Channel-Forming Activity of Immunoaffinity-Purified Connexin32 in Single Phospholipid Membranes†

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ABSTRACT: Connexin32, a member of the family of proteins that forms gap junction channels between cells, was immunoaffinity-purified from rat liver using a monoclonal antibody, under nondenaturing conditions and reconstituted into unilamellar phospholipid liposomes and bilayers. Gel-filtration studies indicate that the connexin32 is purified predominantly in structures of a size consistent with that of single hemichannels and too small to be junctional channels (dimers of hemichannels). Purified connexin formed channels permeable to sucrose and to Lucifer Yellow. The permeability was reversibly reduced by acidic pH and unaffected by several agents that modulate coupling between cells. Modeling of the distribution of the permeability in the liposomes indicates that it is mediated by connexin structures that distribute among the liposomes as single hemichannels. Bilayer recordings of the purified connexin show high conductance channels with asymmetric voltage sensitivity. The results show that immunopurified connexin32 can form channels, in single phospholipid membranes, that have permeability similar to that of gap junction channels and thus can be utilized in studies of permeability and its regulation to investigate its role in normal physiological function, development, and disease.

Connexin proteins form the gap junction channel, which is a regulated and specific pathway for intercellular diffusion of ions, metabolites, and second messengers (Payton et al., 1969; Loewenstein, 1981; Spray & Bennett, 1985; Beyer, 1993). The intercellular communication mediated by gap junction channels is thought to play important roles in many cellular processes, including metabolic homeostasis, regulation of cell growth, and development (Pitts, 1978; Loewenstein, 1979; Warner et al., 1984). Regulatory and signaling molecules that can pass from cell to cell through junctional channels include calcium ions, cyclic nucleotides, inositol trisphosphates, and morphogens [cf. Lawrence et al. (1978), Fraser et al. (1987), Brehm et al. (1989), and Sanderson et al. (1990)]. In muscle and nerve cells, the spread of current through gap junction channels is essential for proper function (Furshpan & Potter, 1959; Bennett, 1977). Recently, connexins have been implicated in several genetic disorders and developmental defects (Paul, 1995), including a human peripheral neuropathy [connexin32; (Bergoffen et al., 1993; Bruzzone et al., 1994b)] and cardiac malformations [connexin43 (Britz-Cunningham et al., 1995)].

There are at least 12 closely related isoforms of connexin, which have tissue-specific distributions (Beyer et al., 1990; White et al., 1995). Hexamers of connexin form membrane-spanning structures called *hemichannels* or *connexons* (Makowski et al., 1977; Unwin & Zampighi, 1980). A gap junction channel is formed by hemichannels, in the plasma membranes of closely apposed cells, that interact end-to-end to create structures spanning both plasma membranes.

Detailed studies of the gating and permeability of connexin channels, and the cellular basis of their regulation, have remained elusive primarily because, unlike other channels, the gap junction channel *in situ* spans two plasma membranes; both ends of the pore, and its modulatory sites, are in cytoplasm. This presents difficulties for accurate assessment of permeability and modulation of connexin channels. Because access to the pore is via cytoplasm (or dialyzed cytoplasm, in whole-cell patch configurations), for selectivity studies one cannot alter permeant species with impunity, and for modulatory studies it is difficult to distinguish direct from indirect effects.

To study the gating, permeability, and modulation of connexin channels at the level of rigor routinely applied to other channels, it would be advantageous to have the direct access to the pore and its modulatory sites possible in reconstituted systems. For connexin channels, such studies may be the best way to gain the fundamental information important for understanding biological function.

Study of reconstitution of double membrane connexin channels is desirable, but reconstitution in a pair of closely apposed phospholipid membranes is technically challenging. However, there is the possibility of study of hemichannel function, since each hemichannel spans a single membrane. Hemichannels often behave in junctional channels as if they contains discrete gating mechanisms that operate in series (Verselis et al., 1987; Bennett et al., 1988; Swenson et al., 1989; Werner et al., 1989).

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Recently, apparent hemichannel currents have been recorded from the plasma membrane of native cells (DeVries & Schwartz, 1992; Malchow et al., 1993; Liu et al., 1995) and expression systems (Paul et al., 1991; Ebihara & Steiner, 1993; Gupta et al., 1994; Ebihara et al., 1995). Recent work describes the properties of connexin channels in excised patches from *Xenopus* oocytes (Trexler et al., 1996). These systems offer opportunities to study connexin channel properties, at least for those connexins capable of forming open hemichannels in cellular membranes. Nevertheless, to understand how different permeabilities arise, and to identify mechanisms by which specific factors affect the physiology of connexin channels, reconstituted systems offer unique advantages, as they have for study of other ion channels.

Reconstitution studies of connexin have been hindered by the difficulty of purifying it under nondenaturing conditions, the difficulty of obtaining a soluble form of connexin for incorporation into liposomes and planar bilayers, and especially the difficulty of identifying observed channels as formed by connexin.

Several reports show channels and/or permeabilities induced in bilayers and liposomes by protein from preparations of junctional membrane from several tissues (Lynch et al., 1984; Girsch & Peracchia, 1985; Nikaido & Rosenberg, 1985; Spray et al., 1986; Walter et al., 1986; Young et al., 1987; Harris et al., 1990, 1991; Campos de Carvalho et al., 1991, 1992; Harris, 1991; Rhee & Harris, 1991; Donaldson & Kistler, 1992; Jarvis & Louis, 1992; Mazet et al., 1992; Buehler et al., 1995). In many of these studies, the protein that forms the observed channel or induces the permeability is not positively identified but correlated with presence or amount of connexin in the prep. It is difficult to identify a reconstituted channel without uniquely identifying properties known to be intrinsic to it (e.g., activation by acetylcholine, toxin effects), particularly when possible contaminating proteins are present, as in preps of junctional membrane. In earlier work we established the channel-forming ability of connexin32 from such material, using a transport-specific selection of connexin32 in liposomes (Harris et al., 1992).

Junctional membranes such as those used in the above studies are not optimal for physiological study. Gap junction membranes are isolated under denaturing conditions and are essentially insoluble (Goodenough & Stoeckenius, 1972; Hertzberg & Gilula, 1979; Fallon & Goodenough, 1981; Baker et al., 1983; Hertzberg, 1984). These methods purify for junctional structures, not for connexin protein, and necessarily yield other components of gap junction membrane. Less than 5% of the junctional membranes present in the tissue is recovered by the detergent method (Ghoshroy et al., 1995). Furthermore, these methods for isolation of junctional membrane can affect the secondary structure of the connexin (Cascio et al., 1990; Ghoshroy et al., 1995). Greater purity can be obtained when the connexin is overexpressed in a heterologous expression system and then purified by somewhat less harsh conditions (Stauffer et al., 1991). Connexin26 purified by this method was recently shown to induce channel activity in bilayers (Buehler et al., 1995).

In the present study, we describe the direct and specific purification of octylglucoside-solubilized connexin32 from native tissue under nondenaturing conditions, using a monoclonal antibody as an affinity reagent. Monoclonal antibodies have the particular advantages of high specificity and the possibility of mild conditions for elution of antigen and have been used to purify functional forms of other membrane proteins [e.g., Parker et al. (1984), Weber et al. (1984), and Yarden et al. (1985)]. We further report the functional reconstitution of purified connexin32 into single phospholipid membranes, where it forms aqueous pores with permeability similar to that of gap junction channels *in situ*. This is the first functional study of channels formed by immunopurified connexin. Preliminary reports have appeared in abstract form (Rhee et al., 1989; Rhee & Harris, 1991; Bevans & Harris, 1995).

MATERIALS AND METHODS

Materials. Egg phosphatidylcholine, bovine phosphatidylserine, and lissamine rhodamine B-labeled phosphatidylethanolamine were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Asolectin (L-α-phosphatidylcholine prepared from soybean), Tween 20, nitro blue tetrazolium (NBT)[1] and diisopropylfluorophosphate (DIFP) were obtained from Sigma Chemical Co. (St. Louis, MO). *N*-Octyl-*β*-D-glucopyranoside (octylglucoside) and Triton X-100 were from Calbiochem Corp. (La Jolla, CA). Bio-Gel (A-0.5m, exclusion limit 500 000 Da) was purchased from Bio-Rad (Richmond, CA). Protein-A affinity chromatography columns were purchased from Bio-Rad and Pierce Chemical Co. (Rockford, IL). Lucifer Yellow was purchased from Molecular Probes (Eugene, OR). Alkaline phosphatase-conjugated goat anti-mouse IgG and 5-bromo-4-chloro-3-indolylphosphate (BCIP) were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). CNBr-activated Sepharose beads were obtained from Pharmacia (Upsala, Sweden) and Immobilon-P transfer membrane from Millipore (Bedford, MA). Rats were obtained from Charles River Laboratory (Wilmington, MA).

Buffers. Homogenization buffer contained 5 mM $NaHCO_3$, 5 mM EDTA, 3 mM NaN_3, and 0.5 mM DIFP, at pH 8.2. Phosphate buffer (PBS) contained 50 mM sodium phosphate, 50 mM NaCl, 5 mM EDTA, 3 mM NaN_3, and 0.5 mM DIFP, at pH 7.0. "Urea buffer" used in the transport-specific fractionation assay contained 10 mM KCl, 10 mM HEPES, 0.1 mM EDTA, 3 mM sodium azide, and 459 mM urea at pH 7.6. "Sucrose buffer" used in the same assay was identical to the urea buffer except that an osmotically equivalent concentration of sucrose (400 mM) was substituted for the urea. Osmolality of urea and sucrose buffers was 500 mOsm/kg, and their specific gravities (D_4^{20}) were measured to be 1.0056 and 1.0511, respectively. For the permeability studies carried out at different pHs, solutions at pH 7.5 and 7.0 were buffered with 10 mM HEPES, and solutions at pH 6.5 and 6.0 with 10 mM MES.

Immunopurification of Connexin32. Connexin32 was affinity-purified from an octylglucoside-solubilized crude

[1] Abbreviations: asolectin, L-α-phosphatidylcholine from soybean; NBT, nitro blue tetrazolium; DIFP, diisopropylfluorophosphate; octylglucoside, *N*-octyl-*β*-D-glucopyranoside; BCIP, 5-bromo-4-chloro-3-indolylphosphate; CNBr, cyanogen bromide; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate buffered saline; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); MES, 2′-([*N*-morpholino)ethanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene fluoride; Tris, tris(hydroxymethyl)aminomethane; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; HPLC, high-performance liquid chromatography; CMC, critical micelle concentration.

membrane fraction of rat liver using a monoclonal antibody. A crude membrane fraction was prepared from livers of 35 day-old female rats (CD strain). Livers were minced in homogenization buffer, disrupted in a mechanical homogenizer (Sorvall) and a hand dounce (B type). The material was filtered through cheesecloth and centrifuged at 11 000*g* in a Sorvall OTD-50 centrifuge for 30 min. The pellet (crude membrane fraction) was suspended in PBS, respun and put aside for solubilization. The crude membrane fraction was solubilized in 80 mM octylglucoside. This corresponds to a ρ (rho) value of ~5 where $\rho = ([\text{detergent}] - \text{CMC})/[\text{phospholipid}]$ and is thus the molar ratio of micellar detergent to phospholipid (Rivnay & Metzger, 1982). The supernatant following centrifugation at 100 000*g* for 45 min was applied to a column containing Sepharose beads to which was attached a murine monoclonal antibody directed against connexin32 (M12.13), which does not cross-react with any other connexin (Goodenough et al., 1988). Following extensive rinsing, bound connexin was eluted from the antibody by brief exposure to pH 4 sodium acetate urea buffer. The eluent was rapidly neutralized by dropping directly into 1 M HEPES (pH 7.5). Time of exposure of connexin32 to the eluting pH was <5 s. Phospholipid (1 mg/mL) and 80 mM octylglucoside were present throughout the purification, and all procedures were carried out at 4 °C.

Gel Electrophoresis, Protein Blots, and Immunoblots. Samples of liposomes for gel electrophoresis were prepared by a methanol-extraction method (Wessel & Flügge, 1984) to concentrate protein and to remove lipid and detergent. The resulting protein pellet was dissolved in SDS sample buffer [10% (v/v) glycerol, 62.5 mM Tris-HCl, 2% (w/v) SDS, 10 mM dithiothreitol, 0.0025% (w/v) bromophenol-blue, pH 6.8]. Discontinuous polyacrylamide gel electrophoresis was performed with a Bio-Rad minigel apparatus by the method of Laemmli (1970) [4% (w/v) stacking gel, 13% (w/v) separating gel]. Protein was electrotransferred to Immobilon PVDF membrane (pore size 0.45 μm) with a semidry blotter (American Bionetics; Emeryville, CA) in standard transfer buffer [25 mM Tris, 192 mM glycine, 15% (v/v) methanol, pH 8.3] at current density of 2.5 mA/cm^2 for 36 min at 4 °C. For staining of total protein, the blot was blocked with 0.3% Tween 20 in PBS and stained with colloidal gold (0.01%, w/v) for at least 2 h at room temperature. For immunostaining, the membrane was pre-incubated with 5% (w/v) nonfat dry milk in PBS-Tween 20 (0.5%, v/v) for 1 h and then incubated with primary antibody (M12.13) (Goodenough et al., 1988) at 5 μg/mL for 2 h with gentle shaking at 37 °C. After washing with PBS-Tween 20 for 30 min with changes every 10 min, the membrane was incubated with secondary antibody (alkaline phosphatase-conjugated goat anti-mouse IgG) at 5−10 μg/mL for 1 h with gentle shaking at 37 °C and then developed in 0.1 mg/mL NBT and 0.05 mg/mL BCIP in alkaline phosphate buffer (10 mM Tris, 10 mM $MgCl_2$, 100 mM NaCl, pH 9.5).

Gel Filtration of Immunopurified Connexin. The immunopurified connexin was analyzed by size-exclusion chromatography. A 1.5 × 20 cm column of Sephadex G-200 was equilibrated with a buffer containing 150 mM KCl, 50 mM Tris-HCl, and 80 mM octylglucoside adjusted to pH 7.5 with KOH. Buffer flow rate was 9 mL/h. Column was calibrated with molecular weight standards (apoferritin, 443 kDa; β-amylase, 200 kDa; albumin, 60 kDa; carbonic anhydrase, 29 kDa). Approximately 1 μg of immunopurified connexin32 was applied to the column. Aliquots of 0.8 mL were collected and analyzed for connexin32 by dot-blotting.

Dot Blots. Aliquots were applied to Immobilon membrane pre-equilibrated with PBS in a dot-blot manifold (Minifold system; Schleicher & Schuell, Keene, NH). The membrane was then blocked with 0.3% Tween-20 in PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.2) for 30 min and visualized using a monoclonal antibody against connexin32 as described in *Gel Electrophoresis, Protein Blots, and Immunoblots* above. Density of the dots was quantified by transmission densitometry at the absorbance peak (545 nm) (Shimadzu CS-9000, Japan). The linearity of the dot-blot densitometry was established over the range of protein blotted (<400 ng per dot).

Reconstitution of Connexin32 into Unilamellar Phospholipid Liposomes. Liposome formation and protein incorporation followed the protocol of Mimms et al. (1981) as modified by Harris et al. (1992). Phosphatidylcholine (PC), phosphatidylserine (PS), and rhodamine-labeled phosphatidylethanolamine (PE) were dissolved in chloroform at a molar ratio of 2:1:0.03. The lipid mixture was dried to a thin film under a stream of argon and desiccated under vacuum to remove residual solvent. The lipid film was suspended at 1 mg/mL in urea buffer containing 80 mM octylglucoside and the immunoaffinity-purified connexin32 at a concentration of 10 μg/mL. The protein−lipid−detergent mixture was incubated on ice for at least 20 min with occasional gentle swirling and then applied to a 1.5 × 20 cm Bio-Gel A-0.5m column pretreated with sonicated liposomes. The column was eluted at a urea buffer flow rate of 9 mL/h at 4 °C. The liposomes were collected in the void volume. The size distribution of liposomes was established by filtration (0.5 mL/min) over a calibrated TSK G6000PW HPLC column (Ollivon et al., 1986) to be highly monodisperse with an approximate mean diameter of 450 Å. In the liposome-forming mixture, the protein/lipid ratio was typically 1:300 (w/w). In the liposomes themselves, the protein/lipid ratio (determined from blotting of protein and rhodamine fluorescence) was approximately 1:60 (w/w), corresponding to an amount of connexin32 equivalent to ~1 hemichannel (6 connexin monomers) per liposome. We intentionally worked at a protein/lipid ratio that produced permeable liposomes and nonpermeable liposomes (as internal controls). Therefore, minor variation in the amount of protein used, the amount damaged in purification, the reconstitution efficiency, the amount of lipid retained on the column, etc., produced variations in the percent of permeable liposomes.

Liposome Trapping of Lucifer Yellow. Liposomes were formed as above except that the liposome-forming solution also contained 3 mM Lucifer Yellow (457 Da). To minimize dilution of Lucifer Yellow during liposome formation, an equal volume of urea buffer containing 3 mM Lucifer Yellow was loaded onto the gel-filtration column just before and just after the liposome-forming solution entered the gel bed. As liposomes formed, they migrated ahead of the untrapped Lucifer Yellow.

Transport-Specific Density Shift Technique. The procedure used to fractionate liposomes into two populations based on sucrose-permeability is described and fully characterized in Harris et al. (1989) (Figure 3A). The principle of using a density-shift to fractionate liposomes was adapted from Goldin and Rhoden (1978). Linear iso-osmolar density

gradients were formed from the urea and sucrose buffers in 4.4 mL ultracentrifuge tubes (Beckman, Palo Alto, CA) using a gradient maker (Hoefer, San Francisco, CA). An aliquot of the liposomes (typically 200 μL) was layered on top of each gradient. Gradients were typically spun at 300 000*g* for 3−5 h in a swinging bucket rotor (Sorvall TST 60.4, Du Pont Co., Newtown, CT) at 4 °C. Gradient fractions of 200 μL were collected. A 50 μL aliquot of each fraction was diluted with 330 μL of PBS−Triton X-100 (0.2%). The distribution of the liposomes in the gradient was determined by measuring the specific intensity of rhodamine fluorescence in each fraction with a Perkin-Elmer 650-10S spectrofluorometer (560 nm excitation; 590 nm emission). Lucifer Yellow fluorescence was measured at an excitation wavelength of 428 nm and an emission wavelength of 540 nm.

The liposomes, which entrap the urea buffer during formation, are centrifuged in the gradients described above. During the centrifugation, liposomes not permeable to sucrose move into the gradient a short distance, being buoyed by the (lighter) trapped internal urea buffer, banding in the upper part of the gradient. Permeable liposomes continuously equilibrate their internal solution with the external solution and move to a lower position in the gradient corresponding to the density of the liposome phospholipid membrane. Because equilibration across the liposome membrane is rapid compared with the duration of the spin (several hours), a single sucrose-permeable channel open for only short times is sufficient to cause the full shift in density.

Calculation of Poisson's Distribution. The average number of connexin structures per liposome (λ) was calculated from the protein to lipid ratio (w/w), the size of the liposomes and the number of monomers in each connexin32 structure (e.g., 6 for a hemichannel). The number of lipid molecules per liposome was calculated from the radii of inner and outer phospholipid leaflets for liposomes with a hydrodynamic radius of 225 Å, assuming a lipid headgroup area of 65 Å^2 (Huang & Lee, 1973). The fraction of sucrose-permeable liposomes predicted by Poisson's distribution was taken as the fractional amount (*P*) calculated to contain *n* or more structures (where *n* is the number of connexin structures required to form a functional pore), according to

$$P = \sum e^{-\lambda}\lambda^k/k!$$

where *k* ranges from *n* to ∞ (Haight, 1967).

Bilayer Technique. Bilayer studies were carried out essentially as previously described (Harris et al., 1992). Planar phospholipid bilayers were formed from bovine liver PE and bovine brain PS at a mole ratio of 1:1, 2% in decane. Bilayer saline was 182 mM KCl, 9 mM HEPES, and 0.1 mM EDTA at pH 7.4 in the *cis* chamber. The KCl was diluted 1:10 in the *trans* chamber to provide an osmotic gradient. The *cis* chamber also contained 30 mM $MgCl_2$. In some experiments, after membrane thinning, microliter amounts of density gradient fractions were puffed at the bilayer through a small (40 μL) pipette. Voltages are relative to the *cis* chamber. High-resolution recordings (15 kHz freq. resp.) were made in a specially designed chamber (Bezrukov & Vodyanoy, 1993) shielded by a high-μ screen from magnetic and electrical fields and were carried out in collaboration with Dr. Sergey Bezrukov (NIDDK, NIH).

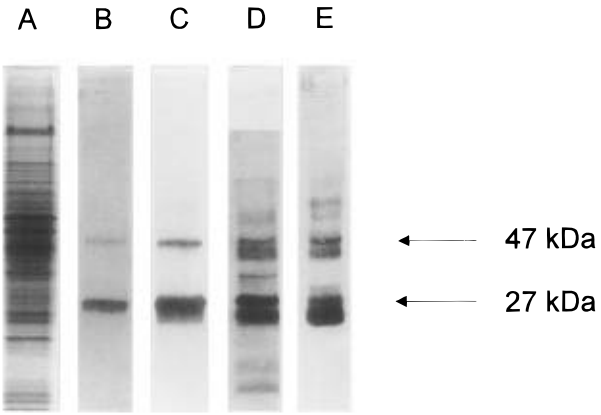

FIGURE 1: Comparison of immunopurified connexin32 with starting material and with isolated gap junctions. (Lane A) Blot of solubilized crude membrane fraction of rat liver homogenate stained for total protein with colloidal gold. (Lane B) Blot of immunopurified connexin32 stained with colloidal gold. (Lane C) Immunoblot of immunopurified connexin32 using monoclonal antibody M12.13. Connexin monomer and dimer bands are indicated. (Lane D) Blot of gap junctions membranes isolated by sarkosyl extraction (Baker et al., 1983) and stained with colloidal gold. (Lane E) Immunoblot of isolated gap junction membranes using monoclonal antibody M12.13. Immunopurified connexin32 is significantly more pure and less proteolyzed than the connexin32 of the isolated junctions.

RESULTS

Immunoaffinity Purified Connexin32. An immunoaffinity protocol employing a monoclonal antibody directed against connexin32 was used to obtain highly pure, solubilized connexin from rat liver membranes (see Materials and Methods). The antibody is directed against an unconserved cytoplasmic epitope and does not cross-react with any other connexin. Western blots of immunopurified material show bands identified as connexin32 at monomer and dimer positions (Figure 1) (Kumar & Gilula, 1986; Paul, 1986; Nicholson & Zhang, 1988). A proteolytic fragment of connexin32 also seen in isolated junctions was occasionally observed (band at ∼22 kDa) (Hertzberg & Skibbens, 1984; Paul, 1986). Though connexin26 was sometimes also recovered (Kordel et al., 1993), the studies discussed here utilized preps that did not contain detectable connexin26.

Typically, no non-connexin protein was detected on colloidal gold-stained Western blots, even from very heavily loaded gels. Actin was an occasional contaminant and could be removed by extensive washing of the immunobeads with 1 M NaCl prior to connexin elution. Blots of immunopurified connexin, and of the starting material and isolated gap junction membranes (included for comparison), are shown in Figure 1. The blots of the immunopurified connexin32 show greater purity and less proteolysis than is seen in isolated junctions. Comparison with gels of junctional membranes isolated by the sarkosyl method (Baker et al., 1983) showed that the immunopurification method reported here yields at least 10 times more connexin32 per rat liver. The total connexin purified is ∼7 μg per gram wet weight of liver, which corresponds to 40% of the connexin in the tissue (Hendrix et al., 1992), or ∼60% of that in the plasma membrane (Rahman & Evans, 1991).

The approximate size of the immunopurified connexin structures was determined by size-exclusion gel chromatography. The connexin eluted from the column in a major peak corresponding to 300 kDa and a minor peak corresponding

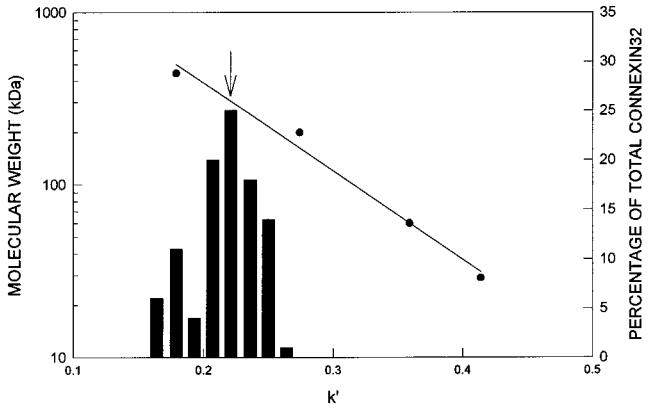

FIGURE 2: Size-exclusion chromatography of immunopurified connexin32. A column of Sephadex G-200 was calibrated with the following standards: apoferritin (443 kDa), β-amylase (200 kDa), albumin (60 kDa), and carbonic anhydrase (29 kDa) (filled circles and regression line; left ordinate axis). Immunopurified connexin was applied to the column and its presence in the eluent assayed by dot blots quantified by transmission densitometry (bars; right ordinate axis). Buffer contained 80 mM octylglucoside, 150 mM KCl, and 50 mM Tris-HCl adjusted to pH 7.5 with KOH. Flow rate was 9 mL/h. Abscissa is the capacity factor k', which equals $(V_e - V_o)/V_o$ where V_e is the elution volume and V_o is the void volume [defined as the elution volume of blue dextran (2000 kDa)]. The major peak of connexin32 is at ~300 kDa (arrow), which is consistent with hexameric connexin32 if it binds ~55% of its weight in detergent. A peak corresponding to monomeric connexin32 was not observed. Water-soluble proteins do not bind significant amounts of detergent, so they can be used as size standards in detergent-containing systems (Muccio & DeLucas, 1985).

to 500 kDa (near the exclusion limit of the gel matrix) (Figure 2). Membrane proteins typically bind detergent equivalent to 30−100% of their weight (Pabst et al., 1984; Muccio & DeLucas, 1985; Nalecz et al., 1986). The major peak is consistent with hexameric connexin32 (192 kDa) if it binds ~55% of its weight in detergent. Therefore the gel-filtration data indicate that the connexin is predominantly in structures the size of hemichannels, with some larger structures that could be junctional channels or other aggregated forms. No peak corresponding to monomeric connexin32 was detected.

Sucrose Permeability of Unilamellar Liposomes Containing Connexin32. The immunopurified connexin was incorporated into unilamellar phospholipid liposomes by gel filtration of a protein/lipid/octylglucoside mixture (Mimms et al., 1981; Ollivon et al., 1986). Liposomes containing functional large channels were identified by a transport-specific liposome fractionation protocol (see Materials and Methods and Figure 3A) (Harris et al., 1989). This technique utilizes isoosmotic density gradients formed by urea and sucrose buffers to cause liposomes significantly permeable to urea and sucrose (i.e., containing large open channels) to become more dense than those that are not. In brief, liposomes not permeable to sucrose move into the gradient a short distance to a position determined by their density. Liposomes that are permeable to sucrose (Stokes radius ~5 Å) become more dense as sucrose enters and urea (the smaller molecule of the two) leaves. They move lower into the gradient, gaining sucrose (and density) as they do so, eventually coming to an equilibrium position in the lower part of the tube, corresponding to the density of the phospholipid membrane. In this way, the functional properties of the channels themselves cause a fractionation of liposomes.

Liposomes formed in the absence of connexin or in the presence of control membrane proteins did not show channel activity by this assay, banding in the upper part of the gradients (Figure 3B) (Harris et al., 1992). In contrast, liposomes formed in the presence of immunoaffinity-purified connexin separated into two bands: a band of impermeable liposomes at the same upper position as the liposomes formed in the absence of connexin, and a band of sucrose-permeable liposomes near the bottom of gradient. To quantify the fraction of the liposomes that were sucrose-permeable, the distribution of liposomes in a gradient was determined from the distribution of fluorescence of rhodamine-labeled PE in the liposome membranes (Figure 4). Under typical conditions, one-third to one-half of the liposomes formed in the presence of connexin were sucrose-permeable by this assay (i.e., contained at least one active channel).

To determine the distribution of connexin32 in the two populations, protein in the sucrose-impermeable and sucrose-permeable liposomes was separated by SDS−PAGE. Western blots were probed with a monoclonal antibody against connexin32 (Figure 5). Typically, all of the detectable connexin32 was in the sucrose-permeable liposomes. Occasionally, a small amount of connexin32 (<15% of total) was detected in the sucrose-impermeable liposomes. This connexin32 apparently does not form functional channels, perhaps due to damage during purification or the reconstitution procedure. We conclude that the immunoaffinity-purified connexin32 forms a sucrose-permeable channel in unilamellar liposome membranes.

Permeability of Liposomes to Lucifer Yellow. To characterize the size of the connexin32-induced permeability pathway, the permeability to the fluorescent dye Lucifer Yellow was assessed. Lucifer Yellow (457 Da, diameter ~12 Å) is a planar molecule near the upper size-permeability limit of gap junction channels *in vivo* (Bennett et al., 1978; Stewart, 1978). Liposomes were formed with and without connexin32 in the presence of 3 mM Lucifer Yellow. Each set of liposomes was fractionated according to permeability as before. As expected, liposomes formed without connexin32 were impermeable and retained Lucifer Yellow (Figure 6, upper graph). Of the liposomes formed in the presence of connexin32, the sucrose-permeable population specifically lost Lucifer Yellow, whereas the sucrose-impermeable liposomes retained it (Figure 6, lower graph). The specific loss of Lucifer Yellow from the sucrose-permeable liposomes demonstrates that the sucrose-permeable pathway formed by connexin32 is also permeable to Lucifer Yellow.

Low pH Reversibly Reduces Sucrose Permeability. When applied to coupled cells, many agents affect junctional conductance. To help identify which act directly on the connexin channels, some of these agents were included in the density gradient buffers. Note that because sucrose equilibration across liposome membrane through a channel is very rapid, changes in the fraction of liposomes defined as sucrose-permeable in this assay corresponds to dramatic changes in open probability and/or closed times.

To determine whether pH affected the connexin32-induced sucrose permeability, the transport-specific fractionation was performed at pHs ranging from 6.0 to 7.5. The fraction of liposomes that were sucrose-permeable was decreased by pH below 7.5. In the experiments summarized in Figure 7, 60% of the liposomes formed in presence of connexin32 were

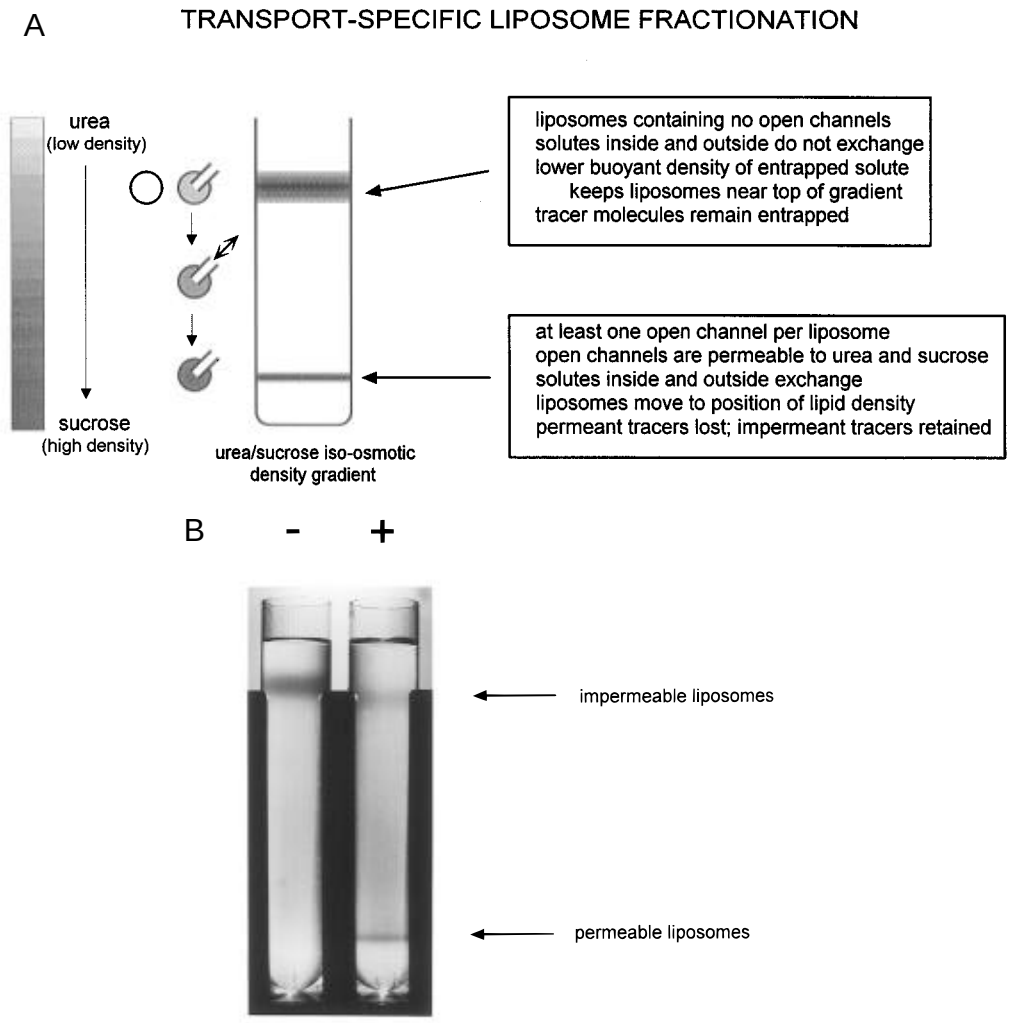

FIGURE 3: Sucrose permeability induced by connexin32 shown by transport-specific fractionation of liposomes. Unilamellar liposomes containing immunopurified connexin32 were separated on the basis of sucrose permeability on isoosmolar density gradients as described in the text (Harris et al., 1989, 1992). (A) Scheme of fractionation of liposomes on basis of permeability. Liposomes permeable to sucrose increase in density (move to a lower position in the gradient; lower band), while those that are not remain at a lighter density near the top of the gradient (upper band). (B) Gradient tubes after a spin showing bands of impermeable and permeable liposomes. Liposomes formed without connexin32 (−) formed a single band near the top of the tube, indicating impermeability to sucrose. Liposomes formed in the presence of connexin32 (+) separated into two populations, one at the upper position characteristic of sucrose impermeability and another, near the bottom of the tube, characteristic of sucrose permeability. Bands of liposomes are colored due to rhodamine-PE in the liposome membrane.

permeable at pH 7.5, while only 16% were permeable at pH 6.0. To determine whether the pH inhibition of sucrose permeability was reversible, liposomes that were impermeable at pH 6.0 were recentrifuged at pH 7.5 (Figure 8, open bars). Compared with matched controls spun twice at pH 7.5, approximately 35% of the expected shift occurred, indicating incomplete recovery of sucrose permeability.

The following compounds that can reduce junctional conductance in cells were tested in the same manner and found to have no effect on connexin channel activity in this assay at 1 mM concentration: glycerrhetinic acid, halothane, metafane, lidocaine, octanol, octanoic acid, heptanol, calcium, cobalt, and manganese. The following compounds were without effect at 10 mM: taurine, bromoethanesulfonate, and propopanesulfonate. The following compounds reported to increase junctional conductance in cells were similarly tested and found to have no effect: quinine (100 μM), melatonin (10^{-8} M). The data indicate that the actions of these agents on cellular junctions are mediated by cytoplasmic and/or membrane components not present in the liposomes and require junctional channels rather than hemichannels to have an effect, or their effects are too subtle to be revealed in this assay.

The Fraction of the Liposomes That Are Sucrose-Permeable Is Consistent with the Pores Being Hemichannels of Connexin32. To characterize the relation between permeability and connexin structures, Poisson's law was used to model the distribution of connexin32 among the liposomes. Given the number of connexin structures and the number of liposomes among which they can independently distribute, Poisson's law can predict the number of liposomes that will contain n such structures. If each structure can form a pore, liposomes that contain one or more structures ($n \geq 1$) will be sucrose-permeable. This calculation places an upper limit on the percentage of liposomes that are permeable, since not all the connexin structures may be functional. Thus, for a given set of conditions and assumptions, the maximum percentage of sucrose-permeable liposomes can be predicted.

For the 450 Å liposomes (size determined by HPLC gel-filtration) and the standard protein/lipid ratio of the liposomes used in these studies (1:60 w/w), the average amount of connexin per liposome is about that of a hemichannel (six

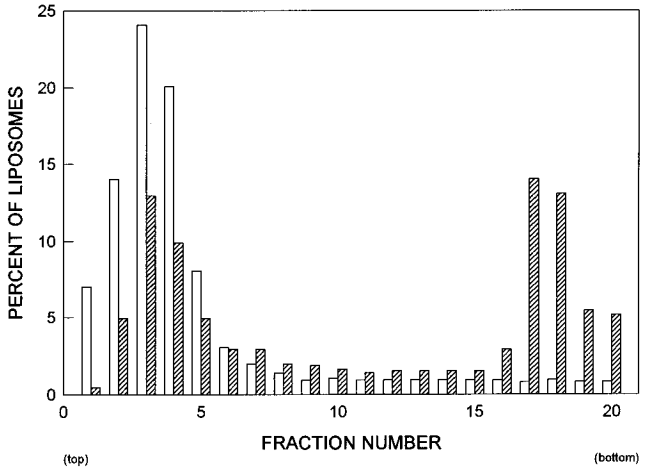

FIGURE 4: Distribution of liposomes after transport-specific fractionation. The distribution of liposomes was determined by measurement of the fluorescence of rhodamine-PE in the liposome membranes. (Open bars) Liposomes formed without connexin32 were in a single peak (centered at fractions 3 and 4). (Hatched bars) Liposomes formed in the presence of connexin32 separated into two peaks; one centered at fractions 3 and 4 as before (sucrose-impermeable) and one centered at fractions 17 and 18 (sucrose-permeable). In this experiment, approximately 60% of the liposomes formed in the presence of connexin32 were sucrose permeable.

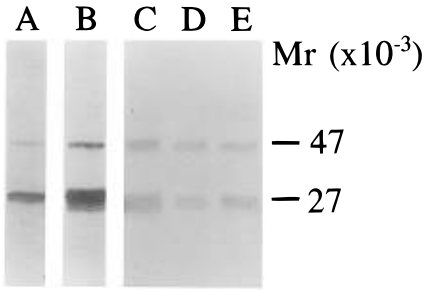

FIGURE 5: Distribution of connexin32 in the liposome populations. (Lane A) Blot of immunopurified connexin32 stained for total protein with colloidal gold. (Lane B) Blot of immunopurified connexin32 stained with monoclonal antibody against connexin32 (M12.13). Note the monomeric and dimeric forms of connexin32, which run at the 27 and 47 kDa positions, respectively. (Lane C) Immunoblot of unfractionated liposomes containing connexin32. (Lane D) Immunoblot of sucrose-impermeable liposomes. (Lane E) Immunoblot of sucrose-permeable liposomes. Typically, no detectable connexin32 was found in the sucrose-impermeable liposomes. The preparation shown here contained the largest amount of connexin seen in sucrose-impermeable liposomes (<15% by transmission densitometry), which may represent nonfunctional connexin32.

connexin monomers). From the gel-filtration data, the connexin exists in units the size of hemichannels. Poisson's law predicts that if the connexin distributes as dodecamers (e.g., as junctional channels) and each forms a pore, no more than 42% of the liposomes should be sucrose-permeable. If the connexin distributes as single hemichannels, and forms a pore, a maximum of ~67% would be sucrose-permeable, but if two hemichannels are required for patent channels ($n \geq 2$), at most only 30% should be sucrose-permeable. Typically, ~60% of the liposomes became sucrose-permeable. Therefore, the number of liposomes that are sucrose-permeable cannot be accounted for by dodecameric channels, and the data are consistent with single hemichannels forming a pore. The slight decrease in percent shifted from the 67% maximum predicted value is most likely due to a small amount of nonfunctional connexin (see Figure 5, lane D).

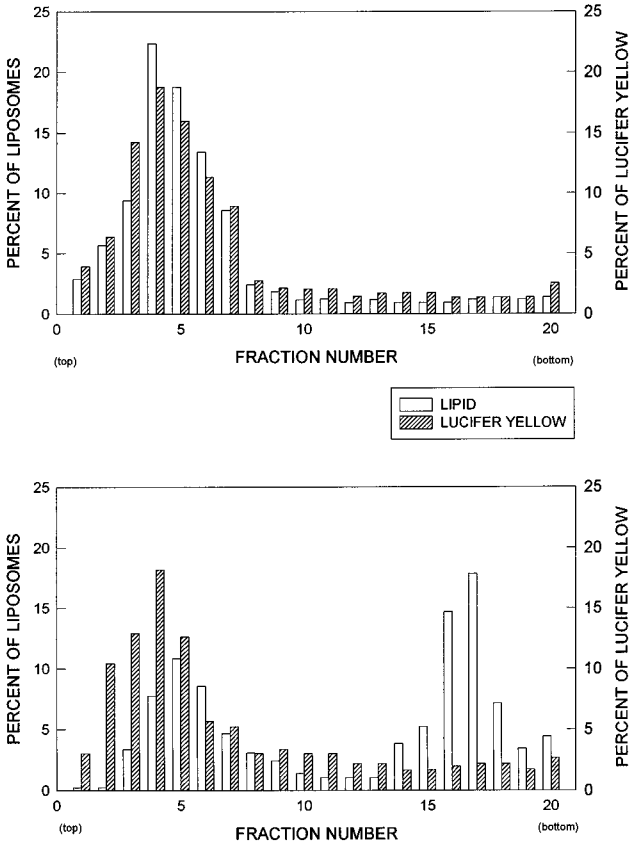

FIGURE 6: Sucrose-permeable liposomes are also permeable to Lucifer Yellow. Liposomes were formed with and without connexin32 in the presence of the fluorescent, gap junction-permeable dye Lucifer Yellow (457 Da, diameter 12 Å). Following transport-specific fractionation, the distributions of lipid (open bars) and of Lucifer Yellow (hatched bars) within the gradients were determined. (Upper graph) In the absence of connexin32, liposomes were sucrose-impermeable and trapped Lucifer Yellow. (Lower graph) In the presence of connexin32, the sucrose-permeable liposomes lost the entrapped Lucifer Yellow, while the sucrose-impermeable liposomes retained it. Therefore, the sucrose-permeable liposomes contain an aqueous pathway at least the diameter of Lucifer Yellow.

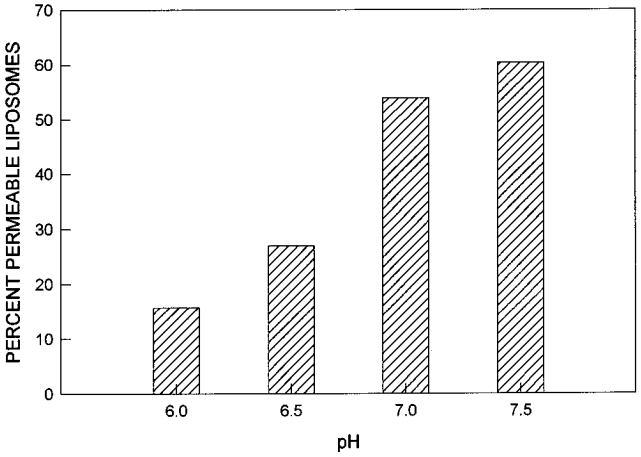

FIGURE 7: Effect of pH on the percentage of connexin-containing liposomes permeable to sucrose. Liposomes formed in the presence of connexin32 were separated on the basis of sucrose permeability over a range of pHs. The fraction of liposomes that were sucrose-permeable decreased with pH below 7.5.

For experiments in which the protein/lipid ratio of the liposomes was changed to 1:90 w/w, the predicted maximal fractions of permeable liposomes are 29% for distribution as dodecamers, 16% for distribution as hexamers and two

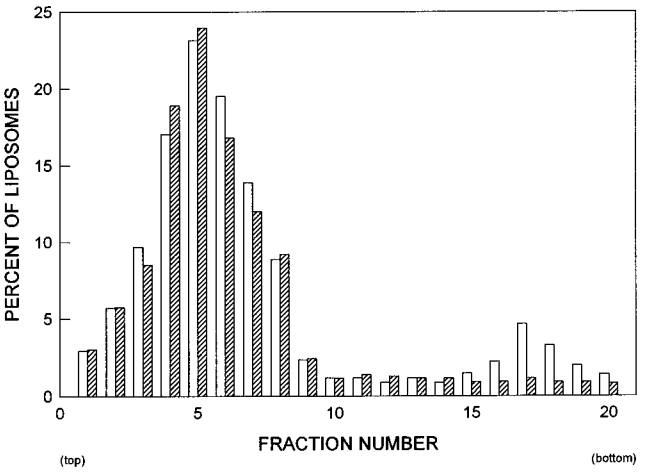

FIGURE 8: Reversibility of pH effect on sucrose permeability. (Open bars) Liposomes that were sucrose-impermeable at pH 6.0 were tested for recovery of sucrose permeability at pH 7.5. There was a small amount of recovery, corresponding to 35% of the liposomes that shifted in matched controls respun at pH 7.5. (Hatched bars) Liposomes that were sucrose impermeable at pH 6.0 were respun at pH 6.0 to control for recovery of sucrose permeability not related to return to neutral pH. No such recovery was observed.

of them required for a functional pore, and 50% for distribution as hexamers that can each form a pore. For this protein/lipid ratio, the observed values were ~35%. Thus, for two different protein/lipid ratios, the fraction of permeable liposomes is inconsistent with distribution as dodecamers and with the functional channels being formed by dodecamers; the data are consistent with the connexin32 distributing as pore-forming hemichannels.

The data can be accounted for by the connexin distributing as monomers and the functional channels being formed by ≤6 monomers. However, the reconstituted channels are not likely to be formed by fewer than six monomers since Lucifer Yellow is near the upper size limit for permeation through junctional channels (Bennett & Goodenough, 1978), and there is no detectable monomeric connexin in the starting material.

Single-Channel Recordings. To confirm the functional basis of the observed permeability, liposomes from the unfractionated, sucrose-permeable, and sucrose-impermeable populations were fused with planar lipid bilayers. With addition of either the unfractionated or the sucrose-permeable liposomes, channels were observed. This activity was not observed with fusion of sucrose-impermeable liposomes (which also contained connexin that was reconstituted but apparently nonfunctional in the liposome permeability assay). Overall, the frequency of observation of clearly identifiable single-channel activity was low, even when liposome fusion with the bilayer was reported by the nystatin method (Woodbury & Miller, 1990). The predominant channel activity was rapid, voltage-dependent bursts of current characterized by high frequency fluctuations in amplitude (Figure 9). Because of the fluctuations of open channel current, unitary conductance was often difficult to ascertain but clustered around multiples of ~180−200 pS. Channels were generally open at 0 mV and closed with one polarity of voltage. Of particular note was the very high frequency and amplitude of the single channel currents. Spectral analysis of open channel currents revealed only a strong 1/*f* character, which is not informative regarding the origin of the fluctuations. The low frequency of occurrence and the

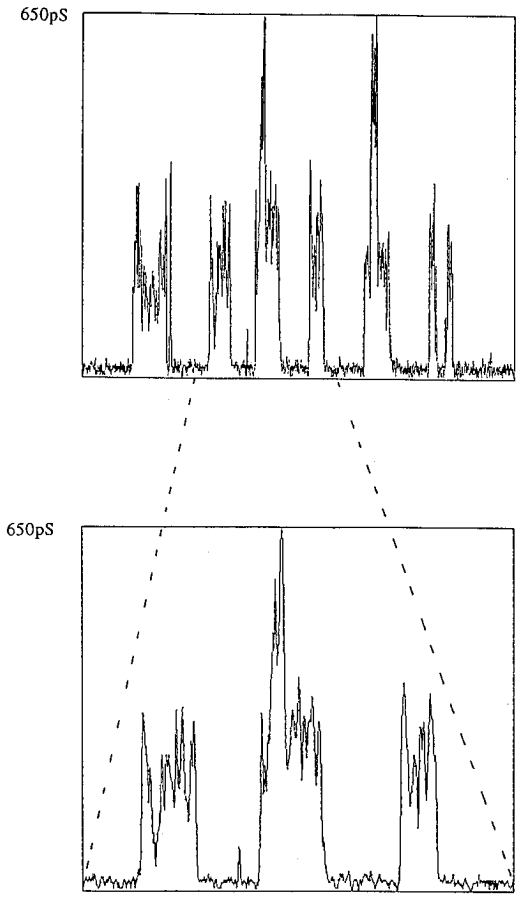

FIGURE 9: Bilayer current transitions induced by immunoaffinity-purified connexin32. High resolution (15 kHz freq. resp.) recordings of channel activity induced by affinity-purified connexin following fusion of sucrose permeable liposomes with the bilayer. Note the discrete gating transitions and the high frequency and high amplitude fluctuations in open channel current. Unitary conductance is difficult to discern but is near 200 pS. Bilayer voltage was 100 mV. The lower trace is an expansion of the indicated section of the upper trace. Upper trace duration is 520 ms; lower trace duration is 200 ms. Recordings made in collaboration with Dr. S. Bezrukov, NIDDK, NIH, using a high-μ chamber (Bezrukov & Vodyanoy, 1993).

somewhat variable properties of these channel currents precluded detailed characterization of their properties and studies of channel modulation in the bilayer system. The data are, however, sufficient to characterize the specific liposome permeability as arising from large connexin channels.

DISCUSSION

We report here that immunoaffinity-purified connexin32 from rat liver forms large channels in unilamellar liposome membranes. The aqueous pore is at least as wide as that of connexin32 junctional channels *in vivo*. The permeability is reversibly inhibited by acidification. The fraction of liposomes made permeable to sucrose by connexin32 corresponds to that expected for channels formed by a single connexin32 hemichannel per liposome. These findings demonstrate that connexin32 can form open single-membrane channels, in reconstituted systems, with properties consistent with functional hemichannels.

Immunoaffinity Purification. The affinity-purification technique employed here avoids overtly denaturing treatments, and yields solubilized protein readily incorporated

into phospholipid membranes for functional studies. It is a one-step procedure yielding highly pure, octylglucoside-solubilized connexin. For biochemical and physiological studies of connexin channels, this purification has particular advantages over the use of preparations of isolated junctional membranes, whether from native cells or heterologous expression systems: (a) Junctional membranes are typically obtained by the use of harsh treatments likely to denature proteins (Baker et al., 1983; Hertzberg, 1984). (b) Junctional membranes are essentially purified on the basis of their insolubility (in detergent or highly alkaline solutions) and so are poorly suited for reconstitution. (c) The yield of purified junctional membrane typically results in the recovery of a small fraction of the connexin present in the starting tissue (Ghoshroy et al., 1995), suggesting that the connexin in isolated junctions may not be representative of the bulk of junctional connexin in the intact tissue. (d) Isolated junctional membranes can contain non-connexin contaminants.

In contrast, immunopurified connexin is isolated under mild conditions in a nonionic detergent. Exposure to acidic pH during elution from the antibody is brief and can be well-controlled. Because of the greater yield and the selection against insoluble structures, immunopurified connexin is more likely to be typical of the connexin in junctions *in vivo*. Most importantly for reconstitution, all the immunopurified connexin is soluble in nonionic detergent. Thus for connexin channels, affinity purification with a monoclonal antibody is of particular value because of the inaccessibility of the channels in their native environment to detailed physiological study, and it has the additional advantages of greater purity, yield, solubility, and nondenaturing conditions, compared with methods that utilize connexin from junctional membrane preps. Connexin43 has been immunopurified for biochemical studies using a monoclonal antibody against a peptide corresponding to a specific sequence (Loo et al., 1995).

Earlier reconstitution studies showed that protein solubilized from rat liver junctional membranes can form open channels in single membranes (Lynch et al., 1984; Spray et al., 1986; Walter et al., 1986; Young et al., 1987; Campos de Carvalho et al., 1991; Harris et al., 1992; Mazet et al., 1992). Our earlier work identified connexin32 as a channel-forming protein in this material by its relative enrichment in liposomes that were permeable to sucrose (Harris et al., 1988, 1992; Harris, 1991). The isolated junctions used in such studies contained non-connexin components and were exposed to denaturing conditions during isolation.

The present study independently shows that connexin32 forms channels in single membranes and that this property is not an artifact of harsh conditions of junction isolation protocols. It also shows that immunopurified connexin32, in the absence of other membrane components, can form pH-sensitive, Lucifer Yellow permeable channels. Furthermore, this study uniquely addresses the properties of a single purified connexin in a well-defined system.

Structural Form of the Connexin. Several points bear on the structural form of the immunopurified connexin. Since only solubilized material is used in the purification (i.e., the supernatant after a 100000*g* spin for 45 min), sheets or small chunks of junctional membrane structures are excluded. *A priori*, the most likely forms are intact junctional channels (end-to-end hemichannels) or single hemichannels.

The gel-filtration data show that monomeric connexin is not evident in the immunopurified material. The major peak is at a position consistent with hexameric connexin32 that binds ~55% of its weight in detergent [a similar amount of octylglucoside is bound by bacteriorhodopsin (Muccio & DeLucas, 1985)]. The major peak is also consistent with pentameric connexin32 binding 88% of its weight in detergent, or septameric connexin32 binding 34%. Since the junctional channel is formed of two hexameric subunits, disruption to form larger or smaller oligomers would produce peaks corresponding to smaller structures (e.g., monomers) which are not seen. The minor peak at ~500 kDa could be formed by aggregated hexameric structures, but the size is ambiguous since it is near the nominal exclusion limit of the gel (600 kDa). We conclude that the immunopurification procedure yields structures that most closely correspond to single hemichannels.

Modeling of the distribution of the protein strongly suggests that the immunopurified protein reconstitutes into the liposomes as if it were in hexameric, and not dodecameric, form. This finding, along with the Lucifer Yellow permeability and the fact that the liposomes are unilamellar, also suggests that the channels are formed by a hemichannel type of structure (i.e., a channel formed by fewer connexin monomers is likely to have a narrowed diameter and thus be impermeable to Lucifer Yellow).

Conductive Hemichannels. Is it surprising that connexin can form open channels in single membranes? On one hand, if open hemichannels exist in plasma membrane, they would adversely affect cell viability, if open for significant periods of time. On the other hand it is clear that in the *Xenopus* oocyte expression system, under conditions of low external calcium ion and/or long depolarizing voltages, expression of certain exogenous connexins induces plasma membrane channels and a permeability to Lucifer Yellow (Paul et al., 1991; Ebihara & Steiner, 1993; Gupta et al., 1994; Ebihara et al., 1995; Trexler et al., 1996), as does an endogenous *Xenopus* connexin (Ebihara, 1995). The endogenous plasma membrane hemichannels are regulated so as to be essentially closed under normal conditions. Apparent hemichannel currents have been recorded from other cells (DeVries & Schwartz, 1992; Malchow et al., 1993; Liu et al., 1995).

Hemichannels cannot not be constituitively open, but regulated openings are certainly possible; many channels that open in the plasma membrane under normal conditions would damage their host cells if they were inappropriately regulated (e.g., the nicotinic acetylcholine receptor). The biological function of plasma membrane connexin channels is unknown. Possibilities include roles in junction formation or sensing tissue damage.

The finding of open connexin channels in single membranes suggests that either single open hemichannels do not exist in plasma membranes (at least not for long), or that they exist and are kept closed most of the time by cytoplasmic or membrane factors not present in the reconstituted system. In the former possibility, during junctional channel formation the two hemichannels may be inserted into the plasma membrane in a coordinated fashion so that they are only briefly open to extracellular space [see Loewenstein (1981)]. For the latter case, the reconstituted system offers a way to precisely identify factors that modulate hemichannel gating.

Of what importance is the physiology of hemichannels? Biochemical, structural, and physiological data indicate that the molecular components that mediate gating transitions and sensitivities are not shared between the two hemichannels of a junctional channel, but exist in each hemichannel (Makowski et al., 1977; Unwin & Zampighi, 1980; Spray et al., 1991). In some cases, junctional channels seem to behave as if each hemichannel operates independently [cf. DeVries and Schwartz (1992)]. In teleost blastomeres, acidification of one cell of a coupled pair produces an asymmetric voltage sensitivity, as if the voltage sensor of one gate were altered and the other unaffected (Verselis et al., 1987). In experiments where mRNAs coding for two different connexins are injected into separate oocytes, the junctions that form between them behave as if they are a linear combination of two independently functioning hemichannels (Swenson et al., 1989; Werner et al., 1989; Ebihara et al., 1995). In the past, some differences in voltage sensitivity in heterotypic channels that had been attributed to structural interactions between hemichannels (Barrio et al., 1991) are now understood to arise from rectification of single channel currents that can be predicted from having two dissimilar conductance pathways in series (Suchyna et al., 1994; Bukauskas et al., 1995).

These data indicate that elucidation of the operation of hemichannels is of direct and fundamental importance to understanding the behavior of junctional channels. In those cases where there is evidence for functional interaction between hemichannels [cf. Hennemann et al. (1992) and Bruzzone et al. (1994a)], comparison with the properties of single hemichannels will identify which properties arise due to cooperativity and which are intrinsic.

Modulatory Sensitivities. The fraction of liposomes that is sucrose permeable decreases when pH is lowered to between 7.5 and 6.0. A similar effect of cytoplasmic pH on junctional conductance has been observed in several cell types [cf. Turin and Warner (1977), Spray et al. (1981, 1986a), and Spray and Bennett (1985)]. The pH sensitivity in the reconstituted system indicates that pH can act directly on connexin channels. This finding contrasts with the lack of pH sensitivity of connexin32 channels obtained from isolated junctional membranes assayed for permeability changes in the same manner (Harris et al., 1992) and other *in vitro* studies using liver junctional membrane preps (Young et al., 1987; Campos de Carvalho et al., 1992). This difference could result from the harsh conditions to which connexin is exposed during junction isolation. Alternatively, it could be that the immunopurified connexin32 is more representative of the junctional connexin32 in the tissue. Isolated junctions are a small portion of the total junctional connexin and an insoluble fraction excluded from the present immunopurification procedure. The pH-insensitive fraction of the liposomes could correspond to an *in situ* subpopulation of connexin32 that is pH-insensitive. The finding of direct action of pH on connexin channel function is consistent with recent mutagenesis experiments on connexin43 (Liu et al., 1993). The specific action of pH on connexin single-channel physiology cannot be determined from the studies reported here.

Several reports describe an absence of effect of pH on cellular junctional conductance. However, they either involve invertebrate systems (Rose & Rick, 1978; Arellano et al., 1986; Peracchia, 1990), which do not express connexin, or cells that do not express connexin32 (Pressler, 1989).

In cells, junctional conductance usually recovers fully when intracellular pH is returned to normal. We found only partial recovery of the permeability. One explanation is that the long duration at low pH (several hours during the spin) causes irreversible damage to some of the channels, especially since at the low ionic strength used the surface pH may be much lower (Aveyard & Haydon, 1973).

The negative findings in our system of several agents that affect junctional coupling in cells suggests, but does not prove, the absence of direct action of these agents on purified connexin32 channels. It should be noted that junctional channel properties can vary with cell type and developmental stage, even for the same connexin [see Spray and Bennett (1985), Noma and Tsuboi (1987), Swenson et al. (1989). Werner et al. (1989), Eghbali et al. (1990), and White et al. (1990)], so connexin32 expressed in other tissues may have different modulatory sensitivities, as one expects other connexins to have as well.

Channel Recordings. The bilayer recordings characterize the permeability of the liposomes as arising from large connexin channels. To the extent that their properties are identifiable, the channels bear some similarities to the properties of connexin26 hemichannels in bilayers in a recent report (Buehler et al., 1995). They are not inconsistent with the data reported in several other studies using protein from liver junctional membrane preps (Spray et al., 1986b; Young et al., 1987; Campos de Carvalho et al., 1991; Mazet et al., 1992). The most striking distinguishing features are the large magnitude high frequency fluctuations of the open channel currents. The fluctuations are of interest because they most likely arise from rapid transitions between closely spaced subconductance states, and alteration of the occupancies of such states is a likely locus of cellular modulation. Rapid "flicker" block is not likely given the size of the components of the bilayer buffer. That such fluctuations have not been reported for single junctional channels recorded by the dual whole-cell voltage-clamp technique is not surprising since the frequency resolution of that system is typically no greater than several hundred Hz, and our recordings were made at 15 kHz (see Materials and Methods).

Most tissues express more than one connexin isoform (White et al., 1995). The different connexins are homologous, and yet every tissue seems to contain a distinct profile of connexins. Differences in amino acid sequences undoubtedly impart distinct physiological properties to each connexin isoform. One promising way to elucidate the structure−function relationship of the connexin proteins is to examine the physiology of channels formed by purified connexins. The opportunity to explore the physiology of purified connexins under defined modifications *in vitro* will facilitate elucidation of the detailed functional properties (gating, permeability) of each connexin and how they are modulated. Since defects in connexin32 are responsible for a demyelinating peripheral neuropathy in humans (Bergoffen et al., 1993), detailed investigation of its permeability properties by the means we describe here has additional importance.

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