Characterization of the Channel
Properties of a Neuronal Acetylcholine Receptor
Reconstituted into Planar Lipid Bilayers

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ABSTRACT An α-toxin-binding membrane protein, isolated from the head and thoracic ganglia of the locust (Locusta migratoria), was reconstituted into planar lipid bilayers. Cholinergic agonists such as acetylcholine, carbamylcholine, and suberyldicholine induced fluctuations of single channels, which suggests that the protein represents a functional cholinergic receptor channel. The antagonist d-tubocurarine blocked the activation of the channels, whereas hexamethonium had only a weak effect; similar properties have been described for nicotinic insect receptors in situ. The channel was selectively permeable to monovalent cations but was impermeable to anions. The conductance of the channel (75 pS in 100 mM NaCl) was independent of the type of agonist used to activate the receptor. Kinetic analysis of the channel gating revealed that, at high agonist concentrations (50 µM carbamylcholine), more than one closed state exists and that multiple gating events, bursting as well as fast flickering, appeared. At very high agonist concentrations (500 µM carbamylcholine), desensitization was observed. Channel kinetics were dependent on the transmembrane potential. Comparing the conductance, the kinetics, and the pharmacology of nicotinic acetylcholine receptor from insect ganglia and fish electroplax reconstituted into bilayers revealed obvious similarities but also significant differences.

INTRODUCTION

The significant advances in understanding the molecular biochemistry of the nicotinic acetylcholine (ACh) receptor, present in muscle and in the muscle-derived electroplax of certain fish, has mainly been due to the availability of electric tissue as an exceptionally rich source of ACh receptor and the use of small polypeptide α-toxins as highly selective labels for the ACh-binding sites (Conti-Tronconi and Raftery, 1982; Maelicke, 1984; Popot and Changeux, 1984). As a consequence, this ACh receptor protein has become one of the best-characterized neurotransmitter receptors and also one of the most thoroughly studied membrane proteins (Changeux et al., 1984). Neuromuscular junctions...
as well as electromotor synapses have evolved for highly specialized functions and thus may differ from neuronal synapses at the molecular level. The observation that there are many neurons in the central nervous system that respond electrically to ACh suggests the existence of nicotinic ACh receptors in nerve cells; it is therefore of interest to compare the molecular identity and microphysiological properties of nicotinic ACh receptors from nervous tissue and peripheral synapses.

The nervous tissue of insects is highly cholinergically innervated (Florey, 1963) and contains a high concentration of α-toxin–binding sites (Dudai, 1979; Breer and Sattelle, 1987). In the insect nervous system, these binding sites probably represent ACh receptors. Recently (Breer et al., 1984, 1985), an α-bungarotoxin–binding protein (Mr 250,000–300,000), a putative neuronal ACh receptor, has been purified from detergent-solubilized ganglionic membrane preparations of locusts using affinity chromatography; it apparently represents an oligomeric complex of four or five similar or perhaps identical polypeptides (Mr, ~65,000). Convincing proof that an isolated protein does in fact represent a functional transmitter receptor (i.e., contains both the agonist-binding site and the channel that it regulates) can only be achieved by reconstitution into artificial model membranes (McNamee and Ochoa, 1982; Montal et al., 1984; Hanke, 1985). Therefore, we have recently started to study the functional properties of the purified α-toxin–binding protein from locust nervous tissue in planar lipid bilayers (Hanke and Breer, 1986). After this protein was inserted into a planar lipid bilayer, we observed single-channel activity induced by cholinergic agonists. This initial characterization of single-channel recordings from insect neuronal ACh receptors revealed properties that appeared quite similar to those reported for the reconstituted ACh receptor isolated from electroplax membranes or for the receptor of muscle cells recorded in situ (Sakmann et al., 1980; Boheim et al., 1981; Labarca et al., 1984a, b). In this study, we have further characterized the channel-forming properties of the purified neuronal nicotinic ACh receptor by analyzing the channel conductance and the lifetimes of its open and closed states in the presence of various cholinergic agonists and antagonists.

MATERIALS AND METHODS

Preparation

An ACh receptor protein from the nervous tissue of locust (Locusta migratoria) was purified by affinity chromatography as described previously (Breer et al., 1984, 1985). Briefly, the head and thoracic ganglia from locust were dissected and stored at ~70°C. After the tissue was homogenized in antiprotease buffer, the nuclei and cell debris were separated by centrifugation at 1,000 g for 10 min. The supernatant was centrifuged at 45,000 g for 30 min. The resulting pellet was extracted with 1% wt/vol sodium deoxycholate. The detergent extract was incubated with an affinity gel (α-bungarotoxin covalently coupled to CNBr-activated Sepharose 4B) overnight. The affinity matrix was then thoroughly washed and the proteins bound were subsequently eluted with 100 mM carbamylcholine. Polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) on a microscale system. Polypeptides were visualized by staining the gel with Coomassie brilliant blue. The native receptor migrated as a single band corresponding to
a molecular mass of ~300,000 daltons; in a sodium dodecyl sulfate gel under reducing conditions, the purified receptor gave a single band at ~65,000. The purified ACh receptor protein was incorporated into membranes of liposomes produced from asolectin/cholesterol (15:2 molar ratio) by extensive dialysis against a large volume of buffer solution (150 mM NaCl, 50 mM sucrose, 10 mM Tris-HEPES, pH 7.4). The preparation was kept at 4 °C during the procedure. The protein-containing vesicle preparation was stored in ice water and was used for a maximum of 3 d.

Planar Bilayer Formation and Channel Insertion

The planar lipid bilayers used were formed according to the technique of Montal and Mueller (1972), by the folding of two stable monolayers across a hole separating two aqueous phases. The Teflon partition was 5.6 μm thick, with hole diameters of 120–150 μm. The Teflon partitions were pretreated with a solution of n-hexane/n-hexadecane, 9:1 vol/vol, and allowed to dry by air. The lipid monolayers used to form the bilayer were spread from a solution of 10 mg lipid dissolved in 1 ml n-hexane on the air-water interface on each side of the divided chamber (Hanke, 1985). Before the bilayer was formed, the hexane of the lipid solution was allowed to evaporate for at least 5 min. Finally, the solution levels in both compartments were raised by the addition of more solution. Formation of the bilayer was monitored by continuously measuring the membrane capacity. The lipids and aqueous solutions used in different experiments are described in the figure legends.

10–50 μl of the liposome solution containing the ACh receptor protein was added to the aqueous solution on one side of the bilayer (2 ml vol). This side was defined as the cis side of the bilayer setup. The chamber was stirred vigorously for a few minutes to increase the rate of fusion of vesicles with the bilayer. In contrast to previous experience (Hanke and Miller, 1983), an osmotic gradient did not significantly increase the fusion rates. Fusion events were monitored by continuous current measurement (Hanke, 1985, 1986) at a constant membrane potential (50 mV). Usually, one or more fusion events could be observed within 30 min. In most experiments, no current fluctuations were observed at this stage. If spontaneous fluctuations did occur, however, the experiment was terminated and a new bilayer was formed. 30 min after the addition of vesicles to the cis side, agonists were added to the aqueous solution. We have added the agonist symmetrically to both sides of the bilayer to make sure that all incorporated channels were activated. Although in general channels were inserted very asymmetrically, “backward” channels occasionally occurred; these could easily be detected in voltage-dependent experiments (see Results). Recordings were started when channel current fluctuations could be observed; this was usually within a few seconds. In cases where no fluctuation occurred within 30 min after agonist addition, the experiment was terminated. All experiments were performed at room temperature (20–22 °C).

Asolectin (type IV, Sigma Chemical Co., St. Louis, MO) was purified (Cook, N., C. Zeilinger, K.-W. Koch, and U. B. Kaupp, manuscript submitted for publication), and cholesterol (analytical grade) was purchased from Fluka (New Ulm, Federal Republic of Germany). In some experiments, we used phosphatidylethanolamine (PE) purified from egg yolk by column chromatography, as well as palmitoyloleoyl-phosphatidylcholine (POPC) and dioleoyl-phosphatidylglycerol (DOPG), both obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). ACh, carbamylcholine, suberyldicholine, d-tubocurarine, and hexamethonium were obtained from Sigma Chemical Co. If not specified otherwise, drugs were added to both sides of the bilayer. All salts and solvents used were at least of analytical grade and the water was double quartz distilled. All aqueous solutions were filtered through filters (pore size, 0.2 μm) before use.
The principles and instrumentation of the electrical setup have been described previously (Hanke and Miller, 1983; Hanke, 1985). An amplifier (LM-EPC7, List-Medical, Darmstadt, Federal Republic of Germany) was used as a current-voltage converter. It was connected to the electrode on the trans side of the bilayer. The cis side of the bilayer was directly grounded. Membrane potentials were applied via the amplifier to the trans side of the bilayer. The data obtained were stored on an FM tape recorder (Store 4 DS, Racal Recorders, Inc., Sarasota, FL) at a bandwidth of 10 kHz (low-pass filter, −3 dB point). In multichannel experiments, current-voltage (I-V) curves were recorded directly onto a pen recorder.

Data Analysis

Single-channel data were replayed on an FM tape recorder and sampled by a Minc 11/23 computer or IBM PC at a rate of 1 kHz and a resolution of 12 bits. Sometimes a higher sampling rate, as specified in the Results, was used. Before digitizing, data were low-pass-filtered at 1 kHz (12 dB per octave, −3 dB point); data with a signal-to-noise ratio of <1:1 were not used. From the digitized traces of channel fluctuations, amplitude histograms were constructed directly by plotting the number of samples as a function of the amplitude. These histograms were fitted by the sum of two Gaussian distributions (if this was not possible, the data were not used), and thus the closed and open states of the channel were determined. From the distance of both maxima, the channel conductance was calculated. The minimum between the two maxima was determined and used to distinguish between the closed and open channel states. The areas under both peaks, \( A_c \) and \( A_o \), were integrated and the probability, \( P_o \), of the open state for the channel was calculated according to

\[
P_o = \frac{A_o}{A_o + A_c}.
\]

Using the value of this minimum, a discriminator (Findline) was set in the original traces, and the amplitude and duration of every single event, for the closed and open states, were determined by the computer. From these results, lifetime histograms for both channel states were constructed. The half-logarithmic plots of these distributions were fitted by the sums of the exponentials. Two exponentials were used only if each component comprised >5% of the amplitude. For the closed-time distribution, this was only found for high agonist concentrations, whereas for the open time, it was mainly observed in experiments where ACh was used as the agonist; those time-distribution data are not considered in this article. For the closed-time distribution, fast components owing to the so-called "flickering" were eliminated by an appropriate filter setting (Methfessel and Boheim, 1982); the occurrence of a second slow time constant in the closed-time distributions is usually interpreted in terms of bursting. Since we were mainly interested in the channel kinetics within the burst, the bursting process was omitted by subtracting the slow process and calculating only the data for the fast process. For this particular case, amplitude histograms and \( P_o \) were calculated again from the original traces, using only the long-lasting bursting intervals. From the lifetime distributions, the mean lifetimes of the channel states were determined and compared with the arithmetic means. In cases of significant discrepancies, the analysis was repeated or the data were not used. From the estimated lifetimes, \( P_o \) was determined according to

\[
P_o = \frac{\tau_o}{\tau_o + \tau_c}
\]

and compared with the numbers determined from the amplitude histograms. Again, data were recalculated and were not used in cases of significant differences. In particular, for experiments using high agonist concentrations, this procedure was used to calculate the values for the inner-burst kinetics. Time constants below 1 ms usually could not be obtained because of the limitations given above. In most of the studies, the data of several experiments (up to 10) were averaged. In other cases, e.g., in attempts to estimate \( P_o = f([\text{carbamylcholine}]) \), the data for each channel were calculated separately and then used to determine a mean slope of the Hill plot. This procedure was particularly necessary because the carbamylcholine concentrations at which \( P_o = 0.5 \) scattered over a wide range;
however, the slope of the Hill plot was nearly constant. At low agonist concentrations, and thus at low $P_o$, the number of channels active in the bilayer cannot unequivocally be estimated; the kinetic values calculated must therefore be considered as upper limits. The various procedures of data analysis employed in this study are illustrated in a flow chart (Fig. 1).

RESULTS

General Properties of the Reconstituted Channel

In the absence of any cholinergic agonist in the aqueous solution, channel fluctuations were usually not observed. In ~5% of the experiments, spontaneous undefined current fluctuations occurred without an agonist present; these currents were never related to defined channels and might have been due to some denatured proteins in the preparations. Agonist-induced channel fluctuations were not found to be significantly dependent on the lipid composition of the bilayer, although small amounts of cholesterol apparently caused longer channel lifetimes; details will be presented in a subsequent article. Under the experimental conditions used in most of our experiments, only one or very few functional channels were incorporated into the planar bilayer during the course of an experiment. Only in rare cases was a higher number of active channels found. A typical example is shown in Fig. 2A. About 30 min after 200 µl of a liposome suspension was added to the cis side of the bilayer, the $I-V$ relation was determined without any agonist. This $I-V$ relation showed a very small, voltage-independent conductance, slightly higher than the conductance of the pure lipid bilayer; this was probably due to some denatured proteins in the preparations. After 50 µM carbamylcholine was added symmetrically to both sides of the bilayer, the $I-V$ relation was determined again. Under this condition, not only was the level of conductance much higher, but the conductance was significantly voltage dependent, increasing toward negative potentials. This observation indicates an asymmetrical insertion of channel proteins. About 75% of the agonist-induced current was blocked after the addition of d-tubocurarine (30 µM), as shown in the third $I-V$ curve. Fig. 2B shows a short fluctuation trace of two independent active channels in one membrane, together with an amplitude histogram of this fluctuation. This trace was recorded at 90 mV in a solution containing 150 mM NaCl, 2.5 mM Tris-HEPES, pH 7.4; activation was achieved by the addition of 5 µM carbamylcholine. The channel conductance was estimated to be ~80 pS in this case. Fig. 3 presents a long continuous trace showing the fluctuation of a single channel under conditions identical to those in Fig. 2B; this recording demonstrates the stability and resolution of the system. An analysis of this recording is given in Fig. 4. Fig. 4A shows an amplitude histogram with a closed (0) and an open (1) state. The evaluated lifetimes of the open (Fig. 4B) and closed (Fig. 4C) states of this channel are demonstrated. Both are given in linear (top) and half-log (bottom) plots. The lifetimes of both states can be described by a single exponential, indicating a mean lifetime of the open state, $\tau_o = 4$ ms, and of the closed state, $\tau_c = 46$ ms. A probability of the channel being in the open state, $P_o = 0.1$, was independently estimated by integrating the amplitude histogram of Fig. 4A.
FIGURE 1. Principles of data analysis as described in the text. The parameters on the right are checked for consistency and are used for evaluation of functional dependence on experimental parameters (voltage, agonist concentration, salt concentration, etc.). In the case of more than one exponential in the closed-state lifetime evaluations (lower left), only one kinetic state was evaluated by selecting parts of the inner-burst fluctuation for further processing. The problem of missing events caused by the limitations in time resolution was omitted by adequately setting the limits of the fit in the lifetime evaluation (lower left).
FIGURE 2. (A) I-V relations of a planar lipid bilayer containing ACh receptor protein purified from insect nervous tissue. The bilayer was made from purified asolectin on a hole of \( \sim 200 \mu m \) diam. The salt solution was identical on both sides of the bilayer and contained 2.5 mM Tris-HEPES, 150 mM NaCl, 1 mM CaCl_2, pH 7.4, 20°C. 200 \( \mu l \) of a proteoliposome preparation was added to the \textit{cis} side of the bilayer and the same volume of salt solution was added to the \textit{trans} side to avoid different levels of solutions. After 30 min, an I-V curve was drawn. Whereas the pure lipid bilayer displayed a conductance that cannot be resolved on the scaling of the diagram, the conductance after fusion with proteoliposomes (A) was small but significantly higher; 5 min after 50 \( \mu M \) carbamylcholine was added symmetrically to both chambers, a clearly different I-V curve was produced (B). The agonist-induced conductance is significantly voltage dependent, increasing toward negative potentials. The addition of 30 \( \mu M \) d-tubocurarine (C) blocked \( \sim 75\% \) of the carbamylcholine-induced current. (B) Single-channel fluctuation of two independent neuronal ACh receptor channels in a planar lipid bilayer. Channels were inserted into solvent-free planar bilayers as described in the Materials and Methods. The lipid used was asolectin, partially purified before use as described (Cook, N., C. Zeilinger, K.-W. Koch, and U. B. Kaupp, manuscript submitted for publication). The amplitude histograms of a long trace and a short fluctuation trace (inset) are shown, recorded at 90 mV. The channels were activated by 5 \( \mu M \) carbamylcholine. The aqueous solution contained 150 mM NaCl and the channels had a mean conductance of \( \Lambda = 80 \) pS. The states of the fluctuation are indicated by 0 (closed), 1 (open_1), and 2 (open_2).
Pharmacology of the Channel

It was found that the neuronal ACh receptor channel can be activated by a variety of cholinergic agonists. In a previous article (Hanke and Breer, 1986), we showed that the channel can be activated by carbamylcholine and suberyldicholine. Fig. 5 shows examples of channel fluctuations induced by adding 0.5 μM carbamylcholine or 0.5 μM ACh to a bath solution. The conductance of the channel was independent of the type of agonist used. The channel properties determined under various conditions are summarized in Table I. The mean

channel conductance was 80 pS in 150 mM NaCl (asolectin/cholesterol) and 75 pS in 100 mM NaCl (asolectin/cholesterol or DOPG/POPC/PE). Suberyldicholine turned out to be the most potent agonist in activating the channel, followed by ACh, and then by carbamylcholine. The lifetime for channel open events became longer from carbamylcholine to ACh and suberyldicholine. We found in some experiments that the open-event lifetimes were not single-exponentially distributed, especially when ACh was used to activate the channel. This observation is presently under more detailed investigation.

Fig. 6 shows the dependence of the channel fluctuations on the agonist concentration. Records were taken at four different concentrations of carbamyl-
choline, as indicated; other conditions were as given in Fig. 2B. At high carbamylcholine concentrations, bursting effects (see below) were omitted as described above (Data Analysis) and only the inner-burst kinetics were evaluated. It is obvious that the probability of the channel being in the open state ($P_o$) increases with increasing carbamylcholine concentrations. All four traces were obtained from the same bilayer at increasing carbamylcholine concentrations. In Fig. 7, lifetimes of the open ($\tau_o$) and the closed ($\tau_c$) states are plotted as a function of the agonist concentration. Whereas the lifetime of the mean open state is only slightly dependent on the agonist concentration, the closed-state lifetime decreases significantly with increasing carbamylcholine concentrations.

We also investigated the effects of antagonists on the neuronal ACh channel. Fig. 8A shows the effect of 0.1 mM of hexamethonium on the ACh receptor channel after activation by 5 μM carbamylcholine. Only ~25% of the activity is blocked at this antagonist concentration, which indicates that the insect receptor is surprisingly insensitive to hexamethonium. We were unable to investigate the effect of hexamethonium at concentrations higher than 0.1 mM because the planar bilayer became unstable at millimolar concentrations. In Fig. 8B, the effects of two different concentrations of d-tubocurarine are shown: 0.5 μM blocked ~35% of the activity and 5 μM blocked ~75% of the activity. We conclude from these data that a d-tubocurarine concentration of ~1 μM is necessary to block 50% of the channel activity. Experiments at the single-channel level revealed that neither hexamethonium nor d-tubocurarine affects the conductance of a channel. As shown in Fig. 9 by two fluctuation traces before and after the addition of d-tubocurarine, the probability of the channel being in the open state is reduced by the drug but the channel conductance is not. Table II summarizes the effects of the antagonists on channel parameters.

Conductance of the Channel

Fig. 10 depicts the I-V relation for a single open channel. This curve is linear over the entire voltage range investigated, indicating an ohmic behavior of the channel with a conductance of 80 pS for that particular experiment. The mean conductance estimated in the presence of 150 mM NaCl was $\bar{G} = 80 \pm 4$ pS ($n = 9$).

In Fig. 11, the conductance of the ACh receptor channel is plotted against the Na$^+$ concentration in the bath solution. The curve saturates at ~100 pS; half-maximal conductance is achieved at a concentration of ~60 mM Na$^+$. In a previous article (Hanke and Breer, 1986), we showed that the channel is not particularly selective for Na$^+$ over K$^+$ and is impermeable to Cl$^-$. In some experiments, conductance substructures (Auerbach and Sachs, 1983) of the ACh receptor channel were observed in addition to the predominant channel conductance, $\bar{G} = 80$ pS. Fig. 12 shows three examples of substructures. In A, a single-channel conductance of ~50 pS in 150 mM NaCl is shown. This set of smaller channel events had a mean conductance of $45 \pm 6$ pS ($n = 9$) in 150 mM NaCl. Only channel events with clearly resolvable open states were considered, thus excluding the problems with channels that were not time-resolved.

In addition to this group of channels with lower conductance, we observed
closing events from the open level of the neuronal ACh receptor channel to different levels of lower conductance; two of these so-called sublevels are shown in Fig. 12B. The conductance of these sublevels scattered over a wide range of conductance with a mean conductance of 80 pS.

**FIGURE 4.** Evaluation of the single-channel properties of the fluctuation trace of the neuronal ACh receptor shown in Fig. 3. **A** In the amplitude histogram of this trace is shown; it clearly exhibits a well-defined closed state (0) and a well-defined open state (1). In the inset, a short fluctuation trace is shown. The channel conductance taken from the histogram is 80 pS, and the mean probability of the channel being in the open state, calculated by interpreting the histograms (see Materials and Methods), is $P_o = 0.1$. **B** The lifetime distribution of the open state of the channel is shown on a linear (top) and a half-logarithmic plot (bottom). The lifetimes are clearly single-exponentially distributed, giving a mean open-state lifetime of $\tau_o = 4$ ms. **C** (opposite) The lifetime distribution of the closed state of the channel is presented in B for the open state. It is single-exponentially distributed with a mean closed-state lifetime of $\tau_c = 46$ ms. From $B$ and $C$, according to Eq. 4, a mean $P_o = 0.08$ can be calculated, which is in good agreement with the value in $A$:

$$P_o = \frac{\tau_o}{(\tau_o + \tau_c)}.$$

(1)
near 25 pS in 150 mM NaCl. However, the probability of finding low-conductance channels or sublevels, as shown in Fig. 12, A and B, was very low (<1% of all events counted).

Voltage Dependence of Channel Kinetics

To demonstrate the voltage dependence of the channel kinetics, four traces of single-channel fluctuations at different voltages are presented in Fig. 13. A significant steep dependence on increasing voltage was not seen; however, the channel seemed to close toward higher potentials with a small voltage dependence (see Fig. 2A for comparison). For better control, we have plotted the open-state
Figure 5. Single ACh receptor channel currents activated by different agonists. A single channel was activated by 0.5 μM ACh (A) and by 0.5 μM carbamylcholine (B). In both parts of the figure, experimental conditions were as given in Fig. 1B. The applied potential was 50 mV. The ACh-activated channel had an open-state lifetime of $\tau_o = 10$ ms and an open-state probability of $P_o = 0.2$; the values for the carbamylcholine-activated channel were $\tau_o = 3$ ms, $P_o = 0.1$. In general, channel fluctuations activated by ACh were more inhomogeneous than those activated by carbamylcholine and, even at low ACh concentrations, were not always single-exponentially distributed in their open lifetimes.

and closed-state lifetimes of one of these experiments as functions of voltage (Fig. 14A). Notice the different scaling for both values. The open-state lifetime became slightly shorter with increasing membrane potential, whereas the closed-state lifetime became longer. Thus, the main part of the voltage dependence is found in the transition from the closed to the open state of the channel. Fig.

Table 1

Dependence of Single-Channel Parameters on the Type of Cholinergic Agonist Used to Activate the Channel

| Agonist                  | $P_o$  | $\tau_o$ | $\tau_c$ |
|--------------------------|--------|----------|----------|
| 0.5 μM carbamylcholine   | 0.05–0.1 | 3        | 100      |
| 0.5 μM ACh               | 0.20   | 10       | 40       |
| 0.5 μM suberyldicholine  | 0.25   | 15       | 44       |

Experimental conditions: 150 mM NaCl, 2.5 mM Tris-HEPES, pH 7.4, 20°C, $V = 50$ mV, asolectin/cholesterol (20:1). The conductance was 80 pS.
ACh receptor channel fluctuation activated by different concentrations of carbamylcholine. The agonist concentration and the open and closed state of the channel are marked at each trace separately. All traces were recorded from the same bilayer and the same channel. Only one channel was active in the bilayer, as can be deduced from the trace at high agonist concentration (500 μM carbamylcholine) (see Materials and Methods). The open-state probability of the channel increased significantly with the agonist concentration. The experimental conditions were the same as given in Fig. 2B. Here only traces within bursts of fluctuations are shown at all agonist concentrations, as we only evaluated the inner-burst kinetics.

14B plots the probability of the channel being in the open state, \( P_o \), as a function of voltage using the equation

\[
\log[P_o/(1 - P_o)] = f(V).
\]

(2)

The data can be fitted quite well by a straight line with a slope indicating that a charge of \( z = 1/2 \) is transferred across the entire membrane during opening of

![Figure 6](image_url)

![Figure 7](image_url)
the channel. A two-state transition, according to Eq. 3, was achieved to calculate this value:

\[ P_o(V) = 1 + \exp\left[-zF(V - V_o)/RT^{-1}\right]. \]

The data presented here were taken from experiments all being performed in 150 mM NaCl and the channels were activated by 0.5 μM carbamylcholine.

**Figure 8.** Effects of antagonists on neuronal ACh receptor-induced multichannel activity. Experimental conditions were the same as given in Fig. 2B, but 5 μM carbamylcholine was used to activate the channels. Membranes containing more than 10 active channels were used. A potential of 50 mV was applied and antagonist was added as indicated. (A) Effect of 0.1 mM hexamethonium added symmetrically to a membrane containing 20–30 active channels. This concentration of antagonist resulted in a 25% block. Additionally, it can be seen in this trace that the amplitude of the fluctuating current is reduced. (B) Effect of two concentrations of d-tubocurarine, 0.5 μM and 5 μM, on a membrane containing ~20 active channels. Again, the antagonist was added symmetrically to the bath solution. 0.5 μM d-tubocurarine caused a 35% block; if the d-tubocurarine concentration was raised to 5 μM, ~75% of channel activity was blocked. Again, in parallel to the integral reduction of the channel-induced current, the current noise is reduced significantly.
Kinetic Effects at Higher Agonist Concentrations

At low concentrations (≤5 μM carbamylcholine), the open- and closed-state lifetimes are usually single exponential, distributed as shown, for example, in Fig. 4. At higher agonist concentrations, this does not hold. When applying carbamylcholine at a concentration of 5 μM, we found pronounced examples of multiple gating events, as shown in Fig. 15. The term “multiple gating” is used to classify the occurrence of small groups of channel events (Boheim et al., 1981).

| Table II |
|---------------------------------|
| **Inhibition of Carbamylcholine-induced Channel Activity of the ACh Receptor by Antagonists** |

| Agonist | Concentration | Channel blocked | Comment |
|---------|---------------|-----------------|---------|
| Hexamethonium | 0.1 mM | 24 | Multichannel experiment |
| | 1.0 mM | 50 | Estimated value due to instability of the bilayer |
| Curare | 0.5 μM | 55 | Multichannel experiment |
| | 1.0 μM | 50 | Single-channel experiment |
| | 5.0 μM | 75 | Multichannel experiment |
| | | 90 | Single-channel experiment |

Experimental conditions: 150 mM NaCl, 2.5 mM Tris-HEPES, pH 7.4, 20°C, V = 50 mV, 5 μM carbamylcholine, asolectin/cholesterol (20:1).
FIGURE 10.  I-V characteristic of a single neuronal ACh receptor channel in 150 mM NaCl. The characteristic is linear over the whole voltage range investigated, with a single-channel conductance of $\bar{\Lambda} = 78$ pS for the given example. All points in this plot were taken from amplitude histograms as shown in Fig. 4.

Here, 2–10 gating events are queued up in series of clearly not statistically independent open events. We have also observed such multiple gating events using other agonists to activate the channel. With 1 $\mu$M suberyldicholine, for example, we found pronounced multiple gating behavior (Hanke and Breer, 1986).

Using even higher agonist concentrations, we found a pronounced bursting behavior of the neuronal ACh receptor channel. Fig. 16 shows an example of such bursting behavior at 50 $\mu$M carbamylcholine. Each burst is marked separately. To more clearly define the results, the evaluations of open- and closed-state lifetimes are presented (Fig. 17). Whereas the open-state lifetime is single-exponentially distributed, with a mean open-state lifetime of $\tau_o = 4$ ms, the closed-state lifetime is clearly double-exponentially distributed. From the plot in Fig. 17, a closed-state lifetime between bursts of $\tau_c = 202$ ms ($N_o$ [total number of events estimated from exponential fits] = 74) and a closed-state lifetime within bursts of $\tau_c = 19$ ms ($N_o = 927$) can be estimated. Close to the experimental

FIGURE 11. Dependence of channel conductance of the neuronal ACh receptor on the salt concentration. Here the dependence on the Na$^+$ concentration is shown. The channel saturates at $\bar{\Lambda} = \sim 100$ pS. The $K_m$ is $\sim 60$ mM. All values were taken at a membrane potential of $V = 90$ mV and an agonist concentration of 5 $\mu$M carbamylcholine.
limits of time resolution, we found a third, very fast group of closing events in our experiments. An example of these so-called flickers is shown in Fig. 18. This fluctuation was activated by 500 μM carbamylcholine. We found a slower component of \( \tau_c = 31 \) ms and, in addition, a very fast component with \( \tau_o \approx 1 \) ms, which is marked by the arrows. Because of our limited experimental resolution, we were not able to analyze or resolve the details of the flickering process, and in all of the other kinetic data presented in this article, we have suppressed it by using a sampling rate of 1 kHz (effective time resolution, 2 ms) in our data analysis. We are going to present data on this process in a subsequent article.

**Figure 12.** Inhomogeneity of the conductance of a single neuronal ACh receptor channel. Under the same conditions given in Fig. 3, the channel exhibits a mean conductance of \( \bar{\lambda} = 80 \) pS. Besides this value, two other classes of events were found. (A) Smaller channel events occur randomly, with \( \lambda = 50 \) pS in the example given. Those events, however, occurred very rarely (≤1%). (B) In some open events, steps down from the main open level, \( \bar{\lambda} = 80 \) pS, to sublevels of lower channel conductance were observed. The conductance of these sublevels varied over a wide range ~25 pS. Again, only a small number of those sublevels were found in long traces of channel fluctuations (see Fig. 3). The experimental conditions were the same as in Fig. 2B.

At very high agonist concentrations (≥500 μM carbamylcholine), we found desensitization effects of the neuronal ACh receptor channel. These effects occurred on a scale of 10 min to 1 h. In long-time recordings, we found prolonged closing intervals between bursts and usually a shortening of the burst duration. Furthermore, channels occasionally closed after a certain time of activity and then never (in the time scale of the experiments) reopened again. As these effects are found only on the time scale we used, we will have to take longer recordings (\( t \geq 2 \) h) to determine whether equilibrium is reached after a long time.
DISCUSSION

A neuronal ACh receptor from ganglia of locusts, purified to homogeneity by affinity chromatography, appeared to represent a membrane protein complex composed of four to five polypeptides (Breer et al., 1985). Reconstitution of this protein in artificial planar membranes has demonstrated its ability to transduce the binding of cholinergic agonists into a transient opening of a cation-selective channel; it thus represents a functional ACh receptor channel. This neuronal ACh receptor exhibits properties (multiple gating of the channel, burst behavior, flickering of the open state) similar to those of the reconstituted receptor from electric tissue (Schindler and Quast, 1980; Nelson et al., 1980; Boheim et al., 1981; Labarca et al., 1984a, b) and to the receptor in muscle cells analyzed in situ (Sakmann et al., 1983). However, very distinct differences were also observed.

![Figure 13](image_url)

**Figure 13.** Voltage dependence of the channel kinetics of a neuronal ACh receptor. Traces of fluctuations at different membrane potentials are shown under the same experimental conditions as in Fig. 2B using 0.5 μM carbamylcholine. The applied potential and the open and closed states are marked for each trace. No significant changes of channel parameters at different voltages are seen in this figure. All traces were taken from the same bilayer and the same channel fluctuation.

It is generally agreed that the peripheral vertebrate receptor opens the channel only if it is doubly bound with agonists. Assuming that the activity seen in Fig. 6 is due to a single channel, the concentration dependence for activating the neuronal insect ACh receptor suggests that one agonist molecule may be sufficient to activate the neuronal receptor. The observation is interesting in view of the possibly homo-oligomeric structure of the receptor complex, since the requirement of two ACh molecules for the peripheral vertebrate receptor has frequently been correlated with the two α-subunits. Some recent data, however (Labarca et al., 1985), suggest that one agonist molecule may be sufficient to activate the vertebrate receptor channel. In some cases, when ACh was used as the agonist, we found that the open-time events were not single-exponentially distributed, which suggests the existence of kinetically distinct open states, which
may be due to the binding of more than one agonist molecule. This aspect is presently under more detailed investigation.

Another major difference between the receptor types concerns their pharmacology. The agonist-activated membrane conductance was found to be inhibited by the classic antagonist d-tubocurarine ($K_i = 10^{-6}$ M); however, hexamethonium showed only very weak effects. Whereas $10^{-5}$ M hexamethonium almost completely blocked the vertebrate receptor (Hanke et al., 1981), concentrations of up to $10^{-4}$ M only slightly (25%) reduced the activity of the neuronal insect receptor. This observation is in good agreement with results from electrophysiological and binding studies, which document a low affinity of hexamethonium

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure14.png}
\caption{Evaluation of kinetic parameters of the channel activity presented in Fig. 13. (A) Mean open-state lifetime, $\tau_o$, and mean closed-state lifetime, $\tau_c$. $\tau_o$ (solid line) is slightly voltage dependent. $\tau_c$ decreases with voltage, while $\tau_c$ (dashed line) increases more significantly in the same direction. Note the different scalings for both values. (B) Dependence of open-state probability ($P_o$) of the neuronal ACh receptor channels on membrane potential. Data are plotted as $\log[P_o/(1 - P_o)] = f(V)$. According to Eq. 3, a charge of $z = 1/2$ can be calculated from this plot, which will be transferred across the entire bilayer upon opening of the channel.}
\end{figure}
for ACh receptors in insect nervous system (David and Sattelle, 1984); this indicates the pharmacological integrity of the purified receptor protein. A comparison of some basic pharmacological properties of the reconstituted receptor proteins from *Torpedo* and *Locusta* is given in Table III.

The receptor channel was found to be weakly voltage dependent, closing toward higher positive potentials; this is similar to results reported for the receptor from electrocytes (Labarca et al., 1984a, b). In this study, we have estimated values for both the open state and the closed state of the channel (Fig. 14).

**Figure 15.** At higher agonist concentrations (here 50 μM carbamylcholine) under conditions otherwise the same as in Fig. 2B, multiple bursting events of non-independent channel openings were found.

**Figure 16.** Bursting of the neuronal ACh receptor channel at high agonist concentrations. Under the same conditions as in Fig. 2B, but with 50 μM carbamylcholine, the channel exhibits pronounced bursting behavior. Channel activity appeared in longer periods of fluctuations separated by long intervals in the closed state. In the traces shown, the closed state is marked to the right of each trace and each burst is marked by a bar over the trace.
FIGURE 17. Half-logarithmic lifetime evaluations of $r_o$ and $r_c$ in a bursting neuronal ACh receptor channel, as shown in Fig. 16. Whereas the open-state lifetime clearly is single-exponentially distributed, with $r_o = 4$ ms, the lifetimes of the closed state are double-exponentially distributed, with a mean closed time between bursts of $r_c = 202$ ms and a mean closed-state lifetime within bursts of $r_c = 19$ ms.

### TABLE III
Comparison of Properties Evaluated for the Reconstituted ACh Receptors from *Torpedo Electric and Locustia Nervous Tissue under Comparable Conditions*

| Channel properties                  | Locusta | Torpedo | Reference                           |
|-------------------------------------|---------|---------|-------------------------------------|
| Maximum conductance ($\rho S$)     | 100     | 95      | Montal et al. (1984)                |
|                                     |         | 90      | Tank et al. (1985)                  |
| $Na^+$ concentration for half-maximal conductance (mM) | 50      | 395     | Montal et al. (1984)                |
| Open-state lifetime at low carbamylcholine (1 $\mu$M) (ms) | 3       | 129     | Tank et al. (1985)                  |
|                                     |         | 5       | Montal et al. (1984)                |
| Apparent cooperativity of agonists  | 1       | 2       | Labarca et al. (1984a, b)           |
| Potency of agonists*                | Suberyldicholine, ACh, carbamylcholine | Suberyldicholine, ACh, carbamylcholine | Labarca et al. (1984a, b) |
| Sensitivity to d-tubocurarine$^\ddagger$ | ++      | ++      | Coronado and Labarca (1984)         |
| Sensitivity to hexamethonium$^\dagger$ | +       | ++      | Hanke et al. (1981)                 |
| Selectivity                         | Na$^+$, K$^+$, Cl$^-$ | Na$^+$, K$^+$, Cl$^-$ | Boheim et al. (1981)                |

There is considerable variability for the values found in the literature; the numbers given in this table should be considered as ranges. The properties of the neuronal locust receptor are based mainly on the results of the present study.

* Agonist action on neuronal ACh receptors; estimated from the lifetime of the channel open state at identical concentrations.
$^\ddagger$ >50% block at $10^{-5}$ M d-tubocurarine (++).
$^\dagger$ >50% block at $10^{-8}$ M hexamethonium (++).
The conductance value determined for the insect ACh receptor channel ($\lambda_{\text{max}} = 100$ pS) appeared to be slightly higher than reported for the *Torpedo* receptor ($\lambda_{\text{max}} = 95$ pS) (Labarca et al., 1984). A higher conductance of cation channels may be typical for insects, since patch-clamp analysis of the insect neuromuscular junction has revealed that the glutamate-activated channels have a higher conductance than the agonist-activated channels in vertebrate muscle (Cull-Candy, 1983). Furthermore, comparing the conductance data at physiological salt concentrations (insect: 150 mM Na$^+$; vertebrate muscle: 150 mM Na$^+$; *Torpedo*: 500 mM Na$^+$), a conductance in the range of 50–80 pS is found for all the ACh receptor channels (Sakmann et al., 1983; Labarca et al., 1984a, b). Sublevels and different classes of channel conductance, which were found in the receptor preparation, have also been reported for ACh receptors from muscle (Hamill and Sakmann, 1981; Sachs, 1983) and electric tissue (Boheim et al., 1982). Sakmann and co-workers (1983) have described two classes of ACh receptor in

![Figure 18](image-url)
muscle cells, which differ significantly in their conductance: the synaptic receptor (70 pS) and the extrasynaptic ACh receptor (45 pS), which have now been identified as different components in one subunit (Mishina et al., 1986). These values are comparable to the conductance subclasses we found in the neuronal preparation. Since synaptic and extrasynaptic ACh receptors are definitely present in the nervous system of insects (David and Sattelle, 1984), we are going to study these neuronal receptor subtypes in more detail. The finding that a nicotinic ACh receptor protein purified from nerve cells can be reconstituted and shows channel properties very similar to those of the hetero-oligomeric vertebrate receptor not only offers the possibility of studying the same receptor in its native membrane (patch clamp) and in artificial membranes (bilayer technique) but may also allow us to study some further comparative aspects. These studies may help us find a minimal model for ACh receptor channel function and may shed some new light on the evolution of the ACh receptor.

We would like to thank Miss G. Hinz for technical assistance and Mrs. G. Moehrke for typing the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 171-C5/C11).

Original version received 21 July 1986 and accepted version received 30 March 1987.

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