Intracellular β-Nicotinamide Adenine Dinucleotide Inhibits the Skeletal Muscle CIC-1 Chloride Channel*

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Background: Weakly voltage-dependent CIC-1 chloride channels regulate skeletal muscle excitability. CIC-1 is the dominant sarcolemmal chloride channel and plays an important role in regulating membrane excitability that is underscored by CIC-1 mutations in congenital myotonia. Here we show that the coenzyme β-nicotinamide adenine dinucleotide (NAD), an important metabolic regulator, robustly inhibits CIC-1 when included in the pipette solution in whole cell patch clamp experiments and when transiently applied to inside-out patches. The oxidized (NAD⁺) form of the coenzyme was more efficacious than the reduced (NADH) form, and inhibition by both was greatly enhanced by acidification. Molecular modeling, based on the structural coordinates of the homologous CIC-5 and CmCIC proteins and in silico docking, suggest that NAD⁺ binds with the adenine base deep in a cleft formed by CIC-1 intracellular cystathionine β-synthase domains, and the nicotinamide base interacts with the membrane-embedded channel domain. Consistent with predictions from the models, mutation of residues in cystathionine β-synthase and channel domains either attenuated (G200R, T636A, H847A) or abrogated (L848A) the effect of NAD⁺.

Results: Intracellular NAD(H) altered the voltage dependence of CIC-1 gating, and specific mutations attenuated this effect.

Conclusion: NAD(H) directly inhibits CIC-1 chloride channels by binding to intracellular domains.

Significance: CIC-1 inhibition by NAD(H) may play an important role in muscle fatigue and the pathophysiology of myotonia congenita.

Approximately 80% of the resting conductance of skeletal muscle membranes is carried by Cl⁻ ions (1), the vast proportion of which are attributable to weakly voltage-dependent CIC-1 channels (2). For action-potential propagation to occur, Na⁺ influx must be sufficient to overcome the electrical “short circuit,” or shunting current, through open CIC-1 channels to depolarize the muscle membrane. With intense activity, however, accumulation of extracellular K⁺ leads to partial membrane depolarization and chronic inactivation of voltage-dependent Na⁺ channels (3). Concurrently, acidification of the muscle leads to decreased chloride conductance, reducing the shunting effect and thereby reducing the magnitude of Na⁺ current required to excite the muscle membrane (4, 5). The molecular mechanism underlying the reduction of muscle chloride conductance with acidification appears to be strong potentiation of CIC-1 inhibition by intracellular ATP at low pH (6, 7).

Members of the CIC family of channels and transporters share a dimeric architecture, supporting two identical conductance pathways. Two gating processes, separable due to the time course of their relaxations, regulate CIC-1 channel activity: a relatively fast “protopore” gate acts separately on each of two identical pores in the channel complex, and a slower “common” gate regulates both pores simultaneously. All eukaryotic CIC proteins have two tandem cystathionine β-synthase (CBS) domains in their intracellular carboxyl-terminal regions that have been proposed to form a binding site for adenosine ligands (8). CIC-1 CBS domains bind ATP, ADP, and AMP with similar apparent affinities, inhibiting the channel by shifting the voltage dependence of the common gate to more positive potentials (6, 7, 9–11). Nucleotide binding to CBS domains also regulates the function of CIC-2 chloride channels (8, 12), the plant CIC NO₃⁻/H⁺ antiporter, AtCICa (13), and the Cl⁻/H⁺ antiporter CIC-5 (14–16). In contrast, crystal structures of carboxyl-terminal fragments of CIC-0, a channel closely related to CIC-1, and CIC-Ka channels do not show ATP binding (17, 18). It is unclear if another ligand apart from ATP binds to the CBS domains of these proteins to regulate function.

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The coenzyme nicotinamide adenine dinucleotide (NAD) is a ubiquitous metabolic regulator that is present in the cytoplasm of cells, consisting of a pyridine and adenine nucleotide joined at their phosphate groups. In addition to its role in redox metabolism, proteins such as the sirtuins are regulated by, and consume, NAD⁺, allowing them to act as metabolic sensors that can regulate downstream metabolic pathways (19). Metabolites of NAD⁺ that result from these enzymatic reactions potentially mobilize intracellular Ca²⁺ stores by acting directly on ryanodine receptors (20) as well as unidentified receptors in other compartments (21) and activate Ca²⁺ influx carried by TRPM2 channels (22). In addition, NAD⁺ and its main metabolite, adenosine 5'-diphosphoribose, also affect calcium signaling via agonism of P2X (23) ligand-gated channels and P2Y G-protein-coupled receptors (24).

We hypothesized that NAD(H) may bind to ClC-1 CBS domains to modulate channel function, thus linking cell metabolism to membrane excitability in muscle cells. Our results show that, at physiologically relevant concentrations, both oxidized (NAD⁺) and reduced (NADH) forms of NAD inhibit ClC-1 channels by binding to CBS domains to regulate common gating and that inhibition is greatly enhanced at low intracellular pH. These findings identify a new molecular pathway coupling ClC-1 activity to the metabolic state of the cell that is entirely consistent with the reduced sarcolemmal chloride conductance that occurs with acidification of skeletal muscle. Our findings also identify a new biological role for NAD in the regulation of membrane excitability of muscle cells and suggest a ubiquitous ligand that may be important in regulation of other ClC proteins.

**EXPERIMENTAL PROCEDURES**

**Channel Expression and Mutagenesis**—For whole-cell experiments human ClC-1 cDNA was subcloned into pCDNA3.1 vector (Invitrogen). Tsa201 cells (a human embryonic kidney cell line that stably expresses the SV40 large T antigen) were transiently transfected with a mixture of the ClC-1 construct and pEGFP-N1 (Clontech) reporter plasmid at a molar ratio of 5:1 using ExtremeGene 9 transfection reagent (Roche) according to the manufacturer’s specifications. Transfected cells were later identified by reporter plasmid-driven expression of the green fluorescent protein. Mutations T636A, H847A, and L848A were introduced into CIC-1 and cloned into the pCIneo (Promega) vector, and mutations G200R and Y261C were introduced into ClC-1 in pCDNA3.1 using the QuikChange (Stratagene) mutagenesis technique as detailed previously (9).

For whole-cell experiments cells were continuously superfused with bath solution containing 140 mM NaCl, 4 mM CsCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES adjusted to pH 7.4 with NaOH. The standard pipette solution contained 40 mM CsCl, 80 mM cesium glutamate, 10 mM EGTA-Na, 10 mM HEPES adjusted to pH 6.8 with NaOH. For low pH internal solutions, 10 mM MES was substituted for HEPES, and the pH was adjusted to 6.2 with NaOH. β-Nicotinamide adenine dinucleotide (NAD⁺), β-nicotinamide adenine dinucleotide phosphate (NADP), reduced β-nicotinamide adenine dinucleotide (NADH, dipotassium salt), and ATP (magnesium salt) were purchased from Sigma, and stock solutions were made at a concentration of 100 mM in distilled water (NAD⁺, NADP, and ATP) or 10 mM NaOH (NADH) and stored at −20 °C. Working solutions containing NAD⁺, NADH, NADP, or ATP were made fresh on the day of the experiment and used immediately. Patch pipettes had a resistance of 1–2 megaohms when filled with the above pipette solution. Series resistance did not exceed 4 megaohms and was 85–90% compensated. After rupturing the cell membrane and achieving the whole-cell configuration, no less than 5 min was allowed for the pipette solution to equilibrate with the intracellular solution before current recordings were made.

Excised patch inside-out experiments were performed on *Xenopus* oocytes using an Axopatch 200B amplifier and Digidata 1440 A/D board controlled by pClamp10 software (Axon Instruments/Molecular Devices). The bath (intracellular) contained 120 mM NMG-Cl, 1 mM MgCl₂, 1 mM EGTA, 0.1 mM 2-mercaptoethanol, 10 mM HEPES, and the pH was adjusted to either 6.8 or 6.2 after the desired concentration of NAD⁺, NADH, or NADP was added. Working solutions containing NAD⁺, NADH, or NADP were prepared from the respective stock solutions immediately before the experiment. Application of solutions containing NAD⁺, NADH, or NADP to the intracellular surface of excised patches was achieved using a SF-77 fast solution exchanger (Warner Instruments). Pipette (extracellular) solution was the same as the bath solution with the exception that 2-mercaptoethanol was omitted, and the pH was adjusted to 7.4. Patch pipettes had a resistance of 0.4–0.6 megaohms when filled with the above solution.

Channel activity was assessed using the same methods as detailed elsewhere (6, 7, 9). For whole-cell experiments test pulses were applied in successive sweeps from −140 to +100 mV in 20-mV increments for a duration of 300 ms. After the test pulse, a 50-ms tail pulse to −100 mV was used to monitor apparent open probability of the channel. The membrane was clamped to −30 mV (whole-cell) or 0 mV (inside-out) for a period of 4 s between each sweep. To measure the open probability of the common gate in isolation, the protopore gate was fully opened by a 400-μs pulse to +170 mV between the test pulse and the tail pulse (27). Instantaneous tail-current amplitude was extrapolated by fitting a double-exponential function, with an offset component, to the tail current relaxation. Inside-out experiments were conducted in the same fashion, except that the voltage steps were from +100 to −140 mV in −20-mV
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increments, and a longer, 200-ms tail pulse was used to monitor open probability. Apparent open probability ($P_o$) was calculated by fitting tail-current amplitudes with a Boltzmann distribution and normalizing to the maximum amplitude ($I_{max}$) obtained from the fit (Equation 1),

$$I(V) = (I_{max} + (I_{max} - I_{min})/(1 + \exp((V_{1/2} - V)/k))) \quad (\text{Eq. 1})$$

where $I_{max}$ and $I_{min}$ are the maximum and minimum current amplitudes, respectively, $V$ is the membrane voltage, $V_{1/2}$ is the half-maximal activation voltage, and $k$ is the slope factor of the voltage dependence. For inside-out experiments, current amplitudes in the presence of NAD$^+$ or NADH were normalized to $I_{max}$ determined for the same patch in the absence of compound. Where applicable, open probability of the protopore gate ($P_{o}^{\text{protopore}}$) was determined by dividing the apparent channel open probability by the open probability of the common gate. The voltage dependence of apparent open probability ($P_{o}(V)$), open probability of the common gate ($P_{o}^{\text{common}}(V)$), and open probability of the protopore gate ($P_{o}^{\text{protopore}}(V)$) were obtained by fitting open probability data with a modified Boltzmann distribution (27, 28),

$$P_{o}(V) = (P_{min} + (1 - P_{min})/(1 + \exp((V_{1/2} - V)/k))) \quad (\text{Eq. 2})$$

where $P_{min}$ is the minimum open probability, and the other symbols have the same meaning as in Equation 1. It is important to note from Equation 2 that $V_{1/2}$ is the half-saturation, or inflection point, of the gating curve and is independent of $P_{min}$. In inside-out experiments where $P_{o}^{\text{common}}$ was monitored continuously, a test voltage of $+40$ mV was applied followed by a $+170$-mV pulse for 400 $\mu$s and finally a tail voltage pulse to $-100$ mV. This protocol was applied to the membrane patch at intervals of 2 s. Exponential curve fitting was performed using AxographX, and the remaining whole-cell data analysis was performed with Microsoft Excel and GraphPad Prism 5. Inside-out data were analyzed using pClamp 10 and Origin 8.0. Data are expressed as the means ± S.E. except where indicated. Where applicable, data were examined using two-way analysis of variance followed by a Bonferroni post test, with $p < 0.05$ taken as the measure of statistical significance.

Homology Modeling of CIC-1 CBS Domains (Model 1)—An homology model of the CIC-1 carboxyl-terminal CBS region was built using the published CIC-5 crystal structure of the same region (PDB ID 2JAJ (14)) and the CIC-1 amino acid sequence (29). The model consists of residue Tyr-601 to Lys-875, corresponding to residues His-578 to Asn-737 in CIC-5. The CIC-1 protein contains a large insertion in the region of the protein linking CBS 1 and CBS 2 (Arg-669–Cys-820), with respect to CIC-5. The linker is resolved in the crystal structure of CIC-5; however, it shares little homology with CIC-1. Critically, the region linking CBS 1 and 2 is unresolved in other CIC CBS domain structures, including CIC-0 residues 619–660 (PDB ID 2D4Z) (18), CIC-Ka residues 606–612 (PDB ID 2FPI) (17), and CmCIC residues Val-600–Val-656 (PDB ID 3ORG) (30). Thus, the region where no structural coordinates were available to base the model on, from Ala-678 to Val-814 of CIC-1, was excluded from the model. In the CIC-5 crystal structures this region does not interfere with the nucleotide binding site, and although we cannot be certain that this is also the case in CIC-1, we have assumed that it has no bearing on the isolated structure of CBS 1 and CBS 2. In the protein region modeled CIC-1 and CIC-5 share 27% identical residues and 47% sequence similarity. A sequence alignment of the relevant protein regions was generated using TCooffee (31), and the CIC-1 model was built on the structural coordinates of CIC-5 in complex with ADP (PDB ID 2JAJ) using Swiss-Model (32) followed by energy minimization in SwissPDB viewer (32). The quality of the model was assessed using Verify3D (33) and found to be satisfactory. NAD$^+$ was docked into the cleft between CBS 1 and CBS 2 using Surflex-Dock 2.1 (34), and the final model was examined using MacPymol 1.4.1.

Homology Modeling of CIC-1 Channel with Intact CBS Domains (Model 2)—An homology model of CIC-1, with intact intracellular CBS domains, was built using the published crystal structure of the homologous regions of CmCIC (PDB ID 3ORG) (30). The model consists of CIC-1 residues Val-110 to Gly-872, corresponding to residues Ser-88 to Asn-710 of CmCIC. For reasons discussed above, residues His-664-Ser-818 of the CBS 1-CBS 2 linker were omitted from model 2. In the protein regions used in construction of model 2, CIC-1 and CmCIC share 30% identical residues and 51% sequence similarity. The model was constructed using the method detailed for model 1. The quality of the model was assessed using Verify3D (33) and found to be satisfactory.

RESULTS

Intracellular NAD(H) Inhibits CIC-1 by Modulating Common Gating—CIC-1 channels are inhibited by intracellular ATP, thus linking cell metabolism to the excitability of skeletal muscle (6, 7, 9–11). The coenzyme NAD(H) has emerged as an important metabolic regulator and through direct action or the action of its metabolites on various ion channels is important in the control of cell calcium. Because of the critical role of calcium signaling in muscle contraction, we hypothesized that NAD(H) could also be involved in regulation of muscle excitability by binding to CIC-1 channels to modulate their function.

We first examined the effect of NAD(H) on CIC-1 function at intracellular pH 6.8, which is close to the resting pH of skeletal muscle. Inclusion of 3 mM NAD$^+$ or NADH in the pipette solution during whole cell experiments shifted the voltage dependence of CIC-1 gating to more positive potentials (Fig. 1). When we separated the contributions of protopore and common gating to the overall open probability of the channel, we found that NAD(H) exerted its effect on CIC-1 by shifting the common-gating curve to the right on the voltage axis and simultaneously reducing the minimum value of $P_o(V)$, whereas protopore gating remained essentially unaffected (Fig. 1, C and D).

Through the actions of NAD kinase, NAD$^+$ may be converted to NADP$^+$ by the addition of a phosphate group to the 2$^\prime$ position of the ribose ring that bears the adenine moiety. In contrast to NAD$^+$ and NADH, the addition of 3 mM NADP$^+$ to the pipette solution had essentially no effect on CIC-1 gating (Fig. 1).

The effect of NAD(H) on CIC-1 gating was dose-dependent and at higher concentrations NAD$^+$ induced a greater shift of the common-gating curve with respect to equivalent concen-
trations of NADH (p < 0.01) (Fig. 2), with both effects appearing to plateau in the low millimolar range (Fig. 2 C). Current estimates place the cytosolic NAD(H) concentration in the range 0.3–0.7 mM (35–37). At intracellular pH 6.8, inclusion of 0.3 mM NAD(+) or NADH in the intracellular solution had only a slight effect on common gating (Fig. 2). Because intracellular ATP has been shown to affect CIC-1 common gating (6, 7, 9–11) in much the same fashion as we observed for NAD(H), we tested the ability of NAD(+) to affect gating in the presence of ATP. Inclusion of 1 mM ATP in the pipette solution shifted the common gating curve to more positive voltages, as expected, but the shift was less than for 1 mM NAD(+) (Fig. 2 C). In the presence of 1 mM ATP, the addition of NAD(+) to the pipette solution resulted in further shifts of common gating curves to the right on the voltage axis (Fig. 2 C).

We next sought to confirm that NAD(+) and NADH act directly on CIC-1 by examining their effect on gating when applied to the intracellular surface of inside-out patches. We first attempted to examine CIC-1 channels in inside-out patches from tsa201 cells and reliably observed inhibition of CIC-1 by application of NAD(+) or NADH (data not shown). Extensive experiments were performed in patches excised from Xenopus oocytes in which more stable, higher quality recording traces were obtained. Fig. 3 shows the effect of transient application of NAD(H) and NADP to the intracellular surface of CIC-1 channels in an oocyte membrane patch. We monitored common gating of CIC-1 by using a voltage protocol designed to fully activate protopore gating before examining peak current at a constant −100 mV test voltage (Fig. 3). Differences in current level in Fig. 3 are, therefore, solely representative of modulation of the open probability of the common gate. Application of solutions containing 1 mM NAD(+) or NADH to the intracellular surface of inside-out patches inhibited CIC-1 common gating (Fig. 3). In contrast, and consistent with our whole-cell data, 1 mM NADP had no appreciable effect. Inhibition occurred rapidly upon perfusion of the excised patch and was washed out just as quickly upon returning to standard solution, consistent with binding of NAD(H) to intracellular domains of CIC-1 to modulate gating. We next examined the effect of 1 mM NAD(+) applied to the intracellular surface of patches on steady-

**FIGURE 1. Intracellular NAD(H) alters the voltage dependence of CIC-1 common gating.** A, representative whole-cell current traces from tsa201 cells expressing CIC-1 with and without 3 mM NAD(+), NADH, or NADP in the pipette solution as indicated. B, overall channel open probability from pooled experiments is shown in panel A, calculated as described under “Experimental Procedures” (n = 4, pH 6.8, 5 (NAD(+)), 4 (NADH), and 3 (NADP). C and D, dissection of channel open probability into protopore (C) and common gating open probability (D), respectively, was calculated as described under “Experimental Procedures” (n as per panel (A)). Solid lines in panels B–D represent fits of Equation 2 to the experimental data points.
state open probability of ClC-1. In agreement with the whole-cell data, 1 mM NAD\(^+\) reduced the open probability of the common gate over the range of voltages tested, whereas protopore gating remained unaltered (Fig. 4).

We also examined the combined effect of NAD\(^+\) and ATP binding on ClC-1 in inside-out patches excised from tsa201 cells and *Xenopus* oocytes (Fig. 5). We have previously shown that the effect of ATP on ClC-1 common gating is almost saturated at 3 mM in inside-out patches from *Xenopus* oocytes (7). At non-saturating concentrations, inhibition of ClC-1 by ATP (0.1 mM) and NAD\(^+\) (1 mM) was additive (Fig. 5). In contrast, in the presence of a saturating ATP concentration (3 mM), application of 1 mM NAD\(^+\) resulted in, essentially, no further inhibition of ClC-1. These results suggest that ATP and NAD\(^+\) exert their effect on ClC-1 gating by binding to the same site.

**NAD(H) Inhibition of ClC-1 Is Potentiated by Acidification**—The resting intracellular pH of human muscle is \(\sim 7.0\) but during intense activity may fall as low as pH 6.2–6.4 (38–42) and recover over a time course of 20–25 min (43–45). Acidification strongly potentiates the effect of ATP on common gating of ClC-1 (6, 7), leading us to examine whether low pH has a similar effect on NAD(H) inhibition of ClC-1. In whole-cell experiments reduction of the internal pH to 6.2 greatly increased the magnitude of the shift of common gating curves for both NAD\(^+\) and NADH when compared with the effect of equivalent concentrations at pH 6.8 (Fig. 6). At pH 6.2, a physiologically relevant concentration of NAD\(^+\) (0.3 mM) shifted the \(V_{1/2}\) of the common-gating curve by \(\sim +108\) mV, in comparison to a shift of only \(\sim +15\) mV at pH 6.8 (Fig. 6C). At pH 6.8 we observed that, for equivalent concentrations, NAD\(^+\) was more effective at shifting the \(V_{1/2}\) of ClC-1 common gating than NADH (Fig. 6C). This trend was far more pronounced at pH 6.2; at concentrations that appeared to be approaching saturation, 1 mM NAD\(^+\) (\(\Delta V_{1/2} \sim +135\) mV) induced a shift of \(V_{1/2}\) some 62 mV greater than for 1 mM NADH (\(\Delta V_{1/2} \sim +73\) mV) (Fig. 6C).

We also observed enhanced inhibition of ClC-1 common gating when NAD\(^+\) or NADH was applied to the intracellular surface of inside-out patches at pH 6.2 (Fig. 7). Consistent with data obtained from whole cells, 1 mM NAD\(^+\) reduced \(P_o^{\text{common}}\) to a greater extent than 1 mM NADH.

**NAD(H) Binds to ClC-1 CBS Domains**—Having observed robust inhibition of ClC-1 by NAD(H), we next sought to exam-
ine the molecular details of NAD\(^+\) binding. All eukaryotic ClC proteins have a cytoplasmic carboxyl-terminal region containing tandem CBS domains that are proposed to form an ATP binding site (8). ClC-1 CBS domains have been shown to bind adenosine ligands (6, 9, 10), and adenine nucleotide binding to CBS domains of ClC-5 has been directly observed using x-ray crystallography (14). Structural coordinates of several ClC CBS domains are available (14, 17, 18, 30), although the only structure involving a complex with nucleotide is the ClC-5 structure. This is very relevant for modeling the binding site, as it has recently been shown that nucleotide binding induces a significant conformational change in isolated ClC-5 CBS domains.

**FIGURE 4.** Effect of NAD\(^+\) on ClC-1 steady-state open probability in inside-out patches excised from Xenopus oocytes. A, shown are current traces taken from the same patch in control intracellular solution (left panels) and during application of 1 mM NAD\(^+\) to the intracellular surface of the patch (right panels). The current traces in the lower panels differ from those above in that a 400-\(\mu\)s pulse to +170 mV is inserted between the variable test voltage and the tail pulse to fully activate the protopore gates (see “Experimental Procedures”). Dotted lines in this panel indicate zero-current level. B, \(P_o^{\text{channel}}\) (top), \(P_o^{\text{common}}\) (bottom), and \(P_o^{\text{protopore}}\) (bottom, inset) calculated from raw data are shown in panel A. Initial tail currents of currents shown in A were normalized to maximal values in control to calculate \(P_o^{\text{channel}}\) and \(P_o^{\text{common}}\). Values of \(P_o^{\text{protopore}}\) were then calculated by dividing \(P_o^{\text{channel}}\) by \(P_o^{\text{common}}\). Solid lines are straight lines connecting experimental data points.

**FIGURE 5.** The inhibitory effects of ATP and NAD\(^+\) on ClC-1 are additive when the ligand concentrations are not saturated. The experiments performed in excised inside-out patches from Xenopus oocytes (A) and transfected tsA201 cells (B) involved continuously monitoring the ClC-1 current at pH\(_i\) = 6.8 by a voltage protocol the same as that shown in Fig. 3. The initial tail-current was plotted as a function of time. Solution exchanges are indicated by solid arrows. Removal of ligands was not labeled. Insets show the comparison of the original recording traces before (black trace) and after (red trace) the application of NAD\(^+\) (1 mM) or NADH (1 mM) in 0.1 mM ATP or in 3 mM ATP (nearly saturated concentration of ATP). In can be seen that although the inhibitions of ClC-1 current by 1 mM NAD\(^+\) are comparable in the absence and presence of 0.1 mM ATP, the inhibition by 1 mM NAD\(^+\) is much smaller in 3 mM ATP compared with the inhibition in the absence of ATP. Recordings from seven other patches (five from oocytes and two from tsA201 cells), all showed similar results.

**FIGURE 6.** NAD(H) modulation of ClC-1 gating is potentiated at low pH\(_i\). A, shown are representative whole-cell current traces from tsa201 cells expressing ClC-1 with and without 1 mM NAD\(^+\) or 1 mM NADH at pH 6.2 as indicated. B, common gating curves were determined from experiments shown as in panel A. Solid lines represent the fit of Equation 2 to the experimental data points. C, half-activation voltages were determined from \(P_o^{\text{common}}(V)\) as shown in panel B, plotted as a function of NAD(H) concentration. Open circles and diamonds are data for NAD\(^+\) and NADH, respectively, at pH 6.8, as shown in Fig. 2. Filled circles and diamonds are data for NAD\(^+\) and NADH, respectively, at pH 6.2. For pH 6.2, \(n = 4\) (0 NAD(H)), 3 (0.1 mM NAD(H)), 3 (0.3 mM NAD(H)), 4 (1 mM NAD\(^+\)), and 3 (1 mM NADH). Solid lines are straight lines connecting experimental data points.
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**FIGURE 7. Enhanced inhibition of CIC-1 at low pH, is preserved in inside-out patches excised from Xenopus oocytes.** The effect of NADH (left), NAD⁺ (middle), and NADP⁺ (right) on \( P_{\text{min}} \) on CIC is shown at pH 6.8 (upper three panels) and pH 6.2 (bottom three panels), \( n = 4 \) – 6 patches. Solid curves are best fits to the Boltzmann equation (Equation 2). Because \( P_{\text{min}} \) changes in different conditions, for the purpose of curve-fitting, the \( P_{\text{min}} \) value was fixed at 0.43 and 0.33 at pH 6.8 (upper three panels) for the data points in control (black) and in the presence of nucleotides (red). At pH 6.2 (bottom three panels), the \( P_{\text{min}} \) was fixed at 0.5 and 0.38 for control (black) and in the presence of nucleotides (red). The fitted values of \( V_{1/2} \), at pH 6.8 (upper three panels) were \(-76.7 \pm 1.3 \text{ mV (control)} \) and \(-39.2 \pm 1.3 \text{ mV (1 NADH) (left)} \), \(-83.6 \pm 1.4 \text{ mV (control)} \) and \(-29.2 \pm 2.8 \text{ mV (1 NAD⁺) (middle)} \), and \(-79.1 \pm 1.4 \text{ mV (control)} \) and \(-75.1 \pm 1.1 \text{ mV (1 NADP⁺) (right)} \). The fitted \( V_{1/2} \) values at pH 6.2 (bottom three panels) were \(-94.9 \pm 1.4 \text{ mV (control)} \) and \(-7.4 \pm 1.6 \text{ mV (1 NADH) (left)} \), \(-108.8 \pm 1.8 \text{ mV (control)} \) and \(+45.6 \pm 3.6 \text{ mV (1 NAD⁺) (middle)} \), and \(-86.5 \pm 1.1 \text{ mV (control)} \) and \(-68.2 \pm 1.0 \text{ mV (1 NADP⁺)} \).

(46), and this is likely to be a conserved feature of nucleotide binding to CBS domains in general (47). Additionally, homology modeling of CIC-1 CBS domains using the CIC-5 structure as a template has recently proven to be highly predictive (10). We, therefore, constructed a model of CIC-1 CBS 1 and 2 based on the structural coordinates of CIC-5 to examine binding of NAD⁺ (Fig. 8). Docked NAD⁺ adopted an orientation where the adenine moiety was buried between CBS 1 and CBS 2, and the nicotinamide group was at the periphery of the CBS domain (Fig. 8A). The predicted NAD⁺ binding pocket is lined by parts of the protein that are indicated in Fig. 8, B and C. Of these residues, side chains from Val-610, Asp-112, Val-634, and Thr-636 in CBS 1 and His-847, Leu-848, Val-860, Glu-864, and Glu-865 in CBS 2 are in close proximity to the docked NAD⁺ molecule (Fig. 8B). We have previously shown that residues Thr-636, His-847, and Leu-848 are important in ATP and AMP modulation of CIC-1 gating (9). We, therefore, examined the importance of these residues for NAD⁺ modulation of CIC-1 by individually mutating them to alanine and measuring the voltage dependence of common gating (Fig. 9). Mutation of residue threonine 636 to alanine (T636A) had little effect on common gating at pH 6.8 and only marginally reduced the effect of 3 mM NAD⁺ on the common-gating curve (\( p < 0.01 \)) (Fig. 9B) (\( \Delta V_{1/2} \) \(+38 \text{ mV for T636A versus } \Delta V_{1/2} \) \(+66 \text{ mV for WT CIC-1} \)). Similarly, mutant H847A channels had common-gating curves mostly indistinguishable from WT CIC-1 at pH 6.8. However, the shift of \( V_{1/2} \) in response to 3 mM NAD⁺ (\( \Delta V_{1/2} \) \(+23 \text{ mV}) was reduced with respect to WT CIC-1 (\( p < 0.01 \)) (Fig. 9C). The most effective of the mutations, L848A, increased the minimum value of \( P_{\text{w}} \) (\( P_{\text{min}} = 0.44 \pm 0.03 \)) with respect to WT CIC-1 (\( P_{\text{min}} = 0.21 \pm 0.02 \)), whereas the half-maximal activation voltage of common-gating remained unchanged (\( V_{1/2} = -54 \pm 4 \text{ mV (L848A)} \), \( V_{1/2} = -51 \pm 2 \text{ mV (WT CIC-1)} \)). In addition, the L848A mutation essentially eliminated any effect of 3 mM NAD⁺ on common gating of CIC-1 channels (\( p < 0.01 \)) (Fig. 9D).

**Myotonic Mutations G200R and Y261C Affect NAD Inhibition of CIC-1**—Our modeling of NAD⁺ binding to the CBS domains of CIC-1 shows that the nicotinamide ring of NAD⁺ is exposed at the periphery of the binding site (Fig. 8A), where it may form contacts with residues contributed by either the extensive CBS 1–2 linker that is omitted from the model (see “Experimental Procedures”) or, alternatively, cytosolic loops of the membrane-embedded channel proper. Indeed, the recently published structure of a eukaryotic CIC transporter, CmCIC, shows that the region of CBS 2 where the nicotinamide group is predicted to lie in our model is in close apposition to the intracellular surface of the membrane-embedded domain (30). To examine the possibility that residues from the membrane-embedded domain participate in interactions with NAD⁺, we constructed a homology model based on the structural coordinates of CmCIC (30). We were unable to dock NAD⁺ into the binding site identified in Fig. 8 into this model. A comparison between the CBS domains of the two models revealed that the binding site in model 2 was obstructed by the loop formed by residues Val-610–Phe-615 (Fig. 10), which forms part of the NAD⁺ binding site shown in Fig. 8. There are plausible arguments to remove this loop from the binding site in the presence of ligand (see “Discussion”), and by removing this loop we were able to dock NAD⁺ into the model in the orientation identified from
Fig. 8 and inspect the molecular details of the binding site in a model with intact channel and CBS domains. This approach identified residues Gly-200, Val-201, and Val-202 as possible candidates to interact with the nicotinamide moiety of NAD\(^+\) (Fig. 10B). These residues are present in the model at the carboxyl-terminal end of \(\alpha\) helix D that, at its amino-terminal end, carries the highly conserved GSGIP motif that forms part of the channel selectivity center (48, 49) (Fig. 10C). The backbone oxygen atom of residue Gly-200 is in hydrogen-bonding distance of the amide group of the nicotinamide moiety (Fig. 10C). We were intrigued by this observation because mutation of this residue to arginine (G200R) causes myotonia congenita (50). In addition, from the modeling presented in Fig. 10, we were able to identify another residue, Tyr-261, that is mutated in myotonia (Y261C) (50) and that, although not directly interacting with NAD\(^+\), is in close proximity to the binding site. We tested the effects of introducing these mutations on the modulation of ClC-1 common gating by NAD\(^+\) and protons.

As reported by others, gating of G200R mutant channels was shifted to more positive voltages with respect to WT ClC-1, whereas Y261C gating curves were unaltered (51) (Fig. 11). Inclusion of NAD\(^+\) in the pipette solution at pH 6.8 shifted the
voltage dependence of common gating of both mutant channels to more positive voltages (Fig. 11B). The maximum effect NAD$^+$ on common gating of the G200R mutant was substantially less than for WT ClC-1, however ($p < 0.01$) (Fig. 11C). Common gating of Y261C mutant channels, in contrast, displayed a dependence on NAD$^+$ concentration that closely paralleled WT ClC-1 (Fig. 11C).

The severity of myotonic symptoms may be alleviated with muscle use by an unknown mechanism often referred to as the “warm-up phenomenon.” Because working muscle becomes acidified, we hypothesized that the pH dependence of ClC-1 inhibition by NAD$^+$ may be altered in the myotonic mutants G200R and Y261C. When we examined the effect of 1 mM NAD$^+$ on common gating at pH$_r$ 6.2, we were surprised to observe that the strong potentiation of inhibition observed for WT ClC-1 (Figs. 6 and 7) was absent in the mutants (Fig. 11D). Common gating of Y261C mutant channels was shifted by 1 mM NAD$^+$ to the same extent at either pH$_r$ 6.8 or 6.2, and gating of G200R mutant channels was actually less sensitive to 1 mM NAD$^+$ at pH$_r$ 6.2 than at pH$_r$ 6.8 ($p < 0.01$) (Fig. 11E).

**DISCUSSION**

We have shown here that intracellular NAD$^+$ directly inhibits ClC-1 channels by shifting the voltage dependence of common gating to more positive voltages and that this inhibition is strongly dependent on intracellular pH. NAD$^+$ is present in the cytoplasm of muscle cells, where energy liberated through glycolysis is transferred to NAD$^+$ by reduction to NADH. NADH is then normally transferred to the mitochondria where it is oxidized in the electron-transport chain as part of ATP generation by oxidative phosphorylation. Under intense exercise conditions, however, pyruvate may be used to oxidize NADH, resulting in lactate accumulation and acidosis (52), reducing cytosolic pH from $7.0$ in resting muscle to as low as pH 6.2–6.4 (38–42). Cytosolic NAD$^+$ concentration is believed to be in the range 0.3–0.7 mM (35–37). In this range NAD$^+$ inhibited ClC-1 channels at pH 6.8, but inhibition was greatly enhanced when pH was reduced to 6.2.

Skeletal muscle acidification leads to decreased sarcolemmal chloride conductance, which acts to maintain excitability of the partially depolarized sarcolemma and opposes the effect of muscle fatigue (4, 5). However, studies of the pH dependence of heterologously expressed ClC-1 have failed to reconcile this decreased chloride conductance with a direct effect of protons on ClC-1 (28, 53, 54). We have previously proposed that potentiation of the inhibitory effect of ATP on ClC-1 by acidification may be the mechanism underlying reduced sarcolemmal chloride conductance (6). The results of this study indicate that at
Our data also show that NAD$^+$ concentrations relevant to those in the cytoplasm of muscle cells, NAD$^+$ also binds to CIC-1 to regulate its activity and imparts a sensitivity to protons that is in keeping with the observed effects of pH on sarcolemmal chloride conductance. Our data also show that NAD$^+$ and ATP act in concert to modulate common gating of CIC-1; however, far more

FIGURE 10. In silico docking of NAD$^+$ to a full-length CIC-1 model. A, shown is a comparison of CBS domains from model 1 and model 2. CBS domain model 1 (as depicted in Fig. 6) is colored in gray and overlaid on CIC-1 CBS domains of model 2 based on the structural coordinates of CmCIC. CBS 1 and CBS 2 of model 2 are depicted in blue and magenta, respectively. Residues Val-610–Phe-615 of model 2 are colored red. NAD$^+$, docked to model 1, is represented as sticks and colored by atom type. B, molecular details of the NAD$^+$ binding site of full-length model 2 are shown. The backbone of CIC-1 is depicted as Cu trace, and side chains of the NAD$^+$ binding site are depicted as sticks. CBS 1 and CBS 2 are colored in blue and magenta, respectively, and shading is the same as in Fig. 7. Residues Gly-200, Val-201, and Val-202 are colored in cyan. For reasons detailed under "Results," residues Val-610–Phe-615, inclusive, are omitted from the model, and for clarity residues Lys-586–Tyr-595 have been omitted from the foreground in this figure. C, NAD$^+$ bound to the full-length model 2 is shown. CIC-1 is depicted in schematic format. For clarity, only one of the subunits, subunit A, is colored in wheat yellow. The other subunit is shaded gray. CBS 1 and CBS 2, of subunit A are depicted in blue and magenta, respectively. $\alpha$-helix D is colored in cyan, and visible elements of the selectivity center are colored in red. Residues Tyr-261 and Gly-200 of subunit A as well as NAD$^+$ are represented as sticks and colored by atom type. This figure was prepared using MacPymol.

FIGURE 11. Effect of myotonia associated mutations G200R and Y261C on NAD$^+$ modulation of CIC-1 common gating. A, shown are representative whole-cell current traces from tsa201 cells expressing G200R (top) and Y261C (bottom) mutants with and without 3 mM NAD$^+$ in the intracellular solution (pH 6.8) as indicated. The voltage protocol used to elicit these currents includes a brief (0.4 ms) step to +170 mV to isolate common gating of CIC-1 before measurement of tail currents. Scale bars are 2 nA (vertical) and 0.1 s (horizontal). B, common gating curves for G200R (n = 3), Y261C (n = 3) derived from pooled experiments are as depicted in panel A. Solid lines represent the fit of Equation 2 to the experimental data points. C, change in half-activation voltage with respect to standard intracellular solution at pH 6.8 was determined from $\Delta V_{0.5}$ as shown in panel B and is plotted as a function of NAD$^+$ concentration. Each data point represents the mean from n = 3–5 experiments. Solid lines are straight lines connecting experimental data points. Asterisks denote statistical significance; at 0.3 mM NAD$^+$ both G200R and Y261C are different to WT but not to one another (p < 0.05), and at 1 and 3 mM NAD$^+$, G200R is statistically different to Y261C and WT, which are not statistically different to one another (p < 0.01). D, shown is change in half-activation voltage of $\Delta V_{0.5}$ induced by 1 mM NAD$^+$ with respect to standard intracellular solution at pH 6.2 and pH 6.8 for WT CIC-1, G200R, and Y261C. Data are the means from n = 3–4 experiments. Asterisks denote statistically significant differences (p < 0.01).
CIC-1 Inhibition by NAD(H)

detailed studies are required to tease out the interplay between binding kinetics and effects on common gating of these two molecules. Our findings add another layer of complexity to the regulation of CIC-1 and its role in regulating the excitability of the muscle membrane. Ultimately the physiological contribution of NAD\(^+\) inhibition of CIC-1 to sarcolemmal excitability must be examined in the context of ATP depletion, changing cytosolic redox state, alteration of the NAD\(^+\)/NADH ratio, membrane depolarization, and acidification in active skeletal muscle.

The structural basis for nucleotide binding to the CBS domains of CIC proteins has been established by X-ray crystallography of isolated CIC-5 cytoplasmic domains (14). Interestingly, low level potentiation of CIC-5 currents in the presence of intracellular NAD\(^+\) indicates that CIC-5 CBS domains also probably bind NAD\(^+\) (16). In the CIC-5 structures ADP and ATP are seen bound to the CBS domains in a “head-on” orientation, where the adenine base is buried in a hydrophobic pocket created between CBS 1 and 2 and the phosphate groups are partly solvated and interact weakly with the protein at the periphery of the binding site. Weak interactions with the phosphate tail are reflected in the indiscriminate effect of ATP, ADP, and AMP on CIC-1 and CIC-5 function. All bind CIC-5 CBS domains with similar affinity (14) and all have similar maximum responses and half-saturation concentrations in the low millimolar range. Conversely, the adenosine moiety is critical for binding, as adenine, IMP, and GTP have essentially no effect on CIC-1 or CIC-5 function (9, 11, 16). Accordingly we expected that NAD(H) would bind to CIC-1 CBS domains in a similar orientation, with the adenine base buried in the binding pocket between CBS 1 and 2 and the nicotinamide base at the outside of the binding domain. The molecular modeling presented in Fig. 8, based on the coordinates of the CIC-5 structure, supports this hypothesis.

The model also offers a satisfying explanation for the lack of effect of NADP (Fig. 1). NADP is unable to adopt the same binding orientation that is shown for NAD\(^+\) or ADP, as the phosphate group on the 2’ position of the adenine nucleotide ribose ring would interfere with an acidic side chain from CBS 2 residue Glu-865 (Fig. 8). Indeed, in many proteins that bind NADP the phosphate group is stabilized by interaction with a basic side chain, whereas conversely, an acidic side chain prevents NADP binding in NAD-dependent enzymes (55). Lending credibility to the model, mutation of residues predicted to be involved in NAD\(^+\) binding either diminished (T636A, H847A) or abrogated (L848A) the effect of NAD\(^+\) on common gating (Fig. 9).

For reasons discussed under “Results,” the CIC-5 based model probably best represents the molecular details of NAD(H) binding by CIC-1 CBS domains. However, it does not yield details of possible interactions between the nicotinamide moiety of NAD(H) and cytosolic elements of the channel domain. To examine these interactions, we constructed a second model with intact CBS and channel domains based on the crystal structure of the CmCIC protein. We were forced to modify this model by removing a flexible loop at the aminoterminal end of CBS 1 in order to dock NAD\(^+\) into the binding site identified above (Fig. 10). This is not surprising given that CmCIC is not known to bind NAD\(^+\) or other ligands and also because occupancy of the binding site is known to induce conformational changes in CBS domains (46, 47). In support of this notion, the isolated CBS domains of CIC-5 were shown to form well diffracting crystals only when complexed with ADP or ATP, suggesting that occupancy of the binding site constrains unstructured regions of the domain (14). Importantly, critical features of the binding site identified from the CIC-5 based model, in particular interactions between bound NAD\(^+\) and His-847 and His-847, are preserved in the CmCIC-based model (Fig. 10).

We have previously speculated that the imidazole moiety of His-847 may interact with the phosphate groups of ATP and that protonation of this moiety may increase the strength of the interaction, leading to the increased effect of ATP at low pH (6). The results from our current study refute this hypothesis. In both models reported here the imidazole group of His-847 is in a stacked arrangement with the pyridine ring of the NAD\(^+\) nicotinamide moiety (Fig. 8A). This orientation is intuitively appealing as it would be expected to lead to favorable π–π or π–π\(\text{cation}\) interactions with NADH and NAD\(^+\), respectively. This hypothesis is inconsistent with the relative enhancement of inhibition we observed for the two species at low pH, however, where the imidazole side chain of His-847 would presumably be positively charged (56). Rather, our data suggest that enhanced inhibition at pH 6.2 results from an allosteric mechanism arising from titration of residues located remotely from the binding site. Consistent with this notion, both myotonic mutations examined in this study, G200R and Y261C, abrogated the potentiating effect of protons on NAD\(^+\) inhibition, and G200R mutant channels were actually less sensitive to NAD\(^+\) inhibition at pH 6.2 than at pH 6.8 (Fig. 11E). Although the G200R mutation reduced the level of inhibition by NAD\(^+\), consistent with the role we propose it plays in NAD\(^+\) binding (Fig. 11C), Tyr-261 does not contribute to the binding site but rather appears to interact with CBS 2 of the adjacent protein subunit in the dimeric complex. In keeping with this observation, NAD\(^+\) inhibition of Y261C mutant channels at pH 6.8 was equivalent to inhibition of WT CIC-1 channels (Fig. 11C).

Altered pH dependence of NAD\(^+\) inhibition of mutants G200R and Y261C raises the possibility that it may be a feature of the pathophysiology of myotonia. Myotonia congenita arises due to defective CIC-1 channels causing the muscle membrane to become hyperexcitable. Myotonia is characterized by an unusual delay in muscle relaxation after muscle contraction that is alleviated by multiple repetitions of the same contraction; a phenomenon often referred to as the “warm-up effect.” It has been suggested that an activity-dependent increase in CIC-1 function may underlie the warm-up effect (57). Under normal circumstances CIC-1 channel activity would be expected to decrease, as working muscle becomes acidified due to enhanced inhibition of CIC-1 by ATP (6, 7) or NAD\(^+\). This is probably the mechanism underpinning the reduced sarcolemmal chloride conductance and increased excitability observed upon intracellular acidification of muscle (4, 5). In the case of the mutant channels examined here, however, acidification of muscle would fail to enhance inhibition of
CIC-1 and would, therefore, be expected to alleviate the effect of pathologically increased excitability of myotonic muscle. The implications of our findings remain speculative, however, and will only become clear when examined in the context of whole muscle.

The molecular basis of common gating is presently obscure, and so we are unable to speculate on how NAD(H) binding affects this mechanism. We are, however, able to suggest possible molecular pathways by which NAD(H) binding may affect gating. Common gating of CIC-0 and CIC-1 channels is accompanied by a conformational change of the intracellular carboxyl terminus that contains the CBS domains (58, 59). Ligand binding is also known to affect the conformation of CBS domains (46, 47). It is reasonable to speculate that NAD(H) binding to CIC-1 CBS domains stabilizes a conformation of CIC-1, which is consistent with the closed state of the common gate. Because the membrane voltage is a potential electrical difference across the cell membrane, it is “invisible” to the intracellular CBS domains. Therefore, for NAD(H) binding to influence the voltage dependence of common gating, binding information must be communicated to the membrane-embedded channel domain. From our CmClC-based CIC-1 model (Fig. 10), we are able to identify possible candidates to mediate this relay of structural information (not shown). CBS 2 of each subunit is positioned in close apposition to the intracellular surface of the membrane-embedded domain. Helix D, which carries the conserved GSGIP motif of the channel pore, makes substantial contacts with CBS 2 at its carboxyl-terminal end. Similarly helix R, which at its amino-terminal end carries the highly conserved Tyr-578 pore-forming residue, also forms contacts with CBS 2 both at its carboxyl-terminal end and by way of the region linking helix R and CBS 1. Finally, the amino-terminal end of membrane-spanning helix G makes intersubunit contacts with CBS 2 of the adjacent subunit. It is possible that a conformational change affecting CIC-1 CBS domains, commensurate with NAD(H) binding, is relayed to the common gating machinery of the channel pore by translation of one or more of these helices.

NAD\(^+\) plays important roles in metabolic regulation (19) and, perhaps of more relevance to the current study, in intracellular calcium signaling by a direct effect of NAD\(^+\) or metabolites thereof on ryanodine receptors (20) and various other receptors and ion channels (21–24). Here we have demonstrated that NAD\(^+\) also regulates the function of skeletal muscle CIC-1 chloride channels by direct binding to intracellular CBS domains. Because intracellular NAD\(^+\) concentration probably changes little, the physiological role of NAD\(^+\) binding to CIC-1 very likely lies in the exquisite sensitivity to pH that it imparts to common gating. The relevance of differential inhibition by oxidized or reduced forms of NAD is unclear and, like the altered inhibition of myotonic mutants G200R and Y261C, requires more detailed study in the context of active skeletal muscle. So too, the relevance of NAD\(^+\) binding to other CIC proteins awaits study; however, we suggest NAD\(^+\)-dependent regulation may become a consistent theme for CIC chloride channels and Cl\(^{-}\)/H\(^+\) antiporters.

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