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# Channel Reconstitution in Liposomes and Planar Bilayers with HPLC-Purified MIP26 of Bovine Lens

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**Summary.** The major intrinsic protein (MIP26) of bovine lens membranes, purified by HPLC, was incorporated into liposomes and planar bilayers. Permeability of MIP26 channels was studied in liposomes by a spectrophotometric osmotic-swelling assay, and channel electrical properties were monitored in planar bilayers following liposome fusion. Particle formation in liposomes was determined by freeze fracture. MIP26 channels were permeable to KCl and sucrose. In planar bilayers, channel-conductance transitions were observed only after addition of liposomes to both chambers and with voltages greater than ±20 mV. Channel open probability decreased progressively as voltage increased, and an open probability of 50% was at 60–80 mV, indicating that the channels are voltage dependent. Histograms of single-channel current amplitudes at 80 mV showed a Gaussian distribution that peaked at 10 pA (~120 pS), after subtraction of 1 pA baseline current. Frequency distributions of open and closed times at 80 mV were single exponential functions with time constants of 0.13 and 1.9 sec, respectively. Open time constants ranged from 0.1 to 0.3 sec, and closed time constants ranged from 1 to 7 sec. $Cs^+$ did not decrease conductance, but reduced mean open time from 0.2 to 0.038 sec and mean closed time from 1.5 to 0.38 sec. The increase in channel flickering with $Cs^+$ occurred in bursts. TEA affected neither conductance nor kinetics. Channel events were also observed in $Na^+$ solutions (zero $K^+$). These data indicate that MIP26 channels are not $K^+$-selective channels. Channel characteristics such as: permeability to molecules larger than small ions, conductance greater than 100 pS, long open and closed time constants, etc., are similar to those of gap junction channels.

**Key Words** lens · MIP26 · planar bilayers · reconstitution · single-channel recording · gap junctions · ion channels

## Introduction

The eye lens, being an avascular tissue with a narrow extracellular space and transport enzymes restricted almost entirely to the most superficial cells, requires an extensive network of cell-to-cell communication for bidirectionally transferring ions and metabolites between deep and superficial fiber cells. Fiber-to-fiber communication is provided by junctions with a structure similar to gap junctions that occupy over 50% of the fiber membrane surface (Benedetti et al., 1976).

The major intrinsic protein (MIP26) of lens fiber cells is a 28.2-kD component (Gorin et al., 1984) that represents over 60% of the membrane proteins (Benedetti et al., 1976). The amino acid sequence of MIP26 has been inferred from cDNA cloning by Gorin et al. (1984) and found to be extremely hydrophobic. The folding probability model of the protein depicts six traverses of the lipid bilayer and places both carboxyl and amino termini at the cytoplasmic surface of the membrane (Gorin et al., 1984).

In recent years, MIP26 fractions of different degrees of purity have been successfully incorporated into artificial lipid systems, and the functional properties of the resulting channels have been described (Girsch & Peracchia, 1985; Gooden et al., 1985; Nikaido & Rosenberg, 1985; Zampighi, Hall & Kreman, 1985; Brewer, 1991; Ehring & Hall, 1991; Ehring et al., 1990; Lea & Duncan, 1991). Channels reconstituted into liposomes were found to be permeable to molecules as large as 1.5 kD (Girsch & Peracchia, 1985; Gooden et al., 1985; Nikaido & Rosenberg, 1985) and were seen to close with $Ca^{2+}$ only in the presence of calmodulin (CaM)(Girsch & Peracchia, 1985). Voltage-dependent channels of various conductances were described in planar bilayers (Zampighi et al., 1985; Ehring et al., 1990; Ehring & Hall, 1991: Lea & Duncan, 1991) and in vesicles formed at the tip of patch pipettes (Brewer, 1990).

This paper reports data on permeability and electrical properties of channels reconstituted into liposomes and planar lipid bilayers with high-performance liquid chromatography (HPLC)-purified MIP26. The data provide new evidence for the capacity of HPLC-purified MIP26 to form channels permeable to molecules as large as sucrose (mol wt = 342) and show that MIP26 forms only one type of channel in planar bilayers. This is possibly the

first example of successful functional reconstitution of a membrane channel purified by reverse-phase HPLC. The channel has a mean conductance of 120 pS, has long open and closed time constants, is voltage dependent and is not a $K^+$-selective channel. A preliminary report of this study has been published (Shen et al., 1991).

## Materials and Methods

### Isolation of Lens Fiber Membranes

The plasma membranes and junctional complexes of bovine lens fiber cells were isolated as previously described (Girsch & Peracchia, 1985). Briefly, after removal of capsule and epithelial layer, 40 lenses were homogenized in 50 mM Tris, 5 mM EDTA, 10 mM $\beta$-mercaptoethanol and 0.1% $Na^+$ azide pH 7.4) and washed three times in the same solution by a 15-min centrifugation at 17,000 × $g$ in the SS-34 rotor of a Sorvall centrifuge (model RC5C, Du Pont de Nemours, Wilmington, DE). The pellets were resuspended twice in 7 M urea and 25 mM Tris (pH 7.4) and collected by centrifugation at 27,000 × $g$ for 20 min, after mixing 1:1 with distilled deionized $H_2O$. The pellets were incubated for 10 min in 0.1 N NaOH and 1 mM $\beta$-mercaptoethanol and centrifuged for 20 min at 44,000 × $g$. The pellets were resuspended in 5 mM $Na^+$-phosphate buffer, centrifuged for 20 min at 44,000 × g and stored at −20°C. All procedures were performed at 4°C. The yield was about 0.5 mg/lens.

### MIP26 Purification by HPLC and Gel Electrophoresis

The stripped lens fiber membranes (200 mg) were suspended in distilled deionized $H_2O$. Small aliquots of 10% octylglucoside were added, and the suspension was vortexed and sonicated (at 4°C) until transparency occurred (~0.5% octylglucoside, close to the critical micellar concentration (Brito & Vaz, 1986)). Small aliquots of suspension (350 $\mu$l, 2–3 mg protein/ml) were loaded onto an HPLC column (Du Pont, Zorbax Bio-series poly-F) equilibrated with 1% acetic acid and processed with an HPLC system (Milton Roy, LDC Division, Riviera Beach, FL). Column parameters were: 1 ml/min flow rate and a head pressure of 350 psi. Column effluent was monitored by UV absorption at 280 nm. After the injection bolus reached the detector (~2 min), a 15-min linear gradient was begun (A = 1% acetic acid, B = 20% isopropanol in acetic acid) reaching 95% of B. Most hydrophilic compounds eluted near the void volume, while MIP26 eluted as a narrow symmetrical peak 8–10 min into the gradient. Fractions (~2 ml) with an optical density greater than 0.4 were collected, mixed with 0.5 ml of glycerol and dried with no heating by a Speed Vac Concentrator (Savant Instruments, Farmingdale, NY). Preparations were stored at −20°C. To quantify MIP26 purity, small aliquots of HPLC-purified material were extracted into 2% sodium dodecyl sulfate (SDS), pH neutralized and prepared for SDS polyacrylamide gel electrophoresis (SDS-PAGE) using the PHAST system (Pharmacia LKB Biotechnology, Piscataway, NJ). Gels were stained with 0.1% Coomassie blue. The gels were scanned at $\lambda$ = 565 nm by a dual-wavelength scanner (CS-930, Shimadzu Scientific Instruments, Braintree, MA). A preliminary report on the HPLC purification of MIP26 has been presented (Girsch, 1988).

### Reconstitution of MIP26 into Liposomes

Aliquots of purified MIP26 were solubilized (3–5 mg/ml) in 4 mM Tris-HCl, 1 mM $CaCl_2$, 200 mM NaCl, 5 mM EGTA and 20% glycerol (vol/vol)(pH 8.0), containing 25 mg of octylglucoside per mg protein. MIP26 was incorporated into liposomes by combining dialysis (Zampighi et al., 1985) with sonication and dehydration/rehydration (Girsch & Peracchia, 1985). L-$\alpha$-diphytanoyl lecithin or egg lecithin (10 mg) were dried either for 1 hr under a stream of nitrogen or for 4 hr with the Speed Vac Concentrator. The dry lipids were suspended in 0.4 ml of 200 mM NaCl and 25 mM N- tris[hydroxymethyl]-methyl-2-aminosulfonic acid (TES, pH 7.0) containing 50 mg of octylglucoside, and the suspension was mixed with different aliquots (0.1, 0.05, 0.01 and 0 mg) of solubilized MIP26. After volume adjustment to 0.5 ml, the suspension was alternately vortex mixed and sonicated, and subsequently dialyzed overnight against buffer (500 ml of 200 mM NaCl, 25 mM TES, pH 7.0, four changes) and $H_2O$ to yield a whitish suspension. Aliquots (70 $\mu$l) of liposome suspension were dried by Speed Vac Concentrator for 8 hr and stored in 0.5-ml plastic vials at −40°C. For spectrophotometric osmotic-swelling assay (Lukey & Nikaido, 1980), the liposomes were rehydrated in 50 $\mu$l of 5 mM Tris-HCl, 0.1 mM $MgCl_2$, 1 mM KCl and 0.02% $Na^+$ azide (pH 7) containing 6% dextran T-10 (final osmolarity = ~60 mOsM), vortex mixed (3 min) and sonicated (10 sec). For determining the presence of channels, the liposomes were exposed to the same solution containing either sucrose (100 mM) or KCl (50 mM) in place of dextran T-10. The osmolarity of all the solutions was measured with a vapor pressure osmometer (5500, Vescor, Logan, UT). For planar bilayer experiments, dried liposomes were rehydrated in 50 $\mu$l of 200 mM NaCl and 25 mM TES (pH 7.0), vortex mixed for 3 min and sonicated for 30 sec.

### Reconstitution of MIP26 into Planar Lipid Bilayers

Planar lipid bilayers were obtained following the painting procedure of Mueller et al. (1962). Phosphatidylethanolamine (0.7 $\mu$l) from *Escherichia coli* (PE, 20 mg/ml, Avanti Polar-Lipids, Pelham, AL) and cholesterol (10 mg/ml) in decane were applied to a 300-$\mu$m hole in a Teflon partition separating the two aqueous chambers. The chambers were filled with 100 mM KCl, 1 mM $CaCl_2$ and 20 mM HEPES (pH 6.0, adjusted with Tris). Membrane capacitance was monitored with a triangular wave of 20 mV peak to peak and 100 Hz until it reached a value consistent with that expected for a single bilayer. The unmodified bilayers had an electrical resistance greater than 100 G$\Omega$ and a capacitance ranging between 200 and 300 pF. MIP26 channels were inserted into the planar bilayer by inducing fusion between the bilayer and channel-bearing liposomes added to both chambers. The fusion process was facilitated by moderate stirring of both chamber solutions with small magnetic bars.

For monitoring single-channel events, a planar bilayer system similar to that described by Alvarez (1986) was used. Holding potentials were applied by a voltage-clamp amplifier. Single-channel recordings were stored on videotape after digital conversion by a PCM audio processor and were analyzed with a laboratory computer.

### Freeze-Fracture Electron Microscopy

Liposome suspensions were mixed 1:1 with a 6% glutaraldehyde solution buffered to pH 7.4 with 0.2 M $Na^+$-cacodylate, and al-

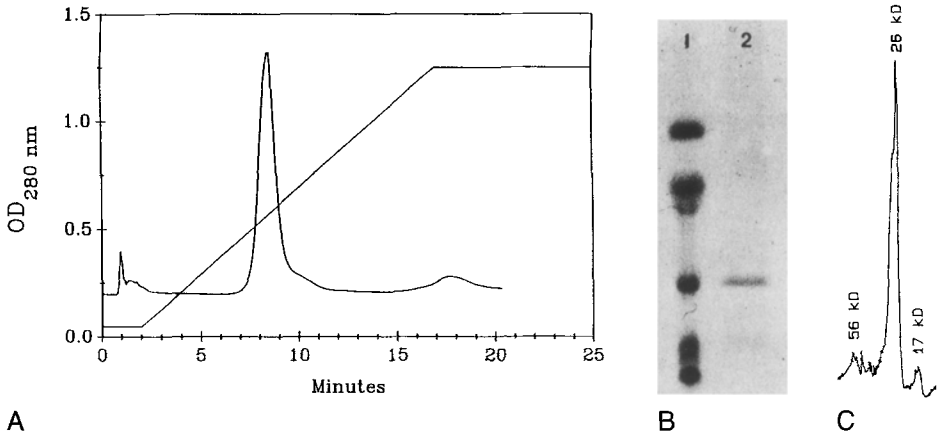


**Fig. 1.** (*A*) HPLC elution profile of lens fiber membrane components detected by UV absorption ($\lambda$ = 280 nm). Elution occurred over 15 min as a solution containing 1% acetic acid was brought to 20% isopropanol in acetic acid by a linear gradient (flow = 1 ml/min). The major component (MIP26) eluted as a narrow symmetrical peak between 7 and 10 min from the beginning of the elution process. To avoid collecting a contaminant protein closely associated with the right shallow shoulder of the main peak, the main fraction was collected at an OD greater than 0.4. (*B*) SDS-PAGE of HPLC-purified MIP26 (lane *2*); the $M_r$ markers, shown in lane *1*, are (from top to bottom) 66, 45, 34, 24, 18 and 14 kD. (*C*) Densitometric scan of SDS-PAGE gel stained with Coomassie blue. The trace shows that HPLC-purified MIP26 monomer was 93% pure. Except for an MIP26 dimer of 56 kD, the major impurity (3.5%) was a 17 kD protein. In contrast, 52% of the crude preparation was composed of MIP26 monomeric and 20.7% of the 17-kD protein (*data not shown*).

lowed to stand for 30 min. The fixed liposomes were centrifuged to a pellet, infiltrated with 30% glycerol and freeze fractured with a Balzers BAF 301 freeze-etch unit as previously described (Peracchia & Peracchia, 1980*a*). The replicas were viewed with an AEI EM 801 electron microscope.

## Results

### Chemical Analysis

HPLC elution of membrane constituents extracted from enriched fractions of lens fiber junctions by octylglucoside resulted in one major and two minor components (Fig. 1*A*). The major component was collected at an optical density greater than 0.4, to avoid a possible contaminant that appears partly associated with it as a shallow right shoulder (Fig. 1*A*). This component, examined by SDS-PAGE (Fig. 1*B*), was found to contain a 26 kD (MIP26) and a 17 kD band. The densitometric tracing of the gel showed that after HPLC purification monomeric MIP26 was 93% pure (Fig. 1*C*). Before HPLC monomeric MIP26 represented 52% of the protein components. The protein yield was 0.5 mg/lens before HPLC and 0.2 mg/lens after HPLC.

### Freeze Fracture of Liposomes Reconstituted with MIP26

Freeze fracture replicas of liposome pellets showed the concave and convex fracture surfaces of vesicles ranging in diameter from 0.2 to 0.8 $\mu$m. Liposomes reconstituted with unpurified MIP26 displayed particles of various sizes (Fig. 2*A*), while particles of liposomes reconstituted with HPLC-purified MIP26 were more homogeneous (Fig. 2*B*). The particles were equally distributed on either surface, and particle density increased with the protein concentration of reconstitution mixtures.

### Spectrophotometric Osmotic-Swelling Assay of Channel Permeability to KCl and Sucrose in Liposomes

By combining dialysis with sonication and dehydration/rehydration, it was possible to control the liposome size to a certain extent. Brief sonications produced larger liposomes (0.2–0.8 $\mu$m) more suitable for spectrophotometric studies (Luckey & Nikaido, 1980). Best results were obtained with a protein/lipid molar ratio of 1:5000. The larger liposomes were probably multilamellar; therefore, the initial rates of swelling, likely to represent the swelling of the external lamellae, more closely reflect the rate of permeant influx (Luckey & Nikaido, 1980).

For determining the presence of channels permeable to KCl and sucrose, liposomes reconstituted in the presence of HPLC-purified MIP26 (MIP26-liposomes) were loaded with the channel impermeant dextran T-10 by rehydration in 5 mM Tris-HCl, 0.1 mM $MgCl_2$, 1 mM KCl and 0.02% $Na^+$ azide (pH 7) containing 6% dextran T-10 (final osmolarity = ~60 mOsM), vortex mixing and sonication. Upon exposure to the same solution in which dextran T-10 was replaced with channel permeants such as sucrose (100 mM) or KCl (50 mM) (final

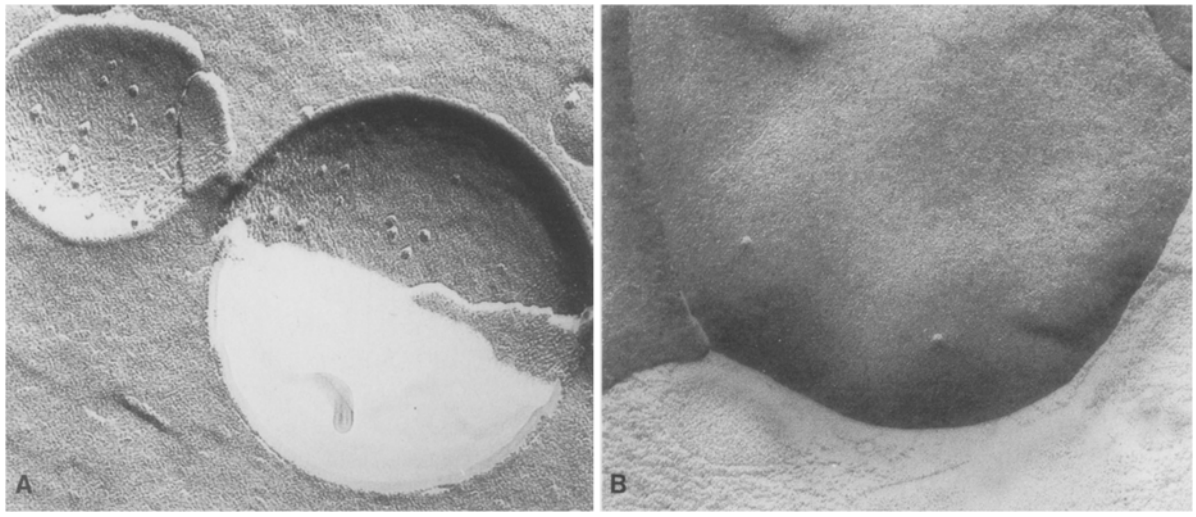


**Fig. 2.** Freeze-fracture replicas of liposomes in which either a nonpurified (*A*) or an HPLC-purified (*B*) MIP26 fraction was incorporated. Liposome diameter ranged from 0.1 to 0.8 $\mu$m. Intramembrane particles and pits were seen on both the convex and concave surface of the liposomes. Liposomes reconstituted with unpurified MIP26 showed particles of various size (*A*), while those reconstituted with HPLC-purified MIP26 displayed particles homogeneous in size (*B*). Magnification = 95,300×.

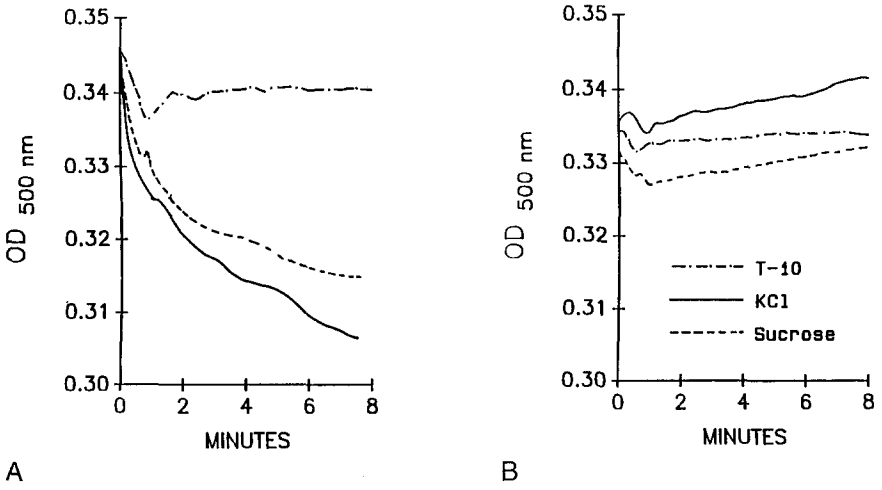


**Fig. 3.** Traces of optical density change (light scattering) in suspensions of liposomes reconstituted with (*A*) and without (*B*) HPLC-purified MIP26. The liposomes were loaded with a channel impermeant (dextran T-10, mol wt = 10,000) and suspended into solutions of channel permeants (KCl or sucrose) slightly hypertonic to T-10. MIP26-liposomes swelled, following a brief initial shrinkage, both in KCl and sucrose solutions, as these permeants diffused through channels into the liposomes, while T-10 remained trapped inside. The resulting increase in liposome osmolarity caused liposome swelling. This resulted in a rapid decrease in optical density (*A*), as the swelling liposomes became less opaque. In contrast, protein-free liposomes did not swell in any of the permeants (*B*), as they had no channels.

osmolarity = ~110 mOsM), MIP26-liposomes shrank transiently and then swelled. The initial osmotic gradient caused rapid water efflux, and consequentially brief liposome shrinkage, while the slower influx of KCl or sucrose caused a gradual increase in internal tonicity and drove water back into the liposomes, resulting in liposome swelling. This process was monitored by a slow decrease in optical density (Fig. 3*A*), following a brief initial increase in density (usually too fast to detect), when the MIP26-liposomes were suspended in hypertonic sucrose or KCl solutions. Swelling occurred more rapidly with KCl than with sucrose (Fig. 3*A*), because of the lower permeability of the latter. As expected, no significant change in optical density was observed when MIP26-liposomes were suspended in the same dextran T-10 solution used for loading (Fig. 3*A*). In contrast, protein-free liposomes did not swell in any of the three solutions (Fig. 3*B*).

## Single-Channel Recording of MIP26 Channels Reconstituted into Planar Bilayers

Successful channel reconstitution in planar bilayers required fresh material and liposomes proven permeable to sucrose by spectrophotometric osmotic-swelling assay. Results were best with small liposomes (obtained by increasing the sonication time, liposome diameter = <0.5 $\mu$m) reconstituted with a protein/lipid ratio of 1:26,000. Channel activity was recorded several minutes after addition of liposomes to both sides of the planar bilayer chamber and required continuous stirring. Attempts to incorporate channels in the bilayer by addition of liposomes to one chamber only were never successful, as previously shown by Zampighi et al. (1985). Most often numerous channels became incorporated, resulting in macroscopic increases in bilayer conductance. These multiple-channel records were not considered because channel analysis would have been considerably less accurate. For this reason, only experiments showing one (Figs. 4–9) or few (Fig. 10) conductance steps were analyzed. Single channel analysis was performed on ten successful experiments of MIP26-channel reconstitution, using two different stocks of HPLC-purified MIP26. In all of these experiments sucrose permeability of liposomes and channel electrical properties in bilayers proved quantitatively reproducible.

Channel opening and closing events were only seen when the voltage applied across the bilayer was greater than ±20 mV. At voltages ranging from ±20 to ±60 mV the channels were preferentially open, and a channel open probability of 50% was at ±60–80 mV. This indicated that the channels were voltage dependent and were open at voltages lower than ±20 mV. A typical continuous record of single-channel activity (Fig. 4*A*) showed current fluctuations of approximately 10 pA (at +80 mV holding potential) and long open and closed time constants. Amplitude histograms representing all points showed two Gaussian distributions that peaked at 11 and 1 pA (Fig. 4*B*), representing channel open (conductance = ~120 pS) and closed states, respectively. By looking at the magnitude of the relative areas under the curve, which reflects the probability of each state, one could tell that at 80 mV holding potential the channel was preferentially closed. The curves describing the frequency distribution of channel open and closed times (Fig. 5*A* and *B*) were well fitted by single exponential functions with time constants of 0.13 and 1.9 sec, respectively. Although the open time constant was quite consistent, ranging in different experiments from 0.1 to 0.3 sec, the closed time constant varied considerably, ranging from 1 to 7 sec.

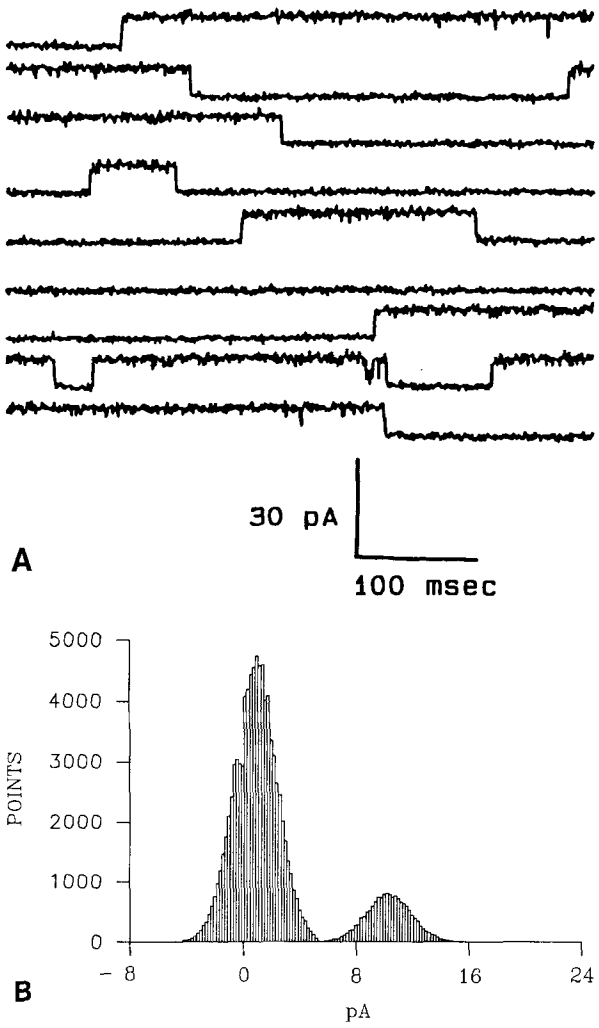


**Fig. 4.** Representative current fluctuations (*A*) and current-amplitude histogram (*B*) recorded from a single channel reconstituted in a PE/cholesterol planar bilayer by fusion with liposomes in which HPLC-purified MIP26 had been incorporated. The bilayer was subjected to an 80 mV holding potential in symmetrical solutions (100 mM KCl, 1 mM $Ca^{2+}$, 20 mM HEPES-Tris, pH 6.0). (*A*) Consecutive current traces filtered at 1.1 kHz and sampled at an acquisition rate of 4.4 kHz (upward deflection indicates channel opening). Note the long open and closed time constants. (*B*) Single-channel current-amplitude histogram ($f_c$ = 300 Hz, sampling = 1.2 kHz). The two Gaussian distribution curves correspond to channel closed (1 pA peak) and open (11 pA peak, corresponding to ~120 pS) states. Judging from the relative areas under the curves, which reflect the probability of each state, one can tell that the channel was preferentially closed at 80 mV.

To eliminate the possibility that the reconstituted channels were $K^+$-selective channels, in some experiments blockers of $K^+$-selective channels, such as $Cs^+$ and TEA, were added to the bath solutions; in others $K^+$ was substituted with $Na^+$. Figure 6 shows current traces and event histograms recorded from the same channel in the presence (Fig. 6*A* and *C*) and absence (Fig. 6*B* and *D*) of 40

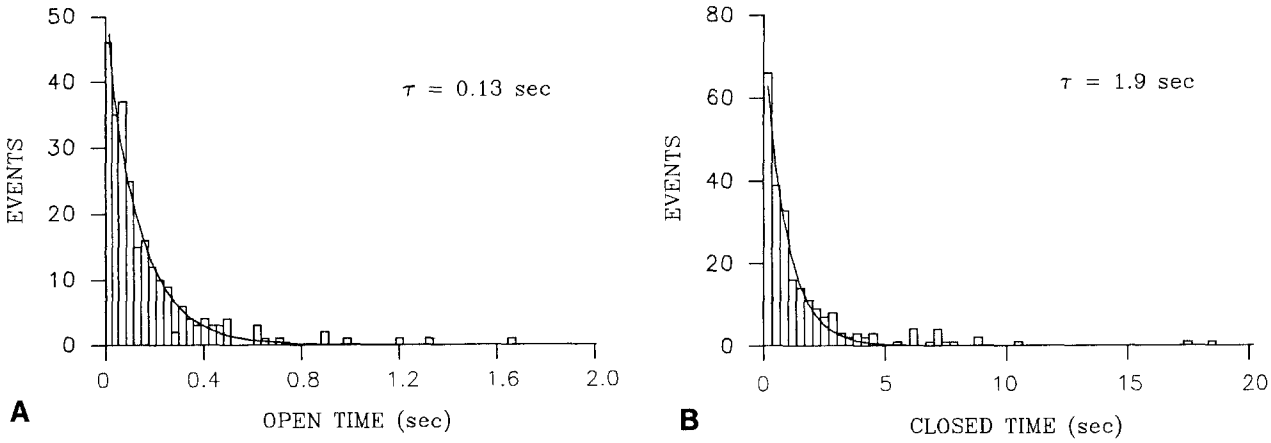


**Fig. 5.** Event histograms of channel open and closed times (80-mV holding potential) taken from three similar bilayers in which HPLC-purified MIP26 channels were reconstituted as described in Fig. 4. The bilayer was exposed to symmetrical solutions (100 mM KCl, 1 mM $Ca^{2+}$, 20 mM HEPES-Tris, pH 6.0). Histogram analysis was set for a threshold of 50% (i.e., only events with amplitudes greater than 50% of mean channel amplitude were analyzed) and an acquisition rate of 1.2 kHz. The frequency histograms are fitted by single-exponential functions with time constants ($\tau$) of 0.13 and 1.9 sec for open and closed events, respectively.

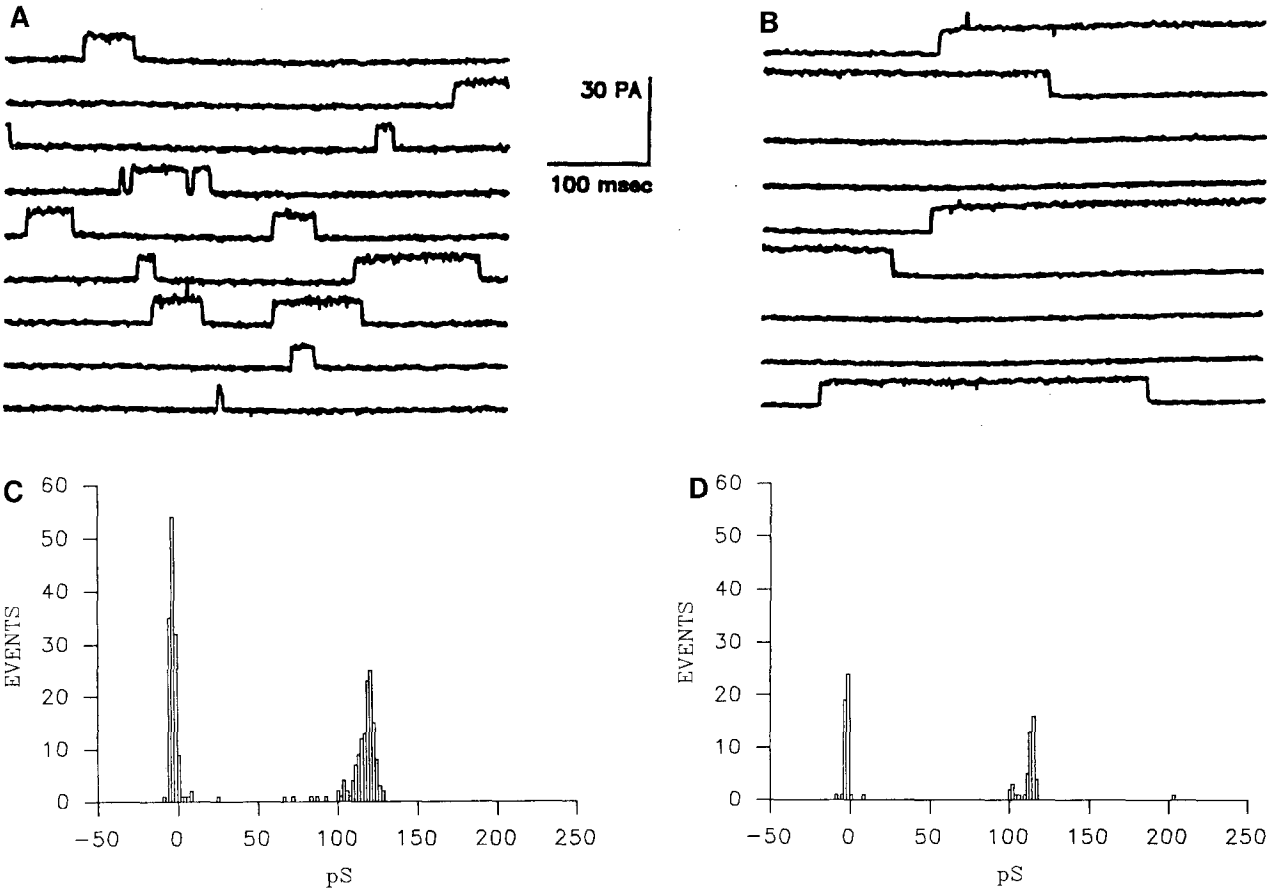


**Fig. 6.** Current traces and channel conductance event histograms recorded from the same channel in the presence (*A* and *C*) and absence (*B* and *D*) of 40 mM $Cs^+$ added to the bath solution of both chambers (100 mM KCl, 1 mM $Ca^{2+}$, 20 mM HEPES-Tris, pH 6.0). $Cs^+$ did not change channel conductance (*compare C* and *D*), but had a significant effect on channel kinetics (*compare A* and *B*). Current traces were filtered at 300 Hz and digitized at 1.2 kHz. Both histograms represented 97-sec recordings.

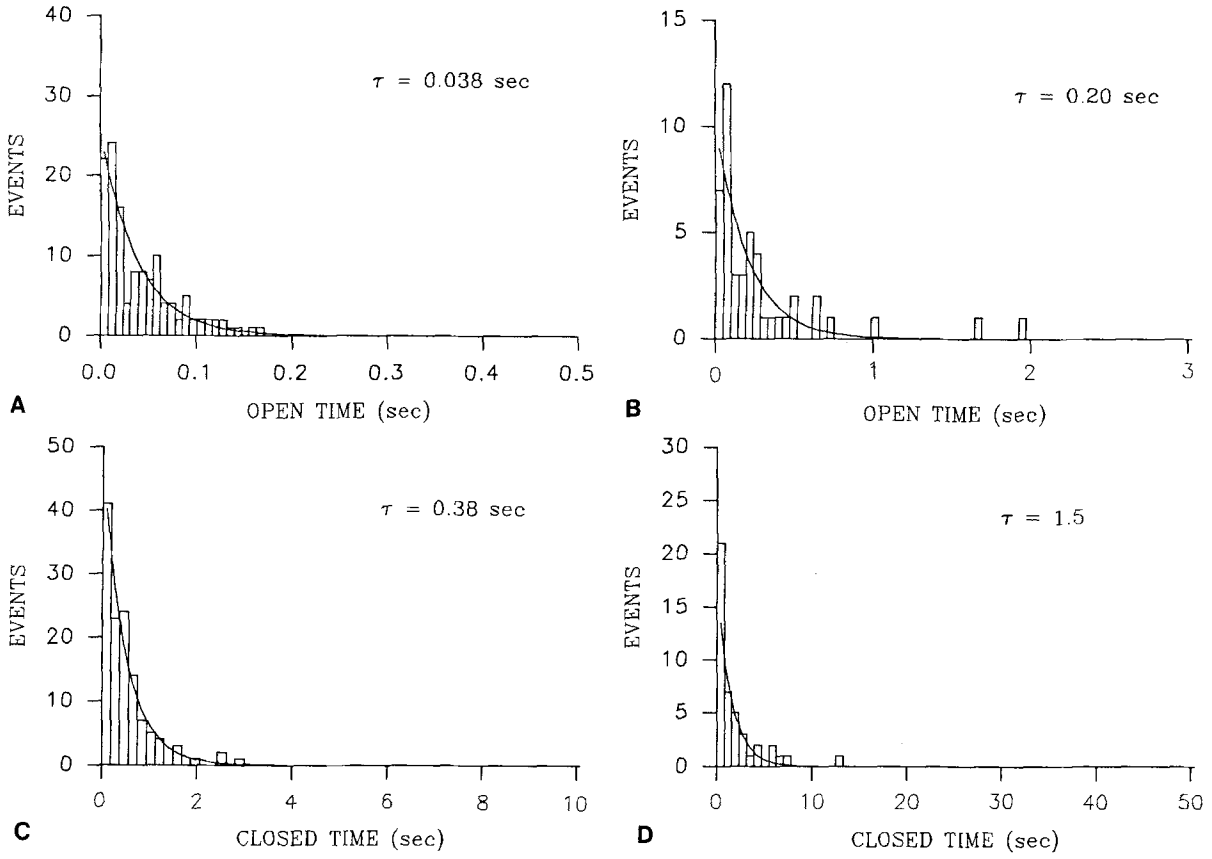


**Fig. 7.** Event histograms of channel open and closed times (80 mV holding potential) in the presence (*A* and *C*) and absence (*B* and *D*) of 40 mM $Cs^+$, as described in Fig. 6. The curves are fitted by single-exponential functions. Note the much shorter time constants ($\tau$) with $Cs^+$.

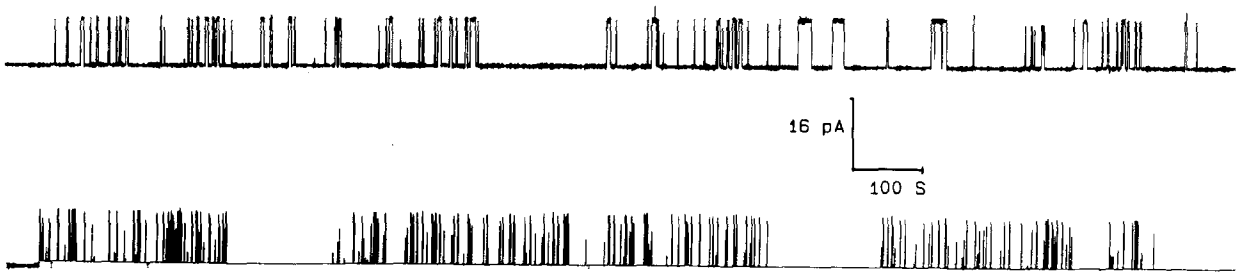


**Fig. 8.** Representative low-speed chart recording of channel events in the presence (lower trace) and absence (upper trace) of 40 mM $Cs^+$, as described in Fig. 6. Note that the increase in flickering with $Cs^+$ occurs in bursts.

mM $Cs^+$. $Cs^+$ did not decrease channel conductance (*compare C* and *D* in Fig. 6), but caused a significant change in channel kinetics (*compare A* and *B* in Fig. 6). As shown in Fig. 7, $Cs^+$ reduced the mean open time from 0.20 sec (*B*) to 0.038 sec (*A*), and the mean closed time from 1.5 sec (*D*) to 0.38 sec (*C*), which resulted in a significant increase in channel flickering. Most often the increase in channel flickering with $Cs^+$ occurred in bursts. This was clearly visible in low speed chart records (Fig. 8). In contrast, addition of TEA to the bath solutions affected neither channel conductance nor channel open and closed time constants. As shown in Fig. 9, the mean open time was 0.06 sec both in TEA and controls, and the mean closed time was 0.57 sec in TEA and 0.59 sec in controls.

Channel events were also observed with bath solutions in which $K^+$ was substituted with $Na^+$.

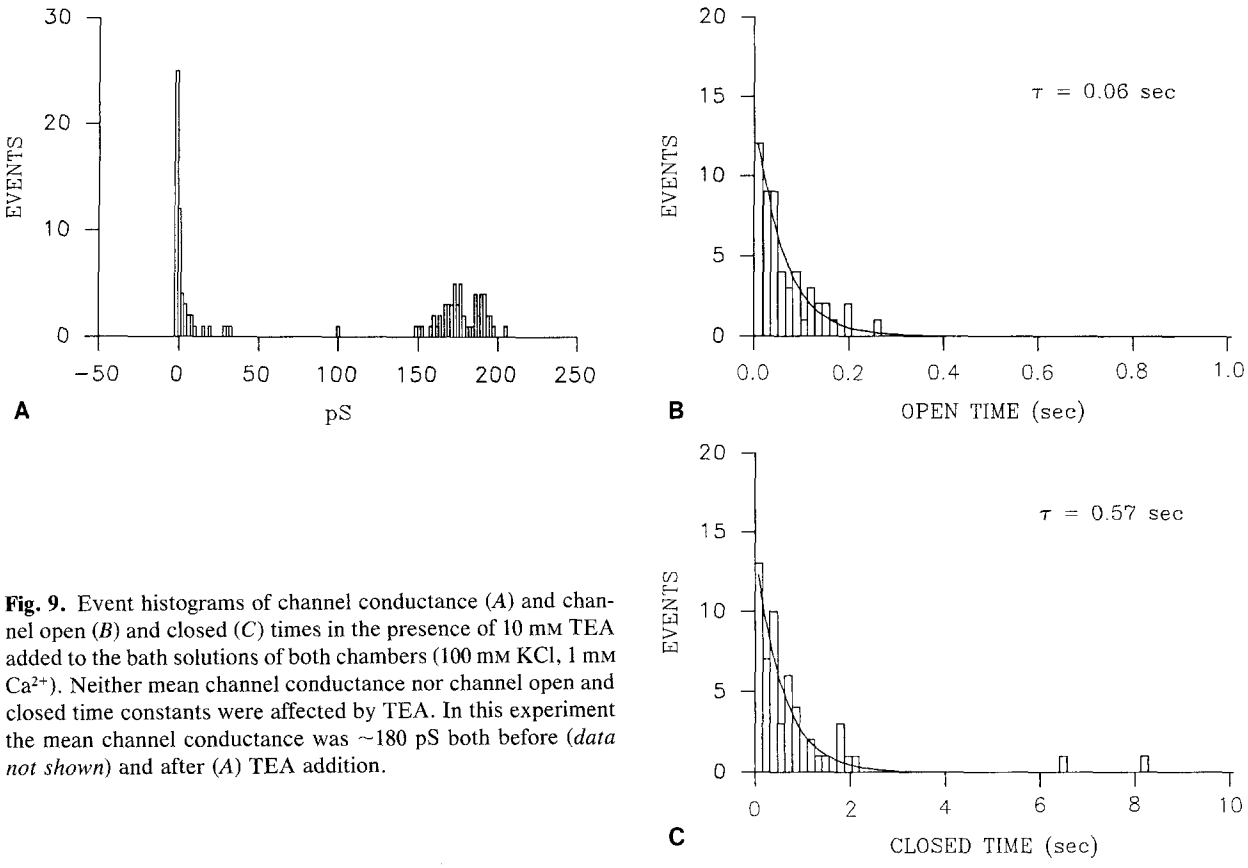


**Fig. 9.** Event histograms of channel conductance (*A*) and channel open (*B*) and closed (*C*) times in the presence of 10 mM TEA added to the bath solutions of both chambers (100 mM KCl, 1 mM $Ca^{2+}$). Neither mean channel conductance nor channel open and closed time constants were affected by TEA. In this experiment the mean channel conductance was ~180 pS both before (*data not shown*) and after (*A*) TEA addition.

This is illustrated in the current-amplitude histogram of Fig. 10, which shows two current peaks of −11 pA (channel open) and -1 pA (channel closed), corresponding to an open channel conductance of 125 pS. However, in $Na^+$ solutions channel activity was significantly reduced, such that bursts were usually rare and short lived.

## Discussion

This study describes functional characteristics of channels reconsituted *in vitro* by incorporating into artificial membranes the lens protein MIP26, purified by HPLC. Data from spectrophotometric monitoring of channel permeability in liposomes and from electrophysiological recording of channel behavior in planar bilayers portray a channel with functional properties similar to those of gap junction channels. They include: permeability to molecules larger than small monovalent metal ions, conductance greater than 100 pS, and long open and closed time constants.

Purification of MIP26 by HPLC is reflected by the homogeneity of intramembrane particles, seen in reconstituted liposomes by freeze fracture, and by the increase in content of MIP26 from 52 to 93%, as monitored by gel electrophoresis. Electrophysiological evidence of a unitary channel conductance in planar bilayers demonstrates that HPLC purification has removed contaminant channel proteins. HPLC does, however, produce relatively low yields, due primarily to protein aggregation that occurs when very hydrophobic proteins such as MIP26 and other channel proteins are exposed to acidic organic-solvent media. This aggregation, noticeable in MIP26 suspensions by the appearance of whitish flocculating material and in gels by the presence of nonmigrating high molecular weight components, increases with storage time. For this reason, reconstitution experiments are generally more successful if freshly purified protein is used.

The observation of liposome swelling in sucrose, as well as in KCl solutions, indicates that channels made of purified MIP26 are permeable to molecules larger than monovalent ions and confirms previous data obtained with less pure MIP26 preparations (Girsch & Peracchia, 1985; Gooden et al., 1985; Nikaido & Rosenberg, 1985). In vertebrate cells, aside from gap junction channels only a few channels are permeable to molecules as large as sucrose. Among ion channels, $Ca^{2+}$-dependent $K^+$

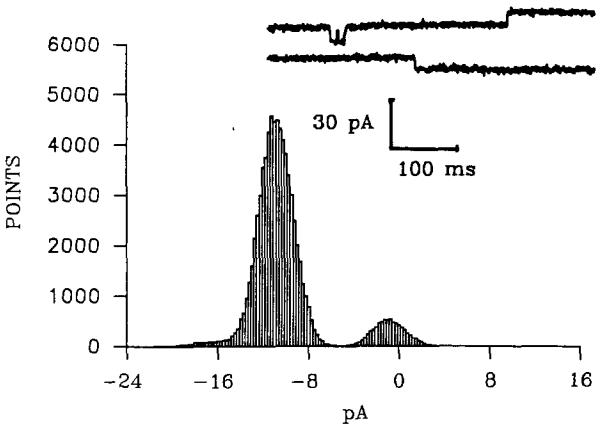


**Fig. 10.** Current-amplitude histogram of single-channel activity recorded in a $K^+$-free NaCl bath solution (100 mM NaCl, 1 mM $Ca^{2+}$, 20 mM HEPES-Tris, pH 6.0). Holding potential = −80 mV. The two Gaussian distribution curves correspond to channel closed (−1 pA) and open (−11 pA) states. $Na^+$ substitution of $K^+$ did not affect channel conductance (~125 pS, in this experiment). The inset shows a representative record of current fluctuations (downward deflection indicates channel opening).

channels, $K^+$ ($Ca^{2+}$), were considered as possible contaminants in view of their large conductance. However, $K^+$ ($Ca^{2+}$)-channel blockers such as TEA and $Cs^+$ did not alter channel conductance, and channels with the same conductance were seen even in $K^+$-free $Na^+$ solutions.

Although $Cs^+$ did not affect single channel conductance, it had an interesting effect on channel kinetics. Both with and without $Cs^+$ the channels showed virtually only two states (open and closed), as subconductance states were rare, but with $Cs^+$ both open-channel and closed-channel time constants were considerably shorter. However, this did not significantly change the open-channel equilibrium probability. Channel fluctuation between closed and open state can be defined by the following kinetic scheme (Colquhoun & Hawkes, 1983):

$$\text{closed} \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} \text{open}$$

in which open-channel equilibrium probability is given by

$$P_o(\infty) = \frac{\beta}{\alpha + \beta}.$$

With this equation, the equilibrium probabilities were similar (~0.1) both before and after addition of $Cs^+$, indicating that $Cs^+$ only speeds the transition between the two states, the result being an increase in flickering. This could be the consequence of channel blocking/unblocking by $Cs^+$. This behavior is very different from that observed by Yellen (1984) in $K^+$ ($Ca^{2+}$) channels. In the latter case, $Cs^+$ decreased the apparent single-channel conductance, as its entry and exit rates were too rapid to be resolved in patch-clamp records.

Previous studies on gap junction channels incorporated into planar bilayers have reported various channel conductances. With crude MIP26 fractions, Zampighi et al. (1985) reported 1500–2000 pS channels in 1 M solutions and extrapolated the conductance to 200 pS at 100 mM. Lea and Duncan, (1991) reported 500 pS channels in 165 mM solutions, and Brewer (1991) described 265-pS channels (hemichannels) in single spherical bilayers and 627 pS channels across two spherical bilayers. Finally, by using HPLC-purified MIP26, a channel conductance of 360 pS in 200 mM solutions was observed by Ehring and Hall (1991), and both 380 and 160 pS channels in 100 mM KCl were reported by Ehring et al. (1990).

With the liver gap junction protein connexin 32 (CX32), Young, Cohen and Gilula (1987) described 140 pS channels in 100 mM solutions, and Harris (1991) observed ~50 and ~130 pS channels in 183 mM solutions. By a different method, involving patch pipettes dipped into suspensions of isolated gap junction fractions, Spray et al. (1986) reported 150 pS channels in a 150 mM solution. In this case, however, complete gap junction channels rather than hemichannels might have been present.

Recently, Brewer (1991) has normalized to a 350 mM ionic strength published conductance values of gap junction channels. At this ionic strength, most of the previously reported conductances of reconstituted channels would range from 220 to 280 pS. Our observation of a mean channel conductance of 120 pS in 100 mM solutions agrees with these values, as this channel would have a conductance of 228 pS in 350 mM ionic strength.

Consistent with previous reports (Zampighi et al., 1985; Ehring et al., 1990) are also both evidence of channel voltage dependence and need of liposome presence on both sides of the bilayer for channel appearance. The reasons for the latter are not clear. The original interpretation of Zampighi et al. (1985), which suggested that gap junction-like channels may have formed by the interaction between channels in the bilayer and channels of liposome fragments adhering to the bilayer, is very provocative because among other things it would help understand the junctional role of MIP26. However, this hypothesis would imply that only junctional channels are functionally active. This is unlikely in view of evidence for permeable channels in liposomes, as morphological data suggest that these channels are most likely hemichannels.

This study is the first to provide data on MIP26-channel kinetics derived from single-channel records. Ehring et al. (1990) only determined "first latencies," defined as the time elapsing between an imposed voltage step and the channel closing, from single-channel recordings. This parameter cannot be meaningfully compared with our data on single-channel open and closed time constants because the latter reflect spontaneous opening and closing phenomena at a steady holding potential, rather than events induced by an imposed voltage step. The single-channel conductance observed in our study (120 pS) is smaller than the conductance steps (160 and 380 pS) reported by Ehring et al. (1990). A possible reason for this is the presence of cholesterol in our bilayer. Indeed, an increase in membrane cholesterol has recently been shown to decrease the conductance of the nicotinic acetylcholine receptor channels (Lasalde & Zuazaga, 1991). Since the plasma membrane of bovine lens fibers is rich in cholesterol (cholesterol/phospholipid molar ratio = 0.6–1.2; Zampighi et al., 1985; Malewicz et al., 1990), our conductance value measured in bilayers with a cholesterol/phospholipid molar ratio of 0.96 may be closer that of *in vivo* MIP26 channels. Cholesterol may modulate channel function by affecting the microviscosity of the lipid bilayer (Lasade & Zuazaga, 1991).

Although permeability, conductance and gating properties of MIP26 channels are similar to those of gap junction channels, the involvement of this protein in cell-to-cell communication is still unclear. MIP26 differs biochemically (Gorin et al., 1984) from typical gap junction proteins of the connexin family (Beyer, Paul & Goodenough, 1990), and immunological data on the localization of MIP26 are controversial. Bok, Dockstader and Horwitz (1982) reported binding of MIP26 polyclonal antibodies to lens junctions only, but Paul and Goodenough (1983) observed antibody binding to nonjunctional membranes as well as to junctions. Furthermore, only thin (13-nm) junctions showed antibody binding, while junctions of normal thickness (17 nm) did not. In contrast, with monoclonal antibodies Fitzgerald, Bok and Horwitz (1982), Sas et al. (1985) and Vallon et al. (1985) localized MIP26 at both types of junctions.

A further complication of this issue has resulted from the recent discovery of another membrane protein that localizes at 17-nm thick lens junctions (Kistler, Kirkland & Bullivant, 1985; Gruijters et al., 1987; Zampighi et al., 1989). This protein (MP70) was found to have some N-terminus sequence similarities with gap junction proteins of the connexin family, and thus, became a primary candidate for lens gap junctions (Kistler, Christie & Bullivant, 1988). However, this protein was localized immunocytochemically only within 100 $\mu$m of the lens surface and, in spite of some evidence for the possible presence of degradation products of MP70 (MP64 and MP38) in deeper cells (Kistler & Bullivant, 1987), the biochemical nature of gap junction channel of these cells, which represents the vast majority of lens fibers cells, is still unknown. Consistent with the idea that MIP26 is involved in cell-to-cell communication are the reported blockage of dye diffusion between lentoid cells by intracellularly injected anti-MIP26 monoclonal antibodies (Johnson et al., 1988) and evidence for both a calmodulin (CaM) participation in the regulation of lens fiber communication (Girsch & Peracchia, 1985; Peracchia & Girsch, 1985; Brewer & Dong, 1990; Gandolfi et al., 1990) and CaM interaction with MIP26 (Hertzberg & Gilula, 1981; Welsh et al., 1982; Van den Eijden-van Raaij, de Leeuw & Broekhuyse, 1985; Van Eldick et al., 1985; Peracchia, 1988; Peracchia & Girsch, 1989; Louis et al., 1990; Girsch & Peracchia, 1991). In addition, Ehring and Hall (1991) have reported preliminary data showing a substantial increase in voltage dependence of the MIP26 channel, associated in some cases with large decrease in bilayer conductance down to values near the bare bilayer level of conductance, upon addition of 10 $\mu$M $Ca^{2+}$ to bathing solutions containing 1 $\mu$M CaM. In contrast, in the absence of CaM, [$Ca^{2+}$] as high as 40 mM was ineffective (Ehring & Hall, 1991). Our present data confirm the absence of a direct effect of $Ca^{2+}$ on the channel gates, as the bilayer bathing solution contained 1 mM $Ca^{2+}$. Experiments with CaM are presently underway and the data available at this stage are too preliminary to be discussed.

Recently, Ehring et al. (1990) have proposed a new role for MIP26 channels in maintaining fluid balance and minimizing the extracellular space between fibers. This model, which assumes that MIP26 channels are hemichannels rather than junctional channels, is entirely based on the observation that MIP26 antibodies bind only to convex surfaces of lens "wavy junctions" (Zampighi et al., 1989). However, these "wavy" membrane appositions are not typical lens fiber junctions; they are found almost exclusively among the deformed fiber cells of the lens nucleus and could very well result from asymmetrical apposition of single junctional membrane following junction membrane separation. Indeed, cortical lens fiber membranes with orthogonal and rhombic particle arrays, typical of MIP26 arrays (Dunia et al., 1987), have been shown to form junctions with precisely matched particle and pit arrays both in isolated fractions (Peracchia, 1978; Peracchia & Peracchia, 1980*a*,*b*) and in intact fiber

cells (Bernardini & Peracchia, 1981); the arrays were found to change from disordered to crystalline with either $Ca^{2+}$ (Peracchia, 1978; Peracchia & Peracchia, 1980*a*; Bernardini & Peracchia, 1981) or $H^+$ (Peracchia & Peracchia, 1980*b*).

In conclusion, this study shows that HPLC-purified MIP26, reconstituted in artificial membranes, makes channels that are permeable to molecules considerably larger than monovalent ions and are endowed with electrical properties similar to those of gap junction channels. To our knowledge this is the first example of successful reconstitution of a functional membrane channel after reverse-phase HPLC purification and demonstrates that functional channels can reform after channel protein purification under harsh conditions. MIP26 channels reconstituted in planar bilayers have a mean conductance of 120 pS, are voltage dependent and show long open and closed time constants. The channels are not $K^+$ channels because they are insensitive to TEA and $Na^+$ substitution for $K^+$, and channel kinetics, but not single-channel conductance, is affected by $Cs^+$.

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