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Colocalization of HCN Channel Subunits in Rat Retinal Ganglion Cells

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Abstract

The current-passing pore of mammalian hyperpolarization-activated, cyclic nucleotide-gated ("HCN") channels is formed by subunit isoforms denoted HCN1-4. In various brain areas, antibodies directed against multiple isoforms bind to single neurons and the current (I_h) passed during hyperpolarizations differs from that of heterologously expressed homomeric channels. By contrast, retinal rod, cone, and bipolar cells appear to use homomeric HCN channels. Here, we assess the generality of this pattern by examining HCN1 and HCN4 immunoreactivity in rat retinal ganglion cells, measuring I_h in dissociated cells, and testing whether HCN1 and HCN4 protein coimmunoprecipitate. Nearly half of the ganglion cells in whole-mounted retinæ bound antibodies against both isoforms. Consistent with colocalization and physical association, 8-bromo-cAMP shifted the voltage-sensitivity of I_h less than that of HCN4 channels and more than that of HCN1 channels, and HCN1 coimmunoprecipitated with HCN4 from membrane fraction proteins. Lastly, the immunopositive somata ranged in diameter from the smallest to the largest in rat retina, the dendrites of immunopositive cells arborized at various levels of the inner plexiform layer and over fields of different diameters, and I_h activated with similar kinetics and proportions of fast and slow components in small, medium, and large somata. These results show that different HCN subunits colocalize in single retinal ganglion cells, identify a subunit that can reconcile native I_h properties with the previously reported presence of HCN4 in these cells, and indicate that I_h is biophysically similar in morphologically diverse retinal ganglion cells and differs from I_h in rods, cones, and bipolar cells.

Keywords

HCN; inward rectification; immunohistochemistry; immunoprecipitation; retina

INTRODUCTION

Hyperpolarization-activated, cyclic nucleotide-gated ("HCN") channels contribute to fundamental properties of neurons, including resting potential, input resistance, dendritic integration, rhythmic voltage changes, and rebound excitation (Santoro and Tibbs, 1999; Biel et al., 2009). The current-passing pore of these channels is formed in mammals by subunit isoforms denoted HCN1-4, and immunoreactivities for multiple isoforms are found in various brain areas and single neurons (Notomi and Shigemoto, 2004; Morris et al., 2004;

Chan et al., 2004; Abbas et al., 2006; Fried et al., 2010). Consistent with self-assembly and coassembly of different isoforms observed in heterologous expression systems (Whitaker et al., 2007), confocal images of the binding of different isoform-specific antibodies show substantial, yet not complete, colocalization. Moreover, the binding of these antibodies has been found to change by different amounts and at different times during development (Vasilyev and Barish, 2002; Surges et al., 2006). These results suggest that single cells may contain mixtures of heteromeric and homomeric HCN channels. The ability to coimmunoprecipitate different isoforms (Much et al., 2003; Brewster et al., 2005; Whitaker et al., 2007), and the inability to predict the gating kinetics, voltage-sensitivity, and modulation by cAMP of whole-cell current (" I_h ") by simple sums of current passing through homomeric channels (Chen et al., 2001; Ulens and Tytgat, 2001), are consistent with presence of heteromeric channels. This is functionally important, as the association of different isoforms not only alters properties of the total current, but may also support membrane expression and cooperative gating (Er et al., 2003; Much et al., 2003; Dekker and Yellen, 2006).

By contrast, retinal rods, cones, and some bipolar cells are thought to use homomeric HCN channels (Demontis et al., 2002; Müller et al., 2003; Cangiano et al., 2007; Knop et al., 2008; Barrow and Wu, 2009). Do retinal neurons generally differ from other central neurons by using only homomeric HCN channels? We test this here in adult rat retina, where nearly half of the ganglion cells bind anti-HCN4 antibodies (Oi et al., 2008) and some somata in the ganglion cell layer were found to bind anti-HCN1 antibodies (Müller et al., 2003; Fyk-Kolodziej and Pourcho, 2007). As a first step, we ask which cells express these HCN subunits. Because previous studies did not test whether HCN1-immunopositive somata are ganglion cells or displaced amacrine cells (Perry, 1981), the immunopositive cells found to date could consist of (i) HCN4-expressing ganglion cells and HCN1-expressing amacrine cells, (ii) separate HCN4-expressing ganglion cells and HCN1-expressing ganglion cells, or (iii) a fraction of the ganglion cell population that expresses both HCN1 and HCN4. We therefore use isoform-specific antibodies and confocal imaging to test for presence of HCN1 and HCN4 in individual ganglion cells, whole-cell patch-clamp recording to test whether cAMP alters I_h as in cells expressing only HCN1 or HCN4, and coimmunoprecipitation to test whether HCN1 and HCN4 heteromerically associate. Furthermore, we test the possibility that presence of these HCN isoforms in nearly half of the ganglion cell population is due to exclusive expression by either on- or off-center ganglion cells (Kuffler, 1953). We did not test for presence of other isoforms, because HCN2 was not found in the ganglion cell layer of rat (Müller et al., 2003; Fyk-Kolodziej and Pourcho, 2007) despite having been found in the mouse ganglion cell layer (Mataruga et al., 2007) and the HCN3 current properties published to date (Stieber et al., 2005; Mistrík et al., 2005) are unlikely to account for the ganglion cell I_h properties we report here.

MATERIALS and METHODS

Animals

Adult rat retinæ were used for the experiments reported here because (i) I_h can be activated in rat retinal ganglion cells (Lee and Ishida, 2007), (ii) ganglion cells of various sizes in all retinal quadrants bind anti-HCN4 antibodies (Oi et al., 2008) and (iii) some ganglion cell layer somata bind anti-HCN1 antibodies (Müller et al., 2003; Fyk-Kolodziej and Pourcho, 2007). Adult Long-Evans rats (female; P60–P120; 150–250 g) were obtained from a commercial supplier (Harlan Bioproducts; San Diego, CA) and housed in standard cages at ~23 °C on a 12-hr/12-hr light/dark cycle. Prior to enucleating eyes for all of the experiments described here, rats were killed by a lethal dose of sodium pentobarbital (150 mg/kg, i.p.). All animal care and experimental protocols were approved by the Animal Use and Care Administrative Advisory Committee of the University of California, Davis.

Antibody characterization

The primary antibodies used in this study are described in Table 1.

The rabbit polyclonal anti-HCN1 antibody was generated against a synthetic peptide corresponding to amino acids 6–24 of rat HCN1. In western blots, this antibody bound to protein with an apparent molecular weight estimated to be 112 kD and, in some blots, a lower weight band at approximately 104 kD. Preincubation of the primary antibody with the immunizing peptide abolished binding to these proteins (see Results).

The rabbit polyclonal anti-HCN4 antibody was generated against a fusion protein of glutathione *S*-transferase (GST) and a sequence corresponding to amino acids 119–155 of human HCN4. In western blots, this antibody bound to protein with an apparent molecular weight estimated to be 135 kD. Preincubation with the immunogen reduced immunostaining below detectable levels in both Western blots and immunohistochemistry (Oi et al., 2008).

The mouse monoclonal anti-HCN1 antibody was raised against a fusion protein of GST and amino acids 778–910 of rat HCN1. In western blots, this antibody bound to protein with an apparent molecular weight estimated to be 112 kD (see Results). Identical immunohistochemical binding patterns were obtained with the mouse and rabbit anti-HCN1 antibodies.

The mouse monoclonal anti-HCN4 antibody was raised against a fusion protein of GST and amino acids 1010–1108 of rat HCN4. In western blots, this antibody bound to protein with an apparent molecular weight estimated to be 135 kD and, in some blots, also around 144 kD. Identical immunohistochemical binding patterns were obtained with the mouse and rabbit anti-HCN4 antibodies (see Results).

The mouse monoclonal antibodies against HCN1 and HCN4 were developed by the UC Davis/NIH NeuroMab Facility. This facility is supported by NIH grant U24NS050606 and maintained by the Department of Neurobiology, Physiology and Behavior, College of Biological Sciences, University of California, Davis, CA 95616.

The goat polyclonal antibody against choline acetyltransferase (ChAT) was generated against ChAT extracted from human placenta. The staining pattern in transretinal ("vertical") sections and flat-mounted retina was identical to that observed by others using this antibody in rat retina (Fyk-Kolodziej and Pourcho, 2007).

The Alexa Fluor 488-conjugated rabbit polyclonal antibody against green fluorescent protein (GFP) was raised against GFP isolated from *Aequorea victoria*, and has previously been used in rodent retina (Xu and Tian, 2008; Liu et al., 2009). We detected no binding of this antibody in rat retina unless GFP expression was induced (results not illustrated).

The rabbit polyclonal antibody against voltage-gated sodium channels (VGSC) was directed against a synthetic peptide corresponding to amino acids 1501–1518 of rat VGSC α subunit. In Western blots, this antibody bound to proteins with apparent molecular weights estimated to be 230 kD and 250 kD, consistent with binding to more than one channel isoform (Boiko et al., 2001).

The mouse monoclonal anti-ABCA4 antibody was raised against the 220 kD rim protein isolated from a bovine source. In western blots, this antibody bound to protein with an apparent molecular weight estimated to be greater than 220 kD, as in other studies of rat retinal homogenates (Bhongsatiern et al., 2005).

The mouse monoclonal antibody against parvalbumin was raised against frog muscle parvalbumin. The staining pattern in vertical sections and flat-mounted retina was identical to that observed in previous studies of rodent retina (Wässle et al., 1993; Partida et al., 2004).

Membrane fraction protein isolation

Retinae were isolated in phosphate-buffered saline (PBS, pH 7.4), collected in micro-centrifuge tubes, and frozen by liquid nitrogen immersion. Ten to twenty retinae were homogenized in lysis buffer (0.32 M sucrose, 0.1 M Tris-HCl, pH 7.4)(see below for the source of all chemicals used in this study). The homogenate was centrifuged at $600 \times g$ for 10 min at 4 °C, and the supernatant was centrifuged at $22,000 \times g$ for 20 min at 4 °C. The final pellet was collected and resuspended in radioimmunoprecipitation (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl, 1% NP-40, 1% Triton X-100, 1 mM EDTA, pH 7.4). Total protein concentration was determined using the Bradford method. Unless otherwise noted, all solutions were ice cold and supplemented with a protease inhibitor cocktail (Complete Mini, 1 tablet per 10 mL).

Western blots

Western blots were formed as described previously (Partida et al., 2004; Oi et al., 2008). Membrane fraction proteins were loaded at 50–100 µg per lane onto a 4–12% gradient Bis-Tris gel (NP0321BOX, Invitrogen; Grand Island, NY), electrophoretically separated in MOPS running buffer at 200 V for 1 hr, and transferred to a polyvinylidene difluoride (PVDF) membrane (162–0174, Bio-Rad; Hercules, CA) at 30 V for 60–90 min. Protein standards (Benchmark and See Blue) were run in lanes adjacent to the samples. The PVDF membrane was divided and incubated on a rocker for 1 hr at room temperature with isoform-specific monoclonal antibody, isoform-specific polyclonal antibody, or isoform-specific polyclonal antibody that had been pre-incubated with a two-fold higher concentration of immunogen on a rocker overnight at 4 °C. All primary antibodies were diluted in PBS. After rinsing in Tris-buffered saline supplemented with 0.1% Tween-20, the membranes were incubated in species-specific secondary antibody conjugated to horseradish peroxidase for 1 hr at room temperature. The secondary antibody was a donkey anti-rabbit IgG conjugated to horseradish peroxidase (NA934V, GE Healthcare Life Science; Piscataway, NJ) for rabbit primary antibodies, and a sheep anti-mouse IgG conjugated to horseradish peroxidase (NA931, GE Healthcare) for mouse primary antibodies. Protein bands were visualized by ECL detection with SuperSignal West Pico Chemiluminescent Substrate.

Immunoprecipitation

Following previously described procedures (Brewster et al., 2005), membrane fraction proteins were diluted to 1 mg/mL in 750 µL RIPA buffer, and incubated with anti-HCN1 or anti-HCN4 antibodies in PBS on a rocker overnight at 4 °C. The resulting antibody-antigen complexes were incubated with immobilized Protein A or immobilized Protein G agarose beads (20333 and 20398, respectively, Thermo Fisher Scientific; Waltham, MA; or, in some experiments, 100.01D and 100.03D, respectively, Invitrogen) on a rocker for 2 hr at 4 °C. After separating the beads from the supernatant, un-bound proteins were washed from these beads by multiple rinses in RIPA buffer and PBS (pH 7.4). Proteins obtained from the supernatant are referred to hereafter as the "flow-through" fraction. A "pull-down" fraction was formed by eluting bound proteins off the beads by boiling in lithium dodecyl sulfate (LDS) sample buffer for 5 min.

Deglycosylation

To test for glycosylation, immunoprecipitated HCN protein was eluted from beads and the collected volume divided in half. Both halves were mixed 1:1 with deglycosylation buffer solution (15 mM Tris-HCl, 20 mM orthophenanthroline, 1% Triton X-100), and PNGase F (1000 U) was added to one of the halves. Both halves were then warmed to 37 °C overnight in a water bath, electrophoretically separated on SDS gels, and western blotted.

Retrograde labeling

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The conjunctiva was cut and the globe retracted with forceps to expose the optic nerve. After nicking the optic nerve with scissors, approximately 2 mg of fixable 10-kD dextran conjugated to fluorescein (D1820, Invitrogen) was deposited against the retinal end of the cut optic nerve fibers. Each rat was allowed to survive 23 to 28 hr to allow the retrograde marker to reach the retina.

Biolistic gene transfer

DNA constructs were generated using standard molecular biology protocols. All constructs were fully sequenced to check for accuracy of the cloning procedure. The plasmid pCAG-eGFP-WPRE contains a hybrid cytomegalovirus enhancer and chicken-beta actin promoter (CAG) driving expression of enhanced green fluorescent protein (eGFP), followed by the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). Plasmid sequences are available upon request. Plasmid DNA (25 µg) was precipitated onto 1.6 µm gold microparticles and delivered *ex-vivo* to freshly dissected rat retina whole mount cultures as described (Koizumi et al., 2007). To achieve sparse eGFP labeling, microparticles were propelled through two 20-µm nylon mesh screens (Small Parts, Inc.; Miami Lakes, FL) that were placed 1 cm above the retina. Immediately following gene transfer, whole mount interphase retina cultures were incubated at 35 °C and 5% CO₂ for 18–20 hr in Ames' culture medium supplemented with 1% horse serum, 1% N2 supplement, 100 U/mL penicillin, 100 U/mL streptomycin, and 0.3 mg/mL L-glutamine.

Immunohistochemistry

Retinae were immunohistochemically processed either as vertical (transretinal) sections or flat mounts. In these experiments, primary and secondary antibodies were diluted in PBS supplemented with bovine serum albumin and Triton-X 100 (0.5% w/v and 0.1% v/v, respectively), and unconjugated Fab fragments were diluted in this buffer along with 5% (v/v) donkey serum.

To form vertical sections, eyes were enucleated, nicked at the corneal limbus, immersed in freshly prepared paraformaldehyde (4% in PBS, pH 8.0–8.5) for 60 min at room temperature, and hemisected. After discarding the anterior portion and removing the lens, the remaining “eyecup” was further fixed in paraformaldehyde (2% in PBS, pH 7.4) for 12–16 hr at 4 °C. Retinae were isolated, rinsed in PBS (pH 7.4), incubated for 30 min in glycine (1% w/v in PBS) to quench residual aldehyde activity, embedded in 3% (w/v) Type VIIA agarose at 34 °C, allowed to set for 30–60 min at 4 °C, and cut into blocks with a razor. These blocks were sectioned vertically at a thickness of 100 µm on a vibrating microtome (VT1000S, Leica Microsystems; Wetzlar, Germany). Free-floating sections were blocked and permeabilized for 4 hr at 4 °C in PBS (pH 7.4) supplemented with 0.5% bovine serum albumin, 0.05% Triton X-100, 0.1% sodium azide, and 5% normal goat serum (see <http://www.lifesci.ucsb.edu/mcdb/labs/fisher/protocols.html>). The sections were incubated in primary antibody overnight at 4 °C, rinsed with PBS, and incubated for 2.5 hr in secondary antibody at room temperature. After rinsing, sections were mounted directly to

glass coverslips (no. 1.5), covered with Vectashield mounting medium, and secured onto glass slides.

To form flat mounts, freshly dissected retinæ were quartered in PBS, placed vitreous side up on a nitrocellulose filter disk (HABG01300, Millipore; Billerica, MA), and flattened onto the filter by applying suction to the opposite side. The flattened retinæ were fixed in modified Davidson's fluid (Latendresse et al., 2002) for 30 min at room temperature. Fixed retinæ were isolated from the filters and thinned by manually slicing with a razor to separate proximal retina (including ganglion cell and inner plexiform layers) from distal retina (Oi et al., 2008). Free-floating pieces of proximal retina were rinsed in TBS, quenched for 30 min in 1% (w/v) glycine, and blocked and permeabilized overnight at 4 °C in PBS (pH 7.4) supplemented with 0.5% bovine serum albumin, 0.05% Triton X-100, and either 5% normal goat serum or 5% normal donkey serum. These pieces of retina were then incubated 1–3 days in primary antibody at 4 °C, rinsed with PBS, and processed for indirect immunofluorescence with secondary antibodies.

Gene-gunned retinæ were immersed for 30 min in paraformaldehyde (4% in Sorensen's phosphate buffer; 17.3 mM sodium phosphate (monobasic), 49.3 mM sodium phosphate (dibasic), pH 7.4, osmolarity adjusted with 150 mM sucrose). To reduce synaptic activity and spiking, this fixative was supplemented with glutamate receptor antagonists (10 μ M RS-CPP, 10 μ M CNQX, 20 μ M L-AP4) and 1 μ M tetrodotoxin. The fixed retinæ were then rinsed in TBS, incubated for 1 hr in a blocking and permeabilizing solution (PBS supplemented with 0.5% bovine serum albumin, 0.5% Triton X-100, and 5% normal donkey serum; pH 7.4), incubated overnight in primary antibody at 4 °C, rinsed in PBS, and incubated with secondary antibodies. During two of these steps (blocking/permeabilizing; secondary antibody incubation), the retinæ were irradiated in a laboratory microwave (PELCO BioWave Pro, Ted Pella; Redding, CA) alternating at 60-sec intervals between 150-W and 0-W power under constant vacuum (22 inches Hg).

The secondary antibodies were species- and isotype-specific, highly cross-adsorbed IgGs. These were derived from goat sera, conjugated to Alexa Fluor 488, 555, 568, or 647 (anti-rabbit: A11034, A21429, A11036, and A21244, respectively; anti-mouse: A11001, A21422, A11004, and A21235, respectively; Invitrogen), and diluted 1:300 (6.7 μ g/mL). Signal attributed to retrogradely filled ganglion cells was amplified by use of Alexa Fluor 488-conjugated anti-fluorescein antibody derived from goat (A11096, Invitrogen), diluted 1:300 (3.3 μ g/mL). Signal due to GFP was amplified by use of a polyclonal anti-GFP antibody conjugated to Alexa Fluor 488. The excitation and emission wavelengths of these fluorophores produced minimal cross-contamination (Partida et al., 2004). Tissues incubated in secondary antibodies without any primary antibodies displayed no fluorescence at the confocal imaging and display settings used to generate any of the figures.

When staining with multiple primary antibodies, pairs of primary and secondary antibodies were applied sequentially by incubating in the first primary antibody, the first fluorophore-conjugated secondary, the second primary antibody and then the second fluorophore-conjugated secondary, with ample PBS rinses in between. To minimize the chance of cross-reactivity, the first primary was stained with anti-mouse Fab fragments (Wessel and McClay, 1986). This was followed by incubation for 4–6 hrs on a rocker at 4 °C in unconjugated donkey-derived anti-mouse monovalent Fab fragments (715-007-003, Jackson ImmunoResearch) diluted 1:1000 (approx. 1.5 μ g/mL). The second primary antibody was stained in turn with whole IgG antibodies derived in donkey and conjugated with DyLight 549 (715-505-151, Jackson ImmunoResearch), diluted 1:300 (5.0 μ g/mL).

Following final rinses in PBS, sections were mounted directly to glass coverslips (no. 1.5), covered with Vectashield mounting medium, and secured onto glass slides.

Negative controls for binding of polyclonal primary antibodies were performed by pre-incubation with either the synthetic peptide that generated the anti-HCN1 antibody (AB5884, Millipore) or the fusion protein that generated the anti-HCN4 antibody (Accession NP_005468). Pairs of sections or flat-mounted pieces of retina were prepared from a single eye or from opposite eyes of a single animal. While one of the so-paired samples was incubated in raw primary antibody, the other was incubated in primary antibody that was pre-incubated with the immunogen for either 6 hr at room temperature or overnight at 4 °C; these paired samples were processed identically at all other stages of each protocol.

Two additional controls were performed on both vertically sectioned tissue and flat-mounted tissue, to test whether more than one monoclonal primary antibody bound to the same secondary antibody. One applied an antibody directed against an HCN subunit isoform, a Fab fragment secondary antibody, an anti-parvalbumin antibody that does not bind to ganglion cells in adult rat retina (Wässle et al., 1993; Partida et al., 2004), and then an IgG secondary antibody. The other control tested whether the second secondary antibody bound to the first primary antibody after incubation in the first secondary antibody by omitting the second primary antibody.

Imaging

After exciting the fluorescent dyes conjugated to the secondary antibodies and introduced dextran, confocal images were acquired on a laser scanning confocal microscope. Excitation was provided by Ar (488 nm), Kr (568 nm), green He-Ne (543 nm), and red He-Ne (633 nm) lasers. Vertical section images were collected on a FluoView FV300 Confocal System (version 4.3, Olympus; Center Valley, PA) interfaced to an Olympus IX-70 inverted microscope. Data were collected using an oil immersion 40× objective in series of optical sections at 0.35- to 1-μm intervals, with 2- or 3-frame Kalman averaging for each section. Whole mounts were imaged on a FluoView FV1000 Confocal System (version 1.6, Olympus) interfaced to an Olympus IX-81 inverted microscope. Analysis of most of the flat-mounted tissue was restricted to the ganglion cell and nerve fiber layers, where data were collected with an oil immersion 60× objective in series of optical sections at 0.44-μm intervals, with 3-frame Kalman averaging for each section. Adjacent optical sections were sometimes merged to form a projected image. Some imaging of the flat-mounted tissue extended into the inner nuclear layer for comparison with the vertically sectioned tissue.

When acquiring images of different fluorophores, we excited each fluorophore sequentially and collected each emission channel independently to minimize bleed-through artifacts and dye cross-reactivity. Images due to each dye were separated and cropped, and changes in color space were applied as needed using the public domain program ImageJ (version 1.43, available on the Internet at <http://rsb.info.nih.gov/ij/>). Any subsequent adjustments to brightness or contrast were performed and overlay images were generated in Photoshop CS4 (Adobe Systems; San Jose, CA). ImageJ was also used to measure ganglion cell somata sizes (in μm) and densities (in numbers per mm²). Cell sizes are reported here as equivalent diameters calculated by averaging the maximum and minimum Feret's diameters (i.e., the long and short axes) of each cell. Cell densities were also measured from these merged images. Partial cells were not counted and their area was subtracted in the calculation.

Analysis of relative levels of immunofluorescence was performed using ImageJ and Photoshop CS4. In ImageJ, z-stacks of each whole mount field were formed by collapsing 10 adjacent optical sections followed by export to Photoshop CS4 as RGB TIFF image files.

Each retrogradely-filled ganglion cell with HCN immunoreactivity was copied from the field, separated into component red, green, and blue channels, and placed into an individual image file on a black background. The mean grey values for the red and green channels of each cell were measured in ImageJ and used to calculate a red:green ratio (R:G) of relative fluorescence. Intensity measures from each cell were normalized against the population mean red and green intensities for cells from their fields of origin to standardize the analysis of tissue from different animals and imaging sessions. In this analysis, HCN1-like immunoreactivity was always assigned to the red channel in RGB color space and HCN4-like immunoreactivity was always assigned to the green channel. The calculated R:G thus represents the relative proportion of HCN1-like immunoreactivity to HCN4-like immunoreactivity.

A subset of cells from one retina was analyzed as above, but spatial resolution was improved by using single optical sections, and only immunoreactivity from the cell membrane was used in calculations to yield membrane R:G. These values were directly comparable and thus did not require normalization. The degree of overlap between red and green pixels in each cell was determined by Manders' overlap coefficient (MOC), a measure of pixel overlap that is independent of average intensity values (Manders et al., 1993; Bolte and Cordelières, 2006). MOC was calculated using the Manders' Coefficients plugin for ImageJ, written by Tony Collins and provided by the Wright Cell Imaging Facility (available on the Internet at <http://www.uhnresearch.ca/facilities/wcif/software/>).

Whole-cell patch-clamp

I_h was recorded from dissociated adult rat retinal ganglion cell somata using dissociation protocols, cell immunopanning by anti-Thy-1 antibody, whole-cell patch electrodes, an Axopatch 200B patch-clamp amplifier (Molecular Devices; Sunnyvale, CA), stimulus generation and data acquisition via pCLAMP software (v. 9.2.1.9, Molecular Devices), perforated-patch recording mode, U-tube microperfusion of solutions, liquid junction potential correction, and grounding via an agar bridge, as described elsewhere (Lee and Ishida, 2007). We previously described the block of this current by Cs^+ and ZD7288, relative permeability to Na^+ and K^+ ions, activation at resting potential, and contribution to rebound excitation (Lee and Ishida, 2007), and did not repeat those measurements here. The recording electrode solution contained (in mM): 115 K-D-gluconic acid, 15 KCl, 15 NaOH, 2.6 MgCl_2 , 0.34 CaCl_2 , 1 EGTA, 10 HEPES, and 5 glutathione (reduced); the pH of this solution was adjusted to 7.4 by adding methanesulfonic acid. Amphotericin B was solubilized in DMSO and included in the pipet solution as the perforating agent at a final concentration of 240 or 480 $\mu\text{g/mL}$. The extracellular solution contained (in mM): 125 NaCl, 26 NaHCO_3 , 1.25 NaH_2PO_4 , 3.5 KCl, 0.1 CaCl_2 , 3.4 MgSO_4 , and 10 D-glucose ; the pH of this solution was adjusted to 7.4 by bubbling with carbogen (95% O_2 , 5% CO_2). The osmolality of these solutions measured 260–280 mmol/kg and 290–310 mmol/kg, respectively. All currents were recorded at 35 °C, because recordings at 37–38 °C became too unstable to assess shifts produced by cAMP.

The voltage-sensitivity of I_h activation was measured from tail currents (Lee and Ishida, 2007). The voltage steps used to activate I_h and the holding potential at which the tails were measured overlap with holding and test potentials used to study voltage-gated Na^+ and Ca^{2+} currents. Therefore, tetrodotoxin (1 μM) was included in the external solution to block voltage-gated Na^+ current, and the external Ca^{2+} and Mg^{2+} concentrations were changed from typical control values to 0.1 mM and 3.4 mM, respectively, to block voltage-gated Ca^{2+} current while maintaining typical total divalent cation concentrations. Addition of 3 mM Cs^+ blocked the time- and voltage-dependent current remaining under these conditions, signifying that the tails were sufficiently isolated to measure the activation range of I_h (see

Fig. 8, 9 of Hayashida et al., 2009). Using a least-squares nonlinear curve fitting routine in KaleidaGraph (v. 4.0J, Synergy Software; Reading, PA), a Boltzmann equation:

$$I_v = I_{\max} / \{1 + \exp[(V - V_{1/2})/s]\}$$

was fitted to the currents recorded after repolarizing cells from various test potentials to a set holding potential, where $I_{\max}$ is the maximum current amplitude, V is the activating voltage (in mV), $V_{1/2}$ is the voltage that activates half of the maximum conductance, and s is a slope factor. As reported for some other tissues (e.g., Yanagida et al., 2000), the activation range found in the present study at 35 °C was more positive than when I_h is measured in the same configuration at room temperature (Lee and Ishida, 2007).

As in other preparations (Santoro et al., 2000; Chen et al., 2001), the increase in amplitude of I_h during hyperpolarizing voltage steps was fitted by the sum of two exponential time functions:

$$A_f e^{-t/\tau_f} + A_s e^{-t/\tau_s} + C$$

where f and s denote fast and slow (respectively), A_x is the maximum amplitude of component x , t is time, τ_x is the time constant for the change in A_x , and C is an offset constant (Lee and Ishida, 2007). Based on the current activated by hyperpolarizations to -92 mV, inverses of the median values of τ_f at 35 °C (Fig. 6) and at 22 °C (Lee and Ishida, 2007) give a temperature coefficient (Q_{10}) of 3.3. Although measurements at different temperatures were not made in single cells, this is well within the range reported for other preparations (e.g., DiFrancesco and Ojeda, 1980).

Reagents

Reagents were obtained from the following sources: Abbott (Chicago, IL): sodium pentobarbital (0074-378-05); Bio-Rad (Hercules, CA): Bradford reagent (500-0006), sodium dodecyl sulfate (SDS; 161-0300); BDH Laboratory Supplies (Poole, UK): CaCl_2 ; Calbiochem (San Diego, CA): tetrodotoxin (584411); GE Healthcare Life Science: glycerol (56-81-5); Invitrogen: phosphate-buffered saline (Ca^{2+} - and Mg^{2+} -free, pH 7.4; 70011-044), Bench Mark protein ladder (10747-012), See Blue Plus2 pre-stained standard (LC5925), LDS sample buffer (NP0007), reducing agent (NP0004), MOPS running buffer (NP0001), transfer buffer (NP0006), 10-kD dextran conjugated to fluorescein (D1820), N2 (17502-048), L-glutamine (25030-081), penicillin/streptomycin (15140-122); Jackson ImmunoResearch: normal goat serum (005000121), normal donkey serum (017000121); New England BioLabs: PNGase F (P0704S); Roche (Indianapolis, IN): Complete Mini protease inhibitor cocktail (11836153001); Sigma-Aldrich (St. Louis, MO): amphotericin B (10041), aprotinin (A6279), benzamidine (B6506), bovine serum albumin (BSA; 8806), 8-bromo-cAMP (B5386), DNase I (D4527), ethylenediamine tetraacetic acid (EDTA; E6758), b glycerol phosphate (G6376), horse serum (H1138-500ml), orthophenanthroline (P-9375), NaF (S6521), sodium orthovanadate (S6508), Ponceau S (P7767), SQ22536 [9-(tetrahydro-2-furanyl)-9H-purin-6-amine] (S153), Tween 20 (P9416), Type VIIA agarose (A0701); Thermo Fisher Scientific: NP-40 (28324), SuperSignal West Pico Chemiluminescent Substrate (34080), Triton X-100 (BP151-100); Tocris Bioscience (Ellisville, MO): CNQX [6-cyano-7-nitroquinoxaline-2,3-dione] (0190), L-AP4 [L-(+)-2-amino-4-phosphonobutyric acid] (0103), (RS)-CPP [(RS)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid] (0173); Vector Laboratories (Burlingame, CA): Vectashield (H-1000). The salts (NaCl , etc.) used for electrophysiological recordings and buffers were all reagent grade, and obtained from Sigma-Aldrich unless otherwise specified.

RESULTS

This study uses immunohistochemical, electrophysiological, biochemical, and anatomical methods to characterize the distribution and disposition of HCN1 and HCN4 subunits in adult rat retinal ganglion cells. Isoform-specific antibodies and confocal imaging are used to test the possibility that HCN1 and HCN4 subunits are present in individual ganglion cells. Whole-cell patch-clamp recording is used to assess HCN channel properties, by measuring how much cAMP shifts the voltage-sensitivity of I_h in ganglion cells and by comparing the activation kinetics in cells of different sizes. Because HCN1- and HCN4-like immunoreactivities colocalize in single cells, and because the properties of I_h are intermediate between those of homomeric HCN1 and HCN4 channels, we use coimmunoprecipitation to test the possibility that HCN1 and HCN4 proteins are physically associated. Lastly, because HCN1 and HCN4 colocalize in ganglion cell somata but are rarely observed in dendrites, we immunohistochemically stain retinæ after inducing expression of green fluorescent protein.

Western blots

To substantiate the isoform-specificity of our immunohistochemical stainings, we used antibodies directed against two amino acid sequences of rat HCN1 and rat HCN4 (cf., Rhodes and Trimmer, 2006). Because the retinal proteins bound by these antibodies have not previously been compared, we tested for binding to protein in western blots prepared from membrane proteins isolated from whole rat retina homogenate. The HCN1 results were obtained with a mouse monoclonal antibody directed against amino acids 778–910 (clone N70/28, NeuroMab) and an affinity-purified rabbit polyclonal antibody directed against amino acids 6–24 (AB5884, Millipore). The mouse monoclonal (Fig. 1, lane 4) and rabbit polyclonal (Fig. 1, lane 5) antibodies both bound rat retinal membrane fraction protein with an apparent molecular weight of around 112 ± 4 kD (mean $\pm$ SD, $n=3$). A band at a lower apparent molecular weight (104 ± 1 kD (mean $\pm$ SD; $n=6$)) was also bound by the polyclonal antibody. Pre-incubating the polyclonal antibody with immunogen reduced its binding to undetectably low levels (Fig. 1, lane 6). In agreement with previously published results (Much et al., 2003; Müller et al., 2003), the higher molecular weight band was not seen when membrane fraction proteins were incubated with an amidase (PNGase F; Fig. 2) and thus appeared to be a glycosylated form of the lower molecular weight band. The latter value agrees with the molecular weight calculated from the amino acid sequence of rat HCN1 (see the SwissProt database at <http://www.expasy.ch/sprot/sprot-top.html>), the apparent molecular weight of rat retinal protein bound by a rat monoclonal antibody directed against amino acids 650–685 of rat HCN1 (Müller et al., 2003), rat retinal protein bound by a rabbit polyclonal antibody directed against the same amino acid sequence as the polyclonal antibody we used (Müller et al., 2003), and rabbit retinal protein bound by a rabbit polyclonal antibody directed against amino acids 819–837 of rabbit HCN1 (Demontis et al., 2002).

The HCN4 results of this study were obtained with a mouse monoclonal antibody directed against amino acids 1019–1108 of the rat HCN4 sequence (clone N114/10, NeuroMab) and an affinity-purified rabbit polyclonal antibody directed against amino acids 119–155 of the human HCN4 sequence (AB5808, Millipore). The mouse monoclonal (Fig. 1, lane 3) and rabbit polyclonal (Fig. 1, lane 2; see also Oi et al., 2008) antibodies bound rat retinal membrane fraction protein with an apparent molecular weight around 135 ± 3 kD (mean $\pm$ SD, $n=6$). These results agree with those obtained with a rat monoclonal antibody against a different amino acid sequence of HCN4 (Müller et al., 2003) and with the molecular weight calculated from the rat HCN4 amino acid sequence (see the SwissProt database at <http://www.expasy.ch/sprot/sprot-top.html>). Occasionally, two bands (144 ± 3 kD and 139 ± 2 kD; mean $\pm$ SEM, $n=4$) were detected when probing with anti-HCN4 antibodies. This was

unexpected because a previous study found that, while HCN4 expressed in HEK293 cells was glycosylated, HCN4 obtained from rat retina was not (Müller et al., 2003). However, treatment as above with PNGase F consistently resulted in loss of the higher molecular weight band. Although the difference in migration between the two anti-HCN4 antibody-binding bands is subtle (Fig. 2), it was statistically significant by a paired t-test ($p < 0.02$), suggesting that the HCN4 we collected included glycosylated and non-glycosylated forms. Moreover, a similarly small shift was recently reported for HCN4 protein obtained from homogenates of mouse olfactory bulb (Fried et al., 2010).

Vertical sections, positive controls, negative controls

We next used the mouse monoclonal primary antibodies, together with fluorophore-conjugated secondary antibodies, to localize HCN1- and HCN4-like immunoreactivity to the major retinal cell layers. Because these antibodies have not previously been used for this purpose, we compared the binding patterns we achieved with those found in other studies (Müller et al., 2003; Fyk-Kolodziej and Pourcho, 2007). As internal controls, we also examined binding by polyclonal antibodies directed against different portions of the HCN1 and HCN4 amino acid sequences (see Materials and Methods). Because the staining of cells in vertical sections and flat mounts by each polyclonal antibody alone was fully consistent with that achieved with the monoclonal antibodies, only monoclonal antibodies were used to produce the confocal images shown here.

Laser scanning confocal imaging of transretinal vibratome sections showed that HCN1-like immunoreactivity and HCN4-like immunoreactivity crisply outlined some somata in both the inner nuclear and ganglion cell layers (Fig. 3A–D), as found with other anti-HCN1 antibodies in rabbit and rat ganglion cell layers (Kim et al., 2003; Müller et al., 2003), photoreceptor inner segments (Demontis et al., 2002; Müller et al., 2003), and other neuronal somata (e.g., Holderith et al., 2003; Doan et al., 2004). In contrast to the binding of both anti-HCN1 and anti-HCN4 antibodies in the inner nuclear and ganglion cell layers, HCN1-like immunoreactivity, but not HCN4-like immunoreactivity, was seen in photoreceptor inner segments (Fig. 3A,B). Photoreceptor outer segments did not display either HCN1- or HCN4-like immunoreactivities. Moreover, while the binding of anti-HCN1 and HCN4 antibodies produced dense stippling at two levels of the inner plexiform layer, HCN1-like immunoreactivity was more intense in the proximal level, and HCN4-like immunoreactivity was more intense in the distal level (Fig. 3; Müller et al., 2003; Fyk-Kolodziej and Pourcho, 2007). We encountered HCN1- and HCN4-like immunoreactivities in a few primary dendrites (e.g., Fig. 4). However, in agreement with the results of previous studies (Müller et al., 2003; Kim et al., 2003), we found few HCN-immunopositive ganglion cell dendrites extending into the sublaminae noted above (Fig. 3).

The difference in staining patterns by the isoform-specific antibodies we used (e.g., binding of anti-HCN1, but not anti-HCN4, antibodies to photoreceptor inner segments) provide positive controls against non-specific binding and agree with the results of Müller et al. (2003). The binding of these antibodies was similarly specific in flat-mounted retinæ, as shown by optical sections collected serially from the ganglion cell layer into the inner nuclear layer (Fig. 3E–G). Negative controls were performed by pre-incubating polyclonal primary antibodies with immunogens. The HCN1 peptide immunogen suppressed binding of the anti-HCN1 antibody (data not illustrated), while the HCN4 fusion protein immunogen suppressed binding of the anti-HCN4 antibody (see also Oi et al., 2008).

Previous studies of retinal HCN1 and HCN4 reported localizations that, for the most part, resemble ours (Demontis et al., 2002; Kim et al., 2003; Müller et al., 2003; Feigenspan et al., 2004; Ivanova and Müller, 2006; Cangiano et al., 2007; Fyk-Kolodziej and Pourcho, 2007; Mataruga et al., 2007). One difference is that these studies show immunoreactivity for

these isoforms in relatively few somata within the ganglion cell layer (e.g., Fig. 2B of Müller et al., 2003; Fig. 7 of Feigenspan et al., 2004; Fig. 5 of Mataruga et al., 2007), while we found HCN4-like immunoreactivity in 40% of the cells we identified as ganglion cells by dextran-backfills (Oi et al., 2008). One would naturally ask whether HCN1 is present in ganglion cells that lack HCN4 or whether individual ganglion cells possess both isoforms.

Colocalization of HCN1- and HCN4-like immunoreactivities in dextran-filled ganglion cells

Roughly half of the somata in the ganglion cell layer of rat retina are amacrine cells rather than ganglion cells (Perry, 1981). Therefore, to test whether HCN1 and HCN4-like immunoreactivities colocalize in single retinal ganglion cells, and to do so in a substantial number of cells per retina, we confocally imaged flat-mounted retinæ after retrograde transport of fluorophore-conjugated, 10-kD dextran via the optic nerve (Fig. 4). Because we were unable to obtain clear double staining of dextran-backfilled retinal ganglion cells with pairs of polyclonal and monoclonal antibodies, we used pairs of monoclonal antibodies. As in other studies (Wessel and McClay, 1986; Vardi et al., 2000), we minimized signal contamination due to non-selective binding between primary and fluorophore-conjugated secondary antibodies, by applying the antibodies sequentially (first primary, first secondary, second primary, second secondary) with the second primary antibody applied after saturating the first primary antibody with fluorophore-conjugated, monovalent Fab fragments. This binding appears to have been efficacious because no fluorescence due to the second secondary antibody was observed if the second primary was not included (Fig. 4I–K). Similar staining patterns were obtained with the pairs of primary and secondary antibodies used here, as with these same antibodies when unconjugated Fab fragment was added after the first pair of primary and secondary antibodies as an additional safeguard against non-specific binding by the subsequently applied antibodies. Moreover, we found no colocalization of first and second secondary antibody binding to ganglion cells when tissue was processed identically as in Figure 4A–D except that the second primary antibody was an anti-parvalbumin antibody which labeled amacrine cells (result not illustrated; see Wässle et al., 1993; Partida et al., 2004). Lastly, similar staining patterns were obtained regardless of whether the first primary antibody was anti-HCN1 or anti-HCN4. For consistency among preparations, we routinely applied anti-HCN1 antibody before anti-HCN4 antibody.

HCN1-like immunoreactivity (i.e., DyLight 649 fluorescence; colored red in Fig. 4A) and HCN4-like immunoreactivity (i.e., DyLight 549 fluorescence; colored green in Fig. 4B) were imaged while focusing on the ganglion cell somata (i.e., the dextran-conjugated fluorescein fluorescence; colored blue in Fig. 4C). To test the possibility that anti-HCN1 and anti-HCN4 antibodies both bind to individual cells, all three fluorescence images (HCN1-like immunoreactivity, HCN4-like immunoreactivity, and dextran-conjugated fluorescein) were superimposed over each other. The intraretinal ganglion cell axon fascicles appeared whitish, due to the addition of red, green, and blue color channels (Fig. 4D). The outlines of some dextran-backfilled somata with HCN1- and HCN4-like immunoreactivity either appeared yellowish due to the addition of red and green color channels, or whitish due to the addition of red, green, and blue color channels (Fig. 4D). It was also possible to find somata appearing slightly more red or slightly more green (e.g., cells in Fig. 4D pointed at by arrows E–H). Regardless of whether these showed dextran backfill (e.g., cells E–G) or not (e.g., cell H), examination for emission by each fluorophore showed that HCN1-like immunoreactivity colocalized to at least some degree with HCN4-like immunoreactivity (Fig. 4E–H).

When the fluorescein fluorescence was not included in the superimposed images, it was easier to see that the merging of green and red onto single pixels produced a yellow appearance in many cells, whereas spatially separate red and green pixels produced either a mottled, predominantly red, or predominantly green appearance in other cells (Fig. 5A–D).

As in other colocalization studies (e.g., Menegola and Trimmer, 2006), we estimated the relative amounts of primary antibody binding by the ratio of red and green fluorescence intensities in each cell, normalized against the mean of the red and green intensities in each population of cells analyzed. When plotted against soma diameter (Fig. 5E), this "red:green" ratio clustered around a value of 1.0 (median=0.99, $n=249$), and roughly equal numbers of ganglion cells were found with higher and lower ratios at all soma diameters. Because cytoplasmic staining contributed to these ratios in some cells, and because the staining along the periphery of each cell profile presumably reflects subunits of channels passing I_h , we also calculated the red:green ratio for cell membranes. The membrane was arbitrarily chosen to be 5 pixels in width, centered along the rim of each cell profile, and the fluorescence intensities were integrated after digitally deleting all other pixels on both the cytoplasmic and extracellular sides. A histogram (Fig. 5F) of the ratio of these fluorescence intensities for all of the cells ($n=70$) in three fields of a single retina (Fig. 5A–C) confirms three impressions gleaned by visual inspection of all of the fields we have imaged. First, the generally yellow, or equally red and green, appearance of most cell profiles is reflected by the clustering of the ratios around unity (Fig. 5E–F). Second, the colocalization of HCN1- and HCN4-like immunoreactivities showed no dependence on retinal eccentricity, as cells of similar colors were found near the optic disk (Fig. 5A), more than 5 mm from the optic disk (Fig. 5C), and in between (Fig. 5B). This is consistent with the previously reported distribution of HCN4-like immunoreactivity across all retinal eccentricities and quadrants (Oi et al., 2008). Third, although no ganglion cells were exclusively red or green (Fig. 4E–G), some cells were either more red or green than yellow. This is reflected by the spread of ratios in Figure 5E–F and exemplified by the ganglion cell profiles in Figure 5D (all selected from Fig. 5A–C). The latter ranged from predominantly green (left) to predominantly red (right) on the basis of their red:green ratios (listed next to each profile). This panel thus shows that cells with ratios smaller than 0.4 are largely green, while those with ratios greater than 6 are largely red. Comparison of these values with the histogram in Figure 5F shows that such predominantly green and predominantly red cells were rare in the fields we examined. For example, cells with ratios either less than 0.4 or greater than 6 altogether constituted 14% of the entire sample of cells (viz., 10 of 70 cells) in these fields. However, despite the overall reddish or greenish appearance of these cells, overlapping pixels from the red and green channels were found in all of the images in Figure 5D. In an intensity-independent measure of pixel overlap (Manders' overlap coefficient; see Manders et al., 1993; Bolte and Cordelières, 2006), at least 21% of the pixels of each membrane profile contained overlapping red and green pixels (range: 0.21 to 0.94, median = 0.71, mean $\pm$ SD = 0.68 $\pm$ 0.18).

Results similar to those in Figure 5 were observed in all of the fields we examined in similarly processed retinæ ($n>10$). As would be expected from colocalization of HCN1 and HCN4, the somata displaying both immunoreactivities resembled those described previously as HCN4-immunopositive ganglion cells (Oi et al., 2008). The equivalent diameters ranged between 6 and 32 μm (mean $\pm$ SD: 15 $\pm$ 3 μm , $n=260$) and thus overlap with most if not all of the ganglion cell sizes found in rat retina (Perry, 1979; Huxlin and Goodchild, 1997). Moreover, these cells populated the fields we analyzed ($n=10$; each approximately 0.045 mm^2 in area) at a density of 580 $\pm$ 147 cells/ mm^2 (mean $\pm$ SD; range: 335 to 781 per mm^2), constituted 45% (range: 23–70%) of the total number of cells that were dextran-filled in these fields, and comprised 60% of the total number of cells that displayed HCN1-like immunoreactivity. The diameters of dextran-backfilled somata displaying no detectable HCN1- or HCN4-immunopositivity ranged between 8 and 25 μm (mean $\pm$ SD: 13.1 $\pm$ 2 μm , $n=319$ cells). While all of the ganglion cells smaller than 8 μm and larger than 25 μm in the fields we imaged thus appeared to have bound HCN1 and HCN4 antibodies, we cannot exclude the possibility that others might not because somata of these diameters are rare.

Shift of $V_{1/2}$ by cAMP

Figures 4 and 5 show that ganglion cells expressing HCN1 also express HCN4. We therefore compared the I_h of retinal ganglion cells with published records of current passing through homomeric HCN1 and HCN4 channels. The biexponential activation kinetics of adult rat ganglion cell I_h (Lee and Ishida, 2007) differ from those recorded under comparable conditions in HCN1-expressing rod photoreceptors and HCN4-expressing type 3 bipolar cells. For example, the fast time constant in ganglion cells at -100 mV (221 ± 9 ms) is slower than that in rods (63 ms, estimated from Fig. 3A of Demontis et al., 2002; see also Knop et al., 2008), while the slow time constant of ganglion cells ($1,212 \pm 70$ ms) is faster than that in the bipolar cells ($2,654 \pm 410$ ms; Müller et al., 2003). In view of this difference, and because whole-cell I_h in cells expressing multiple isoforms generally differs from simple sums of currents passing through homomeric channels (Chen et al., 2001; Ulens and Tytgat, 2001), we did not try to replicate the kinetics of ganglion cell I_h by adding different amounts of current flowing through heterologously expressed homomeric channels. Instead, because cAMP shifts the activation range of current flowing through homomeric HCN channels by different amounts (Santoro et al., 1998; Seifert et al., 1999; Stieber et al., 2005), and because previous studies have not measured effects of cAMP on retinal ganglion cell I_h (Lee and Ishida, 2007; Chen and Yang, 2007), we measured activation curves from ganglion cell I_h tail currents and tested for effects of a membrane-permeable cAMP analog. All currents were recorded at 35°C .

Figure 6 shows the currents recorded in a single ganglion cell hyperpolarized to each of the indicated I_h -activating potentials for 6.5 sec and then repolarized to the holding potential (-62 mV). The upper row of traces show the holding current, the current activated by each hyperpolarization, and the tail currents upon repolarization, when the superfusate flowing over the cell was control solution (A_1), $300\ \mu\text{M}$ 8-bromo-cAMP (A_2), control solution again (A_3), and then $100\ \mu\text{M}$ SQ22536 (A_4). Panels B_1 – B_4 display the initial 1.25 sec of the tail currents, starting at the moment the voltage was returned from each hyperpolarization to the holding potential. These traces are normalized to the largest tail current measured in each solution and displayed with the sum (smooth red lines) of two exponential time functions (Lee and Ishida, 2007). Tail currents can appear to be flat at the holding potential used here and yet be greater than 0% in the activation curve because the activation threshold is more positive than the holding potential, I_h shows little time-dependence near activation threshold, and I_h does not inactivate (Seifert et al., 1999; Moroni et al., 2000; Lee and Ishida, 2007). Little of the inward current at the holding potential is due to unsubtracted leak (see Fig. 8A and 9B of Hayashida et al., 2009), and more positive holding potentials were not used to avoid activating unrelated outward currents (Tabata and Ishida, 1996; Lee and Ishida, 2007).

Figure 6C plots the amplitude of these tail currents measured at 50 msec after each repolarization to the holding potential, against the activating potentials, with the best fits by a Boltzmann equation. In this case, the $V_{1/2}$ (the voltage activating the half-maximal conductance) and s (slope factor) were -72.1 mV and 7.5 in control, and -67.5 mV and 7.6 in 8-bromo-cAMP. The currents and activation curves show that the effects of cAMP were reversed by washing with control saline. Figure 6D plots the data from all of the cells ($n=5$) tested by this protocol and shows that 8-bromo-cAMP shifted $V_{1/2}$ rightwardly without a substantial change in s . The mean ($\pm$ SEM) of the $V_{1/2}$ and s values from the Boltzmann equation fits were -73.9 ± 1.9 mV and 7.0 ± 0.3 in control, and -69.6 ± 2.0 mV and 7.4 ± 0.5 in 8-bromo-cAMP (Fig. 6D). When compared by a paired t -test, these $V_{1/2}$ values differed significantly ($p < 0.005$) whereas the s values did not ($p = 0.43$). This shift in $V_{1/2}$ is intermediate between that found in rod and cone photoreceptors expressing HCN1 (≤ 2 mV; Demontis et al., 2002; Barrow and Wu, 2009) and in HEK cells expressing HCN4 (11–23 mV; Seifert et al., 1999; Stieber et al., 2005). The lack of a change in slope factor indicates that cAMP modulated the current activated by shallow hyperpolarizations as much as it

modulated current activated by larger hyperpolarizations. To test the possibility that the shifts we measured were muted because a constitutively active adenylyl cyclase elevated cAMP levels in control solution, we applied SQ22536 at concentrations used to inhibit adenylyl cyclase activity in other neurons (50–100 μ M; Dibattista et al., 2008). This shifted $V_{1/2}$ leftwardly without a significant change in slope factor, with $V_{1/2}$ and s measuring -77.2 ± 2.2 mV and 6.5 ± 0.5 (mean $\pm$ SEM, $n=5$), respectively. The $V_{1/2}$ values differed significantly in paired t-tests ($p < 0.012$ for cAMP versus SQ; $p < 0.006$ for control versus SQ) whereas the s values did not ($p = 0.12$ for cAMP versus SQ; $p = 0.30$ for control versus SQ). The mean of the differences we measured between $V_{1/2}$ in 8-bromo-cAMP and in SQ22536 (7.5 ± 0.9 mV, mean $\pm$ SEM, $n=5$; Fig. 6D) is intermediate between the shifts produced by cAMP in HCN1-expressing *Xenopus* oocytes (2–6 mV; Santoro et al., 1998; Wainger et al., 2001) and by cAMP and 8-bromo-cAMP in the HCN4-expressing cells cited above. It is also substantially less than the shift produced in HCN2 channel currents by cAMP (Wainger et al., 2001; Stieber et al., 2005). In all except one of the cells we recorded from, 8-bromo-cAMP did not alter the maximum current amplitude during the hyperpolarizations used here (Ludwig et al., 1998; Moroni et al., 2000; Wainger et al., 2001).

I_h in cells of different sizes

In addition to the cells described by Figure 6D, we recorded I_h from 20 other cells in control external solution alone. By combining these with the cells from Figure 6D, and having estimated the whole-cell capacitance of each cell by nulling the slow capacitive current (Marty and Neher, 1995), we obtained information about control I_h for cells ($n=25$) ranging from 6 to 16 pF. If these cells were spherical, these capacitances would correspond to diameters ranging from 13 to 23 μ m. At face value, this suggests that we recorded I_h from cells within the size range of ganglion cells examined immunohistochemically above and in previous studies (Oi et al., 2008; see also Müller et al., 2003).

Fitting the activation kinetics of I_h by the sum of two exponential time functions yielded time constants (τ_f , τ_s) and amplitudes (A_f , A_s) of fast and slow components at each activating voltage (Santoro et al., 2000; Müller et al., 2003; Lee and Ishida, 2007). To examine whether these properties varied with cell size, the fast time constant (τ_f) and the relative contribution of the fast component [$A_f/(A_f + A_s)$] of the current measured at various membrane potentials were plotted against cell capacitance. Fig. 6E–F illustrate these values for hyperpolarizations to -92 mV, with insets plotting the values against the fraction of the cells recorded from. In all four plots, light grey lines are positioned at the median (50th percentile) and dark grey lines are positioned at the 5th and 95th percentiles. The corresponding (5th, 50th, and 95th percentile) values of τ_f are 153, 239 and 505 msec, while those of $A_f/(A_f + A_s)$ are 0.54, 0.7, and 0.85, respectively. The separation between the two largest τ_f values (>505 msec) and the rest of the data (100–350 msec) in Fig. 6E suggests that I_h was particularly slow in some cells. However, these constituted fewer than 10% of our recordings (2 of 25 cells in Fig. 6E), none of these were among the cells plotted in Figure 6D, and we encountered cells with such slow currents too rarely to compare morphologically with the other cells we recorded from. The plot of relative amplitude (Fig. 6F) likewise showed little heterogeneity. Although one or two points lie outside the 5% and 95% lines, the percentile plot in the inset shows that the values from all cells fell within a narrow range (0.55 – 0.84) without a conspicuous gap. Because these ratios were larger than 0.5 in all cases, we did not plot the slow time constants against cell capacitance. Moreover, distributions similar to those in Figure 6E–F were observed at other activating voltages (-87 and -97 mV; data not illustrated).

The cAMP-induced shifts in Figure 6D were recorded from a smaller number of cells ($n=5$). However, these cells ranged in capacitance from 7 to 13 pF, and we found no correlation between these values and the size of cAMP-induced shift on cell capacitance (range: 5.3 –

10.3 mV; correlation coefficient of linear regression = 0.07; data not plotted). The range of capacitances for this subset is similar to the cells plotted in Figure 6E–F, none of the cAMP-induced shifts were as small as in HCN1-expressing retinal neurons, and none were as large as in HCN4 channels (see above). In all of these cells, and as reported for heterologously expressed HCN channels (Ludwig et al., 1998; Seifert et al., 1999; Ulens and Tytgat, 2001), 8-bromo-cAMP altered the gating kinetics of I_h by accelerating the activation and slowing the deactivation. For example, on hyperpolarization from a holding potential of -62 mV to a test potential of -82 mV, the median values of the fast and slow activation time constants were 365 and 2,030 msec in control, respectively, versus 282 and 1,988 msec in 8-bromo-cAMP. When these cells were repolarized from -82 mV to -62 mV, the median values of the fast and slow deactivation time constants were 464 and 2,953 msec in control versus 517 and 3,703 msec in 8-bromo-cAMP.

Coimmunoprecipitation of HCN1 and HCN4

The colocalization of HCN1- and HCN4-like immunoreactivities within single pixels that outlined the profile of single cells (Fig. 4, 5), the moderate size of the cAMP-induced shift in voltage-sensitivity, and the intermediate activation kinetics, could result from heteromeric association of these subunits in single channels or from structural association of homomeric HCN1 and HCN4 channels (see Discussion). We therefore used the antibodies characterized above by western blotting and immunohistochemistry to test whether HCN1 and HCN4 channel subunits coimmunoprecipitate. For this purpose, whole retinal homogenates were incubated, prior to denaturing, with beads bound with antibody directed against one isoform. Thus, proteins were immunoprecipitated from the retinal homogenate either with anti-HCN1 antibody to form an "HCN1 pull-down", or with anti-HCN4 antibody to form an "HCN4 pull-down". Proteins in each of these pull-downs were separated by SDS-PAGE electrophoresis and western blotted. Coimmunoprecipitation of HCN1 and HCN4 was signified by detection of HCN4 in the HCN1 pull-down and by detection of HCN1 in the HCN4 pull-down (Fig. 7). In HCN1 pull-down fractions (Fig. 7A), a band at 134 ± 4 kD (mean $\pm$ SEM, $n=6$) was detected with the anti-HCN4 antibody, and a band at 110 ± 3 kD (mean $\pm$ SEM, $n=7$) was detected with anti-HCN1 antibody. In HCN4 pull-down fractions (Fig. 7B), a band at 139 ± 1 kD (mean $\pm$ SEM, $n=4$) was detected with the anti-HCN4 antibody, and a band at 107 ± 2 kD (mean $\pm$ SEM, $n=5$) was detected with anti-HCN1 antibody. These results would be expected if HCN1 and HCN4 subunits were heteromERICALLY associated in membranes of retinal ganglion cells and/or other retinal cells. In addition to colocalizing in ganglion cells, HCN1 and HCN4 have been found to colocalize in some bipolar cell axon terminals (Müller et al., 2003; Ivanova and Müller, 2006; Fyk-Kolodziej and Pourcho, 2007). However, it has been argued that HCN1 and HCN4 form homomeric channels rather than heteromeric ones in bipolar cells (Müller et al., 2003). We leave the possibility that our coimmunoprecipitations included HCN1 and HCN4 protein from these interneurons to be tested separately.

To test the specificity of the pull-downs by these anti-HCN antibodies, we probed both the flow-through and pull-down fractions for two other membrane proteins. We chose ABCA4 (Illing et al., 1997) because it is present in photoreceptors where HCN4 appears to be absent (Fig. 3; see also Müller et al., 2003), and also voltage-gated Na^+ channel protein because it is present in all ganglion cells and would therefore be present in the ganglion cells where we have localized HCN4 and HCN1. Figure 7 shows that we detected both of these proteins in the flow-through fraction but not in the pull-down fractions. The estimated molecular weight of the protein bound by the anti-ABCA4 antibody was 230 kD. The anti- Na^+ channel antibody bound to bands at 230 kD and 250 kD. The binding observed with this anti- Na^+ channel antibody (e.g., Fig. 7B) is not surprising because it was directed against an amino acid sequence found in multiple Na^+ channel isoforms, it can also bind to glycosylated and

non-glycosylated forms (Messner and Catterall, 1985), and polyclonal antibodies directed against the same amino acid sequence are known to bind multiple Na⁺ channel isoforms in the retina (Boiko et al., 2001).

In addition to the 107–110 kD band identified above, the anti-HCN1 antibody bound to a second band in a few of our HCN1 pull-down fractions. This resulted in bands at 104 and 114 kD in Figure 7A, and 111 and 116 kD in the one other blot showing multiple bands. As noted above, the higher molecular weight band appears to be a glycosylated form of the lower molecular weight band because it was not seen when membrane fraction proteins were incubated with PNGase F (Fig. 2).

Immunostaining of green fluorescent protein-expressing ganglion cells

Previous anatomical studies classified rat retinal ganglion cells on the basis of soma size, dendritic field diameter, branching pattern, and level of arborization in the inner plexiform layer (Brown, 1965; Bunt, 1976; Fukuda, 1977; Perry, 1979; Dreher et al., 1985; Peichl, 1989; Tauchi et al., 1992; Huxlin and Goodchild, 1997; Sun et al., 2002). The soma sizes reported by those studies (8–39 μ m) resemble those found here to bind anti-HCN1- and HCN4-antibodies, suggesting that HCN1 and HCN4 colocalize in multiple types of ganglion cell. However, we could not test this possibility in preparations stained for HCN1 and HCN4 alone because immunopositive dendrites extend into the inner plexiform layer (IPL) from very few immunopositive somata (Fig. 3; see also Kim et al., 2003; Müller et al., 2003; Cangiano et al., 2007; Fyk-Kolodziej and Pourcho, 2007; Knop et al., 2008; Oi et al., 2008). We therefore immunostained retinæ 18–20 hr after inducing expression of green fluorescent protein and examined the dendritic arborization of cells identified as ganglion cells by presence of an axon entering the nerve fiber layer. Expression was induced in relatively few (<20) cells per retina to minimize overlap of fluorescing dendritic and axonal processes from different cells, and cells in the far periphery and near the optic nerve head were not examined (see Peichl, 1989; Sun et al., 2002). The dendritic arborizations were reconstructed from optical sections collected serially between the inner nuclear and ganglion cell layers. In projected flat-mount views of these preparations, the dendrites of individual ganglion cells covered fields that were typically roundish or polygonal and not markedly elongated. We therefore estimated dendritic field diameter by the diameter of a circle having the same area as the smallest polygon circumscribing all of the dendrites of each cell. In side views of each cell (formed by 90° rotations of each z-stack of images), we compared the level(s) at which the ganglion cell dendrites arborized against the inner plexiform layer sublaminae that bound antibodies against the fiducial marker choline acetyltransferase (ChAT; see Masland et al., 1984; Voigt, 1986; Fyk-Kolodziej and Pourcho, 2007). These preparations were immunostained for HCN4 alone because HCN1 and HCN4 colocalize in ganglion cells and because two of the three available detector channels of our confocal system were used to image GFP and ChAT. To our knowledge, this is the first comparison of rat retinal ganglion cell dendritic arborizations with the inner plexiform layer strata marked by ChAT staining.

The morphological features of HCN4-immunopositive ganglion cells in these preparations (n=32 cells; n=17 retinæ) differed in several respects. The somata ranged from 13 to 32 μ m in equivalent diameter. The dendritic fields ranged from 116 to 488 μ m in equivalent diameter (Fig. 8A–D). Consistent with results obtained by other methods, the smallest somata (<18 μ m) exhibited the smallest dendritic fields (116–182 μ m in equivalent diameter), the largest dendritic fields emerged from somata larger than 18 μ m in diameter, fine axons emerged from cells with small somata and either small or medium-sized dendritic fields, and larger caliber axons emerged from cells with larger somata and dendritic fields (Fig. 8A–D; cf. Boycott and Wässle, 1974; Perry, 1979; Dreher et al., 1985; Huxlin and Goodchild, 1997; Sun et al., 2002). We estimate that the largest axon diameters agree with

those reported previously (Dreher et al., 1985; Huxlin and Goodchild, 1997), but did not attempt to measure axon diameters systematically because the finest axons appeared to exceed the resolution limits in our preparations. The dendrites varied in diameter, shape, frequency of branching, presence of spines, and level(s) of arborization in the inner plexiform layer (IPL; Fig. 8,9). The arborizations of most cells were monostратified. Comparison of these arborizations against those binding anti-ChAT antibody showed that they terminated within at least five different levels of the IPL. One was between the amacrine cell layer and the distal ChAT layer (Fig. 9A); the second coincided with the distal ChAT layer (Fig. 9B); the third was between the two ChAT layers (Fig. 9C); the fourth was at the proximal ChAT layer (Fig. 9D); and the fifth was between the proximal ChAT layer and the ganglion cell layer (Fig. 9E). Except for processes ascending vertically through the IPL, bistratified cells arborized in the first and third layer (not illustrated). The dendrites of multistratified cells ramified in all three distal layers (Fig. 9F). We did not test whether dendrites of HCN-immunopositive cells ramify into IPL layers finer than those presented here.

Based on the measurements listed in Table 2, and on the projected tangential views of their dendrites (Fig. 10), the HCN4-immunopositive ganglion cells in our preparations resembled the following previously described types: $RG_{A2\text{ outer}}$ (e.g., Fig. 8C, 9B), $RG_{A2\text{ inner}}$ (e.g., Fig. 9D), RG_{B2} (e.g., Fig. 8A), $RG_{B3\text{ outer}}$ (e.g., Fig. 9C), $RG_{B3\text{ inner}}$, $RG_{C2\text{ outer}}$ (e.g., Fig. 8B), $RG_{C2\text{ inner}}$, $RG_{C4\text{ outer}}$, RG_{D1} , RG_{D2} , outer delta (e.g., Fig. 9A), and multistratified (e.g., Fig. 9F) (Brown, 1965; Bunt, 1976; Peichl, 1989; Huxlin and Goodchild, 1997; Sun et al., 2002). The properties of the RG_{A2} cells we observed also fell within the ranges reported for Type I cells (Perry, 1979), Class I cells (Dreher et al., 1985), and inner and outer alpha cells (Peichl, 1989; Tauchi et al., 2002). The RG_{B1} cells resemble previously described Type II and Class II cells (Perry, 1979; Dreher et al., 1985). Two patterns emerged from this data set. First, every cell of some types was HCN4-immunopositive. These included $RG_{A2\text{ outer}}$ (n=6), $RG_{A2\text{ inner}}$ (n=3), RG_{B2} (n=4), $RG_{B3\text{ outer}}$ (n=3), and outer delta (n=4). The HCN4-immunoreactivity in all cells with somata as large as those of RG_{A2} and outer delta agrees with our observation that the mean diameter of HCN4-immunopositive ganglion cells is significantly larger than that of immunonegative cells (see above for mean, SD, and n; $p < 0.01$, two-tailed type 3 t-test). By contrast, all type RG_{B1} (n=4; e.g., Fig. 11C) and RG_{B4} (n=5, e.g., Fig. 11D) cells, and multistratified cells (n=6) that differed from the multistratified cell in Figure 9, were immunonegative. The dendrites of some of these multistratified cells (n=4) showed numerous spines (Fig. 11A); the dendrites of the others (n=2) lacked spines, arborized over large fields, and ramified in more sublayers of the IPL than in Figure 9. All cells examined in this study were roughly midway between the optic nerve head and far periphery, and we previously found that a similar fraction of the ganglion cell population is HCN4-immunopositive in all retinal quadrants (Oi et al., 2008), indicating that HCN4-expressing cells are not segregated from HCN4-lacking cells into different retinal regions.

Of the other cell types described in previous anatomical studies, we did not find $RG_{C4\text{ inner}}$ cells (Sun et al. 2002), and the following rarely expressed GFP in our preparations: RG_{A1} (n=1, immunonegative; Fig. 11B), $RG_{B3\text{ inner}}$ (n=1, immunopositive; Fig. 10), and $RG_{C4\text{ outer}}$ (n=1, immunopositive). Given the unpredictable hit rate of gene-gunning, the low density of GFP-expressing cells in the best preparations for resolving single cells, and because the cell densities, distributions, and phenotypic variability of each type of ganglion cell in rat retina have not been described in detail (Huxlin and Goodchild, 1997; Sun et al., 2002), we did not attempt to account for the relative numbers of HCN immunopositive and immunonegative cells based on the GFP-expressing cells we have observed.

DISCUSSION

The results of this study provide the first evidence that HCN1 and HCN4 colocalize in individual retinal ganglion cells, that the gating kinetics and cAMP response of I_h are similar among ganglion cells of different sizes and intermediate between those of HCN1 and HCN4 channels, that HCN1 and HCN4 subunit proteins coimmunoprecipitate, and that HCN1- and HCN4-immunopositive ganglion cells differ in terms of soma, axon, and dendritic field diameters and arborize at different levels of the inner plexiform layer. These results show that HCN1- and HCN4-like immunoreactivities are distributed differently in adult rat retinal ganglion cells than suggested by previously published images; show that both this pattern of antibody binding and the hyperpolarization-activated I_h differ from those found in retinal rods, cones, and bipolar cells; and predict that the biophysical properties of I_h may be similar in nearly half of the ganglion cells in this species.

Distribution of HCN1 and HCN4

Our results indicate that HCN1- and HCN4-immunopositive ganglion cells include functionally different units. For example, cells differing in soma, axon, and dendritic field diameters as in Fig. 8 project to different brain regions (Dreher et al., 1985; Martin, 1986). Secondly, the ramification of dendrites of HCN4-immunopositive ganglion cells at levels throughout the IPL (Fig. 9) suggests that HCN channels are available for activation in both on- and off-center rat retinal ganglion cells (see Peichl, 1989; Zhang and Diamond, 2009). Thirdly, because the dendritic fields of ganglion cells generally agree with the diameter of their light-adapted receptive field centers, the variety of dendritic fields of HCN4-immunopositive (Fig. 8, 10) indicate that HCN channels are available for activation in cells maximally sensitive to different spatial frequencies (Lee, 1996).

Because a few types of ganglion cell can account for a large fraction of the ganglion cells in some species (Wässle and Boycott, 1991), one could imagine that binding of HCN isoform-specific antibodies to fewer than half of the ganglion cell layer somata (Müller et al., 2003; Oi et al., 2008) results from HCN subunits expression by only a few types of ganglion cell. However, both the immunopositive and immunonegative somata ranged widely in diameter (6–32 μm vs 8–25 μm , respectively) consistent with the possibility that each were comprised of multiple types. Our results confirmed this possibility for immunopositive cells, perhaps even more than expected, in that anti-HCN4 antibodies bound to cells ramifying in all of the classically recognized sublayers of the inner plexiform layer (Fig. 9) and to cells resembling most of the types reported by previous anatomical studies of rat retinal ganglion cells (Fig. 8, 10). Our results also confirmed the above possibility for immunonegative cells in that three cell types never displayed HCN4-like immunoreactivity ($n=3$ –6 cells per type), two additional cell types were not immunopositive in the few examples we observed (RG_{A1} , $n=1$; RG_{C3} , $n=1$), and additional cells were immunonegative although they did not match any previously described type that we know of (two different bistratified cells, and 1 pair of multistratified cells that do not resemble those illustrated in Fig. 9).

Attempts to account quantitatively for our finding that HCN-immunopositive and immunonegative cells are, respectively, 40% and 60% of the entire ganglion cell population (Oi et al., 2008) will entail knowing every type of ganglion cell in the rat retina and the fraction of the ganglion cell population comprised by each type. Although the type commonly termed 'alpha' (here denoted RG_A) forms 2–4% of the ganglion cells in rat (Peichl, 1989), the corresponding fractions are unknown for the other types recognized here (Huxlin and Goodchild, 1997; Sun et al., 2002). The 'beta' type that forms more than 50% of the ganglion cell population in some species (Illing and Wässle, 1981) is absent in rat (Perry, 1979). In another species which lacks this anatomical cell type (rabbit), cells thought to be functionally analogous form 15% of the ganglion cells (Van Wyk et al., 2006). Thus, it is

difficult to predict which cell types might account for all of the HCN-immunopositive cells, or all of the immunonegative cells, in rat at this point. Although this problem might be solved by testing for the presence of I_h in every ganglion cell within a patch of retina and identifying these cells either before or after recording, we know of no means to ascertain that every type of ganglion cell was encountered.

Disposition of HCN1 and HCN4

How are HCN1 and HCN4 subunits arranged in individual retinal ganglion cells? Our observations that HCN1- and HCN4-like immunoreactivities circumscribe dextran-backfilled somata, and that PNGase F reduces the apparent molecular weight of both HCN1 and HCN4 by amounts consistent with the presence of glycosylated forms, agree well with previous reports that I_h can be activated only in cells displaying surface membrane expression of HCN subunits and that glycosylation is required for surface membrane expression (Proenza et al., 2002; Much et al., 2003; Zha et al., 2008). Thus, the yellow, green, and red pixels in our merged confocal images of ganglion cells imply that HCN1 and HCN4 subunits are in close proximity at many, though not necessarily all, points over the surface of single cells. The simplest interpretation of HCN1 and HCN4 colocalization in ganglion cells, of our having coimmunoprecipitated HCN1 and HCN4 proteins with anti-HCN1 and anti-HCN4 antibodies, and of the shift of I_h voltage-sensitivity by 8-bromo-cAMP without a change in slope factor, is that HCN1 and HCN4 subunits coassemble to form heteromeric channels in ganglion cell membranes and/or that homomeric HCN1 channels are physically associated with homomeric HCN4 channels (Dekker and Yellen, 2006; Whitaker et al., 2007; Biel and Michalakakis, 2009). This is consistent with current recorded from channels formed from covalently linked HCN1 and HCN4 subunits, as the latter show faster activation kinetics and a smaller cAMP response than HCN4 current (Altomare et al., 2003). If, instead, I_h passed through separate homomeric HCN1 and HCN4 channels, and if less negative voltages activated HCN1 but not HCN4 (Viscomi et al., 2001; Stieber et al., 2005), cAMP might be expected to shift the activation range by different amounts at different voltages. This shift would be smaller at voltages activating only HCN1 and larger at voltages which activate HCN4.

One or more other subunit arrangements may have produced red and green pixels in our images. These include homomeric HCN1 channels and HCN4 channels formed by self-assembly (Müller et al., 2003; Whitaker et al., 2007) or, conceivably, combination of HCN1 and HCN4 with other subunits. As mentioned above, HCN2 is not a likely candidate based on previous immunostainings and it is unclear how HCN3 subunits could account for ganglion cell I_h alone or by coexpression with HCN1 or HCN4. Previous studies found that cAMP leftwardly shifts HCN3 current activation curves by no more than 5 mV, that cAMP rightwardly shifts HCN1 current activation curves by similar amounts, and that the initial activation ranges of HCN1 and HCN3 do not differ greatly (Stieber et al., 2005). Thus, cAMP might produce little or no net shift in the activation range of I_h due solely to HCN1 and HCN3. At least two effects might be seen if HCN3 coexpressed with HCN4. If the current activated by small hyperpolarizations was due to HCN3 and not HCN4 (Stieber et al., 2005), cAMP might increase the Boltzmann fit slope by producing a rightward shift at very negative values (due to HCN4) and a leftward shift at less negative values (due to HCN3). Alternatively, if HCN3 and HCN4 activation ranges were as negative ($V_{1/2} = -95$ and -100 mV, respectively) as found by Mistrík et al. (2005), I_h is likely to activate at similarly negative voltages. These trends (no shift; change in slope; very negative $V_{1/2}$) all differ from the cAMP response and initial $V_{1/2}$ we report here for ganglion cell I_h . At the same time, our results do not exclude the possibility that HCN1, HCN3, and HCN4 coexpress in single ganglion cells.

It remains to be seen whether I_h differs in cells that displayed either predominantly HCN1- or predominantly HCN4-like immunoreactivity in our confocal images. These were such a small fraction of the ganglion cells we imaged (Fig. 5F) that we had no way to target these cells for electrophysiological measurements. Even if we could reliably locate such cells, we know of no pharmacological reagents that produce isoform-specific blockade. Likewise, this question would be difficult to answer unequivocally in HCN1 knockout mice because I_h in their ganglion cell somata may be due to other subunits found in wildtype mice (Moosmang et al., 2001; Koizumi et al., 2004; Mataruga et al., 2007), and cells lacking current may correspond either to wildtype ganglion cell layer somata that do not bind HCN antibodies (Mataruga et al., 2007) or to poor membrane expression of HCN channels in the absence of HCN1.

Utilization of HCN1 and HCN4

Gating kinetics and modulatory mechanisms found in other preparations suggest that the combined presence of HCN1 and HCN4 could confer multiple functional advantages on ganglion cells. For example, I_h activates faster (*i*) in cells with mRNA for all four HCN isoforms (hippocampal and neocortical pyramidal neurons) than in cells with mRNA for HCN2-4 but not HCN1 (thalamic relay and dopaminergic substantia nigra neurons) (Franz et al., 2000); (*ii*) in wild-type hippocampal pyramidal neurons than in HCN1 knock-outs (Nolan et al., 2004); and (*iii*) in A-type nodose ganglion cells displaying HCN1, HCN2, and HCN4 immunoreactivities, than in C-type fibers containing HCN2 and HCN4 but not HCN1 (Doan et al., 2004). I_h also activates more quickly during postnatal development as the amount of HCN1 protein increases relative to that of HCN4 in hippocampal CA1 pyramidal neurons (Surges et al., 2006). These observations suggest that HCN1 should enable retinal ganglion cell I_h to activate to a larger degree during brief hyperpolarizations than if only HCN4 formed the I_h -passing channels. Although it is not yet known how brief these hyperpolarizations can be, a model of globus pallidus neurons indicates that HCN channels activate within 25 msec if they form heteromerically from HCN1 and HCN2, but not if they form homomerically from HCN2 alone (Chan et al., 2004). If HCN1 enables ganglion cell I_h to activate at a similar speed, the duration of GABAergic and glycinergic postsynaptic potentials in rat retinal ganglion cells (Protti et al., 1997) would suffice to activate I_h . Moreover, longer hyperpolarizations have been found to alter the gating of heterologously expressed HCN1 channels by slowing the deactivation measured upon repolarization and by accelerating the activation of current upon subsequent hyperpolarizations (Männikkö et al., 2005). If the gating of ganglion cell I_h is similarly history-dependent, volleys of hyperpolarizing inputs would be expected to increase the recruitment of I_h and prolong the effect(s) of doing so.

The contribution of I_h to ganglion cells might also depend on multiple inputs. The modulation of I_h in other preparations by various neurotransmitters, signaling cascades, and auxiliary subunits (Biel et al., 2009), and the presence of some of these cascades in ganglion cells (e.g., Bronstein et al., 1988), suggest that different subunits might increase the number of synaptically activated pathways modulating ganglion cell I_h or, alternatively, establish a combination of pathways that must be coactivated to modulate I_h . Lastly, the robust staining of ganglion cell axons and somata by anti-HCN1 and anti-HCN4 antibodies contrasts with the paucity of dendritic staining, and suggests that electrophysiological recordings *in situ* may reveal additional contributions of I_h to ganglion cell function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Western blots of retinal membrane proteins. Lanes probed with NeuroMab monoclonal (lanes 3, 4) or Millipore polyclonal (lanes 1, 2, 5, 6) anti-HCN4 or anti-HCN1 antibodies. Ticks and numbers at left show migration distance and molecular weight (in kD) of standard proteins. Anti-HCN4 antibody (lanes 2, 3) binds to protein at around 137 kD. Anti-HCN1 antibody (lanes 4, 5) binds to protein at around 104 kD. Paired Millipore lanes (1 vs 2 and 5 vs 6) were processed identically except that lanes 2 and 5 were incubated in raw antibody, while lanes 1 and 6 were incubated in antibody that was preincubated in immunogen. Suppression of binding by immunogen provides negative controls against non-specific binding.

Figure 2.

Western blots of immunoprecipitated HCN1 and HCN4 proteins before and after cleavage by PNGase F. Formatted and labeled as in Figure 1. Retinal membrane proteins were immunoprecipitated with anti-HCN1 and anti-HCN4 antibodies. Samples of each were subjected to cleavage by PNGase F, and electrophoresed on SDS gels with control samples in adjacent lanes. Anti-HCN1 antibody binds to protein at around 108 kD and 100 kD in control HCN1 sample (lane 2) and to a band at around 100 kD in the PNGase F-treated HCN1 sample (lane 1). Anti-HCN4 antibody binds to protein at around 148 kD in control HCN4 sample (lane 3) and to protein at around 141 kD in the HCN4 sample incubated with PNGase F (lane 4). The lanes of each pair (1 and 2, 3 and 4) were adjacent lanes during the SDS-PAGE and not separated during any of the subsequent processing.

Figure 3.

Differential binding of anti-HCN1 and anti-HCN4 antibodies. Transretinal vibratome sections incubated in (A) anti-HCN1 primary antibody and AF568-conjugated goat anti-mouse secondary antibody, or (C) anti-HCN4 primary antibody and AF488-conjugated goat anti-mouse secondary antibody. Paired panels show single optical sections (A, C) of fields under epifluorescence illumination, and after merging these with the same fields under differential interference contrast optics (B, D). Acronyms positioned at the outer segment (os), inner segment (is), outer nuclear (onl), inner nuclear (inl), inner plexiform (ipl), and ganglion cell (gcl) layers. Scale bar in C is 20 μm, and applies to panels A–D. Panels E–G are single optical sections through flat-mounted retina after incubation in anti-HCN1

primary and DyLight 649-conjugated secondary, followed by anti-HCN4 primary and DyLight 549-conjugated secondary. Z-stack extends from the ganglion cell layer (**E**), through the proximal border (**F**) and middle (**G**) of the inner nuclear layer. Fluorescence of DyLight 549 and 649 assigned to the green and red color channels, respectively, and merged to form each panel. Green and red colocalize to some individual ganglion cell layer somata (yellow outlines in **E**), yet localize to separate somata in the inner nuclear layer (**F**, **G**). Green dots in **F** are profiles of bipolar cell axons projecting into the inner plexiform layer from somata in the inner nuclear layer. Scale bar in **G** is 20 μm , and applies to panels **E–G**. A magenta-green copy of this figure is available on-line as a Supplementary figure.

Figure 4.

Colocalization of HCN1 and HCN4 in dextran-backfilled ganglion cells. Panels A–C show a single optical section through the ganglion cell layer after ganglion cells were retrogradely filled with fluorescein-conjugated dextran, and the retina was incubated in anti-HCN1 primary and DyLight 649-conjugated secondary, followed by anti-HCN4 primary and DyLight 549-conjugated secondary. **D** merges panels A–C. Fluorescence from DyLight 649 (**A**), DyLight 549 (**B**), and fluorescein (**C**), assigned to the red, green, and blue color channels, respectively. Yellow in **D** shows colocalization of HCN1- and HCN4-like immunoreactivities, e.g., circumscribing blue somatic profiles. White shows colocalization of fluorescein with HCN1- and HCN4-like immunoreactivities, e.g. in axon fascicles

between the somata. Presence of HCN1 and HCN4 in somata lacking retrograde label (e.g., cell pointed at by arrow labeled **H**) is consistent with binding to displaced amacrine cells (Perry, 1981). Paired panels **E–H** show HCN1- and HCN4-like immunoreactivities of somata pointed out by correspondingly lettered arrows in **D**. Note that both signals are intense in some cells (e.g., **E** and **H**), and that HCN4 can be either more (**G**) or less (**F**) intense and uniform than HCN1 in other cells. Arrowheads in **G** and **F** point at faint membrane staining for HCN1 and HCN4, respectively. Panels **I–K** show a different field of the same retina as in **A–D**, processed as a control for non-specific binding of multiple secondary antibodies. Ganglion cells were retrogradely filled with fluorescein-conjugated dextran, and the retina was incubated in monoclonal anti-HCN1 primary and DyLight 649-conjugated secondary, followed by DyLight 549-conjugated secondary. As in **A–D**, **I** and **J** are single optical sections of the ganglion cell layer with fluorescence from DyLight 649 and DyLight 549 assigned to the red and green color channels, respectively. **K** merges **I** and **J** with the fluorescein fluorescence of the same field (blue color channel). Lack of green in **J**, and of yellow in **K**, indicate lack of binding of the second secondary antibody in the absence of a second primary antibody after saturation of the first primary antibody by the first secondary antibody. Scale bars are 20 μm in **A** (same for **A–C**, **I–K**), 20 μm in **D**, and 10 μm in **H** (same for **E–H**). A magenta-green copy of this figure is available on-line as a Supplementary figure.

Figure 5.

Ratio (R:G) of the mean intensity in the red and green channels of individual HCN-immunopositive ganglion cells. Panels **A–C** show single optical sections through the ganglion cell layer of a flat-mounted retina, immunostained as in Figure 4 and displayed without the fluorescein fluorescence. Central (**A**), mid peripheral (**B**), and far peripheral (**C**) regions (note change in nerve fiber fascicle thickness with eccentricity). Scale bar in **C** is 20 μm, and applies to panels **A–C**. Panel **E** is a scatterplot of R:G of ganglion cells in 10 fields (**A–C** plus fields in other identically processed retinæ) against equivalent soma diameter. These ratios show no dependence on soma diameter ($r^2=0.0067$) and most cells display an R:G of approximately 1 (mean=0.99, SD=10^{0.09}, n=260) regardless of cell size. Panel **D** shows ganglion cell somata (n=19) selected from **A–C**, after digitally deleting pixel color in the cytoplasmic and extracellular spaces. The number below each resulting cell membrane image is its R:G. Membranes appear yellow (or present an equal mix of red and green) in examples presenting R:G between 0.9 and 1.1, predominantly green at ratios less than 0.4, and predominantly red at ratios greater than 6. Panel **F** is a histogram of the membrane R:G for all ganglion cells in **A–C**. The median is 1.08, and the smooth line plots a normal distribution around the mean (mean=1.29, variance=10^{0.14}). A magenta-green copy of this figure is available on-line as a Supplementary figure.

Figure 6.

Effect of cAMP on rat retinal ganglion cell I_h at 35 °C. Perforated-patch whole-cell recordings without leak subtraction. **A–C**, Shift of I_h voltage-sensitivity by 8-bromo-cAMP. I_h activated in a single ganglion cell by hyperpolarizations from the holding potential (–62 mV) to –67, –72, –77, –82, –87, –92, and –97 mV, while the superfusate was changed from control (A_1), to 300 μ M 8-bromo-cAMP (A_2), to control solution again (A_3), and then to 100 μ M SQ22536 (A_4). Steps above current traces show stimulus timing. **B₁–B₄** display the first 1.25 sec of the deactivating portion of these currents in grey, normalized to the largest current measured in each solution and fitted by the sum of two exponential time functions (see Materials and Methods). The smooth black lines plot the first 1.25-sec portion of the fit to the entire record of tail current (A_1 – A_4). **C**, Activation range measured by normalizing the amplitude of these currents against the maximum recorded in each solution, and plotting the normalized amplitudes against activating voltage with best-fits by a Boltzmann equation (smooth curves). Empty circles, grey dots, Xs, and black dots plot current amplitudes in control, 8-bromo-cAMP, wash, and SQ22536, respectively. Amplitudes measured at 50 msec after each repolarization to the holding potential. The fits indicate $V_{1/2}$ and s values of –72.1 mV and 7.5 in control, –67.5 mV and 7.6 in cAMP, and –73.6 mV and 7.4 in wash, and –76.1 and 8.2 in SQ22536. **D**, Open circles, grey dots, and black dots plot mean tail current amplitude for all cells recorded from ($n=5$) in control, 8-bromo-cAMP, and SQ22536, respectively. Error bars plot $\pm$ SEM; those not visible are within the symbols. Fits by Boltzmann equation (smooth curves) yield $V_{1/2}$ and s values of –73.7 mV and 7.1 in control, –69.1 mV and 7.4 in cAMP, and –76.9 mV and 6.7 in SQ22536. **E–F** plot the fast time constant (τ_f) and the relative contribution of the fast component [$A_f/(A_f+A_s)$], respectively, from fits of the sum of two exponential time functions to I_h measured at –92 mV. Values plotted against whole-cell membrane

capacitance of cells recorded in control external solution ($n=25$). Insets plot these values against the fraction of the cells recorded from. Light grey lines mark the median and the dark grey lines show the 5th and 95th percentiles (thus marking the range of values measured in 90% of the cells).

Figure 7.

Coimmunoprecipitation of HCN1 and HCN4. Identically prepared samples of retina membrane proteins were immunoprecipitated with protein G or protein A beads bearing antibodies against HCN1 (mouse monoclonal) or HCN4 (rabbit polyclonal), respectively. The immunoprecipitated proteins ("pull-down" fraction) were then tested for binding of anti-HCN1 and anti-HCN4 antibodies. To assess specificity, the pull-down fraction and the supernatant above the beads ("flow-through") were tested for binding of antibodies against other retinal membrane proteins (ABCA4 and voltage-gated Na⁺ channels). **A**, Immunoprecipitation by NeuroMab anti-HCN1 antibody, then probe with antibodies against proteins listed under each lane. Anti-HCN4 antibody binds to protein at ~138 kD; anti-HCN1 antibodies binds to protein at ~115 kD. A deglycosylated form of the HCN1 subunit protein (~98 kD) also binds the NeuroMab anti-HCN1 antibody (see Fig. 2). **B**, Immunoprecipitation by Millipore anti-HCN4 antibody, then probe with antibody against proteins listed under each lane. Anti-HCN4 antibody (Millipore) binds to protein at ~138 kD; anti-HCN1 antibody (NeuroMab) binds to protein at ~115 kD. Note that anti-HCN1 and anti-HCN4 antibodies both immunoprecipitate both HCN subunit proteins; that neither antibody immunoprecipitates detectable amounts of ABCA4 or voltage-gated Na⁺ channel; and that both of these latter proteins were present in the retinal homogenates (viz., in the flow-through lanes). In some lanes (e.g., **B**), the pan anti-Na⁺ channel antibody bound protein at two migration distances. Regardless of the antibody used to immunoprecipitate the pull down fraction, no-detectable signals were seen when the polyclonal antibodies used to probe for HCN1 and HCN4 were pre-incubated with immunogens (lanes not shown).

Figure 8.

Tangential views. Three views of three ganglion cells (A–C) in flat-mounted retinas. Upper row (A1–C1) shows GFP fluorescence (green) only in projected views of the dendritic field arborizing in the inner plexiform layer (IPL), the soma in the ganglion cell layer (GCL), and the axon in the nerve fiber layer. The middle row (A2–C2) shows the same cells at higher magnification, with the axon extending for at least 100 μm from each soma to the lower right-hand corner of each field. These are displayed without the sections of the dendritic fields and superimposed on single optical sections of the HCN-like immunoreactivity (red). These sections are shown in the bottom row (A3–C3, each cropped for space) without the GFP fluorescence. The blue arrows point at the soma of each cell, showing the HCN4-like

immunoreactivity of the GFP-expressing soma. Comparison of the three cells shows that the soma, dendritic field, and axon diameters in **A1–A2** are all small, those of the cell in **C1–C2** are all large, and those of the cell in **B1–B2** are small, medium, and small, respectively. Identical magnification in **A1–C1**; calibration mark in **A1** is 100 μm . Identical magnification in **A2–C3**; calibration mark in **A2** is 20 μm . **D**: Equivalent diameter of dendritic fields plotted against equivalent diameter of corresponding soma of all GFP-expressing, HCN4-immunopositive cells found in this study. A magenta-green copy of this figure is available on-line as a Supplementary figure.

Figure 9.

Orthogonal rotations. Four views of six large-field ganglion cells (**A-F**). The first three views are formatted as in Figure 8 to show (1) the GFP fluorescence (green) in projected views of the dendritic field, soma, and axon, (2) the soma at higher magnification superimposed on a single optical section of the HCN-like immunoreactivity (red), and (3) the HCN-like immunoreactivity alone. Blue arrowheads point at the GFP-expressing, HCN4-immunopositive somata. The fourth panel for each cell shows the 90° rotation of the GFP-expressing cell superimposed on the fluorescence (purple) localizing choline acetyltransferase-like immunoreactivity (ChAT). The upper and lower sides of these panels are the sclerad and vitread sides of these views, respectively. The round, purple profiles above and below each ganglion cell are the somata of regular and displaced cholinergic amacrine cells, respectively. The dendrites of these cells form the two purple "ChAT bands" in the inner plexiform layer (e.g., right side of **A4** and **B4**, right and left side of **C4** and **D4**, and throughout **E4**). The monostratified ganglion cell dendrites ramify above the distal ChAT band (**A4**), at the same level as the distal ChAT band (**B4**), between the two ChAT bands (**C4**), at the same level as the proximal ChAT band (**D4**), and beneath the proximal ChAT band (**E4**). The multistratified ganglion cell dendrites in **F4** spread laterally in the distal half of the IPL. Dendrites of this cell can be seen ascending and descending through the IPL, i.e. passing toward the amacrine cell layer as well as away from it. The calibration mark in each GFP-only panel (**A1**, **B1**, etc) shows 100 μm. The calibration marks show 20 μm in the HCN4-only panels (**A3**, **B3**, etc.) and are identical for the corresponding GFP-HCN4 fields (**A2**, **B2**, etc.). A magenta-green copy of this figure is available on-line as a Supplementary figure.

Figure 10.

Dendritic fields. Projected views of the dendritic field, soma, and proximal segment of axon of GFP-expressing, HCN4-immunopositive cells. Each profile shows the GFP fluorescence in black, with the cell type based on resemblance of cell soma diameter, dendritic field diameter, dendritic arborization, and dendritic shape and branching pattern, to types identified by Sun et al. (2002), Peichl (1989), and Bunt (1976). The first three properties of each cell here are listed in Table 2. The dendrites vary from sparse branching (RG_{B3} , outer delta) to numerous branchings (RG_{C1} , $RG_{C2\text{ outer}}$, $RG_{C4\text{ outer}}$, RG_{D2}); thick between soma and first branch point ($RG_{A2\text{ outer}}$, $RG_{A2\text{ inner}}$, RG_{C1}) to thin near soma (RG_{B2} , RG_{D1} , multistratified); few if any spines ($RG_{A2\text{ outer}}$, $RG_{A2\text{ inner}}$, outer delta) to numerous, short spines (RG_{B2} , $RG_{C4\text{ outer}}$). Asterisks positioned next to axon of each cell. Calibration bar is 100 μm and applies to all profiles.

Figure 11.

Immunonegative cells, formatted as in Figure 8. The cell types are multistratified (**A**), RG_{A1} (**B**), RG_{B1} (**C**), and RG_{B4} (**D**). As in Figure 8, each GFP panel is a projected view of the dendrites, soma, and axon, and the axon clearly extends beyond the dendritic field (oriented to touch the right edge). The blue arrowheads in the HCN4 panels show the absence of HCN-like immunoreactivity at the position of the GFP-expressing cell soma in the corresponding GFP+HCN4 panels, despite the presence of HCN4-immunopositive somata in the same field. The calibration bars in the GFP-only fields (**A1–D1**) are 100 μ m. For the remaining fields, the calibration bars are 20 μ m (shown in the HCN4-only panel of each pair). A magenta-green copy of this figure is available on-line as a Supplementary figure.

Table 1

Summary of primary Antibodies

Antibody	Immunogen	Source	Species	Concentration
anti-HCN1, clone N70/28	a.a. 778–910, rat HCN1, GST fusion protein	NeuroMab (#75–110)	Mouse IgG1, monoclonal	1 µg/mL (IHC) 2 µg/mL (B)
anti-HCN1	a.a. 6–24, rat HCN1, synthetic peptide	Millipore (#AB5884)	Rabbit IgG, polyclonal	2.5 µg/mL (IHC) 1.5 µg/mL (B)
anti-HCN4, clone N114/10	a.a. 1019–1108, rat HCN4, GST fusion protein	NeuroMab (#73–150)	Mouse IgG1, monoclonal	4 µg/mL
anti-HCN4	a.a. 119–155, human HCN4, GST fusion protein	Millipore (#AB5808)	Rabbit IgG, polyclonal	2.2 µg/mL (IHC) 0.66 µg/mL (B)
anti-ChAT	human placental enzyme	Millipore (#AB144P)	Goat IgG, polyclonal	2 µg/mL
anti-GFP, conjugated to Alexa 488	GFP, <i>Aequorea victoria</i>	Invitrogen (#A21311)	Rabbit IgG, polyclonal	2 µg/mL
anti-VGSC, α -subunit	a.a. 1501–1518, rat VGSC α -subunit, type 1, synthetic peptide	Millipore (#AB5210)	Rabbit IgG, polyclonal	0.3 µg/mL
anti-ABCA4 (Rim 3F4)	220 KD rim protein, bovine	Dr. R.S. Molday University of British Columbia	Mouse, monoclonal	1:400, ascites fluid
anti-parvalbumin, clone PARV-19	muscle parvalbumin, frog	Sigma (#P3088)	Mouse IgG1, monoclonal	1:1000, ascites fluid

GFP - green fluorescent protein, CHAT - choline acetyltransferase, VGSC - voltage-gated sodium channel, IHC - immunohistochemistry, B - biochemistry

Table 2

HCN4-immunopositive cell types

File Name	Soma Diameter (μm)	Dendritic Field Diameter (μm)	Arborization *	Type
0806 2_1	21	367	2	A2 outer ^I
0902P 1_2	25	391	2	
0923 1_4	24	319	2	
0923 2_1**	23	404	2	
1111 1_3	24	350	2	
1019 2_1	25	434	2	
0917 2_1	28	330	4	A2 inner ^I
0923 1_5	32	265	5	
1111 1_2**	28	454	4	
0917 2_3**	26	233	4	B1 ^I
0806 2_2	17	170	3	B2 ^I
0806 2_3**	14	116	3	
0806 2_4	15	164	3	
1112 1_1	18	182	2	
0923 1_1**	20	297	2	B3 ^I
1111 1_1	22	334	2	
1111 1_5	18	289	3	
1112 1_3**	18	381	1	C1 ^I
0923 1_3**	16	240	2	C2 outer ^I
1112 1_4	16	231	2	
0804P 1_2	22	263	4	C2 inner ^I
0804S 1_1**	17	220	4	
1106 3**	16	257	1	C4 outer ^I
11123_1**	15	229	2/4	D1 ^I
0913 1_1**	18	399	2/4	D2 ^I
1106 1	18	401	2/4	
0804P 1_1	24	410	1	outer delta ²
0913 1_2**	22	488	1	
1106 2	18	388	1	
1110 1_1	22	439	1	
0902S 1_2**	18	246	1–3	multistratified ³

* The IPL levels are defined as (1) above the distal ChAT band nearest the INL, (2) at the same level as the distal ChAT band, (3) between the ChAT bands, (4) at the same level as the proximal ChAT band and (5) between the proximal ChAT band and the GCL.

** A projected view of this cell is found in Figure 10.

¹ Sun et al., 2002;

² Peichl 1989;

³ Bunt 1976.