Localization of the α_{1F} Calcium Channel Subunit in the Rat Retina

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PURPOSE. The molecular identity of the calcium channels that mediate glutamate release from photoreceptors is unknown. Mutations in the recently identified, retina-specific α_{1F} calcium channel subunit cause incomplete X-linked congenital stationary night blindness (CSNB2), the phenotype of which is consistent with a defect in neurotransmission within the retina. The purpose of this study was to determine the cellular distribution of the α_{1F} subunit in the retina.

METHODS. Antibodies were raised against a unique peptide from the human α_{1F} sequence. Rat retina sections were labeled with affinity-purified α_{1F} antibodies and the immunofluorescence analyzed by confocal microscopy. The α_{1F} staining was compared with that obtained with a pan-α_{1} antibody, used to reveal the distribution of known voltage-gated calcium channels in the retina. Some sections were double labeled for α_{1F} and the photoreceptor synaptic ribbon marker, bassoon.

RESULTS. Staining of retina sections with anti-α_{1F} resulted in strong punctate labeling in the outer plexiform layer (OPL) and weak punctate labeling in the inner plexiform layer (IPL), consistent with a synaptic localization. Staining was also observed in the outer nuclear layer. Within the OPL, α_{1F} immunoreactivity was clustered in discrete, horseshoe-shaped patches, the shape and dimensions of which are characteristic of rod active zones. Similar structures were labeled with the pan-α_{1} antibody. Localization of α_{1F} immunoreactivity to rod active zones was confirmed by double labeling for bassoon, a component of photoreceptor synaptic ribbons.

CONCLUSIONS. The distribution of α_{1F} immunoreactivity in the OPL suggests that calcium influx through α_{1F} or α_{1F}-like channels mediates glutamate release from rod photoreceptors.

Photoreceptors in the retina are depolarized in darkness and tonically release the neurotransmitter, glutamate, at a high rate. Detection of photons causes a hyperpolarization of the photoreceptor membrane potential, and a decrease in the rate of glutamate release. Ultrastructurally, photoreceptor active zones are characterized by the presence of an electron-dense plate, known as the synaptic ribbon, extending from the active zone into the cytoplasm. Therefore, these synapses are referred to as ribbon synapses. In addition to photoreceptors, ribbon synapses are formed by other sensory neurons that signal by graded transmitter release including retinal bipolar cells and inner ear hair cells.

As at conventional synapses, neurotransmitter release at ribbon synapses is triggered by the influx of calcium through voltage-gated calcium channels. The electrophysiological properties of the calcium channels and their distribution within the nerve terminal are likely to be key determinants of the release properties of a synapse. The calcium channels at phasic synapses in the brain have been well characterized molecularly and physiologically. They open at relatively high voltages and can be physically associated with docked synaptic vesicles. These properties result in tight temporal coupling of transmitter release to action potentials.

The calcium currents of fish and amphibian photoreceptors are sensitive to dihydropyridines (DHPs); thus, they have been classified as L-type. Localization of α_{1C} calcium channel subunits to the synaptic layers of the salamander retina suggests that L-type channels mediate transmitter release in the amphibian retina. In contrast, both the molecular identity and subcellular distribution of the calcium channels of mammalian photoreceptors are unknown. The calcium currents of cone photoreceptors in the tree shrew, and monkey retinas have been characterized electrophysiologically and appear similar to L-type currents, although the activation threshold is more hyperpolarized and the DHP sensitivity lower than for other L-type channels. The synaptic terminals of long-wavelength cones in the tree shrew retina can be labeled with antibodies against the α_{1D} subunit of brain L-type calcium channels, but staining is absent from short-wavelength cones and rods.

Recently, a novel calcium channel gene, CACNA1F, was identified that encodes the α_{1F} subunit of a retina-specific, voltage-gated calcium channel, α_{1F}. Sequence comparisons show that α_{1F} is a member of the L-type family of α_{1} subunits, displaying the greatest amino acid identity (62%) to the α_{1D} subunit of brain L-type calcium channels. Mutations in CACNA1F cause incomplete X-linked congenital stationary night blindness (CSNB2), a recessive nonprogressive visual disease, the phenotype of which is consistent with a defect in neurotransmission within the retina between the photoreceptors and second-order neurons.

The present work shows that α_{1F} immunoreactivity is localized to photoreceptor cell bodies and the synaptic terminals of rod photoreceptors in the rat retina. Moreover, it appears to be colocalized with rod active zones, implicating α_{1F} or α_{1F}-like channels in the release of glutamate at this synapse.

Preliminary results of this work have been reported in abstract form.

MATERIALS AND METHODS

α_{1F} Antiserum

The production of anti-α_{1F} antiserum was performed by Chiron Technologies (Melbourne, Australia), as described previously. Sheep were immunized with a peptide corresponding to amino acids 712 to 730 of human α_{1F} (peptide sequence: SNEKDLQENELVPGVEK) coupled through an additional C-terminal cysteine to diptheria toxin (DT). Immune serum was collected after two immunizations and stored at...
FIGURE 1. The distribution of HVA calcium channels in the rat retina revealed with a pan-α₁ antibody recognizing a conserved epitope on known HVA calcium channel α₁ subunits. (A) Pan-α₁ immunofluorescent staining of a vertical section of rat retina. (B) Pan-α₁ staining in the OPL in a vertical rat retina section. The pan-α₁ antibody stained two types of structures in the OPL: numerous smaller puncta and sparser, but larger patches (C). (C) High-magnification image of pan-α₁ staining in the OPL of an obliquely cut retina section. Arrowheads: labeled crescent-shaped structures that are putative rod active zones. is, inner segments; gcl, ganglion cell layer. Scale bar, (A) 15 μm; (C) 4 μm.

−20°C. Anti-α₁ antibodies were affinity purified against 1 mg α₁ peptide coupled to an N-hydroxysuccinimide (NHS)-activated affinity column (HTrap; Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer’s protocol.

Preparation of Retina Sections
Wistar Kyoto rats 6 to 8 weeks of age were killed by injection with an overdose of pentobarbital and eyes removed. The eyes were cut in half and posterior eyecups fixed in 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS) for 5 to 15 minutes for α₁ staining or 4% (wt/vol) carbodiimide in phosphate buffer (PB) for 30 minutes for pan-α₁ staining. The eyecups were cryoprotected in sucrose, embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA) and sectioned at 12-μm thickness on a cryostat. Sections were collected on gelatin-coated slides, air-dried, and stored at −20°C. All animals were used in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Immunofluorescence Microscopy
Retina sections were blocked by incubation for 30 minutes at room temperature (RT) in antibody incubation solution (AIS; 0.5% Triton X-100, 5% horse serum, 0.05% NaN₃ in PBS). Sections were then incubated in the primary antibody diluted in AIS for either 2 hours or overnight at RT. The sections were washed three times in PBS and then incubated for 1 hour at RT in the secondary antibody diluted in AIS. Sections were again washed in PBS and then coverslipped with Mowiol (Hoechst, Strasbourg, Germany). The following primary antibodies were used at the indicated dilutions: anti-α₁ (1:10³), anti-pan-α₁ (1:50; Alomone Laboratories, Jerusalem, Israel), and anti-bassoon (1:10; StressGen Biotechnologies Corp., Victoria, British Columbia, Canada). The following secondary antibodies were used (all from Jackson ImmunoResearch Laboratories, West Grove, PA): donkey anti-sheep-IgG coupled to carboxyethylindocyanine (Cy3; 1:500), goat-anti-rabbit-IgG-Cy3 (1:500), goat-anti-mouse-IgG-Cy3 (1:500), and donkey-anti-sheep-IgG coupled to fluorescent isothiocyanate (FITC; 1:50). The sections were analyzed with a confocal laser scanning microscope (model TCS 4D; Leica, Heidelberg, Germany) outfitted with a ×40 1.4 numerical aperture oil immersion objective. In the double-labeling experiments, neither cross-labeling of secondary antibodies nor bleed-through between the two filter sets was observed. Images of single optical sections of approximately 0.5-μm thickness were collected and imported into image analysis software for editing (Adobe Photoshop, San Jose, CA). Image enhancement was limited to minor adjustments to image brightness, which were made uniformly over the entire image.

Western Blot Analysis
Rats were anesthetized with a lethal intraperitoneal injection of pentobarbital and their retinas removed. Subcellular retina fractions were prepared by adapting the protocol of Muresan et al., as follows. All steps were performed at 0°C to 4°C. Two rat retinas were immersed in 750 μl ice-cold PB buffer (15 mM phosphate buffer [pH 7.4], 1 mM MgCl₂, 1 mM EGTA, and 0.025% NaN₃). A protease inhibitor cocktail (50 μl; 4-(2-aminoethoxy)benzenesulfonyl fluoride [AEBSF], pepstatin A, E-64, bestatin, leupeptin, aprotinin; Sigma Chemical Co., St. Louis, MO) was added to the buffer (PB) and the tissue was homogenized by hand with 40 up-and-down strokes in a 1-ml glass homogenizer with a Teflon pestle. The homogenate was layered over 500 μl 50% sucrose in PB in a 1.5-ml microcentrifuge tube and centrifuged for 10 min at 15,000g, 4°C in a microcentrifuge (Heraeus Amsersil, Duluth, GA). The membrane fraction at the buffer-sucrose interface was collected and suspended in 1× SDS sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis on precast 4% to 12% Bis-Tris gels (Novex; San Diego, CA) and immunoblotted using chemiluminescence detection (ECL; Amersham International, Amersham, UK) as previously described. Antibodies were used at the following concentrations: 1:1000 anti-α₁ (human), and 1:5000 donkey anti-sheep IgG coupled to horseradish peroxidase (HRP; Jackson ImmunoResearch Laboratories).

RESULTS
Total Calcium Channel Distribution in the Retina
A pan-α₁ antibody recognizing the highly conserved β-subunit binding site on calcium channel α₁ subunits was used to reveal the localization of voltage-activated calcium channels in the rat retina (Fig. 1). At low power the pan-α₁ antibody predominantly stained the synaptic layers of the retina, the outer plexiform layer (OPL), and the inner plexiform layer (IPL). The staining of both plexiform layers appeared punctate, consistent with the localization of calcium channels to retinal synapses. In addition to the synaptic staining, the pan-α₁ antibody labeled the photoreceptor inner segments and, faintly, the neuronal cell bodies in the inner (INL) and outer (ONL) nuclear layers. All pan-α₁ staining was blocked by preincubation of the antibody with the peptide antigen (not shown).

At higher magnification, two types of pan-α₁ staining were observed in the OPL, appearing as numerous elliptical puncta and sparser, large patches (Fig. 1B). Some of the pan-α₁ puncta were crescent-shaped (Fig. 1C). Rods, which constitute approximately 99% of the photoreceptors in the rat retina, have a single active zone, whereas the less numerous cones have larger terminals and, in the primate retina, contain 20 to 30 active zones. Thus, it is possible that the large patches of pan-α₁ staining correspond to cone terminals and the smaller puncta to rod terminals.
Distribution of $\alpha_{1F}$ Immunoreactivity in the Rat Retina

Affinity-purified $\alpha_{1F}$ antibodies against a unique peptide derived from the human $\alpha_{1F}$ sequence were used to determine the distribution of $\alpha_{1F}$-like calcium channels in the rat retina. On a Western blot of rat retinal membranes, these antibodies labeled two prominent bands of 142 and 150 kDa and fainter bands at 125 and 170 kDa (Fig. 2D). Preincubation of the antibody with the $\alpha_{1F}$ peptide completely blocked these bands, thus demonstrating specificity (not shown). Immunofluorescent staining of vertical retina sections with anti-$\alpha_{1F}$ resulted in intense staining of the OPL and fainter staining of the IPL (Fig. 2B). The $\alpha_{1F}$ staining in the IPL, compared with that in the OPL, was somewhat fainter and sparser than was the pan-$\alpha_1$ staining, suggesting that the $\alpha_{1F}$ immunoreactivity may be localized to a subset of IPL synapses. Faint $\alpha_{1F}$ staining was also observed over the ONL. The specificity of the $\alpha_{1F}$ labeling was confirmed by complete block of the staining by preincubation of the antibody with the $\alpha_{1F}$ peptide (Fig. 2C) and by the absence of staining with the preimmune serum (data not shown).

Localization of $\alpha_{1F}$ Immunoreactivity in the OPL

Calcium channel staining in the OPL of the rat is shown at higher magnification in Figure 3. The $\alpha_{1F}$ antibody labeled horseshoe-shaped structures approximately 2 $\mu$m in length, characteristic of rod active zones as defined by the synaptic ribbons. The horseshoe shapes of the $\alpha_{1F}$ staining in the OPL suggest that, rather than being diffusely distributed on the plasma membrane of the rod spherule, the calcium channels are closely associated with the active zone beneath the single synaptic ribbon of the rod photoreceptor. Labeling with the pan-$\alpha_1$ antibody confirmed the presence of calcium channels associated with these structures (Fig. 1C). Because of the low proportion of cone photoreceptors in the rodent retina, it was less clear whether they were also labeled with anti-$\alpha_{1F}$. The large patches of staining, possibly corresponding to cone terminals, seen with the pan-$\alpha_1$ antibody (Fig. 1), were not observed with the $\alpha_{1F}$ antibody (Figs. 3A, 3B). A few aggregates of $\alpha_{1F}$ staining were seen (Fig. 3B, arrowheads) in which discrete horseshoe shapes could not be distinguished. These $\alpha_{1F}$ aggregates may reside in cone terminals, which contain more numerous but smaller active zones than rods, although they are smaller than the patches detected by the pan-$\alpha_1$ antibody.

The localization of $\alpha_{1F}$ immunoreactivity to rod active zones was confirmed by double labeling retina sections with the $\alpha_{1F}$ antibody and an antibody against the active zone marker, bassoon. Bassoon is a 420-kDa protein that is a component of the presynaptic density in conventional synapses, but that has been localized by immunoelectron microscopy to the base of photoreceptor synaptic ribbons in the OPL of the rat retina. The $\alpha_{1F}$ and bassoon immunoreactivities were localized to similar horseshoe-shaped structures in the OPL. The two staining patterns partially overlapped in the OPL (Fig. 4), demonstrating close apposition of the calcium channels with rod photoreceptor active zones.

DISCUSSION

Recently, the CACNA1F gene, encoding the retina-specific $\alpha_{1F}$ calcium channel subunit, has been identified as the site of
mutations causing CSNB2 in humans.\textsuperscript{15,16} The phenotype of the disease implicates the $\alpha_{1F}$ calcium channel in neurotransmission between photoreceptors and inner retinal neurons. Previous work by Morgans et al. (manuscript submitted) shows that at least two $\alpha_{1F}$ isoforms or $\alpha_{1F}$-like channels are present in the retina, one localized to photoreceptor cell bodies and the other to the OPL. The antibody used in the present study labels both the OPL and photoreceptor cell bodies. This study compares the distribution of $\alpha_{1F}$ immunoreactivity to the total distribution of calcium channel $\alpha_{1}$ subunits in the rat retina and shows that $\alpha_{1F}$ immunoreactivity in the OPL is localized to the active zones of rod photoreceptors.

Detailed reconstruction of the rod terminal shows that rods have a single linear active zone approximately 2 $\mu$m long that curves around an invagination of the presynaptic plasma membrane into which the postsynaptic elements protrude.\textsuperscript{25} Curving over the length of the active zone is the synaptic ribbon, an electron-dense, plate-like structure to which numerous synaptic vesicles are tethered.\textsuperscript{25} At high magnification, the $\alpha_{1F}$ immunofluorescence in the OPL (Figs. 3, 4) appeared to consist mainly of numerous horseshoe-shaped arcs approximately 2 $\mu$m long. The $\alpha_{1}$ immunostaining in the OPL was very similar to that observed for proteins associated with photoreceptor synaptic ribbons: KIF1A,\textsuperscript{27} rab interacting molecule (Rim),\textsuperscript{28} B16,\textsuperscript{22,25} and bassoon.\textsuperscript{26} This highly characteristic staining pattern is a strong indication that the $\alpha_{1F}$ immunoreactivity is associated with the rod active zone. This is confirmed in Figure 4 by the overlap between $\alpha_{1F}$ staining in the OPL and staining for the active zone marker, bassoon, located at the base of synaptic ribbons in photoreceptors.\textsuperscript{26}

It was less obvious whether cone terminals in the rod-dominated rat retina\textsuperscript{22} were labeled with the $\alpha_{1F}$ antibody. Unlike the single, large active zone of the rod terminal, cones contain numerous, smaller active zones clustered at the base of the terminal. The pan-$\alpha_{1}$ antibody, which recognizes all known high-voltage-activated (HVA) calcium channels, would be expected to label both rod and cone terminals. Similar to the $\alpha_{1F}$ staining in the OPL, the pan-$\alpha_{1}$ antibody labeled arcs approximately 2-$\mu$m long, characteristic of rod active zones (Fig. 1). In addition, the pan-$\alpha_{1}$ antibody stained sparsely distributed, large patches that are putative cone terminals, based on their low ratio to the small puncta (~1:40–1:80, depending on the tissue section). The $\alpha_{1F}$ antibody also labeled occasional aggregates of smaller puncta that may have been cone terminals, but these patches were smaller than those labeled with the pan-$\alpha_{1}$ antibody. The difference in the pan-$\alpha_{1}$ and $\alpha_{1F}$ staining in the OPL suggests that cone terminals may contain additional calcium channels that are recognized by the pan-$\alpha_{1}$, but not the $\alpha_{1F}$ antibody. Heterogeneity among photoreceptor calcium channels has been described previously in the tree shrew retina\textsuperscript{14} where the synaptic terminals of long wavelength cones were labeled with an $\alpha_{1C}$ subunit antibody, whereas short-wavelength cones and rods were not.

The molecular identity of photoreceptor calcium channels may differ across species. This is suggested by comparison of the present findings with those of Nachman-Clewner et al.\textsuperscript{11} demonstrating immunoreactivity to $\alpha_{1C}$ subunits in photoreceptor terminals in the salamander retina. Within the terminals of dissociated photoreceptors, the $\alpha_{1C}$ staining appeared in hot spots, possibly representing active zones. Differences in the L-type channels expressed by mammalian and amphibian photoreceptors may underlie the apparent differences in the DHP sensitivity of their respective calcium currents.\textsuperscript{8,10,12,13} Nachman-Clewner et al.\textsuperscript{11} also show $\alpha_{1C}$ immunoreactivity in the OPL of the rat retina, although it is not clear whether the staining is in photoreceptor terminals or horizontal cell dendrites, and the specificity of the staining in rat tissue is not demonstrated.

The localization of $\alpha_{1F}$ immunoreactivity to rod, and possibly cone, active zones implicates $\alpha_{1F}$ or $\alpha_{1F}$-like channels in the release of glutamate from photoreceptors. If $\alpha_{1F}$ were the only presynaptic calcium channel in rod terminals, loss-of-function mutations might be expected to eliminate synaptic transmission from rods and result in complete night blindness, rather than the incomplete night-blind phenotype of CSNB2.\textsuperscript{17} The absence of $\alpha_{1F}$ activity during development in individuals with CSNB2 may lead to changes in retinal circuitry that partially compensate for the absence of transmission from rods. For instance, an upregulation of gap junctions between rods and cones might allow the rod signal to be partially transmitted through cones. This could explain the elevated stimulus threshold and delay of the scotopic threshold response (STR) associated with CSNB2.\textsuperscript{51} The $\alpha_{1F}$ immunoreactivity in the ONL suggests that the $\alpha_{1F}$ channel serves an additional function in retinal physiology, besides mediating transmitter release, which may also contribute to the CSNB2 phenotype.

The finding that $\alpha_{1F}$ immunoreactivity is closely associated with the synaptic ribbon in the rod photoreceptor, rather than being diffusely distributed around the synaptic terminal, argues for the plasma membrane at the base of the ribbon being the primary, if not the sole, site of neurotransmitter release. The restriction of the calcium channels to the active zone suggests that they are anchored in place, perhaps as a component of a macromolecular active zone complex, the analysis of which should yield further insight into organization and function of the neurotransmitter release machinery of the ribbon synapse.

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References