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Functional analysis of rod monochromacy-associated missense mutations in the CNGA3 subunit of the cone photoreceptor cGMP-gated channel

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Abstract

Thirty-nine missense mutations, which had been identified in rod monochromacy or related disorders, in the CNGA3 subunit of cone photoreceptor cGMP-gated channels were analyzed. HEK293 cells were transfected with cDNA of the human CNGA3 subunit harboring each of these mutations in an expression vector. Patch–clamp recordings demonstrated that 32 of the 39 mutants did not show cGMP-activated current, suggesting that these 32 mutations cause a loss of function of the channels. From the remaining 7 mutants that showed cGMP-activated current, two mutations in the cyclic nucleotide-binding domain, T565M or E593K, were further studied. The half-maximal activating concentration ($K_{1/2}$) for cGMP in the homomeric CNGA3-T565M channels (160 μM) was 17.8-fold higher than that of the homomeric wild-type CNGA3 channels (9.0 μM). Conversely, the $K_{1/2}$ for cGMP in the homomeric CNGA3-E593K channels (3.0 μM) was 3-fold lower than that of the homomeric wild-type CNGA3 channels. These results suggest that the T565M and E593K mutations alter the apparent affinity for cGMP of the channels to cause cone dysfunction, resulting in rod monochromacy.

Keywords: CNG channel; CNGA3; CNGB3; Missense mutation; Patch–clamp; Rod monochromacy; Retina; Cone photoreceptor cell; cGMP

Cyclic nucleotide-gated (CNG) channels play an important role in signal transduction in both the visual and olfactory systems. In the retina, CNG channels of rod photoreceptors are hetero-tetramers composed of CNGA1 [1] and CNGB1 [2] subunits at a stoichiometry of 3A:1B [3–5]. Cone photoreceptors are also hetero-tetramers but composed of subunits distinct from rod photoreceptors, CNGA3 [6] and CNGB3 [7], at a stoichiometry of 2A:2B [8].

Rod monochromacy (achromatopsia or total color blindness) is a disorder of autosomal recessive trait characterized by cone dysfunction, which leads to a loss of color-vision, very low visual acuity, nystagmus, and photophobia. Gene loci responsible for the disorder, identified to date, are *CNGA3* [9]; *CNGB3* [10,11] and *GNAT2* [12,13], which encodes the α subunit of cone transducin. Among various mutations reported in these genes, 46 and 10 missense mutations have been identified in *CNGA3* [9,14–19] and *CNGB3* [15,17,18,20–22], respectively. These missense mutations are thought to be useful for revealing the pathophysiology of rod monochromacy and also to provide an opportunity for understanding the structure–function relationships of the CNG channels. However, only 11 missense mutations in *CNGA3* [16,23–25] and 4 missense mutations in *CNGB3* [21,26,27] have been functionally studied to date.

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Patel et al. [23] studied 4 mutations: Y181C; N182Y and L186F in the first transmembrane segment (S1); and C191Y in the first loop (Loop 1) of the CNGA3 subunit; to find that none of the 4 mutants showed cGMP-activated current using a patch–clamp technique. Faillace et al. [24] studied 3 missense mutations: R277C; R283W and R283Q; in the fourth transmembrane segment (S4) of the CNGA3 subunit; to find that none of the 3 mutants showed cGMP-activated current. Tränkner et al. [16] studied 2 mutations: T224R in the second loop (Loop 2) and T369S in the pore region of the CNGA3 subunit. The T224R mutant showed no cGMP-activated current, while the T369S mutant showed a decreased affinity for cGMP and a decreased sensitivity to extracellular Ca^{2+} in its homomeric channel. Co-expression of the wild-type CNGB3 protein restored the former sensitivity but not the latter. Liu and Varnum [25] studied 3 mutations: R277C in S4; N471S in the C-linker region and R563H in the cyclic nucleotide-binding domain (CNBD) of the CNGA3 subunit. The R277C mutant showed no cGMP-activated current, consistent with the results observed by Faillace et al. [24], but both N471S and R563H mutants showed an increased affinity for cGMP in their homomeric channels. In all, only 3 mutant proteins out of 11 studied have shown cGMP-activated current and the other 8 are thought to be unable to form a functional channel.

We have analyzed 39 missense mutations of *CNGA3* that were reported by Wissinger's group [9,14], by a patch–clamp technique. In this communication, we present the results of the functional analyses for these 39 mutations, especially on 2 mutations in the CNBD.

Materials and methods

Cloning of CNGA3 and CNGB3 cDNAs and their mutants. Complementary DNAs for human CNGA3 and CNGB3 subunits [21] were cloned into the EcoRI site of an expression vector, pCR3.1 (Invitrogen Corp., Carlsbad, CA). The 39 missense mutations in *CNGA3* reported by Wissinger's group [9,14], and 2 other mutations (E593D and E593R) were each introduced to the wild-type CNGA3 cDNA by the method of Ho et al. [28], and these mutated cDNAs were also cloned into pCR3.1.

Cloning of cDNAs for CNGA3 (wild-type and mutants) and CNGB3 (wild-type) in tag vectors was performed as in the following. The primers used for the CNGA3 series were PCR3.1F, which corresponds to the pCR3.1 vector (5′-TAATACGACTCACTATAGGG-3′); and CNGA ApaR, which has an ApaI site (underlined) followed by the C-terminal complementary sequence of CNGA3 (5′-GCGCGGGCCCCTGTTGTTTGTC-3′). The template was each clone in pCR3.1 and the enzyme used for this accurate PCR was the *Pyrobest* DNA polymerase (Takara Shuzo, Kyoto, Japan). The PCR parameters were: 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min for 25 cycles. The PCR products were digested with HindIII and ApaI, and cloned between the HindIII and ApaI sites of a tag vector, pcDNA3.1/Myc-His A (Invitrogen). The primers used for the CNGB3 subunit were PCR3.1F and CNGBKpnR, which has a KpnI site (underlined) followed by the C-terminal complementary sequence of CNGB3 (5′-GCGCGGTACCTTGCTTAGCCTTTTC-3′). The template was the CNGB3 clone in pCR3.1 and the enzyme used for this accurate PCR was the *Pyrobest* DNA polymerase. The PCR parameters were: 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min for 25 cycles. The PCR products were digested with KpnI, and cloned into the KpnI site of a tag vector, pFLAG-CMV-5a (Sigma–Aldrich, St. Louis, MO).

Table 1
Mutants that showed no cGMP-activated current upon patch–clamp recordings

Region	Mutation	No. of patches examined
N-Terminal cytoplasm	D162V	14
	P163L	8
S1	Y181C	10
	N182Y	13
	L186F	10
Loop 1	C191Y	12
	E194K	10
Loop 2	R223W	10
	T224R	10
S3	D260N	10
Loop 3	G267D	10
S4	R277C	10
	R277H	10
	R283W	12
	R283Q	11
	T291R	16
Pore	S341P	10
	P372S	10
S6	F380S	11
C-linker	R410W	11
	R427C	10
	R436W	10
CNBD	C510S	14
	G513E	10
	G516E	8
	I522T	10
	G525D	10
	V529M	13
	F547L	13
	R563H	10
	R569H	12
	Y573C	11

Cell culture and transfection. HEK293 cells, obtained from Human Science Research Resources Bank (Sennan, Japan), were maintained in DMEM supplemented with 10% fetal bovine serum and were subcultured every 2–3 days. Transfections were performed as previously described [21]. Patch–clamp experiments were conducted 2–3 days after transfection, on green fluorescent protein-positive cells.

Patch–clamp technique and data analysis. Membrane current was recorded in an inside-out mode of the patch–clamp technique with an EPC-8 amplifier (HEKA Elektronik, Lambrecht, Germany). Glass electrodes had a resistance of 2–4 MΩ when filled with the standard high K^+ solution (134 mM KCl, 10 mM Hepes-K (pH 7.2), and 1 mM EGTA). The membrane patches excised from the transfected cells were superfused with standard high K^+ solution without and then with various concentrations of cGMP (0.1–1000 μM). All patch–clamp recordings were conducted at, approximately, 25 °C. A voltage ramp protocol (triangle wave, $dV/dt = \pm 0.5$ V/s) was used to record the macroscopic patch current. The current and voltage signals were digitized at a sampling frequency of 1 kHz using an ITC-16 AD/DA interface (InstruTECH, Port Washington, NY) controlled by Pulse/Pulsefit software (ver. 8.54, HEKA). The current–voltage (I–V) relationship was measured during the descending phase of the voltage ramp and the average of 3–5 consecutive I–V relationships was depicted.

Table 2
Mutations that showed cGMP-activated current upon patch–clamp recordings

Region	Mutant	No. of patches examined	No. of patches showing current
Pore	T369S	33	11
S6-C-linker	M406T	12	8
C-linker	N471S	10	6
	D485V	26	3
CNBD	G557R	20	2
	T565M	29	11
	E593K	46	11

Immunoprecipitation and immunoblot. HEK293 cells were transfected with the cDNA of CNGA3 (wild-type and mutant) in a tag vector (pcDNA3.1/Myc-His A), along with the wild-type cDNA of CNGB3 in another tag vector (pFLAG-CMV-5a). The cells were collected after 2 days, washed twice with phosphate-buffered saline and treated with lysis buffer (25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM $CaCl_2$, 1% Triton X-100, and 2.5 μg/ml each of pepstatin and leupeptin) for 15 min on ice, and then centrifuged at 13,500*g* for 5 min at 4 °C. Anti-FLAG agarose (Sigma–Aldrich) was added to the supernatant and mixed by rotation overnight at 4 °C. The mixture was centrifuged at 1000*g* for 5 min at 4 °C and the pellet was washed twice with lysis buffer. SDS-sample buffer (10 mM Tris–HCl (pH 6.8), 2.3% SDS, 5% β-mercaptoethanol, and 10% glycerol) was added to the pellet and mixed. After centrifugation, the supernatant was subjected to SDS–PAGE (12.5%). Prestained SDS–PAGE standards (broad range; Bio-Rad Lab., Hercules, CA) were used as size markers. The separated proteins were blotted to a PVDF membrane (Millipore Corp., Bedford, MA) using the Mini Trans-Blot Cell (Bio-Rad). The CNGA3 proteins tagged with *myc* were detected using the Anti-*myc*-antibody conjugated with alkaline phosphatase (Invitrogen).

Results and discussion

Analysis of 39 mutations by a patch–clamp technique

Tables 1 and 2 summarize the results of patch–clamp recordings for the 39 mutants. Thirty-two mutants (Table 1) showed no obvious current activation by cGMP at a concentration of 100 μM, which produced a maximum response in the homomeric wild-type CNGA3 channel [21]. Eight mutations: Y181C; N182Y; L186F; C191Y; T224R; R277C; R283W and R283Q; have been reported by other researchers to have shown no cGMP-activated current [16,23–25], which is consistent with our results. Our results and those of others suggest that most of the CNGA3 missense mutations may cause a loss of function of CNG channels, due to a defect in processing, trafficking, and/or channel function itself.

The heterologously expressed wild-type and mutant proteins were associated with the co-expressed wild-type CNGB3 protein, as revealed by immunoprecipitation and immunoblot experiments (Fig. 1). We examined whether the channel function of the 32 mutants was rescued by co-expression of the wild-type CNGB3 protein. In our preliminary experiments on 5–10 patches for each mutant, at least 6 mutants: D162V; Y181C; T291R; R427C; V529M and R563H; showed cGMP-activated current. Patel et al. [23] reported that the Y181C channel was not rescued by the co-expressed wild-type CNGB3 protein, after examination of 12–15 patches. More experiments are obviously needed to examine whether channel function of the remaining 26 mutants are really not rescued by co-expression of the wild-type CNGB3 protein.

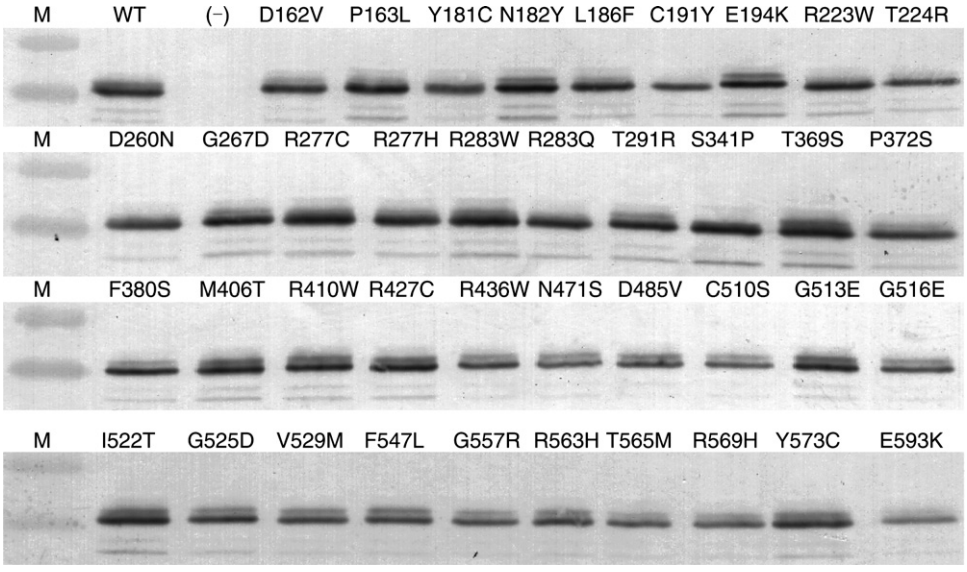

Fig. 1. Immunoblot of *myc*-tagged CNGA3 proteins. HEK293 cells were transfected with the cDNA of CNGA3 subunit (wild-type and mutants) in a tag-vector (pcDNA3.1/Myc-His A), along with the cDNA of wild-type CNGB3 subunit, in a tag-vector (pFLAG-CMV-5a). After 2 days, the cell extract was prepared, to which Anti-FLAG agarose was added. After washing, the proteins associated with the agarose were eluted, separated by SDS–PAGE and blotted to a PVDF membrane. The CNGA3 protein was detected by Anti-*myc*-antibody conjugated with alkaline phosphatase. M, size markers (only 118 and 92 kDa are shown); WT, wild-type CNGA3 protein; (−), transfection of CNGB3 cDNA without transfection of CNGA3 cDNA.

We were unable to detect cGMP-activated current for R563H (Table 1), as observed by Liu and Varnum [25]. In their experiments, the R563H mutant protein was scarcely localized to the plasma membrane as revealed by confocal microscopy and, therefore, the amplitude of the obtained current itself was very small. Thus, there would be no obvious discrepancy between our results and theirs with regard to this mutation. As mentioned above, we could rescue the function of the R563H channel upon co-expression of wild-type CNGB3 protein, and determined that the $K_{1/2}$ for cGMP of the heteromeric CNGA3-R563H/wild-type CNGB channel to be 12.6 μM, which was similar to that (8.18 μM) as determined by Liu and Varnum [25].

Mutants that showed cGMP-activated current as a homomeric channel

We detected cGMP-activated current in 7 of the 39 mutants (Table 2). The T369S mutation has been studied in detail by Tränkner et al. [16] and so we did not investigate this mutation further. The previously unstudied M406T mutation was observed to have a $K_{1/2}$ for cGMP for the homomeric CNGA3-M406T channel of 15.5 μM. The N471S mutation has been studied by Liu and Varnum using *Xenopus* oocytes [25]. They determined that the $K_{1/2}$ for cGMP in the homomeric CNGA3-N471S channel was 2.7-fold lower than that for the homomeric wild-type CNGA3 channel. In our system, using HEK293 cells, the $K_{1/2}$ for cGMP in the homomeric CNGA3-N471S mutant channel (8.4 μM) was similar to that observed in the wild-type homomeric CNGA3 channel (9.0 μM). We cannot explain these discrepancies at present. The homomeric CNGA3-D485V and CNGA3-G557R channels did show cGMP-activated current, but very infrequently (Table 2) for some inscrutable reason, and so we did not further study these mutations.

Detailed analysis of T565M

Fig. 2A shows the *I–V* relationships in the homomeric wild-type CNGA3 and CNGA3-T565M channels. The

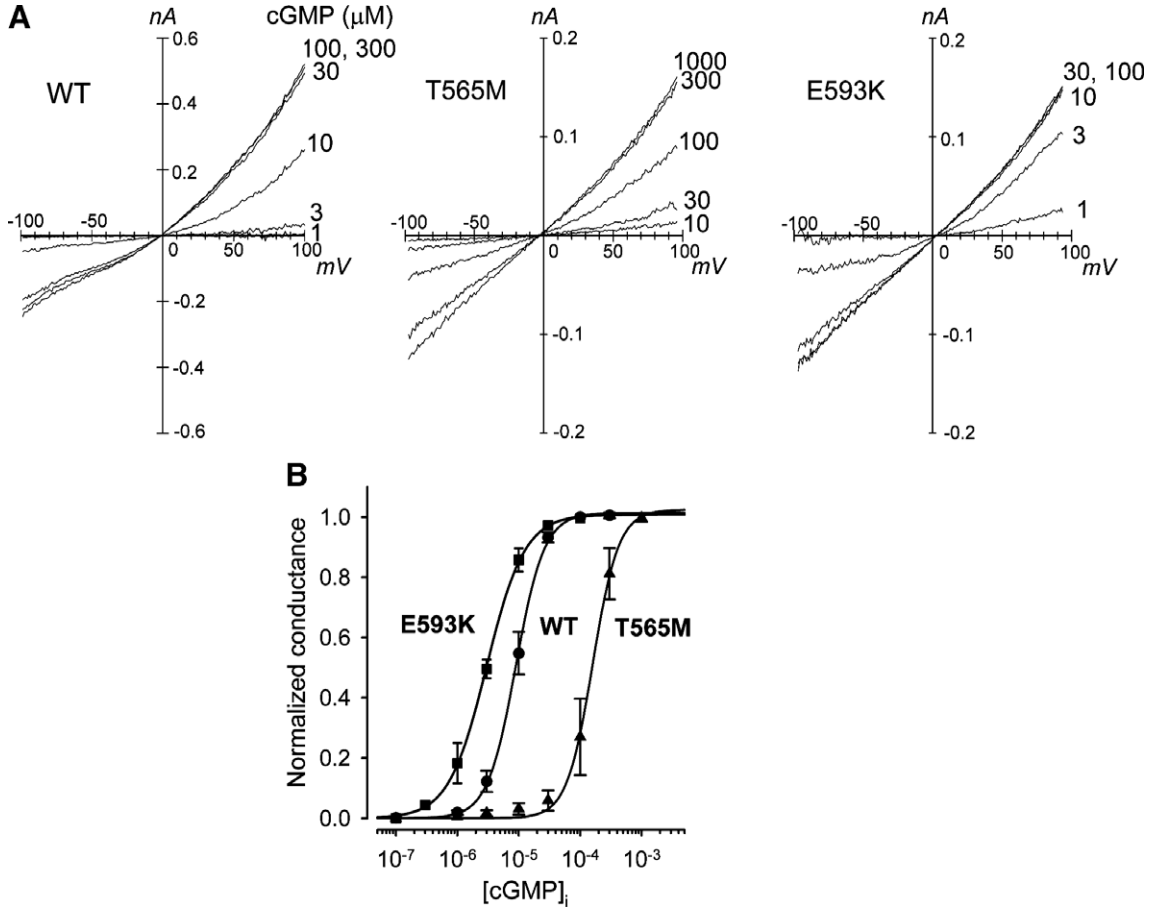

Fig. 2. *I–V* and dose–response relationships in the homomeric CNG channels. (A) Macroscopic patch current was recorded during a voltage ramp protocol in the presence of various concentrations of cGMP. WT, homomeric wild-type CNGA3 channel; T565M, homomeric CNGA3-T565M channel; E593K, homomeric CNGA3-E593K channel. (B) The data points represent the slope conductance of macroscopic current activated by each concentration of cGMP at, approximately, 0 mV normalized with reference to the maximal value evoked by 300–1000 μM cGMP. The lines show a least-squares fit of the Hill equation. WT, homomeric wild-type CNGA3 channel; T565M, homomeric CNGA3-T565M channel; E593K, homomeric CNGA3-E593K channel. The $K_{1/2}$ for cGMP was 9.0 μM for WT, 160 μM for T565M, and 3.0 μM for E563K.

homomeric CNGA3-T565M channel was obviously less sensitive to cGMP concentration than the homomeric wild-type CNGA3 channel. Fig 2B shows the dose–response relationships for the homomeric wild-type CNGA3 and CNGA3-T565M channels. The $K_{1/2}$ for cGMP in the homomeric CNGA3-T565M channel was 160 μM, 17.8-fold higher than that of the wild-type homomeric CNGA3 channel (9.0 μM). The $K_{1/2}$ of the heteromeric CNGA3-T565M/wild-type CNGB3 channel was 135 μM, 8.3-fold higher than that of the heteromeric wild-type CNGA3/wild-type CNGB3 channel (16.3 μM).

Altenhofen et al. [29] mutated the T-560 of CNGA1 protein, which corresponds to the T-565 of the CNGA3 subunit, to A and found a 28.8-fold increase of $K_{1/2}$ for cGMP. They concluded that T-560 forms a hydrogen bond with the cyclized phosphate of the ligand [29]. It would be reasonable to assume a similar functional role for T-565 of the CNGA3 subunit. The impairment of this role by the substitution with M must have caused the decrease in the actual affinity for cGMP in the mutant channel.

We suppose from our results that in rod monochromacy patients with this mutation, CNG channels in their cone cells are always closed, irrelevant to the photon-signals, leading to cone dysfunction.

Detailed analysis of E593K

The E593K mutation caused an increase in the apparent affinity for cGMP (Fig. 2A and B). The $K_{1/2}$ of the homomeric CNGA3-E593K channel (3.0 μM) was 3-fold lower than that of the homomeric wild-type CNGA3 channel (9.0 μM). The heteromeric CNGA3-E593K/wild-type CNGB3 channel showed a $K_{1/2}$ value of 3.1 μM, which was similar to the homomeric CNGA3-E593K channel, in spite of the association of the wild-type CNGB3 protein, and was 5.3-fold lower than that of the heteromeric wild-type CNGA3/wild-type CNGB3 channel (16.3 μM) .

In the CNGA1 subunit, the guanine ring of cGMP was suggested to be recognized by 533-F in the 5th β-roll, and also by 604-D in the α-helix C [30]. The 604-D, which interacts with N1–H and C2–NH_2 of the guanine ring of cGMP via hydrogen bonds, corresponds to 609-D in the CNGA3 subunit. The 593-E is located 16 amino acid residues from the 609-D (four turns ahead of 609-D), at the very N-terminus of α-helix C. Therefore, it is unlikely that 593-E is involved in a direct interaction with cGMP.

We analyzed two other mutations at this position, E593R and E593D (Fig. 3). The homomeric CNGA3-E593R channel showed a similar dose–response curve to the homomeric CNGA3-E593K channel, while the homomeric CNGA3-E593D channel showed a similar curve to the homomeric wild-type CNGA3 channel. These results suggest that either an E or a D can be present at position 593 for normal channel function.

We have previously reported a missense mutation in the CNGB3 subunit found in rod monochromacy, D633G [21]. The position 633 in the CNGB3 subunit corresponds to the

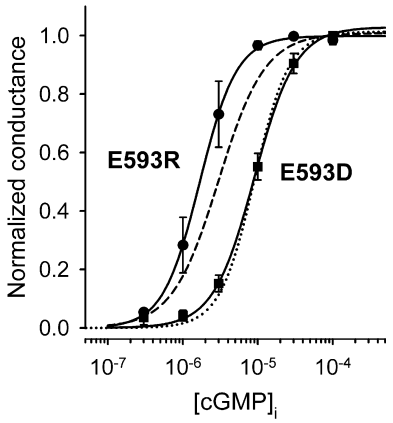

Fig. 3. Dose–response relationships in the homomeric mutant channels. The data points represent the slope conductance of macroscopic current activated by each concentration of cGMP at, approximately, 0 mV normalized with reference to the maximal value evoked by 100 μM cGMP. The lines show a least-squares fit of the Hill equation. E593R, homomeric CNGA3-E593R channel; E593D, homomeric CNGA3-E593D channel. For reference, the dotted and dashed curves represent the dose–response of the homomeric wild-type CNGA3 and the homomeric CNGA3-E593K channels, respectively. The $K_{1/2}$ for cGMP was 1.7 μM for E593R and 9.0 μM for E563D.

position 593 in the CNGA3 subunit. Our data showed that one of the functions of the CNGB3 subunit, to decrease the apparent affinity for cGMP of the heteromeric CNGA3/CNGB3 channel, was abolished by the D633G mutation [21]. If we directly apply these results obtained in the CNGB3 subunit to 593-E in the CNGA3 subunit, this amino acid may have a role in controlling the efficacy of channel-gating when cGMP is bound to the CNGA3 subunit.

We suppose from our results that in rod monochromacy patients with the E593K mutation, the CNG channels in their cone cells are not fully closed even when intracellular cGMP level is decreased by photon-signals, leading to cone dysfunction.

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