Functional consequences of progressive cone dystrophy-associated mutations in the human cone photoreceptor cyclic nucleotide-gated channel CNGA3 subunit

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Liu, Chunming, and Michael D. Varnum. Functional consequences of progressive cone dystrophy-associated mutations in the human cone photoreceptor cyclic nucleotide-gated channel CNGA3 subunit. Am J Physiol Cell Physiol 289: C187–C198, 2005. First published March 3, 2005; doi:10.1152/ajpcell.00490.2004.—Progressive cone dystrophies are a genetically heterogeneous group of disorders characterized by early deterioration of visual acuity and color vision, together with psychophysical and electrophysiological evidence of abnormal cone function and cone degeneration. Recently, three mutations in the gene encoding the CNGA3 subunit of cone photoreceptor cyclic nucleotide-gated (CNG) channels have been linked to progressive cone dystrophy in humans. To investigate the functional consequences of these mutations, we expressed mutant human CNGA3 subunits in Xenopus oocytes, alone or together with human CNGB3, and studied these channels using patch-clamp recording. Compared with wild-type channels, homomeric and heteromeric channels containing CNGA3-N471S or CNGA3-R563H subunits exhibited an increase in apparent affinity for cGMP and an increase in the relative agonist efficacy of cAMP compared with cGMP. In contrast, R277C subunits did not form functional homomeric or heteromeric channels. Cell surface expression levels, determined using confocal microscopy of green fluorescent protein-tagged subunits and patch-clamp recording, were significantly reduced for both R563H and R277C but unchanged for N471S. Overall, these results suggest that the plasma membrane localization and gating properties of cone CNG channels are altered by progressive cone dystrophy-associated mutations, providing evidence that supports the pathogenicity of these mutations.

phosphodiesterase

NORMAL HIGH-ACUITY AND COLOR vision relies on the presence and functional integrity of three types of cone photoreceptors that are optimally sensitive to light of different wavelengths (for review, see Ref. 20). Located in the photoreceptor outer segment, cyclic nucleotide-gated (CNG) channels play a fundamental role in phototransduction by helping to convert sensory input into electrical responses. In the absence of light, the CNG channels are opened by binding of intracellular cGMP (29, 73) and conduct a “dark current” carried mostly by an influx of Na+ and Ca2+ (24). When photons are absorbed by the photopigments, sequential signaling events are initiated, including activation of a photoreceptor G protein transducin and activation of cGMP phosphodiesterase (PDE). The activated cGMP PDE then hydrolyzes and lowers the intracellular cGMP level. Hyperpolarization of the photoreceptors thus results from the closure of CNG channels in response to the lowered cGMP level, and the tonic release of neurotransmitter (glutamate) from the photoreceptor terminal decreases (70). The CNG channels are also crucial for recovery and light adaptation processes by controlling intracellular Ca2+ homeostasis because they are the major pathway for Ca2+ entry into the photoreceptor outer segment (14, 16). Intracellular Ca2+ entry is balanced by Ca2+- Na+/Ca2+-K+ exchangers (for review, see Ref. 51). Within the outer segment, the fall in intracellular Ca2+ concentration due to the closure of CNG channels triggers a negative feedback loop that mediates 1) recovery of the cell to its preactivated state in the case of a transient stimulus or 2) light adaptation in the case of a sustained stimulus (33, 51).

CNG channels are tetrameric proteins (22, 39, 65) composed of some combination of CNGA1, CNGA2, CNGA3, CNGB3, CNGB1, CNGB3 subunits (3, 21, 34, 57). The CNGA1, CNGB2, or CNGB3 subunit can form functional homomeric channels when expressed alone (2, 11, 28, 67). While CNGB1, CNGB3, and CNGB4 subunits do not form functional channels by themselves, they can modulate the channel properties when coassembled with the other subunit types (3–6, 21, 37, 57). Native rod CNG channels are heteromeric proteins formed by assembly of three CNGA1 subunits and one CNGB1 subunit (66, 74, 76). Recent studies suggest that cone CNG channels adopt a different structure, being composed of CNGA3 and CNGB3 subunits in a 2 × 2 configuration (49). Each channel subunit contains six transmembrane regions, cytoplasmic NH2 and COOH termini, a conserved pore domain, and a cyclic nucleotide-binding domain (Fig. 1A) (for review, see Ref. 29). Loss-of-function alterations in the cone photoreceptor CNG channels due to missense mutations, deletions, or splice site disruption in the genes encoding these subunits result in abnormal cone function, leading to daylight and color vision deficiencies (for review, see Ref. 52). The general forms of color blindness, cone dystrophies, can be divided into two broad groups: stationary and progressive cone dystrophy. The stationary form of cone dystrophy is also called “achromatopsia” or “monochromatism” (58). Progressive cone dystrophies are a group of clinically heterogeneous disorders. Patients with these diseases exhibit progressive loss of visual acuity and color vision, together with photophobia and nystagmus, in late childhood or early adulthood (59). Psychophysical and elec-
trophophysiological examinations show abnormal cone function, while rod function is intact (58). The mechanisms underlying the pathophysiology of cone dystrophies are still poorly understood.

Functional characterization of disease-associated mutations in genes encoding CNG channel subunits can provide insight into the molecular mechanisms and pathogenicity of photoreceptor degeneration. Studies of retinitis pigmentosa-associated mutations in the rod CNGA1 subunit suggest that some mutations may lead to absence of functional CNG channels at the plasma membrane (12, 40, 61). Similarly, an achromatopsia-associated frame shift mutation in the cone photoreceptor CNGB3 subunit (31, 60) results in a truncated form of the subunit that is unable to form functional heteromeric channels with CNGA3 subunits (48). This suggests that properly assembled heteromeric channels are critical for normal cone function and survival. Studies also show that an achromatopsia-associated missense mutation in the CNGB3 subunit (S435F) alters the gating properties of heteromeric channels when coexpressed with CNGA3 subunits (44, 48).

Recently, 51 mutations have been identified in the gene encoding the human CNGA3 subunit of cone photoreceptor CNG channels and linked to achromatopsia and progressive cone dystrophy (32, 68). Three of these mutations are present in patients with severe progressive cone dystrophy: R277C (in the S4 domain), N471S (in the C-linker region), and R563H (in the cyclic nucleotide-binding domain, CNBD) (Fig. 1A). An important step toward understanding the development of this disease is to determine how individual mutations alter the functional properties of CNG channels and how abnormal channel function may lead to cone photoreceptor degeneration.

Herein we report the functional consequences of three progressive cone dystrophy-associated mutations in CNGA3 subunits. Our results suggest that these mutations disrupt plasma membrane localization, impair channel protein posttranslational modification, and/or alter the gating properties of cone CNG channels, thus leading to abnormal cone photoreceptor function and ultimately to degeneration.

MATERIALS AND METHODS

Mutagenesis and functional expression. For heterologous expression in Xenopus laevis oocytes, the coding sequence for human CNGA3 (71) was subcloned into pGEMHE (38) and an NH2-terminal tag with green fluorescent protein (GFP) was generated as described.
The human CNGB3 clone was isolated from human retinal cDNA and also subcloned into pGEMHE (47, 48). Mutations in the CNGA3 coding sequence were engineered using overlapping PCR mutagenesis (25, 55). All mutations and the fidelity of PCR-amplified cassettes were confirmed by performing automated DNA sequencing. For expression studies, identical amounts of cDNA were linearized using SphI or NheI, and capped mRNA was transcribed in vitro using the T-7 RNA polymerase mMessage mMachine kit (Ambion, Austin, TX). mRNA concentrations and relative amounts were determined using denaturing gel electrophoresis with 1-D image analysis software (Kodak, Rochester, NY) and by performing spectrophotometry.

Oocytes were isolated as previously described (64, 72) and microinjected with a fixed amount of mRNA for all constructs (~5 ng). For efficient generation of heteromeric channels, the ratio of wild-type or mutant CNGA3 mRNA to CNGB3 mRNA was 1:5 (49). Oocytes were incubated in ND96 (in mM: 96 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2, and 5 HEPES, pH 7.6, supplemented with 10 μg/ml gentamicin). For some experiments, oocytes were incubated in ND96 that also contained 5 μM tunicamycin (EMD Bioscience, La Jolla, CA).

Electrophysiology. Two to seven days after microinjection of mRNA, patch-clamp experiments were performed in the inside-out configuration with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Recordings were made at 20–23°C. Data were acquired using Pulse software (HEKKA Elektronik, Lambruch, Germany). Current traces were elicited by voltage steps from a holding potential of 0 to +80 mV, then to −80 mV, and back to 0 mV. Initial pipette resistances were 0.4–0.8 MΩ. Intracellular and extracellular solutions contained (in mM) 130 NaCl, 0.2 EDTA, and 3 HEPES (pH 7.2). Intracellular solutions were changed using an RSC-160 rapid solution changer (Molecular Kinetics, Pullman, WA). Currents in the absence of cyclic nucleotide were subtracted. For channel activation by cGMP or cAMP, dose-response data were fitted to the Hill equation

\[ I_{\text{max}} = \frac{[cNMP]^n}{K_{n1} + [cNMP]^n} \]

where \( I \) is the current amplitude, \( I_{\text{max}} \) is the maximum current elicited by saturating concentration of ligand, [cNMP] is the ligand concentration, \( K_{n1} \) is the apparent ligand affinity, and \( n \) is the Hill slope. For current block by tetracline, data were fitted to a modified Hill equation in the form

\[ I_{\text{recorded}} = I_{\text{max}} \left( \frac{[\text{cNMP}]^n}{[\text{cNMP}]^n + [\text{tetracaine}]^n} \right) \]

To confirm the formation of heteromeric CNGB3 plus CNGA3 channels, we measured sensitivity to block by applying 25 μM 1-cis-diltiazem (RBI, Natick, MA) to the intracellular face of the patch in the presence of 1 mM cGMP. Data were analyzed using Igor (Wavemetrics, Lake Oswego, OR), SigmaPlot, and SigmaStat software (SPSS, Chicago, IL). All values are reported as means ± SE of n experiments (patches) unless otherwise indicated. Statistical significance was determined using Student’s t-test or the Mann-Whitney rank-sum test, and \( P < 0.05 \) was considered significant.

To describe the gating of homomeric CNGA3 channels, we used a simplified linear allosteric model in which independent ligand-binding steps are followed by a single allosteric transition from the liganded but closed state to the open state (18, 23, 36, 64):

\[
\begin{align*}
C + \text{cNMP} & \rightarrow C_{2k} + \text{cNMP} \\
C_{2k} & \rightarrow C_{k2} \\
C_{k2} + \text{cNMP} & \rightarrow C + \text{cNMP}
\end{align*}
\]

In this kinetic scheme, \( K \) is the equilibrium constant for the initial binding of cyclic nucleotide and \( L \) is the equilibrium constant of the allosteric conformational transition. We used the local anesthetic tetracaine (Sigma, St. Louis, MO), a known state-dependent blocker of CNGB channels that binds to closed channels with nearly 1,000-fold greater affinity compared with open channels (18), to investigate the altered gating properties of mutant homomeric channels. By applying a saturating concentration of cGMP (fully liganded-bound state), we isolated the allosteric conformational change associated with channel opening. Tetracaine sensitivity is a reporter for this equilibrium; thus channels that spend more time in the open state are less sensitive to tetracaine block. We calculated the equilibrium constant for the allosteric transition (L) for cGMP-bound channels using the equation of Fodor et al. (18):

\[
L = \frac{K_d \times (K_{1/2,\text{tetracaine}} - K_a)}{K_a \times (K_d - K_{1/2,\text{tetracaine}})}
\]

In this equation, \( K_d \) (220 nM) and \( K_a \) (170 μM) are dissociation constants for tetracaine binding to closed or open channels, respectively (18). The L value for cAMP-bound channels was determined using the relative agonist efficacy of cAMP compared with cGMP. \( K \) was determined from fits of the simple allosteric model described above to the dose-response data using L values calculated from tetracaine apparent affinity and relative agonist efficacy. The free energy differences for \( L \) and for \( K \) were calculated as follows: \( \Delta G = -RT \times \log (X) \). Also, we calculated \( \Delta G = \Delta G_{\text{mutant}} - \Delta G_{\text{wt}} \).

As described previously (48), confocal images of oocytes expressing GFP-tagged CNGA3 were obtained using a Bio-Rad MRC-1024 confocal laser-scanning system and a krypton-argon laser with a Nikon Eclipse TE 300 inverted microscope with a ×10 lens objective. Four days after injection of mRNA, oocytes expressing homomeric or heteromeric channels were placed in borosilicate coverglass chambers such that the equator was approximately perpendicular to the plane of imaging. GFP fluorescence was determined using an excitation wavelength of 488 nm and a 522 DF 32 emission filter. Laser intensity, pinhole aperture, and photomultiplier gain were the same for all experiments. Images were analyzed using NIH ImageJ software. Surface fluorescence for each oocyte was determined from an area within the animal hemisphere representing ~5% of the circumference in a single plane and expressed as intensity of signal per unit area. Background fluorescence was determined for an equivalent area using a blank region of the same image and subtracted. Values are reported as means ± SE of n oocytes tested.

Protein biochemistry. To assess the overall abundance and processing of CNGB3 subunits expressed in Xenopus oocytes, we performed Western blot analysis of proteins from oocytes expressing GFP-tagged CNGA3. Oocyte lysates were prepared as previously described (47, 53, 54). Brieﬂy, oocytes were placed in lysis buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100 (Surface-Amps X-100; Pierce Biotechnology, Rockford, IL), and a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Oocytes were subjected to trituration followed by cup sonication, repeated a total of three times. The soluble cell lysate was then separated from yolk and other insoluble material by performing centrifugation at 20,000 g and 4°C for 10 min, which was repeated three times. Lysate representing approximately one oocyte per lane was loaded and separated by SDS-PAGE using NuPage 4–12% Tris acetate gels (Invitrogen, Carlsbad, CA). Proteins were then transferred to nitrocellulose membrane using the NuPage transfer buffer system (Invitrogen). Immunoblots were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 (TTBS; Bio-Rad) for 2 h and then probed with anti-GFP Aequorea victoria peptide polyclonal antibody (Clontech, Palo Alto, CA) at a concentration of 1:2,500 in TTBS buffer with 1% nonfat dry milk. GFP-tagged channel subunits were visualized using SuperSignal West Dura substrate (Pierce Biotechnology) and autoradiographic film (Kodak X-Omat Blue XB-1; Eastman Kodak). The molecular weights of the GFP-tagged subunits were estimated using protein standards (SeeBlue Plus2; Invitrogen).
The relative amounts of CNGA3 protein (band density) for all GFP-A3 immunoreactive bands (glycosylated and unglycosylated) were estimated using NIH ImageJ software. To verify that approximately equal amounts of total protein were loaded in each lane, the same blots were probed with MAB1501 pan-actin antibody (Chemicon International, Temecula, CA). The variation in actin signal between lanes, determined using densitometry, was <10% (n = 4 immunoblots).

RESULTS

Functional expression and plasma membrane targeting of mutant human CNGA3 subunit. To investigate the effect of progressive cone dystrophy-associated mutations on plasma membrane localization of homomeric and heteromeric channels, we introduced these mutations into cDNA encoding GFP-tagged human CNGA3 subunits (71) and expressed mutant or wild-type CNGA3 subunits alone or together with the human CNGB3 subunits (31, 47, 60) in Xenopus oocytes.

Surface membrane fluorescence levels in oocytes were determined by performing confocal microscopy after 4 days of incubation. Figure 1, B and C, shows representative confocal images and relative surface membrane fluorescence (F) levels normalized to the levels of wild-type homomeric CNGA3 channels. For both homomeric and heteromeric channels, cell surface expression levels were significantly reduced (P < 0.01) for R277C (F_{homo} = 0.06 ± 0.01, n = 39; F_{hetero} = 0.04 ± 0.01, n = 26) and R563H (F_{homo} = 0.15 ± 0.04, n = 42; F_{hetero} = 0.12 ± 0.02, n = 26) (Fig. 1, B and C) compared with the corresponding wild-type channels. Surprisingly, N471S did not interfere with plasma membrane localization of homomeric or heteromeric channels. Maximum patch-current (I_{max}) levels, determined using patch-clamp recording at a saturating concentration of cGMP (1 mM), were consistent with the observed plasma membrane fluorescence levels (Fig. 1C). Compared with wild-type channels, mean I_{max} for both homomeric and heteromeric channels was reduced for R563H-containing channels (n = 12–13; P < 0.01) but did not change significantly for N471S-containing channels. No current was elicited by a saturating concentration of cGMP for R277C-containing channels. These results suggest that R277C and R563H mutations impaired the plasma membrane expression of cone CNG channel subunits.

Cone dystrophy mutations altered CNG channel protein processing. There are two possible explanations for the reduced plasma membrane localization observed for R277C and R563H subunits. One is that protein folding and/or stability are impaired by the mutations. Another possibility is that the channel proteins are not properly assembled and/or targeted to the plasma membrane. To address these possibilities, we conducted Western blot analysis of oocyte lysates. Immunoblots revealed a reduction in overall protein amount and a change in the processing and maturation of R277C subunits (Fig. 2, A and B). The reduced protein levels for R277C compared with wild-type subunits (Fig. 2, A and B) indicate that the mutation disrupted channel biogenesis and/or stability, which likely accounts for much of the reduction in cell surface expression of these subunits (Fig. 1). In contrast, R563H subunits exhibited only a slight, nonstatistically significant reduction in protein levels, suggesting that this mutation primarily impaired plasma membrane targeting of CNGA3 subunits. Confocal images of intact oocytes display GFP-tagged subunits located at the surface membrane but not the fraction of subunits that remains intracellular (see Ref. 75). Thus, compared with wild-type and N471S subunits, there is a much larger fraction of R563H subunits retained intracellularly and/or retrieved to intracellular compartments.

In addition to the overall reduction in the amount of CNGA3 protein for R277C (Fig. 2, A and B), the upper molecular
weight bands evident for wild-type subunits were also reduced or absent. As shown previously, the generation of mature channels by bovine CNGA1 or CNGA3 subunits is associated with a series of posttranslational modifications involving glycosylation (13, 53). To confirm that the upper molecular weight bands observed in the present study represented glycosylated CNGA3 subunits, oocytes expressing wild-type homomeric and heteromeric CNG channels were treated with tunicamycin, a known N-acetylgalcosaminidase inhibitor that prevents protein glycosylation (56) (Fig. 2). As anticipated, tunicamycin-treated groups lacked the upper molecular weight bands evident in nontreated groups, indicating that these upper molecular weight bands represented N-glycosylated subunits. Impaired N-glycosylation of mutant CNGA3 subunits suggests that the cone dystrophy-associated R277C mutation disrupted CNG channel posttranslational processing and maturation.

Altered gating properties of homomeric CNGA3 channels. The electrophysiological properties of the channels were investigated using patch-clamp recording. Because CNGA3 subunits can form functional homomeric channels when expressed alone, we first examined the effect of these three mutations on the behavior of homomeric channels. Expression of R277C subunits alone did not give rise to cyclic nucleotide-dependent currents even after application of a saturating concentration of cGMP, suggesting that R277C subunits did not form functional CNG channels. Homomeric channels containing N471S or R563H mutations exhibited cyclic nucleotide-activated currents (Fig. 3A) with properties that differed from those of wild-type channels. cAMP is a partial agonist for recombinant and native rod and cone photoreceptor CNG channels, exhibiting a lower efficacy compared with cGMP (29). The initial binding of cAMP is comparable to that of cGMP, but cAMP is much less capable of promoting channel opening once bound (23). For both mutant channels, the relative agonist efficacy for channel activation by a saturating concentration of cAMP compared with maximal activation by cGMP \((I_{\text{max,cAMP}}/I_{\text{max,cGMP}})\) was increased significantly (N471S: 0.35 ± 0.12, \(n = 18, P < 0.01; R563H: 0.64 ± 0.20, n = 12, P < 0.01\)) compared with wild-type channels (0.12 ± 0.01, \(n = 23\)) (Fig. 3, A and B). Furthermore, we calculated the apparent ligand affinity for cGMP and cAMP by fitting the Hill equation to the dose-response relationships for the activation of channels formed by wild-type or mutant subunits (Fig. 3C). The results demonstrated a significant increase in apparent affinity for both cAMP and cGMP compared with WT A3 channels: N471S: 0.57 ± 0.10 mM, \(K_{1/2,cAMP} = 5.25 \pm 0.75 \text{mM}, n_H = 1.89, K_{1/2,cGMP} = 3.04 \pm 0.46 \text{mM}, n_H = 1.49\). Therefore, we determined to fit the Hill equation to the dose-response relationship to the Hill equation. Compared with WT A3 channels, both N471S and R563H exhibited a statistically significant increase in apparent cGMP affinity, \(n = 13–19; *P < 0.01\) compared with WT. R563H also exhibited a statistically significant increase in apparent affinity for both cAMP, \(n = 7–12; *P < 0.01\) compared with WT.

![Fig. 3. Cone dystrophy-associated mutations altered the gating properties of homomeric channels.](http://ajpcell.physiology.org/)

**A** representative current traces are shown for homomeric (A3) cyclic nucleotide-gated (CNG) channels after activation by saturating concentrations of cGMP (1 mM) or cAMP (10 mM). Current traces were elicited using the voltage protocol described in MATERIALS AND METHODS. **B** bar graph showing relative agonist efficacy of cAMP compared with cGMP. \(n = 12–18; *P < 0.01\) compared with WT. **C** representative dose-response relationships for activation of WT (squares), N471S (triangles), or R563H (circles) homomeric A3 channels by cGMP (closed symbols) or cAMP (open symbols). Continuous curves (dashed line for wild type) represent fits of the dose-response relationship to the Hill equation as indicated in MATERIALS AND METHODS. Parameters for each patch shown were as follows: wild type, \(K_{1/2,cAMP} = 13.6 \mu M, n_H = 2.02, K_{1/2,cGMP} = 1.0 \text{mM}, n_H = 1.33\); N471S, \(K_{1/2,cAMP} = 5.72 \mu M, n_H = 2.05, K_{1/2,cGMP} = 1.10 \text{mM}, n_H = 1.31\); R563H, \(K_{1/2,cAMP} = 3.04 \mu M, n_H = 1.86, K_{1/2,cGMP} = 0.57 \text{mM}, n_H = 1.49\). **D** bar graphs showing the apparent ligand affinity \(K_{1/2}\) for cGMP (top) and cAMP (bottom) as determined from fits of the dose-response relationship to the Hill equation. Compared with WT A3 channels, both N471S and R563H also exhibited a statistically significant increase in apparent affinity for cAMP. \(n = 7–12; *P < 0.01\) compared with WT.
cGMP and cAMP with R563H ($K_{1/2,cGMP} = 3.28 \pm 0.63 \mu M; n_{H} = 1.7 \pm 0.2; K_{1/2,cAMP} = 0.64 \pm 0.09 \mu M; n_{H} = 1.1 \pm 0.2; n = 13; P < 0.01$) and an increase in the apparent affinity for tetracaine with N471S ($K_{1/2,\text{tetracaine}} = 5.76 \pm 1.66 \mu M; n_{H} = 2.0 \pm 0.3; n_{L} = 19; P < 0.01$) compared with activation of wild-type homomeric channels ($K_{1/2,cGMP} = 9.26 \pm 1.77 \mu M; n_{H} = 1.9 \pm 0.06; K_{1/2,cAMP} = 1.07 \pm 0.27 \mu M; n_{H} = 1.0 \pm 0.2; n = 18$) (Fig. 3, $D$ and $E$). The Hill coefficients showed no significant change compared with that of wild-type channels. These results suggest that the N471S and R563H mutations made the channel more sensitive to activation by cyclic nucleotide.

The observed increase in ligand sensitivity for mutant channels might result from an increase in the channel’s ability to bind ligand (affinity) or from an increased ability to couple ligand binding to the allosteric conformational change associated with channel opening (efficacy), or both. To quantify the altered gating properties of mutant homomeric channels, we applied the local anesthetic tetracaine, a known closed-state blocker of CNG channels (17, 18), in the presence of a saturating concentration of cGMP (fully ligand-bound state). Channels that are less sensitive to tetracaine block spend more time in the open state. Under these conditions, we calculated the equilibrium constant for the allosteric transition associated with channel opening ($L$) and for the initial binding of ligand ($K$) using a simple allosteric model (18, 23, 36) described in MATERIALS AND METHODS. Consistent with this model and similar to previous results for CNGA1, CNGA2, and A1/A2 chimeric channels (18), wild-type and mutant CNGA3 channels (particularly with mutations outside the cyclic nucleotide-binding domain) exhibited an inverse relationship between cGMP apparent affinity and tetracaine apparent affinity (data not shown).

Figure 4A shows block of wild-type and mutant CNG channels (in 1 mM cGMP) by 50 μM tetracaine. We calculated tetracaine apparent affinity ($K_{1/2,\text{tetracaine}}$) by fitting the dose-response relationships with a modified Hill equation as described in MATERIALS AND METHODS (Fig. 4B). Compared with wild-type homomeric channels ($K_{1/2,\text{tetracaine}} = 55.2 \pm 5.3 \mu M; n = 6$), N471S exhibited a statistically significant decrease in tetracaine apparent affinity ($K_{1/2,\text{tetracaine}} = 102.4 \pm 12 \mu M; n = 8; P < 0.05$), suggesting that this mutant subunit generated channels that spent more time in the open state compared with wild-type channels. R563H-containing channels also exhibited a decrease in tetracaine apparent affinity ($K_{1/2,\text{tetracaine}} = 76 \pm 9.6 \mu M; n = 5$), but this change was not statistically significant ($P = 0.078$).

Figure 5 summarizes the changes in free energy difference ($\Delta G = \Delta G_{\text{mutant}} - \Delta G_{\text{wt}}$), determined for the equilibrium constants $K$ and $L$, relative to those of the wild-type channels ($\Delta G_{L,cGMP} = -3.42 \pm 0.2$ kcal/mol, $\Delta G_{L,cAMP} = 1.09 \pm 0.4$ kcal/mol, $\Delta G_{K,cGMP} = -5.03 \pm 0.1$ kcal/mol, $\Delta G_{K,cAMP} = -4.50 \pm 0.2$ kcal/mol; $n = 6$). A significant decrease ($P < 0.05$ or $P < 0.01$) in the free energy difference of the allosteric transition promoted by cyclic nucleotides ($\Delta G_{L} < 0$) was observed for both N471S and R563H compared with wild-type channels (Fig. 5B). The effect of N471S on $L$ was largely independent of which ligand was bound (no significant difference between $\Delta G_{L}$ for cGMP and cAMP; $P = 0.911$), consistent with a change in the intrinsic gating properties of the channels (9). The free energy difference of initial ligand binding ($\Delta G_{L}$) was not significantly changed for N471S compared with wild type ($P = 0.76$ for cGMP and $P = 0.18$ for cAMP) (Fig. 5C), as expected for a mutation located outside the ligand-binding domain (see Ref. 9; but see Ref. 45). In contrast, R563H, which is located within the ligand-binding domain, exhibited a ligand-specific effect on the free energy difference for $L$ ($P < 0.01$, comparing $\Delta G_{L}$ for cGMP and cAMP): R563H significantly decreased $\Delta G_{L}$ for cAMP-bound channels, yet had no significant effect on cGMP-bound channels ($P = 0.073$ compared with wild-type) (Fig. 5B). R563H also exhibited a ligand-specific change in $\Delta G_{K}$ ($P < 0.01$ comparing $\Delta G_{K}$ for cGMP and cAMP): a significant decrease for cGMP ($P < 0.01$) and no significant effect for cAMP ($P = 0.76$) compared with wild type (Fig. 5C). Thus initial ligand binding of cGMP to the channels was enhanced by R563H.

**Fig. 4.** Cone dystrophy-associated mutations in homomeric channels altered sensitivity to tetracaine block. $A$: representative currents activated by 1 mM cGMP in the absence or presence of 50 μM tetracaine for A3 channels. $B$: representative dose-response relationships for tetracaine block of wild-type (squares), N471S (triangles), or R563H (circles) homomeric A3 channels. Currents were normalized to the maximum cGMP current. Continuous curves represent fits of the dose-response relationship to the modified Hill equation as indicated in MATERIALS AND METHODS. Parameters for each patch shown were as follows: $K_{1/2,cGMP} = 51.9 \mu M; n_{H} = 1.51; K_{1/2,cAMP} = 83.7 \mu M; n_{H} = 1.50; K_{1/2,\text{tetracaine}} = 60.4 \mu M; n_{H} = 1.56$. $C$: bar graph showing the apparent affinity ($K_{1/2}$) for tetracaine block determined from fits of the dose-response relationship to the modified Hill equation. Compared with wild-type A3 channels, N471S exhibited a statistically significant decrease in $K_{1/2}$ for tetracaine. $n = 5–11$.

*p < 0.05.
The decreased free energy difference for the initial ligand binding step and/or the allosteric transition is consistent with the view that both N471S and R563H mutations make channel opening more favorable.

Effect of cone dystrophy mutations on the gating properties of heteromeric channels. While the CNGA3 subunit itself can form functional homomeric channels, native CNG channels in cone photoreceptors are thought to be heteromeric proteins composed of CNGA3 and CNGB3 subunits. To investigate the possible functional consequences of these mutations in heteromeric channels, we coexpressed mutant CNGA3 subunits with CNGB3 subunits in oocytes. To confirm that mutant CNGA3 subunits can form functional heteromeric channels, we used the channel blocker l-cis-diltiazem. For recombinant CNG channels, sensitivity to block by l-cis-diltiazem depends on the presence of CNGB3 or CNGB1 subunits (6, 21, 34, 47). l-cis-diltiazem sensitivity was determined by comparing currents elicited by a saturating concentration of cGMP in the presence or absence of l-cis-diltiazem (\(I_{\text{diltiazem}}/I\)). Consistent with previous studies (47), currents for wild-type heteromeric channels were blocked by \(I_{\text{diltiazem}}/I = 0.43 \pm 0.06; n = 8\), while wild-type homomeric channels were insensitive \(I_{\text{diltiazem}}/I = 0.95 \pm 0.01; n = 6\) (Fig. 6). Both N471S- and R563H-containing heteromeric channels were sensitive to l-cis-diltiazem block (for N471S, \(I_{\text{diltiazem}}/I = 0.45 \pm 0.17; n = 7, P < 0.01\); for R563H, \(I_{\text{diltiazem}}/I = 0.46 \pm 0.1, n = 12, P < 0.01\)), indicating that these mutant CNGA3 subunits can form functional heteromeric channels when coexpressed with CNGB3 subunits. Similarly to findings in experiments performed with homomeric channels, R277C-containing CNGA3 subunits did not generate cyclic nucleotide-dependent currents after application of 1 mM cGMP when coexpressed with CNGB3, indicating that R277C subunits cannot form functional heteromeric channels (Fig. 1C).

Fig. 5. Cone dystrophy-associated mutations altered the free energy difference for channel activation. A: representative dose-response relationships for activation of homomeric wild-type (squares), N471S (triangles), or R563H (circles) channels by cGMP (closed symbols) and cAMP (open symbols). Currents were normalized to the maximum cGMP current. Continuous curves represent fits of the dose-response relationships with the simple allosteric model described in MATERIALS AND METHODS. B and C: bar graphs are shown for the changes in the free energy difference by N471S \((n = 8)\) and R563H \((n = 5)\) compared with that of WT channels, the allosteric transition \((L)\), and the initial ligand binding step \((K)\) for channel activation by cGMP or cAMP.
Electrophysiological measurements demonstrated that R563H containing heteromeric channels exhibited a significant increase in the relative agonist efficacy of cAMP ($I_{\text{max,cAMP}}/I_{\text{max,cGMP}} = 0.79 \pm 0.15$, $n = 12$; $P < 0.01$) compared with wild-type heteromeric channels ($I_{\text{max,cAMP}}/I_{\text{max,cGMP}} = 0.39 \pm 0.07$, $n = 8$) (Fig. 7, A and B). An increase in apparent affinity for cGMP was also observed for both mutant channel subunits (N471S: $K_{1/2,\text{cGMP}} = 12.6 \pm 1.5 \mu \text{M}$, $n = 7$, $P < 0.01$; R563H: $K_{1/2,\text{cGMP}} = 9.44 \pm 3.8 \mu \text{M}$, $n = 11$, $P < 0.001$) (Fig. 7, C and D) compared with wild-type channels ($K_{1/2,\text{cGMP}} = 15.4 \pm 1.5 \mu \text{M}$; $n = 9$). Heteromeric channels containing N471S did not show a significant change in relative agonist efficacy or in apparent affinity for cAMP compared with the wild-type channels, suggesting partial functional rescue when coassembled with CNGB3 subunits. These results indicate that the cone dystrophy-associated mutations N471S and R563H altered the gating properties of the heteromeric channels when coassembled with CNGB3 subunits. The altered gating properties of the heteromeric channels, which more closely resemble the native condition, provide further evidence supporting the pathogenicity of these mutations.

**DISCUSSION**

We have functionally characterized severe progressive cone dystrophy-associated mutations in the gene encoding the human CNGA3 subunit (R277C, N471S, and R563H) and have identified changes in the cell surface expression levels and gating properties of mutant channels. CNGA3 subunits with N471S and R563H mutations formed functional homomeric and heteromeric channels with altered gating properties, displaying increased apparent affinity for cyclic nucleotide and increased relative agonist efficacy for cAMP compared with cGMP. In contrast, R277C subunits did not form functional homomeric or heteromeric channels. Our studies are the first functional characterization of CNGA3 mutations linked to progressive cone dystrophy. Most (40 of 51) of the disease-associated mutations identified in CNGA3 to date are missense mutations at positions that are highly conserved among orthologous sequences (27, 32, 68). It seems that there is little tolerance for structural changes at these positions in the subunits.

Fig. 7. Cone dystrophy-associated mutations altered the gating properties of heteromeric channels. A: representative current traces for heteromeric A3+B3 channels activated by 1 mM cGMP or 10 mM cAMP. Traces were elicited using the voltage protocol described in MATERIALS AND METHODS. B: bar graph showing the relative agonist efficacy of cAMP compared with cGMP, $n = 7–12$; *$P < 0.01$ compared with WT channels. C: representative dose-response relationships for wild-type (squares), N471S (triangles), or R563H (circles) containing heteromeric channels activated by cGMP (closed symbols) or cAMP (open symbols). Currents were normalized to the maximum cGMP current. Continuous curves (dashed line for wild type) represent fits of the dose-response relationship to the Hill equation as indicated in MATERIALS AND METHODS. Vertical dotted line indicates $–2 \mu \text{M}$ physiological intracellular cGMP concentration in the dark (50). Parameters for each patch shown were as follows: wild type, $K_{1/2,\text{cGMP}} = 17.3 \mu \text{M}$, $n_H = 1.89$; $K_{1/2,\text{cAMP}} = 1.35 \mu \text{M}$, $n_H = 1.31$; for N471S, $K_{1/2,\text{cGMP}} = 12.7 \mu \text{M}$, $n_H = 1.77$; $K_{1/2,\text{cAMP}} = 1.07 \mu \text{M}$, $n_H = 1.18$; for R563H, $K_{1/2,\text{cGMP}} = 8.18 \mu \text{M}$, $n_H = 1.40$; $K_{1/2,\text{cAMP}} = 0.67 \mu \text{M}$, $n_H = 1.48$. D: bar graphs showing the apparent ligand affinity ($K_{1/2}$) for cGMP (top) and cAMP (bottom) determined from the fits of the dose-response relationship to the Hill equation. $n = 7–11$; *$P < 0.01$ compared with WT channels.
Loss-of-function phenotype. Previous studies indicate that a lack of functional CNG channels in the photoreceptor outer segment might contribute to cell degeneration. In this regard, animal models provide a useful tool for investigating the molecular mechanisms of photoreceptor degeneration. Cnga3-knockout mice exhibit a progressive loss of cone photoreceptor and cone photoreceptor degeneration with intact rod function (1). This animal model closely resembles human progressive cone dystrophy. Recent studies in Cnga3/Rho double-knockout mice demonstrate normal retinal morphology and photoreceptor response in neonatal mice, yet rod and cone photoreceptor degeneration is observed after 4 wk and progresses to almost complete loss of photoreceptors by 3 mo (8). In addition to Cnga3-deficient mice, constitutive closure of CNG channels in continuous light resembles a loss-of-function phenotype. Progressive photoreceptor degeneration is evident in this model as well (15). Functional studies of disease-associated mutations also provide evidence that the lack of functional CNG channels in the photoreceptor outer segment might contribute to photoreceptor degeneration. Five mutations in the gene encoding the rod channel CNGA1 subunit have been linked to autosomal recessive retinitis pigmentosa, a disease characterized by impaired rod function and rod degeneration (12). Three of the five mutations are null mutations that result in either the synthesis of nonfunctional channel proteins lacking the transmembrane domain and the pore-forming region (E76stop and K139stop) or no protein synthesis in the case of complete gene deletion (12). The other two mutations (S316F and frame shift R654, 1-bp del) are thought to encode functional channels with impaired targeting to the plasma membrane (12, 40, 61).

In the present study, R277C and R563H mutations significantly reduced the availability of functional CNG channels at the cell surface, resembling a loss-of-function phenotype. R277C, which is also a common mutation in patients with complete achromatopsia (68), did not form functional homomeric or heteromeric channels. This result can be accounted for primarily by a reduction in overall protein levels and in subunit maturation. Recently, several mutations in the S4 domain of bovine CNGA3 subunits also have been shown to impair subunit stability and/or processing (13). R563H subunits exhibited decreased plasma membrane localization as well, but without a significant reduction in CNGA3 protein levels or maturation. The functional consequences of these mutations in vitro suggest that high-level expression of working CNG channels in the outer segment is critical for photoreceptor survival.

There are various mechanisms by which disease-associated mutations can impair channel protein expression at the plasma membrane. Chief among these are mutations in the channel subunits that may disturb protein folding or assembly, destabilize synthesized proteins, and/or disrupt targeting to the plasma membrane. The precise cellular mechanisms involved in photoreceptor degeneration for loss-of-function phenotypes remain to be determined. It has been shown previously that the survival of central nervous system neurons, including retinal ganglion cells, depends on physiological levels of electrical activity (42). Does the decrease or absence of functional CNG channel expression at the plasma membrane lead to a lack of proper levels of electrical activity, thus resulting in photoreceptor degeneration? Further work needs to be done to address these questions.

Gain-of-function phenotype. Functional characterization of cone CNG channels containing N471S or R563H mutations in the CNGA3 subunits revealed an increase in apparent ligand affinity and in relative agonist efficacy of cAMP compared with cGMP, consistent with a gain-of-function phenotype. A similar gain-of-function phenotype has been found for an achromatopsia-associated mutation in CNGB3 subunits (S435F) that alters the gating properties of heteromeric channels when coexpressed with CNGA3 subunits (48). CNGB3 S435F-containing heteromeric channels exhibit a more than fourfold increase in cAMP sensitivity and a modest increase in apparent affinity for cGMP. In addition, single-channel recordings reveal an increase in open probability in the presence of saturating concentration of cGMP or cAMP for mutant heteromeric channels (48). In the present study, the increased ligand sensitivity and efficacy suggest that in cone photoreceptors of patients with progressive cone dystrophy, CNG channels may fail to close appropriately as intracellular cGMP (or cAMP) levels fall in response to light stimulation.

What is the likely relationship between the altered gating properties of the mutant channels in the native outer segment membrane and cone photoreceptor degeneration? Under physiological conditions, the free intracellular cGMP level is estimated to be \( \sim 2-4 \mu M \) (50, 51), which is well below the concentration of cGMP expected to elicit half-maximal activation of the channels \( (K_{1/2}) \). This cGMP concentration is sufficient to keep only a small fraction \((<10\%)\) of the channels in the open state in the absence of light (51). A light-induced rapid decrease \((10\) to \(20\) fold) of intracellular cGMP thus results in closure of nearly all of the channels (51). Because the sensitivity of channels to cGMP is so steep, even slight alterations in apparent ligand affinity may be detrimental to normal physiological function. For example, if the apparent affinity of the channels for cGMP changes \([e.g., the K_{1/2} decreases from \sim 15 \mu M (wild-type channel) to \sim 9 \mu M (mutant channel)]\) (see Fig. 7C), nearly 10-fold the number of channels may be open in the absence of light and/or channels may not close properly in response to the fall in cGMP levels after light activation. Similar gain-of-function phenotypes have been discovered for mutations in the genes encoding other critical proteins involved in phototransduction, adaptation, and recovery processes. Recent evidence indicates that mutations which produce constitutively active guanylyl cyclase \((30, 46, 62)\), or loss of rod cGMP PDE activity \((10, 26, 63)\) result in increased intracellular cGMP levels. Increased intracellular cGMP, similarly to an increase in channel sensitivity to cGMP, might lead to inappropriate opening of the channels. Thus more Ca\(^2+\) could enter the photoreceptors through the CNG channels.

We hypothesize that Ca\(^2+\) overload might explain photoreceptor degeneration with gain-of-function mutations in cone CNG channels. One important aspect of CNG channel function is that it is the major pathway for Ca\(^2+\) entry into the outer segment of photoreceptors (16). It is possible that sustained opening of CNG channels, resulting from increased ligand affinity, may lead to abnormally high levels of intracellular Ca\(^2+\). Ca\(^2+\) is a critical second messenger that participates in several intracellular signaling pathways. Investigators in numerous studies have reported that a sustained elevation of intracellular Ca\(^2+\) could result in apoptotic cell death (for
review, see Refs. 7 and 43). In the retina, for example, sustained elevation of intracellular Ca\textsuperscript{2+} has been shown to trigger rod photoreceptor apoptosis and retinal degeneration (19). This general mechanism may underlie other photoreceptor degenerative diseases such as progressive cone dystrophy. At the same time, it has been suggested recently that apoptosis may be triggered by a sustained decrease in intracellular Ca\textsuperscript{2+} levels in rod photoreceptors (for review, see Ref. 35) as expected for rod channel mutations presenting loss-of-function phenotypes. Consistent with this hypothesis, Rpe65-knockout mice, which exhibit impaired synthesis of the opsin chromophore ligand 11-cis-retinal, display light-independent signaling by unliganded opsin, diminished intracellular Ca\textsuperscript{2+} in photoreceptors, and progressive photoreceptor degeneration (69). Furthermore, mutations in RPE65 have been linked to Leber congenital amaurosis (LCA), a severe, early-onset retinal dystrophy (41).

For the cone dystrophy-associated mutations characterized in the present study, both loss-of-function and gain-of-function effects were observed. For example, R563H produced both a reduction in channel cell surface expression levels and an increase in ligand sensitivity. The profound decrease in functional expression for R563H, however, suggests that loss-of-function might play a greater part than the change in channel gating in the pathogenicity of the disease in these patients.

Assembly of N471S-containing CNGA3 subunits with wild-type CNGB3 subunits rescued most functional properties associated with wild-type heteromeric channels, with the exception of increased apparent cGMP affinity. The discrepancy between the mild functional change for N471S-containing heteromeric channels and the severe cone degeneration typically exhibited by patients with this disease implies that an additional, as yet unidentified mutation is necessary for disease progression (68). This additional mutation may be present in a noncoding region of CNGA3, in CNGB3, or in some other allele or modifying gene. Thus further mutation screening is needed to address this possible phenotype-genotype inconsistency.

Overall, our results suggest that complex effects may arise from the progressive cone dystrophy-associated mutations in CNGA3 subunits, including a decrease in plasma membrane localization of the channels; a disruption of channel protein biogenesis, processing, and/or stability; and an increase in ligand sensitivity and/or efficacy. These results provide insight into the molecular pathophysiology and possible cellular mechanisms underlying cone photoreceptor degenerative disease. How these mutations affect cone photoreceptor function and survival in vivo remains an important unanswered question that needs to be addressed using animal models.

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