Functional characterization of the NCC27 nuclear protein in stable transfected CHO-K1 cells

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ABSTRACT **NCC27 belongs to a family of small, highly conserved, organellar ion channel proteins. It is constitutively expressed by native CHO-K1 and dominantly localized to the nucleus and nuclear membrane. When CHO-K1 cells are transfected with NCC27-expressing constructs, synthesized proteins spill over into the cytoplasm and ion channel activity can then be detected on the plasma as well as nuclear membrane. This provided a unique opportunity to directly compare electrophysiological characteristics of the one cloned channel, both on the nuclear and cytoplasmic membranes. At the same time, as NCC27 is unusually small for an ion channel protein, we wished to directly determine whether it is a membrane-resident channel in its own right. In CHO-K1 cells transfected with epitope-tagged NCC27 constructs, we have demonstrated that the NCC27 conductance is chloride dependent and that the electrophysiological characteristics of the channels are essentially identical whether expressed on plasma or nuclear membranes. In addition, we show that a monoclonal antibody directed at an epitope tag added to NCC27 rapidly inhibits the ability of the expressed protein to conduct chloride, but only when the antibody has access to the tag epitope. By selectively tagging either the amino or carboxyl terminus of NCC27 and varying the side of the membrane from which we record channel activity, we have demonstrated conclusively that NCC27 is a transmembrane protein that directly forms part of the ion channel and, further, that the amino terminus projects outward and the carboxyl terminus inward. We conclude that despite its relatively small size, NCC27 must form an integral part of an ion channel complex.—Tonini, R., Ferroni, A., Valenzuela, S. M., Warton, K., Campbell, T. J., Breit, S. N., Mazzanti, M. Functional characterization of the NCC27 nuclear protein in stable transfected CHO-K1 cells. *FASEB J.* 14, 1171–1178 (2000)**

Key Words: single-channel currents · NCC27 · ion channel

A number of ion channels have been identified electrophysiologically on the nuclear membrane of various cell types, though cloned nuclear ion channels are extremely rare. Although the biological roles of nuclear ion channels are yet to be defined in detail, several have been characterized electrophysiologically (1–4). We recently described the molecular cloning and characterization of a novel nuclear chloride ion channel protein, NCC27, which localizes principally to the cell nucleus (5). This protein is a member of a new class of organellar chloride ion channel proteins, the first identified member of which was the bovine chloride channel, p64 (6). Of the several members of this family now cloned, only NCC27 and the newly described CLIC3 (7) are dominantly nuclear in distribution. The localization of NCC27 to the nuclear membrane, and its presence in virtually all cells and tissues we have so far examined, suggested to us that this channel is highly conserved and could play an important role in the function of all or most cells.

NCC27 differs from other putative nuclear ion channels in several ways. It is found predominantly in the nucleoplasm (in soluble form), and only a small proportion of the protein is inserted into the nuclear envelope (5). No other ion channel proteins have been reported to have this unusual distribution in eukaryotic cells. NCC27 is also a very small protein of 27 kDa. These factors led us to consider the possibility that it may represent only a subunit or regulator of an unidentified ion channel rather than a membrane resident channel in its own right. We thought this to be unlikely based on our earlier electrophysiological studies (5), and were further reassured by the knowledge that p64, a close relative of NCC27, has been shown to function as a chloride ion channel in lipid bilayers (8). Nonetheless, we wanted to confirm that NCC27 actually forms part or

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the entire chloride ion channel we described in our earlier report.

The studies we present here were carried out in our model of Chinese hamster ovary cells (CHO-K1) stably transfected with NCC27. This ion channel is constitutively expressed in the nucleus and nuclear membrane of CHO-K1 cells; as we previously reported, when CHO-K1 cells are transfected with an NCC27 containing eukaryotic expression vector, synthesized protein spills over into the cytoplasm. Ion channel activity can then be detected in the plasma as well as the nuclear membrane of the transfected cells (9). We first used conventional single-channel methods to further define the electrophysiological properties of the resulting ion channel. We were able to undertake a detailed comparison of the properties of this channel in its locations on the nuclear and plasma membranes, something that has not previously been possible. We then used a technique, novel to this field, involving the incorporation of epitope tags at both the amino and carboxyl-terminal ends of the NCC27 protein. This then allowed us to use a monoclonal antibody to this tag as a uniquely specific modulator of ion channel properties. In this way we were able to definitively associate the expressed protein with the ion channel activity, and define the orientation of the protein in the plasma membrane.

MATERIALS AND METHODS

Cell culture and preparation of transfected cell lines

Cell lines were obtained from the American Type Tissue Collection (Rockville, Md.). Both nontransfected and transfected Chinese hamster ovary cells (CHO-K1) were grown in DMEM/F12 media (GIBCO BRL, San Giuliano Milanese, Italy) containing 5% fetal calf serum (GIBCO BRL).

Eukaryotic expression of NCC27 and NCC27 tagged with the FLAG peptide (DYKDDDDK) at its carboxyl terminus was performed as described previously (5). A construct incorporating a modified FLAG peptide (DYKDDDDN) at the amino terminus of NCC27 was directionally cloned into pRc/CMV vector (Invitrogen, Groningen, The Netherlands). This construct was transfected into CHO-K1 (80% confluent) for 24 h in 35 mm^2 dishes using 9 μl lipofectamine reagent (Life Technologies, Inc., Milano, Italy) and 1 μg of DNA, as recommended by the manufacturer. Stable transfectants were selected with G418 (Boehringer Mannheim, Mannheim, Germany), followed by subcloning. The subclones were screened by immunofluorescent staining using anti-FLAG M2 (M2-AB, Sigma, Milano, Italy) monoclonal antibody (5).

Isolation of cell nuclei

Nuclei were isolated from CHO-K1 cells transfected with NCC27 cells by shearing 2×10^6 cells in a homogenizer with a Teflon pestle of 0.025 μm clearance and centrifuging the homogenate on a sucrose pad (in mM: 50 Tris HCl, 25 KCl, 5 $MgCl_2$, 250 sucrose) at 4000 rpm in a Labofuge M (Heraeus, Milano, Italy) at 0–4°C. This procedure results in a pellet of pure nuclei, which were resuspended in a standard bath solution (in mM: 120 KCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 1.1 EGTA, 10 HEPES 5 glucose, pH 7.4) at room temperature .

Patch-clamp recording

The patch electrodes were pulled from hard borosilicate glass on a Brown-Flaming P-87 puller (Sutter Instruments, Novato, Calif.). The pipettes were coated with Sylgard (Dow Corning, Seneffe, Belgium) and fire polished to an external tip diameter of 1–1.5 μm. These electrodes had resistances of 7–10 MΩ. We applied standard cell-attached and nucleus-attached patch-clamp techniques to obtain seals of 20–50 GΩ in the single-channel recordings. The bath solution for the CHO-K1 cells contained (in mM): 130 NaCl, 4.8 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 12.5 glucose, 1.2 NaH_2PO_4. For cell-attached and inside-out recordings, the same solution was used in the micropipettes. For outside-out experiments, the patch electrode solution contained (in mM): 10 NaCl, 130 KAsp, 2 $MgCl_2$, 1.3 $CaCl_2$, 10 HEPES, 10 EGTA. The antibody (M2-AB, Sigma) was applied at a concentration of 1.5 μg/ml in both inside-out and outside-out experiments, using a custom-made fast perfusion system. The solution used for patch clamping of isolated nuclei was (in mM): 120 KCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 1.1 EGTA, 10 HEPES 5 glucose, pH 7.4. The same solution was used to fill the electrode in nucleus-attached experiments.

Single-channel currents were recorded with an Axon 200B patch-clamp amplifier (Axon Instruments, Novato, Calif.), digitized, and stored on a VCR (Panasonic, Milano, Italy). Data were analyzed on a PC after filtering at 1000 Hz using both pClamp 7 (Axon Instruments) and custom-made programs developed by W. Gooldby, Department of Anatomy and Cell Biology, Emory University, Atlanta, Georgia.

RESULTS

Single-channel plasma membrane characteristics of NCC27 protein

Single-channel activity recorded from CHO cells transfected with NCC27 protein is illustrated in **Fig. 1**. We found such channel behavior in the plasma membranes of more than 25% of patches in transfected cells (n=50), but in only one patch out of more than 30 experiments with native CHO-K1 cells. This observation suggests that the ion channel activity detected after transfection is related to the functional expression of NCC27 protein. In our experimental conditions a different channel activity, characterized by strong voltage dependence, was occasionally recorded in both transfected and untransfected cells. This ionic pathway is likely to be an endogenous channel and it was easily distinguished from NCC27-associated channel activity by its kinetics and its larger conductance.

The measured resting membrane potential (V_m) of transfected CHO-K1 cells was −35 ± 12.4 mV (n=12). The channel opened outwardly at all potentials and only occasionally conducted inward current. At very positive membrane voltages (above +40-+50 mV), the single-channel current became nonlinear, with current amplitude either becoming

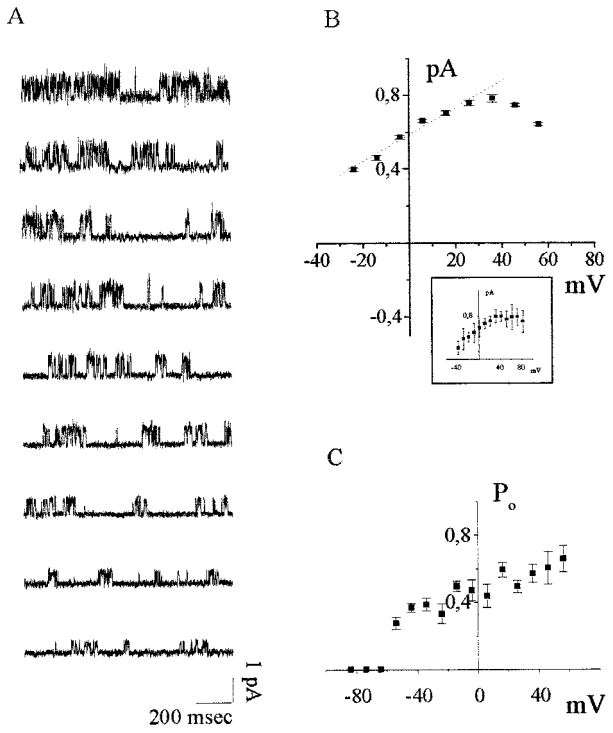

Figure 1. Single-channel characterization of currents recorded from NCC27-transfected CHO-K1 cells. *A)* Cell-attached experiment on a CHO-K1-transfected cell. Sweeps were recorded at 10 mV intervals ranging from −24 mV (bottom) to +56 mV (top). There is a trend for more openings at higher potentials. This is confirmed in panel *C*. *B)* Single-channel current/voltage (i/V) relationship in the same experiment as shown in panel *A*. Data have been pooled from 3 s of recording at each potential. The insert depicts the average i/V plot pooled from identical experiments in 5 different cells. *C)* Plot of mean open channel probability from 5 cells, further illustrating increased channel activity at more depolarized potentials.

constant or in many cases actually declining with increasing depolarization. This is seen in the single-channel current/voltage (i/V) relationship of Fig. 1*B* as well as on averaged i/V data shown in the insert. The single-channel conductance in the example shown (Fig. 1*B*), calculated by fitting the points in the linear part of the i/V plot, was 7.26 ± 0.64 pS and the average conductance in five experiments was 8.05 ± 0.35 pS (Fig. 1*B*, insert). The channel open probability increased slightly with increasing membrane potential and fell to zero at potentials below approximately −60 mV (Fig. 1*C*). Calculated mean open and close time at 0 mV V_m was 8.1 ± 0.4 and 4.3 ± 0.2 ms. The extrapolated reversal potential was −82 ± 18.5 mV. In four experiments we recorded continuously channel activity at +40 mV membrane potential. No significant opening rundown was observed even after more that 20 min of recording. In all these respects, the biophysical properties of the NCC27-associated channels in this transfection model closely resemble both the chloride conductance already described (5) and that reported by others for the closely related channel, p64 (10)

NCC27 conductance is chloride dependent

We noted a wide range of reversal potentials for this channel and also considerable variability in single-channel conductance, which ranged between 6.8 and 9.5 pS in different patches. We hypothesized that this may reflect variations in intracellular chloride concentration. To test this hypothesis, we studied NCC27-associated channels expressed in plasma membrane in the cell-attached mode, before and after intracellular cell perfusion with a 140 mM chloride solution and in inside-out configuration. **Figure 2*A*** illustrates the result of a two-electrode experiment in which a single NCC27-associated channel was observed before (left) and after (right) dialysis of the cell with 140 mM Cl^-. To dialyze the cell, we used a second electrode filled with high chloride solution. The two electrodes were simultaneously placed on the cell in cell-attached mode. After recording of several single-channel current traces using the electrode containing bath solution, we changed the configuration of the patch pipette

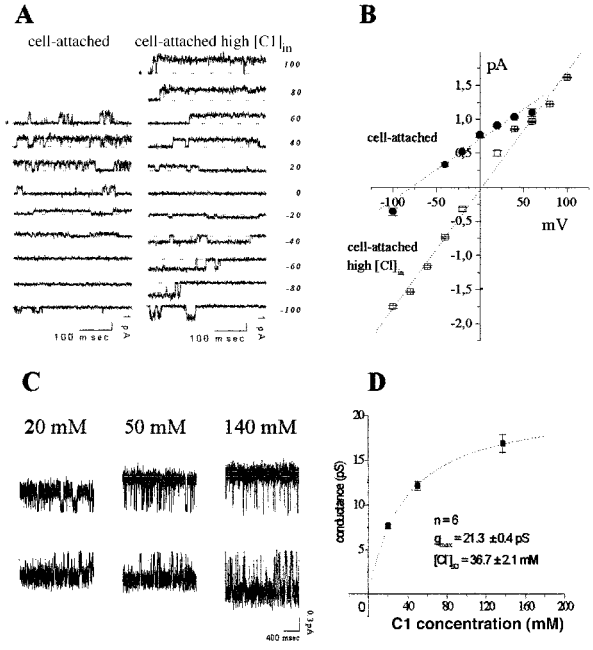

Figure 2. Effect of $[Cl^-]_{in}$ on the electrophysiological properties of NCC27 channel. *A)* Cell-attached single-channel recordings at different membrane voltages before (left) and after (right) intracellular perfusion with 140 mM Cl via a second electrode in whole-cell configuration. *B)* Single-channel i/V relationships in low (dots) and high (squares) $[Cl^-]_{in}$. *C)* Single-channel inside-out experiments in which the external solution (intracellular membrane side) was changed from 20 to 50 and to 140 chloride. *D)* Relationship between single-channel conductance and 'intracellular' chloride concentration recorded using inside-out patches (to allow accurate adjustment of chloride n=6 patches).

filled with 140 mM Cl^- from cell-attached to whole cell. We waited 1–2 min after patch break to collect a single-channel opening in the new configuration. The current-voltage (i/V) relationships are illustrated in Fig. 2*B*; from these we calculated a conductance of 8.8 ± 0.5 pS before and 17.4 ± 0.6 pS after dialysis with 140 mM chloride. Exposure to internal high chloride concentration in two-electrode experiments not only increased the single-channel conductance (16.3±0.85 *vs.* 8.6±0.7; n=3), but also removed the nonlinear behavior described above (illustrated in Fig. 1*B*). In addition, the probability of the channel to conduct inward current increased in high chloride, supporting the hypothesis that there is an active role of the anion in the current flow modulation.

We observed the same chloride dependence of the ionic pathway in inside-out experiments (n=6). In Fig. 2*C* we show an example of channel recording in which the 'cytoplasmic' side of the inside-out patch was serially exposed to 20, 50, and 140 mM Cl at Vp ±50 mV. This resulted in measured channel conductance increasing from 9 ± 0.8 pS at 20 mM Cl^- to 11 ± 1.3 pS at 50 mM Cl^- and 17 ± 1.4 pS at 140 mM Cl^- (Fig. 2*D*).

NCC27-associated channels have similar properties whether expressed on nuclear or plasma membranes

In native cells this protein is predominantly localized in the nucleus. The ionic channel associated with NCC27 expression was frequently detected on the nuclear envelope using patch-clamp on isolated nuclei obtained from transfected CHO-K1 cells. The probability to find a single channel was higher than in the plasma membrane (43% vs. 25%; n=45). Furthermore, in the case of the nuclear envelope as well as plasma membrane, the single-channel current was recorded much more frequently in NCC27 protein-transfected cells compared with channel appearance in native cell isolated nuclei (20%; n=27). We were therefore interested to compare single-channel properties in nuclear and plasma membranes. **Figure 3** (top) depicts current recordings obtained from both cell membrane and the envelopes of isolated nuclei from NCC27-transfected CHO-K1 cells. Whereas active channels were much more frequently detected in the nuclear membrane than in the plasma membrane, most of the biophysical characteristics of the channel were very similar. In the example shown in Fig. 3, the open probabilities at comparable potential differences were 0.4 ± 0.03 and 0.45 ± 0.08 for NCC27 inserted in the plasma membrane and in the nuclear membrane, respectively. There were, however, major differences in single-channel conductance and in the linearity of

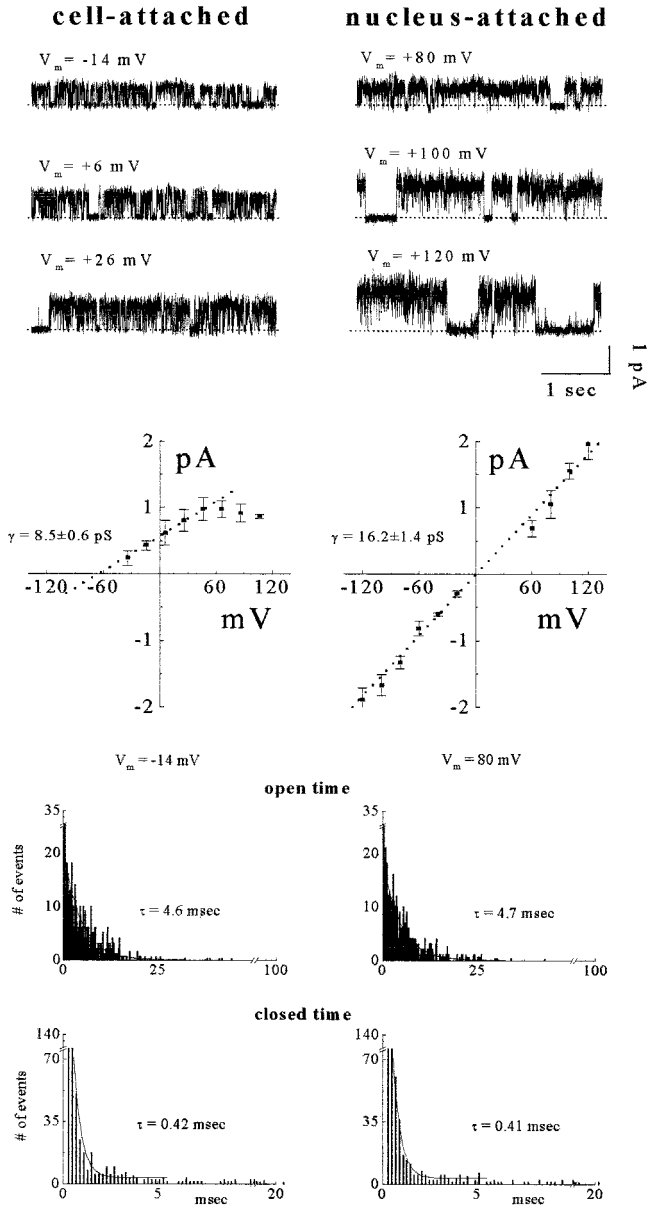

Figure 3. Comparison of single-channel recordings from nuclear envelope and plasma membrane. Single-channel recordings obtained from cell membrane (left) and nuclear envelope of isolated nucleus (right). The upper panels show single-channel recordings at a range of holding potentials. The middle panels illustrate averages i/V relationships (n=6 for cell and n=5 for nucleus attached). Channel conductances are reported next to each fitting. The bottom panels depict open and close times of cell and nucleus single chloride channel. All distributions were fitted by a single exponential function. The potential at which we calculated the two parameters was chosen on the basis of the similarity in the amplitude of the single-channel currents.

the i/V relationship between the two preparations (Fig. 3, middle panels). The cell membrane channel had, on average, a conductance of 8.5 ± 0.6 pS (n=6) and showed a nonlinear i/V relationship at depolarized membrane potentials (Fig. 3, middle left). The i/V for the nuclear channel was quite different (Fig. 3, middle right). This channel con-

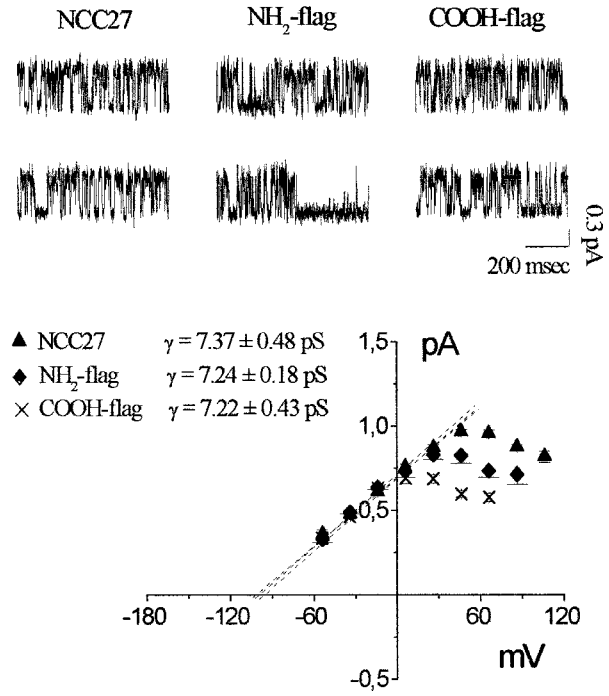

Figure 4. Comparison of electrophysiological properties of NCC27 constructs. Upper panels show two examples each of channel activity recorded at 0 mV from CHO-K1 cells transfected with NCC27. which has either been left untagged (left column), or tagged at the amino terminus (middle) or carboxyl terminus (right) by the FLAG peptide. The lower panel summarizes the i/V relationships for each cell type. These differ only in the voltage at which the voltage dependence slope looses the linearity (see text).

ducts both inward and outward current, with a reversal potential around 0 mV and conductances of 16.2 ± 1.4 pS (n=5). Furthermore, single-channel currents recorded from the nuclear envelope increased linearly with voltage.

Despite the differences in voltage dependence, most of the channel parameters show similar behavior. We can already see from the foregoing text that the open probability of the channel is the same. The bottom of Fig. 3 shows that the calculated open and close time of the channel at a comparable potential are also similar.

Electrophysiological characteristics of FLAG-tagged and unmodified NCC27-associated ionic channel are very similar

These experiments were carried out using two clones in which the transfected NCC27 protein was tagged at either the amino or carboxyl terminus with the eight amino acid FLAG epitope. Studies with the FLAG-tagged, NCC27-transfected CHO-K1 plasma membrane were performed at the single-channel level in cell-attached configuration. The electrophysiological characteristics of both FLAG-tagged and unmodified NCC27-associated channel were very similar. This is demonstrated in **Fig. 4**, which compares recordings made from three cells transfected with nontagged, amino-terminal-tagged, and carboxyl-terminal-tagged NCC27. Two examples of single-channel, cell-attached current recordings (pipette potential = 0 mV) are illustrated at the top of each column. Channel kinetics, mean open times, and open probabilities are very similar in the three NCC27 cell lines and characteristic of this channel (see above). Single-channel conductances calculated from the linear part of the i/V curves (Fig. 4, lower panel) are also very similar. We observed, however, a consistent difference in the voltage at which the current changes slope at positive potential. In nontagged channels, the point of inflection was at a membrane potential of approximately +40 mV, compared to +20 mV for amino FLAG-tagged channel and 0 mV for carboxyl FLAG-tagged channel. This observation suggests that modification of the NCC27 proteins directly altered single-channel current. The eight amino acids positioned either at the amino- or carboxyl-terminal of NCC27 proteins are highly charged and could interfere with the ionic flow.

NCC27 is a transmembrane protein with the amino terminus orientated outward

The FLAG epitope is recognized by a monoclonal antibody (anti-FLAG M2) that identifies only the tagged proteins, as demonstrable on Western blots (5). We undertook a series of inhibition experiments using the FLAG antibody and the plasma membrane of epitope-tagged, transfected CHO-K1 cells (**Fig. 5**). We made recordings in both outside-out and inside-out configurations from plasma membranes of CHO-K1 cells transfected with either the amino FLAG-tagged or carboxyl FLAG-tagged NCC27. Exposure of an outside-out patch of an amino-tagged NCC27 channel to anti-FLAG M2 antibody resulted in complete silencing of channel activity within 5 min (Fig. 5, left; n=7). Average time of channel blockade was 4.8 ± 1.24 min. By contrast, in six similar experiments using the inside-out configuration (n=5), anti-FLAG M2 antibody had no effect on channel activity during 15 min of exposure (not illustrated). Conversely, when six similar experiments were undertaken with the carboxyl FLAG-tagged NCC27 construct, the anti-FLAG M2 antibody blocked channel activity in the inside-out configuration (Fig. 5, right), but had no effect in studies using the outside-out configuration (n=5; not illustrated). The time to stop the single-current flow was higher than outside-out amino FLAG experiments. On average, the current was completely canceled in 6.8 ± 2.16 min. Furthermore, we were unable to detect any

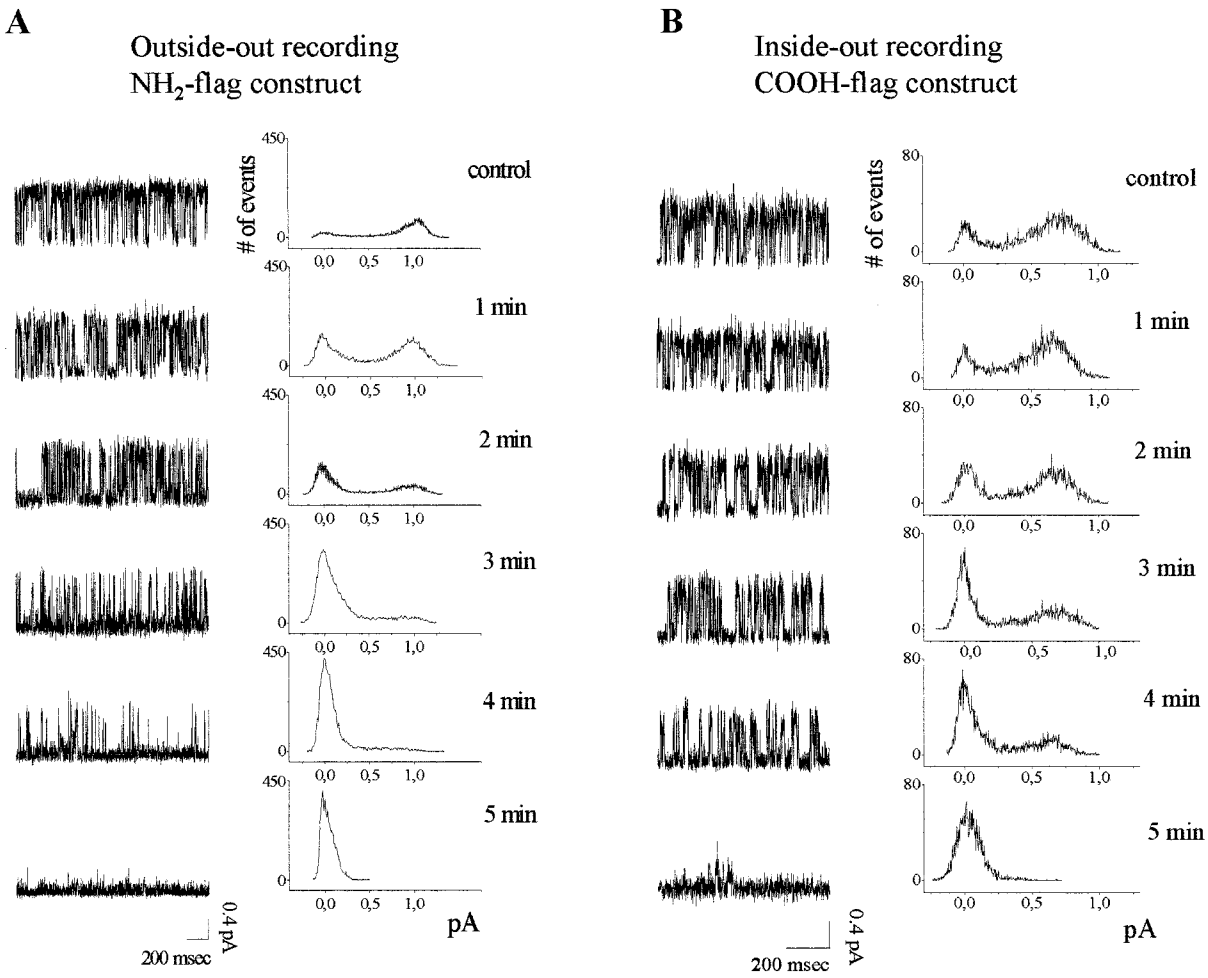

Figure 5. Effect of M2-AB antibody on single-channel recordings in NCC27-FLAG-transfected CHO-K1 cells. Outside-out experiment (left) on amino-terminal-tagged cell. The channel current disappears rapidly after addition of the anti-FLAG monoclonal antibody. A very similar effect is seen, on the right, when carboxyl terminus-tagged NCC27 is exposed to the antibody in an inside-out patch.

effect on single-channel currents in either inside-out or outside-out configurations using either nonspecific antibodies (IgG, UBI; α-tubulin, Sigma) or anti-FLAG M2 antibody already saturated with the FLAG peptide (n=3 and n=4, respectively).

DISCUSSION

Chloride ionic channels are important for cell volume regulation and intracellular pH control (11). Little is known about the role of anion channels present in intracellular compartments, but presumably they serve the same functions (4). Because of its localization, NCC27 could play an important role in nuclear physiology. Most of the protein is concentrated and soluble in the nucleoplasm; only a small percentage is present in the nuclear membranes in association with an ionic channel (12). The possibility for the protein to become inserted in the membranes of the envelope suggests a transient modulatory effect of NCC27. The mechanisms promoting the protein insertion in lipid bilayers are still unclear; the increment of intracellular protein concentration certainly must be one of them. In an overexpression system represented by the NCC27-transfected CHO-K1 cells, not only there is an increase of the proteins in the nuclear envelope, but the probability of finding it in the cytoplasm and cell membrane is also much higher (5). We have previously reported the possibility that NCC27 proteins form a chloride ion channel that localizes to the nuclear envelope. NCC27 is present in a wide variety of cells and cell lines of human origin, the murine cell line RAW 264.7, and untransfected (hamster) CHO-K1 cells; it is also highly conserved across species (5). In the present study we have shown that after transfection of NCC27 protein in CHO-K1 cells, the likelihood of finding chloride channels with the patch-clamp technique in both the cell and the nuclear membrane is increased by many fold. This could suggest that the functional expression of

NCC27 is represented by chloride selective ion channel or that the protein is closely related to it.

The NCC27-associated channel is sensitive to intracellular changes of chloride concentrations. Cytosolic chloride in CHO-K1 cells is on average quite low: ~12 mM (13). We observed a relatively high variability in single-channel conductances and current reversal potentials in our studies of transfected CHO-K1 cells consistent with a range of intracellular chloride concentration in these cells from 4–20 mM. CHO-K1 cell population are actively dividing; not only is it possible to recognize different cell shapes, but by measuring the resting potential it is possible to find values ranging from −30 to −50 mV. We believe this variability could be caused by and at the same time could influence intracellular chloride concentrations that correspond to the presence in culture of cells at various stages of the cell cycle.

Examining Fig. 2*D*, it is clear that the NCC27-associated channel is very sensitive to the chloride concentration changes, particularly at low values. Conductance and reversal potential are not the only parameters modified by the increase of $[Cl^-]_{in.}$ According to the data reported in Fig. 2, the same single channel exposed to 130 mM intracellular chloride increases the open time and the probability of opening for the inward current. Even the nonlinear part of the i/V curve present in cell-attached experiments at a membrane potential above 40 mV is completely abolished in high chloride solutions.

The study of single-channel chloride dependence could be useful in interpreting the data in which we compared cell membrane and nuclear single ionic pathways (Fig. 3). Searching for similarities, the first observation is the marked increase of channel appearance in both plasma and nuclear membranes in CHO-K1-transfected cells: 25% vs. 5% and 43% vs. 25%, respectively. In the kinetics parameters, there is good agreement in the open probability (0.4 for the cell membrane channel and 0.45 for the nuclear envelope conductance) calculated at each potential. Measuring the open and close time at potential in which the single-channel current shows comparable amplitude (Fig. 3, bottom), we obtained analogous values. The big difference is visible in the lower panels of Fig. 3 and concerns the voltage dependence of the current. The i/V relationships of the nuclear envelope and cell membrane channel show different conductances and linearity. However, if we compare nuclear channel i/V with the current/voltage relationship obtained from a single channel recorded in high cytoplasmic chloride solution (Fig. 2*B*), we have an almost perfect match. Not only are the two curves fully linear, but the measured channel conductances of 16.3 and 16.2 pS are almost identical. Taking this into account, we suggest that the nuclear channels recorded in cell-free configuration share the same high chloride ionic solution in the inner membrane side. A previous work has already discussed that during a nuclear isolation procedure there is a disruption of the contacts with the endoplasmic reticulum (14). Since the solution in which the nuclei are isolated contains 140 mM KCl, it is reasonable to think that there is a washout of the contents of the nuclear cisterns. Eventually the nuclear envelope regains integrity *in vitro* after repair of the external membrane of the envelope. However, the solution inside the nuclear cavities is the same high chloride solution used to preserve isolated nuclei.

The mechanism by which intracellular chloride modulates channel activity is unclear, but an intriguing possibility is that it may promote aggregation of channels into multimeric units. We frequently observed multiple conductance states when the inner face of the membrane under study was exposed to a high chloride concentration. At times during these studies it became difficult to distinguish individual single-channel openings, and some of our recordings suggest that single proteins may be combining to form a larger conductance channel. As we reported previously, conductance was not the only property modified by intracellular chloride. The nonlinearity of the voltage dependence of the channel was altered, suggesting that chloride also modifies the gating properties of the channel protein. This observation and our finding that the addition of a small peptide 'tag' to the amino or carboxyl terminus of the NCC27 protein also alters the rectification properties of the channel both support our hypothesis that the NCC27 protein forms either all or a structural component of the ion channel.

The most direct evidence for the latter conclusion is provided by the antibody studies. The monoclonal antibody directed at the FLAG epitope tag rapidly inhibits the chloride conductance, but only in situations where it has access to the FLAG epitope (i.e., only if the tag is facing outward into the buffer containing the antibody). We conclude from this observation that NCC27 must be a transmembrane protein that directly forms part of the ion channel.

Furthermore, based on our observations of the various combinations of amino-tagged and carboxyl-tagged proteins using inside-out and outside-out configurations, we conclude that the amino terminus projects 'extracellularly' and the carboxyl terminus is directed inwardly, at least when NCC27 channels are expressed on the plasma membrane. If we assume an alpha-helical structure for the NCC27 ion channel, the only putative transmembrane segment is that lying between about amino acids 25–46 (see ref (5). This would leave only a short region of ~24 amino acids lying exterior to the membrane and the bulk of the protein projecting intracellularly (or into

the nucleus, as the case may be). By analogy with other well-described ion channel proteins, the small size and the presence of only a single transmembrane domain on NCC27 suggest that it normally coassembles as multimers to form functional ion channels. Our ability to label NCC27 with a peptide to which a monoclonal antibody is available has provided us with a channel 'blocker' of far greater specificity than existing chloride channel-blocking agents. We are unaware of previous reports of the use of this methodology in the elucidation of transfected ion channel behavior. FJ

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