Recombinant pI_{Cln} Forms Highly Cation-selective Channels when Reconstituted into Artificial and Biological Membranes

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ABSTRACT pI_{Cln} has been proposed to be the swelling-activated anion channel responsible for $I_{Cl,\,swell}$, or a channel regulator. We tested the anion channel hypothesis by reconstituting recombinant pI_{Cln} into artificial and biological membranes. Single channels were observed when pI_{Cln} was reconstituted into planar lipid bilayers. In the presence of symmetrical 300 mM KCl, the channels had a high open probability and a slope conductance of 48 pS, and were outwardly rectifying. Reduction of *trans* KCl to 50 mM shifted the reversal potential by -31.2 ± 0.06 mV, demonstrating that the channel is at least seven times more selective for cations than for anions. Consistent with this finding, channel conductance was unaffected by substitution of Cl^- with glutamate, but was undetectable when K^+ was replaced by *N*-methyl-d-glucamine. Reconstitution of pI_{Cln} into liposomes increased $^{86}Rb^+$ uptake by three- to fourfold, but had no effect on $^{36}Cl^-$ uptake. Phosphorylation of pI_{Cln} with casein kinase II or mutation of G54, G56, and G58 to alanine decreased channel open probability and $^{86}Rb^+$ uptake. When added to the external medium bathing Sf9 cells, pI_{Cln} inserted into the plasma membrane and increased cell cation permeability. Taken together, these observations demonstrate that channel activity is due to pI_{Cln} and not minor contaminant proteins. However, these findings do not support the hypothesis that pI_{Cln} is the anion-selective $I_{Cl,\,swell}$ channel. The observed cation channel activity may reflect an as yet to be defined physiological function of pI_{Cln}, or may be a consequence of in vitro reconstitution of purified, recombinant protein.

KEY WORDS: cell volume • swelling-activated anion channels • planar lipid bilayer • liposomes • recombinant protein

INTRODUCTION

An apparently ubiquitous response to swelling in vertebrate cells is activation of an outwardly rectifying anion current termed $I_{Cl,\,swell}$. The general characteristics of this current include an Eisenman type I anion permeability sequence ($I^- > Br^- > Cl^- > F^-$), modest outward rectification, voltage-dependent inactivation at potentials above E_{Cl}, inhibition by a wide variety of compounds including conventional anion transport inhibitors, and block by extracellular nucleotides (Strange et al., 1996; Okada, 1997).

Molecular identification of the protein(s) giving rise to $I_{Cl,\,swell}$ has been controversial and confusing (Strange et al., 1996; Nilius et al., 1997; Okada, 1997; Strange, 1998). Gill et al. (1992) and Valverde et al. (1992) proposed that P-glycoprotein, the product of the multidrug resistance 1 gene, functions as both a drug transporter and the $I_{Cl,\,swell}$ channel. However, numerous laboratories have been unable to reproduce the findings of these investigators, and additional experimental observations have failed to support the original hypothesis (reviewed by Wine and Luckie, 1996; Okada, 1997). Subsequently, it was suggested that P-glycoprotein functions to modulate or regulate $I_{Cl,\,swell}$ (reviewed by Wine and Luckie, 1996; Okada, 1997). It is not clear whether the apparent modulation of $I_{Cl,\,swell}$ by P-glycoprotein reflects a physiologically relevant function, or whether it is simply a consequence of overexpression of the protein induced by transfection or drug selection.

pI_{Cln} is a ubiquitous and abundant 27-kD soluble protein that is localized primarily to the cytoplasm (Krapivinsky et al., 1994; reviewed by Strange, 1998). Because of its ability to induce in *Xenopus* oocytes an outwardly rectifying anion current that superficially resembles $I_{Cl,\,swell}$, pI_{Cln} has been proposed to be either the $I_{Cl,\,swell}$ channel (Paulmichl et al., 1992; Gschwentner et al., 1995) or a channel regulator (Krapivinsky et al., 1994). However, as with P-glycoprotein, key observations supporting these hypotheses have not been reproduced or supported by additional experimental evidence, and

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the role of pI_{Cln} in $I_{Cl,\ swell}$ channel function, if any, remains uncertain (reviewed by Strange, 1998).

To test the hypothesis that pI_{Cln} is an anion channel-forming protein, we reconstituted purified, recombinant protein into artificial and biological membranes. Our results demonstrate conclusively that pI_{Cln} is capable of generating channel activity in vitro, but the channels formed by the protein are highly cation selective. These findings do not support the hypothesis that pI_{Cln} is the $I_{Cl,\ swell}$ channel. The observed cation channel activity may reflect an as yet to be defined physiological function of pI_{Cln}, or it may be a consequence of in vitro reconstitution of recombinant protein. Our findings provide the basis for further physiological investigations of pI_{Cln} and may provide novel insights into the structure of channel-forming proteins and protein–membrane interactions.

MATERIALS AND METHODS

Production and Purification of Recombinant pI_{Cln}

A fusion protein consisting of glutathione S-transferase (GST)[1] and full length pI_{Cln} cloned from rat C6 glioma cells was ligated into the pGEX-4T-1 vector and expressed in BL21 *Escherichia coli* using a commercially available kit (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The GST–pI_{Cln} fusion protein was purified from bacterial lysates using glutathione Sepharose 4B. pI_{Cln} was cleaved from the GST moiety with thrombin, and the thrombin was removed by treatment with benzamidine Sepharose 6B (Pharmacia LKB Biotechnology Inc.). As a control, lysates from bacteria expressing GST alone were subjected to the same purification procedure as the GST–pI_{Cln} fusion protein.

Phosphorylation and Mutagenesis of pI_{Cln}

Casein kinase II (CKII) was used to phosphorylate pI_{Cln} (Sanchez-Olea et al., 1998). Approximately 200 μg of GST–pI_{Cln} was incubated at 30°C with 500 U CKII in 100 μl of reaction buffer (New England Biolabs Inc., Beverly, MA) containing 200 μM ATP. After 30 min, GST–pI_{Cln} was washed with phosphate-buffered saline and subjected to the cleavage procedure described above.

Glycine residues at positions 54, 56, and 58 of C6 glioma cell pI_{Cln} (Sanchez-Olea et al., 1998) correspond to G49, G51, and G53 of Madin-Darby Canine Kidney (MDCK) cell pI_{Cln} (Paulmichl et al., 1992). These residues were mutated to alanine using standard PCR approaches.

Planar Bilayer Studies of Single Channel Activity

Planar lipid bilayers were formed by painting a 10 mg/ml solution of phospholipid (phosphatidylethanolamine [PE]:phosphatidylserine [PS] at a ratio of 1:1) in *n*-decane over a 200-μm aperture in a bilayer chamber. Lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL), and were >99% pure. The *cis* compartment of the bilayer chamber is defined as that compartment to which proteoliposomes were added, and the *trans* compartment is defined as the compartment connected to ground. Bilayer solutions contained 50 or 300 mM KCl and 10 mM 4-morpholinepropanesulfonic acid (MOPS), pH 7.0. For ion substitution experiments, KCl was substituted with either *N*-methyl-d-glucamine (NMDG)-Cl or K-glutamate.

pI_{Cln} was incorporated by sonication into liposomes comprised of PE:PS:phosphatidylcholine:ergosterol (4:1:5:1 ratio by wt) in 1:10 protein:lipid (by wt) ratio. Lipids were purchased from Avanti Polar Lipids, Inc. and were >99% pure. Proteoliposomes were purified by passing through a Sephadex G-50 gel filtration column (Fisher Scientific Co., Pittsburgh, PA) to remove pI_{Cln} remaining in solution. To promote and monitor bilayer fusion, proteoliposomes were doped with nystatin (120 μg/ml liposomes) as described originally by Woodbury and Miller (1990). Channel activity was monitored after addition of proteoliposomes to the *cis* compartment of the bilayer chamber (1 μg protein/ml bilayer solution). Fusion events were detected as transient, nystatin-induced conductance spikes. Single channel currents were measured with a bilayer amplifier (custom made by M. Shen, Physics Lab, University of Alabama, University, AL). Electrical connections between the bath chambers and the amplifier were made using 3 M KCl agar bridges. Data were recorded and analyzed using pCLAMP 6.0.2 software (Axon Instruments Inc., Foster City, CA). Before analysis of dwell times, single channel data were digitally filtered at 100 Hz.

Histogram Analysis

Open and closed time histograms were created with a logarithmic x axis with 10 bins/decade and a lower limit of 10 ms. The maximum likelihood method was used to fit the data with one or two exponentials (pClamp 6.0.2 software; Axon Instruments Inc.). The "goodness" of fit was assessed using the log likelihood ratio test.

Concentrative Tracer Uptake Assay for Study of Channel Activity

A concentrative tracer uptake assay developed by Garty et al. (1983) and modified by Goldberg and Miller (1991) was used to characterize the Cl^- and K^+ transport properties of reconstituted pI_{Cln}. pI_{Cln} was reconstituted into liposomes without nystatin as described above for bilayer experiments. Proteoliposomes were preloaded with 150 mM KCl and external Cl^- was removed by centrifugation through Sephadex G-50 columns equilibrated with Cl-free uptake solution (125 mM K-glutamate, 25 mM Na-glutamate, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glutamic acid, and 20 mM Tris-glutamate, pH 7.6). Radioisotope uptake was initiated and quantified by addition of 1.0 μCi/ml of $^{36}Cl^-$ to the proteoliposome suspension. Intravesicular $^{36}Cl^-$ was assayed at various times after separation of proteoliposomes from the external media using a Dowex 1 mini anion-exchange column (Garty et al., 1983). To assess cation transport properties of pI_{Cln}-containing proteoliposomes, a chemical gradient favoring cation efflux was generated by loading them with 150 mM KCl, and then exchanging the extracellular solution with a K^+-free uptake solution (150 mM NMDG-Cl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glutamic acid, 20 mM Tris-glutamate, pH 7.6). Electrogenic cation uptake was quantified by addition of 1.0 μCi/ml of $^{86}Rb^+$ to the proteoliposomes suspension.

In preliminary studies, we observed that half-maximal uptake of isotope occurred ~60 min after isotope addition. We reasoned that quantification of isotope uptake at 60 min would provide a sensitive measure of any differences in function between wild-type, phosphorylated, and mutant pI_{Cln}. Accordingly, a 60-min uptake period was used in all isotope uptake studies.

[1]*Abbreviations used in this paper:* CKII, casein kinase II; GST, glutathione S-transferase; NMDG, *N*-methyl-d-glucamine.

Light Scattering Measurements

Sf9 cells were grown in long-term suspension culture at 25°C in Grace's Insect cell culture medium (GIBCO BRL, Gaithersburg, MD), supplemented with EX-Cell 401 complete medium (JRH Biosciences, Lenexa, KS), 1:1 by volume. A volume of suspension culture containing $\sim 10^6$ cells was centrifuged and the pelleted cells were resuspended in a cuvette with 2 ml of Na^+- and K^+-free medium containing 143 mM NMDG-Cl, 10 mM glucose, 20 mM HEPES, and 0.5 $CaCl_2$ at pH 7.4. The cuvette was stirred continuously and maintained at 25°C. Right angle light scattering was measured with emission and excitation wavelengths of 400 nm using an F-4000 fluorescence spectrophotometer (Hitachi, Ltd., Tokyo, Japan). Experimental cells were pretreated with either 10 μg/ml gramicidin or 25 μg/ml pI_{Cln} for 10 min before suspension in the Na^+- and K^+-free medium.

Immunofluorescence Microscopy

Sf9 cells were cultured on glass coverslips using the culture conditions described above. Coverslips were incubated with 100 μg/ml pI_{Cln} for 60 min. Both pI_{Cln}-treated and nontreated control cells were fixed for 20 min in 4% paraformaldehyde. Fixed cells were washed in PBS and incubated for 15 min in PBS/1% BSA to block nonspecific background staining. Primary anti–pI_{Cln} (Sanchez-Olea et al., 1998) polyclonal antiserum was applied at a dilution of 1:100 for 90 min at room temperature, followed by incubation with a CY3-conjugated goat anti–rabbit IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 1 h. Control experiments were performed using preimmune serum, followed by CY3-conjugated goat anti–rabbit IgG. Coverslips were mounted in Vectashield antifading solution (Vector Laboratories, Inc., Burlingame, CA), diluted 1:1 in 0.1 M Tris-HCl, pH 8.0. Cells were visualized using an FXA photomicroscope (Nikon Inc., Melville, NY) and an Optronics 3-bit CCD color camera (Optronics Engineering, Goleta, CA). CY3 fluorescence and autofluorescence images were captured separately and merged using IP Lab Spectrum acquisition and analysis software (Scanalytics, Vienna, VA) running on a Power PC 8500. Pictures were printed on a Tektronix Phaser 440 dye sublimation color printer (Tektronix Inc., Wilsonwill, OR).

Statistics

Results are expressed as means ± SEM. Statistical significance was assessed using the log likelihood ratio or Student's *t* tests.

RESULTS

Purification of Recombinant pI_{Cln}

Recombinant pI_{Cln} was produced in *E. coli* as a GST fusion protein and purified using glutathione Sepharose 4B and thrombin cleavage (see MATERIALS AND METHODS). For planar lipid bilayer studies, a control preparation was generated in parallel with the pI_{Cln} preparation by expressing GST alone in *E. coli*. Lysates from GST-expressing bacteria were subjected to the same purification protocol (see MATERIALS AND METHODS) as lysates containing GST-pI_{Cln} fusion protein.

Fig. 1 shows a Coomassie-stained gel and Western blot of the pI_{Cln} preparation. pI_{Cln} is the predominant protein detected in the purified bacterial lysates. When the gel is deliberately overloaded (i.e., 100 μg total protein), extremely faint protein bands were detected below pI_{Cln} (Fig. 1 *A*). These proteins reacted with polyclonal anti–pI_{Cln} antibodies (data not shown), suggesting that they were pI_{Cln} truncation fragments. We also used NH_2-terminal sequence analysis to assess the purity of the preparation. pI_{Cln} was the only protein detected using this method, which indicates that the preparation has been purified to at least 99% homogeneity.

pI_{Cln} Generates Cation-selective Channels when Reconstituted into Planar Lipid Bilayers

pI_{Cln} is a soluble cytoplasmic protein (Krapivinsky et al., 1994). In our initial attempts to reconstitute pI_{Cln}, we added the protein in solution at a concentration of 50 μg/ml to the *cis* side of the bilayer chamber, an approach that has been used successively to reconstitute channel-forming proteins such as VDAC (Xu and Colombini, 1997), bacterial toxins (Wimsen et al., 1990), and porins (Nekolla et al., 1994). Channel activity was detected using this method (e.g., Fig. 2 *B*), indicating that the channel-forming protein present in the purified bacterial lysates can spontaneously insert into the lipid bilayer. However, it was difficult to regulate protein incorporation into the bilayer with this approach. When protein incorporation occurred, multiple channels were usually detected. Single channels were more regularly detected by first reconstituting purified pI_{Cln} into phospholipid liposomes before addition to the bilayer chamber. We also included nystatin and ergosterol into the liposomes to ensure that they were equally fusogenic, and to detect each fusion event as a transient increase in nystatin-induced bilayer conductance (see Woodbury and Miller, 1990). Fig. 2 *C* shows

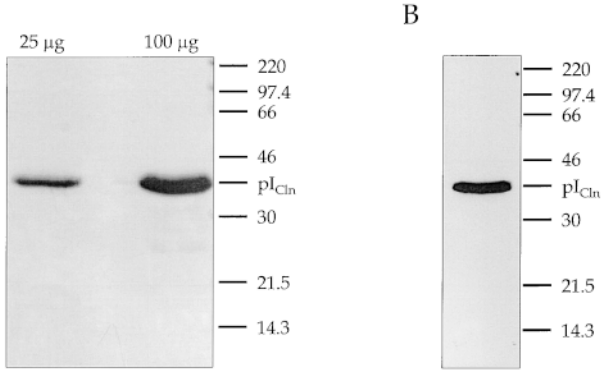

FIGURE 1. Purification of recombinant pI_{Cln}. (*A*) Coomassie-stained gel of pI_{Cln} after cleavage from purified GST–pI_{Cln} fusion protein. The lanes were loaded with either 25 or 100 μg total protein. In the overloaded lane, three or four very faint bands can be seen below pI_{Cln}. These bands reacted with anti–pI_{Cln} polyclonal antibodies (data not shown), suggesting that they were pI_{Cln} truncation fragments. (*B*) Western blot of purified recombinant pI_{Cln}. Gel was loaded with 7.5 ng of total protein.

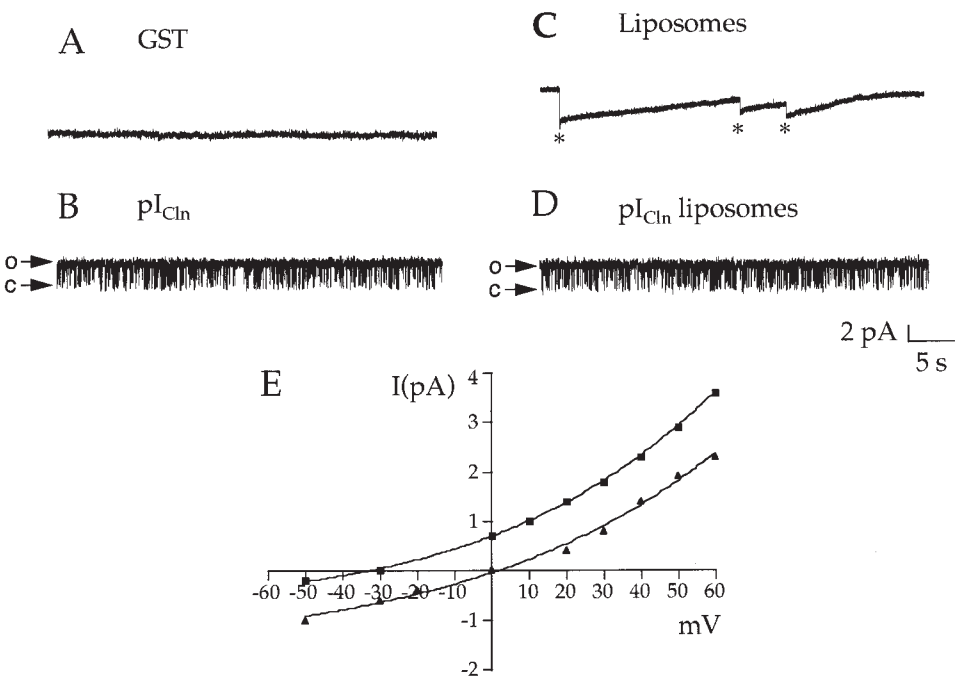

FIGURE 2. Channel activity detected in planar lipid bilayers reconstituted with pI_{Cln} or lysates purified from bacteria expressing only GST. (*A*) Addition of lysates purified from bacteria expressing GST alone (see MATERIALS AND METHODS) directly to the bilayer chamber has no effect on bilayer conductance. (*B*) Channel activity is observed when lysates purified from pI_{Cln}-expressing bacteria are added directly to the bilayer bath chamber. These results indicate that the channel-forming protein can insert spontaneously into the lipid bilayer. (*C*) Example of liposome-bilayer fusion events detected as nystatin-induced transient increases in bilayer conductance (*). Control liposomes were reconstituted with proteins purified from bacteria expressing GST alone. Channel activity was never detected with control liposomes. (*D*) Example of channel activity detected after fusion of bilayer with liposomes reconstituted with pI_{Cln}. (*E*) Current-to-voltage relationship of channels detected after fusion of bilayers with pI_{Cln} liposomes. Slope conductance measured between +10 and +60 mV is 48 pS. Current is outwardly rectifying and reverses at 0 mV when both the *cis* and *trans* bath chambers contain 300 mM KCl. Reduction of KCl in the *trans* bath chamber shifts the reversal potential to −31 mV, demonstrating that the channel is highly cation selective. The cation-to-anion permeability ratio of the channel calculated using the Goldman-Hodgkin-Katz equation is at least 7:1.

an example of liposome-to-bilayer fusion. The asterisks mark transient, nystatin-induced increases in conductance and indicate the fusion of a single liposome with the bilayer.

To assess whether the observed channel activity was specific to pI_{Cln} or due to a minor contaminant present in the bacterial lysates, control liposomes were reconstituted with lysates purified from bacteria expressing GST only. Importantly, in >100 bilayer fusion events observed with the control liposomes, channel activity was never detected (e.g., Fig. 2 *C*). Channel activity was also never observed when lysates purified from bacteria expressing GST were added directly to the bath chamber (e.g., Fig. 2 *A*). In contrast, channel activity was consistently detected with the pI_{Cln}-containing liposomes (Fig. 2 *D*). Channel activity was observed approximately every 10–20 liposome fusion events. Approximately 60% of the successful channel incorporations that occurred provided usable recordings (i.e., channel activity was stable and only a single channel was detected in the bilayer).

Fig. 2 *E* shows the current-to-voltage relationship of the channels reconstituted from the pI_{Cln}-containing liposomes. With symmetrical 300-mM KCl solutions, the current displayed modest outward rectification and reversed close to 0 mV. The slope conductance measured between +10 and +60 mV was 48 pS. When the concentration of KCl in the *trans* bath chamber was reduced to 50 mM, the current reversal potential shifted from 1.1 ± 0.05 to −31.2 ± 0.06 mV (n = 3). The direction of the shift in reversal potential indicates that the channel is highly cation selective. Using the Goldman-Hodgkin-Katz equation, the calculated cation-to-anion permeability ratio of the channel is at least 7:1.

We examined the ion selectivity of the channel further by performing ion substitution experiments. As shown in Fig. 3, *A* and *B*, replacement of Cl^- with glutamate had no effect on channel activity and conductance. In contrast, when K^+ was replaced by the large organic cation, NMDG, channel conductance fell to undetectable levels (Fig. 3 *C*). Taken together, data in Figs. 2 and 3 indicate that a highly cation-selective channel is reconstituted into planar lipid bilayers that have fused with pI_{Cln}-containing liposomes.

Cation Channel Activity Is Due to pI_{Cln} and Not Contaminant Proteins

The above results indicate that pI_{Cln} functions in vitro as a highly cation-selective ion channel. However, it was possible that the channel activity observed was due to incorporation into the bilayer of a minor contaminant protein present in the purified bacterial isolate. To test for this, we assessed the ability of purified pI_{Cln} reconstituted into liposomes to mediate concentrative, electrogenic $^{86}Rb^+$ or $^{36}Cl^-$ flux. The flux assay described in

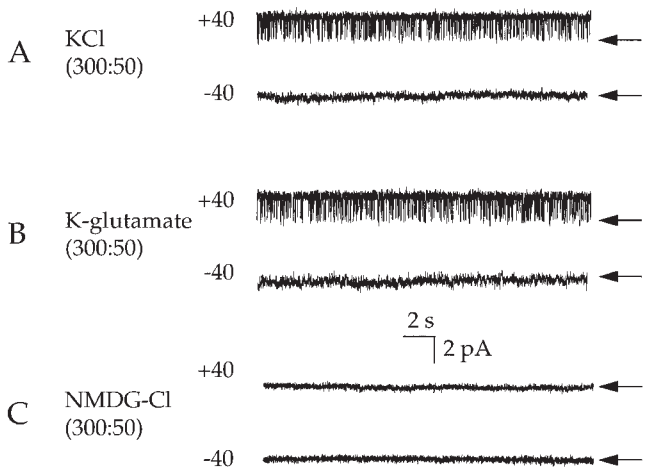

FIGURE 3. Effects of ion substitutions on channel conductance. (*A*) Channel activity observed in the presence of KCl. At −40 mV, a potential close to the reversal potential (Fig. 2 *E*), channel conductance is low. (*B*) Replacement of Cl^- with glutamate has little effect on channel conductance measured at either +40 or −40 mV. (*C*) Channel conductance is undetectable when K^+ is replaced by the impermeant cation NMDG. The *cis* and *trans* bath chambers contained 300 and 50 mM salt, respectively. Ion substitution studies were performed on the same channel by perfusing the bilayer chamber with K^+- or Cl^--free solutions. Traces are representative of four separate experiments.

the MATERIALS AND METHODS section relies on the generation of an electrochemical gradient for cation or anion transport induced by the protein of interest. For example, if purified pI_{Cln} is cation-selective, K^+ should move down its electrochemical gradient out of the proteoliposomes when they are suspended in a K^+-free medium. Potassium efflux will in turn generate an intraliposome negative potential that will drive electrodiffusive uptake of $^{86}Rb^+$ from the extracellular bath.

As shown in Fig. 4 *A*, pI_{Cln} increased electrogenic $^{86}Rb^+$ uptake approximately fourfold above that measured in liposomes without protein. In contrast, $^{36}Cl^-$ uptake was unaffected by the presence of pI_{Cln} in the liposome bilayer (Fig. 4 *B*). The results of the flux assay therefore support the bilayer studies and suggest that the observed cation-selective channel activity is due to pI_{Cln}, which constitutes at least 99% of the protein in the preparation.

To further examine the possibility that a contaminant protein was responsible for the observed cation channel activity, we altered the biochemical properties of pI_{Cln} and reconstituted the altered protein into planar lipid bilayers. pI_{Cln} is phosphorylated by CKII (Sanchez-Olea et al., 1998). As shown in Fig. 5, phosphorylation of the protein significantly ($P < 0.05$) reduced channel open probability (P_o) from 0.82 ± 0.09 to 0.52 ± 0.11 ($n = 5$). Histograms of channel open and closed times for unphosphorylated pI_{Cln} were fit by single exponentials with time constants (τ) of 79 and 10 ms, respectively (Fig. 5 *B*). Phosphorylation of pI_{Cln} had little effect on the channel open time constant, but it caused a decrease in channel open probability by inducing the appearance of a novel long closed state with a time constant of 207 ms (Fig. 5 *B*). These single channel data are consistent with the effect of phosphorylation on pI_{Cln}-induced $^{86}Rb^+$ flux. Reconstitution of phosphorylated pI_{Cln} into liposomes reduced $^{86}Rb^+$ uptake measured over a 60-min period ~50% from 1,945 ± 558 to 920 ± 199 cpm ($P < 0.05$; $n = 3$; Fig. 5 *C*).

Paulmichl et al. (1992) presented compelling evidence to support the hypothesis that pI_{Cln} is a anion

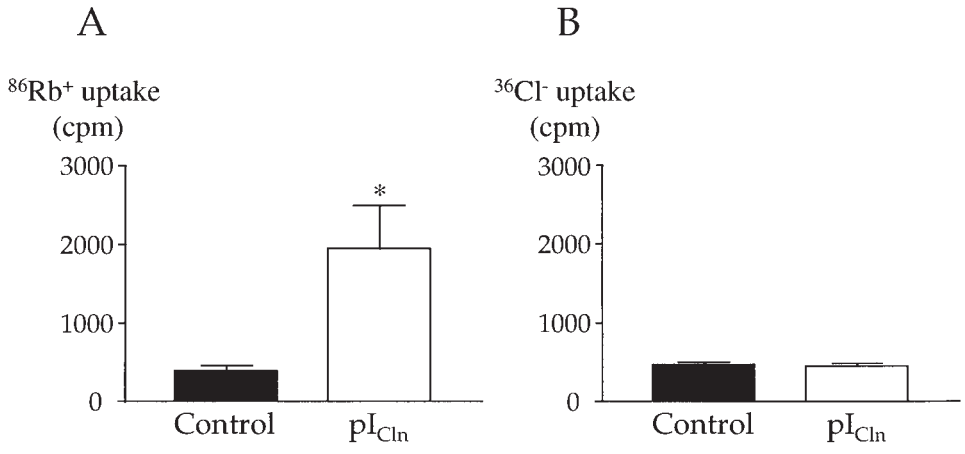

FIGURE 4. Reconstitution of liposomes with pI_{Cln} increases $^{86}Rb^+$ but not $^{36}Cl^-$ uptake. Liposomes were reconstituted in the presence of a low concentration of pI_{Cln} (1:10 protein:lipid by weight). (*A*) Reconstitution of liposomes with pI_{Cln} increases $^{86}Rb^+$ uptake four- to fivefold compared with liposomes with no added protein (*$P < 0.01$). (*B*) Uptake of $^{36}Cl^-$ is unaffected by pI_{Cln} incorporation into the liposome membrane. Values are means ± SEM ($n = 3$–6). Isotope uptake was measured by incubating proteoliposomes with $^{86}Rb^+$ or $^{36}Cl^-$ for 60 min. Preliminary time course studies demonstrated that isotope uptake was half-maximal within ~60 min after addition of isotope to the proteoliposome suspension.

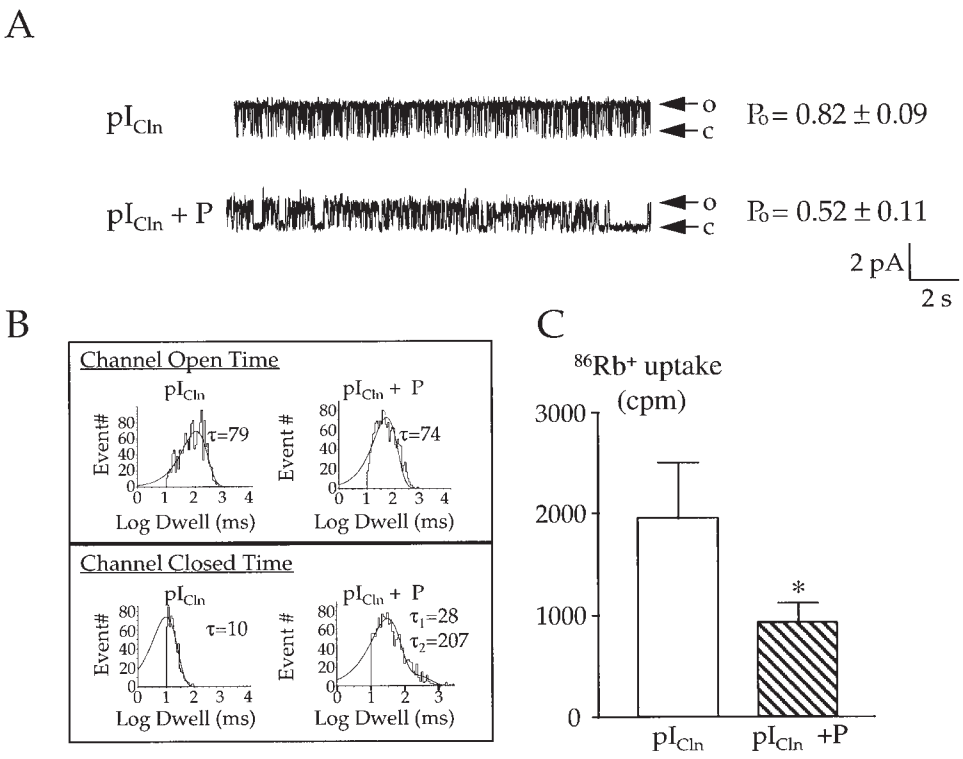

FIGURE 5. Phosphorylation of pI_{Cln} with casein kinase II reduces channel P_o and $^{86}Rb^+$ uptake. (*A*) Mean channel P_o is reduced from 0.82 ± 0.09 to 0.52 ± 0.11 (n = 5) by phosphorylation of pI_{Cln} with CKII. (*B*) Open and closed time histograms of channels observed for unphosphorylated pI_{Cln} and after phosphorylation with CKII (pI_{Cln} +*P*). Histograms for unphosphorylated pI_{Cln} were well fit by a single exponential (τ_{open}, 79 ms; τ_{closed}, 10 ms). Phosphorylation with CKII had no effect on channel open time (τ_{open} = 74 ms), but it increased channel closed time. The closed time histogram was best fit by a double exponential. Both closed time constants were increased compared with unphosphorylated pI_{Cln} (τ_1, 28 ms; τ_2, 207 ms). (*C*) Reconstitution of CKII phosphorylated pI_{Cln} into liposomes reduced $^{86}Rb^+$ uptake ~50% (*P < 0.05). Values are means ± SEM (n = 3). Isotope uptake was measured by incubating proteoliposomes with $^{86}Rb^+$ for 60 min.

channel-forming protein. These investigators identified the three glycine residues between G49 and G53 as being a possible nucleotide binding site. While this site has poor homology with known nucleotide binding motifs (Saraste et al., 1990), its location within the putative channel pore was consistent with the inhibition by extracellular nucleotides of the current induced by expression of pI_{Cln} in oocytes (Paulmichl et al., 1992). Paulmichl et al. (1992) mutated the three glycine residues to alanine ("AAA" mutant) and concluded that the mutant cRNA induced an anion current that was no longer inhibited by nucleotides. However, it has not been possible to reproduce these findings. Voets et al. (1997) have shown that expression of the AAA mutant induces a current identical to that of wild-type pI_{Cln}. Furthermore, Buyse et al. (1997) have identified an endogenous current in oocytes with the characteristics of the current (Paulmichl et al., 1992) attributed to expression of the AAA mutant.

Because of its availability, we used the AAA mutant to ascertain whether it would have any effect on the basic biophysical properties of the reconstituted cation channel. As shown in Fig. 6, functional cation channels were reconstituted from the AAA mutant. However, the mutation significantly (P < 0.05) reduced channel P_o from 0.82 ± 0.09 to 0.50 ± 0.17 (n = 3; Fig. 6 *A*). Histogram analysis revealed that the AAA mutation slowed channel gating. The channel open time constant increased from 79 to 193 ms (Fig. 6 *B*). As with phosphorylation of pI_{Cln} (Fig. 5), the decrease in channel P_o was most likely due to the induction by the mutation of a novel closed state with an increased closed time constant (198 ms; Fig. 6 *B*). Consistent with the reduced P_o of the AAA mutant, reconstitution of this protein into liposomes reduced $^{86}Rb^+$ uptake measured over a 60-min period to 670 ± 49 cpm (P < 0.05, n = 3). The observation that a mutation in pI_{Cln} alters the observed channel activity can only be explained if it is concluded that pI_{Cln} itself, rather than contaminant proteins, forms cation channels in lipid membranes.

pI_{Cln} Increases the Cation Permeability Sf9 Cell Membranes

Data shown in Figs. 4–6 demonstrate that the cation channel activity observed in bilayers is due to pI_{Cln} and not minor contaminant proteins. To further examine this issue, and to ascertain whether pI_{Cln} can function as a cation channel in biological membranes, we assessed the effect of pI_{Cln} on the cation permeability of Sf9 cells.

As shown in Fig. 7, Sf9 cells shrank very slowly ($t_{1/2}$ = 9.8 ± 1.4 min, n = 4) when suspended in Na^+- and K^+-free medium (NMDG replacement) due to the loss of intracellular cations, Cl^-, and osmotically obliged water. However, when the cationophore gramicidin is added to the bathing medium at a concentration of 10

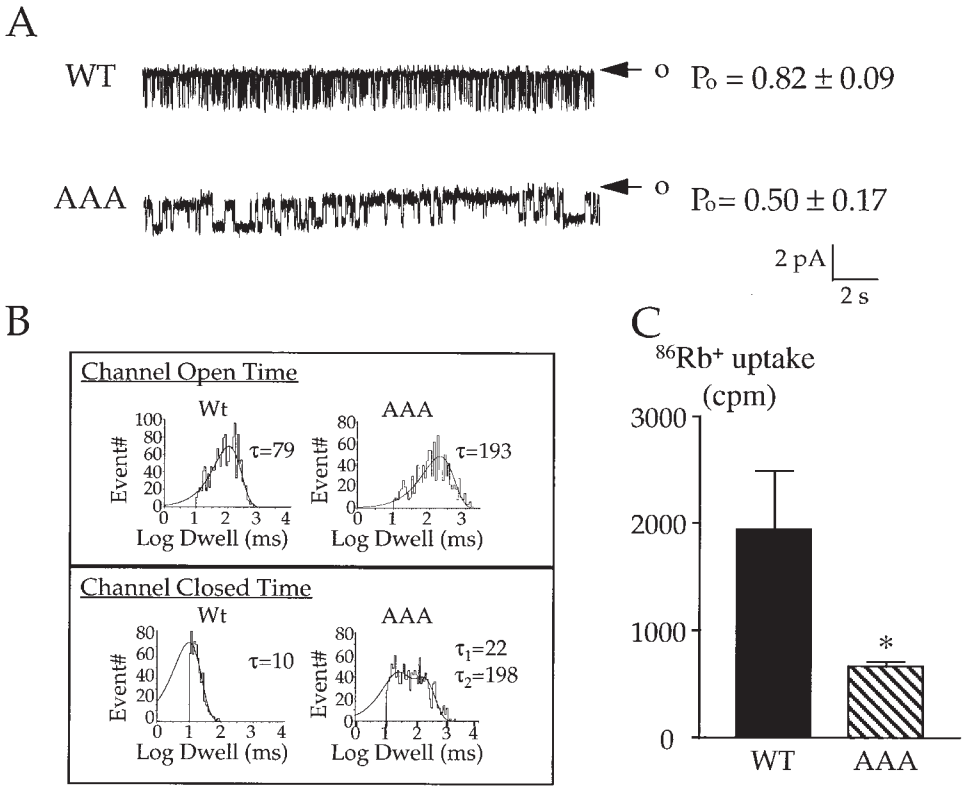

FIGURE 6. Mutation of wild-type (*WT*) pI_{Cln} G54, G56, and G58 to alanine (*AAA*) reduces channel P_o and $^{86}Rb^+$ uptake. (*A*) AAA mutation reduces channel P_o from 0.82 ± 0.09 to 0.50 ± 0.17 (mean ± SEM; n = 3). (*B*) Histogram analysis demonstrated that the AAA mutation slowed channel gating. The channel open time constant increased from 79 to 193 ms. The closed time histogram was best fit by a double exponential. Both the channel closed time constants were increased compared with wild-type pI_{Cln} (τ_1, 22 ms; τ_2, 198 ms). (*C*) Reconstitution of liposomes with AAA mutant pI_{Cln} reduces $^{86}Rb^+$ uptake ~65% compared with wild-type pI_{Cln}. Values are means ± SEM (n = 3). Isotope uptake was measured by incubating proteoliposomes with $^{86}Rb^+$ for 60 min.

μg/ml, the cells underwent a rapid shrinkage ($t_{1/2}$ = 0.52 ± 0.18 min, n = 4). The fact that gramicidin decreases the halftime for shrinkage by ~20-fold indicates that the resting cation conductance of the Sf9 cell membrane is low. Similar findings have been made by Vachon et al. (1995) using fluorescent probes to measure intracellular cation concentrations.

We reasoned that pI_{Cln} should also induce rapid cell shrinkage if it functions as a cation-selective channel. When pI_{Cln} was added to the bath at a concentration of 25 μg/ml, Sf9 cells shrank in a manner similar to that observed with gramicidin ($t_{1/2}$ = 0.54 ± 0.12 min, n = 4; Fig. 7).

If pI_{Cln} is responsible for the enhanced cation conductance of the Sf9 cells, then it should be possible to detect the protein in the plasma membrane. Fig. 8, *A–D*, shows immunofluorescence micrographs of native Sf9 cells and Sf9 cells treated with 100 μg/ml pI_{Cln} for 60 min. Using an anti–pI_{Cln} polyclonal antibody, we observed intense immunostaining of pI_{Cln} in the plasma membrane of pI_{Cln}-treated cells (Fig. 8, *A* and *B*). Importantly, immunostaining was not detected in non–pI_{Cln}-treated cells (Fig. 8 *D*) or in pI_{Cln}-treated cells exposed to preimmune serum (Fig. 8 *C*).

DISCUSSION

When overexpressed in *Xenopus* oocytes, pI_{Cln} induces an outwardly rectifying anion current that superficially resembles a ubiquitous swelling-activated anion current termed $I_{Cl,\ swell}$ (Strange et al., 1996; Nilius et al., 1997; Strange, 1998). pI_{Cln} was proposed initially to be the $I_{Cl,\ swell}$ channel (Paulmichl et al., 1992; Gschwentner et al., 1995). We undertook reconstitution studies using recombinant pI_{Cln} purified to at least 99% homogeneity to directly test the hypothesis that pI_{Cln} is an anion channel–forming protein. As shown in Figs. 2 and 3, channel activity is observed in planar lipid bilayers after fusion with liposomes containing pI_{Cln}. A crucial question that arises from these observations is whether the channel activity is due to pI_{Cln} or minor contaminant protein(s) that copurify with it. We carried out several experiments to address this issue. Reconstitution of pI_{Cln} into liposomes increased the ion permeability of the liposome membrane (Fig. 4). In addition, mutation or phosphorylation of pI_{Cln} altered the gating properties of the channel and radioisotope flux (Figs. 5 and 6). Finally, when added to the external bathing medium, pI_{Cln} incorporated into the plasma membrane of Sf9 cells (Fig. 8) and markedly increased membrane ion permeability (Fig. 7). These studies demonstrate clearly that pI_{Cln}, and not contaminant proteins, is responsible for the observed channel activity.

The pI_{Cln} channel has a high open probability, an intermediate conductance, and is outwardly rectifying. These characteristics superficially resemble those of the $I_{Cl,\ swell}$ channel. However, the pI_{Cln} channel is highly cation selective and has a relative cation permeability

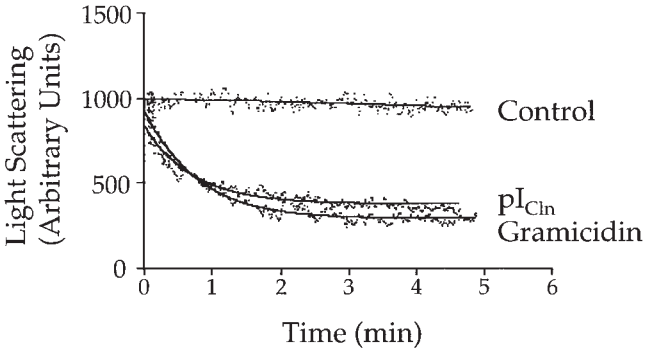

FIGURE 7. pI$_{Cln}$ increases the cation permeability of the Sf9 cell plasma membrane. Cells were suspended in a medium in which Na^+ and K^+ were replaced with the impermeant organic cation NMDG, and cell volume changes were measured by light scattering. As shown in Control, this had little effect on Sf9 cell volume. Cells treated with the cationophore gramicidin underwent a marked shrinkage due to the loss of Cl^-, cations, and osmotically obliged water. These results indicate that the cation permeability of the native Sf9 cell membrane is low. Treatment with pI$_{Cln}$ induces a similar shrinkage as gramicidin, demonstrating that the protein also increases membrane cation permeability.

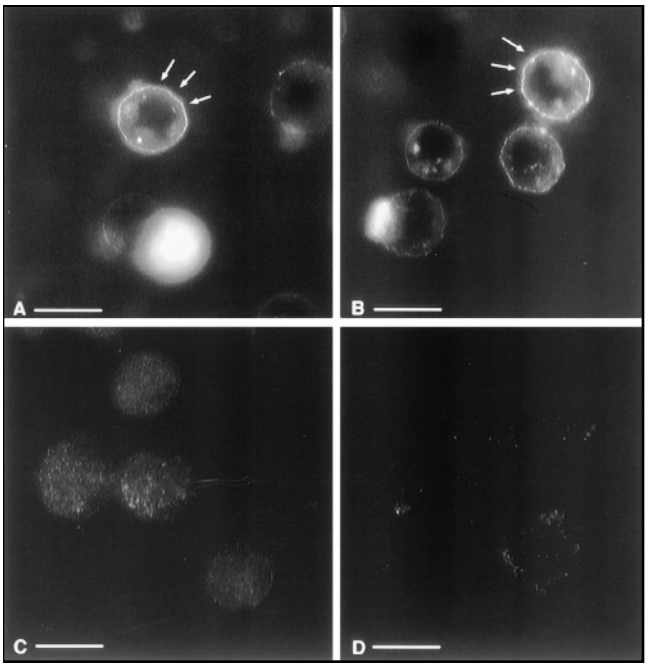

FIGURE 8. pI$_{Cln}$ added to the extracellular medium inserts spontaneously into the plasma membrane of Sf9 cells. Cells were exposed to 100 μg/ml pI$_{Cln}$ for 60 min. After washing and fixation, pI$_{Cln}$ was localized by immunostaining. Autofluorescence images (e.g., *C* and *D*) were acquired to allow visualization of cells. (*A* and *B*) Examples of pI$_{Cln}$-treated cells stained with anti–pI$_{Cln}$ polyclonal antisera and CY3-conjugated goat anti–rabbit IgG. Plasma membrane (*arrows*) shows intense staining for pI$_{Cln}$. (*C*) pI$_{Cln}$-treated cells incubated with preimmune serum show no staining. (*D*) Endogenous pI$_{Cln}$ is not detected in control Sf9 cells stained with anti–pI$_{Cln}$ antiserum.

(P_K/P_{Cl}) of at least 7:1. Consistent with this finding, the conductance of the pI$_{Cln}$ channel was undetectable when K^+ was replaced by *N*-methyl-d-glucamine, but was unaffected by substitution of Cl^- with glutamate (Fig. 3). Furthermore, pI$_{Cln}$ increased the Rb^+ but not the Cl^- permeability of liposomes (Fig. 4). In contrast to the high cation selectivity of the pI$_{Cln}$ channel, the I$_{Cl,\ swell}$ channel in mammalian cells has a P_{cation}/P_{anion} of ~0.03–0.04 (Strange et al., 1996; Okada, 1997). We conclude, therefore, that pI$_{Cln}$ is not itself an anion channel–forming protein.

The cation selectivity of the pI$_{Cln}$ channel is consistent with the biochemical properties of the protein. pI$_{Cln}$ contains two highly acidic domains that are composed of multiple aspartate and glutamate residues (Krapivinsky et al., 1994; Emma et al., 1998; Sanchez-Olea et al., 1998). These domains almost certainly do not interact with the hydrophobic core of the lipid bilayer. If acidic amino acid residues line the channel pore, this would account for the cation selectivity of the pI$_{Cln}$ channel.

In addition to the present studies, several other findings have also challenged the hypothesis that pI$_{Cln}$ is the I$_{Cl,\ swell}$ channel. Voets et al. (1996) have shown that the pI$_{Cln}$-induced current has characteristics distinct from those of I$_{Cl,\ swell}$ (Ackerman et al., 1994; Hand et al., 1997). Buyse et al. (1997) have shown that (*a*) expression in oocytes of an unrelated protein, ClC-6, induces the same current as that induced by expression of pI$_{Cln}$, and (*b*) the pI$_{Cln}$-associated current is observed without cRNA injection in ~5–6% of oocytes (see also Paulmichl et al., 1992). Mutagenesis studies (Paulmichl et al., 1992) that provided compelling support for the hypothesis that pI$_{Cln}$ is a channel-forming protein have not been reproduced (Voets et al., 1997). Expression of AAA mutant pI$_{Cln}$ (Paulmichl et al., 1992) induces the same current as wild-type pI$_{Cln}$ (Voets et al., 1997). Furthermore, in native cells, it has not been possible to unequivocally detect pI$_{Cln}$ in cell membranes (reviewed by Strange et al., 1998). Based on these findings, it has been proposed that heterologous expression of pI$_{Cln}$ in oocytes activates an endogenous anion current distinct from I$_{Cl,\ swell}$ (Voets et al., 1996, 1997; Buyse et al., 1997; Strange, 1998). The anion current thought to be induced by expression of mutant pI$_{Cln}$ (Paulmichl et al., 1992) has also been proposed to arise from an endogenous oocyte anion channel (Voets et al., 1997).

Krapivinsky et al. (1994) proposed that pI$_{Cln}$ is a channel regulator and that expression of the protein in oocytes turns on an endogenous I$_{Cl,\ swell}$ channel. This hypothesis was based in part on the observation that the biochemical properties of pI$_{Cln}$ do not resemble those of typical ion channels, that the protein is localized predominantly to the cytoplasm, and that an anti–

pI_{Cln} antibody inhibits swelling-induced activation of $I_{Cl,\ swell}$ when injected into oocytes. Given the fact that the current induced in oocytes by pI_{Cln} is distinct from $I_{Cl,\ swell}$ (Voets et al., 1996), and that the same current can also be induced by the unrelated protein ClC-6 (Buyse et al., 1997), it seems unlikely that pI_{Cln} is a regulator, in the strictest sense of the term, of the $I_{Cl,\ swell}$ channel. It is clearly possible, however, that pI_{Cln} indirectly affects $I_{Cl,\ swell}$ activity. Disruption of pI_{Cln} function by antibody injection into cells (Krapivinsky et al., 1994), antisense transfection (Gschwentner et al., 1995; Hubert et al., 1998), or overexpression (Hubert et al., 1998) may alter cytoskeletal structure, signal transduction pathways, etc., that ultimately affect $I_{Cl,\ swell}$ activation.

What Is the Function of pI_{Cln}?

The physiological function of pI_{Cln} remains unknown and very controversial. We have suggested previously that pI_{Cln} may function to regulate cytoskeletal properties and/or it may function as a "scaffolding protein" for bringing together and possibly regulating components of signal transduction pathways (Emma et al., 1998; Strange, 1998). Based on the results of these studies, it is clear that pI_{Cln} can also function in vitro as a highly cation-selective channel. Does this imply that pI_{Cln} has a physiological role as a cation channel? This question cannot be answered at present. pI_{Cln} has not been detected unequivocally in the plasma membrane of native cells (reviewed by Strange, 1998), which argues that the protein does not have physiologically relevant channel activity. However, it is possible that small amounts of membrane-associated protein have escaped detection in previous studies, that pI_{Cln} resides in cell membranes only under certain physiological conditions and/or that pI_{Cln} may function as a channel in intracellular membranes. Further and more detailed studies of pI_{Cln}–membrane interactions in vivo are clearly warranted.

It is possible that the pI_{Cln}-induced cation channel activity may simply be a consequence of in vitro reconstitution of a recombinant protein. A number of proteins have been shown to generate channel activity in vitro, but have not been demonstrated unequivocally to do so in vivo. Annexins, for example, are a family of water soluble, calcium-binding proteins that are expressed abundantly in a wide variety of cell types (Kaetzel and Dedman, 1995). These proteins bind to plasma membranes and aggregate as trimers, hexamers, and multimers in response to increases in cell Ca^{2+}. The physiological function of annexins remains uncertain. They have been proposed to play roles in cell differentiation and mitogenesis, initiation of membrane fusion events important for exocytosis and endocytosis and inhibition of phospholipase A_2. Certain members of the annexin family also give rise to Ca^{2+} channel activity when reconstituted into planar lipid bilayers (Pollard et al., 1992; Luecke et al., 1995).

Conclusions

Our studies demonstrate directly that purified, recombinant pI_{Cln} is capable of generating ion channel activity in both artificial and biological membranes. The channels are highly cation selective, a finding that argues strongly against the hypothesis that pI_{Cln} is itself an anion channel–forming protein. Our findings provide a basis and justification for further physiological investigations of pI_{Cln}. Structural studies of pI_{Cln} may provide novel insights into the structure of channel-forming proteins and membrane–protein interactions. In addition, structural studies may provide insight into the native conformation of pI_{Cln}, which, in turn, could provide clues about its physiological function.

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