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Reconstitution of Channels from Preparations Enriched in Lens Gap Junction Protein MP70

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Summary. Detergent-solubilized ovine lens membrane proteins, enriched in the 70-kDa gap junction component (MP70), were reconstituted into planar lipid bilayers and analyzed for channel activities. Three distinct activities were found. Those showing conductance steps of 290 pS (symmetrical 150-mM KCl solutions) had properties similar to those reported earlier for MIP26 (Ehring, G.R., Zampighi, G., Horwitz, J., Bok, D., Hall, J.E. 1990. *J. Gen. Physiol.* **96**:631–664.) of which minor amounts were normally present in the detergent-solubilized preparations. Two novel channel activities had unitary conductances of 90 and 45 pS, were halothane sensitive and did not discriminate between sodium and potassium ions. The 90-pS channel was asymmetrically voltage dependent, and its properties would be consistent with the expected properties of junctional hemichannels.

Key Words reconstitution · planar lipid bilayers · gap junctions · connexons · MP70 · lens

Introduction

Intercellular communication via gap junction channels plays important roles in signal transmission, control of cell growth and tissue homeostasis. The channel-forming proteins of gap junctions belong to the divergent family of connexins. Connexins have apparent molecular weights ranging between 21–70 kDa, are tissue selective and in some cases show temporal changes in expression during development (Beyer, Paul & Goodenough, 1990; Dermietzel, Hwang & Spray, 1990). Structurally, gap junction hemichannels traverse one membrane and are formed from six connexin molecules. These structures, also referred to as connexons, connect with their counterparts in the opposing membrane to form cell-to-cell channels (Unwin & Ennis, 1984; Yaeger & Gilula, 1991). The functional characterization of gap junction channels has been greatly facilitated by the development of double whole-cell patch-clamp technology (Neyton & Trautmann, 1985). These physiological studies of gap junctions indicated a wide range of unitary conductances (22 to 160 pS) consistent with the molecular diversity of the connexin family (Rook, Jongsma & De Jonge, 1989; Brink, 1991).

Gap junction channels provide intercellular pathways for small molecules in the mammalian lens, and disruption of these pathways by closure of junctional channels has been implicated in the early formation of opacities during cataractogenesis (Duncan, 1983; Vrensen et al., 1990). Two connexin-related polypeptides, connexin46 and a 70-kDa membrane protein (MP70), have been identified as components of the lens fiber gap junctions (Kistler, Christie & Bullivant, 1988; Paul et al., 1991). Furthermore, MP70 is converted to a 38-kDa protein (MP38) by age-related cleavage deeper in the lens (Kistler & Bullivant, 1987). The physiological significance of this gap junction processing and its relationship to the maintenance of lens transparency is presently not understood and is an area of potential significance to cataract research.

Unfortunately, the application of double whole-cell patch-clamp techniques to the lens is complicated by the greatly elongated structure of the fiber cells and an inability to form viable cell pairs upon dissociation of the lens. Consequently, lens fiber membrane proteins have been isolated as detergent-soluble complexes and reconstituted either into membrane vesicles (Nikaido & Rosenberg, 1985; Peracchia & Girsch, 1985) or into planar lipid bilayers (Zampighi et al., 1985; Ehring et al., 1990) and then assayed for channel activity. These reconstitution studies were primarily designed to characterize the 26-kDa major intrinsic polypeptide (MIP26) which is the predominant polypeptide of the fiber membranes and which was widely considered the major component of the fiber gap junctions (Sas et al., 1985). Reconstitution of HPLC-purified samples of MIP26 (Ehring et al., 1990) yielded a large

channel which rarely closed and had two preferred conductance states with amplitudes of 380 and 160 pS in 0.1 M KCl. In addition, the channel exhibited strong voltage dependence and was unaffected by changes in pH and calcium concentration. These results together with data from immunocytochemistry (Paul & Goodenough, 1983; Zampighi et al., 1989) and from sequence comparisons (Baker & Saier, 1990; Van Aelst et al., 1991) made it appear unlikely that MIP26 forms the fiber gap junction channels. It has been suggested recently that MIP26 channels traverse only one membrane and play a role in minimizing extracellular space in the lens (Ehring et al., 1990).

We now report on a new series of experiments aimed at the reconstitution of junctional channels into planar lipid bilayers by using preparations in which MP70 has been enriched and MIP26 greatly reduced. This membrane preparation yielded three distinct channel activities, one very similar to previously described MIP26 channels and two novel activities with unitary conductances of 90 and 45 pS (150 mM KCl).

Materials and Methods

Preparation of Lens Fiber Membranes and Protein Solubilization

Sheep lenses were decapsulated (thereby removing the epithelial cell monolayer), and the outer cortex dissected from the bulk of the lenses and collected in buffer A (5 mM Tris-HCl, pH 8, 5 mM EDTA, 5 mM EGTA) as previously described (Kistler & Bullivant, 1987). Tissue was homogenized with a Dounce, and membranes washed three times in buffer A by centrifugation (11,000 rpm, 20 min, Sorvall SS-34 rotor). Crude membranes were extracted with 4 M urea, 5 mM Tris-HCl, pH 9.5, 5 mM EDTA, 5 mM EGTA, pelleted at 17,000 rpm for 60 min, and reextracted with 7 M urea in the same buffer. After pelleting (17,000 rpm, 60 min), membranes were extracted twice with ice-cold 20 mM NaOH, pelleted (17,000 rpm, 45 min), and washed once in buffer B (5 mM Tris-HCl, pH 8.0, 2.5 mM EDTA, 2.5 mM EGTA, 100 mM NaCl). In some cases, alkali stripping was applied to crude membranes and urea treatments were omitted. Urea/alkali or alkali-stripped membranes were resuspended in buffer B with a protein concentration of 6 mg/ml, aliquoted and stored at −90°C until further use.

For solubilized protein preparations, typically 1 ml of membranes was pelleted (17,000 rpm, 30 min) and resuspended in 0.5 ml buffer C (10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.0). An equal volume 0.5% Nonidet NP-40 or 1% Triton X-100 in buffer C was added and the mixture left at room temperature for 10 min. Detergent-solubilized proteins were separated from insoluble material in a Beckman airfuge (20 psi, 30 min) and analyzed by SDS-PAGE (Laemmli, 1970; Kistler & Bullivant, 1987) and by negative stain (1% uranyl acetate) electron microscopy. Aliquots of solubilized protein were used for reconstitution into planar lipid bilayers either fresh or after storage at −90°C.

Bilayer Formation

Bilayers were painted over a 100-μm diameter aperture in the wall of a Delrin cup following the procedures outlined by Hamilton et al. (1989). The bilayer-forming solution was a 3 : 7 mixture of 1-palmitoyl-2-oleoyl phosphatidylcholine (PC) and 1-palmitoyl-2-oleoyl phosphatidylethanolamine (PE) (Avanti Polar Lipids) solubilized at a total lipid concentration of 50 mg/ml in decane. The cup aperture was pretreated by application of a drop (2–3 μl) of decane, dried with N_2 gas, and subsequently a drop of bilayer-forming solution was smeared over the aperture with a glass rod. The pretreated cup was then inserted into a PVC chamber and the *cis* (3 ml) and *trans* (4 ml) chambers filled with electrolyte solution (1 M KCl, 10 mM HEPES buffered to pH 7.4 with KOH). Salt bridges (1 M KCl in 3% agar) were used to connect the chambers to Ag/AgCl electrodes immersed in 1 M KCl. The submerged aperture was painted with the bilayer-forming solution using a thin glass rod, and bilayer formation was assessed by monitoring the membrane capacitance. Typically, membrane capacitance was low immediately following painting but increased to around 120–250 pF within minutes, indicating thinning to bilayer thickness. Once a stable bilayer was formed, 10–50 μl containing 5–20 μg of detergent-solubilized protein were added to either the *cis* chamber only or to both the *cis* and *trans* chambers. Addition of detergent alone had no observable effect on bilayer conductance.

Channel Recording and Data Processing

The voltage-clamp amplifier used in this study was previously described (Hamilton et al., 1989). The command potential was driven by a personal computer as one of two distinct protocols: (i) step protocol—the membrane potential was stepped from 0 mV to a new variable holding potential, data was then recorded before briefly returning to 0 mV and then stepping to a holding potential of opposite polarity, and returned again to 0 mV before stepping to a new holding potential; (ii) ramp protocol—starting from a holding potential of 0 mV, the membrane voltage was swept to 100 mV, then from 100 to −100 mV and finally from −100 mV back to 0 mV at a rate of 10 mV/sec. Channel data were filtered at 500 Hz using an 8-pole Bessel filter (Frequency Devices) and recorded using a TL-1 interface (Axon Instruments) and an IBM-compatible computer. Channel data were also recorded unfiltered on videotape. Single-channel data were analyzed off-line using the pCLAMP suite of programs (Axon Instruments).

Results

MP70-Enriched Preparations

Urea/alkali or alkali-stripped ovine lens fiber membranes were mixtures of junctional plaques and nonjunctional vesicles (Kistler & Bullivant, 1988). MIP26, a nonjunctional channel protein (Zampighi et al., 1989, Ehring et al., 1990), and MP18, an

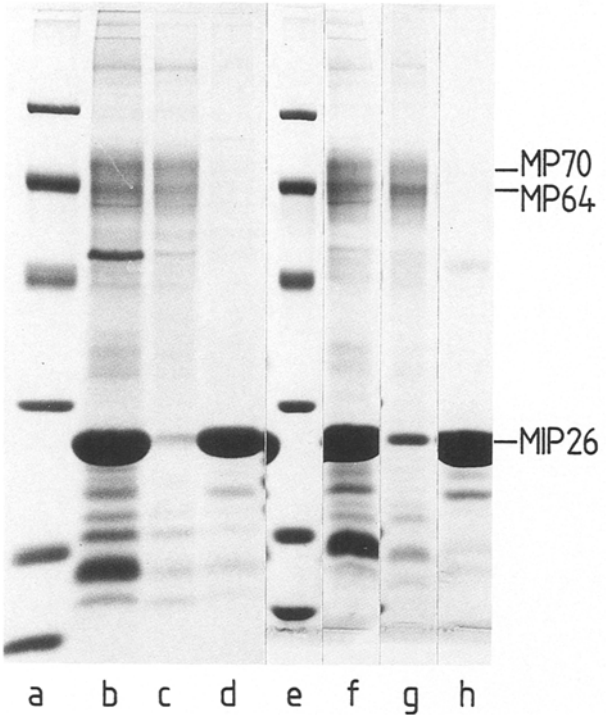

Fig. 1. Enrichment of MP70 by detergent solubilization. SDS-PAGE (10% acrylamide). (*a, e*) Molecular weight markers from top: 97, 66, 45, 31, 21, and 14 kDa. (*b*) Alkali-stripped membranes from sheep lens outer cortex. (*c*) Triton X-100 solubilized membrane proteins. (*d*) Triton X-100 insoluble fraction. (*f*) Membranes extracted with the urea/alkali procedure. (*g*) Nonidet NP-40 solubilized membrane proteins. (*h*) Nonidet NP-40 insoluble fraction.

18-kDa lens fiber specific membrane polypeptide, for which immunolocalization results are still a matter of controversy (Louis et al., 1989, Voorter et al., 1989), dominated the protein profiles on SDS gels (Fig. 1*b* and *f*). A presumably peripheral membrane polypeptide was present as a 50-kDa band on gels of the alkali-stripped preparations but was extracted with the urea/alkali procedure. The gap junction protein MP70 appeared as a 70/64-kDa doublet, as previously described (Kistler et al., 1988). Fractionation of isolated fiber membranes with Triton X-100 or Nonidet NP-40 resulted in the solubilization of predominantly MP70 (Kistler & Bullivant, 1988) and only minor amounts of MIP26 and MP18 (Fig. 1*c* and *g*). These MP70-enriched preparations also contained minor amounts of a 140-kDa polypeptide previously identified as N-cadherin (Maisel & Atreya, 1990). Most of MIP26 and MP18 was insoluble under these conditions. A fraction strongly enriched in MIP26 and its 24-kDa cleavage product was obtained upon re-extraction of the insoluble material with 2% octyl-beta-D-glucopyranoside (Fig. 1*d* and *h*).

Examination of the MP70-enriched preparations by negative-stain electron microscopy (Fig. 2) showed abundant short, double membrane structures (circles) and globular structures with a central stain dot (arrowheads). The short, double membrane structures were variable in length, and the shortest were approximately 10 nm long and 15 nm wide and were consistent with side views of intact connexon pairs (Kistler & Bullivant, 1988). Longer structures probably represent side views of two or three connexon pairs which had not been separated by the detergent treatment. The globular material had dimensions consistent with single connexons viewed in projection. Other structures were also present and were probably due to nonjunctional proteins or detergent micelles. These observations confirmed the enrichment of gap junction derived protein complexes. Sucrose gradient centrifugation and gel filtration experiments also showed that MP70 was present as a larger complex and not solubilized as monomers (*data not shown*).

Identification of Three Distinct Channel Activities

MP70-enriched preparations were analyzed for channel activities using the planar lipid bilayer system. Solubilized protein (5–20 μg) in the presence of detergent was added to either the *cis* side only or to both sides of the painted bilayer. Channel activities usually appeared within 15 min after addition of sample and were recognized as discrete steps in the continuous current records. Channels were seen in 86% of experiments using MP70-enriched preparations with Triton X-100 and addition to the *cis* side of the painted bilayer only. Nonidet NP-40 samples were usually applied to both sides as the appearance of channel activity was less frequent (53%). Multiple and distinct classes of channel activity were most often observed together (Fig. 3*A*), but single-channel classes were also seen in isolation in some experiments. Records from 27 experiments showed that channel activities could be grouped in three distinct classes, independent of which detergent had been used for protein solubilization (Fig. 3*B*). Class I was the largest channel and had a unitary conductance of 2200 ± 60 pS (n = 16) in 1 M KCl and 290 ± 10 pS (n = 3) in symmetrical 150-mM KCl solutions. Class II channels had medium size with a unitary conductance of 650 ± 20 pS (n = 17) in 1 M KCl and 90 ± 5 pS (n = 3) in 150 mM KCl. The frequencies of occurrence were comparable for class I and II channels. Somewhat less frequently observed were the smaller channels grouped in class III with a unitary conductance of 350 ± 10 pS (n = 8) in 1 M KCl and 45 pS in 150 mM KCl.

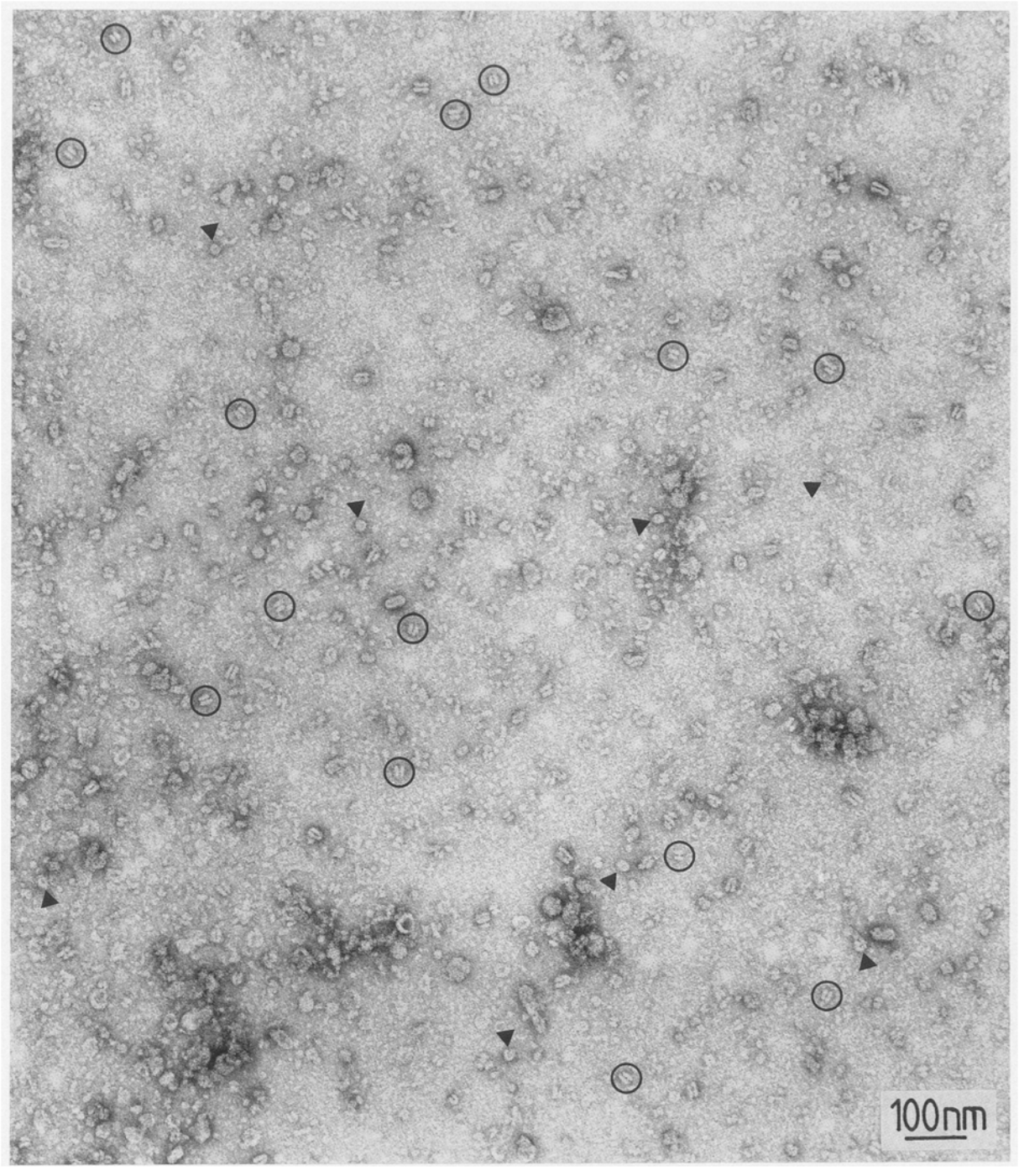

Fig. 2. Negative-stain electron microscopy of MP70-enriched fraction. Preparations were adsorbed to glow discharged carbon/collodion-coated grids and stained with 1% uranyl acetate. Short, double membrane structures (a few are highlighted by circles) are consistent with side views of connexon pairs or small clusters of connexon pairs. Globular structures with a central stain dot (arrowheads) are consistent with connexons viewed in projection. Other structures may be due to nonjunctional proteins or detergent micelles.

A concern with multiple-channel activities is that the smaller conductance steps may represent substates of the larger channel. This is unlikely on the basis of the sample records in Fig. 3*A*, where class II channel openings occur independently of whether the class I channel is open or closed. Furthermore, class II channels but not the larger class I channel activities disappeared within minutes following the addition of

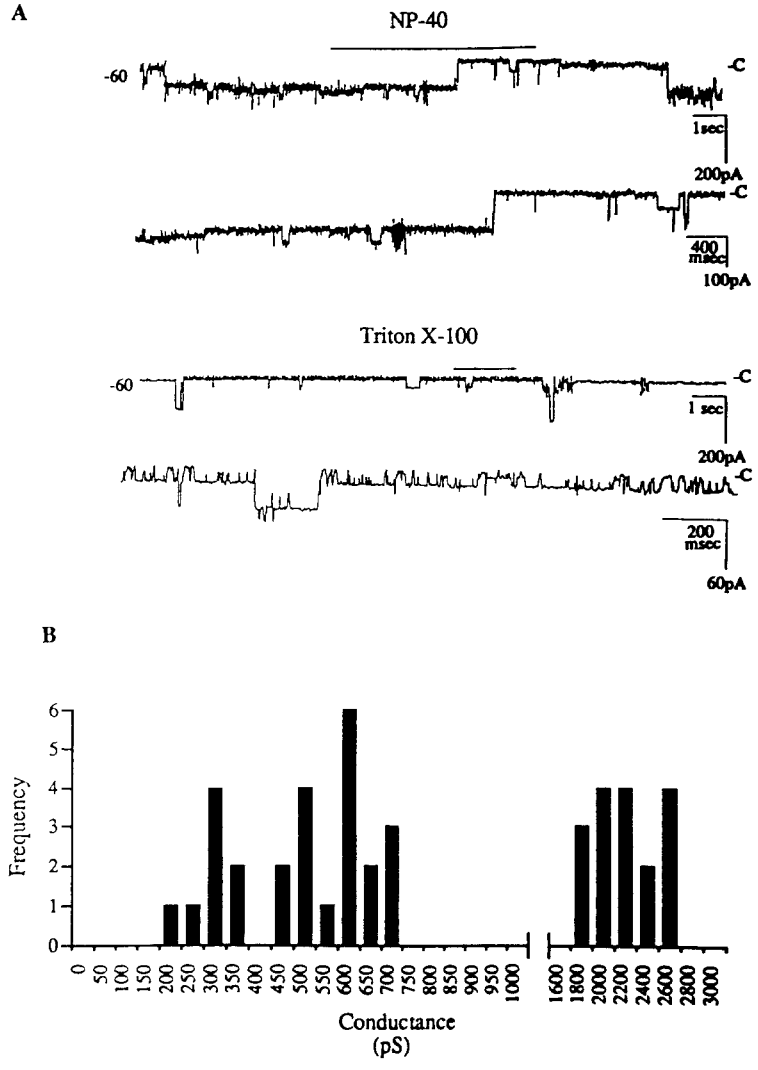

Fig. 3. Classification of channel activities. (*A*) Records of multiple-channel activities following the addition of proteins prepared with Nonidet NP-40 (top panel) or Triton X-100 (bottom panel) to the bilayer system. In both examples the holding potential was −60 mV and symmetrical 1 M KCl solutions were used. The segments of the records overlined with a bar are displayed in expanded form below. The NP-40 trace shows two distinct channel activities (630 and 2180 pS), and the Triton X-100 experiment shows three distinct channel activities (320, 650 and 2070 pS). (*B*) Frequency distribution histogram of unitary conductances from 27 experiments showing three distinct conductance classes of approximately 2000 pS (class I), 600 pS (class II) and 300 pS (class III).

the anesthetic halothane to a final concentration of 5 mM on the *trans* side of the bilayer (Fig. 4). Addition of halothane also resulted in the disappearance of class III channel activity (Fig. 5). This differential halothane sensitivity indicates that class II and III channels are unlikely to be substates of the larger class I channel.

Individual Channel Records

While the majority of experiments produced current records similar to Fig. 3*A*, indicating the simultaneous reconstitution of multiple-channel classes, on a limited number of occasions channel activity from individual channel classes could be recorded in isolation. Records of class I channel activity in isolation (Figs. 6 and 7) showed all the features previously described for MIP26 (Ehring et al., 1990). These authors reported current steps of 2.1 nS (1 M KCl) between predominant minimum (1.6 nS) and maximum (3.7 nS) conductance states, similar to current steps of around 2200 pS in our experiments. Like MIP26, class I channels were found to be voltage dependent in that the channel was predominantly in its maximum conductance state at voltages around 0 mV but shifted to its minimum conductance state at voltages greater than ± 50 mV (Fig. 6*A-C*). The current-voltage relationship for this transition between maximum and minimum conductance states was linear (Fig. 6*D*). Another common characteristic of MIP26 channels and class I channels was their apparent inability to close to the bare bilayer conductance. Only in a few instances was the complete closure of class I channels observed (Fig. 7). In summary, these results on class I channel activity are very similar to those reported from reconstitution studies using HPLC-purified MIP26. Hence, class I channel activity is most likely associated with the minor amounts of MIP26 consistently present in our MP70-enriched membrane preparations.

The medium size class II channels and smaller

size class III channels were not often seen in isolation, and their more detailed characterization was complicated by the usually co-reconstituted, larger MIP26 activity. In records of class II channel activity in the absence of MIP26 channels (Figs. 8 and 9), this channel was observed opening directly over the bare bilayer conductance (Fig. 8*B*), again showing that this channel class was independent of the larger class I activity. However, a small conductance (~150 pS) was also observed, which is most clearly resolved above background noise at holding potentials of 60 and 80 mV. In other very rare occasions this channel activity was observed independently and characterized as a potassium channel (*data not shown*).

The class II channel shown in Fig. 8 expressed distinct asymmetric voltage sensitivity. At positive potentials the activity of the class II channel was reduced by an increase in holding potential, while at negative command potentials the class II channel had virtually a zero open probability (Fig. 8*C*). The exception was at −40 mV where an isolated burst of channel activity of similar amplitude to that recorded at 40 mV was observed, ruling out the possibility that class II channel activity exhibits rectification. In other experiments class II channel activity was recorded that had an identical single-channel conductance but an exactly opposite voltage dependence (Fig. 9). We believe this reflects class II channels inserted into the lipid bilayer with opposite orientation and hence opposite voltage dependence. Class II channels did not discriminate between sodium and potassium (710 ± 20 pS in symmetrical 1 M KCl; 730 ± 40 pS in 1 M NaCl/1 M KCl, $n = 3$) and were inhibited by halothane (Fig. 4).

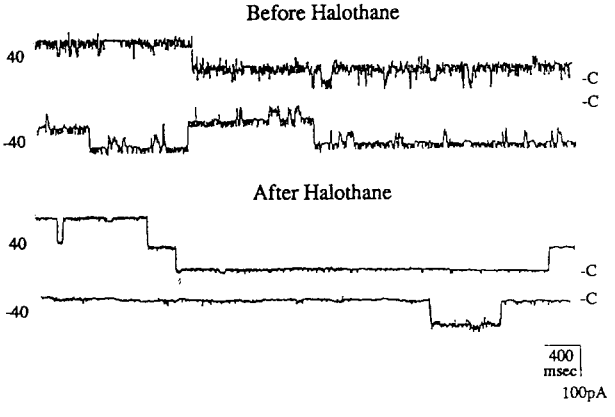

Fig. 4. Inhibition of the class II activity with halothane. Symmetrical 1 M KCl solutions. The upper panel shows records of 640 and 2140 pS channel activities obtained following addition of Triton X-100 solubilized protein to the *cis* chamber. In the lower panel, addition of halothane to a final concentration of 5 mM in the *trans* chamber blocked the 640 pS channel but apparently had no effect on the 2140 pS channel activity.

The smaller class III channels were observed less frequently than the class II channels and only very rarely were records obtained of this channel in isolation (Fig. 10*A*). Class III channels were seen to open directly above the bare bilayer conductance (Fig. 10*B*) and were thus regarded as independent of the other channel classes found in MP70-enriched preparations. Some voltage dependence was indicated (Fig. 10*C*) and was not asymmetric as was the case for the class II channels. The results from a larger number of experiments with multiple-channel activity, showed class III channels to have a linear single-channel current-voltage relationship (Fig. 10*D*). In addition, class III channels did not discriminate between sodium and potassium (320 pS in symmetrical 1 M KCl; 310 pS in 1 M NaCl/1 M KCl, $n = 1$) and were inhibited by halothane (Fig. 5).

In summary, the functional analysis of MP70-enriched preparations consistently showed three distinct channel classes. Large class I channels

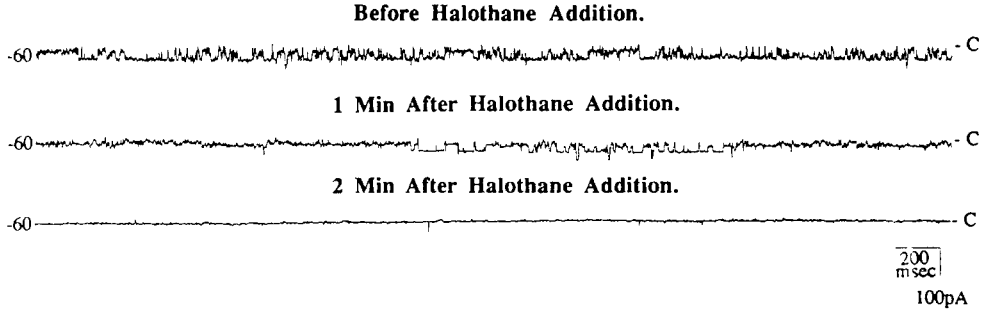

Fig. 5. Inhibition of class III channel activities with halothane. Symmetrical 1 M KCl solutions, holding potential −60 mV. Triton X-100 solubilized protein was added to the *cis* chamber, resulting in the appearance of conductance steps of 340 pS. This class III channel activity disappeared after 2 min following halothane addition to the *trans* chamber.

were identified as MIP26 channels which were frequently part of the records although amounts of this protein appeared minor on SDS gels. Class II and III channels have not been reported before and thus appear to be a novel feature of the MP70-enriched preparations from lens fiber membranes.

Discussion

Experiments in this report were aimed at the functional reconstitution of lens fiber junctional proteins in a planar lipid bilayer system. Our rationale was to use MP70 as a marker protein for the enrichment of junctional channel complexes. MP70 was selec-

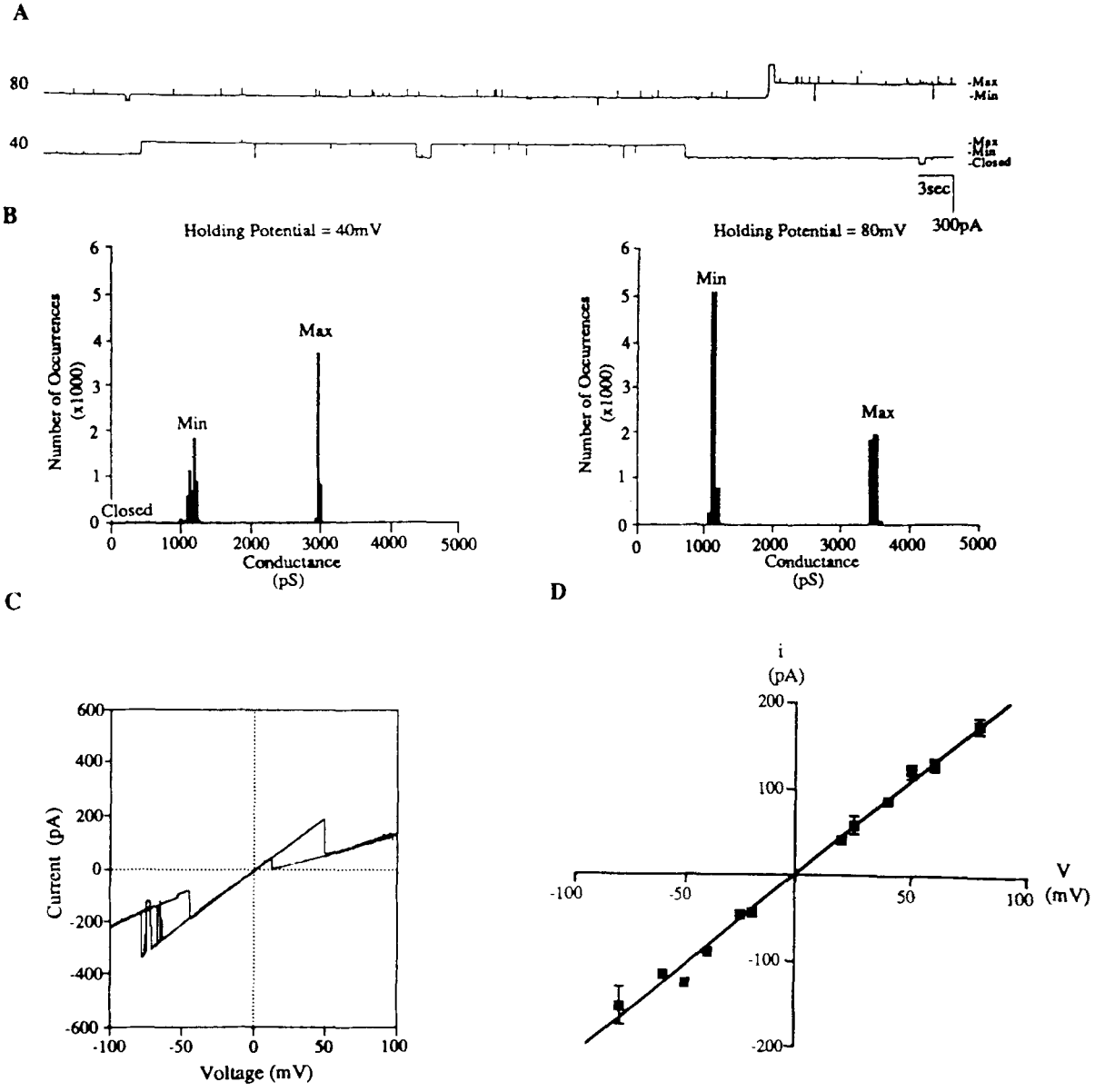

Fig. 6. Voltage dependency of class I channels. Symmetrical 1 M KCl solutions. Triton X-100 solubilized protein was added to the *cis* chamber. (*A*) Current traces at two holding potentials showing the predominant transitions between the minimum and the maximum conductance states. (*B*) Conductance histograms of the current traces displayed in *A*, showing peaks corresponding to a minimum conductance level at around 1000 pS and a maximum conductance level at around 3000 pS. (*C*) Response of a class I channel to the voltage-ramp protocol (0 to 100 mV, 100 to −100 mV and then −100 to 0 mV at a rate of 10 mV/sec). Typically the channel is in its maximum conductance state around 0 mV and shifts to the minimum conductance state at potentials greater than ±50 mV. (*D*) The current-voltage relationship of the transition between the minimum and maximum conductance states is linear and has a slope of 2140 ± 60 pS ($n = 16$).

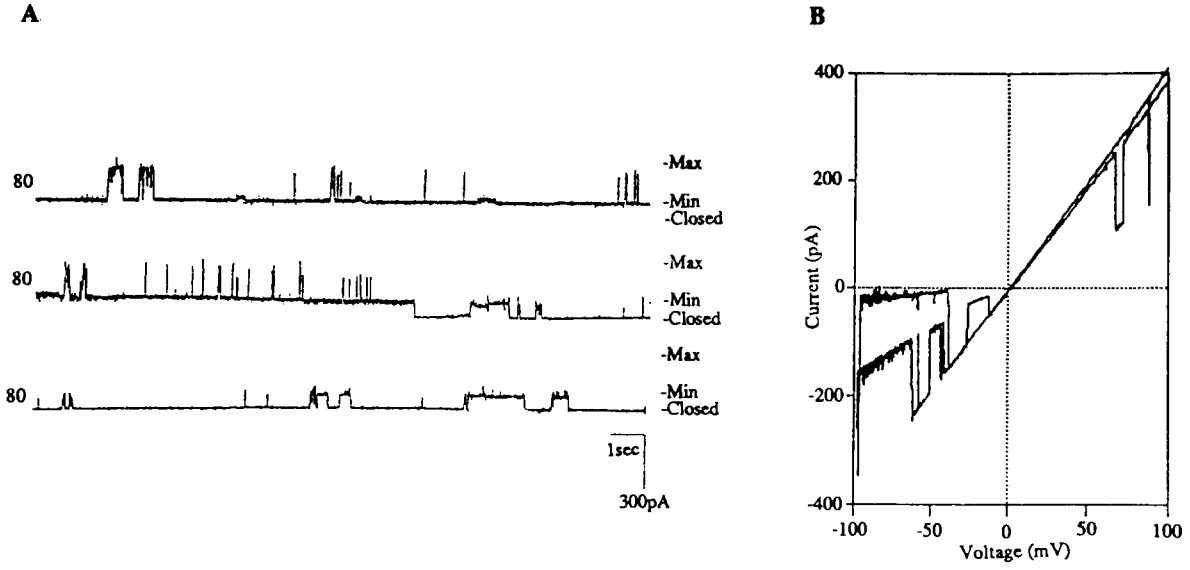

Fig. 7. Rare example of closure of class I channel to the bare bilayer conductance. Symmetrical 1 M KCl solutions. Triton X-100 solubilized protein was added to the *cis* chamber. (*A*) Current traces of class I channel activity at a holding potential of 80 mV. Three major states of the class I channel are observed: a closed state, minimum conductance state and a maximum conductance state. (*B*) Response of the class I channel in *A* to the voltage-ramp protocol, showing transitions between maximum and minimum conductance states and complete closure.

tively solubilized from ovine lens cortical membranes by detergent treatment, and electron microscopy showed that junctional complexes in the form of connexons, connexon pairs and small connexon clusters were indeed abundant in these preparations. However, MP70 may not be the only junctional polypeptide present in the preparations used for reconstitution. Connexin46 has also been localized in the mammalian lens fiber gap junctions and is a protein distinct from MP70 (Paul et al., 1991). Hence, it is possible that connexin46 is co-enriched with MP70 in the detergent extracts. In this event, it is not clear whether connexon structures are heteromers of both junctional polypeptides or whether they represent two distinct populations of homomeric channel structures. We can also not exclude the presence of minor amounts of lens epithelial connexin43 (Beyer et al., 1989), which may extend into the differentiating fiber cells in the outer cortex, in analogy to the situation in the embryonic chick lens (Musil, Beyer & Goodenough, 1990).

Further limitations are set by the nonjunctional impurities evident by SDS-PAGE. Minor amounts of MIP26 and of other lens membrane proteins are consistently present in the detergent extracts. Hence, in the light of the biochemical heterogeneity of these MP70-enriched preparations, we might expect the appearance of a number of different channel activities in the planar lipid bilayer system. Indeed, the unusually large MIP26 channels were frequently apparent in our experiments. Potassium channels were also occasionally observed (*data not shown*). These channel activities were distinct from the novel 90- and 45-pS channels identified in this report.

While the enrichment of connexon complexes in the detergent extracts is suggestive for the occurrence of some junctional channel activities in our records, no firm link can be made without specific antibody-blocking experiments or further protein purification. Yet, the 90- and 45-pS channels have properties which make them candidates for junctional channels. These properties include unitary conductance, sensitivity to halothane, nonselectivity for sodium and potassium ions, and voltage dependence (Spray & Burt, 1990; Moreno et al., 1991). The asymmetric voltage dependence of the 90-pS channel would make it a candidate for a junctional hemichannel. This asymmetrically voltage-dependent 90-pS channel also is comparable with an asymmetrically voltage-dependent 105-pS channel activity which was recently observed in planar lipid bilayers reconstituted with liver connexin32 (conductance adjusted to 150 mM salt; Harris, 1991). In this latter case, a 40-pS channel activity was also observed. Another planar lipid bilayer study using connexin32 also found two predominant channel conductances related to each other by a factor of two (Young, Cohn & Gilula, 1987). By analogy, it appears possible that the 90- and 45-pS channel ac-

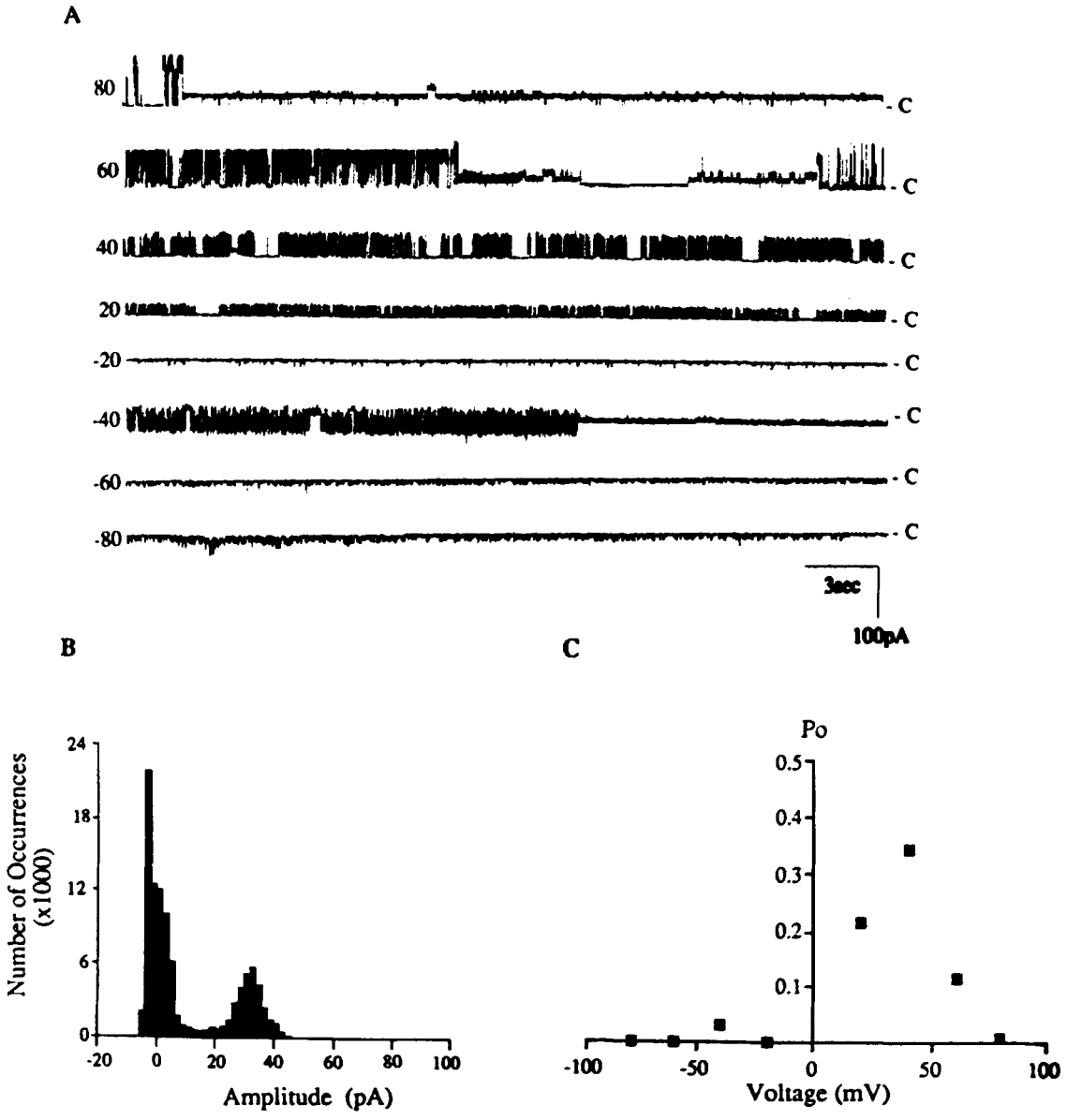

Fig. 8. Characterization of class II channels. Symmetrical 1 M KCl solutions. Triton X-100 solubilized protein was added to the *cis* chamber. (*A*) Records of a class II channel at holding potentials between ±80 mV. An unrelated channel activity is detectable above background noise levels at holding potentials of 60 and 80 mV. (*B*) Amplitude histogram of class II channel activity recorded at 40 mV showing channel closure to the bare bilayer conductance. (*C*) The relationship between the open probability (P_o) of class II channel activity and voltage for the traces displayed in *A*.

tivities in our preparations are in some way related to each other. However, they could also be associated with different polypeptides, thus reflecting the heterogeneity of the MP70-enriched preparations.

Planar lipid bilayer studies offer a genuine alternative to patch-clamp techniques which are difficult to apply to lens fiber cells. The identification of two novel channel activities, which are distinct from MIP26 and lens potassium channels, signals that this approach may be useful for the study of other channel activities, including those associated with the lens fiber gap junctions. It could be a way to establish the functional significance of the age-related processing of fiber junctional proteins and to study the role these channels play in lens transparency.

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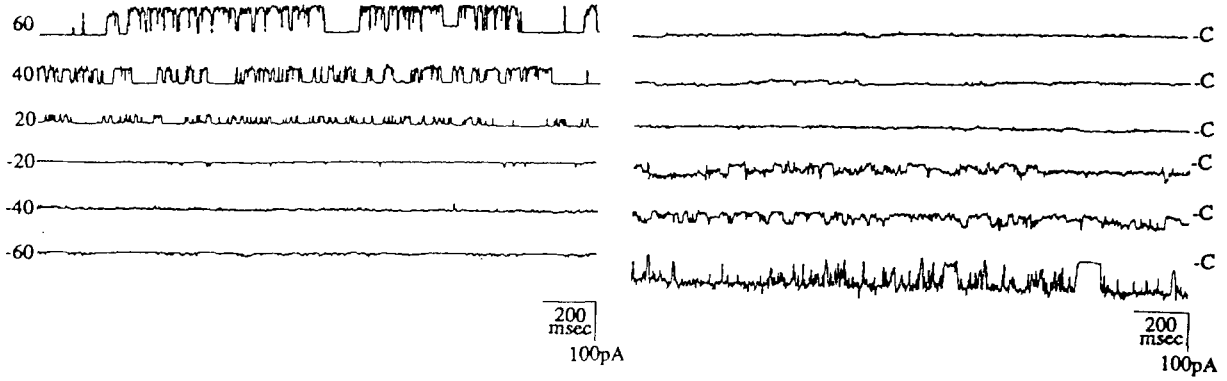

Fig. 9. Asymmetric voltage dependence of class II channels and orientation in the bilayer. Symmetrical 1 M KCl solutions. Triton X-100 solubilized protein was added to the *cis* chamber. Class II current traces from two separate experiments showing channel closure at negative (left panel; data taken from Fig. 8) and positive (right panel) holding potentials. The difference in voltage dependence reflects different orientation of protein complexes in the lipid bilayer.

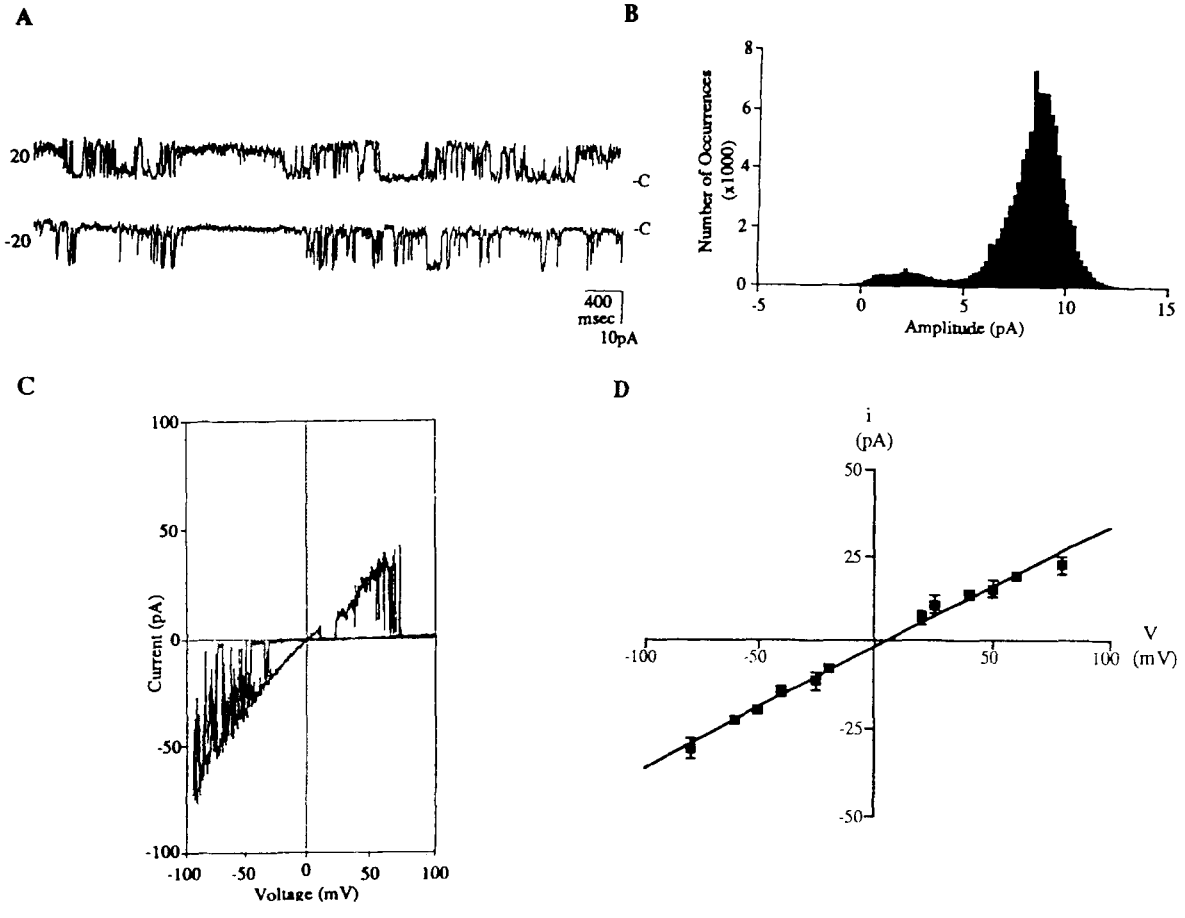

Fig. 10. Recording of individual class III channel activity. Symmetrical 1 M KCl solutions. Triton X-100 solubilized protein was added to the *cis* chamber. (*A*) Current traces at a holding potential of ±20 mV. (*B*) Amplitude histogram of the current trace at 20 mV. (*C*) Response of the class III channel to the voltage-clamp protocol. Some voltage dependence is revealed but is not asymmetric as is the case of class II channels. (*D*) The linear current-voltage relationship from eight pooled experiments has a slope of 350 ± 10 pS.

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