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Functional acetylcholine receptor from *Torpedo marmorata* in planar membranes

(vesicle-derived bilayers/agonist-induced conductance/desensitization/single-channel fluctuations/cation selectivity)

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ABSTRACT **Planar bilayer membranes containing functional acetylcholine receptor were formed from vesicles of *Torpedo marmorata* electric organ without extracting the acetylcholine receptor from its native environment. Native vesicles were transformed into monolayers which subsequently were apposed into planar bilayers. In the absence of agonists the membrane conductance was similar to that of lipid bilayers. Addition of carbamoylcholine or succinylcholine caused increased membrane conductance and this could be competitively inhibited by *d*-tubocurarine and suppressed by α-bungarotoxin. The amplitude of the conductance response was proportional to the number of α-bungarotoxin binding sites in the bilayers. Asymmetric membranes could be formed with the ligand binding sites on only one membrane surface. Desensitization of acetylcholine receptor was evident from equilibrium and kinetic data of the carbamoylcholine-activated conductance. Carbamoylcholine-induced membrane permeability was about 7 times higher for K^+ and Na^+ ions than for Cl^-. At low levels of conductance, single-channel fluctuations of 20–25 pS in conductance and 1.3-msec lifetime were resolved in physiological saline containing carbamoylcholine. The ratio of observed channels to α-bungarotoxin sites present showed that a significant fraction of acetylcholine receptor in the membrane was functional. The quantitative aspects of the cation channel, the desensitization, and the ligand binding properties were in close agreement with established values. This transformation of natural acetylcholine receptor vesicles to planar bilayers conserves the essential properties of the *in vivo* receptor.**

The binding of acetylcholine to the acetylcholine receptor (AcChoR) causes cation-specific membrane channels to open. Electrophysiological experiments have provided a well-documented description of the *in vivo* channel activation (1, 2), but attempts to study channel activation *in vitro* have been handicapped by several problems. Most *in vitro* studies on AcChoR have been performed on preparations from the richest known source of AcChoR, the electric organ of *Torpedo* (3–5), and used the tracer flux assay pioneered by Kasai and Changeux (6). This assay measures the agonist-induced permeability change in AcChoR-enriched vesicles, but it suffers from low time and amplitude resolution, large background efflux, and a complex relationship between the elementary processes and the observed signal (7). However, no other *in vitro* assay has yielded consistent and reliable results (8).

The objective of this study was to measure channel activation of AcChoR *in vitro* by using planar membranes formed directly from *Torpedo* electroplaque vesicle suspensions. This was achieved by means of a new technique for transforming membrane vesicles into monolayers, which are then combined into bilayers (9–11). This method is based on the observation that material from native vesicles or liposomes assembles spontaneously into a lipid–protein monolayer at the solution surface (10). The conditions for successful formation of planar bilayers from such monolayers are well defined (11) and membranous material containing the AcChoR can therefore be organized as a vesicle, a monolayer, or a planar bilayer. The conductance of planar bilayers derived from *Torpedo* electroplaque vesicles was measured in the presence of agonist, and in this report we demonstrate the specificity of the chemically evoked response. Nelson *et al.* (12) present results obtained using AcChoR solubilized and purified from *Torpedo californica* and reconstituted into vesicles.

MATERIALS AND METHODS

Vesicles containing AcChoR were prepared from frozen electric organ of *Torpedo marmorata* (13). Zonal centrifugation (14) was used to increase the specific activity of this vesicle suspension about 4-fold as measured by binding of α-bungarotoxin (α-BuTx). The final specific activity (1.25–2.0 nmol of α-BuTx binding sites per mg of protein) corresponds to ratios of α-BuTx sites/lipid molecule of $2 \pm 0.5 \times 10^{-3}$. AcChoR vesicles (containing routinely 5 μM α-BuTx sites) were suspended in calcium-free Ringer's solution (250 mM NaCl/5 mM KCl/2 mM $MgCl_2$/10 mM Hepes, pH 7.4) and were either used immediately or stored for up to 3 days at 0°C. The concentration of α-BuTx sites in AcChoR vesicles was determined by the method of Schmidt and Raftery (15) with mono[^{125}I]iodo-α-BuTx (16).

The carbamoylcholine (CbmCho)-induced efflux of $^{22}Na^+$ from AcChoR vesicles was assayed as described (6). For the monolayer studies, AcChoR vesicles were incubated with ^{125}I-labeled α-BuTx (^{125}I-α-BuTx) (New England Nuclear) at a 10:1 ratio of toxin sites to toxin. The specific activity of this toxin was 19 μCi/μg (1 Ci = 3.7×10^{10} becquerels) and the radiochemical purity was >95%. Total protein in the vesicles was determined by the method of Bradford (17) with reagents from Bio-Rad. Phospholipid content was measured by organic phosphate determination (18), and lipid concentration was estimated from the phospholipid/cholesterol ratio given by Popot *et al.* (19).

Lipid vesicles were prepared from a mixture of soybean lecithin (Sigma) purified according to Kagawa and Racker (20) and cholesterol (Applied Science Laboratories, State College, PA) at a 6:1 ratio (wt/wt). Lipids (10 mg) were dissolved in 5 ml of hexane, dried under nitrogen to a thin surface film in a 2-liter flask, and resuspended in 10 ml of Ringer's solution (supplemented with 4 mM $CaCl_2$). For studies of single channels or ion selectivity, the lipid film was suspended in buffer A (2 mM Mg Cl_2/4 mM $CaCl_2$/10 mM Hepes, pH 7.4) containing KCl or NaCl as indicated. Lipid vesicles were stored at room temperature (21°C) and sonicated in an MSE (London) sonifier (1 min, position 7) prior to use. The vesicle radius was about 60 nm as determined by coherent light scattering (21). The equilibrium surface pressure at the air/vesicle suspension

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Abbreviations: AcChoR, acetylcholine receptor; CbmCho, carbamoylcholine; α-BuTx, α-bungarotoxin.

interface was 34 ± 1 dynes/cm as determined in a Wilhelmy balance (Krüss, Hamburg).

Planar bilayers were formed as illustrated in Fig. 1 from mixtures of lipid vesicles and AcChoR vesicles. The chamber for bilayer formation, the aperture in the Teflon film and electrical measurements have been described in detail (22). The aperture (2×10^{-4} cm^2) was pretreated with hexadecane (Fluka, Buchs Switzerland) or, for control experiments, with triolein (Sigma). CbmCho or *d*-tubocurarine was added as concentrated solutions through an opening at the bottom of the compartments in aliquots smaller than 10 μl.

Monolayers were used to estimate the AcChoR content of the planar bilayers. Monolayers were formed on the surface of a Langmuir trough (800 cm^2) filled with Ringer's solution. They were spread from a small chamber (8 cm^2) immersed in the trough and containing ^{125}I-α-BuTx-labeled AcChoR vesicles. A series of barriers in the trough with narrow openings for the monolayer impeded the flow of vesicles dragged along by the spreading monolayer. The presence of a monolayer was ascertained from the surface pressure/area curves as described in ref. 10. Surface radioactivity was determined after collection of the monolayer. The total radioactivity of the subphase was routinely less than 20% of the surface radioactivity. From the surface radioactivity and the above value for the specific activity of the ^{125}I-α-BuTx, the total number of toxin sites per cm^2 of monolayer was calculated. The number of lipids per cm^2 of monolayer was estimated by assuming that the monolayer was composed only of lipids. The value of the surface area per lipid molecule was taken from ref. 10.

RESULTS

The vesicle suspensions used for membrane formation contained various weight ratios of AcChoR vesicles to lipid vesicles, hereafter referred to as the vesicle ratio. The range of vesicle ratios investigated was between 0 and 1. At least 40% of the attempts of membrane formation were successful, provided that the monolayer surface pressure exceeded 30 dynes/cm, which was ensured by using lipid vesicles with radii larger than 50 nm (11). Suspensions containing 0.5–1 mg of vesicles per ml were used because the increase of surface pressure under these conditions was complete within 1 min after the membrane cell was filled. Removal of the surface layers by aspiration allowed repeated assembly of fresh monolayers and subsequent formation of bilayers from the same vesicle suspensions. Results were obtained with symmetric membranes—i.e., formed from monolayers of the same composition—bathed in Ringer's solution at 21°C unless stated otherwise. The bilayer character of such vesicle-derived membranes was apparent from the mean measured specific capacitance, 0.7 ± 0.1 μF/cm^2 (23), and from the formation of ion channels by gramicidin (24) which occurs only in bilayers. Membranes were routinely stable for 1–4 hr. Dielectric breakdown voltages decreased from 250 to about 150 mV with increasing vesicle ratio from 0 to 1.

Ligand-induced conductance changes

General Observations. In the absence of specific ligands for AcChoR, the specific conductance of bilayers containing AcChoR was as low as that measured for pure lipid bilayers (1–5 $\times 10^{-8}$ S/cm^2). No significant excess conductance of the AcChoR membrane was observed over a total of 8 hr and with six different membranes at an applied membrane potential of 50–150 mV. CbmCho was the agonist routinely used to elicit conductance changes because it is stable against enzymatic and spontaneous hydrolysis. Fig. 2*a* (upper trace) shows the development of a large conductance increase after addition of 50 μM CbmCho. This induced conductance was inhibited by a specific antagonist of CbmCho, *d*-tubocurarine. The conductance changes obtained by varying the concentrations of CbmCho and *d*-tubocurarine suggested competition between these ligands (25). From these data, the ratio of the equilibrium dissociation constants K_d (*d*-tubocurarine)/K_d (CbmCho) was estimated to be 1.6, in agreement with published values (16). *d*-Tubocurarine was also observed to inhibit conductance increases induced by another specific agonist, succinylcholine.

Demonstration of the chemical specificity of AcChoR channel activation requires several control experiments. (*i*) The lower trace in Fig. 2*a* was obtained with the same AcChoR vesicle preparation as the upper trace but it had been preincubated with α-BuTx to inactivate the AcChoR. This control experiment was repeated with AcChoR vesicle preparations from two different electric organs and over a total of 4 hr at a membrane potential of 50–150 mV. No agonist-induced conductance was observed. (*ii*) CbmCho and *d*-tubocurarine had no effect on membranes derived from lipid vesicles. (*iii*) Pretreatment of the Teflon film with either hexadecane or triglyceride gave AcChoR membranes with the same response to CbmCho and *d*-tubocurarine. (*iv*) Local anesthetics such as procaine, dibucaine, and tetracaine decreased the response of the membrane to CbmCho. However, increased fragility of the membrane prevented a detailed analysis.

Fig. 2*b* shows the effects of CbmCho and *d*-tubocurarine on asymmetric bilayers assembled from one monolayer derived from AcChoR vesicles (the *cis* side) and the other from lipid vesicles (*trans* side). The response was found only after addition of CbmCho or *d*-tubocurarine to the *cis* side. Such bilayers were completely asymmetric with respect to (functional) ligand binding sites but they showed no rectification—i.e., the values of the CbmCho-induced conductance did not differ when the polarity of the applied membrane potential was reversed.

Response Saturation. With increasing CbmCho concentration the membrane conductance increased to saturation. The ligand-induced conductance is saturated above 1 μM CbmCho (Fig. 3). Half-saturation occurred between 50 and 100 nM

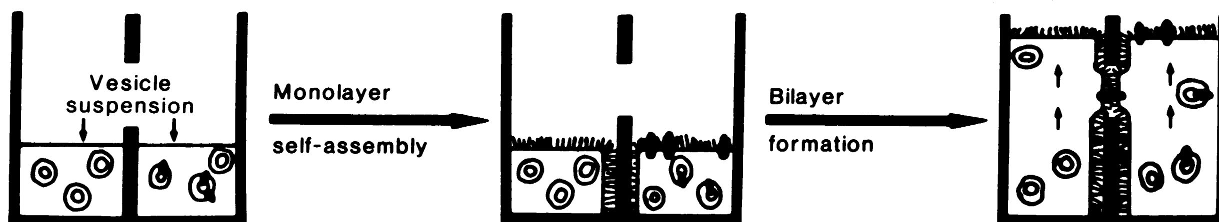

FIG. 1. Transformation of vesicles to planar bilayers. The two compartments of a membrane cell are filled with vesicle suspension to just below the membrane aperture. Monolayers self-assemble at the surfaces of the two solutions. Monolayer composition is similar to vesicle composition, even in the case of natural liposomes (e.g., AcChoR vesicles). Monolayers are easily combined to planar bilayers by raising the water levels successively above the membrane aperture. Spontaneous monolayer formation has been characterized in ref. 10 and the conditions for successful membrane formation are described in ref. 11.

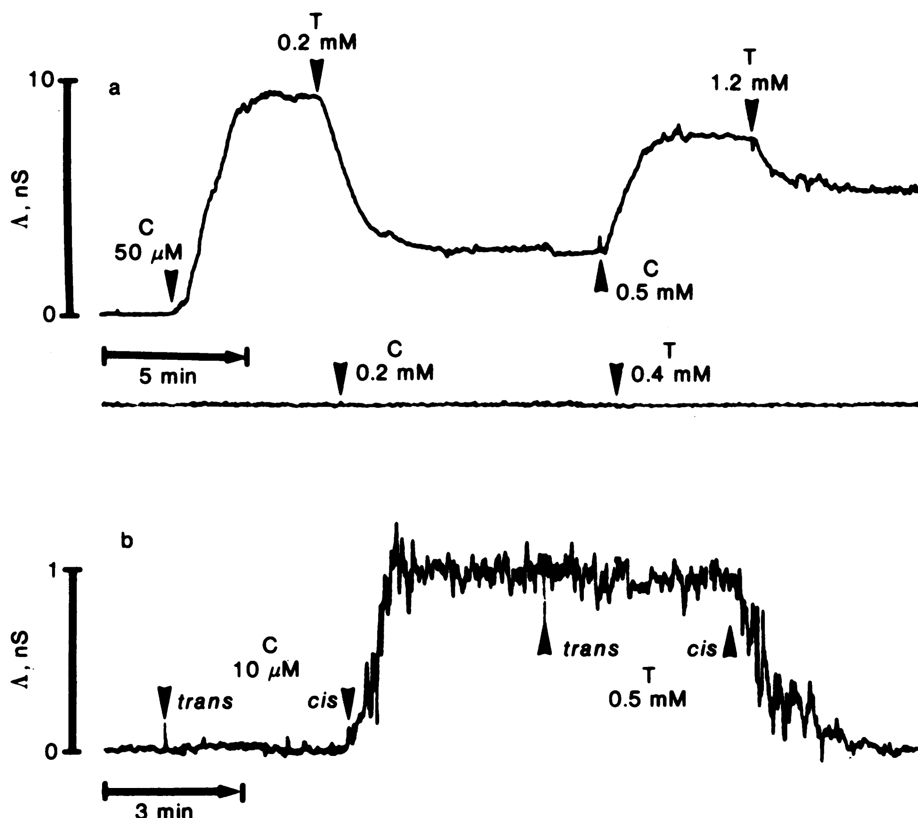

FIG. 2. Effects of CbmCho (C) and *d*-tubocurarine (T) on the conductance of a symmetric (*a*) and an asymmetric (*b*) membrane. Membrane formation preceded the recordings shown by at least 10 min. The recipient chambers for CbmCho and *d*-tubocurarine were stirred at a moderate speed. (*a*) Upper trace. Membrane potential was 50 mV with its higher level on the side of the ligand addition. Vesicle ratio was 1. Lower trace. This trace was obtained under identical conditions and from the same solution as the upper trace except that the AcChoR vesicles had been preincubated with α-BuTx. (*b*) The membrane was derived from two different solutions with vesicle ratio 0.1 (*cis* side) and 0 (*trans* side)—i.e., only the *cis* side contained AcChoR vesicles. The membrane potential was +50 mV with its higher level at the *cis* side.

CbmCho.* The time course of the conductance after a single dose of 1 μM CbmCho showed a pronounced maximum, a typical feature observed at CbmCho concentrations below about 10 μM. Both this maximum and the low dose saturation are indicative of AcChoR desensitization—i.e., a high probability for closing of the AcChoR-associated channel subsequent to channel opening (26–28).

Conductance Steps and Ion Selectivity. At low vesicle ratios, the CbmCho-induced conductance exhibited fluctuations between discrete conductance levels (Fig. 4). These discrete changes are attributed to the opening and closing of individual ion channels. This interpretation is based on the close similarity to single channel traces obtained with established channel-forming ionophores such as gramicidin (24). Analysis of the trace in Fig. 4*B* showed a slight but significant deviation from random channel opening in time. This feature developed into a burst character of open channel occurrence when the vesicle ratio was increased from 2.5×10^{-3}, as used in Fig. 4*B*, to 1.

The observed channels were characterized by a conductance of 90 ± 10 pS and a mean open time of 1.3 msec at 100 mV and 1 M NaCl in buffer A. The single-channel conductance in 0.5

* The dose-response curve (steepness and midpoint) apparently depended on the membrane potential and the vesicle ratio used.

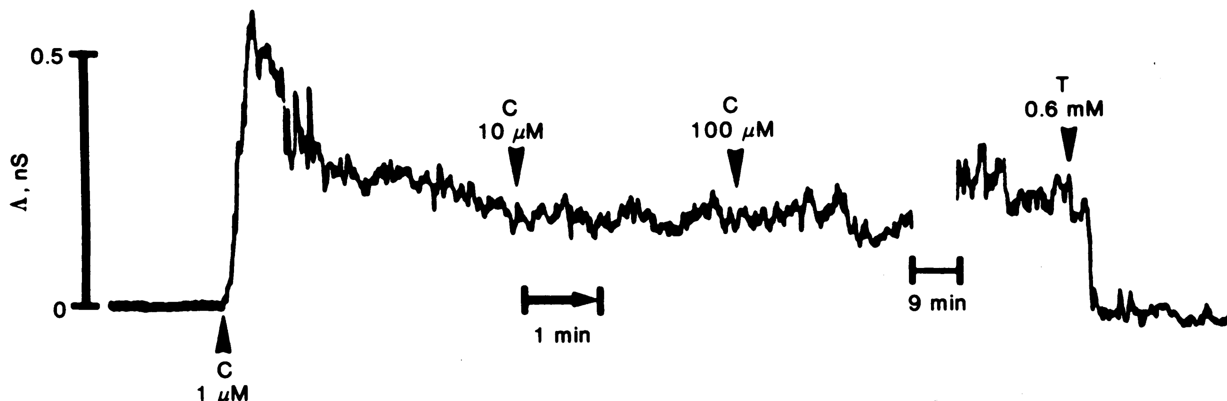

FIG. 3. Conductance saturation. Membrane potential was 50 mV with its lower level at the side of CbmCho addition. The recipient chamber was continuously and vigorously stirred. Vesicle ratio was 0.1. C, CbmCho; T, *d*-tubocurarine.

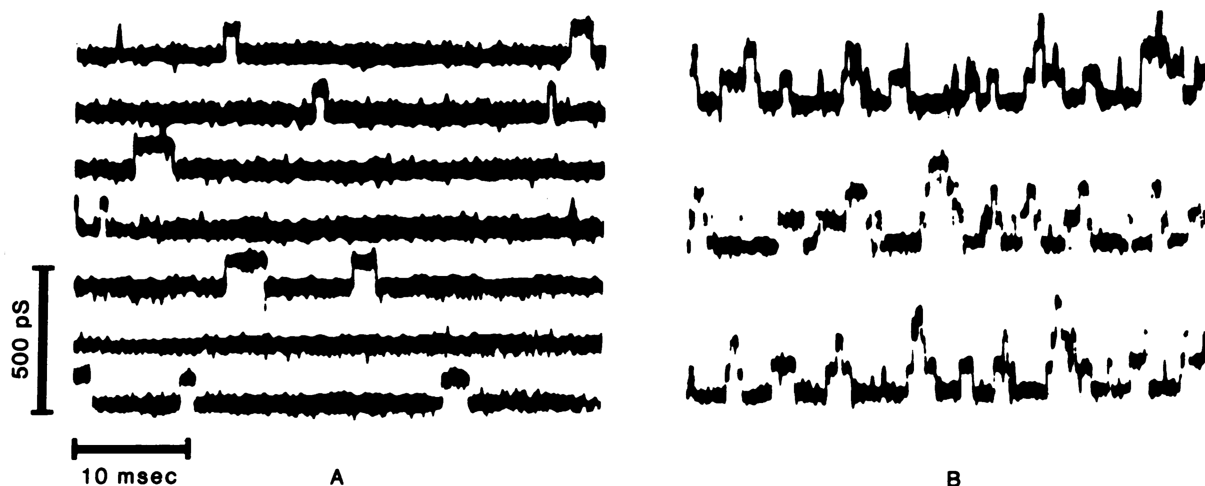

FIG. 4. Single-channel conductance. The membrane was formed from solutions with vesicle ratio 2.5×10^{-3} and containing 1 M NaCl in buffer A. The traces were recorded at 100 mV membrane potential about 1 min (*A*) and 10 min (*B*) after addition of CbmCho to 10 μM. Membrane area was 9.5×10^{-5} cm^2 and time resolution was 0.2 msec.

M NaCl was 45 ± 10 pS. In physiological saline (0.25 M NaCl), the channel conductance could only be estimated from some long-lasting channel opening events after the membrane noise had been reduced at the expense of time resolution. Most values were between 20 and 25 pS. When Na^+ was replaced by K^+ (1 M KCl) the single-channel conductance was 80 ± 15 pS. The channel therefore appears to be almost equally permeable to K^+ and Na^+. The permeability ratios between these cations and Cl^- were determined from Nernst potentials in the presence of at least 10^2 open channels. For a 10-fold salt gradient across the membrane (0.1 and 1 M salt in buffer A), potentials of 38.2 ± 0.6 mV for NaCl and 36 ± 1 mV for KCl were found. The corresponding permeability ratios are 8.0 ± 0.4 for Na^+/Cl^- and 6.8 ± 0.5 for K^+/Cl^-.

Fraction of functional AcChoR and reproducibility

The number of toxin sites present in the planar bilayers was estimated from monolayer studies. For different vesicle ratios in solution, the number of ^{125}I-α-BuTx molecules bound per cm^2 of monolayer was determined at 39 dynes/cm. The resulting values were compared with the number of open channels at CbmCho saturation. The two last columns in Table 1 show data obtained from samples of the 2 electric organs, from the 10 organs tested, that gave the maximal (A) and the minimal (B) response to CbmCho. The data in Table 1 permit the following conclusions.

Table 1. Number of open channels in relation to monolayer toxin sites and AcChoR-vesicle dilution

Solution vesicle ratio*	Monolayer toxin sites per lipid molecule	Bilayer open channels per toxin site†	
		Organ A	Organ B
1	7.7×10^{-4}	2×10^{-4}	3.5×10^{-6}
0.1	8.8×10^{-5}	3×10^{-4}	5.5×10^{-6}
0.01	8.3×10^{-6}	2.2×10^{-4}	2.5×10^{-6}

* The vesicle ratio was defined as the weight ratio of AcChoR vesicles to lipid vesicles in the solutions used for membrane formation.

† The number of toxin sites in half of the bilayer was assumed to equal that in an area of the monolayer corresponding to the bilayer area at 39 dynes/cm. The number of open channels refers to the observed saturation conductance divided by the single-channel conductance.

(*i*) There is an approximately linear relationship between the number of toxin sites and the number of CbmCho-induced channels in membranes from a given preparation.

(*ii*) The fraction of functional AcChoR is estimated in the following way. Only 0.1–0.5% of functional AcChoR channels should be in the open state at equilibrium due to desensitization; the majority are in the closed state (29). The ratios of *open* channels per toxin site in Table 1, therefore, can be expressed in terms of toxin sites per *functional* channel (4–20 for electric organ A). Assuming two toxin binding sites per channel and that 0.2% of the channels were open, one calculates that 25% of the AcChoR in the planar bilayer were functional.

(*iii*) Compared to organ A, the activities of membranes from organ B were 50 times lower. This variability might be due to differences between the fish before death or deterioration of the electric organ with longer storage.

(*iv*) The activity of different bilayers derived from the same AcChoR vesicle sample varied less than a factor of 2, provided that the bilayers were formed within 2 min after monolayer assembly and within a few hours after AcChoR vesicle preparation.

DISCUSSION

A strategy to transform vesicles into planar bilayers (9–11) was applied to vesicles derived from *Torpedo* electric organ. The method permits assembly of planar bilayers containing AcChoR without removing the AcChoR from its native membrane environment. The increase in conductance in response to specific agonist indicated that the AcChoR in these membranes was functional. Specificity was demonstrated by three lines of evidence.

The response was proportional to the number of toxin sites and showed sidedness. The ratio of receptor toxin binding sites to lipids in the monolayer and the membrane conductance at saturation were proportional to the ratio of receptor toxin binding sites to lipid in the vesicle suspension (Table 1). Sidedness was demonstrated by the observation that the bilayers were completely asymmetric with respect to the effect of agonists when only one monolayer contained toxin binding sites. This result implies that, in the monolayer containing AcChoR, all binding sites were facing the solution. However, it is well

known that the AcChoR orientation is not completely asymmetric in the vesicles (13) used to form the monolayer. It is conceivable that the AcChoR molecule may reorient during formation of the monolayer, regardless of its initial orientation in the vesicle. However, it is also possible that those AcChoR molecules in which the bindings sites are exposed to air are inactivated. Both results indicated that the agonist-induced conductance increase was due to a component of the *Torpedo* vesicles.

The ligand-induced effects were in agreement with the pharmacological specificity of the AcChoR. Addition of the agonists CbmCho or succinylcholine led to a saturable increase in membrane conductance that could be completely suppressed by an excess of *d*-tubocurarine. The data further indicated that *d*-tubocurarine may act as a competitive inhibitor of CbmCho with the same relative potency as determined by other methods. Incubation of AcChoR vesicles with α-BuTx in excess reproducibly abolished all ligand-induced response. These results agree with the established pharmacological properties of AcChoR (3, 30).

The quantitative results (e.g., channel characteristics and desensitization) correspond to established observations on the AcChoR. The main characteristics of the observed ion channel (i.e., lifetime, conductance, and cation specificity) are consistent with the detailed knowledge of the AcChoR channel in related membrane systems (1, 2, 31). Asymmetric bilayers showed no rectification, a known feature of *Torpedo* electroplax membranes (32). The following observations are indicative of AcChoR desensitization (26, 27). The observed conductance decrease following activation (Fig. 2) is consistent with a channel-closing step after channel opening. Likewise, both the decay time of $\approx$1 min at 1 μM CbmCho and the occurrence of a conductance maximum at CbmCho concentrations below 10 μM are in agreement with kinetic measurements of CbmCho binding to *Torpedo* membrane vesicles (29). The occurrence of a conductance maximum upon CbmCho addition implies that the time required for CbmCho to diffuse to the membrane and bind to the AcChoR is shorter than the time needed for desensitization. However, the rate of desensitization increases with increasing CbmCho concentration as revealed by CbmCho binding studies (29). The relationship between rate constant and CbmCho concentration is consistent with the observation that a response maximum only occurred for CbmCho concentrations less than 10 μM. As another consequence of desensitization, half-saturation of equilibrium conductance is expected to occur at about 100 nM CbmCho which corresponds to the known equilibrium constant of CbmCho binding to *Torpedo* vesicles (29). This compares well with the CbmCho concentration at half-saturation measured here, 50–100 nM. In contrast, CbmCho concentrations as high as 50 μM at half-saturation are found in electrophysiological measurements (33) and in tracer flux studies (6) in which the experimental conditions allow observation of only the channel opening.

These lines of evidence leave little doubt that the observed conductance changes are due to the chemical activation of the AcChoR-associated channel. The high reproducibility achieved in the assembly of functional membranes allows a more quantitative description of the various aspects of AcChoR channel activation. In particular, measurements of cooperativity, voltage dependence, and kinetics of channel activation should permit a more thorough mechanistic knowledge about the linkage between the binding of AcChoR ligands and the activation of the AcChoR-associated channel.

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