Recombinant CLIC1 (NCC27) Assembles in Lipid Bilayers via a pH-dependent Two-state Process to Form Chloride Ion Channels with Identical Characteristics to Those Observed in Chinese Hamster Ovary Cells Expressing CLIC1*

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To whom correspondence should be addressed: Dipartimento di Bio-

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These authors contributed equally to this work.

An Australian Research Council Australian Research Fellow.

Present address: Dept. of Health Sciences, University of Technology, NSW 2007, Australia.

To whom correspondence should be addressed: Dipartimento di Biologia Cellulare e dello Sviluppo, Universita’ “La Sapienza,” P.le Aldo Moro 5, I-00185, Roma, Italy. Tel.: 39-6-4991-2683; Fax: 39-6-4991-2351; E-mail: michele.mazzanti@uniroma1.it.

CLIC1 (NCC27) is an unusual, largely intracellular, ion channel that exists in both soluble and membrane-associated forms. The soluble recombinant protein can be expressed in Escherichia coli, a property that has made possible both detailed electrophysiological studies in lipid bilayers and an examination of the mechanism of membrane integration. Soluble E. coli-derived CLIC1 moves from solution into artificial bilayers and forms chloride-selective ion channels with essentially identical conductance, pharmacology, and opening and closing kinetics to those observed in CLIC1-transfected Chinese hamster ovary cells. The process of membrane integration of CLIC1 is pH-dependent. Following addition of protein to the trans solution, small conductance channels with slow kinetics (SCSK) appear in the bilayer. These SCSK modules then appear to undergo a transition to form a high conductance channel with fast kinetics. This has four times the conductance of the SCSK and fast kinetics that characterize the native channel. This suggests that the CLIC1 ion channel is likely to consist of a tetrameric assembly of subunits and indicates that despite its size and unusual properties, it is able to form a completely functional ion channel in the absence of any other ancillary proteins.

CLIC1 (formerly NCC27) is a 241-amino acid ion channel protein, which we first identified by subtraction cloning, because of its increased expression with macrophage activation. CLIC1 was the first identified human member of a growing family of organellar ion channels (1). At least five other members of this family have now been cloned, and although they share significant sequence similarity (about 60–75%), they vary in their cellular and subcellular distribution (2–6). CLIC1 is widely distributed, being present in most tissues and cells that have been thus far examined. Immunofluorescence studies of CLIC1-transfected CHO cells indicate that it dominantly localizes to the nucleus and nuclear membrane as well as the cytoplasm (1). However, it has also been visualized within small intracytoplasmic organelles, presumably lysosomes, endosomes, or secretory vesicles (1).

CLIC1 can be identified electrophysiologically on the nuclear and plasma membranes in CLIC1-transfected cells and exhibits similar characteristics in both locations (1, 7, 8). In normal untransfected CHO cells, this translocation of CLIC1 to the plasma membrane occurs in a cell cycle-dependent manner (8). Native CLIC1 channel activity is routinely found only on the plasma membrane of cells in the act of dividing or cells that have just divided (7, 8). However, when cells are transfected with CLIC1, this normal distribution pattern is disrupted, and CLIC1 CI− conductance can be detected on the surface of all cells (7, 8). This suggested a role for CLIC1 in the cell cycle. This view is further reinforced by the observations that CLIC1 blockers increase the percentage of CHO cells in the G1/M phase of the cell cycle (8).

CLIC1 is quite unusual for an ion channel: it is small with only a single putative transmembrane region; it is largely a soluble intracellular protein with only small amounts of protein being found on membranes; and the structure of its soluble form does not resemble that of an ion channel. We have recently determined the high resolution, x-ray crystallographic structure of the soluble form of CLIC1 (9). The protein is

* The abbreviations used are: CHO, Chinese hamster ovary; SCSK, small conductance slow kinetics; HCFK, high conductance fast kinetics; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; GST, glutathione-S-transferase; IAA94, indanyloxyacetic acid 94; pS, picosiemens.
monomeric, structurally homologous to the GST superfamily and has a redox-active site resembling glutaredoxin. Integration of CLIC1 into a membrane is likely to require a major structural rearrangement, probably of the N-domain.

Despite its unusual properties, we and others have previously provided strong data identifying it as an ion channel (1, 7, 8, 10, 11). To further investigate the electrophysiological characteristics of CLIC1 and to better understand its regulation, we describe a series of biochemical, biophysical, and electrophysiological studies performed using soluble, *Escherichia coli*-derived, recombinant CLIC1. We demonstrate that the isolated protein is able to form functional ion channels in an artificial lipid bilayer and that these channels have essentially identical electrophysiological characteristics to those seen in cell membranes. Channel formation involves at least two stages: the initial formation of a small conductance slow kinetics (SCSK) channel followed by the appearance of a high conductance fast kinetics (HCFK) channel, where the latter corresponds to the *in vivo* channel. Our results indicate that the HCFK channel probably results from the assembly of four SCSK conductances. Finally, we demonstrate that the association of CLIC1 with lipids and ion channel formation in bilayers are pH-dependent.

**EXPERIMENTAL PROCEDURES**

**Expression of Recombinant CLIC1**—A recombinant GST-CLIC1 fusion protein was expressed in *E. coli* using the pGEX-4T-1 vector system and purified as described previously (1, 9). Briefly, the fusion protein was immobilized on glutathione-Sepharose (Amersham Biosciences), where it was cleaved with birin-labeled thrombin (Novagen). The thrombin was removed with a streptavidin-agarose affinity matrix, and CLIC1 was further purified by gel filtration on a Superdex G75 column (Amersham Biosciences), where it ran as a monomer.

**Electrophysiology**—We performed patch clamp electrophysiology on inside-out patches of a CLIC1-expressing CHO cell line using standard methods as reported previously (7). Single-channel recordings from lipid bilayers were obtained using the tip-dip method (11, 12). In brief, patch clamp pipettes (Garner Glass 7052) were made using a P97 Sutter Instruments puller (Novato, CA), coated with Sylgard (Dow Corning, Midland, MI) and fire-polished to a tip diameter of 1–1.5 μm and a 5–7 megaohm resistance. The same solution was used both in the bath and in the pipette (140 mM KCl, 10 mM Hepes, pH 6). We varied pH by addition of HCl or KOH. As soon as the pipette tip reached the bath solution, a solution of monopallidylicolympol (phosphatidicholine, Avanti Polar Lipids, Inc., Birmingham, AL) was spread on the surface. The electrode was repeatedly passed through the surface of the solution until the pipette resistance rose above 5 gigohms. Purified recombinant CLIC1 protein (2 μg/ml) was then added to the bath solution. An Axopatch 1D amplifier and pClamp 7 (both from Axon Instruments, Novato, CA) were used to record and analyze single-channel currents. Current recordings were digitized at 5 kHz and filtered at 800 Hz.

**Circular Dichroism and Fluorescence Spectroscopy**—Purified, *E. coli*-derived recombinant CLIC1 was diluted 30-fold from stock solutions of about 5 mg/ml into 20 mM potassium phosphate buffer at either pH 7.0 or pH 6.0 and left at room temperature for 1 h prior to analysis. Far-UV CD spectra were recorded on a Jasco J-720 spectropolarimeter equipped with a Neslab RTE-111 temperature controller. CD data were collected over the wavelength range of 195–260 nm and with a resolution of 0.5 nm, a bandwidth of 1 nm, and a response time of 1 s. Final spectra were the sum of three scans accumulated at a speed of 20 nm/min -1 and were baseline-corrected.

Fluorescence data were collected on a PerkinElmer Life Sciences LS50B luminescence spectrophotometer using 0.5-cm path length cells and 10-nm slit widths. The temperature was maintained at 25 °C using a Bio-Rad E4850 refrigerated recirculator water bath. The excitation wavelength was 295 nm (to specifically excite tryptophan fluorescence), and the emission was monitored from 320 to 380 nm.

**Liposome Preparation**—Liposome synthesis was based on a method from Hase et al. (13) modified as follows: phosphatidycholine (Sigma) and cholesterol (Sigma) were dissolved in chloroform (Ajax Chemicals) at 100 and 10 mg/ml, respectively, and then combined to give a final 9:1 phosphatidylcholine:cholesterol ratio (18 mg of phosphatidylcholine + 2 mg of cholesterol). The lipid mixture was then coated in layers onto the sides of a glass test tube by rotating the tube under a stream of nitrogen. The lipids were dried overnight under vacuum and then resuspended by vortexing in 1 ml of HK buffer (140 mM KCl, 10 mM HEPES, pH adjusted with HCl). The lipid suspension was sonicated with glass beads in a Branson 3200 water bath sonicator until it became translucent (~5–10 min), which is indicative of small, unilamellar liposomes having been formed.

**Measurement of Association of CLIC1 with Liposomes**—To measure association of CLIC1 with liposomes, 1.8 μg of purified recombinant CLIC1 was incubated with 150 μl of liposomes for periods between 10 min and 4 h. In experiments to check the effect of decreased pH, CLIC1 was combined with liposomes, and the pH was then adjusted with HCl. Liposomes were then purified on a discontinuous Ficoll density gradient as follows: liposomes were adjusted to 40% Ficoll and 1 ml of final volume with HK buffer and layered at the bottom of the gradient beneath 3 ml of 30% Ficoll and a 5% Ficoll buffer-only top layer that filled the remainder of the centrifuge tube. The gradient was centrifuged for 2 h at 20,000 rpm in an SW41 swing-bucket rotor at 5 °C. The unincorporated protein was collected from the 40% Ficoll fraction remaining at the bottom of the gradient, and the liposomes were collected from the 30% Ficoll/0% Ficoll interface. The collected liposome fractions were concentrated to a 50-μl volume using a Redivac, being careful not to dry the samples completely, and resuspended in 350 μl of SDS (3 g of SDS/100 ml of H2O). 30 μl of each sample was mixed with 10 μl of reducing 6× Laemmli sample buffer with 20% β-mercaptoethanol, boiled for 5 min, and immediately electrophoresed on a 10% polyacrylamide gel. CLIC1 was detected by Western blotting as described previously (1).

**RESULTS**

**Soluble Recombinant CLIC1 Protein Forms Cl− Channels in Lipid Bilayers with Essentially Identical Biophysical Characteristics to Clic1 on CHO Cell Membranes**

CLIC1 forms Cl− channels in artificial bilayers—Despite its small size and aqueous solubility, CLIC1 can be readily observed to form Cl− channels in tip-dip bilayer experiments. Fig. 1 shows two examples of single-channel recordings obtained at two different pipette potentials (12, 14). Comparable results were obtained regardless of the potential used. Channel recordings always showed the same sequence of events. After bilayer formation on the electrode tip and addition of protein to the external solution, there was a variable time period of null events. Channel activity then started with a few long openings that, during the progression of the experiment, became more consistent in number. In 80% of successful experiments, multiple events turned into one or two larger size channels (as seen in Fig. 1, on the right of the traces) in which not only does the amplitude of each individual current step increase, but also the open and close kinetics turn into fast transitions (flickering).

The analysis of such current recordings is very complicated because even superficial observations indicate that we are dealing with two different ionic conductances. The initial portion of a typical current trace shows the predominance of a small...
conductance channel with slow kinetics, which we call SCSK. These SCSK events show multiple current levels or steps. During a typical experiment, these SCSK events disappear, and they are replaced by a high conductance channel with fast kinetics, which we call HCFK. We have analyzed the SCSK pathway separately from the HCFK ionic pathway.

Fig. 2 shows a detailed analysis of the SCSK channel seen in tip-dip experiments. Both inward and outward current recordings at both ±80-mV membrane voltages showing the presence of 1, 2, 3, or 4 open state levels (top to bottom) are shown. Analysis of single-channel current recordings (left column) at different test potentials produced several amplitude histograms similar to the ones in the figure (center column). From the linear regression of iV plots (right column), we calculated the sublevel conductances, with 7.7 pS being the minimum opening.

Openings of SCSK channel are prominent in tip-dip experiments but can also be found in current recordings from the plasma membranes of CHO cells that have been transfected to express CLIC1 (called CHO-CLIC1). In Fig. 3, we show several examples of single-channel recordings in equimolar 140 mM KCl obtained in tip-dip configuration (left) and in inside-out patches from CHO-CLIC1 (right). In inside-out current traces, smaller current levels are only occasionally evident as part of the single-channel kinetics. Observing native channels, the SCSK type of openings (as judged by their amplitude) show both fast and slow kinetics. This is probably due to the fact that channel subunits are assembled to form a HCFK channel, and current substates are sporadic events occurring randomly. In tip-dip experiments, SCSK openings are produced by proteins that have not yet assembled into a higher order HCFK channel. The recordings presented on the right side of Fig. 3 were chosen from a 22-min continuous recording. By contrast, tip-dip channel current traces clearly showed many small current transitions and (rarely) simultaneous multiple openings or closings in the initial portion of an experiment. However, as soon as the activity of the HCFK channel appears in the tip-dip experiment, SCSK current openings became sporadic, and their frequency of appearance was then comparable with those in inside-out patch experiments.

We note that the conductance of the SCSK channel is 30.5 pS when four current levels are present. This is almost identical to the conductance of the HCFK channel reported previously (9) in both the tip-dip experiments (31.2 ± 1.5 pS) and the recordings of CLIC1 from CHO-transfected cells (29.6 ± 1.9 pS).

In light of these results, we have reason to believe that in the tip-dip experiments, the activity of HCFK channel represents the main conductive state of the fully reconstituted CLIC1 channel, and the SCSK pathway is due to substates of the same
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channel visible only during the channel assembly process. To explore the main channel characteristics, we will then consider only the openings of HCFK channels. Accordingly, Fig. 4 shows a series of shorter single-channel current recordings obtained in equimolar 140 mM KCl at different patch-pipette potentials. They produced the current/voltages relationship shown on the right of the Fig. 4. The mean single-channel conductance for the HCFK was 31 ± 1.8 pS.

CLIC1 in Bilayers Forms an Ion Channel with Similar Kinetic Characteristics to Those in CHO Cell Membrane—It is critical to our understanding of CLIC1 to determine whether its electrophysiological characteristics in lipid bilayers are similar or identical to those in cell membranes. Analysis of the CLIC1 HCFK pathway in lipid bilayers indicates a conductance value of 30.5 pS. In our recent experiments (9), both tip-dip HCFK pathway and inside-out recordings of CLIC1 from CHO-transfected cells (CHO-CLIC1) showed conductances of 31.2 ± 1.5 pS and 29.6 ± 1.9 pS, respectively. This indicates that CLIC1 has essentially identical conductance characteristics on the cell membrane as in lipid bilayers.

To further compare the characteristics of this ion channel in tip-dip bilayer and in CHO-CLIC1 membranes, we analyzed the kinetic characteristics of HCFK single-channel recordings in the two preparations. Fig. 5 depicts open probability (P_o) and open- and closed-time histograms (the example is at V_p = −40 mV) for HCFK single channels in both experimental configurations. P_o was very similar in the two models (0.478 ± 0.035 for inside-out and 0.592 ± 0.026 for tip-dip) and remained constant over a wide range of membrane potentials (Fig. 5, left). From the histograms on the right of Fig. 5, we calculated average open (τ_open) and closed (τ_closed) times of the channel. These properties are virtually identical regardless of whether CLIC1 is functionally expressed in artificial bilayers or when CLIC1 is transfected in CHO cells.

In the experiments shown in Fig. 6a, we applied a ±100-MV voltage ramp to artificial bilayers in which only one HCFK CLIC1 channel was active. Using this voltage ramp protocol, a slight rectification of the channel at high potentials is sometimes evident (10). Using the same voltage protocol, we compared the Cl⁻ dependence of CLIC1 conductance in the inside-out configuration obtained from CHO-CLIC1 (b), which we have reported previously (7), with that produced using the recombinant protein in lipid bilayers (c). The channel conductance in artificial membranes drops to 13 ± 2.3 pS (n = 5) in 20 mM external Cl⁻ with a reversal potential of −60 mV. This is in good agreement with that which we have reported for CHO-CLIC1 (7). Also clearly evident is the shift in current reversal potential in Fig. 6, b and c, as compared with Fig. 6a. Channel openings are seen at 0.026 for tip-dip) and remained 0.026 for inside-out configuration obtained from CHO-CLIC1 (c), which we have reported previously (7).

CLIC1 Current Produced in Artificial Bilayers Is Blocked by IAA94—The CLIC1 ion channel, when expressed in CHO cells, is inhibited by IAA94 and A9C but not DIDS (7). We wanted to determine whether CLIC1 in artificial bilayers exhibited the same characteristics. We found that CLIC1 channels in bilayers have an identical inhibitor profile. Since the blocking action of A9C is irreversible, we confined subsequent studies to the use of IAA94.

Fig. 7 (top) shows the effect of increasing IAA94 concentration during perfusion of a tip-dip patch containing only one functional HCFK Cl⁻ channel clamped at −60 mV. Increasing the concentration from 1 to 50 μM results in a dose-dependent decrease in the channel open probability. In the dose/response graph (Fig. 7, bottom), the normalized channel open probability, P_o, is plotted against the IAA94 concentration. This indicates a maximum effect at concentrations between 50 and 100 μM with a Hill coefficient of 1.4 and an IC₅₀ of 25 μM (n = 4). All these data are in good agreement with our own data from outside-out experiments performed on CHO cells (8) and with the bilayer experiments reported by Tulk et al. (11).

Effect of pH on Both Electrophysiological and Physical Characteristics of CLIC1

Decreasing pH Facilitates the Appearance of CLIC1 Channels in Artificial Membranes—To determine whether ion channel formation in artificial membranes was pH-dependent, we added recombinant CLIC1 to the bath solution at various pH levels and then monitored the time to detect the first event. In Fig. 9, we show three examples of this procedure with the pipette potential held at −50 mV. In all three cases, the initial ion channel activity appears as SCSK channels, as described previously in the legend for Fig. 1. However, as the pH decreases, there is a decrement of the time to observe the first opening. In addition, the probability of observing a 30-pS channel (HCFK) was higher at low pH. In Fig. 10, we have plotted both the average time before the appearance of the first conductance (A), usually an SCSK channel, and the probability of forming a complete HCFK channel (B). The plot on the left of Fig. 10 clearly suggests the existence of a threshold between pH 6 and 6.5 in which the channel changes drastically its ability to insert into the artificial membrane. The time needed to observe a functional SCSK ion channel is almost tripled as the pH of the bath solution increased from pH range 5–6 to range 6.5–7. Probably as a consequence, the histogram plot on the right shows that the formation of HCFK channels has a
higher probability at low pH. However, all attempts to determine whether pH is also involved in the transition of single functional SCSK units to form a HCFK channel in the membrane have failed. A simple explanation of our observations could be that low pH increases the probability of protein insertion into the lipid membrane, increasing the concentration of CLIC1 in the bilayer. This, in turn, favors the aggregation of channel SCSK subunits into a HCFK channel. We have no data at the moment to show a direct influence of the change in the pH of the solution on multimer formation in the membrane.

**Fig. 5.** Open probability and open/closed time constants (at −40 mV voltage in this example) of recombinant CLIC1 protein. In the figure, we compare the kinetic parameters obtained from inside-out (top) and tip-dip (bottom) experiments.

**Fig. 6.** Voltage and Cl⁻ dependence of reconstituted CLIC1. In a, the panel depicts single-channel openings elicited by a voltage ramp from 100 to −100 mV applied to the pipette in equimolar (140 mM) KCl. Below, the same voltage protocol was applied to an inside-out patch obtained from stably transfected CHO cells (b) and to a tip-dip experiment (c), both containing only one channel. In these cases, the pipettes were filled with 140 mM KCl, whereas in the bath solution, the Cl⁻ was 20 mM. Outward openings of the channels can be appreciated at 0 mV.

**Fig. 7.** IAA94 block of CLIC1 single-channel current. The upper panel shows, from the top to the bottom, the effect of increasing concentration of IAA94 in the trans chamber on a single CLIC1 channel held at −60 mV in the patch pipette. Below, we report the average normalized CLIC1 channel open-probability (n = 4).
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Low pH Facilitates the Interaction of CLIC1 with Liposomes—Measurement of the association of CLIC1 with liposomes at pH 6.5 and pH 7.4 indicates that low pH facilitates the interaction of CLIC1 with lipid bilayers (Fig. 11B). To determine the effect of low pH on CLIC1 association with lipid bilayers, CLIC1 was combined with liposomes, and the pH of the mixture was adjusted to pH 7.4 or pH 6.5. The mixture was incubated between 10 min and 4 h, and the liposomes were purified by centrifugation on a discontinuous Ficoll density gradient, which also had the pH adjusted accordingly. Following centrifugation, the liposome fraction was collected, and the amount of CLIC1 it contained was determined by SDS-PAGE and Western blotting followed by densitometry. CLIC1-lipo-

some association was found to increase in a time-dependent manner and to be enhanced at pH 6.5 as compared with pH 7.4 (Fig. 11B).

Low pH Does Not Cause a Major Alteration in the Secondary Structure of Soluble CLIC1—To determine whether the pH-dependent effects on CLIC1 ion channel activity were associated with a conformation change in the protein, we first looked for any changes to secondary structure using CD spectropolarimetry. The spectrum obtained for a CLIC1 in potassium phosphate buffer at pH 7.0 is typical of a protein containing a significant proportion of α-helical structure with minima at 208 and 222 nm (Fig. 12). No differences were observed when the protein was incubated at pH 6.0, suggesting that there are no significant changes in the proportions of secondary structural elements of CLIC1 in this pH range.

To determine whether there were any structural changes that might involve a rearrangement of secondary structural elements, rather than a change in total secondary structure, we looked for changes in tryptophan fluorescence between pH 7.0 and pH 6.0. Changes in the emission wavelength maximum (λmax) or the intensity of fluorescence would indicate a change of environment of the single tryptophan residue at position 35 in CLIC1. At both conditions, CLIC1 exhibited a λmax of 345.5 nm, which is typical for a partially buried tryptophan residue. This suggests that there are no pH-induced structural changes associated with Trp-35.

DISCUSSION

In the present study, we were able to characterize not only the biophysical characteristics of CLIC1 protein, as the essential component of a Cl−-selective anion channel, but also the sequence of events relating to the formation of a functional single channel comparable with the native conductance. The temporal sequence, following addition of protein to the trans solution, was always similar and is well represented in Fig. 1. After an initial delay, SCSS conductances appear in the bilayer. These SCSS modules then appear to undergo a transition to form the HCFK channel, which has four times the conductance of the SCSS and fast kinetics that correspond to the characteristics of the native channel.

Our current model is based on the assumption that either a single 241-amino acid CLIC1 protein or a well defined multimer of CLIC1 is able to span the membrane and to open a channel with very slow opening and closing kinetics, the SCSS conductance. Thus the current traces in most of our experiments are characterized, in particular at the beginning of recording, by the presence of many current levels. Eventually single proteins (or well defined multimer SCSS units) are able to assemble in a complex that duplicates the fast open and closed transition typical of the native CLIC1 (1, 7, 8). According to our data and calculations and based on the conductance measurements (Fig. 6), such a channel needs four SCSS ionic pathways combined to equate in amplitude to a wild-type CLIC1 channel.

This process is not a simple aggregation of subunits; the suggested four elements must also cooperate in determining the opening and closing kinetics of the resultant Cl− channel. This complementary effect of the single subunits is also manifest during the action of the specific channel blocker IAA94. A channel (HCFK) similar to the native form displays an IC50 of 25 μM (Fig. 7). We were not able to calculate the same parameter for the SCSS channel; from our results, it appears that with a drug concentration up to 100 μM, openings remain unchanged (Fig. 8). Above this concentration, (already four times the IC50 calculated for the HCFK channel), openings disappeared suddenly without a gradual decrease of current levels, number, or other parameters such as open time or open
probability of the channels \((n = 6)\). More importantly, although
the 30-pS channel shows prompt reversibility of the IAA94
current block upon washout, we were never able to observe any
more openings of the small SCSK channels after they were
closed by addition of IAA94. This could be interpreted in sev-
eral ways.

**FIG. 10.** CLIC1 single-channel activity versus pH. As shown in A, the time of
the first functional insertion in the lipid bilayer increases markedly as the solu-
tion pH goes from a range of 5–5.5 to one of 6.5–7. B, probability of CLIC1 protein
forming a 30-pS high current fast kinetics HCFK channel. The histogram shows a
higher occurrence of channel aggregates at lower pH. However, it is impossible to
distinguish whether the pH is responsible for this phenomenon or whether the
higher number of sublevels observed at low pH contributes by itself to increase
the probability of 30-pS channel formation.

**FIG. 11.** CLIC1 associates with artificial liposomes. A, Western blot of fractions collected following discontinuous Ficoll density gradient
purification of liposomes: a purified liposome fraction was collected from the 30% Ficoll-0% Ficoll interface, which contains liposome-associated
CLIC1 (lane 1). Soluble CLIC1 that did not migrate through the gradient was collected from the bottom fraction where the protein/liposome
mixture was loaded (lane 2). CLIC1 associates with liposomes, and relative to the soluble fraction, the liposome fraction contains more multimers.
B, time course of CLIC1 association with liposomes. This was determined over 4 h, at pH 7.4 and pH 6.5, by Western blotting of Ficoll
gradient-purified liposome fractions followed by densitometry. At pH 6.5, more CLIC1 is present in the liposome fractions than at pH 7.4, indicating
that low pH facilitates CLIC1-lipid bilayer interaction. Inset, Western blot of time course of CLIC1 association with liposomes at pH 7.4 and pH
6.5; time points are between 10 min and 4 h, as indicated on graph.

**FIG. 12.** Circular dichroism of
CLIC1. CLIC1 was diluted from a stock
solution into potassium phosphate buffer
at either pH 7.0 (solid line) or pH 6.0
(dotted line) and incubated for 1 h at room
temperature, and then CD data were col-
clected over the wavelength range 195–260
nm. The spectra shown were the sum of
three scans and were baseline-corrected.
The simplest interpretation is that the binding characteristics of IAA94 to HCFK and SCSK channels differ. The binding occurring at high concentrations of the channel blocker could be stronger and irreversible for SCSK channels. It is reasonable to think that once IAA94 is linked to the monomer (SCSK) inserted into the membrane, it is also able to prevent multimerization. Alternatively, IAA94 might bind to the CLIC1 molecule in solution, preventing its membrane insertion. HCFK is stable in the membrane and can reopen once IAA94 is washed away. The SCSK channel may be in equilibrium between the membrane and the solution. Once washout occurs, IAA94 alone or IAA94 linked to the protein in solution is rinsed away. This would increase the probability of membrane-inserted CLIC1 molecules in the form of SCSK channels returning to the solution, preventing any possibility of a reappearance of the SCSK channel.

We believe the likeliest ion channel configuration is a complex of four basic units in which each unit consists of either one CLIC1 molecule or a well defined multimer of CLIC1 molecules. This conclusion can be drawn from the experiments illustrated in Figs. 5 and 6 and from previous studies (7). Almost all inside-out recordings show a stable Cl\(^{-}\)-dependent conductance of 30 pS in 140 mM equimolar KCl. Such a conductance is most likely due to the assembly of four SCSKs (conductance of 7 pS each in equimolar 140 mM KCl in lipid bilayers; Figs. 5 and 6) into a single mature HCFK channel. To sustain our point of view, we have to hypothesize that the only change occurring during clustering concerns the kinetics of the channel, with the conductances being simply summed. However, this proposed tetrameric channel appears from our observations to exhibit some variability. In inside-out recordings of the native channel, it is possible to observe current substates corresponding to the contribution of two or three single 7-pS units (Fig. 4). This could be an indication that the cooperative HCFK is in equilibrium with a state corresponding to four independently opening 7-pS channels. It is also not unusual to see the open state conductance equivalent to a doubling or tripling of the standard 30 pS (Fig. 1, bottom traces), suggesting that under some circumstances, much larger cooperative assemblies of HCFK channels can be formed. The various substates identified in these experiments are consistent with our earlier observations of similar substates in CHO cells (7). In this context, we are not surprised to see different conductance values for CLIC1 obtained by other authors in artificial membrane experiments. Tulk et al. (11) found a conductance of 67.5 pS in symmetrical 150 mM KCl. It is possible that their recording system and their experimental conditions allowed multiple aggregation of the CLIC1 protein. Their conductance value corresponds to what we propose could be an eight-unit complex. These authors reported an IC\(_{50}\) for IAA94 in equimolar 300 mM KCl of 86 \(\mu\)M in contrast to our value of 25 \(\mu\)M in 140 mM KCl. Given the sensitivity of CLIC1 to Cl\(^{-}\) concentration (7, 11), this discrepancy is not surprising.

CLIC1 has very similar electrophysiological characteristics in a cell membrane to that with purified E. coli-derived recombinant protein reconstituting the ion channel in artificial lipid bilayers. Not only is the conductance very similar, but the kinetics of the channel are also in good agreement. Tip-dip recordings of HCFK pathways show kinetics parameters very similar to inside-out single-channel current of CHO-transfected CLIC1 (7). Another parameter characteristic of CLIC1 is rectification at extreme membrane voltages, already reported by us for the channel transfected into CHO cells (7) and highlighted by Tulk et al. (11) for the channel inserted into lipid bilayers. In the present study, we did not explore membrane voltages over \(\pm 100\) mV, at which rectification is reported to be important. However, we believe that there is an early hint of this phenomenon visible in the results from the voltage ramp protocol of Fig. 3a. Furthermore, the open probability as a function of membrane voltage in both inside-out and tip-dip experiments (Fig. 4, left) showed a trend for a decrease of the time spent by CLIC1 in the open state at higher potentials.

The low isolectric point of CLIC1 suggested to us that the pH may influence the function of this ion channel, and this appears to be the case. The major effect of decrease in pH was to increase the ability of CLIC1 to interact with lipid and to be inserted into the artificial membrane. This has been demonstrated both biochemically and electrophysiologically. The effect of pH does not appear to involve a major structural change in the soluble form of CLIC1. Both CD and fluorescence spectra are unaffected by pH, although subtle changes, particularly in regions well distant from Trp-35, cannot be excluded in the case of fluorescence. A number of explanations for these findings are possible. As the pH is lowered, it approaches the pI of the protein (pI 4.5). This may in turn lower any electrostatic repulsion effects between channel subunits and thus favor the formation of the active oligomeric ion-channel. This phenomenon may be greater in the lipid environment of the artificial bilayer, where it is likely that some structural change does occur to the protein upon insertion (9). Alternatively, this decrease in pH may decrease the activation barrier hindering the transition from the CLIC1 soluble form to the membrane conformation, which regulates the entry of CLIC1 into the lipid bilayer.

Although pH influences the rate of association with artificial lipid membranes, our experiments indicate that it is less likely that it is involved in modulating the rate of assembly of the individual subunits to form a functional multimeric HCFK channel. We have found a correlation between the time to insertion and pH (Fig. 10) but not between the time to insertion and the appearance of the complete HCFK CLIC1 channel(s). However, channel protein assembly in the lipid bilayer probably depends upon many factors. Certainly the amount of protein able to insert into the membrane plays an important role; more proteins present in a restricted membrane space increase the probability of protein-protein interaction. On the other hand, for reasons that are not clear, the simple presence of the membrane-inserted proteins does not guarantee the formation of HCFK protein complexes. We observed several experiments, at any tested pH, in which even in the presence of many functional current SCSK subunits, the typical HCFK CLIC1 channel(s) never appeared even after 1 h of continuous recording.

In conclusion, our studies show that CLIC1 alone can form a functional Cl\(^{-}\) ion channel with characteristics that are identical to the channel seen in CHO cells. The channel is likely to consist of a tetrameric assembly of CLIC1 subunits. Given that the crystal structure of the soluble form of CLIC1 is unlikely to represent the structure of the integral membrane form, it will be interesting to discover how this protein changes its structure to assemble into an active Cl\(^{-}\) channel.

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Recombinant CLIC1 (NCC27) Assembles in Lipid Bilayers via a pH-dependent Two-state Process to Form Chloride Ion Channels with Identical Characteristics to Those Observed in Chinese Hamster Ovary Cells Expressing CLIC1

Kristina Warton, Raffaella Tonini, W. Douglas Fairlie, Jacqueline M. Matthews, Stella M. Valenzuela, Min Ru Qiu, Wan Man Wu, Susan Pankhurst, Asne R. Bauskin, Stephen J. Harrop, Terence J. Campbell, Paul M. G. Curmi, Samuel N. Breit and Michele Mazzanti

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