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Biochimica et Biophysica Acta 1466 (2000) 105–114

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Modulation of volume regulated anion current by I_{Cln}

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Received 3 December 1999; received in revised form 10 February 2000; accepted 24 February 2000

Abstract

I_{Cln}, a cytosolic protein associated with a nucleotide-sensitive chloride current, may be involved in the regulation of a volume-regulated anion current (VRAC) associated with hypotonic cell swelling. We have determined the nucleic acid sequences of I_{Cln} from human tsA201a, colonic (T84) and myeloma (RPMI 8826) cell lines. The amino acid sequences are highly homologous ($\geq 99\%$) to each other but less homologous to I_{Cln} protein from other species. Using the whole-cell patch clamp technique, we examined the effect of I_{Cln} protein expression levels on VRAC properties during a hyposmotic challenge. Overexpression of T84 or RPMI 8226-derived I_{Cln} protein in tsA201a cells results in a more than 9-fold increase in the rate of VRAC activation over control values, while having no effect on VRAC inactivation properties. Underexpression of endogenous I_{Cln} protein in tsA201a cells using antisense oligonucleotides results in a more than 180-fold decrease in VRAC activation rate as compared to control values. These results indicate that I_{Cln} protein expression modulates VRAC activation but not inactivation.

Keywords: I_{Cln}; Volume-regulated anion current (VRAC); Cell volume regulation; Regulatory volume decrease

1. Introduction

Activation of a volume-regulated anion current (VRAC) during hypotonic swelling occurs in many cell types, although the regulation of VRAC is poorly understood. One protein thought to be associated with regulation of cell swelling is I_{Cln} (protein associated with a nucleotide-sensitive chloride current). I_{Cln} protein is a small, 27 kDa, ubiquitously expressed cytosolic protein whose function in volume regulation remains controversial. Originally cloned in MDCK cells, I_{Cln} was initially thought to be a VRAC based on protein expression in *Xenopus* oocytes [1], although this is no longer the case (see [19] for review).

Studies suggest I_{Cln} protein expression levels may contribute to volume regulatory processes by modulating a volume regulated anion current [2,3]. Underexpression of I_{Cln} protein using antisense oligonucleotides inhibits VRAC activation in bovine nonpigmented ciliary epithelial cells and fibroblasts while I_{Cln} protein overexpression increases VRAC

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0005-2736/00/$ – see front matter
PII: S0005-2736(00)00177-2

activation rate during cell swelling [3,4]. Other recent studies suggest I_{Cln} modulation of VRAC may occur indirectly, through the regulation of cellular processes such as transcription, translation, second messenger cascades and cytoskeletal rearrangement [5]. Alternatively, Li et al. report that I_{Cln} protein insertion in lipid bilayers results in a cation-selective current [6].

The data reported here support the hypothesis that I_{Cln} protein expression level modulates the activation of VRAC, although the properties of voltage-dependent inactivation are not affected. We have determined the amino acid sequences of I_{Cln} from human tsA201a (epithelial), T84 (colon) and RPMI 8226 (myeloma) cells, which are $\geq 99\%$ homologous to other human sequences and display 90% and 92% homology to rat and MDCK I_{Cln}, respectively. I_{Cln} protein expression levels (of either T84 or RPMI origin) modulate VRAC activation in response to cell swelling in tsA201a cells. Overexpression of T84 or RPMI 8226-derived I_{Cln} protein increased the rate of VRAC activation and current amplitude in tsA201a cells, while underexpression of endogenous I_{Cln} protein in tsA201a cells inhibited the rate of VRAC activation. VRAC inactivation properties are *independent* of both protein expression and protein sequence. There was no indication that an increase in I_{Cln} protein expression resulted in the expression of a cation current. Some of the work presented here has been previously presented in abstract form [7–9].

2. Materials and methods

2.1. Cell lines and tissue culture

tsA201a cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine sera (FBS, Gibco BRL), myeloma (RPMI 8226) were grown in suspension with RPMI 1640 supplemented with L-glutamine (300 mg/l) and 20% FBS (Cell Grow of Fisher Scientific/Mediatech, Washington, DC, USA) and T84 epithelial cells were grown in DMEM/F12 (Gibco BRL) and 5% FBS. All media contained penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco BRL) and cells were fed and/or split every 2–3 days.

2.2. Isolation of I_{Cln} cDNA

Total RNA was isolated using a MicroRNA isolation kit from Stratagene (La Jolla, CA, USA) and treated with RNase-free Dnase (RQ1, Promega, Madison, WI, USA) for 30 min at 37°C to remove any genomic DNA contaminants. Reverse transcription (RT) was conducted using random hexanucleotide primers. I_{Cln} cDNA was amplified by PCR (GeneAmp RNA PCR kit, Perkin Elmer, Branchburg, NJ, USA) using primers complementary to the 5′ and 3′ regions of the MDCK I_{Cln} sequence: upstream primer: 5′-CCG GAT CCA TGA GCT TCC TCA AAA GT-3′, downstream primer: 5′-CCG AAT TCG TGA TCA ACA TCT GCA TC-3′.

First strand cDNA was synthesized using 1 μg total RNA and MuLV reverse transcriptase (Perkin Elmer, Branchburg, NJ, USA) in a 100 μl PCR reaction consisting of: 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$ and 15 pmol of each primer. Amplification was carried out using the following protocol: 1 cycle at 95°C for 5 min; 35 cycles at 94°C for 1 min, 64°C for 1 min and 72°C for 4 min; 1 cycle at 72°C for 10 min. The RT-PCR product was run on a 1.0% agarose gel and visualized using ethidium bromide (10 mg/ml). A band of the expected size (715–730 bp) was observed.

The PCR product was ligated into the 5 kb pCR 3.1-Uni or -Bi vector (Invitrogen, Carlsbad, CA, USA) in a 10 μl reaction containing: 120 ng I_{Cln} PCR product, 60 ng pCR 3.1 vector, 1 μl of T4 DNA ligase, 1 μl of 10× ligation buffer and sterile water and incubated overnight at 14°C. The pCR 3.1 vector was subsequently transformed into TOP 10F′ One Shot cells (Invitrogen, Carlsbad, CA, USA) in a reaction consisting of: 2 μl β-mercaptoethanol, 20 ng ligation reaction product and 50 μl One Shot cells and incubated on ice for 30 min. Cells were heat-shocked for 30 s (42°C), cooled on ice, added to SOC (450 ml) media and placed in a shaking incubator for 1 h (37°C). The solution (50–200 μl) was spread onto LB agar plates pre-treated with ampicillin (50 μg/ml) and colonies were grown overnight in a 37°C incubator. Five to ten colonies were selected, added to TYP media and placed in a shaking incubator (37°C) for 14–16 h. Small quantities of the

pCR 3.1 vector (containing I_{Cln} cDNA insert) were isolated from successfully transformed bacterial cells using Wizard Plus Miniprep kit (Promega) and restricted with *Eco*RI, run on a 1.0% agarose gel and visualized using ethidium bromide (10 mg/ml). Restriction enzyme digestion resulted in the expected 5 kb (pCR 3.1 vector) and 715–730 bp (I_{Cln} cDNA) products. Once successfully transformed bacterial colonies were identified, large quantities of I_{Cln} cDNA were isolated using the Maxi-prep kit (5 Prime→3 Prime, Boulder, CO, USA).

2.3. DNA sequencing

I_{Cln} cDNA from tsA201a, T84 and RPMI 8226 cells was ligated in the pCR 3.1-Uni or -Bi vector as described previously and sequenced using Sequenase Version 2.0 DNA Sequencing Kit (USB, Cleveland, OH), based on the chain-terminating dideoxynucleotide method developed by Sanger et al. [10]. Synthesis is initiated by a primer designed to anneal to the M13 region of the pCR 3.1 vector and termination occurs when a 2′, 3′-dideoxynucleoside 5′-triphosphate lacking the 3′-OH is randomly incorporated into the growing nucleotide chain. A set of nested primers was designed to amplify the region between 300–500 bp. The nested primer sequences were as follows: upstream nested primer: 5′-GAT GGC TCT GGA TTA GGA TTCT-3′, downstream nested primer: 5′-TGT CCC CCT GTC CTT GTT CA-3′. [α-^{35}S]dATP is included in the reaction in order to visualize the products via autoradiography following polyacrylamide gel electrophoresis. Because of the extremely high homology of the human sequences, I_{Cln} cDNA from each cell type was sequenced more than once, either in our laboratory and/or at two different commercial sequencing facilities. Sequencing results from all sources agreed.

2.4. I_{Cln} over- and underexpression

tsA201a cells were split and transfected 24 h later with either RPMI 8226 or T84-derived I_{Cln} cDNA using the calcium-phosphate method [11]. Cells were co-transfected with either GFP or CD8 to identify successful transfectants [12]. GFP, isolated from the jellyfish *Aequorea victoria*, is a 27 kDa cytosolic protein which fluoresces when excited with UV light. CD8 is a 32 kDa cell-surface glycoprotein involved in antigen recognition in human T cells. CD8 co-transfected cells were identified using polystyrene beads coated with a primary monoclonal antibody specific for the CD8 antigen.

Underexpression of endogenous I_{Cln} protein was accomplished by transfecting tsA201a cells with a set of previously published antisense oligonucleotides using Lipofectin Reagent (Gibco BRL, Grand Island, NY, USA) [3]. The 31-mer is complementary to the first 31 nucleotides of the MDCK I_{Cln} sequence. A set of sense oligonucleotides consisting of the first 24 nucleotides of MDCK I_{Cln} was used as a control in the underexpression studies. Each base in both nucleotide sequences was phosphorothioated to deter degradation by intracellular nucleases. The sequences are as follows: antisense 5′-CGG CGG CGG GAA ACT TTT TGA GGA AGC TCA T-3′, sense 5′-ATG AGC TTC CTC AAA AGT TTC CCG-3′.

Lipofectin Reagent (Gibco BRL) is a liposome complex composed of the cationic lipid *N*-[1-(2,3-dioleyloxy)propyl]-*n*,*n*,*n*-trimethylammonium chloride (DOTMA) and dioleoly-phosphatidylethanolamine (DOPE) in a 1:1 ratio. A solution containing 1–2 μg cDNA, 200 μl serum-free DMEM and 10 μl Lipofectin Reagent was incubated for 45 min at room temperature. 0.8 ml of serum-free DMEM was added to the cDNA–liposome complex and this mixture was overlaid onto tsA201a cells and incubated for 7–10 h in a 5% CO_2 incubator maintained at 37°C. Mock-transfected cells were treated identically with the exception that cDNA was not added to the liposome complex. Following lipofection, cells were resuspended in 2 ml of serum-containing DMEM and split onto cover slips 24 h later.

2.5. Immunofluorescence

Immunofluorescence was used to confirm under- and overexpression of I_{Cln} protein in transfected tsA201a cells. Adherent cells were grown (tsA201a, T84) on cover slips coated with polylysine and fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS, pH 7.0), washed in PBS three times, 2 min each, permeabilized with 0.1% Triton in PBS, followed by 3% goat sera (Sigma, St. Louis, MO,

USA) in PBS for 2, 30 and 2 min. RPMI 8226 cells, which grow in suspension, were allowed to settle onto coverslips before fixation. Cells were incubated with polyclonal rabbit α-I_{Cln} (1:200, 60 min) followed by fluorescent (Cy-3)-labeled secondary antibody (30 min). All cells were washed in PBS (five times, 3 min each) and mounted using SlowFade (Molecular Probes, Lake Owsego, OR, USA) to prevent fading. Staining and fixing was performed at room temperature (22°C ± 2). Fluorescent images were taken using phase contrast microscopy with a Nikon Diaphot microscope. Fluorescence was quantified by taking images through a center plane of a cell and determining the mean pixel intensity per cell using Scion Image 1.57b. Polyclonal α-I_{Cln} antibodies were generous gifts from Dr. K. Strange and Dr. Jan Eggermont.

2.6. Solutions

The standard extracellular solution contains (in mM): 150 NaCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, pH 7.2. The intracellular solution was composed of (in mM): 150 Cs^+-glutamate, 10 HEPES, 0.1 $CaCl_2$, 1.1 EGTA, 4 ATP, pH 7.2 using CsOH. Cs^+-Glutamate was used in the intracellular solution to block K^+ currents.

The osmolality of the extracellular and intracellular solutions was determined prior to every experiment using a vapor pressure osmometer (Wescor, Logan, UT, USA). VRAC is activated only in the presence of a hyposmotic gradient. Sucrose was added to the intracellular solution so that it was 30–40 mOsm greater with respect to the bath solution. Relative osmolality is defined as: [(extracellular osmolality/intracellular osmolality) × 100]. A hyposmotic gradient of 90% was within physiological relevant range.

2.7. Electrophysiological recording and analysis

Macroscopic currents were recorded using the whole-cell patch clamp technique [13] and analyzed as previously described [14].

3. Results

3.1. Identification of I_{Cln} cDNA

Total RNA from tsA201a, T84 and RPMI 8226 cells was isolated and converted into cDNA. Primers (as described in Section 2) designed to anneal to the published MDCK I_{Cln} sequence were used to amplify a similar sequence from each cell line using polymerase chain reaction (PCR). PCR products were shown to be between 715–735 bp when visualized on a 1.0% agarose gel as expected. The I_{Cln} PCR product was ligated into the pCR 3.1-Uni or -Bi vector for DNA sequence analysis and transient transfections in tsA201a cells. Fig. 1 compares these sequences, translated into amino acids, with previously published human ocular (HuO), rat and MDCK I_{Cln}. I_{Cln} amino acid sequences from T84, RPMI 8226, tsA201a and HuO were ≥ 99% homologous with 90% and 92% homologous to rat and MDCK sequences, respectively.

3.2. Overexpression of I_{Cln} protein

The effect of I_{Cln} protein overexpression on VRAC properties was examined in tsA201a cells overex-

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                195     200   205                      220   225    230    235    240
        ......  |----|*---|          ...............   ---|*--*|----|---*|----|
CK      ......  SQSVSS-QYNM          ...............   YEDGMEVDTTPTVAGQFEDADVDH
RAT     ......  SQSVSS-QYNM          ...............   YEDGMEVETTPTVAGQFEDADVDH
HuO     ......  SQSVSSSQYNM          ...............   YEDGMEVDTTPTVAGQFQDADVDH
T84     ......  SQSVSS-QYNM          ...............   YEDGMEVDTTPTVAGQFEDADVDH
RPMI    ......  SQSVSS-QYNM          ...............   YEDGMEVDTTPTVAGQFEDADVDH
TSA     ......  SQSVSS-QYNM          ...............   YEDGTEVDTTPTVAGQFEDADVDH
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Fig. 1. tsA201a, T84 and RPMI 8226 I_{Cln} amino acid sequences. The C-terminus of amino acid sequences are compared to previously published human ocular (HuO), rat and MDCK sequences. Changes from the MDCK amino acid sequence are indicated by * and are bold in the rat sequence. Differences in the HuO sequence are indicated by open boxes whereas differences in amino acids in, RPMI 8226, T84 and tsA201a cell lines are shown by filled boxes. GenBank accession numbers are tsA201a: AF232708, RPMI 8226: AF232225 and T84: AF232224.

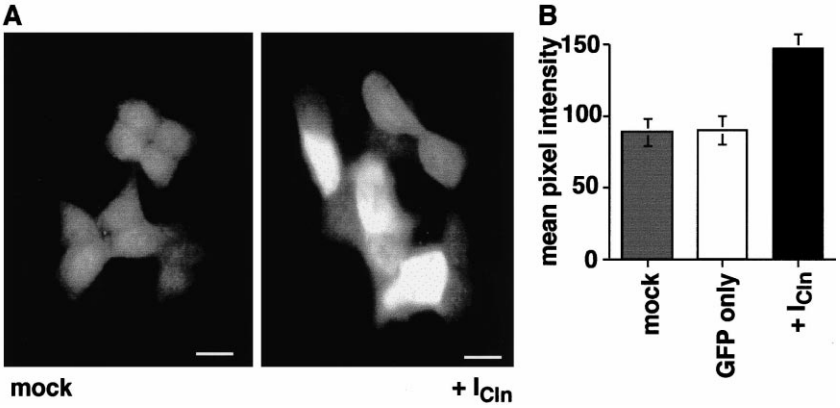


Fig. 2. Overexpression of I_{Cln} protein in tsA201a cells. (A) α-I_{Cln} followed by incubation with a fluorescent (Cy-3) secondary antibody demonstrates an increase in I_{Cln} protein expression in transfected cells expressing a co-expressed marker (GFP) as compared to mock transfected cells. Scale bar indicates 10 μm. (B) Mean pixel intensity (±S.E.M.) per cell under each condition. tsA201a cells co-transfected with GFP and T84 derived I_{Cln} cDNA. Cells were considered to be co-transfected if both red (Cy-3) and green (GFP) fluorescence was detected. Mean pixel intensity was taken from ≥25 cells per condition.

pressing I_{Cln} protein derived from either T84 or RPMI 8226 cells. I_{Cln} protein over-expression was confirmed by incubating fixed transfected tsA201a cells with polyclonal rabbit α-I_{Cln} antibody and visualized using a fluorescent-tagged secondary antibody. Fig. 2 shows cells with two levels of fluorescence intensity. tsA201a cells co-transfected with I_{Cln} and marker (GFP or CD8) showed a higher level of fluorescence intensity, indicating an increased level of I_{Cln} protein expression over endogenous expression. The mean pixel intensity is greater in myeloma-derived I_{Cln} transfected tsA201a than in mock transfectants (147±10 and 88±9, respectively). This effect was the same regardless of the source of I_{Cln}. I_{Cln} protein expression in cells transfected with marker alone were similar to mock transfectants. Note that overexpression does not alter the homogeneous pattern of I_{Cln} protein distribution.

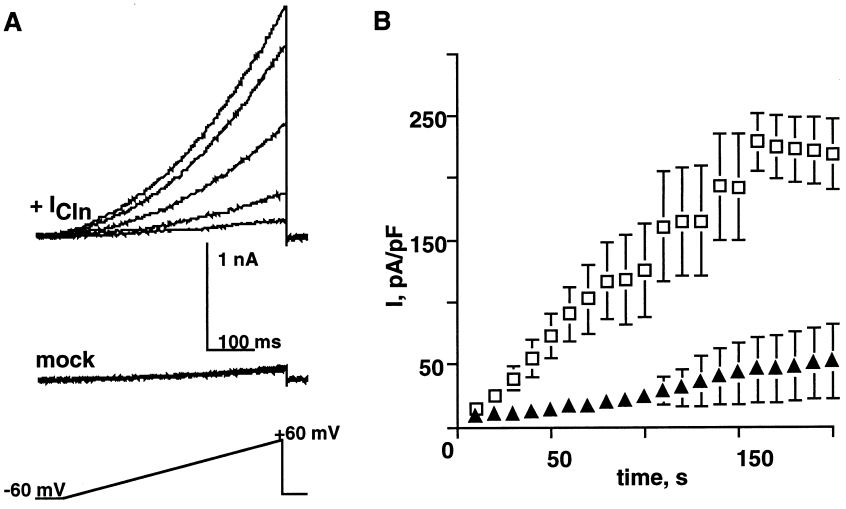
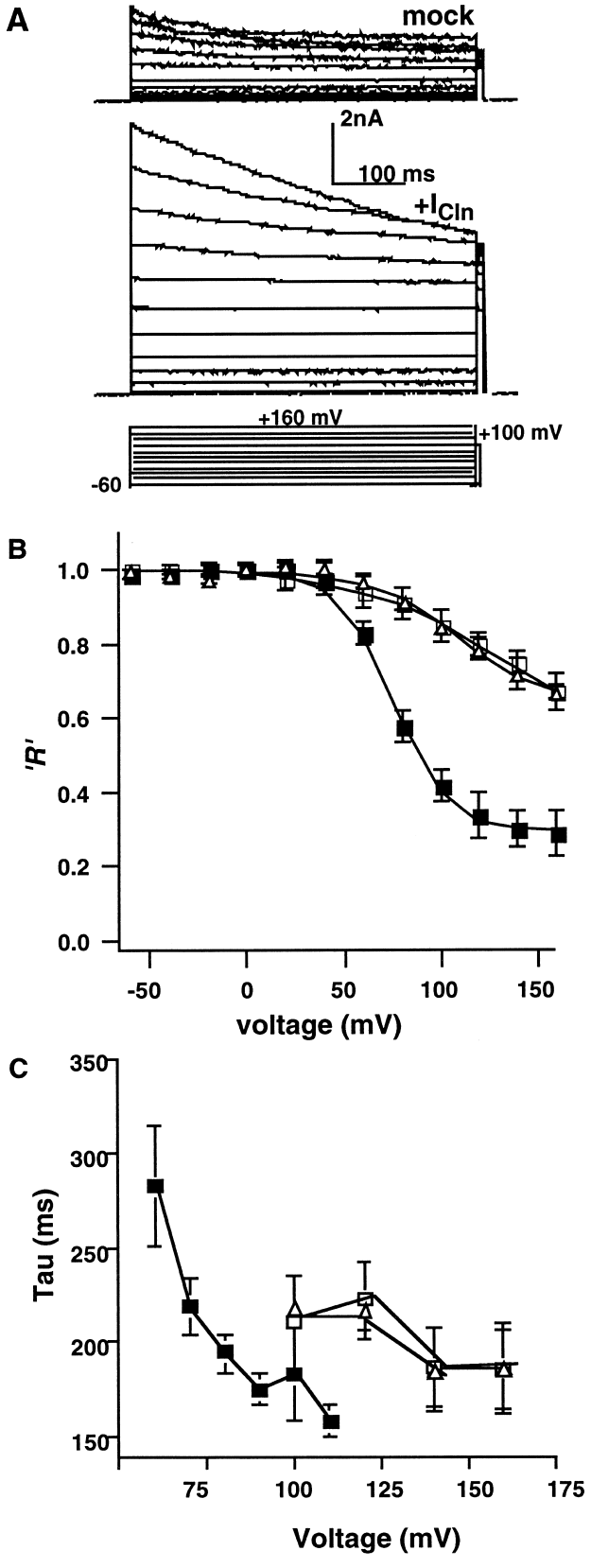


Fig. 3. I_{Cln} protein overexpression increases VRAC activation rate. (A) VRAC was activated in control and tsA201a cells expressing T84 I_{Cln} protein in response to a 90% hyposmotic gradient. Current was elicited in response to a series of voltage ramps from −60 to +60 mV over 500 ms, applied every 10 s. Superimposed currents were taken at times 0, 20, 40, 60 and 80 s. (B) The time course of VRAC current development. The activation rate was 15-fold greater in tsA201a cells transfected with T84 I_{Cln} cDNA (open symbols: 79±24 pA/pF min^{-1}) as compared to CD8-transfected control cells (filled symbols: 5±3 pA/pF min^{-1}). Each point represents an average of recordings made from ≥8 cells±S.E.M.



3.3. *Effect of I_{Cln} protein overexpression on the rate of VRAC activation*

The effect of I_{Cln} protein overexpression on VRAC activation was compared in mock and tsA201a cells transfected with T84 derived I_{Cln}. VRAC activity was initiated with a 90% hyposmotic gradient. Fig. 3A shows current elicited by voltage ramps from −60 to +60 mV in tsA201a cells overexpressing T84 I_{Cln} and mock transfectants. Reversal potential measurements show the current is anion-selective under both conditions (−61 ± 2 and −52 ± 3 mV, respectively), indicating that over-expression of I_{Cln} protein resulted in a large net increase in anion current. If overexpression resulted in a cation current, the reversal potential would be expected to be more positive. The greater negative reversal potential in tsA201a cells overexpressing T84-derived I_{Cln} protein reflects the increased development of anion current in these cells. Transfection with either GFP or CD8 served as an internal control for the overexpression of a small cytosolic protein (GFP) or membrane protein (CD8) and did not alter the reversal potential or alter VRAC properties.

T84-Derived I_{Cln} protein overexpression resulted in a large increase in the rate of VRAC current development (79 ± 24 pA/pF min^{-1}) as compared to CD8- and mock transfectants (5 ± 3 and 8 ± 6 pA/pF min^{-1}, respectively) (Fig. 3B). Similar results

←

Fig. 4. VRAC inactivation properties are independent of I_{Cln} protein expression. (A) Time-dependent VRAC current decay in mock and tsA201a cells transfected with T84 derived I_{Cln}. Voltage-dependent decay was determined using a conditioning pulse protocol. A short test pulse (10 ms) +100 mV was applied following prepulses (500 ms) from −60 to +160 mV. (B) The midpoint of current inactivation ($R=(1+e^{(V-V_{0.5})/k})^{-1}$) is the same for mock (open squares: 121 ± 15 mV) and T84 I_{Cln}-transfected (open triangles: 120 ± 16 mV) tsA201a cells. Inactivation of VRAC in wild-type T84 (filled squares: 74 ± 2 mV) cells are included as a reference. The slope factor in mock and T84 I_{Cln} transfected tsA201a cells is similar ($k=32\pm9$ and 30 ± 3 mV, respectively). The slope factor in wild-type T84 cells is 13 ± 1 mV. (C) Time constant of the exponential decay of currents does not change with expression of T84 I_{Cln} cDNA in tsA201A cells (open squares) and in mock-transfected tsA201a cells (open triangles), but is significantly different from that of wild-type T84 cells (filled squares). Each point represents an average of recordings made from ≥4 cells ± S.E.M.

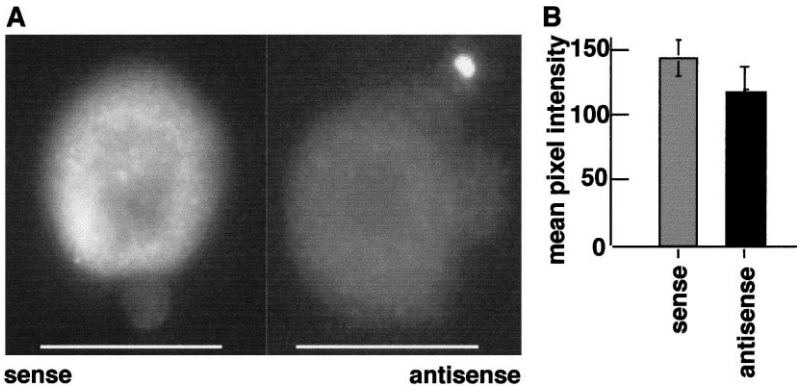


Fig. 5. I_{Cln} protein underexpression using antisense oligonucleotides. (A) α-I_{Cln} followed by incubation with a fluorescent secondary antibody demonstrates underexpression of endogenous I_{Cln} protein in tsA201a cells transfected with antisense oligonucleotides (10 μg) as compared to sense transfected cells. Scale bar indicates 10μm. (B) Mean pixel intensity (±S.E.M.) per cell under each condition, taken from ≥25 cells per condition.

were obtained with overexpression of RPMI-derived I_{Cln} cDNA (14±8 pA/pF min^{-1}). VRAC current did not develop in either control or I_{Cln} transfectants under isosmotic conditions (data not shown).

Overexpression of I_{Cln} protein did not alter VRAC inactivation of the host cell line. Fig. 4 shows current traces of voltage-dependent VRAC inactivation in wild-type tsA201a and T84 I_{Cln} transfected cells in response to a family of voltage pulses. The midpoint of steady-state VRAC inactivation (R) is significantly different in untreated, wild-type tsA201a, T84 and RPMI 8226 cells (V_m = 121±15, 74±2.0 and 105±3 mV, respectively) [14]. Overexpression of T84 I_{Cln} protein in tsA201a cells does not change the midpoint of inactivation (V_m = 120±16 and 121±15 mV, respectively), slope factor (k) (30±3 and 32±9 mV, respectively) or time course of inactivation of the host cell line (Fig. 4). Similarly, there was no change in midpoint of steady-state VRAC inactivation (V_m = 121±4 mV), the slope factor (k = 32±9 mV) or time course of inactivation in tsA201a cells overexpressing RPMI I_{Cln} protein. The similarity of the inactivation properties under these conditions indicates the same VRAC is expressed, independent of the level of I_{Cln} protein expression. Thus, the properties of VRAC inactivation are independent of the level of I_{Cln} protein expression and the differences in V_m in the original cell lines may be due to inherent properties of cell lines.

3.4. I_{Cln} protein underexpression

Antisense oligonucleotides were used to examine the effect of underexpressing endogenous I_{Cln} protein on VRAC properties. tsA201a cells were transfected with antisense oligonucleotides (10 μg) complementary to the published MDCK I_{Cln} sequence (see Section 2). Control cells were transfected with sense oligonucleotides (10 μg) consisting of the first 24 nucleotides of MDCK I_{Cln}.

I_{Cln} protein underexpression in tsA201a cells was confirmed through immunostaining. Fig. 5 shows fluorescence images of cells transfected with antisense and sense oligonucleotides. The mean pixel intensity was greater in sense transfected tsA201a than in antisense-treated cells (146±18 and 118±25, respectively). Fluorescence intensity was decreased in cells transfected with antisense oligonucleotides. The fluorescence of cells transfected with sense oligonucleotides was similar to that of mock transfected tsA201a cells (159±21).

3.5. I_{Cln} protein underexpression inhibits VRAC activation

The effect of I_{Cln} protein underexpression on VRAC activation in tsA201a cells was compared in sense and antisense transfectants. Fig. 6 shows current traces elicited by voltage ramps from −60 to +60 mV under hyposmotic conditions in antisense and sense transfectants. The rate of VRAC current

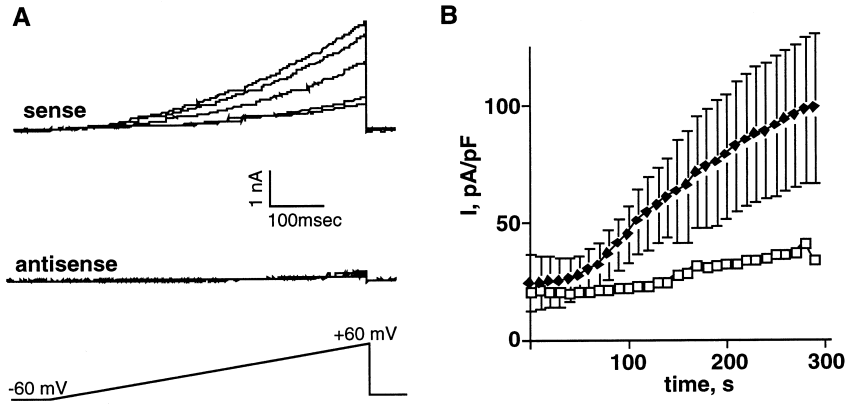


Fig. 6. I_{Cln} protein underexpression inhibits VRAC activation. (A) Activation of VRAC in response to a 90% hyposmotic gradient in tsA201a cells treated with sense and antisense oligonucleotides. Current was elicited in response to a voltage ramp from −60 to +60 mV over 500 ms, applied every 10 s. (B) The time course of VRAC current activation. The rate of VRAC activation was significantly less in tsA201a cells transfected with antisense oligonucleotides (open symbols: 0.1 ± 0.4 pA/pF min^{-1}) as compared to sense transfected cells (filled symbols: 18 ± 5 pA/pF min^{-1}). Each point represents average of recording made from ≥ 6 cells ± S.E.M.

development in antisense and sense transfected tsA201a cells is also shown in Fig. 6. The rate of current development in cells underexpressing endogenous I_{Cln} protein was significantly less than current development in sense transfectants (0.1 ± 0.4 and 18 ± 5 pA/pF min^{-1}, respectively; $n \geq 6$) when challenged with a 90% osmotic gradient. Increasing the osmotic gradient such that the osmolarity of the extracellular solution was 75% that of the intracellular solution did not increase the rate of activation of the current (0.06 ± 1.09 pA/pF min^{-1}; $n = 7$). These results indicate the rate of VRAC activation is dependent on I_{Cln} protein expression levels. It was not possible to compare VRAC inactivation under these conditions because cells treated with antisense oligonucleotides did not develop sufficiently high current amplitude to develop inactivation.

4. Discussion

We determined the amino acid sequence of I_{Cln} protein from three human cell lines, T84, RPMI 8226 and tsA201a. While the I_{Cln} amino acid sequence from human sources was nearly identical ($\geq 99\%$ homology), the human I_{Cln} protein sequences displayed less homology to rat and MDCK I_{Cln} [1,2,15,16]. tsA201a cells expressing I_{Cln} cDNA isolated from T84 cell line results in an increase in both the rate of VRAC activation and current amplitude during hypotonic swelling. Expression of I_{Cln} protein derived from RPMI 8226 cells in tsA201a cells was similar. Underexpression of endogenous I_{Cln} protein using antisense oligonucleotides reduced the rate of VRAC activation during cell swelling. Similar results have been reported in NIH 3T3 fibroblasts and bovine nonpigmented epithelial cells [3,4]. We have found expression of T84- or RPMI 8226-derived I_{Cln} protein has no effect on voltage-dependent VRAC inactivation in tsA201a cells, indicating VRAC inactivation is dependent on as yet indeterminate characteristics of the host cell line rather than on I_{Cln} protein expression levels or source.

I_{Cln} was first identified by expression cloning cDNA from T84 cells in *Xenopus* oocytes [1]. It was first thought that this cDNA encoded a channel protein of novel structure. The cytosolic protein was postulated to incorporate into the membrane in response to osmotic stress. Subsequent work has not supported this hypothesis. In addition, *Xenopus* oocytes have been found to express an endogenous swelling activated current in addition to an endogenous form of I_{Cln} cDNA and that I_{Cln} induced current is distinct from volume regulated anion current [17]. Additional observations suggest that I_{Cln} expression may be a modulator of an unrelated chloride channel protein, ClC-6 [18]. A recent report indicates I_{Cln} protein can form a cation-selective channel when reconstituted in planar lipid bilayers [6]. Although there is no physiological evidence that I_{Cln} protein

forms either an anion- or cation-selective channel in vivo, our results show no indication that I_{Cln} protein expression results in an increase in cation current when expressed in tsA201a cells. The *specific* role of I_{Cln} in volume regulatory processes remains unclear (for more discussion see [19]) but our work and that of other indicates that the expression level of this cytosolic protein in vivo does alter the activity of a volume regulated anion current.

I_{Cln} protein association with actin and other cytosolic proteins (I_{Cln} binding proteins; IBPs) has been documented [2,5]. IBP17 and IBP28, spliceosomal proteins (Sm proteins) which are involved in removing introns from pre-mRNAs as is necessary for the formation of a functional protein [20], were recently shown to be associated with I_{Cln} protein. I_{Cln} protein association with Sm proteins appears to inhibit protein expression by preventing intron splicing, suggesting I_{Cln} protein may regulate transcription and translation processes of protein(s) expressed in response to cell volume regulation.

In lymphocytes, disruption of F-actin in response to a physiological hyposmotic challenge, such as that used here, increases VRAC activation, while F-actin stabilization decreases VRAC activation rate [14]. Cytoskeletal rearrangement in many cell types during cell swelling is well-documented [21–25]. The association of I_{Cln} protein with actin both in vitro and vivo has been reported [5,26,27]. Together, these reports suggest that I_{Cln} protein may interact with the actin cytoskeleton to modulate VRAC activation. Recently, a 72 kDa protein (IBP72) which consistently co-localizes with I_{Cln} protein has been identified as a possible mammalian homologue of yeast Skb1, a protein that may influence cell morphology through interaction with cytoskeletal proteins [28]. IBP72 binds to the carboxyl terminus of I_{Cln}, a region that is highly conserved in different species and cell types, suggesting this region may be functionally significant.

In summary, we show that the amino acid sequences of I_{Cln} from human sources are highly homologous ($\geq 99\%$), while rat and MDCK sequences are less homologous to human I_{Cln}. Our results support the hypothesis that expression levels of I_{Cln} protein modulate VRAC activation and thus may contribute to cell volume regulation. These studies also show that I_{Cln} protein is not likely to contribute to a difference in the voltage-dependent inactivation of VRAC reported in many cell types.

Acknowledgements

We thank the DNA sequencing facilities at MCP Hahnemann University, Philadelphia, PA and University of Chicago, Chicago, IL for their technical assistance. We are indebted to Dr. Strange and Dr. Eggermont for generously providing polyclonal α-I_{Cln} antibodies. We thank Dr. M. White for continuing support. Dr. Garber is an Established Investigator of the American Heart Association. This work was supported by NIDDK46672 and AHA94002340.

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