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Single-Channel Recordings from Purified Acetylcholine Receptors Reconstituted in Bilayers Formed at the Tip of Patch Pipets†

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ABSTRACT: The channel of the purified acetylcholine receptor from *Torpedo californica* electric organ reconstituted in lipid vesicles was assayed by direct electrical recording using patch-clamp pipets. High-resistance seals were obtained by gentle suction of vesicles into the pipet or after the formation of lipid bilayers from monolayers at the tip of the pipet. Single-channel currents were activated by three cholinergic ligands: acetylcholine, carbamylcholine, and suberyldicholine. The single-channel conductance, γ, was 40 ± 5 pS in 0.5 M NaCl, irrespective of the agonist used. The distributions of channel open times were fitted by a sum of two exponentials. The lifetimes of the two exponential components were a factor of 2 longer for suberyldicholine than for acetylcholine or carbamylcholine. At desensitizing concentrations of agonists the single events appeared in paroxysms of channel activity followed by quiescent periods. These results suggest that the full cycle of solubilization, purification, and reconstitution of this membrane receptor can be achieved without impairment of channel function.

Understanding the mechanism of action of channel proteins depends on the convergence of knowledge about the structure of the protein with the detailed characterization of its function at the molecular level. The nicotinic acetylcholine receptor of postsynaptic membranes provides an opportunity to pursue this goal.

Acetylcholine receptor from the electroplax of the ray *Torpedo californica* has been purified and extensively characterized. It is a pentamer of approximate molecular weight 270 000 with a subunit composition of $\alpha_2\beta\gamma\delta$ [reviewed in Anholt et al. (1983), Karlin (1980), Changeux (1981), and Raftery et al. (1980)]. Recently, the amino acid sequences of all four subunits have been elucidated (Noda et al., 1982, 1983; Claudio et al., 1983). The purified receptor has been reconstituted in lipid vesicles [Huganir et al., 1979; Changeux et al., 1979; Lindstrom et al., 1980; Anholt et al., 1980, 1981, 1982; cf. Anholt et al. (1983)] and in planar lipid bilayers (Nelson et al., 1980; Boheim et al., 1981; Labarca et al., 1982, 1983; P. Labarca, J. Lindstrom, and M. Montal, unpublished results). These studies demonstrated that the $\alpha_2\beta\gamma\delta$ subunit structure of the receptor contains both the agonist binding sites and the cation channel that they regulate [cf. Anholt et al. (1983)].

Purified receptors in reconstituted vesicles exhibit two salient features of the postsynaptic membrane, namely, activation and desensitization induced by cholinergic agonists [cf. Anholt et al. (1983)]. These activities are assayed by measuring the translocation of radioactive cations in response to the addition of cholinergic agonists. Although such measurements are convenient and simple, they lack the sensitivity and time resolution needed to study the opening and closing of individual receptor channels. Therefore, it would be of significant interest to study the current fluctuations produced by activation of receptor channels in the same reconstituted vesicles that are used in biochemical assays.

Three recent technical advances allowed us to record the activity of the receptor channel electrophysiologically in reconstituted vesicles: (a) the transfer of *purified* AChR from reconstituted vesicles into monolayers at the air–water interface was demonstrated (Labarca et al., unpublished results); (b) lipid bilayers have been formed from monolayers at the air–water interface in the tip of patch pipets by the successive removal of the pipet from the water and reimmersion through the interface (Wilmsen et al., 1982; Hanke et al., 1983; Coronado & Latorre, 1983); (c) the patch-clamp recording technique (Neher & Sakmann, 1976; Hamill et al., 1981; Horn & Patlak, 1980) has been adapted to record Cl^- channels from crude extracts of *Torpedo* electroplax incorporated into large liposomes (Tank et al., 1982).

Here, we present preliminary results demonstrating the use of patch pipets to obtain single-channel recordings from *purified* receptor reconstituted in vesicles after formation of high-resistance seals (gigaohm range) by gentle suction of the vesicles into the pipet and by the formation of a reconstituted bilayer from monolayers at the tip of the pipet.

Experimental Procedures

Receptor Preparation and Solutions. Receptor from the electric organ of *T. californica* (Pacific Biomarine, Venice,

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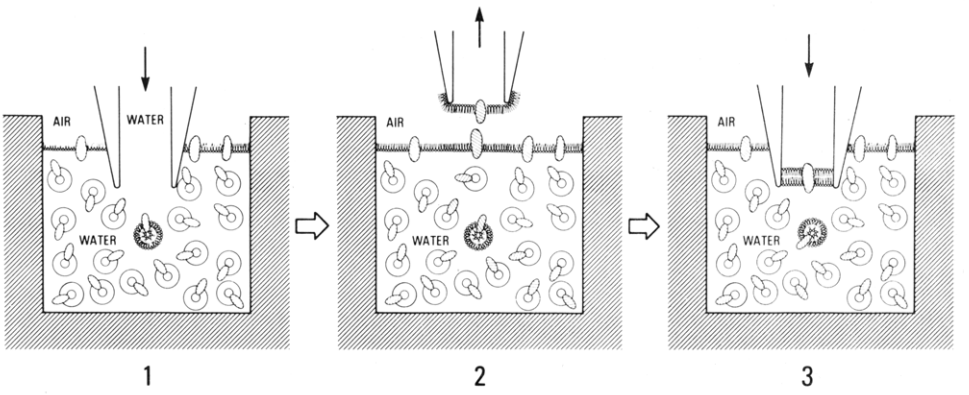

FIGURE 1: Schematic (idealized) representation of the process of bilayer formation from monolayers at the air–water interface at the tip of the patch pipets. Step 1: Monolayers are derived from reconstituted vesicles (one vesicle is shown in more detail). A patch pipet is introduced into the solution under positive pressure. Step 2: The pipet is removed from the solution as the positive pressure is released. The lipid head groups of the monolayers are attached to the pipet while the hydrocarbon tails contact air. Step 3: The pipet is reimmersed into the solution. This leads to the apposition of the hydrocarbon tails of the attached monolayer to those of the original monolayer thereby forming a bilayer. This cartoon does not attempt to depict vesicle size, the mixture of reconstituted vesicles and liposomes known to be present, or the occurrence of one protein per vesicle (Anholt et al., 1981, 1982) (not drawn to any precise scale). It is worth noting that under the conditions routinely used to obtain patch recordings from cells in culture, a monolayer derived from the cells may be formed at the air–water interface (Schindler, 1980; Pattus et al., 1981). Therefore, whenever the patch pipet is removed from and reimmersed into the recording chamber, a bilayer will form at the tip. The composition of such bilayers may be representative of that in the cells. This strategy may prove to be generally valid and significantly useful in membrane biochemical and biophysical investigations.

CA) was solubilized, purified, and reconstituted in lipid vesicles as described in detail previously (Lindstrom, et al., 1980; Anholt et al., 1980, 1981, 1982). The reconstituted soybean lipid vesicles (40 mg/mL) used in this study were supplemented with cholesterol (8 mg/mL) prior to cholate dialysis and were subjected to a freeze–thaw cycle (Kasahara & Hinkle, 1977) as described by Anholt et al. (1982). The functional integrity of the receptor in the reconstituted vesicles was assayed by the carbamylcholine-induced $^{22}Na^+$ uptake (Gasko et al., 1976; Huganir et al., 1979; Lindstrom et al., 1980) before the electrical measurements.

Experiments were conducted at room temperature (22 ± 2 °C) in filtered solutions (Millex-GV, 0.22 μm) that contained 0.5 M NaCl and 2.5 mM Hepes,[1] pH 7.2. Calcium was added to a final concentration of 0.1 or 5 mM. Patch pipets contained the indicated agonist diluted in the same solution.

Patch Pipets. Pipets were produced as described in detail by Hamill et al. (1981) by using hematocrit capillaries made of flint glass (Blu-Tip, plain, i.d. 1.1–1.2 mm, Lancer, St. Louis, MO). The capillaries were cleaned in 1 M nitric acid and rinsed overnight under running deionized water. Before pulling, the capillary tubes were rinsed with methanol and dried by flushing a nitrogen stream. A commercial vertical pipet puller (David Kopf, Model 700C, Tujunga, CA) was used. The pipet tip was fire polished in a microforge to an opening of ≤1 μm and coated with Sylgard (Dow Corning) within 40 μm from the tip.

Electrical Recordings and Data Processing. Membrane currents under voltage clamp were recorded with a commercially available extracellular patch-clamp system (Hamill et al., 1981) (List L/M EPC-5, List Electronic, Darmstadt, Federal Republic of Germany) set at a gain of 10 mV/pA and a filter setting of 10 kHz. The signal coming from the clamp was amplified and recorded on FM tape (RACAL 4DS, bandwidth DC to 2.5 or 5 kHz) and later digitized at a sampling interval of 100 μs for computer analysis. Distributions of dwell times in the open or closed states were analyzed with a PDP 11/34 computer (Digital Equipment Corp.), as described in detail elsewhere (Labarca et al., unpublished results).

[1] Abbreviations: ACh, acetylcholine; CCh, carbamylcholine; SubCh, suberyldicholine; AChR, acetylcholine receptor; γ, single-channel conductance; V, applied voltage; Hepes, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid.

Results and Discussion

Procedures To Obtain a Putative Bilayer at the Tip of a Patch Pipet. Two methods permit patch recordings from reconstituted AChR vesicles. We attempted, first, to obtain liposome-attached patches (Tank et al., 1982). This procedure required a freeze–thaw treatment (Kasahara & Hinkle, 1977; Anholt et al., 1982) to produce vesicles large enough to be manipulated under the microscope. A fire-polished pipet was used to immobilize a vesicle at its tip by applying gentle and constant suction. The recording pipet was then applied to the vesicle. The formation of a bilayer at the tip of the patch pipet was monitored by increases in the DC resistance and in the capacitative current transient in response to a voltage pulse. Seals with resistances ≥1 GΩ formed readily.

The second approach evolved from the first. We observed that when a recording pipet was reimmersed in the vesicle suspension, a seal was formed instantly with resistances ranging from 1 to 10 or more gigaohms. At present, we obtain these seals in ~90% of the trials. These seals only occur in the presence of vesicles and not when this protocol is performed in clean solutions.

Under these conditions, the reconstituted receptor vesicles generate monolayers at the air–water interface (Figure 1, step 1) with a surface pressure of ~30 dyn/cm in the presence of Ca^{2+} [Nelson et al., 1980; Labarca et al., unpublished results; Schindler, 1979, 1980; see also Schindler & Quast (1980)]. This surface pressure is sufficient to support the formation of a bilayer by the interaction of the hydrophobic faces of two apposing monolayers (Montal & Mueller, 1972; Schindler, 1980). Thus, the following sequence of events appears to be the simplest interpretation of the gigaohm seal at the tip of the patch pipet (Figure 1): On removal of the pipet from the aqueous solution through the interface, the lipid head groups of the monolayers attach to the pipet aperture and the lipid hydrocarbon tails get in contact with air (Figure 1, step 2). Reimmersion exposes the hydrocarbon tails of the attached monolayer to those of the parental monolayer leading the formation of a bilayer. The complete bilayer separates two aqueous compartments when it is submerged in the solution (Figure 1, step 3).

We have previously demonstrated that the formation of

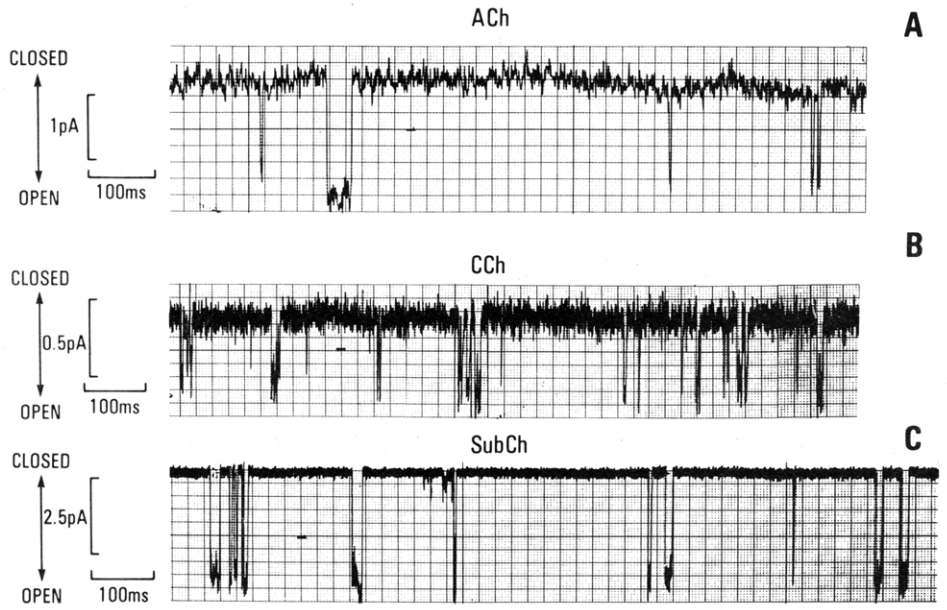

FIGURE 2: Single-channel currents activated by different cholinergic agonists: acetylcholine (10 μM) (upper record), $V = -51$ mV; carbamylcholine (10 μM) (middle record), $V = -10$ mV; suberyldicholine (10 nM) (lower record), $V = -100$ mV. The single-channel conductance can be readily estimated from the amplitude of the current steps divided by the applied voltage. The open-channel conductance was 40 ± 5 pS for all three agonists. The seal resistances were 17, 5, and 10 GΩ, respectively. The records were low-pass filtered at 1 kHz.

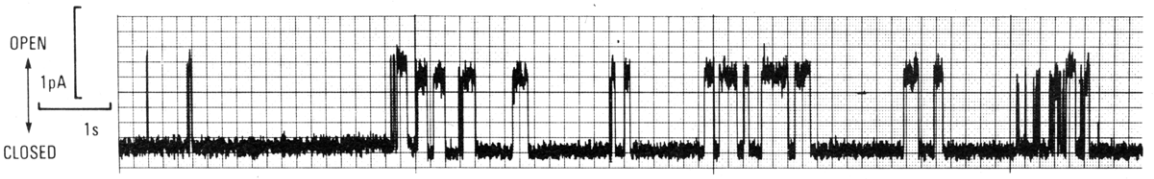

FIGURE 3: Analysis of single-channel currents activated by cholinergic ligands. Single-channel current records, in which only one channel was open at any given time, were analyzed. A recording, obtained with 10 μM acetylcholine at $V = 15$ mV, illustrates this condition. The mean-channel open time is longer at positive than at negative applied voltage (compare with Figure 2A). There are few events that are larger or smaller than the most frequent event; these were also included in the analysis. The seal resistance was 4 GΩ. The records were low-pass filtered at 1 kHz.

monolayers from reconstituted vesicles leads to the transfer of receptor from vesicles to monolayers at the air–water interface (Labarca et al., unpublished results). Similar results were obtained for *T. marmorata* electroplaque vesicles (Schindler & Quast, 1980). Under the conditions described here, the estimated receptor density at the interface is $\sim 10^7$/cm^2 (Labarca et al., unpublished results). Thus, the sampling of the monolayer with the patch pipet (tip area $\simeq 0.8$ μm^2) has a finite probability ($\sim$8%) of trapping a bilayer containing receptor. This is consistent with our ability to record single receptor channel currents in $\sim$10% of all the seals.

Single-Channel Recordings. The currents flowing through individual receptor channels appear as discrete transient steps that fluctuate between, at least, two current levels associated with the closed and open states of the channel (Neher & Sakmann, 1976). Single channels were activated by the cholinergic ligands acetylcholine (ACh) (Figure 2A), carbamylcholine (CCh) (Figure 2B), and suberyldicholine (SubCh) (Figure 2C). The single-channel conductance, γ, was 40 ± 5 pS for the three agonists in 0.5 M NaCl, 5 mM $CaCl_2$, and 2.5 mM Hepes, pH 7.0.

Agonist-activated channels were not recorded in control studies. When bilayers were formed in patch pipets under conditions identical with those described for the reconstituted vesicles but using liposomes devoid of receptor, no channels were observed (25 trials). Neither were channels observed when the reconstituted vesicles were preincubated with an excess of α-bungarotoxin, which specifically inhibits the agonist-activated flux response in vesicles (22 trials) (Lindstrom et al., 1980; Anholt et al., 1982), nor in the absence of agonist in the pipet (16 trials). However, discrete conductance steps were recorded in some seals, which were distinguished from AChR-activated channels on pharmacological evidence. The most salient nonspecific events were small (0.5 pA in amplitude) and slow (mean open time $\simeq$1 s) and appeared at $V = 0$. These results indicate that acetylcholine receptors are responsible for the single-channel currents recorded in the presence of cholinergic agonists.

Single-channel records, in which only one channel was open at any given time (Figure 3), were analyzed to measure the channel dwell times in either the open or the closed states. The open-state lifetime distributions do not follow a single exponential function but are best fitted with the sum of two exponentials, irrespective of the choice of agonist. For example, with 10 μM CCh at a holding potential of −80 mV, the zero-time amplitude (A) and lifetime (τ) of the two exponential components of the open-time distribution (Figure 4A) were $A_1 = 426 \pm 6$ events, $\tau_1 = 0.7 \pm 0.02$ ms and $A_2 = 122 \pm 7$ events, $\tau_2 = 4.5 \pm 0.2$ ms, respectively. The lifetimes of the two exponential components were a factor of 2 longer for SubCh than for either ACh or CCh. The observation of two channel lifetimes reflects the existence of at least two distinct open states of the receptor channel. The distribution of closed times was best fitted with the sum of three exponentials. The exponential component with the longest time constant ($A_3 = 107 \pm 9$ events, $\tau_3 = 26 \pm 2$ ms) was subtracted, and the data

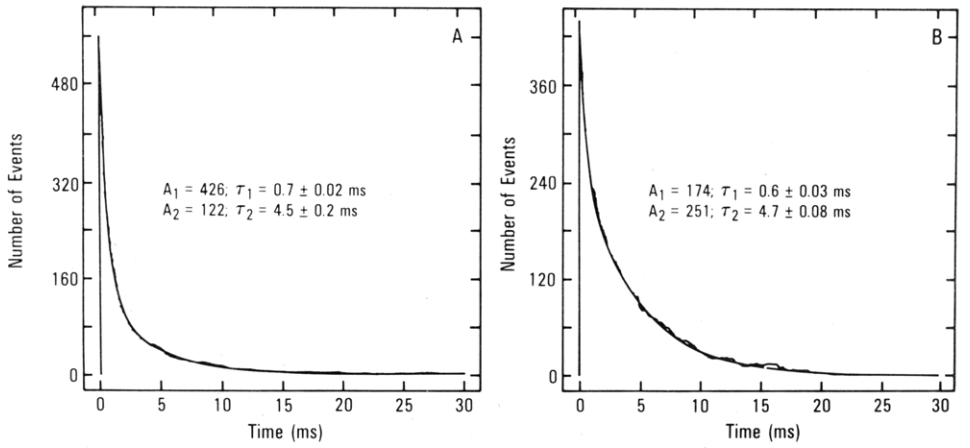

FIGURE 4: (A) Frequency histograms of single-channel open times activated by carbamylcholine (10 μM) at $V = -80$ mV. The histograms were constructed as described elsewhere (Labarca et al., unpublished results). The computer generated a virtual reproduction of the actual signal. The position and the width of the computer-generated rectangular pulses corresponding to the channel openings and closings were stored in the computer. Cumulative open-state or closed-state (Figure 4B) lifetime distributions of the generated rectangular pulses were produced. Time constants were determined by fitting one, two, or three computer-generated exponentials to the data points displayed simultaneously on an oscilloscope screen. The fitted curves (smooth curve) were superimposed on the histograms of the actual data (noisy curve). Semilogarithmic plots of either the actual data or the computer-reconstructed channels were fitted by similar parameter values. (B) Frequency histogram of single-channel closed times activated by carbamylcholine (10 μM) at $V = -80$ mV. Conditions as for (A). The distribution of closed times could not be fitted with a sum of two exponentials. The exponential component with a long time constant (A_3 = 107 ± 9 events, τ_3 = 26.3 ● 2 ms) was subtracted and, thereafter, the curve was fitted with the sum of two exponentials.

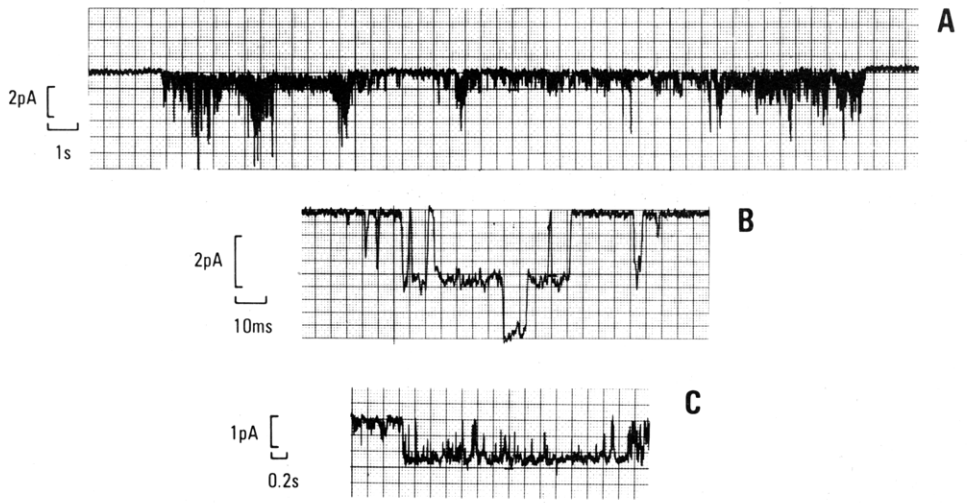

FIGURE 5: Paroxysms of single channels activated by acetylcholine. (A) Single-channel currents were activated by 50 μM acetylcholine at $V = -25$ mV. A downward deflection corresponds to a channel-opening event. The single-channel conductance was 39 pS. The seal resistance was 17 GΩ. The single events appear in bursts of channel activity followed by quiescent periods. The bursts tend to group into clusters. (B and C) Sections of records of single-channel currents activated by desensitizing concentrations of agonist displayed at higher time resolution. (B) The opening and closing of two single channels is clearly discerned. The single-channel conductance was 40 pS and $V = -50$ mV. (C) The channel residence time in the open state is clearly longer than at lower agonist concentration (compare with Figure 2A), indicating a preferential shift to the channel open state. The single-channel conductance was 40 pS and $V = -40$ mV. All the records were low-pass filtered at 1 kHz.

were then fitted with a sum of two exponentials (Figure 4B). The corresponding parameters of the fitted curve for the closed time distribution were A_1 = 174 ● 4 events, τ_1 = 0.6 ± 0.03 ms and A_2 = 251 ± 4 events, τ_2 = 4.7 ± 0.8 ms, respectively. These results are in agreement with those obtained for purified receptor reconstituted in planar lipid bilayers (Labarca et al., 1983, and unpublished results).

A striking pattern of single-channel activity was recorded at ACh concentrations >10 μM. The single events appeared in bursts of channel activity followed by quiescent periods (Figure 5A). Measurements at low-time resolution show that these channel bursts group into clusters (Figure 5A). Displays of records at higher time resolution show that the opening and closing of two channels (Figure 5B) or a single channel (Figure 5C) are discerned clearly. The channel residence time in the open state is longer at 50 μM ACh (Figure 5C) than at 10 μM ACh (Figure 2A), indicating a preferential shift to the channel open state. Similar results were recorded in planar bilayers containing purified receptor (Labarca et al., 1982, 1983, and unpublished results) and are reminiscent of the patterns of channel activity described by Sakmann et al. (1980) in frog muscle at desensitizing concentrations of ACh.

Frequently, vesicles were sealed to the micropipet but no receptor channels were observed. This observation is consistent with the fact that the reconstitution process generates a small number of large vesicles containing receptor and a large number of small liposomes devoid of receptor (Anholt et al., 1981, 1982). The current protocol involves the presence of the cholinergic agonist in the patch pipet. This condition implies that receptors in the sealed vesicle are exposed to agonist as soon as the seal is formed. Therefore, it is likely that receptors will relax into the desensitized conformation characterized by a closed channel. Thus, we attribute the failure to record receptor channels in all sealed vesicles, in part,

to the occurrence of receptor-free liposomes in the preparation and, in part, to receptor desensitization.

Purified receptor in reconstituted bilayers formed at the tip of patch pipets displays channel activity associated with activation and desensitization in the presence of cholinergic agonists. The behavior of receptor channels recorded with both methods of forming bilayers at the tip of pipets is similar; however, the seals obtained by the sequential monolayer transfer technique tend to be more stable and of higher resistance. The recordings obtained with patch pipets have the additional advantage over the conventional planar bilayer (Montal & Mueller, 1972; Nelson et al., 1980; Schindler & Quast, 1980; Boheim et al., 1981; Labarca et al., 1982, 1983, and unpublished results) of allowing measurements with higher signal to noise ratio and faster time resolution. On the other hand, patch recording is limited in that it provides access to one compartment only. As these initial results indicate, patch recordings from reconstituted vesicles appear to be as simple and reliable as those obtained from cells in culture. Reconstituted receptor vesicles permit the use of a wide range of available assays of receptor function, from ligand binding and radiochemical fluxes to spectroscopic measurements and direct electrical recordings. From the above considerations it follows that reconstituted vesicles are an excellent system to study the correlation between receptor structure and its function.

In conclusion, these results indicate that the full cycle of solubilization, purification, and reconstitution of acetylcholine receptors can be achieved without impairment of channel function. Furthermore, the channel activity can be assayed with an electrophysiological method of high resolution and sensitivity under conditions amenable to biochemical manipulations.

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Registry No. ACh, 51-84-3; CCh, 462-58-8; SubCh, 7262-79-5.

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