

Acetylcholine Receptor in Planar Lipid Bilayers

*Characterization of the Channel Properties of the Purified Nicotinic Acetylcholine Receptor from *Torpedo californica* Reconstituted in Planar Lipid Bilayers*

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ABSTRACT The properties of the channel of the purified acetylcholine receptor (AChR) were investigated after reconstitution in planar lipid bilayers. The time course of the agonist-induced conductance exhibits a transient peak that relaxes to a steady state value. The macroscopic steady state membrane conductance increases with agonist concentration, reaching saturation at 10^{-5} M for carbamylcholine (CCh). The agonist-induced membrane conductance was inhibited by *d*-tubocurarine (50% inhibition, IC_{50} , at $\sim 10^{-6}$ M) and hexamethonium ($IC_{50} \sim 10^{-5}$ M). The single channel conductance, γ , is ohmic and independent of the agonist. At 0.3 M monovalent salt concentrations, $\gamma = 28$ pS for Na^+ , 30 pS for Rb^+ , 38 pS for Cs^+ , and 50 pS for NH_4^+ . The distribution of channel open times was fit by a sum of two exponentials, reflecting the existence of two distinct open states. τ_{o1} and τ_{o2} , the fast and slow components of the distribution of open times, are independent of the agonist concentration: for CCh this was verified in the range of 10^{-6} M $< C < 10^{-3}$ M. τ_{o1} and τ_{o2} are approximately three times longer for suberyldicholine (SubCh) than for CCh. τ_{o1} and τ_{o2} are moderately voltage dependent, increasing as the applied voltage in the compartment containing agonist is made more positive with respect to the other. At desensitizing concentrations of agonist, the AChR channel openings occurred in a characteristic pattern of sudden paroxysms of channel activity followed by quiescent periods. A local anesthetic derivative of lidocaine (QX-222) reduced both τ_{o1} and τ_{o2} . This effect was dependent on both the concentration of QX-222 and the applied voltage. Thus, the AChR purified from

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Torpedo electric organ and reconstituted in planar lipid bilayers exhibits ion conduction and kinetic and pharmacological properties similar to AChR in intact muscle postsynaptic membranes.

INTRODUCTION

The acetylcholine receptor (AChR) is by far the best-characterized neurotransmitter receptor, both electrophysiologically and biochemically, and can therefore serve as a model for understanding the structure and function of other neurotransmitter receptors (Karlin, 1980; Changeux, 1981; Anholt et al., 1983, 1984).

The availability of specific toxins that act on the AChR and a rich tissue source for the biochemical isolation and purification of the AChR have facilitated the detailed characterization of its structure. Biochemical studies of the purified protein indicate that the receptor is a noncovalent complex of four distinct integral membrane glycoprotein subunits with subunit stoichiometry of $\alpha_2\beta\gamma\delta$ and a molecular weight of ~270,000 (Reynolds and Karlin, 1978; Lindstrom et al., 1979; Raftery et al., 1980; Noda et al., 1982, 1983; Claudio et al., 1983). The two smallest subunits (40,000 daltons) are responsible for the binding of acetylcholine (Weill et al., 1974; Wolosin et al., 1980; Karlin et al., 1975). The cation channel is an integral component of the receptor, but it is not known which subunits form the channel. Recently, a number of laboratories have succeeded in cloning the genes coding for the various receptor subunits, and the entire amino acid sequence of the receptor subunits has been elucidated (Noda et al., 1982, 1983; Claudio et al., 1983; Devilliers-Thiery et al., 1983).

This detailed knowledge of the structure of the AChR has been accompanied by a detailed characterization of the biophysical events associated with the opening and closing of individual receptor channels. The dynamics of opening and closing have now been characterized through patch recording in the native membrane (Neher and Sakmann, 1976a; Sakmann et al., 1980; Colquhoun and Sakmann, 1981; Jackson et al., 1982, 1983), and more recently the purified protein has been reconstituted into lipid bilayers where the current flowing through individual channels can be recorded (Nelson et al., 1980; Boheim et al., 1981; Labarca et al., 1981, 1982, 1983, 1984; Montal et al., 1984; Tank et al., 1983; see also Schindler and Quast, 1980).

The purified AChR reconstituted in planar lipid bilayers provides a vehicle for combining the biophysical analysis of the receptor channel with the biochemical knowledge about the receptor macromolecule and permits, for the first time, the study of structure and function correlates at the molecular level (Nelson et al., 1980; Labarca et al., 1984; Montal et al., 1984). Clearly, knowledge of the kinetic, pharmacological, and ion conduction characteristics of the purified AChR is necessary to investigate how manipulations in its structure modify these functional properties. Thus, we proceeded to study systematically the channel properties of the purified AChR reconstituted in planar lipid bilayers (Nelson et al., 1980). This approach has the sensitivity and temporal resolution required to collect information on the details of the purified AChR function that are not accessible by other means.

Here we report a detailed characterization of the single channel properties of the purified *Torpedo* AChR: namely, channel conductance and lifetime, as functions of agonist used to activate the receptor, applied voltage, ionic environment, receptor antagonists, and channel blockers. The contribution of receptor desensitization is evaluated from the pattern of single channel activity. The results with purified *Torpedo* AChRs show that the reconstituted membranes display phenomena that are remarkably similar to the events recorded from AChRs in muscle. Preliminary accounts of this investigation have been presented elsewhere (Labarca et al., 1981, 1982, 1983; Montal et al., 1984).

METHODS

Purification of AChRs, Reconstitution in Phospholipid Vesicles, and Assay of Agonist-activated Cation Permeability

These procedures have been described in detail elsewhere (Lindstrom et al., 1980, 1981; Anholt et al., 1980, 1981, 1982).

Briefly, solubilization of electric organ membranes of *Torpedo californica* (Pacific Bio-Marine Laboratories, Inc., Venice, CA) in mixed micelles of sodium cholate (2%; Interchem, Montluçon, France) and crude soybean lipids (5 mg/ml, L- α -phosphatidylcholine type IIs; Sigma Chemical Co., St. Louis, MO) was conducted as described by Anholt et al. (1981). The extract was added to toxin agarose (0.5 mg of *Naja naja siamensis* toxin III/ml of Sepharose Cl 4B) at ~ 5 nmol AChR/ml of agarose and shaken gently for 1 h before being poured into a column. The column was washed with 250–300 column volumes of cholate lipid buffer (1% Na cholate, 2.5 mg/ml of soybean lipid, 100 mM NaCl, 10 mM Na phosphate, 10 mM NaN₃, pH 7.5). Then, a small column of concanavalin A agarose (Sigma Chemical Co.) containing $\frac{1}{10}$ the toxin agarose volume was attached below the toxin agarose column. AChR was eluted by recirculating 1 mM benzoquinonium chloride (a gift of Stirling-Winthrop, Research Institute, Rensselaer, NY) in cholate lipid buffer over the columns for 16 h. AChR eluted from toxin agarose by the benzoquinonium bound to the concanavalin A agarose through its carbohydrate. The concanavalin A agarose was washed free of benzoquinonium with cholate lipid buffer and then AChR was eluted twice with 1 column volume of 1 M α -methyl-D-mannoside and 1 mM EDTA in cholate lipid buffer by shaking the concanavalin A agarose for 2 h at room temperature. The eluate was then made 2% in cholate and 20 mg/ml in soybean lipid and then reconstituted by successive 4-d dialyses, first against 100 vol of 100 mM NaCl, 10 mM NaN₃, 10 mM Na phosphate buffer, pH 7.5, and then against 100 vol of 145 mM sucrose, 5 mM NaN₃, pH 7.5. After reconstitution the vesicles were subjected to a freeze/thaw cycle as described by Anholt et al. (1982). The specific activity of the preparation averaged 8 μ mol of binding sites for ¹²⁵I- α -bungarotoxin (α BGT) per gram of protein, and acrylamide gel electrophoresis in sodium dodecyl sulfate showed that the preparation consisted of the four protein bands characteristic of purified AChR (Lindstrom et al., 1980). The final protein concentration of the reconstituted vesicles was adjusted to be between 100 and 300 μ g/ml and that of AChR between 0.5 and 2 μ M. The reconstituted vesicles were aliquoted in 50- μ l samples and stored frozen at -70°C until use. The integrity of AChR function in the reconstituted vesicles was measured before planar bilayer formation as the amplitude of the integrated uptake of ²²Na⁺ (New England Nuclear, Boston, MA) during a 10-s incubation period in the presence of agonist, as previously described (Epstein and Racker, 1978; Lindstrom et al., 1980; Anholt et al., 1982).

Monolayers at the Air-Water Interface Derived from Suspensions of Membrane Vesicles

The surface pressure of monolayers at the air-water interface was monitored by the method of Wilhelmy in a monolayer trough with two movable barriers and several compartments (Fromherz, 1975) (Mayer Feintechnik, Göttingen, Federal Republic of Germany). The system allows the simultaneous measurement of surface pressure and surface area as well as the compression and transfer of the monolayers from one compartment to another. Monolayer transfer can be achieved with little loss of monolayer, as evidenced by the negligible drop in surface pressure observed during this maneuver (Schindler and Quast, 1980).

A necessary condition for the assembly of planar lipid bilayers from monolayers is that the surface pressure, π , of the monolayer be ≥ 25 dyne/cm (Schindler, 1979, 1980). Surface pressure is exquisitely sensitive to the composition of the aqueous medium (subphase), as described in detail by Schindler (manuscript in preparation). The procedure here selected to derive monolayers from vesicle suspensions with surface pressures sufficient to support the assembly of bilayers was the following: a 50- μ l sample of purified AChR reconstituted vesicles (~ 1 μ M AChR) was incubated in a solution containing an indicated salt concentration buffered with 2.5 mM Tris-glycine, or HEPES, pH 7.4, and 5 mM CaCl_2 in a final volume of 0.5 ml for 20 min at 20°C. Such a suspension was introduced into the bilayer chamber by gently delivering 20- μ l drops (see below).

The transfer of purified AChRs from the reconstituted vesicles to monolayers was determined using ^{125}I - α BGT-labeled AChRs according to Schindler and Quast (1980). Reconstituted vesicles containing 25 mg/ml of soybean lipid and 0.5 μ M AChR toxin binding sites were incubated for 1 h in 0.5 M NaCl, 2.5 mM HEPES, pH 7.4, containing a trace label of ^{125}I - α BGT (20 nM, specific activity 1.97×10^{17} cpm/mol). Vesicles containing purified AChR were separated from liposomes by isopycnic sucrose gradient centrifugation, as described by Anholt et al. (1981, 1982). Approximately 80% of the original receptor was recovered, associated with $\sim 30\%$ of the total lipid. The fraction of AChR labeled with α BGT was estimated assuming one toxin bound per receptor. An aliquot of the labeled reconstituted vesicles (1.5 mg lipid) was incubated for 10 min in 0.5 M NaCl, 5 mM CaCl_2 , 2.5 mM HEPES, pH 7.4 (0.5 ml), and then deposited as droplets into one chamber of the multicompartiment surface trough containing 20 ml of the same buffer. The formation of a monolayer was measured as the increase in surface pressure at the air-water interface. Monolayers at surface pressures of 14–18 dyne/cm were transferred to a vesicle-free compartment. This step was repeated four times (Fromherz, 1975). Finally, the monolayer was compressed to a π of 30 dyne/cm and aspirated into a pasteur pipette for subsequent analysis. The monolayers were removed together with a small volume (≤ 0.5 ml) of aqueous solution. Next, the radioactivity present in the monolayer and in an aliquot of bulk phase was measured. The actual amount of ^{125}I - α BGT present in the monolayer (expressed in counts per minute) was estimated from the difference between the counts per minute in the monolayer plus the associated bulk phase and the counts per minute measured in an equal volume of aqueous solution; the latter was $\sim 10\%$ of the total counts actually present in the monolayer.

To assay the extent of monolayer contamination caused by adsorption of labeled vesicles, lipid monolayers were formed and then exposed to vesicles. Monolayers were spread at the air-water interface from a lipid hexane solution. π was fixed at 30 dyne/cm and the area was measured. Then, ^{125}I - α BGT-labeled vesicles (1.5 mg lipid weight) were injected into the stirred subphase. The addition of vesicles had no effect on π . Monolayers were then transferred four times into clean chambers and recovered. AChR densities in these monolayers were estimated as indicated for monolayers spread from the reconsti-

tuted vesicles. Under these experimental conditions, the presence of labeled AChR was undetectable. Accordingly, the contamination of the monolayers caused by the adsorption of vesicles was considered negligible.

Formation of Planar Lipid Bilayers

Planar bilayers were formed from monolayers and their electrical properties were studied essentially as described before in detail (Montal, 1974; cf. Montal et al., 1981). Monolayers were derived from the reconstituted vesicles (Schindler, 1979, 1980) as described above. The number of AChRs incorporated in the bilayers was varied by diluting the reconstituted vesicles with liposomes (Schindler and Quast, 1980). Planar lipid bilayers were assembled from two monolayers across a $\sim 200\text{-}\mu\text{m}$ -diameter hole in a $12\text{-}\mu\text{m}$ -thick Teflon partition separating two 1-ml capacity Teflon chambers, as described elsewhere (Montal, 1974; Nelson et al., 1980). The hole was coated with $2\ \mu\text{l}$ of 0.5% (vol/vol) hexadecane in hexane or in chloroform/methanol (2:1). The chambers were filled with 0.5 ml of the indicated buffer before the addition of the vesicles. The composition of the electrolyte solution in the two chambers was the same. If symmetric bilayers were formed, the same protocol was applied to both monolayer chambers. Alternatively, when asymmetric bilayers were formed, the AChR-containing monolayer compartment (*cis*) was prepared as previously described, while the only lipid-containing monolayer compartment (*trans*) was derived from liposomes devoid of AChR. The liposomes, from partially purified soybean phospholipids (Kagawa and Racker, 1971), at a concentration of 20 mg/ml in 80 mM Tris-glycine buffer, pH 8.0, were prepared by a 10-min sonication under argon in a water bath sonicator (Branson Sonic Power Co., Danbury, CT). These liposomes, in 50- μl samples, were subjected to the same incubation conditions as the reconstituted AChR vesicles described above. All the planar bilayer experiments reported here were performed on asymmetric bilayers at $22 \pm 2^\circ\text{C}$. After a few minutes, each monolayer achieved a surface pressure of ~ 30 dyne/cm (Fig. 1). Thereafter, the level of the AChR-containing compartment was raised over the aperture in the Teflon septum by introducing additional buffer into the chamber from a reservoir. Next, the second monolayer was apposed to the septum and a bilayer was formed across the aperture. Membrane formation was continuously followed by monitoring the membrane capacitance, C_m . The reconstituted bilayers had a $C_m = 0.8\ \mu\text{F}/\text{cm}^2$. After formation, the membranes were left unperturbed for 10 min. The electrical conductance, g_m , was monitored by applying voltages of up to ± 200 mV. Bilayers with changes in g_m during this period were discarded. Thereafter, the pharmacological agent under study was introduced into the AChR compartment and vigorously mixed with a magnetic stirrer. Single channel recordings were obtained several minutes after the addition of agonists.

Electrical Recordings and Data Processing

The results described were obtained with an improved current amplifier designed for high speed and high resolution. The critical strategy was the minimization of the effective feedback capacitance on the current-to-voltage converter (Analog Devices [Norwood, MA] 528 or Burr-Brown [Tucson, AZ] 3523] or National Semiconductor [Santa Clara, CA] LF357AH). This allows measurements of signals at a gain of 10^{-10} A/V with 1 pA of noise (peak-to-peak; amplifier time constant $\tau = 250\ \mu\text{s}$), and at a gain of 10^{-9} A/V with 2 pA of noise (amplifier $\tau = 120\ \mu\text{s}$). Voltage was applied and current was measured with Ag/AgCl electrodes. Constant voltage was supplied by a DC source and the *trans* side of the membrane was defined as zero voltage. Accordingly, a negative applied voltage corresponds to a depolarization in the electrophysiological convention. Membrane current was recorded directly on a storage oscilloscope or amplified and stored on a Racal (Hythe,

Southampton, England) 4DS tape recorder (frequency response DC–5,000 Hz) for subsequent analysis. The recordings were digitized at sampling intervals between 0.1 and 0.3 ms in a PDP 11/34 computer (Digital Equipment Corp., Marlboro, MA). The digitized records were inspected on a computer-generated display and compared with a matching routine in which the computer generated a virtual reproduction of the actual recording: the computer-generated rectangular pulses were checked for fidelity in matching the channel openings and closings. The duration of the generated rectangular pulses corresponds to the open times, and the intervals between the pulses are the closed times. The parameters of the generated rectangular pulses, position and width, were stored in the computer. After analysis of the data, cumulative open state and closed state lifetime distributions of the generated pulses were automatically produced. Time constants were determined with an exponential fitting routine in which computer-generated exponentials were fitted to the experimental data points and curve parameters were determined by an iterative least-square procedure. Semilogarithmic plots of either the actual data or the computer-reconstructed channels were fitted by similar parameter values. Bandwidth limitations and noise restrict the reliability of our measurements to ≤ 0.5 ms.

RESULTS

Reconstitution of the Purified AChR in Planar Lipid Bilayers

The strategy for reconstituting the AChR in planar lipid bilayers (Schindler, 1979, 1980; Schindler and Quast, 1980) consists of two steps. First, monolayers at the air-water interface are derived from lipid vesicles containing the purified AChR (hereafter called “reconstituted vesicles”). Subsequently, two of these monolayers are joined by hydrophobic apposition to form a planar lipid bilayer (Montal, 1974; Nelson et al., 1980; cf. Montal et al., 1981).

SURFACE PRESSURE REQUIRED TO ASSEMBLE PLANAR LIPID BILAYERS FROM TWO MONOLAYERS The critical surface pressure, π , of the two parental monolayers required to form stable bilayers was ~ 30 dyne/cm (see also Schindler, 1980). The reconstituted vesicles generate surface pressures that are sufficient to sustain a bilayer. Fig. 1 illustrates the time course of change in π upon addition of reconstituted AChR vesicles to a clean air-water interface: π increases with no detectable latency at the resolution of the instrument (≤ 0.5 s) to a value of ~ 28 dyne/cm.

ACHR DENSITY IN MONOLAYERS DERIVED FROM RECONSTITUTED VESICLES

The density of AChR (AChR molecules/cm²), estimated from the monolayer data, and the density of AChR in the reconstituted vesicles were $1.4 \times 10^{10} \pm 0.6 \times 10^{10}$ ($N = 3$) and $1.2 \times 10^{10} \pm 0.4 \times 10^{10}$ ($N = 3$), respectively. Similar results were reported for monolayers from microsacs by Schindler and Quast (1980). Control experiments demonstrated that the contribution of adsorbed vesicles to the AChR density in the monolayer was negligible (see Methods). With the reconstitution protocol described here, the estimated AChR density is of the order of 10^7 AChR/cm².

Cholinergic Agonist-induced Conductances in Planar Lipid Bilayers Containing Purified AChR

CHARACTERISTICS OF MEMBRANE CURRENTS ACTIVATED BY CHOLINERGIC AGONISTS IN PLANAR LIPID BILAYERS CONTAINING PURIFIED AChR (i) *Macroscopic*

responses to the addition of agonists. The response of lipid bilayers containing purified AChR to the addition of agonists is shown in Fig. 2. A transient increase in conductance which spontaneously relaxed to a lower value was elicited by both carbamylcholine (CCh) ($1 \mu\text{M}$) and suberyldicholine (SubCh) ($0.1 \mu\text{M}$). The activation by agonists was accompanied by an increase in the amplitude of the conductance noise (Katz and Miledi, 1972; Anderson and Stevens, 1973). The subsequent addition of curare (10^{-6} M) (Jenkinson, 1960) reduced the membrane conductance and the conductance noise (Fig. 2*B*). This phenomenology was recorded with other AChR agonists such as ACh and with other antagonists such as hexamethonium (see Table I) and gallamine (not shown). Planar bilayers generated from lipid vesicles formed by the same cholate dialysis technique used to reconstitute the AChR in lipid vesicles were electrically stable and gave no responses to agonists. Furthermore, planar bilayers formed from reconstituted vesicles that had been pretreated with an excess of αBGT were unresponsive to the addition of agonist.

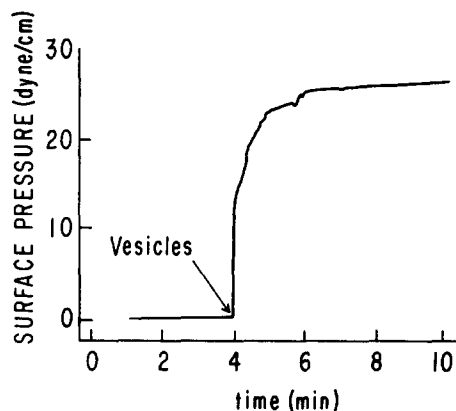


FIGURE 1. Time course of the change in surface pressure caused by the formation of vesicle-derived monolayers at an air-water interface. $100 \mu\text{l}$ of reconstituted vesicles (25 mg soybean lipid/ ml , $0.5 \mu\text{M}$ AChR) was incubated for 10 min in 1 ml of 0.5 M NaCl, 5 mM CaCl_2 , 2.5 mM HEPES, pH 7.4. Aliquots of this suspension ($200 \mu\text{l}$) were added, as droplets, into a chamber containing 20 ml of the same buffer. The time course of the change in π was measured with a Wilhelmy-type surface balance (Fromherz, 1975).

(ii) *The steady state conductance depends on agonist concentration.* The dependence of the steady state membrane conductance on the concentration of CCh was investigated. Successive additions of increasing concentrations of agonists resulted in progressive increases in membrane conductance. This is shown in Fig. 3*A-C*. The dose-response relationship compiled from five studies is presented in Fig. 3*D*. The bilayer conductance increases with CCh concentration and eventually saturates at 10^{-5} M .

(iii) *Effects of curare and hexamethonium on the steady state conductance.* The effects

of curare and hexamethonium, two well-known antagonists of ACh at the postsynaptic membrane (Jenkinson, 1960), on the conductances activated by agonists was investigated. The concentration range used was necessarily limited by the fact that curare and hexamethonium destabilized the bilayers. These antagonists reduced the membrane conductance induced by SubCh (Fig. 2*B*)

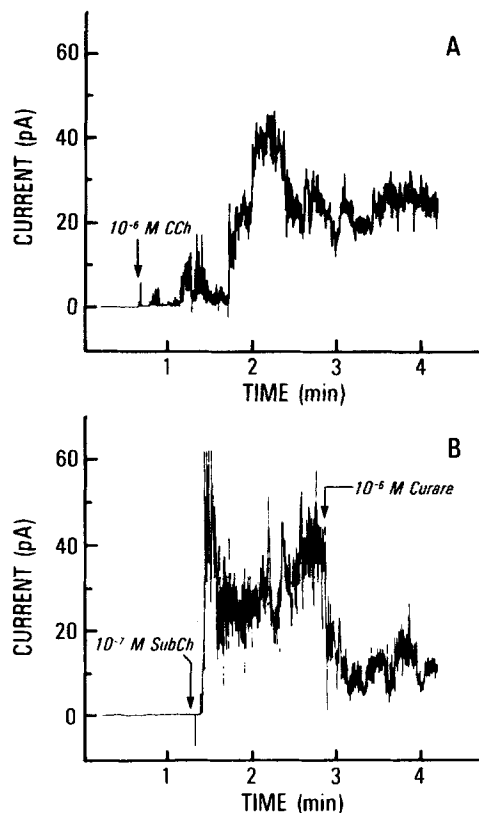


FIGURE 2. Time course of the agonist-activated conductance increase in planar lipid bilayers containing purified AChR. Bilayers were formed in 0.5 M NaCl, 5 mM CaCl₂, 2.5 mM HEPES, pH 7.4. A voltage step of +50 mV was applied across the membrane and held constant throughout the record. The arrows indicate the time at which the agonists or curare was added into the *cis* compartment under vigorous stirring. The response shown in *A* was recorded directly in a chart recorder with a response time of ~0.5 s. Accordingly, the increase in current noise induced by 1 μ M CCh was severely damped. Nevertheless, the increase in membrane noise associated to the conductance increase is apparent when compared with the noise amplitude prevalent before agonist addition. The response shown in *B* was first recorded on an FM tape recorder and then displayed on a chart recorder. Here, the increase in membrane noise following the response is clearly resolved. In *B*, after the response activated by 0.1 μ M SubCh had reached a plateau, 1 μ M curare was added to the *cis* chamber. Note that curare depresses both the membrane conductance and the current noise.

and CCh. The decrease in membrane conductance caused by the antagonists was concentration dependent, as indicated in Table I, exerting 50% inhibition at $\sim 10^{-6}$ M (in the presence of 10^{-6} M CCh).

SINGLE CHANNEL CONDUCTANCE At high current resolution, the opening and closing of individual channels can be monitored. Both the single channel conductance, γ , and the channel open and closed times can be readily measured.

(i) *The single channel conductance, γ .* The currents flowing through individual AChR channels appear as discrete transient steps that fluctuate between two identifiable levels. These levels are associated with the closed and open states of the channel. The channel remains open for a few milliseconds before closing. This is illustrated in Fig. 4. The upper panel is a stretch of a record obtained with 10^{-7} M SubCh at an applied voltage, V , of -100 mV. As indicated, a downward deflection corresponds to a channel opening event and the reverse is true for the closing event. The lower panel is a computer-generated signal used

TABLE I
Effect of Antagonists on the CCh-activated Membrane Conductance in Planar Lipid Bilayers Containing Purified AChR

| Antagonist | Concentration | Fraction of initial conductance |
|---------------|---------------|---------------------------------|
| | <i>M</i> | |
| Curare | 10^{-7} | 0.87 ± 0.1 ($N = 3$) |
| | 10^{-6} | 0.45 ± 0.2 ($N = 3$) |
| Hexamethonium | 10^{-6} | 0.62 ± 0.02 ($N = 3$) |
| | 10^{-5} | 0.40 ± 0.05 ($N = 3$) |
| | 10^{-4} | 0.15 ± 0.05 ($N = 3$) |

Asymmetric bilayers were formed in 0.5 M NaCl buffer; $1 \mu\text{M}$ CCh was added to the *cis* chamber and the steady state conductance was measured at increasing concentrations of curare or hexamethonium. The experimental values are presented as the fraction of membrane conductance measured after antagonist addition \pm SEM. N = number of experiments.

to assist in measuring the bilayer results. The position, width, and height of the generated rectangular pulses were checked for fidelity in matching the channel openings and closings. These parameters were stored for subsequent analysis.

A conductance histogram obtained from a record containing 200 events is illustrated in Fig. 5: two distinct Gaussian distributions are evident, corresponding to the current levels of the closed and open states. The single channel conductance, γ , can be readily estimated from the amplitude difference of the current at the peaks of the two distributions divided by the applied voltage ($V = -100$ mV). For this record, $\gamma = 28$ pS at 0.3 M NaCl.

(ii) *Current-voltage characteristics of single AChR channels.* Fig. 6 shows the single channel current-voltage curve obtained at two concentrations of NaCl, 0.44 and 0.95 M. These results were obtained with 10^{-7} M SubCh. γ is ohmic in the range studied of -100 mV $\leq V \leq 100$ mV. From this plot the slope conductance is estimated as 38 and 62 pS for the two NaCl concentrations, respectively.

(iii) *Selectivity of the single AChR channel conductance.* γ varies with the cationic but not with the anionic species. The values for Na^+ , Rb^+ , Cs^+ , and NH_4^+ are shown in Table II and representative channel recordings in three chloride salts are illustrated in Fig. 7. In contrast, no differences in γ were measured in NaF

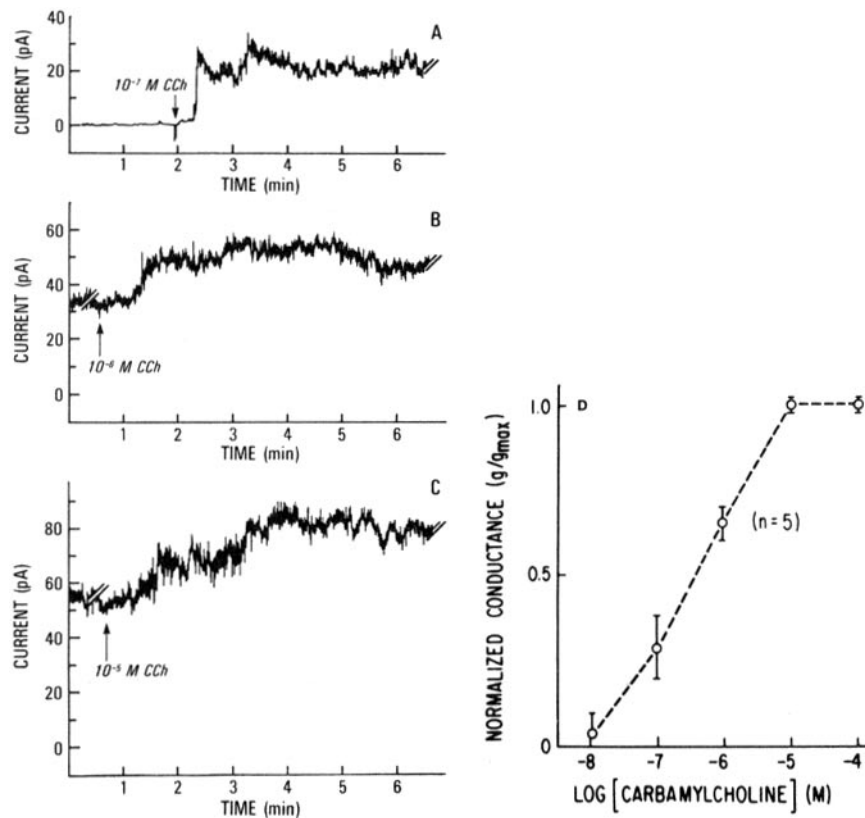


FIGURE 3. Concentration dependence of the CCh-activated membrane conductance. Conditions were the same as for Fig. 2. Agonist addition (indicated by arrows) was done under weak stirring to avoid membrane breakage. Under these conditions, the addition of CCh was followed by a smooth increase in membrane conductance, reaching a steady state in ~ 2 min. Panels A–C are three contiguous sections of the record separated for illustrative purposes. The responses were recorded in a chart recorder with a rise time of ~ 0.3 s. The membrane conductance at each concentration of CCh was estimated as the average conductance measured 3 min after agonist addition. (D) Steady state measurements. The average steady state conductance (\pm SEM) from five different experiments normalized to the conductance at 10^{-4} M CCh was plotted as a function of agonist concentration.

or Na_2SO_4 relative to NaCl. Thus, the apparent selectivity sequence is: $\text{NH}_4^+ > \text{Cs}^+ > \text{Rb}^+ \geq \text{Na}^+ > \text{Cl}^-$, F^- , SO_4^{2-} . Furthermore, γ is the same for SubCh, CCh, or ACh (Montal et al., 1984).

CHANNEL OPEN TIMES (i) *Dependence on agonist.* At room temperature, the

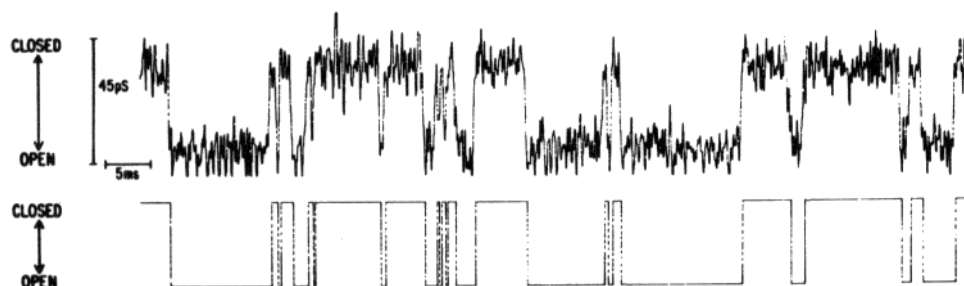


FIGURE 4. Single channel currents activated by agonist in planar lipid bilayers containing purified AChR. Membranes were formed in 0.44 M NaCl, 5 mM CaCl₂, 2.5 mM HEPES, pH 7.4. The agonist was SubCh (0.1 μ M) and $V = -100$ mV. The feedback resistor of the current-to-voltage converter was set to 1 G Ω ; the amplifier time constant was 120 μ s. All other conditions were the same as for Fig. 2. The upper record is a computer-digitized signal from the bilayer amplifier sampled at 0.1-ms intervals after filtering at 1 kHz. A downward deflection is defined as a channel opening event and the next upward step is associated with channel closing. Transitions between closed and open states are indicated by the arrows. The single channel conductance, γ , was 42 pS. The lower record is a computer-generated signal used to assist in measuring the bilayer results as described in Methods.

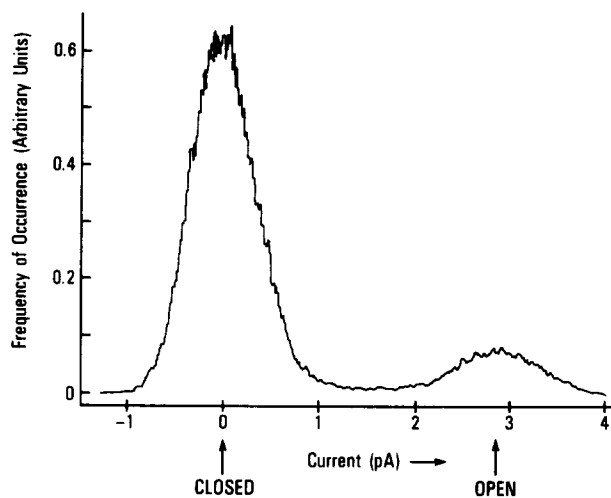


FIGURE 5. Single AChR channel conductance histograms. Membranes were formed in 0.3 M NaCl, 5 mM CaCl₂, 2.5 mM HEPES, pH 7.4. The agonist was SubCh (0.1 μ M) and the applied voltage was -100 mV. The histograms are plots of the relative frequency of occurrence of a given current level. Two Gaussian distributions are clearly discerned: the first one with a mean value (peak) at zero current and the second with a peak at ~ 3 pA. The two peaks correspond to the current levels associated with the closed state and the open state, respectively. γ is the current difference between the two peaks divided by the applied voltage: in these recordings, $\gamma = 28$ pS. The relative areas of closed and open curves are 85 and 15%, respectively, which indicates that the channel tends to be preferentially closed.

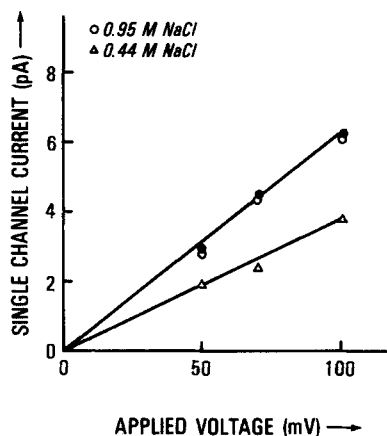


FIGURE 6. Current-voltage relationship of the single AChR channel. Single channel currents activated by SubCh ($0.1 \mu\text{M}$) were measured at the indicated NaCl concentrations and plotted as a function of the applied voltage. Open symbols are for negative and closed symbols for positive applied voltages. The slope conductances were $\gamma = 62$ and 38 pS , respectively.

channel open times are of the order of milliseconds and depend on the agonist used according to the following sequence: SubCh > ACh > CCh (Montal et al., 1984).

(ii) *Analysis of single channel lifetimes.* Single channel current records, in which only one channel was open at any given time, were analyzed as described in Fig. 4. Cumulative open state lifetime distributions were obtained from the computer-generated rectangular pulses. The results of such analysis for SubCh at $V = 100 \text{ mV}$ are presented in Fig. 8. The distribution of open times does not follow a single-exponential function but is best fitted with the sum of two exponentials, yielding values for the fast component, τ_{o1} , of $4.6 \pm 0.2 \text{ ms}$ and for the slow component, τ_{o2} , of $22.8 \pm 1.0 \text{ ms}$, respectively. The results obtained with SubCh at $V = -150 \text{ mV}$ are $\tau_{o1} = 1 \pm 0.02 \text{ ms}$ and $\tau_{o2} = 11.2 \pm 0.5 \text{ ms}$ (not shown).

The distribution of open times can be expressed as:

$$N_{\geq t} = A_1 \exp(-t/\tau_{o1}) + A_2 \exp(-t/\tau_{o2}). \quad (1)$$

TABLE II
Single AChR Channel Conductance in Solutions of Different Monovalent Cations

| X^+ | γ | $\gamma_{X^+}/\gamma_{\text{Na}^+}$ |
|------------------------------|------------|-------------------------------------|
| | pS | |
| Na ⁺ | 28 ± 1 | — |
| Rb ⁺ | 30 ± 2 | 1.10 |
| Cs ⁺ | 38 ± 4 | 1.40 |
| NH ₄ ⁺ | 50 ± 4 | 1.80 |

Single channel currents were activated by SubCh ($0.1 \mu\text{M}$) under the conditions indicated in the legend to Fig. 7. The salt concentration was 0.3 M . γ values indicated are mean \pm SEM.

$N_{\geq t}$ represents the number of events with a channel open time equal to or larger than time t , A_1 and A_2 are the zero time amplitudes, and τ_{o1} and τ_{o2} are the time constants of the fast and slow components of the double-exponential distribution of open times, respectively. The frequency histograms shown in Fig. 8 describe a general feature of the distribution of open times of single channel currents activated by agonists in planar lipid bilayers containing the purified AChR (Labarca et al., 1984). We turn now to describe some characteristics of the two components of the distribution of open times.

(iii) *The time constants of the two components of the distribution of open times are independent of agonist concentration.* The dependence of τ_{o1} and τ_{o2} on the concentration of agonist was investigated. The results for CCh are shown in Fig. 9. The

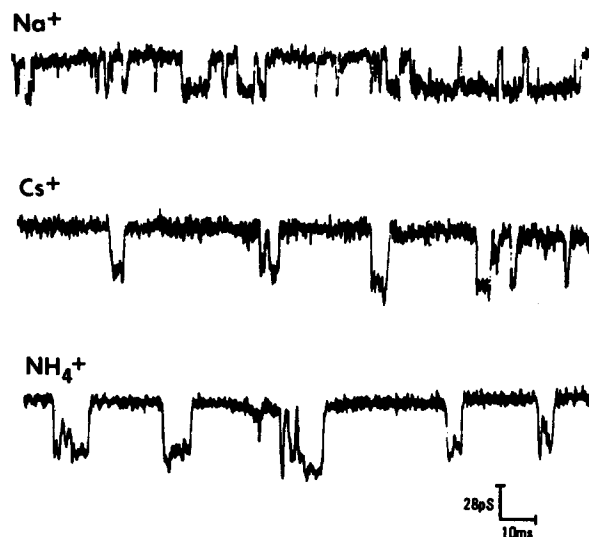


FIGURE 7. Single AChR channel currents in different monovalent cations. Currents were activated by SubCh ($0.1 \mu\text{M}$). Planar lipid bilayers were formed in different salts, at the following activities: NH_4Cl and CsCl , 180 mM; NaCl , 198 mM. In all cases, the aqueous buffer contained 5 mM CaCl_2 , 2.5 mM HEPES, pH 7.4. $V = -70$ mV. The open channel conductances were 28 ± 2 , 38 ± 3 , and 52 ± 2 pS for Na^+ , Cs^+ , and NH_4^+ , respectively.

time constants of the two components are independent of CCh in the range of $10^{-6} \text{ M} < C < 10^{-3} \text{ M}$. Similar results were obtained with SubCh over a more restricted concentration range.

(iv) *The time constants of the distribution of open times depend on the applied voltage.* A detailed study of the voltage dependence for SubCh-activated channels is presented in Fig. 10. The experimental points were fitted by the function:

$$\tau(V)^{-1} = [\tau(0)]^{-1} \exp a \times V, \quad (2)$$

where $\tau(0)$ is the value of either time constant at zero applied voltage and a is a constant in units of reciprocal millivolts (Anderson and Stevens, 1973). $\tau(0)^{-1}$ had values of 0.360 ± 0.040 and $0.048 \pm 0.008 \text{ ms}^{-1}$ (mean \pm SEM) for the fast

and slow components, respectively. On the other hand, the value of the constant a , estimated from the slopes of the straight lines in Fig. 10, was similar for both components, i.e., 0.006 ± 0.001 and $0.005 \pm 0.001 \text{ mV}^{-1}$ (mean \pm SEM), respectively. Similar results were obtained for CCh-activated channels.

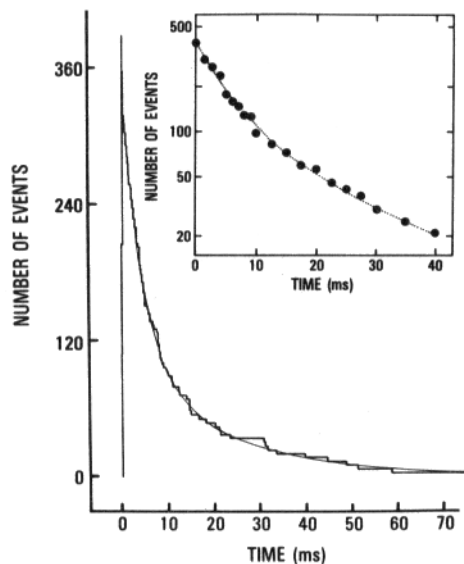


FIGURE 8. Frequency histograms of single AChR channel open times activated by SubCh. Single channel currents were activated with SubCh ($0.1 \mu\text{M}$) at $V = 100 \text{ mV}$. All other conditions were the same as for Fig. 2. The histograms were constructed with the protocol illustrated in Fig. 4. The position and the width of the computer-generated signals corresponding to the channel openings and closings were stored in a PDP 11/34 computer. After analysis of the data, cumulative open state lifetime distributions of the generated rectangular pulses were automatically produced. Time constants were determined by fitting one or two computer-generated exponentials to the data points displayed simultaneously on an oscilloscope screen. The fitted curve (smooth curve) was superimposed on the histograms of the actual data (noisy curve). The distribution of open times could not be fitted with a single exponential (not shown). As illustrated, a sum of two exponentials does generate an adequate fit to the results. The total number of channel openings that were analyzed, N , was 400. The zero-time amplitude (A) and lifetime (τ) of the two components were $A_1 = 280$, $\tau_{o1} = 4.6 \pm 0.2 \text{ ms}$, and $A_2 = 120$, $\tau_{o2} = 22.8 \pm 1.0 \text{ ms}$, respectively. The inset shows a semilogarithmic plot of the actual data. The dotted curve shows the double-exponential function, $N = 280 \exp(-t/4.6) + 120 \exp(-t/22.8)$.

PATTERN OF OCCURRENCE OF SINGLE CHANNEL EVENTS Single channel current records, activated by SubCh at concentrations $\geq 0.1 \mu\text{M}$ or by CCh $\geq 10 \mu\text{M}$, revealed a characteristic pattern of occurrence of single channel activity (Fig. 11). Paroxysms of activity were followed by quiescent periods during which little or no activity was observed. This behavior resembles that of single AChR

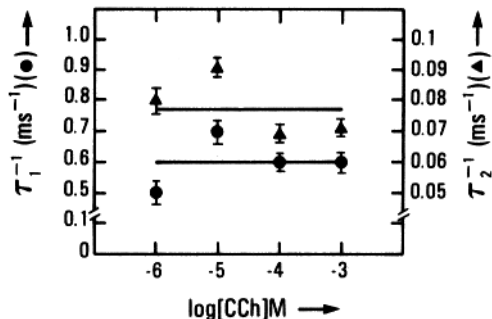


FIGURE 9. τ_{o1} and τ_{o2} as a function of agonist concentration. Single AChR channel currents were activated by CCh over a wide concentration range ($1 \mu\text{M} \leq C \leq 1 \text{ mM}$). All other conditions were the same as in Fig. 3. Records in which only one channel was open at any given time were analyzed. In all cases the distribution of channel open times was adequately fitted by a sum of two exponentials. The inverse of the two components of the distribution of open times (τ_{o1}^{-1} , ●, and τ_{o2}^{-1} , ▲) vs. the log of the CCh concentration are illustrated. Values for τ_{o1} and τ_{o2} shown were measured in the range of $-70 \text{ mV} \leq -100 \text{ mV}$ and normalized to zero applied voltage according to Eq. 2.

channel currents in muscle at desensitizing concentrations of ACh (Sakmann et al., 1980), Paroxysms were composed of many single channel currents. Each open time was interrupted by several brief closings. This is also illustrated in Fig. 11 *b* and *d*, where sections of the records are displayed at higher time resolution.

At concentrations of CCh $\leq 1 \mu\text{M}$, single channel currents appeared more

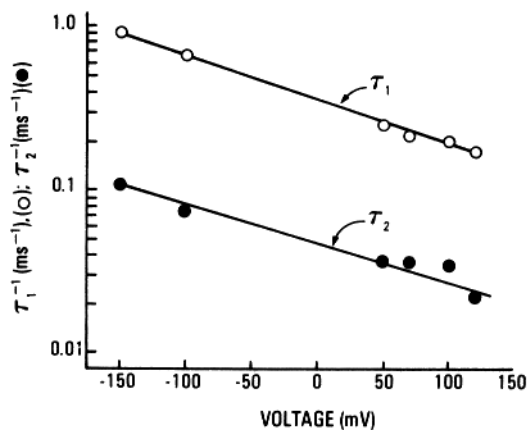


FIGURE 10. Voltage dependence of τ_{o1} and τ_{o2} . Single channel currents were activated by SubCh ($0.1 \mu\text{M}$). Other conditions were the same as for Fig. 4. Records in which only one channel was open at any given time were analyzed. Values for τ_{o1} and τ_{o2} were estimated from the double-exponential distribution of open times. The continuous lines are the best fitting of the experimental points, assuming an exponential dependence of τ_{o1} and τ_{o2} on the applied voltage. The correlation coefficients of the fitted lines are $r_1 = 0.98$ and $r_2 = 0.99$ for τ_{o1} and τ_{o2} , respectively.

evenly distributed throughout the records. A detailed analysis revealed that such was not the case: Fig. 12 *B* shows a histogram of the frequency of channel opening obtained from a record where only one channel was open at any given time. To build the histograms, the record was divided into many equal intervals (100 ms) and the number of openings per period was determined over a large number of periods (>100). The average frequency of channel opening was 1.3 per period and the fraction of the total time that the channel was open was ~5%. The solid bars are the experimental values of P_n , the probability of n (≥ 0) openings. The open bars correspond to the frequencies of openings predicted by a Poisson distribution, using 1.3 as the average number of opening per period. A χ^2 test

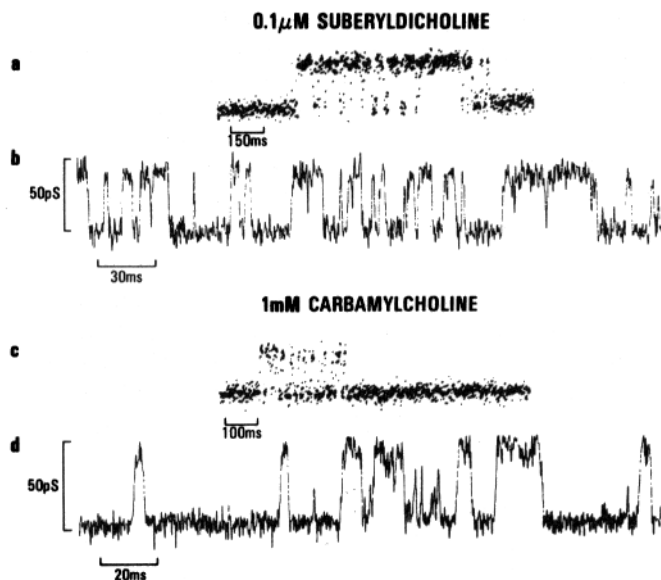


FIGURE 11. Single AChR channel currents activated by desensitizing concentrations of agonists. Single AChR channel currents were activated by SubCh at $V = 100$ mV and CCh at $V = 80$ mV. Other conditions were the same as for Fig. 2. (a) Paroxysm of channel activity activated by $1 \mu\text{M}$ SubCh. In *b*, a section of the same record is displayed at a higher time resolution. (c) Paroxysm of channel activity activated by 1 mM CCh. (d) A section of the same record is illustrated at a higher time resolution.

showed that, at $1 \mu\text{M}$ CCh, the observed frequencies of channel opening deviated significantly from those predicted by a Poisson distribution ($P > 0.001$). At higher CCh concentrations, the clustering of single channel activity reflected in frequency histograms was more prominent and similar to that observed at micromolar concentrations of SubCh. This is illustrated in Fig. 12 *A*. Here the average number of openings per 100-ms period was 1.5. In both histograms, the periods in which zero ($n = 0$) openings occurred, corresponding to the quiescent periods, are in excess over those predicted for a Poisson distribution. Thus, the pattern of single channel activity described for SubCh (Fig. 11) also occurs in

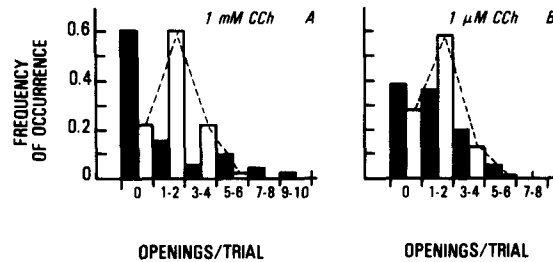


FIGURE 12. Frequency of channel opening in the presence of CCh. Records of single AChR channel currents activated by CCh were analyzed as explained in the text. To build the histograms, the frequencies corresponding to 1–2, 3–4, 5–6, etc. openings were pooled together (solid bars). A χ^2 test of the distribution of frequencies obtained at 1 mM (A) and 1 μ M (B) CCh showed significant departure from the expectations for a Poisson distribution (open bars). $V = 70$ mV.

the presence of CCh (Figs. 11 and 12), although it becomes more evident at concentrations above 10 μ M.

EFFECT OF THE LOCAL ANESTHETIC QX-222 ON THE OPEN CHANNEL LIFETIMES QX-314 and QX-222, two quaternary ammonium derivatives of the local anesthetic lidocaine, are known to block the conduction of ions through the open AChR channel in muscle (Steinbach, 1968; Neher and Steinbach, 1978; Ogden et al., 1981; Ruff, 1982). Blocking by these drugs occurred at micromolar concentrations and, as shown by Neher and Steinbach (1978), the extent of blocking depends on the transmembrane voltage. The effects of QX-222 on the single channel currents activated by agonists can be demonstrated also in planar bilayers. This is shown in Fig. 13. The presence of QX-222 resulted in the appearance of frequent interruptions of the open time (middle panel). This

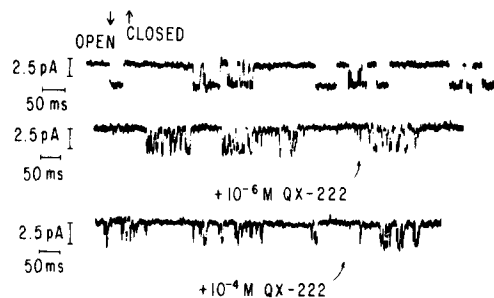


FIGURE 13. Single AChR channel current in the absence and presence of QX-222. Single channel currents were activated by 0.1 μ M SubCh and recorded at $V = -70$ mV. Other experimental conditions were the same as for Fig. 2. The local anesthetic QX-222 was added to the *cis* compartment. Current recordings were stored on analog tape for further analysis (see Table III). The currents shown are photographs of the actual recordings obtained directly from the oscilloscope screen (filtering at 1 kHz). Upper record: control recording in the absence of QX-222. Middle and lower records were in the presence of 1 and 100 μ M QX-222, respectively.

pattern was more prominent at higher concentrations of anesthetic: at 10^{-4} M QX-222 the flickering between open and closed is so fast that distinct events cannot be discerned clearly because of limitations in the time resolution of the current amplifier. Addition of QX-222 to the *cis* side of the membrane reduced the values of both τ_{o1} and τ_{o2} and this effect was concentration dependent (Table III). For 10^{-7} M SubCh at $V = -100$ mV, $\tau_{o1} = 1.5$ ms and $\tau_{o2} = 18$ ms. In the presence of 10^{-3} M QX-222, both the fast and slow components were drastically reduced to ≤ 0.5 and 2.7 ms, respectively. The first value is an upper limit set by bandwidth limitations. Addition of QX-222 to the *trans* side of the bilayer caused no detectable change in τ_{o1} or τ_{o2} (not shown).

The effect of QX-222 on τ_{o1} and τ_{o2} is more pronounced at positive applied voltages (*cis* side positive with respect to the *trans* side). At 1 μ M QX-222 and $V = +100$ mV, τ_{o1} and τ_{o2} were at 1.5 and 5 ms, respectively. These values are four to five times shorter than those measured in the absence of this drug ($\tau_{o1} = 5$ ms, $\tau_{o2} = 25$ ms). In contrast, at $V = -100$ mV, τ_{o1} and τ_{o2} were reduced only by $\sim 20\%$ (Table III).

TABLE III
Effect of Local Anesthetic QX-222 on the AChR Channel Mean Open Times*

| [QX-222] | τ_{o1} | τ_{o2} |
|-----------|----------------|-----------------|
| M | ms | ms |
| — | 1.5 \pm 0.10 | 18 \pm 0.30 |
| 10^{-6} | 1.3 \pm 0.10 | 13.4 \pm 0.30 |
| 10^{-5} | 1.3 \pm 0.03 | 6.2 \pm 0.20 |
| 10^{-3} | 0.5 \pm 0.02 | 2.7 \pm 0.08 |

Channel open times as a function of the concentration of QX-222. Single channel currents were activated by SubCh (0.1 μ M) in planar lipid bilayers containing AChR under the conditions of Figs. 2 and 4, except that the feedback resistor of the current amplifier was 1 G Ω ($\tau = 120$ μ s). Experimental records in which only one channel was open were analyzed. The applied voltage was -100 mV. Frequency histograms of channel open times were fitted by a double exponential and values of τ_{o1} and τ_{o2} were estimated. The estimate of τ_{o1} at 10^{-3} M QX-222 is an upper boundary since bandwidth limitations and noise restrict the reliability of our measurements to ≤ 0.5 ms.

DISCUSSION

The experimental procedure to reconstitute purified AChR with functional ion channels in planar lipid bilayers was defined. This method depends on the transfer of functional AChRs from lipid vesicles into monolayers at the air-water interface (Schindler and Quast, 1980). This strategy allows the assay of the functional integrity of the AChR before bilayer formation. Successful reconstitution in planar lipid bilayers was strictly dependent on the AChR activity in the reconstituted vesicles. The preparations used in this study have been extensively characterized structurally as well as biochemically and pharmacologically (Anholt et al., 1980, 1981, 1982). Anholt and collaborators demonstrated that purified AChRs reconstituted in lipid vesicles displayed both activation and desensitization in the presence of cholinergic agonists. These reconstituted AChR vesicles are

the starting material used to generate monolayers and, in turn, planar lipid bilayers (Nelson et al., 1980).

The transfer of AChRs from the reconstituted vesicles into monolayers at the air-water interface was demonstrated using an interfacial approach (see also Schindler and Quast, 1980): AChRs in vesicles were labeled with α BGT. Monolayers at the air-water interface derived from the vesicles contained labeled AChRs. The contribution of adsorbed vesicles at the interface was determined to be negligible.

The agonist-activated increases in macroscopic membrane conductance exhibited two properties traditionally associated with the postsynaptic membrane: dependence on the agonist concentration and sensitivity to antagonists. The steady state conductance increased with agonist concentration. Similar observations were reported by Sheridan and Lester (1977) in *Electrophorus* electroplaques and were predicted by the classic cyclic model of Katz and Thesleff (1957).

Current measurements with a higher time resolution than previously achieved (Nelson et al., 1980) allowed us to clearly resolve single channel events. The single events display two open states that differ in their mean open times but have similar open channel conductances (see also Labarca et al., 1984).

The open channel conductance (γ) in symmetric electrolyte solutions was ohmic over the range ± 100 mV. This constancy was maintained even at 600 mM salt activity. In contrast, γ increased nonlinearly with the salt activity and approached saturation (Montal et al., 1984). γ saturated also as a function of NH_4^+ activity: $K_{1/2}$ was estimated to be 350 mM and γ_{max} to be 150 pS (unpublished observations).

The magnitude of γ in different salts of sodium was not significantly different, which indicates that anions do not contribute to the single channel currents. This observation is in agreement with the accepted view that anions are poorly permeable through the AChR channel in muscle membranes (Takeuchi and Takeuchi, 1960; Adams et al., 1980, 1981; Dwyer et al., 1980). In contrast, the nature of the cation present in the aqueous solution did affect the magnitude of the single channel conductance: γ was largest for NH_4^+ , and followed the sequence $\text{NH}_4^+ > \text{Cs}^+ > \text{Rb}^+ \cong \text{Na}^+$. The $\gamma_x^+/\gamma_{\text{Na}}^+$ ratios for the cations tested agree well with those obtained by others in muscle membranes (Adams et al., 1981; Hamill and Sakmann, 1981).

Analysis of single channel records indicate that the AChR channel displays two kinetically distinct open states that differ in their mean open times but have similar channel conductances (see Labarca et al., 1984). The occurrence of two components of the distribution of open times in single channel current recordings cannot be attributed to the coexistence in the planar bilayer of a component activated by agonists and a contaminant because (a) channels were observed only after exposure to agonists, (b) γ was the same for the different agonists, (c) the time constants of both components were longer for SubCh than for CCh, and (d) both kinetic components had a similar voltage dependence. These observations, therefore, suggest the occurrence of two kinetically distinguishable components of channel open times (Labarca et al., 1984). The two time constants of the distribution of open times did not change systematically with agonist concen-

tration. The dependence of both time constants on the applied voltage is exponential, with the lifetimes increasing an e-fold for hyperpolarizations of the order of 125 mV. This voltage dependence, although weak, is noticeable since its sign and magnitude are similar to those reported for AChR in muscle (Magleby and Stevens, 1972*a, b*; Neher and Sakmann, 1976*a*) and in *Electrophorus* electroplaques (Sheridan and Lester, 1977). The existence of two kinetic components in the distribution of channel open times has been observed for the AChR (Colquhoun and Sakmann, 1981; Jackson et al., 1982, 1983) and for the glutamate receptor (Cull-Candy and Parker, 1982; Gration et al., 1982) in native membranes.

Single channel currents activated by SubCh and CCh occurred in paroxysms of channel openings separated by quiescent periods. This pattern was more pronounced at higher agonist concentrations and was reflected in histograms of channel opening frequencies. Within a paroxysm, channel openings appeared interrupted by brief closing periods, giving them the appearance of "bursts," as defined by Colquhoun and Sakmann (1981). The paroxysms of channel activity might arise from AChR desensitization (Labarca et al., 1983). According to this interpretation, first proposed by Sakmann et al. (1980), each paroxysm would represent the transit of an AChR through its active conformation, from which it can open and close several times before desensitizing. The fact that clusters of activity occurred at submicromolar concentrations of SubCh, while they became more evident at CCh concentrations above 10 μ M, could be attributed to the higher affinity of SubCh for the AChR (Neher and Sakmann, 1976*b*; Trautman and Feltz, 1980; Jackson et al., 1982).

The local anesthetic QX-222 changed the kinetics of single channel currents, as reflected by the decrease in the values of τ_{o1} and τ_{o2} . Qualitatively, these effects of QX-222 are in agreement with those observed by Neher and Steinbach (1978), who demonstrated that QX-222 transiently blocks the conduction of ions through the open AChR channel (see Ruff, 1982). Our studies indicate that the effect of QX-222 depends on the applied voltage, being more prominent when the *cis* side of the membrane was positive with respect to the opposite side. No detectable effect was measured when this drug was present in the *trans* side of the bilayer. This suggests that if QX-222 enters the channel, it does so only from the extracellular side (Horn et al., 1980).

In conclusion, our studies support the idea that the reconstitution procedure leads to the incorporation of the functional channel protein in a model system that is particularly well suited for electrical studies. This assay demonstrated that the purified channel protein, isolated from its native environment and reconstituted in an extraneous membrane milieu, reproduces the characteristic properties that define, at the molecular level, the process of postsynaptic transmission.

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