Transport-Specific Isolation of Large Channels Reconstituted into Lipid Vesicles

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Summary. To develop a technique for purifying and identifying pore-forming membrane proteins, we used a transport-specific increase in buoyant density to select for lipid vesicles containing voltage-dependent anion channels (VDAC). Monodisperse, single-walled vesicles were formed by gel filtration from a detergent-solubilized mixture of lipid and protein in a urea buffer. The vesicles were layered on a linear iso-osmolar density gradient formed of urea and sucrose buffers. Since VDAC is open at zero *trans*-membrane voltage and is permeable to urea and sucrose, vesicles containing functional VDAC should become more dense as sucrose enters and urea leaves, while those lacking open channels should maintain their original density. Vesicles formed in the absence of VDAC migrated to a characteristic density, while vesicles formed in the presence of VDAC fractionated into two populations in the gradients, one migrating to the same density as the vesicles formed without VDAC, and one at a significantly greater density. In contrast to the lower density vesicles, the higher density vesicles showed a high permeability to calcein, and contained functional VDAC channels (shown by electrophysiological recordings following fusion with a planar bilayer). Thus, vesicles containing open channels were separable from those that did not by a transport-specific shift in density. This technique may be useful for the enrichment of channels of known permeability properties from impure material.

Key Words ion channels · reconstitution · fusion · vesicles · VDAC · transport-selection

Introduction

Examination of detailed properties of membrane transport proteins in reconstituted systems depends on (*i*) purification and identification of relevant proteins, and (*ii*) controlled insertion of purified membrane proteins into phospholipid membranes. Some channel-forming proteins have been purified and/or physiologically identified by use of specific toxins, agonists or affinity reagents (e.g., saxitoxin for voltage-dependent sodium channels, α-bungarotoxin for acetylcholine-activated channels). There are,

however, many other channels for which this is not currently practical. In such cases, incorporation of protein (even highly enriched for one species) into bilayers can yield heterogeneous channel activity since one can detect single molecules.

We developed a technique that makes use of a known functional property of a channel in a purification step: partially purified protein is incorporated into unilamellar phospholipid vesicles, and then vesicles containing the channel of interest are selected on the basis of increased nonelectrolyte permeability. Presence of the channel in the vesicles was confirmed by electrophysiological recordings following fusion with a planar bilayer.

We used the channel-induced increase in permeability to large nonelectrolytes to mediate an increase in vesicle buoyant density, permitting specific isolation of the population of vesicles containing open channels. Vesicles without channels or containing nonfunctional channels remain at a lighter density.

One major consideration was that the technique be rapid, to minimize protein exposure to detergent and lessen the possibility of denaturation. We adapted and concatenated three procedures: (*i*) octylglucoside solubilization, reconstitution, and vesicle formation (Mimms et al., 1981), (*ii*) transport-specific density shift purification (Hess & Andrews, 1977; Goldin & Rhoden, 1978), and (*iii*) fusion of phospholipid vesicles with planar phospholipid bilayers by osmotic gradients (Zimmerberg, Cohen & Finkelstein, 1980; Cohen, Zimmerberg & Finkelstein, 1980).

We used the mitochondrial porin (VDAC; Schein, Colombini & Finkelstein, 1976) to develop the method, and applied it to the reconstitution of channels formed of the gap junction protein connexin32 (A.L. Harris et al., *in preparation*). Some

of this work has been published in abstract form (Harris, Walter & Zimmerberg, 1986; Walter et al., 1986).

Materials and Methods

Buffer Solutions

Urea buffer contained 10 mM KCl, 10 mM HEPES, 0.1 mM EDTA, 3 mM sodium azide and 459 mM urea at pH 7.4. In the sucrose buffer 400 mM sucrose was substituted for the urea. Osmolality for both buffers was 490 mOsm/kg as measured with a vapor pressure osmometer (Wescor, Salt Lake City, UT). The specific gravities (D_4^{20}) of the urea and sucrose buffers were calculated to be 1.0056 and 1.0511, respectively. All densities given in the text are in dimensions of g/cm^3.

Vesicle Formation

The method of protein reconstitution and vesicle formation generally followed that of Mimms et al., 1981. Egg phosphatidylcholine, bovine brain phosphatidylserine (Avanti Polar Lipids, Birmingham, AL) and cholesterol (Sigma, St. Louis, MO) were dissolved in chloroform at a mole ratio of 3:2:5. Cholesterol was recrystallized from ethanol before use. The lipid/chloroform solution was dried under argon to a thin film in clean glass tubes and then held under vacuum for at least 30 min. A trace amount of ^{3}H-dipalmitoylphosphatidylcholine (DPPC) was present to label the vesicles. The dried lipids were dissolved in urea buffer containing 80 mM n-octyl-β-D-glucopyranoside (Calbiochem, La Jolla, CA) to give a final concentration of 5 mg/ml of lipid, and the solution kept on ice for at least 15 min. The lipid/detergent solution was applied to a gel filtration column (1.5 × 20 cm) containing agarose beads (Bio-Gel A-0.5m, exclusion limit 500,000 Da), which had been pretreated with phosphatidylcholine vesicles formed by sonication. The material was eluted with urea buffer at 9 ml/hr at 4°C. The vesicles appeared in the void volume.

Vesicle Formation In Presence of VDAC

The procedure above was followed except that the lipid/detergent solution also contained VDAC, and ^{14}C-DPPC replaced ^{3}H-DPPC. The VDAC was in the form of a outer mitochondrial membrane fraction from *Neurospora crassa*, containing approximately 50% VDAC (wt/wt), graciously provided by Dr. Carmen Mannella. We used VDAC:lipid ratios in the range of 1:1000 to 1:500 (wt/wt). This would yield between one and two VDAC monomers per 750 Å diameter vesicle.

Calcein Experiments

Vesicles were formed as above except that the detergent solution also contained 10 mM calcein (Hach, Iowa City, IO). To minimize dilution of calcein during vesicle formation, an equal volume of urea buffer containing calcein was added to the column after the solubilized (calcein-containing) mixture entered the gel bed. In this way, vesicle formation occurs near the top of the

column in the presence of calcein. As vesicles form, they migrate ahead of the untrapped calcein, which remains in the included volume of the column. The loss of calcein from the trapped volume of the vesicles was measured by comparing the ratio of calcein fluorescence to lipid label. Calcein fluorescence was measured with a Perkin-Elmer MPF44B spectrofluorometer.

Vesicle Sizing

The size distribution of the vesicles was established by gel filtration over a calibrated 1 × 30 cm TSK G6000PW HPLC column at an elution rate of 1 ml/min (Ollivon, Walter & Blumenthal, 1986).

Iso-Osmolar Density Gradients

A transport-specific density shift technique was adapted from Goldin and Rhoden (1978). Continuous linear density gradients were formed from the urea and sucrose buffers in 5-ml ultracentrifuge tubes using a gradient maker (Hoefer, San Francisco, CA). Aliquots of vesicles (100–200 μl) were layered on each gradient. The gradients were typically spun at 300,000 × g for 8 hr in a Beckman Ti55.1 swinging bucket rotor at 4°C unless otherwise noted. Samples were recovered in 250-μl fractions and aliquots taken for liquid scintillation counting to identify lipid distribution.

Vesicle Density Calculations

The density of the vesicles was calculated from the mean diameter of 750 Å, assuming a bilayer thickness of 60 Å and lipid density of 1.02 (Huang & Charlton, 1971; White, Jacobs & King, 1987). The contribution of the protein per vesicle to the density was calculated assuming the mass of the protein was equally divided among intravesicular, extravesicular, and membrane domains. Protein density was taken to be 1.26 (see Tristram-Nagle, Yang & Nagle, 1986). One VDAC channel was assumed to be a dimer of a 32 kDa protein (Forte, Guy & Mannella, 1987). The numbers of vesicles were estimated from the total amount of lipid and vesicle diameter. The amount of lipid per vesicle was calculated assuming a lipid headgroup area of 65 Å^2 (Huang & Lee, 1973).

Permeability of VDAC to Sucrose

The rate of equilibration of external sucrose with the contents of a vesicle through a single VDAC channel was calculated assuming an aqueous pore 20 Å wide and 60 Å long, and a vesicle diameter of 750 Å. The diffusion constant (D) for sucrose was taken as 5.2 × 10^{-6} cm^2/sec (Weast & Astle, 1981). The VDAC permeability coefficient for sucrose (P_{VDAC}) was:

$$P_{VDAC} = \frac{D}{\text{channel length}}. \tag{1}$$

The time constant for exchange (τ) was evaluated as the time-dependent solution to Fick's first law with initial conditions of a homogeneous concentration of solute within the vesicle, and an infinite dilution outside the vesicle. Then:

$$\tau = \frac{\text{vesicle volume}}{(\text{channel cross-sectional area})(P_{VDAC})}. \quad (2)$$

For a vesicle containing one VDAC pore, we calculate a τ of 0.6 msec for equilibration with external sucrose.

BILAYER EXPERIMENTS

Planar phospholipid bilayers were formed from bacterial phosphatidylethanolamine (PE) and bovine phosphatidylserine (PS) at a mole ratio of 1:1, 2% in decane (Fluka, Ronkonkoma, NY) according to Mueller et al., 1963. Saline was 271 mM KCl, 5 mM MES, and 0.1 mM EDTA at pH 6.0. After membrane thinning, aliquots of density gradient fractions were added to the front chamber, followed by mixing. If the bare bilayer conductance remained low and noise free, $CaCl_2$ was added to the front chamber to a concentration of 40 mM, providing both an osmotic gradient and divalent cations to the vesicle-containing compartment. These conditions are known to promote fusion of vesicles to planar bilayers (Cohen et al., 1980; Zimmerberg et al., 1980). All additions were followed by several minutes of thorough mixing. Voltages were imposed relative to the rear chamber. Bilayer currents were measured by standard techniques using an operational amplifier (AD515LH, Analog Devices, Norwood, MA) and a $5 \times 10^8 \ \Omega$ feedback resistor. Data were recorded on a chart recorder (BD40 Kipp & Zonen, Delft, Holland).

PROTEIN ASSAY

Protein in the gradient fractions was determined by either an amido black method (Schaffner & Weissmann, 1973) following dot-blotting onto Millipore filters, or by a colloidal gold detection system (Aurodye; Janssen, Belgium) following dot-blotting onto Immobilon PVDF membrane (Enprotech, Hyde Park, MA).

Results

VESICLE CHARACTERIZATION

Since the expected density changes were fairly subtle and sensitive to the relative aqueous and lipid volumes of the vesicles, it was essential that the vesicle population have a monodisperse and reasonably narrow size distribution. The formation of single-walled vesicles from octylglucoside/lipid solutions on gel filtration columns is well established (e.g., Mimms et al., 1981). Vesicle sizes were determined by gel filtration on HPLC. Vesicle populations formed in the presence and absence of VDAC were monodisperse (Fig. 1). In the absence of VDAC, vesicle mean diameters ranged from 730 to 800 Å. In the presence of VDAC, average apparent diameters were 5–10% larger, though this could be due to a change in vesicle elution characteristics rather than a genuine diameter increase. Even if real, these size differences would change the buoyant density of the vesicles in urea buffer no more
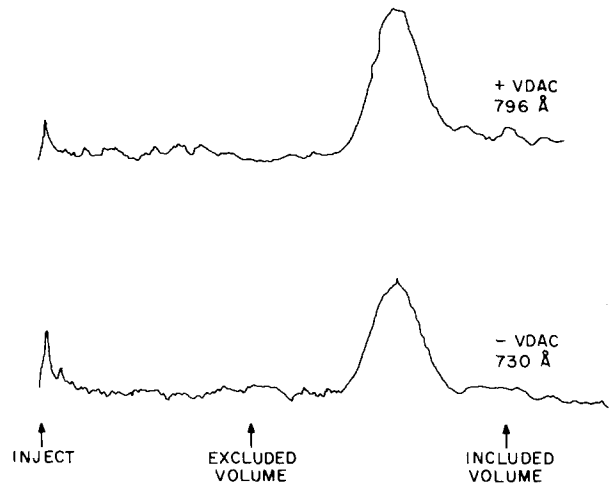

Fig. 1. Size determination of vesicles. The size distributions of vesicles were determined by gel filtration with an HPLC, using a calibrated TSK G6000PW column (Ollivon et al., 1986). Vesicle elution was detected by $O.D._{280}$. The vesicle sizes were similar and monodisperse. The mean diameters shown represent the extremes of divergence in vesicle size

than 1.6% of the range of density used in the density gradients below (typically each gradient fraction represented 5% of the density gradient). We calculate the density of our vesicles, assuming spherical shape and 750 Å diameter, in urea buffer, to be 1.0155 (see Materials and Methods).

TRANSPORT-SPECIFIC VESICLE DENSITY SHIFTS

Vesicles prepared in urea buffer were layered onto linear density gradients formed of the urea buffer and an identical buffer in which the urea was replaced by iso-osmotic sucrose. The density ranged from 1.0056 at the top to 1.0511 at the bottom. Since the calculated initial vesicle density was 1.0155, the vesicles should enter the sucrose-containing region of the gradient. The internal concentrations of those vesicles permeable to sucrose and urea will equilibrate with those of the external solution at each level in the gradient. These vesicles will become more dense as sucrose enters (and urea leaves), and, therefore, move to a lower position. This process of exchange and density increase will continue until equilibrium is reached. In contrast, vesicles whose membranes are impermeable to urea and sucrose will show no increase in density; these vesicles will remain at their original density (1.0155), about $\frac{1}{5}$ into the gradient. Vesicles permeable to urea, but not to sucrose, will undergo osmotic shrinkage, and should come to a position no more than $\frac{1}{3}$ into the gradient (see Discussion).
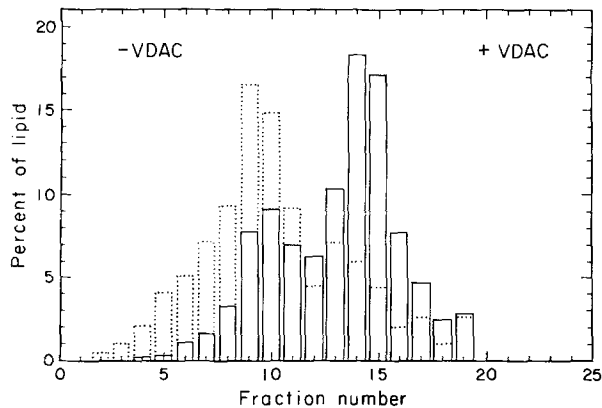
Fig. 2. Density distribution of vesicles. Vesicles were centrifuged on linear iso-osmolar density gradients composed of urea and sucrose buffers (8 hr; 300,000 × g). Lipid distribution was monitored by radiolabel. Vesicles formed without VDAC were in a peak centered at fractions 9 and 10 from the top of the gradient. Vesicles formed in the presence of VDAC separated into two peaks, a minor one at fractions 9 and 10, and a major one at fractions 14 and 15. The shift of most of the vesicles formed with VDAC to a position of higher density suggests they were permeable to sucrose. We interpret the lower density peak of the VDAC vesicles to represent vesicles without functionally reconstituted VDAC channels

For centrifugation of vesicles containing no protein on iso-osmolar density gradients, the lipid was observed in a single peak of lipid about $\frac{1}{3}$ to $\frac{1}{2}$ into the gradient (Fig. 2). However, vesicles formed in the presence of VDAC showed two peaks: one at the same position as the vesicles formed without protein, and a second peak at a significantly lower position, $\frac{2}{3}$ to $\frac{3}{4}$ into the gradient. By calculation, this position shift corresponded to approximately 80% exchange of vesicular urea for sucrose.

The observation of two vesicle populations at characteristic densities was highly reproducible ($n > 40$). For different preparations, the relative proportion of lipid (as indicated by radiolabel) in each peak varied. Vesicles made without VDAC never gave two significant peaks, and vesicles made with VDAC always gave two peaks, one at the density of the lipid-only vesicles, and one at a characteristic greater density.

Since VDAC will be quantally distributed among a large number of vesicles, with a range of means between one and two monomers per vesicle, we used Poisson statistics to predict the ratio of shifted to nonshifted vesicles, and found excellent agreement with the data. For example, in the experiment shown in Fig. 2, the protein to lipid ratio was 1:1000, corresponding to 1.08 VDAC monomers per vesicle. For one VDAC monomer per channel, Poisson statistics predict 66% of the vesicles should

shift. We estimated the areas under each peak and found that 65% of the vesicles shifted.

We interpret the higher density peak to be composed of vesicles whose density increased in the gradient, and, therefore, were permeable to urea and sucrose, and contained at least one functional VDAC channel spanning their membranes. To test this hypothesis, it was necessary to (i) correlate the density increase with permeability to large molecules, and (ii) establish that the density increase was not due to the protein causing nonspecific membrane permeability changes or increasing the density by virtue of its own mass.

CORRELATION OF PERMEABILITY WITH DENSITY SHIFT

To show explicitly that the density increase correlated with permeability of the vesicle membrane to a large molecule, vesicles were formed in the presence of the fluorescent, membrane impermeable dye calcein (662 Da), and the movements of the dye followed. The density shifted VDAC vesicles had 79–95% less calcein per lipid than did the unshifted VDAC vesicles. Vesicles made without VDAC showed the same high calcein to lipid ratio as the unshifted VDAC vesicles. The results show a clear fractionation of the vesicles into two populations: one of increased density, which lost calcein, and one of unchanged density, which did not. The correlation between calcein loss and density shift suggests that the vesicles in the shifted peak were permeable to large hydrophilic solutes, as indicated by calcein efflux.

DENSITY SHIFT IS NOT DUE TO DENSITY OF PROTEIN IN VESICLES

Calculations based on the amount of lipid and vesicle size show that adsorption of one VDAC monomer would increase the density of a vesicle approximately 0.00009 or 0.2% of the gradient used, simply because protein is more dense than lipid or the buffers. In our experiments, the VDAC to lipid ratio in the starting material corresponded to between one and two VDAC monomers per vesicle. Since most vesicles shifted, it is unlikely that the shift was due to the density contribution of the protein. The observed increase in density would correspond to adsorption of 250 inactive VDAC channels per vesicle.

To provide experimental evidence that protein incorporation *per se* did not cause density shifts, vesicles were formed in the presence of glycophorin or bacteriorhodopsin, integral membrane proteins
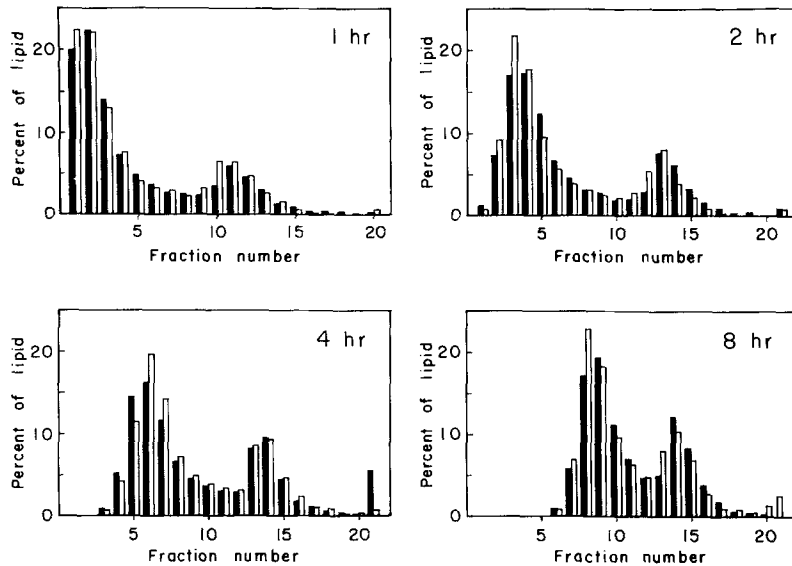
Fig. 3. Time course of density shift. The kinetics of the fractionation of the VDAC vesicles into two populations are shown. Lipid distribution was monitored by radiolabel. Vesicles were spun in the standard iso-osmolar density gradients at 300,000 × g for increasing times. The filled and open bars are for duplicate gradients run simultaneously. At 1 hr, the vesicles were already fractionated into light and heavy subpopulations. At 2 hr, the separation between peaks was unchanged, but both were further down the gradient. At 4 and 8 hr, the shifted, heavier peak remained at the same position, but the lighter, "unshifted" population was at a lower position in the gradient, decreasing the separation. These results are consistent with a rapid movement of vesicles on the basis of specific transport properties, followed by slower movement due to nonspecific vesicle permeabilities

which can be reconstituted into vesicles under these conditions (Mimms et al., 1981; Jackson & Litman, 1985). Density gradients showed only a single peak at the unshifted position even when vesicles were formed with 5 to 15 times the molar amount of glycophorin or bacteriorhodopsin per lipid than was used in forming the VDAC vesicles. Protein assay confirmed that the mass of glycophorin or bacteriorhodopsin incorporated per vesicle was 20–30 times greater than that of one VDAC channel, yet the position of the vesicles in the gradient did not shift.

KINETICS OF THE DENSITY SHIFT

The time course of the movement of the vesicle populations in the density gradients was determined to maximize separation between the vesicle populations and to minimize the time required. Aliquots of vesicles containing VDAC were spun on gradients for different times, and the vesicle positions determined (Fig. 3). The separation of the two populations was virtually complete after 1 hr, with the lighter and heavier peaks about $\frac{1}{2}$ gradient apart. At 2 hr, the separation between the peaks was unchanged, but both peaks were 10% further down the gradient. After 4 or 8 hr, the position of the heavier peak was unchanged, but the lighter peak moved down, so that at 8 hr the separation was about $\frac{1}{3}$ to $\frac{1}{4}$ of the gradient.

These findings are consistent with relatively rapid movement (2 hr) of sucrose-permeable vesicles to a maximally shifted position, and a slower, continual drift of other vesicles to positions of greater density due to a low, nonspecific vesicle membrane permeability to sucrose and urea.

Calculations based on the rate of diffusion of sucrose through a single pore the size of VDAC indicate that equilibration of external sucrose with vesicle contents occurs in about a millisecond (*see* Materials and Methods). Therefore, the rapid separation of vesicles containing reconstituted channels was determined by the hydrodynamics and densities of the vesicles, not by the kinetics of sucrose and urea fluxes across the membranes.

BILAYER EXPERIMENTS

To test for incorporation of functional VDAC, vesicles from each population were fused with planar bilayers under controlled conditions (*see* Cohen et al., 1980) to ensure that conductance changes could be correlated with vesicle-bilayer fusion. These studies were performed single-blind, so that the experimenters did not know which fraction was added to the bilayer chamber.

The VDAC vesicles that had not shifted position on the density gradient either had no effect or gave rise to nonspecific conductances and occasional channels (*not shown*). These channels inserted spontaneously into the planar bilayer without the conditions known to be necessary for vesicle-bilayer fusion (no osmotic gradient, no divalent cations). These nonspecific conductances are characteristically seen with denatured VDAC (J. Zimmerberg, *unpublished observations*).

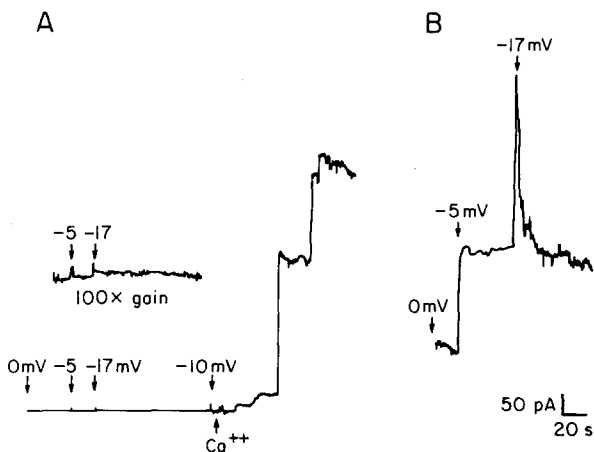On the other hand, the shifted VDAC vesicles consistently showed channel activity dependent

A   B

Fig. 4. Fusion of transport-selected VDAC vesicles with planar bilayer. (A) A planar bilayer membrane (1 : 1 PE : PS) was formed at V = 0 in symmetrical solutions (3 ml of 271 mM KCl, 5 mM MES, 0.1 mM EDTA, pH 6.0). Seven min before the start of the record shown, 50 μl of pooled transport-selected VDAC vesicles were added to the front (cis) compartment. At the arrows, the transmembrane voltage was changed from 0 mV to −5 mV, −17 mV, and −10 mV to test for VDAC activity. Following this, CaCl₂ was added to the cis compartment to a final concentration of 40 mM to give both osmotic and chloride gradients across the planar bilayer. Discrete jumps in membrane current ensued, indicating fusion of vesicles containing functional channels. (B) Two min after the end of record A the channels in the membrane were tested for voltage-dependent gating. A sequence of 0 mV, −5 mV, and −17 mV was applied. At V = 0 mV, the current (165 pA) was trans to cis, indicating an anionic reversal potential. At −17 mV, the current rapidly decayed in a manner characteristic of voltage-dependent VDAC turn off. The position of zero current is the same for both traces A and B, and is given by the 20-sec calibration mark.

upon vesicle fusion into the bilayer (Fig. 4). This was demonstrated by the following criteria: (i) Channels were *never* seen unless divalent cations were present and there was an osmotic gradient across the bilayer. (ii) The channel activity followed very clear fusion events. A heterogeneous distribution of conductance jump sizes (numbers of channels per vesicle fused into the bilayer) was observed, in accordance with a Poisson distribution of VDAC in the vesicles. We show two large jumps in Fig. 4 to demonstrate the simultaneous insertion of channels, the criterion of fusion.

The channel activity was characteristic of VDAC with regard to voltage dependence, kinetics and selectivity (Schein et al., 1976). Therefore, it seems reasonable to conclude that the vesicles that shifted position in the gradient contained VDAC that had been functionally reconstituted into the vesicle membrane.

Discussion

Fusion of protein-containing vesicles to planar bilayers often results in heterogeneous channel activity, raising the possibility that the observed activity is due to contaminants. It would be useful to purify the protein on the basis of its known functional properties prior to addition to the bilayer.

The density-shift protocol described here is a reliable method for separating vesicles that contain at least one large open channel from those that do not. The vesicles significantly increased density only when a channel permeable to urea and sucrose was incorporated into their membranes. These simple criteria permit selection of vesicles containing a specific channel without use of specific ligands such as affinity reagents, blockers or agonists.

The use of transport-specific density shifts has allowed the isolation of functional forms of several membrane transport proteins (e.g., Hess & Andrews, 1977; Goldin & Rhoden, 1978; Papazian, Rahamimoff & Goldin, 1979; Goldin, Rhoden & Hess, 1980). We adapted the concept to select on the basis of permeability to nonelectrolytes, and not on the basis of gating or vectorial transport properties. In addition, vesicle density changed on the gradients, rather than during an incubation step prior to separation on a gradient. This feature permits the use of diverse selection criteria, as discussed below.

In our method, high permeability to both gradient-forming solutes was required for the observed increase of vesicle density. It is instructive, however, to examine the consequences of permeability to only one of the permeant substances. For example, vesicles significantly more permeable to urea than sucrose (as may be the case for unmodified vesicles) would shrink due to osmotic forces as urea exited and sucrose was unable to enter. As a result of shrinking, the density of such vesicles would become less a function of the density of the intravesicular aqueous volume, and would approach the density of the membrane (about ⅓ down from the top of the gradients used in this study, or fraction 7). However, vesicles were not expected to lose their entire internal volume because of constraints imposed by minimal radius of curvature of the membrane.

For a spherical vesicle of given size, it is possible to calculate the internal volume for "shrunken" structures containing the same lipid volume and limited by the minimal radius of membrane curvature. The limiting diameter for phospholipid vesicles measured by hydrodynamic methods is 210–250 Å (Huang, 1969; Huang & Lee, 1973). Vesicles that are osmotically shrunk will most likely form

structures approaching biconcave disks (A. Walter, P. Vinson, and Y. Talmon, *unpublished results*). Upper and lower limits for the internal volume of such a structure can be approximated by those of pancake and torus structures, respectively.

For the lipid volume of a 375 Å radius spherical vesicle (9×10^7 Å^3), a torus with a minimal radius of 105–125 Å will have internal volume 16–26% that of the sphere, which corresponds to the density within gradient fraction 6. For a pancake structure with the same constraints and amount of lipid, the internal volume is 38–53% of the sphere, corresponding to the density within gradient fraction 5.[1]

These calculations show that (*i*) the density of the vesicles that shift position cannot be accounted for by osmotic shrinking, and (*ii*) for the conditions of our experiments, vesicles highly permeable to only one permeant substance cannot be distinguished from those highly permeable to neither. Under appropriate conditions, however, it should be possible to take advantage of the fact that selection is done on the gradient to make this distinction. For example, a sucrose to raffinose gradient could separate vesicles containing channels permeable to sucrose and raffinose, from those permeable to both and neither (because the density of the lipid would be less than that of either buffer solution).

For the density shift studies, it was necessary for the vesicles to have a monodisperse size and density distribution. Multilamellar vesicles are not appropriate because (*i*) differing numbers of lamellae give rise to different densities, and (*ii*) the trapped volume available for permeant exchange would vary from vesicle to vesicle. Techniques for vesicle formation from detergent solutions such as rapid dilution (Jackson & Litman, 1985) can give rise to several sizes of vesicles (A.L. Harris, A. Walter & J. Zimmerberg, *unpublished results*). We chose to use the gel filtration method of vesicle formation because it is a rapid and proven method for forming single-walled vesicles of uniform size and for incorporating membrane proteins (Mimms et al.,

1981). Other methods based on detergent removal such as slow dialysis or use of hydrophobic resins (e.g., Ueno, Tanford & Reynolds, 1984) are also likely to give acceptable results. One advantage of the gel filtration method, however, is that unincorporated protein can be readily separated from the vesicle preparation.

The protocol we present in this report has several features of interest to the study of membrane transport phenomena in reconstituted systems, even in those cases where a specific agonist can be used to identify a particular channel.

1) Contaminating membrane protein is diluted out in the solubilization/vesicle formation step. With this selection method, even with substantial amounts of contaminating protein in the starting material, significant purity of a channel can be achieved by insuring that (*i*) protein molecules that are adjacent in the native membrane are solubilized into separate detergent micelles, and that (*ii*) there is sufficient lipid so that the vesicles contain an average of less than one protein molecule per vesicle. When these conditions are satisfied, specific associations between the channel of interest and other proteins are eliminated. Solubilization using a vast excess of lipid may be used to achieve these conditions. The density-selection method can then be used to isolate those vesicles that contain the (channel-forming) protein of interest, and to select against those that contain others (as well as those that do not contain any protein at all). In this way, the advantages of near-infinite dilution of channel-forming proteins can be achieved without the disadvantages of extreme dilution, if the vesicles are transport-selected prior to biochemical or electrophysiologic study.

2) Every vesicle selected by increased density contains at least one functional channel. This means that every vesicle-planar bilayer fusion event is likely to lead to usable channel recordings. Therefore, experiments can proceed with much lower rates of fusion to the bilayer (e.g., due to constraints on lipid composition or amount of material) than would otherwise be possible. The amount of irrelevant membrane protein (including undesired channels) in the bilayer during an experiment can be drastically reduced, leading to electrically tighter and more stable planar bilayers, and quieter recordings. Recent findings show that vesicles containing protein can adhere virtually irreversibly to planar bilayers under some conditions used to promote fusion (Niles & Cohen, 1987). This "clogging" of the membrane and its attendant problems of noise and instability could be reduced as well.

3) The separation technique not only aids in

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[1] A circular torus is formed by the rotation of a circle about an axis in the plane of the circle, with r the radius of the revolving circle and R the distance of its center from the axis of rotation. For the calculations above, total volume was $2\pi^2 R r^2$ where r is the minimal radius of membrane curvature. Internal volume was calculated from the same expression where r was replaced by $r - 60$ Å. Lipid volume was the difference between total volume and internal volume. For given r, R was chosen to give the required lipid volume of the structure. Total volume of the pancake was calculated as the sum of the volume of a cylinder of radius R and one-half the volume of a torus where R and r have meanings as above. For calculation of internal volume, r was replaced by $r - 60$ Å as above.

protein purification, but also yields information about the permeability of the vesicles to nonelectrolytes. This aids in setting the osmotic parameters for the fusion of these vesicles with planar membranes (Cohen, 1986). An osmoticant will be effective in promoting fusion only if it is channel permeant (Niles & Cohen, 1987). For example, knowing that vesicles are permeable to glucose, but not to sucrose, will identify glucose as the appropriate osmoticant for fusion. In addition, having a population of vesicles selected for a specific permeability can aid the study of that permeation pathway. For example, pharmacologic or immunologic reagents can be assayed for effect on the transport-dependent density changes.

In conclusion, the advantage of this approach is that it yields reconstituted channel material in a well-characterized form that is optimal for fusion to bilayers. The vesicles are of homogeneous size and contain at least one functional channel per vesicle. We have used this technique to identify the gap junction protein connexin32 as a channel-forming protein in bilayers (Harris et al., 1988; A.L. Harris et al., *in preparation*).

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