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Single-Channel Current Recordings of Acetylcholine Receptors in Electroplax Isolated from the *Electrophorus electricus* Main and Sachs' Electric Organs

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Summary. Extensive chemical kinetic measurements of acetylcholine receptor-controlled ion translocation in membrane vesicles isolated from the electroplax of *Electrophorus electricus* have led to the proposal of a minimum model which accounts for the activation, desensitization, and voltage-dependent inhibition of the receptor by acetylcholine, suberyldicholine, and carbamoylcholine. Comparison of chemical kinetic measurements of the dynamic properties of the acetylcholine receptor in vesicles with the properties of the receptor in cells obtained from the same organ and animal have been hampered by an inability to make the appropriate measurements with *Electrophorus electricus* electroplax cells. Here we report a method for exposing and cleaning the surface of electroplax cells obtained from both the Main electric organ and the organ of Sachs and the results of single-channel current recordings which have now become possible. The single-channel current recordings were made in the presence of either carbamoylcholine or suberyldicholine, as a function of temperature and transmembrane voltage. Both the channel open times and the single-channel conductance were measured. The data were found to be consistent with the model based on chemical kinetic measurements using receptor-rich membrane vesicles prepared from the Main electric organ of *E. electricus*.

Key Words acetylcholine receptors · single-channel currents · *Electrophorus electricus* Main and Sachs' electric organs · temperature dependence

Introduction

Electrophorus electricus has three distinct organs, each containing large amounts of acetylcholine receptor protein: the Main organ, the Sachs' organ and the Hunter's organ. The Sachs' organ occupies the most caudal part of the electric eel and is composed of large cells (electroplax) that are suitable for electrophysiological studies (Keynes & Martins-Ferreira, 1953; Altamirano et al., 1955; Schoffeniels & Nachmansohn, 1957; Schoffeniels, 1957, 1959). The Main organ occupies most of the body of the eel and has proved to be a rich source of acetylcholine receptors for studies of acetylcholine-dependent ion flux in membrane vesicles (Kasai & Changeux, 1971; Hess & Andrews, 1977; Hess et al., 1983) and of the binding of ligands to the receptor (Kasai & Changeux, 1971; Meunier & Changeux, 1973; Bulger et al., 1977; Fu et al., 1977). The electroplax of the Main organ are more irregular in shape and are much smaller than the cells in the organ of Sachs.

Membrane vesicles prepared from the *E. electricus* Main organ were used to elucidate the mechanism of action of acetylcholine and other activating ligands that, upon binding to the acetylcholine receptor, induce transmembrane ion flux (Hess et al., 1979; Aoshima et al., 1980; Cash & Hess, 1980; Cash et al., 1980, 1981; Pasquale et al., 1983). Taking advantage of rapid mixing and chemical kinetic techniques it was shown that in the absence of ligands the acetylcholine receptor is mostly in an active state. Upon binding of two molecules of ligand the channel associated with the receptor undergoes a transition allowing ions to move through the cell membrane. On a time scale of milliseconds the ligand induces another transition of the receptor to an inactive (desensitized) state which apparently cannot form transmembrane channels. In *E. electricus* this inactivation process decreases the rate of the receptor-mediated ion flux. Thus, the use of chemical kinetic techniques allowed the study of the ion flux mediated by the large number of acetylcholine receptors present in a membrane vesicle preparation. The equilibrium and rate constants pertinent to the model relating ligand binding to the acetylcholine receptor and ion flux were evaluated using carbamoylcholine (Hess et al., 1979; Aoshima et al., 1980, 1981), acetylcholine (Cash et al., 1980, 1981), suberyldicholine (Hess et al., 1982, Pasquale

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et al., 1983) and *trans* Bis-Q (3-3′-bis(trimethylammonio-methyl)azobenzene bromide) (Delcour & Hess, 1986).

The single-channel current recording technique, which allows the detection of ion flux through individual transmembrane channels (Neher & Sakmann, 1976; Sakmann & Neher, 1984), represents a complementary method for the study of the transmembrane channels. The results of these measurements can be compared to chemical kinetic measurements (Hess et al., 1984). An electroplax preparation suitable for making single-channel current measurements has not existed although it has been occasionally possible to obtain such measurements with such cells (Hess et al., 1984). Here we report a method for exposing and cleaning the surface of electroplax that makes these cells suitable for single-channel current measurements. We describe single-channel current recordings used to detect suberyldicholine- and carbamoylcholine-induced channels in electroplax dissected from the *E. electricus* Main organ and the organ of Sachs. The data obtained allow a comparison to be made between the receptors from the two organs and are consistent with the minimum mechanism previously proposed on the basis of chemical kinetic measurements made with membrane vesicles prepared from the *E. electricus* Main electric organ (for a review *see* Hess et al., 1983).

Materials and Methods

E. electricus were obtained from World Wide Scientific Animals, Apopka, FL and kept up to a week in shallow water. The electroplax were dissected immediately after killing the electric eel and stored in eel Ringer's solution (mM: 169 NaCl, 5 KCl, 3 $CaCl_2$, 1.5 MgCl, 1.5 sodium phosphate, pH 7.0) at 4°C for a maximum of 3 days. Tetram [0,0-diethyl-S-[2-(diethylamino)ethyl] thiophosphate was a gift from Dr. R.D. O'Brien and suberyldicholine diiodide was a gift from Dr. Ungar, University of Edinburgh. Carbamoylcholine chloride, collagenase type IV from *Clostridium histolyticum*, and hyaluronidase type IV-S from bovine testes were purchased from the Sigman Chemical Co.

Single-Channel Current Recordings

TW 150-6 borosilicate glass capillary tubes from W-P Instruments, Waltham, MA, were used to make the patch pipettes, which were then fire-polished. Gigaseals (5 to 100 GΩ) were obtained by applying negative pressure to the inside of the recording patch pipette after touching the cell membrane (Neher & Sakmann, 1976) and typically lasted 1 to 2 hr. Occasionally gigaseals developed just by pressing the pipette tip against the cell membrane. In most recordings the electroplax membrane was not intact or the patches were excised so that the transmembrane voltage was equivalent to the voltage applied to the inside of the recording electrode. The single-channel current recording system used to apply a holding voltage to the pipette interior and to record single-channel currents was similar to that described by Hamill et al. (1981). The bath was held at zero voltage. The electroplax membranes were observed with a Zeiss IM35 phase-contrast microscope (400× magnification). The data were stored on analog tape using a Racal Store 4DS recorder. For analysis, the data were replayed (at a slower speed) onto an oscillographic chart recorder (Hewlett-Packard 7402A) and then analyzed by hand. The effective filter bandwidth was 1600 Hz for most of the traces used in the analysis.

Results

Dissection of the Electroplax

The organ of Sachs in *E. electricus* consists of longitudinal rows of electroplax separated by thick collagen septa (Couceiro & De Almeida, 1961). The electroplax is a long flat cell (about 2 to 3 mm wide, 0.1 mm thick and 1 to 2 cm long) derived embryologically from a muscle cell in which the contractile fibers do not develop. Each electroplax is contained in a compartment limited by two thin collagen sheets perpendicular to the thicker longitudinal septa. The caudal face of each electroplax is innervated, is rich in acetylcholinesterase and acetylcholine receptors, and is seen to have short invaginations. The membrane of the rostral noninnervated face of each electroplax shows long invaginations (Couceiro & De Almeida, 1961; Schoffeniels, 1961). Most of the volume of the compartments is filled with a gelatinous extracellular matrix containing mucopolysaccharides (Couceiro & De Almeida, 1961). The electroplax are, therefore, separated from each other in the longitudinal rows by large amounts of gelatinous substance. The morphology of the organ of Sachs is particularly suitable for the dissection of individual cells or layers of cells for electrophysiological measurements.

A transverse slice, 1.5 cm thick, was removed from the tail of an electric eel immediately after killing it (Fig. 1*A*). A sector 5 cells thick was then isolated from the slice and anchored in a dissecting chamber with thread (Fig. 1*B*). Under a dissection microscope, the sector was then divided into layers of 5 electroplax by cutting through the extracellular spaces separating the cells, in the direction of the length of the compartments (Fig. 1*C*). Finally the layers of the electroplax were completely separated by cutting through the the skin (on the external side of the sector) and the connective tissue (on the interior of the sector). Each layer, composed of 5 electroplax attached side by side and with their innervated membranes facing upward, was placed in a

35-mm plastic culture dish (Fig. 1*D*). Thread was tightened at the four corners of the layer to hold it in place. A collagenous sheet (Fig. 2*E* and *F*) covering each cell was then removed by dissection.

Figure 2*A*, *B* and *C* illustrate the cell at this stage. A crucial step in the preparation of electroplax suitable for single-channel current recording is described next. Careful observation (Fig. 2*B*) indicated that the innervated membrane of the electroplax was still covered by fibrous material that prevented the microelectrodes from reaching the surface of the cell and forming a gigaseal. Harsh treatment with collagenase and protease in an attempt to remove this material from the cell surface damages the electroplax membrane and may affect the receptor function. However, after 20 to 60 min of treatment with 0.1% collagenase and 0.1% hyaluronidase, at room temperature, a second thin sheet, presumably collagenous, previously tightly associated with the cell membrane, could be removed by dissection. This sheet, composed mostly of intertwined collagenous fibers, has never been described before and is shown in Fig. 2*B*, *D*, *E* and *F*. Gigaseals were then obtained on the exposed electroplax membrane (Fig. 1*F*). During the dissection the electroplax were bathed in outside eel Ringer's solution.

The procedure for the dissection of electroplax from the Main electric organ was essentially the same as described above for the electroplax of the organ of Sachs. However, the morphology of the Main organ is not as favorable for the dissection of layers of cells. The electroplax of the Main organ are in fact tightly and irregularly packed, and are smaller and more delicate than the cells from the Sachs' organ (Keynes & Martins-Ferreira, 1953). The electroplax were dissected from a transverse slice that was as caudal as possible. The cells in the caudal part of the Main organ are more regular. The layer of cells placed in the 35-mm culture dish was 2 cells thick (and therefore contained 10 cells). After treatment for 10 to 20 min with 0.1% collagenase and 0.1% hyaluronidase both collagenous sheets covering the cell membrane were dissected together, leaving the cell membrane exposed and ready for single-channel current recording.

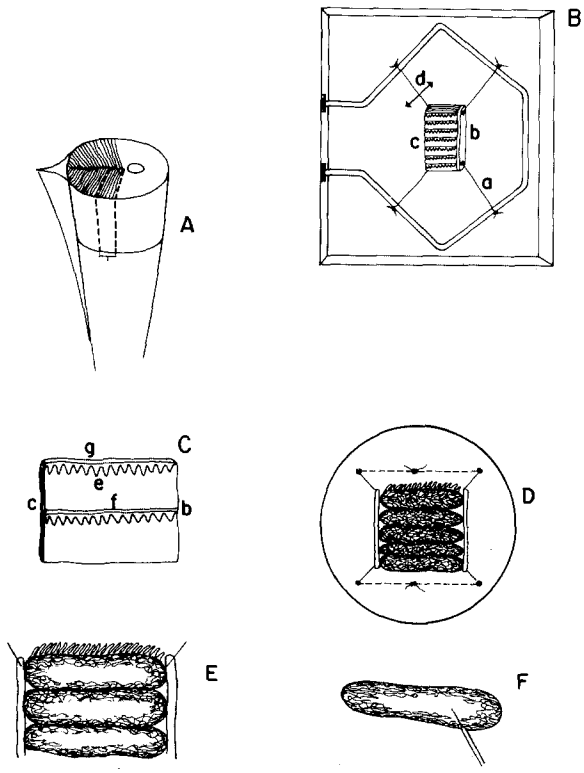
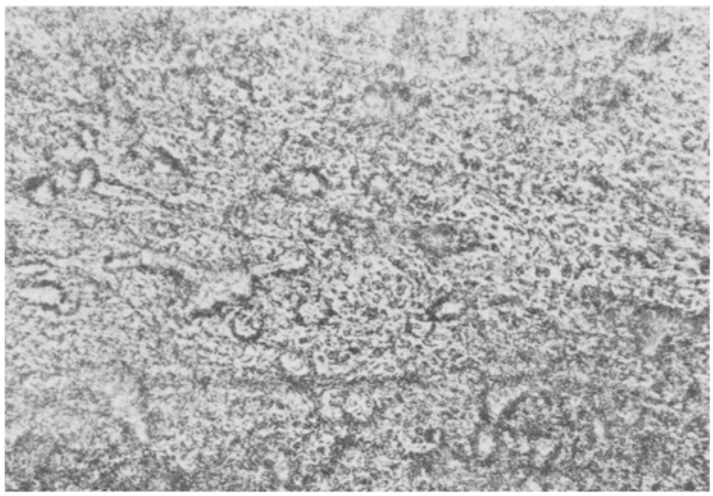
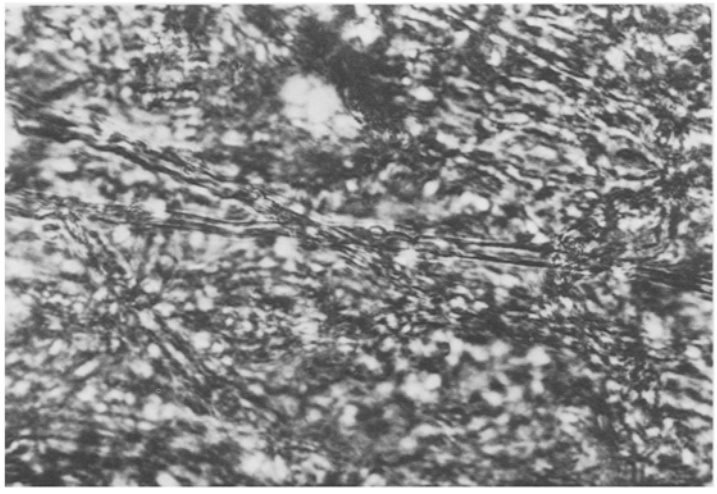


Fig. 1. Dissection of the electroplax. (*A*) A transverse slice was cut from the caudal part of *E. electricus* and a section 5 cells in thickness was removed. (*B*) The section was placed in a dissecting chamber by means of thread. *a*, thread; *b*, skin; *c*, connective tissue; *d*, thickness of 5 cells. (*C*) Two cells in their compartment, same view as in (*B*). *g*, innervated face covered by collagenous sheets; *e*, long papillae of the noninnervated face; *f*, intercellular matrix. (*D*) Slice 1 cell thick and 5 cells wide placed, by means of thread, in a 35-mm culture dish. (*E*) Same view as in (*D*), the cells have had part of the thick collagenous sheet removed by dissection. (*F*) The thin collagenous sheet has been removed and a patch pipette has been placed on the cell membrane for gigaseal formation

Single-Channel Current Recording

Examples of the single-channel current data obtained using *E. electricus* electroplax and suberyldicholine are shown in Fig. 3. The amplitude of the single-channel currents is dependent on the transmembrane voltage and on temperature, as summarized in Fig. 4*A* and *B*. The conductance calculated from the current amplitudes at transmembrane voltages between −50 and −150 mV is about 80 pS at 24°C and about 50 pS at 12°C in the Main organ as well as in the Sachs' organ. The data from the two organs have been combined in Fig. 4*A* and *B* because they are indistinguishable. Figure 5 indicates that the open times of the suberyldicholine-induced channels have a single exponential distribution and, therefore, that the receptor present in the electroplax membranes has only one open-state form. The lifetime of the open state τ_o depends upon transmembrane voltage and temperature as illustrated in Fig. 6*A* and *B*, using 50 and 500 nM suberyldicholine. τ_o is independent of the suberyldicholine concentration (Fig. 6*A* and *B*), as is expected if the channels close without a change in the number of suberyldicholine molecules that are bound (Hess et





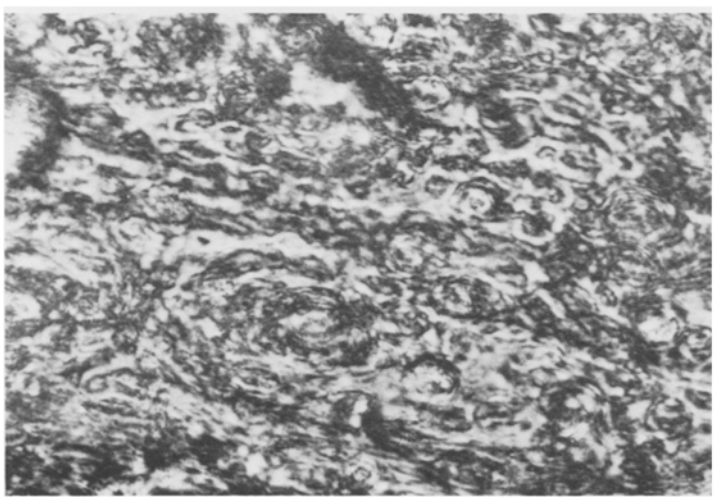
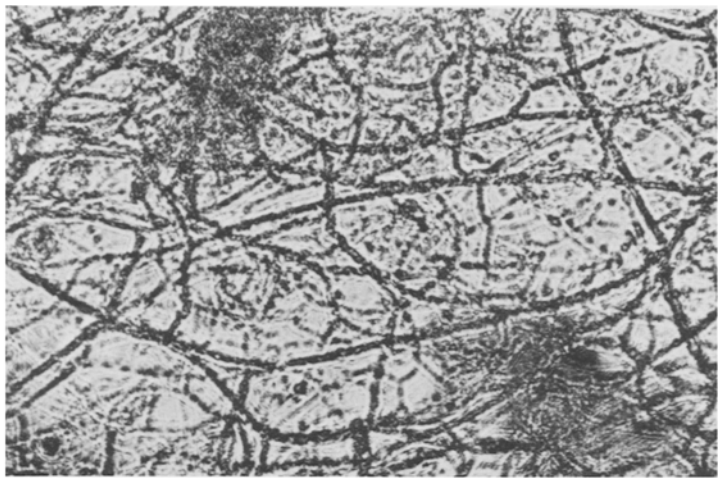
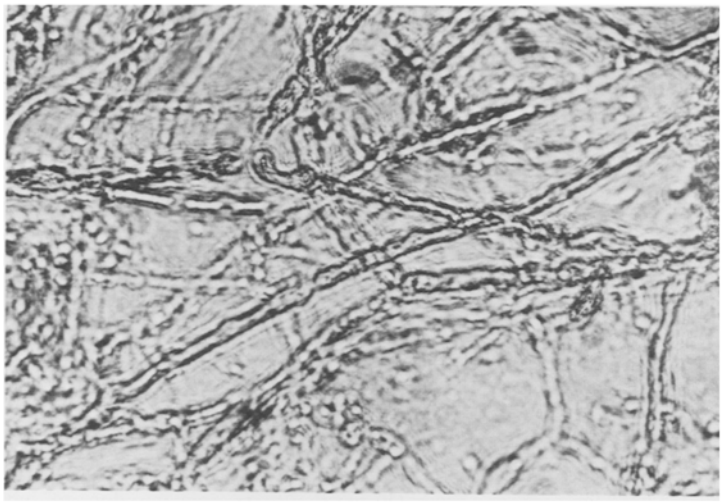


Fig. 2. Light microscope photographs of *E. electricus* electroplax from the organ of Sachs. (*A*) to (*C*) Electroplax still covered by the thin collagenous sheet tightly associated with the cell membrane (*see* Results section). Photograph taken from the innervated face. The circular formations are the long papillae of the noninnervated face.





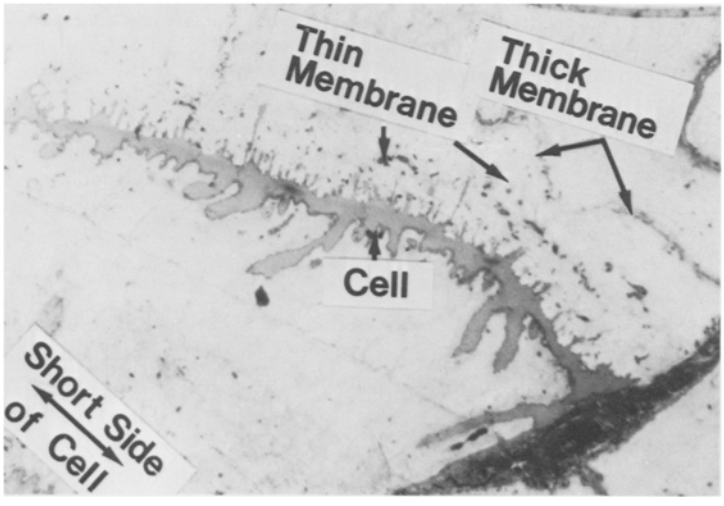


Fig. 2. *continued.* In (*B*) some of the fibers covering the cell are clearly visible and in (*D*) and (*E*) the network of collagenous fibers that constitutes the thin sheet has been isolated. At the bottom of (*D*) on the right, part of the thicker collagenous sheet is left. (*F*) Toluidine-Idol-stained sections embedded in Epon 8/2 for electron microscopy 1 μm thick

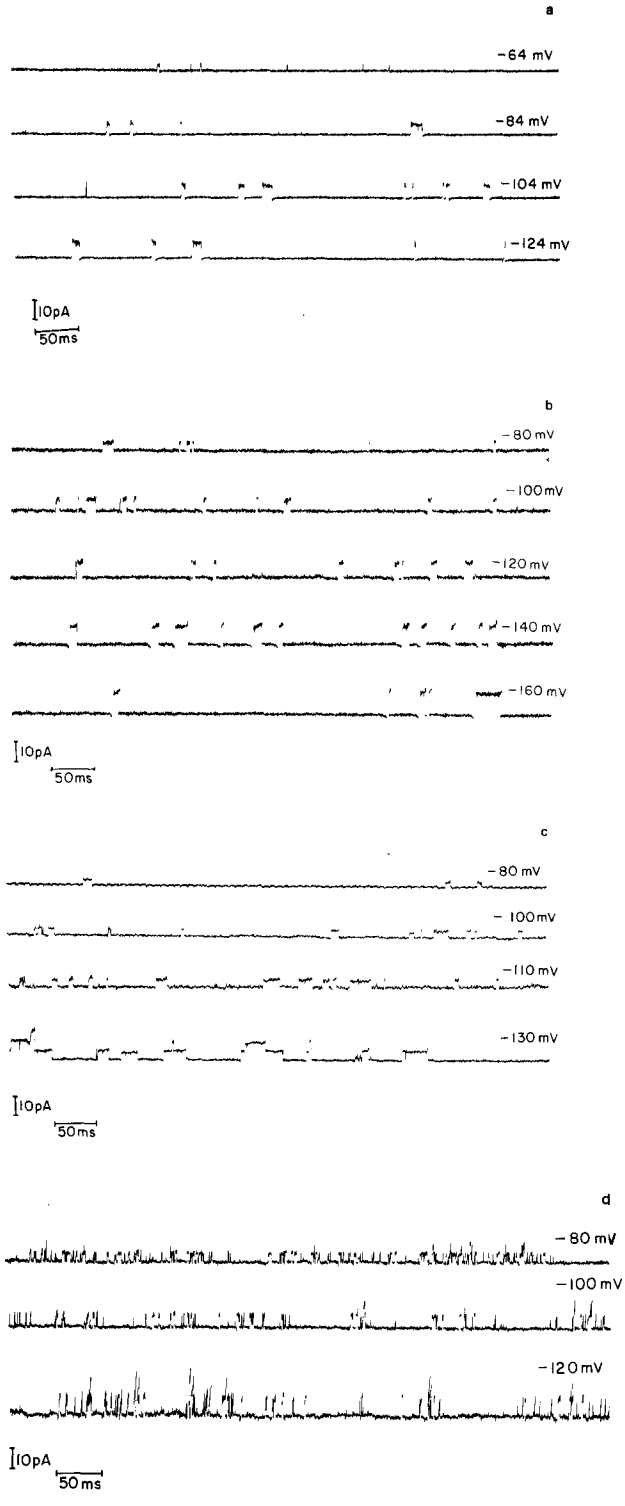


Fig. 3. Single-channel current recordings at various transmembrane voltages. (*a*) Data obtained with electroplax from the *E. electricus* Main organ at 24°C in the presence of 50 nM suberyldicholine. (*b*) Data obtained with the Sachs' organ at 24°C in the presence of 50 nM suberyldicholine. (*c*) Data from the Sachs' organ at 12°C in the presence of 20 nM suberyldicholine. (*d*) Data from the Sachs' organ at 24°C in the presence of 30 μM suberyldicholine

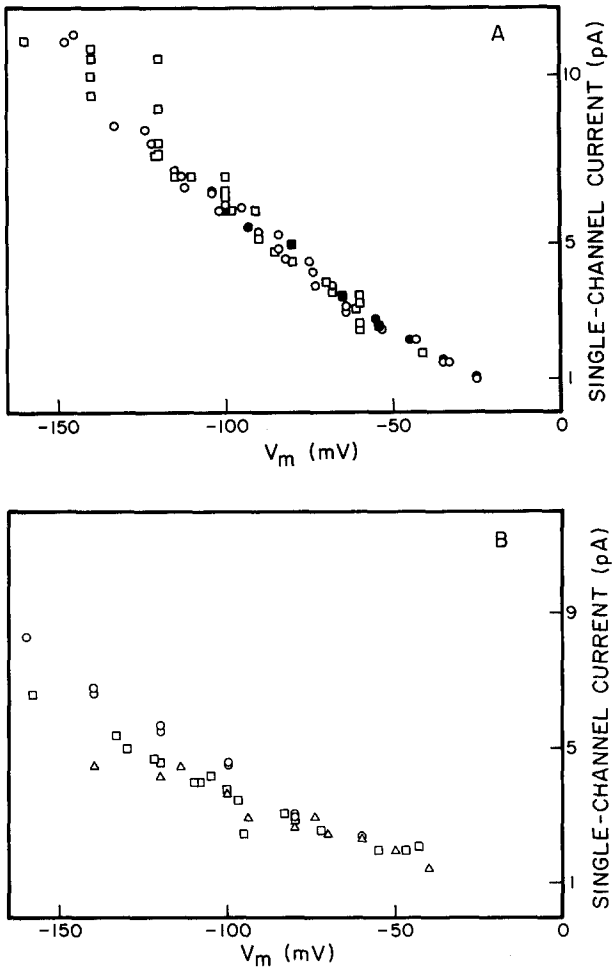


Fig. 4. Single-channel current dependence on voltage. (*A*) Data obtained with the Main (○) or Sachs' (□) organ at 24°C in the presence of various (50 nM to 30 μM) concentrations of suberyldicholine. $\gamma \sim 80$ pS, as measured from the data between −50 and −150 mV. ●, ■, 2 or more determinations with the same value. (*B*) Data obtained with the Main (○) or Sachs' (□, △) organ at 12°C in the presence of various concentrations of suberyldicholine (○, □) (20 nM to 30 μM) or carbamoylcholine (△) (50 and 0.5 μM). $\gamma \sim 50$ pS, as measured from the data between −50 and −150 mV. All current measurements were done on excised patches and in all cases the solution on either side of the patch was *E. electricus* Ringer's solution (mM: 169 NaCl, 5 KCl, 3 $CaCl_2$, 1.5 $MgCl_2$, 1.5 sodium phosphate buffer, pH 7.0)

al., 1982). The open times are about twice as long at 12°C as they are at 24°C and the data obtained with the Sachs' organ and with the Main organ are very similar. A voltage decrease of about 60 mV corresponds to a twofold increase in τ_o.

Carbamoylcholine-induced channels close about 2.5 times more rapidly than suberyldicholine-induced channels at 12°C (at ~100 mV, τ_o is 3.5 msec with carbamoylcholine and 9.1 msec with suberyldicholine) but the single-channel current amplitudes

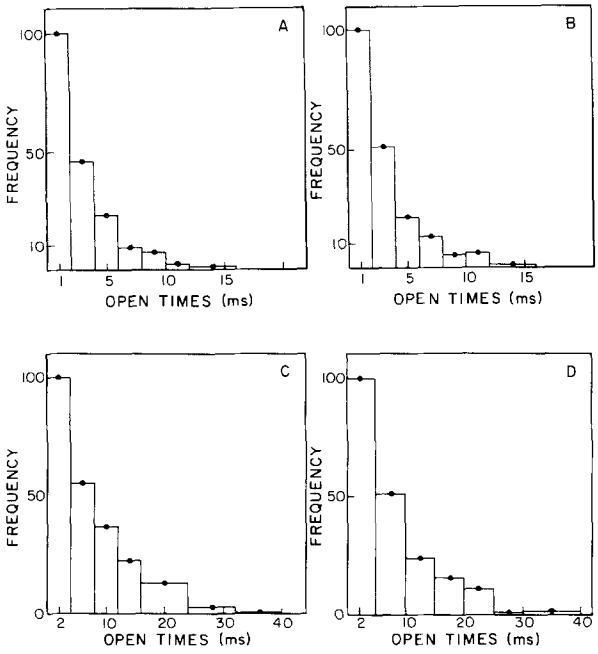


Fig. 5. Distribution of open times of the acetylcholine receptor in presence of suberyldicholine. (*A*) Data obtained from the Main organ, at 24°C, −104 mV in the presence of 50 nM suberyldicholine, τ_o = 2.7 ± 0.1 msec, reduced χ^2 = 0.13. (*B*) Data obtained from the Sachs' organ, at 24°C, −100 mV in the presence of 50 nM suberyldicholine. τ_o = 3.05 ± 0.23 msec, reduced χ^2 = 0.32. (*C*) Data obtained from the Main organ at 12°C, −100 mV in the presence of 20 nM suberyldicholine. τ_o = 7.52 ± 0.34 msec, reduced χ^2 = 0.15. (*D*) Data obtained from the Sachs' organ at 12°, −100 mV in the presence of 20 nM suberyldicholine τ_o = 9.0 ± 0.76, reduced χ^2 = 0.11. Approximately 400 events were analyzed in each histogram

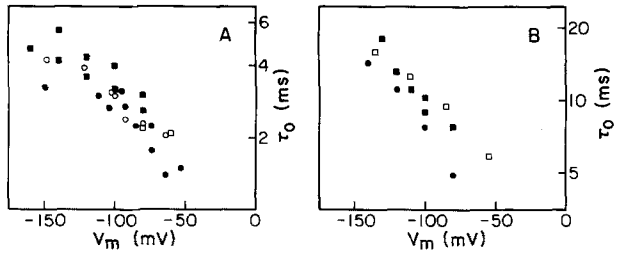


Fig. 6. Voltage dependence of the lifetime τ_o of the open form of suberyldicholine-induced channels. Each point is obtained from a histogram similar to those shown in Fig. 5. ●, ○ data obtained with the Main organ, ■, □ from the Sachs' organ. (*A*) 24°C; ■ and ●, 50 nM and □ and ○, 500 nM suberyldicholine. (*B*) 12°C; ■ and ●, 20 nM and □ and ○, 500 nM suberyldicholine

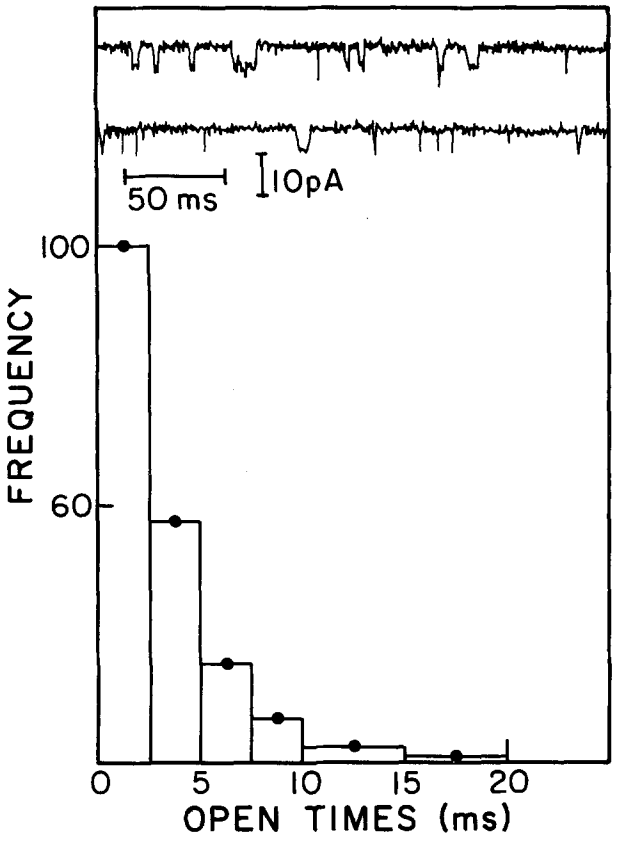


Fig. 7. Single-channel current recording and distribution of open times of the acetylcholine receptor in presence of 50 μM carbamoylcholine at 12°C and −94 mV. Data from the organ of Sachs. τ_o = 3.5 ± 0.3 msec, reduced χ^2 = 0.36. Approximately 400 events were analyzed

are the same for the two ligands (Fig. 7). The open times measured in the presence of carbamoylcholine are also exponentially distributed (Fig. 7).

Discussion

The electroplax cells obtained from the *E. electricus* organ of Sachs have been used previously for electrophysiological investigations (Keynes & Martins-Ferreira, 1953; Altamirano et al., 1955; Schoffeniels & Nachmansohn, 1957; Schoffeniels, 1957, 1959). They are very rich in acetylcholine receptors, can be individually isolated by dissection from the electric organ and their large dimensions allow the use of intracellular microlectrodes. Recently they have also been utilized for single-channel current recordings (Hess et al., 1984), but the signal-to-noise ratio was about 1/50 of the experiments reported here. In those experiments the single electroplax preparation of Schoffeniels (1959) was used and we were not aware of the collagenous fibers (Fig. 2*B–E*) which we remove in the experiments reported here. As a consequence we obtained, at best, one suitable recording per electric eel.

We have developed a method for exposing and cleaning large areas of the innervated electroplax

membranes, using both the organ of Sachs and the Main organ as a source of cells. The intact electroplax, treated with minimum amounts of hyaluronidase and collagenase, are suitable for convenient and reproducible single-channel current recordings. An inverted microscope is sufficient to obtain a clear view of the electroplax membrane from which a patch is to be obtained. The layers of electroplax cells used are only one (Sachs' organ) or two (Main organ) cells thick. The ability to obtain inside-out isolated patches allows a precise determination of the voltage across the innervated membrane. The temperature and ionic composition of the solution bathing the electroplax during the single-channel current recordings can be easily controlled with our method.

It was important to establish whether or not the acetylcholine receptors obtained from the organ of Sachs and from the Main organ have similar characteristics. Much of the available information, at the molecular level, on the *E. electricus* acetylcholine receptor was obtained by using chemical kinetic techniques and membrane vesicles prepared from the Main electric organ (e.g. Hess et al., 1983). However, electroplax of the organ of Sachs are much more suitable for electrophysiological recordings, including single-channel current measurements.

We have shown here that the conductance and the open times of the acetylcholine receptor channel from the two organs are essentially the same. Therefore, chemical kinetic techniques using membrane vesicles prepared from the Main organ and electrophysiological techniques utilizing electroplax isolated from the organ of Sachs are complementary in the elucidation of the mechanism of action of the nicotinic acetylcholine receptor (Hess et al., 1984).

The single-channel conductance γ is related to the value of the specific reaction rate for the ion translocation process $\bar{J}$ measured with chemical kinetic techniques and membrane vesicles (Hess et al., 1984). From the value of γ at 24°C (about 80 pS) a value of 7×10^7 M^{-1} sec^{-1} is obtained for $\bar{J}$, and from the value of γ at 12°C (about 50 pS) a value of 4×10^7 M^{-1} sec^{-1} is obtained for $\bar{J}$. The single-channel conductance γ is dependent on temperature and increases by a factor of about 1.5 for a 10°C rise in temperature in the range of our measurements (12 to 24°C). The value of 3×10^7 M^1 sec^{-1} found for $\bar{J}$ at 1°C using chemical kinetic techniques is, therefore, consistent with the value of γ calculated from single-channel current recordings.

The single-channel current amplitudes measured at about −100 mV using carbamoylcholine or suberyldicholine are the same, again in agreement with previous results with membrane vesicles, indicating that the value of $\bar{J}$ is independent of the ligand used (Hess et al., 1981, 1982). Previous patch-clamp results obtained using acetylcholine receptors from other sources also showed that the value of γ is the same in the presence of different ligands (Suarez-Isla et al., 1983; Gardner et al., 1984; Hess et al., 1984).

The lifetime of the open form of the acetylcholine receptor τ_o is about 2.5 times longer in the presence of suberyldicholine than in the presence of carbamoylcholine. This confirms the previous observation that the channel-closing equilibrium constant Φ has a value about 2.5 times higher with carbamoylcholine-induced channels than with suberyldicholine-induced channels (Hess et al., 1982; Suarez-Isla et al., 1983). The values of τ_c, the lifetime of the closed form of the receptor, do not depend on the transmembrane voltage (Neher & Sakmann, 1976; Udgaonkar & Hess, *manuscript in preparation*).

The observed dependence of τ_o upon voltage and temperature using *E. electricus* acetylcholine receptors is consistent with previous findings related to acetylcholine receptors from other sources (Neher & Steinbach, 1978; Jackson & Lecar, 1979; Nelson & Sachs, 1979).

Only one lifetime τ_o is observed for the open-channel form of the *E. electricus* receptor: the distribution of the channel open times can be fitted to a single exponential (Figs. 5 and 7). Similarly, current amplitude histograms consisting of approximately 400 measurements indicate the presence of only one type of receptor channel. The electroplax cells contain, therefore, a single type of acetylcholine receptor with a single, unique open conformation, as was proposed on the basis of chemical kinetic measurements (Aoshima et al., 1980; Cash et al., 1980). The electroplax are, in this respect, a convenient source of acetylcholine receptors for single-channel current recordings. Many other preparations present more complex distributions of channel open times (Colquhoun & Sakmann, 1981; Jackson et al., 1983; Sachs, 1983; Suarez-Isla et al., 1983; Tank et al., 1983) or a mixture of channels with different conductances (Colquhoun & Sakmann, 1981; Hamill & Sakmann, 1981; Auerbach & Sachs, 1983; Sachs, 1983; Tank et al., 1983).

An additional advantage of the acetylcholine receptors from *E. electricus* is related to their incomplete inactivation. The acetylcholine receptors from *T. californica* and from muscle cells become completely inactive within a few seconds, in the presence of high concentrations of ligands (Katz & Thesleff, 1957; Sakmann et al., 1980; Hess et al., 1982; Tank et al., 1983). In *E. electricus*, some re-

ceptors remain in the active state for over one hour even at high concentrations of ligand (Aoshima et al., 1981). Single-channel current recordings can be obtained over a very wide range of ligand concentrations, allowing the elucidation of various concentration-dependent properties of the acetylcholine receptor and of properties observed only at high concentrations of ligand. As an example, in Fig. 3*d* are shown recordings obtained in the presence of 30 μM suberyldicholine at 24°C. The apparent open times are several times shorter than the open times shown in Figs. 3*B* and 6*A*, obtained in the presence of 50 and 500 nM suberyldicholine. High concentrations of ligands inhibit the acetylcholine receptor in a voltage-dependent fashion, as was shown using chemical kinetic techniques (Pasquale et al., 1983; Takeyasu et al., 1983). The ion flux rate is decreased with increasing concentration of suberyldicholine in the μM to mM range. A regulatory site to which a molecule of ligand binds with inhibitory effect was proposed to explain the observed decrease in ion flux (Pasquale et al., 1983). In the single-channel current recordings this inhibition can be observed when the receptor is in the open conformation. Ion flux is prevented for brief periods of time as a consequence of the binding of an inhibitory molecule of ligand to the regulatory site. Consequently the lifetime of the open form τ_o appears to be shorter at high suberyldicholine concentrations. A similar inhibitory process is observed in single-channel current recordings when local anesthetics are present together with an activating ligand of the acetylcholine receptor (Neher & Steinbach, 1978; Neher, 1983). Sine and Steinbach (1984) have shown that acetylcholine blocks acetylcholine channels in single-channel measurements.

The single-channel current recording results reported here are entirely consistent with the model previously proposed to relate ligand binding to the acetylcholine receptor and transmembrane ion flux. Some characteristics of the *E. electricus* acetylcholine receptors (e.g., incomplete inactivation, presence of one type of channels) make the single-channel current measurements of receptor from this source very promising for the elucidation of important features of the nicotinic acetylcholine receptor function.

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