aryl or other hydrophobic groups. They may be competitively or allosterically modulated by agonists, toxins, or other blockers (Eldefrawi & Eldefrawi, 1980; Hucho, 1986), and some of them promote receptor desensitization through interactions with high- or low-affinity sites (Heidmann et al., 1983; Blanchard et al., 1979).

The characterization of membrane electrical properties has been greatly facilitated by measurements utilizing hydrophobic ions [see, for example, Bakeeva et al. (1970), McLaughlin (1977), Kamo et al. (1979), Cafiso and Hubbell (1981), and Flewelling and Hubbell (1986)]. In some cases, these measurements provide information on the electrostatic interactions of proteins with membranes (Cafiso & Hubbell, 1980; Cafiso, 1984). In membranes containing the AcChR, measurements using hydrophobic cations, such as the triphenylalkylphosphoniums, are complicated by their action as blockers of the AcChR channel. They bind to the receptor, compete with phencyclidine (PCP), and block ion fluxes in receptor-containing vesicles (Lauffer & Hucho, 1982). Triphenylmethylphosphonium ($TPMP^+$) has also been observed to block signal transmission at the neuromuscular junction (Spivak & Albuquerque, 1985). $TPMP^+$ and its paramagnetic derivatives bind to the receptor in an agonist-dependent fashion (Lauffer & Hucho, 1982; Davis et al., 1983; Cafiso, 1984). The binding of radiolabeled derivatives of the phosphoniums to subunits of the AcChR has been employed to identify regions in the AcChR complex where this apparent channel blocker is localized (Muhn et al., 1984; Fahr et al., 1985; Oberthur et al., 1986).

In order to obtain structural, electrostatic, and other physical information about specific sites and regions on the AcChR, we have been interested in developing electrical and site-specific probes for the AcChR. In this paper, we examine the behavior of a series of paramagnetic and fluorescent phosphonium ion probes in AcChR-rich membranes isolated from the electroplax tissue of *Torpedo nobiliana* and *Torpedo californica*. The series of hydrophobic ion probes we use here are



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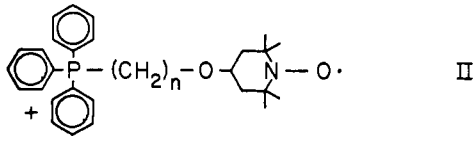
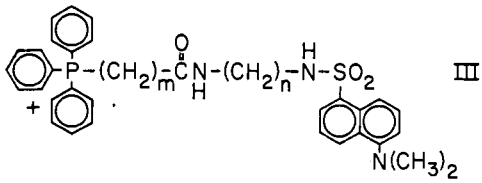


II(n)

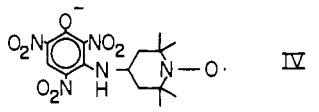


III (m,n)

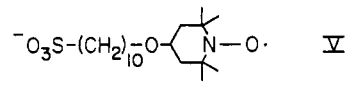
We investigate the time-dependent binding of these probes to the AcChR using electron paramagnetic resonance (EPR) and fluorescence spectroscopy. We also examine the specificity of the phosphonium binding and the ability of these probes to desensitize the AcChR as a function of probe hydrophobicity; information that proves useful for determining binding site accessibility. Finally, we demonstrate, using a number of negatively charged paramagnetic amphiphiles, that agonist-induced changes in the binding of the phosphonium are not due to electrostatic changes in the receptor membrane. While the behavior we find is consistent with their role as channel blockers, the phosphoniums are unique in their stoichiometry when compared to other well-characterized channel blockers such as histrionicotoxin (HTX) or PCP.

Materials and Methods

Spin-Labeled and Fluorescent Ligands or Probes. The amide-linked spin-labeled alkylphosphonium nitroxide I was synthesized as previously described (Cafiso & Hubbell, 1980). The series of ether-linked alkylphosphonium nitroxides II(n) was synthesized by producing a bromoalkyl ether nitroxide (from the appropriate dibromoalkane and TEMPOL) and then reacting this bromoalkyl ether with triphenylphosphine as previously described (Flewelling & Hubbell, 1986). The fluorescent agonist NBDA-C_5-acylcholine was synthesized according to published procedures, and its identity was confirmed with ^1H NMR spectroscopy (Jurss et al., 1979). The fluorescent dansyl phosphonium III(4,4) was synthesized as previously described (Raines & Cafiso, 1985). The negatively charged spin-labeled trinitrophenolate IV was a gift of Wayne



IV



V

Hubbell. The negatively charged alkanesulfonate label V was synthesized as previously described (Hartsel & Cafiso, 1986).

Other Receptor Ligands. Erabutoxin B, quinacrine mustard, α-bungarotoxin, and FITC-labeled α-bungarotoxin were obtained from Sigma Chemical Co. (St. Louis, MO). Radiolabeled *N*-[^3H]propionyl-α-bungarotoxin was obtained from Amersham (Arlington Heights, IL). D-Tubocurarine was obtained from Calbiochem (La Jolla, CA). Isodihydrohistrionicotoxin (isoHTX) was generously provided by Dr. John Daly, and phencyclidine was a gift of Dr. Patrice Guyenet. Alkyltriphenylphosphoniums were synthesized from the appropriate alkyl halide and triphenylphosphine, as previously described (Denney & Smith, 1962).

Isolation of Nicotinic Postsynaptic Membranes. Live *Torpedo californica* or *Torpedo nobiliana* were obtained from Pacific Biomarine (Venice, CA) or Biofish Associates (Boston, MA), respectively. Receptor-rich vesicles were isolated from freshly dissected electric organ by a procedure similar to that described previously (Jeng et al., 1981). The initial homogenization buffer contained 5 mM EDTA, EGTA, 0.1 mM PMSF, 0.1 mM iodoacetamide, and 0.02% NaN_3. The addition of these agents prevented the proteolysis of the receptor during the isolation procedure and did not noticeably affect the binding or kinetics of the ligands examined here. Receptor-rich vesicles were purified on a discontinuous sucrose gradient and were used immediately or stored in isopycnic sucrose in a liquid nitrogen refrigerator. Unless otherwise noted, all AcChR vesicle suspensions were washed free of sucrose before use and suspended in a *Torpedo* Ringer's solution (250 mM NaCl, 5 mM KCl, 3 mM CaCl, 2 mM MgCl, and 5 mM sodium phosphate, pH 7.0) containing sodium azide (0.02%).

The concentration of receptor was determined by assaying the binding of ^3H-labeled or fluorescent α-bungarotoxin to

AcChR vesicle suspensions. We used a centrifugation assay similar to those described previously (Krodel et al., 1979; Heidmann et al., 1983). In some cases the concentration of receptor was determined by quantitating the binding of NBDA-C_5-acylcholine, with a K_D of 0.26 μM for the desensitized state. This was found to give identical results with the toxin assay. Total protein concentrations were determined according to the method of Lowry et al. (1951). The AcChR suspensions were found to have ~0.5–3 nM toxin binding sites/mg of protein.

Desensitization of the AcChR was measured by assaying the time-dependent binding of NBDA-C_5-acylcholine (Prinz & Maelicke, 1983a,b; Jurss et al., 1979) or by measuring the time-dependent binding of ^{3}H-labeled α-bungarotoxin following preincubation with Carb, as described previously (Weiland & Taylor, 1979).

Extracted lipids from native AcChR preparations were prepared as previously described (Folch et al., 1957). These lipids were sonicated in *Torpedo* Ringer's, and the resulting vesicle suspension was used to determine the line shape for lipid-associated phosphonium nitroxides.

EPR and Fluorescence Spectroscopy. Electron paramagnetic resonance spectroscopy was carried out on a Varian E-109 or a modified Varian V-4500 spectrometer equipped with a pneumatically driven mixing device described previously (Cafiso & Hubbell, 1982). EPR spectra were typically taken of 20 μM label (unless otherwise noted) in a 70-μL quartz flat cell at a power level of 10 mW and modulation field of approximately 1 G peak to peak. Fluorescence measurements were made on an SLM-4000 (SLM Instruments). Excitation and emission wavelengths of 342 and 500 nm, respectively, were used to measure the fluorescence from the dansyl phosphonium III. In some experiments, a Corning glass filter (3-75) was included in the 90° emission beam to reduce artifacts due to scattering of the incident excitation beam. Fluorescence of the NBDA-C_5-acylcholine was measured with excitation and emission wavelengths of 484 and 538 nm, respectively.

Analysis of EPR Spectra. For nitroxide labels that partition between membrane and aqueous phases, the population of the aqueous probe was determined by calibrating the amplitude of the high-field resonance ($m_I = -1$) of the EPR spectra in terms of the concentration of aqueous probe. By use of a procedure described previously (Cafiso & Hubbell, 1982), the ratio of membrane-bound to aqueous probe λ was determined from

$$\lambda = (A_{f^\circ} - A)/[A - (\beta/\alpha)A_{f^\circ}] \quad (1)$$

Here, A is the high-field resonance amplitude in the presence of AcChR vesicle suspensions, and A_{f° is the amplitude of this resonance in the absence of membranes. The parameters β and α reflect the contributions made by equal quantities of bound and aqueous spin to the high-field resonance amplitude, respectively. The bound line shape for the high-field resonance of labels I and II(n) is sufficiently broad so that the parameter β/α is essentially zero. This approximation is not altered by the presence of protein-bound label.

In the case where changes in the partitioning of hydrophobic ions or surface amphiphiles are a result of membrane electrical changes, λ can be used to estimate changes in surface, transmembrane, or interfacial potential as described previously (Cafiso & Hubbell, 1981). The negatively charged dinitrophenol spin-label IV can be used to estimate both transmembrane and interfacial potentials. The alkanesulfonates can be used to estimate surface potentials as described previously (Hartsel & Cafiso, 1986).

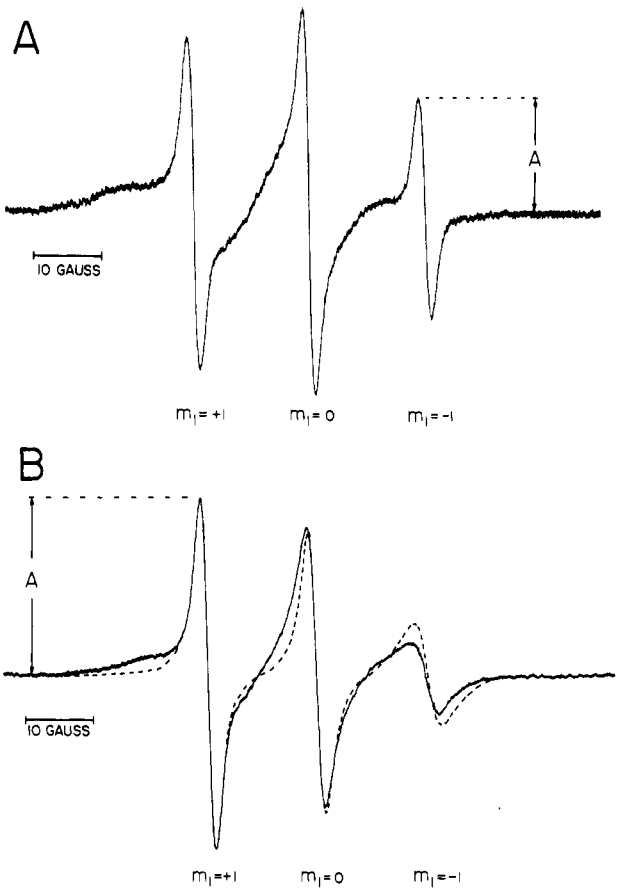


FIGURE 1: (A) An EPR spectrum of 20 μM alkylphosphonium nitroxide I at equilibrium in an isolated receptor suspension containing ca. 10 μM AcChR. This spectrum is a composite of both membrane-associated and aqueous spin populations similar to that seen previously for this probe (Cafiso & Hubbell, 1980). An additional broad component, associated with a dynamically restricted probe population, is also apparent in this spectrum. We used the amplitude of the high-field ($m_I = -1$) nitroxide resonance, A, in the spectrum of probes I, II(4), and II(8) to quantitate the aqueous probe concentration as described in the text. A decrease in the amplitude, A, along with an increase in the magnitude of the broad spectral component is seen with the addition of these labels to AcChR vesicle suspensions. (B) An EPR spectrum of 20 μM alkylphosphonium nitroxide II(12) in equilibrium with receptor-rich membranes (ca. 10 μM AcChR). No aqueous population of probe can be detected, due to the large negative free energy of transfer for this probe to the membrane hydrocarbon. This spectrum, as seen above for probe I, contains a broad spectral component arising from a dynamically restricted probe population. Superimposed over this spectrum is a spectrum of probe II(12) in the presence of vesicles formed from extracted *Torpedo* lipids at a concentration of 10 mg/mL (--). We estimate the population of probe associated with the bulk lipid by calibrating the amplitude, A, of the low-field resonance ($m_I = +1$) for a standard sample of spin bound to vesicles formed from lipids extracted from native AcChR-rich membranes. When probe II(12) or II(10) is added to AcChR suspensions, a decrease in the amplitude A is observed along with an increase in the population of dynamically restricted probe.

RESULTS

Association of Paramagnetic Phosphoniums with the AcChR. At receptor concentrations in the range of 5–20 μM AcChR (approximately 2–10 mg/mL total membrane lipid), the EPR spectra of the phosphonium labels I, II(4), and II(8) are a composite of both membrane-associated and aqueous probe populations. Shown in Figure 1A is a spectrum of label I. Also apparent in this spectrum is a broad component with a line shape similar to that seen for other paramagnetic probes incorporated into AcChR-containing vesicles [see, for example, Ellena et al. (1983) and Earnest et al. (1984)]. For the longer

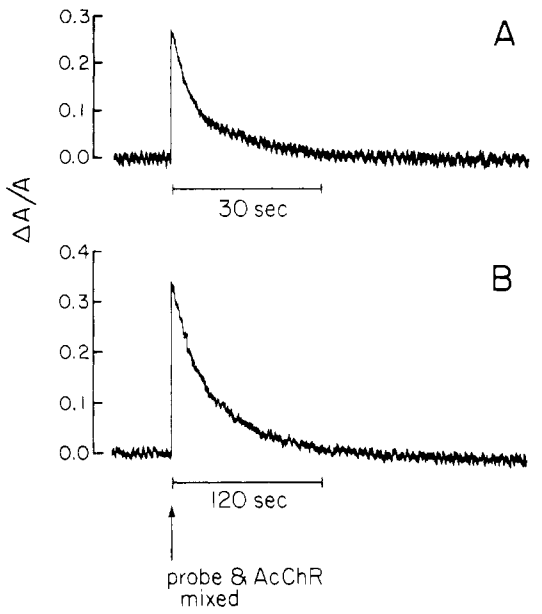


FIGURE 2: (A) Fractional change ($\Delta A/A$) in the high-field resonance amplitude for 20 μM label II(8) as a function of time following mixing of the probe with an AcChR vesicle suspension (ca. 2 μM AcChR). The decrease in signal corresponds to a decrease in the aqueous population of probe. (B) Fractional change ($\Delta A/A$) in the low-field resonance amplitude of 20 μM II(12) as a function of time following mixing of the probe with an AcChR vesicle suspension (ca. 2 μM AcChR). The decrease in signal intensity for this label is a result of a decrease in the probe population in the bulk lipid phase. For the series of probes II(n), the time scale for these intensity changes increases as n is increased.

chain alkylphosphoniums, II(10) or II(12), no aqueous population of probe can be detected. In this case, Figure 1B, a large broad spectral component is also apparent. The narrower spectral component is identical with the spectrum obtained for probe associated with the extracted lipids from the AcChR membrane (see Figure 1B). The broad spectral components seen in Figure 1 are the result of a dynamically restricted population that apparently results from protein-associated probe.

In the absence of agonist, we observe time-dependent changes in the binding of the phosphonium ions following mixing with AcChR vesicles. Shown in Figure 2A is a recording of the high-field resonance amplitude of spin-label II(8) when mixed with native AcChR vesicles. The decrease in amplitude of this resonance is the result of a time-dependent binding to AcChR vesicles and does not result from spin reduction. These rates are dependent upon the concentration of phosphonium and AcChR used. A similar change is observed in the spectrum of labels II(10) and II(12), which remain totally membrane associated at the concentration of vesicles used here. For these bound probes, the low-field resonance ($m_I = +1$), Figure 1, can be used to monitor the population of probe associated with the bulk membrane lipid. This population of probe decreases with time following mixing of the probe with AcChR membranes (see Figure 2B). Within our experimental error, the time-dependent binding appears to be first order. The rates of binding of these phosphonium probes are enhanced with agonist, and the rates vary greatly, with the more hydrophobic probes showing much slower kinetics.

The fastest binding rates for these probes are observed when agonist is added simultaneously with probe to AcChR vesicles. In Table I are shown the apparent half-times for the association of the phosphoniums when this probe is simultaneously mixed with Carb into AcChR vesicles. Also shown are the rates when AcChR-containing vesicles are preincubated with agonist. Preincubation or simultaneous addition of agonist does not change the number of moles of phosphonium probe bound to the AcChR membrane at equilibrium.

Table I: Approximate Half-Times for the Binding of Phosphonium Nitroxides to AcChR from *T. californica*[a]

	half-time (s)				
probe	no additions	Carb/ preinc[b]	Carb/simult[b]	EbTx[c]	isoHTX[c]
II(12)	46	39.0	16.8	59	49
II(10)	10.8	3.1	2.0		
II(8)	4.5	1.2	0.8	5.0	2.5
I	5.4	1.0	0.6		
II(4)	3.3				

[a] Probe concentrations were ≈20 μM; AcChR concentration was ≈4 μM. Half-times are reported in seconds and have an error of ±10% or less. An approximately equal number of binding sites for these labels is found in the presence and absence of agonist. A decrease in phosphonium binding of approximately one site per receptor is observed following incubation of the receptor with isoHTX. [b] Carb concentrations were 200 μM for both preincubated and simultaneously mixed AcChR samples. [c] AcChR vesicle preparations were preincubated with erabutoxin and isodihydrohistrionicotoxin at concentrations of 10 μM.

There are several possible sources for the time-dependent binding changes observed in the spectra of these paramagnetic phosphoniums. Time-dependent phase partitioning changes are observed following mixing of spin-labeled phosphoniums with sonicated lipid vesicles. These changes are the result of a transmembrane migration of probe (Cafiso & Hubbell, 1982). In the present case, the changes observed in Figure 2 can not be the result of a transmembrane movement of probe, since the addition of tetraphenylboron (an efficient transmembrane carrier for the phosphonium) does not alter the rates of binding seen in Figure 2. Changes in binding rates of labels I and II to AcChR vesicles with agonist could be associated with changes in membrane electrostatics. However, measurements made with negatively charged probes IV and V reveal no significant changes in transmembrane, surface, or interfacial potential with agonist addition, under the conditions used here.[2]

Previous reports indicate that $TPMP^+$ binds to the high-affinity channel blocking site (Lauffer & Hucho, 1982; Muhn & Hucho, 1983). The slow binding we see here could be due to the binding of our paramagnetic labels to this site. We tested the ability of known channel blockers of the AcChR to displace the phosphonium probes from AcChR membranes. In Figure 3 is shown the effect of adding isoHTX or the non-spin-labeled phosphonium triphenyldodecylphosphonium ($TPDP^+$) on the high-field resonance amplitude of label II(8). The increase in signal intensity is a result of the dissociation of probe II(8) from AcChR membrane vesicles. The addition of 200 μM $TPDP^+$ displaced virtually 100% of the probe that associated slowly with the AcChR membrane. In all cases, isoHTX and PCP (data not shown) could displace only 30 ± 5% of the spin-label that was displaced by $TPDP^+$. The

[2] The phase partitioning changes for labels IV and V can be used to estimate changes in potential that occur at the membrane interface. From the experimental error of the measurements made here, we can place limits on the magnitude of any surface charge or interfacial charge movement occurring with the desensitization of the receptor following agonist addition. Under the conditions of our experiments, we estimated charge density changes using a procedure similar to that described previously (Cafiso & Hubbell, 1980). These changes are less than ca. 0.2 mC/m^2. This is less than ca. one charge per receptor from values obtained previously for AcChR densities in receptor rich membranes (Gonzalez-Ros et al., 1982).

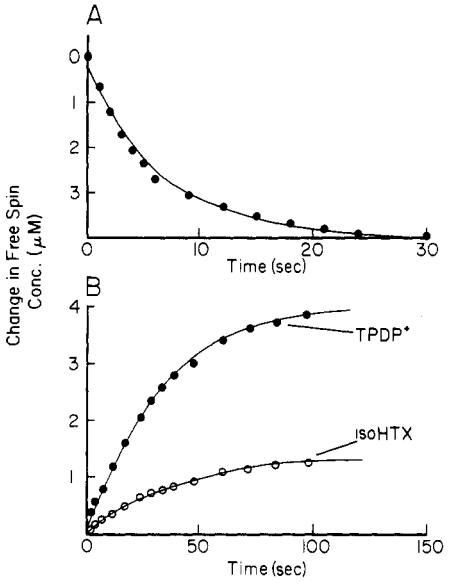


FIGURE 3: (A) Change in the aqueous probe concentration following mixing of 20 μM II(8) with AcChR suspensions (ca. 1.5 μM AcChR) (●). The aqueous concentration change is determined as described in the text. The solid line (—) is an exponential fit to these data. (B) Change in the aqueous concentration of II(8) when 100 μM $TPDP^+$ (●) or 100 μM isoHTX (○) is added to AcChR suspensions (ca. 1.5 μM AcChR) that are preequilibrated with 20 μM II(8). These curves are a result of the dissociation of II(8) from AcChR membranes following the addition of these unlabeled competing ligands. The lines (—) are exponential fits to these dissociation data and were used to determine the first-order dissociation rate constant, k_{-1}, for II(8).

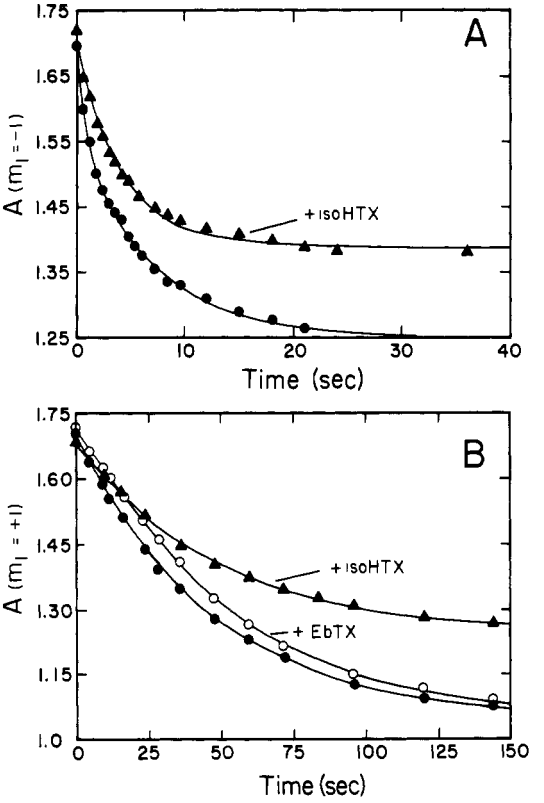


FIGURE 4: (A) High-field resonance amplitude, $A(m_I = -1)$, for ≈20μM I(8) following mixing of the label with AcChR suspensions (ca. 1.5 μM). Here, $A_{f^\infty} = 3.5$. (B) Low-field resonance amplitude, $A(m_I = +1)$, of 20 μM II(12) following mixing of the label with AcChR suspensions (ca. 1.5 μM AcChR). Time-dependent amplitudes are shown for receptor-rich vesicles with no additions (●), following incubation with 20 μM erabutoxin b (○) and incubation with 20 μM isoHTX (▲). The points were fitted to single exponentials (—) by a nonlinear least-squares curve fitting procedure. The half-times are shown in Table I.

first-order dissociation rate constant for the labeled phosphoniums was identical (within experimental error) whether the displacement was initiated by $TPDP^+$ or isoHTX. We also examined the effect of preincubating AcChR membrane vesicles with these compounds preceding the addition of probe. Preincubation of AcChR membranes with 20 μM isoHTX (Figure 4), 200 μM PCP, or 100 μM quinacrine mustard prevented approximately 30 ± 5% of the time-dependent binding of phosphonium labels I and II(n). We also tested the effects of erabutoxin b on the binding rates of the phosphonium (Figure 4B and Table I). This toxin has been reported to stabilize the resting state of the AcChR (Oswald et al., 1983). Erabutoxin b slows the binding rate slightly without affecting the extent of phosphonium binding.

A more quantitative analysis of the phosphonium binding data for probe II(8) yielded the second-order association rate constant k_1, defined previously (Maelicke et al., 1977). From assays of AcChR concentration with both fluorescent agonist and bungarotoxin binding, a stoichiometry of two to three phosphoniums per AcChR is found. Assuming this stoichiometry, k_1 for II(8) is found to be 1×10^4 M^{-1} s^{-1}. From the dissociation data we also calculated a value for the reverse rate constant k_{-1}. The value for $K_d = k_{-1}/k_1$ was found to be approximately 2.8 μM. We also estimated the value of K_d by measuring the number of moles of II(8) that bind to AcChR membrane vesicles as a function of the concentration of II(8). From this binding, which is clearly saturable, we estimate an apparent K_d of 10 μM for label II(8) and 5 μM for label II(12). The number of moles of II(8) displaced by saturating levels of isoHTX (50 μM) was found to be approximately one per receptor complex.

The association rate constant measured here is considerably slower than diffusion-limited binding (10^7–10^8 M^{-1} s^{-1}) and is similar to values obtained for the binding of PCP or perhydrohistrionicotoxin to the AcChR channel blocking site (Oswald et al., 1983; Aronstam et al., 1981). The value of K_d is consistent with that found for other noncompetitive channel blockers of the high-affinity site (Heidmann et al., 1983) and similar to the value of ca. 13 μM found for $TPMP^+$ to the resting state of the AcChR.

These results provide strong evidence that the phosphonium labels used here bind in a time-dependent manner to the high-affinity channel blocking site of the AcChR. In addition, these probes show a slow binding to one or two additional sites distinct from the channel blocking site, as defined by isoHTX and PCP.

Desensitization of the AcChR by Alkylphosphonium Nitroxides. The electophysiological effects of the phosphonium ion $TPMP^+$ indicate that it may act to desensitize the AcChR (Spivak & Albuquerque, 1985). We directly investigated the ability of the phosphonium probes I and II(n) to desensitize the AcChR by examining the effect that these probes had on the rate of binding of NBDA-C_5-acylcholine to AcChR membrane vesicles.

When added to the AcChR in the resting state, NBDA-C_5-acylcholine fluorescence is quenched as it associates with the primary agonist binding site and desensitizes the AcChR (Prinz & Maelicke, 1983a,b; Jurss et al., 1979). The binding of this probe appears to be similar to that for dansylamino-C_6-acylcholine. For both these probes, preincubation of the receptor with agents that promote desensitization is found to accelerate the probe binding rate (Prinz & Maelicke, 1983a,b; Heidmann & Changeux, 1979a,b). In Figure 5A, the fluorescence decay when AcChR membrane vesicles are mixed with NBDA-C_5-acylcholine is shown.[3] The addition of excess

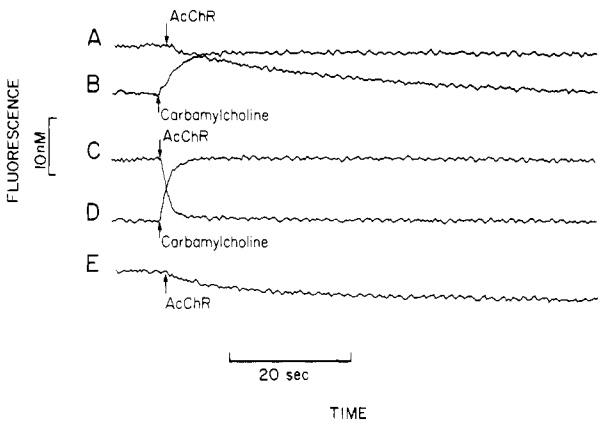


FIGURE 5: Time-dependent fluorescence intensity of 50 nM NBDA-C_5-acylcholine. AcChR vesicle suspensions contain ca. 20 nM AcChR. (A) Fluorescence of aqueous NBDA-C_5-acylcholine upon the addition of the AcChR suspension. (B) Fluorescence changes for the sample in (A), at equilibrium, following the addition of 200 μM Carb. (A) and (B) are plotted on the same relative fluorescence scale. (C) Fluorescence intensity of aqueous NBDA-C_5-acylcholine upon the addition of an AcChR suspension that was incubated with 5 μM II(8). (D) Fluorescence changes for the sample in (C), at equilibrium, upon the addition of 200 μM Carb. (C) and (D) are plotted on the same scale of relative fluorescence. (E) Fluorescence of aqueous NBDA-C_5-acylcholine following the addition of an AcChR suspension that was treated with 10 μM isoHTX. A calibration is shown indicating the fluorescence amplitude change corresponding to 10 nM NBDA-C_5-acylcholine.

Carb quantitatively reverses the fluorescence decay (Figure 5B). In the presence of the phosphonium probes II(n), the rate of binding of the fluorescent agonist is dramatically accelerated (Figure 5C). The phosphonium probes also slightly increase the extent of NBDA-C_5-acylcholine binding. In the presence of high levels of phosphonium probe (up to 50 μM), no direct competition of the hydrophobic ions for the NBDA-C_5-acylcholine binding site is detected. At the concentrations of AcChR used here (approximately 20 nM), the rate of NBDA-C_5-acylcholine binding is identical with its control level when the concentration of added probe [e.g., probe II(8)] is below ca. 100 nM.

At AcChR concentrations above 100 nM, probes II(8), II(10), and II(12) phase partition to AcChR vesicles so that II(12) is almost totally membrane associated. Under these conditions, the phosphoniums vary in their ability to accelerate the binding rate of NBD-A-C_5-acylcholine. The order of their effectiveness is II(8) > II(10) > II(12), where the rate of NBDA-C_5-acylcholine binding is approximately 5 times greater in the presence of 1 μM II(8) than in the presence of 1 μM II(12). At lower concentrations of AcChR (10 nM), there is no significant binding of these probes to the membrane, and even the more hydrophobic phosphonium, II(12), exists primarily in the aqueous phase. Under these conditions, no difference is observed between probes II(8), II(10), and II(12) in their effectiveness at accelerating the binding of NBDA-C_5-acylcholine.

The influence of the channel blocker isoHTX on NBDA-C_5-acylcholine binding in the presence of the phosphonium nitroxides is quite dramatic. As shown in Figure 5E, preincubation of the AcChR vesicle suspension with 10 μM isoHTX virtually abolishes the phosphonium-induced increase in NBDA-C_5-acylcholine binding rate.

These results strongly suggest that phosphoniums can desensitize the AcChR. As an additional check for receptor desensitization, we examined the binding rate of radiolabeled α-bungarotoxin (in the presence of agonist) to AcChR vesicle suspensions. The rate of α-bungarotoxin binding is decreased by approximately 2-fold when 0.45 μM AcChR suspensions are preincubated with 5 μM phosphonium I before toxin addition. When the suspensions are preincubated with 3 μM Carb, the rate of toxin binding is decreased by approximately 3-fold.

Association of a Dansyl Phosphonium to the AcChR. We examined the behavior of the fluorescently labeled phosphonium III(4,4) in AcChR-containing vesicles to determine its properties compared with $TPMP^+$ and the spin-labeled phosphoniums I and II(n) shown above. As previously shown, this probe phase partitions to membranes and exhibits voltage-dependent fluorescence changes (Raines & Cafiso, 1985). When AcChR-containing membranes are added to a buffer containing probe III(4,4), an increase in fluorescence is seen (see Figure 6). This is associated with a partitioning of the probe to the AcChR-containing membranes. At the concentration of AcChR vesicles used here, the probe is not completely membrane bound but is in equilibrium with an aqueous population of probe. Here, the ratio of membrane to aqueous probe, λ, is ≈ 1. After the addition of vesicles, the fluorescence signal from this probe is relatively stable with time, showing only a slight increase in fluorescence during the time course of this experiment. As shown in Figure 6, the addition of Carb results in an increase in fluorescence signal intensity; in addition, the emission spectrum of this probe undergoes a slight blue shift (ca. 20 nm). This spectral shift indicates a change in probe environment, apparently resulting from an agonist-dependent association of the probe with the AcChR. The increase in fluorescence can be completely reversed by the

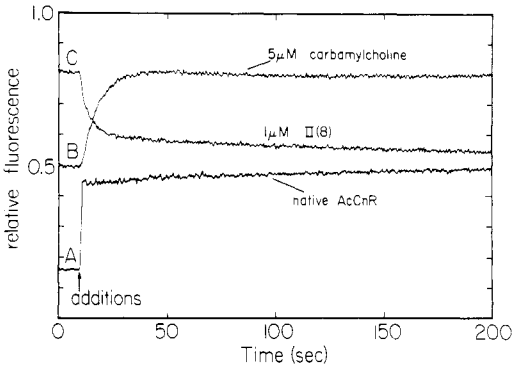


FIGURE 6: Time-dependent fluorescence intensity of 100 nM dansyl phosphonium III(4,4). (A) Fluorescence increase following the addition of an AcChR vesicle suspension (ca. 20 nM AcChR) to aqueous III(4,4). The AcChR suspension is added at t = 10 s. (B) Fluorescence increase seen upon the addition of 5 μM Carb to a sample containing III(4,4) equilibrated for ca. 4 min with the AcChR suspension. Carb is added at t = 10 s. (C) The fluorescence decrease associated with the addition of II(8) to a sample containing III(4,4) equilibrated with the AcChR suspension and Carb for ca. 4 min. II(8) is added at t = 10 s. The fluorescence decrease is not associated with Stern–Volmer quenching since nonparamagnetic analogues, i.e., $TPDP^+$, lead to similar fluorescence changes.

[3] Both slow and fast exponential processes are observed in the binding of NBDA-C_5-acylcholine and dansylamino-C_6-acylcholine (Prinz & Maelicke, 1983a,b; Heidmann & Changeux, 1979a,b); the fast process appears to reflect binding to receptor present in the desensitized form, and the slow process is a result of desensitization promoted by the probe. Using bimolecular association rate constants of 9.5×10^7 M^{-1} s^{-1} and 3.5×10^5 M^{-1} s^{-1} to desensitized and resting states, respectively (Heidmann & Changeux, 1979a), we expect the fast process to occur on the order of a few hundred milliseconds and the slow process to occur on the order of seconds (for the conditions used here). Thus, the data in Figure 5 primarily reflect the slow process seen previously. Both the fast and slow rates are accelerated by agents that desensitize the receptor, and an independent quantitation of the contributions made by each process was not required for the qualitative conclusions made here.

addition of 1 μM II(12) and partially reversed (~30–40% of the signal intensity) by 2 μM isoHTX. These results are qualitatively similar to the agonist-dependent behavior reported previously for $TPMP^+$ in AcChR suspensions (Lauffer & Hucho, 1982; Davis et al., 1983) and are analogous to results obtained with quinacrine (Grünhagen et al., 1977). Agonist-induced increases in the binding of labels I and II(12) were previously observed under experimental conditions where significant desensitization of the AcChR had not occurred (Cafiso, 1984). At the concentration of probe used here (100 nM), phosphonium probes do not accelerate the binding of NBDA-C_5-acylcholine and thus are not at high enough concentrations to desensitize the AcChR.

The differences between the behavior of the fluorescent phosphonium III(4,4) (Figure 6) and the paramagnetic phosphoniums, II(n), are a result of the lower concentrations of fluorescent probe used here. This allows the binding of this receptor ligand to be examined at concentrations that do not saturate the available binding sites or desensitize the receptor. At higher concentrations of III(4,4), larger time-dependent increases in fluorescence are seen with the addition of AcChR vesicles (data not shown). Thus, the spin-labeled and fluorescent probes appear to be consistent in their behavior. As was the case for the spin-labeled probes, isoHTX does not completely dissociate III(4,4) from the AcChR vesicle.

Discussion

The data that are presented here provide strong evidence that the spin-labeled phosphoniums I and II(n) associate with the AcChR slowly when incubated with AcChR vesicle suspensions. While 2–3 mol of probe appears to associate with 1 mol of the receptor complex, 1 mol of probe is associated with the high-affinity HTX binding site (as would be expected from the stoichiometry of HTX sites; Aronstam et al., 1985; Albuquerque et al., 1980). From our kinetic data, we were not able to distinguish separate sites; thus, within our experimental error, these two or three sites must have similar affinities for the phosphoniums.

The observation made here of more than one binding site for the phosphonium is not inconsistent with the stoichiometry seen for other noncompetitive channel blockers [see, for example, Elliot and Raftery (1979) and Eldefrawi et al. (1980)]. Labeling of the AcChR by the phosphonium $TPMP^+$ occurs at more than one site. $TPMP^+$ labels the α-subunit in an agonist-independent fashion and labels the δ-subunit with agonist stimulation (Muhn et al., 1984; Fahr et al., 1985). Other noncompetitive channel blockers also show labeling at sites that are independent of the high-affinity HTX or PCP site. For example, the α-subunit can be efficiently labeled by [^{3}H]chlorpromazine, and this labeling is insensitive to the addition of PCP or perhydro-HTX at concentrations that should only affect the high-affinity sites (Heidmann & Changeux, 1984; Heidmann et al., 1983; Oswald & Changeux, 1981).

The increase in the rate of NBDA-C_5-acylcholine binding seen in the presence of alkylphosphonium ions and the decrease in the rate of α-bungarotoxin binding provide strong evidence that these hydrophobic ions can desensitize the receptor. Desensitization of the AcChR was previously evoked to explain the equilibrium blockade of the receptor channel by $TPMP^+$ (Spivak & Albuquerque, 1985).

As shown above, isoHTX is remarkably effective at preventing desensitization of the AcChR by the phosphonium. This observation is similar to that noted by Heidmann et al. (1983), where PCP- or trimethisoquin-mediated desensitization of the receptor could be prevented by perhydro-HTX. These data indicate that the phosphonium acts to desensitize the AcChR at the high-affinity isoHTX binding site. It is also possible that isoHTX simply stabilizes the resting state of the receptor; however, the slow binding rate of NBDA-C_5-acylcholine (which reflects desensitization) is only weakly affected by the addition of isoHTX (see Figure 5E), making this latter possibility seem less likely.

Here, we find that the rate of binding of the paramagnetic phosphonium labels II(n) to the AcChR is a strong function of the length of the alkyl chain on the probe. The more hydrophobic probes (which are totally membrane associated) bind much more slowly than the shorter chain probes, which have significant aqueous concentrations. This is not simply due to a limited dissociation rate for the long-chain probes from the bulk membrane lipid. The rates of dissociation of probes I and II(n) from lipid membranes are extremely rapid compared to the time scales seen in Figure 3. It has been suggested that the binding of some noncompetitive blockers such as PCP and perhydro-HTX is rate limited by steric effects (Eldefrawi et al., 1980; Oswald et al., 1983). While this may, in part, account for the slow time scales we see, it does not appear to account for the binding rate dependence we see on alkyl chain length. For example, probe I is much more rapid in its binding rate than probes II(10) or II(12), yet it is comparable in size. The free energy of binding for probe I to the membrane is similar to that for probe II(8), presumably due to the presence of the polar amide linkage. Thus, similar binding rates are seen for probes that have similar membrane/aqueous partition coefficients. Under conditions similar to that for the EPR experiment, the binding rate of the fluorescent agonist NBDA-C_5-acylcholine shows the same qualitative dependence upon the alkyl chain length of probes II(8)–II(12). The simplest explanation for this behavior is that the sites at which this probe binds and desensitizes the receptor are accessible only from the aqueous phase. The aqueous concentration of ligand, which is determined by the probe phase partitioning, determines the association rate of the probe to the AcChR.

Another fascinating, but unexplained, observation made here is the dramatic acceleration of the binding rate of these probes when mixed simultaneously with agonist and AcChR vesicles. This type of behavior was previously observed for the binding of channel blockers such as PCP, perhydro-HTX, and chlorpromazine (Heidmann & Changeux, 1984; Aronstam et al., 1981). Neuromuscular blockade by $TPMP^+$ is also dramatically enhanced in the presence of agonist (Spivak & Albuquerque, 1985). The interpretation usually given for this behavior is that the activation free energy for binding of the blocker is lowest in the open channel state (Karpen & Hess, 1986). The factors controlling this association rate are not understood but are potentially extremely important for a molecular understanding of AcChR channel regulation.

The EPR and fluorescent phosphoniums described here complement techniques using radiolabeled blockers and can readily be used to make measurements of the kinetics of channel blockers. Clearly, there are strengths and weaknesses to these probe techniques. For example, for simple binding assays, the paramagnetic probes require relatively large quantities of receptor-rich membrane, when compared to assays using radiolabeled blockers. On the other hand, it is relatively easy with EPR to distinguish between bilayer- and receptor-associated probe. It is also possible with EPR and fluorescent probes to obtain information on the physical properties of the channel blocking site. In principle, these types of probes can be used to obtain information on the molecular dynamics and electrostatics of specific receptor sites.

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Registry No. I, 89505-18-0; II (8), 107959-92-2; II (10), 107940-97-6; II (12), 89505-17-9; III, 97228-12-1; IV, 107940-98-7; V, 104778-50-9; HTX, 34272-51-0; isoHTX, 34272-52-1; NBDA-C_5-acylcholine, 70214-86-7; α-bungarotoxin, 11032-79-4; phencyclidine, 77-10-1; quinacrine mustard, 4213-45-0.

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