Molecular Cloning and Expression of a Chloride Ion Channel of Cell Nuclei*

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Ion channels are known to be present on the plasma membrane of virtually all cells and have been found on the membranes of various intracellular organelles. However, until recently they were believed not to occur at the nuclear membrane. In this study we describe the molecular cloning and characterization of a nuclear ion channel protein, designated nuclear chloride channel-27 (NCC27), from the human myelomonocytic cell line, U937. NCC27 is a novel chloride ion channel protein that was found to localize principally to the cell nucleus. Its only known homologue is a bovine chloride ion channel protein (p64) believed to localize to internal organelles. NCC27 therefore represents the first human member of a new class of organellar chloride ion channel proteins.

Whereas ion channels have been found on the membranes of various intracellular organelles, it has only been recently that patch clamping studies have suggested their existence at the nuclear membrane. The nuclear pore complexes have been considered the site of communication and exchange between the nucleus and cytoplasm (1, 2). Studies of traffic across the nuclear envelope have in general conformed to the paradigm that ions and small metabolites with diameters of less than 3–4 nm passively diffuse across the nuclear envelope (3). Thus, the concept of nuclear membrane ion channels seems at variance with the generally accepted views of the nuclear envelope, with this fact.

The first demonstration of ionic conductances in the nuclear membrane were in mouse zygote pronuclei (4). Further evidence then followed with the demonstration of ion-selective channels in avian erythrocytes (5), in mouse oocyte germinal vesicles, in nuclei from two-cell embryos and liver (6), in the nuclei of cardiac myocytes (7), and in rat hepatocyte nuclei (8). Mak and Foskett (9) described the presence of inositol 1,4,5-trisphosphate-dependent receptor channels in isolated nuclei which were activated by inositol 1,4,5-trisphosphate, inhibited by heparin and selective to calcium ions. Similarly, Pasyk and Foskett (10), have shown chloride channel activity in isolated nuclei from CHO1 cells. More recently, following fractionation and reconstitution of inner and outer nuclear membrane fractions into lipid bilayers, Rousseau et al. (11) have shown the presence of two types of chloride channels. The use of calcium ion imaging techniques has also demonstrated variations in calcium ion concentrations between the nucleus and cytoplasm, again suggesting a selective uptake or retention of these ions by the nucleus (12, 13).

With growing electrophysiological data for the existence of nuclear ion channels, it is clear that the cloning and isolation of these proteins will greatly assist in determining their structure and function. This paper describes the molecular cloning and characterization of what we believe to be the first chloride ion channel protein of the nuclear membrane and only the second cloned ion channel found at this location. As part of a project investigating gene expression in “activated” monocyte cells, a subtracted cDNA library designed to enrich for activation-associated genes was prepared using U937 cells. U937 cells were used to generate the cDNA library, since they are a human histiocyte lymphoma cell line with monoblastic characteristics that can undergo in vitro differentiation into a cell with monocyte-like characteristics (14). Exposure of U937 cells to various factors including all-trans-retinoic acid (RA) or interferon-γ, can induce this change. These differentiated cells can be activated with phorbol 12-myristate 13-acetate (PMA), much like normal monocytes.

Screening of the subtracted cDNA library for genes associated with activation resulted in the isolation of clone 4, which was found to code for a novel 241-amino acid protein, designated nuclear chloride channel-27 (NCC27). It is homologous to a cDNA clone, BOVCCP64A (GenBank™ accession number L16547), which codes for a bovine chloride channel protein, p64, believed to localize to internal organelles (15). NCC27 is a novel chloride channel protein that localizes principally to the nucleus of all cells studied. Patch clamp studies of CHO-K1 cells transfected with NCC27 revealed an increased level of chloride ion channel activity at their nuclear membrane. NCC27 was found to be widely expressed in various cells and cell lines, and its gene expression was regulated by activation stimuli.

Experimental Procedures

Subtraction Library Production—A subtraction library was constructed using U937-derived mRNA. The driver cDNA library (U937-RA) was synthesized from U937 differentiated with 1 μM RA for 3 days,

* The abbreviations used are: CHO, Chinese hamster ovary; RA, all-trans-retinoic acid; PMA, 12-myristate 13-acetate; FCS, fetal calf serum; IL, interleukin; MOPS, 4-morpholinepropanesulfonic acid; GST, glutathione S-transferase; NCC27, recombinant NCC27; PBS, phosphate-buffered saline; kb, kilobase(s).

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and the tester cDNA library (U937-RT) was synthesized from U937 cells treated for 3 days with 1 μM RA, followed by 160 nM PMA activation for 3 h. The CDNA was cloned into the EcoRI site of the ZAP II vector (Stratagene) using the ZAP-cDNA Gigapak II Gold Cloning Kit (Stratagene). Each library was then converted to pHuscript (Pharmacia) via plasmid cloning. A second construct (clone 4FLAG) was also cloned into pReCMV vector, which incorporated the sequence for the 8-amino acid FLAG peptide (DYKDDDDK) at the carboxyl terminus of the recombinant protein NCC27. The insert-containing pReCMV constructs were then transfected into monolayers of CHO-K1 cells. CHO-K1 cells (80% confluent) were transfected for 24 h in 35-mm dishes using 9 μl of Lipofectamine reagent (Life Technologies, Inc.) and 1 μg of DNA, as recommended by the manufacturer. Stable transfectants were selected with 1000 μg/ml G418 (Boehringer Mannheim). Subclones were isolated and then screened by immunofluorescent staining using anti-m2 (anti-FLAG) monoclonal antibody (IBI) and anti-NCC27 polyclonal antiserum.

**Immunocytochemistry and Antibody Blocking Study—**Cells were seeded in 8-well glass chamber slides (NUNC) one day prior to staining, washed twice with PBS, and either fixed and permeabilized for 15 min at room temperature in PBS containing 3.5% (v/v) formaldehyde and 0.1% (v/v) Triton X-100 for internal cell staining or fixed with 4% paraformaldehyde for 30 min for surface staining. The cells were stained with primary antibody for 30 min at 4 °C. Antibodies used were anti-FLAG antibody (1:1000 dilution), control class-matched monoclons antibody, anti-CD4 (OKT4) (550 mg/ml), a gift from Dr. Margaret Cooley (CFI, Australia) (1:50), anti-NCC27 rabbit polyclonal antisera (1:1000), and control preimmune rabbit sera (1:1000). A further control of anti-NCC27 polyclonal antibody (1:2000), which had been preincubated for 30 min at room temperature with 50 μg of purified rNCC27 protein, was also included. Following the reaction with primary antibody, the cells were washed in PBS and then incubated with secondary sheep anti-mouse or sheep anti-rabbit fluorescein isothiocyanate-conjugated polyclonal antibody (Silenus Laboratories, Australia) for 45 min at room temperature. Cells were then mounted and viewed using UV fluorescence microscopy, with excitation at 488 nm and by confocal microscopy using a Sarastro 2000 CLSM (Molecular Dynamics), with a plan apochromat × 60/1.40 NA oil immersion lens and an argon-ion laser (Leica, B.V.).

**Western Blotting—**SDS-polyacrylamide gel electrophoresis and Western blotting were performed by standard techniques (23). Cells were solubilized on ice for 15 min, in 1 × lysis buffer (50 mM Tris-HCl, pH 7.0, 0.5% (v/v) Nonidet P-40, 3.7% (w/v) EDTA) including leupeptin and phenylmethylsulfonyl fluoride (Boehringer Mannheim). Samples were clarified by centrifugation at 10,000 × g for 5 min. 6 × SDS sample buffer (9 M urea, 6.8% SDS, 10% Tris, pH 6.8, 0.8% glycine, and 0.0125% (v/v) bromphenol blue) was then added to protein from 1 × 10^6 cells and loaded onto a 15% SDS-polyacrylamide gel (Bio-Rad, Australia). This was followed by tank electrotransfer of the samples to nitrocellulose membrane (Bio-Rad). Western blots were blocked for 1 h at 37 °C (or overnight at 4 °C) with 3% (w/v) bovine serum albumin, 0.05% (v/v) Tween-20 in PBS and then probed for 30 min at room temperature using either anti-NCC27 (1:2000) or anti-FLAG antibody (IBI) (1:1500). Control antibodies were anti-GST polyclonal (1:5000) (AMRAD-Pharmacia) and preimmune rabbit sera (1:2000). After washing, secondary biotin-labeled anti-mouse Ig or anti-rabbit Ig polyclonal antibodies were applied (1:2000) (Amersham) for 30 min at room temperature, after which time blots were again washed. This was followed by a 30-min incubation at room temperature with streptavidin-horseradish peroxidase-conjugated horseradish peroxidase complex (1:2000) (Amersham). After final washing, the staining was visualized using Renaissance chemiluminescence reagent (DuPont) according to the manufacturer's instructions and exposure of the blots to XAR-5 x-ray film (Kodak).
NONIDET P-40. Following lysis, the nuclei were centrifuged through a 30% sucrose cushion at 800 × g for 2 min to remove the remaining Optiprep. The pellet was resuspended in 200 μl of hypotonic buffer and freeze-thawed three times, and the insoluble material was washed three times. The insoluble nuclear fraction was resuspended in 100 μl of hypotonic buffer containing 1 unit of DNase I and incubated for 30 min at 37 °C. The insoluble fraction was solubilized in nonreducing SDS electrophoresis sample buffer and separated on a 15% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with polyclonal anti-NCC27 antibody as described above.

Electrophysiological Recording—Single ion channel activity in isolated nuclei from CHO-K1 were recorded using a "nucleus-attached" configuration at room temperature (23 °C) (4, 25). In other experiments, whole-cell and single-channel currents were recorded from the plasma membrane of intact CHO-K1 (26). Patch pipettes with a tip opening of 30-40 μm were used, and associated A/D hardware (pClamp 6.0/Digidata 1200, Axon Instruments Inc. and Scientific Solutions Inc.). Single-channel open probability was determined from steady-state recordings of 2-min duration, with the nucleus clamped at the resting membrane potential, and quantified as follows,

\[ P_o = \sum_{m=1}^{M} \sum_{n=1}^{M} M \cdot P_\text{m} \]  

\[ (\text{Eq. 1}) \]

where \( P_o \) represents single-channel open probability, \( M \) is the apparent number of channels in the patch, \( m \) is each channel in the range 1 to \( M \), and \( P_\text{m} \) is the open probability associated with the \( m \)th channel in the patch. The apparent number of channels in each patch was determined as the number of discrete levels of current observed in the patch clamp recordings. The values for \( P_o \) were obtained as the area under the curve for gaussian curves fitted to each of the channel peaks in amplitude histograms constructed for each patch (28). All data are presented as the mean ± S.E., with the number of different cells used to obtain the recordings indicated in parentheses (see "Results"). The solution used for superfusion of the intact CHO-K1 contained 180 mM NaCl, 4.8 mM KCl, 1.2 mM MgCl2, 1.2 mM NaH2PO4, 10 mM HEPES, 12.5 mM glucose, 1 mM CaCl2, bovine albumin (0.5 mg/ml, fraction V, Sigma), pH 7.4, with NaOH. The solution used for superfusion of the isolated nuclei contained 140 mM KCl, 1.2 mM MgCl2, 5 mM EGTA, and 10 mM HEPES, pH 7.2, with KOH. The same solution was used to fill the recording pipette for both the "nucleus-attached" and whole-cell recordings. In separate experiments using the whole-cell configuration and clone 4-transfected CHO-K1 cells to determine the permeability of the channels to different anions, the chloride in the superfusing solution was substituted for nitrate, isothiocyanate, iodide, acetate, bicarbonate, sulfate, or fluoride. The permeability ratio of the anion (X−) relative to Cl− (PX/PCl) was calculated using the Goldman-Hodgkin-Katz equation from the resultant shift in reversal potential of the IV curves.

RESULTS

Nuclear Ion Channel

Isolation of Nuclei for Electrophysiological Studies—Nuclei were isolated based on the method of Wotton et al. (24). Briefly, confluent CHO-K1 were detached with 0.02% EDTA and then washed twice in ice-cold PBS. Cells were resuspended in 2 ml of the nuclear suspension underlaid with 2 ml of 30% Optiprep, followed by 1 ml of 35% Optiprep. The sample was centrifuged for 40 min at 10,000 × g at 4 °C, and the nuclei were collected at the 25–30% interface. The nuclear suspension was diluted 4-fold in hypotonic buffer and centrifuged at 800 × g for 2 min to remove the remaining Optiprep. The pellet was resuspended in 200 μl of hypotonic buffer and freeze-thawed three times, and the insoluble material was washed three times. The insoluble nuclear fraction was resuspended in 100 μl of hypotonic buffer containing 1 unit of DNase I and incubated for 30 min at 37 °C. The insoluble nuclear fraction was solubilized in nonreducing SDS electrophoresis sample buffer and separated on a 15% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with polyclonal anti-NCC27 antibody as described above.

Electrophysiological Recording—Single ion channel activity in isolated nuclei from CHO-K1 were recorded using a "nucleus-attached" configuration at room temperature (23 °C) (4, 25). In other experiments, whole-cell and single-channel currents were recorded from the plasma membrane of intact CHO-K1 (26). Patch pipettes with a tip opening of between 0.9 and 1.5 μm (27) and impedance of 3–7 megaohms were fabricated from thin walled borosilicate glass (Vitrex Microhematocrit Tubes, Denmark). The reference electrode was connected to the super- fusate bath with a KCl salt bridge for experiments in which the Cl− was either reduced in concentration or substituted by a different anion. The channel currents were amplified and filtered at 1 kHz (−3 db point) using an Axopatch 1D amplifier (Axon Instruments) and sampled on line by a microcomputer (IBM 486 compatible) using commercial software and associated A/D hardware (pClamp 8.0/Digidata 1200, Axon Instruments Inc. and Scientific Solutions Inc.). Single-channel open probability was determined from steady-state recordings of 2-min duration, with the nucleus clamped at the resting membrane potential, and quantified as follows,

\[ P_o = \sum_{m=1}^{M} \sum_{n=1}^{M} M \cdot P_\text{m} \]  

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RESULTS

Nuclear Ion Channel

Isolation and Characterization of Clone 4—A 1223-base pair cDNA clone (clone 4), was isolated from the U937 subtracted cDNA library, and comparison of its DNA sequence to the GenBankTM and EMBL data bases revealed significant homology to a bovine chloride channel clone, BOVCCP64A (15), of 1561 base pairs in size (full-length cDNA, 6160 nucleotides). The longest open reading frame of clone 4 extends from nucleotide 219 to 944 (Fig. 1A). The initiation codon is flanked by sequences that concur with the consensus sequence for the initiation of translation (29).

mRNA Expression—Northern blots of total RNA from various cell lines and cell lines were probed using the complete clone 4 insert. This resulted in the detection of two mRNA species of approximately 1.2 and 1.0 kb. In the monocytoid cell line U937, clone 4 mRNA is up-regulated by PMA after 24–48 h, with a disproportionate increase in expression of the 1.2-kb band compared with the 1.0-kb band. This response could be further enhanced by pre-exposure of the cells to RA (Fig. 2A). This response appears to be PMA-specific, since lipopolysaccharide had no noticeable effect (data not shown). Similarly, pretreatment of human culture-derived macrophages with interferon-γ for 9 h followed by IL-2 for 3 h, up-regulates the 1.2-kb band with no detectable 1.0-kb band expression (Fig. 2B).

To determine whether the two mRNA transcripts represented two closely related genes or alternate products of the same gene, Northern blots of total RNA from U937 cells were probed separately with the three EcoRI restriction digest-generated fragments from clone 4 (Fig. 1A). All three fragments were found to hybridize to both mRNA transcripts. The Northern blots were then probed with two shorter fragments generated by PCR, which represent the 5′- (primers T3.4 and T7.6) (Fig. 1A) and 3′- (primers T3.5 and T7.7) (Fig. 1A) untranslated sequences of clone 4. It was found that only the 3′ probe detected both mRNA species, while the 5′ probe only detected the 1.2-kb species. It was therefore inferred that the two mRNA transcripts varied in at least the first 120 base pairs of the longer species. This would suggest that the two transcripts are due either to alternate transcription start sites or alternate transcripts.

Clone 4 mRNA was found to be expressed in all of the following cells and cell lines, including the monocytoid cell lines U937, HL-60, THP-1, and KG-1, the mouse monocyte cell line RAW264.7, peripheral blood monocytes and lymphocytes, the B cell lines Daudi, Raji, and U266B1, and the T cell line HUT 78 as well as human umbilical vein endothelial cells, the fibroblast line CCD-365LU, and K562 cells (results not shown).

Predicted Amino Acid Sequence of the Clone 4 Translation Product, NCC27—The longest open reading frame of clone 4 codes for a polypeptide of 241 amino acids with a predicted molecular mass of 26,900 Da and pI of 4.85. Comparison of the predicted amino acid sequence of NCC27 with the available protein data bases NBRF and SwissProt again confirmed its close homology to the bovine chloride channel protein p64, with scores of 57% identity and 72% similarity. The 241 amino acids of NCC27 align with p64 at positions 194–437, which repre-
sent the carboxyl-terminal half of the protein p64 (Fig. 1B). Extensive searches and multiple alignment comparisons of these two protein sequences to other ion channel proteins revealed no similarities.

Hydrophobicity analysis (19) shows two strongly hydrophobic regions that are long enough to represent transmembrane domains, from amino acids 21–39 and 170–189 (Fig. 1A). Comparison of the hydrophobicity plots for NCC27 with p64 reveals a similar pattern of hydrophobic domains, with the two longest regions found at the same relative position in both proteins (Fig. 1C). Furthermore, analysis of NCC27 for possible motifs revealed two putative nuclear localization sequences, KRR and KKYR, at positions 49–51 and 192–195, respectively (Fig. 1A).

There is one putative N-glycosylation site at asparagine 42, one cAMP phosphorylation site at threonine residue 49, five possible casein kinase II phosphorylation sites, four putative protein kinase C phosphorylation sites at serines 27 and 163 and threonines 48 and 77, and five possible N-myristoylation sites.
Expression and Localization of NCC27 in CHO-K1 Cells—To localize NCC27, CHO-K1 cells transfected with clone 4, the clone4FLAG construct, or the vector alone were stained using anti-FLAG. The antibody showed bright staining of the clone4FLAG-transfected cells, while there was no visible staining in the other two transfectants (Fig. 3). The antibody staining was brightest throughout the nucleus, including the nuclear membrane, with a small amount of staining also apparent in the cytoplasm and on the plasma membrane (Fig. 3). Staining of fixed but not permeabilized cells with the anti-FLAG antibody revealed a pattern of staining of the clone4FLAG-transfected cells, suggestive of surface membrane distribution (Fig. 3). There was no staining seen in the control-transfected cells (Fig. 3).

The three CHO-K1 transfectants discussed above were also visualized using the polyclonal antibody raised to the GST-NCC27 fusion protein. This showed binding to the nucleus and nuclear membrane in all three of the transfectants (Fig. 3) as well as nontransfected CHO-K1 (data not shown). This suggested constitutive expression of the native protein in transfected and nontransfected cells. A control anti-GST antibody (data not shown) and rabbit preimmune sera showed no comparable staining of any of the cells (Fig. 3). Blocking studies undertaken by preincubation of the anti-NCC27 antibody with purified rNCC27 protein confirmed the specificity of the antibody with no binding being observed in cells stained with blocked anti-NCC27 (Fig. 3).

To provide further evidence supporting the presence of NCC27 on the nuclear envelope, a crude nuclear membrane fraction was analyzed. Purified nuclei were freeze-thawed to disrupt the membranes of the nuclear envelope and washed to remove any remaining nucleoplasmic contents. This fraction is expected to contain the inner and the outer nuclear envelopes, along with the nuclear pore complexes, and the underlying nuclear lamina. NCC27 was found to be present in this insoluble nuclear material (Fig. 4A). This is consistent with the

**Fig. 2.** A, Northern blot analysis of total RNA from U937 (20 μg/lane) from nontreated and treated U937. Blot a, probed with clone 4; blot b, probed with an oligonucleotide to 28 S ribosomal RNA. Lane 1, nontreated; lane 2, RA for 1 day; lane 3, RA for 2 days; lane 4, RA for 3 days; lane 5, RA for 1 day and PMA for 12 h; lane 6, RA for 2 days and PMA for 12 h; lane 7, RA for 3 days and PMA for 12 h; lane 8, RA for 4 days and PMA for 12 h. B, Northern blot analysis of total RNA from human peripheral blood monocytes cultured for 5 days in serum-free media (20 μg/lane). Blot a, probed with clone 4; blot b, probed with an oligonucleotide to 28 S ribosomal RNA. Lane 1, nontreated; lane 2, INFγ for 9 h; lanes 3–7, all cells pretreated with INFγ for 9 h followed by treatments as listed (IL-2 for 3 h (lane 3), IL-4 for 3 h (lane 4), and IL-4 for 9 h (lane 5)). C, Northern blot analysis of total RNA (10 μg/lane) from transfected and nontransfected CHO-K1. Lane 1, control nontransfected CHO-K1; lane 2, CHO-K1 transfected with Clone4FLAG.
occurrence of either a transmembrane form of the protein or its association with a multiprotein channel complex.

To further examine NCC27 protein expression in CHO-K1 cells, Western blots of whole cell lysates were probed with anti-NCC27 antibody. This showed the presence of a 27-kDa protein in all samples including U937 (Fig. 4A) and nontransfected CHO-K1 (data not shown). Identical blots stained with the anti-FLAG antibody showed binding to a similar sized protein band in only those CHO-K1 cells transfected with the clone4FLAG construct (Fig. 4B). Identical blots probed with control preimmune sera and anti-GST polyclonal antibody showed no specific binding (data not shown).

To further confirm that the purified rNCC27 protein was the same as the 27-kDa protein detected by Western blot analysis of U937 whole cell lysates, CNBr cleavage was undertaken. Since the predicted NCC27 protein sequence encodes only two methionine residues, with the second at amino acid position 32, CNBr cleavage would result in a molecular weight reduction to 23.5 kDa. Samples of both purified rNCC27 and soluble protein from U937 whole cell lysates were CNBr-treated. The resultant products analyzed by Western blotting clearly indicated that the macroscopic ion currents (ordinate) in response to variations in the potential at which the cell was voltage-clamped (abscissa), A, current-voltage relation of the ion currents in a CHO-K1 cell transfected with clone 4 (○). Dilution of the ion gradient across the cell (■) shifted the equilibrium potential of the current-voltage relation from 0 mV to +25 mV, which was toward the Nernst potential for Cl− under the experimental conditions, indicating that the macroscopic currents were carried by Cl− ions. B, control CHO-K1 cell transfected with vector only (○) showed only extremely small currents, while dilution of the gradients across the cell (■) did not shift the equilibrium potential of the current-voltage relation, confirming these as leakage currents, not carried by ion channels.

To confirm that NCC27 was constitutively expressed in CHO-K1 cells, Northern blots of total RNA from both clone 4-transfected CHO-K1 and nontransfected control CHO-K1 were probed with the entire clone 4 insert. Both samples were found to express the clone 4 mRNA 1.2-kb transcript (Fig. 2C). This was in keeping with the studies using the antibody to rNCC27, which suggested that native protein as well as recombinant protein were detected in the clone 4-transfected cells.

**Electrophysiological Characterization of NCC27—** Ionic currents in both clone 4-transfected and vector only control-transfected CHO-K1 were recorded using patch clamp electrophysiology. Using the whole-cell configuration, the macroscopic conductance of the cell membrane of intact cells was 4.6-fold greater in clone 4-transfected cells (3.70 ± 0.06 nanosiemens, n = 14) compared with the vector only control-transfected cells (0.81 ± 0.06 nanosiemens, n = 3). Single-channel recordings of the clone 4-transfected cells revealed single channels with a conductance of 22 ± 5 picosiemens (n = 6, 95% confidence interval, 9–35 picosiemens; Fig. 6). Thus, transfection of CHO-K1 with clone 4 introduced ion channel activity that was not normally present at the plasma membrane of these cells. This was found to be due to the activity of chloride ion channels, since simultaneous dilution of the extracellular cations and anions shifted the reversal potential of the current-voltage relation toward the Nernst potential for Cl− (Fig. 5). Furthermore, the chloride channels were permeable to other anions to varying degrees. The permeability sequence, with the relative permeability to Cl− in parentheses, was as follows: SCN−...
(1.45) > F\(^-\) (1.28) > Cl\(^-\) (1.0) > NO\(_3\)\(^-\) (0.82) ~ I\(^-\) (0.81) = HCO\(_3\)\(^-\) (0.81) > acetate (0.67).

Patch clamp recordings were also undertaken of intact isolated nuclei from both clone 4- and vector only control-transfected cells. The isolated nuclei, were seen as intact spheres when viewed under high power phase contrast microscopy. Some nuclei showed varying degrees of cytoplasmic debris associated with them. Only those nuclei that showed a sharp outline were chosen for the patch clamp experiments. Single-channel recordings of the nuclear membrane obtained from clone 4-transfected CHO-K1 revealed single channels with a conductance of 33 ± 4 picosiemens (n = 6, 95% confidence interval, 23–43 picosiemens). The conductance of these channels was not significantly different from those recorded from the plasma membrane (p = 0.12, not significant, Student’s t test). Although electrophysiologically similar channels were also present in the nuclear membrane of control CHO-K1 transfected with the expression vector only, their activity was 9.5-fold less than in the clone 4-transfected cells (Fig. 6). Specifically, the single-channel open probability for clone 4-transfected cells was 0.19 ± 0.08 (n = 7) compared with 0.02 ± 0.02 (n = 5) in cells transfected with the vector only (p = 0.02, Mann-Whitney test). Furthermore, in none of the vector-only transfections was there more than one level of single-channel current transitions, whereas in the nuclei from clone 4-transfected cells, integral multiples of the transition level were commonly observed, indicating the presence of several active ion channels in the patch of membrane sealed by the recording micropipette.

**DISCUSSION**

Several lines of evidence indicate that NCC27 acts as an ion channel or forms part of an ion channel complex. These include its protein structure, which is consistent with that of other ion channel proteins, its strong homology to p64, a bovine chloride channel protein of internal organelles (15), and electrophysiological and immunohistochemical studies of transfected CHO-K1 showing plasma membrane and nuclear membrane chloride ion channels.

NCC27 localizes principally to the nucleus, including the nuclear membrane, of both transfected and control-transfected CHO-K1, although small amounts were also noted in the cytoplasm and on the cell membrane. Patch clamp studies of isolated nuclei from transfected and control-transfected cells revealed chloride ion channels at the nuclear membrane with significantly more activity noted in the nuclei of clone 4-transfected cells. The detection of chloride channel activity in the nuclei of control-transfected cells coincides with the immunofluorescent staining and Western blot studies, which indicate that NCC27 is constitutively expressed in CHO-K1. Analysis of the NCC27 putative protein sequence revealed two nuclear localization motifs that may be acting as the target sequences for the migration and retention of NCC27 in the nucleus.

The ion channel activity detected in clone 4-transfected cells at their plasma membrane is likely to be largely due to spillover resulting from overexpression of the recombinant protein, since control-transfected and nontransfected CHO-K1 showed no ion channel activity at this location. This, nonetheless, proved useful for electrophysiological studies, since the channel-forming properties of NCC27 appeared to be the same at both locations, with the conductance of single channels at the cell membrane found to be not significantly different from that of the single channels recorded from the nucleus. This allowed initial characterization of the channel to be carried out at the plasma membrane, which is more easily accessible.

The immunohistochemical staining pattern observed in the CHO-K1 also revealed some cytoplasmic staining, suggesting...
that NCC27 may also be localizing to other intracellular organelles, as was reported for p64, where staining was noted in association with the Golgi and other compartments toward the periphery of the cell (30).

NCC27 and p64 are not homologous to any other ion channel proteins in the protein data bases, which would suggest that together they constitute a new class of ion channel proteins associated with internal organelles. NCC27 is not, however, the human homologue of p64, since Northern blot analysis of mRNA from human tissue reveals that the apparent human homologue of p64 has an mRNA of 6.5 kb (15) whereas NCC27 mRNA is only 1.2 kb. Furthermore, bovine p64 is a much larger protein (64 kDa) than NCC27 (27 kDa), and the amino-terminal half of p64 bears no homology to NCC27.

Our electrophysiological data also support the hypothesis that NCC27 and p64 constitute a new class of ion channel proteins. For example, the anion permeability sequence of the NCC27 chloride channel differs from that of the cystic fibrosis transmembrane regulator chloride channel (31) and other chloride channels that are associated with secretion in exocrine glands (32, 33, 34, 35). Furthermore, a chloride channel with a single channel conductance of 30 picosiemens from the inner recently been described (11).

The presence of free NCC27 protein in the nucleoplasm is a puzzling observation and is unusual for an ion channel protein. Furthermore, it is unclear how the protein is regulated between these two states, in a free form in the nucleoplasm and then translocating to the nuclear membrane. One possibility is that insertion of NCC27 into the nuclear membrane is a calcium-dependent process, as occurs in the annexin family (36). Alternatively, NCC27 may be acting as a regulatory subunit of a multiprotein chloride ion channel complex and therefore itself might not represent the ion-conducting unit of the chloride channel complex.

The two mRNA transcripts detected by clone 4 are up-regulated by various activation stimuli, including PMA and IL-2, which also results in the preferential expression of the 1.2-kb transcript. This regulation was also found to be associated with the maturational state of the cell. For example, the response of U937 cells to PMA activation was enhanced in those cells that had prior exposure to RA (a known differentiating agent of monocytoid cells) compared with those that had no prior RA treatment. While the two mRNA transcripts of NCC27 are believed to be alternate transcripts or alternate start sites, they are unlikely to involve the coding region of the protein and thus have no effect on the structure of the ion channel protein itself. The sum total of this data suggests that an increased number of NCC27 channel protein molecules may in some way be required for cell activation.

Northern blot analysis of clone 4 also revealed that the gene is expressed in a wide variety of cells and cell lines of human origin as well as in the murine cell line RAW 264.7 and in CHO-K1. Furthermore, the fact that the anti-NCC27 antibody cross-reacts with native protein in CHO-K1 indicates that the protein is not only widely expressed throughout various tissues and cells but is also highly conserved across species.

The wide distribution of clone 4 mRNA and its regulation by activation stimuli, as well as the fact that the encoding protein localizes to the nucleus and nuclear membrane, suggest that NCC27 is involved in some basic biological function in all cells. Such a role could be involvement in the regulation of either cell replication or gene transcription, where alteration of the nuclear chloride ion concentration may either serve to limit or facilitate this process. Its localization to the nuclear envelope and function as a nuclear ion channel suggest that it would act to control the ionic concentrations in the perinuclear cisternae as well as controlling transmembrane potentials.

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Molecular Cloning and Expression of a Chloride Ion Channel of Cell Nuclei
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