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Phosphorylation Modulates the Voltage Dependence of Channels Reconstituted from the Major Intrinsic Protein of Lens Fiber Membranes

G.R. Ehring†, N. Lagos,‡*, G.A. Zampighi‡, and J.E. Hall†

†Department of Physiology and Biophysics, University of California, Irvine, California 92717, and ‡Department of Anatomy and Cell Biology, Jerry Lewis Center, University of California, Los Angeles, California 90024

Summary. Major intrinsic polypeptide (MIP), a 28-kDa protein isolated from lens fiber cell membranes, forms large, nonselective channels when reconstituted into lipid bilayers. MIP channels are regulated by voltage, such that these channels close when the potential across the membrane is greater than 30 mV. We have investigated the modulation of the voltage-dependent closure of MIP channels by phosphorylation. In this report, we describe the isolation of two isomers of MIP from lens fiber cell membranes. These isomers differ by a single phosphate at a protein kinase A phosphorylation site. The phosphorylated isomer produces channels that close in response to applied voltages when reconstituted into bilayers. The nonphosphorylated isomer produces voltage-independent channels. Direct phosphorylation with protein kinase A converts voltage-independent channels to voltage-dependent channels *in situ*. Analyses of macroscopic and single-channel currents suggest that phosphorylation increases the voltage-dependent closure of MIP channels by increasing closed channel lifetimes and the rate of channel closure following the application of voltage.

Key Words ion channels · phosphorylation · modulation of ion channels · lens fibers · reconstitution · intercellular junctions · major intrinsic protein · gap junctions

Introduction

Highly elongated cells, called fiber cells, extend from the posterior to the anterior surfaces of the lens and are the principal cells of the vertebrate lens. These cells, which constitute the bulk of the developed lens, derive from the layer of simple cuboidal epithelium that underlies the anterior and equatorial capsules of the lens (Kuwabara, 1975). During differentiation fiber cells lose most of their cytoplasmic organelles, increase in length and volume, and synthesize a unique complement of cytoplasmic and membrane proteins (Kuwabara, 1975; Maisel et al., 1981; Bloemendal, 1982; Kistler & Bullivant, 1989). It is the properties of the terminally differentiated fiber cells that confer the attributes of transparency and refraction on the lens.

A large proportion (50–60%) of the plasma membrane protein from lens fiber cells consists of a 28-kDa intrinsic protein called MIP (Broekhuyse, Kuhlmann & Stols, 1976; Bloemendal et al., 1977; Broekhuyse, Kuhlmann & Winkens, 1979). MIP's structure and membrane topology have been studied using biochemical methods (Takemoto et al., 1978; Broekhuyse & Kuhlmann, 1980; Horwitz & Wong, 1980; Horwitz & Bok, 1987); its primary sequence has been determined by cDNA cloning (Gorin et al., 1984), and its localization in plasma membrane and in specialized junctions between fiber cells has been characterized by immunocytochemistry (Bok, Dockstader & Horwitz, 1982; Paul & Goodenough, 1983; Sas et al., 1985; Zampighi et al., 1989). However, its role in the physiology of the lens is still poorly understood. Several possible functions have been suggested for MIP; these include gap junctional channel (Bok et al., 1982; Fitzgerald, Bok & Horwitz, 1982; Girsch & Peracchia, 1985*a*,*b*; Sas et al., 1985), cell adhesion molecule (Lo & Harding, 1984; Costello, McIntosh & Robertson, 1989; Gruijters, 1989), and single-membrane channel (Ehring et al., 1990).

The channel-forming properties of MIP were suggested by the observation that when MIP was reconstituted into liposomes, the permeability of these liposomes to molecules less than 1.5 kDa in mol. wt. increased (Girsch & Peracchia, 1985*a*; Gooden et al., 1985*a*; Gooden, Takemoto & Rintoul, 1985*b*; Nikaido & Rosenberg, 1985; Peracchia & Girsch, 1985*c*; Scaglione & Rintoul, 1989). We have previously reported that MIP forms large, relatively nonselective, voltage-dependent channels when re-

* *Present address:* Departamento de Fisiologia y Biofisica, Facultad de Medicina, Universidad de Chile, Casilla 70055 Santiago 7, Chile.

constituted into planar bilayers (Zampighi, Hall & Kreman, 1985; Ehring et al., 1990). Reconstituted MIP channels have a single-channel conductance of 360 pS in 100 mM KCl and close with membrane voltages greater than 30 mV (Ehring et al., 1990). While the open and closed probabilities of reconstituted MIP channels are regulated by membrane voltage, little is known regarding the mechanisms for the regulation of these channels in vivo. In this report, we investigate the possibility that phosphorylation modulates function of MIP channels.

MIP is phosphorylated in vivo (Lampe & Johnson, 1990) and has been shown to be a substrate for several protein kinases (Johnson et al., 1985; Louis et al., 1985; Lampe et al., 1986; Lampe & Johnson, 1989). There is strong evidence that altering the level of phosphorylation in cells modulates the function of some ion channels (for reviews *see* Levitan 1985, 1988). Several studies have implicated direct phosphorylation of channel-forming proteins as a mechanism for modulating channel function (Ewald, Williams & Levitan, 1985; Shuster et al., 1985; Flockerzi et al., 1986). Because MIP is endogenously phosphorylated in the lens and because of the precedents cited above, it seemed quite possible that MIP channels in vivo could be modulated by phosphorylation.

In this paper, we show that two isomers of MIP can be isolated from bovine lens membrane and that one of the isomers is phosphorylated at a protein kinase A phosphorylation site. We show that the two isomers also differ functionally. The phosphorylated isomer reconstituted into bilayers forms voltage-dependent channels similar to those previously described (Ehring et al., 1990). But the nonphosphorylated isomer reconstituted into bilayers forms voltage-independent channels. We further show that voltage-independent channels reconstituted into planar bilayers from nonphosphorylated MIP can be converted into voltage-dependent channels by direct phosphorylation. In multi-channel membranes, phosphorylation increased the fraction of voltage-dependent current without significantly affecting the maximum membrane conductance. Single-channel data showed that the phosphorylation-induced enhancement of the voltage-dependent closure of MIP channels resulted from an alteration in channel kinetics rather than a change in single-channel conductance.

Materials and Methods

Reagents

Alkaline phosphatase from bovine pancreas and protein kinase A, type II, from skeletal muscle were purchased from Sigma Chemical (St. Louis, MO). Protein kinase A catalytic subunit was purchased from Sigma Chemical (St. Louis, MO) or obtained as a gift from Dr. Irwin Levitan (Brandeis University, Boston, MA). Phosphatidylethanolamine and phosphatidylcholine were purchased from Avanti Polar Lipids (Birmingham, AL). Squalane was purchased from Atomergic Chemicals (Plainview, NY). All inorganic salts, reagents and buffers were analytical reagent grade or better.

Biochemistry

Lenses from three-month-old calves or adult cows, quick frozen in liquid nitrogen at the slaughterhouse and shipped in dry ice, were obtained from Pel-Freeze Biologicals (Roger, AR). Plasma membranes were isolated using previously described techniques (Zampighi et al., 1982). MIP was purified from the membranes by detergent solubilization and anionic exchange HPLC. Plasma membranes were dissolved with octyl-glucopyranoside at a detergent : protein ratio of 20 : 1 (wt/wt) in 10 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1 mM $CaCl_2$, 0.1 mM DTT, 25 mM NaCl and 10% glycerol (vol/vol) (buffer A). The solution was centrifuged at 100,000 × *g* for 30 min, and the resulting pellet discarded. The supernatant was injected (500–700 μg of protein in 140 μl) into an analytical MA7Q column (Bio-Rad) and eluted with a linear gradient (0.025 to 0.5 M NaCl in buffer A containing 34 mM detergent) at a flow rate of 1.5 ml/min. Fractions of about 1 ml were collected and their protein concentration was monitored by absorbance at A_{280}. The fractions containing proteins were concentrated to 1–2 mg/ml by centrifugation at 5,000 × *g* using a microconcentrator tube (Centricon-10, Amicon, Danvers, MA).

To obtain preparations enriched in the nonphosphorylated isomer of MIP, approximately 100 μl of isolated membranes (2–3 mg/ml) were incubated with 1,000 units of alkaline phosphatase in 0.1 M glycine, 0.01 M $MgCl_2$, pH 10.4 for 30 min at room temperature. The treated membranes were then dissolved and chromatographed as described above. To obtain preparations enriched in phosphorylated MIP, alkaline phosphatase dephosphorylated MIP (65 μg) was incubated at 30°C for 30 min in 9 mM Na acetate buffer pH 6.0, 5 mM $MgCl_2$, 25 μM dibutyl-cAMP, 0.5 mM ATP and 200 units of protein kinase A. When phosphate incorporation into MIP was studied, nonradioactive ATP was replaced by 0.5 mM gamma ^{32}P-ATP with a specific activity of 335 mCi/mmol.

Protein concentrations were measured according to Bradford (1976), and phosphate concentration was measured according to Bartlett (1959). The protein compositions of isolated membranes and purified fractions were determined by SDS-PAGE in 12.5% acrylamide or 4–20% gradient acrylamide gels (Laemmli, 1970). MIP-containing fractions were identified by Western blots (Burnette, 1981) using a monoclonal antibody prepared against MIP (Paul & Goodenough, 1983). The aggregation number of the solubilized and purified MIP was determined by crosslinking with glutaraldehyde. Purified MIP (60 μg) was treated with 16 mM glutaraldehyde in 10 mM Tris-HCl pH 8.0 for 45 min at room temperature. The reaction was quenched with eight-fold molar excess glycine. The different molecular weight aggregates formed by crosslinking were identified by Western blot analysis.

Electrophysiology

MIP was reconstituted into unilamellar phosphatidylcholine vesicles using previously published methods (Zampighi et al., 1985). MIP-containing vesicles were then used to induce MIP channels

in phosphatidylethanolamine bilayers (Zampighi et al., 1985; Ehring et al., 1990; Ehring & Hall, 1991). Briefly, planar bilayers were formed on a 200–300-μm hole in a 20-μm thick Teflon partition. The partition divided a Teflon chamber into two compartments each containing approximately 2 ml of 200 mM KCl, 20 mM 2-(N-morpholino) ethanesulfonic acid, pH 5.8. After formation of the bilayer, solution containing MIP reconstituted into vesicles (10–20 μl) was added to both compartments of the bilayer chamber. After the addition of vesicles, both compartments were stirred for 3 min. Channel incorporation, which occurred within 30 min, was detected by increases in membrane conductance.

The electrical properties of channels reconstituted from either nonphosphorylated or phosphorylated MIP were compared under voltage clamp. Nonphosphorylated MIP was obtained by collecting the HPLC peak eluting with a retention time of 0:39 (min:sec). In some cases, the yield of nonphosphorylated MIP was enhanced by pretreating the membranes with alkaline phosphatase. Phosphorylated MIP was obtained by incubating the purified nonphosphorylated protein 40 μg/ml with 500 units of protein kinase A catalytic subunit, in 9 mM sodium acetate, pH 6.0, containing 1.0 mM Na-ATP and 5.0 mM $MgCl_2$ for 2 hr at 30°C. After phosphorylation, the protein was chromatographed as described above, and the peak with a retention time of 1:18 (min:sec) collected for reconstitution.

In situ phosphorylation was accomplished by the addition of a phosphorylating mixture to the bilayer chamber after the incorporation of channels from nonphosphorylated MIP. The mixture contained either protein kinase A holoenzyme or protein kinase A catalytic subunit. Enzyme concentrations of 0.03 to 2.5 μg/ml added to each compartment of the bilayer chamber were found effective in these experiments. The *in situ* phosphorylation mixture also contained 5 mM $MgCl_2$, 0.5 mM ATP, 25 μM cAMP in 200 mM KCl, 20 mM 2-(N-morpholino) ethanesulfonic acid, pH 5.8. Cyclic AMP was omitted from the phosphorylation mixture when catalytic subunit was used instead of the holoenzyme. Except as noted below, the phosphorylation mixture was added to both sides of the membrane.

The membrane current-voltage relationship was determined by applying 5-sec voltage pulses from 0 mV to voltages from 20 to 80 mV in 20-mV increments. Voltage-clamp data were collected using a FORTRAN-based data acquisition system (J.E. Hall). Summarized data are presented as the mean ± the SEM. Statistical comparisons of group means were performed using Student's *t* test. Single and double exponential fits were done with a nonlinear least-squares fitting routine (Clampfit, Axon Instruments, Foster City, CA). Analysis of single-channel conductances, latency to first closure, and open and closed channel lifetimes were done with Fetchan and Pstat (Axon Instruments, Foster City, CA).

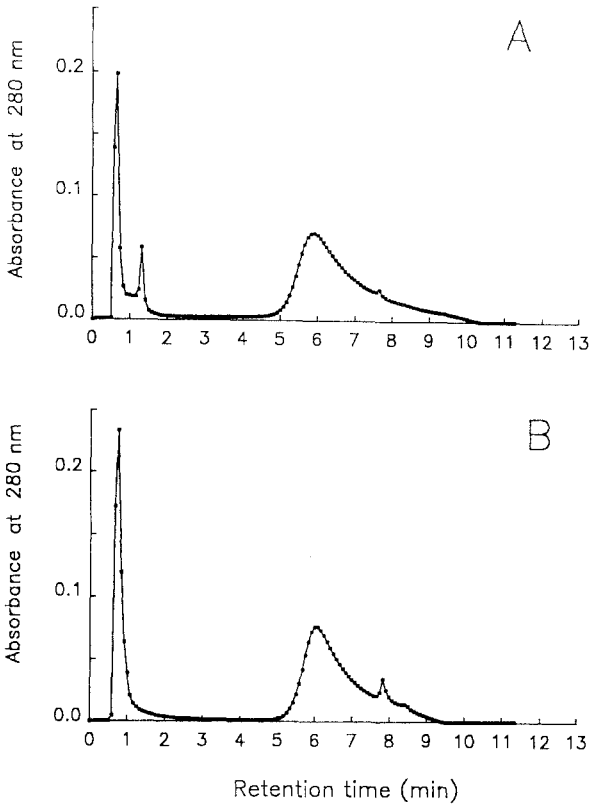

Fig. 1. Absorbance profile of solubilized lens fiber cell membrane proteins eluted from a strong anionic exchange HPLC. Isolated lens junctions were dissolved in buffer containing octyl-glucopyranoside at a detergent : protein ratio of 20 : 1 (wt/wt). The solution was centrifuged at 100,000 × *g* for 30 min, and the supernatant chromatographed in a MA7Q column (Bio-Rad). The solubilized lens proteins eluted with a linear gradient (0.025 to 0.5 M NaCl containing 34 mM detergent). MIP eluted in two peaks near the void volume (*A*). These peaks, designated $t_{0:39}$ and $t_{1:18}$, had retention times of 0:39 (min:sec) and 1:18 (min:sec), respectively. In addition to the peaks containing MIP, the elution profile contained a broad peak where the remaining proteins eluted. The treatment of isolated junctions with alkaline phosphatase prior to solubilization eliminated the $t_{1:18}$ peak (*B*).

Results

Isolation of MIP Isomers

The detergent-solubilized plasma membrane proteins were separated into three distinct peaks when chromatographed in a strong anion exchange column (Fig. 1*A*). Two sharp peaks, each containing only MIP, eluted with short retention times. The first peak eluted just after the void volume with a retention time of 0:39 (min:sec) and was designated peak $t_{0:39}$. The second peak eluted with a retention time of 1:18 (min:sec) and was designated peak $t_{1:18}$. A third peak containing the rest of the proteins eluted with a broad absorbance maximum and had a retention time of approximately 6:00 (min:sec). That the two sharp peaks contained only MIP was confirmed with SDS-PAGE *see* (Fig. 3*A*) and Western blot analysis (*not shown*). Peak $t_{0:39}$ contained 80% of the total MIP, and peak $t_{1:18}$ contained 20%.

Conversion of Isomers

Since ion exchange chromatography separates polypeptides on the basis of charge, the elution of MIP in two peaks suggested that the membranes contained

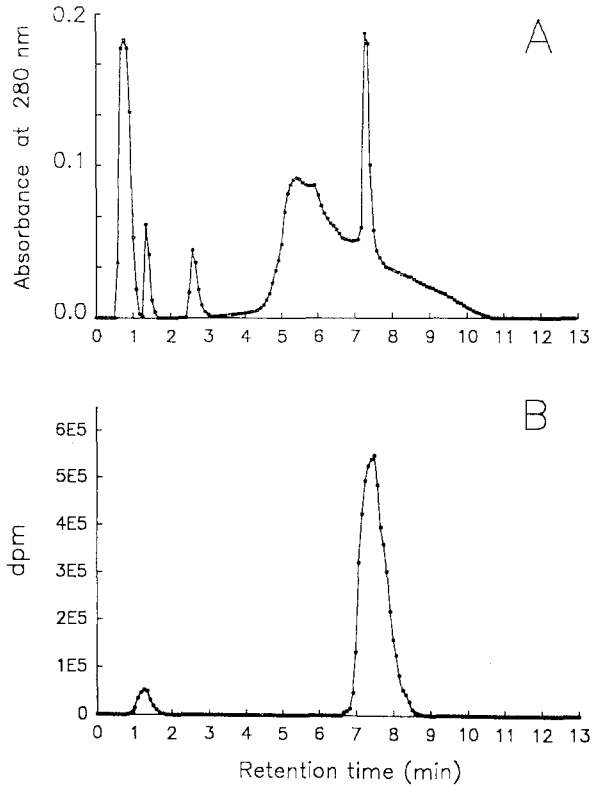

Fig. 2. HPLC chromatogram of in vitro phosphorylated MIP. Nonphosphorylated MIP was incubated in phosphorylating buffer containing gamma ^{32}P-ATP. (*A*) An absorbance profile of peaks containing phosphorylated MIP, nonphosphorylated MIP, ADP and ATP. (*B*) Incubation with gamma ^{32}P-ATP resulted in two radioactive peaks corresponding to phosphorylated MIP and gamma ^{32}P-ATP.

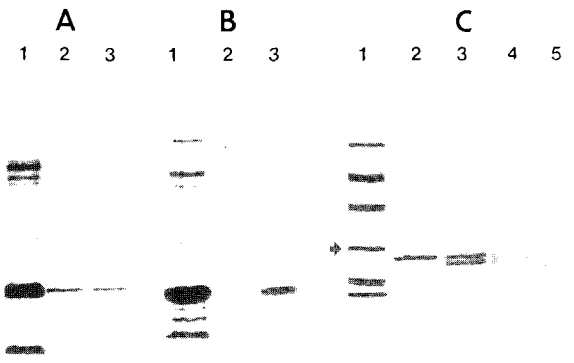

Fig. 3. Phosphorylation and crosslinking of purified MIP. (*A*) Silver-stained polyacrylamide gels. Lane *1* was loaded with solubilized protein from isolated junctional membranes (4 μg protein). MIP migrates as a 27-kDa mol wt band, the largest on the gel. Lane *2* was loaded with 0.8 μg protein from peak $t_{0:39}$, and lane *3* with 0.8 μg protein from peak $t_{1:18}$. The single bands visible in lanes *2* and *3* were identified as MIP by Western blots with a monoclonal antibody to MIP (*not shown*). (*B*) A SDS-PAGE gel stained with Coomassie blue and its autoradiogram. Lane *1* shows isolated junctional membranes (5–6 μg), lane *2* the protein that eluted in peak $t_{1:18}$ and lane *3* the autoradiogram of lane *2*. Lanes *2* and *3* demonstrate that the MIP contained in the $t_{1:18}$ peak is phosphorylated. (*C*) A Coomassie blue-stained gradient gel (4–20%) showing crosslinking of MIP with glutaraldehyde. Lane *1* shows molecular weight standards (black arrow at 31 kDa). Lane *2* shows that before crosslinking purified MIP migrates in a single band. Lane *3* shows that after crosslinking most of the protein appeared in a split band migrating at 27 and 25 kDa. Lane *4* shows a Western blot before crosslinking, and lane *5* shows a Western blot after crosslinking.

isomers of the protein differing in their net charge, and thus, possibly in their phosphorylation state. Therefore, we examined the influence of phosphorylation and dephosphorylation reactions on the elution profile of MIP.

Treatment of isolated membranes with alkaline phosphatase before solubilization eliminated the $t_{1:18}$ peak (Fig. 1*B*). This result suggested that the $t_{1:18}$ peak might be phosphorylated and that at least 20% of MIP was endogenously phosphorylated. Further evidence that the $t_{1:18}$ peak corresponds to phosphorylated MIP was obtained by in vitro phosphorylation. In these experiments, MIP from the peak $t_{0:39}$ was incubated with a phosphorylating mixture containing nonradioactive ATP. This resulted in the reappearance of peak $t_{1:18}$ (Fig. 2*A*). The addition of gamma ^{32}P-ATP to the mixture made peak $t_{1:18}$ radioactive (Fig. 2*B*). SDS-PAGE and autoradiography (Fig. 3*B*) and Western blotting (*not shown*) demonstrated that MIP was the substrate of the phosphorylation reaction.

The ratio of specific activities of phosphorylated MIP to gamma ^{32}P-ATP was 0.85, suggesting a phosphorylation stoichiometry of 1 : 1 by the kinase. A similar estimate of the stoichiometry was obtained from measurement of the amount of ADP generated by ATP hydrolysis. Thus, our results confirm those of Lampe and Johnson (1990) and show that only one of the serines on MIP was phosphorylated by protein kinase A.

Purified MIP was further characterized in terms of its phosphorus content and its aggregation number. Inorganic phosphorus determination of the purified MIP eluting in peak $t_{0:39}$ shows that there was 10.5 ± 2 (n = 4) mol of P_i per mol of MIP. Assuming that all the MIP in peak $t_{0:39}$ is nonphosphorylated, this result suggests that there are about 10 mol of phospholipids per mol of detergent-solubilized MIP.

Glutaraldehyde crosslinking of purified MIP showed that octyl-glucopyranoside solubilized MIP mostly as 28-kDa monomers (Fig. 3*C*). Higher molecular weight aggregates, consistent with dimers and trimers of MIP, were also observed by Western blot analysis. High molecular weight

aggregates represented a small fraction of the solubilized protein. Thus, in our hands, a solution containing a 20 : 1 (wt/wt) ratio of octylglucoside to protein dissolved MIP to monomers. However, if after the protein was dissolved in detergent solution, it was kept at room temperature or at 4°C for long periods of time (many hours), the fraction of high molecular weight aggregates in solution increased. Our results confirm a similar observation reported by Manenti et al. (1988). In contrast to these results, Aerts et al. (1990) using a similar solubilization protocol, but a different method of protein purification, found that MIP was soluble as tetramers in octylglucoside.

Function of MIP Isomers

To determine if phosphorylation altered the function of MIP channels, we reconstituted both isomers of MIP into planar lipid bilayers. We examined phosphorylation-induced differences in the conductance properties of multi-channel and single-channel membranes. Multi-channel data provide a large sample for statistical comparisons between phosphorylated and nonphosphorylated MIP channels, while single-channel data provide information on the effects of phosphorylation on MIP channels at the molecular level.

In multi-channel membranes containing channels reconstituted from nonphosphorylated MIP, voltage pulses elicited current steps proportional to the applied voltage (Fig. 4*A*). In addition, the current observed was nearly constant throughout pulses with durations varying from 5 to 60 sec at applied voltages from 20 to 80 mV. Thus, channels formed from nonphosphorylated MIP were largely time and voltage independent. In contrast, multi-channel membranes containing channels reconstituted from phosphorylated MIP produced currents which were both time and voltage dependent. In these membranes, voltage pulses only 5 sec in duration elicited currents that declined exponentially with time during the pulse and the magnitude of the decline was greater the larger the voltage applied to the membrane (Fig. 4*B*). Thus, while the instantaneous current-voltage relationships were similar for nonphosphorylated and phosphorylated channels, their steady-state current-voltage relationships differed. The time and voltage dependence of MIP currents for both the nonphosphorylated and the phosphorylated channels varied between different reconstitutions. In some reconstitutions, during pulses to 80 mV, currents from nonphosphorylated MIP channels decreased during the pulse. However, this decrease in current was always less than that observed for phosphorylated channels. Thus, while the ratio of steady-state current (I_{ss}) to the instantaneous current (I_0) was variable for both isomers, the difference in ratios between isomers was statistically significant at voltages with absolute values above 20 mV (Fig. 4*D*). A slight asymmetry in the current-voltage relationship from the channels reconstituted with material that eluted with the $t_{1:18}$ peak was noted. This asymmetry was not statistically significant.

Voltage-independent channels were converted to voltage-dependent channels by direct phosphorylation in the bilayer (Fig. 4*C*). After voltage-independent channels were reconstituted into bilayers, a phosphorylation mixture containing protein kinase A, cyclic AMP, $MgCl_2$ and ATP was added to both sides of the bilayer chamber. The resulting increase in voltage dependence required the presence of all components of the mixture except that protein kinase A catalytic subunit could be substituted for the combination of protein kinase A and cAMP. Comparison of commercially available catalytic subunit with highly purified protein kinase A catalytic subunit showed higher activity of the purified subunit but no qualitative differences in effect on the channels. After adding the phosphorylation mixture to both sides of the bilayer, the steady-state current-voltage curves obtained from the multi-channel membranes were symmetric, and the steady-state current was significantly less than from the same channels before phosphorylation (Fig. 4*D*). Thus, when phosphorylated MIP was reconstituted into the bilayer, or when nonphosphorylated MIP was reconstituted into the bilayer and then treated with a phosphorylation mixture, similar voltage-dependent channels were produced.

After the addition of the phosphorylation mixture to the bilayer chambers, the currents became progressively more voltage dependent. Maximum voltage dependence was attained in 10–45 min. The time dependence observed in these experiments could have been produced in either of two ways. First, phosphorylation could affect each channel in an all-or-none fashion, i.e., produce a discrete increase in the probability of channel closure. In this case, the progressive increase in the voltage dependence of the multi-channel membrane would result from a gradual increase in the population of channels that close in response to voltage pulses. Or second, phosphorylation could enhance the voltage dependence of the channels incrementally so that the overall increase in voltage dependence would result from gradual increases in the degree of voltage dependence of all the channels in the membrane. To distinguish between these two possibilities, we analyzed two types of experimental data: the steady-state conductance-voltage curves and the kinetics of the currents observed during the application of voltage pulses.

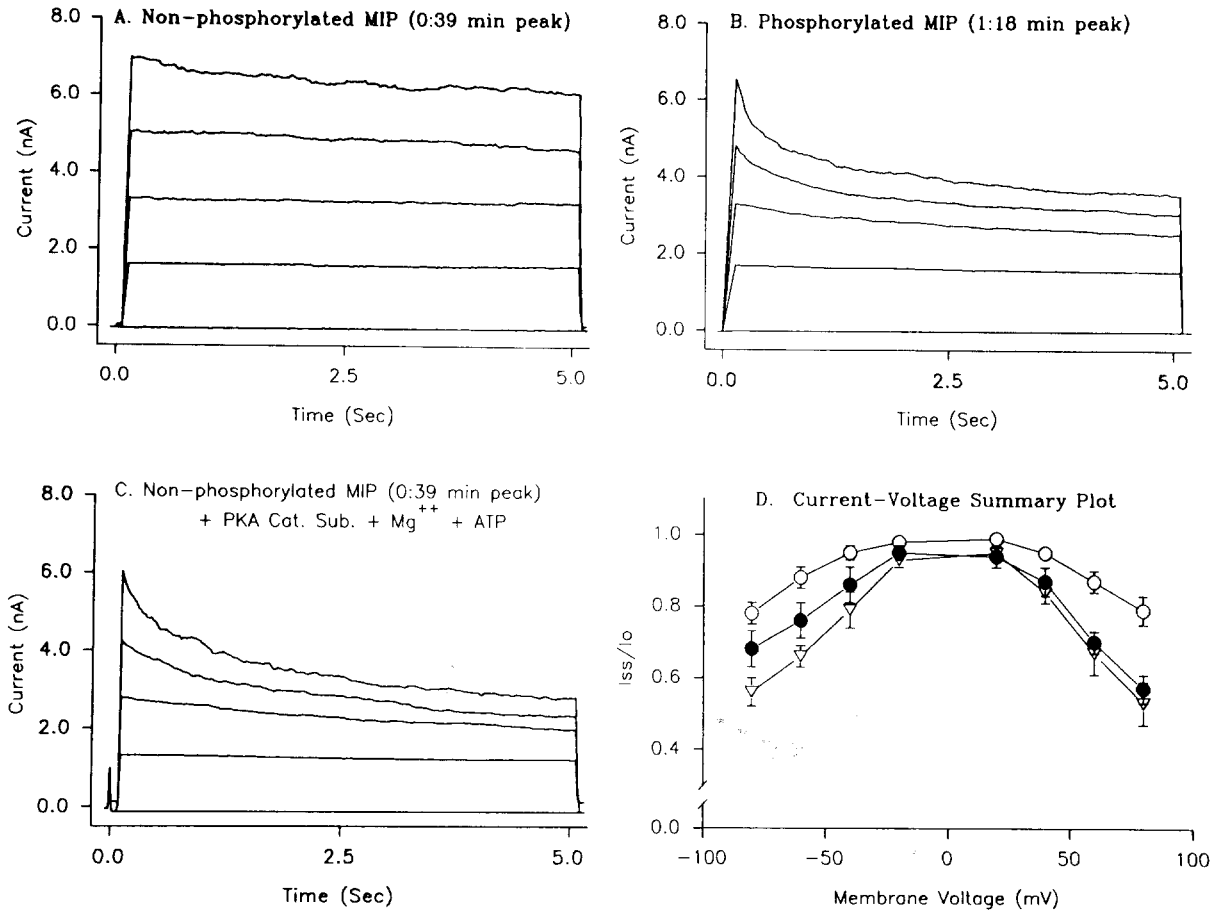

Fig. 4. Current-voltage relations for nonphosphorylated and phosphorylated MIP channels. *(A–C)* Multi-channel currents in 200 mM KCl. Five-sec pulses were applied from a holding potential of 0 mV and the resulting currents recorded. Successive pulses were applied from 20 to 80 mV in 20-mV increments with 1-sec intervals between pulses. *(A)* Nonphosphorylated MIP-induced currents that exhibited little time or voltage dependence. *(B)* Phosphorylated MIP-induced currents that depended markedly on both time and voltage. *(C)* The conversion of the nonphosphorylated type of current response to the phosphorylated type of current response. After the current records were taken for *A*, a phosphorylation mixture was added to both sides of the membrane. The records in *C* were taken approximately 60 min following the addition, and show a marked increase in both the time and voltage dependence of the measured currents. All components of the phosphorylation mixture were necessary to convert voltage-independent to voltage-dependent currents. *(D)* Steady-state current-voltage curves for phosphorylated and nonphosphorylated MIP channels. The current at the end of the pulse (I_{ss}) was divided by the current at the beginning of the pulse (I_0) to calculate the ratio I_{ss}/I_0. The ratio of I_{ss}/I_0 was plotted against membrane voltage to facilitate comparison of membranes of different conductivity. Currents from membranes (n = 9) with channels reconstituted from the nonphosphorylated MIP (open circles) were least voltage dependent. Currents from membranes (n = 9) with channels reconstituted from phosphorylated MIP (filled circles) and currents from membranes (n = 6) with channels reconstituted from nonphosphorylated MIP and phosphorylated *in situ* (open triangles) show enhanced voltage dependence. At voltages with absolute values greater than 40 mV, the steady-state currents from phosphorylated MIP channels were significantly less than those from nonphosphorylated channels ($P \leq 0.05$).

Steady-state conductance-voltage curves for MIP channels can be well fit by the sum of a two-state Boltzmann distribution and a voltage-independent conductance (Fig. 5). This relation takes the following form:

$$G_{ss} = [G_{max} \exp(-ne(V - V_0)/kT)) + G_{min}]/ [1 + \exp(-ne(V - V_0)/kT)] + G_{ind}.$$

The variables in this equation are G_{ss}, the measured steady-state conductance, and V, the applied voltage. The physical constants are, e, elementary charge, and k, Boltzmann's constant. T is the temperature in degrees Kelvin. The model parameters are, n, the valence of the gating charge, V_0, the voltage at which half the voltage-dependent channels are in the closed state, G_{max}, the maximum value of the voltage-dependent conductance, G_{min}, the minimum value of the voltage-dependent conductance, and G_{ind}, the value of the voltage-independent

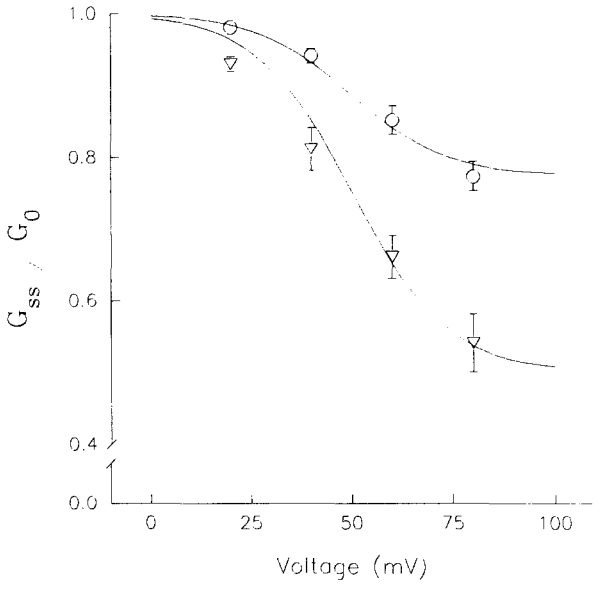

Fig. 5. Comparison of the conductance-voltage relationships of MIP channels before and after phosphorylation. The mean and SE (n = 6) for the ratio of the steady-state conductance to maximum membrane conductance (G_{ss}/G_0) were plotted for voltages from 20 to 80 mV. In membranes reconstituted with nonphosphorylated MIP (open circles), a slight decrease in conductance was observed at higher voltages. The conductance-voltage relationship was well fit by the sum of a two-state Boltzmann distribution and a voltage-independent conductance (*see* equation in the text). The best-fit values of the parameters that define the voltage dependence of the conductance were n = 2.12 e, and V_0 = 50 mV. The ratio of the voltage-independent conductance to the voltage-dependent conductance was 0.77. After the membrane was treated with a phosphorylation mixture, the steady-state membrane conductance was reduced at higher membrane voltages (open triangles). The equation still fit the conductance-voltage relationship for the membrane. There was no significant change in parameters that described the voltage-dependent conductance (n = 1.93 e, and V_0 = 50 mV), but the ratio of the voltage-independent conductance to voltage-dependent conductance decreased to 0.54.

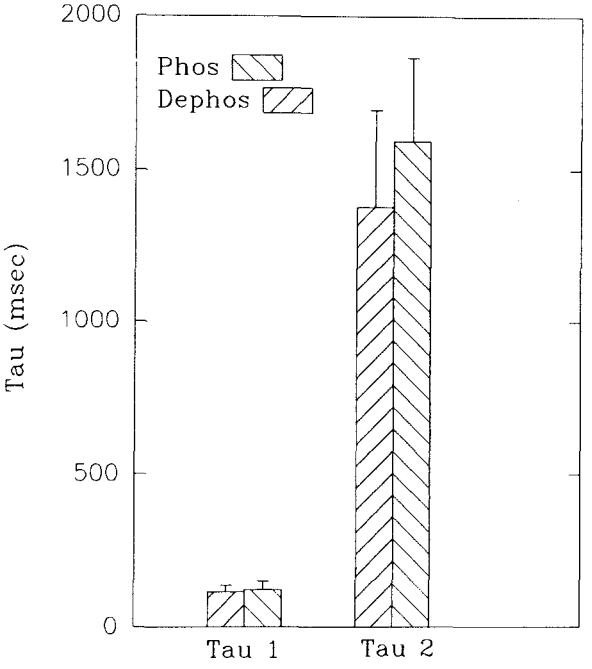

Fig. 6. Analysis of multi-channel kinetics. During test pulses to 80 mV, current through nonphosphorylated and phosphorylated MIP channels decreased exponentially with time. The data were best fit with double exponentials. In eight experiments with nonphosphorylated channels, the two time constants (tau) were 122 ± 28 and 1594 ± 272 msec. In eight experiments with phosphorylated channels, they were 116 ± 21 and 1378 ± 318 msec. Thus, the time constants were not significantly affected by phosphorylation.

conductance. An increase in n or a decrease in V_0 would be expected if the voltage dependence of each channel was altered incrementally by phosphorylation. By contrast, the ratio of G_{ind} to G_{max} would decrease if the fraction of channels capable of voltage-dependent closure were increased by phosphorylation. A comparison of the data and the predictions of the model (Fig. 5) show that the only parameter altered by phosphorylation is the ratio of G_{ind} to G_{max}, i.e., the percentage of voltage-dependent and voltage-independent channels.

Analysis of the kinetics in multi-channel membranes showed that phosphorylation altered the fraction of current which turned off at a given voltage, but had little effect on the rate at which the current decreased during voltage pulses. In membranes reconstituted with channels from phosphorylated MIP, the currents during 5-sec pulses to voltages from 40 to 80 mV were best fit by double-exponential functions, which were similar to those previously reported (Ehring et al., 1990). In membranes with channels reconstituted from nonphosphorylated MIP, it was not possible to determine the rate of decrease in current during pulses to voltages less than 80 mV, since the size of the decrease was too small to accurately fit the data with exponentials. Figure 6 shows that the time constants determined during pulses to 80 mV for currents from channels reconstituted from nonphosphorylated and phosphorylated MIP did not differ significantly. This result suggests that the primary effect of phosphorylation was to increase the number of voltage-dependent channels, rather than to increase the rate of closure of channels that were already partially voltage dependent. Therefore, analysis of the kinetic and steady-state properties of multi-channel membranes suggest that phosphorylation has an all-or-none effect on channel-gating parameters.

After the reconstitution of nonphosphorylated MIP, addition of the phosphorylation mixture to

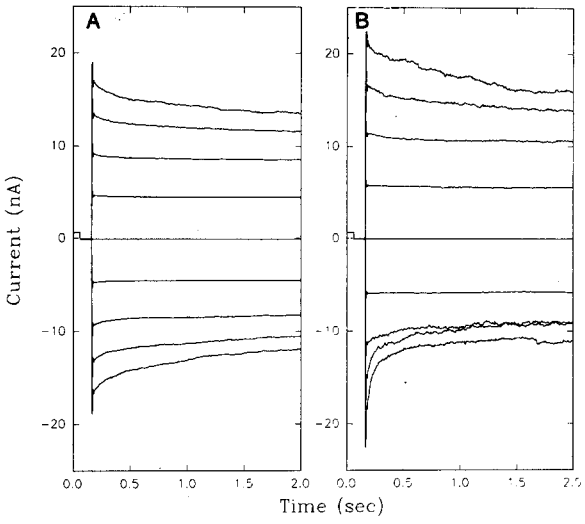

Fig. 7. Asymmetric phosphorylation results in asymmetric voltage dependence. (*A* and *B*) Multi-channel currents in 200 mM KCl. Five-sec pulses were applied from a holding potential of 0 mV and the resulting currents recorded. Successive pulses were applied from +80 to −80 mV in 20-mV increments with 1-sec intervals between pulses. (*A*) Nonphosphorylated MIP-induced currents that exhibited little time or voltage dependence. (*B*) Currents recorded after the addition of a phosphorylation mixture to only one compartment of the bilayer chamber. Pulses to positive voltages resulted in currents that were similar to those obtained before the addition of the mixture. Pulses to negative voltages resulted in currents that were both time and voltage dependent.

only one side (*cis*-compartment) of the bilayer chamber resulted in channels with an asymmetric voltage dependence (Fig. 7). When the *cis*-compartment was made negative with respect to the *trans*-compartment, the channels closed as the absolute magnitude of the voltage increased. When the *cis*-compartment was made positive, the channels did not close with increased voltage.

Single-Channel Analysis

The increase in voltage dependence induced by phosphorylation could result from a change in the lifetimes of the open and closed states or from a change in single-channel conductance of the open or closed state. Figures 8*A* and *B* show the single-channel currents before and after *in situ* phosphorylation. The primary peaks observed in conductance histograms of this data were fit by the sum of five gaussians, indicating that the membrane contained five channels. The single-channel conductances measured from these histograms were 715 ± 36 pS (n = 5) before phosphorylation and 726 ± 37 pS after phosphorylation. Thus phosphorylation did not significantly affect single-channel conductance (Fig. 8*C* and *D*). While phosphorylation did not alter the single-channel conductance, it substantially decreased the number of channels open at the end of a 5-sec pulse to 80 mV. The probability of observing all five channels open throughout the pulse was sevenfold greater for the nonphosphorylated channels than for the phosphorylated channels. Conversely, the probability that only one of the five channels would be open for some period during the test pulse was 10-fold greater after phosphorylation.

The latency to first closing was measured during pulses from 0 to 80 mV. Cumulative histograms of the interval between onset of the voltage step and the first channel closure were plotted for channels reconstituted from nonphosphorylated MIP before and after the addition of a phosphorylation mixture to the bilayer chamber (Fig. 9). Single exponentials fit to these histograms showed that the rate of channel closure after phosphorylation was approximately twice that for channels before phosphorylation. Additional experiments (*not shown*) demonstrated that before the addition of the phosphorylation mixture, channels reconstituted from nonphosphorylated MIP had longer open-state dwell times and shorter closed-state dwell times than the same channels after phosphorylation. Thus, *in situ* phosphorylation enhanced the probability that the channel entered the closed state and stabilized the channel once in that state.

Discussion

Different Isolation Principle: Same Channel

Reconstitution into bilayers is an extremely sensitive technique for the study of membrane protein function. However, its very sensitivity makes it difficult to rule out that the channels seen are not due to a contaminant. We have previously shown using HPLC purification methods based on molecular size that the likely candidate for the channel-forming protein in our reconstitutions was MIP. In this study, we used ion exchange HPLC, an entirely different separation principle, to obtain purified MIP. Even though the purification principle was entirely different, the channels seen were identical to those seen with gel filtration HPLC (Ehring et al., 1990). This result supports our previous conclusion that MIP is most likely the channel-forming protein in our lens membrane isolate. This conclusion is further strengthened by the parallels between the phosphor-

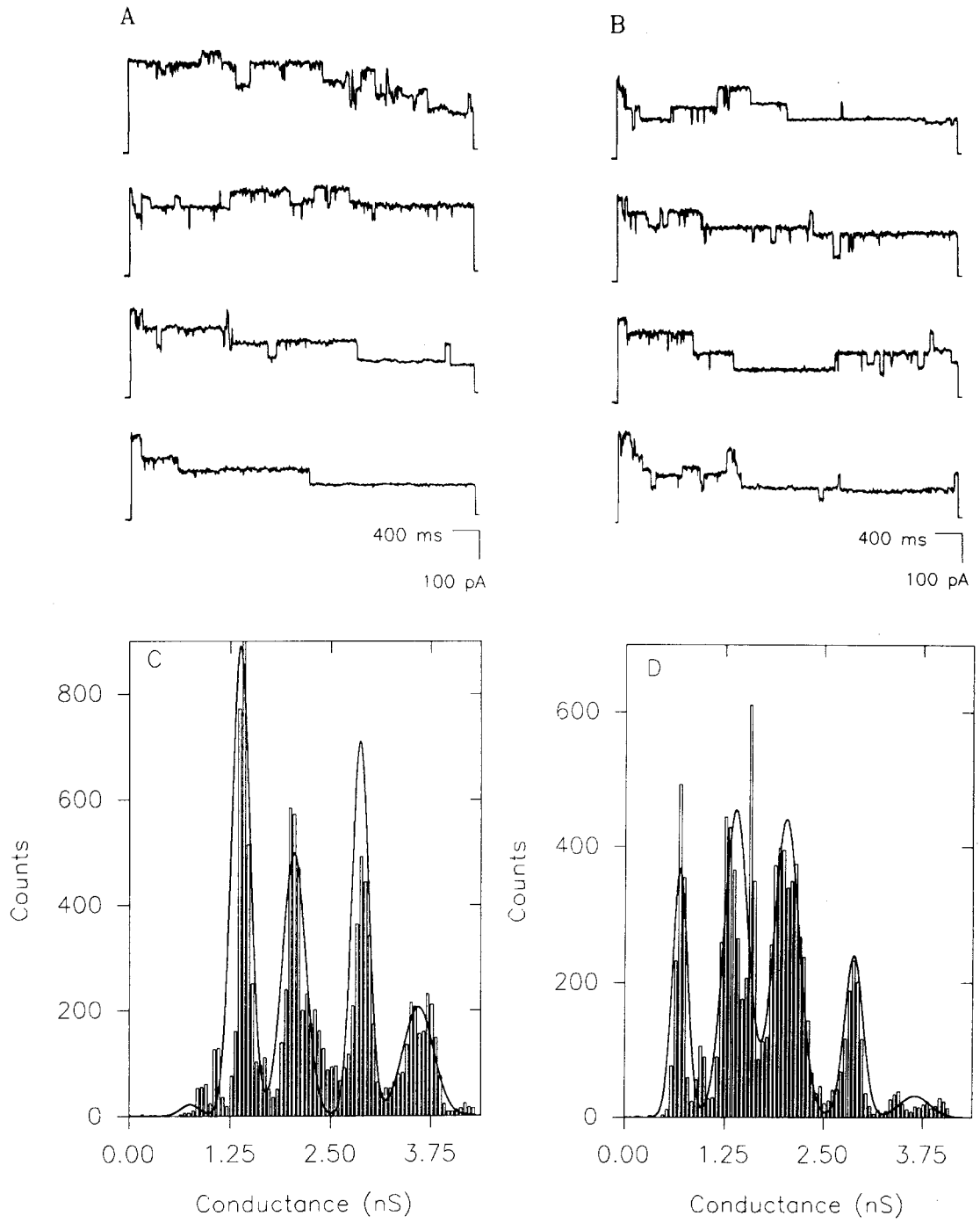

Fig. 8. Analysis of single-channel conductance. (*A* and *B*) Representative current recordings during pulses to 80 mV in a membrane containing five MIP channels. (*A*) Current records of channels induced by nonphosphorylated MIP. After the current records were taken for *A*, a phosphorylation mixture was added to both sides of the membrane. (*B*) Records taken 30 min following the addition of the phosphorylation mixture. (*C* and *D*) Conductance amplitude histograms taken from the current records shown in *A* and *B*. As described in the text, the major conductance levels in histograms were fit by a sum of five gaussians. Additional conductance levels resulting from channels entering less probable substates were not fit in this analysis. The positions of the gaussian peaks were not shifted by phosphorylation, suggesting that the single-channel conductance of MIP channels was unaffected by phosphorylation. Single-channel conductances were estimated from the amplitudes of each of the 5 gaussians. The single-channel conductance after phosphorylation (726 ± 37 pS) was not significantly different from the single-channel conductance before phosphorylation (715 ± 36 pS). After addition of the phosphorylation mixture, the area under the gaussian with a mean conductance of 3.6 nS (5 channels open) decreased and the area under the gaussian with a mean conductance of 0.7 nS (1 channel open) increased. This result suggests that phosphorylation increased the probability that MIP channels would close during pulses to 80 mV.

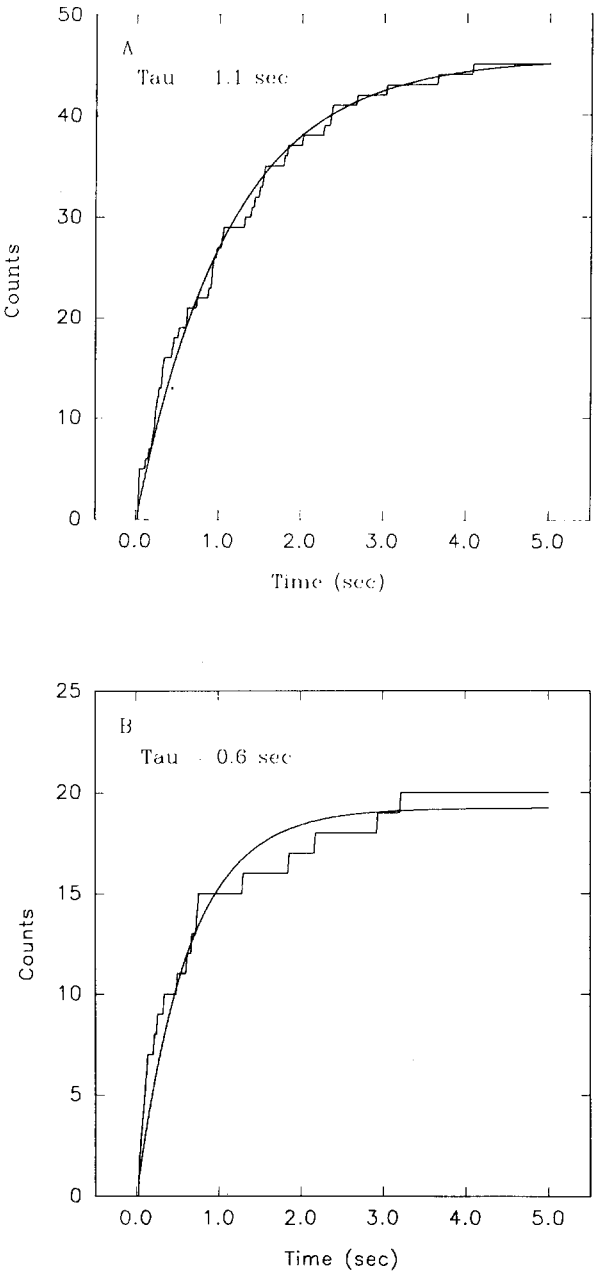

Fig. 9. Analysis of single-channel closing kinetics. Single channels were reconstituted into membranes using nonphosphorylated MIP. The latencies to first closure after the application of 80-mV pulses were measured for single-channel membranes. The channels were then phosphorylated in the bilayer by the addition of 0.03 μg/ml protein kinase A catalytic subunit, 5 mM $MgCl_2$ and 0.5 mM ATP to the bilayer chamber. The latencies to first closure were remeasured 30 min to 1 hr after the addition of the phosphorylation mixture. The figure shows cumulative histograms for latencies before and after *in situ* phosphorylation. The distribution of latencies was best fit by a single exponential for both cases. The time constant for nonphosphorylated channels was 1.1 sec (*A*), and the time constant for phosphorylated channels was 0.6 sec (*B*).

ylation state of MIP and channel voltage dependence. Putative contaminants must now share the phosphorylation properties of MIP in addition to having to copurify with MIP by two different methods and run in the same band as MIP on SDS-PAGE. We regard the existence of a contaminant satisfying these properties as unlikely.

A Significant Proportion of Endogenous MIP Is Phosphorylated

Our results show that at least 20% of the MIP in the plasma membrane isolated from bovine lens is phosphorylated. Because our experimental conditions do not minimize possible effects of phosphatases during isolation, this estimate is a lower limit on the fraction of phosphorylated MIP. However, Lampe and Johnson (1990) found a similar percentage of phosphorylated native MIP in their study. MIP aggregates as tetramers in the lens (Zampighi et al., 1989) and when reconstituted into unilamellar vesicles (Dunia et al., 1987; Ehring et al., 1990). Thus it is possible that the channels reconstituted into planar lipid bilayers are formed from tetramers of MIP. If phosphorylation of only one of the MIP monomers in a tetrameric channel were required to confer voltage-dependent gating and all the MIP in the membrane formed channels, a significant proportion of the channels would be voltage dependent.

Phosphorylation Increases the Voltage Dependence of MIP Channels

That the same protein forms both the voltage-dependent and voltage-independent channels is confirmed by the conversion of voltage-independent channels incorporated in the bilayer to voltage-dependent channels by *in situ* phosphorylation with protein kinase A or its catalytic subunit. That phosphorylation is required for the change in voltage dependence is indicated by the observation that the complete phosphorylation mixture is required to induce voltage dependence. ATP, Mg^{2+}, cAMP, the holoenzyme of protein kinase A and the catalytic subunit were tested independently and did not alter channel voltage dependence.

In multi-channel membranes, the conversion of voltage-independent channels to voltage-dependent channels proceeds with a variable time course depending on the amount of enzyme present. The increase in voltage dependence following the addition of the phosphorylation mixture could be explained entirely by an increase in the proportion of channels closed by voltage. This is suggested

by both steady-state conductance-voltage relationships and kinetics. Neither the effective gating charge nor the voltage at which half the channels closed was affected by phosphorylation. The time constants for the exponential decrease in current during a voltage pulse were also unaffected by phosphorylation. These two results support the hypothesis that the effect of phosphorylation is essentially all or none. Thus variability in voltage dependence between isolates probably arises from variation in the relative proportion of the two channel types present in a given preparation.

Channels could exist in several different phosphorylation states. If the channels are indeed tetrameric, as previously suggested, from one to four monomers could be phosphorylated. In principle, each phosphorylation state could yield a different voltage dependence, but in practice variation in channel properties seems limited to whether or not a channel is voltage dependent at all. This could be explained in several ways. First only one state of phosphorylation could yield a voltage-dependent channel. This state would likely be that of maximum phosphorylation, i.e., all monomers making up the channel would be phosphorylated. The second possibility is that voltage dependence requires the phosphorylation of only one monomer and subsequent phosphorylations of additional monomers have little effect on the parameters of the voltage dependence.

Change in Voltage Dependence is Apparent at the Single-Channel Level

The primary effect of phosphorylation on the single-channel properties of MIP channels was to increase the probability of a channel entering the closed state with increased voltage. This is shown by the decreased latency to first closure and decreased open channel lifetimes. In addition, phosphorylation appeared to stabilize the channel in the closed state as shown by the increased closed channel lifetime. While phosphorylation altered channel-state lifetimes it did not alter the single-channel conductance. This suggests that the phosphorylated charge is probably more than a Debye length (about 10 Å in 100 mM salt) from the channel mouth when the channel is in the open state.

It is important to note that, while it was possible to alter the gating properties of single channels reconstituted from nonphosphorylated MIP by *in situ* phosphorylation, it was not possible to determine the initial phosphorylation state of a given single channel. Single channels from both phosphorylated and nonphosphorylated material were seen to close, and channels reconstituted from both types of material had the same conductance. As noted above, material that eluted from the HPLC in the $t_{0.39}$ peak, while enriched for nonphosphorylated MIP, may have contained some proportion of the phosphorylated isomer. Therefore, we did not compare single-channel properties of channels reconstituted from MIP eluting from the HPLC in the phosphorylated and nonphosphorylated fractions. We did, however, demonstrate that *in situ* phosphorylation altered the gating properties of MIP single channels in a manner that was entirely consistent with the effects of phosphorylation on multi-channel membranes.

In some membranes, reconstitution of nonphosphorylated MIP resulted in single channels that closed with one sign of voltage but not with the other. In these cases, addition of the phosphorylating mixture to both sides of the membrane resulted in channel closure with both signs of voltage. Moreover, the parameters of channel closure for the sign of voltage which initially showed closure were not altered by the addition of the phosphorylating mixture. After phosphorylation, the parameters of closure for both signs of voltage were similar. It seems likely that the difference between channels having symmetric or asymmetric voltage dependence is a difference in sideness of phosphorylation. That is, a channel which displays asymmetric voltage dependence is one which is phosphorylated on only one side of the membrane. The single-channel conductance of open phosphorylated channels and of nonphosphorylated channels is the same. This implies that both channels share a common structure, a conclusion strengthened by the observation that nonphosphorylated channels can be converted to phosphorylated ones *in situ*.

Regulation Appears to Be at the Carboxy Terminal

Models derived from the sequence of MIP suggest a largely hydrophobic protein with six transmembrane segments with both the amino and carboxy terminals on the cytoplasmic side (Fig. 10). Lampe and Johnson (1990) have shown that serine 243 and serine 245 near the carboxy terminal are phosphorylated endogenously and that serine 243 can be phosphorylated by protein kinase A. We have previously shown that age-related endogenous proteolysis of MIP, which results in the loss of the carboxy terminal, correlates with the loss of voltage dependence of reconstituted MIP channels (Ehring, Zampighi & Hall, 1988). From these observations we infer that the phosphorylated form of serine 243 is required for voltage-dependent gating of MIP channels. Studies

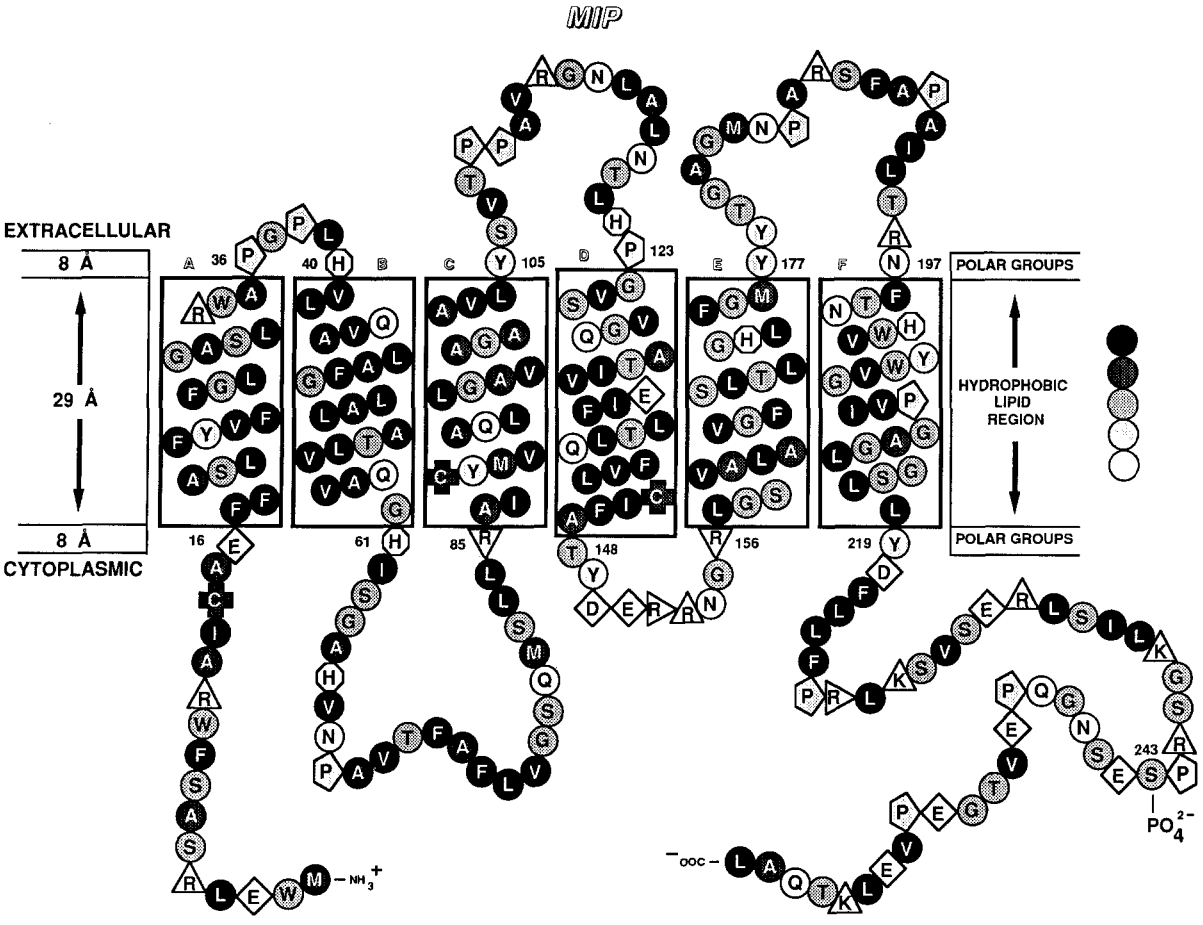

Fig. 10. Model transmembrane structure of MIP. This figure is based on the model of MIP proposed by Gorin et al. (1984). The primary amino acid sequence is represented by the standard one-letter code. The relative hydrophobicities of the amino acids are illustrated by a 5-step grey scale, with black being the most hydrophobic. In this model, there are six putative transmembrane domains (*A-F*) and both the carboxy and amino terminals are on the cytoplasmic side of the membrane. Domain *F* includes a putative amphiphilic helix, which Gorin et al. (1984) suggest may form part of a channel pore. Lampe and Johnson (1990) have shown that the serine at location 243 near the carboxy terminal is phosphorylated by protein kinase A. We propose that phosphorylation of serine 243 is required for the voltage-dependent gating of MIP channels.

of the effects of calcium and calmodulin on the permeability of MIP-containing liposomes provide additional evidence that the carboxy terminal may be involved in the gating of MIP channels (Girsch & Peracchia, 1985*a*; Peracchia & Girsch, 1985*a,b*).

We cannot determine from the present experiments how phosphorylation results in enhanced voltage dependence. It is possible that the added phosphate at serine 243 directly contributes to the voltage sensor for the channel, but it is equally possible that phosphorylation of MIP results in a global change in the structure of MIP that alters the effectiveness of its voltage sensor.

Nevertheless, it appears likely that the effect of protein kinase A on the voltage-dependent gating of MIP channels is mediated by the phosphorylation of the carboxy terminal serine 243. Moreover, the lens also has a substantial endogenous protein kinase A activity (Takats et al., 1978). Thus phosphorylation of MIP, already demonstrated to occur in the lens, may regulate MIP channels in vivo.

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