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Reconstitution of purified acetylcholine receptors with functional ion channels in planar lipid bilayers

(membranes/synapse/signal transduction/neurotransmitters/fluctuation analysis)

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ABSTRACT **Acetylcholine receptor, solubilized and purified from *Torpedo californica* electric organ under conditions that preserve the activity of its ion channel, was reconstituted into vesicles of soybean lipid by the cholate-dialysis technique. The reconstituted vesicles were then spread into monolayers at an air–water interface and planar bilayers were subsequently formed by apposition of two monolayers. Addition of carbamoylcholine caused an increase in membrane conductance that was transient and relaxed spontaneously to the base level (i.e., became desensitized). The response to carbamoylcholine was dose dependent and competitively inhibited by curare. Fluctuations of membrane conductance corresponding to the opening and closing of receptor channels were observed. Fluctuation analysis indicated a single-channel conductance of 16 ± 3 pS (in 0.1 M NaCl) with a mean channel open time estimated to be 35 ± 5 ms. Thus, purified acetylcholine receptor reconstituted into lipid bilayers exhibited the pharmacological specificity, activation, and desensitization properties expected of this receptor in native membranes.**

The acetylcholine receptor (AcChoR) transduces the binding of the neurotransmitter acetylcholine into an increase in the cation permeability of the postsynaptic membrane (1, 2). Because the AcChoR is by far the best characterized membrane receptor, both physiologically (3) and biochemically (4–6), it provides an archetype for understanding the structure and function of signal transducing membrane proteins.

The AcChoR from *Torpedo californica* is an integral membrane protein of which the monomeric form is composed of four glycopeptide subunits in the mole ratio of $\alpha_2\beta\gamma\delta$ (7, 8). The α subunits (M_r 40,000) are known to contain the acetylcholine-binding sites (9), and the AcChoR monomer has been shown to be active in cation flux assays (10). However, the exact functions of the β (M_r 50,000), γ (M_r 60,000), and δ (M_r 64,000) subunits are still unknown.

Functional reconstitution of purified AcChoR into model membranes provides a valuable approach for investigating its mechanism of signal transduction at the molecular level. Epstein and Racker (11) recently introduced a simple and reproducible procedure for the reconstitution of AcChoR from crude AcChoR-enriched membrane preparations into vesicles by cholate dialysis in the presence of supplementary soybean lipids. This work enabled subsequent purification of the AcChoR in a functionally active form (12–15). The ion channel activity of purified AcChoR in reconstituted lipid vesicles can be assayed by measuring the translocation of radioactive cations in response to the addition of carbamoylcholine (CbmCho) (10–15). Although such measurements are rapid and simple, they lack the time resolution and sensitivity needed to study the kinetics of the response and to observe the opening and closing of single channels. Planar bilayers overcome these limitations, because the response can be monitored electrically. However, past attempts to reconstitute the AcChoR in planar bilayers have encountered many difficulties, mainly due to lack of reproducibility (6, 16).

Here we report a reproducible procedure to reconstitute purified AcChoR in planar lipid bilayers. In order to minimize denaturing effects on the AcChoR molecule, we avoided the use of organic solvents and directly spread the AcChoR incorporated in lipid vesicles into monolayers at the air–water interface (¶, 17–20). Planar bilayers were then formed by apposition of two monolayers (cf. ref. 16). This protocol enables us to measure the translocation of cations by radiochemical means in reconstituted vesicles and subsequently apply the electrophysiological assay in planar bilayers to observe the fine details of the response. We show that addition of CbmCho to the latter induces an increase in membrane conductance. In addition, this response exhibits spontaneous reversal—i.e., desensitization. Schindler and Quast (20) present results obtained by using crude vesicles from *Torpedo marmorata* enriched with AcChoR. The combined results of these preliminary phenomenological studies indicate that functional AcChoR can be reconstituted in planar lipid bilayers. Furthermore, purified AcChoR reconstituted into both vesicles (10, 12) and planar bilayers provides a powerful approach for studying the mechanism of AcChoR function.

MATERIALS AND METHODS

Purification of Functionally Active AcChoR. We have purified functionally active AcChoR from *Torpedo californica* by five methods (12). AcChoR purified by all of these procedures can be reconstituted into vesicles and subsequently into planar bilayers. All the experiments reported in this paper were performed with AcChoR purified by one of these methods. Briefly, AcChoR-rich membranes are solubilized in sodium cholate (Interchem, Montlucon, France) (2%) in the presence of soybean lipids (L-α-lecithin, Sigma) (5 mg/ml) and applied to concanavalin A conjugated to Sepharose 4B (Con A-agarose, Sigma). After extensive washing of the gel in the continuous presence of soybean lipids at 5 mg/ml, most of the contaminants, which bind with relatively low affinity to Con A-agarose, can be removed by a pre-elution with 0.2 M methyl α-D-mannoside (Sigma). Approximately 17% of the bound

Abbreviations: AcChoR, acetylcholine receptor; Con A-agarose, concanavalin A conjugated to Sepharose 4B; CbmCho, carbamoylcholine; ACF, autocorrelation function.

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AcChoR is then eluted with 1.0 M methyl α-D-mannoside in 10 mM sodium phosphate buffer, pH 7.5/100 mM NaCl/1 mM EDTA/5 mM NaN_3/2% sodium cholate/5 mg of soybean lipids per ml. The specific activity of this preparation averages 8.0 nmol of binding sites for ^{125}I-labeled α-bungarotoxin per mg of protein, and acrylamide gels in sodium dodecyl sulfate show that the preparation consists predominantly of the four protein bands characteristic of purified AcChoR, with only some contamination of high molecular weight material remaining (12).

Reconstitution of AcChoR into Lipid Vesicles. The eluate from the Con A-agarose was adjusted to a lipid concentration of 25 mg/ml, using a sonicated stock dispersion of soybean lipids at 150 mg/ml in distilled water. Final concentrations of the other components in the reconstitution mixture were 4 mM sodium phosphate buffer (pH 7.5), 100 mM NaCl, 2% sodium cholate, and approximately 1–2 μM AcChoR. After shaking for 10 min at 4°C, the mixture was centrifuged for 15 min in an Eppendorf microcentrifuge, and the supernatant was dialyzed for 16–18 hr at 4°C against 500 vol of 4 mM sodium phosphate buffer/100 mM NaCl, pH 7.5, and then for the same period against 500 vol of 10 mM sodium phosphate buffer/145 mM sucrose/10 mM NaN_3, pH 7.5. The final protein concentration ranged between 100 and 300 μg/ml and that of AcChoR between 1 and 2 μM. The reconstituted vesicles could be stored frozen at −70°C.

Assays. Protein was determined by the method of Lowry *et al.* (21), using bovine serum albumin as standard. AcChoR concentration was determined as the concentration of ^{125}I-α-bungarotoxin-binding sites by radioimmunoassay (22).

Spreading of Vesicles and Formation of Planar Bilayers. Liposomes from partially purified soybean phospholipids (23) at a concentration of 40 mg/ml in 80 mM Tris–glycine buffer, pH 8.0, were prepared by 10-min sonication under argon in a Bransonic waterbath sonicator. A 50-μl sample of reconstituted vesicles (at ≈1–2 μM AcChoR) was supplemented with 100 μl of liposomes. This mixture was sonicated for 5 min, diluted to 1.0 ml in 2.5 mM Tris–glycine, pH 7.7/0.1 M NaCl/10 mM $MgCl_2$, and then incubated for 10 min on ice. During this incubation the suspension becomes progressively turbid. Samples (100–200 μl) of the turbid suspension were spread over a clean air–water interface by gently delivering 10-μl drops onto the surface. The aqueous phase contained 10% sucrose in 2.5 mM Tris–glycine at pH 7.7, 0.1 M NaCl, and 10 mM $MgCl_2$. An equilibration period of between 15 min and 1 hr is required before stable bilayers can be formed.

Planar bilayers were formed from monolayers and their electrical properties were studied as described (24, 25). All bilayer experiments were done at room temperature. A Teflon film (12.5 μm thick) with an aperture (diameter 0.25 mm) was sealed with silicone grease to the septum of a two-compartment Teflon chamber; the volume of each compartment was 5.0 ml and the area was 3.5 cm^2. Bilayers were formed by sequentially raising the water level in the two compartments above the aperture. Calomel or silver/silver chloride electrodes were used throughout.

Current was measured with a no. 515 operational amplifier (Analog Devices); the feedback resistance was 10 GΩ and the time constant was 3 ms. Membrane current was recorded directly on a storage oscilloscope or amplified and stored on a RACAL 4DS tape recorder (frequency response dc-5000 Hz) for subsequent analysis. The autocorrelation function (ACF) (26) of current fluctuations was calculated by using a Honeywell–Saicor 43 A correlator after band-pass filtering (0–150 Hz) and digitization at 1-ms sample intervals over 32 × 1024 summations. The ACFs of the current fluctuations were stored in a PDP 11/34 computer. Time constants were determined by fitting one or two computer-generated exponentials to the data points displayed simultaneously on an oscilloscope screen.

RESULTS

AcChoR, purified by affinity chromatography on Con A-agarose in the continuous presence of soybean lipid and then reconstituted into lipid vesicles, showed a CbmCho-induced dose-dependent uptake of $^{22}Na^+$ which was subject to desensitization, as is described in detail elsewhere (12). Uptake of $^{22}Na^+$ in response to 0.1 mM CbmCho was routinely assayed for all vesicle preparations before they were used to spread monolayers.

Planar bilayers, formed by apposition of two monolayers, were observed for at least 15 min before further manipulation. Only stable bilayers that covered the hole in the Teflon septum completely and showed a membrane capacitance (±SD) of 0.75 ± 0.1 μF/cm^2 were used for experimentation. These membranes were often stable for up to about 3 hr. The initial bilayer conductance ranged from 10 pS to ≈4000 pS, with the occurrence of spontaneous conductance fluctuations (≈50 pS, see Fig. 1*B*). Membranes with an initial conductance of <10 pS gave no response to CbmCho.

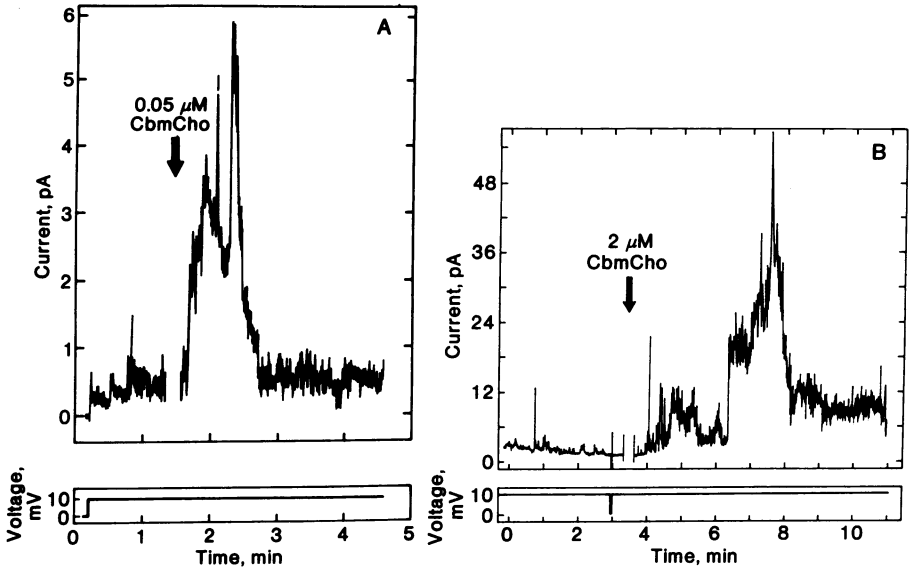

FIG. 1. Time course of the CbmCho-induced conductance increase obtained from purified AcChoR incorporated in symmetric planar lipid bilayers. A voltage step of 10 mV, positive in the side of CbmCho addition, was applied across the membrane and held constant throughout the record as shown in the lower trace. The current flowing across the bilayer in response to the applied voltage is displayed in the upper trace. The arrow indicates the time at which CbmCho was added. Samples (10 μl) of the concentrated solution were added to final CbmCho concentration of 0.05 μM (*A*) or 2 μM (*B*) and mixed by five up-and-down strokes of the Pipetman pipetter. The electrical artifact introduced by the stirring maneuver was deleted from the record as indicated by the interruption in the trace.

Table 1. Concentration dependence of the CbmCho-induced conductance response of AcChoR in symmetric planar lipid bilayers

CbmCho, μM	Increase in membrane conductance at peak, pS*	Channels opened*†
0.05	412 ± 118 (5)	26 ± 7 (5)
0.2	1700 ± 1017 (5)	106 ± 63 (5)
2	6680 ± 1920 (5)	417 ± 120 (5)

Experimental conditions as in Fig. 1 legend.
* Values expressed as mean ± SD (number of experiments).
† Estimate based on a single-channel conductance of 16 pS.

Addition of CbmCho to one of the aqueous compartments caused a large increase in the membrane conductance, which spontaneously relaxed back to the initial level. This decay corresponds to the physiological phenomenon known as desensitization (27). Fig. 1 demonstrates the dose dependence of this response. Doses of CbmCho as low as 25 nM produced a small, but distinct, change in conductance, which was of short duration. The response to 50 nM CbmCho is shown in Fig. 1*A*. At 2 μM, CbmCho evoked large increases in the membrane conductance that were rapidly limited by desensitization (Fig. 1*B*). The desensitization process could be enhanced by addition of another dosage of CbmCho at the peak of the response (data not shown). Larger concentrations of CbmCho sometimes caused the conductance to increase to such an extent that rupture of the membrane occurred before the peak response could be observed. Table 1 shows the results of the macroscopic response at the concentrations of CbmCho tested, averaged over five different experiments.

Asymmetric bilayers formed by apposing two monolayers composed of AcChoR–lipid and lipid without AcChoR showed asymmetric responses to CbmCho, as is illustrated in Fig. 2*A*. When CbmCho was applied to the compartment limited by the lipid monolayer, small (Fig. 2*A*) or no (data not shown) changes in conductance resulted. Subsequent addition of the same concentration of CbmCho to the AcChoR–lipid side then gave rise to the characteristic response. This demonstrates that the AcChoR displays a preferential orientation in the system. Furthermore, this experimental strategy provides a built-in control as far as discarding unspecific pharmacological effects.

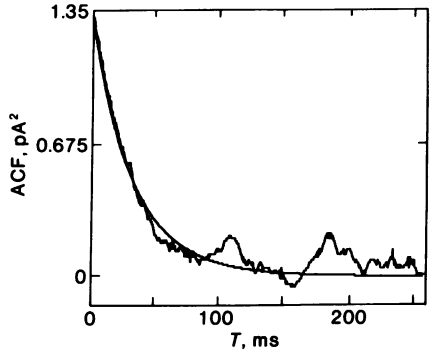

FIG. 3. ACF of the CbmCho-induced current fluctuations in planar lipid bilayers containing AcChoR. Data points represent values of the ACF(T) for relative time displacement T. The ACF is fitted by a single exponential with a time constant τ = 32.5 ms. The experimental conditions were as in Fig. 1*B*. The applied voltage was +10 mV. Estimation of the single-channel conductance from the ratio of the amplitude of the ACF and mean value of the membrane conductance gives a value of 19 pS for this set of data points.

The CbmCho-induced increase in membrane conductance could be competitively inhibited by low concentrations of curare (cf. ref. 28). Fig. 2*B* shows such an experiment performed in a symmetric system. Administration of 0.2 μM CbmCho to one compartment elicited a transient conductance increase. Subsequent addition of 1.25 μM curare prevented the response to 0.2 μM CbmCho. The inhibitory effect of curare could be overcome by an excess of CbmCho (10 μM).

The CbmCho response was always accompanied by an increase in the conductance noise. Analysis of these fluctuations provides information about the size and duration of the single AcChoR channel (29, 30). This information can be extracted from the ACF of membrane conductance fluctuations, illustrated in Fig. 3. The decay time constant of the ACF of the

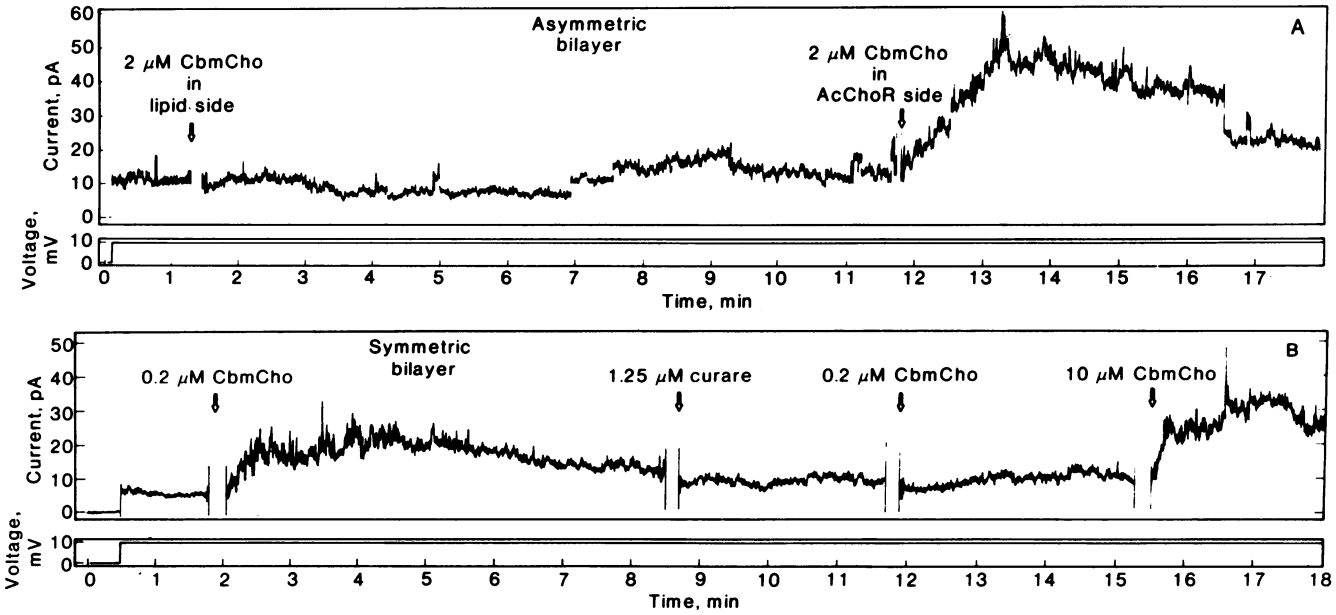

FIG. 2. Time courses of CbmCho-induced conductance responses of lipid bilayers. Conditions were as for Fig. 1 except as noted. (*A*) Response obtained from purified AcChoR incorporated in an asymmetric planar lipid bilayer. No rectification was detected when the polarity of the applied voltage was inverted. (*B*) Effect of curare on the response of a symmetric planar lipid bilayer.

conductance fluctuations represents the average lifetime of the open channel and was estimated to be 35 ± 5 ms. The single-channel conductance can be estimated from the ratio of the amplitude of the ACF and the mean value of the membrane conductance: the mean conductance increase caused by the opening of a single channel is calculated to be 16 ± 3 pS in 0.1 M NaCl (Fig. 3).

The bilayer responses hitherto described arise from membranes containing 10–1000 channels. In order to directly measure the single-channel conductance, the experimental conditions were modified: the number of channels incorporated in the bilayer was reduced by increasing the lipid-to-protein ratio (100-fold) and the sensitivity of the current measurement was enhanced by forming the membranes in a solution of higher conductivity (0.5 M NaCl). The latter condition favors the assembly of high-conductance membranes, which are frequently unstable. In stable membranes that had low conductance, it was possible to resolve defined conductance fluctuations between discrete levels; these transitions were of approximately constant amplitude but varying duration, as shown in Fig. 4. The conductance fluctuated rapidly between low and high values, indicating rapid transitions of the channel between closed and open states (31). The single-channel conductance recorded with an applied voltage of +10 mV is 60 pS (in 0.5 M NaCl). The estimated mean channel open time is approximately 50 ms. Although these values are limited by the amplifier time constant due to low signal-to-noise ratio and are subject to change as the recording conditions are improved, they are in fair agreement with the values estimated by fluctuation analysis.

DISCUSSION

Attempts to reconstitute AcChoR in planar lipid bilayers have met with considerable difficulties (6, 16). These could be attributed to the inactivation of the AcChoR ion channel. We have shown that delipidation of the AcChoR irreversibly denatures the channel without profound effects on ligand binding (12). Attempts to reconstitute AcChoR in bilayers with methods analogous to those used with rhodopsin (17, 32), involving the extraction of a protein–lipid complex in an organic solvent, did not achieve a reliable reconstitution of channel activity despite the reproducible recovery of substantial concentrations of ^{125}I-α-bungarotoxin-binding sites in the extract (unpublished data). Here we employ the procedure developed by Schindler (19), which avoids the use of organic solvents, and we spread monolayers from a reconstituted AcChoR–lipid complex, the functional integrity of which can be assessed in advance. This strategy allows us to reproducibly integrate the AcChoR into planar bilayers in a functionally active form. Twelve preparations of AcChoR purified by using Con A-agarose have been studied, and in all of them we observed transient increases in membrane conductance after addition of CbmCho accompanied by an increase in the conductance noise. We have observed similar responses upon using AcChoR purified in intact membranes by alkaline extraction or purified by affinity chromatography using *Naja naja siamensis* toxin conjugated to agarose (12). The latter preparation contains only four subunits characteristic of the AcChoR complex, confirming the notion that these four subunits are sufficient to mediate the CbmCho-induced transient permeability response. These effects are, therefore, independent of the actual procedure by which purified functionally active receptors were obtained. Such responses were never observed when concentrations of CbmCho as high as 10 mM were added to lipid bilayers formed by the same technique but exclusively of phospholipids without AcChoR. These dose-dependent spontaneously relaxing increases in conductance, which could be competitively inhibited by curare, therefore lead us to conclude that the observed conductance changes are indeed measurements of functional agonist-regulated ion channels in the purified AcChoR preparations.

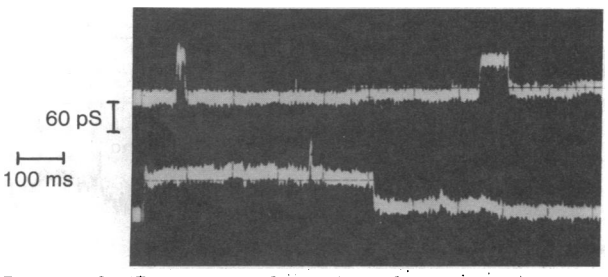

FIG. 4. Oscilloscope recordings of membrane current at an applied voltage of +10 mV from a symmetric planar lipid bilayer containing purified AcChoR. An upward deflection of the trace indicates the opening of a single channel. The record was obtained after addition of 25 nM CbmCho. The lipid-to-protein ratio was 100-fold larger than indicated in *Materials and Methods*. The bilayer was formed in 0.5 M NaCl instead of 0.1 M NaCl; all other conditions were as for Fig. 1.

The observation that the desensitization process could be enhanced by administration of an additional dosage of CbmCho at the peak of the response is consistent with the notion that the binding of the agonist induces a conformation of the protein (33) that is associated with the closing of the channel.

In the reconstituted vesicles 74 ± 4% of the total ^{125}I-α-bungarotoxin sites face outward (12). This preferred orientation appears to be preserved in the monolayer and in the planar bilayer, because the ratio of the response amplitudes in asymmetric bilayers was ≤6, higher in the compartment containing the AcChoR-reconstituted vesicles (Fig. 2*B*).

Our aim here has been to develop a reliable technique to study purified AcChoR in planar bilayers. The experimental conditions were not optimized for maximum response. Current major deficiencies reside in the application of drugs under inadequate stirring and in the requirement of faster electrical recording with better signal-to-noise ratio than hitherto obtained. The first defect accounts for the lag time observed after addition of CbmCho which, therefore, does not accurately represent the latency of the response but is due to slow diffusion of the agonist. Lack of mixing in the chambers is probably also responsible for the fact that multiple transient responses often occurred after the addition of CbmCho. Refined current measurements are mandatory for a rigorous study of the kinetics of single-channel opening and closing and for analysis of the ion selectivity and voltage dependence of the single channels (29–31, 34).

Low signal-to-noise ratio made it difficult to obtain satisfactory single-channel recordings for analysis. Conditions that increase this ratio such as large clamping voltages (>100 mV instead of the 10 mV used) and aqueous media of high conductivity (1 M instead of the 0.1 M used) tend to destabilize the bilayers and introduce artifacts. Thus, it was considered prudent to derive preliminary information about the properties of the single AcChoR channel by fluctuation analysis. The ACF calculated from the conductance fluctuations provides an estimate of the single-channel conductance that is similar to that reported for receptors from different sources (34), but the channel lifetime is longer. Prolongation of the mean channel open time may be a result of the particular conditions used in our system, such as the nature of the lipid environment, the composition of the bath solution, and the applied voltage.

The planar bilayer system described in this report, in contrast to radiochemical assays for $^{22}Na^+$ flux in vesicles, allows the

study of the kinetics of the AcChoR response to agonists in the μs time range and, therefore, enables investigation of the fine details of the response. Planar bilayers display high sensitivity in that concentrations of CbmCho as low as 25 nM are sufficient to elicit a measurable change in conductance. Purified AcChoR reconstituted in vesicles is convenient for biochemical manipulations and will be useful in identifying the function of AcChoR subunits (12–15). Hence, planar bilayers in conjunction with reconstituted vesicles constitute a powerful approach for investigating the mechanism of signal transduction of the AcChoR at the molecular level.

Note Added in Proof. The reconstituted vesicles described in this paper were recently used, in collaboration with H. Schindler, to prepare membranes as described in ref. 20. We resolved single channels with a conductance of 90 ± 15 pS and a mean open time of ≈5 ms at an applied voltage of 100 mV (in 1.0 M NaCl without sucrose). Thus, AcChoR derived both from native vesicles and from purified reconstituted vesicles exhibits the same channel properties when studied under identical conditions.

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