J. Membrane Biol. 154, 239–249 (1996)

The Journal of
Membrane
Biology

Properties of Chicken Lens MIP Channels Reconstituted into Planar Lipid Bilayers

E. Modesto[1], P. D. Lampe[2], M. C. Ribeiro[1], D. C. Spray*, A. C. Campos de Carvalho[1]

[1]Instituto de Biofisica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil

[2]Fred Hutchinson Cancer Research Center, Seattle, Washington

Received: 27 March 1996/Revised: 5 August 1996

Abstract. Membrane fractions highly enriched in chicken lens MIP (MIP28) were found to form ion channels when incorporated into planar lipid bilayers. The channels displayed prominent unitary conductances of about 60 and 290 pS in symmetric 150 mM KCl solution and were slightly anion selective. For both depolarizing and hyperpolarizing voltages, voltage sensitivity of the MIP28-induced conductance could be fit by a Boltzmann relation, symmetric around zero mV, with $V_0 = 18.5$ mV, $n = 4.5$ and $g_{min}/g_{max} = 0.17$. Channel properties were not appreciably altered by pH in the range of 5.8 to 7, although channel incorporation was observed to occur more frequently at lower pH values. Calcium, at millimolar concentrations, decreased the channel mean open time. Partial proteolysis of MIP28 to yield MIP21 did not appreciably affect single-channel conductance or voltage sensitivity of the reconstituted channels. MIP28 was not phosphorylated by cAMP dependent protein kinase (PKA). Although unitary conductance and selectivity of the chicken MIP channel are similar to those reported for the bovine MIP (MIP26), the voltage sensitivity of MIP28 was higher than that of the bovine homologue, and voltage sensitivity of MIP28 was not modulated by treatments previously shown to affect MIP26 voltage gating (partial proteolysis and protein phosphorylation by PKA: (Ehring et al., 1990). The existence of such strikingly different functional properties in highly homologous channel isoforms may provide a useful system for exploration of the structure-function relations of MIP channels.

Key words: Single channels — Voltage gating — Phosphorylation — Proteolysis — MIP

Introduction

MIP, the main intrinsic protein of the lens, constitutes almost 50% of the total membrane protein in lens fiber cells (Benedetti et al., 1976). The extensive intercellular coupling in this tissue, together with the biochemical and immunological detection of MIP in areas of membrane apposition, initially rendered MIP the logical candidate as the protein forming lenticular gap junction channels (Benedetti et al., 1976; Goodenough, 1979; Kusak, Maisel & Harding, 1978). However, numerous studies using diverse techniques have challenged the authenticity of MIP as a junctional protein. Detailed examination of MIP distribution in the membranes of fiber cells first showed that it is also present in high amounts in nonjunctional membrane areas (Paul & Goodenough, 1983). Subsequent additional findings which have been interpreted as evidence against a role for MIP in forming intercellular channels include the lack of sequence homology between MIP and members of the connexin multigene family (Gorin et al., 1984; Paul, 1986; Kumar & Gilula, 1986; Beyer et al., 1987; Willecke et al., 1991), the absence of junctional communication in oocytes injected with mRNA encoding MIP (Swenson et al., 1989), the ultrastructural finding that MIP channels are not always coaxially aligned across the bilayers of adjoining cells (Zampighi et al., 1989), and the identification of other membrane proteins in lens fiber cells that share sequence homology with the connexin family and thus appear to be more legitimate candidates for gap junction proteins in this tissue (Kistler, Christie & Bullivant, 1988; Paul et al., 1991; White et al., 1992).

Nevertheless, a role for MIP in intercellular com-

* *Permanent address:* Departments of Neuroscience and Medicine, Albert Einstein College of Medicine, Bronx, NY 10461

Correspondence to: D.C. Spray

munication in the lens has not been rigorously excluded. In a tissue that has an extremely high extracellular resistance (Mathias and Rae, 1985), the presence of large nonselective channels in the membranes of adjacent cells could indeed effectively couple these cells as suggested by Ehring et al. (1990), in a model analogous to ephaptic transmission in the nervous system (Bennett, 1966). One of the basic premises of such a model is that MIP is able to form ion channels, and membrane fractions of bovine and chicken lens membranes enriched for MIP have exhibited this property when incorporated into planar lipid bilayers (Zampighi et al., 1985; Modesto et al., 1990; Shen et al., 1991). In addition, bovine lens membrane preparations incorporated into liposomes have been found by a number of laboratories to increase liposome permeability to high molecular weight compounds (Girsch & Peracchia, 1985*a,b;* Nikaido & Rosenberg, 1985; Gooden e al., 1985*a,b;* Scaglione & Rintoul, 1989).

Another likely role for MIP in the lens is related to volume regulation. To most effectively focus light, it is essential that extracellular space in the lens be very restricted. The presence of large water permeable channels in the membrane of the fiber cells could prevent fluid accumulation in that space due to the presence of an osmotic gradient favoring fluid transport into the crystallin-rich fiber cell cytoplasm. In this regard it is interesting to note the recently reported homology between MIP and a number of integral membrane proteins of animal, bacteria and plant origin that appear to function as transporters (Rao, Jan & Jan, 1990; Muramatsu & Mizuno, 1989; Smith & Chater, 1988; Sandel & Marcker, 1988; Johnson, Hofte & Chrispels, 1990). Of special interest is the high degree of homology between MIP and the cloned water channel proteins CHIP28 (Preston & Agre, 1991; Preston et al., 1992; Zhang et al., 1993) and WCH-CD (Fushimi et al., 1993), which reinforce the suggestion that MIP may play a role in volume regulation in the lens. In fact, Mulders et al. (1995) have recently shown that oocytes expressing MIP exhibit a four- to fivefold increase in the coefficient of osmotic water permeability.

Lens-intrinsic membrane proteins are highly homologous throughout the evolutionary scale. Rat and bovine MIP are 93% identical over the last 130 amino acid residues of the carboxyl-terminus (Shiels et al., 1988) and partial clones of the chicken protein indicate more than 80% identity for the C-terminal 113 amino acid residues (Kodama et al., 1990) of the total 263 residues that constitute bovine MIP (Gorin et al., 1984). However, bovine, rat and chicken MIP migrate as distinct proteins in SDS-PAGE (Johnson et al., 1985*b*), and certain monoclonal antibodies to rat MIP do not cross-react with the bovine homologue (Paul et al., 1991).

Even though the degree of homology is very high, at least for the carboxyl-terminus, there are important structural and functional differences between bovine and chicken MIP proteins. According to Kodama et al. (1990) helix F, the sixth putative transmembrane domain of chicken MIP, is more hydrophobic due to substitutions of glycines and serines with alanines, this helix is thus unlikely to be the pore-lining domain, the topology suggested by Gorin et al. (1984) for bovine MIP. Moreover, chicken MIP lacks the Ser243 residue of the bovine sequence, which in the bovine sequence was demonstrated by Lampe & Johnson (1990) to be phosphorylated by cAMP dependent protein kinase, causing a major change in voltage dependent gating of bovine MIP channels (Ehring, Zampighi & Hall, 1991).

We have now extended the observations regarding functional differences between the bovine and chicken MIP by studying the channel properties of the chick isoform, MIP28, incorporated into planar lipid bilayers. Our results indicate that this protein forms channels with unitary conductance and selectivity that are very similar to those of the bovine isoform. However, voltage-dependent gating and regulation of MIP26 and MIP28 channels differ substantially. The chicken MIP28 channels were found to be more sensitive and were more completely closed by voltages of either polarity. In addition, MIP28 was not phosphorylated by cAMP dependent protein kinase, and its voltage sensitivity was not affected by partial proteolysis of the native protein, which yields MIP21. These findings contrast with the significant decrease in voltage sensitivity seen following proteolysis of the bovine isoform MIP26 (Ehring et al., 1991).

Materials and Methods

Purification of Lens Membrane

Lenses were removed from chicken heads that were obtained at a slaughterhouse and transported on ice to the lab. Membrane fractions highly enriched for MIP were obtained following the procedure described by Goodenough (1979). In brief, 100 lenses were decapsulated, cut into small pieces and homogenized in bicarbonate buffer (1 mM $NaHCO_3$, 5 mM EDTA, pH 8.0; 24 ml/lens). Adequate disruption of the tissue was verified by the absence of intact cells in phase microscopy. Homogenate was distributed in twelve 200 ml bottles and spun at 7000 rpm for 30 min (2×10^5 g-min) in a GSA rotor. The resulting pellets were resuspended in 5 mMTRIS, 1 mM EDTA, 1 mM $CaCl_2$, pH 9.0 (TEC buffer) and respun at 7000 rpm. The pellets were then resuspended in 4 M urea in TEC buffer (200 ml final volume) and spun in a SS-34 rotor at 7800 rpm for 30 min. Supernatants were carefully decanted and the pellets resuspended in 7M urea in TEC, brought up to a final volume of 225 ml. Samples were then pelleted in a SW28 rotor at 23,500 rpm for 60 min. The six pellets were resuspended in 20.1 ml TEC plus 40.1 ml 68.5% sucrose, to yield a 50% sucrose-TEC suspension. Sucrose gradients were formed in SW28 tubes, from bottom to top: 10 ml sample in 50% sucrose, 9 ml 45% sucrose-TEC, 9 ml 41% sucrose-TEC and 9 ml 25% sucrose-TEC. The gradients were spun at

23,500 rpm for 2 hr and the 41–25% interface collected from each tube. Each sample was diluted to a final volume of 36 ml with TEC and mixed thoroughly. After pelleting (SW28, 23,500 rpm, 1 hr) samples were resuspended in TRIS buffer at concentrations of 1–3 mg/ml, as determined by Lowry assay, and stored at −70°C for not more then three months before use.

Electrophoresis and Western Blot

After isolation, lens membrane fractions were electrophoresed in SDS-PAGE (12.5% gels). Gels were loaded with varying amounts of total protein and stained with Coomassie Blue to check for enrichment in MIP28. Western blotting was performed after transfer of electrophoresed proteins to nitrocellulose membranes using a monoclonal antibody kindly supplied to Dr. Ross Johnson (University of Minnesota) and previously characterized (Sas et al., 1985). Blots were stained with anti-mouse IgG conjugated to alkaline phosphatase (Sigma Chemical). MIP presence was detected by reaction with 5-bromo-4-chloro, 3 indoyl phosphate/nitro blue tetrazolium and blue color development. Alternatively, blots were incubated with a rabbit anti-mouse IgG and then exposed to ^{125}I protein A.

Solubilization and Reconstitution of MIP

After membrane isolation, MIP was solubilized in detergent and reconstituted into liposomes. Samples of the membrane preparations (0.3 ml, ~3 mg/ml) were solubilized overnight at 4°C in 300 μl of 4% solutions (v/v in 300 mM K_2SO_4, 5 mM MES, pH 5.8 with 1 mM azide) of the following detergents: octyl-β-D-glycopyranoside (OG), CHAPS, and Triton X-100 (TX). Solutions were centrifuged at 100,000 × *g* for 60 min and supernatants were then added to 5 ml of a solution containing 5 mM MES pH 5.8, 1 mM DTT, 50 mg asolectin (Sigma, type II) and either 10% OG, 0.6% CHAPS or 5% TX. The lipid protein mixture was placed in a dialysis bag (M_r cutoff 3.5 kD; Spectrum Medical) and dialyzed twice for 48 hr at 4°C against 2 liters of a solution with the following composition (in mM): 300 K_2SO_4 or 150 KCl, 5 MES pH 5.8. The liposomes thus formed were used for incorporation of MIP into the planar bilayers.

Proteolysis of MIP

Lens membrane preparations were proteolyzed with trypsin as previously described (Keeling et al., 1983). In short, 50 μg of membranes was mixed with 25 TRIS pH 7.6, 5 $CaCl_2$ and 2 μg of trypsin for 2 hr at 37°C. The reaction was stopped by the addition of 20 μg of soybean trypsin inhibitor and the membranes pelleted at 16,000 × *g* for 30 min. Samples of the digested proteins were then submitted to electrophoresis before reconstitution into liposomes.

Phosphorylation of Purified Lens Membrane

Chicken and bovine lens membranes were isolated as described above. Membranes treated with 10 units of alkaline phosphatase (molecular biology grade, Boeringer Mannheim) were incubated in 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (Sigma), 100 mM Tris-HCl, pH 8.0 at 37°C for 3 hr, washed with 7M urea, 50 mM Tris-HCl, pH 7.6 and then washed twice with the same solution without urea. Membranes were treated with cAMP-dependent protein kinase (Sigma, P5511, 100 μg/ml) in a phosphorylation reaction mixing containing 50 mM HEPES-pH 7.5, 10 mM $MgCl_2$, 5 μM cAMP [Sigma, A6885], 500 μm ATP (Sigma, A5394-supplemented with Amersham, PB170 − (γ^{32}P)ATP to 0.25 Ci/mmol], and lens membranes (1 mg/ml). Kinase incubations were carried out at room temperature for 30 min and were terminated by pelleting in a microcentrifuge (12,000 × *g*), washing once in buffer and recentrifuging. Pelleted membranes were analyzed by SDS-PAGE on 12.5% gels with Coomassie Blue staining. After drying the gels under vacuum, autoradiography was performed overnight using Kodak XAR 5 film.

Bilayer Recordings

Planar lipid bilayers were formed in 200–300 μm orifices in teflon cups from a solution of asolectin (Type II, Sigma Chemical, St. Louis, MO) in decane (30% w:v), using the painting technique. Membrane resistance was checked before the addition of protein and usually exceeded 100 gigaohms. After membrane thinning, liposomes containing membrane fractions highly enriched for MIP were added to the CIS compartment (total volume = 2 ml) under conditions of constant stirring with an imposed voltage of 50 mV, CIS side positive. The voltage clamp amplifier was used assembled from a current to voltage converter (Burr-Brown OPA104) with a feedback resistor of 10^9 Ω, a booster circuit to increase frequency response, and a variable gain output stage (*see* Alvarez, 1986). A pulse generator, driven by batteries, supplied DC voltages of either polarity or voltage ramps of varying amplitude across the membrane. When ramps were used the potential was swept from negative to positive values at a variable rate; at the end of the ramp the potential was quickly reversed to the original negative voltage. When not pulsed, the membrane was always held at zero mV transmembrane potential. The transmembrane voltage was applied to the CIS compartment, while the TRANS compartment was held at virtual ground. The transmembrane voltage and current were moni-

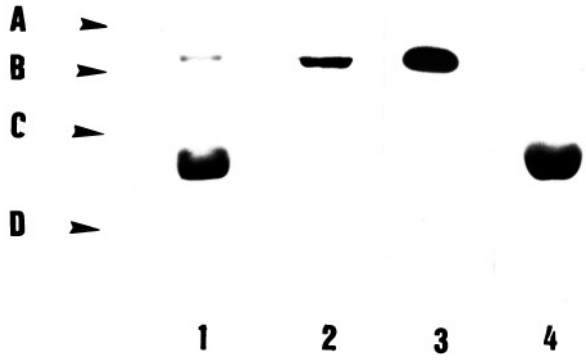

Fig. 1. Major protein composition of MIP28-enriched membrane fractions isolated from chicken lens. Lane 1 shows Coomassie Blue stained SDS PAGE of the isolated membrane fraction. Note the presence of two proteins with distinct M_r in this sample. Lane 2 shows a Western blot of the sample shown in lane 1 using an antibody specific for δ-crystalline. Note that the only protein recognized by this antibody corresponds in M_r to the lower mobility protein in lane 1. Lane 3 shows a Coomassie Blue stained gel of a sample of δ crystalline purified from chicken lens. Note that this protein runs on SDS PAGE with a mobility corresponding to the only band in lane 2 and the upper band in lane 1. Lane 4 shows a Western blot of the MIP28 enriched preparation (lane 1) using a monoclonal antibody specific for MIP28 at 1:20 dilution for 2 hr at RT. The membrane was then exposed to ^{125}I protein A (1:1000) for 1 hr at RT. Note that the single stained band has mobility similar to that of the lower band in lane 1. Lettered arrowheads to the left indicate M_r of 20, 29, 45, 66 kDa (bottom to top) obtained from Coomassie Blue stained SDS-PAGE of M_r standards.

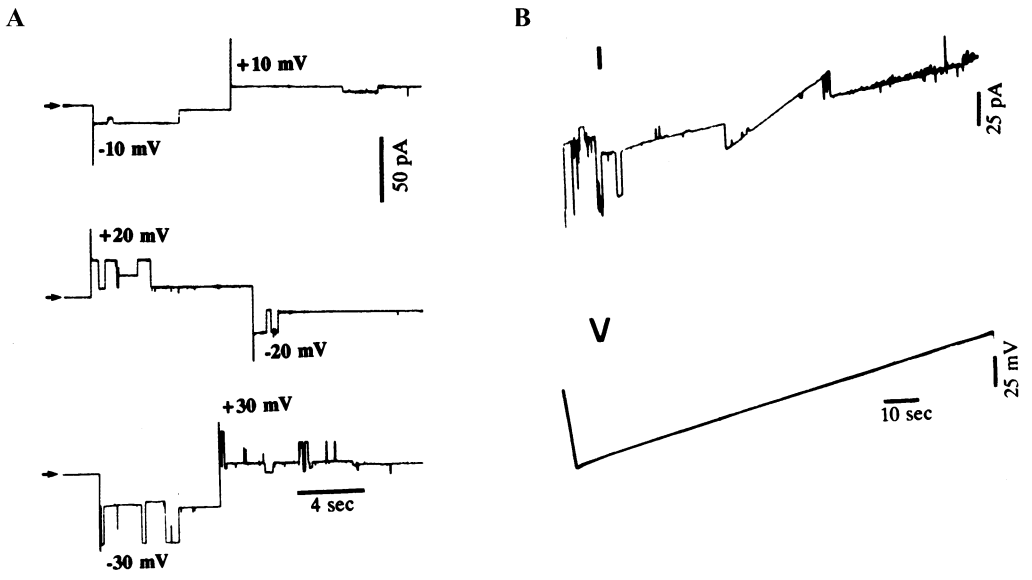

Fig. 2. Channels incorporated into planar lipid bilayers from membrane fractions enriched in MIP28 (as shown in lane 1 of Fig. 1). Records were obtained in symmetric solutions containing 300 mM K_2SO_4, 5 mM MES, pH 5.8. In *A*, the transmembrane currents were recorded at the voltages indicated alongside the capacitive artifacts corresponding to the changes in voltage. The arrows to the left indicate the zero current level in each record. Gating by voltage is reflected by the decrease in current after the onset of the transmembrane potential. At +30 mV, closures to bare bilayer conductance can be detected. Subconductance states are present in almost all records shown. In *B*, a voltage ramp (*V*) from −50 to +50 mV was applied to the bilayer. The current trace (*I*) shows voltage-dependent closure of the MIP28 channels when transbilayer potentials of either polarity reach the 20 to 30 mV range.

tored on a 2-channel oscilloscope (Meguro, MOS 1251) and recorded using a Gould (2200) chart recorder. In some experiments, data were also recorded on tape after pulse code modulation (Neurocorder, model 384) for subsequent analysis. Most recordings were obtained in solutions containing varying concentrations of KCl or K_2SO_4 under symmetric or asymmetric conditions (indicated in figure legends). In experiments carried out at pH 5.8 mM 2-(N-morpholino)ethanesulfonic acid (MES), was used as a buffer; for the experiments carried out at pH 7 we used 5 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES). In some experiments calcium (1–5 mM) was added to the solutions.

Results

Characterization of Intact and Proteolyzed Lens Membrane Preparations

Figure 1 shows a Coomassie Blue stained gel (lane 1) of the lens membrane preparations used for reconstitution of MIP into liposomes. Two bands are easily identified in this heavily loaded gel (100 μg/lane): a minor band at 54 kDa and a more prominent band running at about 28 kDa. The 28 kDa protein is MIP, as detected by Western blots using a monoclonal antibody to MIP (lane 4). The 54 kDa band is recognized in Western blot using a monoclonal antibody specific for δ-crystalline, the major protein in the lens cytoplasm (lane 2), and the major protein in a purified δ-crystalline preparation runs as a band of

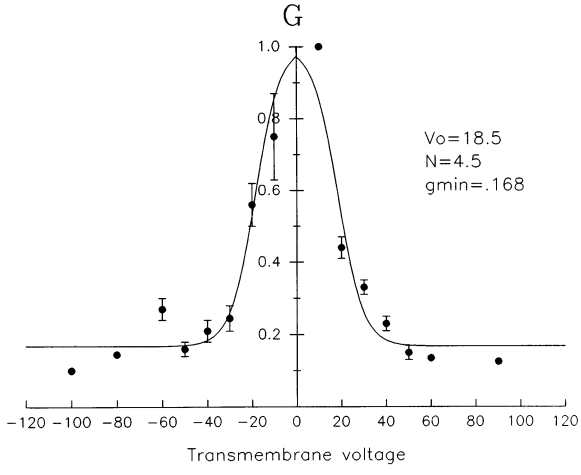

Fig. 3. Normalized steady-state conductance (*G*) as a function of transmembrane potential. Values for *G* were obtained from five different experiments in which MIP28 was incorporated into asolectin painted bilayers (30 mg/ml in decane) under symmetric solutions of 150 mM KCl, 5 mM MES, pH 5.8. Experiments were independently normalized and each point in the graph represents the mean of the normalized values obtained at a particular voltage. The bars are the standard deviations, and where not present are smaller than the width of the symbols. Superimposed smooth curves represent the best fit of the Boltzmann equation (*see text*) to the experimental data.

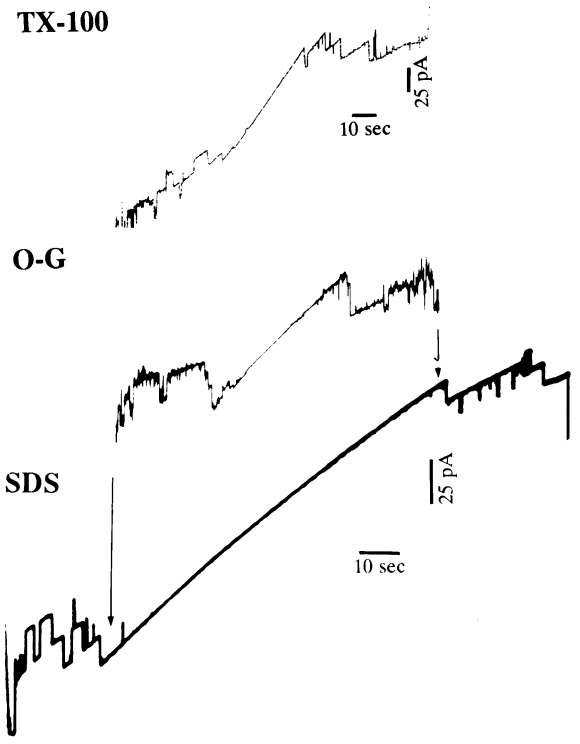

Fig. 4. MIP 28 enriched membrane fractions were solubilized in either 4% Triton X-100 (TX-100), 4% n-octyl-β-D-glucopyranoside (O-G) or 1% sodium dodecyl sulfate (SDS) and then reconstituted into liposomes after extensive detergent dialysis (*see* Materials and Methods). Liposomes formed in this way were then incorporated into asolectin painted bilayers (30 mg/ml in decane) and voltage ramps were applied to the bilayer. Voltage dependence is quite pronounced when TX-100 or O-G are used to solubilize the membrane fractions. However, under SDS voltage gating is only detected when transmembrane voltages in excess of ±80 mV are reached (*see* arrows). For the two upper records the voltage ramps used swept the membrane potential from −50 to +50 mV, while for the last record the voltage varied from −100 to +100 mV. All records were obtained in asymmetric solutions of the following composition (in mM): 300 K_2SO_4, 5 MES, pH 5.8 (CIS compartment), 70 K_2SO_4, 5 MES, pH 5.8 (TRANS compartment).

similar mobility in a Coomassie Blue stained SDS-PAGE (lane 3). This 54 kDa protein is thus very likely to correspond to δ-crystallin.

Voltage Dependence of MIP28 Channels

We examined voltage-dependent gating of the MIP channels by using both voltage pulse and ramp stimulus protocols. Figure 2*A* shows single-channel records obtained during the application of ±10, ±20 and ±30 mV voltage pulses. The largest and most active of these channels exhibited voltage dependence, such that the channel remained at the highest conductance level most of the time at ±10 mV, and spent more time at lower conductance levels with increasing voltages of either polarity. The latency to first closure of the largest channel also decreased as the voltage was increased. Intermediate conductance levels of various amplitudes and durations are also present in most of these records. In Figure 2*B* we show the voltage dependence of the channel when a voltage ramp was applied across the bilayer, driving the transmembrane potential from −50 to +50 mV. The first 20 sec of this recording is characterized by brief openings of large conductance channels, due to the polarity reversal from +50 mV at the end of the previous ramp to −50 mV at the beginning of this voltage ramp. Thereafter, a stable low conductance state is reached. As the voltage approached −20 mV the channels opened and remained open until the ramp reached positive voltages around 25–30 mV, when the channels closed once again. Thus, with regard to pulse protocols (Fig. 2*A*), and with voltage ramps over most of the voltage ranges (Fig. 2*B*), the steady state conductance of MIP28-containing membranes varied symmetrically with voltage, as was previously described for the homologous bovine lens protein, MIP26 (Zampighi et al., 1985). To compare the voltage dependent behavior of MIP28 channels to that of MIP26 (Zampighi et al., 1985), we plotted the steady-state conductance of membranes containing MIP28 as a function of membrane voltage (Fig. 3). The points in this figure represent the mean ± SD of 5 experiments carried out under symmetric conditions in 150 mM KCl, 5 mM MES,

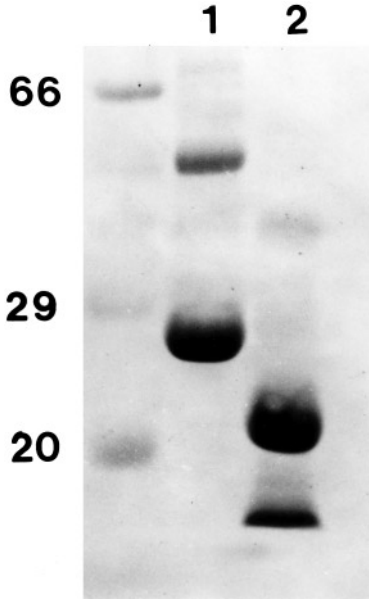

Fig. 5. Coomassie Blue stained 12.5% SDS-PAGE of MIP28 enriched membrane fractions from chicken lens before (lane 1) and after (lane 2) proteolytic digestion with trypsin. Membrane fractions enriched in MIP28 display two prominent bands, at about 54 kDa and at about 28 kDa (lane 1). Proteolysis (lane 2) cleaves the proteins into a major 21 kDa product and smaller peptide fragments. Both lanes were loaded with 100 μg of protein. Molecular weight markers, with their respective mass in kDa are shown to the left.

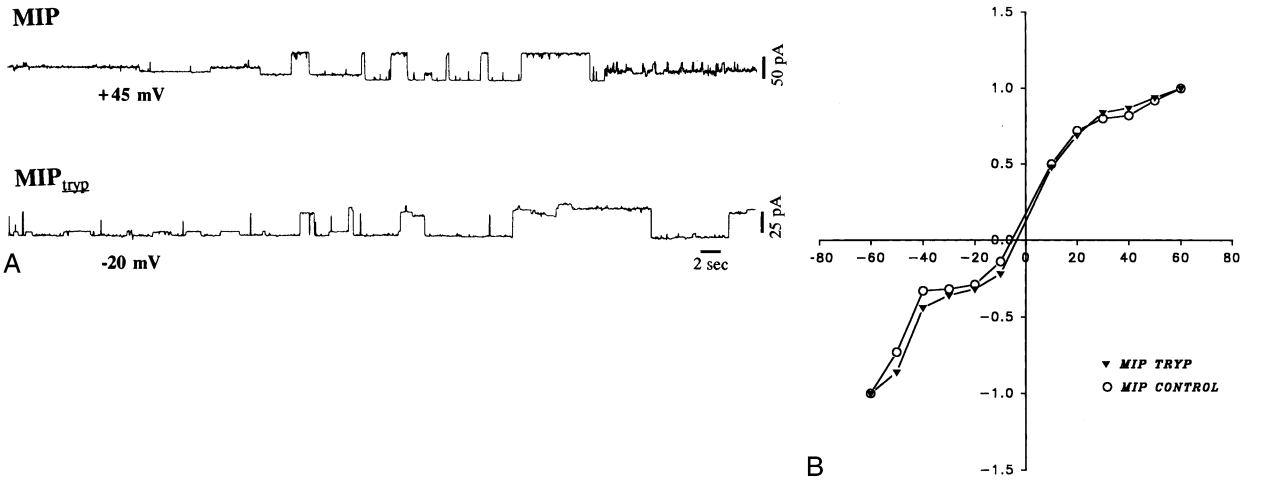

Fig. 6. Removal, by trypsinization, of a large portion of the C-terminal region of MIP28 (*see* Fig. 5, lane 2) does not alter its channel properties. Records of transmembrane currents were obtained in symmetric solutions of 300 mM K_2SO_4, 5 mM MES, pH 5.8. The native protein was trypsinized, as described in Materials and Methods, resulting in a 21 kDa fragment that was then incorporated into the bilayer. In *A*, transmembrane currents were measured at the indicated potentials when the native (MIP) and the trypsinized (MIP_{tryp}) proteins were reconstituted into asolectin painted bilayers. Trypsinization did not alter significantly the amplitude of the single-channel transitions (1.55 nS for the largest transition in MIP and 1.50 nS in MIP_{tryp}) or the occurrence of substate levels. Calibration bars indicate the currents in pA (vertical) and the time in sec (horizontal). In *B*, average transmembrane currents were recorded as eight consecutive voltage ramps swept the membrane potential from −60 to +60 mV. Current was then normalized to the maximum current value obtained when control and trypsinized MIP were reconstituted into bilayers to allow comparison of the voltage sensitivities under these conditions. The ordinate represents the normalized average current and the abscissa the transmembrane voltate in mV. Voltage-dependent gating is not influenced by trypsinization as indicated by the quasi-superimposable *I-V* relations.

pH 5.8. The solid line was obtained according to the Boltzmann equation[1]:

$$G_\infty = (g_{max} - g_{min})/(1 + \exp(n/kT\,(V - V_0))) + g_{min} + g_{min}$$

The best fit was determined by linear regression of the experimental values obtained for $\ln (g - g_{min})/(g_{max} - g)$ *vs.* transmembrane voltage, where g is normalized steady-state bilayer conductance, g_{max} is maximal g and g_{min} is the voltage-insensitive residual conductance reached at very high voltages (Spray, Harris & Bennett, 1981; Harris, Spray & Bennett, 1981). The Boltzmann parameters obtained were $V_0 = 18.5$ mV, $n = 4.5$ and $g_{min} = 0.17$, indicating a rather steep voltage dependence for the MIP28 channels. To exclude the possibility that detergent effects might influence the voltage dependence of MIP28 channels, we reconstituted MIP28 solubilized by octylglucoside (0-G in Fig. 4) and Triton X-100 (TX-100 in Fig. 4) and examined voltage gating using ramp protocols. These studies did not reveal striking differences between membranes solubilized using

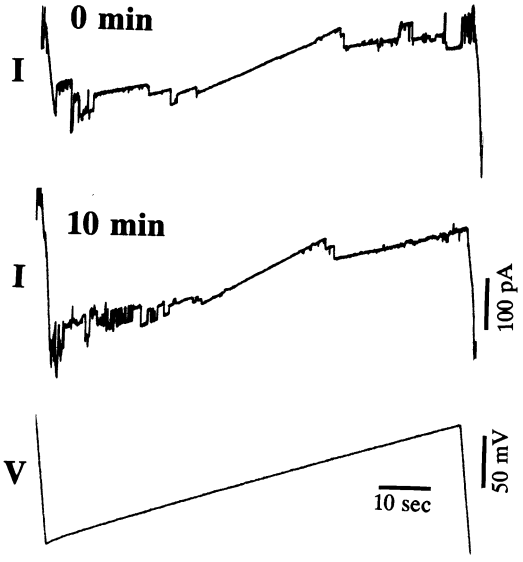

Fig. 7. Records of transmembrane currents (*I*) obtained in symmetric solutions of 300 mM K_2SO_4, 5 mm MES, pH 5.8 as the transmembrane voltage (*V*) was varied from −65 to +65 mV. The upper current trace (0 min) is a control record, taken after incorporation of 15 mg of MIP28, the native protein, to the bilayer. The lower current trace (10 min) was obtained 10 min after the addition of 0.5 μg trypsin to both recording chambers and illustrates that voltage dependent gating is not significantly altered by the presence of the enzyme.

[1] Although strictly this relation should only apply to two-state channels in which transitions from one state to another are reversible and where the transitions from closed to open state are a function of the electric field across the bilayer, it has been usefully employed to compare properties of different types of gap junction and VDAC channels, where substates certainly exist (e.g., Schein et al., 1976; Spray et al., 1981).

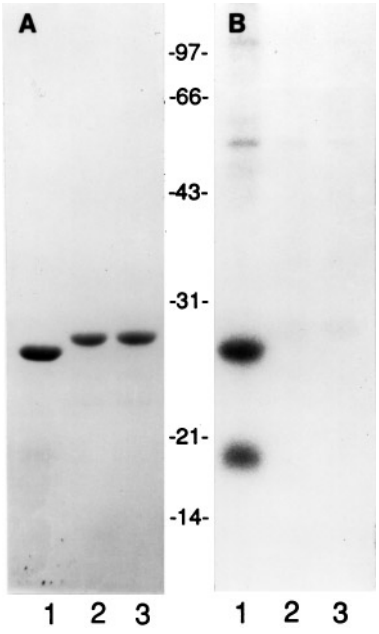

Fig. 8. Phosphorylation of bovine but not chicken lens membranes (10 μg of protein) by cAMP dependent protein kinase. The Coomassie Blue stained gel (*A*) and corresponding autoradiograph (*B*) are shown. Numbers in the center indicate molecular standard masses in kDa. Lane 1 shows bovine lens membranes. Lanes 2 and 3 show chicken lens membranes. The membranes in lane 3 were treated with alkaline phosphatase prior to cAMP dependent phosphorylation (*see* Materials and Methods), note that phosphate incorporation was not detected in chicken lens membranes.

these detergents and those solubilized using CHAPS (Fig. 2*B*). We also incorporated MIP28 into neutral membranes (PE:PC, 10 mg/ml in decane, Avanti Chemicals) and observed voltage gating using the same range of transmembrane voltages (*results not shown*). However, when MIP28 was solubilized in SDS, voltage gating of the reconstituted channels was significantly altered, the channels closing only in response to voltages in excess of ±80 mV (Fig. 4).

Proteolysis Did Not Affect Single-Channel Conductance or Voltage Dependence of MIP28 Channels

Digestion of membrane preparations enriched in MIP28 resulted in cleavage of the protein into a 21 kDa peptide (lane 2, Fig. 5) and smaller peptide fragments that were not well resolved on 12.5% SDS PAGE. Preparations obtained in this way were incorporated into lipid bilayers in order to compare properties of the normal and proteolyzed proteins. When the proteolyzed MIP28, designated as MIP21 (*see* Fig. 5, lane 2) was reconstituted into bilayers, events of various unitary conductances were recorded (Fig. 6*A*, lower trace) that did not differ markedly from those obtained with the native protein (Fig. 6*A*, upper trace; Fig. 2*A*). When the voltage dependence was examined using a ramp protocol driving the membrane potential from −60 to +60 mV, we also saw no major differences between the behavior of channels formed by MIP28 and MIP21, as is illustrated in Fig. 6*B* in which currents recorded during eight consecutive ramps were averaged to compare macroscopic voltage sensitivities of native and trypsinized MIP. In addition to these experiments in which MIP21 was directly incorporated into the bilayer, we have performed experiments in which the native protein (MIP28) was first incorporated into the bilayer and then the proteolytic enzyme was added to both bilayer chambers. At 10 min after 0.5 μg of trypsin was added to the bathing solution containing 15 μg native protein (equivalent to the enzyme: substrate ratio that resulted in virtually complete proteolysis of MIP28: Fig. 5), the voltage dependence was not appreciably altered (Fig. 7).

MIP28 Was Not Phosphorylated by Protein Kinase A

Lens membranes from both calf and chicken were incubated with cAMP-dependent protein kinase as described in Materials and Methods. The major protein band in each of these preparations was MIP (Fig. 8*A*). Phosphate

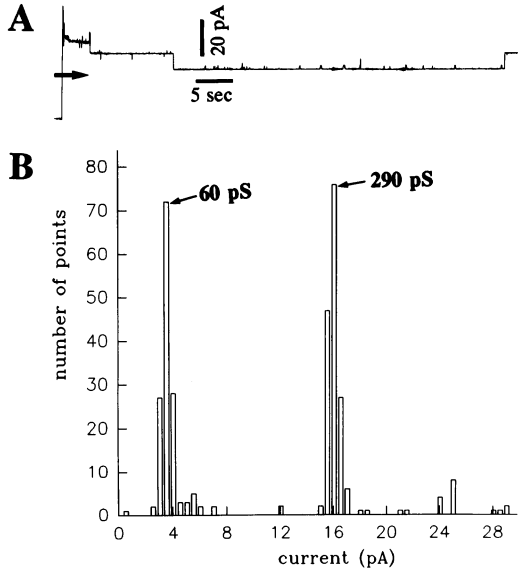

Fig. 9. Unitary conductances of MIP29 channels. (*A*) Unitary currents recorded in symmetric solutions of 150 mM KCl, 5 mM MES, pH 5.8. After setting the transmembrane potential to +55 mV transmembrane current varies due to the voltage-dependent closure and opening of MIP28 channels. The arrow points to the zero current level. (*B*) Frequency histogram of the time spent in each current level for the record illustrated in *A*. Two main conductances levels are prominent at 290 and 60 pS.

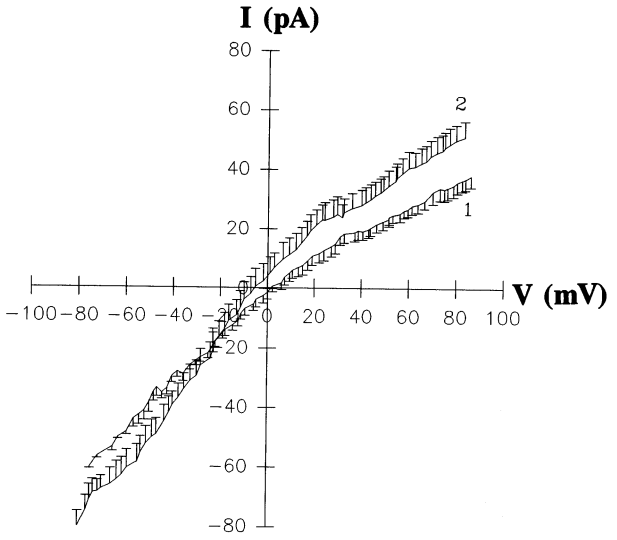

Fig. 10. *I-V* relation for chicken MIP incorporated into asolectin painted bilayers under symmetric (curve 1) and asymmetric (curve 2) salt solutions. Curve 1 was obtained with 0.1 M KCl in both compartments and curve 2 when the salt concentration in the TRANS compartment was raised to 0.2 M KCl. Voltage ramps driving the transmembrane potential from −80 to +80 mV were applied and the resulting currents recorded. The solid lines represent the mean values, calculated after digitizing the transmembrane currents of four different experiments. Error bars are indicated for each curve. The reversal potential after increasing the salt concentration in the TRANS compartment was −5.1 mV, resulting in a calculated P_{Cl}/P_K ratio of 1.87.

incorporation into bovine lens membranes as a result of cAMP-dependent protein kinase was localized in two major phosphoproteins, MIP26 and a minor band at 19 kDa (MP19, Fig. 8*B*, lane 1) as has been reported previously (Johnson et al., 1985; Garland & Russel, 1985; Louis et al., 1985; Johnson et al., 1986; Lampe et al., 1989). Chicken MIP (MIP28) exhibited slightly lower electrophoretic mobility than bovine MIP (compare lanes 1 and 2 in Fig. 8*A* and *see* Sas et al., 1985), and apparently did not contain MP19 (Fig. 8*B*, lane 2). Chicken MIP, in contrast to bovine MIP, was not phosphorylated by cAMP-dependent protein kinase to any significant extent (compare lanes 1 and 2 in Fig. 8*B*). Treatment of chicken membranes with alkaline phosphatase, which effectively removed phosphate from immunoprecipitates of connexin43 under similar conditions (Moreno et al., 1994), did not increase the level of subsequent phosphorylation with protein kinase A (lane 3, Fig. 8). Treatment of bovine lens membranes with alkaline phosphatase only released a very small amount of phosphate (*results not shown; see* Louis et al., 1985), which is consistent with the low level (8–20%) of endogenous phosphorylation of bovine MIP (Lampe et al., 1990; Ehring et al., 1991).

Other Properties of MIP28 Channels

In 150 mM KCl, the single-channel conductance of MIP28 was found to be approximately 230 pS as illustrated in Fig. 9. In *A* we show a 140-sec duration unitary current record obtained at +55 mV, and in *B* the resulting histogram for time spent in each current level. Two preferred conductance states are evident in this Figure (at 290 and 60 pS), but other conductance levels are also present. Although closures to bare bilayer are not shown in this recording, such closures did rarely occur. To evaluate selectivity, we measured the shift in reversal potential (E_r) after doubling salt concentration in the trans compartment, as illustrated in Fig. 10. We only included in our analysis those experiments where the junction potential at the end of the experiment varied by less than 0.5 mV, a value corresponding to 10% of the average shift in E_r observed in five different experiments (mean $E_r = -5.1$ mV). A slight anion selectivity was also observed for the chicken MIP isoform with $P_{Cl}/P_K = 1.87$, a value which is quite similar to that of 1.8 previously described for the bovine homologue (Ehring et al., 1990).

Calcium (millimolar range) and pH (from 5.8 to 7.0) did not affect single-channel conductance (*not shown*). At millimolar concentrations, however, calcium did decrease the open probability of the channel. As seen in Fig. 11, in an experiment where at least 3 channels were present in the bilayer, the addition of 5 mM calcium to both chambers led to a significant decrease in the amount of time all three channels were found in the open state, in contrast to the control situation (0 Ca) where no calcium was added to the solutions and therefore free calcium concentration should have been in the micromolar range.

Discussion

A major issue in studies in which membrane proteins are reconstituted into lipid bilayers relates to the nature of the channel forming protein, especially when analyzing single or few channel current records. Trace amounts of contaminating proteins, undetected by gel staining techniques, might account for the observed channel activity. The absence of specific physiological or pharmacological properties for lens MIP channels renders the task of attributing the observed channel activity to a specific protein even more difficult. However, we argue that MIP28 is the channel-forming protein in our reconstitution experiments based on the following: using the statistical arguments raised by Zampighi et al. (1985) the protein responsible for channel formation should account for at least 20% of the total protein present in lens membrane preparations. Even in our heavily loaded gels (*see* Fig. 1) only two products reach that high proportion of the total protein: MIP28 and the 54 kDa protein. We believe that the 54 kDa protein is either δ-crystallin or an MIP dimer, but to verify that the other band was the channel forming protein we reconstituted the electro-eluted 28 kDa band in liposomes. Figure 12 shows the channel activity recorded from the electro-eluted MIP28

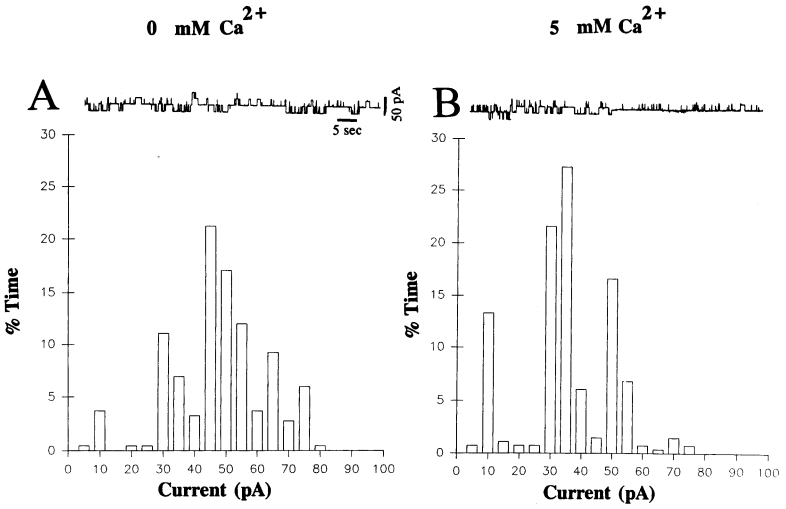

Fig. 11. A. Records of transmembrane current in asymmetric solutions of K_2SO_4 (300 mM CIS; 70 mM TRANS), 5 mM MES, pH 5.8, before (0 Ca) and after the addition of 5 mM $CaCl_2$ to both compartments of the bilayer chamber. Three channels are present in the record. Channel openings are represented by downward reflections and therefore the uppermost current levels correspond to the closed state for all three channels. After adding Ca^{2+} the probability of finding of all channels in the open state is decreased, as illustrated by the histograms in the lower portions of the figure, which display the % time spent at each current level.

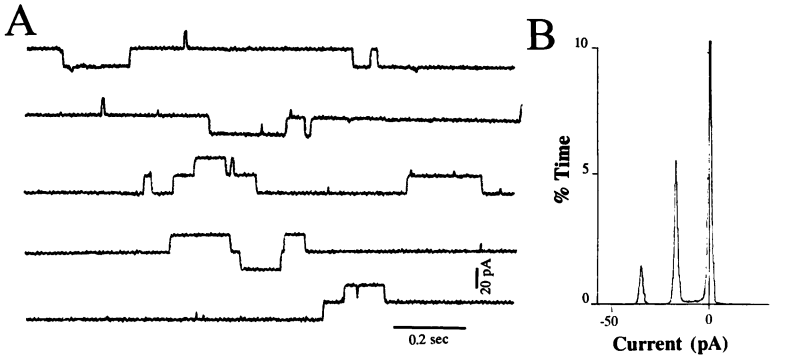

Fig. 12. Recording of transmembrane current (*A*) and corresponding all points histogram (*B*) obtained after the incorporation of electro-eluted MIP28 into an asolectin painted planar bilayer (30 mg/ml in decane), in asymmetric solutions containing K_2SO_4 (300 mM CIS; 70 mM TRANS), 5 mM MES, pH 5.8. Transmembrane potential was set at −75 mV.

reconstituted into liposomes after a detergent exchange procedure as described by Hanke et al. (1990). The single-channel conductance was quite similar to that obtained from our membrane preparations under identical reconstitution conditions. Electro-elution and reconstitution of the 54 kDa band using the same procedure did not result in channel activity, thus leading to the conclusion that MIP28 was most likely responsible for the observed channel activity in our lens membrane preparations. It also seems unlikely that the presence of a contaminating connexin might form the channels which we have studied here, because reconstitution of gap junction hemichannels would be expected to produce asymmetric $g_j - V_j$ relations (Ebihara & Steiner, 1993; DeVries & Schwartz, 1992; Harris et al., 1992).

Comparison of the properties of chicken and bovine MIP emphasizes similarities and striking differences. Both proteins form channels with approximately the same unitary conductances and ionic selectivities. Calcium and pH do not affect MIP28 single-channel conductance, as was also reported for MIP26 (Ehring et al., 1990); however we found that mM levels of calcium significantly reduced the mean open time of the chicken channels, while Ehring et al. (1990) reported no effect of varying calcium concentration in the range of pCa 2 to 7.

Voltage gating is strikingly different for the chicken and bovine MIP channels. Our present studies on MIP28 indicate that it is more voltage sensitive and that the channel is more completely closed by large voltages of both polarities. The Boltzmann parameters for the chicken MIP28 channel are $V_0 = 18.5$ mV, $n = 4.5$, $g_{min}/g_{max} = 0.17$, while those for the bovine MIP28 homologue are $V_0 = 57$ mV, $n = 2.6$, $g_{min}/g_{max} = 0.49$ (Ehring et al., 1990). Unfortunately, a full length cDNA for the chicken MIP is not yet available, so that complete sequence comparison cannot be made. However, MIP28 and MIP26 are more than 80% identical in their carboxyl-terminus halves as deduced by comparison of the full length clone for bovine cDNA (Gorin et al., 1984) and the partial length clone for chicken cDNA (Kodama et al., 1990). There are, however several amino acid substitutions even in this highly homologous part of the protein and some of them have clear functional predictions. MIP28 lacks the Ser243 of the bovine sequence, an important phosphorylation site for c-AMP-dependent protein kinase. In this paper we have demonstrated that MIP28 is not phosphorylated by protein kinase A. Additionally, Ehring et al. (1991) have shown that phosphorylation regulates the voltage dependence of bovine MIP. Taken together, these data would also explain why

partial proteolysis of the bovine MIP (which removes the cAMP phosphorylation site) reduces its voltage dependence (Ehring et al., 1988), while applying the same treatment to the chicken homologue does not have such an effect. Based on these functional comparisons, one may speculate that although in the bovine homologue an important voltage-dependent regulation site is present in the C-terminal cytoplasmic tail of MIP, this region probably does not contain the voltage-sensing region of the protein. Actually, the structural similarity and the reported differences in properties between the two proteins should provide helpful insights in choosing regions to mutate in either the bovine or chicken homologue in studies aimed at unravelling the functionally significant domains of the MIP proteins.

The high degree of homology between the lens MIP and a number of transporters in plants, bacteria and animal cells has given rise to the concept of an ''MIP family'' of integral membrane proteins (Pao et al., 1991; Preston & Agre, 1991). Of special interest in this family are Big Brain, a *Drosophila* neurogene product (Rao et al., 1990), and the erythrocyte and kidney water channels: CHIP28 (Preston & Agre 1991) and WCH-CD (Fushimi et al., 1993). mRNAs encoding CHIP28 and WCH-CD have been injected into *Xenopus* oocytes resulting in the expression of functional water channels (Preston et al., 1992; van Hoek & Verkman, 1992; Fushimi et al., 1993). However, the injected oocytes showed no alteration in membrane conductance, suggesting that the water channels do not function as ion channels. A number of laboratories have shown that oocytes injected with MIP mRNA also fail to exhibit alterations in total membrane conductance (D.L. Paul, G. Dahl) *personal communication;* Mulders et al., 1995; Kushmerick et al., 1995). Furthermore, Mulders et al. (1995) reported that oocytes expressing MIP displayed increased osmotic permeability coefficients, although with permeability values 6-fold smaller than those displayed by aquaporin-expressing oocytes. The observation that reconstitution of HPLC-purified (Ehring et al., 1990; Shen et al., 1992) and electroeluted MIP (Fig. 12, present paper) into planar lipid bilayers results in ion channel activity is difficult to reconcile with the oocyte expression experiments. Either the oocyte system fails to correctly process the translated protein, as has been reported for connexin46 (Paul et al., 1991), or the biochemical procedures used to isolate and reconstitute MIP into artificial membranes induce conformational changes in the present that render it ion permeable. The use of mammalian expression systems should help in attributing to MIP a definitive role as an ion or a water channel.

This work was supported by National Institutes of Health grants EY 08969 to DCS and ACCC, GM37230 to PDL, and a Program Project grant from FINEP to ACCC. EM and MCR received fellowships from the National Research Council (CNPq). ACCC is a career investigator of CNPq.

References

Alvarez, O. 1986. How to set up a bilayer system. *In:* Ion Channel Reconstitution. C. Miller, editor. pp. 115–129 Plenum Press, NY

Benedetti, E.L., Dunia, I., Bentzel, C.J., Vermorken, A.J.M., Kibbelaar, M., Bloemendal, H. 1976. A portriat of plasma membrane specializations in eye lens epithelium and fibers. *Biochim. Biophys. Acta* **457:353–384**

Bennett, M.V.L. 1966. Physiology of electrotonic junctions. *Ann. N.Y. Acad. Sci.* **37:**509–539

Beyer, E.C., Paul, D.L., Goodenough, D.A. 1987. Connexin43: a protein from rat heart homologous to a gap junction protein from liver. *J. Cell Biol.* **105:**2621–2629

DeVries, S.H., Schwartz, E.A. 1992. Hemi-gap junction channels in solitary horizontal cells of the catfish retina. *J. Physiol.* **445:**201–230

Ebihara, L., Steiner, E. 1993. Properties of a nonjunctional current expressed from a rat connexin46 cDNA in *Xenopus* oocytes. *J. Gen. Physiol.* **102:**59–74

Ehring, G.R., Zampighi, G.A., Hall, J.E. 1988. Properties of MIP26 channels reconstituted into planar lipid bilayers. *In:* Gap Junctions. R.G. Johnson and E.L. Hertzberg, editors. pp. 335–346. A.R. Liss, New York

Ehring, G.R., Zampighi, G., Horwitz, J., Bok, D., Hall, J.E. 1990. Properties of channels reconstituted from the major intrinsic protein of lens fiber membranes. *J. Gen. Physiol.* **96:**631–664

Ehring, G.R., Lagos, N., Zampighi, G.A., Hall, J.E. 1991. Phosphorylation modulates the voltage dependence of channels reconstituted from the major intrinsic protein of the lens fiber membranes. *J. Membrane Biol.* **126:**75–88

Fushimi, K., Uchida, S., Hara, Y., Hirata, Y., Marumo, F., Sasaki, S. 1993. Cloning and expression of apical membrane water channel of rat kidney collecting tubule. *Nature* **361:**549–552

Garland, D., Russel, P. 1985. Phosphorylation of lens fiber cell membrane proteins. *Proc. Natl. Acad. Sci. USA* **82:**653–657

Girsch, S.J., Peracchia, C. 1985*a.* Lens cell-to-cell channel protein: I. Assembly into liposomes and permeability regulation by calmodulin. *J. Membrane Biol.* **83:**217–225

Girsch, S.J., Peracchia, C. 1985*b.* Lens cell-to-cell protein: II. Conformational change in the presence of calmodulin. *J. Membrane Biol.* **83:**227–233

Gooden, M.M., Takemoto, L.J., Rintoul, D.A. 1985*b.* Reconstitution of MIP26 from single human lenses into artificial membranes. I. Differences in pH sensitivity of cataractous *vs.* normal human lens fiber cell proteins. *Curr. Eye Res.* **4:**1107–1115

Gooden, M.M., Rintoul, D.A., Takehana, M., Takemoto, L. 1985. Major intrinsic polypeptide (MIP) from lens membrane: Reconstitution into vesicles and inhibition of channel forming activity by antiserum. *Biochem. Biophys. Res. Commun.* **128:**993–999

Goodenough, D.A. 1979. Lens gap junctions: A structural hypothesis for nonregulated low-resistance intracellular pathways. *Invest. Ophthalmol. Vis. Sci.* **18:**1104–1122

Gorin, M.B., Yancey, S.B., Cline, J., Revel, J.P., Horwitz, J. 1984. The major intrinsic protein (MIP) of the bovine fiber membrane: Characterization and structure based on cDNA cloning. *Cell* **39:**49–59

Hanke, W., Andree, J., Strotmann, J., Kahle, C. 1990. Functional renaturation of receptor polypeptides eluted from SDS polyacrylamide gels. *Eur. Biophys. J.* **18:**129–134

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

Harris, A.L., Walter, A., Paul, D., Goodenough, D.A., Zimmerberg, J. 1992. Ion channels in single bilayers induced by rat connexin32. *Molec. Br. Res.* **15:**269–280

Johnson, K.D., Hofte, H., Chrispeels, M.J. 1990. An intrinsic tonoplast protein of protein storage vacuoles in seeds is structurally related to a bacterial solute transporter (GlpF). *The Plant Cell* **2:**525–532

Johnson, K.R., Panter, S.S., Johnson, R.G. 1985*a*. Phosphorylation of lens membranes with a cyclic AMP-dependent protein kinase purified from the bovine lens. *Biochim Biophys. Acta* **844:**367–76

Johnson, R.G., Frenzel, E., Johnson, K., Klukas, K., Lampe, P., Sas, D., Biegon, R., Louis, C. 1985*b*. Is lens MP26 a gap junction protein? A phosphoprotein? *In:* Gap Junctions. M.V.L. Bennett and D.C. Spray, editors. pp. 91–105. Cold Spring Harbor, NY

Johnson, K.R., Lampe, P., Hur, K.J., Louis, C.F., Johnson, R.G. 1986. A lens intercellular junction protein, MP26, is a phosphoprotein. *J. Cell Biol.* **102:**1334–1343

Kistler, J., Christie, D., Bullivant, S. 1988. Homologies between gap junction proteins in lens, heart and liver. *Nature* **331:**721–723

Kodama, R., Agata, K., Mochi, M., Eguchi, G. 1990. Partial amino acid sequence of the major intrinsic protein (MIP) of the chicken lens deduced from the nucleotide sequence of a cDNA clone. *Exp. Eye Res.* **50:**737–741

Kumar, N.M., Gilula, N.B. 1986. Cloning and characterization of rat liver cDNAs coding for a gap junction protein. *J. Cell Biol.* **103:**767–776

Kushmerick, C., Rice, S.J., Baldo, G.J., Haspel, H.C., Mathias, R.T. 1995. Ion, water and neutral solute transport in *Xenopus* oocytes expressing frog lens MIP. *Exp. Eye Res.* **61:**351–362

Kuszak, J., Maisel, H., Harding, C.V. 1978. Gap junctions of chick lens fiber cells. *Exp. Eye Res.* **27:**495–498

Lampe, P., Johnson, R.G. 1989. Phosphorylation of MP26, a lens junction protein, is enhanced by activators of protein kinase C. *J. Membrane Biol.* **107:**145–155

Lampe, P., Johnson, R.G. 1990. Amino acid sequence of in vivo phosphorylation sites in the main intrinsic protein (MIP) of lens membrane. *Eur. J. Biochem.* **144:**541–547

Louis, C.F., Johnson, R.G., Johnson, K.R., Turnquist, J. 1985. Characterization of the bovine lens plasma membrane substrates for cAMP-dependent protein kinase. *Eur. J. Biochem.* **150:**276–286

Mathias, R.T., Rae, J.L. 1985. Transport properties of the lens. *Am. J. Physiol.* **249:**C181–C190

Modesto, E., Barcellos, L., Campos de Carvalho, A.C. 1990. MIP28 forms channels in planar lipid bilayers. *Braz. J. Med. Biol. Res.* **23:**1029–1032

Moreno, A.P., Sáez, J.C., Fishman, G.I., Spray, D.C. 1994. Human connexin43 gap junction channels. Regulation of unitary conductances by phosphorylation. *Circ. Res.* **74:**1050–1057

Mulders, S.M., Preston, G.M., Dien, P.M., Guggino, W.B., van Os, C.H., Agre, P. (1995) Water channel properties of major intrinsic protein of lens. *J. Biol. Chem.* **270**(15):9010–9016

Muramatsu, S., Mizuno, T. 1989. Nucleotide sequence of the region encompassing the *glpKF* operon and its upstream region containing a bent DNA sequences of *Escherichia coli*. *Nucl. Acids Res.* **17:**4378

Nikaido, H., Rosenberg, E.Y. 1985. Functional reconstitution of lens gap junction proteins into proteoliposomes. *J. Membrane Biol.* **85:**87–92

Pao, G.M., Wu, L.F., Johnson, K.D., Hofte, H., Chrispeels, M.J., Sweet, G., Sandal, N.N., Saier, M.H. 1991. Evolution of the MIP family of integral membrane transport proteins. *Mol. Microbiol.* **5:**33–37

Paul, D.L. 1986. Molecular cloning of cDNA for rat liver gap junction protein. *J. Cell Biol.* **103:**123–134

Paul, D.L., Ebihara, L., Takemoto, L.J., Swenson, K.I., Goodenough, D.A. 1991. Connexin46, a novel lens gap junction protein, induces voltage-gated currents in nonjunctional plasma membrane of *Xenopus* oocytes. *J. Cell Biol.* **115:**1077–1089

Paul, D.L., Goodenough D.A. 1983. Preparation, characterization, and localization of antisera against bovine MP26, an integral protein from lens fiber plasma membrane. *J. Cell Biol.* **96:**625–632

Preston, G.M., Agre, P. 1991. Isolation of the cDNA for erythrocyte integral membrane protein of 28 kilodaltons: Member of an ancient channel family. *Proc. Natl. Acad. Sci. USA* **88:**11110–11114

Preston, G.M., Carroll, T.P., Guggino, W.B., Agre, P. 1992. Appearance of water channels in *Xenopus* oocytes expressing red cell CHIP28 protein. *Science* **256:**385–387

Rao, V., Jan, L.V., Jan, L.Y. 1990. Similarity of the product of the *Drosophila* neurogenic gene big brain to transmembrane channel proteins. *Nature* **345:**163–167

Sandel, N.N., Marcker, K.A. 1988. Soybean noduli 26 is homologous to the major intrinsic protein of the bovine lens fiber membrane. *Nucl. Acids Res.* **16:**9347

Sas, D.F., Sas, J.M., Johnson, K., Menko, A.S., Johnson, R.G. 1985. Junctions between lens fiber cells are labelled with a monoclonal antibody shown to be specific for MP26. *J. Cell Biol.* **100:**216–225

Scaglione, B.A., Rintoul, D.A. 1989. A fluorescence quenching assay for measuring permeability of reconstitued lens MIP26. *Invest. Ophthal. Vis. Sci.* **30:**961–966

Schein, S.J., Colombini, M., Finkelstein, A. 1976. Reconstituting in planar lipid bilayer of a voltage-dependent anion-selective channel obtained from *Paramecium* mitochondria. *J. Membrane Biol.* **30:**99–120

Shen, L., Shrager, P., Girsch, S.J., Donaldson, P.J., Peracchia, C. 1991. Channel reconstitution in liposomes and planar bilayers with HPLC-purified MIP26 of bovine lens. *J. Membrane Biol.* **124:**21–32

Shiels, A., Kent, N.A., McHale, M., Bangham, J.A. 1988. Homology of MIP26 to Nod26. *Nucl. Acid Res.* **16:**9348

Smith, C.P., Chater, K.F. 1988. Structure and regulation of controlling sequences for the *Streptomyces coelicolor* glycerol operon. *J. Mol. Biol.* **204:**569–580

Spray, D.C., Harris, A.L., Bennett, M.V.L. 1981. Equilibrium properties of a voltage-dependent junctional conductance. *J. Gen. Physiol.* **77:**75–94

Swenson, K.I., Jordan, J.R., Beyer, E.C., Paul, D.L. 1989. Formation of gap junctions by expression of connexins in *Xenopus* oocyte pairs. *Cell* **57:**145–155

van Hoek, A.N., Verkman, A.S. 1992. Functional reconstitution of the isolated erythrocyte water channel CHIP28. *J. Biol. Chem.* **267**(26): 18267–18269

White, T.W., Bruzzone, R., Goodenough, D.A., Paul, D.L. 1992. Mouse C×50, a functional member of the connexin family of gap junction proteins, is the lens fiber protein MP70. *Mol. Biol. Cell* **3:**711–720

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

Zampighi, G.A., Hall, J.E., Kreman, M. 1985. Purified lens junctional protein forms channels in planar lipid films. *Proc. Natl. Acad. Sci. USA* **82:**8468–8472

Zampighi, G.A., Hall, J.E., Ehring, G.R., Simon, S.A. 1989. The structural organization and protein composition of lens fiber junctions. *J. Cell Biol.* **108:**2255–2276

Zhang, R., Skach, W., Hasegawa, H., van Hoek, A.N., Verkman, A.S. 1993. Cloning, functional analysis and cell localization of a kidney proximal tubule water transporter homologous to CHIP28. *J. Biol. Chem.* **120:**359–369