The Cholesterol Dependence of Activation and Fast Desensitization of the Nicotinic Acetylcholine Receptor

Saffron E. Rankin, George H. Addona, Marek A. Kloczewiak, Birgitte Bugge, and Keith W. Miller
Department of Anesthesia, Massachusetts General Hospital and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02114 USA

ABSTRACT When nicotinic acetylcholine receptors are reconstituted into lipid bilayers lacking cholesterol, agonists no longer stimulate cation flux. The kinetics of this process are difficult to study because variations in vesicle morphology cause errors in flux measurements. We developed a new stopped-flow fluorescence assay to study activation independently of vesicle morphology. When receptors were rapidly mixed with agonist plus ethidium, the earliest fluorescence increase reported the fraction of channels that opened and their apparent rate of fast desensitization. These processes were absent when the receptor was reconstituted into dioleoylphosphatidylcholine or into a mixture of that lipid with dioleoylphosphatidic acid (12 mol%), even though a fluorescent agonist reported that resting-state receptors were still present. The agonist-induced channel opening probability increased with bilayer cholesterol, with a midpoint value of 9 ± 1.7 mol% and a Hill coefficient of 1.9 ± 0.69, reaching a plateau above 20–30 mol% cholesterol that was equal to the native value. On the other hand, the observed fast desensitization rate was comparable to that for native membranes from the lowest cholesterol concentration examined (5 mol%). Thus the ability to reach the open state after activation varies with the cholesterol concentration in the bilayer, whereas the rate of the open state to fast desensitized state transition is unaffected. The structural basis for this is unknown, but an interesting corollary is that the channels of newly synthesized receptors are not fully primed by cholesterol until they are inserted into the plasma membrane—a novel form of posttranslational processing.

INTRODUCTION

The role of cholesterol in modulating the properties of phospholipid bilayers has been extensively studied, but its role in lipid-protein interactions is less well understood (reviewed in Barrantes, 1993). For example, in the reconstituted (Ca^{2+}-Mg^{2+})-ATPase from sarcoplasmic reticulum, a cholesterol analog could either inhibit or activate the enzyme, depending on the composition of the surrounding phospholipids (Lee et al., 1994). Similarly, in a ligand-gated ion channel, the nicotinic acetylcholine receptor (nAcChoR) from *Torpedo*, cholesterol, or the hemisuccinate is required for activation of the channel (Fong and McNamee, 1986). In neither protein is cholesterol's action associated with its effect on bulk lipid bilayer fluidity (Lee et al., 1994; Sunshine and McNamee, 1994), although an action on the lipid adjacent to the protein, suggested for the (Ca^{2+}-Mg^{2+})-ATPase from sarcoplasmic reticulum (Squier and Thomas, 1988), remains a possibility (Sunshine and McNamee, 1994). In both proteins evidence has been presented for the existence of nonannular, or interstitial, sites within the protein that are distinct from the lipid-protein interface (Jones and McNamee, 1988; Narayanaswami and McNamee, 1993; Lee et al., 1994). However, there is no evidence directly supporting a functional role for the putative nonannular cholesterol sites. Indeed, activity is maintained in the nAcChoR when a variety of hydrophobic compounds are substituted for cholesterol. This observation challenges the nonannular site hypothesis, which is now required to postulate very lax structure-activity requirements (Sunshine and McNamee, 1992).

The aim of this study was to investigate quantitatively the role of cholesterol in the agonist-induced rapid state transitions associated with channel opening in the AcChoR. Rapid cation flux measurements are the obvious way to extend the previous phenomenological observations (Walker et al., 1982; Fernandez et al., 1993; Fernandez-Ballester et al., 1994), but they suffer from severe experimental drawbacks because the measured flux rate depends on the morphology of the vesicles, which may vary with lipid composition (Anholt et al., 1982). Specifically, controls would need to be performed to correct for variation in vesicle internal volume and receptor surface density, as well as for inhomogeneities in the vesicle population, and there is no rapid and secure way of doing so (Udgaonkar and Hess, 1986). Furthermore, sealed vesicles are not formed when the receptor is reconstituted into some lipid bilayers (Pradier and McNamee, 1992). Therefore, we developed an assay using the fluorescent probe ethidium bromide, whose interaction with the AcChoR has been characterized (Schimerlik et al., 1979; Herz et al., 1987, 1991; Pedersen, 1995). We find that after rapid mixing of receptors with agonist and ethidium, the earliest fluorescence increase reports the fraction of channels that opened and the rate of fast desensitization. Exploiting this new assay, we show that the ability to reach the open state after agonist binding depends on the

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Address reprint requests to Dr. Keith W. Miller, Department of Anesthesia, Massachusetts General Hospital, White 4, Room 430, Fruit St., Boston, MA 02114-2636. Tel.: 617-726-8985; Fax: 617-726-5845; E-mail: k miller@helix.mgh.harvard.edu.

Dr. Rankin's present address is Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, England.

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cholesterol concentration in the bilayer, whereas the rate of fast desensitization is unaffected.

MATERIALS AND METHODS

Materials

Torpedo nobiliana were obtained live from Biofish Associates (Georgetown, MA). All phospholipids were purchased from Avanti Polar lipids (Alabaster, AL), cholesterol was from Calbiochem (La Jolla, CA). Affigel 102, acrylamide, bis-acrylamide, and Coomassie brilliant blue were from Biorad (Richmond, CA). Spectra/Por tubing for dialysis was from Spectrum Medical Industries (Houston, TX). $^{86}Rb^+$ and [^{3}H]acetic anhydride were obtained from New England Nuclear (Billerica, MA). Dithiothreitol (DTT) and bromoacetyl bromide were purchased from Fisher Biotech (Pittsburgh, PA). Choline bromide, EDTA, EGTA, phenylmethylsulfonyl fluoride, and dns-choline (1-(5-dimethylaminonaphthalene-1-sulfonamido) ethane-2-trimethylammonium) were purchased from Sigma (St. Louis, MO), and all other chemicals were obtained from Aldrich (Milwaukee, WI), unless otherwise stated.

Bromoacetylcholine bromide was synthesized from choline bromide and bromoacetyl bromide (Damle et al., 1978). [^{3}H]Acetylcholine iodide was synthesized from [^{3}H]acetic anhydride and β-dimethylaminoethanol (Anjaneyulu, 1985). Dns-C_6-Cho ([1-(5-dimethylamino naphthalene)sulfonamido]*n*-hexanoic acid b-(*N*-trimethylammonium bromide) ethyl ester) was synthesized by a published method (Waksman et al., 1976).

Preparation of nAcChoR-rich membranes from *Torpedo nobiliana*

Receptor-rich membranes were obtained from the electric organ of *Torpedo nobiliana* and prepared for reconstitution in homogenization buffer (10 mM NaH_2PO_4, 5 mM EDTA, 5 mM EGTA, 0.02% NaN_3 at pH 7.4) made 0.1 mM in phenylmethylsulfonyl fluoride and 10 mM in iodoacetamide immediately before use (McCarthy and Moore, 1992). Native nAcChoR membranes were prepared as previously described (Braswell et al., 1984). Membrane suspensions were kept frozen at −80°C until used.

Purification and reconstitution of nAcChoR by affinity chromatography

nAcChoR was purified and reconstituted into lipid vesicles by a modification of a published procedure (Ellena et al., 1983) in which Affi-gel 102 was used in place of Affi-gel 401. It was derivatized with DL-*N*-acetyl homocysteine thiolactone and then modified with bromoacetylcholine bromide. Rereconstitution by lipid substitution was performed according a published method (Ochoa et al., 1983). The ratio of phospholipid to receptor was generally in the range of 250–500:1 (mol:mol), but a few preparations were prepared at higher and lower lipid ratios (4000–140:1). Depletion of cholesterol from reconstituted nAcChoR samples by using cholesterol-free liposomes was performed as described previously (Leibel et al., 1987).

Characterization of reconstituted nAcChoR

Protein concentration (Markwell et al., 1978), phospholipid content (McClare, 1971), and cholesterol content (Rudel and Morris, 1973) were determined by standard methods. All bilayer compositions are given in mol%. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli, 1970) was performed under denaturing conditions to verify the identity and purity of the receptor. Two methods were used to determine the concentration of agonist-binding sites. First, by competition with the fluorescent cholinergic ligand, (dansylaminoethyl) trimethylammonium perchlorate (Dns-Cho), by acetylcholine (Neubig and Cohen, 1979) and, second, by a [^{3}H]AcCho-binding assay (Braswell et al., 1984). Receptor concentration refers to the oligomer unless agonist sites are specified (two agonist sites per oligomer).

Electron microscopy

Reconstituted nAcChoR samples were visualized by electron microscopy. Small squares of carbon-coated mica were cut out, and a drop of dilute nAcChoR in buffer A was placed on the edge of each square, with capillary action forcing the sample down between the carbon and the mica. The mica square was floated on stain solution, 1% sodium silicotungstate in water (the carbon with the sample floated on the surface while the mica sank to the bottom), and a copper grid was placed over the carbon on the stain surface and the sample allowed to stain for ~1 min. The copper grid with the sample adhering to it was removed and air dried.

Steady-state fluorescence experiments with ethidium

Steady-state fluorescence experiments were carried out according to a published method (Herz et al., 1987). In brief, ethidium (1 μM) was dissolved in *Torpedo* physiological saline (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM sodium phosphate, and 0.02% NaN_3 at pH 7.0), and an emission spectrum was acquired with an SLM DMX-1000 spectrofluorimeter. Ethidium fluorescence was stimulated by using an excitation wavelength of λ_{ex} = 500 nm; each emission spectrum was scanned from 525 to 700 nm. Experiments were performed at 20°C. An aliquot of native membranes (final concentration 2 μM acetylcholine sites) was then added to the quartz cuvette and an emission spectrum acquired. Carbachol (carbamylcholine; final concentration 1 mM) was then added, and the corresponding emission spectrum recorded. A final spectrum was acquired in the presence of 500 μM phencyclidine (PCP) to determine the extent of nondisplaceable ethidium binding. Samples were corrected for dilution effects.

Stopped-flow fluorescence energy transfer experiments were performed with a BioSX-17 MV spectrofluorimeter, with a 150-W xenon lamp equipped with a input monochromator and with a filter at the collection port; a T-mixer is integrated into the quartz optical cell (Applied Photophysics, Leatherhead, England). All experiments were carried out at 20 ± 0.4°C in *Torpedo* physiological saline. Equal volumes of fluorophore (plus ligands where appropriate) solution and nAcChoR vesicle suspension were rapidly mixed to give a final receptor concentration of 0.2 μM, equivalent to 0.4 μM in acetylcholine-binding sites. Tryptophan residues were excited at 290 nm (0.5-mm slit), and the binding of ethidium or Dns-C_6-Cho was recorded with a 530-nm high-bandpass cutoff filter. Between four and eight traces were acquired digitally for each set of conditions and then signal averaged. Each trace (2000 data points) was acquired for 500 s in the logarithmic timebase mode. Data collection was continued if the reaction did not reach equilibrium within this time.

When required, brief preincubations of receptor with one of the ligands was carried out with the BioSX-17 MV configured in the sequential mixer mode.

Traces of fluorescence intensity versus time were fit to an equation of the sum of the appropriate number of exponentials as described previously (Raines et al., 1995). If the rates and amplitudes of each exponent were determined as a function of agonist concentration, they were fit by a nonlinear least-squares routine to the logistic equation

$$x = (x_{max} - x_{min}) \cdot \left(\frac{[A]^n}{[A]^n + EC_{50}^n} \right) + x_{min} \qquad (1)$$

where x, x_{max}, and x_{min} are the rates ($x = k$) or amplitudes ($x = F$) observed at the experimental, [A], highest, and lowest agonist concentrations, respectively; n is the Hill coefficient; and EC_{50} is the half-effect concentration. Errors given are standard deviations unless otherwise noted.

Channel inhibition

The net efflux of $^{86}Rb^+$ from sealed native vesicles elicited during 10-s exposure to carbachol (10 mM) at 4°C was measured in the presence of the increasing concentrations of ethidium to determine its inhibition curve as previously described (Forman et al., 1987).

RESULTS

Establishing the kinetic paradigm

Titration of carbachol-induced state transitions

When nAcChoRs (0.2 μM) were rapidly mixed with ethidium (0.2 μM) and increasing concentrations of carbachol (2.5 nM to 25 mM), a series of traces were obtained (Fig. 1). Low concentrations of carbachol (≤2.5 μM) resulted in fluorescence traces resembling those obtained for ethidium alone, in which the major fraction of the intensity only began to develop after 10 s. However, at higher concentrations (>10 μM carbachol), fluorescence increased strongly, with a major increase occurring between 100 ms and a few seconds, after which a plateau was achieved and maintained until ~100 s had elapsed. Beyond this, a further slow increase occurred, paralleling, with a slight lag, that seen at lower carbachol concentrations. The traces at low carbachol concentrations were best fit to the sum of two exponentials (called "fast" (time constant of 2 s) and "slow" (time constant of 140 s)), but above ~10 μM carbachol a third exponent (designated "early") was necessary. The slow component of ethidium fluorescence represents slow desensitization and has been characterized previously (Quast et al., 1979; Herz et al., 1987). In the present work, we wish to focus on the rapid, agonist-induced component, particularly the early one.

The early component

The fractional amplitude of the early component was insignificant below 2.5 μM carbachol, but increased rapidly above 10 μM, to reach a plateau of 20–30% of the total amplitude (Fig. 2). The apparent rate of this component also increased with carbachol concentrations above 10 μM to reach a plateau above 1 mM (Fig. 3). The carbachol concentration for the half-maximum rate was 0.22 ± 0.046 mM, and the Hill coefficient was 0.9 ± 0.16.

When the carbachol concentration was held constant at 25 mM and the ethidium concentration varied, the observed rate was independent of ethidium concentration in both native and reconstituted membranes. Because the nonspecific binding was so low, the relationship was best examined in reconstituted receptors. In two preparations with the composition PC:phosphatidic acid:Chol (30 mol%), the observed rate was independent of the ethidium concentration from 0.2 to 25 μM; a linear fit had a slope of −1.6 (± 1.1) 10^4 M^{-1} s^{-1} (p = 0.2) and an intercept of 2.9 ± 0.32 s^{-1}. Similar results were obtained for native membranes over the

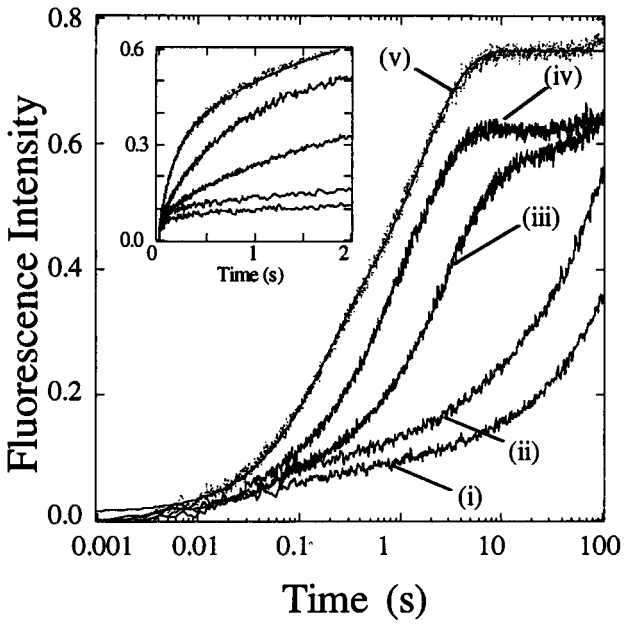


FIGURE 1 Effect of carbachol on fluorescence traces of ethidium-labeled native nAcChoR membranes. Native membranes were rapidly mixed to give 0.2 μM receptors with 0.2 μM ethidium plus the following carbachol concentrations in the stopped-flow fluorimeter's cell: (*i*) no carbachol, (*ii*) 2.5 μM, (*iii*) 25 μM, (*iv*) 250 μM, and (*v*) 25 mM. All traces were corrected for nondisplaceable binding and have been normalized to the maximum fluorescence obtained with 2.5 μM carbachol at 2000 s. The line through the 25 mM data is the nonlinear least-squares fit. The inset shows the same traces represented on a linear time scale.

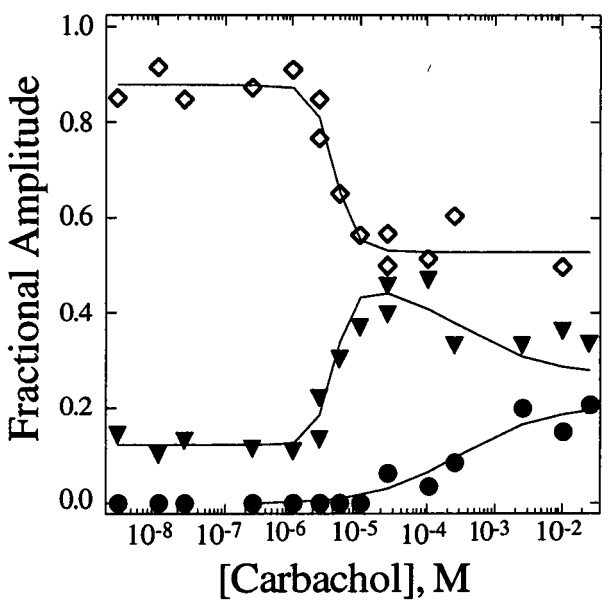


FIGURE 2 The fractional amplitudes of the three components of ethidium fluorescence as a function of carbachol concentration. The traces, such as those in Fig. 1 from native membranes, were fit to an equation that was the sum of three exponentials after nondisplaceable binding had been subtracted. ●, Early component; ▼, fast component; ◇, slow component. The curves for the slow and early components were determined by nonlinear least-squares fitting; the curve for the fast component represents the remaining relative amplitude.

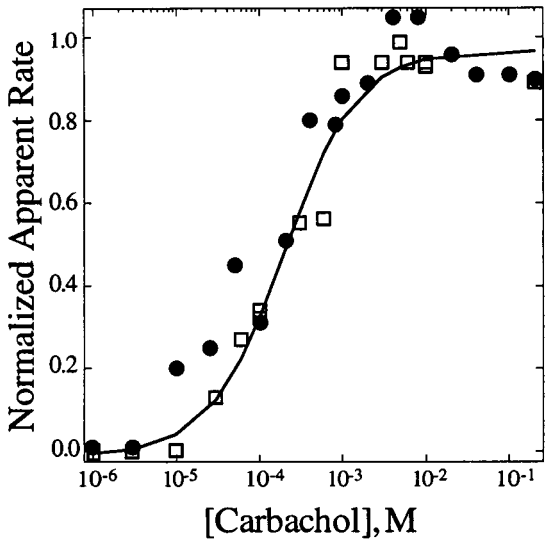


FIGURE 3 Carbachol concentration dependence of the apparent rate of the early component. The traces, such as those in Fig. 1, were fit to an equation that was the sum of three exponentials after nondisplaceable binding had been subtracted. Results for native membranes (□) are from two separate titrations not presented in Fig. 2, and those for reconstituted membranes (●; DOPC:DOPA:Chol (36%)) are from a single experiment. The data have been normalized to the maximum rate to aid comparison. Parameters of the native membrane curve (Eq. 1): maximum rate 7.3 ± 0.36 s^{-1}, carbachol concentration at half-maximum rate (EC_{50}) 220 ± 46 μM, and Hill coefficient 0.9 ± 0.16. Comparable parameters for the reconstituted membrane: 2.7_5 ± 0.14, 120 ± 45 μM, and 0.8 ± 0.21.

range 0.1–5 μM ethidium ($p = 0.6$). The absolute amplitude increased sigmoidally, but the relative amplitude remained constant.

The fast component

Unlike the early component, the fast component was even observed in the absence of carbachol. It contributed 12 ± 1.8% of the total fluorescence up to 1 μM carbachol, above which the relative amplitude increased sharply, with a midpoint of 5 μM and a maximum of 45 ± 2.6% of the total amplitude at 100 μM carbachol. The increasing fractional amplitude of the fast component mirrors the decreasing amplitude of the slow component as the carbachol concentration increases. Their midpoints and the absolute values of their Hill coefficients were identical within error. Above 100 μM carbachol, the absolute amplitude of the fast component was constant, whereas the relative amplitude fell because the amplitude of the early component increased over the same concentration range (Fig. 2). The apparent rate of this component also increased sigmoidally from 0.19 ± 0.029 s^{-1} up to 1 μM carbachol to 0.66 ± 0.037 s^{-1} above 100 μM carbachol, with a half-maximum rate at 26 ± 5.4 μM and a Hill coefficient between 1 and 2.

When ethidium was titrated in native and reconstituted membranes in the presence of high concentrations of carbachol as described in the above section, the fast component's apparent rate was also independent of ethidium concentration. Its absolute amplitude increased sigmoidally with increasing ethidium concentration, but its relative amplitude (35 ± 5.6%, Fig. 2) was independent of ethidium concentration. However, in the absence of carbachol, its relative amplitude was only 17 ± 3.6% and the observed rate independent of ethidium concentration.

Effect of preconditioning the nAcChoR with carbachol

With the stopped-flow fluorimeter in sequential mixing mode, nAcChoRs and carbachol were rapidly mixed and forced into an aging tube, where their concentrations were 0.4 μM and 30 mM, respectively. After 10 s the contents of the aging tube were rapidly mixed with carbachol and ethidium at the second mixer, to give final concentrations in the optical cell of 0.2 μM nAcChoR, 1.0 μM ethidium, and 30 mM carbachol. Fig. 4 compares 1) the simultaneous mixing of receptors with carbachol and ethidium and 2) receptors preconditioned with carbachol and then mixed with ethidium. The early component, which had a total amplitude in the control experiment of 117 ± 1.4 mV, was completely abolished by preincubation with carbachol for 10 s.

Similar results were obtained using nAcChoRs reconstituted into 1,2-dioleoyl-sn-glycero-3-phosphocholine/1,2-dioleoyl-sn-glycero-3-phosphate/cholesterol (DOPC/DOPA/cholesterol) vesicles with a molar ratio of 63/12/25 under the same conditions as above.

Ethidium inhibits agonist-induced cation flux

Ethidium's apparent affinity for the open state was estimated from its ability to inhibit maximum-carbachol stim-

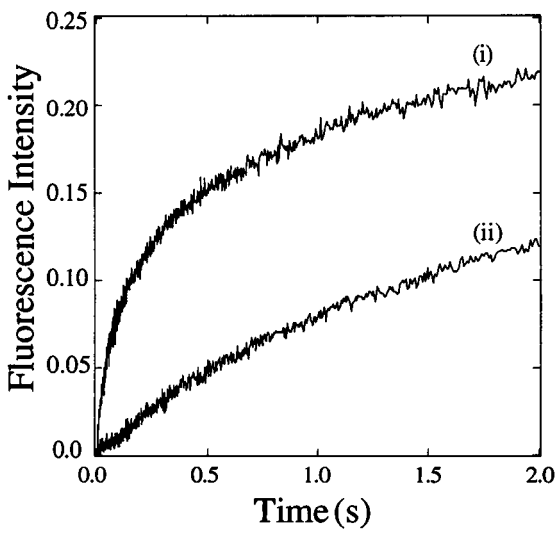


FIGURE 4 The effect on ethidium fluorescence of preconditioning the nAcChoR with carbachol. Trace i is native membranes rapidly mixed with ethidium and carbachol to give 0.4 μM sites, 1 μM ethidium, and 30 mM carbachol. Trace ii is native receptor incubated with carbachol for 10 s and then rapidly mixed with ethidium (final concentrations were 0.4 μM ACh sites, 30 mM carbachol, and 1 μM ethidium). Traces have been corrected for nondisplaceable binding.

ulated $^{86}Rb^+$ efflux. Using a 10-s manual assay (Forman et al., 1987), it was found that above 10 μM, ethidium caused a concentration-dependent inhibition, which was essentially complete at 300 μM and had an IC_{50} of 40 $\pm$ 12 μM and a Hill coefficient of 1.4 $\pm$ 0.39.

Nondisplaceable binding of ethidium

Nonspecific binding of ethidium was resolved either by preincubating nAcChoRs with 500 μM PCP, with or without 10 mM carbachol, and rapidly mixing them with ethidium, or by adding all of the ligands simultaneously to the nAcChoRs. The two methods gave identical results. Fluorescence followed a single exponential. Nondisplaceable binding in native membranes was ~6% and 20% of the total amplitude at 0.2 and 2 μM ethidium, respectively. In reconstituted samples, nondisplaceable binding was ~9% of the total amplitude at 2 μM ethidium, considerably lower than in native membranes, and the rate of the early component was independent of ethidium concentration, justifying the strategy of using 2 μM ethidium to enhance the signal of scarce reconstituted preparations.

The effects of lipid bilayer composition

Characteristics of reconstituted membranes

Affinity-purified and reconstituted membranes contained only the four AcChoR subunits plus minor high-molecular-weight components at $\geq$100 kDa. Electron micrographs of these membranes revealed only the well-known rosette-shaped receptor independent of the lipid composition. Native membranes formed large vesicles that were inhomogeneous in size, whereas those formed with receptors in DOPC:EPA:Chol (60:12:28) were smaller. Receptors reconstituted in DOPC:EPA (88:12) exhibited a mixture of both sausage-shaped vesicles and sheets, whereas in DOPC only sheets were seen. This lipid-dependent morphology is consistent with reports in the literature (Pradier and McNamee, 1992).

Role of lipid in carbachol-induced state transitions

In an initial survey, receptors (0.2 μM) were rapidly mixed with ethidium (2 μM) plus carbachol (0, 3 μM or 10 mM; all final concentrations). At the lower concentration most of the fluorescence derived from the slow component, whereas at the higher concentration the early and fast components were dominant. The four representative reconstituted receptors surveyed had lipid compositions of DOPC, DOPC:Chol (28%), DOPC:DOPA, and DOPC:DOPA:Chol (25%), where the molar proportion of DOPA was kept constant at 12% of the total lipid because this reproduces the average charge density in native membranes, assuming that the average negative charge is 1.5 per molecule of DOPA (Pott et al., 1995).

At 0 and 3 μM carbachol, the composition of the bilayer had very little effect on the kinetics (not shown). In contrast, 10 mM carbachol (Fig. 5) revealed dramatic differences between the four reconstituted membranes at subsecond times: only those containing cholesterol supported fast agonist-induced transitions independent of the presence of DOPA. Over the full 2000 s of the experiment, the traces for the cholesterol-containing bilayers had to be fit to four exponentials, whereas the others could be fit to three. The rates and amplitudes of all components were all comparable to those for native membranes.

Cholesterol titration

Receptors were reconstituted into bilayers of DOPC:DOPA:Chol in the proportions ($\{88 - x\}$:12:x), where x was varied from 0 to 50 mol% and confirmed experimentally. Typical traces from single experiments with 10 mM carbachol illustrate the effect of increasing the mol% of cholesterol in the bilayer from 0 to 24% (Fig. 6). In the region where the early component was fully established (~0.5–1 s), the amplitude of the traces increased with cholesterol content most markedly at the lowest cholesterol contents examined.

To verify that there was no residual activity in reconstituted receptors lacking cholesterol, we examined six different preparations containing no cholesterol, three each of DOPC and DOPC:DOPA. Nonspecific binding was subtracted in all cases; it was small and usually less than 5% of total amplitude. To confirm these subtractions, we subtracted the uncorrected traces with and without carbachol. Both subtraction methods failed to reveal any early component in the absence of cholesterol.

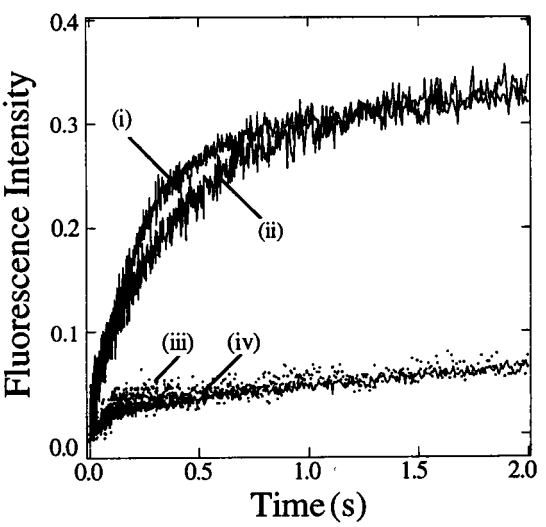


FIGURE 5 The effect of lipid composition on the state transitions induced by a saturating concentration of agonist (10 mM carbachol) detected with 2 μM ethidium. Final AcChoR concentration was 0.2 μM. Traces are averages of 4–6 shots and have been normalized to the maximum fluorescence obtained at 2,000 s. Trace i is DOPC:DOPA:Chol (63:12;25); trace ii is DOPC:Chol (72:28); trace iii (*dotted trace*) is DOPC:DOPA (88:12); trace iv is DOPC.

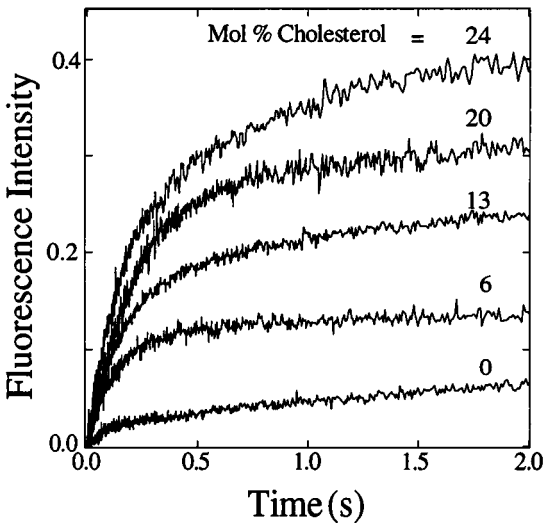


FIGURE 6 Effect of cholesterol on state transitions induced by a saturating concentration of agonist (10 mM carbachol). AcChoRs were reconstituted into a bilayer of DOPC:DOPA:Chol in the proportions ({88 − x}:12:x), where x has the value shown. Final AcChoR and ethidium concentrations were 0.2 μM and 2 μM, respectively; traces have been normalized to the maximum fluorescence obtained at 2000 s with 2.5 μM carbachol.

To provide controls for the process of reconstitution, a number of samples were obtained by postpurification manipulations. For example, a preparation with 42% cholesterol was depleted of cholesterol by incubation with DOPC vesicles to give samples of 30 and 5 mol% cholesterol. Similarly, the two highest cholesterol concentrations were achieved by lipid substitution (rereconstitution) from a DOPC sample. None of these samples differed notably from similar samples prepared on the column.

Another variable surveyed briefly was the role of polyunsaturation. In one sample with 28% cholesterol, egg phosphatidylcholine was substituted for DOPC (there was no phosphatidic acid (PA)), and in another with 43% cholesterol EPA was substituted for DOPA. Neither of these variations had any effect.

Carbachol titration

The carbachol dependence of the apparent early rate in a DOPC:DOPA:Chol (36%) membrane was very similar to that of native membranes (Fig. 3) that have a half-maximum rate at 120 ± 45 μM carbachol and a Hill coefficient of 0.8 ± 0.21. The fast component rate also showed a carbachol dependence very similar to that of native membranes that have a half-maximum rate at 32 ± 20 μM carbachol and a Hill coefficient of 1.1 ± 0.1.

Studies with a fluorescent agonist

Receptors reconstituted in DOPC, DOPC:DOPA, or DOPC:DOPA:Chol (21%) were rapidly mixed with Dns-C_6-Cho to give final concentrations of 0.2 and 2 μM, respectively, yielding traces that could be fit to four exponentials. The two fastest exponentials represent Dns-C_6-Cho binding to the preexisting slow and fast desensitized conformations of the receptor (Raines et al., 1995). The amplitudes of these two exponentials varied little with lipid composition and were close to those previously reported for native membranes; that of the first exponent was 15–24% and that of the second exponent was 12–30%. The remaining amplitude reflects receptors that slowly convert from the resting to the high-affinity desensitized states. Thus the percentage of receptors initially in the low-affinity resting state was calculated to be 47 ± 2.9, 54 ± 6.5, and 61 ± 2.1 for DOPC, DOPC:DOPA, and DOPC:DOPA:Chol, respectively, compared to 57% in native membranes under the same conditions.

DISCUSSION

Rapid ethidium kinetics

The early component

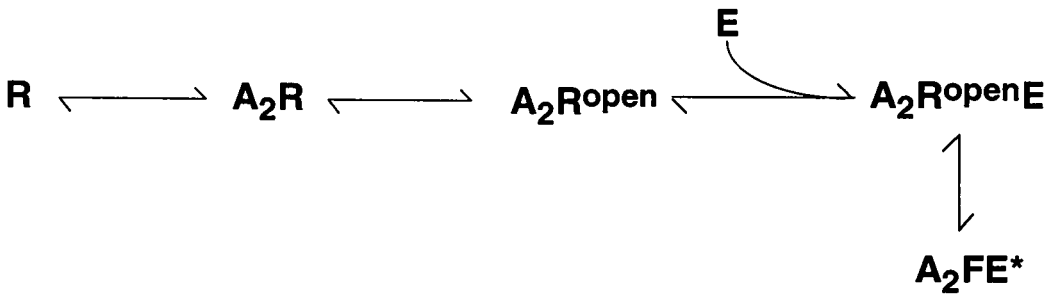
The early component's rate was independent of ethidium concentration and therefore indicates a conformational change. The maximum observed rate is very close to that for fast desensitization, which we have determined in rapid flux studies in *Torpedo nobiliana* membranes (Forman and Miller, 1988). Similar rates have been reported for *Electrophorus* (Hess et al., 1982) and for *Torpedo californica* (Walker et al., 1982). Such a carbachol-induced increase in quantum yield would be analogous to that detected without change in [^{3}H]ethidium binding on the desensitized state (Schimerlik et al., 1979). The carbachol dependence of fast desensitization (via flux studies) in *T. californica* membranes reconstituted into asolectin or in *Electrophorus* has a half-point of ~1 mM carbachol for fast desensitization (Walker et al., 1982; Hess et al., 1982), somewhat higher than the value we observe. The origin of the small difference might be either the different species and conditions or that flux reports a global change in all of the subunits, whereas ethidium reports more selectively.

At what point before the quantum yield increase accompanying the $A_2R^{open} \rightarrow A_2F$ step does ethidium bind? Based on our data, it seems likely that ethidium is a slow open channel blocker. We have found that the early component's amplitude is decreased by procaine and by self-inhibitory concentrations of carbachol (>100 mM), which suggests a location on the open state of the receptor (Forman and Miller, 1989) such that its quantum yield increases upon desensitization as the aqueous pore closes (Scheme I). This likely explains the high apparent IC_{50} reported by our flux assay, because during the assay the majority of the $^{86}Rb^+$ is released in the first 30 ms. Procaine's on rate at this site is $2.5 \times 10^5\ M^{-1}\ s^{-1}$ (Forman and Miller, 1989), and a much larger molecule like ethidium would be expected to be considerably slower. The resting state can be excluded both because it is estimated by radiolabel and fluorescence binding assays to have an affinity for ethidium of only 0.1–1

mM (Schimerlik et al., 1979; Cohen et al., 1985; Herz et al., 1987) and because brief preincubation with ethidium did not enhance the early component. Furthermore, we can expect that carbachol binding and channel opening will occur in the deadtime of our instrument (<1 ms). We did observe enhanced ethidium fluorescence in the deadtime, which increased with ethidium concentration, but this binding was nonspecific and not displaced by PCP. The only ligand affecting deadtime fluorescence was carbachol, which caused a small decrease consistent with ethidium's reported low-affinity binding to the agonist site (Pedersen, 1995). Thus it is clear that the early component allows us to achieve our first goal of monitoring the occurrence of the transient states induced by high carbachol concentrations without the necessity of conducting flux experiments. The fact that we are monitoring transient conformational states is clear when we force all of the receptor into the fast desensitized state by a 10-s preincubation of carbachol with the receptor (Fig. 4). The addition of ethidium at this point reveals the total loss of the early component, indicating that the transient state to which it binds is no longer available.

bilayer clearly revealed, especially in the fast state transitions. There was no evidence for substantial changes in the distribution of states existing in the absence of agonist due to the differing lipid compositions examined. The most dramatic effect was the absolute dependence of the early component's amplitude on cholesterol content but not on other variables, such as polyunsaturation, lipid:protein ratio, surface charge, or the membrane changes associated with the reconstitution procedure (Figs. 5–7). This effect was clearly present at the lowest cholesterol concentration we examined (5 mol%). In contrast, the rate of the early component, representing fast desensitization, was insensitive to any manipulation (Fig. 7).

Our evidence suggests that carbachol binds the resting state receptors but fails to open the channel unless cholesterol is present. Referring to Scheme I and recalling that the proportion of R state receptors is not strongly affected by lipid composition, the absence of cholesterol could either prevent the transition from the activated to the fast desensitized state or the attainment of the activated state. The



Scheme 1

The fast component

This component was observed in the absence of carbachol, where it represents ~15% of the total fluorescence amplitude; with carbachol the maximum amplitude was ~36%. In each case the observed rate is independent of ethidium concentration. The dependence of the observed rate on carbachol concentration had a half-maximum of ~30 μM carbachol. Similarly, the slow component of ethidium fluorescence was also independent of ethidium concentration but was dependent on carbachol, with a half-maximum rate at 4 μM carbachol (Rankin, 1996). It is possible that these processes represent conformational changes associated with preexisting fast and slow desensitized receptors, but more detailed assignments were not attempted because this component was found not to vary with lipid composition.

The role of lipids in rapid state transitions

In the absence of agonist, all of the lipid mixtures exhibited interactions with ethidium, but only when activating concentrations of agonist were added was the role of the lipid

former explanation is unlikely because the rate and amplitude of the early component depend on cholesterol in different ways and because prior experiments indicate that no agonist-dependent transmembrane cation flux is observed in the absence of cholesterol (Fong and McNamee, 1986). The early component might, therefore, be absent because carbachol fails to activate resting-state receptors through lack of either efficacy or affinity. The latter possibility is difficult to rule out completely, because at the high concentrations required to test this hypothesis, carbachol binds to the channel inhibition site on the receptor with an apparent dissociation constant of ~200 mM (Forman et al., 1987). However, in cholesterol-free membranes we found that concentrations of carbachol as high as 200 mM failed to promote the early component. Therefore it follows that cholesterol is intimately involved in receptor activation, and lack of it lowers carbachol's efficacy to zero.

Slow flux studies (Fong and McNamee, 1986; Sunshine and McNamee, 1992; Perez-Ramirez, 1994) and preliminary rapid flux studies (Fernandez et al., 1993; Fernandez-Ballester et al., 1994) have also demonstrated the need for

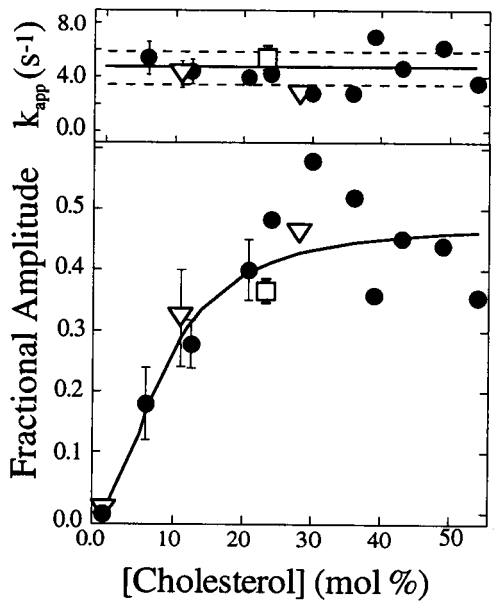


FIGURE 7 The amplitude, but not the rate, of the early component depends on the mol% of cholesterol in the bilayer. Traces, such as those in Fig. 6, were fit to the sum of three or four exponentials after nondisplaceable binding had been subtracted. The curves shown in the lower panel were determined by nonlinear least-squares fitting. In the upper panel the solid line is the mean and the dashed lines the standard deviations of the reconstituted membranes (4.7 ± 1.2; n = 19); the comparable values in native membranes were not significantly different. ●, DOPC:DOPA:Chol in the proportions ({88 − x}:12:x); ▽, DOPC:Chol in the proportions ({100 − x}:x); □, native membranes. Symbols without error bars (standard deviations) represent a single experiment. The six zero cholesterol points are means of three preparations each for DOPC:DOPA and DOPC. The curve has a maximum fractional amplitude of 0.46 ± 0.052, a cholesterol concentration at half maximum amplitude (EC_{50}) of 9 ± 1.7 mol%, and a Hill coefficient of 1.9 ± 0.69.

cholesterol for agonist-induced channel activation. However, because flux assays are sensitive to the morphology of the vesicles, these workers were unable to make detailed comparisons between different lipids. The advantage of using ethidium is particularly clear when AcChoRs are reconstituted into DOPC, which forms sheets incapable of trapping ions. It is now possible to conclude unequivocally that channel activation does not occur in DOPC membranes.

Our work differs in one other respect from previous studies: we find that the presence of DOPA in DOPC confers no particular advantage, whereas others have found that the slight flux response of DOPC:Chol reconstitutions was dramatically enhanced by the addition of DOPA (Fong and McNamee, 1986). The explanation for this may lie in vesicle morphology, because negatively charged lipids tended to increase the internal volume of vesicles.

Mechanistic basis for cholesterol's action

A priori, a number of mechanisms might explain the role of cholesterol in agonist-induced activation. These include 1) lipid fluidity; 2) aggregation; 3) binding of cholesterol to specific sites on the receptor but not in the lipid-protein interface (nonannular or interstitial sites); 4) an action in the lipid-protein interface, such as hydrophobic mismatching between the dimensions of the bilayer and the hydrophobic region of the receptor (Bloom et al., 1991); and 5) a change in intrinsic curvature brought about by conformationally induced perturbation. Our data only allow us to comment on some of these models.

Lipid fluidity was originally suggested to explain the dependence of channel activity on lipid composition (Fong and McNamee, 1986) but has subsequently been ruled out by two studies (Sunshine and McNamee, 1992; Fernandez-Ballester et al., 1994). Our data support this conclusion. For example, changes from ~20 to 50 mol% cholesterol did not have any effect, and in one sample (43% cholesterol), when egg PA was substituted for DOPA, neither the rate nor the amplitude of the early component changed significantly. We cannot rule out a "buffering" effect on fluidity in the lipid-protein interface, which has been postulated to occur during slow desensitization but not during activation (Narayanaswami and McNamee, 1993; Sunshine and McNamee, 1994).

Aggregation has been implicated as an explanation of lipid modulation of sarcoplasmic reticulum Ca-ATPase activity (Cornea and Thomas, 1994). There is some evidence that in alkali-extracted AcChoR membranes fused with lipid vesicles, cholesterol may modulate the receptor's rotational correlation time, perhaps because of aggregation (Rousselet et al., 1981). This mechanism is unlikely to apply here, because varying the ratio of phospholipid to receptor at a constant phospholipid:cholesterol ratio had no effect. For example, preparations with 400 and 4100 total lipids per receptor and 12 and 13 mol% cholesterol, respectively, had very similar rates and fractional amplitudes for the early component.

Another mechanism, binding of cholesterol, is lent strong support by our data. The independent evidence is that when brominated cholesterol is titrated into receptors reconstituted into dibromo-DOPC, the intrinsic fluorescence is quenched, with a half-point of ~10 mol% cholesterol, and the effect saturates at ~30 mol% cholesterol. Because the dibromo-DOPC was expected to quench the intrinsic fluorescence at the lipid-protein interface, the additional quenching by brominated cholesterol was interpreted to represent binding of cholesterol to nonannular or interstitial sites. The authors suggested that 5–10 cholesterol binding sites might be responsible (Jones and McNamee, 1988). When we normalize the cholesterol titration (Fig. 7) to the fluorescence quenching data, the agreement of the data is convincing within experimental error (Fig. 8).

Although there is other evidence for a special role for cholesterol (it is not possible to remove all of the cholesterol from native membranes when it is repeatedly extracted into lipid vesicles (Leibel et al., 1987), AcChoRs have a high affinity for cholesterol in lipid monolayers (Popot et al., 1978) and bilayers (Marsh, 1990), and theoretical studies consistent with nonannular sites have been presented (Or-

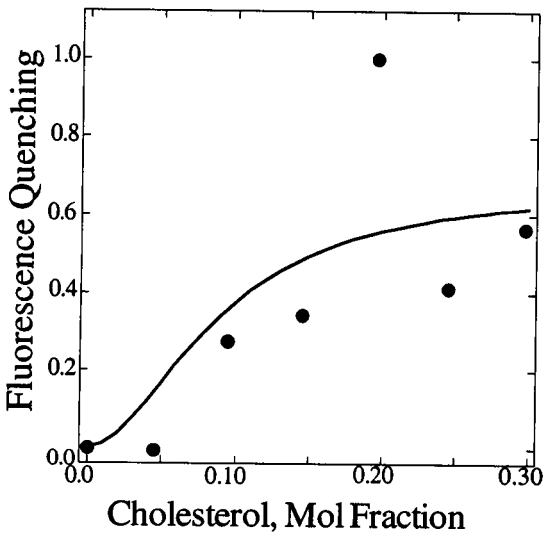


FIGURE 8 Do nonannular cholesterol sites account for cholesterol's effect on channel activation? The experimental data (●) shows the increase in quenching of the nAcChoR's intrinsic fluorescence when bromocholesterol is added to nAcChoRs reconstituted into dibromo-DOPC (arbitrary scale). This additional quenching has been attributed to nonannular cholesterol sites (Jones and McNamee, 1988). The solid line has the same parameters (EC_{50}: 0.09; Hill coefficient: 1.9; minimum: 0.0) of the functional cholesterol titration shown in Fig. 7, except that a nonlinear least-squares procedure was used to normalize the maximum to the fluorescence quenching data points.

tells et al., 1992)), one salient observation argues against a functional role for such sites. That is the remarkable observation that many other neutral "lipids," such as squalene and α-tocopherol, can support AcChoR activation in the absence of cholesterol (Kilian et al., 1980; Sunshine and McNamee, 1992). Either the putative cholesterol sites have very loose structural requirements, or some other model is required. One possible mechanism for cholesterol's action is the ability for cholesterol to freely flip from one leaflet of the bilayer to the other to relieve the mechanical stress caused by membrane bending, if receptor activation were to involve a conformation change that expanded one leaflet of the bilayer more than the other. This model would also accommodate the aforementioned lipids, which can substitute for cholesterol and support receptor activation, because all of the them can flip across the bilayer as well.

Although the physical mechanism remains unknown, an interesting corollary is that the channels of newly synthesized receptors are not fully primed by cholesterol until they are inserted into the plasma membrane—a novel form of posttranslational processing.

CONCLUSIONS

We have overcome the experimental difficulties imposed by morphological variation in monitoring agonist-induced channel activation in reconstituted AcChoRs by developing a new fluorescence assay based on ethidium. Cholesterol is absolutely required for activation to occur, but the presence of a negatively charged lipid is unimportant. Lack of channel activation is not accounted for by accelerated fast desensitization, because the rate of this process is insensitive to cholesterol. About half of the receptors are in the resting state irrespective of lipid composition; therefore they do not account for lack of activation in the absence of cholesterol. The number of channels that opened in response to high agonist concentrations increased with the percentage of cholesterol in the bilayer, reaching a maximum close to the fraction of resting-state AcChoRs. The half-point of this effect is close to 10 mol% cholesterol, which is comparable to that for saturating the postulated interstitial, or nonannular, cholesterol sites on the receptor (Jones and McNamee, 1988). In contrast, the subsequent slower conformational change into the fast desensitized state, A_2F, is not modulated by cholesterol.

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