



Institut für Biologische Informationsverarbeitung
Institut 1: Zelluläre Signalverarbeitung

***Systematic analysis of hyperpolarization-
activated and cyclic nucleotide-gated (HCN)
channels in photoreceptors and bipolar cells
of the rat retina***

Elena N. Ivanova

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Abstract

Hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels were studied in the rat retina. The expression pattern of the four HCN channel isoforms HCN1 – 4 was studied using immunocytochemistry with isoform-specific antibodies and confocal microscopy. Using the patch-clamp technique, functional properties of HCN currents were recorded in 240 voltage-clamped bipolar cells and 20 photoreceptor cells in a retinal slice preparation. While recording, cells were filled with Lucifer Yellow. On the basis of morphological and electrophysiological criteria, recorded and filled bipolar cells could be classified as 11 types of cone bipolar cells and one type of rod bipolar cell. Two of the cone bipolar cell types had not been identified in previous studies.

Based on immunohistochemical and electrophysiological results, HCN1 was found in rod and cone photoreceptors. Cones also expressed HCN3 at the cone pedicle base. Type 3 bipolar cells expressed HCN4, type 5 and type 6a bipolar cells HCN1 and HCN4. These isoforms were found throughout the bipolar cells. Strikingly, HCN2 was highly concentrated at the axon terminal systems of type 3, 5, 6, 7, 8, and 9 cone bipolar cells and the rod bipolar cell. No HCN immunoreactivity was found in type 1, 2, and 4 cone bipolar cells. In the electrophysiological recordings, the activation kinetics and the mid-point potential of activation were studied in detail. In general, the properties of HCN currents recorded from the different bipolar cell types in the retinal slice agreed reasonably well with those of the respective isoforms expressed heterologously in HEK293 cells. All HCN currents could be blocked by application of external cesium. Cones and cone bipolar cells type 5 and 6a expressed more than one HCN isoform, however, electrophysiologically, no evidence for the formation of heterooligomeric channels was found. In some instances, functional properties of HCN channels in retinal cells differed from those of the heterologously expressed channel isoforms, indicating that the channels might be modulated in the retina. Preliminary experiments suggest that intracellular calcium and cAMP might be involved in the modulation of the channels.

The widespread expression of HCN channels in the retina suggests that they are involved in retinal information processing at several stages. The most striking feature of HCN channel distribution was the synaptic localization of HCN2 and HCN3. This might indicate that the channels are involved in regulation of synaptic transmission.

Kurzfassung

Die Arbeit beschreibt die Verteilung und die funktionellen Eigenschaften hyperpolarisationsaktivierter und zyklisch Nukleotid-gesteuerter (engl. hyperpolarization-activated and cyclic nucleotide-gated, HCN) Kanäle in der Rattenretina. Die Verteilung der vier HCN-Kanal-Untereinheiten HCN1 – 4 wurde mittels Immunhistochemie mit isoformspezifischen Antikörpern und Konfokaler Mikroskopie untersucht. In retinalen Gewebsschnitten („slices“) wurden Nervenzellen im Ganzzellmodus der patch-clamp Technik elektrophysiologisch abgeleitet. Dabei wurden die funktionellen Eigenschaften der HCN-Kanäle in insgesamt 240 Bipolarzellen und 20 Photorezeptorzellen untersucht. Während der Ableitung wurden die Zellen mit Lucifer Yellow gefüllt. Anhand morphologischer und elektrophysiologischer Kriterien konnten die abgeleiteten und gefüllten Bipolarzellen in 11 Typen von Zapfenbipolarzellen und einen Typ von Stäbchenbipolarzelle eingeteilt werden. Zwei der Zapfenbipolarzelltypen wurden erstmals beschrieben.

Sowohl immunhistochemisch als auch elektrophysiologisch wurde die Untereinheit HCN1 in Zapfen und Stäbchen gefunden. Zapfen exprimieren auch HCN3 an der Basis des Zapfenendfußes. Typ 3 Bipolarzellen exprimieren HCN4, Typ 5 und Typ 6a Bipolarzellen HCN1 und HCN4. Diese Isoformen wurden überall in der Zelle gefunden. HCN2 dagegen wurde hoch konzentriert nur an den Axonterminalsystemen der Bipolarzelltypen 3, 5, 6, 7, 8 und 9 sowie der Stäbchenbipolarzelle lokalisiert. In den Bipolarzelltypen 1, 2 und 4 wurde keine HCN-Immunreaktivität beobachtet. In den elektrophysiologischen Ableitungen wurden die Aktivierungskinetik der Kanäle und die Spannung halbmaximaler Aktivierung detailliert untersucht. Die HCN-Kanäle der Bipolarzellen stimmten in ihren Eigenschaften meist gut mit den entsprechenden Isoformen überein, die in HEK293 Zellen heterolog exprimiert wurden. Alle HCN-Ströme wurden durch extern appliziertes Cäsium blockiert. Zapfen und die Bipolarzelltypen 5 und 6a exprimierten mehr als eine HCN-Kanal-Isoform. Die elektrophysiologische Untersuchung ergab allerdings keine Hinweise auf die Bildung heterooligomerer Kanäle. In einigen Retinazelltypen unterschieden sich die funktionellen Eigenschaften der HCN-Kanäle von denen heterolog exprimierter Kanäle. Das könnte bedeuten, dass HCN-Kanäle in der Retina moduliert werden. Vorläufige Experimente weisen darauf hin, dass intrazelluläres Calcium und cAMP an dieser Modulation beteiligt sind.

Die weite Verbreitung der HCN-Kanäle in der Retina lässt vermuten, dass sie an mehreren Stellen an der retinalen Informationsverarbeitung beteiligt sind. Ein besonders interessantes Ergebnis ist die synaptische Lokalisation von HCN2 und HCN3. Es könnte bedeuten, dass HCN-Kanäle die synaptische Transmission regulieren.

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II Abbreviations

ATP	Adenosine 5'-triphosphate
AC	<u>A</u> macrine <u>C</u> ell(s)
BAPTA	1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
bv	blood vessel(s)
cAMP	Adenosine 3',5'-cyclic monophosphate
Chat	<u>C</u> holine <u>a</u> cetyl <u>t</u> ransferase
cDNA	<u>c</u> omplementary DNA
cGMP	Guanosine 3',5'-cyclic monophosphate
cm	centimeter
CNS	<u>C</u> entral <u>N</u> ervous <u>S</u> ystem
CP	<u>C</u> one <u>P</u> edicle
Cy3, Cy5	Carbocyanine derivatives
DIC	<u>D</u> ifferential <u>I</u> nterference <u>C</u> ontrast
DNA	<u>D</u> eoxyribon <u>u</u> cleic <u>a</u> cid
D1	Dopamine D1 receptor(s)
ECACC	European Collection of Cell Cultures
EDTA	<u>E</u> thylene <u>d</u> iamine <u>t</u> etra <u>a</u> cetic acid
EGTA	<u>E</u> thylene glycol-bis(β -aminoethyl ether) N,N,N',N'- <u>t</u> etra <u>a</u> cetic acid
e.g.	<i>exempli gratia</i> , for example
et al.	<i>et alii</i> , and others
Extr.	Extracellular
Fig.	Figure
FITC	<u>F</u> luorescein <u>i</u> sothiocyana <u>t</u>
Flp	recombinase from <i>Saccharomyces cerevisiae</i>
Flp-hHCN1	HEK293 stable cell line expressing human HCN1 channels
Flp-hHCN4	HEK293 stable cell line expressing human HCN4 channels
FRT	<u>F</u> lp <u>R</u> ecombination <u>T</u> arget site
GABA	γ -aminob <u>u</u> tyric <u>a</u> cid
CabP	<u>C</u> alcium <u>b</u> inding <u>P</u> rotein
GC	<u>G</u> anglion <u>C</u> ell(s)
GCL	<u>G</u> anglion <u>C</u> ell <u>L</u> ayer
GFP	<u>G</u> reen <u>F</u> luorescent <u>P</u> rotein
GLT-1	<u>G</u> lutamate <u>T</u> ransporter-1
GTP	Guanosine 5'-triphosphate
GluR1	Ionotropic glutamate receptor 1
G Ω	10 ⁹ Ohm
h	hour
HC	<u>H</u> orizontal <u>C</u> ell(s)
HCN	<u>H</u> yperpolarization-activated and <u>C</u> yclic <u>N</u> ucleotide-gated channels
HCN1	Mammalian HCN channel isoform 1
HCN2	Mammalian HCN channel isoform 2
HCN3	Mammalian HCN channel isoform 3
HCN4	Mammalian HCN channel isoform 4
HEDTA	N-(2- <u>H</u> ydroxyethyl) <u>e</u> thylene <u>d</u> iamine-N,N',N'- <u>t</u> ri <u>a</u> cetic acid
HEK293 cells	<u>H</u> uman <u>E</u> mbryonal <u>K</u> idney cells
HEPES	4-(2- <u>H</u> ydroxyethyl)piperazine-1- <u>e</u> thanesulfonic acid
IC	<u>I</u> nterplexiform <u>C</u> ell(s)
i.e.	<i>id est</i> , that is

I_h, I_f, I_q	Current, mediated by HCN channels h for hyperpolarization-activated f for funny q for queer
I_{Kx}	Current, mediated by K_x potassium channels
I_m	Current passing through the cell membrane; in current-clamp mode is equal to the command current
INL	<u>I</u> nn <u>e</u> r <u>N</u> uclear <u>L</u> ayer
Intr.	Intracellular
IPL	<u>I</u> nn <u>e</u> r <u>P</u> lexiform <u>L</u> ayer
IS	<u>I</u> nn <u>e</u> r <u>S</u> egment(s)
Kd	dissociation constant
kHz	10^3 Hertz
$K_{1/2}$	Michaelis constant, concentration leading to half-maximal effect
LY	<u>L</u> ucifer <u>Y</u> ellow, 6-Amino-2,3-dihydro-1,3-dioxo-2-hydrazinocarbonylamino-1H-benz[d,e]isoquinoline-5,8-disulfonic acid
M	Molar
M10	modified MEM
MC	<u>M</u> üller <u>C</u> ell(s)
MEM	<u>M</u> inimal <u>E</u> ssential <u>M</u> edium
MinK	<u>M</u> inimal <u>K</u> ⁺ -channel, β subunits from the KCNE family of single transmembrane spanning proteins
MIRPs	<u>M</u> inK- <u>R</u> elated <u>P</u> eptides, β subunits from the KCNE family of single transmembrane spanning proteins
mGluR6	metabotropic glutamate receptor 6
min	minute(s)
ml	10^{-3} liter
μ l	10^{-6} liter
mV	10^{-3} volt
M Ω	10^6 Ohm
n.a.	not applicable
n.d.	not determined
NFL	<u>N</u> erve <u>F</u> ibre <u>L</u> ayer
NMDA	<u>N</u> - <u>m</u> ethyl- <u>D</u> - <u>a</u> spartate
Nr.	Number
OFF-cell	cell that shows hyperpolarizing light response
ON-cell	cell that shows depolarizing light response
OPL	<u>O</u> uter <u>P</u> lexiform <u>L</u> ayer
OS	<u>O</u> uter <u>S</u> egment(s)
PA	<u>P</u> araformaldehyde
pA	10^{-12} ampere
PDE	<u>P</u> hosphodiesterase(s)
pF	10^{-12} farad
PB	<u>P</u> hosphate <u>B</u> uffer
pH	Negative logarithm of the effective hydrogen-ion concentration
PKA	cAMP-dependent protein kinase A
PKC	<u>C</u> a ²⁺ -dependent protein <u>k</u> inase
RB	<u>R</u> od <u>B</u> ipolar cell
rHCN1	HCN channel isoform 1 from rat
rHCN2	HCN channel isoform 2 from rat
rHCN3	HCN channel isoform 3 from rat

rHCN4	HCN channel isoform 4 from rat
RT	<u>R</u> oom <u>T</u> emperature (~+22°C)
S	Slope of Boltzmann curve
s	second(s)
SD	<u>S</u> tandard <u>D</u> eviation
sol.	solution
Tab.	Table
TEA	<u>T</u> etra <u>e</u> thylammonium
VIP	<u>V</u> asoactive <u>I</u> ntestinal <u>P</u> olypeptide
V _m	membrane voltage; in voltage-clamp mode is equal to the command potential
V _{glut}	<u>V</u> esicular <u>G</u> lutamate <u>T</u> ransporter
V _{rest}	resting potential or zero-current potential of the cell
V _{rev}	reversal potential of the channels
V _{1/2}	mid-point potential of channel activation
Ø	diameter

1 Introduction

1.1 The fundamental plan of the retina

The retina is a true part of the brain, deriving in the early embryogenesis from the evaginations of the forebrain. The retina lines the back of the eye and, hence, is easily accessible. The retina is an approximately 200 μm thick, transparent and well-layered tissue, which can be easily removed intact from the back of the eye – a "natural brain slice".

All vertebrate retinæ are organized according to the same basic plan (Fig. 1.1). Three cellular layers, the ONL, the INL, and the GCL (Outer and Inner Nuclear Layers and Ganglion Cell Layer) harbour the somata of six basic classes of retinal neurons: photoreceptors (rods and cones) build up the ONL; somata of horizontal (HC), bipolar (BC), amacrine (AC), and interplexiform (not shown) cells form the INL; ganglion cell (GC) and displaced amacrine cell (not shown) somata occupy the GCL. The interaction among the cell types takes place in two synaptic layers, OPL and IPL (Outer and Inner Plexiform Layers). In the OPL, photoreceptors axons contact bipolar cells and horizontal cells. In the IPL, the axons of bipolar cells either feed directly into the dendrites of ganglion cells or make synapses with amacrine cells, which in turn contact ganglion cells. The ganglion cell axons leave the eye and project to the higher visual centres of the brain. The dominant type of glial cells in the vertebrate retina is the Müller cell (MC). The cells extend vertically through the retina, from the distal margin of the outer nuclear layer to the inner margin of the retina.

Light, entering the eye (Fig. 1.1, arrows), passes through the transparent retina and is finally captured by the visual pigment-containing outer segments of the photoreceptors. The two kinds of photoreceptors show different functional properties (for review see Dowling, 1987). Rods are very sensitive and can reliably detect the absorption of single photons. They are responsible for vision at low light levels, such as moonlight. Cones are much less sensitive and are suited for daylight and colour vision. In the dark, both rods and cones are depolarized and release glutamate as transmitter at their synapses. Upon illumination, photoreceptors hyperpolarize and glutamate release is decreased (for review see Müller and Kaupp, 1998).

Cones synapse onto different types of cone bipolar cells: some bipolar cells depolarize upon illumination and are thus called "ON-bipolar" or "depolarizing bipolar" cells. The axons of ON-bipolar cells stratify in the inner half of the IPL (closer to the GCL), the so-called ON-sublamina. Other bipolar cells hyperpolarize in light, but depolarize in the dark, the so-called "OFF-bipolar" or "hyperpolarizing bipolar" cells. OFF-bipolar cells stratify in the outer half of

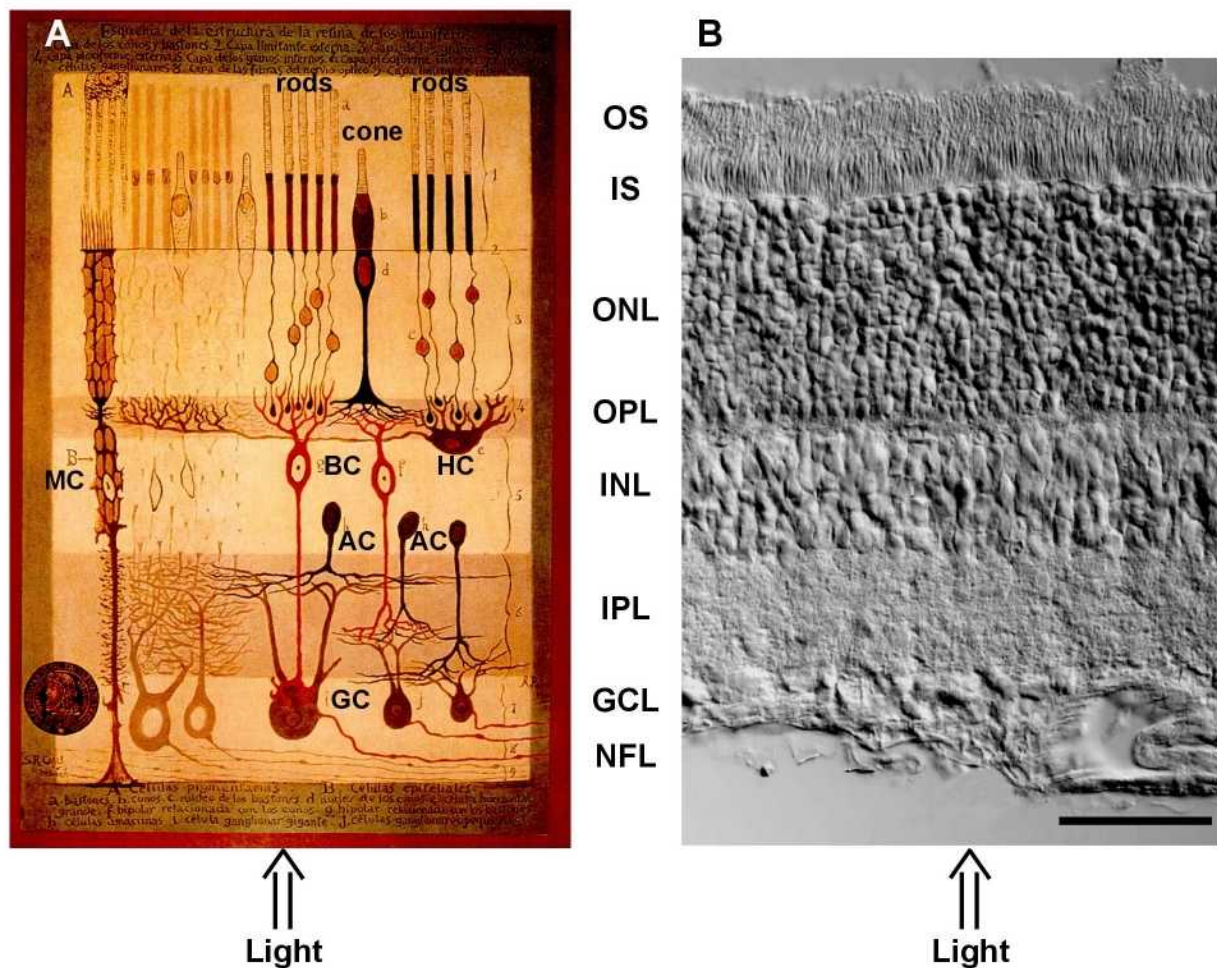


Fig. 1.1: Cellular organization of the retina.

(A) Schematic drawing of a vertical section through the retina by Cajal (taken from Hubel, 1988). The scheme is based on observations of cells impregnated by the Golgi method. In the picture, the cell types are marked: rods and cone; HC, horizontal cell; BC, bipolar cell; AC, amacrine cell; GC, ganglion cell; MC, Müller cell.

The following layers are shown: OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fibre layer. Arrows represent light passing through the retina.

(B) Differential Interference Contrast (DIC) image of a vertical section through the rat retina. Scale bar 50 μm .

the IPL, in the OFF-sublamina. Morphologically, cone bipolar cells are further subdivided into 8-11 different types (in rabbit, Famiglietti, 1981; in cat, Famiglietti, 1981; Kolb et al., 1981; McGuire et al., 1984; Cohen and Sterling, 1990; in monkey, Boycott and Wässle, 1991; in rat, Euler and Wässle, 1995; Hartveit, 1997). The different responses of ON- and OFF-bipolar cells are determined by different glutamate receptors on their dendrites: in the OFF-bipolar cells, glutamate released from the photoreceptors in the dark causes sodium influx through ionotropic receptors; in the ON-bipolar cells, metabotropic receptors link glutamate to the closure of cation channels (Nawy and Jahr, 1991; Yamashita and Wässle, 1991). This dichotomy is maintained in the subsequent circuitry; ON-bipolars excite ON-ganglion cells, and OFF-bipolars excite OFF-ganglion cells. As a result, a transient increase in light intensity

is transmitted to the brain by a rapid burst of spikes from ON-ganglion cells, whereas a transient decrease in intensity is encoded mostly by a burst of spikes from OFF-ganglion cells (Hartline, 1938).

In the mammalian retina, rods connect to a single type of bipolar cell (Boycott and Dowling, 1969; Dacheux and Raviola, 1986; Wässle et al., 1991), the rod bipolar (RB) that shows depolarizing light responses. Mammalian rod bipolar cells do not feed directly into ganglion cells. Upon illumination, the rod bipolar excites the AII amacrine cell, which in turn excites depolarizing cone bipolar cells through electrical junctions, and inhibits hyperpolarizing cone bipolar cells through glycinergic synapses (Kolb and Famiglietti, 1974; Famiglietti and Kolb, 1975; Kolb and Nelson, 1983; Pourcho and Goebel, 1985; Müller et al., 1988). Under low light level, i.e. in the scotopic range, the rod signals are conveyed by this pathway to the ON- and OFF-ganglion cells.

For higher light levels, in the mesopic range, it has been proposed that rod signals can also travel by another route that circumvents the rod bipolar cell – namely, by passing through gap junctions to cones and from there to the cone ON- and OFF-bipolar cells. Electrical connections between rod and cone cells have, in fact, been documented both physiologically (Schneeweis and Schnapf, 1995; Nelson, 1977) and anatomically (Smith et al., 1986).

A third, minor rod pathway, so far investigated only in rodents, seems to utilize one type of OFF-cone bipolar cell (Soucy et al., 1998; Hack et al., 1999; Tsukamoto et al., 2001).

Hence, there are several vertical pathways for visual processing in the retina. In addition, the information is processed by lateral pathways in both plexiform layers.

In the OPL, both rods and cones receive lateral inhibitory input from horizontal cells, that leads to contrast enhancement between adjacent light and dark regions and to the formation of receptive fields with excitatory center and inhibitory surround. In rat and mouse, only one type of horizontal cells has been described (Peichl and Gonzalez-Soriano, 1994).

In the IPL, light-responses are strongly modulated by amacrine cells that provide lateral and feedback inhibitory input to bipolar cells and feedforward inhibition to ganglion cells. Amacrine cell circuits are essential prerequisites for the formation of complex receptive field properties of ganglion cells, such as motion and directional selectivity. Twenty-nine types of amacrine cell have been described in mammals (for review see Masland 1988; Vaney, 1990) which are thought to have distinct dedicated functions - they carry out narrow tasks concerned with shaping and control of ganglion cell responses. Ten to fifteen types of ganglion cells have been found in mammals (in cat, Boycott and Wässle, 1974; Kolb et al., 1981; in rabbit, Amthor et al., 1983, 1984, 1989; Famiglietti, 1987; in rat, Peichl, 1989). Being the output

neurons of the retina, the different ganglion cell classes can be considered as neuronal filter elements that extract different aspects of information from the complex image that is projected onto the retina. For detailed review and further discussion of retinal cell types see Wässle and Boycott (1991) and Masland (2001).

To fully understand the function of the retina, we need to gain knowledge on two different aspects of information processing. First, we need to analyze the synaptic circuitry to understand the properties of the retinal network. Second, we must understand the shaping and modulation of signals within the individual elements of the network – the cells. For this, we must investigate the inventory of ion channels that determine the functional properties of a cell. -

One class of voltage-activated cation channels present in variety of retinal cells is activated by hyperpolarization and gated by cyclic nucleotides (HCN channels).

1.2 HCN channels

HCN channels were well characterized in a variety of native tissues and in heterologous expression systems (for review see: Pape, 1996; Kaupp and Seifert, 2001).

HCN currents were discovered during the late 1970s in sino-atrial node cells where they determine the rate of the heart beating, and were termed "pace-maker" currents (Brown et al., 1979; Brown and DiFrancesco, 1980; DiFrancesco, 1981a and 1981b; Yanagihara and Irisawa, 1980). The same currents were found in hippocampal pyramidal cells (Halliwell and Adams, 1982; Maccaferri et al., 1993) and in photoreceptors cells (Fain et al., 1978; Baylor et al., 1984; Hestrin, 1987). At the time of discovery, the properties of the channels seemed to be unique, especially the activation upon hyperpolarization; currents were, therefore, termed I_h (h for hyperpolarization-activated), I_f (f for funny) or I_q (q for queer).

Recently, genes coding for HCN channels from different vertebrates (mouse, rat, human, rabbit) and invertebrates (*Strongylocentrotus purpuratus*, *Helionthes viscerens*, *Drosophila melanogaster*) have been identified (Gauss et al., 1998; Ishii et al., 1999; Krieger et al., 1999; Ludwig et al., 1999; Marx et al., 1999; Seifert et al., 1999; Shi et al., 1999; Vaccari et al., 1999). In vertebrates, the HCN channel family comprises four members (HCN1-4).

The four different mammalian genes display high sequence homology. Hydropathy analysis and sequence comparison classify HCN channels as members of the superfamily of voltage-gated cation channels. The channels are related (20 - 25 % overall sequence identity) to

eag K⁺ channels and to the cyclic nucleotide-gated channel class. Some similarities were found with inwardly rectifying K⁺ channel from *Arabidopsis thaliana* (KAT channels) (for review see Warmke and Ganetzky, 1994; Kaupp and Seifert, 2001; Biel et al., 2002).

HCN channels contain six membrane-spanning helices (S1-S6), including a positively charged voltage-sensing S4 segment, and an ion conducting pore between S5 and S6. In the C terminus, the channels carry a cyclic nucleotide-binding domain. Most likely, four subunits assemble to form a functional channel. The homooligomeric channels can be functionally expressed in heterologous systems (HCN1, Santoro et al., 1998 and 2000; Ishii et al., 2001; Ulens and Tytgat, 2001a and b; Chen et al., 2001; HCN2, Ludwig et al., 1998 and 1999; Santoro et al., 2000; Moroni et al., 2000; Ulens and Tytgat, 2001a and b; Chen et al., 2001; HCN3, Moosmang et al., 2001; HCN4, Ishii et al., 1999; Seifert et al., 1999; Ludwig et al., 1999; Ishii et al., 2001).

The hallmarks of native and expressed HCN channels are: activation upon membrane hyperpolarization, conduction of Na⁺ and K⁺, blockage by extracellular Cs⁺, and modulation by cyclic nucleotides.

1. Activation of the channels. Upon hyperpolarization, all HCN currents develop with a characteristic sigmoidal time course, which hints at a complex series of voltage-dependent activation steps (Kaupp and Seifert, 2001). The channel isoforms exhibit marked differences in their activation kinetics. HCN1 channels show the fastest, HCN4 channels the slowest activation.

The voltage of half-maximal activation (mid-point potential, $V_{1/2}$) is a measure of the voltage range within which the channel operates. The mid-point potentials differ among the channel isoform.

2. Ion selectivity. HCN channels pass both Na⁺ and K⁺. The relative permeability ratio P_{Na}/P_K is dependent on the extracellular potassium concentration and varies from 0.15 to 0.40 (Edman et al., 1987; Herstin, 1987; Maricq and Korenbrot, 1990; Wollmuth and Hille, 1992; Gauss et al., 1998; Ludwig et al., 1998; Santoro et al., 1998). Under physiological gradients of monovalent ion concentrations, the reversal potential of the channels ranges from -20 mV to -30 mV (Seifert et al., 1999; Gauss et al., 1998; Ludwig et al., 1998).

3. Cesium block. HCN channels are blocked by millimolar concentrations of external Cs⁺ ($K_{1/2} = 2.19$ mM, in Purkinje fibres, DiFrancesco, 1982). The block is voltage dependent. In contrast to inward rectifier channels like I_{kir} , millimolar concentrations of Ba²⁺ did not significantly affect HCN channels (Ludwig et al., 1998; Pape, 1996).

4. *Modulation by cyclic nucleotides.* The activity of HCN channels is modulated directly by binding of the cyclic nucleotides cAMP ($K_{1/2} = \sim 0.5 \mu\text{M}$, measured for HCN2; Ludwig et al., 1998) and cGMP ($K_{1/2} = \sim 6 \mu\text{M}$, measured for HCN2; Ludwig et al., 1998) by shifting the activation curve towards more positive voltages. HCN channel isoforms are set apart from each other by the range of modulation. The shift of $V_{1/2}$ by cAMP appears to be smallest in HCN1 ($\sim 2 \text{ mV}$, Santoro et al., 1998; $\sim 4 \text{ mV}$, Chen et al., 2001). HCN2 displays a shift of 12-14 mV (Ludwig et al., 1998 and 1999) and HCN4 exhibits a shift of 11-23 mV (Ishii et al., 1999; Ludwig et al., 1999; Seifert et al., 1999).

In summary, the time constants of activation, mid-point potentials, and modulation by cyclic nucleotides are specific for each channel isoform and allow its identification.

1.3 Immunocytochemical detection of HCN channels in the retina

Monoclonal and polyclonal antibodies were used to study the distribution of the four HCN channel isoforms in the rat retina. Fig. 1.2 gives a brief overview of their cellular localization.

Outer retina. In the outer retina, photoreceptors were stained with antibodies against HCN1 (Fig. 1.2A). It will be shown later, that both rods and cones express HCN1. Prominent HCN3 labeling was observed in the OPL, associated with the endfeet of cones, the cone pedicle synapses (Fig. 1.2D, arrows mark individual cone pedicles). Blood vessels (bv) were labeled unspecifically by the secondary antibody.

Inner retina. In the inner nuclear layer, bipolar cells (long arrow) were positive for HCN1 (Fig. 1.2A). They send dendrites to the outer plexiform layer and axons to dense bands (band 2 and 3) in the middle of the inner plexiform layer. Band 1 seems to originate from amacrine cells rather than bipolar cells (Fig. 1.2A, band 1), because it was not labeled by an antibody against the vesicular glutamate transporter 1, which is a good marker for bipolar axon terminals (data not shown). In fact, many amacrine somata (short arrow) labeled with antibodies against HCN1 were found at the border of INL and IPL.

In the ganglion cell layer, some somata, presumably ganglion cells, were strongly labeled with HCN1 antibodies. Axon bundles were labeled in the nerve fibre layer.

HCN4 was detected in two types of bipolar cells (long arrow), whose axons projected to two different strata in the IPL (Fig. 1.2B, bands 1 and 2). A strongly immunoreactive stratum was observed in the OFF sublamina of the IPL (band 1). A weaker band was detected in the middle of the IPL (band 2) where HCN1-positive bipolar cells stratify (Fig. 1.2A, band 2 and band 3), suggesting that HCN1 and HCN4 channels might be coexpressed in the same cells.

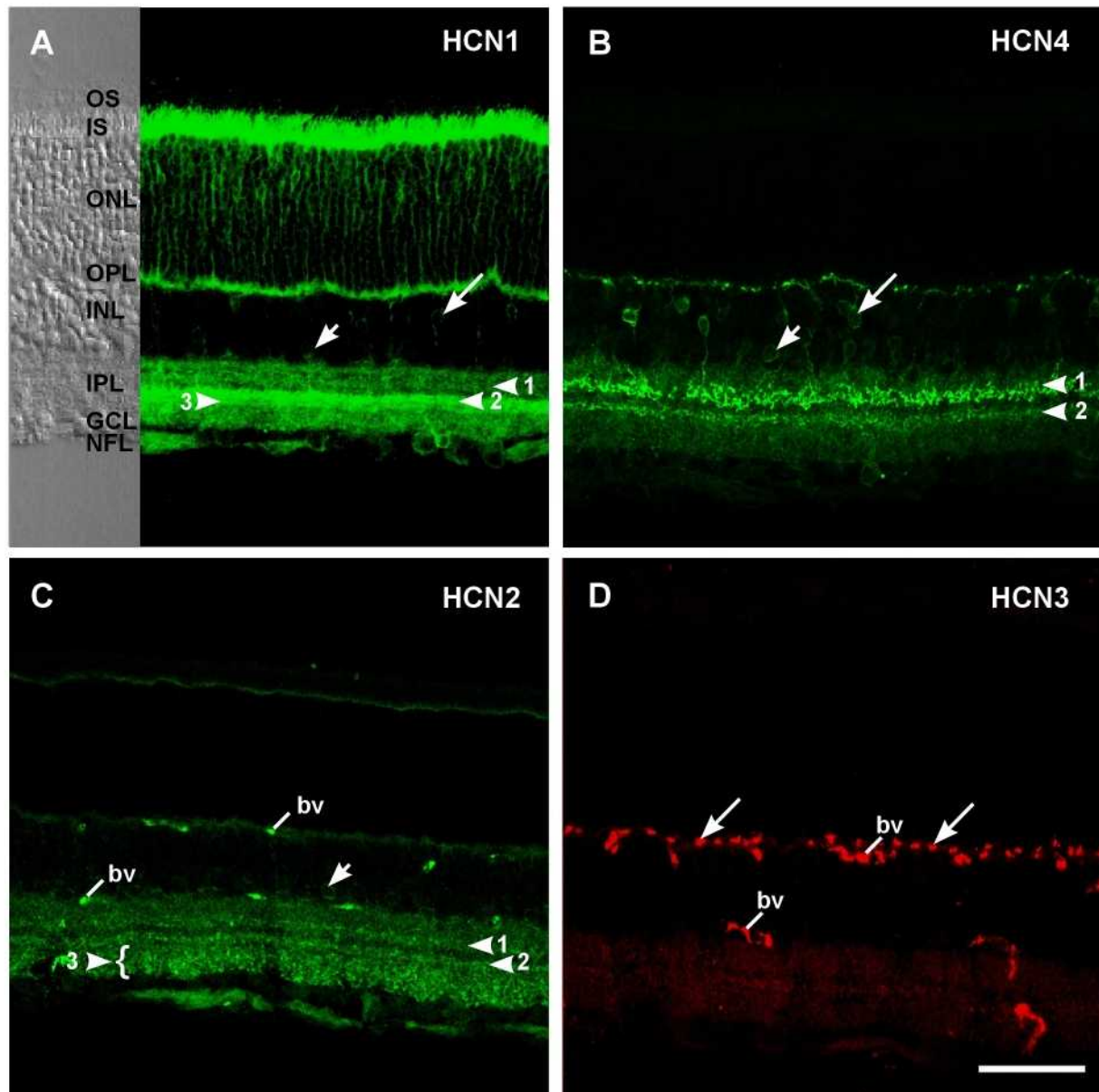


Fig. 1.2: Immunohistochemical localization of HCN1-4.

(A) Left panel: DIC image of a vertical retinal section. Right panel: HCN1-specific antibody labeled photoreceptors. Cone bipolar cells (long arrow) whose axons terminate in the middle of the IPL and amacrine cells (short arrow), some somata in the GCL and ganglion cell axons in the NFL were also labeled. Several bands of immunofluorescence were detected in the IPL (indicated by numbers).

(B) Two bipolar cell types (long arrow) that terminate in the OFF-sublamina (band 1) or in the middle of the IPL (band 2) and some amacrine cells were labeled for HCN4 (short arrow).

(C) HCN2 staining was found in several bands in the IPL (marked by numbers) and in some amacrine cell bodies (short arrow).

(D) Antibodies against HCN3 strongly labeled cone pedicle synapses (arrows) in the OPL and, although much weaker, processes in the IPL.

Scale bar 50 μ m. OS, outer segments; IS, inner segments; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fibre layer; bv, blood vessel.

Few amacrine cells and somata in the GCL were also HCN4-immunoreactive.

HCN2 was detected in several bands in the IPL, in small, lobular structures that were reminiscent of axon terminal systems of bipolar cells (Fig. 1.2C, bands are marked by

numbers). Few amacrine cell bodies (arrow) and, very rarely, some bipolar cell bodies and cone photoreceptors were faintly labeled (not visible at the low resolution used in Fig. 1.2C). Blood vessels (bv) were labeled unspecifically by the secondary antibody.

In summary, the four HCN channel genes are differentially expressed in the retina. HCN channels are expressed in variety of retinal cells and could play an important role in retinal signal transduction. In rod and cone photoreceptors, HCN channels shape the light response. The channels become activated during hyperpolarization in bright light and depolarize the cell toward the dark membrane potential, making the light response transient (Fain et al., 1978; Baylor et al., 1984; Hestrin, 1987).

Currents mediated by HCN channels were recorded in ganglion cells (in goldfish, Tabata and Ishida, 1996; in cat, O'Brien et al., 2002); and were suggested to transiently augment ganglion cell excitability after termination of hyperpolarizing light stimuli (Tabata and Ishida, 1996).

Little is known about HCN currents in bipolar cells. HCN currents were recorded in fish bipolar cells (in goldfish, Kaneko and Tahibana, 1985; in bass, Lasater, 1988; in zebrafish, Connaughton and Maguire, 1998). In mammals, HCN currents have been found in rat rod bipolar cells by Karschin and Wässle (1990) and in some unidentified mouse bipolar cells by Kaneko and coworkers (1989). The function of I_h in these cells is not known. In mammalian cone bipolar cells, HCN currents have not been studied. Fig. 1.2 suggests that three HCN channel isoforms are expressed in subsets of bipolar cells. This is the first indication that different bipolar cell types may differ in their inventory of ion channels and, therefore, might have distinct functional properties. The aim of the current study will be the electrophysiological characterization of HCN channels in rat retina bipolar cells.

1.4 Rat retinal bipolar cells

Nine types of cone bipolar cells and one type of rod bipolar cell have been described in the rat retina (Euler and Wässle, 1995; Hartveit, 1997). The level of stratification in the IPL is the most important morphological criterion to identify the bipolar cell type. Each cell type forms his own stratum in the IPL (Fig. 1.3).

In the IPL, cholinergic ON- and OFF-amacrine cells form two dendritic strata that can be visualized with antibodies against cholineacetyltransferase and which are commonly used as "landmarks" in the IPL (in chicken, Baughman and Bader, 1977; in rabbit, Masland and Mills, 1979; Hayden et al., 1980; in rat, Voigt, 1986). To distinguish bipolar cell types, it is very

helpful to relate their level of axonal stratification to the ON-cholinergic band, which is slightly below the middle of the IPL (dotted line in Fig. 1.3 and the following figures).

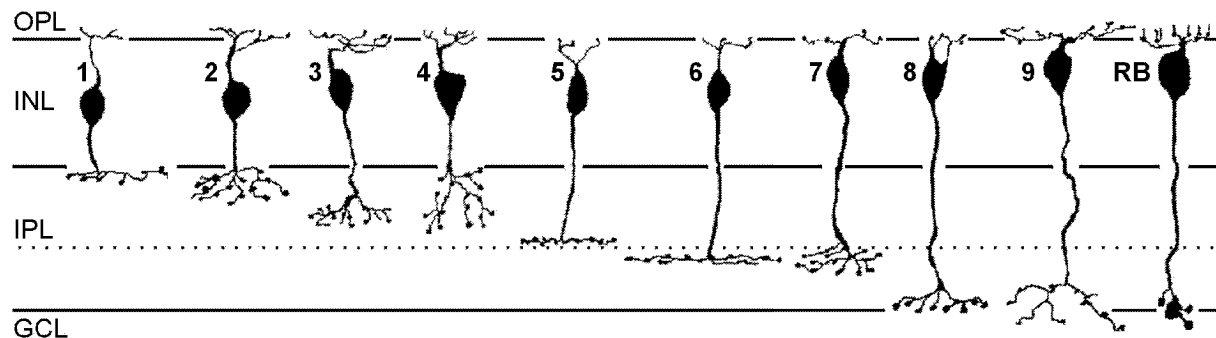


Fig. 1.3 : Morphological classification of bipolar cells in the rat retina.

Classification scheme of bipolar cells in the rat retina (adapted from Euler and Wässle, 1995). Cone bipolar cell types (labeled by numbers) and rod bipolar cell (RB) are marked. The borders of the IPL are depicted by solid lines. The ON-cholinergic band is shown by a dotted line.

If a certain current is to be recorded from a neuron, it is important to ask which other currents are present in the cell, that might interfere with the recording. Several types of voltage-activated channels have been found in the rat retinal bipolar cells.

Depolarization-activated channels. In rod bipolar cells depolarization-activated outward currents with transient (presumably inactivating potassium channels) and sustained (probably delayed rectifier) components were described. Currents were reduced by internal Cs^+ and by application of external TEA. Currents were further suppressed by addition of 5-10 mM Co^{2+} to the bathing medium, indicating the presence of calcium-activated potassium channels (Karschin and Wässle, 1990). In cone bipolar cells potassium currents were not characterized. High- and low-voltage-activated calcium channels were described in rod and cone bipolar cells (Protti and Llano, 1998; Pan, 2000). Sodium channels were found in some cone bipolar cells by Pan and Hu (2000).

Hyperpolarization-activated channels. HCN currents were recorded in rod bipolar cells of rat (Karschin and Wässle, 1990). There is one report on hyperpolarization-activated chloride channels in rod bipolar cells (Enz et al., 1999). It is not known, whether inwardly rectifying channels are expressed in bipolar cells.

Thus, most currents described in bipolar cells are not active within the voltage range of HCN channel activation. Channels active in the hyperpolarizing voltage range can be distinguished by their sensitivity to channel blockers. Inward rectifier currents are blocked by extracellular barium, which does not influence HCN currents. HCN currents are sensitive to extracellular

cesium and can thus be distinguished from chloride currents. Hence, using a small set of pharmacological tools, it should be possible to record and unequivocally identify HCN currents in retinal bipolar cells.

1.5 The aims of the current study

The aim of the present thesis is to study the expression of HCN channels in bipolar cells of the rat retina. The outcome is interesting for several reasons. First, rat bipolar cells have been classified based on morphological criteria, but otherwise, we know little about their properties and their function. Why are there so many bipolar cell types in the retina? Do they only differ morphologically or also functionally? Differences in the inventory of ion channels might give a first indication for functional diversity in retinal bipolar cells. Moreover, this systematic approach might even reveal further subpopulations of bipolar cells that have not been identified in previous studies.

Second, because of their unique properties, HCN channels have intrigued physiologists for more than two decades. However, several interesting issues could not be investigated because the molecular identity of the underlying proteins was unknown. Now, that the genes coding for HCN channels have been cloned and all HCN channel isoforms can be identified using specific antibodies as molecular tools, these issues can be addressed. For example, does a given cell type express a single HCN channel isoform, or several? Are different HCN channel subunits targeted to different compartments of the cell, or do they colocalize? This would allow the formation of heterooligomeric channels and, hence, might increase the functional diversity among HCN channels. Can we find evidence for the formation of heterooligomeric channels in recordings of neurons? Bipolar cells of the retina provide an ideal system, to study these questions. Bipolar cells are highly polarized interneurons. In retinal slices, bipolar cells can easily be recorded and identified after filling intracellularly with fluorescent dyes. Thus, the analysis can be performed on individual, identifiable cell types. Moreover, for many bipolar cell types, specific antibody markers are available, that allow detailed immunohistochemical analysis.

In the past, comparison of the biophysical properties of native and recombinant channels has proven extremely fruitful in the discovery of novel subunits and modulatory mechanisms, e.g.

of phosphorylation. In this study, I will follow this kind of approach. First, the HCN channel isoforms that are expressed in a given bipolar cell will be identified by immunohistochemical analysis. Second, the properties of HCN currents in this cell type can be predicted, based on the functional properties of the respective isoforms recorded in a heterologous system. Third, HCN currents in the bipolar cell will be recorded and compared to the predicted currents. Deviations from the prediction will indicate, whether HCN channels in bipolar cells are modified by additional subunits or other mechanisms to match the physiological demands of the bipolar cell.

2 Materials and Methods

2.1 Heterologous expression of HCN channels in HEK293 cells

2.1.1 Transient expression in HEK293 cells

Heterologous expression of rat HCN1, HCN2, and HCN4 was carried out in HEK293 cells (Human Embryonal Kidney cells, ECACC Nr. 85120602). The cells were cultivated in Petri dishes in MEM medium (Minimal Essential Medium, GIBCO BRL, Cat. Nr. 41090) supplemented with 10 % fetal calf serum, 2 mM L-Glutamin, 1 % Antibiotics/Antimycotics (GIBCO BRL). Further on, this modified MEM medium will be called "M10".

For transient expression, the full-length cDNAs were cloned into the expression vector pcDNA1 Amp (Invitrogen). The HCN channel construct was cotransfected with a plasmid containing the cDNA encoding a mutated GFP (Green Fluorescent Protein, pBOB1.5, gift from Thomas Hughes, Yale University, USA). The excitation maximum of the GFP is shifted by the mutation S65T to 488 nm, thus, the cells coexpressing GFP and HCN channels can be visualized using conventional fluorescence optics with visible light. The cells were transiently transfected with a mixture of the two vectors according to the calcium phosphate transfection protocol (Chen and Okayama, 1987). The following solutions were used:

Tab. 2.1: Solutions for transfection.

Stock solutions	Transfection mixture for Ø 5 cm dish ~4x10 ⁵ cells
HCN channel construct 0.6 - 1 µg DNA/µl	6-10 µg DNA of HCN channel construct + 1 µg DNA of GFP construct
GFP construct 0.5 µg DNA/µl	
H ₂ O	added up to 124 µl
CaCl ₂ 1 M	41 µl
2 x BBS (pH 6.95)*	165 µl

*2 x BBS (pH 6.95) - 50 mM N,N-bis-(2-Hydroxyethyl)-2-Aminoethansulfonacid (BES); 280 mM NaCl; 1.5 mM Na₂HPO₄

The transfection mixture was incubated for 20 min (RT) and then pipetted into the Ø 5 cm Petri dish with logarithmically growing HEK293 cells (~4x10⁵ cells in 3 ml M10). During 20 - 22 h incubation at 35°C, 3 % CO₂ and 95 % humidity the calcium phosphate-DNA precipitate was formed. After washing with 3 ml PBS (RT), the cells were treated with 2 ml PBS/EDTA (0.5 % EDTA in PBS, pH 7.4) at RT to dissolve the precipitate. The cells were detached from the dish with 0.3 ml trypsin/EDTA (0.05 % trypsin/0.02 % EDTA, GIBCO BRL) at 37°C and resuspended in 4.7 ml M10. Approximately 200 µl of the cell

suspension was transferred onto coated glass cover slips in a multiwell plate. The cover slips had been coated with poly-L-lysine (0.1 mg/ml H₂O, Sigma) overnight, washed two times with PBS and covered with 200 µl M10 medium. The cells were incubated at 37°C, 5 % CO₂ and 95 % humidity. Four hours after plating, sodium butyrate (0.5 M in PBS, Sigma) was added at a final concentration of 3 mM to foster expression. Before the patch-clamp experiments, the expression of HCN channels was checked by immunocytochemistry (2.3.2.2). The inspection of the cells indicated a transfection efficiency of about 15 %. More than 80 % of GFP-positive cells coexpressed HCN channels (Fig. 2.1). The cells were used for patch-clamp experiments during the following two days.

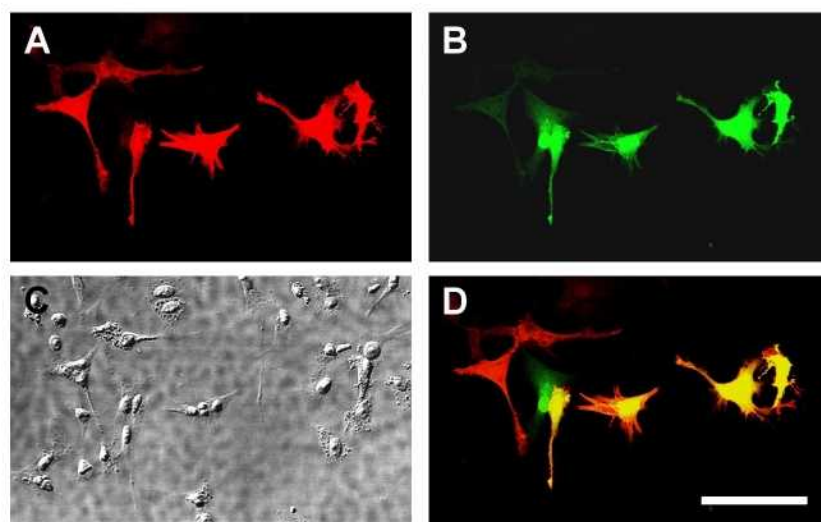


Fig. 2.1: GFP and HCN2 channels cotransfected in HEK293 cells. HCN2-positive cells are shown in **A** (red), and GFP-positive cells in **B** (green). The cells coexpressing both proteins are yellow in the overlay (**D**). (**C**): Differential Interference Contrast (DIC) image of the same region. Scale bar: 50 µm.

PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM K₂HPO₄, pH=7.4-7.5

2.1.2 Stable expression in FLP-INTM-293 cells

Two stable cell line were used: Flp-hHCN1 and Flp-hHCN4. The cell lines were generated by cotransfection of HEK293 - FlpIn cells (FLP-INTM-293, Invitrogen) with plasmid vector (pcDNA 5-FRT, Invitrogen) bearing the gene of interest, and the plasmid (pOG44, Invitrogen) encoding Flp recombinase. Transfection was made following the standard protocol. In the genome of these cells, FRT sites (Flp Recombination Target site) flank the zeocin resistance gene. The FRT site serves as the binding and the cleavage site for the Flp recombinase. Upon cotransfection, Flp recombinase mediates a homologous recombination

event between the FRT sites such that the pcDNA 5-FRT construct is inserted into the genome. The plasmid vector with the gene of interest also contains the hygromycin resistance gene. Insertion of the vector into the genome inactivates the zeocin resistance gene and confers the hygromycin resistance to the cells. The cells with the gene of interest can be selected for hygromycin resistance and zeocin sensitivity.

The cells were cultivated in Petri dishes in DMEM medium (GIBCO BRL, Cat. Nr. 41965) supplemented with 10 % fetal calf serum, 2 mM L-Glutamin, 1 % Antibiotics/Antimycotics (GIBCO BRL). Hygromycin was added into the medium at a final concentration of 100 µg/ml. The expression of HCN channels was checked by immunocytochemistry and electrophysiology methods.

2.2 Electrophysiology

2.2.1 Setup

The specimens were viewed under an up-right microscope (Axioskop FS, Zeiss) with x 40 water immersion objective (Zeiss, 40/0.75 W) and DIC (Differential Interference Contrast) optic. To visualize Lucifer Yellow (LY) filled bipolar cells in the retina or GFP in HEK293 cells, I employed epifluorescence using a mercury fluorescent lamp (Osram) and LY filter set (Zeiss, Nr.5).

The electrical measurements were performed with two Ag/AgCl electrodes. The reference electrode was an Ag/AgCl pellet, the second electrode was an Ag/AgCl wire placed into the glass pipette. I used soft glass (Blaubrand Cat. Nr. 7087-33, Brand) and borosilicate filament glass (Hilgenberg, Malsfeld). The pipettes were pulled on a horizontal DMZ-Universal puller (Zeitz, Augsburg). The tips of the borosilicate pipettes were heat-polished (Microforge L/M-CPZ 101, Darmstadt).

The electrodes were connected with the headstage of a patch-clamp amplifier (Axopatch 200A, Axon Instruments). The headstage (CV 201A) was mounted onto a micromanipulator (SM 1, Luigs & Neumann). Clampex 6 software was used for stimulus generation and simultaneous data acquisition. Holding and command potentials of the applied stimulus protocols were converted by a 12-bit digital-to-analog converter. After filtering with 5 kHz low-pass filter, signals were digitized by a 12-bit 125 kHz analog-to-digital converter (TL-1, Axon Instruments). Data were stored at a sampling rate of 1 kHz on the hard disk of a personal computer (PC SD-LC 486).

2.2.2 Whole-cell experiments on retina slices

2.2.2.1 Solutions

All compounds were purchased from Sigma or from Merck. All solutions were prepared with bidistilled water. The pH of solutions was adjusted after all compounds had been added. Solutions were aliquoted and kept frozen at -20°C .

Extracellular solution Nr. 1 (Extr. sol. Nr. 1) was used as the standard solution and was exchanged for Nr. 2 only in the experiments where I used barium or cobalt. These divalent cations precipitate with sulphates, phosphates and carbonates present in Extr. sol. Nr. 1. Cobalt, cesium and barium chloride were prepared as 1 M or 2 M stock solutions and added at a final concentration of 4 mM, 1 mM, 0.3 mM, respectively, to Extr. sol. Nr. 2 before the experiment.

The Nernst potential was -83 mV , $+65\text{ mV}$, -53 mV for potassium, sodium, and chloride ions, respectively.

Tab. 2.2: Solutions for the experiments on slices.

Compound	Extracellular solutions		Intracellular solution
	Nr. 1 [mM]	Nr. 2 [mM]	Nr. 1 [mM]
NaCl	136.89	136.89	10.00
$\text{KC}_6\text{H}_{11}\text{O}_7$	-	-	126.00
KCl	5.36	5.36	4.00
CaCl_2	1.25	0.81	1.00
MgCl_2	-	1.25	1.00
Hepes	10.00	10.00	10.00
D-glucose	5.55	5.55	-
Phenol Red	0.03	0.03	-
Na_2HPO_4	0.34	-	-
KH_2PO_4	0.44	-	-
MgSO_4	0.81	-	-
EGTA	-	-	10.00
ATP 1 mol Mg^{2+} /mol	-	-	1.00
GTP 3 mol Na^{+} /mol	-	-	1.00
Lucifer Yellow (LY)	-	-	0.15
Biocytin	-	-	10.00
	pH 7.4, adjusted with NaOH		pH 7.3, adjusted with KOH ~2 mM KOH/mM EGTA

The low chloride concentration of the intracellular solutions created a liquid junction potential (measured according to Neher, 1992) of 10 mV. The value, calculated with "Junction Potential Calculator" (MS Windows version, Axon Instruments) was 14 mV. Membrane voltages were corrected Off-line for the measured liquid junction potential of 10 mV.

2.2.2.2 Preparation of retina slices

Adult rats (Wistar) 5 and 8 weeks of age were deeply anesthetized with isoflurane (CuraMED Pharma GmbH) and decapitated. The eyeball was dissected free and transferred into a small Petri dish under a binocular microscope. The eye was completely submersed in Extr. sol. Nr. 1, which had been bubbled with oxygen for 30 min. With fine-tipped forceps the eye was held at the conjunctiva and a small incision was made in the eyeball at the equator with an injection needle. This incision was then cut further open with small dissecting scissors. After opening the eye completely, the cornea, the lens and the vitreous were carefully removed. The peripheral pieces of the retina were cut off. To ease flattening of the half-spherical retina, three small incisions from the periphery towards the center were made. The retina was isolated from the rest of the eyecup and transferred in a small spoon into a custom made chamber for cutting slices.

With two fine brushes the retina was mounted onto a millipore filter (Schleicher and Schuell, NC10, Dassel, Germany) with the photoreceptor side up. Beneath the filter, a sintered glass was located. A small amount of the solution from the chamber was sucked through the filter and sintered glass. By this the retina was attached to the filter. Vertical slices of 150 μm thickness were cut together with the filter by rolling with a curved scalpel blade over the retina (Boos et al., 1993). From a retina, on average 15 good slices were obtained.

DNA released from the cells which had been damaged by the slicing procedure, prevented the formation of the gigaseal. Therefore, slices were treated with DNase I (Roche, Molecular Biochemicals; 50 $\mu\text{g/ml}$ in Extr. sol. Nr. 1) for 10 min to remove DNA.

During patch-clamp experiments, only one or two slices were present in the recording chamber, others were kept in darkness in a closed Petri dish perfused with moistened oxygen. Slightly covered with extracellular solution, slices had sufficient oxygen supply and could be well maintained for at least five hours at RT.

2.2.2.3 Recording chamber

Electrophysiological measurements were performed in a plexiglass chamber with a glass bottom (Fig. 2.2). The chamber had a very small volume ($\sim 200 \mu\text{l}$). On two opponent borders, incisions were made to fix the slices. The recording chamber was superfused continuously at a rate of $\sim 2 \text{ ml/min}$. Four different kinds of continuously oxygenized solutions could be delivered into the chamber by gravity force through plastic tubes that fed into four joined

together needles connected to the chamber. Solution was removed from the chamber by a peristaltic pump (Varioperpex II pump, LKB) at the opposite end of the chamber. The reference electrode was situated in a close proximity to the input of the solutions into the recording chamber.

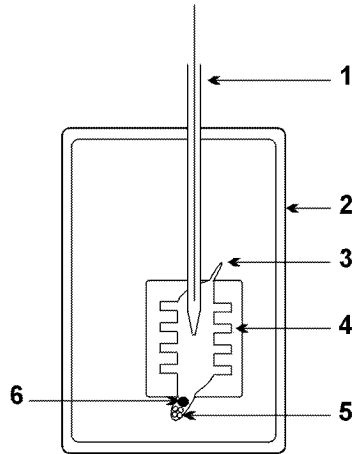


Fig. 2.2: Schematic drawing of the recording chamber for retina slices. 1 – patch pipette with the electrode inside; 2 - border of the chamber holder; 3 - output from the chamber where solution is removed by the pump; 4 - incisions for fixing slices; 5 - input of the solutions; 6 - reference electrode.

2.2.2.4 Identification of bipolar cells and photoreceptors

To record from bipolar cells, I patched somata in the middle and distal parts of the INL. Cone bipolar cell somata are mostly situated at the middle of the INL and rod bipolar cell somata are localized mainly at the outer border of the INL (Euler and Wässle, 1995). With a certain practice, I could recognize rod bipolar cell and type 5 cone bipolar cell somata because of their characteristic morphology and position in the INL. Other types could be identified only after filling the entire cell with LY. After patch-clamp recordings, the slices were counterstained with specific antibody markers (2.3.2.3).

To record from cones, large elongated somata in the outer half of the ONL were patched. Small rounded somata in the ONL were patched to record from rods. Cell identity was confirmed after filling of the cell with LY.

2.2.2.5 Experimental protocols

Pipettes were made from the soft glass and had resistances of $\sim 13 \text{ M}\Omega$. The measurements start with a pipette being pressed against an intact cell to form a gigaseal between the pipette

and the cell membrane. In my measurements, the seal resistance was at least 10 G Ω . All electrophysiological recordings were carried out in darkness. The light was turned off as soon as the gigaseal had been achieved. After electrical compensation of pipette capacitance, the membrane patch under the pipette was ruptured by gentle suction, and the whole-cell configuration was established. In retinal cells, series resistance and membrane capacitance were usually not compensated (2.2.2.6).

The membrane potential of the bipolar cells was clamped to a holding value of $V_m = -50$ mV (which is close to the resting potential of most bipolar cells) and HCN currents were recorded during families of 500 ms hyperpolarization steps ranging from -55 mV to -115 mV, in -15 mV increments. In some cases, an additional voltage step to -40 mV was applied. At $V_m = -50$ mV, HCN channels have a finite open probability; moreover, when V_m is stepped back to -50 mV after hyperpolarization, closure of HCN channels is rather slow. Therefore, after each hyperpolarizing step, the membrane was clamped to the holding voltage $V_m = -50$ mV for 250 ms to measure tail currents and then to $V_m = +10$ mV for 150 ms. During this step HCN channels closed rapidly; 100 ms later, the next hyperpolarizing step was applied.

Two parameters of the channels were investigated in detail: the activation kinetics and the mid-point potential of activation.

The activation kinetics of the channels was used as a criterion to identify the isoforms of HCN channels, expressed in the particular cell type. I used 14 s hyperpolarization steps to $V_m = -100$ mV, long enough to reach steady state conditions in all HCN channel isoforms. Only one step was used in order not to damage the cells during the application of non-physiologically negative membrane potentials. In all recordings of the current study, the activation kinetics of the channels was measured within the first minute after establishing the whole-cell configuration. During this time, no noticeable "rundown" of channel activity was observed. When the current underwent abrupt changes, the recording was stopped.

The second parameter which was analyzed in detail is the mid-point potential of activation, $V_{1/2}$. For the $V_{1/2}$ measurements, 5 s hyperpolarizing voltage steps ranging from -16 mV to -114 mV in -7 mV increments were applied. After each hyperpolarizing step, the tail currents from the HCN channels were measured at $V_m = -114$ mV. Then the membrane voltage was clamped to $V_m = -50$ mV for 200 ms and to $V_m = +10$ mV for 250 ms to close the channels.

The usual mode of operation of patch-clamp was voltage-clamp. In the voltage-clamp mode the membrane potential was controlled, and the current needed to maintain the potential was

recorded. In some experiments I used the current-clamp mode, in which the membrane current was controlled while the membrane voltage was recorded. The current of the cell was clamped to $I_m = 0$ pA, which corresponds, by definition, to the resting potential of the cell. Current injections were made in the FAST-CLAMP current-clamp mode of the amplifier. The increment size of the current steps was -2.5 pA.

2.2.2.6 Evaluation of the space clamp

Rat bipolar cell axons are 15 to 60 μm long, depending on the cell type. Rod bipolar cells have the longest axon of ~ 60 μm and the question may arise whether the axon terminal system is properly clamped. In mouse bipolar cells, a space constant of 700 μm was estimated (De la Villa et al., 1998). Karschin and Wässle (1990) found a space constant of 340 μm in rat rod bipolar cells.

In my recordings the cell capacitance was around 3-4 pF, which corresponds to the cell surface of 400 μm^2 . Assuming a membrane resistance of 10 $\text{G}\Omega$, a cytoplasm resistance of 2 $\Omega\cdot\text{m}$ (data were taken from De la Villa et al., 1998), and an axon diameter of ~ 1 μm , a space constant of 450 μm could be calculated. The series resistance was maximally ~ 35 $\text{M}\Omega$, and the current recorded at -100 mV was around -100 pA. The voltage error produced by the series resistance was less than 5 % and, therefore, was not compensated. It seems likely that the axon terminal was fairly well clamped.

2.2.3 Whole-cell experiments on HEK293 cells

2.2.3.1 Comparative study of HCN channels heterologously expressed in HEK293 cells

I studied heterologously expressed HCN channels in HEK293 cells under conditions identical to those used for the retinal experiments (see 2.2.2). Soft glass pipettes with a resistance of 10 $\text{M}\Omega$ were used.

I studied human HCN1 and HCN4 channels that were expressed in stable cell lines, and rat HCN1, HCN2, HCN4 channels that were transiently expressed. Concerning the activation kinetics and $V_{1/2}$ measurements, no significant differences were observed between rat and human HCN1, and rat and human HCN4, respectively, and data were combined for the

analysis.

In HEK293 cells, HCN currents were usually less than 200 pA at $V_m = -100$ mV. In the recordings, series resistance was less than 25 M Ω . In the case of a small current (≤ 200 pA), series resistance and membrane capacitance were not compensated. The voltage error resulting from uncompensated series resistance was less than 5 %. In all others cases, up to 70 % of compensation was achieved.

$V_{1/2}$ of HCN2 was measured with the same protocol as in the retina. For the $V_{1/2}$ measurements of HCN1 channels, 2 s voltage steps from -10 mV to -120 mV in -10 mV increments were applied and the tail current was measured at $V_m = -90$ mV. For HCN4 channels, 5 s voltage steps from -10 mV to -130 mV in -10 mV increments were applied and the tail current was measured at $V_m = -90$ mV. In all protocols, after each hyperpolarizing voltage step, membrane voltage was clamped to $V_m = -50$ mV for 200 ms and then to $V_m = +10$ mV for 250 ms to close HCN channels.

The reversal potential (V_{rev}) of the channels was measured with the following voltage protocol: HCN channels were activated with 600 ms hyperpolarization steps to $V_m = -110$ mV. Tail currents were measured at different membrane voltages ranging from -2 mV to -32 mV in -3 mV increments. Tail currents were plotted against the voltage. Tail currents depended linearly from the voltage and V_{rev} was determined as -19.0 ± 0.8 mV ($n = 6$).

2.2.3.2 Investigation of the influence of intracellular calcium on HCN2 channels

To investigate the influence of intracellular calcium on HCN channel properties, I recorded two sets of HEK293 cells expressing HCN2 channels with either 0 μ M (Intr. sol. Nr. 2, Tab. 2.3) or 10 μ M (Intr. sol. Nr. 3, Tab. 2.3) free calcium concentration in the pipette solution. The calcium concentration was adjusted with BAPTA ($K_{d_{BAPTA}} = 210$ nM).

EGTA and BAPTA are widely used calcium buffers. EGTA is considered to be a slow calcium buffer, which can not control fast changes in the calcium concentration. BAPTA is a very fast calcium buffer, which is sufficient to control rapid changes in the calcium concentration. Both buffers allow to precisely adjust the calcium concentration in similar concentrations ranges ($K_{d_{BAPTA}} = 210$ nM, buffering range is from 69 nM to 690 nM; $K_{d_{EGTA}} = 110$ nM, buffering range is from 34 nM to 340 nM; data were taken from MaxChelator program, corrected 2.10 version, Stanford University).

Thus, while both buffers are suitable to adjust very low calcium concentrations, the buffering range does not cover the desired concentration of 10 μM calcium. Hence, using one of these buffers, this calcium concentration will be less well defined. Alternatively, for the high calcium concentration, another buffer substance with lower affinity might be used, e.g. HEDTA ($K_{\text{dHEDTA}} = 5 \mu\text{M}$, buffering range is from 1.6 μM to 16 μM ; data were taken from MaxChelator program, corrected 2.10 version, Stanford University). However, there is a disadvantage when comparing the results of experiments with BAPTA on one hand and HEDTA on the other hand: not only the calcium concentration is changed, but also the buffer substance. It cannot be ruled out that either one substance might have unspecific effects directly on the HCN channels or on the membrane properties of the HEK293 cells. Therefore, in the present study, I performed two sets of experiments. In one set, the concentration of 10 μM calcium was adjusted with BAPTA, in the second with HEDTA. The results of both experiments are quite comparable. In the following, I will only show the experiments with BAPTA.

In these experiments, HCN2 and GFP were cotransfected in HEK293 cells, and the cells, expressing HCN channels, were visualized using epifluorescence. The GFP expression level seemed proportional to the expression level of HCN channels. Only cells with strong GFP fluorescence could be found, thus, the HCN currents recorded in these cells were very large ($>1 \text{ nA}$). In recordings from such cells, the voltage drop due to series resistance could be significant and the cell membrane might be not properly clamped. Thus, the extracellular potassium concentration was reduced (Extr. sol. Nr. 3, Tab. 2.3) to decrease the size of HCN currents. Borosilicate pipettes with large tip opening were used, the pipette resistance was between 5 and 7 $\text{M}\Omega$. In all cases, 70 % of compensation was routinely achieved.

The holding potential of the cells was $V_{\text{m}} = -10 \text{ mV}$. For the $V_{1/2}$ measurements I used voltage protocols with 1.6 s hyperpolarizing steps. The steps ranged from -5 mV to -115 mV in -15 mV increments; when necessary an additional voltage step to -130 mV was performed. The tail current was measured at -100 mV . After tail current measurements, the membrane voltage was clamped to $V_{\text{m}} = +10 \text{ mV}$ for 250 ms to close the channels. This relatively short voltage protocol allowed to keep the cell alive for a very long time with a minimal "rundown" of the channel activity.

The high calcium concentration in the pipette solution favours the closure of the membrane after rupturing. This prevents the dialysis of the cell with the pipette solution. The access was continuously controlled by monitoring the series resistance and cell capacitance during dialysis of the cell with the pipette solution. Correctness of compensation was proved before

and after application of each voltage protocol.

In these experiments, I recorded data 1 min (time needed for the series resistance and pipette capacitance compensation) and 10 min (time needed for dialysis of the entire cell with the pipette solution) after the whole-cell configuration had been established.

Extr. sol. Nr. 3 contained EGTA and was used to eliminate any possible calcium influx into the cells.

Tab. 2.3: Solutions for studying the influence of intracellular calcium on HCN2 channels.

Compound	Extracellular solution Nr. 3 [mM]	Intracellular solutions	
		Nr. 2 (0 μ M of free Ca^{2+}) [mM]	Nr. 3 (10 μ M of free Ca^{2+}) [mM]
NaCl	137.89	10.00	10.00
$\text{KC}_6\text{H}_{11}\text{O}_7$	-	126.00	126.00
KCl	1.00	4.00	4.00
CaCl_2	-	-	4.91
MgCl_2	4.00	1.01	0.88
Hepes	10	10.00	10.00
BAPTA	-	5.00	5.00
EGTA	1	-	-
ATP 1 mol Mg^{2+} /mol ATP	-	1.00	1.00
	pH 7.4, adjusted with NaOH, ~2 mM NaOH/mM EGTA	pH 7.3, adjusted with KOH, ~4 mM KOH/mM BAPTA	

The intracellular free calcium concentration was calculated using MaxChelator (MaxChelator, corrected 2.10 version, Stanford University) from the total concentration of the corresponding calcium buffer (EGTA, BAPTA) and divalent cations, assuming that ATP is also a buffer for divalent cations.

2.2.4 Outside-out recordings of HCN2 channels in HEK293 cells

Current recordings were performed from membrane patches in the outside-out configuration. For these experiments, another chamber was used (Fig. 2.3). The chamber was supplemented with glass barrels connected with plastic tubes to 10 ml syringes which were fixed 25 cm above the chamber. Test solution from the syringes were gravity-fed to the chamber. All parts of the chamber were made from either plastic or glass, no metal connections were used.

The cells were continuously superfused with the bath solution at a rate of ~1 ml/min. The borosilicate heat-polished pipettes had resistances of ~4 M Ω . Slowly pulling the pipette away from the cell in the whole-cell configuration yielded a patch in the outside-out configuration.

The patch was kept at a holding potential of +10 mV and was exposed to two different test solutions. The test solutions were identical to Extr. sol. Nr. 2, except that potassium concentration was elevated to 30 mM by adding KCL to increase the HCN currents. To one solution, 4 mM CoCl_2 was added. The pipette solution was Intr. sol. Nr. 1 with additional 20 mM NaCl. Hyperpolarizing voltage steps of 1.6 s, ranging from -40 mV to -115 mV in -15 mV increments were applied to activate the channels.

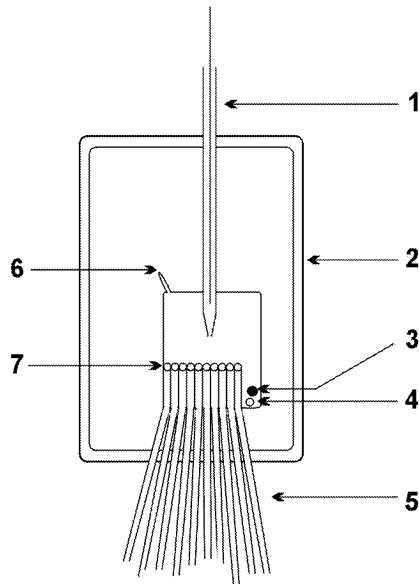


Fig. 2.3: Schematic drawing of the recording chamber for outside-out experiments. 1 - pipette with the electrode inside; 2 - border of the chamber holder; 3 - reference electrode; 4 - input of the bath solution; 5 - tubes connected with syringes; 6 - output from the chamber where solution is removed by the pump; 7 - openings from the perfusion.

2.2.5 Data analysis

Data were analyzed with Clampfit 6 (Axon Instruments Software) and the final figures were created with Sigma-Plot software.

All data were corrected off-line for the liquid junction potential of 10 mV (2.2.2.1) and filtered at 300 Hz, using a Gaussian low-pass filter. Only cells with negligible leak currents were included in the analysis. Leak currents were not subtracted.

Time constants of activation were determined by fitting the late rising phase of HCN currents, activated at $V_m = -100$ mV, with a single, double or triple exponential functions. The initial lag of the rising phase was excluded from the fitting procedure. The size of HCN currents given in Tab. 3.2 and Tab. 3.3 was calculated by subtracting the amplitude of the instantaneous current from the amplitude of steady state currents.

Tail currents were plotted as a function of the hyperpolarization voltage step and fitted with the Boltzmann equation:

$$I = A + \frac{I_{\max}}{1 + \exp\left(\frac{-(V_m - V_{1/2})}{S}\right)},$$

where

I	-	the tail current,
A	-	the offset,
$I_{\max}$	-	the maximal current,
V_m	-	the applied test voltage,
$V_{1/2}$	-	the mid-point potential,
S	-	the slope of Boltzmann curve.

In some cases I used the steady state current at the end of hyperpolarization steps to obtain the activation curves. Steady state current amplitudes measured at the end of hyperpolarizing steps were plotted as a function of the applied voltages and fitted with the Boltzmann equation for steady state currents:

$$I = A + \frac{G_{\max} * (V_m - V_{\text{rev}})}{1 + \exp\left(\frac{-(V_m - V_{1/2})}{S}\right)},$$

where

$G_{\max}$	-	the maximal conductance,
V_{rev}	-	the reversal potential of the channels.

Fits from Boltzmann curves of n experiments were normalized and averaged.

All data are reported as means $\pm$ SD. The number of the cells tested is shown in parenthesis.

2.3 Immunocytochemistry

2.3.1 Confocal microscopy

All fluorescent specimens were viewed by using a Leica TSC SP confocal inverted microscope. The microscope was suited for conventional microscopy with transmitted light and epifluorescence, as well as for confocal laser scanning microscopy. An argon-krypton laser was used that emitted light at the following wavelengths: 476 nm, 488 nm, 568 nm and 647 nm. Three of these lines were used to excite the fluorophores of the secondary antibodies used in this study: 488 nm for FITC and Alexa488; 568 nm for Alexa568 and Cy3; 647 nm for Cy5. Three confocal detectors allowed simultaneous imaging of different fluorescent stainings. Transmitted light was used to create a DIC image of the specimen. Laser lines and emission filters were optimized with the Leica TSC Power Scan software. Concentration of

primary and secondary antibodies, laser intensity and filter settings were carefully controlled to completely rule out crosstalk between the fluorescence detection channels. In multi-label experiments, the different fluorophores were scanned sequentially. The confocal principle yields optical sections of thick specimens. Stacks of confocal images can be obtained by changing the confocal plane. Using the Power Scan software, images of such stacks could be "projected" onto a single image with an extended depth of focus. If not indicated otherwise, all images shown resulted from such projection. Images were processed using Adobe Photoshop or Corel Draw.

2.3.2 Immunostaining

The staining was carried out according to the standard protocol (Tab. 2.6). All incubation steps were done at RT in darkness. In some cases, the staining was intensified by using a secondary antibody raised in goat, followed by a tertiary antibody donkey-anti-goat, that carried the same fluorophore.

For double or triple labeling, primary antibodies were mixed and applied simultaneously. All secondary antibodies were highly crossabsorbed and carefully tested to exclude reactions with the wrong primary antibody. Control experiments included the omission of one primary antibody, resulting in labeling by the remaining primary antibody only, or omission of both primary antibodies resulting in nonspecific background staining.

Tab. 2.4: List of primary antibodies.

Antibodies	Specificity	Working dilution	Source
7C3	Rat anti-HCN1	1:50	E. Kremmer, GSF, München, Germany
Anti-HCN1	Rabbit anti-HCN1	1:400	Alomone, Jerusalem, Israel
4A6	Rat anti-HCN2	1:5	E. Kremmer, GSF, München, Germany
6C5	Rat anti-HCN3	1:5 – 1:20	E. Kremmer, GSF, München, Germany
1E5	Rat anti-HCN4	1:5 – 1:20	E. Kremmer, GSF, München, Germany
73	Rabbit anti-HCN4	1:400	A. Scholten, IBI-1, Jülich, Germany
PKC α	Rabbit anti-PKC α	1:40,000	Santa Cruz, Heidelberg, Germany
PKC γ	Mouse anti-PKC γ	1:1000	Transduction Laboratories, Inc., Freiburg, Germany
Recoverin	Rabbit anti-recoverin	1:40,000	K.W. Koch, IBI-1, Jülich, Germany
Chat	Mouse anti-cholineacetyltransferase	1:400	Chemicon, Hofheim, Germany
mGluR6	Rabbit anti-mGluR6	1:7,500	S. Nakanishi, Kyoto, Japan
Kinesin II	Mouse anti-kinesin II	1:50	Berkeley Antibody Company, Richmond, USA
CabP	Mouse anti-calbindin	1:300	Sigma, Deisenhof, Germany
Anti-LY biotinylated	Rabbit anti-LY	1:200	Molecular Probes, Leiden, Holland
GLT-1	Rabbit anti-GLT-1	1:500	S. Haverkamp, MPI, Frankfurt, Germany

Tab. 2.5: List of secondary antibodies and conjugates.

Antibodies	Working dilution	Source
Goat-anti-rabbitAlexa488	1:500	Molecular Probes, Göttingen, Germany
Goat-anti-ratAlexa488	1:500	Molecular Probes, Göttingen, Germany
Goat-anti-mouseAlexa488	1:500	Molecular Probes, Göttingen, Germany
Goat-anti-ratAlexa568	1:500	Molecular Probes, Göttingen, Germany
Donkey-anti-ratCy3	1:500	Dianova, Hamburg, Germany
Donkey-anti-mouseCy3	1:100	Dianova, Hamburg, Germany
Donkey-anti-goatFITC	1:100	Dianova, Hamburg, Germany
Donkey-anti-rabbitCy3	1:1000	Dianova, Hamburg, Germany
Donkey-anti-mouseCy5	1:100	Dianova, Hamburg, Germany
StreptavidinAlexa488	1:1000	Molecular Probes, Göttingen, Germany

Tab. 2.6: Standard protocol for immunocytochemistry.

Incubation step	Incubation conditions: solution / time			
	Retina			HEK293 cells
	Cryostat sections	Wholemout	Slices	
Preincubation	CT / 1 h	CT / 3 h	CT / 2 h	CT / 20 min
Incubation with primary antibodies	CTA / 15 h	CTA / 4 days	CTA / 24 h	CTA / 1 h
Washing	PB / 3 x 10 min	PB / 3 x 1 h	PB / 5 x 10 min	PB / 3 x 2 min
Incubation with secondary antibodies	C / 1 h	CA / 15 h	CA / 12 h	C / 30 min
Washing	PB / 3 x 10 min	PB / 3 x 20 min	PB / 5 x 10 min	PB / 3 x 2 min
Incubation with tertiary antibodies	C / 1 h	-	CA / 6 h	-
Washing	PB / 3 x 10 min	-	PB / 5 x 10 min	-

PB: 19 mM Na₂HPO₄, 81 mM NaH₂PO₄, pH 7.4

CT: 5 % chemiblocker (Chemicon), 0.5 % Triton-X 100 in PB

CTA: 5 % chemiblocker, 0.5 % Triton-X 100, 0.05 % NaN₃ in PB

CA: 5 % chemiblocker, 0.05 % NaN₃ in PB

C: 5 % chemiblocker in PB

2.3.2.1 Immunostaining of retina sections

All immunocytochemical stainings on retina sections were done in collaboration with F. Müller (IBI - 1, Forschungszentrum Jülich).

Adult rats were deeply anesthetized with isoflurane and decapitated. Eyes were enucleated and opened by an encircling cut at the limbus. The retinae in the eyecup were immersionfixed for 10 - 15 min in 4 % PA in 0.1 M PB (RT) and washed in PB several times. For the staining with GLT-1 antibodies, the fixation time was increased to 1 h. Tissue was incubated in 10 % sucrose in PB for 30 min, followed by 30 % sucrose for 3 hours. The retina was flat embedded and frozen in OCT compound (Miles Scientific). Twenty µm thick vertical sections were cut on a cryostat (Reichert-Jung) and collected on gelatinized slides* (2.3.2.1.1). The staining was carried out according to the standard protocol (Tab. 2.6). Stained sections were

coverslipped with Prolong antifade kit (Molecular Probes; Gillingen, Germany) or Aqua Polymount (Polysciences; Eppelheim, Germany).

For the wholemount staining, the fixed and cryoprotected retina was freeze-thawed several times and then stained and flat embedded.

***Slides** (Superfrost, Menzel) were washed overnight in absolute ethanol and then in distilled water. The slides were coated with gelatin (0.5 % in distilled water, for 30 s) at 50°C, and dried at 60°C.

4 % PA: 4 % paraformaldehyde, dissolved in PB at 60°C

2.3.2.2 Immunostaining of HEK293 cells

Cover slips with transfected HEK293 cell were washed twice with Extr. solution Nr. 1. Cells were fixed with 4 % PA in 0.1 M PB for 10 min at RT and washed in PB several times. The stainings were carried out following the standard protocol (Tab. 2.6).

The cover slips with the stained cells were put upside down onto microscope slides with a small drop of Aqua Polymount.

2.3.2.3 Immunostaining of retina slices

After recordings, the slices with LY- and biocytin-filled cells were fixed for 10 min with 4 % PA in 0.1 M PB at RT in a multiwell plate and washed in PB several times. Free-floating slices were stained following the standard protocol (Tab. 2.6). In order to increase the fluorescence of the filled cells, I used a biotinylated rabbit anti-LY antibody followed by donkey anti-rabbitAlexa488 and StreptavidinAlexa488. Biocytin was visualized using StreptavidinAlexa488. When slices were stained for recoverin, biocytin was visualized using StreptavidinAlexa488. No anti-LY antibodies were used in these experiments.

Stained slices were transferred into a custom made chamber filled with PB and examined with the inverted confocal microscope.

2.3.3 Electron microscopy

Electron microscopical images of HCN3 channel distribution in the retina were kindly provided by S. Haverkamp (Max-Planck-Institut für Hirnforschung, Frankfurt).

Adult rats were killed and retinae were prepared as described above (2.3.2.1). Retinae were fixed in 4 % PA for 40 min. Retinae were dissected out, cryoprotected in 30 % sucrose overnight and shock frozen in liquid nitrogen. Vibratome sections were cut and processed as described previously using the ABC Detection Kit (Vector) and diaminobenzidine as chromogen, followed by silver intensification (Sassoè-Pognetto et al., 1994).

certain peak and then began to rise. The duration of the initial lag phase differs among the channel isoforms and was voltage dependent. The initial lag phase was followed by the rising phase, at the end of which the currents reached a steady state plateau. This complex activation kinetics shows a sigmoidal shape and is a characteristic feature of HCN currents.

After stepping back to the holding voltage $V_m = -50$ mV, tail currents (Fig. 3.1A, marked by arrows), of the slowly closing HCN channels were observed, whose amplitudes were dependent of the membrane voltage of the preceding hyperpolarizing step.

The activation kinetics as well as the tail current kinetics were strongly voltage dependent and characteristic for each channel isoform. HCN1 channels displayed the fastest kinetics. In the last three voltage steps to $V_m = -85$ mV, -100 mV, -115 mV, the currents reached a steady state plateau. The initial lag phase was the shortest among HCN channels (barely visible in the current traces). Deactivation of the tail currents was very fast.

HCN4 channels showed the slowest kinetics. In the last three steps very slowly activating currents were observed. At the end of 500 ms voltage steps, currents were still far away from steady state conditions. The initial lag phase was very prominent. Deactivation of the tail currents was very slow.

The kinetics of HCN2 channels was somewhat intermediate between HCN1 and HCN4 channels. The channels are clearly active already at the second voltage step, but only in the very last voltage step the current was close to reaching steady state conditions. The initial lag phase was very prominent.

Fig. 3.1B shows currents of HCN1, HCN2, and HCN4 channels recorded in 14 s voltage steps to $V_m = -100$ mV which were long enough to completely activate all HCN channel isoforms. The holding potential was $V_m = -50$ mV. The HCN currents were normalized and plotted together with the same time scale (Fig. 3.1B). HCN4 channels (shown in green) were one order of magnitude slower than HCN1 channels (red), HCN2 channels (cyan) had an intermediate time course. Initial lag phases can be clearly seen for HCN2 and HCN4. For HCN1 the initial lag phase is difficult to see under this time scale.

When the initial lag phase is excluded from the analysis, the time course of current activation can be satisfactorily described by either a single or a double exponential function (for review see Kaupp and Seifert, 2001). The multiphasic kinetics may originate from multiple gating process within a channel (Kaupp and Seifert, 2001). Some investigators use a single exponential function to describe the activation kinetics of HCN channels (Santoro et al., 1998; Seifert et al., 1999; Ishii et al., 2001; Moosmang et al., 2001; Ludwig et al., 1998). Others consider the two component fit to be more accurate (Santoro et al., 2000; Moroni et al., 2000;

Ulens and Tytgat, 2001a; Chen et al., 2001). The fit is not improved after addition of a third component.

In Fig. 3.1C, the same current traces as in B are plotted. I fitted the rising phase of the currents curves with single or with double exponential functions. For convenience, I plotted the currents with different time scales, such that the differences between single and double exponential fits for all isoforms can be seen.

One exponential functions allow to roughly appreciate the time constants, τ , of channel activation and show the prominent differences among the channel isoforms. At $V_m = -100$ mV, HCN1 channels activated with a time constant of ~ 100 ms, HCN2 channels with ~ 390 ms, and HCN4 channels with ~ 2000 ms (Fig. 3.1C; single exponential fits are shown by arrows).

When fitted by a double exponential function, the first time constant of HCN1 channels was $\tau_1 = 54 \pm 6$ ms: this time constant accounted for 60 ± 11 % of the total current amplitude. The second time constant was $\tau_2 = 242 \pm 55$ ms (40 %). For HCN2 channels the following time constants were found: $\tau_1 = 157 \pm 14$ ms (41 ± 7 %) and $\tau_2 = 552 \pm 130$ ms (59 %). For HCN4 channels time constants were $\tau_1 = 580 \pm 110$ ms (26 ± 6 %) and $\tau_2 = 2400 \pm 220$ ms (74 %). The results are summarized in Tab. 3.1.

The time constants agree reasonably well with time constants described by other authors: for HCN1 channels the time constants were found to be 100 to 300 ms (at -130 to -100 mV); HCN2 channels have time constants between 200 and 500 ms (at -140 to -100 mV); HCN4 time constants range between a few hundred millisecond (at -150 mV) up to 30 s (at -70 mV) (data were taken from Kaupp and Seifert, 2001).

The second parameter which was analyzed in detail is the mid-point potential of activation, $V_{1/2}$. $V_{1/2}$ is the membrane voltage, at which channel activation is half-maximal, i.e. the relative open probability is 0.5.

The mid-point potential can be derived from the analysis of either tail currents (long arrows in Fig. 3.2A) or steady state currents (short arrows; for details see 2.2.5). Fig. 3.2B shows the activation curves created by both methods by plotting the normalized currents versus the membrane voltage for the cells shown in Fig. 3.2A. Curves with dots were obtained from the analysis of tail currents, curves without dots from the analysis of steady state currents. A good agreement was found between activation curves derived from both methods for every individual cell (Fig. 3.2B). Thus, both methods produce similar activation curves and the data obtained from both methods can be combined.

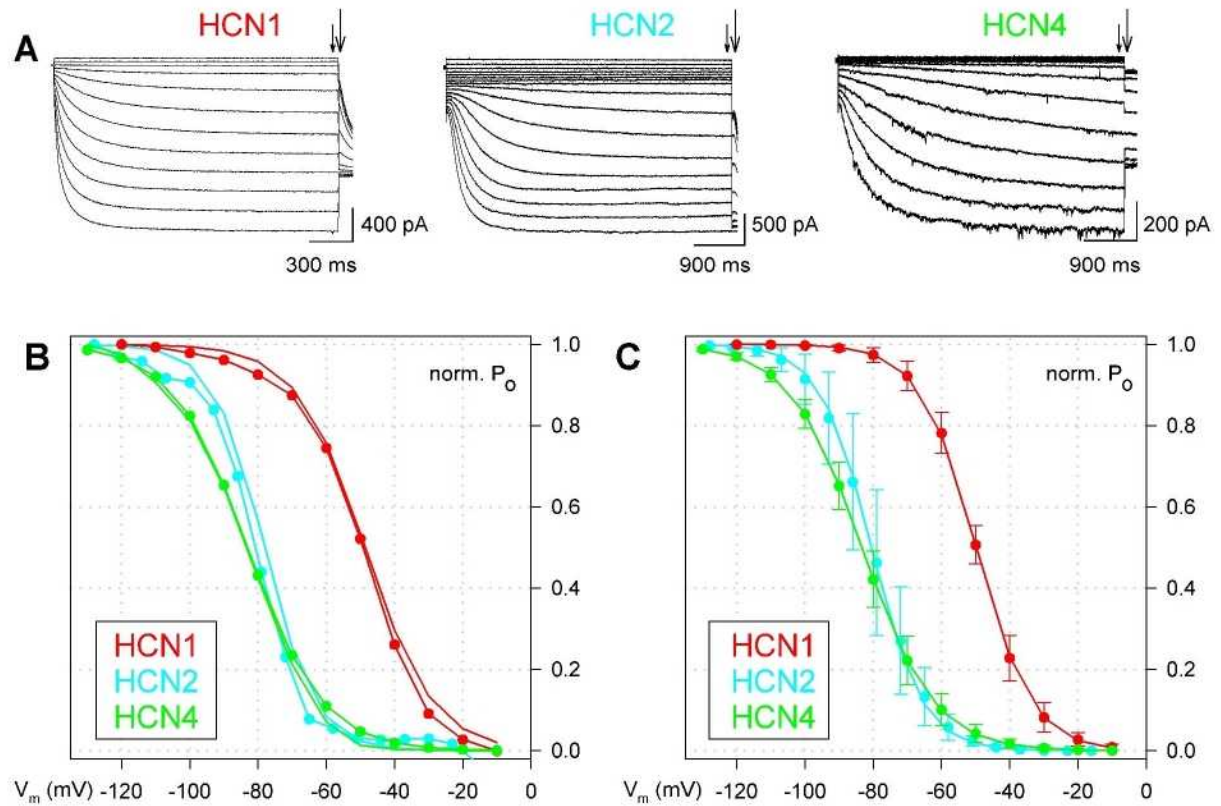


Fig. 3.2: Activation curves of HCN channels heterologously expressed in HEK293 cells.

(A) The cells were clamped to $V_m = -30$ mV. Different voltage protocols were applied for HCN1, HCN2 and HCN4 channels. After each hyperpolarizing step, a 250 ms voltage step to $V_m = -30$ mV was applied followed by a 150 ms voltage step to $V_m = +10$ mV to fully close HCN channels (not shown).

HCN1: 2 s voltage steps ranged from -10 mV to -120 mV in -10 mV increments; tail currents were measured at $V_m = -90$ mV.

HCN2: 5 s voltage steps ranged from -16 mV to -128 mV in -7 mV increments; tail currents were measured at $V_m = -128$ mV.

HCN4: 5 s voltage steps ranged from -10 mV to -130 mV in -10 mV increments; tail currents were measured at $V_m = -90$ mV.

Short arrows indicate the time at which the steady state current amplitudes were taken; long arrows mark the time at which the tail current amplitudes were taken.

Tail currents from the cells in A, were normalized (norm. P_o) and plotted in B (curves with dots). For the same cells activation curves obtained from steady state amplitudes are shown (curves without dots). There is a good agreement between activation curves achieved by two methods for an individual cell.

Normalized and averaged Boltzmann fits are plotted in C. The $V_{1/2}$ were -50.0 ± 1.4 mV for HCN1; -80.4 ± 5.0 mV for HCN2; -83.0 ± 2.9 mV for HCN4 channels. Dots represent the measuring points.

For a large number of cells, the following $V_{1/2}$ and slope factors (S), were found:

$V_{1/2} = -50.0 \pm 1.4$ mV and $S = 8.0 \pm 1.6$ mV for HCN1; $V_{1/2} = -80.4 \pm 5.0$ mV and $S = 7.6 \pm 1.0$ mV for HCN2; $V_{1/2} = -83.0 \pm 2.9$ mV and $S = 10.5 \pm 1.0$ mV for HCN4 channels.

The averaged activation curves are shown in Fig. 3.2C and the data are summarized in Tab. 3.1.

In general, the following mid-point potentials were reported: from -72 to -60 mV for HCN1 (Santoro et al., 2000; Ishii et al., 2001; Ulens and Tytgat, 2001b; Chen et al., 2001) and from -92 to -78 mV for HCN2 (Santoro et al., 2000; Moroni et al., 2000; Ulens and Tytgat,

2001b; Chen et al., 2001). For HCN4, at RT $V_{1/2}$ ranged from -85 mV (7.5 s steps) to -75 mV (60 s steps, Seifert et al., 1999). At 35°C , $V_{1/2}$ was -62 mV (Ishii et al., 2001).

As indicated already in Fig. 3.1A, a small instantaneous current can be observed immediately after the application of a hyperpolarization voltage step. The amplitude of these instantaneous currents varied from cell to cell and was found to be independent of the isoform of the channels expressed. Are these instantaneous currents conducted by HCN channels? Similar currents were found in HEK293 cells, transfected with an empty transfection vector. In these cells, the instantaneous current could be completely blocked with 0.3 mM Ba^{2+} or 4 mM Cs^{+} similar to currents from inwardly rectifying potassium channels (data not shown). Indeed, endogenous inward rectifier channels were described in HEK293 cells (Zhu et al., 1998; Yu and Kerchner, 1998; Zhang et al., 2001).

In contrast, in HEK293 cells expressing HCN channels, the instantaneous current could only partially be blocked with 0.3 mM Ba^{2+} but was completely blocked with 4 mM Cs^{+} which blocks inward rectifier channels and HCN channels (data not shown). Probably, the instantaneous current which was not blocked by barium originated from HCN channels.

Several investigations confirm the existence of an instantaneous fraction of HCN currents (Proenza et al., 2001; Macri et al., 2002). In the current study I do not further investigate whether or not a part of the instantaneous current belongs to HCN channel activity.

HCN3 channels could not be functionally expressed (own study; R. Seifert, IBI-1, Forschungszentrum Jülich, personal communication). So far, there is only one paper about functional expression of HCN3 channels (in HEK293 cells, Moosmang et al., 2001). They showed that functional properties of HCN3 channels are very close to those of HCN2 channels.

Tab. 3.1: Functional properties of HCN channels heterologously expressed in HEK293 cells.

Isoform	Activation constants measured at $V_m = -100$ mV [ms]	Fraction of total HCN current [%]	Slope (S) and $V_{1/2}$ [mV]
HCN1	$\tau_1 = 54 \pm 6$ $\tau_2 = 242 \pm 55$ (16)	60 ± 11 40 ± 11	$S = 8.0 \pm 1.6$ $V_{1/2} = -50.0 \pm 1.4$ (5)
HCN2	$\tau_1 = 157 \pm 14$ $\tau_2 = 552 \pm 130$ (12)	41 ± 7 59 ± 7	$S = 7.6 \pm 1.0$ $V_{1/2} = -80.4 \pm 5.0$ (7)
HCN3	no functional expression		
HCN4	$\tau_1 = 580 \pm 110$ $\tau_2 = 2400 \pm 220$ (6)	26 ± 6 74 ± 6	$S = 10.5 \pm 1.0$ $V_{1/2} = -83.0 \pm 2.9$ (4)

Data are reported as mean $\pm$ SD, the number of cells measured is shown in parenthesis.

3.2 HCN channels are differentially expressed in retinal bipolar cells

The electrophysiological results are based on recordings from 240 bipolar cells, all of which were identified morphologically after intracellular filling with Lucifer Yellow (LY). Cells were classified according to criteria established by Euler and Wässle (1995) and Hartveit (1997). For brevity, the term "cone bipolar cell type X" will be shortened to "type X cell".

The immunohistochemical analysis of HCN channel expression in the retina was done in collaboration with F. Müller (IBI-1, Forschungszentrum Jülich).

The results are presented in form of a catalogue with separate chapters for each bipolar cell type and are summarized in Tab. 3.2 and Tab. 3.3 at the end of this chapter.

3.2.1 OFF-bipolar cells

Types 1, 2, 3, and 4 cells were classified as OFF-bipolar cells based on the pharmacological profile of their glutamate receptors (Euler et al., 1996; Hartveit, 1997). Their axon terminals stratify in the outer part of the IPL, above the ON-cholinergic band. Like photoreceptors, OFF-cells hyperpolarize in response to light. Therefore, one might speculate that they express HCN channels that sharpen the light response. Of those four cell types, only type 3 cells showed unequivocal expression of HCN channels.

3.2.1.1 Type 3 cone bipolar cells express HCN4

In the outer region of the IPL, strong HCN4 immunofluorescence was observed (Fig. 3.3A). HCN4 channels were detected in two types of bipolar cells, whose axons projected to two different strata in the IPL (indicated by numbers). A strongly immunoreactive stratum was found in the OFF-sublamina of the IPL (band 1), a second, weaker stratum was found in the middle of the IPL (band 2). In the INL, numerous bipolar cell bodies were stained (long arrows), few amacrine cells (short arrow) were also immunoreactive. In the OPL, the dendrites of bipolar cells were stained (open arrowheads).

The upper band in the INL (band 1) is built up by "treelike" axon terminal systems. The position of the band 1 in the IPL and the shape of the stained axon terminal systems resemble those of type 3 cells. Fig. 3.3B and C shows type 3 cells described by Euler (B) and Hartveit

(C), respectively. I recorded from 18 cells, which after filling with LY showed a type 3-like morphology. After recording and filling of the cells, the pipettes were removed and the slices were fixed in PA and counterstained with HCN4 antibodies. In D, such a LY-filled cell counterstained with HCN4 antibodies is shown. The axon terminal of the filled cell stratified exactly in the band 1 of HCN4 staining.

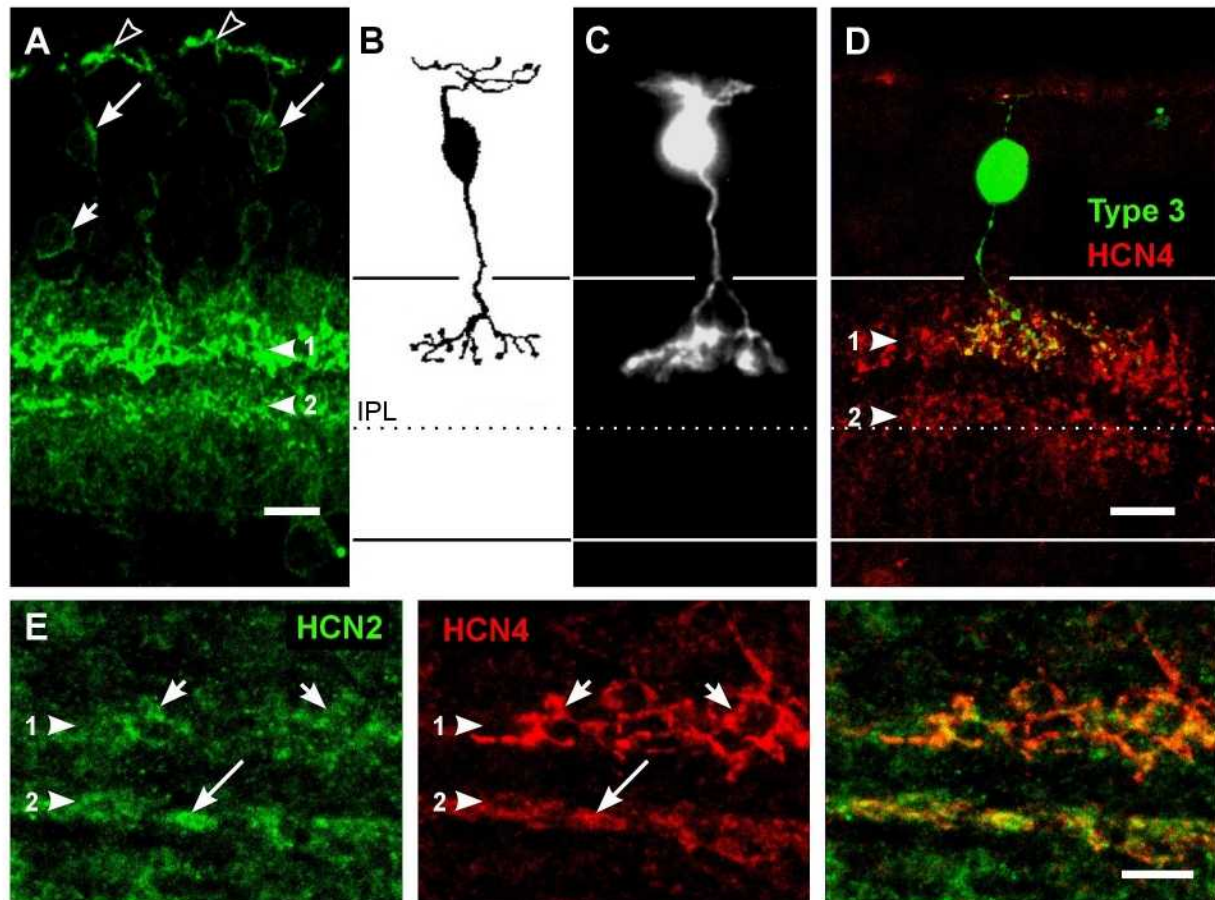


Fig. 3.3: Type 3 cells predominantly express HCN4.

(A) HCN4-specific antibodies labeled two bands (shown by numbers) in the IPL. The first band represents the axon terminal system of type 3 cells. The second weaker band is in the middle of the IPL and build up by type 5 cell axon terminals. Numerous bipolar cell bodies are stained in the INL (long arrows). An amacrine cell is marked by a short arrow. In the OPL, the dendrites of bipolar cells were stained (open arrowheads).

(B) Schematic representation of type 3 cell (adapted from Euler and Wässle, 1995).

(C) A LY-filled type 3 cell (adapted from Hartveit, 1997).

(D) A LY-filled cell (green) counterstained with HCN4 antibodies (red) after patch-clamp recording. The cell stratified exactly in the HCN4-positive band 1.

In B, C and D the borders of the IPL are marked by solid lines, the ON-cholinergic stratum is shown by a dotted line. Scale bar 10 μm in A and D.

(E) Double labeling of the IPL for HCN4 (red) and HCN2 (green) channels. The right panel shows the merged image. Two bands are seen with punctate staining of HCN2. The HCN2-positive dots were localized at the axon terminal systems of type 3 and type 5 cells stained with HCN4 (arrows mark similar structures in HCN4 and HCN2 immunolabeling). Scale bar 5 μm.

Unfortunately, antibody stainings were always weaker on slices than on cryostat sections. On slices stained with HCN4 antibodies no stained somata were observed in the INL and the

fluorescence in the IPL was weak (Fig. 3.3D). The slices were much thicker than sections and were not embedded during the scanning procedure (see material and methods) which could strongly affect the quality of the image.

The axon terminal system of the LY-filled cell was only partially labeled with HCN4 antibodies. It is conceivable that LY masked the epitopes of HCN4 channels in the filled cell and that the antibody could not recognize the antigen. Similar technical problems have been described for other immunostainings (see e.g. Voigt, 1986). In spite of the problems listed above, the cell shown in Fig. 3.3D is clearly of the same type as the bright cells from Fig. 3.3A, whose axon terminal systems formed the band 1 of HCN4 immunofluorescence.

Bands 1 and 2 of HCN4 immunoreactivity also showed weak punctate HCN2 staining (Fig. 1.2). Fig. 3.3E shows double labeling with HCN2 (green) and HCN4 (red) antibodies in the IPL. Two bands can be recognized in both stainings. HCN2-positive dots were localized at the axon terminal systems in both HCN4-positive bands (Fig. 3.3E; similar structures are marked by arrows). While HCN4 immunoreactivity was found throughout the axon terminal systems, HCN2 staining was prominent at restricted patches. No bipolar cell bodies were stained in the INL with HCN2 antibodies (see Fig. 1.2C).

Thus, from immunocytochemistry, a high level of HCN4 expression and a very low level of HCN2 can be expected in type 3 cells. I compared hyperpolarization-activated currents recorded from type 3 cells in retinal slices with currents recorded from HEK293 cells expressing HCN4 channels (Fig. 3.4).

In HEK293 cells expressing HCN4, hyperpolarizing steps produced inward currents that activated with a slow time course (Fig. 3.4A). Currents reached a steady state only during hyperpolarization for several seconds (lower panel, $V_m = -100$ mV). At $V_m = -100$ mV, current activation was best fitted by a double exponential function with the following time constants: $\tau_1 = 580 \pm 110$ ms (26 ± 6 %), $\tau_2 = 2400 \pm 220$ ms (74 %) (Fig. 3.4B, lower panel, the fit is shown by a white line on top of the trace). Hyperpolarization-activated currents in type 3 cells showed a similarly slow time course: $\tau_1 = 430 \pm 30$ ms (38 ± 13 %), $\tau_2 = 2654 \pm 400$ ms (62 %), (Fig. 3.4A, lower panel, the fit is shown on top of the trace).

Slowly decaying tail currents were observed after stepping V_m back to the holding potential. The tail currents in type 3 cells had a transient and a sustained component. While the sustained component originated from HCN channels, the transient component was due to T-type calcium channels. The strong hyperpolarization of the membrane used to activate HCN channels removed the inactivation from these transient calcium channels. After stepping back to -50 mV, T-type channels became activated. The kinetics and amplitudes of the calcium

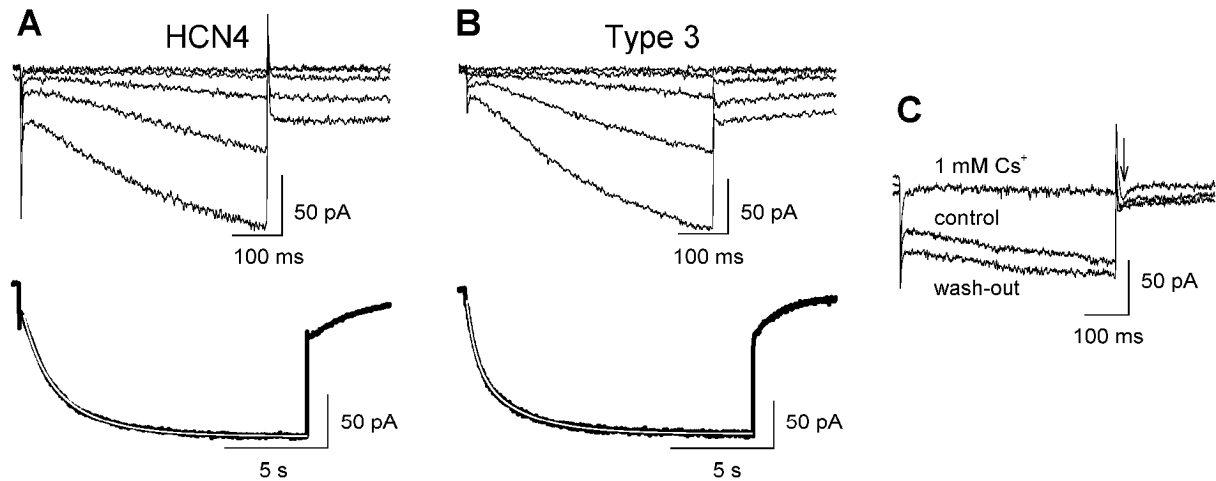


Fig. 3.4: Electrophysiological recording in type 3 cells and HEK293 cells expressing HCN4 channels.

(A) Currents mediated by HCN4 channels in HEK293 cells. Upper panel: V_m was stepped from -50 mV to hyperpolarizing levels (-55 to -115 mV, 15 mV increments) for 500 ms. HCN4 currents activated with a slow time course. At the end of each hyperpolarization step, V_m was clamped to -50 mV for 250 ms, and then for 150 ms to $V_m = +10$ mV to close HCN channels (not shown).

Lower panel: V_m was stepped from -50 mV to -100 mV for 14 s. The current was fitted by a double exponential function: $\tau_1 = 540$ ms (35%), $\tau_2 = 2490$ ms (65%).

(B) HCN currents recorded in a type 3 cell. Currents were remarkably similar to those of HCN4 homomeric channels. Conditions as in (A). Lower panel: the current was fitted by a double exponential function: $\tau_1 = 460$ ms (46%), $\tau_2 = 2300$ ms (54%).

(C) HCN currents ($V_m = -100$ mV) in type 3 cells were reversibly blocked by 1 mM extracellular cesium. The transient component of the tail current was not blocked by cesium (arrow).

currents seemed to be dependent on the membrane voltage applied in preceding step. T-type channels have been described in all rat bipolar cells. Functional properties of these calcium channels were different among the bipolar cell types (Pan, 2000).

The hyperpolarization-activated currents as well as the sustained component of the tail currents in type 3 cells were reversibly blocked by 1 mM extracellular Cs^+ , which is regarded as a hallmark of HCN currents (Fig. 3.4C). The transient component of the tail current originating from T-type calcium channels was not blocked by cesium (Fig. 3.4C, transient component is marked by an arrow).

HCN immunofluorescence and, hence, channel density, is higher at the axon terminal of type 3 cells than at the soma. In the whole-cell recording, voltage attenuation along the bipolar cell axon might be expected to shift the apparent activation range of HCN channels. Although this cell type was not studied in detail, I did not observe significant differences between HCN4 in HEK293 cells and HCN4 in type 3 cells, probably, because bipolar cells are electrically quite compact.

The first time constant was slightly smaller and accounted for a larger fraction of current in type 3 cells than in HCN4 in HEK293 cells. It is possible that this reflects the presence of

HCN2 channels in type 3 cells. Based on immunocytochemistry, HCN2 channels constitute only a minute fraction of HCN channels in this cell type. Probably, this fraction is too small to be detected as a separate component in the whole-cell recordings, but it might slightly accelerate HCN currents in type 3 cells.

3.2.1.2 Type 1 and 4 cone bipolar cells do not express HCN channels

Fig. 3.5 shows type 1 and 4 cells (A, adapted from Euler and Wässle, 1995; B, from Hartveit, 1997). C represents two corresponding LY-filled cells from my patch-clamp recordings. For type 1 and 4 cells no specific antibody markers are available.

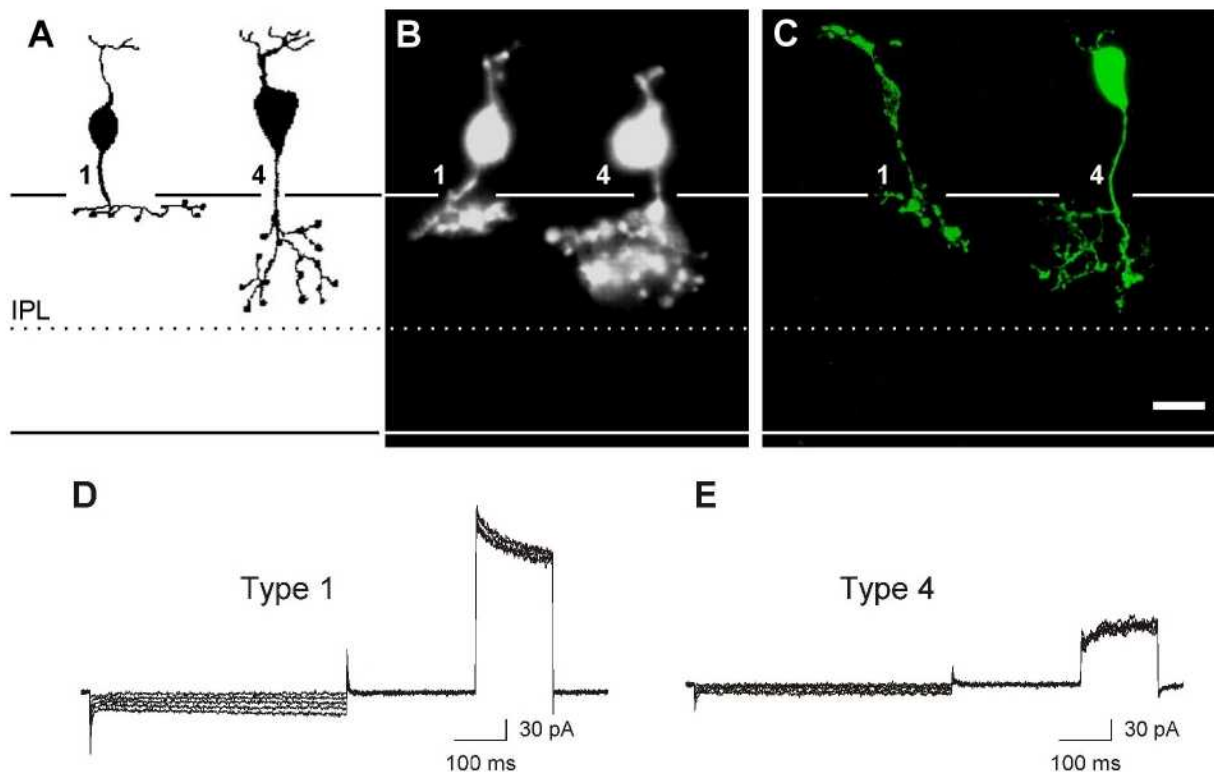


Fig. 3.5: Type 1 and type 4 CB cells do not show HCN currents.

(A) Schematic representation of type 1 and type 4 cells (adapted from Euler and Wässle, 1995).

(B) LY-filled type 1 and type 4 cells (adapted from Hartveit, 1997).

(C) LY-filled cells of both types from my patch-clamp recordings. The borders of the IPL are marked by solid lines, the ON-cholinergic stratum by a dotted line. Scale bar 10 μm.

(D) Type 1 cell. The standard voltage protocol was applied: V_m was stepped from -50 mV to hyperpolarizing levels (-55 to -115 mV, 15 mV increments) for 500 ms. No HCN currents were detected. At the end of the hyperpolarizing steps, the membrane was clamped to $V_m = -50$ mV for 250 ms followed by a depolarizing step to $V_m = +10$ mV which evoked large outward currents; presumably a mixture of inactivating and delayed rectifier potassium channels.

(E) The same voltage protocol as in D was applied in type 4 cells. No HCN current was observed. A depolarizing step at the end of the protocol activated a small potassium current, probably, a delayed rectifier.

Type 1 cell is a cone bipolar cell with a flat stratification at the outer border of the IPL. Type 4 cell has a very diffuse stratification, the axon terminal occupies nearly the whole OFF-sublamina of the IPL. No immunostaining that resembled the stratification of type 1 or type 4 cell axon terminal systems was observed with any of the HCN channel antibodies.

In Fig. 3.5D and E the recordings from these cell types are shown. In type 1 cells, very small hyperpolarization-activated currents were occasionally recorded. Currents were too small for a detailed investigation. In type 4 cells, no hyperpolarization-activated currents were recorded. In contrast, outward currents were easily recordable in these cells, indicating that the whole-cell mode had been established and the cells were healthy. Type 1 cells had very prominent outward currents which are, probably, a mixture of two types of potassium currents, a delayed rectifier current and an inactivating A-type potassium current. Type 4 cells had smaller outward currents, which seem to be mostly from delayed rectifier potassium channels. Both types of cells were rare in my study as well as in other investigations (Euler and Wässle, 1995; Hartveit, 1997).

3.2.1.3 Are there HCN channels in type 2 cone bipolar cells?

Type 2 cells have very "bushy" axon terminal systems, that stratify in the outer half of the OFF-sublamina in the IPL.

Type 2 cells are immunoreactive for the calcium-binding protein recoverin (Euler and Wässle, 1995). In Fig. 3.6A, recoverin staining is found in somata of photoreceptors in the ONL and in their endfeet in the OPL. In the INL, type 2 cells (open arrowhead) are labeled, whose axons stratify in the OFF-sublamina of the IPL (short arrows).

Type 8 cells (closed arrowhead) stratify in the ON-sublamina close to the GCL (long arrow).

In Fig. 3.6B and C, axons of type 8 cells are marked by long arrows (type 8 cells will be discussed later). Compared to other bipolar cell types, type 2 cells have a somewhat larger soma located usually in the middle of the INL.

HCN2- and HCN4-immunoreactivity in the IPL was observed close to the region of stratification of type 2 cells. Fig. 3.6B and C shows double labeling performed with recoverin antibodies to label type 2 cells and with HCN2 and HCN4 antibodies. In B, axon terminals of type 2 cells (recoverin, red) and HCN4 immunofluorescence (green) are shown. No colocalization was observed. The axon terminal systems of recoverin-positive cells terminate above the HCN4-positive band 1. Occasionally, yellowish dots at the ends of the axon

terminals are observed, that originate from the crossing of red and green processes, and, therefore, do not represent colocalization of both antigens in the same cell.

In Fig. 3.6C, the axon terminals of recoverin-positive type 2 cells (red) and HCN2-immunofluorescence (green) are shown. As with HCN4, no colocalization was observed. The HCN2-positive band was clearly below the axon terminal systems of type 2 cells. Thus, by immunocytochemistry, neither HCN2 nor HCN4 isoforms were found in type 2 cells.

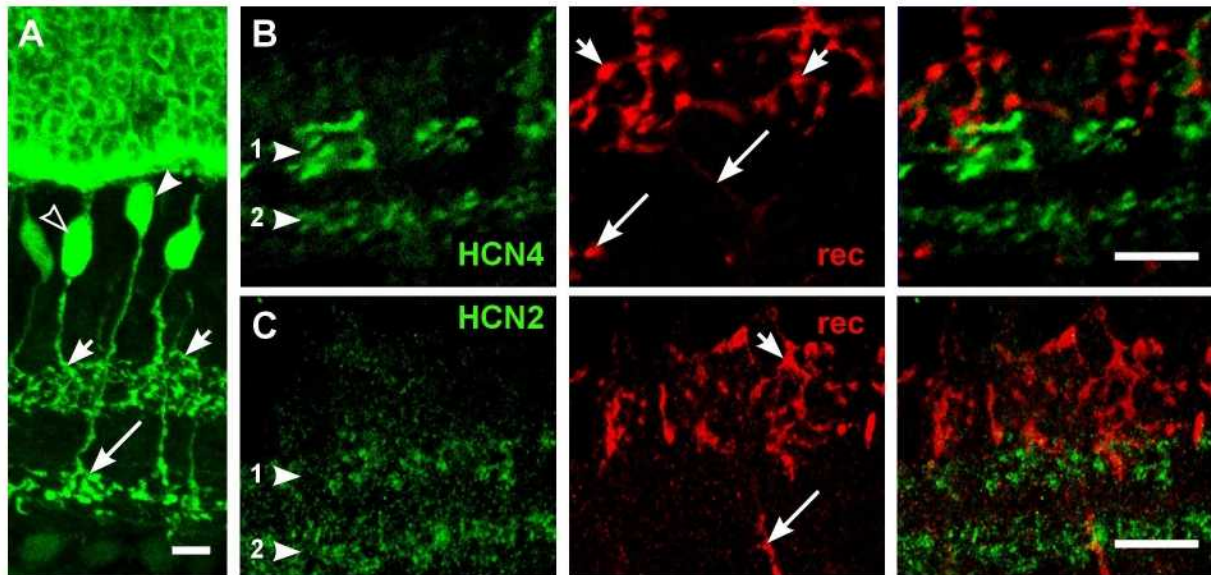


Fig. 3.6: Immunocytochemistry study of type 2 cells.

(A) Recoverin-specific antibodies labeled photoreceptors in the ONL and their endfeet in the OPL. In the INL, type 2 cells (open arrowhead) are labeled, whose axons stratify in the OFF-sublamina of the IPL (short arrows). Type 8 cells (closed arrowhead) stratify in the ON-sublamina close to the GCL (long arrow).

(B) Double labeling of the IPL for recoverin (red) and HCN4 channels (green). The right panel shows the merged image. The axon terminal systems of type 2 cells are marked by short arrows, from type 8 cells are shown by long arrows. Two HCN4-positive bands represent the axon terminals of type 3 cells (band 1) and type 5 cells (band 2). No double labeling was observed. Axon terminals of type 2 cells stratified clearly above HCN4-positive band 1.

(C) Double labeling of the IPL for recoverin (red) and HCN2 channels (green). The right panel shows the merged image. The axon terminal systems of type 2 cells are marked by short arrows, the axons from type 8 cells are shown by long arrows. Two HCN2-positive bands represent the axon terminals of type 3 cells (band 1) and type 5 cells (band 2). No double labeling was observed. Axon terminals of type 2 cells stratified clearly above HCN2-positive band 1.

Scale bars 10 μm .

Surprisingly, however, hyperpolarization-activated currents can be recorded in this cell type. Fig. 3.7 compares type 2 cells from Euler and Wässle (1995, A), Hartveit (1997, B) and from my study (C). In type 2 cells, I found hyperpolarization-activated currents that were reminiscent of HCN channels in type 3 cells: the kinetics is slow, the channels start to activate at $V_m = -85$ mV (Fig. 3.7D). Currents in type 2 cell had much smaller amplitude than in type 3 cells. This cell type was rare in my recordings and only in one cell the long protocol could be applied to measure the activation kinetics. The activation of the current was fitted best by a

double exponential function with the following time constants: $\tau_1 = 390$ ms (67 %), $\tau_2 = 1950$ ms (33 %), i.e. currents developed with a slightly faster time course than those in the type 3 cells.

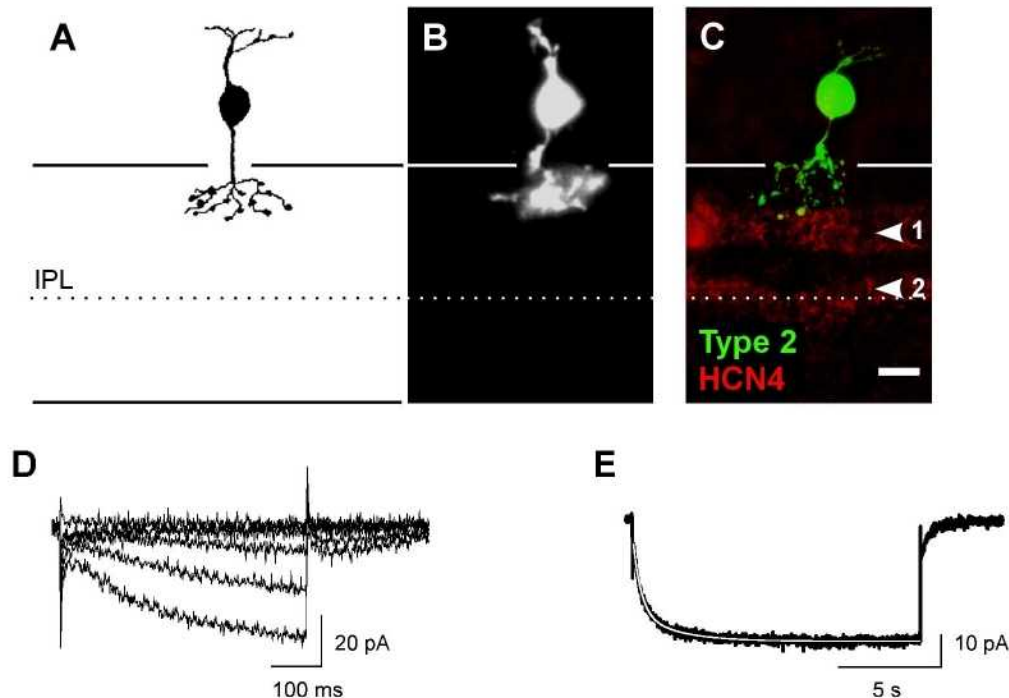


Fig. 3.7: Electrophysiological recordings in type 2 cells.

(A) Schematic representation of type 2 cell (adapted from Euler and Wässle, 1995).

(B) A LY-filled cell (adapted from Hartveit, 1997).

(C) A LY-filled cell (green) counterstained after patch-clamp recording with HCN4 antibody (red). The axon terminal of the LY-filled cell stratified above the HCN4-positive band.

In A, B and C the borders of the IPL are marked by solid lines, the ON-cholinergic stratum is shown by a dotted line. Scale bar 10 μ m.

(D) Hyperpolarization-activated currents recorded in a type 2 cell. V_m was stepped from -50 mV to hyperpolarizing levels (-40 to -115 mV, 15 mV increments) for 500 ms. At the end of each hyperpolarization step, the membrane was clamped to $V_m = -50$ mV for 250 ms, and then for 150 ms to $V_m = +10$ mV to close HCN channels (not shown).

(E) V_m was stepped from -50 mV to -100 mV for 14 s. The current was fitted by a double exponential function with the following time constants: $\tau_1 = 390$ ms (67 %), $\tau_2 = 1950$ ms (33 %). The fit is shown on top of the current trace.

Based on the kinetics, the currents found in this cell could result from a mixture of HCN4 and HCN2 currents. Therefore, after patch-clamp recordings, I counterstained the LY-filled cell with HCN4 antibodies (Fig. 3.7C). The axon terminal system of the LY-filled cell stratified clearly above the HCN4-positive band in the IPL.

How can the discrepancy between the immunohistochemical results (Fig. 3.6B and C and 3.7C) and the electrophysiological results be explained? First, currents might be unrelated to I_h . It seems unlikely, because the activation kinetics closely resembles that of HCN channels. Unfortunately, it could not be tested, whether currents were blocked by cesium. Currents

might originate from chloride channels. Chloride currents similar to HCN currents were found in rat sympathetic neurons (Clark et al., 1998), but have not been described in cone bipolar cells. Second, the currents in these cells were quite small. If one assumes that the channels are distributed throughout the whole cell, the channel density might be too low to be detected with the antibodies.

3.2.2 Bipolar cells that stratify in the middle of the IPL

The middle of the IPL is the transition zone between OFF-sublamina (outer part of the IPL) and ON-sublamina (inner part of the IPL). In Fig. 3.8A, HCN1-positive bipolar cells (long arrows) are shown that terminate in the middle of the IPL (band 2). Euler and Wässle (1995) described two bipolar cell types with very flat axon terminal systems, that stratify in this region. Their type 5 cells stratify exactly in the middle of the IPL, while type 6 cells stratify a few μm closer to the GCL. The strata of both cells are separated by the dendritic stratum of the ON-cholinergic amacrine cells. According to Euler and Wässle, type 5 and type 6 cell types are immunoreactive for the glutamate transporter-1 (GLT-1) and their axon terminal systems form two very thin GLT-1-positive strata. The axon terminal processes do not cross the ON-cholinergic band. In this chapter, I will discuss evidence from my studies suggesting that, probably, four bipolar cell types terminate in the middle of the IPL, which I named 5a, 5b, 6a and 6b, respectively.

To distinguish between type 5 and 6 cells, retina sections were double labeled for cholineacetyltransferase (Fig. 3.8B, Chat, red) and HCN1 (green). Cholinergic ON- and OFF-amacrine cells form two dendritic strata. The lower stratum is formed by the dendrites of the ON-cholinergic cells. Three HCN1-positive bands were detected in the IPL. Band 1 was narrow, and most likely originated from dendrites of amacrine cells (see 1.3). A thick band of HCN1 immunofluorescence was observed in the middle of the IPL (Fig. 3.8B, green, band 2); a thin band (band 3) was found closer to the GCL. HCN1-positive axon terminals of band 2 were observed preferentially above the ON-cholinergic stratum, indicating that they originated from type 5 cells. However, some processes were found within the cholinergic band, which is not typical for Euler type 5 cells. Weakly stained axon terminals form the thin HCN1-positive band 3, located below the ON-cholinergic band, suggesting that type 6 cells also express HCN1 channels.

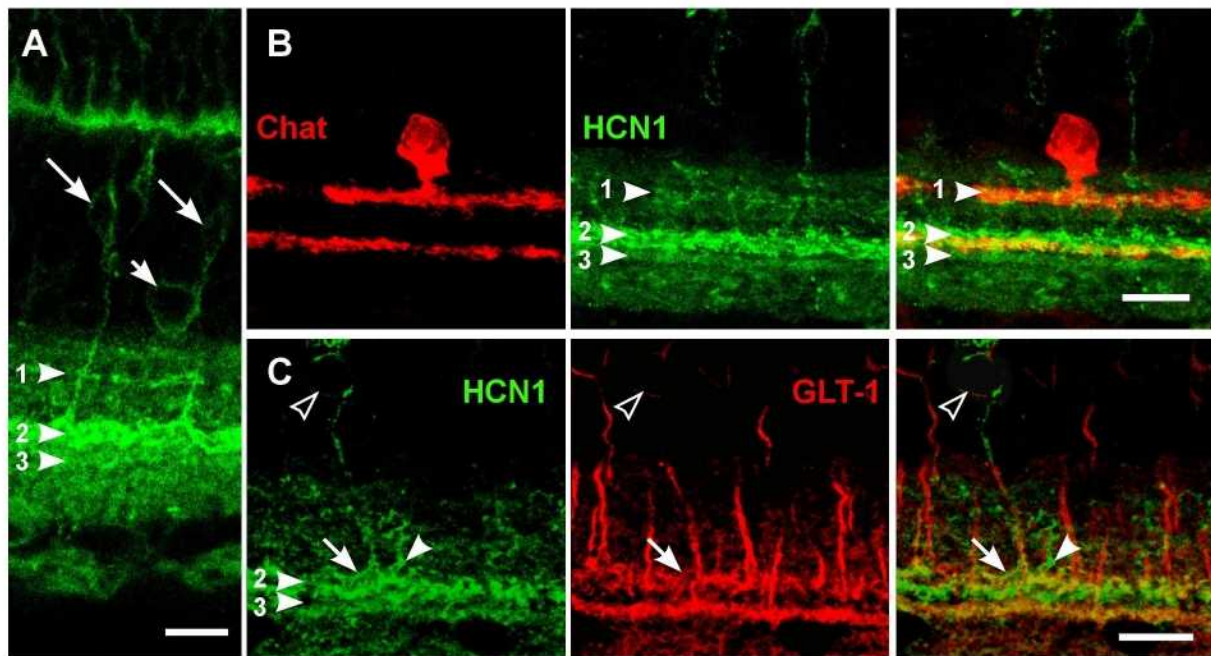


Fig. 3.8: HCN1 channels are localized in type 5 and type 6 cells.

(A) HCN1-positive bipolar cells (long arrows) terminate in the middle of the IPL. Three bands of HCN1 immunofluorescence were detected in the IPL (bands are labeled by numbers). Band 1 presumably originates from amacrine cells, bands 2 and 3 are axon terminals of bipolar cells. An amacrine cell is shown by a short arrow.

(B) Double labeling of the IPL and the INL for Chat (red, left) and HCN1 (green, middle). The right panel shows the merged image. In the Chat staining an amacrine cell and the OFF-cholinergic (upper) and ON-cholinergic (lower) stratum in the IPL can be distinguished. HCN1-positive axon terminal systems from type 5 cells are mostly located above (band 2), labeled type 6 axon terminals below the ON-cholinergic stratum (band 3).

(C) Double labeling of the IPL for HCN1 (green, left) and GLT-1 (red, middle). The right panel shows the merged image. In HCN1 staining, two axon terminal systems of type 5 cells are shown (arrow and arrowhead). A soma is positive in the INL (open arrowhead). Two thin bands were stained with GLT-1 in the IPL. In the overlay, one axon terminal was positive for GLT-1 (arrow), the other one was negative for GLT-1 (arrowhead). The bipolar cell body in the INL was double labeled for HCN1 and GLT-1 (open arrow). Band 3 was counterstained with GLT-1. Scale bars 10 μ m.

A double labeling of the rat retina for HCN1 (green) and GLT-1 (red) is shown in Fig. 3.8C. GLT-1 antibodies strongly labeled two thin bands in the middle of the IPL, and to a lesser extent, axons and somata of bipolar cells. The soma of an HCN1- and GLT-1-positive bipolar cell is marked by an open arrowhead. The upper band, formed by the axon terminal systems of Euler type 5 cells, was clearly thinner than band 2 of HCN1 immunolabeling (Fig. 3.8C). Indeed, in the overlay only the upper part of band 2 was positive for GLT-1. Two HCN1-positive axon terminal systems that stratify in band 2 are marked. One of them was positive for GLT-1 (arrow), the second one was negative for GLT-1 (arrowhead). Band 3 of HCN1 immunoreactivity was colocalized with the lower band of GLT-1 staining, i.e. this band represents the axon terminals of Euler type 6 cells.

Thus, it seems as if HCN1-band 2 is formed by the axon terminals of two types of bipolar cells. First, the shape of HCN1-positive/GLT-1-negative processes argue for bipolar cell

processes rather than amacrine or ganglion cell dendrites. Second, in preliminary experiments (data not shown) all HCN1-positive processes in band 2 were also positive for Vglut (vesicular glutamate transporter), a good marker for the glutamatergic bipolar cell axon terminal systems. Finally, I found two bipolar cell types with morphologically slightly different axon terminals that stratify in band 2 (Fig. 3.9B and D). The cell in B has a relatively flat axon terminal system, similar to GLT-1-positive Euler type 5 cell (Fig. 3.9A). I call this cell: type 5b. The cell in D has a more bushy axon terminal system, more like the cell described by Hartveit (1997; Fig. 3.9C). This cell is GLT-1-negative and is called type 5a. Interestingly, Euler describes his type 5 cells as OFF-bipolar cells (Euler et al., 1996), while Hartveit claims that type 5 cells are ON-bipolar cells (Hartveit, 1997). Whether this discrepancy can be explained by the existence of two subpopulations of type 5 cells, remains to be investigated.

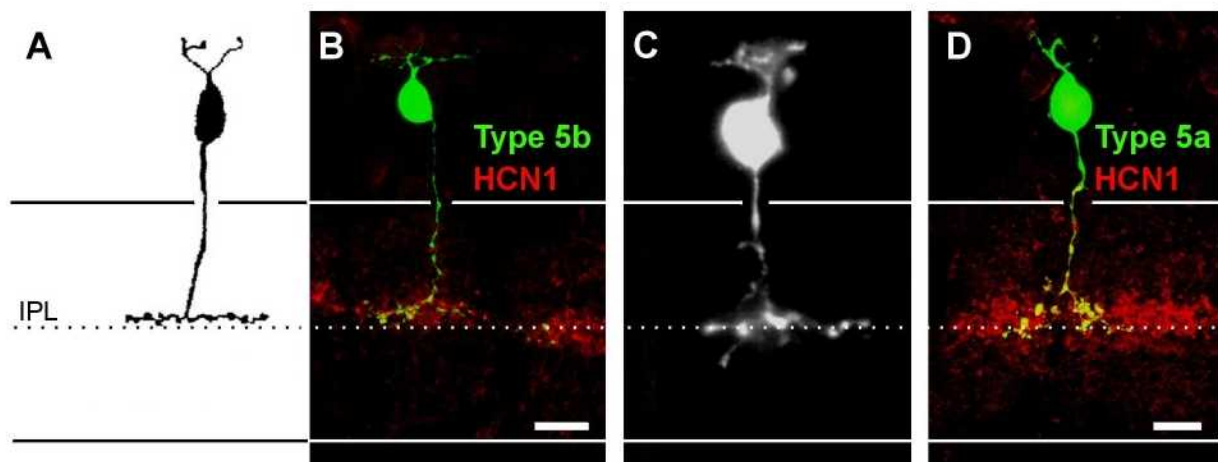


Fig. 3.9: Type 5a and 5b cells.

(A) Schematic drawing of type 5 cell (adapted from Euler and Wässle, 1995).

(B) A LY-filled cell (green) counterstained after patch-clamp recording with HCN1 antibody (red). The cell stratified in the HCN1-positive band. The cell resembles that in A.

(C) A LY-filled cell (adapted from Hartveit, 1997).

(D) A LY-filled cell (green) counterstained after patch-clamp recording with HCN1 antibody (red). The cell stratified in the HCN1-positive band, but had a "bushy" axon terminal system. The morphology of the cell is closer to the cell shown in C, than in A.

The borders of the IPL are marked by solid lines, the ON-cholinergic stratum is shown by a dotted line.

Scale bars 10 μ m.

As reported earlier, HCN4 and HCN2 were also found in processes in the middle of the IPL (see Fig. 1.2B and Fig. 3.3A and E), that might originate from type 5 cells. Double labeling with antibodies against HCN1 (red) and HCN4 (green) revealed that type 5 cells indeed coexpress both HCN isoforms (Fig. 3.10). Strictly spoken, to verify whether both type 5a and type 5b coexpress HCN4 and HCN1 channels, counterstaining for GLT-1 is required.

Unfortunately, these stainings were technically problematic, because, GLT-1 and HCN4 antibodies were both raised in rabbit, thus they cannot be used for a double labeling; moreover, GLT-1 staining works better on well-fixed tissue, while HCN1 antibodies stain best in a shortly-fixed tissue.

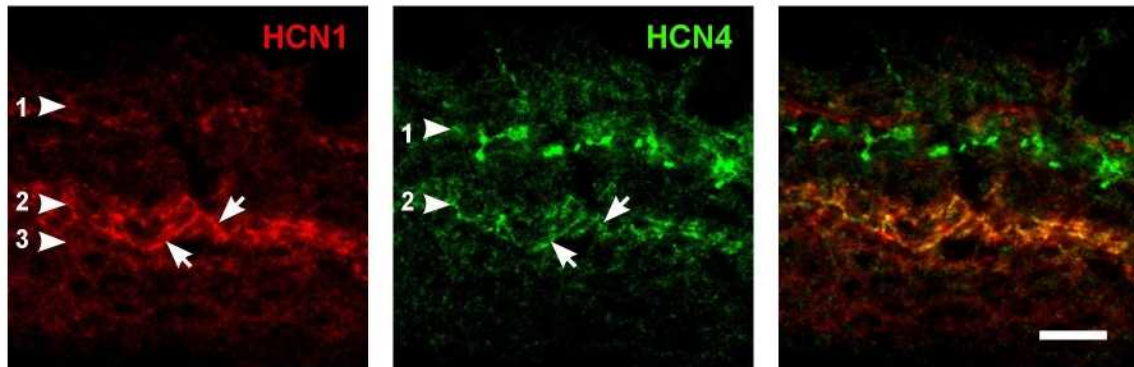


Fig. 3.10: Type 5a and 5b cells express both HCN1 and HCN4 channels.

Double labeling of the IPL for HCN1 (red) and HCN4 (green) in a single confocal section. In the axon terminals of type 5a and 5b cells, HCN1 and HCN4 were colocalized. Two processes that can be recognized in both stainings are marked (arrows). Scale bar 10 μm .

In Fig. 3.10, HCN1 immunofluorescence was most prominent in axon terminals of type 5 cells (arrows). Type 6 cells are barely visible in this section. It seems that all HCN1-positive structures in band 2 also express HCN4. Processes have the same shape in both stainings and appear yellowish in the overlay (arrows). Hence, immunocytochemistry suggests the presence of HCN1 and HCN4 channels in type 5a and type 5b cells.

This colocalization is consistent with the idea that HCN1 and HCN4 coassemble to form heterooligomeric channels, but not necessarily proves this point. Alternatively, homooligomeric channels consisting of either HCN1 or HCN4 might be located in close proximity. To test these hypotheses, I compared HCN currents recorded from type 5 cells and from HCN1 or HCN4 in HEK293 cells.

3.2.2.1 Type 5a and 5b cone bipolar cells express both HCN1 and HCN4 channels

I recorded currents from a number of type 5a and type 5b cells. In both cell types, profound "rundown" of channel activity was observed during the measurements. As in all recordings of the current study, the activation kinetics of the channels was measured within the first minute of the whole-cell configuration. During this time no noticeable "rundown" occurred, therefore, these data are not affected. At the end of the voltage protocol for $V_{1/2}$ estimation, the kinetics

of the channels did not resemble that in the initial measurements: the amplitude of the current was reduced dramatically and the development of the current was much slower (data not shown), thus, the $V_{1/2}$ data from type 5 cells should be interpreted with caution.

Fig. 3.11 shows the hyperpolarization-activated currents of type 5a (D) and type 5b (E) cells in comparison with the currents of HCN1 (A) and HCN4 (B) channels expressed in HEK293 cells. No considerable difference was found between the hyperpolarization-activated currents of type 5a and 5b cells and data from both cell types were combined.

As described earlier in HEK293 cells, HCN1 currents activated much faster than HCN4 currents (compare Fig. 3.11A and B). In Fig. 3.11C the HCN1 and HCN4 currents were measured at $V_m = -100$ mV and plotted together. Current activation of HCN1 was best described by two time constants $\tau_1 = 54 \pm 7$ ms (60 $\pm$ 11 %) and $\tau_2 = 242 \pm 56$ ms (40 %; $n = 16$), activation of HCN4 by $\tau_1 = 580 \pm 110$ ms (26 $\pm$ 6 %) and $\tau_2 = 2400 \pm 220$ ms (74 %; $n = 6$).

Hence, HCN1 and HCN4 currents can be readily distinguished by their different time course of activation. In both channels two time constants are needed to describe current activation. Assuming that type 5 cells are furnished with homooligomers of HCN1 and HCN4, then HCN currents in these cells can be described by the algebraic sum of HCN1 and HCN4 currents. Therefore, currents should show a very fast kinetic component (τ_1 of HCN1), a very slow component (τ_2 of HCN4), and an intermediate component that corresponds to the mixture of τ_2 of HCN1 and τ_1 of HCN4, which are too similar to be fitted independently.

These expectations are born out by experiments (Fig. 3.11F). Type 5 cells exhibited HCN currents that developed with the time course similar to HCN1; however, steady state was reached only during long steps. A fit of HCN currents in type 5 cells ($n = 27$) required three exponents: $\tau_1 = 63 \pm 5$ ms, which is close to τ_1 of HCN1; $\tau_2 = 379 \pm 32$ ms, and $\tau_3 = 2945 \pm 592$ ms, which is close to τ_2 of HCN4 ($V_m = -100$ mV). In the particular type 5a cell shown in Fig. 3.11F, I fitted the current with the following parameters: $\tau_1 = 65$ ms (42 %), $\tau_2 = 348$ ms (40 %), and $\tau_3 = 3400$ ms (18 %) (the fit is shown on top of the black current trace). In HEK293 cells, the fast current component (τ_1) of HCN1 accounts for 60 % of the total HCN1 current. The slow current component (τ_2) of HCN4 accounts for 74 % of the total HCN4 current. If one assumes that these values are also true for HCN1 and HCN4 currents in type 5 cells (which is not necessarily true), one can calculate that the fraction of HCN1 current from the total HCN current in the type 5 cell would be 70 %; the fraction of HCN4 current from the total HCN current would be 25 %. This would account for 95 % of the total current, a reasonably good value. In a second step, I tried to reconstruct the current of the type 5 cell by adding normalized and averaged current traces of HCN1 and HCN4 channels (see

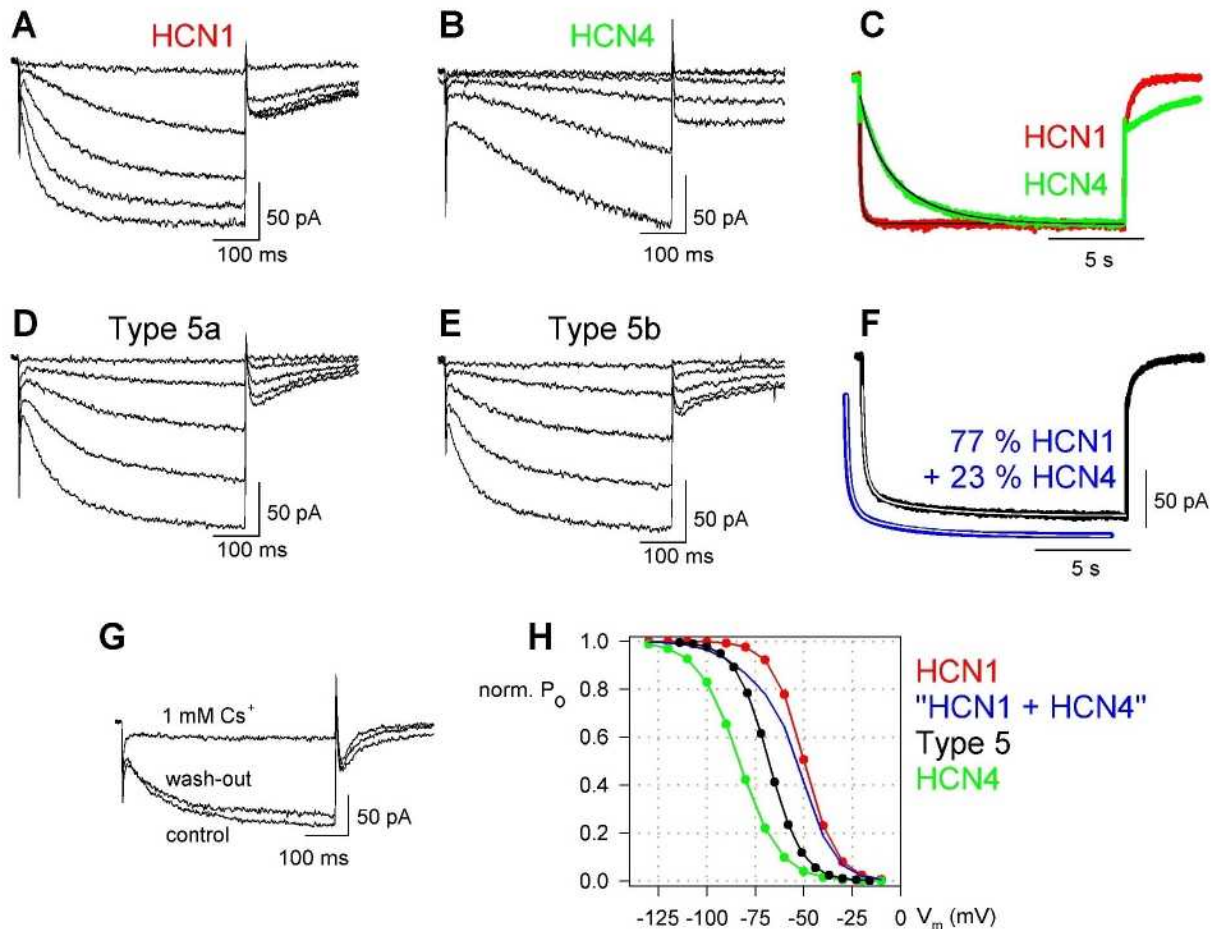


Fig. 3.11: Electrophysiological characterization of HCN channels in type 5a and 5b cells.

(A) HCN1 currents in a HEK293 cell. V_m was stepped from -50 mV to hyperpolarizing levels from -55 to -115 mV for 500 ms. Currents at $V_m = -100$ mV and -115 mV reached steady state after 400 ms. After the hyperpolarizing steps, the membrane was clamped to $V_m = -50$ mV for 250 ms and then to $V_m = +10$ mV for 150 ms (data not shown).

(B) HCN4 currents in a HEK293 cell. Conditions as in A. Currents did not reach steady state plateau.

(C) HCN1 (red) and HCN4 (green) currents at $V_m = -100$ mV. Normalized current traces were fitted by double exponential functions: $\tau_1 = 54$ ms (74 %), $\tau_2 = 240$ ms (26 %) for HCN1; $\tau_1 = 540$ ms (34 %), $\tau_2 = 2500$ ms (66 %) for HCN4. The fits are shown in black on top of the current traces.

(D) and (E) HCN currents recorded in type 5a cell and type 5b cells, conditions as in A. Currents activated with a time course similar to HCN1, but did not reach steady state levels during the short hyperpolarization steps.

(F) HCN currents (black) recorded in a type 5a cell. Conditions as in (C). The current could be fitted best by a three exponential function: $\tau_1 = 65$ ms (42 %), $\tau_2 = 348$ ms (40 %), and $\tau_3 = 3400$ ms (18 %), the fit is shown on top of the black current trace. The blue trace is from a "theoretical cell" calculated by adding 77 % of HCN1 and 23 % of HCN4 currents. For details see text.

(G) HCN currents in type 5 cells were reversibly blocked by Cs^+ . A transient component in the tail currents, probably a T-type Ca^{2+} current persisted blockage.

(H) Activation curves of HCN1 (red) and HCN4 (green) measured in HEK293 cells, HCN channels in type 5 cells (black, $V_{1/2} = -68.0 \pm 4.0$ mV and $S = 8.5 \pm 0.5$ mV) and the calculated activation curve of a "theoretical cell" that expresses HCN1 : HCN4 in a ratio of 80 : 20 (blue, $V_{1/2} = -56$ mV).

Tab. 3.1) from HEK293 cells in different proportions. The current was best imitated, if it consisted of 77 % HCN1 and 23 % of HCN4 current. The "reconstructed current" is shown in Fig. 3.11F, lower trace (blue). The fit of the type 5 cell is shown on top of the "reconstructed current" trace.

Thus, the data are consistent with the hypothesis that HCN1 and HCN4 form independent homooligomeric channel populations in type 5 cells, and there is no need to invoke the existence of heterooligomeric channels to explain the electrophysiological data. However, it cannot be ruled out that a fraction of heterooligomeric channels exists, that activate with the intermediate time constant τ_2 .

Fig. 3.11H shows the activation curves of HCN channels in type 5 cells (black, $n = 3$), HCN1 (red) and HCN4 (green) measured in HEK293 cells, and the calculated activation curve of a "theoretical cell" in which HCN1 and HCN4 currents were added in the proportions of HCN1 = 80 % to HCN4 = 20 % (blue). The calculated $V_{1/2} = \sim -56$ mV did not match the measured $V_{1/2} = -68.0 \pm 4.0$ mV of type 5 cells. Several explanations for this discrepancy can be given. First, as was discussed earlier, a "rundown" of the channels was observed in type 5 cells thus, it might be that the mid-point potentials that I measured shows a systematic error in type 5 cells. Second, the channels could be modified in the retina (or in HEK293 cells). As I will show later, putative HCN1 channels of rod photoreceptors also have a more negative mid-point potential than HCN1 channels in HEK293 cells.

3.2.2.2 Two "type 6" cone bipolar cells

Two morphologically distinct bipolar cell types, that stratify directly below the On-cholinergic stratum were found in the current study (Fig. 3.12C and D). In C, the cell was counterstained with HCN1 antibodies. The axon terminal of the LY-filled cell stratified exactly in the band 3 of HCN1 staining (arrowheads). The axon terminal system was flat and with large lateral extension. The shape of this cell and the stratification of its axon terminal closely resemble those of GLT-1-positive type 6 cells described by Euler and Wässle (1995); Fig. 3.12A) and Hartveit (1997; Fig. 3.12B). Further on, these cells will be called type 6a cells. Immunocytochemistry suggests that these cells express HCN1 channels.

In my experiments, another cell type which stratifies in this region of the IPL was found (Fig. 3.12D). The axon terminal of this cell type was rather diffuse and had a large lateral extension. The processes of the axon terminal systems reached beyond the limits of the HCN1-positive band 3 (not shown). The axonal swellings were large and robust. It seems, that these cells had not been characterized by Euler or by Hartveit; I will term them type 6b cells. Electrophysiologically, type 6a and 6b cells can be identified as separate cell types.

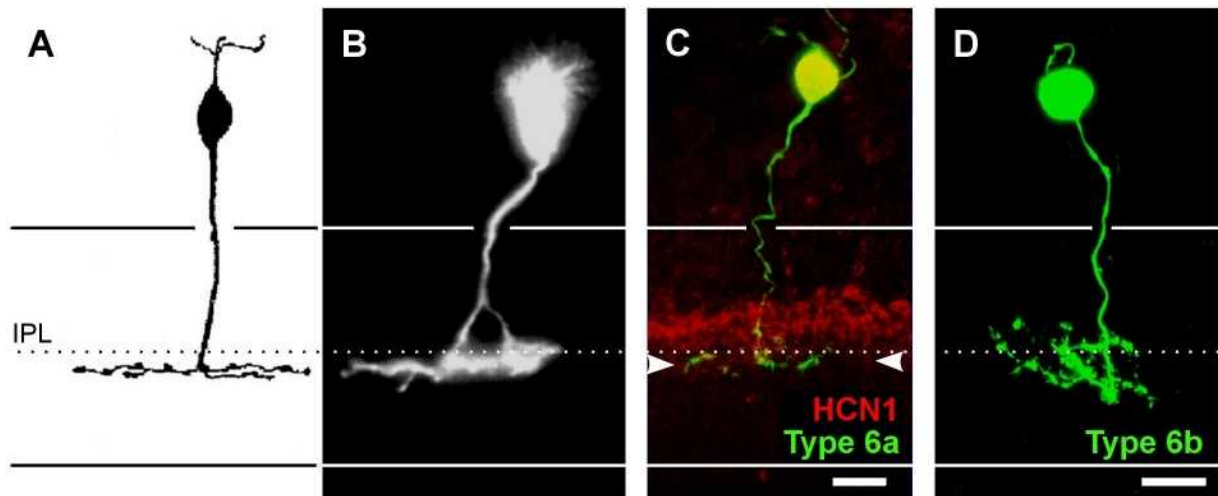


Fig. 3.12: Two morphological subtypes of type 6 cells.

(A) Schematic drawing of the type 6 cell (adapted from Euler and Wässle, 1995).

(B) A LY-filled cell (adapted from Hartveit, 1997).

(C) A LY-filled cell (green) counterstained after patch-clamp recording with HCN1 antibodies (red). The cell stratified in the HCN1-positive band 3 and resembled those in A and B.

(D) A LY-filled cell with a diffuse axon terminal system and large swellings at the ends. Morphologically, the cell is distinct from the cells shown in A, B, and C. The borders of the IPL are marked by solid lines, the ON-cholinergic stratum is shown by a dotted line. Scale bars 10 μm .

3.2.2.2.1 Type 6a cone bipolar cells

Fig. 3.13 presents the hyperpolarization-activated currents of type 6a cells. In A and B, the recordings from two different type 6a cells are shown. Upon hyperpolarization, the currents developed with a complex time course. The cells showed large instantaneous currents, which rapidly declined. In the last two voltage steps, currents first declined, but started to rise again at the end of the steps, indicating that a declining and a slowly rising current were superimposed. Application of cesium abolished the slowly rising current, indicating that it was I_h (Fig. 3.13A, lower panel). The instantaneous, rapidly declining current was affected neither by cesium, nor by barium (Fig. 3.13B).

The instantaneous, rapidly declining currents likely originate from Ca^{2+} -activated chloride channels. In the "short" protocol used in A, after each hyperpolarizing step a 150 ms step to $V_m = +10$ mV was performed, followed 100 ms later by the next hyperpolarization. The +10 mV step does not only close HCN channels, it also activates voltage-gated Ca^{2+} channels at the axon terminal and, therefore, leads to Ca^{2+} influx. Although EGTA was present in the pipette solution, it seems difficult to effectively control the Ca^{2+} concentration. Especially the remote axon terminal system of the bipolar cells may be difficult to load with EGTA from the pipette. Two experiments in line with this hypothesis are shown in Fig. 3.13B and C.

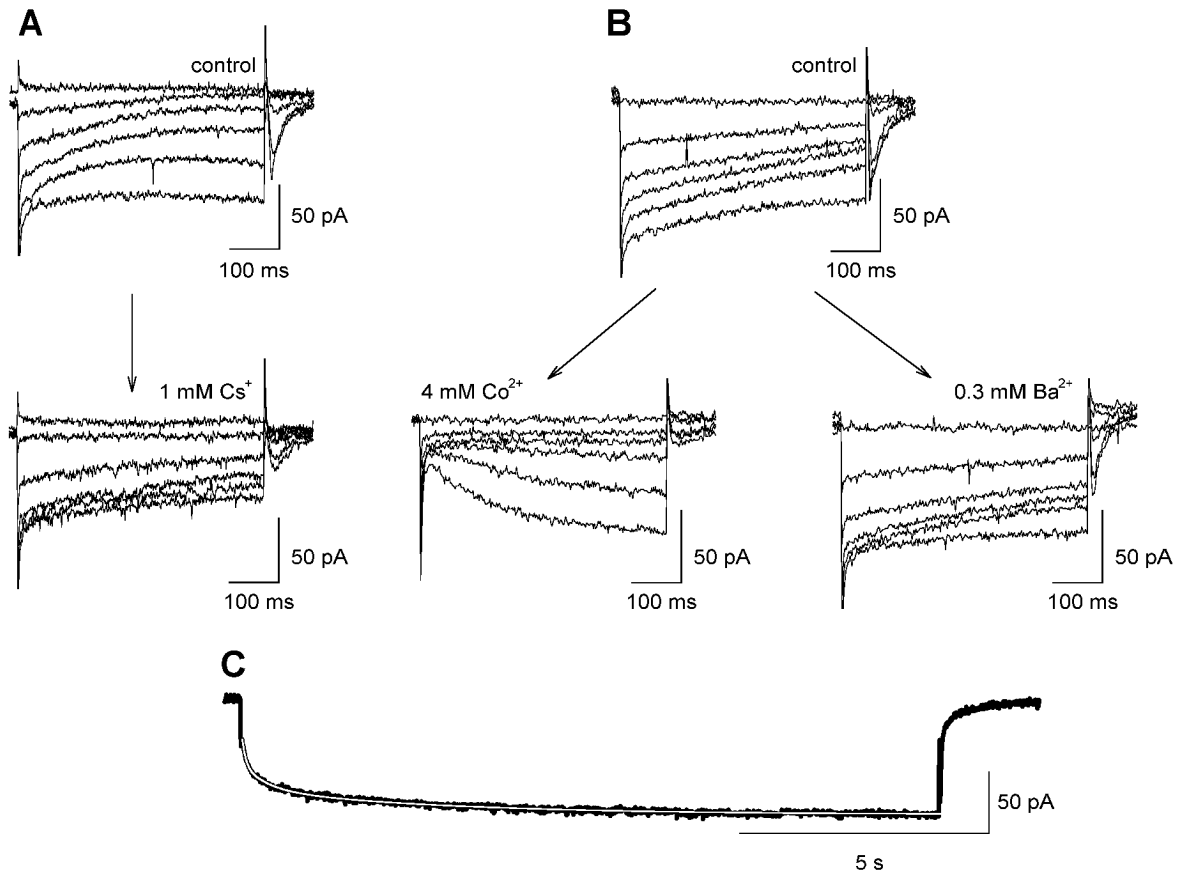


Fig. 3.13: Electrophysiological characterization of HCN channels in types 6a cells.

(A) and (B), upper panels. Hyperpolarization-activated currents were recorded in type 6a cells: V_m was stepped from -50 mV to hyperpolarizing levels from -40 to -115 mV for 500 ms. After hyperpolarizing voltage steps, the membrane was clamped to $V_m = -50$ mV for 250 ms and then to $V_m = +10$ mV for 150 ms (data not shown). (A), lower panel. 1 mM extracellular cesium blocked the late rising component of hyperpolarization-activated currents.

(B) lower left panel. 4 mM extracellular cobalt abolished the instantaneous and decaying components of the currents, slowly activating HCN-like currents persist. T-type calcium channels were blocked (see tail currents). (B), lower right panel. 0.3 mM extracellular barium did not significantly affect any component of the currents.

(C) HCN currents were measured at $V_m = -100$ mV and fitted by a three exponential function: $\tau_1 = 70$ ms (33 %), $\tau_2 = 467$ ms (33 %), and $\tau_3 = 3900$ ms (34 %). The fit is shown on top of current trace.

At the presynaptic terminal of goldfish retinal bipolar cells, Ca^{2+} -dependent Cl^- currents have been described (Okada et al., 1995). The current was completely suppressed by the external application of 3.5 mM cobalt, which effectively blocks voltage-activated Ca^{2+} influx. The presence of 4 mM Co^{2+} also completely abolished the putative Ca^{2+} -activated Cl^- currents in my experiments (Fig. 3.13B, please note that T-type Ca^{2+} currents in the tail currents are also blocked). Only the second, slowly activating current component could be recorded, with activation properties typical for I_h .

In Fig. 3.13C, the type 6a cell was held at -50 mV for several seconds, before it was hyperpolarized to -100 mV. During this time, the internal Ca^{2+} concentration seems to have dropped to the basal level and no Ca^{2+} -activated Cl^- currents could be recorded. Only HCN

currents were observed, that were best fitted by a three exponential function: $\tau_1 = 74 \pm 4$ ms (37 ± 10 %), which is close to τ_1 of HCN1; $\tau_2 = 524 \pm 55$ ms (33 ± 2 %), and $\tau_3 = 3360 \pm 400$ ms (30 ± 7 %), which is close to τ_2 of HCN4 ($n = 3$). This suggests that, similar to type 5 cells, HCN1 and HCN4 are expressed as homooligomeric channels in type 6a cells.

The absolute HCN current at $V_m = -100$ mV was -63 ± 4 pA, which was half as large as HCN currents in type 5 cells. Based on a similar calculation as in the previous chapter, in type 6a cells, HCN1 channels account for ~ 38 pA and HCN4 channels for ~ 25 pA of the total current. In comparison, in type 5 cells, HCN1 accounts for ~ 97 pA and HCN4 for ~ 25 pA of the total current amplitude. Hence, the number of HCN1 channels in type 6a cells seems to be 2.5 times lower than in type 5 cells, which is in agreement with the much weaker HCN1 immunofluorescence in type 6a cells. In contrast, the size of HCN4 current in type 6b cells seems to be the same as in type 5 cells, but no clear HCN4 immunofluorescence was observed in the axon terminals of type 6a cells. It might be that HCN4 channels are distributed more uniformly throughout the entire type 6a cell and hence, the density of the channels is too low to be detected by immunocytochemical methods.

3.2.2.2 Type 6b cone bipolar cells

Hyperpolarization-activated currents in type 6b cells are characterized by large instantaneous currents followed by small slowly activating currents (Fig. 3.14). Both components were largely blocked by cesium (Fig. 3.14A, lower panel). In contrast, 0.3 mM barium mostly blocked the instantaneous component (probably from inward rectifier channels) and typical HCN currents could be seen (Fig. 3.14B, lower panel). Application of cobalt did not affect hyperpolarization-activated currents, but blocked T-type calcium channels (Fig. 3.14C, lower panel).

HCN currents in type 6b could be fitted by a double exponential function: $\tau_1 = 212 \pm 60$ ms (56 ± 12 %), and $\tau_2 = 1400 \pm 500$ ms (44 %), τ_1 was close to τ_1 of HCN2 channels, however, τ_2 was somewhat slower than τ_2 of HCN2. No antibody marker for type 6b cells is known that would allow to confirm HCN2 expression in this cell type immunohistochemically. However, the axon terminals of type 6b cells overlap with the band 3 of HCN2 immunoreactivity (compare Fig. 1.2 and 3.15).

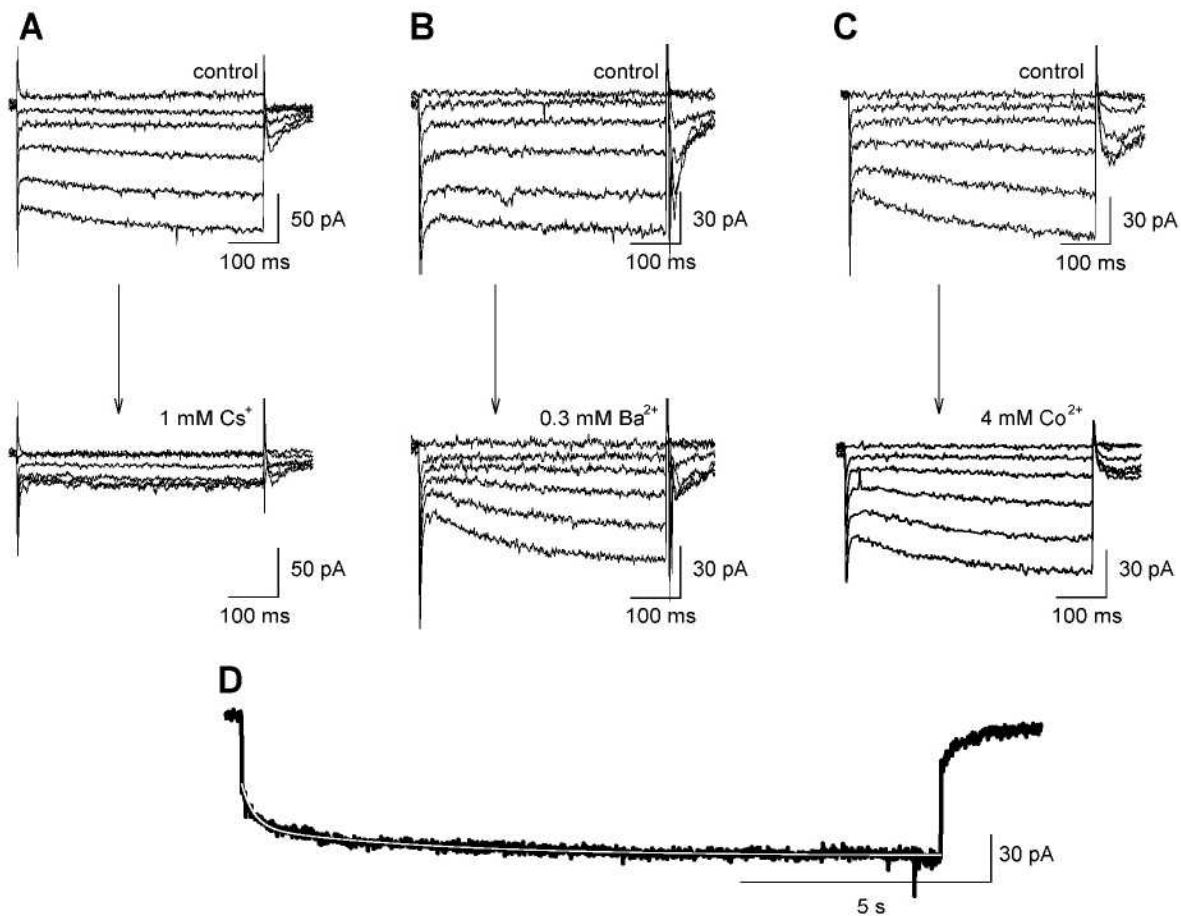


Fig. 3.14: Electrophysiological characterization of HCN channels in type 6b cells.

(A), (B), and (C), upper panels. Hyperpolarization-activated currents in type 6a cells: V_m was stepped from -50 mV to hyperpolarizing levels from -40 to -115 mV for 500 ms. After hyperpolarizing voltage steps, the membrane was clamped to $V_m = -50$ mV for 250 ms and then to $V_m = +10$ mV for 150 ms (data not shown). (A), lower panel. 1 mM extracellular cesium largely blocked hyperpolarization-activated currents. (B), lower panel. 0.3 mM extracellular barium significantly suppressed the instantaneous component of the currents. Typical HCN currents persist. (C), lower panel. 4 mM extracellular cobalt did not significantly affect any component of the hyperpolarization-activated currents. T-type calcium channels were blocked (see tail currents). (D) HCN currents at $V_m = -100$ mV were fitted by a double exponential function: $\tau_1 = 200$ ms (61 %), $\tau_2 = 2000$ ms (39 %). The fit is shown on top of the current trace.

3.2.3 ON-bipolar cells which stratify in the inner part of the IPL express HCN2 channels

Type 7, 8, and 9 cells and rod bipolar cells are ON-cells (Euler and Wässle, 1995; Hartveit, 1997). The axons of these cells stratify in the inner part of the IPL, below the ON-cholinergic stratum. HCN2 immunoreactivity was detected in this region in lobular structures, resembling bipolar cell axon terminal systems (Fig. 3.15, band 3). It appears that most, if not all, terminals of bipolar cells located in this sublamina are HCN2-positive. Moreover, the HCN2-immunoreactive structures can be counterstained with antibodies against the vesicular

glutamate transporter, Vglut. Among these bipolar cells, the rod bipolar cells is the most common and most easily identifiable cell type.

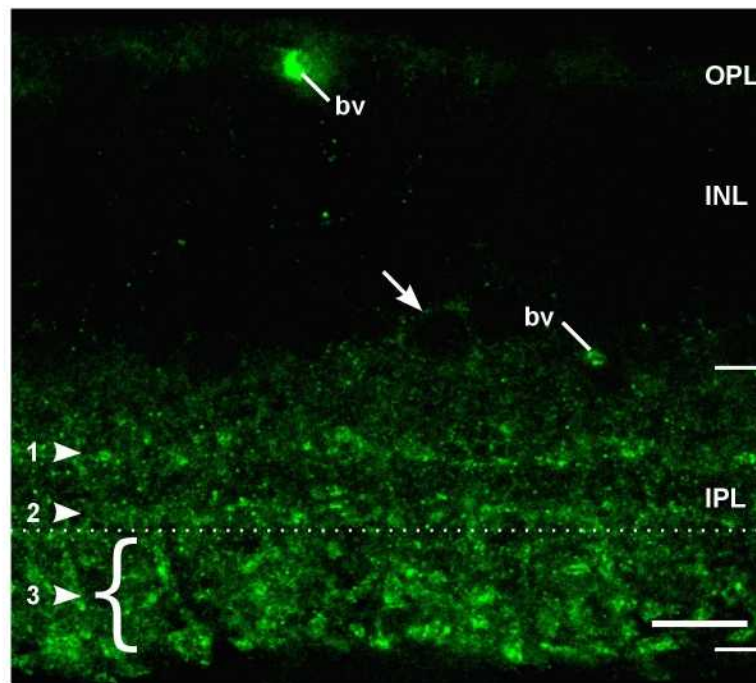


Fig. 3.15: HCN2 immunofluorescence in the IPL and the INL of the rat retina.

HCN2 immunoreactivity was found in three regions of the IPL (labeled by numbers). Band 1 originates from the axon terminals of type 3 cells; band 2 from type 5 cells. The third band occupies the inner part of the IPL, where the axons of types 7-9 and RB stratify. In addition, some amacrine somata were stained (arrow). OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; bv, blood vessel. ON-cholinergic band is depicted by a dotted line, the borders of the IPL by solid lines. Scale bar 10 μ m.

3.2.3.1 Rod bipolar cells

In rat, rod bipolar cells represent around 50 % of all bipolar cells (Euler and Wässle, 1995). The cells have large somata located in the outer part of the INL and long axons stratifying at the inner border of the IPL. In Fig. 3.16, examples taken from Euler and Wässle (A, 1995), Hartveit (B, 1997), and from my experiments (C) are shown.

Rod bipolar cells can be selectively labeled with antibodies against the α isoform of protein kinase C (PKC) in all mammalian retinæ tested, including rat (Greferath et al., 1990). Double labeling revealed that HCN2 is localized in axon terminals of rod bipolar cells (Fig. 3.16D). In a confocal section through the IPL, axons of rod bipolar cells labeled for PKC (red) and HCN2 (green), terminate close to the GCL. At the axonal swellings, HCN2 staining was prominent at restricted patches of the plasma membrane (arrows). Therefore, in a given plane

of section, not all rod bipolar terminals showed HCN2 staining. What might be the function of HCN channels at the axon terminal?

Studies in crayfish and hippocampal neurons suggest that HCN channels modulate synaptic transmission either directly by some unknown mechanisms or by cAMP-dependent depolarization (Beaumont and Zucker, 2000; Mellor et al., 2002). The triple staining in Fig. 3.16E suggests that HCN2 channels might subserve such a function in bipolar cells. The bipolar cell synapse displays a characteristic electron-dense structure, called the synaptic ribbon (Dowling and Boycott, 1966), which can be visualized by antibodies against kinesin II (Muresan et al., 1999; Ghosh et al., 2001). Rod bipolar terminals were identified by PKC staining (Fig. 3.16E, left panel, blue). Numerous synapses, appearing as red dots by the kinesin II labeling, exist on the rod bipolar terminals (arrows). A few ribbons do not colocalize with the PKC staining; presumably they originate from terminals of other types of bipolar cells. The overlay of images of the ribbon staining (red) and the HCN2 staining (green) demonstrates that HCN2 channels are found in close proximity to ribbon synapses.

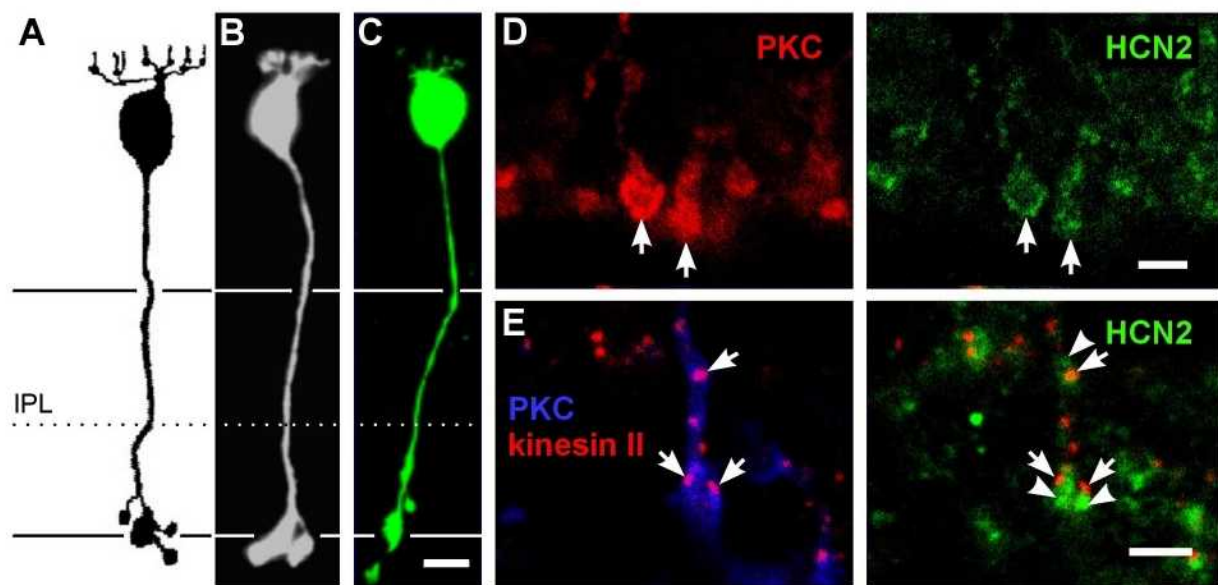


Fig. 3.16: HCN2 is highly localized on the axon terminals of rod bipolar cells.

(A) Schematic representation of a RB cell (adapted from Euler and Wässle, 1995).

(B) A LY-filled RB cell (adapted from Hartveit, 1997).

(C) A LY-filled RB cell from my experiments. Scale bar 10 μ m.

In A, B and C the borders of the IPL are marked by solid lines, the ON-cholinergic stratum by a dotted line.

(D) Single confocal section through the IPL, double labeled for PKC (red) and HCN2 (green). HCN2 staining was found on the membrane of rod bipolar axon terminals (arrows). Scale bar 5 μ m.

(E) Single confocal section through the IPL, triple labeled for PKC (blue), kinesin II (red) and HCN2 (green). In the left panel, kinesin II-positive red ribbons were found on rod bipolar axon terminals (arrows). In the right panel, HCN2 was often found in close proximity to the ribbons (double arrows). Scale bar 5 μ m.

No staining in rod bipolar cells was found with antibodies against other HCN isoforms, hence HCN2-like currents are to be expected in rod bipolar cells. Fig. 3.17 compares HCN2 currents in HEK293 cells (HCN2, A and C) with HCN currents recorded from a rod bipolar cell (RB, panel B and D). The current in HEK293 cells developed with a pronounced sigmoidal onset (upper panel) and reached steady state only after prolonged hyperpolarization. The sigmoidal onset of currents is much less pronounced in the bipolar cell. While currents in rod bipolar cells and HEK293 cells can be described by two exponents of similar values (RB: $\tau_1 = 137 \pm 14$ ms, $\tau_2 = 696 \pm 119$ ms; HCN2 in HEK293 cells: $\tau_1 = 157 \pm 14$ ms, $\tau_2 = 553 \pm 131$ ms), the kinetics components have different proportions in the two cell types. The fast and slow components account for 41 ± 8 % and 59 ± 8 % ($n = 12$), respectively, of the total current in HCN2 channels, and for 68 ± 13 % and 32 ± 13 % ($n = 28$) in rod bipolar cells.

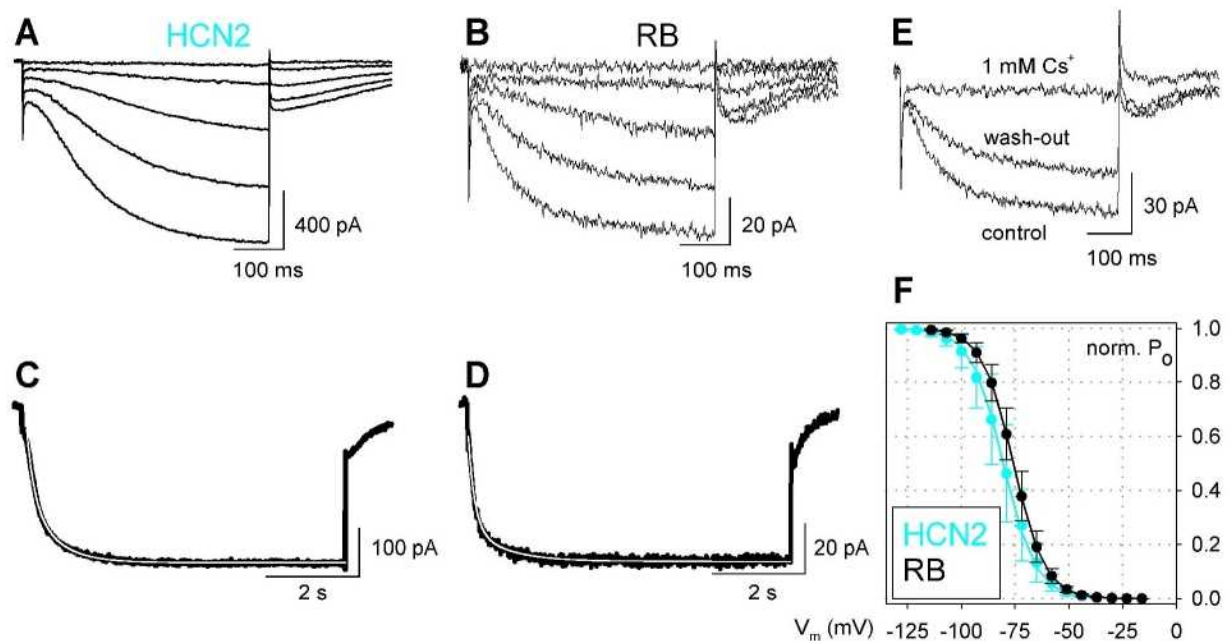


Fig. 3.17: Comparison of HCN currents in RB cells and HCN2 currents in HEK293 cells.

(A) Currents mediated by HCN2 channels in HEK293 cells. V_m was stepped from -50 mV to hyperpolarizing levels (-55 to -115 mV, 15 mV increments) for 500 ms. At the end of each hyperpolarization step, the membrane was clamped to $V_m = -50$ mV for 250 ms, and then for 150 ms to $V_m = +10$ mV (not shown).

(B) HCN currents recorded in a RB cell. Conditions as in (A).

(C) HCN2 current in a HEK293 cell. V_m was stepped from -50 mV to -100 mV for 14 s. The current was fitted by a double exponential function: $\tau_1 = 158$ ms (46 %) and $\tau_2 = 574$ ms (54 %). The fit is shown on top of the current trace.

(D) In RB cell, the current was fitted by a double exponential function: $\tau_1 = 135$ ms (68 %), $\tau_2 = 750$ ms (32 %). Conditions as in (C). The fit is shown on top of the current trace.

(E) HCN currents ($V_m = -115$ mV) in rod bipolar cells were reversibly blocked by 1 mM extracellular cesium.

(F) Averaged activation curves of HCN2 channels expressed in HEK293 cells (cyan): $V_{1/2} = -80.4 \pm 5.0$ mV and $S = 7.6 \pm 1.0$ mV; and of HCN channels measured in RB cells (black): $V_{1/2} = -76.0 \pm 2.0$ mV and $S = 7.4 \pm 0.5$ mV. Dots represent the measuring points.

The mid-point potential of HCN channels in rod bipolar cells approached that of HCN2 channels expressed in HEK293 cells (Fig. 3.17F, HCN2 channels (cyan): $V_{1/2} = -80.4 \pm 5.0$ mV and $S = 7.6 \pm 1.0$ mV ($n = 7$); HCN channels in RB cells (black): $V_{1/2} = -76.0 \pm 2.0$ mV and $S = 7.4 \pm 0.5$ mV ($n = 4$)).

HCN2 immunoreactivity in RB cells was only found at the axon terminals of the cells. I patched a number of cells, that had been axotomized to various degrees during the slicing procedure. After recording, slices were fixed and stained with antibodies against PKC. Double labeled cells could thus be identified as rod bipolar cells. In these cells, I recorded at $V_m = -115$ mV the following HCN currents: -13 ± 5 pA ($n = 5$) from the soma and dendrites (axon completely lost); -33 ± 3 pA ($n = 2$) from the soma plus axon, but without the axon terminal. When recorded in the entire cells, -58 ± 9 pA of current was obtained at $V_m = -115$ mV ($n = 18$). This suggests that 43 % of HCN2 channels are localized at the very end of the axon terminal system, 35 % of the channels are localized at the axons and only 22 % of the channels are distributed throughout the somata and the dendrites of the cells. This channel density seems to be below the immunocytochemical detection threshold.

3.2.3.2 Type 7 cone bipolar cells

The cells depicted in the scheme by Euler and Wässle (Fig. 1.3) are "ideal" cells; in these scheme, type 7 cells can not be mistaken for type 8 or type 6 cells. In many instances, however, morphological criteria alone were not sufficient to unequivocally classify a given bipolar cell as type 6b, 7 or 8, and the question could arise, whether we observe variations within one cell class or these cells really represent distinct types. This is particularly problematic in slice preparations, because it cannot be ruled out that axon terminals become damaged or slightly distorted during slicing. In the following, I will show that the combination of morphological data and electrophysiological data on the unique combination and functional properties of voltage-activated channels will help to identify these cells as distinct cell types.

No specific marker protein is known for type 7 cone bipolar cells, therefore, HCN channels expression could not be investigated by immunocytochemical methods in this cell type.

Fig. 3.18A and B show type 7 cells from Euler and Wässle (1995), and Hartveit (1997), respectively. In some cases, when the cells were difficult to identify, it was helpful to stain the

slice with the filled bipolar cell with antibodies against recoverin, to label type 8 cells (and type 2 cells; Euler and Wässle, 1995). Such a staining is shown in Fig. 3.18C.

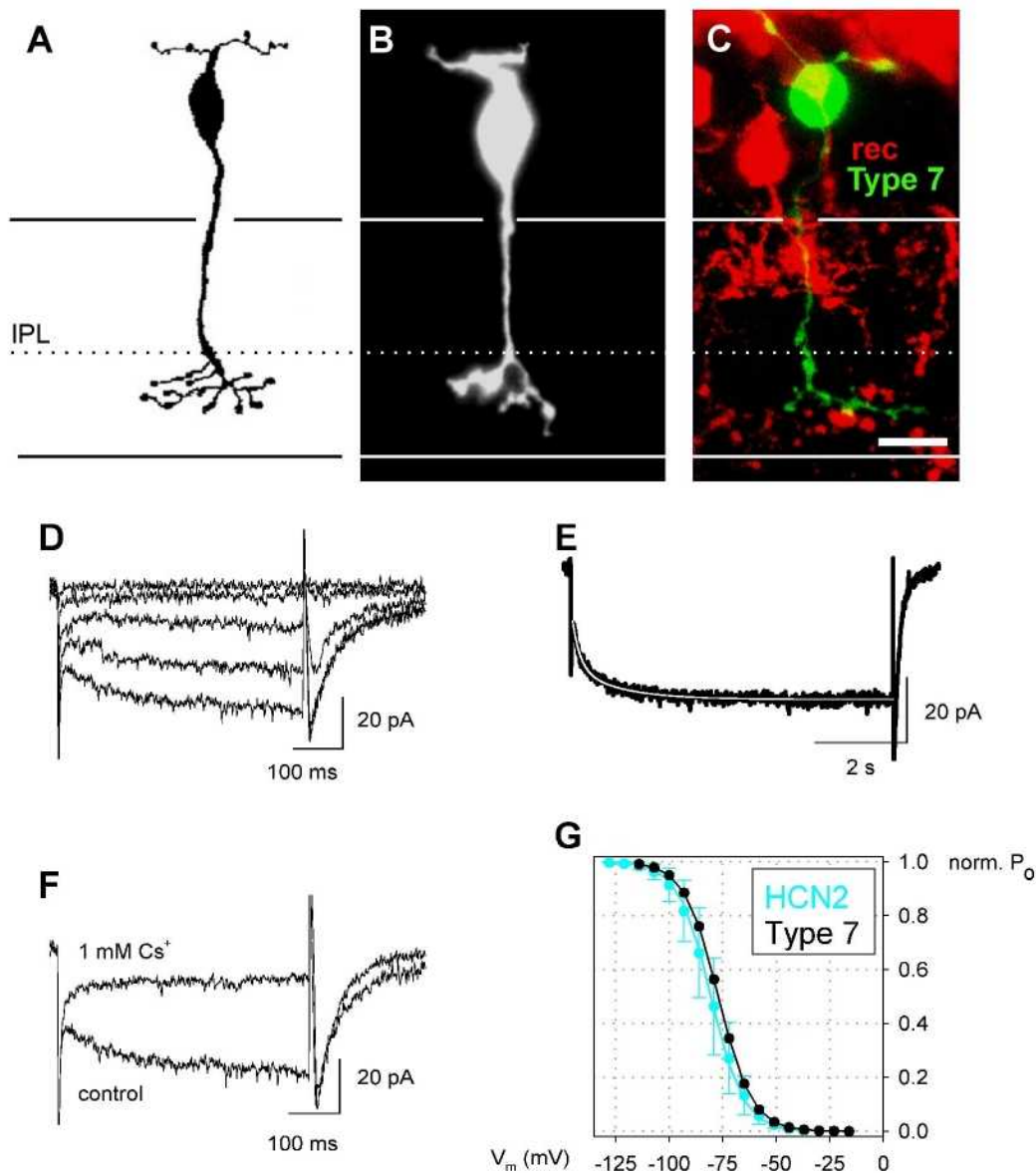


Fig. 3.18: Electrophysiological study of HCN currents in type 7 cells.

(A) Schematic representation of a type 7 cell (adapted from Euler and Wässle, 1995).

(B) A LY-filled type 7 cell (adapted from Hartveit, 1997).

(C) A LY-filled type 7 cell (green) in a slice, stained after patch-clamp recording with antibody against recoverin (red). The cell stratified within the upper part of the type 8 recoverin band. Scale bar 10 μm .

In A, B and C the borders of the IPL are marked by solid lines, the ON-cholinergic stratum by a dotted line.

(D) Currents mediated by HCN2 channels in type 7 cell. V_m was stepped from -50 mV to hyperpolarizing levels (-55 to -115 mV, 15 mV increments) for 500 ms. At the end of each hyperpolarization step, the membrane was clamped to $V_m = -50$ mV for 250 ms, and then for 150 ms to $V_m = +10$ mV (not shown). Very large T-type calcium currents were observed when the membrane voltage was stepped back to the holding level.

(E) V_m was stepped from -50 mV to -100 mV for 14 s. The current was fitted by a double exponential function: $\tau_1 = 168$ ms (60 %), $\tau_2 = 975$ ms (40 %). The fit is shown on top of the current trace.

(F) HCN currents ($V_m = -115$ mV) in type 7 cells were reversibly blocked by 1 mM extracellular cesium.

(G) The activation curves from HCN2 expressed in HEK293 cells (cyan, $V_{1/2} = -80.4 \pm 5.0$ mV and $S = 7.6 \pm 1.0$ mV) and HCN currents measured in a type 7 cell (black, $V_{1/2} = -76.0$ mV and $S = 7.8$ mV). Dots represent the measuring points.

Typically, the axon terminals of type 7 cells start to stratify slightly above the terminals of type 8 cells and show some overlap with the type 8 recoverin band. The cell in C, however, stratified well within the type 8 band. Hence, based on purely morphological criteria, this cell might have been described as type 8. Based on the properties of its currents, however, it must be regarded clearly as a type 7 cell.

Type 7 cells are characterized by a combination of small HCN currents and very large T-type calcium currents. HCN currents could be fitted best by a double exponential function: $\tau_1 = 165 \pm 22$ ms (51 ± 17 %, very similar to τ_1 of HCN2 in HEK293 cells), $\tau_2 = 1040 \pm 280$ ms (49 %, which is longer than τ_2 of HCN2 in HEK293 cells) ($n = 4$, Fig. 3.18E). The activation curve of the channels was similar to those of HCN2 channels expressed in HEK293 cells (Fig. 3.18G; $V_{1/2} = -76.0$ mV and $S = 7.8$ mV, $n = 1$). In conclusion, the HCN currents recorded in type 7 cells most likely originate from HCN2 channels.

3.2.3.3 Type 8 cone bipolar cells

Fig. 3.19A shows a schematic drawing of a type 8 bipolar cell (Euler and Wässle, 1995). In B, a type 8 cell filled with LY (Hartveit, 1997) is shown. The comparison of these two cells reveals the morphological diversity within the cell type. In A, the cell has a relatively flat axon terminal system stratifying close to the GCL. In B, the axon terminal system is more diffuse, spreading from slightly below the ON cholinergic band towards the GCL. Indeed, diffuse staining in both type 2 and type 8 bands was obtained with the antibodies against recoverin (Fig. 3.19C). In C, a slice with a filled type 8 cell was stained with recoverin antibodies. The axon terminal system of the LY-filled cell closely resembles that of the neighboring recoverin-positive type 8 cell (arrow).

HCN2-positive spots (Fig. 3.19D, green) were found at the axon terminal systems of type 8 cells (recoverin, red), similar to the distribution of HCN2 on RB cell axon terminals. HCN currents in type 8 cells were fitted by a double exponential function: $\tau_1 = 147 \pm 18$ ms (58 ± 19 %), $\tau_2 = 692 \pm 140$ ms (42 %) ($n = 9$, Fig. 3.19F). The activation parameters were similar to those of HCN channels in RB cells and to HCN2 in HEK293 cells. Summarizing, HCN2 channels were found in type 8 cells by immunocytochemistry and electrophysiology.

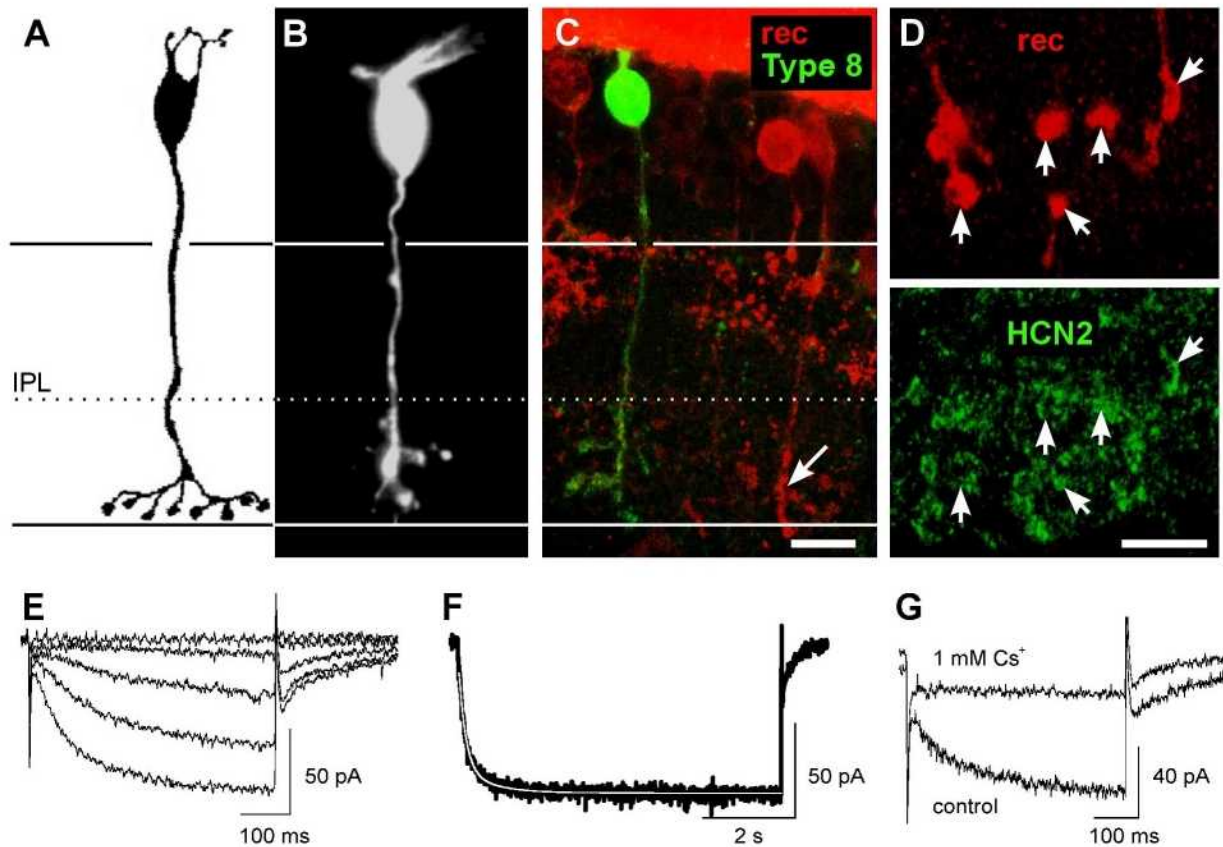


Fig. 3.19: Electrophysiological and immunocytochemical study of HCN channels in type 8 cells.

(A) Schematic representation of a type 8 cell (adapted from Euler and Wässle, 1995).

(B) A LY-filled type 8 cell (adapted from Hartveit, 1997).

(C) A LY-filled type 8 cell (green) doublestained after patch-clamp recording with antibodies against recoverin (red). The axon terminal system of the filled cell stratified throughout the entire recoverin-positive type 8 band and closely resembled that of the neighboring recoverin-positive cell (arrow). Scale bar 10 μm . In A, B and C, the borders of the IPL are marked by solid lines, the ON-cholinergic stratum is shown by a dotted line.

(D) Single confocal section through the IPL double-labeled for recoverin (red) and HCN2 (green). HCN2-positive spots were found on type 8 recoverin-positive axon terminals (arrows). Scale bar 5 μm .

(E) Currents mediated by HCN2 channels in a type 8 cell. V_m was stepped from -50 mV to hyperpolarizing levels (-55 to -115 mV, 15 mV increments) for 500 ms. At the end of each hyperpolarization step, the membrane was clamped to $V_m = -50$ mV for 250 ms, and then for 150 ms to $V_m = +10$ mV to close HCN channels (not shown). T-type calcium currents were observed as transient components of the tail currents when the membrane voltage was stepped back to the holding level.

(F) V_m was stepped from -50 mV to -100 mV for 14 s. The current was fitted by a double exponential function: $\tau_1 = 148$ ms (77 %), $\tau_2 = 652$ ms (23 %). The fit is shown on top of the current trace.

(G) HCN currents ($V_m = -115$ mV) in type 8 cells were blocked by 1 mM extracellular cesium. The transient component of the tail current persisted.

3.2.3.4 Type 9 cone bipolar cells

Type 9 cells have very diffuse axon terminal systems which extend into the GCL (Fig. 3.20, the soma of the cell was displaced while removing the patch pipette). Their dendritic tree covers a wide range of the OPL. These cells were rarely encountered in a previous study (Euler and Wässle, 1995), as well as in my study. Because of the low density and the very

large dendritic tree, this bipolar cell type was suggested to contact blue cones. Blue cones in rat retina represent 0.05 % of all photoreceptors (Szel and Rohlich, 1992), thus, a large dendritic tree of type 9 cells would enable them to contact several blue cones (Euler and Wässle, 1995). So far, no specific marker has been found for type 9 cells.

I recorded from several cells of this type (Fig. 3.20D). The HCN current was fitted by a double exponential function: $\tau_1 = 200 \pm 30$ ms (50 ± 14 %), $\tau_2 = 900 \pm 200$ ms (50 %) (n = 6, Fig. 3.20E) and was completely and reversibly blocked by cesium (Fig. 3.20F). The HCN current in type 9 cells is most likely mediated by HCN2 channels.

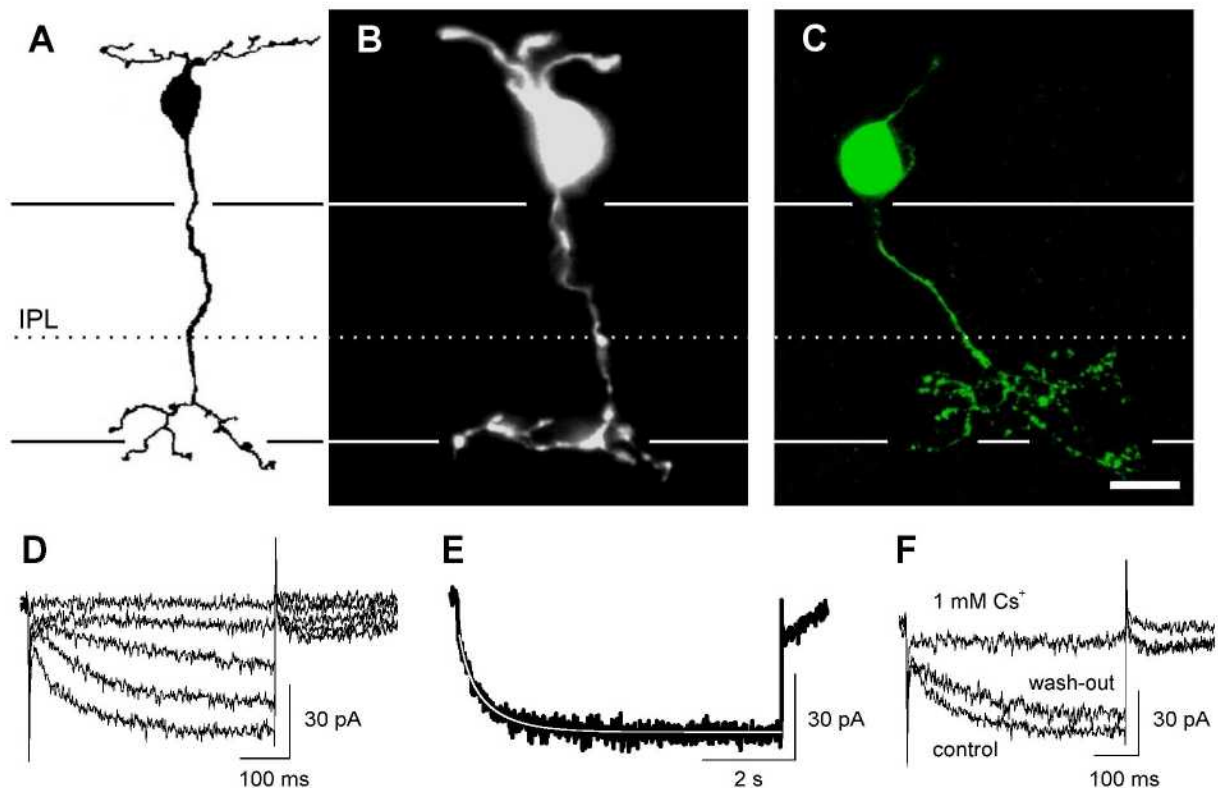


Fig. 3.20: Electrophysiological study of HCN currents in type 9 cells.

(A) Schematic representation of a type 9 cell (adapted from Euler and Wässle, 1995).

(B) A LY-filled type 9 cell (adapted from Hartveit, 1997).

(C) A LY-filled type 9 cell of my study after patch-clamp recording. The soma of the cell was displaced towards the inner INL while removing the pipette. Scale bar 10 μ m. In A, B and C the borders of the IPL are marked by solid lines, the ON-cholinergic stratum is shown by a dotted line.

(D) Currents mediated by putative HCN2 channels in a type 9 cell. V_m was stepped from -50 mV to hyperpolarizing levels (-55 to -115 mV, 15 mV increments) for 500 ms. At the end of each hyperpolarization step, the membrane was clamped to V_m = -50 mV for 250 ms, and then for 150 ms to V_m = +10 mV (not shown). No prominent T-type calcium current was observed.

(E) V_m was stepped from -50 mV to -100 mV for 14 s. The current was fitted by a double exponential function: $\tau_1 = 190$ ms (33 %), $\tau_2 = 700$ ms (67 %). The fit is shown on top of the current trace.

(F) HCN currents (V_m = -115 mV) in type 9 cells were reversibly blocked by 1 mM extracellular cesium.

3.3 How many bipolar cell types do we find in the retina?

Fig. 3.21A shows nine types of cone bipolar cells and one type of rod bipolar cell which have been classified in the rat retina on the basis of morphological criteria (Euler and Wässle, 1995). Basically, Hartveit (1997) described the same cell types. In some instances, however, his cells differ slightly from those shown by Euler. These differences have been pointed out in the previous chapters. The results of my study suggest that the scheme must be extended by at least one, maybe by two, bipolar cell classes (Fig. 3.21B).

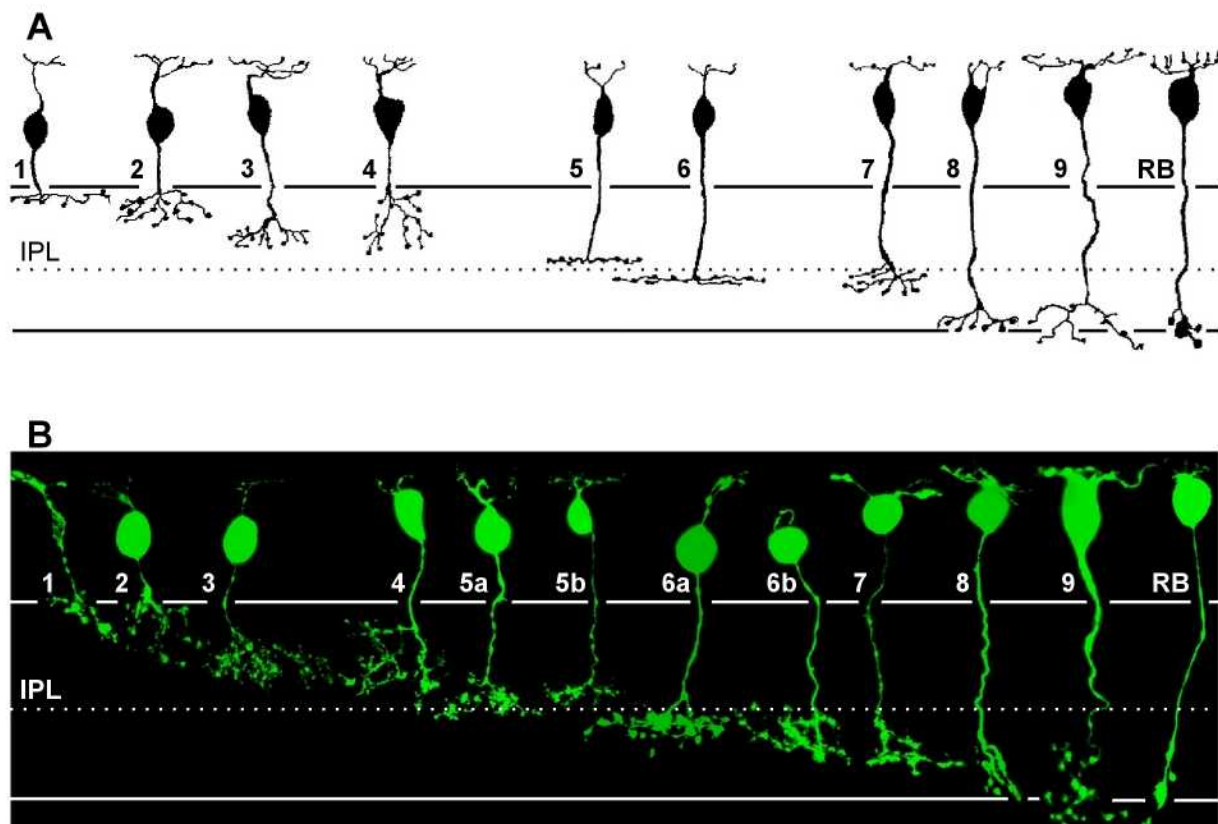


Fig. 3.21: Morphological classification of bipolar cells in the rat retina.

(A) Classification scheme for bipolar cells in the rat retina (adapted from Euler and Wässle, 1995).

(B) LY-filled bipolar cells described in the current study.

The borders of the IPL are marked by solid lines, the ON-cholinergic stratum is shown by a dotted line.

There is one big problem when classifying cell types. Within each cell class, cells will show certain variations, making it difficult to draw clear borders between cell classes. For example, compare the types 6a, 6b, 7, and 8 of my scheme. All four cell types stratify below the ON-cholinergic band and their axon terminal systems show considerable overlap. Based on morphological criteria alone, it is difficult to decide whether they are variants of one cell type or whether they represent distinct cell types. Most probably, my type 6b was mistaken as either type 6a or type 7 cells in the previous studies. In the following, I will show that the four

cell types can be distinguished on the basis of their voltage-activated currents. Type 6a cells show unique inward currents, originating from superimposed HCN currents and (most likely) calcium-activated chloride currents (see Fig. 3.13). Electrophysiologically, 6a cells cannot be mistaken for another cell type. Types 6b, 7 and 8 are more difficult to distinguish. For example, all three types show HCN2-like currents. Fig. 3.22 compares representative current traces of type 6b, 7, and 8 cells.

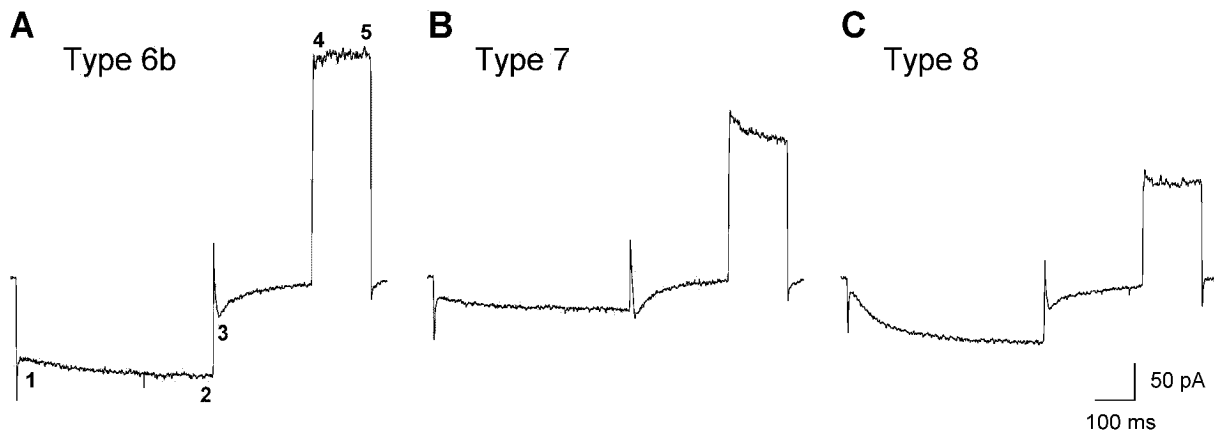


Fig. 3.22: Comparison of voltage-gated channels in type 6b, 7, and 8 cells.

(A) Currents measured in a type 6b cell. The currents were evoked by one episode of the standard protocol: the membrane was clamped to $V_m = -50$ mV for 16 ms, then to $V_m = -115$ mV for 500 ms followed by a 250 ms step to $V_m = -50$ mV and a 150 ms step to $V_m = +10$ mV.

The amplitudes of the current were calculated by subtracting the amplitude of the current at the holding level (in the 16 ms step, $V_m = -50$ mV) from the amplitudes of the currents measured in the indicated points. Numbers indicate the amplitude of instantaneous current (1); instantaneous current and slowly developing HCN current (2); current mediated by T-type of calcium channels and tail current of HCN channels (3); outward current at the beginning of depolarizing voltage step to +10 mV (4) and at the end of this step (5).

(B) Currents measured in a type 7 cell. Conditions as in A.

(C) Currents measured in a type 8 cell. Conditions as in A. All current traces were plotted with the same scales.

Traces were taken from the standard short protocol, which was applied to each bipolar cell in this study. Cells were held at -50 mV, then V_m was stepped to -115 mV to activate HCN channels. After 500 ms, V_m was stepped back to -50 mV for 250 ms to measure tail currents. Finally, a depolarizing step to $+10$ mV was performed to completely close HCN channels. During this step, outward currents carried mostly by voltage-activated potassium channels can be observed. At the positions indicated by numbers in the first trace, current amplitudes were measured (peak minus holding current). Note that current amplitudes differ strongly between the three traces (scales are identical for all traces). For example, the instantaneous current (at position 1) is -106 pA in the type 6b cell, but only -22 pA in the type 7 cell shown. Averaged currents for all cell types are given in Tab. 3.2. Moreover, the proportions of the different currents differ strongly between cell types. Currents were normalized to the steady state HCN current (position 2). The normalized instantaneous current (1) is 0.82 ± 0.06 in type

6b cells, 0.54 ± 0.09 in type 7 cells and 0.28 ± 0.07 in type 8 cells. Using this single parameter, most of 45 bipolar cells analyzed ($n = 45$) were unequivocally classified as either type 6b, 7, or 8, respectively. In few cases, the normalized tail current peak (position 3) was needed as a second parameter to decide whether a given cell was type 7 (0.85 ± 0.22) or type 8 (0.36 ± 0.07). Unfortunately, this kind of analysis did not help to clearly distinguish type 5a from type 5b cells. Neither on morphological, nor on electrophysiological criteria can it clearly be decided, whether all type 5 cells belong to one cell type, but show large variations in the thickness and form of their axon terminals, or whether two subpopulations of type 5 cells exist. The strongest argument for two distinct classes is the mismatch between the GLT-1 and HCN1 staining (see Fig. 3.8). Euler classified type 5 cells as OFF-bipolar, Hartveit as ON-bipolar cells. This might reflect the presence of two separate cell types stratifying in this sublamina of the IPL. Clearly, more investigations are needed to settle this question.

Tab. 3.2: Comparison of voltage-gated channels in type 6b, 7, and 8 cells.

Type of cell	Current amplitude measured at the points indicated in Fig. 3.22:								
	1		2	3		4		5	
	[pA]	rel.*		[pA]	rel.*	[pA]	rel.*	[pA]	rel.*
6b (15)	-73±17	0.82±0.06	-90±23	-33±15	0.40±0.26	+340±90	3.97±1.53	+308±71	3.66±1.47
7 (15)	-27±10	0.54±0.09	-51±19	-41±13	0.85±0.22	+207±59	4.43±1.54	+166±47	3.55±1.24
8 (15)	-20±7	0.28±0.07	-70±19	-25±8	0.36±0.07	+130±32	1.88±0.36	+114±23	1.69±0.43

Data are reported as mean $\pm$ SD, the number of cells measured is shown in parenthesis.

Relative currents* are normalized to the current in point 2.

3.4 HCN channels in photoreceptors

HCN currents were first detected in rod photoreceptors of the retina (Fain et al., 1978), where they counteract the hyperpolarizing response at high light intensities by allowing an inward current that depolarizes the cell.

Fig. 3.23 shows the immunocytochemical investigation on HCN channel expression in photoreceptors. The most intensive immunofluorescence was detected with HCN1 antibodies (Fig. 3.23A), in both rod and cone photoreceptors. Rodent retinae are rod dominated; only $\sim 1\%$ of the photoreceptors are cones (Jeon et al., 1998). Several cone somata are shown by arrows. The somata of cones are located mostly in the outer layers of the ONL and are more elongated and larger than rod somata. In both kinds of photoreceptors, HCN1 immunostaining was prominent in the inner segments, in the somata and axons, and in the axon terminals.

Fig. 3.23B shows HCN2 immunofluorescence in the outer retina. HCN2 immunofluorescence in cones (arrows) was weak and only slightly brighter than the background. This suggests that the expression level of HCN2 channels in cones is much lower than for HCN1. No HCN2 immunofluorescence was detected in rods.

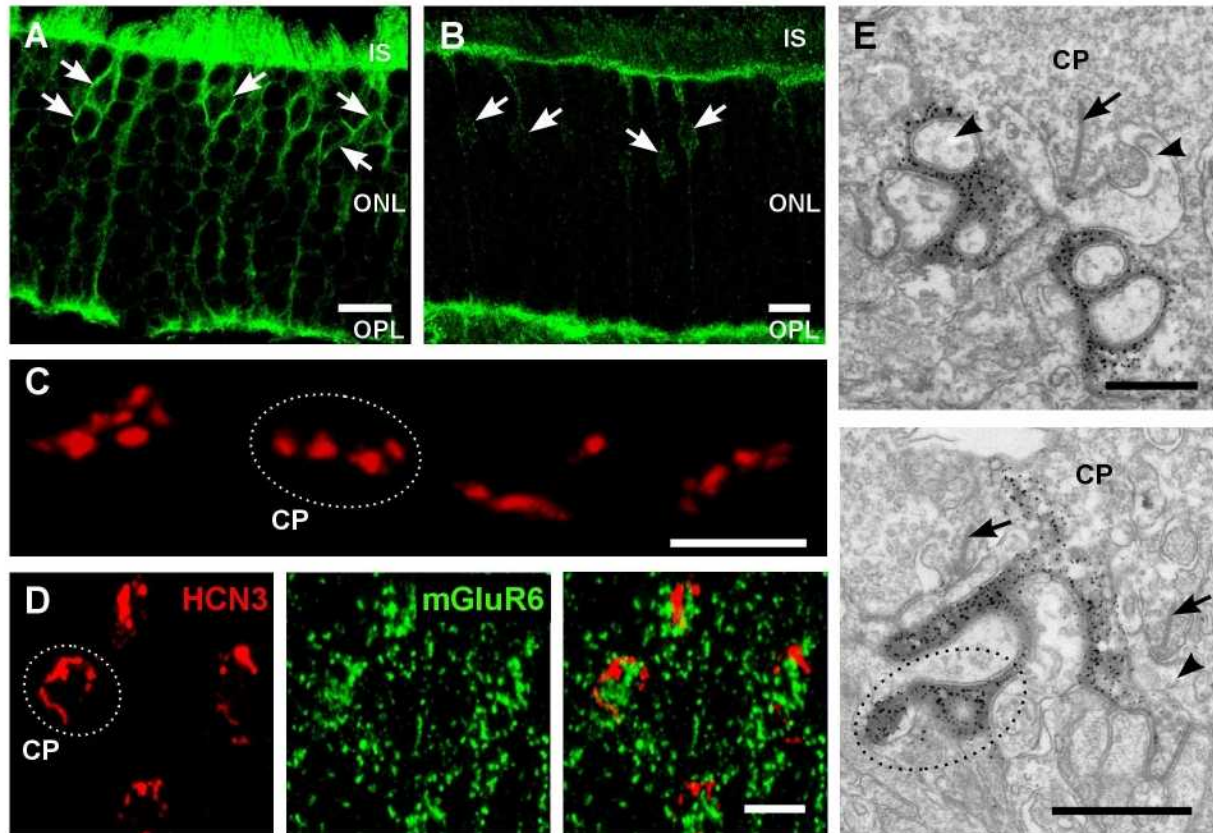


Fig. 3.23: HCN channel distribution in rat photoreceptors.

(A) HCN1 immunostaining is found in the inner segments, somata, axons and endfeet of photoreceptors. A number of cone somata are marked with arrows. Inner segments (IS), outer nuclear layer (ONL), outer plexiform layer (OPL). Scale bar 10 μ m.

(B) HCN2 immunostaining in the outer retina. A weak staining was detected in a number of cones (marked with arrows). No fluorescence was observed in rods. Scale bar 10 μ m.

(C) HCN3 at cone pedicle synapses in the OPL (in a vertical section). One cone pedicle (CP) is encircled. Scale bar 5 μ m.

(D) Wholemount preparation labeled for HCN3 (red) and mGluR6 (green) with the focus in the OPL. The right panel shows the merged image. mGluR6-positive ON-bipolar cell dendrites were clustered at cone pedicles (one is encircled), but they did not overlap with HCN3 staining. Scale bar 5 μ m.

(E) Electronmicroscopic localization of HCN3 at the cone pedicle base and at finger-like extensions (encircled process). Arrows mark synaptic ribbons, arrowheads mark invaginating processes. Scale bar 0.5 μ m.

HCN3 staining was most noticeable in the OPL at the axon terminal of cones, the cone pedicle (Fig. 3.23C and D). The cone pedicle is a very complex synapse, in which the cone contacts up to ten different cell types: 9 types of cone bipolar cells and one type of horizontal cells (for review see Haverkamp et al., 2000). HCN3 channels might be expressed either presynaptically in cones or in any one of the postsynaptic processes. To address this question, retinal sections, as well as retinal wholemounts, were double labeled for HCN3 and for

markers of dendritic tips of either ON-bipolar cells (mGluR6 – the metabotropic glutamate receptor 6), or horizontal cells (CabP – the calcium binding protein), or some types of OFF-bipolar cells (GluR1 – the ionotropic glutamate receptor 1).

Fig. 3.23D shows a whole-mounted retina stained for HCN3 and mGluR6; the focal plane was in the OPL. HCN3 (red) was localized in circular or oval structures ($\varnothing$ of $\sim 5 \mu\text{m}$). The green dots represent mGluR6 in dendritic tips of ON-bipolar cells. The dots clustered at high density within the area encircled by HCN3. However, no colocalization of mGluR6 and HCN3 was observed. Similarly, no colocalization was observed between HCN3 and either CabP or GluR1 (data not shown), indicating that HCN3 might be localized presynaptically in cones rather than in postsynaptic processes.

This question was fully solved by electron microscopy (in collaboration with S. Haverkamp, Max-Planck-Institut für Hirnforschung, Frankfurt). Two cone pedicles (CP) are shown in electron micrographs in Fig. 3.23E. In both cases, typical ultrastructural features of cone pedicle synapses, like the ribbons (arrows) and invaginating processes of postsynaptic cells (arrowheads) can be identified. HCN3 immunoreactivity was observed as silver grains presynaptically at the cone membrane that surrounds invaginating processes and in finger-like extensions (encircled process) at the base of the cone pedicle. Interestingly enough, similar staining was observed with antibodies against dystrophin and beta-dystroglycan (Koulen et al., 1998; Blank et al., 1999) and with antibodies against the NMDA receptor subunit NR1C2 (Fletcher et al., 2000). Two kinds of cones have been described in the rat: short wavelength sensitive cones ("blue-cones") and middle-long wavelength sensitive cones ("red-green cones", Széll et al., 1996). In the rat retina, red-green cones are strongly stained by HCN3 antibodies, while blue-cones show no or, in few cases, very weak HCN3 immunoreactivity (F. Müller, IBI -1, Forschungszentrum Jülich, personal communication).

Summarizing the immunocytochemical results, HCN1 is the only HCN isoform expressed in rods and is the dominant isoform in cone photoreceptors. HCN2 channels were found only in cones at apparently low expression level. HCN3 channels were localized presynaptically exclusively in cone pedicle synapses.

I recorded hyperpolarization-activated currents from both kinds of photoreceptors. The differences in hyperpolarization-activated currents and the different shapes of the axon terminal systems as revealed by LY-filling (large triangular pedicles for cones and small round spherules for rods), allowed to clearly distinguish rods from cones.

In rods, hyperpolarizing voltage steps activated instantaneous currents followed by rapidly activating currents that developed with a sigmoidal time course characteristic for I_h .

(Fig. 3.24A). After stepping back to the holding level, small rapidly deactivating tail currents were recorded. In rods, no T-type calcium channels have been described, consequently, the tail currents are most likely mediated by HCN channels.

The rising phase of the currents could be closely fitted by a double exponential function with the following time constants: $\tau_1 = 57 \pm 3$ ms (89 ± 6 %), $\tau_2 = 271 \pm 16$ ms (11 %) (Fig. 3.24B). The values of the time constants in rods are very similar to those found for HCN1 channels expressed in HEK293 cells ($\tau_1 = 54 \pm 6$ ms and $\tau_2 = 242 \pm 55$ ms), nevertheless, clear differences are observed between the currents recorded in the two cell types. In rods, the first kinetic current component contributes much more to the total HCN current than in HCN1 channels expressed in HEK293 cells. As a consequence, the HCN currents in rods reached steady state conditions much easier than HCN1 currents in HEK293 cells.

Another difference was found between the mid-point potential of HCN channels in rods and of HCN1 channels in HEK293 cells (Fig. 3.24H). The mid-point potential in rods was measured from the tail currents at $V_m = -50$ mV after 500 ms hyperpolarizing prepulses (the same voltage protocol as in Fig. 3.24A; the 500 ms pulses should have been sufficiently long for a reliable estimation of $V_{1/2}$, because currents had reached steady state plateau). The mid-point potential of rod HCN1, $V_{1/2} = -64.2 \pm 3.1$ mV, was much more negative than that of HCN1 channels in HEK293 cells, $V_{1/2} = -50.0 \pm 1.4$ mV. In guinea-pig rods, $V_{1/2}$ ranged from -66 mV to -76 mV (Demontis et al., 1999).

Hyperpolarization-activated currents in rods were abolished by cesium (Fig. 3.24C). The instantaneous currents in rods were very large (at $V_m = -115$ mV, they accounted for more than 50 % of the total steady state currents). In one cell tested, roughly 50 % of the instantaneous current was blocked by barium (at $V_m = -115$ mV), suggesting that this current component originate from inward rectifier channels (Fig. 3.24D). So far, inward rectifier channels were only observed in *Xenopus* rods (Akopian and Witkovsky, 1996), but not in mammalian rods.

I recorded hyperpolarization-activated currents also in mouse rods (data not shown). The currents were virtually indistinguishable in shape and amplitude from those in rat rods (data not shown).

Fig. 3.24E shows a whole-cell recording of a cone. The hyperpolarizing voltage steps induced large inward currents with fast activation kinetics. Similar currents were found in monkey cones (Yagi and Macleish, 1994). The amplitude of HCN currents was the largest among the retinal cells recorded in this study. The currents were well fitted by a double exponential function, with $\tau_1 = 55$ ms (67 %) and $\tau_2 = 205$ ms (33 %) (Fig. 3.24F). Time constants as well

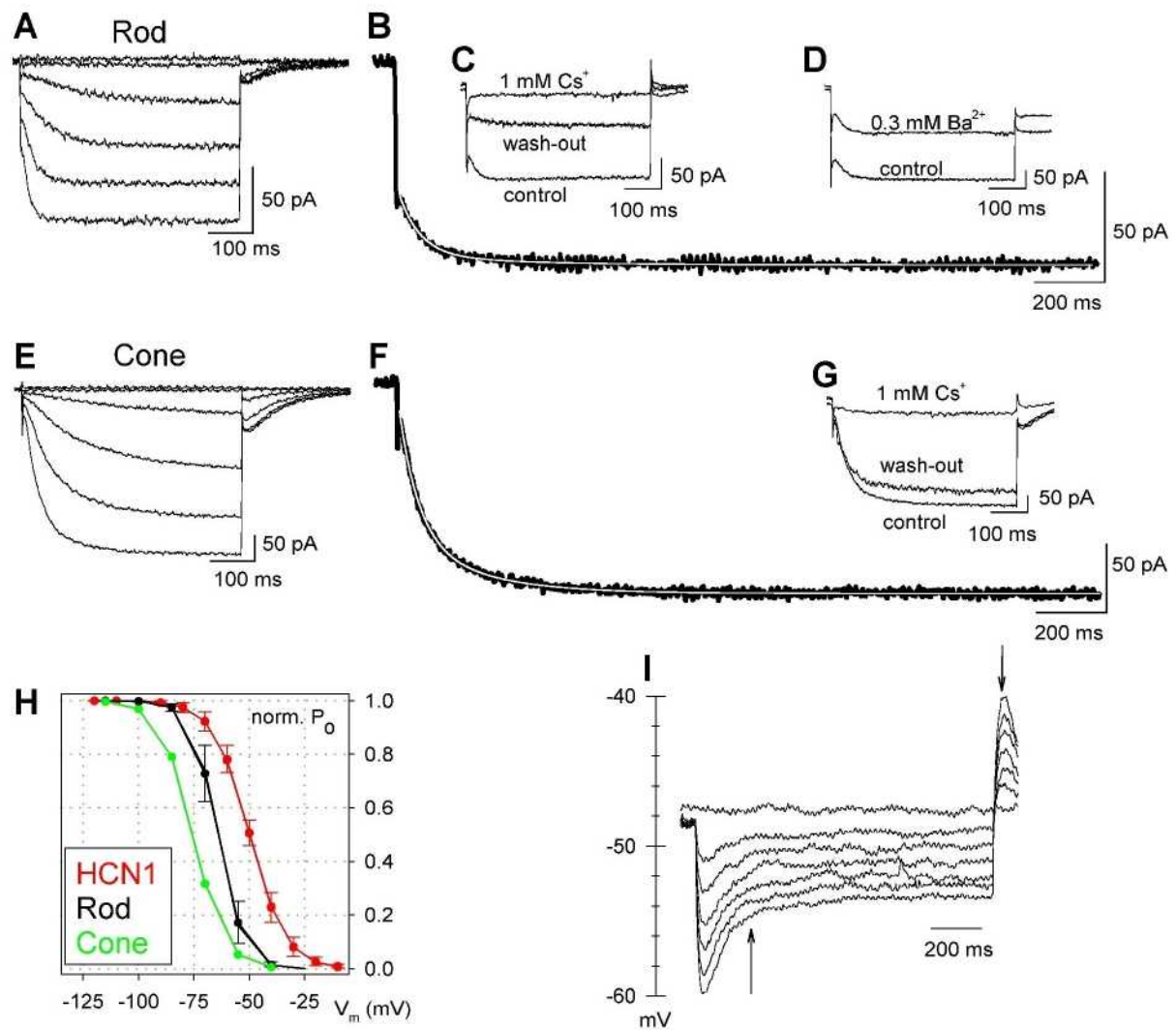


Fig. 3.24: Electrophysiological recordings in rods and cones.

(A) Hyperpolarization-activated currents in a rod. V_m was stepped from -50 mV to hyperpolarizing levels (-40 to -115 mV, 15 mV increments) for 500 ms. At the end of each hyperpolarization step, the membrane was clamped to $V_m = -50$ mV for 250 ms, and then for 150 ms to $V_m = +10$ mV (not shown).

(B) V_m was stepped from -50 mV to -100 mV for 14 s. The current was fitted by a double exponential function: $\tau_1 = 60$ ms (84 %), $\tau_2 = 283$ ms (16 %). The fit is shown on top of the current trace.

(C) Hyperpolarization-activated currents ($V_m = -115$ mV) in rods were reversibly blocked by 1 mM extracellular cesium.

(D) Instantaneous component of hyperpolarization-activated current ($V_m = -115$ mV) in rods were partially blocked by 0.3 mM barium. For a reason unrelated to the barium experiment, after the hyperpolarizing voltage step the membrane voltage was clamped to $V_m = -80$ mV.

(E) HCN currents recorded in a cone. Currents were remarkably similar to those of HCN1 homomeric channels. Conditions as in A.

(F) The current was fitted by a double exponential function: $\tau_1 = 55$ ms (67 %), $\tau_2 = 205$ ms (33 %). The fit is shown on top of the current trace. Conditions as in B.

(G) Hyperpolarization-activated current ($V_m = -115$ mV) in a cone was reversibly blocked by 1 mM extracellular cesium.

(H) Activation curves obtained from HCN1 currents in HEK293 cells (red) and HCN currents in rods (black) and cones (green). In rods and cones, the $V_{1/2}$ was measured from the tail current amplitude at $V_m = -50$ mV after 500 ms hyperpolarizing voltage prepulse. $V_{1/2}$ was: -50.0 ± 1.4 mV for HCN1 in HEK293 cells; -64.2 ± 3.1 mV in rods and -75.5 ± 2.1 mV in cones.

(I) Current-clamp experiment in a rod. The current of the cell was clamped to $I_m = 0$, the measured membrane voltage corresponded to the resting potential of the cell (-47 mV). Currents ranging in amplitude from 0 to -15 pA in -2.5 pA increments were injected for 1.2 s. After induced hyperpolarization, the membrane potential displayed a characteristic sag (long arrow) due to HCN channel activation. After current offset, an overshoot in membrane voltage was observed (short arrow).

as the proportions of the two current components were very similar to those of heterologously expressed HCN1 channels.

In contrast to rods, the instantaneous current component was very small. In one cone tested, I_h current was completely and reversibly blocked by cesium (Fig. 3.24G).

The mid-point potential in cones was measured with the same voltage protocol that I used for rods. In contrast to HCN currents in rods, in cones the currents reached steady state plateau only in the last voltage steps. Therefore, the mid-point potential, $V_{1/2} = -75.5 \pm 2.1$ mV ($n = 2$), estimated under these conditions might be more negative than if measured with longer hyperpolarizing steps. The activation curves of HCN channels in rods (black), in cones (green) and HCN1 channels expressed in HEK293 cells (red) are plotted in Fig. 3.24H.

The function of I_h in photoreceptors is to oppose strong hyperpolarization of the membrane induced by bright light. I mimicked this situation in a current-clamp experiment (Fig. 3.24I). The current of the rod was clamped to $I_m = 0$, the measured membrane voltage of -47 mV corresponded to the resting potential of the cell. A series of hyperpolarizing currents was injected into the cell, simulating the light-induced hyperpolarization of the membrane, and changes in the membrane voltage were recorded. Immediately after the onset of current injection, the membrane hyperpolarized, but then depolarized again due to the activation of HCN channels. Note the characteristic sag (long arrow). After the offset of current injection, an overshoot in membrane voltage was observed, because HCN channels did not close immediately, but still conducted a depolarizing current (short arrow).

3.5 Functional properties of HCN channels expressed in photoreceptors and bipolar cells of the rat retina

Tab. 3.3 summarizes the functional properties of HCN currents recorded in rods, cones, and in twelve types of bipolar cells of the rat retina. Moreover, it compares the identification of HCN channel isoforms by electrophysiological and immunohistochemical analysis. In general, electrophysiological and immunohistochemical data match very well. "?" indicates that the isoform could not be unequivocally identified, e.g. because no specific antibody marker was available for double labeling experiments in a particular cell type. "(HCN2)" indicates that only weak or punctate immunofluorescence was observed, while another isoform dominated in the staining.

Tab. 3.3: Functional properties of HCN channels in photoreceptors and bipolar cells of the rat retina.

Type of cell	V_{rest} [mV]	Number of experiments with Cs^+	Steady state HCN current at -100 mV [pA]	Activation constants measured at -100 mV [ms]	Fraction of total HCN current [%]	S and $V_{1/2}$ [mV]	Isoforms of HCN channels found by	
							patch-clamp	immuno-histochemistry
1 (5)	-30 (1)	-	-	n. a.		n. a.	none	none
2 (5)	-30±0 (2)	-	-32±3 (2)	$\tau_1 = 389$ $\tau_2 = 1940$ (1)	67 33	n. d.	HCN2? HCN4?	none
3 (18)	-35 (1)	3	-100±20 (8)	$\tau_1 = 430±30$ $\tau_2 = 2654±400$ (8)	38±13 62±13	n. d.	HCN4	HCN4 (HCN2)
4 (5)	-38±8 (2)	-	-	n. a.		n. a.	none	none
5a, 5b (59)	-46±4 (11)	18	-122±27 (27)	$\tau_1 = 62±5$ $\tau_2 = 379±32$ $\tau_3 = 2945±590$ (27)	52±11 30±11 18±7	$S = 8.5±0.5$ $V_{1/2} = -68.0±4.0$ (3)	HCN1 HCN4	HCN1 HCN4 (HCN2)
6a (16)	n. d.	3	-63±4 (3)	$\tau_1 = 74±4$ $\tau_2 = 524±55$ $\tau_3 = 3360)400$ (3)	37±10 34±2 29±7	n. d.	HCN1 HCN4	HCN1
6b (18)	-50±3 (3)	2	-28±7 (6)	$\tau_1 = 212±60$ $\tau_2 = 1400±500$ (6)	56±12 44±12	n. d.	HCN2	HCN2?
7 (18)	-51±5 (3)	3	-33±10 (4)	$\tau_1 = 165±22$ $\tau_2 = 1040±280$ (4)	51±17 49±17	$S = 7.8$ $V_{1/2} = -76.0$ (1)	HCN2	HCN2?
8 (23)	-53±4 (3)	4	-41±15 (9)	$\tau_1 = 147±18$ $\tau_2 = 692±140$ (9)	58±19 42±19	n. d.	HCN2	HCN2
9 (8)	-52 (1)	3	-29±5 (6)	$\tau_1 = 200±30$ $\tau_2 = 900±200$ (6)	50±14 50±14	n. d.	HCN2	HCN2?
RB (65)	-49±5 (14)	16	-44±10 (28)	$\tau_1 = 137±13$ $\tau_2 = 695±118$ (28)	67±12 32±12	$S = 7.4±0.5$ $V_{1/2} = -76.0±2.0$ (4)	HCN2	HCN2
Rod (18)	-47 (1)	3	-25±8 (2)	$\tau_1 = 57±3$ $\tau_2 = 271±16$ (2)	89±6 11±6	$S = 5.6±0.3$ $V_{1/2} = -64.2±3.1$ (6)	HCN1	HCN1
Cone (2)	n. d.	1	-135 (1)	$\tau_1 = 55$ $\tau_2 = 205$ (1)	67 33	$S = 7.1±0.2$ $V_{1/2} = -75.5±2.1$ (2)	HCN1	HCN1 HCN3 (HCN2)

Data are reported as mean ±SD, the number of cells measured is shown in parenthesis.

n. d. – not determined; n. a. – not applicable.

The amplitude of the steady state HCN current was calculated by subtracting the amplitude of the instantaneous current from the amplitude of the steady state current measured at the end of a 14 s hyperpolarization step to $V_m = -100$ mV. For further details see text.

3.6 Cobalt changes the activation kinetics and $V_{1/2}$ of HCN channels in rod bipolar cells

The most striking feature of retinal HCN2 and HCN3 channels is their high density on synaptic terminals. What might be the functional significance of this channel distribution? HCN channels were suggested to modulate synaptic transmission (Beaumont and Zucker, 2000; Mellor et al., 2002). Alternatively, HCN channels might be modulated by events occurring at the synapses, such as Ca^{2+} influx.

Both L-type and T-type of calcium channels are found at the axon terminal systems of bipolar cell (Protti and Llano, 1998; Pan, 2000). Hence, Ca^{2+} entry could occur in close proximity to HCN channels and might trigger modulation of HCN channels, e.g. by calcium-activated kinases or phosphatases. Moreover, the voltage protocol that I used to measure HCN channels favours calcium influx through T-type calcium channels (in the tail current) and through L-type calcium channels during the depolarizing voltage pulses to $V_m = +10$ mV, which were applied to close HCN channels between the hyperpolarizing steps. The pipette solution contained EGTA to buffer intracellular calcium. However, when bipolar cells are patched on the soma, dialysis of the axon terminal is limited due to the long axon. Moreover, EGTA is a relatively slow buffer. Thus, the intracellular calcium concentration at HCN channels in close proximity to calcium channels in the axon terminals might be elevated during the measurements (see also 3.2.2.2.1).

To investigate, whether intracellular calcium influences the properties of HCN channels, I recorded HCN channels in a rod bipolar cell under control conditions and in the presence of 4 mM Co^{2+} . This concentration of cobalt blocks L-type and T-type Ca^{2+} channels bipolar cells (Pan, 2000), but was reported not to affect the properties of HCN currents (Galligan et al., 1990).

I used the standard voltage protocol (see 2.2.2.5, protocol for $V_{1/2}$ measurement; Fig. 3.25A). After this control measurement, 4 mM Co^{2+} in an otherwise identical extracellular solution was applied. After 4-5 min of cobalt application, the mid-point potential was measured again (Fig. 3.25B). It shifted from $V_{1/2} = -76.0 \pm 2.0$ mV ($n = 4$) under control conditions (black) by ~ 12 mV to $V_{1/2} = -64.1 \pm 3.3$ mV ($n = 3$) in the presence of 4 mM cobalt (Fig. 3.25C, red). Such shifts were never observed in control experiments without cobalt. Moreover, HCN channels activated faster in the presence of cobalt than under control conditions (not analyzed in detail).

According to the basic idea of the experiment, Co^{2+} blocked Ca^{2+} influx into the rod bipolar cell. Hence, low intracellular Ca^{2+} concentration upon Co^{2+} application would lead to fast

HCN channels kinetics and relatively positive $V_{1/2}$. High intracellular Ca^{2+} concentration under control conditions would slow down channel kinetics and shift $V_{1/2}$ towards more negative membrane voltage. On the other hand, Co^{2+} as a divalent cation might have "unspecific" effects on ion channel properties due to the screening of membrane surface charges (for review see Hille, 1992).

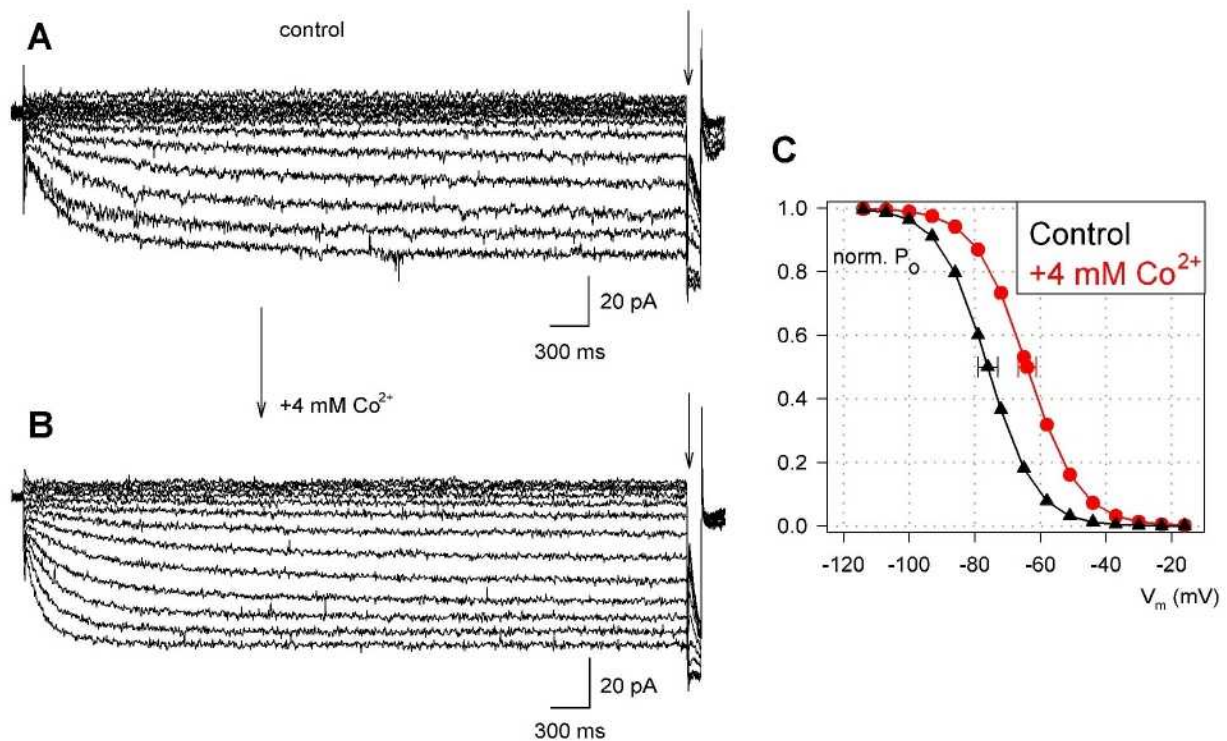


Fig. 3.25: Functional properties of HCN2 channels in rod bipolar cells are altered by cobalt.

(A) The mid-point potential of HCN channels was measured under control conditions. Currents were activated by 5 s hyperpolarizing voltage steps ranged from -16 mV to -114 mV in the -7 mV increments. Only voltage steps from -16 mV to -100 mV are shown. The amplitudes of tail currents were taken at $V_m = -114$ mV (arrow).

(B) The same cell as in A, in the presence of 4 mM Co^{2+} for 5 min (conditions as in A). The activation kinetics was faster than in the control experiments.

(C) Activation curves obtained under control conditions (black) and at the presence of cobalt (red). $V_{1/2} = -76.0 \pm 2.0$ mV and $S = 7.4$ 0.5 mV under control conditions ($n = 4$); $V_{1/2} = -64.1 \pm 3.3$ mV and $S = 7.9 \pm 0.4$ mV at the presence of 4 mM cobalt ($n = 3$).

The effect of cobalt on I_h is discussed controversially in the literature. Galligan and coworkers (1990) found no influence of millimolar concentrations of extracellular cobalt on I_h in guinea pig enteric neurons. In lobster stretch receptor neurons, addition of cobalt into the extracellular solution or increase of the extracellular calcium concentration shifted the activation curve of I_h (Edman and Grampp, 1991). Similar effects were observed by increased calcium concentrations on I_h in cultured pacemaker cells from the rabbit sinoatrial node (Fermini and Nathan, 1990).

A detailed analysis of the action of divalent cations on HCN channel properties was beyond the scope of the present study. However, a set of preliminary experiments suggests that external cobalt effects can account only partially for the shift of 12 mV in $V_{1/2}$ observed in rod bipolar cells. Experiments were performed on outside-out patches of HEK293 cells expressing HCN2. Outside-out patches allow the strict control of both external and internal solution and rule out possible modulation of channels by intracellular factors. Internal and external solutions were comparable to the experiments on the rod bipolar cell, except that the potassium concentration had to be raised to 30 mM, to increase the current amplitude in the patches. One solution contained the divalent cation concentration used in whole-cell experiments (0.81 mM magnesium and 1.25 mM calcium), in the second solution 4 mM cobalt had been added. Outside-out patches ($n = 5$) were exposed to both external solutions and activation curves were measured under identical conditions. In the presence of cobalt, $V_{1/2}$ was shifted by 7 mV towards more positive voltages (data not shown). While for a detailed analysis more experiments are needed, obviously, this shift is smaller than the 12 mV shift observed in rod bipolar cells. Probably, in the rod bipolar experiment, external cobalt effects and internal effects, due to blockage of calcium influx and, therefore, reduced internal calcium concentration, worked synergistically. The following chapter describes experiments performed to analyze the effect of internal calcium concentration on functional properties of HCN2 channels expressed in HEK293 cells.

3.7 Intracellular calcium modulates functional properties of HCN2 channels heterologously expressed in HEK293 cells

To demonstrate the influence of intracellular calcium on HCN channel properties, I recorded two sets of HEK293 cells expressing HCN2 channels with either 0 μ M or 10 μ M free calcium concentration in the pipette solution. The calcium concentration had to be adjusted with appropriate calcium buffers (see 2.2.3.2 for detailed discussion of the experimental conditions).

Fig. 3.26 shows experiments performed on two HEK293 cells expressing HCN2 channels. The extracellular calcium concentration was adjusted with EGTA to 0 μ M to avoid any putative influx of calcium into the cells. In A and B, the pipette solution contained 0 μ M calcium. In C and D, the pipette solution contained 10 μ M free calcium. Exactly one minute after rupturing the membrane, currents were recorded in both cells (one minute was chosen,

because the proper adjustment of series resistance and membrane capacitance took 30 – 40 seconds). Activation kinetics and $V_{1/2}$ measured in both cells were very similar (compare A ($V_{1/2} = -72.0$, $S = 6.6$ mV) and C ($V_{1/2} = -74.0$ mV, $S = 5.6$ mV)). The mid-point potentials averaged from many experiments were: $V_{1/2} = -74.0 \pm 3.6$ mV, $S = 6.4 \pm 1.0$ mV, with 0 μ M calcium in the pipette ($n = 9$) and $V_{1/2} = -74.3 \pm 3.3$ mV, $S = 6.8 \pm 1.4$ mV, with 10 μ M free calcium in the pipette ($n = 14$, Fig. 3.26E).

The time needed to achieve complete equilibration of the cells interior with the pipette solution depends on the composition of the solution, the cell size and the pipette resistance. In experiments comparable to the one described here, equilibration took about 8 min (measured with fluorescent dyes; S. Frings, IBI-1, Forschungszentrum Jülich, personal communication). Exactly 10 min after establishing the whole-cell mode, the measurements were repeated in both cells. HCN currents remained largely unchanged in the cell dialyzed with 0 calcium (compare A and B), except that the instantaneous current was slightly smaller in all cells ($n = 9$). In contrast, in the cell dialyzed with 10 μ M calcium, HCN channel activation was slowed down significantly. Moreover, while at the beginning, HCN currents had been observed in the last four voltage steps (C), at the end of the experiment, currents were recorded only in the last three voltage steps. If an additional voltage step was applied (increment -15 mV, dotted current trace in D), currents were similar to the last step in C, indicating a shift in $V_{1/2}$ of about -15 mV in this cell.

With 10 μ M intracellular calcium, the activation kinetics was altered in a complex way by changing time constants, as well as the proportion of the two current components. The data set is too small, to analyze these changes in detail. To roughly evaluate the changes in the activation kinetics of the channels, the currents were fitted by a single exponential function (data not shown). During the experiment with 0 μ M intracellular calcium, the time constant increased from 185 ± 29 ms to 192 ± 23 ms ($V_m = -115$ mV), while the amplitude of the HCN current (measured by subtracting the instantaneous current component from the steady state current) increased by ~ 14 %. In contrast, during the experiment with 10 μ M intracellular calcium, the time constant increased from 170 ± 20 ms to 282 ± 99 ms ($V_m = -115$ mV), while the amplitude of the HCN current decreased by ~ 11 %.

The averaged activation curves from the experiments with 0 μ M calcium (B) and the experiments with 10 μ M calcium (D) are shown in Fig. 3.26F. The following parameters were estimated: $V_{1/2} = -77.6 \pm 2.1$ mV with $S = 5.9 \pm 0.5$ mV ($n = 9$) for the experiments with 0 μ M calcium; $V_{1/2} = -84.6 \pm 5.2$ mV with $S = 6.3 \pm 1.4$ mV ($n = 14$) for the experiments with 10 μ M intracellular calcium, yielding a difference of 7 mV. This value, however, has to be

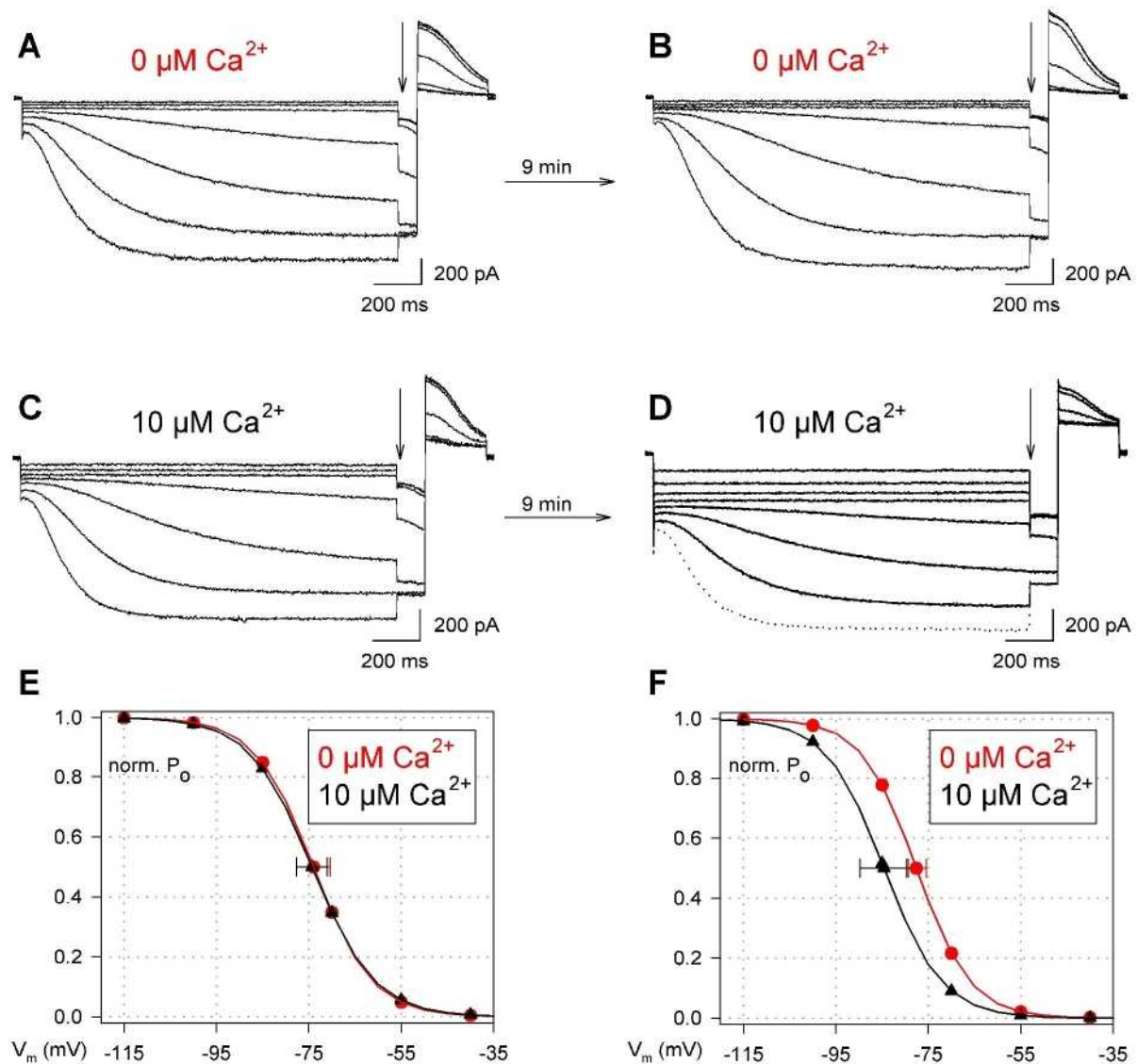


Fig. 3.26: Effect of intracellular calcium on functional properties of HCN2 channels.

(A) The cell was clamped to $V_m = -10$ mV. 1 min after establishing the whole-cell configuration a family of hyperpolarizing steps ranging from -25 to -115 mV in -15 mV increments for 1.6 s was applied. Activation curves were obtained from tail current amplitudes measured at $V_m = -100$ mV (arrow). The intracellular solution contained 0 μM free calcium (Intr. sol. Nr. 2), extracellular solution was Extr. sol. Nr. 3. Calculated mid-point potential and slope factor were $V_{1/2} = -72.0$ mV and $S = 6.6$ mV.

(B) Currents were measured in the same cell as in A, 10 min after establishing the whole-cell configuration. Conditions as in A. Calculated mid-point potential and slope factor were $V_{1/2} = -76.0$ mV and $S = 5.3$ mV.

(C) HCN currents were measured as in A. The intracellular solution contained 10 μM free calcium (Intr. sol. Nr. 3). Calculated mid-point potential and slope factor were $V_{1/2} = -74.0$ mV, $S = 5.6$ mV.

(D) Currents were measured in the same cell as in C, 10 min after establishing the whole-cell configuration. Conditions as in C. An additional voltage step to $V_m = -130$ mV was applied to completely activate the channels (dotted trace). Calculated mid-point potential and slope factor were $V_{1/2} = -90.0$ mV and $S = 5.2$ mV.

(E) Averaged activation curves obtained from 9 cells tested as in A (red) and from 14 cells as in C (black). Dots on the curves represent the measuring points, mid-point potentials are shown by dots together with the standard deviations. Calculated mid-point potential and slope factor were $V_{1/2} = -74.0 \pm 3.6$ mV and $S = 6.4 \pm 1.0$ mV (0 μM free calcium); $V_{1/2} = -74.3 \pm 3.3$ mV and $S = 6.8 \pm 1.4$ mV (10 μM free calcium).

(F) Averaged activation curves obtained from the same cells as in E, 10 min after establishing the whole-cell configuration. Calculated mid-point potential and slope factor were $V_{1/2} = -77.6 \pm 2.1$ mV and $S = 5.9 \pm 0.5$ mV (0 μM free calcium, red); $V_{1/2} = -84.6 \pm 5.2$ mV and $S = 6.3 \pm 1.4$ mV.

corrected for a technical reason. $V_{1/2}$ is only measured correctly, if channels reach steady state conditions, i.e. during long hyperpolarizing steps. Unfortunately, cells loaded with 10 μM calcium were very sensitive and only tolerated short steps. Thus, $V_{1/2}$ will be underestimated. At the beginning of the experiment, channel kinetics and, therefore, the error in $V_{1/2}$ were rather similar for both calcium concentrations. At the end of the experiment, channel kinetics in cells loaded with 10 μM calcium was significantly slower, hence, the error in $V_{1/2}$ increased. Is the observed difference of 7 mV solely due to this error? Currents were measured at the end of 1600 ms steps. For cells loaded with 10 μM calcium, this is 5.67 times longer than τ (282 ms at -115 mV). In cells with 0 μM intracellular calcium, τ was 192 ms, hence, currents measured at 1089 ms (5.67×192 ms) should yield a similar error in $V_{1/2}$. Using currents measured at 1089 ms, $V_{1/2}$ in cells with 0 μM calcium was calculated as -80.6 mV, i.e. 3 mV more negative than the initial value. Based on this correction, $V_{1/2}$ in cells with 10 μM calcium on average is 4 mV more negative than $V_{1/2}$ in cells with 0 μM calcium.

In summary, from the experiments on rod bipolar cells, described in chapter 3.6 it was proposed that, HCN2 channel activation was slowed down and $V_{1/2}$ was shifted to more negative membrane voltage by an increased intracellular calcium concentration. The experiments described in this chapter show the same effect in HEK293 cells. How could calcium trigger this modulation? Preliminary experiments on inside-out patches of HEK293 cells expressing HCN2 suggest, that neither calcium nor complexes of calcium/calmodulin directly modulate HCN2 channel function (data not shown). Most likely, intracellular calcium activates a cellular pathway which leads to a modification, e.g. phosphorylation, of the channel protein. Further experiments are required, to identify the biochemical pathway involved in this modulation.

4 Discussion

4.1 HCN channels are differentially expressed in photoreceptors and bipolar cells of the rat retina

All four HCN channel isoforms are expressed in the rat retina, however, the expression patterns differ between the isoforms and between different cell types (for details, see Tab. 3.3). HCN1 and HCN4 channels were usually expressed throughout the entire (or nearly the entire) cell. In both rod and cone photoreceptors, HCN1 was found from the inner segments down to the endfeet. In type 5 bipolar cells, HCN1 was detected throughout the entire cell, from the dendrites to the axon terminal. The same was true for HCN4 in type 3 and type 5 bipolar cells. Nevertheless, in these bipolar cells, HCN channel immunofluorescence was clearly stronger at the axon terminal than in the soma. Hence, these isoforms show a certain tendency for synaptic localization. HCN2 and HCN3 immunoreactivity was highly localized in synaptic regions, but was only rarely observed in somata. HCN2 was strongly concentrated at axon terminal systems of many bipolar cell types. In rod bipolar cells, HCN2 immunoreactivity was shown close to the synaptic ribbons. Even in type 3 or 5 bipolar cells, that expressed other HCN channel isoforms throughout the cell, HCN2 was found in very small spots at the axon terminals only. This strongly suggests, that the function of HCN2 channels differs from that of other HCN channel isoforms expressed in these cells. A similar conclusion can be drawn for the function of HCN3. HCN3 was mostly restricted to the pedicles of red-green cones, while HCN1 was expressed throughout the cone cell. An important aspect in future work will be the search for scaffolding proteins, that interact with HCN channels, and that are responsible for the precise channel targeting.

Some cell types seem to express only one isoform of HCN channels. This holds true for rods, that express HCN1 only, and for most ON-bipolar cells, that express HCN2 only. If a cell expresses two or three isoforms, one isoform is clearly dominant. For example, in type 3 bipolar cells, HCN4 is strongly expressed, whereas only small spots of HCN2 immunoreactivity were found on the axon terminal systems. Type 5 cells express three HCN isoforms: HCN1 is the predominant isoform (80 %), followed by HCN4 (20 %), and by traces of HCN2 at the axon terminals. The distribution of HCN channels in other parts of the CNS has not been studied in such detail, as in the retina. It will be interesting to compare the expression pattern of HCN channels in other brain regions with those described here. Of

particular interest is the question, whether HCN channels are concentrated at synaptic sites in the brain.

The approach followed in this study was simple and fruitful. The immunohistochemical detection of HCN channel isoforms in the retina predicted the properties of HCN currents for a given cell type. These predictions were based on the electrophysiological properties of the respective HCN isoforms expressed in HEK293 cells. In general, the properties of HCN currents recorded in the retinal cells matched the predictions quite well. However, in several instances, interesting differences were found. Let's consider HCN1. Channel activation was characterized by four parameters: the time constants τ_1 and τ_2 , the contribution of each kinetic current component to the total current, and $V_{1/2}$. In cones, activation of HCN1 is nearly indistinguishable from HCN1 in HEK293 cells, with one exception: $V_{1/2}$ is much more negative. In rods, two parameters differ from those found in HEK293 cells: $V_{1/2}$ is more negative, but similar to $V_{1/2}$ in cones; and the contribution of the second kinetic component is negligibly small. Hence, it seems as if HCN1 channels might exist in several variations, each of them optimized in its properties to match the physiological demands of a given cell. Such variations might be created either by inclusion of other subunits in heterooligomeric channels (see 4.2) or by modulation of HCN1 homooligomeric channels (see 4.4).

HCN currents in cones are worth discussing for another reason. Red-green cones do not only express HCN1, but also show HCN3 immunoreactivity at their pedicle. Little is known about the activation parameters of HCN3. It could not be heterologously expressed in the present study and in a previous study (R. Seifert, IBI-1, Forschungszentrum Jülich, personal communication). Moosmang and coworkers (2001) reported functional properties of HCN3 close to HCN2. HCN currents in cones were very similar to HCN1. No current component indicating HCN3 channel activity was observed. Filling of the recorded cones with Lucifer Yellow indicated that the pedicle (and therefore HCN3) had not been cut off during slicing. It is also unlikely that all recorded cones were blue-cones (which do not express HCN3), because this cone type is rather rare. Most likely, the contribution of HCN3 is too small in cones to be detected as a separate current component. Alternatively, HCN3 channels might be modulated such, that they cannot be distinguished from HCN1 in the activation parameters examined in this study.

4.2 Do HCN channels exist as homo- or heterooligomers?

Overlapping expression patterns of HCN isoforms revealed by *in situ* hybridization (Santoro et al., 1998; Ludwig et al., 1998; Moosmang et al., 1999) as well as results from single-cell PCR (Franz et al., 2000) are consistent with the hypothesis, although do not prove it, that HCN subunits form heteromultimers. Moreover, in heterologous expression systems coexpression of HCN1 and HCN2 produced channels with kinetic properties that cannot be reproduced by the algebraic sum of the kinetics of HCN1 and HCN2 homomeric channels (Ulens and Tytgat, 2001; Chen et al., 2001). In the following, I argue that, in bipolar cells of the mammalian retina, HCN isoforms for the most part do not co-assemble. Type 3 bipolar cells strongly express HCN4, and HCN currents in these cells are very similar to those of HCN4 in a cell line. HCN currents in type 5 as well as in type 6a cells can be reproduced by the algebraic sum of two independent populations of homomeric HCN1 and HCN4 channels. Thus, there is no evidence for the formation of heteromeric channels in these cell types.

In type 3 and type 5 cells, HCN2 immunoreactivity is observed in very few and small spots at the axon terminals, suggesting that the contribution of HCN2 to the total currents is too small to be identified in the recordings. Although the existence of heterooligomeric channels cannot be ruled out at these few sites, they certainly would account for only a minute fraction of HCN channels.

Type 6b, 7, 8, and 9 cone bipolar cells and RB cells express HCN2 channels whose activation kinetics differ among these cell types. Immunocytochemical data suggest that HCN2 is the only HCN isoform expressed in these cells (see 3.2.3). The channels might be modulated by unknown intracellular factors (see 4.4). Alternatively, HCN channels in some of these cell types might include yet unknown subunits which could modulate the properties of the channels. In fact, co-expression of MIRP1 (MinK-Related Peptide 1) with HCN1 and HCN2 in *Xenopus* oocytes was reported to accelerate the kinetics of current activation (Yu et al., 2001). In RB cells, HCN2 channels have the fastest activation kinetics. Whether they express MIRP subunits needs to be investigated in the future.

Rods seem to express HCN1 only. Cones express HCN1, HCN2, and HCN3. The immunoreactivity for HCN1 is strong in the inner segment, the soma and axon of cones, but much weaker in the pedicle. The distribution of HCN2 is similar, although HCN2 immunoreactivity was very weak and barely above background. HCN3 is restricted to cone pedicles. In most cases, HCN3 and HCN1 staining showed little overlap (data not shown). In conclusion, my results provide no evidence for the formation of heterooligomeric HCN channels in bipolar cells and photoreceptors.

4.3 Functional roles for HCN channels in the retina

HCN channels shape the light responses in hyperpolarizing cells

HCN currents were first detected in rod photoreceptors of the retina (Fain et al., 1978), where they counteract the hyperpolarizing response at high light intensities by allowing an inward current that depolarizes the cell. In order to quickly resist the hyperpolarization, rapidly activating HCN isoforms might be most suited. In both rods and cones HCN1, the isoform with the fastest activation kinetics was found. This finding agrees with results obtained in other species (in mouse, Moosmang et al., 2001; in guinea-pig, Demontis et al., 1999; in rabbit, Demontis et al., 2002). The mid-point potentials of HCN1 in rat rods ($V_{1/2} = -64.2 \pm 3.1$ mV) and cones ($V_{1/2} = -75.5 \pm 2.1$ mV), as well as in guinea-pig rods ($V_{1/2}$ ranged from -66 mV to -76 mV; Demontis et al., 1999) and in rabbit rods ($V_{1/2} = -75.0 \pm 0.3$ mV; Demontis et al., 2002) were much more negative than that of HCN1 channels in HEK293 cells ($V_{1/2} = -50.0 \pm 1.4$ mV). If the mid-point potential of HCN1 channels in photoreceptors were as positive as in HEK293 cells, then HCN channels would be open at the dark resting potential and any light-induced membrane hyperpolarization would be truncated immediately. Hence, HCN channels in photoreceptors seem to be modulated such that their properties exactly match their function in these cells.

HCN channels might have a similar function in OFF-bipolar cells and shape the light response. Of the four OFF-bipolar cells (type 1-4) (Euler et al., 1996; Hartveit, 1997), HCN currents were recorded in type 3 cells. In type 2 cells, a hyperpolarization-activated current was observed, that yet needs to be identified as HCN current. A transient OFF-bipolar cell, classified as either type 2 or type 3, was recently described (Euler and Masland, 2000). However, it is worth debating, whether a channel activating as slow as HCN4 (in type 3 cells) can substantially truncate the light responses of bipolar cells (at body temperature and in the physiological voltage range, τ is a few hundred milliseconds (Ishii et al., 2001). A recent study suggested that sustained and transient OFF responses are generated by different types of glutamate receptors at the OFF-bipolar dendrites (DeVries, 2000).

What is the function of HCN channels in cells with depolarizing light responses?

All ON-bipolar cells express HCN channels. ON-bipolar cells depolarize upon illumination, hence, one might argue that HCN channels cannot shape their light responses. What is the function of HCN channels in these cells? First, in most ON-bipolar cells, HCN channels are localized at the synaptic terminal and may serve a specialized function. This aspect will be

discussed later. Second, ON-bipolar cells hyperpolarize in darkness, when the photoreceptors release glutamate. During prolonged darkness, activation of HCN channels might help to set a suitable range of membrane voltages in ON-bipolar cells. Moreover, when this hyperpolarization is terminated by a depolarizing input, HCN channels will still be open and may contribute to the depolarizing current. This could lead to the augmentation of the depolarizing stimulus and to a shortening of the response latency. The light response would speed up and would become more transient. In the rat retina, Euler and Masland (2000) showed that axotomized rod bipolar cells had longer light response latencies than intact cells. This may reflect the loss of HCN channels, which are predominantly expressed at the axon terminal systems of rod bipolar cells. Similarly, I_h was shown to transiently augment retinal ganglion cell excitability after termination of the membrane hyperpolarization (Tabata and Ishida, 1996).

Under physiological conditions, photoreceptors and bipolar cells respond to light stimuli that change in a complex manner. Demontis and coworkers (1999) studied changes in the membrane voltage of photoreceptors during injection of sinusoidal currents. The membrane voltage of the photoreceptors followed the current injection in a complex way. Depending on the initial membrane voltage and on the frequency of the sinus function, the signal was amplified or reduced. Moreover, the voltage peak could precede the current peak, or it could lag behind. The authors attributed this effect to the combination of hyperpolarization-activated depolarizing currents (I_h) and depolarization-activated hyperpolarizing currents (I_{kx}) found in photoreceptors. The effect will depend on the overlap in the activation range of both currents and on the proportions of the current amplitudes. My results suggest, that different bipolar cell types differ in their combination of HCN currents and other voltage-gated currents (see e.g. 3.3). It is tempting to speculate that different bipolar cell types represent different "channels" to transmit signals within a certain temporal frequency domain. Each of these channels might be optimized in its inventory of ion channels to subserve this function.

Do bipolar cells spike?

In many neuronal cell types, HCN channels are involved in the generation of rhythmic spiking activity. At negative membrane voltages, HCN channels become activated and conduct an inward current that depolarizes the cell towards the threshold of T-type calcium channels. T-type channels open and depolarize the cell further until L-type channels are activated. Activation of calcium channels can lead to calcium spikes or a burst of Na^+/K^+ action potentials (for review see Kaupp and Seifert, 2001).

Bipolar cells were generally believed to be non-spiking neurons. Recent evidence, however, suggests that at least some bipolar cells can generate spikes. In unidentified cone bipolar cells of the rat, sodium spikes were observed (Pan and Hu, 2000). Protti and coworkers (2000) reported that calcium spikes were generated at the axon terminal of a certain type of goldfish bipolar cell in dark-adapted, but not in light-adapted retina. Each spike started with an initial slow depolarization, that was suggested by the authors to originate from HCN currents. HCN currents were described in goldfish bipolar cells (Kaneko and Tachibana, 1985). My study, as well as others, show that HCN channels, T-type and L-type calcium channels, and potassium channels are present in bipolar cells of the rat. This set of ion channels might form the basis of spike generation. The number of current clamp experiments in my study was small, nevertheless, occasionally I could observe spike-like events in bipolar cells. Further experiments are needed to reveal, whether some bipolar cell types in the rat retina use spikes for information coding.

Why are HCN channels concentrated at synaptic sites?

The most striking feature of retinal HCN channels is their high density on synaptic terminals. HCN2 is highly clustered on bipolar cell axon terminals. HCN3 is restricted to the base of the cone pedicle. What might be the functional significance of this channel distribution? Recent studies show that HCN channels might directly modulate synaptic transmission (Beaumont and Zucker, 2000; Mellor et al., 2002; but see also Chevaleyre and Castillo, 2002). Beaumont and Zucker (in crayfish, 2000) suggested that, by a yet unknown mechanism, activation of HCN channels enhances mobilization of synaptic vesicles to a readily releasable pool. Mellor et al. (in hippocampal neurons, 2002) proposed that HCN channels can depolarize the membrane far enough to evoke calcium influx into the cells and hence, transmitter release. In bipolar cells, HCN channels might depolarize the membrane until low-threshold T-type calcium channels are opened. T-type calcium channels can directly mediate neurotransmitter release in rat retinal bipolar cells (Pan et al., 2001). A second reason, why HCN channels might be localized to synaptic sites, is discussed in the following chapter.

4.4 Are HCN channels modulated in the retina?

The impact of HCN channels on the electrical properties of bipolar cells depends on how far the voltage range of HCN channel activation overlaps with the physiological voltage range of bipolar cells. Under my experimental conditions, HCN channels started to activate at about

–50 to –60 mV. ON-bipolar cell resting potentials in my slice preparation ranged from –42 to –60 mV (see Tab. 3.3), therefore I found overlap of the two voltage ranges in some, but not in all bipolar cells. In dark-adapted rat retinal slices, Euler and Masland (2000) described average resting potentials of –45 mV for rod bipolar cells and –36 to –39 mV for cone ON- and OFF-bipolar cells. Therefore, it seems as if the average membrane voltage of bipolar cells is too depolarized to allow significant HCN channel activation. Why should bipolar cells express channels, that have no function? There are two solutions for this conundrum. The first one may reflect a technical problem. The membrane voltage of bipolar cells in the intact retina might be more negative than the average values reported from slice recordings. In fact, in my preparations, resting potentials were on average more negative than those described by Euler and Masland. Moreover, Euler and Masland found individual bipolar cells with resting potentials as negative as –61 mV. Finally, the standard deviations given for their average values of –39 and –36 mV are large (10–14 mV). This may reflect different strength of hyperpolarizing inputs to different bipolar cells in the slice. Probably, bipolar cells tend to be more depolarized in slices than in intact retinæ, because long range inhibitory input coming from the wide-field GABAergic amacrine cells may be reduced during the slicing procedure. The second solution to this conundrum is of great physiological interest. Binding of cAMP and cGMP to HCN channels could shift the threshold of activation towards the physiological voltage range of bipolar cells. Indeed, in preliminary experiments I found strong shifts of HCN2 channel activation after internal dialysis of rod bipolar cells with cAMP (data not shown).

A strong increase in cGMP production in presumably type 5, 6, and 7 bipolar cells of the rat retina was observed upon stimulation of soluble guanylate cyclases with nitric oxide donors (Gotzes et al., 1998). At the axon terminal systems, bipolar cells receive strong input from amacrine cells. A variety of neurotransmitters and neuropeptides that control cAMP levels, such as dopamine, somatostatin, vasoactive intestinal polypeptide (VIP), and enkephalin are present in amacrine cells and were shown to modulate ion channels in photoreceptors, bipolar cells, and amacrine cells (Akopian and Witkovsky, 1996; Yazulla et al., 2001; Feigenspan and Bormann, 1994; Veruki and Yeh, 1994; Petrucci et al., 2001). The effective coupling between cAMP synthesis and HCN channel modulation may require that receptors, adenylyl cyclase, and HCN channels are in close proximity at the synaptic terminal. In fact, dopamine D1 receptors were colocalized with HCN4 channels in type 5 cells (F. Müller, personal communication). Thus, stimulation of D1 receptors would activate adenylyl cyclase and would trigger modulation of HCN channels in this cell type.

Preliminary experiments suggest that high intracellular calcium concentration slowed down HCN2 channel activation and shifted $V_{1/2}$ to more negative values in HEK293 cells and rod bipolar cells. In principle, the modulation might be mediated by three different mechanisms: 1. by direct binding of calcium or calcium/calmodulin to the channel. Preliminary experiments suggest that this is not the case; calcium and calcium/calmodulin had no direct effect on HCN2 channels in inside-out patches of HEK293 cells (data not shown). 2. by a calcium-mediated decrease in the intracellular cyclic nucleotide concentration. At present, it is unknown, whether calcium/calmodulin-inhibited adenylyl cyclases or calcium/calmodulin-activated phosphodiesterases are present in HEK293 cells and rod bipolar cells, that could account for the observed effects. 3. by a calcium-activated phosphorylation or dephosphorylation of the channel protein. Modulation of HCN channels by phosphorylation has been studied in a variety of cells. Unfortunately, the HCN channel isoforms that were expressed in these cells, are not known. Phosphorylation of HCN channels by the calcium-dependent protein kinase C (PKC) lead to reduction of the maximal conductance, while $V_{1/2}$ was little affected (Cathala and Paupardin-Tritsch, 1997). In Purkinje fibres and isolated ventricular myocytes, treatment with phosphatase inhibitors resulted in a positive shift of $V_{1/2}$ of HCN channels (Yu et al., 1993, 1995). HCN channel activity was up-regulated by cAMP-dependent protein kinase A (PKA) in rat olfactory neurons by shifting $V_{1/2}$ and increasing the maximal conductance of HCN channels (Vargas and Lucero, 2002).

Interestingly, HCN channels in photoreceptors of the *Xenopus* retina were down-regulated upon stimulation of dopamine D2 receptors (Akopian and Witkovsky, 1996). While D2 receptors are usually negatively coupled to adenylyl cyclase, the authors claim that the effects were cAMP-independent, but depended on calcium. If the intracellular calcium concentration was buffered with BAPTA to 0 μ M, the inhibitory effect was abolished.

Hence, there are several ways to modulate HCN channels through intracellular signaling cascades, and calcium may be a key element in many of them. Whether calcium will cause up- or down-regulation of the channels may depend on the presence or absence of signaling pathways in a given cell type. Indeed, contradictory effects of calcium on the regulation of HCN channels were observed: a positive shift of HCN channel mid-point potential by calcium increase was shown by Lüthi and McCormick (1999) in thalamocortical neurons; no effect of calcium was observed in thalamic neurons (Budde et al., 1997); a negative shift in $V_{1/2}$ was reported for HCN channels in neurons of the trigeminal mesencephalic nucleus (Khakh and Henderson, 1998).

4.5 Outlook

HCN channels are differentially expressed in the retina and their widespread expression suggests, that they are involved in retinal information processing at several stages. In some cells, like in photoreceptors, the function of HCN channels seems quite well understood. In many cell types, however, we can only speculate. This study can serve as a basis to ask questions and direct future investigations on HCN channel function in the retina. How do HCN channels shape or modulate light responses in bipolar cells? Why do different bipolar cell types show different expression patterns of HCN channels? Do different bipolar cell types show different physiological responses? These questions must be addressed by recording of light responses in dark-adapted retinal slice preparations. Why are HCN channels located at synaptic sites? Do they control synaptic transmission? For this issue, paired recordings of bipolar cells and their postsynaptic targets, i.e. amacrine or ganglion cells must be combined with pharmacological blockade of HCN channels. Finally, this study provides clues for the design of HCN channel knock-outs in transgenic animals and the analysis and interpretation of the respective phenotype.

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