Cellular Processing of Cone Photoreceptor Cyclic GMP-gated Ion Channels

A ROLE FOR THE S4 STRUCTURAL MOTIF

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We examined cellular protein processing and functional expression of photoreceptor cyclic nucleotide-gated (CNG) ion channels. In a mammalian cell line, wild type bovine cone photoreceptor channel α subunits (bCNGA3) convert from an unglycosylated state, at 90 kDa, to two glycosylated states at 93 and 102 kDa as they transit within the cell to their final location at the plasma membrane. Glycosylation per se is not required to yield functional channels, yet it is a milestone that distinguishes sequential steps in channel protein maturation. CNG ion channels are not gated by membrane voltage although their structure includes the transmembrane S4 motif known to function as the membrane voltage sensor in all voltage-gated ion channels. S4 must be functionally important because its natural mutation in cone photoreceptor CNG channels is associated with achromatopsia, a human autosomal inherited loss of cone function. Point mutation of specific, not all, charged and neutral residues within S4 cause failure of functional channel expression. Cellular channel protein processing fails in every one of the non-functional S4 mutations we studied. Mutant proteins do not reach the 102-kDa glycosylated state and do not arrive at the plasma membrane. They remain trapped within the endoplasmic reticulum and fail to transit out to the Golgi apparatus. Coexpression of cone CNG β subunit (CNGB3) does not rescue the consequence of S4 mutations in CNGA3. It is likely that an intact S4 is required for proper protein folding and/or assembly in the endoplasmic reticulum membrane.

Cyclic nucleotide-gated ion channels (CNG)1 are localized in the plasma membrane of sensory transduction neurons, such as retinal photoreceptor and olfactory sensory cells where they play a central role in signal transduction (for review see Refs. 1–3). Photoreceptor CNG channels are heteromers constituted by α and β subunits in 3:1 stoichiometry (4–6). Whereas specific details of CNG protein cellular processing have previously not been reported, it is likely these channels follow the cellular processing pattern typical of other channel proteins (reviewed in Ref. 7). Nascent channel proteins first attach to endoplasmic reticulum (ER) membranes, targeted to these membranes by a signal sequence. In the ER, proteins fold and translocate across the lipid bilayer and are core glycosylated by linking of N-acetylgalactosamine (GlcNAc) to Asn residues in the consensus sequence NX-S or -T. Frequently, core-glycosylated channel proteins are processed by additional glycoylation in the ER and/or Golgi apparatus.

Various single point mutations in the α subunit of CNG channels of cone photoreceptors have been identified in humans afflicted by achromatopsia, also known as total color blindness or rod monochromic, an autosomal, inherited disease characterized by complete loss of cone photoreceptor function (8). Two of the most frequent mutant alleles in achromatopsia are mutations from charged to neutral amino acids in the S4 structural motif of the cone-specific CNG channel α subunit (CNGA3) (8). This motif consists of 4–9 sequential repeats of the tandem Arg-Lys-X-X in the 4th transmembrane α helix, where X-X are neutral amino acids. Its three-dimensional structure has been recently proposed (9). The motif is present in every known voltage-gated ion channel (K+ Na+, and Ca2+), and its existence in CNG ion channels is one of the arguments first used to identify CNG channels as members of the same gene superfamily as voltage-gated K+ channels (10, 11).

In voltage-gated channels S4 functions as a voltage sensor (12–14) and its voltage-dependent translocation within the membrane is linked to channel gating (Refs. 15–19, reviewed in Ref. 20). In addition to its biophysical function, however, the S4 structure also plays a role in the cellular biogenesis of voltage-gated channels (for review see Refs. 7, 21, and 22). In voltage-gated K+ channels, neutralization of some charged amino acid residues in S4 change the voltage dependence of gating, whereas mutation of others cause channel functional failure (23–25). In HCN channels, which are gated by voltage and modulated by cyclic nucleotides, S4 plays a similar dual role: some residues affect the voltage dependence in gating, whereas others interfere with their arrival at the plasma membrane (19, 26).

Although CNG channels contain an S4 domain they are not voltage-gated. That is, their probability of opening is independent of membrane voltage and depends only on cyclic nucleotide concentration. This is despite the fact that the CNG channels’ S4 domain can perform as a voltage sensor when placed in a permissive structural environment. Chimera experiments demonstrate that replacing the authentic S4 motif in ether-a-go-go K+ channel with the olfactory S4 motif (CNGA2) maintains the voltage-dependent behavior of the K+ channel (27). On the other hand, CNG channels do exhibit several voltage-dependent functions. 1) The ligand sensitivity is voltage dependent. The ligand concentration necessary to half-maximally activate the channels is about 10 μM higher when measured at -50 mV than at +50 mV (in cones 28, in rods 29). 2) The
lifetime of the channel open state in rod CNG is slightly longer at depolarized than at hyperpolarized voltages, causing slight outward rectification in the open channel I-V curve (30). 3) Open channels are blocked by divalent cations, but the extent of block is voltage-dependent: block is relieved as the voltage moves away from the reversal voltage (in cones 31 and 32, in rods 29 and 33). Ion permeation is well described by an Eyring rate theory model that assumes ions bind to sites in the pore of the channel and binding is dependent on membrane voltage (in cones 34, in rods 35–37). S4 mutations in CNGA3, hence, can cause channel malfunction because of either defective protein biogenesis or a defect in a voltage-dependent function. To explore the role of S4 in CNG channel function, we investigated the consequences of point mutations in the S4 domain of the CNG channel a subunit cloned from bovine cone photoreceptors (bCNGA3). We measured the electrical properties of transfected mammalian cells, as well as the cell-processing and expression patterns of the channel proteins. We have found that normal channels exist in various states of glycosylation as they transit within cells to the plasma membrane. S4 plays a crucial role in channel biogenesis. Specific, not all, point mutations in S4 cause failure of cellular channel protein processing. Mutant channel proteins are not glycosylated and do not reach the surface plasma membrane; they remain trapped within the endoplasmic reticulum, likely misfolded.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis and Hemagglutinin (HA) Tag Insertion**—The cDNA of bovine CNGA3 (accession NM_174279, Refs. 36 and 37) and mouse CNGB3 were the kind gift of Dr. M. Biel (University of Munich). The cDNA was inserted in-frame with the cytomegalovirus promoter in a bacterial plasmid (pcDNA 3, Invitrogen, Carlsbad, CA). Site-directed mutagenesis of the cDNA was executed using the QuickChange® kit (Stratagene, San Diego, CA).

To track cellular processing of bCNGA3 we tagged the protein with HA peptide (YPYDVPDYA) by inserting the coding sequence of the HA peptide (YPYDVPDYA) by inserting the coding sequence of the peptide in-frame with the channel sequence at three specific locations using the QuickChange kit. 1) bCNGA3-N-HA in the amino terminus, between Leu238 and Ser239. 2) bCNGA3-C-HA in the carboxyl terminus, between Pro699 and Gln700 (seven residues from the end of the protein). 3) bCNGA3-S1S2-HA in the extracellular loop that connects transmembrane helices S1 and S2, between residues Leu200 and Gln201. All mutants and chimeras were sequenced in full to ensure that unintended, random mutations did not occur because of polymerase errors.

**Cell Transfection—tsA-201 cells** (transformed from HEK 293 cells to constitutively expressing T-antigens) were grown on plastic wells in Dulbecco's modified Eagle's H-21 media (with 10% fetal bovine serum and penicillin/streptomycin) at 37 °C under 5% CO2. When they reached 70% confluence they were transfected with plasmid cDNA introduced as a calcium phosphate precipitate using a commercial kit (Speciality Media, Phillipsburg, NJ). 9.6-μm wells were simultaneously transfected with 12.5 μg of channel cDNA plasmid and 3 μg of pEGFP, a pcDNA 3 plasmid that promotes cytoplasmic expression of EGFP. 20 h later the DNA-calcium phosphate precipitate was washed away. Cells were cultured for another 48 h before subjecting them to electrophysiological, biochemical, or imaging studies.

**Electrophysiological Measurements**—Cells were suspended in a simple Ringer’s solution consisting of (in mM): NaCl (140), KCl (2.5), NaHCO3 (3.5), Na2HPO4 (1), CaCl2 (1), MgCl2 (1), glucose (10), minimal essential medium amino acids and vitamins (1%), and HEPES (10), pH 7.4, osmotic pressure: 310 mosmol. To produce these suspensions, cell layers growing on plastic were gently trypsinized (0.05% trypsin, 0.02% EDTA) and plated on a poly-L-lysine-coated (0.1 mg/ml) glass coverslip that formed the bottom of an electrophysiological recording chamber. The chamber was held on the stage of a modified patch clamp amplifier (Axopatch 1D, Axon Instruments, Foster City, CA). Analog outputs were low pass filtered below 100 Hz with an eight pole Bessel filter (Kronh-Hite, Avon, MA) and digitized on line at 200 Hz (pClamp, Axon Instruments, Union City, CA).

Electrode filling solution was freshly prepared under dim red light and used within 3 h or discarded. We used a salt solution consisting of (in mM): glumatic acid (130), triethanolamine-CI (20), HEPES (10), MgCl2 (0.7), GTPNa+ (1), ATPNa+ (2), 1,2-histidine-2-aminophenyl ether-N,N,N′,N′-tetraacetic acid (2) titrated to pH 7.3 with CaOH. Under deep red light we added MCM-caged 8-PCPT-cGMP (7-methoxyisocoumarin-methyl-4-yl)-methyl-caged 8-(4-chlorophenylthio)guanosine cyclic 3′,5′- monophosphate) from a 25 mM in Me2SO stock to a final concentration of 15 μM. The compound was the kind gift of Dr. V. Hagen, who has described its synthesis (39). An immunolabelling analysis of HA-tagged Channel proteins—Transfected cells were lysed with non-denaturing lysis buffer: 100 mM Tris/HCl, pH 8.0, 100 mM NaCl, 0.5% Triton X-100, and 1 and /ml of solution of a protease inhibitor mixture (Complete Mini EDTA-free, Roche Applied Science). 25-μg samples of total protein were separated by size using SDS-PAGE on 6% gels, and then transferred to nitrocellulose membranes by semidyeblotting. Membranes were incubated overnight in blocking buffer: 100 μM phosphate-buffered saline, 0.1% Tween 20, 0.5% nonfat dried milk, and 1% goat serum at 4 °C with continuous agitation. They were then incubated for 1 h at room temperature in 15 ml of the same buffer, now containing anti-HA rat polyclonal antibody at 100 ng/ml (3F10 clone, Roche Applied Science). Bound primary antibody was detected by reaction with a secondary anti-rat goat antibody conjugated to horseradish peroxidase (1:1000 in blocking buffer) (Jackson ImmunoResearch Laboratories, West Grove, PA). Horseradish activity was exposed either with diaminobenzidine and nickel substrate or with ECL reagents (Amersham Biosciences). Immunoblots were repeated at least three times.

**Immunocytochemistry**—In these experiments tsA-201 cells were grown on poly-d-lysine-coated (1 mg/ml) glass number 1 coverslips placed in plastic wells. Cells were grown on 2-cm2 wells and transfected with plasmid DNA scaled in concentration to maintain the weight ratios described above for cells grown on larger wells. 4 h after transfection we added the DNA-calcium phosphate precipitate fixed with 4% paraformaldehyde for 10 min at room temperature, washed thoroughly with phosphate-buffered saline, and then blocked by incubation in phosphate-buffered saline containing 0.1% Tween 20, 2% bovine serum albumin, and 2% goat serum for at least 1 h at room temperature. Coverslips were then incubated overnight with anti-HA rat monoclonal antibody to 200 ng/ml added to the blocking solution (3F10 clone). The anti-HA antibody was detected with a fluorescent secondary goat anti-rabbit antibody conjugated to rhodamine (1:5000). Membrane compartments—Labeling of Transformed Cells—Cells grown on poly-d-lysine-coated coverslips in 2-cm2 wells were simultaneously transfected with channel cDNA (2.6 μg) and 0.65 μg each of one of several DNA constructs designed to express EGFP fusion protein at specific subcellular compartments. To label the plasma membrane we transformed cells with pEGFP-F protein (BD Biosciences, Courth, Palo Alto, CA). This construct promotes the expression of a farnesyl signal in fusion with EGFP, the protein product is palmitoylated and targeted specifically to the cell plasma membrane. To label the endoplasmic reticulum we transformed cells with pEGFP-ER, a construct consisting of the residues –30 to 8 of bovine preprolactin (40) in fusion with EGFP. To label Golgi apparatus we transformed with pEGFP-TG, a construct consisting of residues –77 to –31 of human β-1,4-galactosyltransferase (41) in fusion with EGFP. The later two constructs were the kind gift of Dr. A. Verkman (University of California, San Francisco), who has described their construction and application (42).
Uncaging pCPT-cGMP by bright flash illumination caused a large inward current at −75 mV in cells transfected with wt bCNGA3 (Fig. 1A). In cells tested at holding voltages between −65 and −25 mV, 27 of 29 exhibited uncaging flash-activated currents. The few failures almost certainly reflect cells in which EGFP was expressed (the criterion to identify transformed cells), but channels were not. The absolute amplitude of the current varied from cell to cell, likely reflecting variance in the level of channel protein expression. At −75 mV mean cyclic nucleotide-dependent current amplitude was −74 ± 50.5 (range −36 to −170, n = 6).

Three independent controls make evident that the current generated by uncaging the cyclic nucleotide analog reflects specific activation of bCNGA3 channels. 1) Flash illumination of transformed cells not loaded with caged nucleotides or untransformed cells loaded with the nucleotides did not generate currents. 2) The I–V characteristics of the flash-activated current are the same as those of the native cone CNG channels under similar ionic solutions (Fig. 1D). The composition of intra- and extracellular solutions were designed to minimize the amplitude of small delayed rectifier K⁺ and Ca²⁺-dependent Cl⁻ currents endogenous to tsA-201 cells. The 8-pCPT-cGMP-dependent difference I–V curve is typical of CNGA3 channels and demonstrates the well documented voltage-dependent relief of channel blocking by divalent cations. 3) Currents were of identical features whether activated by uncaging caged-8-pCPT-cGMP or caged-cGMP (not shown).

**Functional Consequence of Various Point Mutations in S4**—We transformed cells with bCNGA3 cDNA modified to mutate R296Q, this is the second Arg in the S4 motif of the

Microscopy and Image Analysis—Fluorescent cell images were captured with a confocal microscope (Zeiss, model LSM 510) using a ×63/1.3 NA objective. Spatial resolution was further improved by deblurring the confocal images with the use of deconvolution algorithms (C-Imaging Systems, Compix Inc., Cranberry Township, PA). The same software was used for image analysis and overlay in pixel-by-pixel operations at 12 bit resolution.

**RESULTS**

We investigated the electrical properties and cellular protein processing of the bCNGA3. Electrical function was assayed in transformed tsA-201 cells by assessing whether, under voltage clamp, membrane currents were activated upon photo release of cyclic nucleotide from a caged precursor compound. The analog we used, 8-pCPT-cGMP is the most potent CNG channel agonist so far described (about 80-fold higher than cGMP (43)), thus yielding a highly sensitive assay of function. Caged nucleotide was loaded into cells by allowing time for its diffusion from the lumen of a tight-seal electrode into the cell cytoplasm. Typically, the effect of photoreleased 8-pCPT-cGMP was assessed 3.5 min after attaining the whole cell mode, a time sufficient for equilibration between the cytoplasm of the cell and electrode filling solution. At this time, cells transformed with wt α subunits of bCNGA3 exhibited a mean holding current at −40 mV of −14.3 ± 17.6 pA (n = 22). Because endogenous K⁺ channels in tsA-201 were partially blocked by the ionic solutions we used in these experiments, holding current magnitude principally reflects cell integrity and the quality of the seal resistance and offers an excellent tool to discard damaged cells. Indeed, cells were accepted for analysis only if their holding current was within the statistical range reported above.

**Fig. 1.** Currents generated by flash-release of caged 8-pCPT-cGMP in tsA-201 cells transformed to express bCNGA3. Cells were voltage-clamped and held at −40 mV. 3.5 min after attaining whole cell mode, membrane voltage was stepped to −75 mV and a bright, brief light flash delivered to the cell. In a cell transformed with wt CNGA3 uncaging of the nucleotide caused a large increase in inward current (panel A). The I–V curve of a different cell transformed with wt CNG3 shows that in darkness the cell sustained only small currents at voltages between −75 and 60 mV; flash illumination increased current amplitude at all voltages and the difference I–V curve (light-dark) is typical of cGMP-dependent channels (panel D). A point mutation in the S4 motif that changed R296Q caused failure of functional channel expression (panel B). Reversion of the mutation in the cDNA back to the wt sequence, Q296R, restored normal functional channel expression.
channel (Table I). In these cells the holding current at −40 mV was −9.6 ± 9.6 pA (n = 10). None of these cells exhibited detectable changes in current upon cytoplasmic flash release of cyclic nucleotides (Fig. 1B). The failure of functional channel expression is specifically because of the point mutation, because cells transformed with the very same plasmid mutated back to the wt sequence resulted in electrical behavior identical to that of wt channels (Fig. 1C). In cells transformed with

### Table I

| Position | COOH-tagged | Mutation | I hold at −40 mV | ΔI flash at −75 mV | Range | Responsive/total cells |
|----------|-------------|----------|------------------|-------------------|-------|-----------------------|
| wt       | HA          | Gln      | −6.8 ± 6.4       | −74.0 ± 50.5      | −36 to −170 | 6/7 |
| Arg<sup>293</sup> | HA          | Gln      | −13.8 ± 13.9     | −84.6 ± 94        | −15 to −280 | 0/4 |
| Phe<sup>294</sup> | Leu, Ala   | Gln      | −10.7 ± 8.4      | −84.6 ± 94        | −28 to −400 | 0/7 |
| Arg<sup>296</sup> | HA          | Gln      | −9.6 ± 9.6       | −84.6 ± 94        | −28 to −400 | 0/10 |
| Arg<sup>296</sup> | HA          | Gln      | −5.0 ± 3.5       | 0                 | 0/4 |
| Arg<sup>296</sup> | HA          | Gln      | −10.6 ± 4.5      | 0                 | 0/3 |
| Arg<sup>296</sup> | HA          | Cys      | −3.0 ± 1.0       | 0                 | 0/5 |
| Leu<sup>297</sup> | Ala         | Gln      | −7.4 ± 9.4       | 0                 | 0/5 |
| Ly<sup>299</sup> | Gln         | Gln      | −30 ± 22         | 0                 | 0/2 |
| Ly<sup>299</sup> | HA          | Gln      | −11.5 ± 16.9     | 0                 | 0/4 |
| Arg<sup>302</sup> | HA          | Trp      | −6 ± 5.5         | 0                 | 0/1 |
| Arg<sup>302</sup> | HA          | Trp      | −9.6 ± 5.5       | 0                 | 0/5 |
| Leu<sup>303</sup> | Ala         | Gln      | −7.0 ± 2.6       | 0                 | 0/6 |
| Arg<sup>306</sup> | HA          | Cys      | −6.0 ± 0.8       | 0                 | 0/5 |
| Arg<sup>306</sup> | HA          | Gln      | −9.6 ± 9.6       | 0                 | 0/10 |
| Arg<sup>306</sup> | HA          | Gln      | −5.0 ± 3.5       | 0                 | 0/4 |
| Arg<sup>306</sup> | HA          | Gln      | −10.6 ± 4.5      | 0                 | 0/3 |
| Arg<sup>306</sup> | HA          | Cys      | −3.0 ± 1.0       | 0                 | 0/5 |
| Leu<sup>297</sup> | Ala         | Gln      | −7.4 ± 9.4       | 0                 | 0/5 |
| Ly<sup>299</sup> | Gln         | Gln      | −30 ± 22         | 0                 | 0/2 |
| Ly<sup>299</sup> | HA          | Gln      | −11.5 ± 16.9     | 0                 | 0/4 |
| Arg<sup>302</sup> | HA          | Trp      | −6 ± 5.5         | 0                 | 0/1 |
| Arg<sup>302</sup> | HA          | Trp      | −9.6 ± 5.5       | 0                 | 0/5 |
| Leu<sup>303</sup> | Ala         | Gln      | −7.0 ± 2.6       | 0                 | 0/6 |
| Arg<sup>306</sup> | HA          | Cys      | −6.0 ± 0.8       | 0                 | 0/5 |
| Arg<sup>306</sup> | HA          | Gln      | −9.6 ± 9.6       | 0                 | 0/10 |
| Arg<sup>306</sup> | HA          | Gln      | −5.0 ± 3.5       | 0                 | 0/4 |
| Arg<sup>306</sup> | HA          | Gln      | −10.6 ± 4.5      | 0                 | 0/3 |

<sup>a</sup> Counting cells transformed with wt bCNGA3 and tested at voltages other than −75 mV; 27/29 EGFP-positive cells exhibited currents upon flash release of pCPT-cGMP.

<sup>b</sup> Same mutations as found in congenital achromatopsia.

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**Fig. 2. Analysis of the function and cell processing pattern of HA-tagged bCNGA3 wt channel proteins.** Cells were transformed with one of three constructs: 1) bCNGA3-C-HA, tag inserted in the cytoplasmic carboxyl-terminal; 2) bCNGA3-S<sub>1S2</sub>-HA, tag in the extracellular loop between transmembrane helices 1; and 3) bCNGA3-N-HA, tag in the cytoplasmic amino-terminal. In panel A are shown the electrical properties of the cells. Cells were voltage-clamped and held at −40 mV. 3.5 min after attaining whole cell mode, membrane voltage was stepped to −75 mV and a bright, brief light flash delivered to the cell. Only the bCNGA3-C-HA yielded functional channels. In panel B are confocal images of cells treated with rhodamine-labeled anti-HA antibody. The size marker is 10 μm. The functional channel proteins expressed by bCNGA3-C-HA are localized at the surface of the cell. Indeed, as we demonstrate elsewhere in this report, they are localized in the plasma membrane. Non-functional bCNGA3-S<sub>1S2</sub>-HA, in contrast, fails to drive protein expression altogether. In panel C is an immunoblot of total proteins from cells transfected with each of the constructs, as well as cells transfected with wt channels lacking the HA tag. HA-tagged channel proteins are identified with anti-HA and an enzyme-linked secondary antibody. As expected the untagged channel protein is functional, but does not yield detectable bands in the blot. bCNGA3-S<sub>1S2</sub>-HA does not drive protein expression at all and, hence, protein bands are not detected. The expression pattern of amino- and carboxyl-terminal-tagged proteins is identical: two sharp bands at about 90 and 93 kDa and a diffuse band centered at about 102 kDa.
revertant Q296R cDNA, the holding current at −40 mV was −5.6 ± 3.7 pA and peak uncaging flash-generated current at −75 mV was −59.3 ± 36.8 pA (range −20 to −96 pA, n = 3). That is, our mutagenesis protocol introduced the desired point mutations in S4 without causing unintended changes in the cDNA and neutralizing the charge of one specific amino acid in S4 caused failure of functional channel expression.

We explored the functional consequence of individually mutating most of the residues in the S4 motif. The wt sequence in bCNGA3 and the mutations we tested are listed in Table I. The data presented are the mean holding current at −40 mV, the mean and range of current amplitude activated by flash uncaging at −75 mV, and the ratio of the number of cells that exhibited flash-activated current over the total number of EGFP-positive cells sampled. The holding current was similar in value in every one of the cells analyzed, independently of CNG channel function. Effects of the mutations were of only two kinds: either channel cDNA failed to express detectable currents, or it expressed currents that were indistinguishable from wt currents.

As documented in Table I, replacement of any of the charged amino acids (Arg293, Arg296, Lys299, or Arg302) by neutral ones, one at a time or all four simultaneously, caused failure of functional expression. This was true regardless of the identity of the neutral amino acid, channels in which Arg296 was replaced by Gln or Cys or Arg302 by Gln or Trp did not function. We tested the Cys and Trp mutations in particular because these are the natural mutations found in achromatopsia. Channel functional expression was also affected by mutation of some, but not all, of the neutral residues in S4. We found that conservative replacements of neutral amino acids by other neutral amino acids were without consequence in some positions (Phe294, Leu297, and Leu303), whereas in other positions they caused functional channel failure (Asn295 and Leu297).

Functional Assay of HA-tagged bCNGA3 Constructs—We developed a tool to track the biochemical processing and cellular expression of the CNG channel proteins. We modified wt cDNA to drive the in-frame expression of the HA epitope at three different locations: 1) amino terminus (bCNGA3-N-HA); 2) carboxyl terminus (bCNGA3-C-HA); and 3) extracellular loop between transmembrane helices 1 and 2 (bCNGA3-S1S2-HA). Cells were transformed with each of the three constructs and channel function was assayed electrophysiologically, while protein processing and expression were studied with immunoblots and immunocytochemistry using anti-HA antibodies.

The HA tag in the carboxyl-terminal allowed functional channel expression indistinguishable from that of untagged wt channels. In cells transformed with bCNGA3-C-HA, the mean holding current at −40 mV was −13.8 ± 13.4 pA (n = 11) and the mean flash-generated current at −75 mV was −44.6 ± 94.2 pA (n = 11, range −15 to −280 pA) (Fig. 2A, top). Immunoblots of these cells revealed that in the steady-state channel protein exists in three states characterized by apparent molecular masses of about 90, 93, and 102 kDa in SDS gels (Fig. 2C). The lower mass bands always appeared sharp and there was little uncertainty in their molecular weight; the heaviest band was always wide and diffuse and the apparent molecular mass we describe is that at the middle of the band. High-resolution, deconvoluted confocal images indicate that functional channel proteins were exclusively detected in the surface of the cell (Fig. 2B, top). As we show below they are, indeed, localized in the plasma membrane.

Electrophysiological characteristics of cells transformed with bCNGA3-C-HA constructs mutated in S4 are the same as those of cells transformed with S4 mutants of untagged channels. Table I presents detailed measurements of the holding current and the response to uncaging light flashes in cells transfected with various S4 bCNGA3-C-HA mutants. Within the statistical uncertainty of our data, the effects of S4 mutations in HA-tagged and untagged bCNGA3 channels are indistinguishable.

The other two HA-tagged constructs failed to yield functional channels. bCNGA3-S1S2-HA was unable to command tran-
scription and/or translation. Cells transformed with this construct had typical holding currents at −40 mV, mean −11.6 ± 7.9 pA, n = 5, but did not exhibit current changes upon flash illumination (Fig. 2A, middle). Furthermore, in these cells there was no detectable expression of HA-containing proteins in either immunoblots (Fig. 2C) or cell images (Fig. 2B, bottom). Although bCNGA3-N-HA did not yield functional channels (n = 6) (Fig. 2A, bottom), the protein expression pattern and cellular transport were the same as that of functional channels (Fig. 2C). The channel protein existed in 3 states of molecular masses 90, 93, and 102 kDa and they were detected on the cell surface. In fact, as we show below, at the plasma membrane.

N-linked Glycosylation Is Not Required for Wild Type bCNGA3 Functional Channel Expression—Experimental evidence demonstrates that the 327NDT sequence in mammalian rod CNG channels (CNGA1) is the only site of core glycosylation (44). Glycosylation at this site, however, is not required to yield functional ion channels. Thus, glycosylation of CNGA3 proteins is not required to yield functional ion channels.

The gly(−)-bCNGA3 mutant yields functional channels, but their protein processing pattern is different from wt channels. Whereas immunoblots of wt-transformed cells exhibit three channel protein bands (Fig. 2C), non-glycosylated proteins appear only as a single sharp band at about 90 kDa size, the same as the very smallest of the bands in wt channel (Fig. 3C). The difference in expression pattern between gly(−) and wt constructs suggests that wt channel proteins are glycosylated in the course of normal cellular processing. They convert from an unglycosylated state, at 90 kDa, to two other glycosylated states of different masses, 93 and 102 kDa. Although they are processed differently, both wt and gly(−) channel proteins arrive at the plasma membrane in Fig. 3B we present confocal images of cells transfected with wt or gly(−)-bCNGA3-C-HA. Protein expressed by both constructs is localized at the surface of the cell, in fact, as we show below, at the plasma membrane. In brief, then, core glycosylation and changes in mass of a 90-kDa state are not required per se to yield functional CNGA3 channels, these events, nonetheless, are milestones that mark the passage of the channel protein through distinct cellular maturation steps.

The Functional Effect of S4 Mutations Is Correlated with Abnormal Cell Protein Processing—To investigate cellular protein processing of S4 mutants we compared cell immunoblots of various HA-tagged bCNGA3 S4 mutants (Fig. 4). The cells, regardless of whether channels were functional, expressed every one of the mutant proteins. The protein expression pattern was of only two types, one common to all functional mutants and the other to all non-functional mutants. Functional mu-
tants expressed a protein pattern identical to wt channels: two sharp bands at about 90 and 93 kDa and a diffuse one at 102 kDa. Non-functional channels express only the two small, sharp bands, unglycosylated at 90 kDa and glycosylated at 93 kDa. Overexposure of ECL blots affirmed that only the 90- and 93-kDa bands are expressed by S4 mutant proteins (Fig. 4B). S4 mutations that cause channel function failure are improperly processed and do not reach the 102 kDa. We note, however, that successful conversion from light to heavy glycosylated states does not warranty channel function, because the bCNGA3-N-HA mutant has a wt-like protein conversion, but does not yield functional channels (Fig. 2C, lane 4).

Immature S4 Mutant Proteins Do Not Arrive at the Plasma Membrane—Mutant channels could be in the plasma membrane and inoperative or might have failed to arrive at the plasma membrane. We assessed intracellular protein traffic using immunocytochemistry. We determined the subcellular distribution of the protein by testing whether the channel protein did or not colocalize with independent markers of specific cellular compartments.

In Fig. 5 we present images of cells simultaneously transformed with pEGFP-F and various tagged bCNGA3 constructs. pEGFP-F promotes the exclusive expression of EGFP in the plasma membrane where it appears as a sharp, narrow fence at the perimeter of the cell. The wt channels are functional, and of course, are expressed in the plasma membrane where they colocalize with EGFP-F. The HA amino-terminal-tagged channel (bCNGA3-N-HA) is not functional, yet also colocalized with EGFP at the plasma membrane. Recall that the non-functional bCNGA3-N-HA is glycosylated indistinguishably from the wt protein (Fig. 2). These results indicate that the HA tag on the amino-terminal does not interfere with cellular protein traffic and the channels arrive at the plasma membrane, but they are inoperative.

As would be expected, every one of the protein S4 mutants that yielded functional channels colocalized with EGFP-P at the plasma membrane. These include wt, unglycosylated (gly(−)), or functional S4 mutants (F294L). In contrast, every one of the non-functional S4 mutants we tested, whether mutated in charged (R296Q) or neutral (N295Q) amino acids, where not at the plasma membrane, but localized in spatially restricted compartments within the cytoplasm of the cell. That is, the S4 mutation interfered with normal intracellular protein traffic and prevented transfer to the plasma membrane.

Molecular Defects in Achromatopsia-like CNG Ion Channels—We explored the mechanisms of the functional defect in S4 mutants transformed to match the genetic alteration frequently found in humans afflicted by achromatopsia (8). We measured the electrophysiological properties and channel protein expression pattern in cells transfected with R296C and R302W. The electrical properties of the cells are listed in Table I. As in other mutations of charged residues, the achromatopsia-like channels were not functional and did not exhibit currents upon flash release of cyclic nucleotides (Fig. 6B). Similarly, immunoblots and cell images demonstrated that the mutant channels were expressed, but processed abnormally. Channel proteins were core glycosylated and reached the 90- and 93-kDa states, but failed to mature into the 102-kDa state (Fig. 6A). The incompletely processed proteins did not localize in the plasma membrane, but remained trapped within spatially restricted intracellular compartments (Fig. 6).

Coexpression of β Subunits Does Not Rescue the Functional Defect in S4 Mutants—β Subunits in voltage-gated K+ channels can function as weak chaperones and facilitate the intracellular assembly and transit of normal channels (46, 47). Because S4 mutants are defective in their cellular assembly/traffic we tested whether cone CNG channel β subunits (designated as CNGB3) might affect the behavior of a subunits. We simultaneously transformed cells with equal weight amounts of plasmid DNA to code wt mouse CNGB3 and various bCNGA3 constructs.

Coexpressing wt α and β subunits in the same cell yielded currents electrically indistinguishable from those produced by α subunits alone. In these cells, holding currents at −40 mV was −6.3 ± 4.3 pA and flash release of cyclic nucleotide at −75 mV generated a current of mean amplitude −164.3 ± 254.6 pA (range −10 to −667 pA, n = 6). The I–V curve and reversal voltage of the nucleotide-dependent currents were identical. We tested the effects of β on three different α subunit S4 mutants, each representing a specific class of mutation. For
every mutant class tested, we list the mean holding current at -40 mV and the number of cells tested: 1) charged to neutral amino acids, R296Q (Im = -0.5 pA, n = 2); 2) neutral to neutral amino acid, L297A (Im = -2.3 pA, n = 4); and 3) human natural mutation: R302W (Im = -10 pA, n = 3). In every cell tested uncaging cyclic nucleotide failed to activate the detectable current at -75 mV. Thus, the functional phenotype of S4 mutants was not changed by coexpression of S4 subunits.

S4 Channel Mutant Proteins Are Trapped within the Endoplasmic Reticulum—To determine the intracellular compartments in which non-functional S4 mutants are trapped we assessed colocalization of tagged channel proteins and EGFP specifically targeted to either endoplasmic reticulum or Golgi apparatus. In Fig. 7, we present fluorescent images of cells simultaneously transformed with pEGFP-ER and various tagged bCNGA3 constructs. pEGFP-ER promotes the exclusive expression of EGFP in the endoplasmic reticulum. The functional channels, wt, unglycosylated (gly(-)), or S4 mutant F294L are not localized in the endoplasmic reticulum. In contrast, every one of the non-functional channel mutant proteins is colocalized with EGFP-ER in the endoplasmic reticulum. In Fig. 8 we present fluorescent images of cells simultaneously transformed with various tagged bCNGA3 constructs and pEGFP-TG, a plasmid that drives the expression of EGFP in Golgi apparatus. Every one of the functional channels, wt, unglycosylated (gly(-)), or S4 mutant F294L, are not localized in the Golgi. Also, every one of the non-functional mutants did not colocalize with EGFP in the Golgi. That is, alteration of the S4 domain in CNG channels causes the protein to remain trapped in the endoplasmic reticulum.

DISCUSSION

Specific point mutations in the S4 motif cause failure of functional maturation of a subunit of the bCNGA3. In a mammalian cell line, normal channel proteins are first core glycosylated at asparagine 357, and then processed to a heavier glycosylated form as they transit through the cells on their way to the plasma membrane (reviewed in Ref. 7). Mutations of specific, not all, charged and neutral amino acids in S4 cause functional channel failure. Although loss of function could arise from many different mechanisms, every one of the S4 mutant proteins we tested fail by the same mechanism: they are translated and core glycosylated, but fail to be processed further along the normal cellular maturation pathway and remain trapped within the endoplasmic reticulum. Mutation in the S4 motif of CNGA3 proteins similar to those we investigated here occur naturally in the human population and are associated with achromatopsia, a congenital disease characterized by complete loss of cone photoreceptor function (8). Achromatopsia, however, is an end state phenotype caused by several other natural mutations, including some in the cone CNG channels (48, 49).

In the normal course of cellular processing, CNGA3 proteins undergo a sequence of N-linked glycosylations that tag the protein as it matures, but glycosylation is not absolutely required for channel function. In agreement with a previous study of CNGA1 (45), we have found that despite lack of gly-
cosylation, CNGA3 proteins can transit through the cell, reach the plasma membrane, and function as normal ion channels. Of course, we cannot determine whether the pathway of transit in the absence of glycosylation is the same as for \textit{wt} proteins, but proteins lacking N-linked glycosylation arrive at the correct end point in a properly assembled state. Moreover, glycosylation alone does not assure normal function. The amino HA-tagged channel protein is properly glycosylated and transported to the plasma membrane just as the \textit{wt} protein, but it does not yield functional channels. The exact mechanism of functional failure in this mutant is unknown; although proteins are seemingly processed and transported normally, the tag in the cytoplasmic amino-terminal interferes with gating. Gordon and Zagotta (50) and Mottig \textit{et al.} (51) have suggested that linkage between nucleotide binding at the carboxyl-terminal of the channel and gating involves allosteric structural changes in several different regions of the protein, both in transmembrane and cytoplasmic domains.

The role of N-linked glycosylation in channel protein traffic and function cannot be generalized, even within the superfamily of voltage-gated K⁺ channels. Whereas N-linked glycosylation is not required for functional expression of Shaker or Kv K⁺ channels (52, 53), in HCN (54) and HERG channels (55) it is required for proper cell surface targeting and function.

Experimental studies of the function of S4 in CNG channels have not been previously reported. We expect that functional behavior similar to that of the cone photoreceptor CNGA3 will be true of CNG \(\alpha\) subunits of other neurons, such as rod photoreceptors (CNGA1) and olfactory sensory cells (CNGA2), because this motif is highly conserved among the various sensory neurons (Fig. 9). Conservancy of S4 among CNG channels is not only in its sequence, but also in the number of repeat tandems, 4, and their location along the 4th transmembrane helix (Fig. 9).

We began an exploration of structure-function relationships of S4 in CNG ion channels because, while not voltage-gated,
these channels exhibit a number of weak voltage-dependent functions. Whether the structure of S4 affects these functions remains to be determined, but it plays a critical role in channel maturation. In voltage-gated K^+ channels it has also been found that some of the S4-charged residues are important in guiding channel protein maturation. There is, however, a remarkable difference between CNG and voltage-gated channels. In CNG channels neutralizing any or all of the charged residues causes failure of maturation, whereas in voltage-gated K^+ channels the majority of the residues can be neutralized without affecting functional expression, although voltage-gated function is altered. In Shaker-type voltage-gated K^+ channels, for example, neutralizing 2 of 7 S4 charged residues causes failure to mature, but the others do not interfere with functional surface expression (13, 23). Similarly, in HCN channels, mutation of only one charged residue (out of 8) causes complete failure of channel maturation and changes at two other sites cause significant, but not absolute failure of surface expression (19, 26).

The S4 motif is, in fact, not a single structure within the superfamily of voltage-gated K^+ channels. In every instance, S4 consists of the tandem repeat of a R- or K-XX sequence and it is always in the 4th transmembrane helix. Detailed structure, however, varies in complexity among various channels, as depicted in Fig. 7. Three general statements seem consistent with the data accumulated to date. 1) S4 always has a role in the folding/trafficking of channel proteins, but it may or may not serve as a voltage sensor. 2) Channels in which S4 acts as a voltage sensor have no less than 5 repeats of the tandem amino acid repeat theme. 3) The portion of the S4 sequence associated with protein cellular maturation (folding/traffic?) does not have a unique, consistent localization along the thickness of the lipid bilayer of the membrane.

The structural changes in S4 that matter in guiding proper processing are specific. Not all amino acids within S4 in CNG ion channels are important in guiding protein functional maturation. Certainly, replacement of any or all of the charged residues by neutral ones causes failure, regardless of the neutral amino acid used for the substitution. Among the wt neutral amino acids, however, some can and some cannot be changed without loss of functional maturation (Table I). The identity of the critical versus non-critical neutral amino acids is not related to their hydrophilicity, but their position. The neutral amino acids whose change causes functional failure are absolutely conserved among CNG channels, independently of the cell type or species in which they are expressed (Fig. 9).

A refined and detailed understanding of the molecular event(s) that fail while cells process CNG channel S4 mutants...
requires further investigation. Nonetheless, evidence in the literature identifies three specific events as plausible causative candidates (reviews in Refs. 7, 21, and 22). 1) The S4 sequence plays a role as a translocation signal helping the nascent channel protein interact with a translocon to facilitate peptide integrating into the ER membrane (25). This mechanism is unlikely to explain our findings because the mutant channels do undergo correct glycosylation. Hence they are likely to have been properly targeted and translocated across the ER membrane. 2) It is energetically unfavorable to insert charged amino acids into the low dielectric environment of the lipid bilayer. Elegant experiments on Shaker K+ channels indicates that neutralization of the positive charges in S4 by near neighbor negative charges in transmembrane domains 2 and 3 is required for proper channel functional maturation (24). In the mutants we have constructed, and the natural mutations associated with human achromatopsia, the loss of this charge neutralization is unlikely to be the sole cause of functional loss because conservative mutations of some of the neutral amino acids in S4 have the same consequence as mutation of the charged amino acids.

CNG channels in the plasma membrane are heteromeric and composed of 3 α and 1 β subunits (4–6). The subunits interact with each other in the course of normal protein maturation and cell transit. This interaction is made evident by recent work of Trudeau and Zagotta (1) who discovered a rod α subunit mutant (CNGA1-RP) that fails to form functional channels when coexpressed with the β subunits (CNGB1), but not when expressed alone. S4 α mutants could conceivably fail to form proper homomers. Coexpression of a β cone subunit does not repair the functional defect in S4 α mutants. This is consistent with findings in humans afflicted by achromatopsia because of natural mutations in S4 of the α, in whom there is no evidence that β is defective, and yet cone photoreceptors are not functional. In contrast, a recent report indicates that some mutations in cone α subunits, which alter the functional features of the channel when compared with wild type channels, are rescued (revert to wild type phenotype) when β subunits are coexpressed with them (49).

The imaging data affirms the electrical finding that functional channel proteins, whether glycosylated or not, are expressed in the plasma membrane (as opposed to being in the endoplasmic reticulum membranes, as evidenced by their subcellular localization and the fact they are core-glycosylated. However, they remain trapped in the endoplasmic reticulum and fail to transit to the Golgi apparatus. An intact S4 seems required for proper channel protein folding and/or assembly in the ER membranes. This requirement is absolute whether the channel assembly consists of α subunits alone or both α and β subunits.

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