Achromatopsia-associated Mutation in the Human Cone Photoreceptor Cyclic Nucleotide-gated Channel CNGB3 Subunit Alters the Ligand Sensitivity and Pore Properties of Heteromeric Channels*

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Changhong Peng, Elizabeth D. Rich, and Michael D. Varnum‡
From the Department of Veterinary and Comparative Anatomy, Pharmacology, and Physiology and Program in Neuroscience, Washington State University, Pullman, Washington 99164-6520

Cone photoreceptor cyclic nucleotide-gated (CNG) channels are thought to form by assembly of two different subunit types, CNGB3 and CNGB3. Recently, mutations in the gene encoding the CNGB3 subunit have been linked to achromatopsia in humans. Here we describe the functional consequences of two achromatopsia-associated mutations in human CNGB3 (hCNGB3). Co-expression in Xenopus oocytes of human CNGB3 (hCNGB3) subunits with hCNGB3 subunits containing an achromatopsia-associated mutation in the S6 transmembrane domain (S435F) generated functional heteromeric channels that exhibited an increase in apparent affinity for both cAMP and cGMP compared with wild type heteromeric channels. In contrast, co-expression of a presumptive null mutation of hCNGB3 (T383fsX16) with hCNGB3 produced channels with properties indistinguishable from homomeric hCNGB3 channels. The effect of hCNGB3 S435F subunits on cell-surface expression of green fluorescent protein-tagged hCNGB3 subunits and of non-tagged hCNGB3 subunits on surface expression of green fluorescent protein-hCNGB3 S435F subunits were similar to those observed for wild type hCNGB3 subunits, suggesting that the mutation does not grossly disturb subunit assembly or plasma membrane targeting. The S435F mutation was also found to produce changes in the pore properties of the channel, including decreased single channel conductance and decreased sensitivity to block by L-cis-diltiazem. Overall, these results suggest that the functional properties of cone CNG channels may be altered in patients with the S435F mutation, providing evidence supporting the pathogenicity of this mutation in humans. Thus, achromatopsia may arise from a disturbance of cone CNG channel gating and permeation or from the absence of functional CNGB3 subunits.

Cyclic nucleotide-gated (CNG) ion channels are fundamental to sensory transduction in retinal photoreceptor cells and in olfactory receptor neurons. CNG channels couple a change in intracellular cyclic nucleotide concentration in these cells into an electrical response underlying sensory signaling. CNG channels are part of a superfamily of ion channels whose members include voltage-gated potassium channels and channels gated by intracellular ligands (1, 2). There are six genes encoding CNG channel subunits in mammals: CNGA1, CNGA2, CNGB3, CNGB4, CNGB1, and CNGB3. Native CNG channels are thought to be tetrameric proteins formed by at least two of these subunit types. Each of these subunits exhibits six putative transmembrane domains, intracellular amino and carboxyl termini, a conserved pore domain, and a cyclic nucleotide binding domain. In cone photoreceptor cells, CNG channels are composed of CNGB3 (α) and CNGB3 (β) subunits. Similar to their rod photoreceptor counterparts, heterologous expression of CNGB3 subunits alone can generate functional homomeric channels (3–5), but CNGB3 subunits cannot (6, 7). Compared with the proteins forming rod and olfactory CNG channels, the protein subunits forming cone photoreceptor CNG channels are not as well characterized: only recently has the molecular identity of the cone CNG channel β subunit been revealed (6, 8, 9).

Mutations in the CNGA3 and CNGB3 genes encoding human cone photoreceptor CNG channel subunits have been found to segregate with patients having complete and incomplete achromatopsia (8–12). Achromatopsia is an autosomal recessive disease characterized by absent or limited cone function (but intact rod function), compromised visual acuity, nystagmus, and photophobia. For CNGA3-deficient mice, an animal model of complete achromatopsia, loss of cone function correlates with progressive degeneration of cone photoreceptors (13). Complete achromatopsia in humans, however, is thought to be a stationary disorder without obvious cone degeneration (but see also Ref. 14). One disease-associated mutation in humans (S435F), located in the putative S6 transmembrane domain of CNGB3, has been linked to the unusually frequent occurrence of complete achromatopsia among Pingelapese islanders (8, 9), a population described by Oliver Sachs in The Island of the Color-blind (15). The mechanisms underlying achromatopsia remain poorly defined. An important step toward understanding the development of the disease is determining how individual mutations in the genes that encode cone photoreceptor CNG channel subunits may alter the functional properties of these critical proteins.

Here we report the functional consequences of two mutations in hCNGB3 associated with achromatopsia in humans. Our results demonstrate that co-expression of hCNGB3 with hCNGB3 subunits containing an achromatopsia-associated frameshift mutation that truncates the pore and the carboxyl-
Effects of Achromatopsia Mutations in Human CNGB3

**EXPERIMENTAL PROCEDURES**

**Molecular Biology**—The human retinal cone CNG channel β subunit clone, hCNGB3, was isolated from human retinal cDNA as previously described (7). The coding sequence of this clone differs from the complete published sequence for CNGB3 of Kohl et al. (9) (AP272900) at two positions (nucleotide 1789g→a and nucleotide 1834a→g), representing either sequence polymorphisms or Tαq polymerase errors; neither nucleotide change alters the amino acid sequence. It was subcloned into pGEME (16) for heterologous expression in *Xenopus* oocytes. Human CNGA3 (4), a generous gift of Dr. K.-W. Yao, was also subcloned into pGEME. To generate amino-terminal fusions of enhanced green fluorescent protein (GFP) with CNGB3 and CNGA3, PCR-amplified GFP (Qigene, Carlsbad, CA) including an additional linker sequence (LAG, RARLPA) was subcloned in-frame with cDNA encoding CNGCAG3 and hCNGB3 at amino acids Asp-24 and Gly-107, respectively. The essential properties of hCNGA3 and hCNGB3 plus hCNGB3 channels were unaltered by the GFP fusion (data not shown). Although the GFP tag may still have a slight influence on expression level or plasma membrane targeting, we estimate that the effect of the tag on expression level (patch current density) is small. Mutations in the hCNGB3 coding sequence were engineered using overlapping PCR mutagenesis (17). All mutations and the fidelity of PCR-amplified cassettes were confirmed by automated DNA sequencing. For expression studies, identical amounts of cDNA were linearized using *SalI* 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 by denaturing gel electrophoresis and one-dimensional image analysis software (Kodak, NY) and spectrophotometry.

**Electrophysiology**—For functional expression studies, *Xenopus laevis* oocytes were isolated and microinjected with 5–10 ng of mRNA as previously described (18). The ratio of wild type or mutant hCNGB3 mRNA to hCNGA3 mRNA was typically 5:1. Two to 7 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 (HEKA Elektronik, Lambrecht, Germany). Initial pipette resistances were 0.4 MΩ. In addition, anhydrous EDTA was substituted for MgCl2 in the bath solution to reduce the background current. Currents in the absence of cyclic nucleotide were subtracted. Data were acquired using Igor (Wavemetrics, Lake Oswego, OR), SigmaPlot, and SigmaStat (SPSS Inc.). All values are reported as the mean ± S.E. of *n* experiments unless otherwise indicated. Statistical significance was determined using a Student’s *t* test or Mann-Whitney rank sum test, and a *p* value of <0.05 was considered significant.

**Confocal Microscopy**—Confocal images were collected using a ×10 objective on a Nikon Eclipse TE 300 inverted microscope equipped with a Bio-Rad MRC-1024 confocal laser-scanning system and a krypton-argon laser. Oocytes expressing GFP-tagged CNG channel subunits 64–72 h after injection of mRNA were placed in boriculate coverglass chambers such that the equator was approximately perpendicular to the plane of imaging. GFP fluorescence was measured using an excitation wavelength of 488 nm and a 522 DF 32 emission filter. Laser intensity, pinhole aperture, and photo-multiplier gain were kept constant for all experiments. Oocytes injected with mRNA for non-tagged subunits were included as controls for auto-fluorescence. Images were analyzed using NIH ImageJ software. Surface fluorescence was determined using a defined area positioned at the animal pole of the oocyte and expressed as intensity of signal per unit area; surface fluorescence was then normalized to the mean surface fluorescence of GFP-hCNGA3-expressing oocytes within the same experiment.

**Biochemistry**—To assess the overall abundance of cone CNG channel subunits expressed in *Xenopus* oocytes, we used Western blot analysis of proteins from oocytes expressing GFP-tagged hCNGA3 or hCNGB3. Oocyte proteins were prepared using a protocol adapted from Rosenbaum and Gordon (22) and others (23). Briefly, oocytes were placed in buffer containing 20 mM Heps (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100 (Surface-Amphi-X-100; Pierce) and a protease inhibitor mixture (Roche Applied Science). Oocytes were subjected to trituration followed by cup sonication using a Branson Sonifier (500 W; Branson Ultrasonics, Danbury, CT) repeated a total of three times. The soluble cell lysate was then separated from yolk and other insoluble material by centrifugation at 20,000 × *g* and 4 °C for 10 min and repeated three times. Material representing approximately two oocytes was loaded per lane and separated by SDS-PAGE using NuPage 3–8% Tris acetate gels (Invitrogen). Proteins were then transferred to nitrocellulose using the NuPage transfer buffer system (Invitrogen). Immunoblots were probed with anti-GFP *Aequorea victoria* peptide polyclonal antibody (Clontech, Palo Alto, CA) at a concentration of 1:250 in Tris-buffered saline with 1% nonfat dry milk. GFP-tagged channel subunits were visualized using SuperSignal West Dura substrate (Pierce) and autoradiography was performed using Kodak X-OMAT AR film (Eastman Kodak Co.). The approximate molecular weights of the GFP-tagged subunits were calculated by interpolation using the linear relationship between the log of molecular weight for protein standards (Invitrogen), as reported by the manufacturer for these buffer conditions, and the migration distance of the proteins.

**RESULTS**

**Functional Expression of Mutant Human CNGB3 Subunits**—To investigate the functional consequences of two achromatopsia-associated mutations in the human cone photoreceptor CNG channel β subunit gene (CNGB3) (8, 9), we introduced these mutations into cDNA encoding the human CNGB3 subunit and co-expressed mutant or wild type CNGB3 with human cone channel α subunits (hCNGA3) (4) in *Xenopus* oocytes. Fig. 1A illustrates the approximate positions in hCNGB3 of the two mutations examined in this study, 1) S435F, located within the S6 transmembrane domain, and 2) T383fs.A3C, a 1-base pair deletion at nucleotide 1148 generating a frameshift and premature stop codon at Leu-395 with truncation of the carboxyl terminus of the CNGB3 subunit (8, 9). Similar to other CNG channel β subunits (6, 24–26), human CNGB3 failed to generate functional cyclic nucleotide-activated channels when expressed alone in oocytes (data not shown). As previously demonstrated (7), co-expression of wild type human CNGB3 subunits with human CNGA3 subunits...
Fig. 1. Functional characterization of recombinant cone CNG channels containing achromatopsia-associated mutations in hCNGB3. A, schematic of human CNGB3 subunit topology with the approximate locations of two mutations (* linked to achromatopsia in humans, T383fs.C and S435F. B, current families recorded from inside-out patches excised from Xenopus oocytes expressing homomeric hCNGA3 (hA3) channels or heteromeric channels generated by co-injection of mRNA encoding wild type or mutant hCNGB3 (hB3) with hCNGA3 subunits. Current sweeps were elicited at a saturating concentration of cGMP (1 mM) or cAMP (10 mM) by voltage steps from 0 mV to potentials between −100 mV and 100 mV, in 20-mV increments. Currents in the absence of cyclic nucleotide were subtracted. C, representative dose-response relationships for channel activation by cGMP for homomeric hCNGA3 channels (filled circles), wild type heteromeric hCNGA3 + hCNGB3 channels (open circles), heteromeric channels containing hCNGB3-S435F subunits (filled squares), and channels formed after co-expression of hCNGB3-T383fs.C with hCNGB3 subunits (open squares). Currents were measured after voltage steps to +80 mV and were normalized to the maximum current elicited by a saturating concentration of cGMP. Continuous curves represent fits of the dose-response relation with the Hill equation, \( I/I_{\text{max}} = (c^{nH}/K_{1/2}^{nH}) \). For these fits, the following parameters were used. For homomeric hCNGA3 channels, \( K_{1/2} = 11 \mu M, n = 2.1 \); for wild type hCNGA3 + hCNGB3 channels, \( K_{1/2} = 18 \mu M, n = 2.0 \); for hCNGA3 S435F + hCNGB3 channels, \( K_{1/2} = 7.6 \mu M, n = 1.1 \); for hCNGA3-T383fs.C + hCNGB3, \( K_{1/2} = 10 \mu M, n = 2.0 \). D, representative dose-response relationships for activation of homomeric and heteromeric channels by cAMP; symbols are the same as in C. Currents were normalized to the maximum current elicited by a saturating concentration of cGMP. For these fits, the following parameters were used. For homomeric hCNGA3 channels, \( K_{1/2} = 1.20 \mu M, n = 1.4, I_{\text{max cAMP}}/I_{\text{max cGMP}} = 0.16 \); for wild type hCNGA3 + hCNGB3 channels, \( K_{1/2} = 954 \mu M, n = 1.5, I_{\text{max cAMP}}/I_{\text{max cGMP}} = 0.35 \); for hCNGA3 S435F + hCNGB3 channels, \( K_{1/2} = 170 \mu M, n = 1.3, I_{\text{max cAMP}}/I_{\text{max cGMP}} = 0.45 \); for hCNGA3-T383fs.C + hCNGB3, \( K_{1/2} = 1214 \mu M, n = 1.3, I_{\text{max cAMP}}/I_{\text{max cGMP}} = 0.20 \).
tively unaffected by the presence of hCNGB3-T383fs.ΔC subunits; under the same experimental conditions, patch current density was $591 \pm 137 \mu A/\mu m^2$ ($n = 9$) for hCNGA3 plus hCNGB3 T383fs.ΔC and $589 \pm 156 \mu A/\mu m^2$ ($n = 18$) for hCNGA3 alone. Thus, not only did the basic properties of CNG channels generated by co-injection of mRNA encoding hCNGA3 and hCNGB3-T383fs.ΔC subunits resemble homomeric hCNGA3 channels, but also no apparent dominant-negative effect of the truncated hCNGB3 subunit was evident. These results are consistent with the idea that complete functional absence of the modulatory CNGB3 subunit can be critical for the development of achromatopsia and that homomeric hCNGA3 channels are not sufficient for normal cone photoreceptor function.

**Plasma Membrane Targeting of Recombinant Cone CNG Channel Subunits**—The pathological basis of many disease-associated mutations in the genes that encode ion channels has been attributed to impaired folding, assembly, and/or plasma membrane targeting of channel subunits or complexes. In addition, photoreceptor dysfunction and degeneration have been associated with mutations that disrupt the processing and trafficking of proteins normally targeted to the photoreceptor outer segment (for review, see Ref. 27). Furthermore, a mutation in CNGA1 linked to retinitis pigmentosa has been shown to dramatically alter the plasma membrane targeting of recombinant rod CNG channel subunits (28–30). Thus, we were interested in determining whether achromatopsia-associated mutations in hCNGB3 impaired the assembly and cell-surface localization of recombinant cone CNG channels.

To address this question we engineered gene fusions of GFP with the amino-terminal regions of the respective human cone CNG channel subunits, generating GFP-hCNGA3 and GFP-hCNGB3. GFP-tagged subunits provide a convenient means to assess cell-surface localization of CNG channel subunits in oocytes using confocal microscopy. The functional properties of GFP-tagged subunits in the context of both homomeric (for hCNGA3) and heteromeric channels were indistinguishable from those of non-tagged subunits (data not shown). We first investigated the effect of co-expression of wild type or mutant hCNGB3 subunits on the surface expression of GFP-tagged hCNGA3 subunits (Fig. 2, A and B). GFP-hCNGA3 mRNA was injected into oocytes either alone or in combination with mRNAs encoding non-tagged hCNGB3 subunits. For these experiments, the amount of GFP-hCNGA3 mRNA injected was held constant, and the ratio of GFP-hCNGB3 to non-tagged hCNGB3 or control mRNA was fixed at 3:1, a ratio fully effective for the formation of heteromeric channels (data not shown). Compared with the expression of hCNGA3 subunits alone, co-expression of wild-type hCNGB3 subunits attenuated plasma membrane localization of the GFP-tagged hCNGA3 subunits (Fig. 2, A and B). hCNGB3-S435F subunits similarly attenuated surface fluorescence of GFP-hCNGA3 subunits, indicating that these mutant β subunits are competent for assembly with α subunits. In contrast, co-expression of hCNGB3 T383fs.ΔC did not significantly alter GFP-hCNGA3 surface fluorescence, suggesting that the carboxyl-terminal region of hCNGB3 may be important for assembly with hCNGA3 subunits or for trafficking control of cone CNG channels. Co-injection of either of an irrelevant mRNA species transcribed from a lacZ gene construct in the same vector or of mRNA for non-tagged hCNGB3 had no significant effect on surface fluorescence of GFP-hCNGA3 (Fig. 2B). These controls confirm that attenuation of GFP-hCNGA3 surface expression by hCNGB3 did not arise from a nonspecific effect, such as overwhelming the oocyte protein synthesis machinery.

Reduced surface fluorescence of GFP-hCNGA3 subunits may result from intracellular retention or recycling of channel complexes or from reduced hCNGA3 protein levels. To help discriminate between these possibilities, we used immunoblots of total solubilized oocyte protein probed with anti-GFP antibody to qualitatively assess the relative levels of GFP-hCNGA3 protein in the absence or presence of hCNGB3 subunits. Western blotting suggested that GFP-hCNGA3 protein levels were not substantially altered by co-expression of wild type or mutant hCNGB3 subunits ($n = 3$; Fig. 2C). Together, these results imply that CNGB3 subunits may provide a brake, regulating
trafficking of heteromeric cone CNG channels.

Next we examined the cell-surface localization of wild type and mutant GFP-hCNGB3 subunits in the absence or presence of non-tagged hCNGB3 subunits. For these experiments, the amount of wild type or mutant GFP-hCNGB3 mRNA injected was held constant, and the ratio of GFP-hCNGB3 mRNA to non-tagged hCNGB3 mRNA was 5:1. Only low level surface fluorescence for GFP-hCNGB3 was detected when these subunits were expressed alone (Fig. 2, D and E). This result implies that hCNGB3 subunits are only marginally competent to traffic to the plasma membrane of oocytes in the absence of hCNGB3 subunits, perhaps contributing to their failure to form functional channels when expressed alone. Co-expression of hCNGB3 subunits promoted surface expression of GFP-hCNGB3 subunits. GFP-tagged hCNGB3 S435F subunits also exhibited little surface fluorescence when expressed alone, and surface expression of S435F subunits was similarly enhanced by hCNGB3 (Fig. 2, D and E). Surface fluorescence of GFP-hCNGB3 S435F subunits was reduced compared with wild type GFP-hCNGB3 subunits under identical conditions both in the absence and presence of hCNGB3 subunits, paralleling the reduced level of functional expression observed for S435F macroscopic currents. Furthermore, immunoblot of total oocyte protein probed with anti-GFP antibody suggested that the relative amounts of GFP-tagged hCNGB3 and hCNGB3-S435F protein were qualitatively similar and that protein levels were obviously changed by co-expression of hCNGB3 (n = 3; Fig. 2F). In contrast to hCNGB3 wild type and S435F subunits, GFP-tagged hCNGB3 T383F and ΔC subunits exhibited greater surface fluorescence when expressed alone, and surface fluorescence was not significantly enhanced by hCNGB3 subunits. Overall, results shown in Fig. 2 indicate that (a) hCNGB3 subunits promote or stabilize plasma membrane localization of hCNGB3 subunits, (b) the S435F mutation in hCNGB3 does not impede assembly with hCNGB3 subunits but may instead hinder trafficking of subunits to the plasma membrane, and (c) the carboxyl-terminal region of hCNGB3 may participate in channel assembly and/or may be important for subunit trafficking.

Pore Properties of Wild Type and Mutant Heteromeric Cone CNG Channels—Sequence homology between hCNGB3 and related potassium-selective channels such as KcsA (31) and MthK (32) (Fig. 3A) suggests that Ser-435 may contribute to the pore of cone CNG channels. Thus, we were interested in determining if the S435F mutation in hCNGB3 influences the pore properties of recombinant cone CNG channels. First we examined the monovalent cation selectivity of homomeric and heteromeric cone CNG channels by measuring the reversal potential under symmetrical bi-ionic conditions, substituting intracellular Na⁺ with K⁺, Li⁺, Rb⁺, or Cs⁺ (Fig. 3, B–D). The relative monovalent cation permeabilities for recombinant cone CNG channels, determined using the Goldman-Hodgkin-Katz equation, are shown in Table I. For homomeric hCNGB3 channels, Na⁺ ≥ Na⁺ ≥ Rb⁺ ≥ Li⁺ ≥ Cs⁺; for heteromeric hCNGA3 + hCNGB3 channels, Rb⁺ ≥ K⁺ ≥ Na⁺ ≥ Li⁺ ≥ Cs⁺; for hCNGB3 S435F + hCNGB3 channels, Na⁺ ≥ K⁺ ≥ Rb⁺ ≥ Li⁺ ≥ Cs⁺. The relative conductance sequences for outward currents measured at +80 mV were as follows: for homomeric hCNGB3 channels, Na⁺ > K⁺ > Li⁺ > Rb⁺ > Cs⁺; for heteromeric hCNGB3 + hCNGB3 channels, Na⁺ > K⁺ > Li⁺ > Rb⁺ > Cs⁺; for hCNGB3 S435F + hCNGB3 channels, Na⁺ > K⁺ > Li⁺ > Rb⁺ > Cs⁺. Overall, channels formed with hCNGB3-S435F subunits exhibited subtle differences in relative monovalent ion selectivity and conductance compared with both wild type heteromeric channels and homomeric hCNGB3 channels. Small changes in ion selectivity and conductance might arise from a local structural perturbation of the pore due to the S435F mutation. Minimally, these results are consistent with the participation of mutant hCNGB3 subunits in the formation of the cone CNG channel ion conduction pathway.

We also investigated the effect of the S435F mutation on the single channel properties of recombinant cone CNG channels. The single channel current amplitude at +80 mV for wild type hCNGB3 plus hCNGB3 heteromeric channels was 3.26 ± 0.08 pA (n = 4 patches) (Fig. 4A). The corresponding single channel conductance (~41 picosiemens) was similar to that reported previously for mouse CNGB3 plus CNGA3 channels (6). For S435F-containing heteromeric channels, the single channel current amplitude at +80 mV was reduced to 2.55 ± 0.19 pA (n = 4 patches) (Fig. 4B), representing a single channel conductance of ~32 picosiemens. Furthermore, for activation of heteromeric channels by a saturating concentration of ligand, the maximum open probability (P_max) increased from 0.76 ± 0.02 in 1 mM cGMP and 0.31 ± 0.02 in 10 mM cAMP for wild-type heteromeric channels (Fig. 4A) to 0.85 ± 0.03 and 0.39 ± 0.04, respectively, for channels containing S435F subunits (Fig. 4B). Thus, the absolute agonist efficacy of cAMP and of cGMP increased for S435F-containing heteromeric channels, but the agonist efficacy of cAMP relative to cGMP (P_max cAMP/P_max cGMP) remained similar to that of wild type heteromeric channels (~0.4) and was in reasonable agreement with the...
relative agonist efficacy observed with macroscopic current recordings. These results indicate that the achromatopsia-associated S435F mutation decreased the single channel conductance and increased the open probability (at saturating ligand concentrations) of recombinant heteromeric cone CNG channels.

Next we examined the sensitivity of wild type and mutant cone CNG channels to block by L-cis-diltiazem. L-cis-Diltiazem, applied to the cytoplasmic face of the membrane, blocks native photoreceptor CNG channels in a voltage-dependent manner, consistent with this agent binding within the membrane electric field (33–35). For recombinant CNG channels, sensitivity to L-cis-diltiazem depends on the presence of CNGB1 or CNGB3 subunits (6, 7, 24, 36). Like homomeric hCNGA3 channels (4, 7), channels formed after co-injection of mRNA encoding hCNGB3 T383f.s. and hCNGA3 were insensitive to L-cis-diltiazem (Fig. 5, A and B). Surprisingly, heteromeric channels generated by co-expression of hCNGB3-S435F and hCNGA3 subunits were less sensitive to L-cis-diltiazem block than channels containing wild type hCNGB3 subunits (7) but more sensitive than hCNGA3 homomer channels (Fig. 5), suggesting that the mutation may direct or indirectly alter the binding site for the drug. In addition, S435F-containing channels were found to be more sensitive to block by 25 μM L-cis-diltiazem in low [cGMP] than in the presence of a saturating concentration of cGMP (Fig. 5C). Consistent with this observation, S435F-containing heteromeric channels were also more sensitive to L-cis-diltiazem in the presence of a saturating concentration of the partial agonist cAMP; in 10 nM cAMP, I_{cAMP}/I was 0.42 ± 0.11 (n = 4) (data not shown). Homomeric hCN3 channels were also somewhat more sensitive to L-cis-diltiazem in low [cGMP] (Fig. 5C). Wild type heteromeric channels were blocked equally well in low or high [cGMP]. These results provide evidence indicating that a block by L-cis-diltiazem of cone CNG channels can exhibit closed-state dependence, as is the case in the tetracaine block of homomeric CNG channels (37), and that the altered gating properties of S435F-containing heteromeric channels may contribute to decreased sensitivity to L-cis-diltiazem.

**DISCUSSION**

We have characterized achromatopsia-associated S435F and T383f.s.ΔC mutations in human CNGB3 using heterologous expression in *Xenopus* oocytes and have identified changes in both ligand sensitivity and pore properties for S435F-containing channels. CNGB3-S435F subunits formed functional heteromeric channels with altered properties when co-expressed with hCNGA3 subunits, whereas CNGB3-T383f.s.ΔC subunits did not participate in channel formation. One of the most dramatic changes observed with S435F-containing channels was a greater than 4-fold increase in cAMP sensitivity. S435F-containing channels also exhibited a modest increase in apparent affinity for cGMP. In addition, single channel recordings revealed an increase in open probability for both cGMP- and cAMP-bound mutant heteromeric channels. These results indicate that the gating properties of heteromeric channels are altered by the S435F mutation.

Most members of the superfamily of ion channels that includes potassium-selective channels and CNG channels present small volume amino acid side chains (Ala, Ser, or Gly) at positions aligning with Ser-435 in hCNGB3 (Fig. 3A). The Van der Waals volumes (in Å³) of the amino acids alanine, serine, and glycine are 67, 73, and 48, respectively (38). For the recently described crystal structure of the open MthK potassium channel, the residue in MthK (Ala-88, Fig. 3) that aligns with Ser-435 in hCNGB3 forms the narrowest part of the MthK intracellular “entryway” (32). Jiang et al. (32) argue that a small side chain at this position seems important to prevent interference with ion conduction. If Ser-435 is similarly positioned in open cone CNG channels, then the reduced single channel conductance (Fig. 4) and altered sensitivity to block by L-cis-diltiazem (Fig. 5) that we have observed for mutant heteromeric channels may arise at least in part from the increased size of the substituted amino acid (135 Å³ for phenylalanine) in the hCNGB3 subunit. Furthermore, the inner helix (S6) bundle of CNG channels is thought to undergo a conformational change during channel activation (39, 40) that may be similar to the outward displacement proposed for K⁺ channels (32, 41, 42). We hypothesize that S435F may effectively destabilize the closed state of the channel if the bulky aromatic side chain is more difficult to accommodate in the closed conformation, perhaps because the smaller side chain normally found at this position in CNG channels is packed or buried when channels close (see Ref 43). This would account for the increase in ligand sensitivity seen with S435F-containing heteromeric channels.

The increase in ligand sensitivity reported here for recombinant cone CNG channels containing the S435F mutation in the CNGB3 subunit would be consistent with a phenotype of enhanced channel activity. This suggests that in cone photoreceptors of patients with this mutation, CNG channels may fail to close appropriately as intracellular concentrations of cGMP (or cAMP) fall in response to light stimulation or other, slower adaptive processes. Elevated photoreceptor cyclic nucleotide levels have been previously associated with other congenital retinal disorders (44). In addition, native cone CNG channels and recombinant homomeric CNGA3 channels display remarkably high calcium permeability (45–49). Possible cellular mechanisms for impaired cone photoreceptor function in achromatopsia may depend on altered calcium homeostasis. Because calcium is a universally critical intracellular signaling molecule and plays a particularly important role for recovery and adaptation in cone photoreceptors (50, 51), even subtle changes in CNG channel function may have profound cellular consequences. Also, future studies should address the question of whether the S435F mutation in hCNGB3 may alter the calcium permeability of heteromeric CNG channels.

| Ion   | hCNGA3 | hCNGB3 + hCNGA3 | hCNGB3 S435F + hCNGA3 |
|-------|--------|----------------|----------------------|
|       | \(P_f/P_{Na}\) | \(G_{Na}/G_{Na}\) | \(P_f/P_{Na}\) | \(G_{Na}/G_{Na}\) | \(P_f/P_{Na}\) | \(G_{Na}/G_{Na}\) |
| Na⁺  | 1.0    | 1.0            | 1.0                  | 1.0                  | 1.0                  |
| K⁺   | 1.04 ± 0.04 | 0.66 ± 0.14 | 1.06 ± 0.09 | 0.97 ± 0.06 | 0.97 ± 0.06 | 0.68 ± 0.07 |
| Li⁺  | 0.56 ± 0.02 | 0.36 ± 0.18 | 0.74 ± 0.10 | 0.48 ± 0.10 | 0.61 ± 0.03 | 0.35 ± 0.07 |
| Rb⁺  | 0.98 ± 0.08 | 0.20 ± 0.11 | 1.27 ± 0.11 | 0.45 ± 0.10 | 0.94 ± 0.30 | 0.31 ± 0.11 |
| Cs⁺  | 0.37 ± 0.15 | 0.13 ± 0.12 | 0.55 ± 0.07 | 0.14 ± 0.03 | 0.43 ± 0.15 | 0.13 ± 0.08 |

TABLE I

**Permeability and conductance ratios**

Data are the mean ± S.D.; \(n = 6–12\) patches.
more, no dominant-negative effect on functional hCNGA3 expression was observed, in contrast to the current suppression effects reported for similarly truncated K+ channel subunits (52).

One possible explanation is that the cytoplasmic carboxy-terminal domain of CNGB3 may be critical for assembly with CNGA3 subunits. In relation to this possibility, it has been proposed previously that intersubunit interactions between cytoplasmic amino- and carboxyl-terminal domains may be involved in assembly of olfactory (53) and rod (22, 29, 54, 55) CNG channels. In addition, homotypic interactions among the carboxyl-terminal domains of CNGA1, CNGA2, or CNGA3 subunits have been recently demonstrated (56). Further experiments are needed to define the important intersubunit contacts that control the assembly of CNG channels composed of both CNGA3 and CNGB3 subunits. Thus, in patients with this frame-shift mutation, cone dysfunction may arise from the complete lack of serviceable hCNGB3 subunits. In contrast to the current suppression effects reported for similarly truncated K+ channel subunits (52).
brane localization of CNGB3 subunits in the absence of CNGA3 subunits. Because CNGB3 subunits do not form functional homeric channels, we do not expect these truncated CNGB3 subunits to form tetramers. It is possible that aberrant targeting of hCNGB3-T833fs.ΔC subunits in cone photoreceptors may contribute to the development of achromatopsia. Elegant experiments with inwardly rectifying potassium channels have recently identified distinct signals that control the trafficking of the subunits to the plasma membrane; these signals include sequence motifs for endoplasmic reticulum retention/retrieval (57) and other motifs that promote endoplasmic reticulum exit and cell-surface expression (58, 59). Such signals are thought to be vital for the control of channel makeup (heteromultimerization) and of the density of channels at the plasma membrane (57, 59). Human CNGB3 subunits may possess similar trafficking signals within the carboxyl-terminal domain that lead to intracellular retention of CNBG3 subunits (in the absence of CNGA3 subunits) and of incompletely assembled channels. Overall our results indicate that T833fs.ΔC is effectively a null mutation, whereas the pathogenicity of the S435F mutation may arise from a complex of effects, an increase in ligand sensitivity, changes in the pore properties, and a decrease in functional expression level of heteromeric channels. A subtle decrease in patch current density and cell-surface localization after heterologous expression, however, cannot be readily extrapolated to predict the effect of mutations on cone CNG channel density or makeup in vivo. Thus, animal models expressing achromatopsia-associated mutations may be required to address this question more definitively. Results presented here provide a rational basis for the generation of mutant mice containing select achromatopsia mutations in the CNGB3 subunit.

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