Functionally Important Calmodulin-binding Sites in Both NH2- and COOH-terminal Regions of the Cone Photoreceptor Cyclic Nucleotide-gated Channel CNGB3 Subunit*

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Changhong Peng‡, Elizabeth D. Rich‡, Christopher A. Thor‡, and Michael D. Varnum‡§

From the §Department of Veterinary and Comparative Anatomy, Pharmacology and Physiology, ¶Program in Neuroscience, Washington State University, Pullman, Washington 99164-6520

Whereas an important aspect of sensory adaptation in rod photoreceptors and olfactory receptor neurons is thought to be the regulation of cyclic nucleotide-gated (CNG) channel activity by calcium-calmodulin (Ca2+-CaM), it is not clear that cone photoreceptor CNG channels are similarly modulated. Cone CNG channels are composed of at least two different subunit types, CNGA3 and CNGB3. We have investigated whether calmodulin modulates the activity of these channels by direct binding to the CNGB3 subunit. Heteromeric channels were formed by co-expression of human CNGB3 with human CNGA3 subunits in *Xenopus* oocytes; CNGB3 subunits conferred sensitivity to regulation by Ca2+-CaM, whereas CaM regulation of homomeric CNGA3 channels was not detected. To explore the mechanism underlying this regulation, we localized potential CaM-binding sites in both NH2- and COOH-terminal cytoplasmic domains of CNGB3 using gel-overlay and glutathione S-transferase pull-down assays. For both sites, binding of CaM depended on the presence of Ca2+. Individual deletions of either CaM-binding site in CNGB3 generated channels that remained sensitive to regulation by Ca2+-CaM, but deletion of both together resulted in heteromeric channels that were not modulated. Thus, both NH2- and COOH-terminal CaM-binding sites in CNGB3 are functionally important for regulation of recombinant cone CNG channels. These studies suggest a potential role for direct binding and unbinding of Ca2+-CaM to human CNGB3 during cone photoreceptor adaptation and recovery.

Cyclic nucleotide-gated (CNG)1 channels play a fundamental role in both visual and olfactory transduction by helping to convert sensory input into electrical responses that are ultimately processed as sensory information (1). In mammals, at least six genes encode CNG channel subunits (2, 3). Native CNG channels are tetrameric proteins (4, 5) composed of at least two structurally related subunit types, α (CNGA1, CNGA2, CNGA3, and CNGA4) and β (CNGB1 and CNGB3). Each subunit contains six putative transmembrane domains, cytoplasmic NH2 and COOH termini, a cyclic nucleotide-binding domain, and a conserved pore region (1, 6). Whereas heterologous expression of α subunits alone (except CNGA4) forms functional homomeric channels, the co-assembly of appropriate α and β subunits creates heteromeric CNG channels with properties that more closely resemble native CNG channels (7–12).

CNG channels are highly permeable to Ca2+, and Ca2+ entry through CNG channels provides a negative feedback signal that assists in adaptation and recovery in photoreceptors and olfactory sensory neurons (13–15). Calmodulin (CaM) is thought to participate in these processes by binding Ca2+ and then targeting to different cellular components including CNG channels (16). Numerous studies have described Ca2+-CaM regulation of the ligand sensitivity of rod and olfactory CNG channels (17–25). Olfactory CNG channels are composed of CNGB2 (26), CNGB4 (8, 9), and CNGB1b subunits (10, 11); Ca2+-CaM modulates olfactory channel activity via direct binding to NH2-terminal CaM-binding sites of CNGB2 (20) and probably CNGB1b subunits, whereas the presence of CNGB4 and CNGB1b subunits tunes the kinetics of the response to CaM (27, 28). Rod CNG channels contain CNGB1 (29) and CNGB1 (7) subunits. For these channels, Ca2+-CaM modulation is comparatively weaker, and the CNGB1 subunit provides the CaM-binding site in the NH2-terminal cytoplasmic domain that is critical for regulation (30–32).

For cone CNG channels, Ca2+-feedback mechanisms are less well defined. Ca2+-dependent regulation of apparent cGMP affinity is robust for native striped bass cone CNG channels and is thought to be of greater significance for cone photoreceptor adaptation than the corresponding changes in rods (33–35). Application of CaM to patches excised from cone outer segments inhibits these channels, but CaM only partially mimics the activity of an endogenous modulator(s) (33, 34). In contrast, CaM has no effect on CNG channels in patches excised from catfish cone outer segments (36). Recombinant homomeric CNG channels composed of chick cone CNGA3 subunits are modulated by Ca2+-CaM (37). However, homomeric channels formed by the human or bovine orthologs of CNGA3 are insensitive to CaM regulation, even though these subunits possess an NH2-terminal CaM-binding site (38–40). Thus, there appear to be species differences in sensitivity to regulation by Ca2+-CaM both for native cone photoreceptor CNG channels and for heterologously expressed homomeric CNGA3 channels. With recent cloning of the cone photoreceptor β subunit (CNGB3) for mice (12) and humans (41, 42), the importance of this subunit for modulation of cone CNG channels by Ca2+-CaM can now be examined.

In this study, we demonstrate that the human CNGB3 (hCNGB3) subunit forms functional heteromeric channels with human CNGA3 (hCNGA3) when co-expressed in *Xenopus* oo-
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Cytes. These recombinant heteromeric channels exhibited electrophysiological properties that differed from those of homomeric hCNGA3 channels, including sensitivity to regulation by Ca2⁺-CaM. Human CNGB3 plus CNGA3 heteromeric channels are shown to be unique compared with other CNG channel types, in that CaM regulation involves functionally important CaM-binding sites in both the NH2- and COOH-terminal cytoplasmic domains of hCNGB3.

EXPERIMENTAL PROCEDURES

Molecular Biology—The human retinal cone CNG channel β subunit clone, hCNGB3-c2, was isolated using nested PCR from human retinal cDNA ( Marathon Ready-to-Clone cDNA; Clontech) with adapter- and gene-specific primers. The coding region for this clone differs from the published sequence for human CNGB3 of Kohl et al. ( AF272900) (42) at two positions (nucleotides 1743 G → A and nucleotides 1788 A → G); these likely sequence polymorphisms do not alter the encoded amino acid sequence. Human CNGA3 ( AF065314) (38, 43) was a generous gift of Professor K.-W. Yau. Both hCNGB3 and hCNGA3 were subcloned into pGEMHE (44) for heterologous expression in Xenopus oocytes. A human calmodulin cDNA clone ( hCaM) was also isolated from human retinal cDNA using nested PCR with gene-specific primers. hCaM was modified to include a FLAG epitope (DYKDDDDK) at the COOH terminus and subcloned in-frame with polyhistidine at the NH2 terminus ( pQE-30; Qiagen, Valencia, CA). Constructs for polyhistidine-representing NH2- and COOH-terminal cytoplasmic domains of hCNGA3 ( amino acids 6–206, respectively) and hCNGB3 ( amino acids 1–214 and 441–809, respectively) were generated as in-frame gene fusions with GST ( pGEX-5X-2; Amersham Biosciences) using PCR. Deletions were engineered by overlapping PCR, or by restriction digestion followed by blunt end generation using T4 DNA polymerase (New England Biolabs, Beverly, MA) and in-frame ligation. All mutations and the fidelity of PCRamplified cassettes were confirmed by fluorescence-based automated DNA sequencing.

CaM Interaction Assays—Recombinant protein expression in bacteria and purification were carried out as previously described (25). Briefly, cells were harvested and resuspended in buffer S ( 50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween 20, 1% milk, and either 1 mM EDTA or 5 mM MgCl₂) or sonicated. Soluble proteins were isolated by centrifugation at 20,000 × g for 30 min. GST fusion proteins were purified using glutathione-Sepharose beads (Amersham Biosciences). His-tagged CaM was puriﬁed using Ni²⁺-charged Sepharose (Probond resin; Invitrogen), eluted with 0.5 M imidazole (pH 6.0), and then dialyzed with buffer S. GST pull-down assays were performed using immobilized GST fusion proteins and 2 μM recombinant hCaM-FLAG, in the presence of either 2 mM Ca2⁺ or 10 mM EDTA. Pull-down assays were incubated overnight in buffer S at 4 °C with 100 or 200 μg of GST beads or 50 μg of Ni²⁺-charged Sepharose beads. Beads were centrifuged at 20,000 × g for 30 min at 700 °C for 30 min. Beads were washed 5 times in 100 μl of buffer S (including either Ca2⁺ or EDTA), and suspended in SDS-PAGE sample buffer containing 2-mercaptoethanol. Bound proteins were separated using 12 or 4–20% SDS-PAGE and transferred to nitrocellulose. Gel overlay assays were performed using similarly blotted GST fusion proteins with 1 μg/ml hCaM-FLAG in 20 mM Tris (pH 7.5), 0.15 mM NaCl, 0.1% Tween 20, 1% milk, and either 1 mM CaCl₂ or 5 mM EDTA ( maintained throughout the assay and blot processing). For both assays, interacting hCaM-FLAG was visualized using a chemiluminescent detection system (SuperSignal West Femto ECL Substrate; Pierce). Interaction assays were repeated at least three times with reproducible results. CaM-binding site search and analysis were performed using the Calmodulin Target database of M. Ikuja and co-workers.2

Electrophysiology—For functional expression of hCNGA3 and hCNGB3, Xenopus laevis oocytes were isolated and microinjected with 5–10 ng of in vitro transcribed mRNA (mMessage mMachine; Ambion, Austin, TX) as previously described (45). The ratio of hCNGB3 to hCNGA3 cRNA was 1.5. Two to 7 days later when microinjection of mRNA into oocytes, patch clamp experiments were performed with an Axopatch 200B patch clamp amplifier (Axon Instruments, Foster City, CA) in the inside-out configuration. Initial pipette resistances were 0.45–0.75 MΩ. Currents were low-pass filtered at 2 kHz and sampled at 10 kHz. Intracellular and extracellular solutions contained 130 mM NaCl, 0.2 mM EDTA, and 3 mM HEPES (pH 7.2). Cyclic nucleotides were added to intracellular solutions as indicated. Intracellular solutions were changed using an RSC-160 rapid solution changer (Molecular Kinetics, Pullman, WA). Currents in the absence of cyclic nucleotides were subtracted. Recordings were made at 20 to 22 °C. For electrophysiological experiments involving CaM, bovine CaM ( Calbiochem, San Diego, CA) was used at 250 nm EDTA was substituted by 2 mM nitrotriatic acid and 704 μM CaCl₂ for 50 μM buffered free Ca2⁺ in the intracellular solution. Excised patches were first exposed to 5 mM EDTA for 2–10 min to remove soluble, endogenous Ca2⁺-dependent modulators. In addition, any changes because of Ca2⁺ alone were permitted to occur before CaM was added (10–40 min). After CaM experiments, the channels were typically tested for sensitivity to l-cis-diltiazem ( Research Biochemicals International, Natick, MA and Sigma Chemical Co, St. Louis, MO) and applied in the intracellular solution to verify the subunit composition of the channel, i.e. whether hCNGB3 and deletion constructs of hCNGB3 participated in the formation of the expressed channels. Dose-response relationships were obtained by plotting the current as a function of cyclic nucleotide concentration.

RESULTS

The Properties of Heteromeric CNG Channels Containing hCNGB3 Subunits Differ from Homomeric hCNGA3 Channels—To examine the potential functional signiﬁcance of the human CNGB3 subunit, hCNGB3 was co-expressed with hCNGA3 in Xenopus oocytes and macroscopic cGMP- and cAMP-activated currents were recorded from excised, inside-out membrane patches. As expected, hCNGB3 did not form functional CNG channels when expressed alone ( data not shown). Co-expression of hCNGB3 and hCNGA3 generated recombinant heteromeric channels that demonstrated dramatically altered functional properties compared with homomeric hCNGA3 channels ( Fig. 1, Table I). First, the fractional activation of the channels by a saturating concentration of cAMP compared with saturating cGMP ( IcAMP/IcGMP) was signiﬁcantly increased ( p < 0.001) for heteromeric CNG channels ( Fig. 1A). Second, a statistically signiﬁcant increase in the apparent affinity for cAMP ( p < 0.05) and decrease in the apparent afﬁnity for cGMP ( p < 0.05) were observed for channels containing hCNGB3 subunits compared with the homomeric channels ( Fig. 1B). Third, sensitivity to block by l-cis-diltiazem applied to the cytoplasmic face of the patch was also examined. As expected, homomeric hCNGA3 channels were insensitive to block by l-cis-diltiazem ( Fig. 1C, left, D; Table I). Heteromeric human cone CNG channels demonstrated much greater sensitivity to l-cis-diltiazem (Fig. 1C, right, D; Table I): the K50 for the l-cis-diltiazem block of hCNGB3 + hCNGA3 channels was 7.4 ± 5.2 μM, n = 0.92 ± 0.13 ( n = 16). The voltage dependence and K50 for block by diltiazem were roughly similar to that reported for native catfish cone CNG channels (46), which results show that the presence of the hCNGB3 subunit modiﬁed the ligand sensitivity of the expressed channels and dramatically enhanced susceptibility to block by l-cis-diltiazem. Thus, the functional properties of recombinant heteromeric channels formed by hCNGB3 and hCNGA3 subunits recapitulated some of the characteristics commonly shared with native CNG channels.

hCNGB3 Plus hCNGA3 Heteromeric Channels Are Inhibited by Ca2⁺—We examined the sensitivity of both homomeric hCNGA3 channels and heteromeric hCNGA3 + hCNGB3 channels...2 calcium.uhres.utoronto.ca/cdb/pub_pages/classify/index.htm.
nels to regulation by Ca\(^{2+}\)-CaM. Consistent with previous studies (39), homomeric channels formed by hCNGA3 subunits alone were not inhibited by 250 nM CaM in the presence of 50 \(\mu\)M Ca\(^{2+}\) (Fig. 2, A and C, left). In contrast, heteromeric hCNGA3 + hCNGB3 channels displayed significant inhibition by Ca\(^{2+}\)-CaM under identical conditions (Fig. 2, B and C, right). The time course for maximum current inhibition by Ca\(^{2+}\)-CaM varied between seconds and minutes. The ratio of the current elicited by 10 \(\mu\)M cGMP at +80 mV with and without 250 nM CaM, i.e. \(I_{\text{CaM}}/I\), was 0.96 ± 0.01 (n = 2) for homomeric hCNGA3 channels and 0.62 ± 0.09 (n = 8) for heteromeric hCNGA3 + hCNGB3 channels. Inhibition by Ca\(^{2+}\)-CaM was relieved by perfusion of the patch with calcium-free solution (Fig. 2, B and C, right). The apparent affinity of the heteromeric channel for cGMP was decreased slightly by Ca\(^{2+}\)-CaM (Fig. 2D), from a \(K_{\text{uGMP}}\) of 19.0 ± 2.7 \(\mu\)M, \(n = 20 ± 0.2\) (n = 5) in the absence of CaM (see also Table I) to 25.8 ± 6.7 \(\mu\)M, \(n = 2.1 ± 0.2\) (n = 5) in the presence of CaM (p < 0.05, paired t test). No change in the maximum current at a saturating concentration of cGMP (1 mM) was observed with Ca\(^{2+}\)-CaM compared with Ca\(^{2+}\) alone (data not shown). These results indicate that the presence of the cone photoreceptor hCNGB3 subunit conferred sensitivity to regulation by Ca\(^{2+}\)-CaM in the context of recombinant heteromeric channels.

**Identification of Region in the hCNGB3 NH\(_2\)-terminal Domain Important for Interaction with Ca\(^{2+}\)-CaM**—To test the hypothesis that hCNGB3 subunits provide sites for direct binding of Ca\(^{2+}\)-CaM, we used epitope-tagged human CaM in gel overlay and pull-down interaction assays with GST fusion proteins representing cytoplasmic domains of hCNGB3 and hCNGA3. hCNGB3 subunits are known to possess a CaM-binding site in the NH\(_2\)-terminal cytoplasmic domain that is functionally silent in the context of homomeric channels (39). As expected, the NH\(_2\)-terminal cytoplasmic domain of hCNGB3 (hCNGB3 N) also was able to physically associate with Ca\(^{2+}\)-CaM (Fig. 3, B and C). Next, we localized the CaM-binding site within the hCNGB3 NH\(_2\)-terminal domain. GST fusion proteins representing various hCNGB3 N deletion constructs were generated and tested for interaction with CaM in vitro (Fig. 3A). Deletion of amino acids 2 to 107 of hCNGB3 N did not disrupt binding of Ca\(^{2+}\)-CaM. However, deletion of amino acids 108–159 in hCNGB3 N completely abolished the interaction with Ca\(^{2+}\)-CaM (Fig. 3, B and C). Moreover, a GST fusion protein including only amino acids 108–154 of hCNGB3 N was able to associate with Ca\(^{2+}\)-CaM. Thus, these results show that this region of hCNGB3 N is both necessary and sufficient for direct binding of Ca\(^{2+}\)-CaM. No CaM binding was observed for any of the fusion proteins in the absence of Ca\(^{2+}\), confirming that the interaction was entirely Ca\(^{2+}\)-dependent (Fig. 3, B, middle, and C, bottom). Sequence alignment of the probable CaM-binding site in the hCNGB3 NH\(_2\)-terminal domain with those of CNGB1, CNGA3, and CNGA2 is shown in Fig. 3D. This site in hCNGB3 N resembles a 1–14 CaM-binding motif, exhibiting bulky hydrophobic residues at positions 1 and 14, a net charge of +4, and a predicted propensity to form an amphipathic a-helix (47, 48). This pattern in hCNGB3 N appears to conform better to these common

**Table 1**

| Deletion | cGMP | CaM |
|----------|------|------|
|          | \(K_{uGMP}\) | \(K_{uAM}\) | \(I_{\text{CaM}}/I\) | \(I_{\text{CaM}}/I\) |
| hA3      | 13.5 ± 2.5 (39) | 2.2 ± 0.2 | 1329 ± 369 (39) | 1.3 ± 0.2 | 0.12 ± 0.04 (39) | 0.94 ± 0.04 (6) |
| hA3 + hB3| 19.9 ± 3.8 (56) | 2.0 ± 0.2 | 897 ± 177 (56) | 1.6 ± 0.2 | 0.27 ± 0.09 (56) | 0.26 ± 0.11 (15) |
| hA3 + hB3N| 16.1 ± 3.4 (20) | 2.0 ± 0.2 | 767 ± 196 (18) | 1.5 ± 0.3 | 0.34 ± 0.08 (18) | 0.26 ± 0.03 (5) |
| hA3 + hB3C| 22.0 ± 4.9 (13) | 1.9 ± 0.3 | 973 ± 468 (13) | 1.4 ± 0.2 | 0.30 ± 0.09 (13) | 0.31 ± 0.08 (4) |
| hA3N + hB3N| 17.4 ± 4.0 (10) | 2.1 ± 0.2 | 1156 ± 367 (8) | 1.6 ± 0.3 | 0.34 ± 0.08 (9) | 0.30 ± 0.11 (1) |
| hA3N + hB3NCC| 24.4 ± 4.4 (14) | 2.0 ± 0.2 | 1124 ± 283 (14) | 1.4 ± 0.2 | 0.21 ± 0.07 (13) | 0.32 ± 0.07 (4) |

**Fig. 1.** Recombinant heteromeric CNG channels containing hCNGB3 subunits differ from homomeric hCNGA3 channels. A, representative current traces are shown for homomeric hCNGA3 (hA3, left) and heteromeric hCNGA3 + hCNGB3 (hA3 + hB3, right) channels after activation by 1 mM cGMP or 10 mM cAMP (bold). Current traces were elicited by voltage steps from a holding potential of 0 to +80 mV, then to −80 and 0 mV. Leak currents in the absence of cyclic nucleotide were subtracted for all recordings. B, dose-response relationships for the activation of homomeric channels (open symbols) and heteromeric channels (filled symbols) by cGMP (circles) and cAMP (squares) at +80 mV. Currents were normalized to the maximum current in saturating cGMP. Continuous curves represent fits of the dose-response relation to the Hill equation in the form: \(I = I_{\text{max}}/\left(1 + \left[I_{\text{uGMP}}/K_{\text{uGMP}}\right]^n\right)\). For fits to the activation of homomeric channels by cGMP and cAMP, the following parameters were used: \(K_{\text{uGMP}} = 9.2 \mu\)M, \(n = 2.0\); \(K_{\text{uAM}} = 1201 \mu\)M, \(n = 1.3\). For activation of heteromeric channels: \(K_{\text{uGMP}} = 17.8 \mu\)M, \(n = 2.0\); \(K_{\text{uAM}} = 664 \mu\)M, \(n = 1.5\). C, currents elicited by 100 \(\mu\)M cGMP in the absence and presence (bold) of 25 \(\mu\)M 1-cis-diltiazem for homomeric hCNGA3 (left) and heteromeric hCNGA3 + hCNGB3 channels (right). Current traces were elicited by the same protocol described in A, D, dose-response curves for block of homomeric (open triangles) and heteromeric channels (filled triangles) by 1-cis-diltiazem in the presence of 100 \(\mu\)M cGMP. Continuous curves represent fits of the dose-response relation to the Hill equation in the form: \(I_{\text{CaM}}/I = I_{\text{max}}/\left(1 + \left[I_{\text{uAM}}/K_{\text{uAM}}\right]^n\right)\). For heteromeric channels, the \(K_{\text{uAM}} = 5.4 \mu\)M and \(n = 0.92\). Homomeric channels were insensitive to 1-cis-diltiazem (\(K_{\text{uAM}} >> 100 \mu\)M).
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Calmodulin-binding motifs than the previously characterized “unconventional” CaM-binding site in the NH2-terminal domain of CNGB1 (30, 31).

Localization of a CaM-binding Site in the hCNGB3 COOH-terminal Domain—Because a functionally silent CaM-binding site was previously identified in the COOH-terminal cytoplasmic domain of the rod CNG channel β subunit (CNGB1) (30, 31), we investigated the possibility that the carboxyl-terminal domain of hCNGB3 (hCNGB3 C) may also bind Ca2+-CaM. Gel-overlay experiments with full-length hCNGB3 C demonstrated an interaction with Ca2+-CaM in vitro (Fig. 4B). Similar to hCNGB3 N, CaM binding to hCNGB3 C depended on the presence of calcium. As expected, the hCNGB3 COOH-terminal domain did not interact with CaM. To localise which regions of hCNGB3 C were necessary for binding of Ca2+-CaM, GST fusion proteins representing hCNGB3 C deletion mutants were engineered and tested using in vitro protein-protein interaction assays (Fig. 4). Assays carried out with fusion proteins including the C-linker region and the cyclic nucleotide-binding (CNB) domain, or the CNB domain alone, revealed no binding to Ca2+-CaM. Instead, Ca2+-CaM specifically interacted with a fusion protein representing the region of hCNGB3 C distal to the CNB domain (amino acids 637–809). A fusion protein limited to amino acids 661–757 of hCNGB3 was sufficient for interaction with Ca2+-CaM, whereas proteins including amino acids 637–681 or 680–809 of hCNGB3 C were unable to bind CaM. Similar results were obtained using GST pull-down assays (data not shown). In all cases, CaM binding did not occur in the absence of calcium (Fig. 4B, middle). These results, summarized in Fig. 4A (right), identify a region distal to the cyclic nucleotide-binding domain that is necessary for Ca2+-CaM binding in vitro. Consistent with these findings, a search for likely CaM-binding sites in hCNGB3 C highlighted this sequence as a candidate CaM target (Fig. 4A, bottom). This Ca2+-CaM-binding site in hCNGB3 is positioned similar to the functionally silent CaM-binding site in the COOH-terminal domain of CNGB1, but this region of hCNGB3 exhibits little sequence homology with CNGB1.

Effects of hCNGB3 NH2- and COOH-terminal Deletions on Ca2+-CaM Modulation of Heteromeric CNG Channels—To determine the functional importance of the CaM-binding sites identified within hCNGB3 NH2- and COOH-terminal cytoplasmic domains, hCNGB3 deletion mutants were engineered and individually co-expressed with hCNGA3 in Xenopus oocytes. As shown in Table I, the functional properties of these channels, as indicated by the K1/2 for cGMP, the K1/2 for cAMP, and I4/15

\[ I_{CaM} = \frac{K_{1/2}}{K_{1/2} + cAMP} + \frac{I_{CaM}}{cAMP} \]
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FIG. 4. Localization of Ca$^{2+}$-CaM-binding site in hCNGB3 COOH-terminal domain. A, schematic representation of GST fusion proteins of hCNGB3 C deletion constructs. Box to the right summarizes the results from interaction assays with Ca$^{2+}$-CaM. Below, diagram of the hCNGB3 COOH-terminal region with sequence of the putative CaM-binding site: S6, putative sixth transmembrane domain; open box, putative CaM-binding site. B, gel overlay assays using CaM-FLAG were performed as described in the legend to Fig. 3 in the presence (top) or absence of Ca$^{2+}$-CaM of hCNGA3 and hCNGB3. The mechanism for CaM regulation appears to be similar for L-cis-diltiazem block of currents at +80 mV for hCNGA3 and hCNGB3 ΔNΔC channels (6.6 ± 4.2 μM, n = 0.74 ± 0.17; n = 5) was similar to that of wild-type heteromeric channels. These results confirm that hCNGB3 ΔNΔC subunits were fully competent for assembly into functional heteromeric channels. Yet, the basic properties of hCNGB3 ΔNΔC-containing channels differed somewhat from wild-type heteromeric channels. In particular, there was a significant decrease in both apparent affinity for cAMP (p = 0.001) and relative agonist efficacy, I_{AMP}/I_{cAMP} (p < 0.01) (Table I). These changes indicate that deletion of both CaM-binding sites in hCNGB3 has an effect on channel gating that parallels the effect of Ca$^{2+}$-CaM on the activation of wild-type heteromeric channels.

**DISCUSSION**

We have investigated Ca$^{2+}$-CaM regulation of channels formed by human retinal cone CNGB3 and CNGB3 subunits and found that the CNGB3 subunit confers sensitivity to modulation. Our results are consistent with a mechanism whereby direct binding of CaM to CNGB3 subunits of heteromeric channels reduces the sensitivity of these channels to cyclic nucleotide. The mechanism for CaM regulation appears to be similar to that of olfactory and rod CNG channels: an allosteric effect of bound CaM on the opening transition of the channel. In this way, the ligand sensitivity of human cone photoreceptor CNG channels can be regulated via a Ca$^{2+}$-feedback mechanism that involves binding of CaM to sites located within the NH$_2$ and COOH-terminal cytoplasmic domains of hCNGB3. CaM regulation of the ligand sensitivity of cone CNG channels is expected to contribute to an extension of the operating range of cone photoreceptors (49).

With completion of this study and comparison to previous studies (for review, see Ref. 50), similarities and differences in CaM binding and regulation among olfactory, rod, and cone CNG channels are now evident. All three sensory transduction
channels contain one or more subunits with NH2-terminal CaM-binding sites located a short distance from the first putative transmembrane domain: CNGA2 and CNGB1b for the olfactory channel, CNGB1 for the rod channel, and CNGA3 and CNGB3 for the cone channel. The cone channel is in several species unique among these CNG channel types. First, the NH2-terminal CaM-binding site of the human CNGB3 subunit is functionally silent not only for homomeric channels (39), but also, as shown in this paper, for heteromeric channels containing hCNGB3 subunits. Second, the sequence of the hCNGB3 NH2-terminal CaM-binding site conforms better to typical IQ-type motifs than the NH2-terminal CaM-binding site of CNGB1, which has been previously described as unconventional in that its sequence is in part similar to an IQ-type motif also, as shown in this paper, for heteromeric channels containing hCNGB3 subunits. Third, whereas CNGB3 and CNGB1 subunits both possess a COOH-terminal CaM-binding site located distal to the CNB domain, only the site in the CNGB3 subunit is functionally important for Ca2+-CaM regulation. For the rod CNG channel, the CNGB1 COOH-terminal CaM-binding site is not necessary or sufficient for regulation by Ca2+-CaM (30, 31). In contrast, the COOH-terminal CaM-binding site of the CNGB3 subunit contributes to the sensitivity of the cone channel to Ca2+-CaM regulation and is sufficient for regulation in the absence of the NH2-terminal CaM-binding site. Thus, our results suggest that both NH2- and COOH-terminal CaM-binding sites of hCNGB3 are equally important for regulation. Because single deletions of the individual CaM-binding sites gave rise to heteromeric channels that exhibited sensitivity to modulation roughly similar to that of wild-type channels, we favor a model where each CaM-target site in hCNGB3 is independently capable of conferring regulation, rather than the two sites acting in a synergistic or additive manner.

CaM also decreases the ligand sensitivity of rod, cone, and olfactory CNG channels to different extents. Ca2+-CaM reduces the apparent affinity of the olfactory CNG channel for cAMP by up to 20-fold (18, 20), whereas it has a more modest effect on channels formed by rod photoreceptor CNGB1 plus CNGA1 subunits (19, 22, 30–32). The magnitude of cone channel modulation by Ca2+-CaM is much smaller than that of olfactory CNG channels but similar to that of rod channels. Although the sensitivity of cone photoreceptor CNG channels to regulation by CaM may vary with different species, our results indicate that channels formed by human CNGB3 and CNGA3 subunits are modulated by Ca2+-CaM to an extent similar to recombinant bovine and human rod CNG channels. As previously suggested (25, 27, 32, 39, 51), CNG channel sensitivity to regulation by Ca2+-CaM is dependent not merely on the presence, number, or location of CaM-binding sites but also on other structural features and interactions.

As is the case for CNGA2 homomeric channels (20, 25), deletion of the CaM-binding sites in hCNGB3 subunits alters the activation properties of heteromeric channels in a way that parallels the effect of Ca2+-CaM binding on gating of wild-type channels. Previous evidence suggests that an interaction occurs between NH2- and COOH-terminal cytoplasmic domains of olfactory CNGA2 subunits that promotes channel opening and helps control gating of the channels. Ca2+-CaM modulates olfactory CNG channels by direct binding to an NH2-terminal autoinhibitory domain (20, 52, 53), disrupting interdomain coupling with the COOH-terminal CNB domain (25). For rod CNG channels, Ca2+-CaM binding to an NH2-terminal site in CNGB1 also disrupts an interaction between this domain and the distal COOH-terminal region of CNGA1 (32). It remains to be determined if a similar mechanism is involved in CaM regulation of cone CNG channels.

Whereas the simplest explanation for the observations described in this paper is that Ca2+-CaM can regulate the activity of cone CNG channels by direct binding to NH2- and COOH-terminal CaM-binding sites of hCNGB3 subunits, other mechanisms remain possible. Ca2+-CaM may alter channel activity by binding to a channel-associated protein rather than, or in addition to, docking at the hCNGB3 subunit itself. In this regard, hCNGB3 deletions may prevent interactions between the channel and proteins other than CaM. Another possibility is that Ca2+-CaM may stimulate patch-associated CaM-dependent kinase activity, which in turn may modulate cone CNG channels via phosphorylation. CaM kinase II activity has been implicated in circadian regulation of the ligand sensitivity of native chick cone CNG channels (54); whether the reported effect is because of direct phosphorylation of the channel protein or occurs via an indirect pathway remains to be determined. Muller and co-workers (40) have demonstrated that the ligand sensitivity of homomeric bovine CNGA3 channels is regulated by protein kinase C-mediated phosphorylation. Therefore, like rod CNG channels (Refs. 55 and 56, for review, see Ref. 57), cone CNG channels may be the targets of several regulatory pathways that include but are not limited to direct binding of CaM.
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An additional consideration is that at least in some species other physiologically important effectors for Ca$^{2+}$-dependent regulation of cone CNG channels may act in concert with CaM. There is evidence suggesting that a Ca$^{2+}$-binding protein other than CaM may regulate native CNG channels of striped bass cone photoreceptors, particularly because CaM application to patches excised from cone outer segments does not completely recapitulate the effect of the endogenous calcium-dependent modulator(s) (33, 34). For CNG channels of intact cone outer segments, changes in cytoplasmic Ca$^{2+}$ concentration can alter apparent cGMP affinity over a 4-fold range (34). The more modest extent of CaM-mediated inhibition of heterologously expressed CNGB3 plus CNGA3 channels suggests that CaM can modulate for some but not all of this Ca$^{2+}$-dependent modulation of ligand sensitivity. It has also been suggested that an unknown endogenous factor may be important for Ca$^{2+}$-dependent regulation of native rod (21) and olfactory (58) CNG channels. Warren and Molday (59), however, have recently shown that soluble extract from bovine rod outer segments that have been depleted of endogenous CaM do not contain an additional endogenous factor sufficient to regulate rod CNG channel activity. Several other Ca$^{2+}$-binding proteins related to CaM are expressed in the retina (60). One of these, CaBP1, has recently been shown to compete with CaM for binding to a region within the cytoplasmic COOH-terminal domain of neuronal Ca$_{2+}$,1 channels (61). Further studies are needed to identify and characterize potential interactions between CNG channels and other calcium-sensitive regulatory proteins that may augment or compete with the action of CaM.

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Functionally Important Calmodulin-binding Sites in Both NH2- and COOH-terminal Regions of the Cone Photoreceptor Cyclic Nucleotide-gated Channel CNGB3 Subunit
Changhong Peng, Elizabeth D. Rich, Christopher A. Thor and Michael D. Varnum

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