Single Channel Behavior of Recombinant β_2 Gap Junction Connexons Reconstituted into Planar Lipid Bilayers

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ABSTRACT The β_2 gap junction protein (Cx26) was expressed in an insect cell line by infection with a baculovirus vector containing the rat β_2 cDNA. Isolated β_2 gap junction connexons were reconstituted into planar lipid bilayers. Single channel activity was observed with a unitary conductance of 35–45 pS in 200 mM KCl. Channels with conductance values of 60 pS and 90–110 pS also coexisted with the lower conducting channel suggesting that there are channels with different conductance properties within a population of connexons. Channel activity was observed at voltages of up to 150 mV. Furthermore, the characterization of these channel properties from the β_2 connexons that were generated by this heterologous expression system has provided the basis for identifying an endogenous β_2 connexon channel in material reconstituted from native rat liver gap junctions.

INTRODUCTION

Gap junctions (GJs) form communication channels between neighboring cells (for reviews see Loewenstein, 1981; Kumar and Gilula, 1992; Beyer, 1993). GJ proteins, termed connexins, oligomerize to form GJ hemi-channels (connexons). Connexons from two adjacent cells form a double-membrane gap junction channel by association at their extracellular surfaces. Electron microscopy (EM) and x-ray diffraction data (Caspar et al., 1977; Makowski et al., 1977; Unwin and Zampighi, 1980) have provided evidence that GJs can contain large numbers of connexons. Furthermore, these large, often compact arrays of many GJ particles participate in efficient metabolic and electrical coupling, two basic functional properties of GJs in cell-cell communication (Gilula et al., 1972).

The application of the double voltage-clamp technique (Spray et al., 1979) to cells of early amphibian embryos provided evidence for a symmetric decline of transjunctional current with increasing voltage (Spray et al., 1981) for GJ channels with time constants of several hundred milliseconds (Harris et al., 1981). This characteristic voltage dependence, which is independent of the resting potential of the cell membrane, has been demonstrated subsequently for all mammalian and amphibian GJ proteins studied (Bennett et al., 1991). In insect cells, however, changes in membrane potential do affect transjunctional conductances (Obaid et al., 1983). The identification, cloning, and sequencing of 13 different genes that code for GJ proteins (Kumar and Gilula, 1992; Willecke et al., 1991) and their subsequent expression in heterologous cell systems used for the characterization of macroscopic transjunctional currents has made it possible to define a number of the electrophysiological properties for these channel proteins. Double whole cell patch-clamp recordings expanded the range of observable transjunctional currents to microscopic dimensions for which individual current steps could be resolved and correlated to the presence of active, single GJ channels (Veenstra and DeHaan, 1986; Chow and Young, 1987; Chen and DeHaan, 1992; Chanson et al., 1993).

β_2 GJ protein, or Cx26, is the smallest connexin (26,000 mol wt) among all members of the GJ multigene family, having an unusually short (13-amino-acid) COOH-terminal cytoplasmic domain (Zhang and Nicholson, 1989). The protein does not appear to be phosphorylated (Sáez et al., 1990; Traub et al., 1989), suggesting it is insensitive to kinase-mediated second messenger pathways. The dye transfer properties of the protein have been studied in rat pinealocytes (Sáez et al., 1991), and its electrophysiological properties have been characterized in *Xenopus* oocytes (Barrio et al., 1991; Rubin et al., 1992a). Interestingly, β_2 GJ channels not only show a typical symmetric steady-state current-voltage dependence, but they also exhibit a fast, voltage-dependent component that confers a slight asymmetry onto the instantaneous current-voltage relation, indicating a response to cell membrane potentials (resting membrane potential dependence). This asymmetry has been shown in *Xenopus* oocytes to influence the steady-state current properties of the slow voltage-dependent component of heterotypic Cx32/Cx26 GJs (Rubin et al., 1992b; Suchyna et al., 1993). Single-channel conductances of 40–70 pS have been reported from PC12 cells that have been transfected to express β_2 GJ protein (Spray et al., 1992).

In hepatocytes, β_2 GJ protein is expressed along with β_1 GJ protein, and both proteins have been co-localized to the same gap junctional plaque (Nicholson et al., 1987). Functional reconstitution of purified rat liver GJ channels into planar lipid bilayers has been reported (Young et al., 1987; Harris et al., 1992; Mazet et al., 1992). However, separation of β_1 from β_2 connexons during purification is quite difficult,

Received for publication 19 May 1994 and in final form 30 January 1995.
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0006-3495/95/05/1767/09 $2.00

especially if they exist as heteromeric complexes. Consequently, purified β_1 has been reconstituted in only one study (Young et al., 1987). Therefore, it is most advantageous to isolate and purify GJ proteins from a heterologous expression system that does not express endogenous vertebrate GJ proteins. For this purpose, the baculovirus expression system has been used previously to obtain large quantities of homomeric β_1 connexons (Stauffer et al., 1991).

In this study, we report for the first time the functional reconstitution of recombinant, homomeric β_2 GJ connexons into planar lipid bilayers. Connexons were produced by baculovirus infection of insect cells, enriched by sucrose step-gradient centrifugation, further purified by gel filtration chromatography, and then reconstituted in planar membranes for determination of intrinsic channel activity.

MATERIALS AND METHODS

Materials

Soybean lipid type II-S was purchased from Sigma Chemical Co. (St. Louis, MO), and synthetic lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanol amine (POPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) were purchased from Avanti Polar Lipids (Alabaster, AL). Nonionic detergents octyl-β-D-gluco-pyranoside (OG), dodecyl-β-D-maltoside (DoDM), and decyl β-D-maltoside (DeM) were purchased from Calbiochem (La Jolla, CA).

Construction and isolation of recombinant baculovirus

The rat β_2 GJ cDNA (Kren et al., 1993) was inserted into the *Bam*HI/*Bgl*II site of pVL1392. The β_2 cDNA integration into the baculovirus genome was accomplished by cotransfection of the transfer plasmid and wild-type baculovirus DNA into Sf9 cells with a calcium phosphate transfection scheme (Summers and Smith, 1987). Recombinant virus containing the β_2 cDNA and displaying the characteristic occlusion-negative plaque morphology was plaque purified three times.

β_2 expression in Sf9 and Hi5 insect cells

Insect Sf9 and Hi5 cells were cultured following the protocol of Summers and Smith (1987). Cells were grown in TNM-FM medium supplemented with 10% fetal calf serum (FCS), 2.5 μg/ml amphotericin B, and 50 μg/ml gentamicin. Batches from suspension culture of up to 1 l were infected at densities of 2×10^6/ml with recombinant virus at a multiplicity of infection of >10. Cells were harvested 65 h after infection by centrifugation at 10^3 *g* for 10 min. Cell pellets were washed once in 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.2, quickly frozen in liquid nitrogen, and stored at −20°C.

Isolation and purification of connexons

Isolation of GJs and solubilization and purification of connexons from Sf9 cells were done as described previously (Stauffer et al., 1991). A shorter procedure was sufficient when using Hi5 cell-derived membranes. Purification was performed in a single fast protein liquid chromatography (FPLC) gel filtration chromatography step. Frozen sucrose gradient-enriched GJ membrane preparations were thawed and pelleted for 15 min in a microcentrifuge. Pellets, usually 1–2 mg, were resuspended by a 5-s pulse of sonication in 0.6 ml of 100 mM glycine, 2 M NaCl, 10 mM EDTA, 10 mM dithiothreitol (DTT), pH 10, containing either 8% OG or 2% DoDM. Solubilization of membranes into its components was allowed for 2 h at 4°C. Nonsoluble particles were pelleted, and the supernatant was run over a Superose 6 column (Pharmacia, Uppsala, Sweden) preequilibrated with 50 mM HEPES, 1 M NaCl, 10 mM EDTA, 0.005% NaN_3, 10 mM DTT, pH 8.0, in 1% OG or 0.25% DoDM. Fractions containing pure β_2 protein (as examined by SDS-PAGE) in the form of connexons (negative stain EM; data not shown) were used directly for reconstitution into bilayers. Samples were pooled and concentrated in a Centricon 30 microconcentrator to obtain protein concentrations of at least 50 μg/ml. Protein yield was determined by a micro-BCA protein assay (Pierce, Rockford, IL).

Isolation and purification of GJ channels from rat liver

The preparation of rat liver plasma membranes and isolation of GJs by alkaline extraction (Hertzberg, 1984) was carried out by the procedures described in Zimmer et al. (1987). Purification of connexons by FPLC was as described above.

Analytical gel electrophoresis and immunoblotting

SDS-PAGE with 12.5% polyacrylamide gels and immunoblots were performed as described by Milks et al. (1988). Affinity-purified antibodies prepared against synthetic peptides corresponding to the cytoplasmic loop between transmembrane segments M2 and M3 for both β_1 and β_2 were used for this study. Procedures for their production have been described previously (Risek et al., 1990).

Reconstitution and single-channel recordings in planar lipid bilayers

Membranes were formed from soybean lipid or synthetic POPE/POPS (4:1) on the tip of a patch pipette as described (Suarez-Isla et al., 1983). Protein was added from detergent extracts (DoDM, DeM, or OG) to the membrane. If the detergent concentration was diluted at least fivefold below the critical micellar concentration, the bilayer stability was not affected. Alternatively, connexons were reconstituted into soybean lipid vesicles by a detergent dialysis protocol. Connexons solubilized in 1% OG were mixed with lipid and dialyzed five times against a 200X volume of buffer (200 mM KCl, 10 mM HEPES, pH 7.5) for at least 24 h. Vesicles were fused to bilayers by adding 5–10 μl of solution to the bilayer in the presence of 5 mM Ca^{2+} and a sucrose gradient (100 mM sucrose in subphase, no sucrose in pipette). Single or multilevel currents were amplified with an EPC/7 patch-clamp amplifier (List Medical, Darmstadt, Germany) and stored on tape (DAT Sony two-channel recorder; Unitrade Co., Philadelphia, PA). Channel recordings were analyzed by using pClamp acquisition and analysis software (Axon Instruments, Foster City, CA) on a Gateway 2000 486/50DX computer. Mean conductance values for single channels were obtained from Gaussian fits of all points amplitude histograms. Alternatively, channel transitions were analyzed visually allowing for a few millisecond plateaus on either side of the transition for an event to be included. Sampling rate was 0.1 ms at 2 kHz low pass filtering (corner frequency, 8-pole Bessel filter, Frequency Devices, Haverhill, MA). Current recordings ranged from several seconds to minutes (longest channel activity was 30 min).

RESULTS

GJ expression in cultured insect cells

GJs were generated by infecting insect cells with a baculovirus containing the cDNA for rat liver β_2 protein. GJ structures were isolated from membranes that contained homo-oligomeric complexes of β_2 GJ protein. Gel filtration chromatography of detergent-treated junctional membrane fractions was used to obtain enriched preparations of β_2 GJ

protein as determined by analytical gel electrophoresis and immunoblotting (Fig. 1 *A*). In addition, comparison of the elution volume of β_2 with molecular weight markers and with similar preparations of β_1 connexin (Stauffer et al., 1991) was consistent with the majority of the protein present as connexons rather than whole channels. Individual single membrane 8.5-nm connexons were observed in these preparations by EM analysis (data not shown). Rat liver GJ preparations (Fig. 1 *B*) were analyzed by immunoblotting for the presence of β_1 and β_2 GJ proteins.

Single-channel activity of recombinant β_2 connexons

When β_2 connexons were reconstituted from detergent extracts into planar bilayers, channel activity was observed randomly after the addition of protein extract to the membrane. Resultant single-channel currents were analyzed. Fig. 2 *A* contains a current recording of β_2 connexons derived from Sf9 insect cells exhibiting a single-channel conductance of 40.4 ± 5.8 pS (n = 51; n is number of transitions). Fig. 2 *B* shows a single-channel recording of β_2 connexons that was obtained from a Hi5 insect cell preparation, with a major conducting unit of 45.8 ± 4.8 pS (n = 22). A higher conductance of 61.8 ± 5.6 pS (n = 17) appeared with various frequencies in most recordings, examples of which are given in Fig. 2 *A* (bottom trace) and Fig. 2 *B* (second trace). Only one open channel was observed at any given time, possibly as the result of the low open probability of the observed channels. However, even during periods of increased channel activity (see burst of the 46-pS channel; Fig. 2 *B*, top trace; open probability = 0.52), no additional channel opened, suggesting that one single β_2 connexon may occupy one (40 pS) or the other (60 pS) of two distinctly different conducting levels. All points amplitude histograms (Fig. 2 inset) illustrate the noise in the closed state caused by brief openings (Fig. 2 *B*) and the variability in the open level of the channel shown in Fig. 2 *A*.

Channels with single-channel conductances of 90 pS and higher coexisted sporadically with the 40-pS and 60-pS channels. Fig. 3 contains an example of one such recording that lasted 45 s and contained both a 35.6 ± 4.2-pS (n = 46) and a 104.0 ± 6.8-pS (n = 4) channel. The 104-pS channel had a low open probability of 0.006 as compared with the 35-pS channel with an open probability of 0.24. However, the low open probability of the 104-pS channel does not allow one to distinguish between two open states of the same channel or two independent channels.

As reducing conditions (with DTT) during isolation and purification of GJ channels were reported to facilitate the dissociation of GJs into connexons (Stauffer et al., 1991), the presence of nondissociated, full GJ channels is expected to be minimal in the starting material (although reassembly of connexons into full channels during reconstitution might occur). Fig. 2 contains single-channel recordings of recombinant β_2 connexons isolated and purified in the presence (Fig. 2 *A*) or absence (Fig. 2 *B*) of the reducing agent DTT. Omitting the reducing agent during channel preparation generally reduced the yield of connexons (measured as amount of protein), but it had no detectable effect on channel conductance.

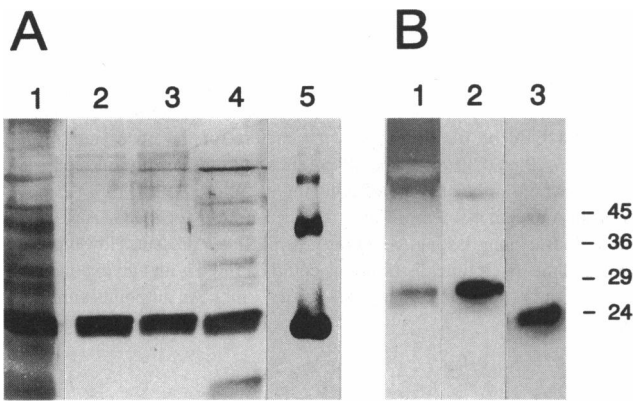

FIGURE 1 β_2 GJ protein purification. (*A*) SDS-PAGE of FPLC-purified, recombinant β_2 GJ protein. (*Lane 1*) Silver-stained gel lane of total plasma membrane fraction from Sf9 insect cells infected with baculovirus containing the β_2 rat liver cDNA; (*lanes 2–4*) silver-stained gel lanes of FPLC eluate fractions (peak) containing purified β_2 GJ protein; (*lane 5*) immunoblots of total plasma membrane fraction treated with β_2J antibodies showing mono-, di-, and trimeric forms of solubilized protein. (*B*) FPLC-purified rat liver GJ protein. (*Lane 1*) Silver-stained sample; (*lane 2*) immunoblot of sample treated with β_1S antibodies; (*lane 3*) immunoblot of sample treated with β_2J antibodies. Molecular mass markers on the right are given in kDa.

β_2 channel component of rat liver GJs

Single-channel recordings (Fig. 4) of recombinant, homomeric β_2 connexons derived from the heterologous baculovirus expression system were compared with those obtained from purified rat liver connexons. Single-channel conductances of 35–45 pS were observed for connexons derived from rat liver junctional membranes (Fig. 4 *B*) as well as from β_2-infected Sf9 insect cells (Fig. 4 *A*). This suggests that β_2-containing connexons from rat liver GJs were successfully reconstituted into planar lipid bilayers. However, the majority of channel conductances (65%) observed in 200 mM KCl with rat liver connexons were in the range of 100 pS and larger, consistent with previously reported conductance values for β_1 connexin in planar lipid bilayers (Young et al., 1987; Harris et al., 1992) and in cell pairs (Moreno et al., 1991; Spray et al., 1992). A single-channel recording of a 150-pS channel is shown in Fig. 4 *C*. The brief openings of less than 10 ms are typical for this channel type. Longer openings in the range of several hundreds of milliseconds or seconds have not been observed, in contrast to such long lasting openings of the 40-pS channel.

Connexon unitary conductances

The distribution of averaged conductances from individual single-channel recordings obtained with connexon channels derived from both insect cells and rat livers shown is shown in Fig. 5. Fig. 5 *A* shows a histogram of mean γ_i values for

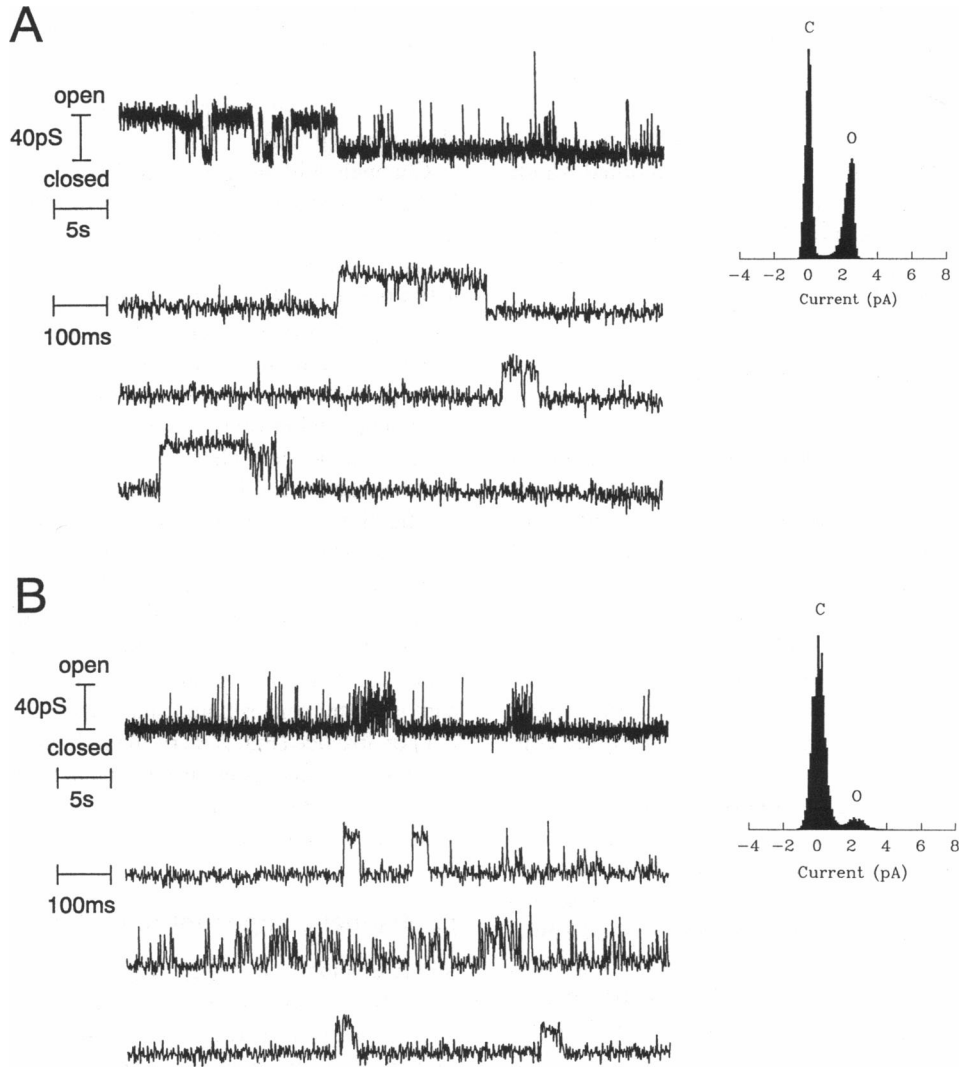

FIGURE 2 Single-channel recordings and open probability of β_2 *connexons*. Single-channel recordings were obtained in symmetric 200 mM KCl solution at +50 mV. A total of 45 s is shown in the top panels (acquisition rate, 1 ms). Three recordings on an expanded time scale of 1 s each show complete channel transitions between closed and open states at a high time resolution (acquisition rate, 0.1 ms). (*A*) β_2 connexons from Sf9 insect cells in OG were reconstituted into POPE/POPS (4:1) membranes. The bathing buffer contained 200 mM KCl, 10 mM HEPES, 5 mM $MgCl_2$ and 5 mM EGTA, pH 7.5. The channel was initially in its open state, interrupted by brief closings. After 16 s, the channel switched to a mainly closed state with an open probability of <0.15. Open dwell times resided in the millisecond range. Dwell time distributions could be fitted with two parameters for the open state (τ_{o1} = 0.44 ± 0.01 ms; τ_{o2} = 3.32 ± 0.29 ms) and closed state (τ_{c1} = 0.43 ± 0.01 ms; τ_{c2} = 7.29 ± 0.50 ms). The all points amplitude histogram represents 45 s of recording as shown in the upper trace. (*B*) β_2 connexon from Hi5 insect cells purified in the absence of reducing agent (without DTT). Channels were reconstituted from OG into soybean lipid membranes. The bathing solution was as in A except that 1 mM $CaCl_2$ replaced 5 mM $MgCl_2$/EGTA. Channel conductance was 45.8 ± 4.8 pS, and, less frequently, 61.8 ± 5.6 pS. There were no periods of long lasting (>1 s) open dwell times present. However, two bursts of the 46-pS channel are apparent. The open probability during the burst is 0.52, and the dwell time distributions could be fitted with two exponentials for the open state (τ_{o1} = 0.43 ± 0.04 ms; τ_{o2} = 3.38 ± 3.24 ms) and the closed state (τ_{c1} = 0.59 ± 0.01 ms; τ_{c2} = 8.34 ± 2.53 ms). An all points amplitude histogram represents the first 20 s of recording (*upper trace*) excluding the two bursts.

recombinant β_2 GJ connexons. The peak conductance was 35–45 pS in 200 mM KCl referred to here as the unitary conductance of the recombinant, homomeric β_2 connexon. The broad distribution between 20 and 60 pS, however, shows an inherent heterogeneity within a population of β_2 connexons. Single-channel conductances in 200 mM NaCl were 24.4 ± 1.3 pS (n = 2; n is number of membranes), 41.9 ± 0.8 pS (n = 2), and 63.0 ± 4.9 pS (n = 4). Mean conductance values were determined from short periods (up to 1 min) of single-channel activity that exhibited only one open level. The total conductance range observed for rat liver connexons is shown in Fig. 5 *B*. Clearly, there is a broader variability of conductances found, possibly reflecting the more heterogeneous composition of rat liver connexons as compared with the more homogeneous insect cell-derived material. The control histogram (Fig. 5 *C*) plots the frequencies of mean conductance values found when reconstituting plasma membrane fractions that have not been purified by gel chromatography from noninfected Sf9 insect cells. Peak conductance of the channels in the control fraction was 10 to 20 pS.

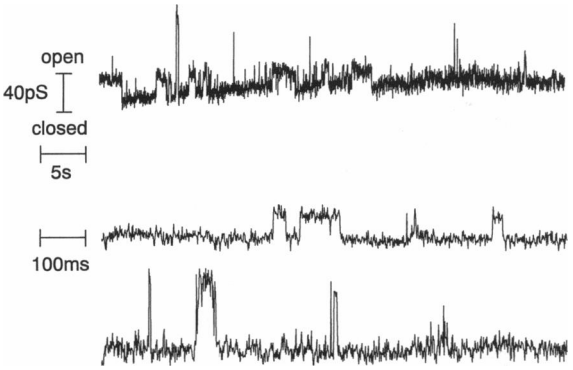

FIGURE 3 Conductance heterogeneity. Current recording showing the coexistence of two different channels with conductances of 35.6 ± 4.2 pS and 104.0 ± 6.8 pS, respectively. The upper trace shows 45 s of recording. The two lower traces are selected stretches on an expanded time scale (50×) showing (from top to bottom) the 40-pS channel and the 110-pS channel. Homomeric β_2 connexons were solubilized and purified in OG from Sf9 insect cells and reconstituted into POPE/POPS (4:1) membranes. The bathing buffer contained 200 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, and 10 mM HEPES, pH 7.4. Channel activity was recorded at +110 mV. The probability to see both channels open simultaneously is 0.0014. This corresponds to 63 ms of 45 s of recording.

Channel gating

Single-channel open probabilities for recombinant β_2 connexons were plotted as a function of voltage (Fig. 6 *A*). Channel activity was measured at continuously clamped membrane potentials to measure open probabilities under steady-state conditions. A linear regression analysis for data at positive voltages (in the range +40 to +150 mV) gives a correlation coefficient $r = -0.049$, indicating no dependence of the open probability on voltage in this higher voltage range. However, some voltage dependence is evident by the absence of channel activity at low voltages. Two experiments with rat liver channels (40 pS) showed open probabilities of 0.32 (+70 mV) and 0.18 (+80 mV), which lie within the range of open probabilities observed for recombinant β_2 connexons. On the basis of the occurrence of random open probability values for recombinant connexons, there is insufficient data for making any conclusions regarding the voltage-dependent behavior of rat liver connexons.

The distribution of mean open dwell times versus voltage for β_2 connexons is plotted in Fig. 6 *B*. A linear regression analysis of τ_{o1} versus voltage (closed symbols) gives a slope of almost zero ($r = 0.694$), confirming the finding for open probabilities that there is no voltage dependence over the range measured (−100 mV to +150 mV). In 9 of 56 membranes, channel recordings showed a conditional open probability, i.e., a decrease in open probability with time. An example is given in Fig. 2 *A*. The channel is in its open state and closes after approximately 20 s, showing brief openings as does the channel shown in Fig. 2 *B*. The open probability changed from approximately 0.8 to less than 0.15. However, such decreases in open probabilities were not reversible by clamping the voltage to low values (<30 mV).

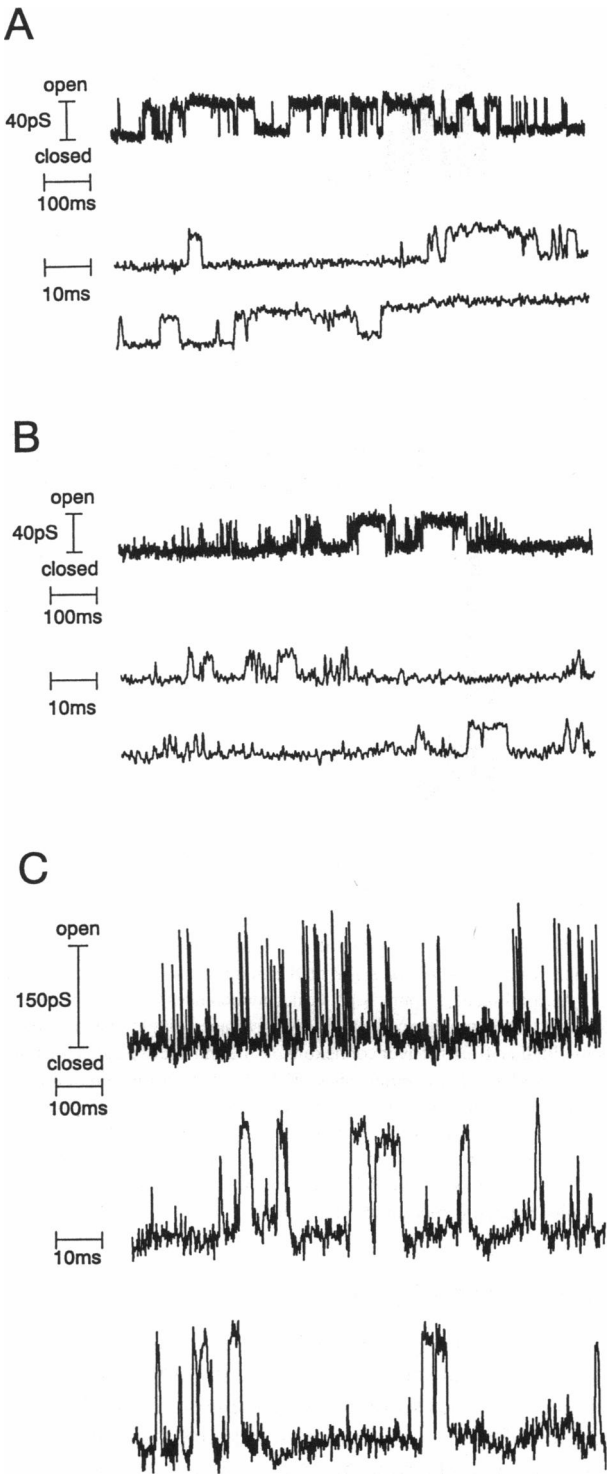

FIGURE 4 Identification of rat liver GJ channel activity on the basis of recombinant channel activity. All panels contain a 1-s recording (top) and two 100-ms stretches (below) on an expanded time scale. (*A*) Single-channel activity from Hi5 insect cell-derived β_2 connexons obtained in 200 mM KCl at +80 mV. (*B*) Single-channel activity from rat liver-derived GJ channels in 200 mM KCl at +80 mV. The channel is indistinguishable from the recording in A. (*C*) Single-channel activity from rat liver-derived GJ channels in 200 mM KCl at +110 mV. Channel conductance of 150 pS is clearly distinct from recombinant β_2 connexon activity.

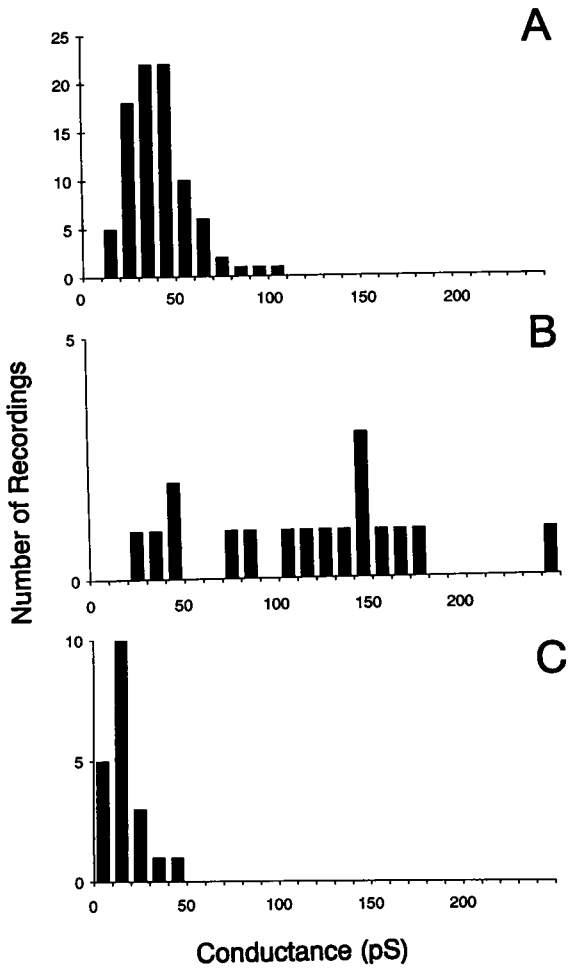

FIGURE 5 Distribution of mean conductance values of β_2 *single channels.* (*A*) Conductance histogram of averaged single-channel conductances obtained from 56 membranes (32 of 87 membranes had no activity) containing recombinant β_2 connexons. Peak conductances were 35–45 pS in 200 mM KCl. (*B*) Conductance histogram of averaged single-channel conductances obtained from rat liver connexons. (*C*) Control Sf9 plasma membrane preparations had a peak conductance of 10–20 pS. Control membranes were derived from noninfected insect cells. For these controls, plasma membrane preparations that have not been purified by gel filtration chromatography were reconstituted. Conductance values were either determined from Gaussian fits to all points amplitude histograms or as the arithmetic mean ± SD of single transitions measured with a ruler.

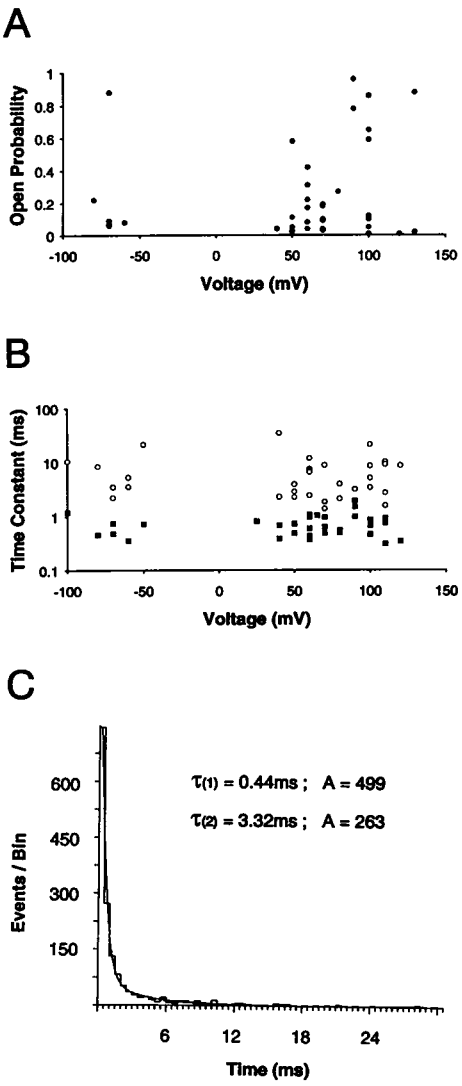

FIGURE 6 Correlation of single-channel open probability and open mean time with voltage. (*A*) Distribution of channel open probabilities as a function of the membrane potential. Open probabilities were determined from amplitude histograms fitted with two Gaussians. Each data point represents a single-channel recording from a different membrane. Note the absence of channel activity at low membrane potentials. (*B*) Distribution of open mean time parameters τ_1 (*closed symbols*) and τ_2 (*open symbols*) as a function of membrane potentials. The first time constant averages 79%, with the lowest value 52% and the highest 98%. (*C*) Open time histogram of a recombinant β_2 connexon at +50 mV (from experiment shown in Fig. 2 *A*; the actual recording used for dwell time analysis lasted 145 s, thus including more data than represented in the upper trace). The amplitude of τ_1 and τ_2 are 65% and 35%, respectively.

Intrinsic channel properties

Changing various parameters during recombinant β_2 connexon reconstitution as summarized in Table 1 showed that channel properties were not dependent on them. Using both Sf9 and Hi5 insect cell lines gave indistinguishable results in terms of channel properties and average yield of channel activity. The effects of three detergents, OG, DoDM, and DeM, on channel reconstitution were examined. All three are nonionic detergents and suitable for reconstitution of proteins into planar lipid bilayers when adding microliter quantities of protein/detergent extracts to the subcompartment of a membrane without affecting its stability. Furthermore, these nonionic detergents were equally effective in the yield of reconstituted channel activity. However, for vesicle reconstitution, a detergent such as OG was used as it has a higher critical micellar concentration (0.58%) than DoDM (cmc ~0.007%) and it can be removed easily by detergent dialysis. DoDM was preferentially used for optimal solubilization of junctional membranes, and it was subsequently exchanged with OG during gel filtration followed by dialysis. Subsequently, proteoliposomes were fused to preformed planar lipid bilayers. The channel activities that were obtained were indistinguishable from those reconstituted from detergent extracts. Similarly, adding 10–20% cholesterol, a

TABLE 1 Effect of experimental conditions on the appearance of ion channel activity in planar lipid bilayers

Parameter	% membranes with single channel activity*
GJ expressed in:	
Hi5 insect cell	55 (N=39)
Sf9 insect cell	50 (N=28)
GJ plaques solubilized in:	
OG	57 (N=27)
DoDM	44 (N=20)
DeM	50 (N=16)
Reconstitution procedure:	
Detergent extract	47 (N=54)
Proteoliposome fusion	70‡ (N=13)
Membrane lipid composition:	
POPE/POPS	47 (N=46)
POPE/POPS/Cholesterol	55 (N=11)

*Channel activity was detected in 52% of all experiments.
‡Independent parameters, when analyzed separately and compared pairwise (e.g., fusion versus subphase addition of detergent extracts), fulfilled the criterion of the null hypothesis of the Fisher exact test. Therefore, variations in channel activity observed as listed in the table are not statistically significant.

major lipid component in native GJ plaques (Malewicz et al., 1990), to the planar membranes did not significantly affect the yield of channel activity or the properties observed.

DISCUSSION

Connexon channel activity

In this study, it has been possible to demonstrate that the recombinant β_2 connexons form ion channels across phospholipid bilayers. Both the unitary conductance of 35–45 pS (in 200 mM KCl) and open probabilities of less than 0.4 over a voltage range of −100 to +150 mV were independent of the reconstitution procedure, lipid composition, or physical state of the membrane. The approach used in this study is unique in that it was possible to determine the conductance properties of recombinant, homomeric connexons in the absence of other mammalian GJ proteins. This is important because the isolation of pure β_2 connexin from tissue sources is difficult as tissues often contain more than one type of GJ protein. Consequently, the observation of a channel conductance from rat liver GJ membranes that is indistinguishable from recombinant material suggests that there are β_2-containing connexons in native rat liver that can be functionally reconstituted in planar lipid bilayers. In addition, another channel conductance was observed in rat liver material that is likely to be related to the β_1 channel activity as reported previously (Young et al., 1987; Harris et al., 1992).

An insect cell line-based expression system was used to successfully produce β_2 GJ protein in the absence of other vertebrate connexins. Detergent solubilization and gel filtration chromatography were applied to produce an enriched connexon preparation as analyzed by silver staining of SDS-PAGE. Although we cannot exclude the possibility of contamination in these preparations with insect cell proteins, these are unlikely to be responsible for the observed channel activity as control membranes from the noninfected insect cells did not show these activities. In addition, immunoblot analysis with antibodies to α_1 (Cx43), α_3 (Cx46), β_1 (Cx32), and β_3 (Cx31) connexins were negative (data not shown), indicating the absence of these connexins in the β_2 connexon preparations used.

Connexon conductance variability

The full range of single-channel conductances observed for β_2 connexons was 20–110 pS, with the majority of events ranging from 20–60 pS. This functional heterogeneity suggests that there is probably some structural heterogeneity. Conductance heterogeneity for recombinant, homomeric β_2 connexons can be interpreted in at least two ways. First, single-channel recordings may contain two different and independent channels (Fig. 3). This observation may be explained by a structurally heterogeneous population of connexons. For example, there may exist more than one stable open state (40 pS and 110 pS) for two simultaneously active identical oligomeric complexes or the connexon population may contain a mixture of oligomers that differ in their degree of post-translational modifications or the number of subunits in the oligomer. Second, no doublet activity was observed in the recordings in Fig. 2. Thus, in these cases, it seems likely that, instead of two channel units of different structure being active, only one active connexon was present in the membrane, being able to open randomly from its closed state(s) into one of two possible open states (40 and 60 pS). As no direct transitions between 40- and 60-pS levels have been detected, the lower conducting channel is unlikely to be a substate of the larger channel (Fox, J.A., 1987). Finally, the conductance heterogeneity in rat liver preparations could result from the possible existence of heteromeric connexons composed of β_2 and β_1 subunits. As reassembly of connexons into full channels during reconstitution cannot be completely excluded, both homotypic and heterotypic channels may, in addition, contribute to the observed conductance heterogeneity of rat liver connexons.

The connexon as a functional channel unit

GJ channels are unique because they span two membranes in vivo. Therefore, a discussion of conductance variability should take into account the transition from a connexon (hemi-channel) to a GJ channel (full channel). If a GJ channel were to exist in single planar lipid bilayers, it would be predicted to have a conductance comparable with those reported from cell pair studies (which was observed in this study) as well as a symmetric decrease in current with increasing membrane potential of either polarity (which was not observed in this study). A hemi-channel, however, is expected to yield a larger conductance (ideally twice as large) compared with a single full channel in cell pairs (which we find, but connexon conductances of 70–90 pS constitute a minor fraction of channel activity) and an asymmetric rectification during which the channel remains open mainly at one polarity (open probability ideally of 1) and shows decreasing open probability with increasing voltage at the opposite polarity (which

we do not find). The last argument assumes that all connexons are incorporated into the membrane in the same orientation, as the correlation of open probabilities and mean open times with membrane potential includes single-channel recordings from different experiments. Although the connexons were purified by FPLC gel filtration and selected according to the molecular weight of the channel complex, it cannot be unequivocally excluded that full channels are present in our preparation (e.g., by reassociation of connexons into full channels in the bilayer). As the voltage-gating properties of reconstituted connexons differ from observed transjunctional current-voltage relations in cell pairs and do not show the expected hemi-channel voltage-gating behavior (Harris et al., 1981), the observed voltage dependence cannot be used to discriminate hemi-channel from full channel reconstitution. However, it might be taken as evidence against the presence of full channels after reconstitution into bilayers. Finally, the finding of a similar value for connexon conductance as compared with single-channel conductance in cell pairs (double connexons) will be addressed in the following paragraph.

The connexon single channel conductances described here could be explained adequately by considering nonlinear electrical properties of the connexon with respect to the full channel. Indeed, we may assume that the open channel resistance does not simply depend on changes in pore dimension when separating GJs into halves. To understand the structure-function relationship for β_2 connexons (and other GJ types), it is important to consider differences that exist in the channel architecture of the two GJ channel forms, i.e., as a hemichannel (connexon) or a full channel (double connexon). Furthermore, special consideration should be given to the relative distribution of fixed charges within the pore. Connexons have been predicted earlier to contain a pair of charged rings contributed by the sequence motif K(R)-X-X-X-E in transmembrane segment M3 (Milks et al., 1988). Chen and Eisenberg (1993) have calculated current-voltage relations for single open channels of one conformation with a known distribution of permanent charge using the Poisson-Nernst-Planck (PNP) theory. When applied to the double and single connexons discussed here (Chen, Buehler, and Eisenberg, personal communication), the PNP theory predicts single open channel conductances quite close to those measured, because the resistance of a channel in this theory (for these parameters) is not a simple function of channel length. Rather, it is determined chiefly by the distribution of potential and concentration in the channel's pore and at its interface with the bathing solutions. GJ channels are a potentially suitable system to test the PNP theory once hemichannel and full channel conductances can be measured under conditions in which the transition from full channel to hemi-channel can be controlled. A more detailed analysis will be submitted separately in the future.

Disrupting the protein-protein interaction in an in vitro system and incorporation into a nonnative membrane environment may result in the unique channel behavior of β_2 connexons. Channel properties of connexons can depend on the type of the paired connexon as shown in *Xenopus* oocyte double voltage clamp experiments (Rubin et al., 1992b; Suchyna et al., 1993; Bruzzone et al., 1994), supporting the notion that protein-protein interaction in junctional membranes contributes to novel channel properties. In heterotypic β_1/β_2 junctions, the β_1 connexon gating properties are obscured in favor of the fast responding voltage gate of the β_2 connexon (mainly because of their opposite voltage-gating polarities; Verselis et al., 1994). The slow, voltage-dependent decrease in junctional current in these heterotypic junctions is different from the β_1 or β_2 channels in homotypic junctions. A slight dependence of transjunctional gating properties on connexon interaction is therefore evident. However, for other GJ channels (Bruzzone et al., 1993), the transjunctional current-voltage relationship is related to each connexon, with each connexon behaving as an independent voltage-gated unit. Understanding the connexon as a unique functional unit and comparing its properties with that of full GJ channels will eventually help us to understand how the properties of two connexons can be influenced by their association across the extracellular gap (White et al., 1994). The application of a reconstitution system with the capacity to utilize specific recombinant-derived connexons may ultimately lead to the biophysical characterization of GJ channels that can complement studies involving cell pairs.

We thank A. F. Schinder for helpful discussion of the manuscript, R. Safarik and J. B. Miller for their technical support for insect cell cultures and protein purification, and Linda Callahan for her secretarial assistance.
This work was supported by National Institutes of Health grants GM 37904 to N.B.G. and GM 37907 to N.B.G. and N.M.K. and by a grant from the Lucille P. Markey Charitable Trust.

REFERENCES

Barrio, L. C., T. Suchyna, T. Bargiello, L. X. Xu, R. S. Roginski, M. V. L. Bennett, and B. J. Nicholson. 1991. Gap junctions formed by connexins 26 and 32 alone and in combination are differently affected by applied voltage. *Proc. Natl. Acad. Sci. USA.* 88:8419–8414.

Bennett, M. V. L., L. C. Barrio, T. A. Bargiello, D. C. Spray, E. Hertzberg, and J. C. Sáez. 1991. Gap junctions: new tools, new answers, new questions. *Neuron.* 6:305–320.

Beyer, E. C. 1993. Gap junctions. *Int. Rev. Cytol.* 137C:1–37.

Bruzzone, R., J.-A. Haefliger, R. L. Gimlich, and D. L. Paul. 1993. Connexin 40, a component of gap junctions in vascular endothelium, is restricted in its ability to interact with other connexins. *Mol. Biol. Cell.* 4:7–20.

Bruzzone, R., T. W. White, and D. L. Paul, 1994. Expression of chimeric connexins reveals new properties of the formation and gating behavior of gap junction channels. *J. Cell Sci.* 107:955–967.

Caspar, D. L. D., D. A. Goodenough, L. Makowski, and W. C. Phillips. 1977. Gap junction structures. I. Correlated electron microscopy and x-ray diffraction. *J. Cell Biol.* 74:605–628.

Chanson, M., K. J. Chandross, M. E. Rook, J. A. Kessler, and D. C. Spray. 1993. Gating characteristics of steeply voltage-dependent gap junction channels in rat Schwann cells. *J. Gen. Physiol.* 102:925–946.

Chen, Y., and R. L. DeHaan. 1992. Multiple-channel conductance states and voltage regulation of embryonic chick cardiac gap junctions. *J. Membr. Biol.* 127:95–111.

Chen D. P., and R. S. Eisenberg. 1993. Charges, currents, and potentials in ionic channels of one conformation. *Biophys. J.* 64:1405–1421.

Chow, I., and S. H. Young. 1987. Opening of single gap junction channels during formation of electrical coupling between embryonic muscle cells. *Dev. Biol.* 122:332–337.

Fox, J. A. 1987. Ion channel subconductance states. *J. Membr. Biol.* 97:1–8.

Gilula, N. B., O. R. Reeves, and A. Steinbach. 1972. Metabolic coupling, ionic coupling and cell contacts. *Nature.* 235:262–265.

Harris, A. L., D. C. Spray, and M. V. L. Bennett. 1981. Kinetic properties of a voltage-dependent junctional conductance. *J. Gen. Physiol.* 77:95–117.

Harris, A. L., A. Walter, D. A. Goodenough, and J. Zimmerberg. 1992. Ion channels in single bilayers induced by rat connexin 32. *Mol. Brain Res.* 15:269–280.

Hertzberg, E. L. 1984. A detergent independent procedure for the isolation of gap junctions from rat liver. *J. Biol. Chem.* 259:9936–9943.

Kren, B. T., N. M. Kumar, S. Wang, N. B. Gilula, and C. J. Steer 1993. Differential regulation of multiple gap junction transcripts and proteins during rat liver regeneration. *J. Cell Biol.* 123:707–718.

Kumar, N. M., and N. B. Gilula. 1992. Molecular biology and genetics of gap junction channels. *Semin. Cell Biol.* 3:3–16.

Loewenstein, W. R. 1981. Junctional intercellular communication: the cell-to-cell membrane channel. *Physiol. Rev.* 61:829–913.

Makowski, L., D. L. D. Caspar, W. C. Phillips, and D. A. Goodenough. 1977. Gap junction structures. II. Analysis of the x-ray diffraction data. *J. Cell Biol.* 74:629–645.

Malewicz, B., V. V. Kumar, R. G. Johnson, and W. J. Baumann. 1990. Lipids in gap junction assembly and function. *Lipids.* 25:419–427.

Mazet, J.-L., T. Jarry, D. Gros, and F. Mazet. 1992. Voltage dependence of liver gap-junction channels reconstituted into liposomes and incorporated into planar bilayers. *Eur. J. Biochem.* 249:249–256.

Milks, L. C., N. M. Kumar, N. Houghten, N., Unwin, and N. B. Gilula. 1988. Topology of the 32-kDa liver gap junction protein determined by site-directed antibody localization. *EMBO J.* 7:2967–2075.

Moreno, A. P., B. Eghbali, and D. C. Spray. 1991. Connexin 32 gap junction channels in stably transfected cells: equilibrium and kinetic properties. *Biophys. J.* 60:1267–1277.

Nicholson, B., R. Dermietzel, D. Teplow, O. Traub, K. Willecke, and J.-P. Revel. 1987. Two homologous protein components of hepatic gap junctions. *Nature.* 329:732–734.

Obaid, A. L., S. Socolar, and B. Rose. 1983. Cell-to-cell channels with two independently regulated gates in series: analysis of junctional conductance modulation by membrane potential, calcium and pH. *J. Membr. Biol.* 73:69–89.

Risek, B., S. Guthrie, N. Kumar, and N. B. Gilula. 1990. Modulation of gap junction transcript and protein expression during pregnancy in the rat. *J. Cell Biol.* 110:269–282.

Rubin, J. B., V. K. Verselis, M. V. L. Bennett, and T. A. Bargiello. 1992a. A domain substitution procedure and its use to analyze voltage dependence of homotypic gap junctions formed by connexins 26 and 32. *Proc. Natl. Acad. Sci. USA.* 89:3832–3824.

Rubin, J. B., V. K. Verselis, M. V. L. Bennett, and T. A. Bargiello. 1992b. Molecular analysis of voltage dependence of heterotypic gap junctions formed by connexins 26 and 32. *Biophys. J.* 62:183–195.

Sáez, J. C., V. M. Berthoud, R. Kadle, O. Traub, B. J. Nicholson, M. V. L. Bennett, and R. Dermietzel. 1991. Pinealocytes in rats: connexin identification and increase in coupling caused by norepinephrine. *Brain Res.* 568:265–275.

Sáez, J. C., A. C. Nairn, A. J. Czernik, D. C. Spray, E. L. Hertzberg, P. Greengard, and M. V. L. Bennett. 1990. Phosphorylation of connexin 32, a hepatocyte gap-junction protein, by cAMP-dependent protein kinase, protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II. *Eur. J. Biochem.* 192:263–273.

Spray, D. C., A. L. Harris, and M. V. L. Bennett. 1979. Voltage dependence of junctional conductance in early amphibian embryos. *Science.* 204: 432–434.

Spray, D. C., A. L. Harris, and, M. V. L. Bennett. 1981. Equilibrium properties of a voltage-dependent junctional conductance. *J. Gen. Physiol.* 77:77–93.

Spray, D. C., A. P. Moreno, B. Eghbali, M. Chanson, and G. I. Fishman. 1992. Gating of gap junction channels as revealed in cells stably transfected with wild type and mutant connexin cDNAs. *Biophys. J.* 62:48–50.

Stauffer, K. A., N. M. Kumar, N. B. Gilula, and N. Unwin. 1991. Isolation and purification of gap junction channels. *J. Cell Biol.* 115:141–150.

Suarez-Isla, B. A., K. Wan, J. Lindstrom, and M. Montal. 1983. Single channel recordings from purified acetylcholine receptors reconstituted in bilayers formed at the tip of patch pipettes. *Biochemistry.* 22:2319–2323.

Suchyna, T. M., L. X. Xu, F. Gao, C. R. Fourtner, and B. J. Nicholson. 1993. Identification of a proline residue as a transduction element involved in voltage gating of gap junctions. *Nature.* 365:847–849.

Summers, M. D., and G. E. Smith. 1987. A manual of methods of baculovirus vectors and insect cell culture procedures. *Tex. Agri. Exp. St. Bull.* 1555.

Traub, O., J. Look, R. Dermietzel, F. Bruemmer, D. Hulser, and K. Willecke. 1989. Comparative characterization of the 21kD and 26kD gap junction proteins in murine liver and cultured hepatocytes. *J. Cell Biol.* 108:1039–1050.

Unwin, P. N. T., and G. Zampighi. 1980. Structure of the junction between communicating cells. *Nature.* 283:545–549.

Veenstra, R. D., and R. L. DeHaan. 1986. Measurement of single channel currents from cardiac gap junctions. *Science.* 233:972–974.

Verselis, V. K., C. S. Ginter, and T. A. Bargiello. 1994. Opposite voltage gating polarities of two closely related connexins. *Nature.* 368:348–351.

White, T. W., R. Bruzzone, D. A. Goodenough, and D. L. Paul. 1994. Voltage gating of connexins. *Nature.* 371:208–209.

Willecke, K., H. Hennemann, E. Dahl, S. Jungbluth, and R. Heynkes. 1991. The diversity of connexin genes encoding gap junctional proteins. *Eur. J. Cell Biol.* 56:1–7.

Young, J. D., Z. A. Cohn, and N. B. Gilula. 1987. Functional assembly of gap junction conductance in lipid bilayers: demonstration that the major 27kd protein forms the junctional channel. *Cell.* 48:733–743.

Zhang, J.-T., and B. J. Nicholson. 1989. Sequence and tissue distribution of a second protein of hepatic gap junctions, Cx26, as deduced from its cDNA. *J. Cell Biol.* 109:3391–3401.

Zimmer, D. B., C. R. Green, W. H. Evans, and N. B. Gilula. 1987. Topological analysis of the major protein in isolated intact rat liver gap junctions and junction-derived single-membrane structures. *J. Biol. Chem.* 262:7751–7763.