CLIC1 inserts from the aqueous phase into phospholipid membranes, where it functions as an anion channel

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Received 17 August 2001; accepted in final form 19 December 2001

Tulk, Barry M., Shefalee Kapadia, and John C. Edwards. CLIC1 inserts from the aqueous phase into phospholipid membranes, where it functions as an anion channel. Am J Physiol Cell Physiol 282: C1103–C1112, 2002. First published January 2, 2002; 10.1152/ajpcell.00402.2001.—CLIC1 is a member of the CLIC family of proteins, which has been shown to demonstrate chloride channel activity when reconstituted in phospholipid vesicles. CLIC1 exists in cells as an integral membrane protein and as a soluble cytoplasmic protein, implying that CLIC1 might cycle between membrane-inserted and soluble forms. CLIC1 was purified and detergent was removed, yielding an aqueous solution of essentially pure protein. Pure CLIC1 was mixed with vesicles, and chloride permeability was assessed with a chloride efflux assay and with planar lipid bilayer techniques. Soluble CLIC1 conferred anion channel activity to preformed membranes that is indistinguishable from the previously reported activity resulting from reconstitution of CLIC1 into membranes by detergent dialysis. The activity is dependent on the amount of CLIC1 added, appears rapidly on mixing of protein and lipid, is inhibited by indanyloxyacetic acid-94, N-ethylmaleimide, and glutathione, is inactivated by heat, and shows sensitivity to pH and to membrane lipid composition. We conclude that CLIC1 in the absence of detergent spontaneously inserts into preformed membranes, where it can function as an anion-selective channel.

p64; indanyloxyacetic acid-94; intracellular chloride channel; diphtheria toxin

CLIC proteins are a recently described family of proteins related to the bovine intracellular chloride channel p64 (4, 7, 8, 12, 16, 18, 22, 24, 33). The family consists of seven distinct members: CLIC1, CLIC2, CLIC3, CLIC4, CLIC5, p64, and parchorin. The family is defined by a COOH-terminal core segment of ~230 amino acids that is highly conserved among all family members. Sequences upstream of this core vary considerably among the various family members in length and sequence.

CLIC1 has been most intensely studied (30–33). This protein has only a few amino acids upstream of the conserved core that defines the CLIC family. CLIC1 is expressed to some extent in most tissues and cell types that have been studied and is particularly highly expressed in muscle (31). It is expressed in a punctate intracellular vesicular pattern in a variety of cultured cells, in the apical domain of renal proximal tubule cells, in an intracellular distribution in placenta (4), and in nuclei of Chinese hamster ovary cells (31, 33).

CLIC1 clearly functions as a chloride channel. Expression of CLIC1 in cultured cells has been reported to lead to increased chloride channel activity in plasma and nuclear membranes (30, 33). Recombinant CLIC1 expressed in bacteria and purified to apparent homogeneity yields a unique indanyloxyacetic acid-94 (IAA)-inhibitable chloride channel when reconstituted into phospholipid membranes (32).

Thus, pure CLIC1, in the absence of other subunits or modifying factors, can function as a chloride channel in vitro. However, CLIC1, like other CLIC family members, has physical properties that are quite atypical for ion channel proteins. On disruption of cells or tissues, some of the total CLIC1 behaves as a typical integral membrane protein, fractionating with cell membranes and showing membrane association resistant to washes with alkali or chaotropic agents. However, more than one-half of the total CLIC1 in homogenized kidney does not fractionate with membranes but, instead, behaves as a soluble cytoplasmic protein, partitioning with the aqueous extract in the absence of any detergent or chaotropic agent. Thus CLIC1 exists in cells in both a membrane-associated form and a soluble form (31).

Biogenesis of membrane proteins has been extensively studied (2). Typical membrane proteins are synthesized on ribosomes bound to the rough endoplasmic reticulum, with the protein inserting into the membrane cotranslationally. Such proteins are then targeted to their appropriate membrane compartment through a series of vectorial vesicular transport events, during which the molecule passes through the Golgi complex and the trans-Golgi network to the final membrane destination, such as the plasma membrane, nuclear membrane, or lysosomal membranes. In this process, the protein never exists as a free molecule in solution but remains inserted in the lipid bilayer from the moment of synthesis throughout the life of the protein.

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Cotranslational insertion into the rough endoplasmic reticulum is undoubtedly the mechanism by which most membrane proteins enter the lipid bilayer. However, there is a growing body of evidence that a small group of integral membrane proteins enter the membrane by a completely different mechanism. Such proteins are synthesized as soluble proteins and insert posttranslationally, directly into target membranes from a soluble pool. These proteins include some bacterial toxins and porins (23) and the eukaryotic proteins Bcl-2, Bax (27, 28), and voltage-dependent anion channel (5). Several of these proteins function as channels after membrane insertion, indicating that they not only associate with the membrane but also must completely cross the lipid bilayer and form a pore.

Our observations that CLIC proteins exist in both soluble cytoplasmic and membrane-associated forms led us to ask whether CLIC1, like these atypical membrane proteins noted above, might enter the lipid bilayer directly from the aqueous phase. Here we demonstrate that purified recombinant CLIC1 in the absence of detergent confers chloride-selective permeability to preformed phospholipid vesicles. This chloride permeability is inhibited by IAA and the sulphydryl-reactive agents N-ethylmaleimide (NEM) and glutathione, is dependent on the amount of CLIC1 added to the vesicles, and shows a marked dependence on the lipid composition of the target vesicles. In planar lipid bilayer studies, we find that the CLIC1 chloride channel resulting from soluble insertion is essentially indistinguishable from the CLIC1 activity we had previously described resulting from reconstitution of CLIC1 into lipid vesicles by detergent dialysis. We conclude that soluble CLIC1 can directly insert into preformed phospholipid vesicles, where it functions as an anion-selective channel.

MATERIALS AND METHODS

Purification of CLIC1

CLIC1 was expressed in bacteria as a glutathione S-transferase (GST) fusion protein, as previously described (32). Initial steps of the purification were identical to our previously reported methods (32), except 10 mM 2-mercaptoethanol was substituted for dithiothreitol (DTT) in all the solutions up to and including the digestion with thrombin. CLIC1 released by thrombin digestion was diluted threefold with 10 mM Tris (pH 8.0), 5 mM DTT, and 1.4% n-octyl glucoside and loaded onto a 1-ml Mono-Q cartridge (Bio-Rad). The column was washed with 10 column volumes of 10 mM Tris (pH 8.0) and 5 mM DTT and then eluted with 0.5 M NaCl, 10 mM Tris (pH 8.0), and 5 mM DTT. The peak fractions were pooled (usually 2.5-3 ml) and dialyzed against two 1-liter exchanges of 175 mM NaCl, 10 mM Tris (pH 8.0), and 5 mM DTT. The spent dialysis buffer after the second exchange was used as the control buffer in the chloride efflux experiments. For the lipid bilayer experiments, the protein was further purified through a gel filtration column. The pooled peak fraction (1 ml) from the Mono-Q eluate was passed through a 40-ml Sephacryl S-100 column, which was equilibrated and eluted with 150 mM NaCl, 10 mM Tris (pH 8.0), and 1 mM DTT. Peak fractions were pooled and used directly in the planar lipid bilayer experiments.

Protein concentrations were determined using the bicinchoninic acid protein assay (Fierce).

Chloride Efflux Assays

Selective chloride permeability of lipid vesicles conferred by soluble CLIC1 was assayed using a variant of the chloride-selective electrode assay described previously (9, 32). Asolectin (type IV, catalog no. P-3644, Sigma) or purified phospholipids (Avanti Polar Lipids) were dissolved in chloroform and dried under a stream of nitrogen in a glass tube. Lipid was brought up to 20 mg/ml in 200 mM KCl and 2 mM HEPES (pH 8.0) and allowed to hydrate for ≥30 min. The resulting lipid suspension was put through 10 rapid freeze-thaw cycles and then passed through a 400-nm filter 15 times to yield a relatively homogeneous population of unilamellar vesicles. Vesicles (100 μl) were mixed with protein in a total volume of 200 μl and incubated at room temperature for 5 min. The protein-lipid mixture was applied to a 3-ml Biogel P6 (Bio-Rad) spin column that had been equilibrated in 330 mM sucrose and chased through the column with 300 μl of 330 mM sucrose. The osmolality of the sucrose was adjusted to match the osmolality of the KCl solution in which the vesicles had been prepared. The effluent from the spin column (~0.5 ml) was added to a cup containing 2 ml of 10-5 M KCl in 330 mM sucrose; the free chloride concentration was continuously monitored with a chloride-selective electrode. The output of the electrode was interfaced through a pH meter and a Digidata analog-to-digital converter to a personal computer running Axoscope 8.0 (Axon Instruments). At 40 s after addition of vesicles to the cup, 2.5 μl of 1 mM valinomycin in ethanol was added, and recording was continued. After 30 s, 10% Triton X-100 (25 μl) was added to release all remaining intravesicular chloride. Output of the pH meter (in mV) was converted to chloride concentration by using a standard curve generated with each set of experiments. The difference between the final chloride concentration after addition of Triton X-100 and the initial chloride concentration immediately before addition of valinomycin is taken as the initial total intravesicular chloride. The rate of fractional chloride release was determined from the first 3 s after addition of valinomycin and is taken as the valinomycin-dependent chloride permeability. Data were analyzed using analysis of variance, and significance of difference between means was determined using the two-tailed t-test (3).

IAA was dissolved at 10 mM in Tris (pH 9.5). NEM was dissolved at 1 M in ethanol. Reduced glutathione (GSH) and oxidized glutathione (GSSG) were dissolved in water at 100 mM. Solutions were prepared immediately before each experiment. Neither vehicle alone had any effect on activity.

Treatment with protein kinase A catalytic subunit was carried out as follows. Catalytic subunit (Sigma Chemical) was solubilized at 2 U/μl in 40 mM DTT according to the manufacturer’s instructions. One hundred fifty units of kinase were mixed with 120 μg of CLIC1 in a total volume of 300 μl of 150 mM NaCl, 10 mM Tris (pH 8), 5 mM DTT, and 1 mM MgCl2. The sample was divided into two 150-μl aliquots. ATP was added to one aliquot to a final concentration of 1 mM, and both aliquots were incubated at 30°C for 30 min. Each sample was then assayed for ability to confer valinomycin-dependent chloride efflux to a solectin vesicles as described above. To confirm phosphorylation, 10 μl of the reaction mixture without ATP were spiked with 50 μCi of [γ-32P]ATP before the 30-min incubation at 30°C, separated by SDS-PAGE, and detected by autoradiography.
Lipid Bilayer Experiments

Planar lipid bilayer experiments were performed as described previously (32). Membranes were formed from asolectin [type II phosphatidylethanolamine (PC); Sigma] or 7.3 phosphatidylethanolamine (PE)-phosphatidylserine (PS; Avanti Polar Lipids) in the presence of a 300 mM (cis)-50 mM (trans) KCl gradient and buffered at pH 7.0 with 1 mM HEPES. The cis chamber also contained 1 mM CaCl2 and 1 mM DTT. Membranes were clamped at −50-mV holding potential referenced to the trans chamber (ground) using an Axopatch 200B integrating patch-clamp amplifier (Axon Instruments) that was interfaced with a personal computer via a Digidata 1200 analog-to-digital converter (Axon Instruments). Data acquisition and analysis were accomplished using the pCLAMP7 suite of programs (Axon Instruments). In this configuration, positive current represents chloride movement from the trans to the cis chamber. All recordings were performed at room temperature, which was assumed to be 25°C.

CLIC1, purified through Sephacryl S-100 chromatography, was added to the cis chamber directly or after incubation with preformed asolectin vesicles containing 300 mM KCl, 300 mM glycerol, 10 mM HEPES (pH 7.0), and 1 mM DTT. After addition of CLIC1, the cis chamber solution was mixed at regular intervals and continuously monitored for the presence of channels. Once active channels were observed, currents were recorded at several holding potentials between +150 and −150 mV in the presence of asymmetric and symmetric salt solutions. Reversal potentials and conductance were determined from current-voltage plots of the data, as previously described (32). Permeability ratio was calculated on the basis of the Goldman-Hodgkin-Katz equation (13). KCl concentrations were converted to activity using standard methods (26) for use in calculation of the permeability ratio.

RESULTS

Purification of Soluble CLIC1

Soluble CLIC1 was prepared using a variant of the previously reported method (32). Results of a representative preparation are shown in Fig. 1. GST-CLIC1 fusion protein is expressed in bacteria and released by lysis with lysozyme. After treatment with DNase and RNase, the material is allowed to bind to a resin with immobilized GSH. Unbound proteins are washed away, and the resin with bound fusion protein is digested with thrombin in the presence of n-octyl glucoside, releasing CLIC1 (Fig. 1, lane 1), which demonstrates its characteristic apparent molecular weight of 34,000 on SDS-PAGE (31). Subsequent elution of the resin with GSH released GST (27,000 mol wt) and residual undigested GST-CLIC1 fusion protein (54,000 mol wt), as shown in lane 2. The released CLIC1 was applied to a Mono-Q ion-exchange column. The material was washed extensively with detergent-free solution while bound to the column and then eluted with NaCl, yielding material that appears as a single band on an overloaded Coomassie-stained gel (lane 3). This material was dialyzed against two exchanges of a detergent-free saline solution before use in the chloride efflux assay. A typical preparation from 0.5 liter of bacterial culture yielded 2.5–3 ml containing ∼1–1.5 mg/ml protein. The protein proved to be stable in solution in the absence of detergent and has not shown any tendency to precipitate. Further purification on Sephacryl S-100 was performed before planar lipid bilayer experiments. The pooled peak from the Sephacryl column is shown in Fig. 1, lane 4.

Soluble CLIC1 Confers Chloride Permeability to Preformed Phospholipid Vesicles

KCl-loaded phospholipid vesicles were mixed with soluble CLIC1 or protein-free control buffer and incubated at room temperature for 5 min. Extravesicular chloride was removed by passage through a desalting spin column, and the resulting vesicle suspension was added to a cup containing 10−5 M KCl in sucrose. The chloride concentration in the cup was continuously monitored with a chloride-selective electrode. The raw output from the electrode (in mV) is plotted against time in Fig. 2A, and these same data converted to chloride concentration are plotted in Fig. 2B. Clearly, a dramatic increase in valinomycin-dependent chloride efflux is conferred by addition of soluble CLIC1. To determine rates of efflux, data as in Fig. 2B were converted to fractional chloride released after addition of valinomycin as plotted in Fig. 2C. Initial rates of valinomycin-dependent efflux were determined from the first 3 s after addition of valinomycin, and these rates are taken as the chloride-selective permeability of the vesicles. In this example, the CLIC1-dependent rate is ∼10-fold higher than the background chloride leak of the protein-free vesicles. We found clear CLIC1-dependent chloride permeability with each of six independent preparations of soluble CLIC1, although the...
absolute rates of valinomycin-dependent efflux per microgram of protein varied from preparation to preparation. For these six preparations, control vesicles yielded a mean valinomycin-dependent chloride efflux rate of 0.54 ± 0.08%/s (mean ± SE, range 0.26–0.80), while vesicles that had been mixed with 20 µg of CLIC1 yielded a rate of 2.76 ± 0.31%/s (range 1.98–3.62). The difference between control and CLIC1 vesicles is highly significant (P = 0.00048). Thus soluble CLIC1 confers selective chloride permeability to preformed phospholipid vesicles.

**Detection of Soluble CLIC1 Channel Activity in a Planar Lipid Bilayer**

To determine whether the efflux activity noted above is due to appearance of discrete channels, we used a planar lipid bilayer approach. A lipid bilayer was prepared by painting lipid (asolecin or a PE-PS mixture) dissolved in decane across the 0.25-mm-diameter aperture of a lipid bilayer chamber in the presence of a 300 mM cis-50 mM trans KCl gradient, as described previously (32). Soluble CLIC1 was added to the cis chamber directly or after incubation with preformed vesicles, and the solution was briefly stirred. Once a channel was detected in the membrane, currents were recorded at several holding potentials to determine the current-voltage relationship. The trans chamber solution was then adjusted to 300 mM KCl, and activity was again recorded at several holding potentials.

Representative data are shown in Fig. 3. In six separate preparations, addition of purified soluble CLIC1 to the cis chamber resulted in the appearance of a chloride-selective channel with an average single-channel conductance in symmetric 300 mM KCl of 150 ± 4 (SE) pS (n = 5) and an average reversal potential in the 300 mM cis-50 mM trans KCl gradient of 29 ± 4 mV (n = 6), yielding a chloride-to-potassium permeability ratio of 8:1. The open probability of the channel is dramatically decreased at potentials above +100 mV or below −100 mV. As we described for reconstituted CLIC1 channels (31), we also often observed channels that remained open almost continuously and rarely transitioned between open and closed states. Soluble CLIC1 channels were inhibited by IAA. In three separate experiments, addition of 50 µM IAA to the cis chamber inactivated the channel completely (data not shown).

The activity associated with soluble CLIC1 was never observed in control vesicles or without the addition of soluble CLIC1 and is essentially identical to the reported characteristics of the CLIC1 channel resulting from material purified in the presence of detergent and reconstituted into vesicles by detergent dialysis.

Initial experiments were performed by direct addition of soluble CLIC1 to the cis chamber, which requires the protein to insert directly into the very small area of membrane separating the two chambers at the 0.25-mm-diameter pore. As an alternative to this approach, subsequent experiments were performed in which soluble CLIC1 was added to a suspension of

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**Fig. 2. Chloride efflux assay of CLIC1 channel activity.** Preformed asolecin vesicles were mixed with control buffer or 20 µg of CLIC1 in control buffer in a total of 200 µl, incubated at room temperature for 5 min, passed through a spin column equilibrated in 330 mM sucrose, and added to a cup containing 10 M KCl in 330 mM sucrose that was monitored with a chloride-selective electrode. At 40 s after vesicles were added, valinomycin was added to 1 µM. After 30 s, Triton X-100 was added to 0.1%. A: raw output from chloride-selective electrodes. B: data from A converted to chloride concentration ([Cl]) in A and B, first arrow, addition of vesicle suspension to the cup; second arrow, addition of valinomycin to initiate voltage-driven chloride efflux; third arrow, addition of Triton X-100 to lyse vesicles and release any remaining intravesicular chloride. C: plot of cumulative fraction of initial intracellular chloride released, starting at the point of valinomycin addition. Dashed lines, initial rates of valinomycin-dependent fractional chloride release: 2.98%/s for CLIC vesicles and 0.30%/s for control (Con) vesicles.
preformed asolectin vesicles as in the efflux experiments. After incubation at room temperature, aliquots of the vesicle suspension were added to the cis chamber, and the bilayer was monitored for evidence of membrane fusion. This approach yielded the typical CLIC1 activity more reliably than the direct addition of soluble CLIC1 directly to the bilayer chamber.

Characterization of CLIC1-Mediated Chloride Conductance

**Protein concentration.** Chloride efflux assays were carried out over a range of CLIC1 concentrations, and the fractional valinomycin-dependent efflux rates were determined (Fig. 4A). Increasing amounts of protein up to 25 μg/ml led to increasing chloride efflux rates. The efflux was approximately linear with protein at lower concentrations and appeared to begin to saturate at higher concentration. There is no suggestion of a higher-order dependence of channel activity on the concentration of CLIC1.

**Time dependence.** To address the kinetics of channel formation by CLIC1, protein and vesicles were mixed and incubated for various times before passage through the spin column and chloride efflux assay.

![Diagram](http://ajpcell.physiology.org/)

**Fig. 3.** Single-channel properties of activity resulting from addition of soluble CLIC1 to preformed membranes. Purified CLIC1 (100 μg/ml) was mixed 1:1 with large unilamellar vesicles and added to the cis chamber of a planar lipid bilayer setup at 5-min intervals in 5-μl aliquots. On addition of the third aliquot (at which point the cis chamber CLIC1 concentration was 0.75 μg/ml), channel activity was observed and recorded at several holding potentials. **A:** single-channel transition at the indicated holding potentials in 300 mM (cis)-50 mM (trans) KCl gradient. **B:** single-channel transitions in symmetric 300 mM KCl. In **A** and **B,** arrows denote closed state. **C:** current-voltage relationships in 300 mM (cis)-50 mM (trans) KCl (○) and symmetric 300 mM KCl (□). Reversal potential in 300 mM (cis)-50 mM (trans) KCl gradient was +33.3 mV; single-channel conductance in 300 mM KCl was 120 pS.

![Diagram](http://ajpcell.physiology.org/)

**Fig. 4.** Concentration and time dependence of channel activity. **A:** dependence of activity on CLIC1 concentration ([CLIC1]). Assays were carried out over a range of CLIC1 concentrations, and initial rates of valinomycin-dependent chloride efflux were determined. **B:** time course of appearance of CLIC1 activity. Protein plus vesicles (■) or control buffer plus vesicles (○) were mixed and incubated for various times before passage through the spin column and efflux assay. There are no significant differences among efflux rates due to CLIC1 at the various times assayed. In **A** and **B,** each point represents average of 2 efflux assays, and error bars represent SE. Error bars smaller than symbols are not visible.
Results are shown in Fig. 4B. The appearance of chloride permeability appears to be complete within the ~2 min required to pass the material through the spin column and introduce it into the assay vessel. There is no significant change in total efflux activity with increased time of incubation up to 1 h.

**Divalent cations.** Presence of 1 mM CaCl₂ or 1 mM MgCl₂ in the reaction mixture had no effect on the increased time of incubation up to 1 h.

**Inhibitors.** IAA is an indanyloxyacetic acid derivative that was originally identified as an inhibitor of microsomal chloride channel activity and was used for affinity purification of the first CLIC protein discovered, p64 (16). IAA is known to inhibit reconstituted CLIC1 with an apparent IC₅₀ of ~9 μM (32). To test the effect of IAA on activity resulting from soluble CLIC1, vesicles were prepared as usual, except IAA was added to 50 μM after the last freeze-thaw cycle and before extrusion, thus ensuring that the drug would be inside and outside the vesicles. Protein was added to the vesicle-IAA mixture and assayed as usual. The appearance of chloride efflux activity (data not shown).

The results are presented in Fig. 5B. Efflux rates were as follows: 0.83 ± 0.05%/s (mean ± SE, n = 3) for control vesicles, 1.92 ± 0.36%/s (n = 2) for CLIC1 vesicles, and 0.48 ± 0.05%/s (n = 2) for CLIC1-IAA vesicles. IAA at 50 μM significantly inhibited the CLIC1-mediated valinomycin-dependent chloride efflux (P < 0.02).

In devising the purification of CLIC1, we had noted that the presence of reducing agent during the purification was essential and that increasing DTT from 1 to 5 mM tended to yield higher levels of channel activity, suggesting that sulphhydryl groups within CLIC1 may be important. To determine whether sulphhydryl groups are essential, we treated CLIC1 with the sulphhydryl-modifying reagent NEM before mixing with the lipid vesicles. CLIC1 [in 175 mM NaCl, 10 mM Tris (pH 8.0), and 5 mM DTT] was brought to 10 mM NEM and incubated at room temperature for 5 min before it was mixed with vesicles for the chloride efflux assay. This experiment was done simultaneously with the IAA experiment described above and used the same positive and negative controls. The results are presented in Fig. 5A. The rate of efflux after NEM pretreatment of CLIC1 was 1.1 ± 0.06%/s (n = 2). Preincubation with NEM dramatically inhibited valinomycin-dependent chloride efflux (P < 0.05). As expected, NEM at concentrations below that of the DTT already present in the sample had no effect, since NEM will be consumed by reaction with excess DTT (not shown).

On the basis of the recently noted structural similarity between CLIC proteins and GST (6, 11), we assessed whether glutathione has an effect on the channel activity of soluble CLIC1. One microgram of CLIC1 was diluted into 100 μl of 200 mM KCl, 10 mM HEPES (pH 8.0), and no additives, 2 mM GSH, 2 mM GSSG, or 2 mM DTT. The sample was incubated at room temperature for 5 min before it was mixed with 100 μl of KCl-loaded vesicles for the standard valinomycin-dependent chloride efflux assay. Results are shown in Fig. 5B. Efflux rates are as follows: 0.363 ± 0.002%/s (n = 2) for control vesicles (no protein), 2.0 ± 0.1%/s (n = 2) for CLIC1, 0.42 ± 0.03%/s (n = 2) for GSH-treated CLIC1, 0.33 ± 0.04%/s (n = 2) for GSSG-treated CLIC1, and 1.8 ± 0.4%/s (n = 2) for DTT-treated CLIC1. GSH and GSSG significantly inhibited CLIC1-mediated chloride efflux (P < 0.001).

To confirm that the CLIC1 activity is consistent with that of a protein, CLIC1 was heated to 95°C or kept on
ice for 5 min before standard assay. Results are shown in Fig. 5C. Efflux rates are as follows: 0.40 ± 0.06%/s (n = 2) for control vesicles (no protein), 1.63 ± 0.07%/s (n = 2) for CLIC1 vesicles, and 0.652 ± 0.004%/s (n = 2) for heat-denatured CLIC1 vesicles. The chloride efflux rate resulting from the heat-denatured sample is significantly less than that of the non-denatured sample (P < 0.001) and is not significantly different from that of the no-CLIC1 control.

Hydrogen ion concentration. We investigated whether the activity of CLIC1 resulting from direct insertion varied with pH. Vesicles were prepared in 200 mM KCl with 2 mM buffer (MES at pH 5.0 or 6.0, HEPES at pH 7.0 or 8.0, or borate at pH 9.0). CLIC1 was brought to the desired pH by addition of appropriate buffer to 20 mM immediately before the experiment. Vesicles and protein were mixed at the desired pH, incubated for 5 min at room temperature, and assayed for valinomycin-dependent chloride efflux. The results are shown in Fig. 6. Activity is lowest at pH 7, with significantly greater activity at lower and higher pH values.

Lipid composition. To enter the lipid bilayer from the aqueous phase, a protein would need to interact with the surface of the lipid bilayer. The distribution of fixed charges on the surface of the membrane would be expected to have an influence on these interactions and perhaps affect insertion and/or activity. Our initial experiments had been performed with asolectin, a crude preparation of soybean phospholipids, the composition of which is not well defined. To determine whether the polar head groups of the membrane phospholipids could have an effect on CLIC1 activity, we compared rates of valinomycin-dependent chloride efflux conferred by CLIC1 from vesicles prepared from defined combinations of purified phospholipids. In initial experiments, we found that vesicles prepared from purified phospholipids alone tended to have significant endogenous permeability to chloride. This protein-dependent chloride leak could be suppressed by addition of cholesterol to the phospholipid mixture, and subsequent experiments were carried out with vesicles comprised of 10% cholesterol unless otherwise noted. We examined various mixtures of neutral (PC and PE) and anionic [phosphatidic acid (PA) and PS] phospholipids. The results are shown in Fig. 7. None of the defined mixtures of phospholipids supported CLIC1-associated chloride permeability as effectively as the crude asolectin. PC-cholesterol and PC-PE-cholesterol vesicles supported little or no CLIC-dependent chloride permeability. In contrast, PC-cholesterol vesicles containing 10% PA or 10% PS supported reasonable efflux activity, although not as robust as that seen with the asolectin vesicles (valinomycin-dependent efflux rates about one-third of that seen with asolectin vesicles). Increasing the fraction of cholesterol to 30% in PC-PA-cholesterol vesicles greatly suppressed the activity.

Protein kinase A. CLIC1 has been noted to contain a consensus sequence for phosphorylation by protein kinase A (33). To determine whether phosphorylation by protein kinase A regulates the CLIC1 channel activity, we treated purified CLIC1 with catalytic subunit of protein kinase A in the presence or absence of ATP. CLIC1 was then mixed with vesicles and assayed for valinomycin-dependent chloride efflux. We found that pretreatment with protein kinase A catalytic subunit and ATP had no effect on CLIC1-mediated valinomycin-dependent chloride efflux rates compared with protein that was exposed to protein kinase A catalytic

![Graph](image)

**Fig. 6.** Effect of pH on CLIC1 activity. Efflux assays were carried out over a range of pH values with 2 μg of CLIC1 (■) or an equal volume of control buffer (○). Each point represents average of 2 assays. Error bars represent SE. Rate at pH 7 is significantly different from rates at all other pH values (P < 0.05). Error bars smaller than symbols are not visible.

![Graph](image)

**Fig. 7.** Dependence of CLIC1 activity on vesicle phospholipid composition. Phospholipid vesicles of various composition were prepared and assayed for valinomycin-dependent chloride efflux activity with control (Con) buffer or 10 μg of CLIC1 in control buffer. Aso, soybean asolectin vesicles; PC:ch, 90% PC-10% cholesterol; PC:PE:ch, 80% PC-10% PE-10% cholesterol; PC:PS:ch, 80% PC-10% PS-10% cholesterol; PC:P:Ach10; 80% PC-10% PA-10% cholesterol; PC:PA:ch30, 60% PC-10% PA-30% cholesterol. Values represent average of 2 assays. Error bars represent SE. *Significantly greater than control (P < 0.05).
subunit in the absence of ATP (data not shown). This experiment does not rule out the possibility that CLIC1 channel activity could be regulated by protein kinase A phosphorylation after insertion in the membrane.

DISCUSSION

We have demonstrated that the intracellular chloride channel CLIC1 remains stable in solution after exhaustive removal of detergent and that this protein can directly enter preformed phospholipid vesicles, where it functions as an anion-selective channel. The single-channel properties demonstrated by this activity are indistinguishable from that of CLIC1 purified in the presence of detergent and reconstituted into lipid vesicles by detergent dialysis. The activity shows a linear dependence on the amount of CLIC1 added to the vesicles at low protein concentrations, appears rapidly on combination of protein and lipid, is inhibited by IAA and NEM, shows strong pH dependence with minimal activity at pH 7.0, and shows marked dependence on the lipid composition of the vesicles into which it is inserting. The activity is not affected by the presence or absence of divalent cations, nor is it affected by phosphorylation by the catalytic subunit of protein kinase A.

The evidence that the protein not only associates with membranes but also inserts and spans the membrane is unequivocal. The observation of well-defined channels resulting from protein added to only one side of the membrane could not occur if the protein did not enter and span the membrane. The single-channel properties, the dependence on protein concentration, the inhibition by IAA and NEM, and the dependence on pH and membrane composition support the notion that the observed chloride permeability is not some nonspecific disruptive effect of a peculiar protein on the lipid but is, instead, due to the formation of a defined, organized channel.

The properties of the chloride permeability resulting from addition of soluble CLIC1 to preformed vesicles lead to a number of inferences about this channel activity. However, it is important to realize that the assumption that the protein has entered and spans the membrane. The single-channel properties, the dependence on protein concentration, the inhibition by IAA and NEM, and the dependence on pH and membrane composition support the notion that the observed chloride permeability is not some nonspecific disruptive effect of a peculiar protein on the lipid but is, instead, due to the formation of a defined, organized channel.

The activity shows an essentially linear relationship between protein concentration and channel activity at low protein concentration with some apparent saturation at higher concentration. The linear relationship at low concentration indicates that no rapid self-association or multimerization is required for insertion or activity. This observation would support models in which CLIC1 monomers insert into the membrane or CLIC1 in solution is already in the state of multimerized that is required for insertion and activity. The saturation at higher concentrations is at least partly because these high rates are approaching the limit of the response time of the chloride-selective electrode.

Support for this interpretation comes from the observation that initial rates of change in electrode output due to the valinomycin-dependent efflux at high protein concentration are approaching the initial rates of change due to the addition of Triton X-100 at the end of the experiment, an intervention that should result in essentially instantaneous release of chloride. Thus it is possible that the actual rate of chloride release at high protein concentration is more rapid than we can measure with these methods. A second potential contributing factor to the observed saturation could be a limitation by the size of the target vesicle pool. At low protein-to-lipid ratios, it is unlikely that any vesicle will have more than one channel inserted. As the protein-to-lipid ratio rises, the possibility of multiple insertion events in a single vesicle becomes greater. As the number of vesicles with multiple channels rises, the rate of efflux per channel would be expected to fall. Thus the apparent saturation of fractional chloride efflux rates at high protein concentration does not necessarily indicate a real saturation of the relationship between CLIC1 concentration and the extent of channel formation.

Channel activity does not vary with the time of incubation of protein with vesicles, indicating that insertion is rapid. Similar essentially instantaneous insertion and appearance of channel activity have been reported with Bel proteins (29).

Inhibition by IAA is consistent with previously reported properties of the CLIC1 channel. Inhibition by NEM and the need for DTT in the preparation to maintain activity suggest that a reduced sulfhydryl group is essential. There are six cysteines in the CLIC1 sequence, three of which are conserved among all CLIC family members. Maintenance of at least one of these cysteines in the sulfhydryl form must be essential for the ability of this protein to insert into the membrane or form an active channel.

Hydrogen ion concentration has a complex effect on the channel activity resulting from addition of soluble CLIC1 to preformed vesicles. The valinomycin-dependent chloride efflux is least at pH 7 and greater at higher and lower pH values. Titration of ionizable groups on the protein would be expected to have profound effects on the interaction of the protein with the surface of the membrane and on the ability of segments of the protein to enter and cross the hydrophobic core of the lipid bilayer. Certain phospholipids also have ionizable groups that would be titrated through the range of pH values we investigated, and a resulting change in the surface charge on the membrane would be expected to have profound effects on interaction of a protein with the membrane. The biphasic shape of the curve suggests that multiple titratable groups may be important in the activity, with protonation of certain groups at low pH enhancing activity and deprotonation of other groups at higher pH also enhancing activity. At this point, we do not know whether these pH effects are due to titration of groups on the protein or on the membrane or whether these effects result from changes in the ability of the protein to bind to the membrane,
insert in the membrane, or form a channel once it has inserted.

The chloride channel activity resulting from direct addition of CLIC1 to preformed phospholipid vesicles shows a marked dependence on the identity of the polar head groups within the lipid membrane. We found the most abundant activity using a crude preparation of phospholipids from soybean. This lipid mixture contains ~40% PC and 20% PE with the remainder of the phospholipids undefined. Using mixtures of pure phospholipids from animal sources, we found that neutral phospholipids failed to support CLIC1 activity, while mixtures containing 10% of a phospholipid with net negative charge (PA or PS) support CLIC1 activity. Increasing cholesterol to 30% in the presence of 10% PA suppressed the activity almost entirely. The dependence of fixed negative charges on the surface of the target membrane suggests that positively charged residues on the protein may need to interact with the surface of the membrane before insertion. The suppressive effect of cholesterol may explain, in part, the selective presence of CLIC1 in intracellular membranes, where the cholesterol concentration tends to be much lower than in the plasma membrane, where cholesterol concentration can be as high as 30% (10).

A Model for Spontaneous Membrane Insertion of CLIC1

We have demonstrated that addition of soluble CLIC1 in aqueous solution to preformed phospholipid vesicles results in insertion of CLIC1 into the vesicles with formation of chloride-selective channels. The structural features of CLIC1 that allow it to demonstrate this remarkable behavior are unknown. However, several proteins that are capable of direct insertion into membranes have been studied extensively, and some insight into the mechanism of membrane insertion has been obtained. These molecules include the bacterial proteins diphtheria toxin, colicin A1, and δ-endotoxin (23), as well as the eukaryotic apoptosis-related proteins Bcl-2 and Bax (27–29). Each of these proteins contains a long antiparallel α-helical loop, which is thought to insert into the membrane and form the transmembrane portion of the molecule (reviewed in Ref. 23). In aqueous solution, the hydrophobic character of this loop is shielded by portions of the rest of the molecule. To insert into the membrane, the protein undergoes a transition in which this loop is exposed and partitions into the lipid bilayer. For several of these molecules, the event that triggers the structural transition is unknown. In the case of diphtheria toxin, it appears that a fall in pH causes the structural transition that leads to membrane insertion.

Two models of CLIC structure have been proposed. Initially, solely on the basis of Kyte-Doolittle hydropathy analysis, it was proposed that CLIC family molecules could have two typical α-helical transmembrane domains that would lie within two highly conserved hydrophobic stretches within the CLIC sequence (18). However, neither of these hydrophobic stretches is likely to form an α-helix as determined by a variety of secondary structure prediction methods. Furthermore, these two hydrophobic stretches are separated by a 130-amino acid hydrophilic segment. Spontaneous insertion of such a molecule into a membrane would require the translocation of this entire hydrophilic segment across the lipid bilayer, a process that seems unlikely to be favored. No experimental evidence supporting this model has been published to our knowledge.

More recently, it was noted that CLIC family proteins demonstrate a distant but real homology to GST proteins (6). The crystal structure of soluble CLIC1 was recently solved (11). These authors propose that the NH2-terminal domain of CLIC1 unfolds and crosses the membrane to yield the membrane-inserted form of the molecule. On the basis of the crystal structure, we propose an alternative model of membrane insertion. Within the structure there is a single antiparallel α-helical loop that would be long enough to span the lipid bilayer. This loop stretches from amino acids 101 to 145 in the CLIC1 sequence. On the basis of its similarity to the structures of the toxins noted above, we propose that this loop forms the transmembrane structure that allows CLIC1 to function as a pore. Both α-helical portions of this loop show amphilopathic character, with charged amino acids clustered to one side of each helix. These antiparallel α-helices contain five positively charged amino acids and five negatively charged amino acids, which could contribute to ion selectivity of a channel formed by this loop. Whether this structure is responsible for the CLIC1 pore remains to be determined, and, as with the original model for CLIC proteins, no experimental evidence for its validity has been published.

Potential Biological Role of Posttranslational Insertion of a Chloride Channel

A handful of channel proteins have been shown to enter the lipid bilayer from the aqueous phase. At least for some of these proteins, it is thought that the partitioning between soluble and membrane-inserted forms is a critical means of regulation. Several CLIC proteins have been noted to exist in cells in membrane-associated and soluble forms, and physiological stimuli affecting cellular transport have been shown to alter the distribution between membrane-associated and soluble forms for at least one family member, parchorin (22). Thus regulation of the distribution of CLIC proteins between a soluble pool and a membrane-inserted active channel could be a key means of regulation of the chloride permeability of the target membranes for these proteins. This model is particularly attractive for those CLIC family members such as p64, CLIC1, CLIC3, and CLIC 4, which are thought to reside primarily in intracellular membranes. Chloride conductance of these intracellular membranes is thought to be critical in determining the steady-state pH of the compartments they define (1). If chloride channels were delivered to these membranes by the usual method of
vesicular transport, these channels would be continuously passing through each of the intermediate compartments on their way to their final destination, while other, presumably very similar, channel proteins responsible for the chloride conductance of the intermediate compartments themselves reside there. Appropriate regulation of the resident channels without interference from the transient channels could be a problem. A potential solution would be to eliminate the passage of potentially disruptive intracellular channels through each of the intermediate compartments. Instead, channels responsible for chloride conductance of intracellular compartments could be inserted directly into the membrane of each compartment as chloride conductance is needed. Possible signals recruiting channel from a soluble pool could include phospholipid and/or protein composition of the membranes themselves, in a method very similar to the way phospholipid and/or protein composition of the membranes associates with the actin cytoskeleton of placental microvilli.

We thank Dr. Q. Al-Awqati (Columbia University) for providing the IAA and E. Schuff for excellent technical assistance. This work was supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-44838 and by a Department of Veterans Affairs Merit Award.

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