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Reassembly of protein–lipid complexes into large bilayer vesicles: Perspectives for membrane reconstitution

(liposomes/rhodopsin/photosynthetic reaction centers/cytochrome *c* oxidase/acetylcholine receptors)

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ABSTRACT **Protein–lipid complexes in apolar solvents reassemble into large bilayer protein–lipid vesicles (PLVs) with diameters of several micrometers. PLVs form spontaneously upon hydration of the protein–lipid complex residue after solvent removal. This procedure has been applied to the following membrane proteins: bovine and squid rhodopsin, reaction centers from *Rhodopseudomonas sphaeroides,* beef heart cytochrome *c* oxidase, and acetylcholine receptors from *Torpedo californica.* PLVs have a large internal aqueous space (e.g., 790 μl/mg of lipid for cattle rhodopsin vesicles). Freeze-fracture replicas of PLVs revealed that both internal and external leaflets contained numerous intramembranous particles with diameters between 80 and 120 Å, depending on the specific protein incorporated in the membrane. The optical spectral properties of rhodopsin and reaction centers in PLVs were similar to those recorded in the respective natural membrane. Furthermore, bovine rhodopsin in PLVs was chemically regenerable with 9-*cis*-retinal. Actinic illumination induced proton efflux from reaction center vesicles that was abolished by proton ionophores. Therefore, this method is suitable for the incorporation of some membrane proteins in their functional state. PLVs were penetrated with microelectrodes and visualized by the injection of a fluorescent dye. Preliminary electrical recordings were obtained by sealing PLVs to a hole in a septum separating two aqueous compartments. These studies suggest that PLVs assembled by this procedure permit the simultaneous analysis of reconstituted membranes by chemical, optical, and electrical techniques.**

In recent years, phospholipid bilayer vesicles, liposomes, have gained wide acceptance as a model system to examine structure–function relationships in membrane biology. Efforts have been undertaken to produce large vesicles in order to apply electrophysiological techniques and to take advantage of a larger inner volume (1–7). Reeves and Dowben (3) reported the formation of vesicles several micrometers in diameter; these were formed by drying lipids from a chloroform/methanol solution and allowing them to swell in water. Recently, Antanavage *et al.* (4) obtained preliminary electrical recordings from large vesicles either by microelectrode puncture or by sealing the vesicles to an aperture in a polyethylene septum that separated two aqueous compartments. This important advance in the experimental amenability of bilayer vesicles for electrical measurements prompted us to assess the feasibility of reassembling protein–lipid complexes (PLCs) of membrane proteins into large vesicles by extending our own previous techniques (8). Here, we report that PLCs from several membrane proteins can be extracted into apolar solvents and after solvent removal can be reassembled into large protein–lipid vesicles (PLVs) by a modification of the Reeves and Dowben (3) procedure. We describe some structural and functional properties of the preparation that suggest the suitability of this procedure for the simultaneous analysis of reconstituted membranes by chemical, optical, and electrical techniques.

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METHODS

Membrane Preparations. Cattle and squid rhodopsin-containing membranes and acetylcholine receptor-enriched membranes from *Torpedo californica* were purified as described (8–10). Reaction centers (RCs) from *Rhodopseudomonas sphaeroides* R-26 (11) and beef heart cytochrome *c* oxidase (12) were purified by published techniques. All procedures, up to and including vesicle formation, were performed at 4°C, unless otherwise specified, and for the rhodopsins and RCs were carried out under dim red or green light, respectively.

Transfer of PLCs into Apolar Solvents. All proteins were extracted into apolar solvents with soybean phospholipids (13). Bovine rhodopsin was directly transferred from rod outer segment membranes preferentially into ether as described (14). Squid rhodopsin (8), cytochrome *c* oxidase (12), and RCs (15) were extracted into hexane as described, and acetylcholine receptor into ether by modifications of procedures detailed in ref. 14 (R. Anholt and J. Lindstrom, personal communication).

Formation of Large Vesicles. The hexane or ether extract composed of protein and 1–15 mg of lipid was evenly spread on the bottom of a flat-bottomed flask 6–16 cm in diameter, and the solvent was slowly evaporated with argon or nitrogen. Care was taken during solvent removal not to perturb the surface of the solution to ensure that the resulting protein-lipid film would be uniformly distributed. Thereafter, 4–8 ml of H_2O was gently layered over the film. The flask was sealed under argon or nitrogen and left undisturbed for 48 hr. Large vesicles were purified from the resulting suspension by layering the material on 1% sucrose (wt/vol) and centrifuging in a Sorvall HB-4 swinging bucket rotor (1500 × *g* for 10 min). A volume equal to that initially deposited was removed from the top of the tube for subsequent studies.

RESULTS AND DISCUSSION

Formation of large bilayer vesicles from PLCs

PLCs from membrane proteins extracted into hexane or ether reassemble into large bilayer vesicles. The choice of the solvent used in the PLC extraction is determined by the preservation of biological activity and optimum yield. Hexane is suitable for all the proteins here described, whereas ether is adequate only for cattle rhodopsin and cytochrome *c* oxidase because it ap-

Abbreviations: PLC, protein–lipid complex; PLV, protein–lipid vesicle; RC, reaction center.

pears to denature the others. Vesicle formation involves the hydration of the PLC after the apolar solvent is evaporated. The vesicles "grow" in time, and after 48 hr at 4°C the majority of the starting material is suspended.

Inspection of the suspension under the light microscope reveals the presence of large (5–300 μm in diameter) spherical structures as well as amorphous material. When visualized by dark-field illumination, by Nomarski optics, or by the fluorescence of vesicles formed in the presence of 0.1 μM perylene, a lipid probe (16), it is possible to distinguish two populations of spherical structures. The material of interest consists of 5- to 100-μm vesicles appearing to have one or a few layers. Viewed with Nomarski optics, these vesicles display a distinct ridge along their perimeter (see Fig. 4*A*), and with dark field, or perylene fluorescence, only a ring corresponding to the vesicle border is delineated. These large protein–lipid vesicles are identified as PLVs.

In contrast, the predominant population of rounded structures consists of multilamellar vesicles and amorphous material showing uniform brightness in dark field or fluorescence. Their Nomarski images have less defined edges. These denser structures may be separated from PLVs by sedimentation through sucrose.

The yield of PLVs is strongly influenced by the ionic strength of the hydrating medium (3, 4). The amount of [^{14}C]phosphatidylcholine incorporated into floated vesicles is 2–30% when the vesicles are formed in deionized water and an order of magnitude lower when 25 mM KCl is used as the hydrating medium.

Structure and composition of the vesicles

Electron micrographs of freeze-fracture replicas of vesicles containing specific membrane proteins are illustrated in Fig. 1. Cross-fractured PLVs occasionally contained smaller vesicles.

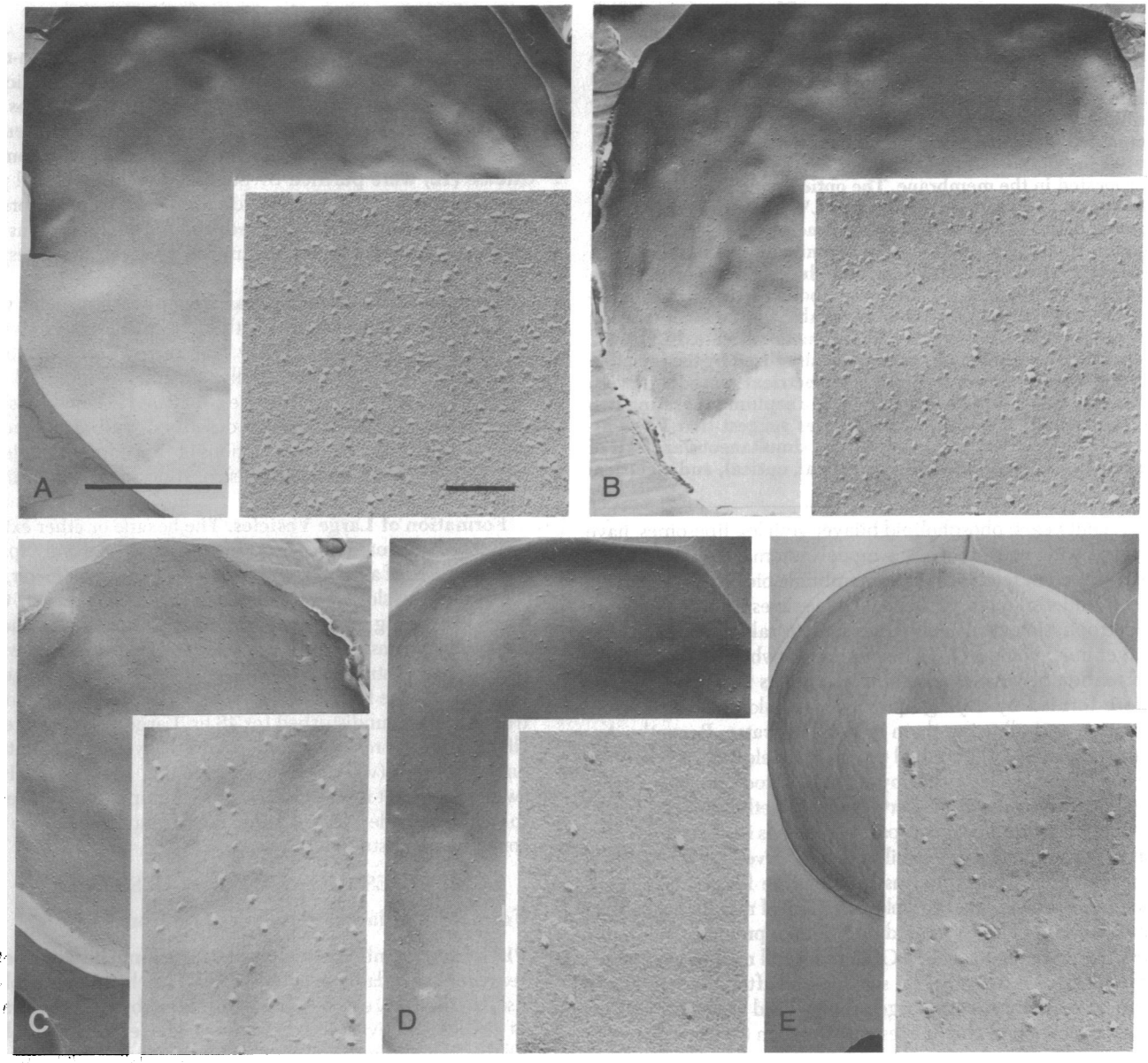

FIG. 1. **Freeze-fracture replicas of large PLVs.** The freeze-fracture appearance of the different vesicle preparations is shown at low magnification; a sector at high magnification illustrating the presence of intramembranous particles is inserted in each right-hand quadrant. Freeze-fracture replicas were obtained as described (8). (*A*) Bovine rhodopsin vesicles. (*B*) Squid rhodopsin vesicles. (*C*) RC vesicles. (*D*) Cytochrome oxidase vesicles. (*E*) Acetylcholine receptor vesicles. (Low magnification ×20,000; bar, 1 μm. High magnification *Insets* all ×100,000; bar, 1000 Å.)

At high magnification (*Insets*) all membranes revealed the presence of intramembranous particles uniformly distributed in both concave and convex fracture faces of the bilayer vesicles without evidence of phase separation. The ultrastructural appearance of each type of protein experimentally incorporated into the vesicles was distinct: bovine and squid rhodopsin preparations showed particles 80 Å in diameter (Fig. 1 *A* and *B*); the RC membranes contained particles ≈100 Å in diameter, with some "rod-shaped" particles that were ≈200 Å long (Fig. 1*C*); cytochrome *c* oxidase appeared as large 110-Å particles (Fig. 1*D*); the acetylcholine receptor samples revealed heterogeneously sized particles averaging 90–100 Å in diameter (Fig. 1*E*). The density of intramembranous particles depends on the protein-to-lipid ratio of the extracts: bovine rhodopsin PLVs derived from ether extracts with high ratios appeared more densily packed. Vesicles formed without protein under identical conditions displayed smooth membrane surfaces with no particles.

The internal aqueous space of PLVs after sucrose floatation is large, amounting to ≈790, ≈320, and ≈180 μl/mg of lipid for cattle rhodopsin, squid rhodopsin, and RCs, respectively. This was determined by gel exclusion chromatography through Ultrogel AcA34 (LKB) after vesicles had been formed in the presence of [^{14}C]phosphatidylcholine and [^{3}H]inulin. Phospholipid vesicles prepared by following the same protocol also exhibited a large inner volume (880 μl/mg of lipid). These values should be compared with those reported for multilamellar vesicles (<4 μl/mg of lipid, ref. 5), sonicated unilamellar vesicles (0.5 μl/mg of lipid, ref. 17), or vesicles prepared by reverse phase evaporation (8–14 μl/mg of lipid, ref. 5). The internal volumes of PLVs correspond to estimated unilamellar vesicle diameters of 31, 13, and 7 μm for cattle rhodopsin, squid rhodopsin, and reaction center vesicles, respectively (7). This is consistent with the sizes observed in the light microscope, suggesting that the PLVs do not generally contain more than a few layers. Because sucrose floatation and gel exclusion chromatography may induce breakdown of the vesicles, the measured trapped volume should be considered a lower limit.

Functional studies

The extent of incorporation of cattle and squid rhodopsin and of reaction centers into PLVs as well as the preservation of the spectral integrity of the proteins in the vesicles was measured by optical absorbance; this is illustrated in Fig. 2. The dark absorption spectrum of bovine rhodopsin exhibits the characteristic maximum absorbance at 500 nm that disappears upon illumination (Fig. 2*A*). The ratio of absorbances at 280 nm and 500 nm is conventionally used as a purity criterion for rhodopsin preparations. Comparison of this ratio between native disc membranes and floated vesicles indicated that more than 90% of rhodopsin was preserved during vesicle formation. Furthermore, bovine rhodopsin incorporated into the large vesicles was chemically regenerable (78%) with 9-*cis*-retinal (22). These spectral features indicate that rhodopsin in the large vesicles is photochemically active.

PLVs containing squid rhodopsin retain the characteristic dark absorption spectrum with an absorption maximum at ≈495 nm (23) (Fig. 2*B*). Upon illumination at pH 11.0, rhodopsin bleaches to form alkaline metarhodopsin; this is indicated by the absorbance peak at around 380 nm. More than 80% of the rhodopsin present in the hexane extract was preserved during vesicle formation.

Absorbance spectra of RCs incorporated in PLVs indicate the preservation of both the spectral integrity and the activity of the protein; this is illustrated in Fig. 2*C*. The dark spectrum exhibits the three peaks in the infrared region at 760, 802, and 865 nm, which are characteristic of purified RCs (11). The most prominent light-induced absorbance change in this spectrum is the bleaching at 865 nm, which accompanies the primary photochemical reaction.

Light-induced electron transport in bacterial photosynthetic membranes results in proton translocation across the membrane (24), as predicted by the chemosmotic hypothesis (25). Isolated photosynthetic RCs, which accomplish in the light the primary photochemical reaction, translocate protons across the membrane when reconstituted in PLVs. The upper trace in Fig. 3 shows the light-induced acidification of the suspension resulting

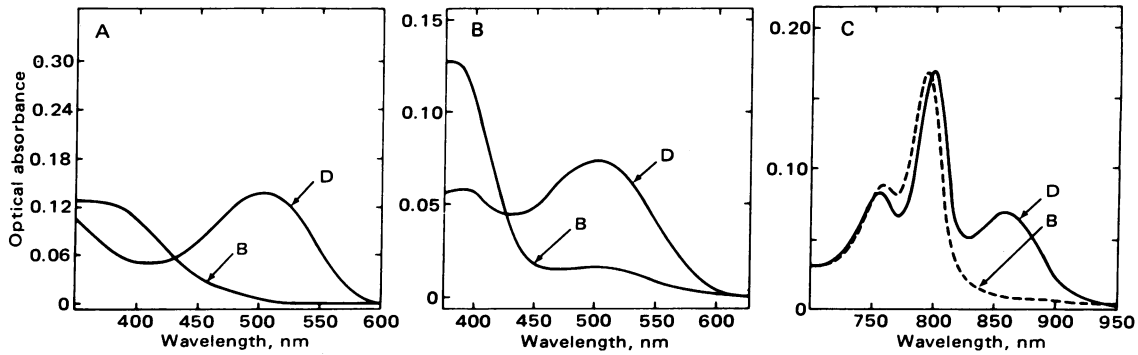

FIG. 2. Optical absorbance spectra in the dark (D) and after bleaching illumination (B) of bovine rhodopsin, squid rhodopsin, and RCs from *R. sphaeroides* R-26 derived from floated vesicles. (*A*) The ether extract used for vesicle formation contained 0.28 mg of cattle rhodopsin and 1 mg of phospholipid. The amount of rhodopsin incorporated into floated vesicles represents 24 ± 12% (mean ± SD, n = 3) of the total in the ether extract. Floated vesicles were collected by centrifugation in a Beckman Ti65 rotor at 30,000 rpm for 2 hr, then solubilized in 50 mM octyl glucoside (18)/0.2 M NaCl/1 mM $CaCl_2$/10 mM imidazole·HCl, pH 6.9, and their dark and bleached spectra were recorded. (*B*) The hexane extract contained 0.3 mg of squid rhodopsin and 10 mg of phospholipid. The PLVs harvested by centrifugation as in *A* were solubilized in 2% sucrose lauroyl ester (19)/50 mM Na_2CO_3, pH 11.0, and their dark spectra were recorded. Rhodopsin was partially bleached at 0°C through a 530-nm interference filter. The spectra were corrected for the contribution of lipid absorbance after determination of the sample lipid content by direct phosphate analysis (20). The amount of squid rhodopsin incorporated into the floated vesicles was 20% of the total in the hexane extract. Less than 5% of the 495-nm absorbance in purified membranes could be attributed to retinochrome as determined by the sensitivity of the dark spectrum to hydroxylamine (21). (*C*) The hexane extract was composed of 2 μM RCs and 1.4 mM phospholipid (15). The amount of RCs incorporated into the floated vesicles represented 35% of the total in the hexane extract. The vesicles were dissolved in 0.5% dodecyldimethylamine oxide/10 mM Tris·HCl, pH 8. The light-induced reversible changes in the spectrum were monitored by using the IR-2 mode of the Cary spectrophotometer, which provides a high-intensity measuring beam (I = 0.6 W/cm^2). All spectra were recorded at 22°C, except that of squid rhodopsin at 18°C, using a Perkin–Elmer 555 or a Cary 14 spectrophotometer; pathlength was 1 cm.

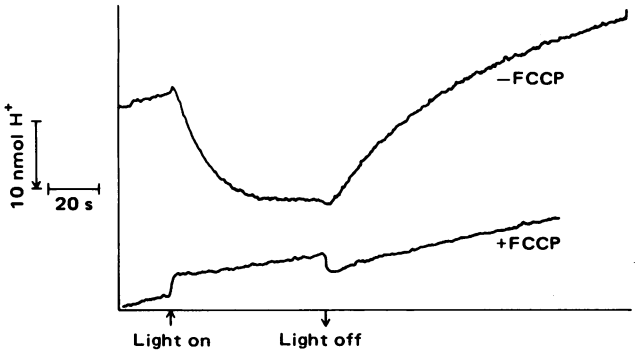

FIG. 3. Light-induced proton translocation in a suspension of RCs from *R. sphaeroides* R-26 incorporated into purified large vesicles. pH changes were monitored by a Radiometer combination electrode (type GK2320 C) connected to a Keithley electrometer (610B). The changes were recorded by a potentiometric recorder and calibrated by comparison to the electrode response to additions of standards of HCl. Actinic light was provided by a 500-W slide projector and filtered through a heat filter, Corning CS 7-69 filter and 5 cm of water. The light intensity at the position of the vesicle suspension was 25 mW/cm^2. The reaction mixture contained floated vesicles equivalent to 0.2 μM RCs, 30 μM reduced horse heart cytochrome *c*, 250 μM ubiquinone 0, 250 μM diaminodurene, and 1 μM fluoro carbonyl cyanide phenylhydrazone (FCCP) where indicated. The final volume was 1 ml and the temperature was 22°C.

from proton translocation. The medium was supplemented with an exogenous electron donor (reduced cytochrome *c*) and acceptor (ubiquinone 0, i.e., 2,3-dimethoxy-5-methyl-1,4-benzoquinone) as well as with diaminodurene (2,3,5,6-tetramethyl-*p*-phenylenediamine), which probably mediates the closing of the redox cycle and the actual transfer of protons out of the vesicles. When the light was turned off, the pH resumed its baseline value with a decay time constant that was primarily determined by the intrinsic proton permeability of the vesicle membrane. Addition of a proton ionophore—i.e., fluoro carbonyl cyanide phenylhydrazone (26)—collapsed the light-induced pH gradient as soon as it was generated and no net accumulation of protons was recorded (lower trace in Fig. 3). The residual small amplitude signal of opposite direction to the control arises from photochemical reactions of the exogenous reactants with RCs; this also occurs with detergent-solubilized RCs and is, therefore, unrelated to transmembrane proton translocation. The extent of proton efflux (100–200 mol of H^+/mol of RC) and the initial rate (2–4 mol of H^+/mol of RC per sec) are comparable to reported values for reconstituted sonicated vesicles (27).

It was established that cytochrome *c* oxidase extracted into solvents retains enzymatic activity (12). Upon solvent evaporation and subsequent hydration the PLC reassembled into vesicles with incorporated functionally active cytochrome oxidase (12). Cytochrome oxidase has also been incorporated into large vesicles by a different procedure (P. Mueller, personal communication).

The retrieval of function in the acetylcholine receptor preparations (Fig. 1*E*) has not yet been accomplished. Results indicate that the ether extraction inactivates a considerable fraction of α-bungarotoxin binding activity and, therefore, must be improved.

A particularly attractive feature of the vesicles is their large size, which suggests the possibility of making direct electrical measurements. This possibility indeed received support from experiments in which a low molecular weight tracer was iontophoretically injected into a vesicle and was retained in the intravesicular space. Fig. 4*A* illustrates a rhodopsin vesicle penetrated with a microelectrode, viewed with Nomarski optics; the electrode contained the fluorescent dye Lucifer Yellow CH (28). After impalement, steady negative current was passed through the electrode, filling the PLV with dye. The vesicle was then visualized by exciting Lucifer Yellow with UV light and observing the emitted fluorescence (Fig. 4*B*).

Unfortunately, the electrodes containing dye were of high resistance (often $\approx 10^9\ \Omega$) and unsatisfactory for measurements of vesicle membrane resistance. However, parallel experiments under Nomarski visualization, using low-resistance electrodes (100 MΩ, filled with 3 M potassium acetate) and a bridge circuit, allowed electrical recording from PLVs. Preliminary experiments indicate that the membrane resistance is high (>100 MΩ), but more refined measurements to rule out spurious effects and tests of protein-gated permeabilities must await further experimentation.

The electrical properties of PLVs were studied by the technique of Antanavage *et al.* (4); this is illustrated in Fig. 5. A two-compartment cell with a thin Teflon septum possessing a hole was used. PLVs were added to one aqueous compartment and a small hydrostatic pressure gradient was applied. The beginning of the record in Fig. 5*A* shows the current flowing through the open hole in response to a voltage pulse of 110 mV; the resultant conductance was 61 nS in 2 mM NaCl. As the vesicles flowed through the hole the current decreased transiently (spikes) until a vesicle larger than the hole occluded it and a sharp decrease in current was recorded. At this stage the vesicle fused with the septum, bringing the current almost to zero. This was shown by switching the voltage to zero and back to 110 mV; the conductance across the sealed vesicle was less than 0.5 pS (Fig. 5*B*). Antanavage *et al.* (4) showed that application of short ($\approx$1 ms) high voltage (0.5 V) pulses across a vesicle sealed to the hole breaks part of the vesicle, leaving a single planar bilayer covering the hole.

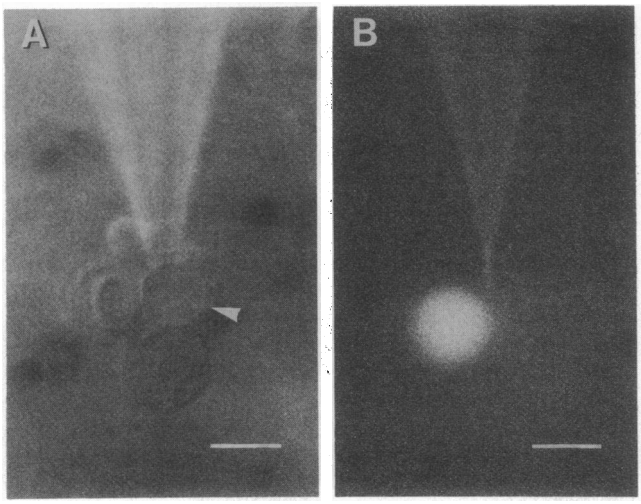

FIG. 4. Photomicrographs of PLVs impaled with glass capillary microelectrodes. A suspension of bovine rhodopsin PLVs in water was placed in a chamber, allowed to settle to the bottom, and viewed with Nomarski interference contrast optics. (×370; bars indicate 25 μm.) Vesicles were impaled with glass capillary microelectrodes containing 5% aqueous Lucifer yellow dye (28). Dye was ejected from the electrodes with small ($\approx$10 pA) constant negative currents. The fluorescence of the dye was viewed by excitation with an HBO 100 W/2 mercury lamp, using a BG12 exciter and LP520 barrier filters. (*A*) Bright-field view of electrode in large vesicle (arrow); interference contrast optics. Smaller vesicles are clustered around the central vesicle. (*B*) Ultraviolet illumination reveals fluorescence of different electrode and vesicle filled with Lucifer Yellow dye. Control observations indicate that PLVs had no autofluorescence and did not take up dye ejected iontophoretically onto their surface.

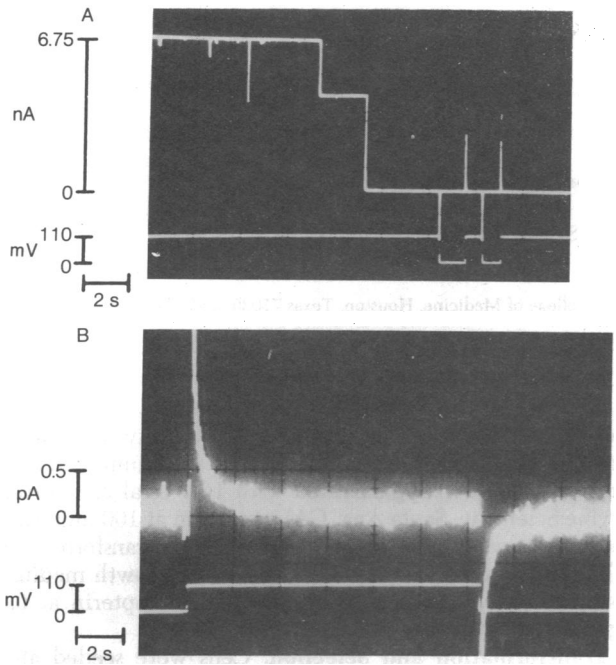

FIG. 5. Occlusion and sealing of vesicle to a hole. (*A*) Membrane current (upper trace) in response to a voltage pulse of 110 mV (lower trace). (*B*) Corresponding amplified record after vesicle sealing to the hole. The conductance across the sealed vesicle was less than 0.5 pS. The chamber containing the hole (14-μm diameter) in the Teflon septum, as well as the recording equipment, was analogous to that described (29). Rhodopsin vesicles were added (100 μl) to one aqueous compartment (final volume 1.5 ml) and a small hydrostatic pressure (1–2 mm H_2O) was applied between the two compartments. The septum was preconditioned with 1% petroleum jelly in hexane (centrifuged), supplemented with hexyl bromide at a volume ratio of 1:1000 with respect to hexane. The electrolyte was 2 mM NaCl and the temperature 20°C.

CONCLUSION

We have shown that membrane proteins can be incorporated into large lipid vesicles of several micrometers diameter and display biological activity. The large vesicles offer advantages over other available model membranes. Their large inner space allows the entrapment of large markers and widens the experimental scope of the vesicle system by enabling the study of the electrical properties with microelectrodes (Fig. 4) or other techniques (Fig. 5). In addition, the osmotic fragility of PLVs may prove useful to spread them at air-water interfaces into monolayers; this provides another strategy for incorporating proteins into planar bilayers suitable for electrical measurements (29, 30). This is especially attractive because the vesicle system seems to be favorable for functional reconstitutions: many proteins have been successfully reconstituted in vesicles but not in planar bilayers (cf. ref. 31). The vesicle system allows a more direct correlation of membrane composition parameters, such as protein concentration, with functional parameters. This may aid in relieving serious concerns in planar bilayer studies arising from the uncertainty of the composition of black films and the contribution of phase separations in the monolayers that are used to assemble the bilayer (cf. ref. 31). Because PLCs in apolar solvents are the starting material for the formation of both the large vesicles (8) and planar bilayers (29), the methods may be used in conjunction to complement each other. Therefore, the large vesicle system emerges as a promising powerful tool in membrane reconstitution studies.

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