Regulation of CNGA1 channel gating by interactions with the membrane

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ABSTRACT

Cyclic nucleotide-gated (CNG) channels are expressed in rod photoreceptors and open in response to direct binding of cyclic nucleotides. We have previously shown that potentiation of CNGA1 channels by transition metals requires a histidine in the A' helix following the S6 transmembrane segment. Here, we used transition metal ion FRET and patch-clamp fluorometry with a fluorescent, noncanonical amino acid (Anap) to show that the potentiating transition metal Co\(^{2+}\) binds in or near the A' helix. Adding high-affinity metal-binding sites to the membrane (C18-NTA) increased potentiation for low Co\(^{2+}\) concentrations, indicating that the membrane can coordinate metal ions with the A' helix. These results suggest that restraining the A' helix to the plasma membrane potentiates CNGA1 channel opening. Similar interactions between the A' helix and the plasma membrane may underlie regulation of structurally related HCN and KCNH channels by plasma membrane components.

Cyclic nucleotide-gated (CNG) channels are voltage-independent, nonselective cation channels that are activated by the direct binding of cGMP and cAMP (1, 2). They are expressed in photoreceptors and olfactory receptors, where they mediate visual and olfactory transduction, respectively (3). They are also expressed throughout the brain (4, 5), where they can modulate neuronal excitability (6) and long-term potentiation (7). CNG channels are structurally similar to hyperpolarization-activated cyclic nucleotide-gated (HCN) and voltage-gated potassium subfamily H (KCNH) channels (1, 8). They are composed of four subunits forming around the central ion-conducting pore (Figure 1A). Each subunit has a C-terminal cyclic nucleotide-binding domain (CNBD), which is connected to the channel pore by a C-linker region (Figure 1B) (9). In CNG and HCN channels, cyclic nucleotide binding to the CNBD causes a conformational change in the C-linker, which then favors opening of the channel pore.

While structural rearrangements of the CNBD have been extensively studied, rearrangements of the C-linker are less well understood. The C-linker is the site of virtually all of the intersubunit interactions in the C-terminal region. It is composed of several alpha helices, the most N-terminal of which is the A' helix, directly following the S6 transmembrane segment (Figure
Regulation of CNGA1 channels by membrane interactions

(9). Based on the structure of the isolated C-linker and CNBD of HCN2, the A’ helix is thought to run nearly parallel to the membrane near the cytosolic surface.

The first evidence that the A’ helix plays a role in channel gating came from studies of transition metal ion effects on CNGA1 (rod) and CNGA2 (olfactory) channels. Micromolar concentrations of transition metals were found to potentiate activation of rod channels, increasing the currents in response to partial agonists and subsaturating concentrations of full agonists (10–12). This effect was localized to a single histidine at position 420 in the A’ helix of CNGA1 channels (10). Similarly, a nearby histidine in the A’ helix of CNGA2 channels (equivalent to 417 in CNGA1) was required for transition metal inhibition in these channels (13). The high apparent affinity for transition metal indicates that multiple ligands must coordinate metal ions to produce potentiation. Intersubunit coordination by multiple H420 residues, however, cannot easily explain the high-affinity for transition metal, as the X-ray crystal structure of the tetrameric C-linker and the CNBD of HCN2 show these residues to be ~30 Å apart (9). Whether H420 interacts directly with the transition metal and what other ligands are involved remain unclear.

One powerful method for measuring structural dynamics in proteins is transition metal ion Förster resonance energy transfer, or tmFRET (14–21). In tmFRET, a transition metal ion (Ni^{2+}, Co^{2+}, or Cu^{2+}) serves as a FRET acceptor for a nearby fluorescent donor whose emission overlaps with the absorption of the metal ion. As a result, FRET between the fluorescent donor and the metal ion acceptor is observed as a quenching of donor fluorescence when the donor is near the metal. The degree of quenching is the FRET efficiency, which reports the distance between donor and acceptor. Compared to traditional FRET, tmFRET measures shorter distances (~10-20 Å), does not have orientation dependence, and allows metal to bind reversibly to engineered binding sites within proteins or the membrane. In addition, different metals with varied absorption properties can be used to report different distance ranges, depending upon the amount of overlap with the fluorophore emission.

A promising approach for specific labeling of channels is amber codon suppression to incorporate a fluorescent noncanonical amino acid. One such noncanonical amino acid is 3-(6-acetylnaphthalen-2-ylamino)-2-aminopropanoic acid (Anap), which is only slightly larger than a tryptophan residue (Figure 1C) and has fluorescence in the visible range (Figure 1D) (22, 23). The size, fluorescent properties, and spectral overlap with the transition metal ion Co^{2+} (Figure 1D), make Anap well-suited for tmFRET measurements within ion channel proteins (24).

In this study, we combined tmFRET with patch-clamp fluorometry (PCF) to study the mechanism of metal potentiation. We tested the hypothesis that, in CNGA1 channels, the A’ helix coordinates metal ions with a membrane-resident ligand. Using PCF, we measured metal binding with Anap fluorescence and simultaneously controlled and measured the channel function with patch-clamp recording. We found that Co^{2+} quenched the fluorescence of Anap incorporated into a site in the A’ helix (position 417). The degree of quenching indicated that Co^{2+} binds close to Anap on the A’ helix. tmFRET between Co^{2+} and Anap at position 417 was not observed in H420Q channels, suggesting that Co^{2+} ions bind directly to H420. Additionally, adding high-affinity metal ion-binding sites to the membrane enhanced potentiation in an H420-dependent manner, supporting our hypothesis that a Co^{2+}-binding site on the membrane participates in coordination. Taken together, these results indicate that restraining the A’ helix at H420 near the membrane promotes channel opening.

**EXPERIMENTAL PROCEDURES**

Molecular biology The bovine CNGA1 channel with a C-terminal YFP was cloned into the
pGEMHE plasmid optimized for oocyte expression (25). The C-terminal YFP constructs were used for all fluorescence experiments. Standard site-directed mutagenesis PCR techniques were used to make Q417tag and H420Q constructs. All regions that were subject to PCR were sequenced for verification (Genewiz, Seattle, WA). Channel RNA was transcribed in vitro from linearized DNA using the mMessage mMachine T7 Ultra transcription kit (Ambion, ThermoFisher). Defolliculated *Xenopus laevis* oocytes, as described previously (26), were given cytoplasmic injections of channel RNA (CNGA1-WT, CNGA1-Q417tag, CNGA1-Q417tag,H420Q; 50 nL injection) 0.5-2 days following surgical removal. To express Anap aminoacyl tRNA synthetase and Anap tRNA, oocyte nuclei were injected with pAnap plasmid DNA (13 nL injection of 50 ng/µL; obtained from Addgene) (22) at the same time as RNA injections (27). For Anap incorporation, oocytes were incubated with L-Anap methyl ester (30 µM from 3 mM stock solution in EtOH stored at -20 °C; AsisChem, Waltham, MA) in the bath after injection until recording.

**Patch-Clamp Recording** Inside-out patches were excised from oocytes between two and seven days post-injection. The pipette (300-800kΩ) and bath solutions each contained: 130 mM NaCl, 3 mM HEPES, 0.2 mM EDTA, pH 7.2. Currents were recorded using EPC10 amplifier and Patchmaster software (HEKA Elektronik). For electrophysiology experiments in Figures 2, 3, and 6, patches were held for ~10 minutes before data acquisition to allow for run-up of currents. Currents with saturating ligand (1 mM cGMP or 16 mM cAMP) were leak-subtracted using identical voltage protocols in the absence of ligand to remove leak and any endogenous currents. For C18-NTA experiments, oocytes were preincubated with 3 µM Co²⁺-C18-NTA (Toronto Research Chemicals; (28) in the bath solution for at least 15 minutes. Some patches were directly perfused with 3 µM Co²⁺-C18-NTA, and no differences were seen for the two application strategies of C18-NTA and so the datasets were combined. Electrophysiology data were analyzed using Patchmaster and Igor Pro (Wavemetrics).

**Fluorescent Microscopy** Patches were imaged using a Nikon Eclipse TE2000-E microscope with a 60x water immersion objective. Anap was excited using epifluorescence with a Lambda SC Smart shutter controller (Sutter Instruments) and 376/30 nm excitation filter and 485/40 nm emission filter, and YFP with 490/10 nm excitation and 535/30 nm emission. YFP fluorescence was used to focus on the patch. Before Anap image acquisition, patches were given five 1-s prebleaching exposures to remove a fast-decaying background signal, (see Figure S1). Anap in channels was then imaged with 100 ms exposures using an Evolve 512 EMCCD camera (Photometrics) and Metamorph software (Molecular Devices). To analyze patch fluorescence, a region of interest was drawn around the patch, and background subtracted using a similar region of interest in a region of the pipette not containing the patch. For quenching experiments, 100-1000 ms exposures were used, and fluorescence was normalized to a control image. Patches were only used if currents were >1 nA and patches did not contain any yolk granules, which were fluorescent. Only ~5-10% of patches fulfilled these requirements.

**Fluorometry** L-Anap methyl ester (Asis Chem, Waltham, MA) was dissolved at 100 nM in a buffer used for electrophysiology. Spectra were acquired with a Spex Fluorolog-3 spectrofluorometer with FluorEssence software (Horiba Jobin Yvon, Edison, NJ). For the Anap emission spectrum, the excitation wavelength was 360 nm with a 5 nm slit width, and emission measured in 1 nm increments from 425 to 600 nm. For the Anap excitation spectrum, emission was measured at 490 nm (5 nm slit window) and excited from 260 to 450 nm (2 nm slit window). Co²⁺ absorption spectrum is of Co²⁺ coordinated by two histidines in a peptide and was previously published (29).

**Data Analysis** All data were analyzed using Microsoft Excel and Wavemetrics IgorPro6. For statistical analyses, Students’ t-test was computed in Excel for comparisons between two datasets, and significance was considered for *p* < 0.05. For comparisons across multiple conditions, a two-way ANOVA was computed using Origin software. Pearson correlation coefficient was calculated using IgorPro.
RESULTS

CNGA1 potentiation by Co$^{2+}$ It has been shown previously that several transition metal ions potentiate opening of CNGA1 channels, greatly increasing the current with the partial agonist cAMP or subsaturating concentrations of the full agonist cGMP (10–12). For fluorescence experiments, we used CNGA1 channels fused to a C-terminal YFP (25) expressed in Xenopus oocytes, and the transition metal Co$^{2+}$, which was shown to be an optimal FRET acceptor for Anap (24).

We characterized potentiation by Co$^{2+}$ by measuring CNGA1 currents in response to saturating concentrations of the partial agonist cAMP (16 mM) relative to the full agonist cGMP (1 mM) ($I_{cAMP}/I_{cGMP}$). Inside-out excised patches were held at a holding potential of 0 mV and current was driven through open channels using steps to $\pm$ 60 mV. In wild-type CNGA1 (CNGA1-WT) channels, saturating cAMP (Figure 2A, top, red traces) elicited only a small fraction of current compared to saturating cGMP (Figure 2A top, green traces). With the addition of 1-10 µM Co$^{2+}$, however, the cAMP-activated current increased relative to the cGMP-activated current in a concentration-dependent manner (Figure 2A, top, blue traces). The increase of cAMP-activated current reversed upon removal of Co$^{2+}$ from the bath and addition of EDTA (data not shown). These results are summarized in Figure 2B (white bars), where 1, 3, and 10 µM Co$^{2+}$ increased $I_{cAMP}/I_{cGMP}$ relative to that measured in EDTA. The extent of potentiation with 10 µM Co$^{2+}$ is consistent with previous reports of CNGA1-WT channel potentiation with Ni$^{2+}$ (10). Additionally, Co$^{2+}$ potentiation of CNGA1-WT channels without and with C-terminal YFP was indistinguishable ($I_{cAMP}/I_{cGMP} = 38.6 \pm 5.1\%$ without and 35.4 \pm 3.1\% with C-terminal YFP), and was combined in the CNGA1-WT condition. We conclude that Co$^{2+}$ is an adequate transition metal for examination of metal potentiation in CNGA1 channels.

We have previously shown that a histidine on the A’ helix at position 420 is required for metal potentiation (10). We tested whether the mutation of this residue (H420Q) eliminated potentiation by Co$^{2+}$. As shown in Figure 2A (bottom traces), no potentiation was observed with three Co$^{2+}$ concentrations for CNGA1-H420Q channels. $I_{cAMP}/I_{cGMP}$ was slightly elevated in CNGA1-H420Q compared to CNGA1-WT channels, but no potentiation was measured with the addition of Co$^{2+}$ (Figure 2B, gray bars). The effect of Co$^{2+}$ on $I_{cAMP}/I_{cGMP}$ current was significantly greater for CNGA1-WT versus CNGA1-H420Q channels (Two-way ANOVA significant interaction: $F(3, 58) = 60.07, p < 0.01$). These results indicate that H420 is necessary for potentiation by Co$^{2+}$ ions.

Anap incorporation into CNGA1 To test whether H420 represents part of the binding site for Co$^{2+}$, we used tmFRET to determine if the potentiating Co$^{2+}$ would quench a fluorophore near H420 on the A’ helix. This approach requires the specific labeling of the A’ helix with a fluorophore. To do this, we incorporated the fluorescent noncanonical amino acid, Anap (Figure 1C) at position 417 using amber codon suppression (23) (Figure 3A). The nuclei of Xenopus oocytes were injected with plasmid DNA encoding an orthogonal tRNA/aminoacyl tRNA synthetase pair developed previously for Anap (pAnap) (22, 27). We also injected cRNA encoding CNGA1 with a tag codon at position 417 (CNGA1-Q417tag) into the cytoplasm of these same oocytes. The oocytes were then cultured in medium containing L-Anap-ME, a cell-permeable form of Anap.

We tested whether Anap incorporated at position 417 on the A’ helix expressed as functional, cyclic nucleotide-activated channels with properties similar to wild-type channels. Figure 3B shows examples of currents from inside-out patches activated by 1 mM cGMP. CNGA1-WT channels produced large (~5-30 nA) currents (Figure 3B, top left; 3C, left column, circles). When expressing CNGA1-Q417tag alone (without pAnap), no cyclic nucleotide-activated current was detected (Figure 3B, top right; 3C, second column). In addition, no cyclic nucleotide-activated current
was detected when pAnap was coexpressed with CNGA1-Q417tag, but L-Anap-ME was omitted from the culture medium (Figure 3B, left bottom; 3C, third column). In contrast, coexpression of CNGA1-Q417tag and pAnap in oocytes cultured in L-Anap-containing medium produced robust, cyclic nucleotide-activated currents (Figure 3B, bottom right; 3C, right column). These data indicate that L-Anap was specifically incorporated at position 417, and that the L-Anap-incorporated channels were functional (CNGA1-Q417Anap channels). Although mean current amplitude from CNGA1-Q417Anap channels was reduced by approximately 80% compared to CNGA1-WT channels (Figure 3C, black bars), many patches had large currents. Additionally, we measured the cGMP dependence of CNGA1-Q417Anap channels (Figure 3D), and found it was not different from CNGA1-WT channels (average EC50 at +60 mV for cGMP: CNGA1-WT, 45.4±4.0 μM, n = 5 versus CNGA1-Q417tag, 55.1±4.8 μM, n = 5, p > 0.05). We conclude that CNGA1-Q417Anap channels behave similarly to CNGA1-WT channels.

To determine whether Anap fluorescence from patches was sufficient for PCF, we examined the fluorescent properties of CNGA1 channels with Anap in inside-out patches. We used channels fused to a C-terminal YFP to find and focus on the patch. We observed YFP fluorescence in patches expressing CNGA1-WT and CNGA1-Q417Anap (Figure 4A, middle). Interestingly, fluorescence in the Anap color channel was observed in patches from cells cultured with L-Anap-ME in the medium but not injected with pAnap or CNGA1 cRNA (Figure S1). This non-specific fluorescence has also been reported for mammalian cells cultured in L-Anap-ME-containing medium, where it was reported to bleach more rapidly than Anap incorporated into a membrane protein (24). We measured the kinetics of decay of the non-specific Anap fluorescence and the fluorescence due to Anap incorporated into CNGA1 channels. We found that the non-specific fluorescence decayed much more rapidly than the CNGA1-associated Anap fluorescence (Figure S1). For all experiments, patches were therefore prebleached using five 1-second exposures to Anap excitation light (24). Following the prebleaching exposures, Anap fluorescence was observed in CNGA1-Q417Anap (Figure 4A bottom right), but not CNGA1-WT channels (Figure 4A, top right), indicating that the Anap fluorescence was sufficient for PCF experiments.

If the fluorescence signals are specific to channels expressed in the patches, then the fluorescence should correlate to the number of channels in the patch, in other words, the amount of current measured per patch. For CNGA1-WT channels, as expected, YFP fluorescence (green, left axis), but not Anap fluorescence (blue, right axis) was correlated with current (Figure 4B, top left; for YFP, r² = 0.88, p < 0.0001; for Anap, r² = 0.05, p = 0.84). Similarly, the small amount of Anap fluorescence was uncorrelated with YFP fluorescence (Figure 4B, top right; r² = 0.01, p = 0.68). For CNGA1-Q417Anap channels, however, both YFP and Anap signal correlated with current amplitude (Figure 4B, bottom left; for YFP, r² = 0.78, p < 0.0001; for Anap, r² = 0.56, p < 0.001), and there was a correlation between YFP and Anap fluorescence (Figure 4B, bottom right; r² = 0.59, p < 0.001). This correlation confirms that the Anap fluorescence measured is from Anap incorporated into CNGA1 channels.

**tmFRET with Anap** To determine whether H420 in the A' helix is in close proximity to the Co²⁺-binding site involved in potentiation, we tested whether Anap incorporated one helical turn away, at position 417, could act as a donor for tmFRET with Co²⁺ bound to the potentiating site as an acceptor (Figure 5A). As discussed above, because transition metals absorb but do not fluoresce, tmFRET between Anap and Co²⁺ will be observed as a quenching of the Anap fluorescence. Application of 10 μM Co²⁺ reversibly quenched patch fluorescence of CNGA1-Q417Anap channels, as shown in the example patch in Figure 5A. However, this was not always the case, and this experiment was repeated three times.
5B (left). In contrast, the H420Q mutation eliminated quenching (Figure 5B, right). These experiments are summarized in Figure 5C, where normalized patch intensity (see Methods) is shown. For CNGA1-Q417Anap, application of cGMP did not change patch fluorescence, but 10 μM Co²⁺ reduced it by ~40%, both in the presence and absence of cGMP. Quenching was reversed by application of EDTA. Furthermore, quenching by Co²⁺ was significantly reduced in channels containing the H420Q mutation (for CNGA1-Q417Anap vs. CNGA1-Q417Anap, H420Q channels, \( p < 0.05 \), for Co²⁺ and cGMP + Co²⁺ conditions). These results indicate that Co²⁺ remains bound to the channel in the absence of cGMP, and that it binds close to position 417. These experiments support the idea that H420 directly coordinates Co²⁺ to promote channel opening.

Role of the membrane in potentiation

As the affinity of an imidazole, the sidechain of histidine, for transition metals is in the millimolar range (30), the high affinity of transition metal potentiation indicates that the transition metal is coordinated by multiple ligands. It has been previously suggested that multiple H420 residues on adjacent subunits coordinate transition metal ions (31, 32). However, the tetrameric structure of the related HCN2 C-terminal fragment reveals that the H420 equivalent position in the A’ helices of neighboring subunits are too far to support metal ion coordination in CNGA1 channels (9). The structure also showed that H420 is near the presumed location of the membrane. If the metal ions coordinated between H420 and the membrane are the basis for metal potentiation, then application of higher affinity binding sites to the membrane should yield higher-affinity metal potentiation (Figure 6A). We have previously shown that stearoyl-nitrilotriacetic acid (C18-NTA), a lipid with a metal-chelating head group, can be incorporated into cell membranes and binds Co²⁺ as a high-affinity tmFRET acceptor for a membrane-resident dye (28). The affinity of the C18-NTA for Co²⁺ is ~20 nM (33) much higher than the micromolar concentration of metal needed for potentiation. We measured cGMP- and cAMP-activated currents with 1-10 μM Co²⁺ without and with application of C18-NTA (Figure 6B, left). After C18-NTA application, \( L_{AMP}/L_{GMP} \) was significantly larger with 1-3 μM Co²⁺ (Two-way ANOVA significant interaction: \( F(3, 60) = 4.61, p < 0.01 \)) (Figure 6C, left). These results indicate that a site on the membrane and H420 can together coordinate Co²⁺ to produce potentiation.

To determine whether the potentiation of Co²⁺ bound to C18-NTA and Co²⁺ bound to endogenous sites occurred via the same mechanism, we tested whether potentiation by Co²⁺ bound to C18-NTA involved H420. We found that C18-NTA did not confer Co²⁺ potentiation on CNGA1-H420Q channels (Figure 6B, right). Co²⁺ application did not significantly alter \( I_{cAMP}/I_{cGMP} \), with or without C18-NTA (Two-way ANOVA interaction: \( F(3, 32) = 0.44, p > 0.05 \)) (Figure 6C, right). The results of these experiments indicate that H420 can coordinate metal ions with C18-NTA in the membrane, and that this interaction makes channel opening more favorable. The similar dependence on H420Q and the nonadditive nature of the potentiation is most simply explained if the endogenous ligand is also on the membrane.

DISCUSSION

Metal potentiation has been described previously with Ni²⁺, Cd²⁺, Zn²⁺, and Mn²⁺ (10–12), and H420 was shown to be necessary for metal potentiation (10). Using a fluorescent noncanonical amino acid (Anap) and PCF to measure tmFRET in functional channels, we showed that Co²⁺ binds directly to H420 in the A’ helix. Furthermore, by adding a custom-synthesized lipid (C18-NTA) to coordinate metal ions on the membrane, we provide evidence that coordination of a metal ion between H420 on the A’ helix and the intracellular leaflet of the bilayer can potentiate CNGA1 channels. The presumed location of the A’ helix along the inner surface of plasma membrane suggests that transition metals restrain the A’ helix near the
plasma membrane and this potentiates opening of CNGA1. This mechanism, responsible for transition metal potentiation of CNGA1 channels, may also underlie the lipid regulation of other channels with an A’ helix in the cyclic nucleotide-regulated channel family(34–36).

We combined several approaches to study the A’ helix: tmFRET, amber codon suppression for incorporation of Anap, PCF, and the use of a custom metal chelator, C18-NTA, that integrates into the membrane. tmFRET has been used previously to show movements of the CNBD in C-terminal fragments of HCN2 (19, 29), but this is the first time tmFRET has been used in functional channels, while assaying the channel state with electrophysiology. The use of the noncanonical amino acid Anap allowed for specific labeling of the channel with reduced background labeling compared to cysteine-reactive fluorophores. Because Anap is environmentally sensitive, it has been previously used to measure local changes in other proteins (27, 37, 38). The combination of Anap with tmFRET is a promising approach for measuring changes in ion channel protein structure in intact functional channels in the membrane that do not rely on local environmental changes. The use of PCF allows for simultaneous measurements of channel function and control of membrane voltage. We have also recently shown that C18-NTA can be used to quench fluorescence of a membrane dye (28) and Anap fluorescence in TRPV1 (24). The combination of these techniques here showed that the A’ helix interacts with the membrane, and this interaction influences gating.

To determine that H420 has a direct role in Co²⁺ coordination rather than an indirect effect that allows for potentiation, we showed that Co²⁺ can quench Anap fluorescence at position 417. The calculated FRET R₀, or the characteristic distance producing 50% FRET efficiency, for Anap and Co²⁺, when Co²⁺ is coordinated by two histidine residues (29), is 12.0 Å. The 40% quenching with 10 μM Co²⁺ we observed corresponds to a distance of ~13 Å. It is possible that the coordination site is not fully occupied by Co²⁺, however, which could produce an underestimate of quenching and distance measurement. Given the location of Anap with respect to H420, only three residues away on an α helix, and because we do not precisely know the rotameric position of Anap, the histidine sidechain, and the membrane ligand, 13 Å is a reasonable distance between Anap and Co²⁺ coordinated by H420. The short distance dependence of tmFRET supports the direct coordination of Co²⁺ by H420, rather than Co²⁺ binding elsewhere in an H420-dependent manner.

Because a single histidine residue binds transition metals with millimolar affinity (30), but metal potentiation is accomplished with 1-10 μM Co²⁺, another ligand must participate in coordinating Co²⁺. It has been suggested previously that H420 residues on adjacent subunits coordinate metal ions (31, 32), but the homology model of CNGA1 from the tetrameric HCN2 C-terminal region crystal structure shows that the residues are not close enough to support metal ion coordination. Instead we found that adding a metal-chelating lipid to the membrane (C18-NTA) increased the affinity of Co²⁺ for potentiation. This result is consistent with the idea that, in native membranes, phospholipids with negatively charged headgroups may participate in coordination, and Co²⁺ binds to these headgroups and H420 with approximately micromolar affinity, producing potentiation. This result, combined with the quenching of Q417Anap with and without cGMP, suggest that this metal coordination between H420 and the membrane restrains the A’ helix near the membrane, promoting activation by cyclic nucleotides. Although the free concentration of endogenous potentiating metal ions would likely not be high enough to potentiate CNGA1 channels physiologically, understanding the molecular mechanism of potentiation aids in understanding channel activation by cyclic nucleotides and regulation by lipids.

Because the membrane acts as a ligand with the A’ helix to coordinate metal ions, we have
a greater understanding of the role the A’ helix plays in CNGA1 channel activation. Since cyclic nucleotide regulation is potentiated when the A’ helix is coordinating metal ions with the membrane, it is plausible that upon cyclic nucleotide binding, the A’ helix moves towards the membrane, and this movement makes the channel more likely to open. In a recent crystal structure of a C-terminal fragment of a bacterial CNG channel, SthK, the A’ helix is moved upward, toward the presumed location of the membrane, when bound to an agonist (cAMP) but not to an antagonist (cGMP) (39).

The A’ helix also may need to be rotated in a specific position relative to the membrane to promote opening. In olfactory CNG channels (CNGA2) a histidine in a slightly different position (at the position equivalent to 417 in CNGA1) causes transition metals to inhibit channel opening (13). Similarly, histidine residues introduced along the A’ helix of CNGA1 channels cause transition metals to have distinct effects on channel opening depending on their position, with inhibiting and potentiating residues located on different faces of the helix (40). The locations of these residues suggest that restraining the A’ helix to the membrane can promote either channel opening or channel closure depending on the rotation of the helix.

The A’ helix is also involved in modulation of channel gating in other channels with a C-linker. In HCN2 channels, interactions of the more proximal part of the A’ helix with the S4-S5 linker stabilize the closed state (41). PIP2 inhibition of sea urchin HCN channels (34) and ELK channels of the KCNH family (35) is thought to occur via the proximal A’ helix. CNG channels have also been shown to be inhibited by PIP2 (36), and it is possible that the A’ helix is involved in this regulation. These studies indicate that the A’ helix is a strong modulator of gating in the cyclic nucleotide-regulated channel family, and may be an important site for channel regulation in photoreceptors, olfactory receptors, and other neurons.

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Author Contributions: TKA designed, conducted, analyzed experiments and wrote the paper. SEG and WNZ designed and analyzed experiments, and wrote the paper.
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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. CNGA1 channels and Anap. A, Homology model of CNGA1 C-terminal region based on HCN2 structure (PDB: 1Q3E_B; (9)) with cartoon of transmembrane region and YFP. B, C-terminal region from one subunit of structure shown in A. C, Structure of Anap. D, Anap excitation (dark blue) and emission spectra (light blue) on left axis shown with absorbance of Co$^{2+}$ bound to a dihistidine motif (29) (gray) on right axis.

FIGURE 2. Co$^{2+}$ potentiates CNGA1 channels. A, Representative traces for CNGA1-WT (top) and CNGA1-H420Q (bottom) channels with 1 mM cGMP, 16 mM cAMP, and 16 mM cAMP + 1-10 µM Co$^{2+}$. Voltage steps were applied to an inside-out patch to -/+ 60 mV for 10 ms. B, Summary data showing mean cAMP current as a function cGMP current at +60 mV for CNGA1-WT (white bars) and CNGA1-H420Q (gray bars) channels in 0.2 mM EDTA, 1 µM Co$^{2+}$, 3 µM Co$^{2+}$, and 10 µM Co$^{2+}$ (CNGA1-WT: N = 18, 8, 3, 9; CNGA1-H420Q: N = 10, 5, 4, 9 respectively).

FIGURE 3. Anap incorporates into CNGA1 channels. A, Predicted structure of the A’ and B’ helices with cartoon of the membrane indicating the position of Anap and H420. B, Current traces with steps between -100 and +100 mV in 10-mV increments in the conditions indicated. C, Summary data showing mean (black bars) and individual current amplitudes (points) from patches with CNGA1-WT channels (N = 10), CNGA1-Q417tag channels (N = 15), CNGA1-Q417tag channels + p-Anap (N = 9), CNGA1-Q417tag + pAnap + L-Anap (CNGA1-Q417Anap, N = 72). D, cGMP concentration-dependence for CNGA1-WT (N = 5) and CNGA1-Q417Anap channels (N = 5).

FIGURE 4. Anap and YFP fluorescence are correlated with current in CNGA1-Q417Anap channels. A, brightfield (left), YFP (middle), and Anap (right) images of recording pipette containing patch with either CNGA1-WT (top) or CNGA1-Q417Anap channels. Scale bar = 10 µm. B, Left, YFP (green, left axis) and Anap (purple, right axis) patch fluorescence plotted as a function of current amplitude for CNGA1-WT (top; N = 23) and CNGA1-Q417Anap (bottom; N = 44 for YFP; 33 for Anap) channels. Right, Anap fluorescence in individual patches plotted as a function of YFP fluorescence for CNGA1-WT (top; N = 23) and CNGA1-Q417Anap (N = 33) channels.

FIGURE 5. Co$^{2+}$ quenches CNGA1-Q417Anap fluorescence with, but not without, H420. A, Cartoon depicting Q417Anap, with and without H420 and Co$^{2+}$. B, Images of patches containing CNGA1-Q417Anap (left) and CNGA1-Q417Anap, H420Q (right) channels. Scale bar = 20 µm. C, Normalized patch fluorescence for indicated conditions, with CNGA1-Q417Anap (open triangles) and CNGA1-
FIGURE 6. C18-NTA increases Co$^{2+}$ potentiation. A, Cartoon illustrating C18-NTA (yellow) in membrane coordinating Co$^{2+}$ with H420 (left) but not with H420Q mutation (right). B, For CNGA1-WT (left), and CNGA1-H420Q (right) channels, current traces from representative patches with 1 mM cGMP and 16 mM cAMP (+/- Co$^{2+}$) without (top) and with (bottom) C18-NTA. C, Summary for cAMP currents relative to cGMP currents with 0-10 µM Co$^{2+}$ for control without C18-NTA (white) and after C18-NTA application (red). CNGA1-WT summary is on left and CNGA1-H420Q summary on right. (CNGA1-WT, for EDTA, 1, 3, 10 µM Co$^{2+}$: Control, N = 18, 8, 3, 9; C18-NTA, N = 15, 8, 4, 3; CNGA1-H420Q for EDTA, 1, 3, 10 µM Co$^{2+}$: Control, N = 10, 5, 4, 9; C18-NTA, N = 5, 4, 4, 1).
Figure 1

A

B

C

D

Anap

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\text{H}_2\text{N} - \text{HN} - \text{CO}_2\text{H}
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Figure 2

A

![Voltage responses for WT and H420Q constructs with different cAMP and cGMP concentrations.

B

![Bar graph showing the ratio of I_{cAMP}/I_{cGMP} for WT and H420Q constructs with EDTA and different Co^{2+} concentrations.]
Figure 3

A

B

C

D

CNGA1-WT  CNGA1-Q417tag
CNGA1-Q417tag  CNGA1-Q417tag

CNGA1-Q417tag  CNGA1-Q417tag

pAnap  pAnap

pAnap  L-Anap  pAnap + L-Anap

1 nA
10 ms

| CNGA1-WT | + | - | - | - |
|----------|---|---|---|---|
| CNGA1-Q417tag | - | + | + | + |
| pAnap | - | - | + | + |
| L-Anap | - | - | - | + |
Figure 5

A

Q417Anap

Q417Anap, H420Q

B

brightfield

YFP

Anap

ccontrol

+ Co^{2+}

EDTA wash

C

normalized F

control cGMP cGMP Co^{2+} wash

Q417Anap Q417Anap, H420Q

* *
Figure 6

A

WT

H420Q

to S6

to S6

B

Voltage (mV)

-60

60

Control

C18-NTA

cGMP

cAMP

cAMP + 1 μM

cAMP + 1 μM

1 μM

3 μM

10 μM Co²⁺

3 μM

10 μM Co²⁺

C

\[
\frac{I_{cAMP}}{I_{cGMP}}
\]

Control

C18-NTA

EDTA

1 μM Co²⁺

3 μM Co²⁺

10 μM Co²⁺

EDTA

1 μM Co²⁺

3 μM Co²⁺

10 μM Co²⁺

H420Q Control

H420Q C18-NTA
