A Secondary Structural Transition in the C-helix Promotes Gating of Cyclic Nucleotide-regulated Ion Channels*

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**Background:** Cyclic nucleotide-regulated channels are involved in sensory transduction and repetitive electrical activity.

**Results:** Transition metal ion FRET and electrophysiology demonstrated a coil-to-helix transition within the ligand binding domain.

**Conclusion:** Agonist binding stabilized the structure of the C-helix, triggering channel gating.

**Significance:** This transition is important for ion channel activation and may be necessary for the activation of other cyclic nucleotide-regulated proteins.

Cyclic nucleotide-regulated ion channels bind second messengers like cAMP to a C-terminal domain, consisting of a β-roll, followed by two α-helices (B- and C-helices). We monitored the CAMP-dependent changes in the structure of the C-helix of a C-terminal fragment of HCN2 channels using transition metal ion FRET between fluorophores on the C-helix and metal ions bound between histidine pairs on the same helix. cAMP induced a change in the dimensions of the C-helix and an increase in the metal binding affinity of the histidine pair. cAMP also caused an increase in the distance between a fluorophore on the C-helix and metal ions bound to the B-helix. Stabilizing the C-helix of intact CNGA1 channels by metal binding to a pair of histidines promoted channel opening. These data suggest that ordering of the C-helix is part of the gating conformational change in cyclic nucleotide-regulated channels.

Cyclic nucleotide-gated (CNG)² and hyperpolarization-activated cyclic nucleotide-modulated (HCN) channels are members of the superfamiliy of voltage-activated ion channels (1, 2). CNG and HCN channels are unique in the voltage-gated channel superfamily in that their gating from closed to open is promoted by direct binding of the cyclic nucleotides cAMP and cGMP. CNG channels are responsible for the primary electrical signals in vision and olfaction (1). HCN channels contribute to pacemaking in the heart as well as repetitive firing, resting membrane potential, and dendritic integration in some neurons (1, 3).

Cyclic nucleotides bind to a conserved domain in the cytoplasmic C-terminal region of each subunit (1). Several structures of the C-terminal region in complex with cyclic nucleotide have been solved for eukaryotic HCN channels (4–8). The structures contain a novel helical domain called the C-linker, which is attached directly to the ion channel pore in intact channels and is the site of tetramerization of the cytoplasmic domain (Fig. 1, A and B). Immediately following the C-linker is a cyclic nucleotide-binding domain (CNBD) that is very similar to the binding domains of the bacterial MloK1 potassium channel, the *Escherichia coli* transcription factor CAP, the guanine nucleotide exchange factor Epac, and the regulatory domains of the kinases PKA and PKG (9–15). The core of the CNBD consists of an eight-stranded β-roll followed by two α-helices, the B- and C-helices (Fig. 1B). Extensive contacts are made between the cyclic phosphate and ribose moieties of the cyclic nucleotide and residues in the β-roll and a short helix between the sixth and seventh β-strands called the P-helix. The C-helix caps the ligand-binding site by forming interactions with the purine ring of cAMP or cGMP.

A mechanism for the activation of eukaryotic cyclic nucleotide-regulated channels has been proposed based on electrophysiological and biochemical studies (1). In this model, initial contact is made between the ligand and the β-roll/P-helix. Mutations in this region of the channel have effects on cyclic nucleotide affinity, with little effect on the efficacy of the ligand to promote channel opening (4, 16–19). Subsequent to ligand binding, the C-helix is thought to translate toward the binding pocket and form contacts with the ligand. Mutations in the C-helix have effects on the ability of ligand to promote channel activation, suggesting that C-helix movement is coupled to the opening conformational change of the channel pore (4, 16–21).

Direct structural evidence for this hypothesis in eukaryotic channels is limited. The ligand-free (apo) structure of the mouse HCN2 C-terminal region has been solved (6). Overall, the apo-structure is very similar to the ligand-bound structure, but it differs in two key regions. The F’-helix of the C-linker adopts a nonhelical coil structure in the absence of agonist. Although the position of the proximal C-helix is quite similar between the apo- and ligand-bound HCN2 structures, the distal half of the C-helix is not resolved in the apo-structure. Furthermore, circular dichroism spectra reveal a marked increase in the helical content of the HCN2 C terminus in the presence of cAMP (22). These results raise the intriguing possibility that the C-helix becomes more ordered upon cyclic nucleotide binding and that this secondary structural transition may be coupled to the gating conformational change that opens the channel.

In this study, we used transition metal ion fluorescence resonance energy transfer (tmFRET) and electrophysiology to
investigate the hypothesis that cyclic nucleotide binding stabilizes the secondary structure of the C-helix. tmFRET is a FRET technique that uses small, covalently attached fluorophores as donor molecules and colored transition metal ions (e.g. Ni²⁺ and Co²⁺) bound to pairs of histidine residues as acceptors (6, 23–25). This method can report very short distances (8–20 Å), virtually eliminates the orientation dependence of classical FRET, and allows the experimenter to tune the dynamic range of distance measurements by changing the acceptor ion. Previously, a comparison of tmFRET measurements in the absence and presence of cAMP demonstrated that the C-helix undergoes a translation toward the β-roll in the presence of cyclic nucleotide (6). Furthermore, the transition metal ion Ni²⁺ bound more tightly to pairs of histidines in the C-helix spaced four residues apart in the presence of cAMP than in its absence, suggesting cAMP causes a stabilization of the secondary structure of the C-helix (6). In this study, we used tmFRET within the C-helix and between the B- and C-helices of the purified HCN2 C-terminal domain to demonstrate further that the C-helix undergoes a secondary structural transition, becoming more ordered upon binding cAMP. Using patch clamp electrophysiology on intact, functioning ion channels, we show that this secondary structural transition is coupled to channel opening and is therefore part of the gating conformational change in cyclic nucleotide-regulated channels that translates ligand binding into channel opening.

EXPERIMENTAL PROCEDURES

Molecular Biology

The gene encoding the cysteine-free C-terminal fragment (amino acids 443–640) of the mouse HCN2 ion channel (HCN2) was synthesized by Blue Heron Biotechnology (Bothell, WA) and cloned into the pMALc2T vector (New England Biolabs, Ipswich, MA) (6). The vector encodes an N-terminal maltose-binding protein tag that is separated from the gene product of interest by a thrombin-cleavable linker. CNGA1Q was subcloned into the pGEMHE vector (26). This construct contains the full-length CNGA1 channel with all of the native cysteines mutated, with the exception of Cys-481 and the native Ni²⁺ potentiation site mutated to a glutamine (H420Q) (16, 27). All mutations were created using standard PCR techniques and verified using automated sequencing (Genewiz, Seattle, WA). To make RNA for Xenopus oocyte expression experiments, cDNAs encoding CNGA1Q constructs were linearized with NheI (New England Biolabs) and transcribed in vitro using the mMessage mMachine Ultra kit with T7 polymerase (Ambion, Austin, TX).

Protein Purification

Soluble HCN2 constructs were prepared as described previously (28). Briefly, all constructs were expressed in BL21 (DE3) bacteria (New England Biolabs), grown in 2-liter cultures, and induced with 1 mM isopropyl thio-β-D-galactopyranoside. Bacteria were lysed and centrifuged to remove insoluble cellular material. MBP-HCN2 constructs were purified on an amylose affinity column. The maltose-binding protein tag was removed by thrombin digest followed by ion exchange chromatography.

Protein Labeling

Labeling of cysteine residues inserted into the HCN2 construct via mutagenesis was carried out during purification after the thrombin digest but prior to ion exchange chromatography. Protein was eluted from the amylose affinity column into 20 ml of buffer containing 150 mM KCl, 50 mM D(+)-malto monohydrate, 10% glycerol, and 30 mM HEPES, pH 7.2. Fluorophores
C-helix Secondary Structure Changes

(monobromobimane or dibromobimane) were added to the buffer at a final concentration of 100 μM from a 10 mM stock in DMSO, which was stored at −80 °C. At this concentration, the fluorophore was in molar excess of the cysteines intended for labeling. The solution was placed on an orbital shaker (60–80 rpm) for 1 h at 20–25 °C. Unincorporated fluorophore was removed during the ion exchange step. Labeling efficiency was not quantitated for the subsequent experiments as any unlabeled protein would not be expected to contribute to the fluorescence signal.

UV-Visible Spectroscopy

Absorbance spectra of 1 mM Ni²⁺ and Co²⁺ bound to the peptide ACAAKAAKHAHAAHKA (3 mM) were acquired in a Beckman DU460 spectrophotometer.

Fluorometry

Fluorescence spectra were acquired using a Spex Fluorolog-3 spectrofluorometer outfitted with a MicroMax plate reader (HORIBA Jobin Yvon, Edison, NJ). The excitation wavelength was 390 nm, and emission was measured between 440 and 600 nm in 1-nm increments with a 0.1-s integration time and slit widths set between 5 and 14 nm on the excitation and emission monochromators. For FRET experiments, peak emission was averaged over a 7-nm window, usually between 477 and 483 nm. Bimane-labeled HCN2 constructs were diluted to a concentration between 30 nM and 60 μM. Experiments were conducted in a buffer containing 150–300 mM KCl, 10% glycerol, and 30 mM HEPES, pH 7.2, at 20–25 °C. Data were acquired from 250-μl samples in black, flat-bottom 96-well plates (Corning; Low-Backell, MA) through fiber optics in the plate reader connected to the sample compartment of the fluorometer. To reduce the amount of scattered excitation light collected by the emission fiber optic, a 425-nm long pass filter was placed in line with the emitted light path. To each sample was added 7.5 μl of water or cAMP (sodium salt, monohydrate) diluted to the desired concentration from a 10 mM aqueous stock. Unless otherwise noted, the final cAMP concentration was 300 μM. 2.5 μl of water or aqueous stocks of NiCl₂·6H₂O or CoSO₄·7H₂O were also added to each well.

Fluorescence anisotropy measurements were conducted using 200-μl samples in a standard quartz cuvette. The Fluorolog-3 was outfitted with Glan-Thompson polarizers. Data recordings were carried out in the inside-out patch clamp configuration with an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA) and digitized with an ITC-16 analog/digital converter (Instrutech/HEKA, Bellmore, NY). Data were low pass filtered at 2 kHz and sampled at 10 kHz. All data were acquired using Pulse software (HEKA). Both pipette and bath solutions contained 130 mM NaCl, 3 mM HEPES, pH 7.2, with N-methyl-D-glucamine. Bath solutions were supplemented with cGMP or inosine 3′,5′-cyclic monophosphate sodium salt (cIMP) and NiCl₂·6H₂O as indicated. Pipettes were pulled from borosilicate glass and fire polished to a resistance of 0.2–0.6 megohms. Rapid solution exchange was achieved using an RSC-100 solution changer (Biologic, Claix, France). All recordings were performed at room temperature (20–25 °C). CNGA1 currents exhibit a time-dependent run-up following patch excision (31). To account for this phenomenon, the patches were repetitively pulsed in the presence of 200 μM cGMP until the current magnitude stabilized, typically 15–20 min.

Data Analysis

Fluorescence—All data were blank-corrected by subtracting spectra recorded from solutions with the same components but no fluorescent protein. Because we used a plate reader for our fluorescence measurements of HCN2 constructs, it was impossible to determine the exact path length of the excitation and emission light and therefore not possible to mathematically correct for the inner filter effect of the absorbance of ions and fluorophore in our samples. Rather, we corrected for this experimentally by dividing normalized fluorescence data from constructs containing bimane-conjugated cysteines and introduced di-histidine metal-binding sites by normalized fluorescence data from constructs containing the same fluorophore configuration but no metal-binding site. Not only did this partially account for the inner filter effect, but it eliminated the low affinity quenching component observed in proteins lacking an exogenously introduced metal-binding site, which is likely the result of quenching by ions in solution.

Corrected fluorescence data were plotted as a function of total metal (Ni²⁺ or Co²⁺) concentration and fit with a single binding site model as shown in Equation 1,

\[ \frac{F}{F_{\text{no site}}} = \frac{-[M^{2+}] + [\text{protein}] - K_d + \sqrt{([M^{2+}] - [\text{protein}] + K_d)^2 + 4 \cdot K_d \cdot [\text{protein}]}}{2 \cdot [\text{protein}]} \cdot E_{\text{FRET}} + 1 - E_{\text{FRET}} \]  

(Eq. 1)

where \( F/F_{\text{no site}} \) is corrected fluorescence, \([M^{2+}]\) is the total transition metal ion concentration, \([\text{protein}]\) is the total concentration of the HCN2 protein being used, \(K_d\) is the apparent metal binding affinity, and \(E_{\text{FRET}}\) is the FRET efficiency, i.e., the amount of quenching at saturating metal concentrations.

The cAMP concentration dependence of tmFRET was fit with Equation 2,

\[ \frac{F}{F_0} = \frac{-[\text{cAMP}] + [\text{protein}] - K_d + \sqrt{([\text{cAMP}] - [\text{protein}] + K_d)^2 + 4 \cdot K_d \cdot [\text{protein}]}}{2 \cdot [\text{protein}]} \cdot \left(\frac{1 - F_{\text{cAMP}}}{F_0}\right) + \frac{F_{\text{cAMP}}}{F_0} \]  

(Eq. 2)
where [cAMP] is the total cAMP concentration; \(F_o\) is the fluo-
rescence in the absence of cAMP; \(F_{cAMP}\) is the fluorescence
in the presence of saturating cAMP, and all other terms are as
defined above.

Distances between FRET donors and acceptors were cal-
culated using the Förster (Equation 3),

\[
R = R_0 \cdot \left( \frac{1}{E_{\text{FRET}}} - 1 \right)^{\frac{1}{2}}
\]

(Eq. 3)

where \(R\) is the distance between the donor and acceptor; \(R_0\) is
the distance at which FRET efficiency is 0.5, and \(E_{\text{FRET}}\) is the
FRET efficiency. \(R_0\) for each FRET pair was calculated as
described previously (6, 29, 32).

Electrophysiology—Current records were leak- and capaci-
tance-corrected by subtracting off currents from the same
patch in a solution with no cyclic nucleotide. Current magni-
tudes reported were the mean currents from steps to −60 mV
for cyclic nucleotide concentration-response relationships and
steps to −100 mV for Ni\(^{2+}\) potentiation experiments. Currents
in every patch were normalized to the current magnitude at 10
mM cGMP.

Cyclic nucleotide concentration-response relationships
were fit with the Hill Equation 4,

\[
\frac{I}{I_{\text{max,cGMP}}} = A_{\text{cGMP}} \frac{[\text{cNMP}]^n}{K_d + [\text{cNMP}]^n}
\]

(Eq. 4)

where \(I_{\text{max,cGMP}}\) is the maximal current response in 10 mM
cGMP; \(A_{\text{cGMP}}\) is the fractional activation in cGMP; [cNMP] is
the free cyclic nucleotide concentration; \(K_d\) is the apparent
affinity for cyclic nucleotide, and \(n\) is the Hill coefficient.

Ni\(^{2+}\) concentration-response relationships were deter-
mined in the presence of 16 mM cIMP. Ni\(^{2+}\) concentration-response
relationships for the CNGA1\(_Q\) construct, which exhibited
block, but no potentiation, were fit with a single binding site
model of Equation 5,

\[
\frac{I_{\text{Ni}^{2+}}}{I_{\text{no Ni}^{2+}}} = \frac{K_p}{[\text{Ni}^{2+}] + K_p}
\]

(Eq. 5)

where \(K_p\) is the apparent affinity for Ni\(^{2+}\) block. Ni\(^{2+}\) concen-
tration-response data for the CNGA1\(_Q\) E595H,Q599H con-
struct were fit with Equation 6, which describes two indepen-
dent components, Ni\(^{2+}\) potentiation and Ni\(^{2+}\) block,

\[
\frac{I_{\text{Ni}^{2+}}}{I_{\text{no Ni}^{2+}}} = \left( \frac{K_p}{[\text{Ni}^{2+}] + K_p} \right) \cdot \left( 1 + \frac{[\text{Ni}^{2+}]}{[\text{Ni}^{2+}] + K_p} \right)
\]

(Eq. 6)

where \(P\) is the fractional potentiation by Ni\(^{2+}\), and \(K_p\) is the
apparent affinity for Ni\(^{2+}\) potentiation. Because the E595H,
Q599H mutation should not affect the Ni\(^{2+}\) block of the channel
pore, \(K_p\) was fixed to the value determined from the
CNGA1\(_Q\) fit to Equation 5, reducing the fit of Equation 6 to only
two free parameters.

To determine the \(\Delta \Delta G\) for stabilization of the open state of
CNGA1\(_Q\) E595H,Q599H by Ni\(^{2+}\) binding to the C-helix, we
first had to calculate \(L\), the equilibrium constant for channel
opening. Because the Ni\(^{2+}\) potentiation experiments were
conducted at saturating concentrations of the partial agonist
clMP, the gating scheme was simply represented as shown in
Equation 7,

\[
\text{C} \leftrightarrow \text{O}
\]

(Eq. 7)

In this scheme, \(L\) for each agonist is defined by Equation 8,

\[
L \equiv \frac{P_{\text{open}}}{1 - P_{\text{open}}} = \frac{I_{\text{max}}}{1 - \frac{I}{I_{\text{max}}}}
\]

(Eq. 8)

where \(P_{\text{open}}\) is the probability of channel opening, and \(I_{\text{max}}\) is
the current at maximal open probability. In wild type CNGA1
channels, maximal current is measured in saturating cGMP and
1 \(\mu\)M Ni\(^{2+}\), where \(P_{\text{open}}\) has been directly measured to be near
unity (33). In the CNGA1\(_Q\) constructs, this determination was
not possible because the native Ni\(^{2+}\) potentiation site was
mutated (H420Q). To determine \(I_{\text{max}}\), we assumed that the rela-
tive efficacy of cGMP and clMP was preserved between wild
type and our mutant constructs. This is a reasonable assump-
tion as we did not mutate any of the amino acids that were
expected to contact the ligand. We then used Equation 9 to
calculate \(I_{\text{max}}\),

\[
\frac{L_{\text{cIMP}}}{L_{\text{cGMP}}} = \frac{1 - \frac{I_{\text{cIMP}}}{I_{\text{max}}}}{1 - \frac{I_{\text{cGMP}}}{I_{\text{max}}}}
\]

(Eq. 9)

where \(L_{\text{cIMP}}/L_{\text{cGMP}}\) was set equal to 0.06, based on the value
reported in the literature (18). Equation 9 was rearranged to
make Equation 10,

\[
I_{\text{max}} = \frac{0.94 \cdot I_{\text{cGMP}}}{I_{\text{cIMP}} - 0.06 \cdot I_{\text{cGMP}}}
\]

(Eq. 10)

Our experimentally determined values of \(I_{\text{cGMP}}\) and \(I_{\text{cIMP}}\) were
then used to calculate \(I_{\text{max}}\) and \(L\) for CNGA1\(_Q\) E595H,Q599H with
and without Ni\(^{2+}\). \(\Delta \Delta G\) was then calculated using Equation 11,

\[
\Delta \Delta G = -RT \ln \left( \frac{L_{\text{Ni}}}{L} \right)
\]

(Eq. 11)

where \(R\) is the gas constant, and \(T\) is temperature in Kelvin.

Statistics and Fitting

Data were analyzed and fit using Origin 8.0 (OriginLab
Corp., Northampton, MA) and Microsoft Excel (Microsoft
Corp., Redmond, WA). Data were reported as the mean ± S.E.
For fluorescence anisotropy measurements, one-way analysis
of variance was used to determine statistical significance with
the threshold set to \(p \leq 0.05\) after determining that the data
were normally distributed (Fig. 3D). For comparison of distances in the presence and absence of cAMP, we used a paired samples t test with a significance threshold of \( p \leq 0.05 \) (Fig. 4B).

**Chemicals and Reagents**

All chemicals were purchased from Sigma except for monobromobimane and dibromobimane, which were obtained from Invitrogen.

**RESULTS**

tmFRET between Donors and Acceptors in the C-helix of Soluble HCN2—We previously suggested that the C-helix of the CNBD in HCN2 channels undergoes a coil-helix transition upon binding of cAMP (6). As a more stringent test of this hypothesis, we sought to measure distances between locations within the C-helix with and without cAMP. If there is a change in the secondary structure of the C-helix rather than just a rigid body movement induced by agonist binding, then there should be a change in dimensions of the helix. To this end, we engineered cysteines into the C-helix of an otherwise cysteine-free, soluble HCN2 C-terminal construct (which we will refer to here as HCN2), and we modified them with a cysteine-reactive fluorophore. We chose monobromobimane (mBBr) as our donor because of its small size and short linker (Fig. 2A). mBBr reacts via nucleophilic substitution with cysteine, which forms a covalent bond between the methylene carbon on bimane and the sulfur of cysteine, with Br\(^-\) as the leaving group. We inserted histidines, spaced at \( i \) and \( i + 4 \) into the same helix to serve as metal-binding sites. The emission spectrum of bimane overlaps with the absorbance spectra of the transition metals Ni\(^{2+}\) and Co\(^{2+}\) when they are coordinated by histidine pairs in a helical peptide, making them suitable FRET acceptors (Fig. 2B). The calculated \( R_0 \), the distance at which FRET efficiency is half-maximal, is 9.2 Å for Ni\(^{2+}\) with bimane and 11.7 Å for Co\(^{2+}\) allowing us to measure short intrahelical distances over two different dynamic ranges (Fig. 2C).

We first sought to confirm that we were able to measure distances within an α-helix of a protein. We expressed and purified mutants of the soluble HCN2 C-terminal construct with a single cysteine on the N-terminal side of the C-helix labeled with bimane and metal-binding sites positioned one (A624C\(_{\text{mb}}\)T627H,D631H) or two (A624C\(_{\text{mb}}\)I630H,D634H) helical turns away from the donor fluorophore (Fig. 2A). At the concentrations used in our experiments, these constructs are monomeric (8). As a control, we expressed a construct with the cysteine, but no exogenous metal-binding sites (A624C\(_{\text{mb}}\)). Fig. 2D (left panel) shows the emission spectra of the control construct with increasing concentrations of Co\(^{2+}\) in the presence of 300 μM cAMP. Some quenching of the bimane emission occurred at high Co\(^{2+}\) concentrations (\( > ~ 1 \) mM). The apparent \( K_d \) for quenching was 23 mM. This reduction in fluorescence was likely the result of quenching by ions in solution rather than specific binding. In contrast, the construct with a metal-binding site positioned one helical turn away from the fluorescent donor (A624C\(_{\text{mb}}\)T627H,D631H) showed a much greater amount of quenching and quenched at a much lower Co\(^{2+}\) concentration (Fig. 2D, right panel).

This quenching results from FRET between the bimane and the bound Co\(^{2+}\) ion.

The concentration dependence for Ni\(^{2+}\) and Co\(^{2+}\) quenching of A624C\(_{\text{mb}}\)T627H,D631H and A624C\(_{\text{mb}}\)I630H,D634H in the presence of 300 μM cAMP is shown in Fig. 2E. All data were normalized to quenching in the control construct (A624C\(_{\text{mb}}\)), which has no introduced metal-binding sites, and fit with a single binding site model (see under “Experimental Procedures”). The fits to the data produced two parameters, the FRET efficiency (the fraction of quenching at high metal concentration) and the apparent metal binding affinity. With both Ni\(^{2+}\) and Co\(^{2+}\), there was a greater FRET efficiency (more quenching) for bimane and the closer metal-binding site (A624C\(_{\text{mb}}\)T627H,D631H) than the farther site (A624C\(_{\text{mb}}\)I630H,D634H) as expected from the distance dependence of FRET (Figs. 2E and 3A). Also, there was a greater FRET efficiency for both constructs with Co\(^{2+}\) than with Ni\(^{2+}\), consistent with the calculated \( R_0 \) values, although Ni\(^{2+}\) bound with a higher apparent affinity (Fig. 2E). Distances determined from FRET measurements are the most accurate and precise when they are near \( R_0 \) where the changes in FRET are largest per unit of distance. This underscores an important feature of tmFRET; the metal ion can be chosen to suit the desired distance range. The distances measured here are closer to the \( R_0 \) of Co\(^{2+}\) than Ni\(^{2+}\). Therefore, although we have obtained similar results with Ni\(^{2+}\), we will use Co\(^{2+}\) as the acceptor for the remainder of these tmFRET experiments.

The Förster equation was used to convert the measured FRET efficiencies into distances (Fig. 3A). Because bimane covalently bound to a cysteine has a number of possible rotamers, it is difficult to predict the absolute distances measured from tmFRET from the crystal structure of HCN2. Therefore, we calculated the difference between the distances to the two metal-binding sites (Δ distance), making the assumption that bimane should adopt the same set of conformations for the constructs with the two different metal-binding sites (Fig. 3B). This distance should approximate the distance between the two sites because the bimane and metal sites are all predicted to lie on the same face of the α-helix. The Δ distance determined from the tmFRET measurements was 3.6 ± 0.2 Å, within 1 Å of the 4.5 Å distance predicted based on an average spacing of 1.5 Å per residue of an α-helix.

To extend our distance measurements, we repeated the above experiment using the bBBr, which has two reactive moieties and can react with neighboring cysteines or cysteines placed three residues apart on an α-helix (28). By using bifunctional bimane, we could lock the fluorophore in two different orientations relative to the helix and greatly decrease the mobility of the fluorophore (Fig. 3C). We expressed and purified HCN2 C-terminal constructs containing the same metal-binding sites as before, but with pairs of cysteines reacted with bifunctional bimane, either at adjacent positions (R623C\(_{\text{mb}}\)A624C) or three residues apart (M621C\(_{\text{mb}}\)A624C) on the C-helix (Fig. 3C). The emission spectrum of bifunctional bimane conjugated to cysteine pairs in these constructs was indistinguishable from that of bimane attached at A624C. In all of these constructs, the closest point of attachment of the bifunctional bimane relative
to the metal ion-binding site was the same as for bimane (A624C).

To show that the bifunctional bimane reacted with both cysteines and decreased its mobility, we measured fluorescence anisotropy (Fig. 3D). As expected, attaching bimane to a single cysteine on the C-helix of HCN2 (A624C<sub>mb</sub>, T627H,D631H) greatly slowed its tumbling rate, resulting in a much higher anisotropy compared with mBBr in water (a change from 0.008 ± 0.001 to 0.117 ± 0.001). Covalently attaching bimane on both sides of the molecule by reacting bifunctional bimane with cysteine pairs further reduced its mobility. The anisotropy of bifunctional bimane attached to cysteines spaced three residues apart (M621CbBA624C,T627H,D631H) was 0.174 ± 0.001, and the anisotropy of bifunctional bimane attached to cysteines spaced four residues apart was 0.206 ± 0.001.
neighboring cysteines (R623C bBA624C,T627H,D631H) was further increased to 0.205 ± 0.001, a value approaching that for mBBr in glycerol (0.228 ± 0.002). Both values for bifunctional bimane were significantly greater than for bimane with one reactive functional group. These data indicate that bifunctional bimane reacted with both cysteines on the C-helix, reducing its rotational degrees of freedom.

Despite the difference in orientation and mobility of bimane when it is covalently attached to two residues, the distances measured using tmFRET were very similar. We measured the concentration-dependent quenching of bifunctional bimane in two different orientations by Co²⁺ bound to two different di-histidine sites within the same helix. n = 6 for all measurements. B, distance between the two di-histidine metal-binding sites (T627H,D631H and I630H,D634H) calculated by subtracting the absolute distances reported in A for each orientation of bimane. The dashed line indicates the theoretical distance between the two metal-binding sites. C, schematics illustrating different orientations of bifunctional bimane attached to the C-helix either between M621C and A624C or between R623C and A624C depicted with the di-histidine metal-binding site T627H,D631H. D, plot of fluorescence anisotropy of bimane and bifunctional bimane attached to different HCN2 constructs as indicated as well as mBBr in water and glycerol. n = 5–21. * denotes p ≤ 0.05, one-way analysis of variance.

cAMP-dependent Conformational Changes in the C-helix—We measured tmFRET between Co²⁺ bound at the T627H, D631H site and bimane attached in three different arrangements, A624CmB, M621CbiA624C, and R623CbiA624C in the presence and absence of cAMP (Fig. 4A). For every combination tested, the metal binding affinity increased in the presence of cAMP. An increased affinity for metal binding to two histidines in helical register suggests a stabilization of the α-helical structure, i.e. an ordering of the C-helix in the presence of cAMP (6, 34–36). Fig. 4C shows the increase in Co²⁺-binding energy (ΔΔG) for the T627H,D631H site as a result of agonist binding. Co²⁺ binding was stabilized between 0.8 and 1.6 kcal/mol. We observed a greater cAMP-dependent stabilization of transition metal binding in the construct with bimane attached to a single cysteine (A624CmB,T627H,D631H) than in either construct in which bifunctional bimane was reacted with two cysteines within the C-helix. This may be the result of reduced flexibility of the helix when the conformational space that can be sampled is restricted by cross-linking two residues.

If agonist binding is accompanied by a secondary structural change in the C-helix, it might result in a change in the distance between the donor and acceptor sites on the helix. In addition to the stabilization of metal binding gained by the addition of cAMP, we also observed a change in the distance between the
donor fluorophore and the acceptor metal ion in the C-helix. Again, regardless of the configuration of bimane used (A624CmB, M621CbBA624C or R623CbBA624C), we measured a cAMP-dependent change in the FRET efficiency such that there was always significantly greater FRET in the presence of agonist than in its absence (Fig. 4, A and B). From the FRET efficiencies, we calculated distances with and without cAMP present and expressed these values as a change in distance upon addition of agonist (Fig. 4D). With bimane attached at position 624 (A624C), the fluorophore moved 0.52 ± 0.08 Å closer to the di-histidine-binding site (T627H,D631H) upon addition of cAMP. A similar value was obtained with bifunctional bimane attached to cysteines spaced three residues apart (M621CbBA624C) where the donor and acceptor moved 0.52 ± 0.26 Å closer together. Although small, these significant distance changes occurred within a single turn of the helix and likely reflect some disorder of the helix at this location in the absence of agonist.

Surprisingly, the agonist-dependent change in distance between the donor and acceptor site was much greater when measured with bifunctional bimane reacted with neighboring cysteine residues (R623CbBA624C,T627H,D631H; Fig. 4D). These donor and acceptor sites moved 2.72 ± 0.14 Å closer in the presence of cAMP. This increase in the agonist-induced distance change arose largely from the greater distance separation between the donor and acceptor site in this construct in the absence of agonist (Fig. 4, A and B). Most likely, tethering adjacent cysteines together distorted the structure of the C-helix with no agonist present, and this distortion was overcome by the ordering of the helix in the presence of cAMP. Whatever the case, the dramatic FRET changes observed for R623CbBA624C,T627H,D631H further support the hypothesis that cAMP induces a conformational change within the C-helix.

Dependence of FRET Changes on cAMP Concentration—The changes in tmFRET within the C-helix due to cAMP binding could be used to measure the apparent affinity for cAMP. Fig. 5 shows the quenching of R623CbBA624C,T627H,D631H by a nearly saturating Co2+ concentration (1 mM), as a function of the total cAMP concentration. The apparent $K_d$ values for cAMP binding derived from fits to the data were 2.8 ± 1.5 μM. This value is in the concentration range reported from fluorescence and calorimetry studies of monomeric HCN2 proteins
C-helix Secondary Structure Changes

(130 nM to 13.2 μM), indicating that the mutations and modifications of the C-helix did not dramatically alter the cAMP binding (22, 37, 38). In the absence of metal, 300 μM cAMP had no significant effect on the fluorescence emission of R623C\textsubscript{sH}A624C (data not shown).

**cAMP Increases the Distance between the B-helix and the Distal C-helix**—If the C-helix secondary structure was altered by cAMP binding, we also expect to observe changes in distance between residues on the C-helix and its nearest neighbor, the B-helix. We measured FRET between bimane at position 635 on the distal C-helix and Co\textsuperscript{2+} bound to a pair of histidines on the B-helix (R635C\textsubscript{mB},N612H,E616H, see Fig. 6A). Co\textsuperscript{2+} bound to the B-helix quenched bimane in a concentration-dependent fashion in both the absence and presence of 300 μM cAMP (Fig. 6B). Unlike Co\textsuperscript{2+} binding to histidines on the C-helix, there was no change in the free energy of Co\textsuperscript{2+} binding to the B-helix upon addition of cAMP (Fig. 6C). There was, however, a large increase in the distance between the distal C-helix and the B-helix upon addition of cAMP. In the absence of cAMP, bimane at position 635 was 13.3 ± 0.1 Å away from Co\textsuperscript{2+} bound to the B-helix. In the presence of cAMP, this distance was increased to 15.4 ± 0.2 Å. Such a distance change would not be possible if the C-helix moved as a rigid body. The most likely explanation for this observation is that in the absence of cAMP, the C-helix adopted a flexible coil conformation, allowing bimane tethered to the helix to more closely approach a Co\textsuperscript{2+} ion bound to the B-helix.

**Ordering of the C-helix Promotes Gating in Intact Channels**—We have shown that there is a change in the secondary structure of the C-helix of the C-terminal fragment of HCN2 induced by agonist binding. Does this conformational change occur in intact functioning channels? If there is a transition of the C-helix from a less ordered state to a more stable helical conformation, is this structural change simply a consequence of agonist binding, or is it coupled to opening of the ion channel pore, i.e. is it a transition associated with agonist binding or channel gating?

To answer these questions, we turned to intact CNG channels. CNG channels are highly homologous to HCN channels (31.7% identical in the C-terminal region, see Fig. 1C), and available data suggest that their C-terminal regions are structurally very similar (39, 40). However, cyclic nucleotides have a
much larger energetic effect on the gating of CNG channels than they do on HCN channels (1). Furthermore, although cGMP is a full agonist of CNG channels, cIMP is a partial agonist of CNGA1 channels, i.e. it does not fully activate channels at saturating concentrations. This can be useful for differentiating between binding effects and gating effects. Finally, the effects of metal binding to CNGA1 channels have been well characterized, and the endogenous metal-binding site can be removed (27, 41–43).

To test the hypothesis that ordering of the C-helix is coupled to gating of cyclic nucleotide-regulated channels, we inserted a di-histidine metal-binding site into the C-helix of a mutant CNGA1 channel with its native Ni\textsuperscript{2+} potentiation site removed (CNGA1\textsubscript{Q}, see under “Experimental Procedures”). The di-histidine mutation, CNGA1\textsubscript{Q}, E595H, Q599H, was placed in an analogous position to the T627H, D631H site in HCN2 based on sequence alignment and homology modeling (Fig. 7A). We reasoned that if the conformational change induced by cyclic nucleotide-stabilized metal binding to a di-histidine site in the C-helix, then binding metal to the di-histidine site in the C-helix should promote the conformational change induced by agonist binding. To separate the effects of Ni\textsuperscript{2+} on initial cyclic nucleotide binding from the effects on gating the channel, we used a saturating concentration of the partial agonist cIMP. If metal binding to the di-histidine site caused an increase in the current response to cIMP relative to cGMP when all the cyclic nucleotide-binding sites were occupied, then the increase in current must have come from an increase in the favorability of the opening transition, i.e. from promotion of the gating conformational change and not a change in the initial binding affinity for agonist.

Fig. 7B shows CNGA1\textsubscript{Q} E595H, Q599H currents in response to a voltage pulse from a holding potential of 0 to −60 and +60 mV in the presence of a saturating concentration of the full agonist cGMP (10 mM) and the partial agonist cIMP (16 mM). Similar data were compiled to generate a concentration-response curve. Data were fit as described under “Experimental Procedures.” The solid gray line represents a fit to the CNGA1\textsubscript{Q} data in which only block by Ni\textsuperscript{2+} was observed. K\textsubscript{a} = 2.4 mM. The black line is a two-component fit to the CNGA1\textsubscript{Q} E595H, Q599H data, with a blocking component (fixed to the same values determined from CNGA1\textsubscript{Q}) and a second component representing Ni\textsuperscript{2+} potentiation. K\textsubscript{a} = 5.0 μM, p = 0.61. The dashed gray line is the potentiation component of the fitted curve. n = 3 for CNGA1\textsubscript{Q}. For CNGA1\textsubscript{Q} E595H, Q599H, n = 7 at 1 and 10 μM Ni\textsuperscript{2+}, n = 6 at 100 μM Ni\textsuperscript{2+}, and n = 3 at 1 mM Ni\textsuperscript{2+}.

We then examined the effect of Ni\textsuperscript{2+} on the current at saturating concentrations of cIMP. Fig. 7D shows CNGA1\textsubscript{Q} control currents resulting from steps to −100 and +60 mV from a holding potential of 0 mV in 16 mM cIMP in the absence and presence of 100 μM Ni\textsuperscript{2+}. In the absence of an introduced metal-binding site, the current magnitude did not change in response to this concentration of Ni\textsuperscript{2+}. We tested multiple concentrations of Ni\textsuperscript{2+} and found no effect on current magnitude in CNGA1\textsubscript{Q} except at 1 mM, where some degree of Ni\textsuperscript{2+} block was evident, as described previously (Fig. 7E) (43). In contrast, the current magnitude in CNGA1\textsubscript{Q} E595H, Q599H channels
was significantly enhanced upon binding of 100 μM Ni\(^{2+}\) (Fig. 7D). We observed an increase in cIMP-induced currents as the Ni\(^{2+}\) concentration was increased (Fig. 7E). Again, there was a decrease in current at 1 mM Ni\(^{2+}\). We fit these data with a model that takes into account both the potentiation and block by Ni\(^{2+}\) to determine the maximal effect of Ni\(^{2+}\) on channel gating (see under “Experimental Procedures”). Ni\(^{2+}\) bound to CNGA1, E595H,Q599H with an apparent affinity of 5.0 μM, similar to the binding affinity of the homologous site in the C-helix of HCN2 (Fig. 2E). From our fits, we determined that stabilizing the C-helix by Ni\(^{2+}\) binding to the E595H,Q599H site potentiated the current in saturating cIMP by a factor of 1.6. This corresponded to a stabilization of the opening gating transition by 0.47 kcal/mol, on the same order of magnitude as the values we measured for stabilization of Co\(^{2+}\) binding to the T627H,D631H site in the C-helix of HCN2 in response to cAMP (Fig. 4C). These results indicate that ordering of the C-helix is tightly coupled to the opening conformational change in these cyclic nucleotide-regulated channels.

**DISCUSSION**

These data led us to a revised model for the agonist-induced conformational changes in eukaryotic cyclic nucleotide-regulated channels (Fig. 8). In the absence of cyclic nucleotide, the C-helix does not form a tight helical structure, and the channel pore is closed (unbound/closed). Agonist initially binds to residues in the P-helix and β-roll, with the ion channel pore still closed (bound/closed). Subsequent to agonist binding, there is an ordering of the C-helix as well as the F’-helix. Additionally, the C-helix moves closer to the agonist-binding site. This ordering of the C-helix, and possibly the translational movement as well, is coupled to the opening conformational change in the pore (bound/open).

Previous work in HCN channels has suggested that there is an ordering of the C-helix induced by agonist binding (6). In the apo state crystal structure of HCN2, there is no density resolved for at least half of the C-helix. This may indicate that the helix is disordered or adopts multiple conformations. However, crystal packing interactions at the C-helix may have distorted the structure. There is also the possibility that Br\(^{-}\) ions in the agonist-binding pocket acted as pseudo ligands. We also previously demonstrated that Ni\(^{2+}\) bound more tightly to several histidine pairs in helical register on the C-helix when cAMP was present. In this study, we extended this observation by directly examining distance changes between locations within the C-helix with tmFRET. The cAMP-dependent increases in FRET efficiency observed between bimane and bifunctional bimane attached to the N-terminal end of the C-helix and transition metal ions bound to a site roughly one helical turn away are best interpreted as a change in conformation within the helix itself. We again observed an increase in metal affinity for a di-histidine-binding site in the C-helix in the presence of cAMP. We also examined the distance between the distal end of the C-helix and the neighboring B-helix, which was significantly shorter in the absence of cAMP than in its presence. The simplest explanation for these observations is that the C-helix adopts a flexible, coiled conformation in the absence of agonist. Finally, working in intact CNG channels, we demonstrated that stabilizing the helical conformation of the C-helix promoted channel opening in a saturating concentration of the partial agonist cIMP. This result suggests that the transition in the C-helix from a less ordered structure to a stable helix is directly coupled to channel gating.

Agonist-induced secondary structural transitions in the C-helix have been reported for other CNBD-containing proteins. NMR studies comparing the apo- and cyclic nucleotide-bound forms of the *E. coli* transcription factor CAP clearly demonstrate a coil-to-helix transition in this region subsequent to agonist binding (44). Crystal structures of the apo state of the prokaryotic, cyclic nucleotide-activated K\(^{+}\) channel MloK1 show a variety of conformations of the C-helix, including some structures in which density of part of the C-helix is absent (9). However, a recent NMR structure of MloK1 suggests that although both the B- and C-helices move closer to the binding site following agonist binding, they move as rigid bodies, with no accompanying change in secondary structure (45). Although the details vary, large conformational changes of this sort have also been reported for the kinases PKA and PKG as well as for Epac (46). Therefore, although we believe there is a rearrangement of the C-helix associated with cyclic nucleotide binding in almost all CNBD-containing proteins, the details of the conformational change may differ for different proteins.

This study demonstrates further the utility of tmFRET to measure very short distances and small distance changes in proteins. Using the small fluorophore bimane and transition metal ion acceptors, we were able to reproducibly measure changes in FRET efficiencies between adjacent rungs of an α-helix. This measurement would not have been possible using
classical FRET methods where long $R_0$ values of commonly used donor/acceptor pairs would make it necessary to place the donor and acceptor on the order of 30–50 Å apart to be in the proper range for distance measurements from FRET. To suit our particular distance range, we chose Co$^{2+}$ among multiple transition metals to improve the accuracy and precision of our measurements. Finally, we used bifunctional bimane to reduce the flexibility of the fluorophore and demonstrated that tmFRET measurements are not strongly influenced by reduced flexibility or fixed orientation of the fluorescent donor. This orientation independence likely resulted, at least in part, from the multiple absorption dipoles of the acceptor ion. tmFRET has allowed for increased insight into the conformational transitions in the CNBD of cyclic nucleotide-regulated channels and offers hope for measurements of the structural dynamics in many proteins.

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