Giant Liposomes: A Model System in Which To Obtain Patch-Clamp Recordings of Ionic Channels†

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ABSTRACT: Cell-size, giant liposomes have been formed by submitting a mixture of asolectin lipid vesicles and native membranes from *Torpedo*, highly enriched in acetylcholine receptor (AcChR), to a partial dehydration/rehydration cycle [Criado, M., & Keller, B. U. (1987) *FEBS Lett. 224*, 172–176]. Giant liposomes can be prepared in bulk quantities, in the absence of potentially damaging detergents or organic solvents, and their formation is mediated by membrane fusion phenomena. In fact, fluorescence microscopy and freeze–fracture data indicate that protein and lipid components of the initial membranes and lipid vesicles are homogeneously distributed in the resulting liposomes. Giant liposomes containing AcChR have been used as a model to evaluate whether this system can be used to monitor the activity of ionic channels by using high-resolution, patch-clamp techniques. Excised liposome patches in an "inside-out" configuration have been used in this work. We find that the most frequent pattern of electrical activity in response to the presence of acetylcholine in the patch pipet corresponds to a cation-specific channel exhibiting a dominant conductance level and a sublevel of approximately 78 and 25 pS, respectively. Such channel activity exhibits the pharmacological specificity, ion channel activation, ion selectivity, and desensitization properties expected from native *Torpedo* AcChR. Thus, it appears that the giant liposome technique offers a distinct advantage over other reconstitution procedures in that it provides a unique opportunity to undertake simultaneous biochemical, morphological, and electrophysiological studies of the incorporated ionic channel proteins.

Ionic channels are integral membrane proteins that, upon activation, allow a passive flow of specific ions down thermodynamic gradients established between both sides of the membrane. Thus, assessing the functionality of isolated molecular components comprising these channels requires their reconstitution into model membranes in which translocation of ions may be measured [see, for instance, Miller (1983a, 1984)]. Nevertheless, monitoring ionic channel responses is difficult since channel activation promotes very rapid changes in the permeability of the membrane to certain ions. Initially, ion fluxes were mostly monitored by using radioactive ion tracers in assays of poor time resolution (ultrafiltration, ion-exchange chromatography, etc.). Electrical methods, having higher sensitivity and time resolution, are a better alternative to quantitate flow of ions, and thus, two basic systems have been developed to combine membrane reconstitution of ionic channels and electrical monitoring of ion flow: planar bilayers and liposomes (Mueller & Rudin, 1968; Schindler, 1980; Montal et al., 1981; Eytan, 1982; Miller, 1984).

Planar bilayers have been extensively used since they can be voltage-clamped and provide easy access to both sides of the reconstituted membrane. However, one has little control on the insertion of proteins into the bilayer (Miller, 1983b). Also, because the planar bilayer is not amenable to biochemical measurements, it is difficult to know how many channels are inserted at a given time. Other limitations of conventional planar bilayers regarding the resolution of single-channel currents have been circumvented by forming bilayers of much smaller diameter at the tip of patch-clamp pipets (Suarez-Isla et al., 1983; Coronado & Latorre, 1983; Hanke et al., 1984). However, here the bilayer is formed from lipid monolayers at the air–water interface, and it is an open question whether ionic channels could be perturbed when assembled into the monolayers.

Reconstituted liposomes can be formed in bulk quantities and analyzed biochemically (Miller, 1984). Nevertheless, even the largest reconstituted liposomes, produced by the commonly employed detergent elimination procedures (Allen, 1984), are too small to be used in electrical monitoring of incorporated ionic channels. Alternatively, small liposomes can be fused into larger liposomes by freezing and thawing (Tank et al., 1983), but so far, this has been confined to a limited number of samples. More recently, the formation of cell-size, giant liposomes presenting active, reconstituted ion channels has been reported (Criado & Keller, 1987; Keller et al., 1988). This technique uses a dehydration/rehydration procedure to induce liposome formation from mixtures of native membranes and lipid vesicles and is based on previous observations by Mueller et al. (1983) on the formation of similar structures by hydration of dried lipid mixtures. Although these reports lack sufficient quantitative information on the ionic channels tested, they clearly suggest that the giant liposomes could be an ideal system to prepare these proteins in a suitable form for the application of conventional patch-clamp methods. To validate this possibility, however, one would have to demonstrate that the incorporation into giant liposomes does not cause alteration in known features of the ionic channel of interest. With this purpose, we report here studies on giant liposomes made from Torpedo membranes highly enriched in acetylcholine receptor (AcChR).[1] *Torpedo* AcChR is one of the best known ionic channels up to date, and functional information on the AcChR

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[1] Abbreviations: AcChR, acetylcholine receptor; α-Bgt, α-bungarotoxin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; OG, octyl β-D-glucoside; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; FITC, fluorescein isothiocyanate; TMRITC, tetramethylrhodamine isothiocyanate; TTX, tetrodotoxin; IMPs, intramembrane protein particles; asolectin, crude extracts of phosphatidylcholine from soybean.

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ranges from macroscopic radioactive cation fluxes in crude membrane preparations to single-channel recordings of the purified, reconstituted protein [see for a review Barrantes (1983), Hess et al. (1983), Popot and Changuex (1984), Labarca et al. (1984), McCarthy et al. (1986), McNamee et al. (1986), and Jones et al. (1987)]. Our results indicate that known properties of the AcChR regarding ligand binding specificity, agonist-induced desensitization, ion channel activation, and ion selectivity are preserved in the giant liposomes. Also, we provide evidence to support that giant liposome formation is mediated by a membrane fusion process, which implies that protein and lipid components present in the starting membranes and lipid vesicles have been randomly redistributed in the resulting giant liposomes.

Materials and Methods

Sodium cholate, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), acetylcholine chloride, *d*-tubocurarine, and crude extracts of phosphatidylcholine from soybean (type 2-S, asolectin lipids) were purchased from Sigma. Octyl β-D-glucoside (OG) was from Calbiochem. Fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TMRITC) were from Molecular Probes. Tetrodotoxin (TTX) was a gift from Dr. B. Soria, from this Institute.

^{125}I-α-Bungarotoxin (^{125}I-α-Bgt) was purchased from New England Nuclear, and ^{125}I-α-Bgt binding was measured by using a DEAE-cellulose filter disk assay (Schmidt & Raftery, 1973). Nonradioactive α-Bgt was purified to homogeneity from *Bungarus multicinctus* venom (Miami Serpentarium) as previously described (Ferragut et al., 1984). Labeling of α-Bgt with TMRITC for fluorescence microscopy was performed as in Ravdin and Axelrod (1977).

Preparation of AcChR Membranes. The electric organ of *Torpedo marmorata* was used to prepare membranes highly enriched in AcChR, as previously described, including alkaline extraction of peripheral membrane proteins (Artigues et al., 1987). These membranes were suspended in 10 mM Hepes buffer, pH 7.4, containing 100 mM NaCl and had specific activities of approximately 4 nmol of α-Bgt bound/mg of protein. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate indicated that ~70–80% of the protein bands present in these membranes corresponded to the characteristic polypeptide pattern of purified AcChR (Artigues et al., 1987). Protein and lipid phosphorus concentrations were determined as in Lowry et al. (1951) and Kyaw et al. (1985), respectively. Membrane suspensions were divided into 100-μL aliquots and stored at −80 °C.

Occasionally, AcChR membranes (3 mL at ~2.5 mg of protein/mL) were fluorescently labeled by reaction with FITC (~1.4 mg) in 90 mM sodium bicarbonate buffer, pH 9.5. After 30-min incubation at room temperature, unbound fluorescence products were eliminated by centrifugation. Labeled membranes were finally resuspended in 10 mM Hepes, pH 7.4, and 100 mM NaCl.

Formation of Lipid Vesicles. Asolectin lipids were suspended in 10 mL of distilled water, at ~100 mg/mL, by sonication in a probe-type Artek 300 apparatus. The almost transparent lipid suspension was diluted 10-fold with 10 mM Hepes, pH 7.4, and 100 mM NaCl, and solid CHAPS (or other detergents) was added to a final concentration of 1% (w/v). Lipid vesicles were formed by elimination of the detergent by exhaustive dialysis. The dialyzed sample was divided into 0.8–1.0-mL aliquots and stored at −80 °C.

Formation of Giant Liposomes. The procedure used in these studies is a modification of that previously described by Criado and Keller (1987). Routinely, a AcChR membrane aliquot containing 100 μg of protein was mixed with 1.7 mL of a 13 mM (in terms of lipid phosphorus) suspension of dialyzed asolectin vesicles. The mixture was centrifuged for 30 min, at 50000 rpm, in a Kontron TFT-70 ultracentrifuge rotor, and the resulting pellet was resuspended, at 4 °C, in 75 μL of Hepes buffer, pH 7.4, containing 5% (v/v) ethylene glycol, by forcing the suspension through the needle of a small syringe until an apparently homogeneous suspension was produced. The suspension was deposited as small drops (~20 μL) on clean, delipidated glass microscope slides and submitted to partial dehydration for 3 h and at 4 °C, in a desiccator containing anhydrous $CaCl_2$. The samples were rehydrated by adding 20 μL of a 50 mM NaCl solution (or just distilled water) on top of each dehydrated drop. Giant liposomes were observed shortly after addition of the rehydration solution, under a regular light microscope. As a routine, however, samples were rehydrated overnight in a cold chamber, inside the large petri dishes containing a wet paper pad on the bottom. The resulting giant liposomes were pipetted off the rehydrated drops. Alternatively, when a more efficient collection of liposomes was required, the whole glass slide, containing several drops, was rinsed off with buffer and the liposome suspension collected in a test tube. For "semipreparative" purposes, larger amounts of samples were prepared and deposited as wide stripes, rather than drops, on several microscope slides for the dehydration/rehydration cycle.

Freeze–Fracture Electron Microscopy. For freeze–fracturing, giant liposomes were centrifuged in Eppendorf tubes, and the resulting pellets were fixed in 1% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0, at room temperature for 2 h. Fixed samples were washed several times with buffer, soaked for 2 h in buffer containing 20% glycerol, coated with poly(vinyl alcohol), frozen in Freon 22 cooled with liquid nitrogen, fractured at −110 °C, and shadowed with platinum/carbon in a Balzers 400D apparatus. A total of three to six replicas were prepared from each pellet. More details on the handling of replicas and on the quantitation of IMPs have been previously described (Garcia-Segura et al., 1986).

Patch-Clamp Recording of Ion Channels. Aliquots (3–15 μL) of giant liposomes were deposited into 3.5-cm Petri dishes, mixed with 200–300 μL of a DEAE-Sephadex A-50 suspension (~3 mg of dry gel/mL) previously equilibrated in 4 mM Hepes buffer, pH 7.4, containing 50 mM NaCl and 0.1 mM $CaCl_2$, and incubated for 15–30 min at room temperature. This treatment anchors the liposomes to the gel beads and to the bottom of the dish, thus allowing extensive washing with the buffer of choice for electrical recording (bath solution) while leaving a high number of liposomes remaining in the dish. Single channel recordings were obtained by using patch-clamp techniques as described by Hamill et al. (1981). Giga seals (10–20 GΩ) were formed on giant liposomes with regular patch pipet microelectrodes (10–20-MΩ resistance for the open electrode) made from hematocrit capillaries (glass blue tip; Lancer, St. Louis, MO) by apposing the pipet tip to the exposed liposome surface with the help of a micromanipulator and applying gentle suction to the pipet interior. After the pipet was sealed, careful withdrawal of the pipet from the liposome surface and, if necessary, quickly passing the tip through the air/water interface resulted in an excised patch ("inside-out" configuration). In most experiments, both the pipet and the bath solutions were identical (referred to as "symmetrical" conditions) and contained 4 mM Hepes buffer,

pH 7.4, 100 mM NaCl, and 0.1 mM $CaCl_2$, with or without the presence of cholinergic agonist or other ligands, as indicated under Results. An EPC-5 patch amplifier (List Medical Electronics) was used, at a gain of 20 mV/pA and a filter setting of 10 kHz. The holding potential was applied to the interior of the patch pipet, and the bath electrode was maintained at virtual ground. The signal from the clamp was visualized on a storage oscilloscope, digitized, and stored on a video recorder. All experiments were conducted at room temperature. For "off-line" computer analysis of the data, the recordings were filtered at 1 kHz and digitized by a Cambridge Electronic Design 1401, at a sampling interval ranging from 100 to 500 μs. The computer software for the analysis of single-channel data was provided by Dr. J. Dempster from the University of Strathclyde (Dempster, 1988). Routinely, for each holding potential, current amplitude values of channel opening events were plotted to produce Gaussian amplitude histograms (number of events vs current amplitude) and used to determine the mean amplitude values of open-channel states and the probability of channel opening under those conditions. Mean amplitude values at the different holding potentials were used to calculate the open-channel conductance from the slope of current amplitude vs voltage plots.

Results

Formation of Giant Liposomes. Submitting a mixture of membranes and lipid vesicles to a partial dehydration/rehydration cycle as described under Materials and Methods resulted in the formation of cell-size, reconstituted giant liposomes (Criado & Keller, 1987; Keller et al., 1988). These liposomes formed rapidly upon rehydration of the dehydrated mixtures, and their appearance could be monitored under a regular light microscope (Figure 1). Experimental variables such as the length of the dehydration and rehydration periods did not seem to significantly affect the formation of the giant liposomes. In fact, we have obtained giant liposomes by dehydrating the membrane/vesicle mixtures anywhere from 1 to 5 h or rehydrating them from 1 to 36 h. It is apparent, however, that changes in other variables, including temperature, ionic strength, type of lipids used, and the way lipid vesicles are prepared, were critical and affected quite dramatically the formation and the properties of the giant liposomes. For instance, rehydrating in high ionic strength media resulted in giant liposomes with a "jelly"-like consistency, which made seal formation impossible. Also, when the dehydration/rehydration cycle was carried out at room temperature, or when purified egg phosphatidylcholine, instead of asolectin, was used in the experiments, the number and the size of the resulting liposomes were greatly decreased. Furthermore, use of asolectin lipid vesicles prepared by dialysis of different detergents also affected the shape, size, and number of the resulting giant liposomes. Use of vesicles obtained by dialysis of CHAPS or OG resulted in abundant liposome formation, but while the former gave raise to mostly spherical liposomes, use of the latter resulted in a mixture of spherical liposomes and closed, long tubules. Vesicles obtained by cholate dialysis, on the other hand, produced fewer and smaller liposomes, which were comparable to those obtained from asolectin vesicles prepared by sonication, in the absence of any detergent. Giant liposome formation is, therefore, a complex process which can be affected by many variables, each of which needs further investigation. Nevertheless, when defined experimental conditions such as those described under Materials and Methods were used, giant liposomes were easily and reliably formed.

Use of FITC-labeled *Torpedo* membranes to form giant liposomes indicated that the fluorescence from the labeled components of the membranes was uniformly distributed in the giant liposome (data not shown), similarly to previous reports by others (Criado & Keller, 1987). Also, incubation of standard giant liposomes with TMRITC-labeled α-Bgt suggested an apparently homogeneous distribution of the AcChR (Figure 2). Both types of experiments indicated that uniform mixing and redistribution of components between the membranes and the asolectin vesicles occurred during giant liposome formation. This was confirmed by freeze–fracture

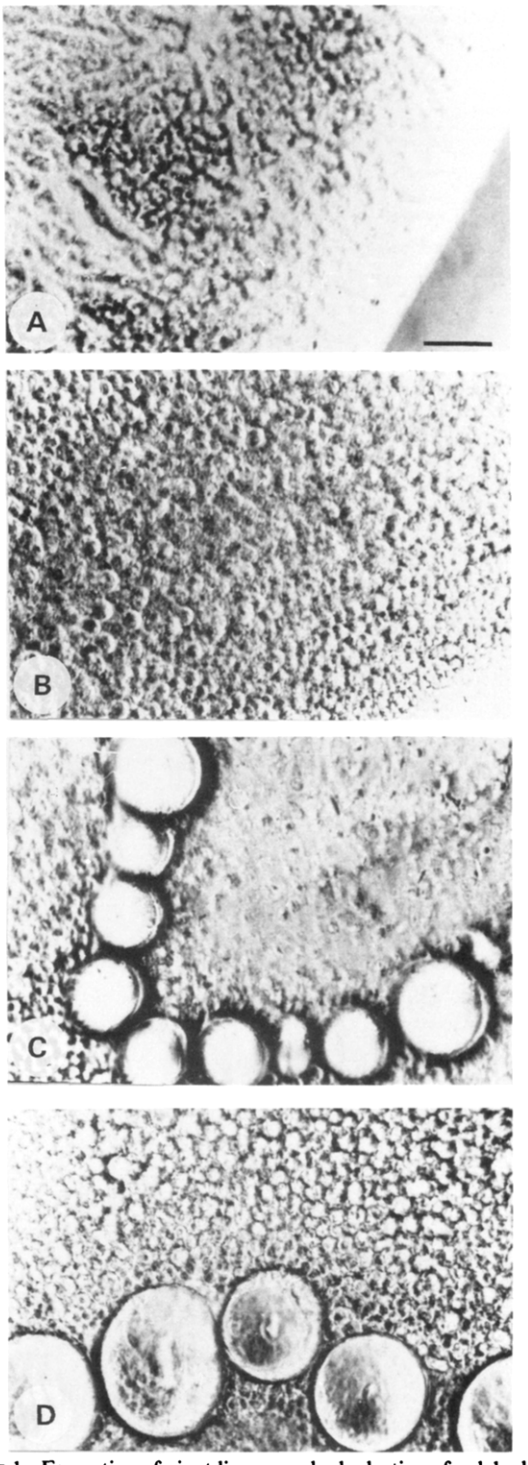

FIGURE 1: Formation of giant liposomes by hydration of a dehydrated mixture of *Torpedo* membranes and asolectin vesicles (panel A). To illustrate the rate of giant liposome formation, panels B, C, and D show the same sample as panel A, but after 1 min, 1 h, and 3 h, respectively, following addition of the hydration solution (see Materials and Methods). The bar equals 25 μm.

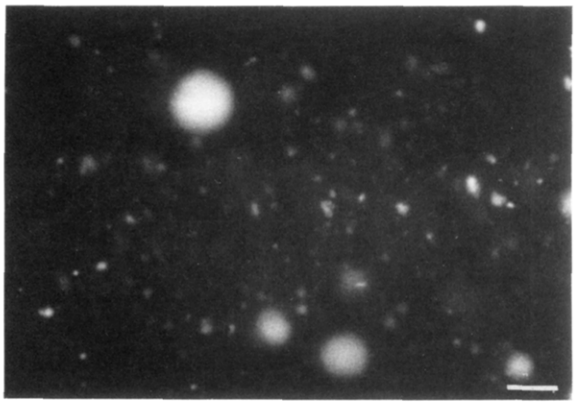

FIGURE 2: Formation of AcChR/α-Bgt fluorescence complexes by incubation of standard giant liposomes with TMRITC-labeled α-Bgt. The liposome samples were diluted in 4 mM Hepes buffer, pH 7.4, containing 100 mM NaCl, incubated with the TMRITC-labeled α-Bgt at room temperature for 2–3 h, washed several times with buffer to eliminate unbound fluorescence products, and viewed under a Nikon Diaphot TMD-EF fluorescence microscope by using phase-contrast or rhodamine fluorescence equipment. All liposomes observed were fluorescently labeled, as shown in the micrograph. On the contrary, giant liposomes made from asolectin lipids, but in the absence of AcChR membranes, did not produce fluoresence images upon incubation with the labeled neurotoxin (data not shown). The bar equals 25 μm.

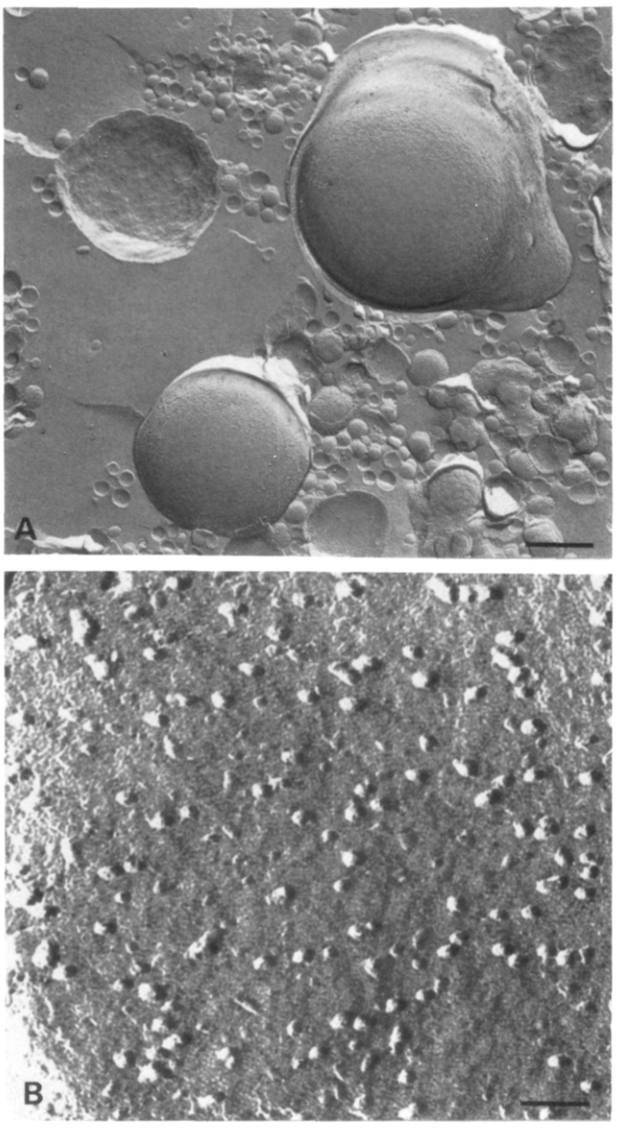

FIGURE 3: Freeze–fracture electron microscopy images of giant liposomes obtained under the standard conditions described under Materials and Methods. Panoramic view to show liposomes of different size (bar equals 0.8 μm). High magnification of a freeze–fracture replica of a liposome membrane showing the distribution of IMPs (bar equals 0.1 μm).

experiments (Figure 3), in which numerous intramembrane protein particles (IMPs) were present uniformly distributed in all the giant liposomes observed (565 ± 47 IMPs/μm^2, n = 77). Ultrathin sections of osmium tetroxide treated, fixed giant liposome samples were also observed by transmission electron microscopy (data not shown). These studies revealed that the larger liposomes were composed of many membrane bilayers, producing myelin-like figures under the electron microscope. On the contrary, the smaller liposomes (less than 2–5 μm in diameter) were mostly unilamellar.

Protein and lipid components (estimated in terms of α-Bgt binding sites and lipid phosphorus, respectively) present in the starting membranes and asolectin vesicles mixtures were recovered associated into giant liposomes with a 60–70% yield. This is a minimum estimate for liposome formation since part of the rehydrated sample stuck to the glass slide where the liposomes were formed, and could not be quantitatively removed by rinsing with the aqueous buffer. Nevertheless, this apparent loss of material was nonselective, as suggested by the fact that AcChR/phospholipid ratios in the starting mixture and in the liposome samples were identical. Giant liposome samples used in most of the experiments described here contained 100 000–200 000 mol of phospholipid/mol of α-Bgt binding sites.

Patch-Clamp Recording of Ion Channels. Protein-free, giant liposomes made from asolectin vesicles, but in the absence of AcChR membranes, were adequate to produce seals of high resistance and inside-out, excised patches. No electrical activity was detected (n = 10) at pipet holding potential ranging −150 to +150 mV.

Standard, protein-containing giant liposomes also formed stable patches and in the absence of acetylcholine in the pipet solution exhibited a characteristic ion channel activity, which could be detected only in 11% of the cases (n = 9). The main features of this ion channel, detected in the absence of cholinergic activation, were that it fluctuated between two open states (indicated as O_1 and O_2, respectively, in Figure 4A) of identical amplitude, but with a different opening probability. As the pipet potential was made increasingly positive, the probability of channel opening for the O_1 level increased, at the expense of a decrease of that corresponding to O_2 openings. The open conductance, estimated from the slope of current amplitude vs voltage plots, was 21.3 ± 2.5 pS (Figure 4B). These features, including the characteristic "double-level" opening pattern, rapid gating, and high opening probability at positive holding potentials, as shown in Figure 4A, are similar to those reported by Miller and co-workers for the *Torpedo* chloride channel (Miller, 1983a; Tank-Miller, 1983) (open conductance of 18 pS in 150 mM NaCl, pH 7.5), which is also independent of cholinergic activation. Identical recordings of this channel and similar probabilities of finding active patches were also obtained in giant liposomes previously incubated with excess α-Bgt, with (n = 6) or without (n = 7) the presence of 5 μM acetylcholine in the pipet solution, or under conditions in which acetylcholine and a 10–100-fold molar excess of d-tubocurarine (n = 2) coexisted in the pipet (Figure 4B).

More complex situations were produced under conditions of cholinergic activation. The presence of 5 μM acetylcholine in the solution contained in the patch pipet and the use of 100 mM NaCl symmetrical conditions resulted in recordings ex-

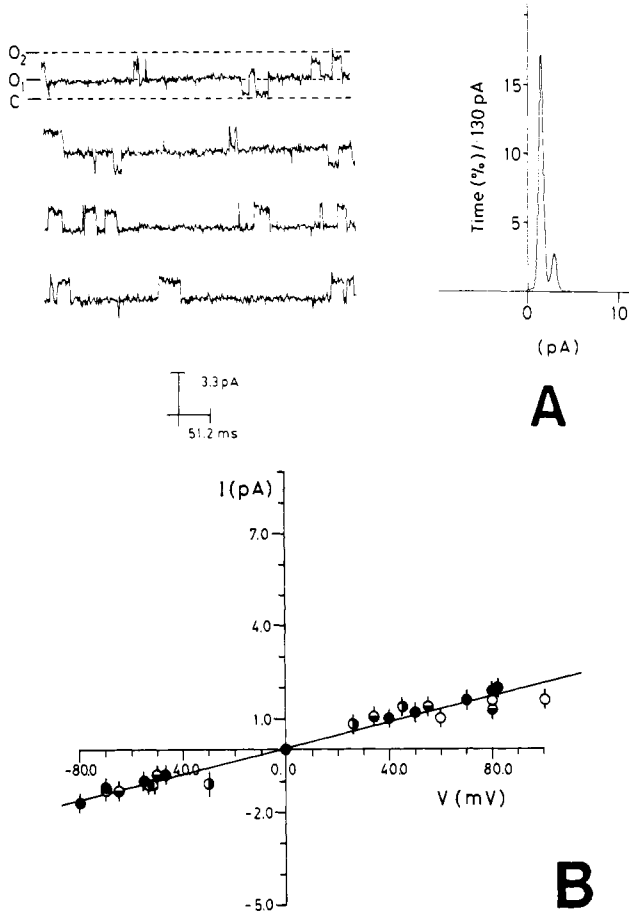

FIGURE 4: Ion channel activity exhibited by excised patches from giant liposomes made from *Torpedo* membranes and asolectin vesicles, in the absence of cholinergic stimulation. Single-channel records obtained at a holding potential at +100 mV (panel A) and the corresponding amplitude histogram are shown. Panel B represents the single-channel current vs voltage curve in a symmetrical 4 mM Hepes buffer, pH 7.4, containing 100 mM NaCl and 0.1 mM $CaCl_2$ (○). Other symbols in panel B are used to illustrate a similar behavior of this ion channel observed in the presence of 5 μM acetylcholine and 50 μM *d*-tubocurarine coexisting in the pipet solution (◒) or in giant liposomes preincubated with an excess of α-Bgt with (◑) or without (●) the presence of 5 μM acetylcholine in the pipet solution.

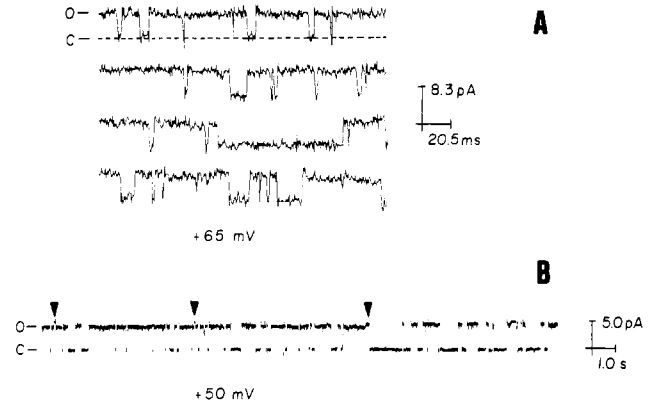

FIGURE 5: Typical single-channel records exhibited by excised patches from giant liposomes in response to cholinergic stimulation (5 μM acetylcholine in the pipet solution). (A) Single-channel activity observed at a holding potential of +65 mV and using the symmetrical buffered solution indicated in Figure 4. (B) Channel activity observed on a paper recorder at a filter setting of 3 kHz. Buffered solutions used here were as in Figure 4, except that the bath solution contained 100 mM KCl, instead of 100 mM NaCl. A holding potential of +50 mV was used in this experiment. The arrowheads indicate the opening and closing of an associated current sublevel.

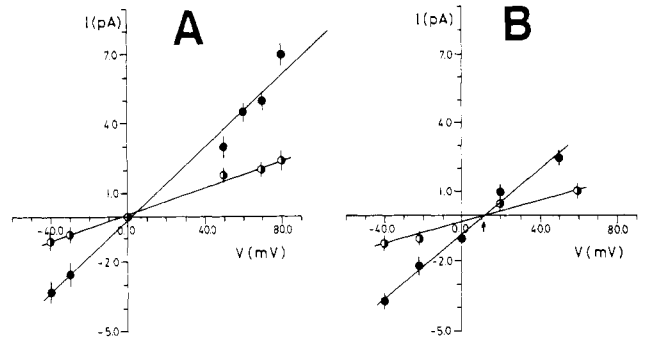

FIGURE 6: Single-channel current vs voltage curves for the main current level (●) and sublevel (◑) shown in Figure 5. For panel A, both sides of the liposome patch were bathed by the 100 mM NaCl symmetrical solution indicated in Figure 4, except that 5 μM acetylcholine was added into the pipet. Panel B corresponds to asymmetrical conditions in which the pipet solution was identical with that used in panel A, while the bath solution differed in that it contained a higher concentration of NaCl (190 mM). The arrow in panel B indicates the reversal potential for the main current level and sublevel under these asymmetrical conditions.

hibiting a complicated pattern of electrical activity during the first seconds or even minutes following patch formation, after which the number of fluctuations decreased and it was possible to obtain recordings corresponding to discrete transient fluctuations between two or more current levels, likely to be associated with the closing and opening of an ion channel. At the protein concentration indicated under Materials and Methods to prepare the giant liposomes, we found these ion channel activities in response to the presence of 5 μM acetylcholine in 21 out of a total of 41 patches recorded, i.e., 50% of the cases. Use of acetylcholine at a lower concentration (1–2 μM) did not change significantly the probability of finding responsive patches, but when higher concentrations of the agonist were used, such probability decreased dramatically so that at 10 μM acetylcholine only ~20% of the liposome patches were active and at 50 μM acetylcholine all patches were electrically inactive. Furthermore, decreasing and increasing the initial amount of protein resulted, respectively, in a decreased and increased probability of finding active patches. Higher protein concentrations, however, made it difficult to distinguish single-channel currents, and, therefore, for the experiments described below, we chose to use the protein and agonist concentrations described under Materials and Methods. Sealed patches from standard giant liposomes which were not responsive to the presence of acetylcholine in the pipet, or patches not previously exposed to cholinergic agonist, never responded to addition of acetylcholine to the bath solution, indicating that adequate acetylcholine binding sites were not exposed to the bath solution in our inside-out excised patches. This suggested that the "right-side-out" orientation of AcChR in the starting native membranes was preserved upon giant liposome formation.

The most frequent pattern of electrical activity (found in 67% of the responding patches) caused by the presence of 5 μM acetylcholine in the patch pipet is shown in Figure 5. Single-channel recordings corresponding to discrete transient fluctuations between two currents levels (open and closed states) were mostly observed. Also, a subconducting current was detected, with an amplitude corresponding to approximately one-third of that exhibited by the dominant current and a much lower opening probability at the binding potentials used in the experiments. This smaller current was never detected alone, and it was present in almost all the patches exhibiting the main current. Figure 6A shows current vs voltage plots obtained from mean single-channel amplitudes

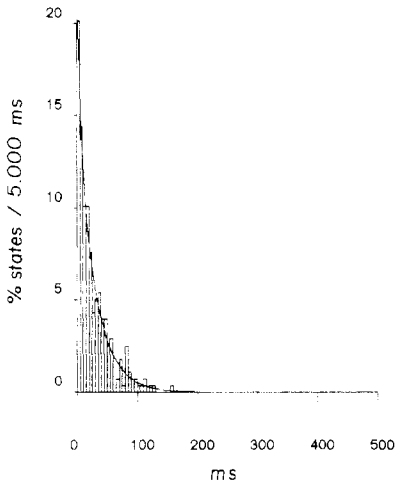

FIGURE 7: Representative frequency histogram of channel open times derived from single-channel records at a holding potential of +65 mV. Experimental conditions and solutions used here were those indicated in the legend to Figure 5A.

determined at different holding potentials. Both the dominant current level and the sublevel were ohmic within the voltage range studied, and in 100 mM NaCl symmetrical solutions, the conductance values estimated were 78.6 ± 10.8 pS (n = 14) and 24.9 ± 4.1 pS (n = 9) for the dominant level and the sublevel, respectively.

Similar experiments conducted in the presence of 5 μM acetylcholine but using asymmetrical solutions (100 mM NaCl in the pipet and 190 mM NaCl in the bath) (Figure 6B) were used to determine the reversal potential for both the main current level and the sublevel. The reversal potentials for both conductances were equal to +12 mV, which are close to the expected reversal potential for sodium under these conditions (+16 mV, at 20 °C). Under symmetrical or asymmetrical ionic conditions, the presence of 4 μM TTX either at one or at both sides of the liposome patch had no detectable effects on the observed ion channel activity. Furthermore, substituting Na^+ for K^+ in the bath solution to produce bionic conditions (100 mM NaCl inside the pipet and 100 mM KCl in the bath) produced current vs voltage plots in which the reversal potentials for both conductances were close to zero, thus indicating that the permeabilities of the channel for both monovalent cations were similar. This was further confirmed by using symmetrical conditions in which 100 mM KCl solutions, instead of NaCl solutions, were at both sides of the liposome patch. The open-channel conductance estimated under these conditions was similar to that determined in the 100 mM NaCl symmetrical conditions from above. Also, increasing the ionic concentration of Na^+ and/or K^+ in the bath solution to produce asymmetrical conditions always resulted in experimental values for the reversal potential similar to the expected reversal potential for the cations present (data not shown).

Single-channel recordings presenting only the dominant current level were processed to study the open-state lifetime distribution as in Figure 7. At all conditions attempted, the distribution of opening events was best described by the summation of two exponential terms. For instance, using 5 μM acetylcholine, a pipet holding potential of +65 mV, and symmetrical 100 mM NaCl solutions at both sides of the patch, which are the conditions used in Figure 7, the lifetimes corresponding to the two exponential terms were 4.86 ± 1.58 and 29.4 ± 1.92 ms (278 events analyzed), respectively, while at a holding potential of +90 mV, such lifetimes were 0.42 ± 0.08 and 3.22 ± 0.23 ms, respectively (488 events analyzed). The observed values as well as the differences between the shorter and the longer lifetimes, at a given holding potential, were similar to those reported by others for the AcChR reconstituted into "freeze-thaw" liposomes (Tank et al., 1983) or planar bilayers (Labarca et al., 1984, 1985) and suggested the existence of two kinetically distinct open states of different mean open times but identical conductance.

Discussion

The functional properties of *Torpedo* AcChR incorporated into giant liposomes have been used as a model to evaluate the possible advantages offered by this technique in the characterization of ionic channel proteins. Native membranes have been used in this work, instead of the purified AcChR, to illustrate better possible applications of the giant liposome technique to study other ionic channel proteins which may be difficult to be purified but can be obtained in a membrane-bound form.

Giant liposomes can be prepared in large quantities and at the desired density of ion channels by adjusting the initial protein to lipid ratio. Freeze–fracture and fluorescence microscopy data indicate that the incorporated proteins are distributed quite homogeneously in all giant liposomes observed. Also, the size of the giant liposomes, along with the anchoring effect of the added DEAE-Sephadex beads, facilitates perfusion of the bath solution used in electrical monitoring and easy production of high-resistance seals using standard patch pipets. Furthermore, giant liposomes containing no protein do not have artifactual ion channel-like activities, thus providing an excellent signal to noise ratio when ion channel proteins are incorporated.

Under the conditions chosen for most of the experiments described here, the probability of having ionic channel activity, in the absence of cholinergic agonist, is low (~11%). The activity detected in these cases corresponds to a channel with conductance and gating patterns similar to those described previously for a chloride channel present in the noninnervated face of *Torpedo* electrocytes (Miller, 1983a; Tank & Miller, 1983). However, since this activity was detected in the absence of acetylcholine, we did not attempt any further characterization.

The presence of cholinergic agonist at moderate concentration results in an increased probability (~51%) of finding responsive liposome patches. The most common pattern of activity in response to cholinergic stimulation (~67% of the responsive patches) corresponds to an ion channel with a main conductance of approximately 78 pS and a substate of 25 pS, in 100 mM NaCl. We idenitified this main conductance and its sublevel with the AcChR, based on evidence which includes the following: (1) it is activated by acetylcholine, and its reversal potential in NaCl indicates specificity to translocate sodium; 2) it is not sensitive to TTX, and it can be inhibited by α-Bgt or *d*-tubocurarine, both of which are specific ligands of the nicotinic AcChR; (3) the permeabilities for sodium and potassium ions are about the same, as would be expected from the little selectivity exhibited by the native AcChR for these monovalent cations (Lewis & Stevens, 1983); (4) the high probability of finding this channel activity correlates with the high abundance of AcChR in the starting membrane material, as indicated by the characteristic profile in electrophoretic analysis and by the high specific activity in α-Bgt binding assays. Furthermore, the decreased probability of finding patches responsive to acetylcholine as the agonist concentration is increased, the kinetics of channel opening including the existence of long shut periods, and the higher electrical activity detected in the initial period following seal formation suggest the occurrence of characteristic AcChR desensitization pro-

cesses, which must have been preserved upon incorporation into giant liposomes. In fact, since freeze–fracture data indicate that it would be unlikely for a membrane patch ($\sim$1-μm diameter) to be free of protein, we speculate that AcChR desensitization by prolonged exposure to the cholinergic agonist contained in the patch pipet may be the main reason for detecting AcChR ion channel activity only in 51% of the cases.

Evidence to support that the 25-pS conductance is a substate of the main level and not an independent channel (Fox, 1987) is based on that the smaller conductance has never been detected independently of the main conductance and on that both conductances respond identically when confronted to cholinergic antagonist and α-neurotoxin and exhibit identical selectivity for the different monovalent cations assayed. In fact, a subconducting state of similar characteristics has been reported associated to the AcChR main conductance is cultured muscle cells (Hamill & Sakmann, 1981) and in reconstituted AcChR preparations (Tank et al., 1983).

Previously reported values for the AcChR single-channel conductance obtained in cultured muscle cells or in other reconstituted systems exhibit a wide range of variation (from 16 to 80 pS, under ionic conditions similar to those used here) (Nelson et al., 1980; Schindler & Quast, 1980; Hamill & Sakmann, 1981; Tank et al., 1983; Brehm et al., 1984a,b; Labarca et al., 1984; Sakmann et al., 1985; Mishina et al., 1986; Brehm & Kullberg, 1987; Colquhoun et al., 1987). Our conductance estimates are higher than most reported previously for reconstituted AcChR from *Torpedo*. Nevertheless, our values are similar to those reported more recently in cultures from mouse skeletal muscle (70 pS; Brehm & Kullberg, 1987) and Xenopus muscle cells (64 pS; Brehm et al., 1984a,b), or in oocytes expressing AcChR from *Torpedo* (80 pS; Imoto et al., 1988). On the basis of the latter similarities, it is tempting to speculate that the giant liposome technique could perhaps be less damaging to the AcChR channel than more conventional procedures of reconstitution, implying use of detergents or organic solvents. Nonetheless, it is likely that channel conductance depends upon factors including subtle differences in the properties of the lipid matrix used in reconstitution and, thus, it should not be taken as the only criteria to identify ion channels in reconstituted systems.

In conclusion, we have functionally characterized *Torpedo* AcChR incorporated into giant liposomes formed by fusion of native AcChR membranes and asolectin vesicles. The incorporated AcChR exhibits the pharmacological specificity, cation channel activation, and desensitization properties expected of this receptor in native membranes. A disadvantage of this technique when compared to reconstitution into planar bilayers is that, because of the inherent limitations of the "inside-out" patches used here, monitoring of channel activation in giant liposomes is complicated by desensitization caused by the prolonged exposure to the agonist present in the pipet solution. On the other hand, giant liposomes offer distinct advantages over other reconstitution systems, particularly in regard to (i) reproducibility, (ii) allowing a much better control of experimental variables, such as the density of ion channels, and (iii) avoiding unnecessary exposure of ion channel proteins to potentially damaging detergents or organic solvents, which are required in other reconstitution procedures. Furthermore, the giant liposome technique provides a unique opportunity to undertake *simultaneous* biochemical, morphological, and electrophysiological studies in the same sample, and, in combination with other biochemical and molecular biology techniques, it could help to address questions such as the functional consequences of point mutations or posttranslational modifications in this and other ion channel proteins.

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Effect of Unsaturated Phosphatidylethanolamine on the Chain Order Profile of Bilayers at the Onset of the Hexagonal Phase Transition. A ^{2}H NMR Study†

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ABSTRACT: The quadrupolar splitting profiles of methylene groups along the acyl chains of perdeuteriated dimyristoylphosphatidylcholine (DMPC-d_{54}) in mixtures with dioleoylphosphatidylethanolamine (DOPE) were studied by ^{2}H NMR. The quadrupolar splittings, obtained for lipid mixtures in the bilayer state, were measured as functions of temperature and PE:PC ratio and were used to obtain the approximate gauche probabilities at a given chain position, p_B. Ratios (R) of p_B for C13, C12, and C11 relative to that of the plateau region were used to characterize the effect of increasing PE on the gauche content of PC chains. At all temperatures studied (including the bilayer to hexagonal phase transition region), for each ratio R (e.g., $R_{C13/P}$), the relative gauche content of the DMPC chains was similar over the range of 25–85% PE. DOPE is viewed in simple terms as having a "conical" shape; if this geometry applies to the acyl chain region of the molecule, a greater lateral pressure would be expected toward the center of the bilayer as the PE content is increased, resulting in a decreased gauche content, relative to the plateau, of those methylene groups of PC. The failure to observe the predicted increase in lateral pressure has ramifications for the cone-shape molecular model. The overall "cone shape" of PE is seen to arise from the smaller size of the head-group relative to the acyl chains; however, the acyl chain region itself is not rigidly cone-shaped and is better represented by a flexible "balloon". These results were supported by small-angle X-ray diffraction, which showed a decreasing trend in the area per molecule with increasing PE content.

All biological membranes contain some phospholipids that, when isolated, will adopt nonbilayer structures under physiological conditions. The function of these lipids in membranes is not well understood, but it is believed that they help to maintain the membranes at a marginally stable state in order to optimize their functional condition (Wieslander et al., 1986). Many biological membranes, especially the highly active ones such as the inner mitochondrial membranes, the membranes of the photoreceptors, and the thylakoid membranes, have a high content of non-bilayer-preferring lipids. However, none of the biological membranes express the nonbilayer form which would destroy their ability to function as compartmental barriers.

The preference of some lipids for a nonbilayer state is usually viewed in terms of the geometric shape of these lipid molecules. The self-assembly theory of lipid aggregates (Israelachvili et al., 1976, 1980), based on the concept of geometric parameter or "molecular shape", has been widely used to interpret the lipid polymorphism (Cullis & De Kruijff, 1979; Rilfors et al., 1984). Most non-bilayer-preferring lipids, when not otherwise constrained, have unequal cross-sectional areas at the hydrophilic and hydrophobic ends (the *cone-shape* molecular model). Packing of these lipids in a planar bilayer is not energetically favorable as they prefer structures of finite

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