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Altered gating and regulation of a carboxy-terminal ClC channel mutant expressed in the Caenorhabditis elegans oocyte

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Members of the ClC superfamily of voltage-gated Cl\(^{-}\) channels are present in all phyla and function in diverse and essential physiological processes (20). Determination of bacterial ClC crystal structures by MacKinnon and co-workers (12, 13) marked a breakthrough in understanding ClC channel gating. ClCs are homodimers, and each monomer is comprised of 18 \(\alpha\)-helical domains, 17 of which are intramembrane (12). Monomers form a single, independently gated pore or protopore. Protopore gating is a fast process and is thought to be mediated by the movement of a highly conserved glutamate residue located adjacent to the selectivity filter (13) and perhaps larger-scale structural rearrangements of the pore (1). Many ClC channels also exhibit a slow or common gating process that opens and closes the two protopores simultaneously (Refs. 17, 22; for review, see Ref. 20). The molecular basis of slow gating is unknown but may involve large-scale channel conformational changes (5, 26, 31).

Eukaryotic ClCs have extensive cytoplasmic NH\(_2\) and COOH termini that are absent from crystallized bacterial ClC proteins. In addition, the COOH termini of all eukaryotic ClC channels contain two cystathionine-\(\beta\)-synthase (CBS) domains (3, 20). The bacterial ClC crystal structures indicate that the last \(\alpha\)-helix immediately preceding the cytoplasmic COOH terminus contributes directly to the coordination of Cl\(^{-}\) within the channel selectivity filter (12). Dutzler et al. (12) postulated that the interaction of this \(\alpha\)-helix with cytoplasmic structures could provide a mechanism for regulating channel-gating properties and activity. Consistent with this idea, mutagenesis studies have demonstrated that cytoplasmic NH\(_2\) and COOH termini play important roles in ClC gating (8, 15, 16, 19, 30). COOH-terminal mutations in ClC-1 and ClC-2 are associated with myotonia and epilepsy (4, 18, 25). Recent studies have suggested that CBS domains may function in slow gating (14). However, despite their obvious importance, the precise structural and functional relationships of cytoplasmic NH\(_2\) and COOH termini are poorly defined.

Six ClC-type anion channel-encoding genes, termed clh-1--clh-6 or cecle-1--cecle-6, are present in the Caenorhabditis elegans genome (2, 23, 29). The six nematode ClC genes are representative of the three major subfamilies of mammalian ClC genes. Two CLH-3 splice variants, termed CLH-3a and CLH-3b, have been cloned from C. elegans (23, 29). These proteins have identical intramembrane domains but differ significantly in their cytoplasmic NH\(_2\) and COOH termini. The major differences include a 71-amino acid NH\(_2\)-terminal extension on CLH-3a and a 270-amino acid extension of the CLH-3b COOH terminus. CLH-3b is expressed in the C. elegans oocyte and gives rise to a swelling- and meiotic cell cycle-regulated Cl\(^{-}\} current (9).

CLH-3a and CLH-3b exhibit striking differences in voltage sensitivity, activation kinetics, and sensitivity to extracellular \(\mathrm{pH}\) and Cl\(^{-}\} concentration (9). On the basis of structural and functional insights gained from crystallized bacterial ClC proteins (12, 13), we postulated that alternative splicing of the COOH terminus may account for these differences by altering the accessibility and/or function of pore-associated ion-binding sites (9).
We recently identified a worm strain harboring an 841-nt deletion in a region of clh-3 encoding the predicted cytoplasmic COOH terminus of CLH-3a and CLH-3b. The deletion removes 101 amino acids that are unique to CLH-3b and another 64 amino acids shared by both splice variants. Mutant CLH-3b channels are functionally expressed in the C. elegans oocyte. To begin defining the role of cytoplasmic domains in regulating CLH-3b gating and activity, we characterized the biophysical properties of the mutant channels in their native cellular environment. We have demonstrated in the present study that the deletion mutation of the COOH terminus disrupts volume- and phosphorylation-dependent channel regulation and dramatically alters voltage sensitivity, sensitivity to extracellular H\(^+\) and Cl\(^-\), and inhibition by Zn\(^{2+}\). These studies provide new insights into the role of the cytoplasmic domains in the regulation of CIC gating and activity and provide the foundation for future site-directed mutagenesis and heterologous expression studies.

### MATERIALS AND METHODS

_C. elegans culture and strains_. Nematodes were cultured at 16°C using standard methods (6). Wild-type worms were of the Bristol N2 strain. _clh-3(ok768)_ worms were provided by the _C. elegans_ Gene Knockout Project at the Oklahoma Medical Research Foundation, which is part of the International _C. elegans_ Gene Knockout Consortium (http://www.mutantfactory.ouhsc.edu/).

PCR analysis. _clh-3(ok768)_ mutant worms were outcrossed three times, and homozygous animals were identified using PCR analysis of genomic DNA isolated from single worms (24) and the primer set 5’-GTCATTTGCATATTGGTT-3’ and 5’-GGTCAGAAACGG-GAAAACCAA-3’ (Fig. 1A). To determine the mutant mRNA sequence, oligo(dT) primed cDNA was prepared from RNA isolated from Bristol N2 and _clh-3(ok768)_ worms using standard techniques. A partial open reading frame was PCR amplified, cloned, and sequenced using the primer set 5’-CCGTGATACCCATACACGGTC-3’ and 5’-TCAGAATTTTTTCGTGATGAA-3’. These primers annealed in exon 11 and exon 19 immediately before the stop codon (see Fig. 1B).

Patch-clamp recording of whole cell Cl\(^-\) currents in isolated _C. elegans_ oocytes. Gonads were isolated by placing single nematodes in egg buffer (in mM: 118 NaCl, 48 KCl, 2 CaCl\(_2\), 2 MgCl\(_2\), and 25 HEPES, pH 7.3; 340 mosM) and cutting them behind the pharyngeal bulb and in front of the spermatheca using a 26-gauge needle. Isolated gonads were transferred to a patch-clamp bath chamber mounted on the stage of an inverted microscope. Late-stage oocytes were released spontaneously from the cut end of the gonad.

Patch-clamp electrodes were pulled from 1.5-mm-outter-diameter silanized borosilicate microhematocrit tubes. Currents were measured using an Axopatch 200B (Axon Instruments, Foster City, CA) patch-clamp amplifier with control bath solution containing (in mM) 116 NMDG-Cl, 2 CaCl\(_2\), 2 MgCl\(_2\), 25 HEPES, and 71 sucrose (pH 7.3; 340 mosM) and a pipette solution containing (in mM) 116 NMDG-Cl, 2 MgSO\(_4\), 20 HEPES, 6 CsOH, 1 EGTA, 48 sucrose, 2 ATP, and 0.5 GTP (pH 7.2; 315 mosM). For low-Cl\(^-\) solutions, we used a NaCl-based bath solution that was otherwise identical to the NMDG-Cl\(^-\) bath solution. Low-NaCl solutions were prepared by isosmotic substitution of sucrose for NaCl. Oocytes were swollen by exposure to a hypotonic (260 mosM) bath solution that contained no added sucrose. Metabolic inhibitors were dissolved as stock solution in DMSO and then added to pipette or bath solutions at final DMSO concentration of \(\leq 0.01\%\).

Electrical connections to the patch-clamp amplifier were made using Ag/AgCl wires and 3 M KCl-agar bridges. Data acquisition and analysis were performed using pCLAMP 8 software (Axon Instruments).

RNA interference. RNA interference (RNAi) was performed as described previously (27). Briefly, a DNA template corresponding to the first 847 bp of the open-reading frame of _clh-3_ was obtained by PCR, and sense and antisense RNA were synthesized using T7 polymerase (MEGAscript; Ambion, Austin, TX). Template DNA was digested with DNase I, and RNA was precipitated using 3 M sodium acetate and ethanol. Precipitated RNA was washed with 70% ethanol, air dried, and dissolved in water. RNA size, purity, and integrity were assayed on agarose gels. Double-stranded RNA (dsRNA) was formed by annealing sense and antisense RNA at 65°C for 30 min. Annealed dsRNA was diluted into potassium citrate buffer for injection. Worms were injected in one gonad arm with ~1,000,000 molecules of dsRNA.

Statistical analysis. Data are presented as means ± SE. Statistical significance was determined using Student’s two-tailed _t_-test. _P_ ≤ 0.05 was assumed to indicate statistical significance.

### RESULTS

_clh-3(ok768)_ allele is a 165-amino acid deletion in the _CLH-3_ COOH terminus. PCR analysis of genomic DNA from wild-type and _clh-3(ok768)_ heterozygous and homozygous worms revealed that the _ok768_ allele is an ~800-bp deletion (Fig. 1A). DNA sequencing demonstrated that the deletion spanned a region of 841 nt that included part of exon 12 and all of exons 13 and 14, which are present only in CLH-3b (23) (Fig. 1B). PCR analysis of the mutant mRNA demonstrated that it coded for a protein where a cryptic site in exon 12 was spliced in frame to exon 15 (Fig. 1B). Amplification using primers specific for CLH-3a did not yield a PCR product (data not shown).

The mutant transcript lacked sequence coding for COOH-terminal amino acids _V604–E768_ of the CLH-3b protein (Fig. 1C). We termed the deletion mutant protein CLH-3bΔC. Amino acids _R668–E768_ are unique to CLH-3b and are encoded by exons 13 and 14. Amino acids _V604–S667_ comprise a domain that is conserved in both CLH-3a and CLH-3b (Fig. 1C). Eleven of the amino acids deleted in this region are present in the first CBS domain.

CLH-3bΔC forms channels with altered voltage sensitivity and activation kinetics. To determine whether CLH-3bΔC channels are functional, we recorded whole cell Cl\(^-\) currents in oocytes isolated from wild-type and _clh-3(ok768)_ worms. As shown previously (9, 27, 28), wild-type meiotic cell cycle-arrested, nonswollen oocytes exhibit a small inwardly rectifying CLH-3b current (I\(_{\text{CLH-3b}}\)) that is activated by strong hyperpolarization. Figure 2A shows mean whole cell current traces...
recorded in nine wild-type oocytes. The mean amplitude of this current recorded during the last 20 ms of the 1-s test pulse to 
−100 mV was 13 pA/pF (Fig. 2B).

Nonswollen, cell cycle-arrested oocytes from clh-3(ok768) worms also exhibited inwardly rectifying Cl\(^−\) currents. Figure 2A shows mean whole cell current traces recorded from 18 clh-3(ok768) oocytes. The mean steady-state CLHb-3C current (\(I_{\text{CLH-3bC}}\)) amplitude measured during the last 20 ms of the −100-mV test pulse was 26 pA/pF (Fig. 2B), which was significantly (\(P < 0.02\)) greater than that observed in wild-type worms.

To test whether this current was due to the activity of a clh-3-encoded channel, we performed RNAi experiments. clh-3 RNAi disrupts CLH-3 channel expression in the C. elegans oocyte (9, 27, 28). Steady-state current amplitude recorded at −100 mV in oocytes isolated from clh-3(ok768) worms injected with clh-3 dsRNA was inhibited 95% (\(P < 0.01\)) to a mean ± SE value of 1.2 ± 0.8 pA/pF (\(n = 3\)). These results demonstrate that whole oocyte Cl\(^−\) currents in clh-3(ok768) worms are carried by CLH-3bΔC channels.

The kinetics of hyperpolarization-induced activation of \(I_{\text{CLH-3bC}}\) were considerably more rapid than those of \(I_{\text{CLH-3b}}\). Figure 2C shows average current traces recorded at −100 mV from wild-type and clh-3(ok768) oocytes. The \(I_{\text{CLH-3b}}\) trace is scaled by a factor of 2.1 and superimposed over the \(I_{\text{CLH-3bC}}\) trace for comparison. The zero-current level is indicated by a dashed line.

![Figure 2](image)

Fig. 2. Cl\(^−\) currents recorded from nonswollen, meiotic cell cycle-arrested wild-type and clh-3(ok768) oocytes. A: mean whole cell CLH-3b (\(n = 9\)) and CLH-3bΔC (\(n = 18\)) currents. Currents were evoked by voltage clamping oocytes between −100 and +20 mV for 1 s in 20-mV increments from a holding potential of 0 mV. Each test pulse was followed by a 1-s recovery period at 0 mV. B: current-voltage (I-V) relationships for inwardly rectifying CLH-3b current (\(I_{\text{CLH-3b}}\)) and mean steady-state CLHb-3ΔC current (\(I_{\text{CLH-3bC}}\)). Values are means ± SE (\(n = 9–18\)) of current values measured during the last 20 ms of each test pulse. C: comparison of \(I_{\text{CLH-3b}}\) and \(I_{\text{CLH-3bC}}\) activation kinetics at −100 mV. The \(I_{\text{CLH-3bC}}\) trace has been scaled by a factor of 2.1 and superimposed over the \(I_{\text{CLH-3b}}\) trace for comparison. The zero-current level is indicated by a dashed line.

![Figure 3](image)

Fig. 3. Regulation of \(I_{\text{CLH-3bC}}\) by oocyte swelling and ATP depletion. A: relative amplitude (\(I_{\text{hyp}}/I_{\text{iso}}\)) of \(I_{\text{CLH-3bC}}\) recorded during the last 20 ms of a −100-mV test pulse after hypotonic oocyte swelling for 10 min. Values are means ± SE (\(n = 6–9\)). * \(P < 0.01\) vs. \(I_{\text{CLH-3b}}\). B: I-V relationships for \(I_{\text{CLH-3bC}}\) recorded from metabolically poisoned oocytes or DMSO controls. Values are means ± SE (\(n = 8\)).
biexponential process described by fast and slow time constants at $-100$ mV of $\approx13$ ms and $\approx58$ ms, respectively (discussed in detail below).

As shown previously (27, 28), oocyte swelling dramatically increases $I_{CLH-3b}$ amplitude. Figure 3A shows that hypotonicity induced oocyte swelling for 10-min-activated $I_{CLH-3b} \sim 23$-fold. In marked contrast, $I_{CLH-3b_{AC}}$ amplitude increased only 1.2-fold in response to oocyte swelling for 10 min (Fig. 3A).

ATP depletion activates CLH-3b by inducing net protein dephosphorylation (28). We depleted clh-3(ok768) oocytes of ATP by exposing them to 5 mM 2-deoxyglucose (2-DG) and 1 mM rotenone in bath solution. After being incubated for 20–30 min, oocytes were patch clamped using an ATP-free pipette solution containing (in µM) 40 oligomycin, 20 rotenone, and 5 iodoacetate. As shown in Fig. 3B, ATP depletion had no significant ($P > 0.4$) effect on $I_{CLH-3b_{AC}}$ amplitude.

Oocyte shrinkage inhibits $I_{CLH-3b}$ that has been activated by either oocyte swelling or meiotic cell cycle progression (27). However, $I_{CLH-3b_{AC}}$ amplitude was not significantly ($P > 0.8$) altered by cell shrinkage. Mean ± SE relative current amplitude measured at $-100$ mV after 10-min exposure of clh-3(ok768) oocytes to hypertonic saline was $0.98 \pm 0.08$ ($n = 6$). We conclude that the COOH-terminal deletion mutation drastically reduces CLH-3b volume sensitivity, prevents dephosphorylation-induced channel activation, and alters the kinetic properties of the channel.

Oocyte swelling alters the voltage sensitivity and kinetics of hyperpolarization-induced activation of $I_{CLH-3b}$ (9, 27). Figure 4A shows mean whole cell currents recorded in wild-type and clh-3(ok768) oocytes swollen for 10 min. Mean CLH-3b and CLH-3bAC current densities at $-100$ mV were $-175$ pA/pF and $-25$ pA/pF, respectively.

The apparent voltage sensitivities of $I_{CLH-3b}$ and $I_{CLH-3b_{AC}}$ recorded in swollen oocytes were considerably different. Figure 4B shows Boltzmann relationships for $I_{CLH-3b}$ and $I_{CLH-3b_{AC}}$. As described previously by us (9) and by Schriever et al. (29), CLH-3 channels close too rapidly at depolarized potentials for tail currents to be measured reliably. Furthermore, the depolarization-induced potentiation of $I_{CLH-3b_{AC}}$ (see below) prevented tail current analysis using a negative test pulse. Consequently, we were unable to estimate the voltage dependence of CLH-3bAC open probability using tail current analysis. We therefore normalized steady-state current values recorded at each test potential to those measured at $-100$ mV and fitted this relationship using a Boltzmann function. As shown in Fig. 4B, $I_{CLH-3b}$ was activated at more depolarized potentials of $V = 100\text{ mV}$ and $V = 80\text{ mV}$.

$\text{Fig. 4. Voltage- and time-dependent properties of } I_{CLH-3b}$ and $I_{CLH-3b_{AC}}$. A: mean whole cell $I_{CLH-3b}$ ($n = 9$) and $I_{CLH-3b_{AC}}$ ($n = 6$) recorded in worm oocytes swollen for 10 min by exposure to hypotonic bath solution. Voltage-clamp protocol is the same as that described in Fig. 2. B: Boltzmann fits of normalized $I-V$ relationships for $I_{CLH-3b}$ and $I_{CLH-3b_{AC}}$. Steady-state current amplitude recorded at each test potential was normalized to that measured at $-100$ mV. Fits were performed using the equation

$$I(V_m) = \frac{A_1 - A_2}{1 + e^{d(V_m - V_{0.5})}} + A_2,$$

where $V_{0.5}$ is the half-activation potential and $d$ is the slope factor. Values are means ± SE ($n = 6–9$). C: comparison of fast-activation time constants for $I_{CLH-3b}$ and $I_{CLH-3b_{AC}}$ at $-100$ mV and $-80$ mV. * $p < 0.0007$ and ** $p < 0.00006$ compared with values measured at $-100$ mV. Statistical analyses were performed with paired data. D: slow-activation time constants for $I_{CLH-3b}$ and $I_{CLH-3b_{AC}}$ at $-100$ and $-80$ mV. Values in C and D are means ± SE ($n = 6–9$). Time constants were derived from biexponential fits of the first 200 ms of hyperpolarization-induced current activation.
potentials than $I_{\text{CLH-3bAC}}$, causing a rightward shift in its current-voltage ($I$-$V$) relationship. The mean ± SE half-activation voltages ($V_{0.5}$) and slope factors ($k$) derived from the Boltzmann curves for $I_{\text{CLH-3b}}$ and $I_{\text{CLH-3bAC}}$ were $-67 ± 0.7$ mV and $17 ± 0.6$ mV$^{-1}$ ($n = 9$) and $-80 ± 0.4$ mV ($n = 6$) and $9 ± 0.4$ mV$^{-1}$ ($n = 6$), respectively. Similar $V_{0.5}$ and $k$ values for $I_{\text{CLH-3bAC}}$ were observed in nonswollen oocytes (data not shown).

The kinetics of channel activation were estimated by fitting two-term exponential functions that described $\tau_{\text{fast}}$ and $\tau_{\text{slow}}$ components of the activation portion of current traces. Because of the small current amplitude of $I_{\text{CLH-3bAC}}$ at $-60$ mV, reasonable exponential fits could be performed only at $-80$ mV and $-100$ mV. The $\tau_{\text{fast}}$ values for both currents were voltage dependent and increased significantly ($P < 0.0007$) with depolarization (Fig. 4C). In contrast, $\tau_{\text{slow}}$ values for $I_{\text{CLH-3b}}$ and $I_{\text{CLH-3bAC}}$ were voltage independent ($P > 0.2$) over the voltage range tested (Fig. 4D).

We also observed statistically significant ($P < 0.0001$) differences in $\tau_{\text{fast}}$ and $\tau_{\text{slow}}$ values of $I_{\text{CLH-3b}}$ compared with $I_{\text{CLH-3bAC}}$. However, because the amplitude of $I_{\text{CLH-3bAC}}$ is much greater than that of $I_{\text{CLH-3bAC}}$ (Fig. 4A), the relevance of such apparent differences is unclear at present.

$CLH-3bAC$ exhibits predepolarization-induced potentiation.

The slower activation kinetics and hyperpolarizing shift in the $I$-$V$ relationship of $I_{\text{CLH-3bAC}}$ are reminiscent of the biophysical characteristics of heterologously expressed CLH-3a (9). A striking characteristic of CLH-3a is its sensitivity to conditioning predepolarization. Both we (9) and Schriever et al. (29) showed previously that hyperpolarization-induced activation of CLH-3a is potentiated by prior membrane depolarization. However, native CLH-3b in the nematode oocyte as well as heterologously expressed CLH-3b are virtually insensitive to depolarizing prepulses (9). Given the similarities of voltage-dependent activation of CLH-3bAC and CLH-3a, we tested the effect of conditioning prepolarization on $I_{\text{CLH-3bAC}}$. Oocytes isolated from clh-3(ok768) worms were clamped for $3$ s at conditioning potentials between $-20$ mV and $+60$ mV in $20$-mV increments and then stepped to $-100$ mV for $2$ s to activate $I_{\text{CLH-3bAC}}$. Figure 5A shows that hyperpolarization-induced activation of $I_{\text{CLH-3bAC}}$ was potentiated by conditioning prepolarization. For example, $I_{\text{CLH-3bAC}}$ amplitude recorded at $-100$ mV after a conditioning potential of $60$ mV was $-1.5$-fold that after a conditioning potential of $-20$ mV (Fig. 5B).

Conditioning prepolarization also induces a slow inactivation process at hyperpolarizing test voltages in CLH-3a but not in CLH-3b (9). The current traces shown in Fig. 5A demonstrate that depolarizing prepulses induced a similar inactivation process in CLH-3bAC. Figure 5C summarizes the voltage dependence of inactivation of $I_{\text{CLH-3bAC}}$. The degree of inactivation was calculated by normalizing the pseudo-steady-state current ($I_{\text{ss}}$) amplitude recorded during the last $20$ ms of the $-100$-mV test pulse to the peak current ($I_{\text{peak}}$) amplitude recorded between $170$ and $270$ ms. This time domain was chosen because it bracketed the peak $I_{\text{CLH-3bAC}}$ amplitude after depolarized conditioning pulses (see Fig. 5A). $I_{\text{CLH-3bAC}}$ inactivation was rarely observed after a conditioning potential of $-20$ or $0$ mV but became prominent with stronger depolarizing prepulses (Fig. 5C). After a conditioning pulse to $+60$ mV, $I_{\text{CLH-3bAC}}$ was inactivated by $-15\%$ after the current was activated by hyperpolarization to $-100$ mV.

$CLH-3bAC$ exhibits altered sensitivity to extracellular $Cl^-$ and $pH$. Heterologously expressed CLH-3a is significantly more sensitive than CLH-3b to changes in extracellular $Cl^-$ and $H^+$ concentration (9). We therefore examined the effects of extracellular Cl$^-$.
of changes in bath Cl⁻ concentration and pH on I_{CLH-3bAC}.

Whole cell currents in clh-3(ok768) oocytes were recorded in control bath (124 mM Cl⁻) and 30 s after switching to a 16 mM Cl⁻ bath solution. Figure 6A shows mean ± SE currents recorded between −100 mV and +40 mV in control and low-Cl⁻ bath solutions. Reduction of Cl⁻ inhibited I_{CLH-3bAC}. At −100 mV, for example, I_{CLH-3bAC} amplitude was reduced ~24% (P < 0.0001). A similar change in bath Cl⁻ concentration has no effect on heterologously expressed I_{CLH-3b} or I_{CLH-3b} measured in wild-type oocytes (9).

Figure 6, B and C, shows the effect of changing bath pH on I_{CLH-3bAC}. Reduction of bath pH from 8.1 to 5.9 increased steady-state I_{CLH-3bAC} amplitude ~6.3-fold at −100 mV. In contrast, a similar reduction of bath pH increases the amplitude of native and heterologously expressed I_{CLH-3b} only approximately twofold (9).

Kinetics of inhibition by Zn²⁺ are altered in CLH-3bAC. I_{CLH-3b} in the C. elegans oocyte is inhibited by >90% by bath application of 10 mM Zn²⁺ (27). Because I_{CLH-3b} and I_{CLH-3bAC} exhibit different sensitivities to extracellular Cl⁻ and pH, we tested whether they also differ in their sensitivity to extracellular Zn²⁺. Oocytes were held at 0 mV and stepped to −100 mV every 2 s for 1 s during continuous perfusion of control bath or bath containing 5 mM Zn²⁺. Figure 7, A and C, shows typical time course experiments for Zn²⁺-induced inhibition of I_{CLH-3b} and I_{CLH-3bAC}, respectively. Zn²⁺ inhibited I_{CLH-3b} with a biphasic time course that consisted of a rapid initial phase followed by a much slower secondary phase (Fig. 7A). The time course of Zn²⁺ inhibition of I_{CLH-3b} could be fitted with a biexponential function describing τ_{fast} and τ_{slow} (Fig. 7B). Mean ± SE amplitudes of the fast (A_{fast}) and slow (A_{slow}) components were 0.69 ± 0.05 and 0.27 ± 0.04 (n = 4), respectively. In contrast, Zn²⁺ inhibited I_{CLH-3bAC} with a rapid monoexponential time course that lacked the slower phase observed during inhibition of I_{CLH-3b} (compare Fig. 7, A and C). The time constant for Zn²⁺-mediated inhibition of I_{CLH-3bAC} is shown in Fig. 7D. Mean ± SE amplitude was 0.90 ± 0.01 (n = 7). Mean ± SE inhibition of I_{CLH-3b} and I_{CLH-3bAC} with 5 mM Zn²⁺ at −100 mV was 86 ± 2% (n = 4) and 90 ± 1% (n = 7), respectively. The extent of inhibition in the two channels was not significantly different (P > 0.07). Block of both I_{CLH-3b} and I_{CLH-3bAC} was reversible, and Zn²⁺ washout followed similar monoexponential time courses that were not significantly different for the two channels (P > 0.6) (Fig. 7, B and D). Mean ± SE amplitudes of current recovery for I_{CLH-3b} and I_{CLH-3bAC} were 0.11 ± 0.01 (n = 4) and 0.1 ± 0.03 (n = 3), respectively.

Predepolarization-induced potentiation of I_{CLH-3bAC} is blocked by Zn²⁺. In addition to observing inhibition of total channel activity, we also found that Zn²⁺ blocked predepolarization-induced potentiation of I_{CLH-3bAC}. Figure 8A shows mean current traces from three clh-3(ok768) oocytes recorded in control bath and in the presence of 100 µM Zn²⁺, which blocks prepotentiation completely. A dose-response curve for Zn²⁺-mediated inhibition of prepotentiation is shown in Fig. 8B. IC₅₀ for Zn²⁺ inhibition of I_{CLH-3bAC} prepotentiation was 10 µM. Interestingly, Zn²⁺ concentrations up to 250 µM failed to block hyperpolarization-activated I_{CLH-3bAC}. Mean ± SE steady-state I_{CLH-3bAC} amplitudes recorded at −100 mV in control saline and in the presence of 250 µM Zn²⁺ were −21 ± 8 pA/pF and −21 ± 9 pA/pF (n = 3), respectively. These values were not significantly different (P > 0.9).

**DISCUSSION**

We showed previously that heterologous expression of CLH-3a and CLH-3b gives rise to currents with distinct functional properties (9). Several characteristics of I_{CLH-3a} and
The properties of CLH-3bΔC resemble but do not fully recapitulate those of heterologously expressed CLH-3a. It is noteworthy that the stimulatory effects of bath acidification, elevated bath Cl−, and a 3-s predepolarization to 60 mV on CLH-3a are about twice those observed for CLH-3bΔC (Table 1). This suggests that the CLH-3a NH2 terminus may be required for full sensitivity to these parameters or that sensitivity to depolarization and extracellular ions is also modulated by the 169-amino acid extension of the CLH-3b COOH terminus. Site-directed mutagenesis and heterologous expression studies are required to fully define the role of NH2 and COOH termini in CLH-3 channel gating.

Bacterial CIC crystal structures (12, 13) have demonstrated that the last α-helix, or R helix, immediately preceding the cytoplasmic COOH terminus contributes a tyrosine residue that protrudes into the channel pore and functions in Cl− coordination. Amino acid residues thought to be involved in CIC fast gating and pore Cl− binding (12, 13) are fully conserved in CLH-3a and CLH-3b. We suggested previously (9) that the differences in extracellular H+ and Cl− sensitivity of the splice variants (see Table 1) might be due to structural changes in the CLH-3b COOH terminus that secondarily alter the structure of the R helix and the accessibility and/or function of pore-associated ion-binding sites. Recent studies by Hebeisen and Fahlke (17) are consistent with this idea and have shown clearly that truncation of the COOH terminus alters the conformation of the outer vestibule of CIC-1.

We also suggested previously (9) that the effect of depolarization on channel activation could be due to effects of changes in local Cl− concentration on a fast gating mechanism. How-
et al. (9). All currents were recorded at −100 mV. Identical voltage-clamp protocols and changes in bath Cl– levels and pH were used in HEK cell and C. elegans oocyte experiments. *Values are relative to a conditioning potential (CP) of −20 mV. †Values are relative to those measured at a bath pH of 8.1.

Table 1. Comparison of the effects of depolarization and bath pH and Cl– concentration on HEK cells expressing CLH-3a and CLH-3b and on CLH-3b and CLH-3bΔC expressed in the C. elegans oocyte

<table>
<thead>
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<th></th>
<th>Potentiation by +60-mV CP*</th>
<th>Inactivation After +60-mV CP*</th>
<th>Activation by pH 5.9 Bath†</th>
<th>Inhibition by Low Bath Cl–</th>
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<tr>
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<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>C. elegans oocyte CLH-3bΔC</td>
<td>1.5-fold</td>
<td>15%</td>
<td>6-fold</td>
<td>24%</td>
</tr>
</tbody>
</table>

Data on human embryonic kidney (HEK) cells expressing CLH-3a and CLH-3b and on Caenorhabditis elegans oocyte CLH-3b are summarized from Denton et al. (9). All currents were recorded at −100 mV. Identical voltage-clamp protocols and changes in bath Cl– levels and pH were used in HEK cell and C. elegans oocyte experiments. *Values are relative to a conditioning potential (CP) of −20 mV. †Values are relative to those measured at a bath pH of 8.1.
interactions and/or regulatory interactions within the channel protein itself.

In conclusion, we have characterized a CLH-3b COOH-terminal deletion mutant in its native cellular environment, where regulatory interactions with other proteins are presumably minimally perturbed. Our results demonstrate clearly that the cytoplasmic COOH terminus plays important roles in channel gating and regulation. Site-directed mutagenesis studies are clearly warranted to begin defining the underlying structural and functional relationships of the COOH terminus. Our findings contribute to a growing body of evidence indicating that NH2- and COOH-terminal cytoplasmic domains are essential for the function of eukaryotic ClCs and likely contribute significantly to the evolution and physiological diversification of these channels.

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