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Functional Reconstitution of Ion Channels from *Paramecium* Cortex into Artificial Liposomes

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Abstract. Toward isolating channel proteins from *Paramecium,* we have explored the possibility of functionally reconstituting ion channels in an artificial system. Proteins from *Paramecium* cortex reconstituted with soybean azolectin retained several channels whose activities were readily registered under patch clamp. The most commonly encountered activities were three: (i) a 71-pS cation channel that opens at all voltages unless di- or trivalent cations were added to close them, (ii) a 40 pS monovalent cation channel, and (iii) a large-conductance channel that prefers anions and exhibits many subconductance states. These channels survived mild detergent treatments without observable functional alterations. The possible origin of these channels from internal membranes, the possible role of 71-pS channel in internal Ca^{2+} release, and the prospects of their purification are discussed.

Key words: Channel reconstitution — Ca^{2+}-release channel — *Paramecium* — Liposome — Internal membrane

Introduction

An extensive electrophysiological study of *Paramecium* has been carried out (*see* Saimi et al., 1994, Martinac et al., 1994, for reviews). This study has been motivated by the use of this excitable single-cell animal as a model system to genetically dissect membrane excitation in biophysical and biochemical terms. In the course of this study, at least thirteen different membrane currents have been documented either as macroscopic currents in vivo recorded with a two-electrode voltage clamp (Oertel et al., 1977) or as microscopic currents in vitro recorded from patches excised from surface blisters (Saimi & Martinac, 1989). Activities of two types of divalent-cation currents have also been observed after incorporating a *Paramecium* ciliary membrane fraction into planar lipid bilayer (Ehrlich et al., 1984). However, the molecules that conduct these various currents have yet to be identified. Although some putative *Paramecium* channel genes can be identified through their homologies to those in metazoa (Jegla & Salkoff, 1994), it remains to be determined whether and which currents these gene products conduct. A systematic way to find *Paramecium* channel proteins and to clone their genes would be very welcome.

Most genes are identified through homology by comparison to genes already cloned in current research. Cloning genes with no known homologs remains a challenge, although such original findings are clearly important. Three strategies have been proven successful in cloning original ion-channel genes. The first strategy begins with a mutant organism with a behavioral phenotype. For example, a K^+-channel gene in *Drosophila* was first cloned through chromosome walk towards a locus, mutations at which cause leg shaking under anesthesia (Papazian et al., 1987). The second strategy begins with a purified protein. For example, a Na^+-channel protein was first purified originally by following its binding to a toxin. Partial peptide sequences from the protein then guided the design of degenerate oligonucleotides used as probes in library search (Noda et al., 1984). The third strategy begins with the functional expression of the target channel, i.e., the current or ion flux. Such expression cloning commonly employs *Xenopus* oocytes, where nucleic acid pools expressing the desired electric currents are sorted to identify the single responsible cDNA species (Rudy & Iverson, 1992).

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A combination of the second and the third strategy has also been successful. Channel proteins can first be identified by their functional expression upon reconstitution in vitro. For example, Cook et al. (1987) first purified the cGMP-dependent cation channel from the rod photoreceptor before peptide sequences were determined and gene cloned (Kaupp et al., 1989). Here the channel protein was identified by functionally reconstituting it into azolectin liposomes and measuring channel activity spectrophotometrically in terms of cGMP-induced release of Ca^{2+}. Recently, Sukharev et al. (1993) showed that a large-conductance mechanosensitive ion channel from *E. coli* can be functionally reconstituted in liposome as assessed by patch-clamp sampling. By following this activity through protein enrichment, the channel protein was identified and the corresponding gene then cloned (Sukharev et al., 1994*a,b*).

Toward identifying *Paramecium* channel proteins and genes, we have examined the possibility of functionally reconstituting *Paramecium* cortical proteins into azolectin liposome. "Cortex" refers to the outer layer of the *Paramecium* cell. It includes the plasma membrane, the ciliary basal bodies, the alveolar sacs, the epiplasm, and certain cytoskeletal elements near the surface. The alveolar sacs are membrane enclosed structures that apparently store Ca^{2+} (Stelly et al., 1991). In pure form, cortical preparation excludes cilia, mitochondria or other organelles. When these liposomes were sampled with patch clamp pipettes, we discovered that activities of at least three kinds of ion channels can readily be detected. Here, we describe the reconstitution procedures and report on the activities of three types of channels, one analyzed in some detail.

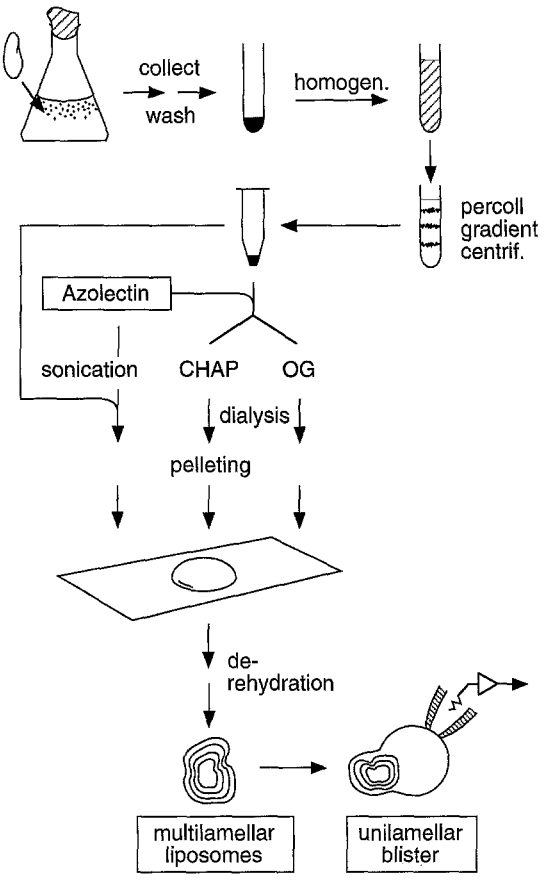

Fig. 1. A schematic diagram of the experimental procedure from the *Paramecium* culture (upper left) to patch-clamping liposome membrane just before patch excision (lower right). *See* Materials and Methods for detail.

Materials and Methods

Paramecium Stock and Culture

A strain of *Paramecium tetraurelia*, nd6, (Lefort-Tran et al., 1981) unable to discharge its trichocysts was used throughout to avoid trichocyst contamination during subcellular fractionation. *Paramecium* cultures were maintained at room temperature in wheat-grass medium that had been inoculated with *Enterobacter aerogenes* the night before use (Sonneborn, 1970). At harvest, cell density was 2.5 to 5×10^3 cells per ml.

Preparation of *Paramecium Cortex*

Preparation and reconstitution of cortical material are summarized in Fig. 1. The protocol for *Paramecium* cortex isolation was modified from that of Stelly et al. (1991). Three to 10 liters of culture were harvested to yield several ml of packed cells. Cells were extensively washed by repeated resuspension and centrifugation in Dryl's solution (Dryl, 1959) to get rid of contaminating bacteria. The cells were finally washed and packed in (mM) 250 sucrose and 20 Tris-HCl, 3 potassium EDTA (ethylenediaminetetraacetate) pH 7.8, and resuspended in two packed-cell volumes of this sucrose-Tris-EDTA solution enriched with 1 mM PMSF (phenylmethylsulfonyl fluoride) and 10 μg/ml of leupeptin to inhibit endogenous proteases. This sucrose, Tris, EDTA, PMSF, and leupeptin mixture is referred to as STEP below. The cell suspension was transferred to a Potter hand-homogenizer prechilled on ice. All procedures hereon were performed on ice or at 4° C. Homogenization was gauged by repeated microscopic observation of the samples until most of the cells were broken. The homogenate was washed and centrifuged with double volume of STEP for five times (Sorvall SS34, 1,500 rpm, 5 min), and the pellet was resuspended in 1 ml of STEP. 0.15 ml each of this suspension was then layered onto 14 ml of 25% percoll in STEP and ultracentrifuged at 30,000 rpm for 40 min. The middle of the three visible bands was collected and added to 10–30 volumes of STEP before centrifugation at 10,000 rpm for 10 min. The precipitate was resuspended in the same volume of STEP and recentrifuged in the same manner. After two such washes, the material was finally collected in a 1.5 ml Eppendorf tube and pelleted in a microfuge. The precipitate was resuspended in 0.5 ml of STEP and examined under a microscope to be sure that it was free of bacterial contamination. Contaminating paramecium organelles were few in number and were not quantified. This final preparation was then dispensed in 20 μl aliquots and stored at –80° C. There were no significant differences in reconstituted channel activities between material frozen briefly and that frozen for over three months.

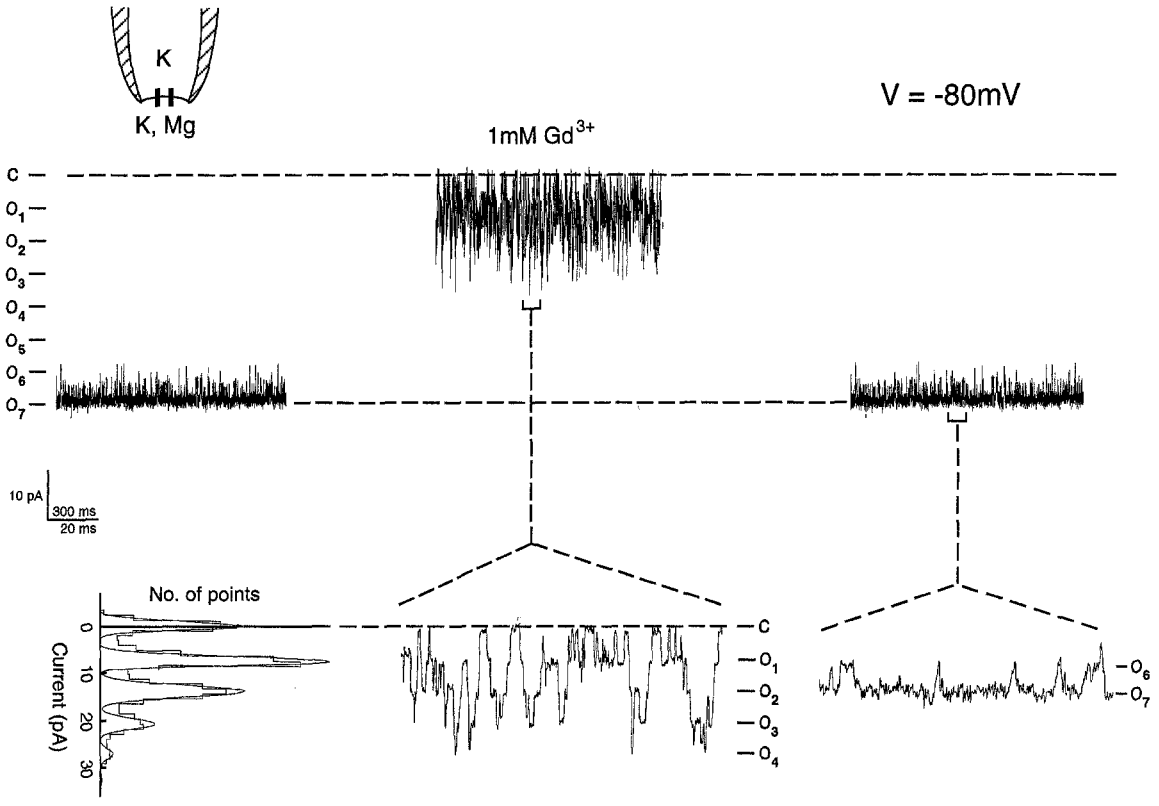

Fig. 2. Currents through open channels dominate the activities in a typical patch excised from liposome reconstituted with *Paramecium* cortical material. The patch, first excised into a bath of 100 mM KCl and 10 mM $MgCl_2$ (left trace), usually showed 1–2 gigohm resistance. Brief single-unit closures could be discerned but not easily resolved since most closures lasted less than 1 msec. Addition of 1 mM $GdCl_3$ to the above K-Mg-bath solution caused most units to close (middle trace). Stochastic opening of a few units in the same patch became evident and could be clearly resolved, as shown by the bottom inset of the middle trace and the corresponding amplitude histogram. This histogram shows the time distribution of the current at 4 to 0 units (0_4 to C). Closure by $GdCl_3$ was reversible. Returning to the Gd^{3+}-free bath of 100 mM KCl and 10 mM $MgCl_2$ (right trace and inset) restored the original behavior of this patch, where the current dwelled mostly at a level comparable to 7 open channel units (0_7). Perfusion of 10 mM Gd^{3+} abolished the Type 1 activities without changing the basal current level (*data not shown*). This level is taken as the closed (C) level here. Pipette contained 100 mM KCl. All pipette or bath solutions in this experiment as well as those in the rest in this paper, except Fig. 5, also contained 10^{-5} M $CaCl_2$, 5 mM HEPES, and were adjusted to pH 7.2.

Reconstitution of *Paramecium* Channels in Azolectin Liposomes

The methods of Delcour et al. (1989) for reconstituting *E. coli* material into azolectin liposomes were used with a few modifications. 75 µl stock azolectin (100 µg/µl previously dissolved in chloroform with a 5% weight ratio of cholesterol) was dried in a stream of nitrogen gas, and then dissolved in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.2), with either 2% CHAPS (3-[(3-chloamidopropyl)dimethylammonio]-1-propanesulfonate) or 2% OG (octylglucoside, Fig. 1). Thawed *Paramecium* cortical preparation (*above*) was added at approximate the desired protein-to-lipid ratio and mixed thoroughly. The mixture was then dialyzed against (in mM): 10 Tris, 100 NaCl, 0.2 EDTA, 0.02% NaN_3, pH 7.2 for 24 hr with one change of buffer near midway. After dialysis, the mixture was spun at 50,000 rpm, 1 hr, and the precipitate was resuspended in 10 mM MOPS (3-[N-morpholino]propanesulfonic acid), 5% ethylene glycol, pH 7.4. This material was added on microscope slides and then subjected to dehydration overnight in a desiccator at 4° C, followed by rehydration in (mM): 150 KCl, 0.1 EDTA, 10^{-5} M $CaCl_2$, 5 HEPES (N-2-hydroxyethylpiperazine-N′-2 ethanesulfonic acid), pH 7.2, at 4° C for 4hr. The rehydrated material was then added to the patch-clamp recording chamber with a bath of the Mg^{2+}-containing "giga-seal solution" (*below*). Unilamellar blisters that emerged in 0.5 hr were sampled with the patch-clamp pipettes (*below*).

We have also used successfully an alternative method of reconstitution besides the use of detergents. Azolectin in TE buffer was sonicated (Branson, Shelton, Conn.) for 5 min in a water bath at room temperature. A thawed sample of cortical preparation was then mixed, spun down, resuspended in MOPS/ethylene glycol, dehydrated, rehydrated, and blisters induced as above.

Electric Recording

Patch-clamp experiments were performed according to standard methods (Hamill et al., 1981). The patch clamp pipettes (Boralex, Rochester Sci. N.Y.), filled with a "K^+ solution" (100 mM KCl, 10^{-5} M $CaCl_2$, 5 mM HEPES, pH 7.2.), had resistances of 3 to 5 megohm. The perfusible bath first contained the "gigaseal solution" (100 mM KCl, 40 mM $MgCl_2$, 10^{-5} M $CaCl_2$, 5 mM HEPES, pH 7.2. After seal formation, the membrane patch was excised from the liposome. All experiments were performed at room temperature (19–23° C) with a patch clamp

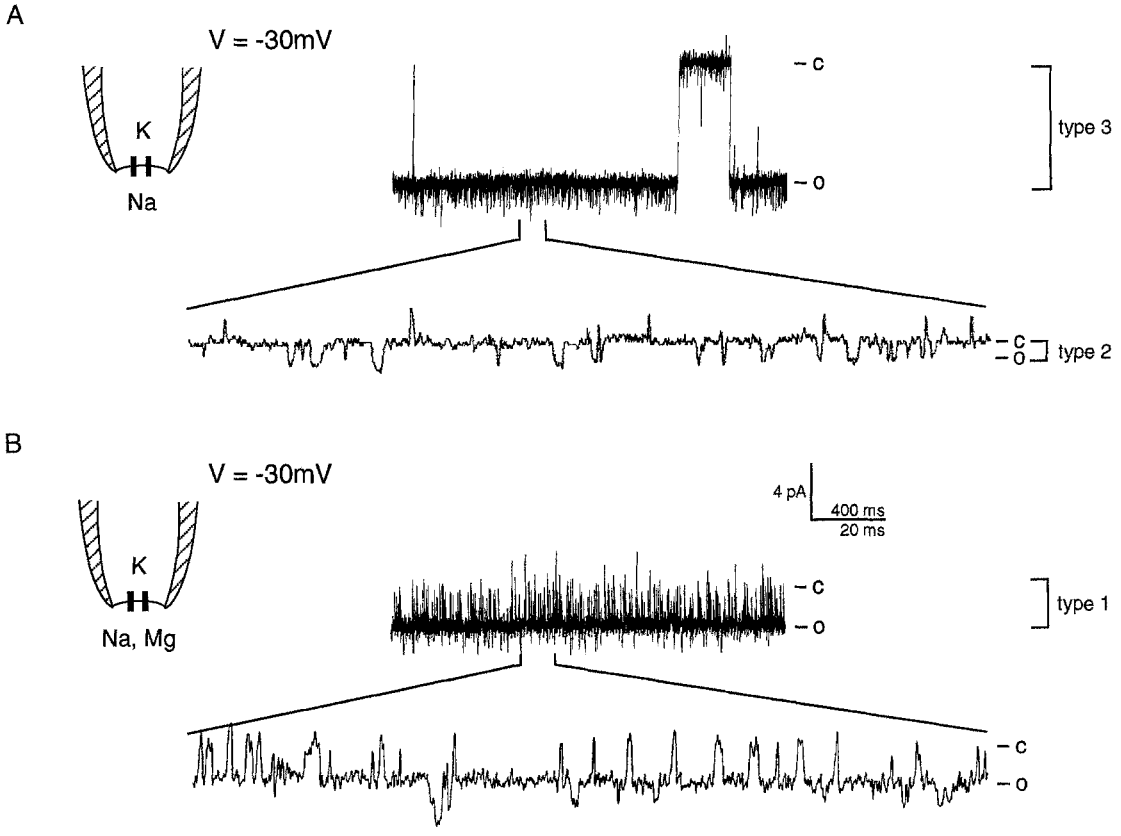

Fig. 3. Three types of channel activities were commonly observed in patches excised from liposomes reconstituted with cortical proteins. This patch was excised into a bath of 100 mM NaCl (top), although similar results have been observed in 100 mM KCl as well. The open probability of Type 1 channels was very large at any voltage applied, when the bath contained no di- or trivalent cations. On the base line current through several open Type-1 channels, the activities of a cation channel of a smaller conductance (Type 2) and those of an anion channel of a large conductance (Type 3) could be observed (*A*). When 10 mM $MgCl_2$ was added to the bath that bathed the same patch, brief closures of Type-1 channel became evident (*B*). Pipette: 100 mM KCl. "C" and "O" are the closed and the open level respectively.

system (EPC-7, List Electronic, Darmstadt, Germany) in the excised patch mode. Membrane currents were recorded on chart (Gould, Cleveland, OH) and on tape (Vetter, Rebersburg, PA). Signal was digitized at 10 kHz, filtered at 1 kHz (8-pole Bessel filter) before analysis with pCLAMP Program (Axon Instruments, CA). All experiments began with symmetric solutions, in which the reference voltage level was determined. Stated in this paper are the pipette voltages with respect to the grounded bath.

Results

Functional Reconstitution of *Paramecium* Ion Channels

Using *Paramecium* cortex as the source of protein and soybean azolectin as lipid, we readily formed gigohm seals and observed channel activities in patches excised from reconstituted liposomes. At a protein-to-lipid ratio of approximately 1:100, we encountered channel activities in about ⅕ of the patches. At a ratio of approximately 1:20, activities were observed in about ⅓ of the patches. At a protein-to-lipid ratio of approximately 1:10, we often had difficulties forming blisters from the multilamellar liposomes. There was no obvious difference in channel activities whether the *Paramecium* cortex had been mixed with sonicated lipid or first treated with either detergent (CHAPS or octylglucoside) before dialysis and reconstitution (*see* Materials and Methods and Fig. 1). This report summarizes our experience from over 750 patches in five sets of experiments with cortical proteins. Each set consisted of an individual cortex preparation and three treatments (sonication, CHAPS, or octylglucoside). Each treatment was replicated five times, and 10–20 patches were sampled each time.

Reconstituted Liposome Patches Contain Open Channels

Patches excised from these liposomes often had low resistances. We discovered that the low resistance was due to open channels in the patches and not due to poor seals. We found that the abundant open channels (mostly Type

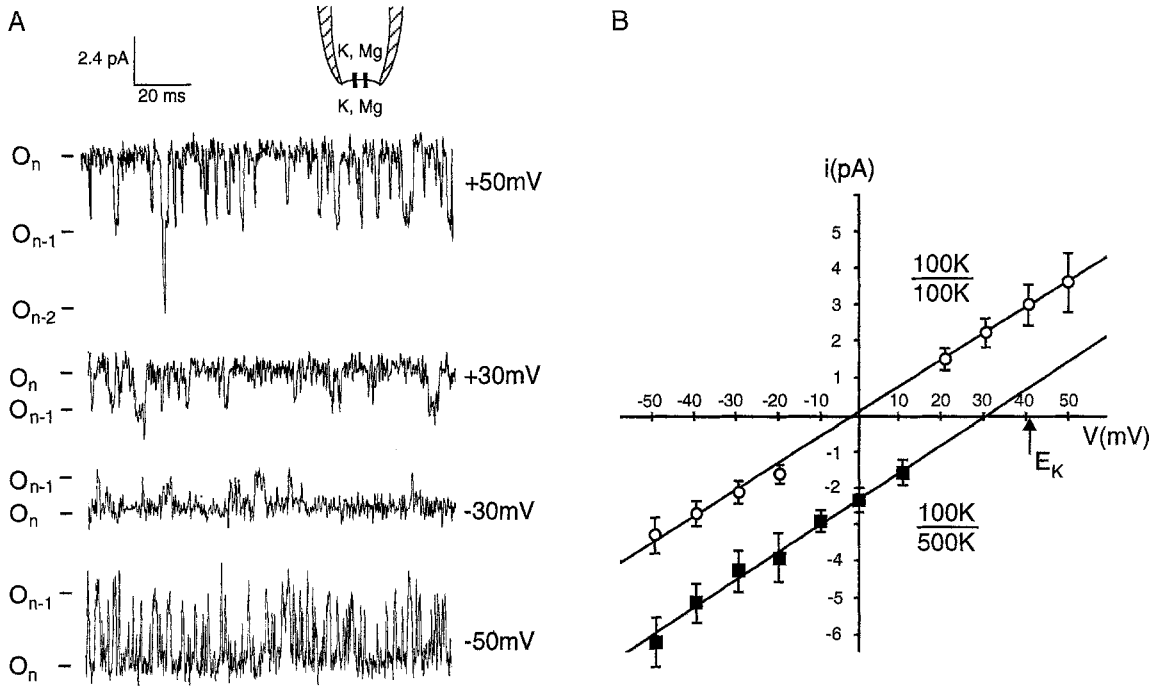

Fig. 4. Unit conductance and cation selectivity of Type-1 channels. The activities through several such channels at four transmembrane voltages are shown (*A*), under symmetric conditions of 100 mM KCl and 10 mM $MgCl_2$. The brief closures were occasionally long enough to allow measurements from flat-top unit currents. Under these conditions, the current-voltage plot from three independent experiments (*B*), open circles, mean ± SD, $n = 3$) reverses near 0 mV and has a slope conductance of 71 pS. Increasing the bath KCl to 500 mM did not change the slope of the *I-V* curve from these experiments but moved it to the right (*B*, filled squares). The new reversal potential of 30.3 ± 2.5 mV indicates cation selectivity. Selectivity is not perfect since this reversal potential is lower than the calculated equilibrium potential of K^+ of 41 mV (E_K, marked).

1, *see below*) could be closed briefly by lower, and for long times by higher concentrations of di- or trivalent cations. For example, Fig. 2 shows that, during continuous recording of the same patch, an application of Gd^{3+} reversibly closed channels and thereby revealed the background current level (the "closed" level, marked C). Brief closures from the open level due to the added Mg^{2+} (Fig. 2, left and right trace) and the brief openings from the closed level in the presence of Gd^{3+} (middle trace) indicate the quantal nature to the single-channel currents. Prolonged closures in Gd^{3+} allowed a clearer resolution of the unit currents as evidenced by a current amplitude histogram (Fig.2, lower left).

Types of Channel Activities Reconstituted

Note that the experiment shown in Fig. 2 was performed in baths with 10 mM Mg^{2+} throughout. In the absence of any di- or trivalent cations, we often observed activities of other channels riding on a steady current through Type 1 channels (Fig. 3*A*). Once Mg^{2+} was added, however, the traces became dominated by the events of closing and reopening of the Type 1 channels (Fig. 3*B*).

Three types of channel activities were consistently encountered hundreds of times in our extensive study of liposomes reconstituted with *Paramecium* material. That they differ in unit conductance, ion selectivity, and kinetics suggests that they reflect different channel entities and are unlikely to be alternative states of the same entity. Below, we first describe Type 1 channel activities in detail and then Type 2 and Type 3 briefly.

Type 1: A 71-pS Cation Conductance

This was the most abundant type of channel activity, observed in about half of active patches. About five units in the open state were usually encountered in each patch; as many as tens of such units have been observed in some patches. Their individual activities could be made evident upon the addition of di- or trivalent cations (Fig. 2, Fig. 3*B*). The conductance units in the same patch appeared to behave independently from each other, since current amplitude histograms can be approximated with Poisson distributions (Fig. 2, lower left). These are apparently activities of channels in a cluster captured in a patch and not substate behaviors of a single channel entity.

Without significant amount of di- or trivalent cations

in the solutions, the open probability at all voltages was near 100% regardless of the direction or magnitude of the transmembrane voltage. Mg^{2+} was therefore added in the bath so that unit currents at different voltages could be measured. As shown in Fig. 3*B*, prolonged conduction of K^+ current through all units was interrupted by brief closures usually of only one unit. Even in the presence of 10 mM Mg^{2+}, most closures were less than 1 msec and could not be resolved clearly. Nonetheless, rarer longer unit closure allowed measurement of unit current. We began the investigation in 100 mM KCl, 10 mM $MgCl_2$, in both the pipette and the bath. (All solutions used in this report, except Fig. 5, also contained 10^{-5} M Ca^{2+} and buffered to pH 7.2 with 5 mM HEPES.) Fig. 4*B* plots the unit currents at different voltages in this symmetric condition, yielding a slope conductance of 71.3 ± 3.9 pS (mean ± SD, $n = 10$). When the bath was changed to 500 mM KCl, the *I-V* curve (closed squares) showed a parallel shift, changing the reversal potential from near 0 mV to +30.3 ± 2.5 mV ($n = 3$). From these results, the permeability ratio of K^+ to Cl^- was calculated to be greater than 10:1, showing that Type 1 is a cation channel.

This 71 pS conductance discriminated poorly among monovalent cations. By examining the reversal potentials in biionic conditions (*data not shown*), we have determined the permeability ratio of K^+:Cs^+:Na^+ = 1.0:1.0:0.9. Curiously, this channel also passed divalent cations even though they also tended to close it. Both effects are shown in Fig. 5*A*. Under biionic conditions here, where Mg^{2+} was the cation in the bath, increasing the Mg^{2+} concentration increased the unit current driven into the pipette by negative voltages, consistent with Mg^{2+} being the charge carrier. At the same time, the mean closed time also increased resulting in a clearer resolution of one or two unit currents entering the pipette (Fig. 5*A* right, bottom trace), consistent with Mg^{2+} also being the agent that closes the channel. At a bath concentration of 100 mM Mg^{2+}, the reversal potential was +10.3 ± 1.5 mV ($n = 3$). A 5-fold increase in the bath Mg^{2+} concentration caused a right shift of the reversal potential to +39.6 ± 6.5 mV ($n = 3$) (Fig. 5*B*). This shift corresponds to a permeability ratio of K^+:Mg^{2+} of about 1.0:1.2, according to the expanded Goldman-Hodgkin-Katz equation (Spangler, 1972). The prediction from this calculated permeability ratio (straight line, Fig. 5*C*) fits well the experimentally determined relation between the reversal potential and the bath Mg^{2+} concentration ranging from 100 to 1,000 mM (Fig. 5*C*, triangles).

A similar set of experiments with bath Ca^{2+} instead of Mg^{2+} gave similar results (Fig. 6), where the reversal potential was 42.5 ± 4.5 mV ($n = 3$) in 100 mM KCl in the pipette and 500 mM $CaCl_2$ in the bath. The permeability ratio of K^+ to Ca^{2+} was determined to be 1.0:1.6. Thus, Type 1 is potentially an effective Ca^{2+} release channel.

Type 2: A 40 pS Monovalent Cation Conductance

In patches excised from liposomes reconstituted with cortical proteins, the Type 1 71 pS activity was very often accompanied by a different activity. This second activity has a smaller conductance and lower open probability (Fig. 3*A*). When present, one or two such conductances were found in an excised patch. In symmetric 100 mM KCl solutions, the channel conductance was found to be about 40 pS (Fig. 7*A* left, 7*B* open circles). Replacing the bath solution with 500 mM KCl caused a right shift of the *I-V* plot from near zero (−2.3 ± 1.5 mV, $n = 3$) to 35.0 ± 2.5 mV ($n = 3$) (Fig. 7*B*, closed squares). This shift corresponds to a permeability ratio of K^+:Cl^- ≈ 20:1. Replacing the bath with 100 mM NaCl caused a discernable decrease in the unit current from the bath into the pipette (Fig. 7*A*, lower right) and a small but significant ($P < 0.002$) left shift of the reversal potential to −7.7 ± 2.5 mV ($n = 3$), suggesting a slight channel preference for K^+ over Na^+. Unlike Type 1 channel, this Type 2 channel did not conduct divalent cations. Replacing the bath solution with 100 mM $MgCl_2$ caused a left shift of the reversal potential by more than 30 mV as extrapolated from the outward K^+ currents (*data not shown*). Even with 10 mM $MgCl_2$ channel, Type 2 activities seemed to reflect flickering blocks by Mg^{2+} (Fig. 3*B*).

Type 3: An Anion Conductance

In about one-fifth of the patches, we encountered a conductance drastically different from Type 1 and 2. First, this Type 3 channel has slow kinetics: open and closed times longer than 100 msec were commonly observed (Fig. 8*A*). Second, it has multiple subconductance states. In patches where apparently only one unit was captured, the presumed full-conducting unit in symmetric 100 mM KCl was found to be 400 pS (Fig. 8*A*). Full conducting levels, marked by "Os", were the current maxima during tens of seconds). Discrete subconducting states at 100, 160, 240, 280, and 320 pS were evident (Fig. 8*A*, asterisks). Full channel opening and closing did not appear to vary drastically at different voltages, though a possible voltage dependence of the complex distribution among the full and the subconductance states has not been explored thoroughly. Third, it is not a cation-selective channel. Increasing the bath KCl concentration by five times the symmetric condition caused a left shift of the *I-V* curve of the maximal conducting unit. The new reversal potential, −14.3 ± 2.1 mV ($n = 3$) corresponds to a permeability ratio of K^+ to Cl^- = 0.4:1. Thus Type 3 appears to be a nonselective channel that prefers anions. Anion replacement experiments showed that it discriminates poorly between chloride and glutamate

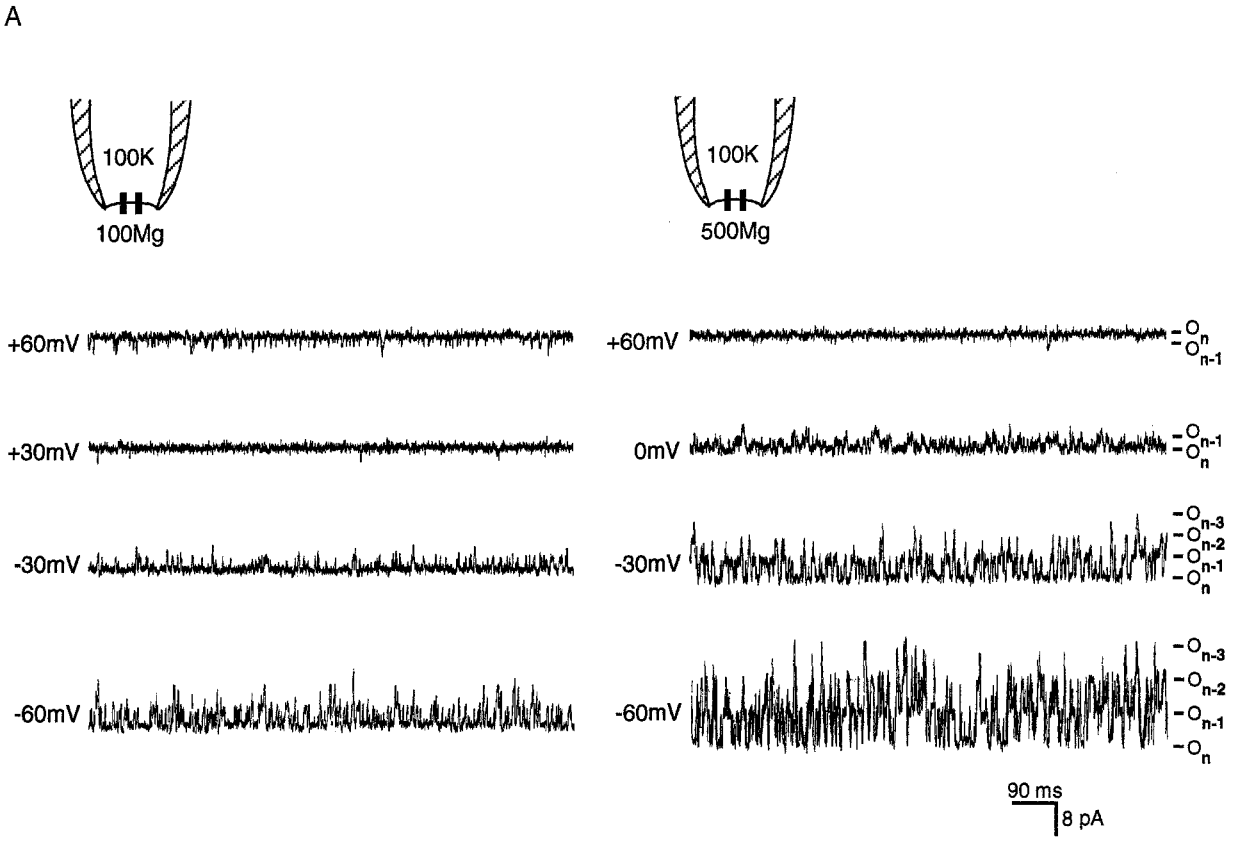

Fig. 5. Mg^{2+} permeates Type 1 channel. The 71-pS conductances in patches excised from liposomes incorporated with cortical material were examined. Under a bi-ionic condition with 100 mM KCl in the pipette and 100 mM $MgCl_2$ in the bath, both inward and outward currents were evident upon voltage manipulations (*A*, left). When the bath was changed to 500 mM $MgCl_2$, unit currents entering the pipette became larger. Unit events also became more distinct due to longer closure in the presence of a higher concentration of Mg^{2+} (*A*, right). The *I-V* plot in the 100 mM $MgCl_2$ (*B*, open circles, mean ± SD, $n = 3$) could be approximated with a straight line. This *I-V* curve has a reversal potential at a small positive voltage, consistent with the channel's slight preference for Mg^{2+} over K^+. The *I-V* curve showed a prominent shift in the direction of positive voltage when bath $MgCl_2$ was increased to 500 mM (*B*, filled squares). The reversal potentials at four different external Mg^{2+} concentrations (*C*, triangles) plotted in a semilogarithmic scale agree with prediction based on the P_K:P_{Mg} ratio (*C*, straight line) calculated based on an expanded Goldman-Hodgkin-Katz equation of Spangler (1972): $E_r = 58 \log\{[-P_K[K]_i + (P_K{}^2[K]_i{}^2 + 16\, P_{Mg}[\mathrm{Mg}]_o P_K[K]_i)^{1/2}]/2\, P_K[K]_i\}$ *mV*

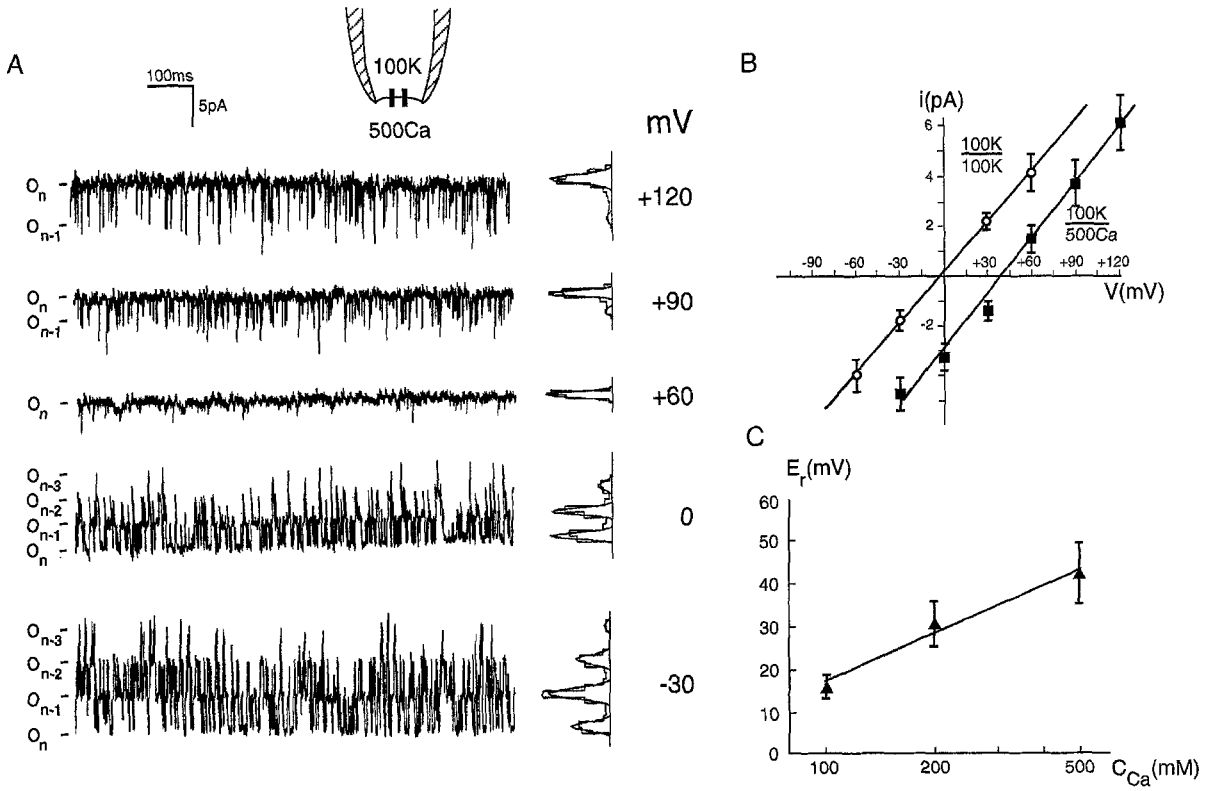

Fig. 6. Ca^{2+} permeates Type-1 channel effectively. Sample currents and their corresponding amplitude histograms are shown (*A*). These currents were recorded with 100 mM KCl in the pipette and 500 mM $CaCl_2$ in the bath. (*B*) shows the *I-V* plot in symmetric 100 mM KCl (open circles) and in 500 mM bath $CaCl_2$ (filled squares). The reversal potentials at three different external Ca^{2+} concentrations (*C*, triangles) agree with the prediction based on the P_K:P_{Ca} ratio (*C*, straight line) calculated as below (Spangler, 1972): $E_r = 58 \log\{[-P_K[K]_i + (P_K{}^2[K]_i{}^2 + 16\, P_{Ca}[Ca]_o P_K[K]_i)^{1/2}]/2 P_K[K]_i\}$ *mV*

(*data not shown*). Like the Type 1 and Type 2, this channel was effectively closed or blocked by divalent cations. For example, Fig. 8*B* shows that an addition of 50 mM $MgCl_2$ revealed currents that dwelled at deeper closed levels not observed before the addition.

Channel Activation

None of the three types of channels showed any clear activation or inactivation by voltage in the range of −100 to +100 mV tested.

Attempts to activate or inactivate channel activity with Ca^{2+} (10^{-3} to 10^{-8} M), IP_3 (10 to 200 μM), cAMP (0.1 to 1 mM), cGMP (0.1 to 1 mM) or ryanodine (1 to 100 μM) were also unsuccessful. Suctions applied to the patches exceeding 100 mm Hg also had no effects on the channel activities showing that these are not mechanically activatable channels.

Discussion

The purpose of this investigation is to see whether any channels from the *Paramecium* cortex can be functionally reconstituted. The ultimate aim is to purify, or at least enrich, channel proteins by following the reconstituted activities through protein fractionation, as has been successfully done in the case of mechanosensitive channel protein of *E. coli* (Sukharev et al., 1994*a,b*). We have been successful to this end, since at least three types of channel activities were readily observed upon reconstitution. Unlike those reported upon reconstituting ciliary membrane onto planar lipid bilayers (Ehrlich et al., 1984), the activities we observed in patches excised from liposomes reconstituted with cortical material have conductances of 10s to 100s pS, well above background noise. We also found that the activities survive solubilization by 2% CHAPS or octylglucoside. The relatively large signals, the high frequency of encountering these activities prior to any protein enrichment, and their ability to withstand mild detergents make hopeful the enrichment or even purification of channel proteins through fractionations.

The three types of activities we found in patches reconstituted with cortical material were not among those described for plasma and ciliary membrane channels (Saimi et al., 1994, Martinac et al., 1994). In addi-

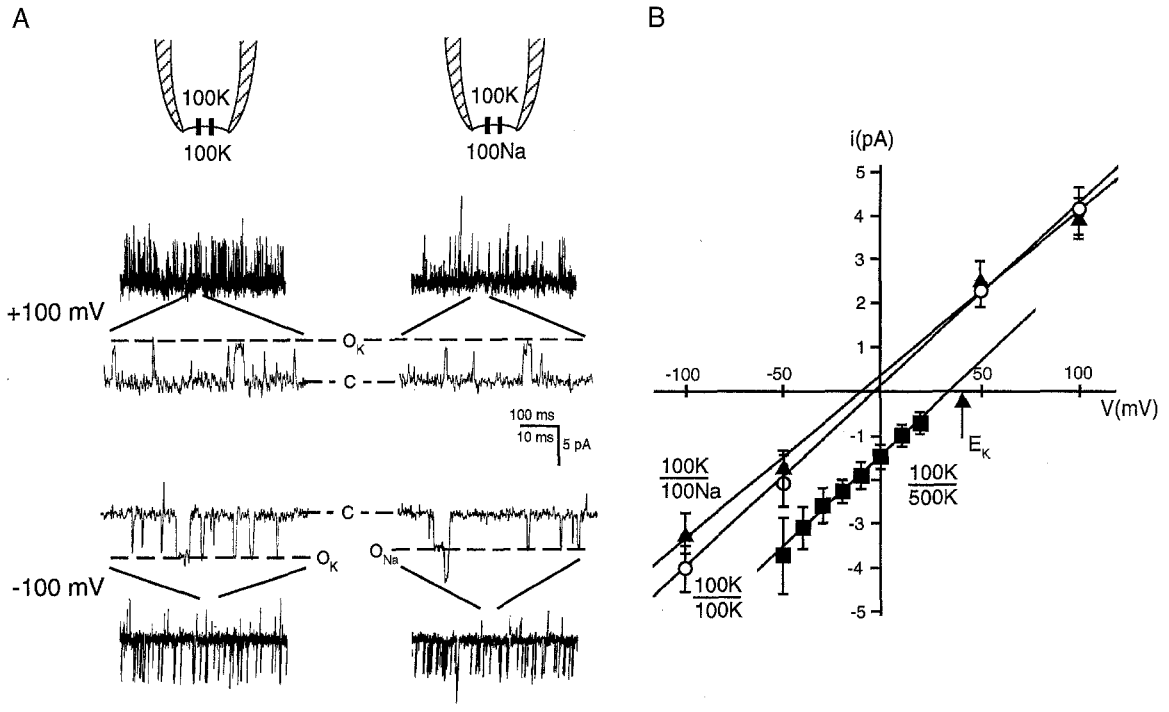

Fig. 7. Characteristics of Type-2 channel. Sample traces of current through this type of channel in symmetric 100 mM KCl are shown in *A*, left. The unit currents plotted against voltage show a slope conductance of 40 pS (*B*, open circles). Increasing bath KCl concentration to 500 mM caused a right shift of the *I-V* plot (*B*, filled squares, mean ± SD, $n = 3$) with a reversal potential very close to the calculated equilibrium potential of K^+ (E_K, marked), indicating strong K^+ preference over Cl^-. Replacing the bath with 100 mM NaCl did not alter the general appearance of the unit currents (*A*, right column), though the unit currents entering the pipette became smaller (*A*, lower right, *B*, filled triangles). This replacement also caused a small left shift in the reversal potential (*B*, filled triangles). These results indicate permeation of Na^+ and a slight preference of K^+ over Na^+.

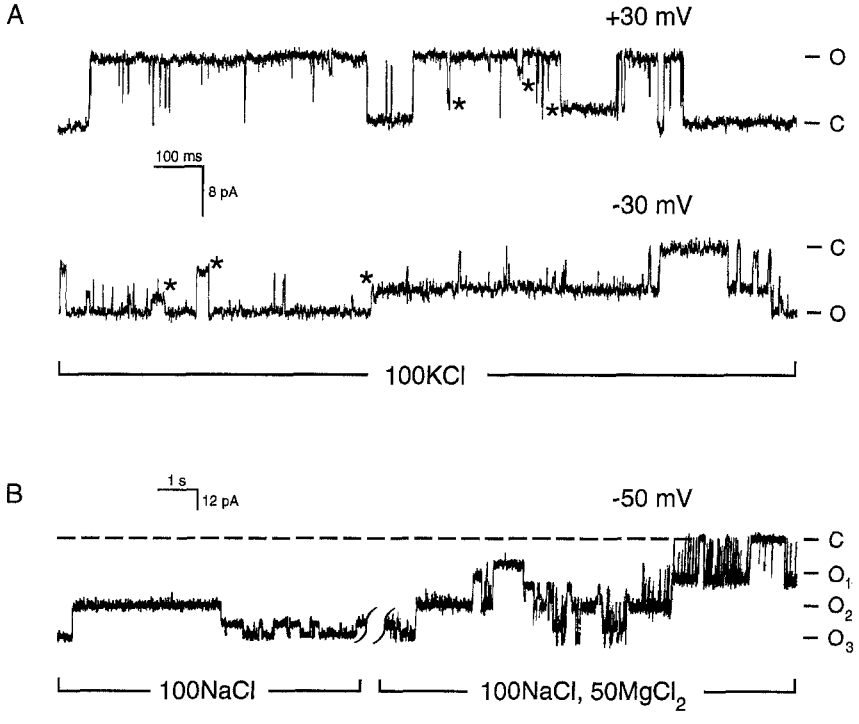

Fig. 8. Characteristics of Type 3 conductance. (*A*) shows sample currents in symmetric 100 mM KCl condition at +30 or −30 mV. The full unit current indicates a conductance of 400 pS. However, the current also dwells at several different levels below the maximum (*A*, asterisks), indicating substate behavior of the channel. (*B*) shows currents through the same type of channels in a different patch at a slow time scale. In a bath of 100 mM NaCl, closure events to different substates of only one full unit (between 0_3 and 0_2) were registered. After a bath change to 100 mM NaCl with 50 mM $MgCl_2$, closure events to all three full unit levels (0_3, 0_2, 0_1, C) as well as various substate levels became evident.

tion, we observed the activities of the same channels regardless of whether we reconstituted *Paramecium* cortices prepared from ciliated or deciliated cells. We therefore need to consider the possibility of their being originated from internal membranes. That the reconstituted activities have little or no voltage dependence also suggests that the channels reside in a compartment(s) without a transmembrane voltage drop. *Paramecium* cortex includes the alveolar sacs. Stelly et al. (1991) showed that the alveoli are associated with a strong Ca^{2+} pump and is likely a vast Ca^{2+} store, which may function in cortical morphogenesis. In addition, Erxleben and Plattner (1994) have recently showed a Ca^{2+} release from the alveoli that appears to correlate with trichocyst discharge. Of the three activities we commonly observed, Type 1, though cation nonspecific, has a largest permeability for Ca^{2+} among cations tested and may reflect a Ca^{2+} release channel of the alveolar sacs. It seems unlikely that such a channel is left open at all or most time as their activities in artificial liposome membrane patches have indicated. We were unable to discover ligands that gate these channels, although cNMP, Ca^{2+}, and IP_3 have been tested. It remains possible that these channels are gated by other second messengers or through protein-protein interaction with a GTP-binding protein or calmodulin. It is also possible that the plasmamembrane channels have been inactivated and the alveolar channel protein(s) has been altered during cortical preparation or reconstitution. The loss of a regulatory subunit that normally accompanies the pore-forming subunit, for example, cannot be ruled out. In any event, the conducting pores provide positive electric signals, albeit not necessarily completely physiological. Such signals are required so that the activities can be followed during fractionation, enrichment and eventually protein purification.

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