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Two Types of Cone Bipolar Cells Express Voltage-Gated Na⁺ Channels in the Rat Retina

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Abstract

Two groups of retinal cone bipolar cells (CBCs) in rats were found to express voltage-gated Na⁺ channels. The axon terminals of the first group stratify in sublamina 2 of the inner plexiform layer (IPL) and partially overlap with the OFF-cholinergic band. This group was identified as type 3 CBCs. The axon terminals of the second group stratify in sublamina 3 of the IPL, slightly distal to the ON-cholinergic band. Cells of this second group resemble type 5 CBCs. In addition, we observed another group of ON-type CBCs with terminal stratification similar to that of the second group. However, this latter group did not show any Na⁺ current, instead exhibiting a large hyperpolarization-activated cyclic nucleotide-gated cation current, suggesting the existence of two subclasses of physiologically distinct type 5 CBCs. Both groups of Na⁺-expressing bipolar cells were capable of generating a rapid TTX sensitive action potential as revealed by current injection. Multiple spike-like potentials were also observed in some of these cells. Results of this study provide valuable insights into the function of voltage-gated Na⁺ channels of retinal bipolar cells in retinal processing.

Keywords

Na⁺ current; bipolar cells; retinas; rats

INTRODUCTION

Voltage-gated Na⁺ channels play an essential role in the initiation and propagation of fast action potentials in CNS neurons (Yu & Catterall, 2003). Na⁺ current has also been found in retinal bipolar cells (Pan & Hu, 2000; Zenisek *et al.*, 2001), the second-order retinal neurons that are commonly believed to use slow graded potentials for signal transmission (Werblin & Dowling, 1969; Kaneko, 1970). To date, voltage-gated Na⁺ current has been reported in bipolar cells of the rat (Pan & Hu, 2000), goldfish (Zenisek *et al.*, 2001), tiger salamander (Ichinose *et al.*, 2005), and ground squirrel (Saszik & DeVries, 2005), which suggest that Na⁺ channel expression may be a general property of bipolar cells. In lower vertebrates, the Na⁺ channels of retinal bipolar cells have been reported to amplify the light response of ganglion cells (Ichinose *et al.*, 2005) and mediate light adaptation (Ichinose & Lukasiewicz, 2007). More recently, blockade of Na⁺ channels in ON cone bipolar cells has been reported to affect the ERG b-wave in the rat (Mojumder *et al.*, 2007; 2008). Thus far, however, voltage-gated Na⁺ currents have only been observed in a subclass of bipolar cells. The detailed expression pattern of Na⁺ channels in bipolar cells and their potential diverse roles in retinal processing, especially in mammalian retinas, still remain to be investigated.

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Retinal bipolar cells relay visual information from photoreceptors to retinal third-order neurons, and they comprise multiple types that are believed to be the important anatomical substrates for segregating visual information into parallel pathways (Wu et al., 2000; Wässle, 2004). Bipolar cells are classified into ON- and OFF-types based on their response polarity to light, and they are also subdivided into rod and cone bipolar cells based on their synaptic inputs. In mammals, only a single type of rod bipolar cell (Boycott & Dowling, 1969; Boycott & Kolb, 1973; Dacheux & Raviola, 1986; Greferath *et al.*, 1990) and at least nine or ten types of cone bipolar cells (CBCs) have been described, differing primarily in the terminal stratification at the inner plexiform layer (IPL; Famiglietti, 1981; Kolb *et al.*, 1981; Pourcho & Goebel, 1987; Euler & Wässle, 1995). It has been proposed that the information transmitted from bipolar cells to third order neurons is organized anatomically in the IPL according to its content (Wu *et al.*, 2000; Roska & Werblin, 2001; Pang *et al.*, 2004). Thus, the identification of the bipolar cell type(s) that express Na⁺ channels could provide valuable insights into the functional roles of Na⁺ channels of bipolar cells in retinal processing.

In this study, we performed whole-cell patch-clamp recordings and immunohistochemical staining to identify the type(s) of bipolar cells that express voltage-gated Na⁺ channels in the rat retina. Two morphologically distinct types of CBCs were identified to express voltage-gated Na⁺ channels.

Methods

Animals and retinal slice preparations

Retinal slices were prepared from Long-Evans rats ≥ 4 weeks of age by procedures described previously (Werblin, 1978; Cui et al., 2003). All animal-handling procedures were approved by the Institutional Animal Care Committee at Wayne State University and were in accord with the NIH Guide for the Care and Use of Laboratory Animals. Briefly, animals were deeply anesthetized with CO₂ and killed by decapitation. Retinas were removed and placed in Hanks' solution (in mM): NaCl, 138; Na₂HPO₄, 0.3; KCl, 5; KH₂PO₄, 0.3; CaCl₂, 2.5; MgSO₄, 0.5; MgCl₂, 0.5; HEPES-NaOH, 5; glucose, 22.2; pH7.2. Retinal slices ~ 150 μ m thick were cut and mounted in a glass-bottomed recording chamber. For light response recordings, animals were dark-adapted for more than 2 hrs before the experiment, and retinal slice preparation was performed under dim red illumination. The recording chamber was mounted on the stage of an upright microscope equipped with a 40x water immersion objective with differential interference contrast and epifluorescence optics. The recording chamber was continuously superfused with oxygenated Hanks' solution at a rate of ~ 2 ml/min, and the recordings were performed at room temperature ($\sim 22^{\circ}\text{C}$). In the recordings of light responses, the chamber was superfused with oxygenated Ames' solution (Sigma-Aldrich, St. Louis, MO, USA) heated to $30 \sim 33^{\circ}\text{C}$.

Patch-clamp recordings

Patch electrode recordings in the whole-cell configuration were made by standard procedures with an EPC-9 amplifier and PULSE software (Heka Elektronik, Germany). Electrodes were coated with SYLGARD (Dow Corning, Midland, MI, USA) and fire polished. Pipette resistance was 13–16 M Ω when filled with a K-gluconate-based electrode solution and 8–10 M Ω when filled with a Cs-acetate-based electrode solution. Whole-cell access resistance was not compensated. The resistance with the K-gluconate-based electrode solution usually ranged from 20 to 40 M Ω and cells with access resistance >40 M Ω were not included in the analyses. The mean value of the access resistance for the cells used in the analyses was 28.2 ± 8.3 M Ω (mean $\pm$ SD; $n = 141$). Cell capacitance was canceled, and the capacitance values were recorded by PULSE software. The sampling rate of the recordings was usually 2–10 kHz. The filter frequency (low-pass) was at least one-half to one-third of the sampling rate. The electrode

solution contained (mM): K-gluconate, 133; KCl, 7; MgCl₂, 4; EGTA, 0.1; HEPES, 10; Na-GTP, 0.5; and Na-ATP, 2. The pH was adjusted with KOH to 7.4. For recording currents evoked by puffing L-AP4, the electrode solution contained (mM): Cs-acetate, 130; TEA-Cl, 10; MgCl₂, 1; EGTA, 0.1; HEPES, 10; and cGMP, 1; Na-GTP, 0.5; and Na-ATP, 2. The pH was adjusted with CsOH to 7.4. Liquid junction potentials were corrected. The fluorescent dye Alexa 488 was added to the electrode solution at a concentration of 100 μ M. Fluorescence images of most recorded cells were taken with a digital camera (SPOT; Diagnostic instruments, Sterling Heights, MI, USA).

Light responses were evoked by an LED, which was mounted above the recording chamber and controlled by an analogue output of the EPC-9 amplifier.

Immunocytochemistry

After recordings, retinal slices were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) for 2 hrs. The slices were rinsed three times with 0.1 M PB, and blocked for 2 hrs in a solution containing 3% normal donkey serum and 0.5% Triton X-100 in PB (pH 7.4) at room temperature (RT). The slices were incubated in a mixture of primary antibodies including anti-Alexa 488 (rabbit) and anti-choline acetyltransferase (anti-ChAT; goat) at a dilution of 1:200 for 3–4 days at 4°C. After washing in PB, the slices were treated with a mixture of secondary antibodies (Donkey anti-rabbit Alexa 488 and Donkey-anti-goat Alexa 555) at a dilution of 1:500 for 1 hr in the dark at RT. Specimens were viewed using a Zeiss Axiophot microscope. Fluorescence images were taken with a CCD camera. The images were adjusted using Adobe Photoshop.

Chemical agent application and data analysis

When applying L-AP4, a puffer pipette (diameter 0.5–1 μ m; air pressure ~ 15 psi) was placed 5–10 μ m away from the somas of the recorded cells. Other chemical agents were applied by bath superfusion. Alexa dyes were purchased from Invitrogen (Carlsbad, CA, USA). Tetrodotoxin (TTX) was purchased from Alomone Lab (Jerusalem, Israel). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Data were analyzed off-line using PULSEFIT (Heka Elektronik, Germany) and ORIGIN (Microcal Software, Northampton, MA, USA) programs. Data are presented as mean $\pm$ SD unless otherwise stated.

RESULTS

Morphologies of two groups of Na⁺ channel-expressing cone bipolar cells

Combining whole-cell patch-clamp recordings and fluorescent dye filling, two morphologically distinct groups of CBCs were found to show voltage-gated Na⁺ current. Figure 1A shows a representative fluorescence image of CBCs from the first group ($n = 41$). The axon terminals of these CBCs stratified within sublamina 3 of the inner plexiform layer (IPL) or near the middle of the IPL as revealed by fluorescent dye Alexa 488. To determine the relationship between their terminal stratification and the processes of cholinergic starburst amacrine cells, the retinal slices that contained dye-filled CBCs were double labeled with antibodies against Alexa 488 and choline acetyltransferase (ChAT). As illustrated in Figure 1B for the same cell shown in Figure 1A, the axon terminals (in green) of these cells stratified slightly distal to the ON-cholinergic band (in red). Some of their terminal varicosities may overlap with the ON-cholinergic band. As will be described in more detail below, we observed another group of CBCs that show similar axon terminal stratification but no detectable voltage-gated Na⁺ current. For simplicity of description, we will refer to this group of Na⁺ channel-expressing CBCs as CB3a because their axon terminals are confined within sublamina 3. A similar scheme has been recently used in the classification of bipolar cells in mice (Pignatelli & Strettoi, 2004).

The second group of CBCs that were found to display Na^+ current had a different terminal stratification. Figure 1C shows a representative fluorescence image of the cells in this group ($n = 33$). The somas of these CBCs were slightly larger than those of the CB3a, while their axons were shorter. Their axon terminals had a tree-like branching pattern and stratified in sublamina 2 of the IPL. Figure 1D shows the double labeling for ChAT and Alexa 488 for the same cell shown in Figure 1C. The axon terminals of these CBCs ramified proximal to and partially overlapped with the OFF-cholinergic band. We will refer to this group of CBCs as CB2 because their axon terminals are confined within sublamina 2.

Voltage-gated Na^+ current and other voltage-gated membrane currents in CB3a

To examine voltage-gated Na^+ current, cells were held at -80 mV and depolarized by a series of test pulses ranging from -60 mV to 10 mV. A representative recording of CB3a is illustrated in Figure 2A-C ($n = 41$). A fast inward current was activated at the onset of the test pulses of > -50 mV (Fig. 2A). Application of TTX (0.5 - 1 μM) abolished the fast transient inward current (Fig. 2B) ($n = 12$). The TTX sensitive Na^+ current is shown in Figure 2C, and the I-V relationship for the current is shown in Figure 2D. The average peak Na^+ current was 131.6 ± 37.5 pA (mean $\pm$ SD; $n = 12$), and the average current density obtained by dividing the peak current by the cell membrane capacitance was 65.9 ± 19.0 pA/pF ($n = 12$).

It should be noted that the recordings of Na^+ current in this group were always shown a sign of escape from voltage control (see Figs. 2A and 2C; also see Discussion). This was not observed in the second group of Na^+ current expressing bipolar cells (see Fig. 6).

In addition to the Na^+ current, CB3a cells generally exhibited a second component of the inward current, which showed slower activation and inactivation than that of the Na^+ current. This is illustrated in Figure 2E. This latter current was found to be voltage-gated calcium current; it was not sensitive to TTX (see Figure 2B), but to Cd^{2+} or Co^{2+} (data not shown). The average peak inward calcium current measured in the presence of TTX was -42.7 ± 16.8 pA (mean $\pm$ SD; $n = 12$).

Previous studies reported a differential expression of inwardly rectifying K^+ current (I_{Kir}) and hyperpolarization-activated cyclic nucleotide-gated (HCN) cation current (I_h) in bipolar cells (Ma *et al.*, 2003; Müller *et al.*, 2003). Therefore, we also examined the hyperpolarization-activated currents in CB3a. Representative recordings are shown in Figure 3. The currents were evoked by a series of test pulses ranging from -70 to -120 mV from the holding potential of -60 mV. As shown in Figure 3A, the inward currents had two components: one that showed instantaneous or time-independent activation, with a second component exhibiting time-dependent activation. These time-independent and time-dependent current have been shown to be I_{Kir} and I_h , respectively, in the acutely dissociated cell preparation (Ma *et al.*, 2003), but the contamination of a leak current in this time-independent component could not be excluded because of a lower whole-cell seal resistance that could be achieved in the slice preparation than in the acutely dissociated cell preparation. Therefore, these two currents will be referred to as $\text{I}_{\text{t-indep}}$ and $\text{I}_{\text{t-dep}}$ in this study. Figure 3B shows the I-V plots for the time-independent current, $\text{I}_{\text{t-indep}}$, measured at the beginning of the test steps (square), and the peak current, $\text{I}_{\text{t-indep}}$ plus $\text{I}_{\text{t-dep}}$, measured at the end of the test steps (circle). The average amplitudes of $\text{I}_{\text{t-indep}}$ and $\text{I}_{\text{t-dep}}$, evoked by a 2-second hyperpolarizing step to -120 mV, were -41.5 ± 19.8 pA and -10.8 ± 4.5 pA (mean $\pm$ SD; $n = 11$), respectively (Figures 3C and D).

The presence of a second subclass of CB3 that does not express voltage-gated Na^+ channels

In addition to the cell types delineated above, we encountered another group of CBCs that had similar terminal stratification as CB3a, but exhibited no apparent voltage-gated Na^+ current. Figures 4A and B depict the representative fluorescence image and double labeling image for

ChAT and Alexa 488, respectively, for CBCs in this group. Like CB3a, the axon terminals of these CBCs also ramified within sublamina 3 of the IPL, slightly distal to the ON-cholinergic band.

However, the physiological properties of these cells are different from those of CB3a in several respects. First, as stated above, no voltage-gated Na^+ current was observed in these cells (see a representative recording in Figure 4C). The lack of voltage-gated Na^+ current was first ascertained due to the lack of an apparent fast inward current (left panel in Fig. 4C; $n = 44$). This was confirmed after the application of TTX (right panel Fig. 4C; $n = 22$) since no TTX-sensitive current was found (-0.60 ± 11.5 pA; mean $\pm$ SD; $n = 22$). It should be mentioned that we did not find any significant difference in the recording conditions between cells with and without Na^+ current. For example, the mean values of the whole-cell access resistance between CB3a (29.6 ± 8.8 ; $n = 41$) and CB3b (27.9 ± 8.7 ; $n = 44$) are not statistically different. Second, voltage-gated calcium current was only observed in a small portion of the cells in the presence of TTX (7 out of 22). The current was less prominent than that of CB3a. The average peak inward calcium current was -16.1 ± 4.8 pA (mean $\pm$ SD; $n = 7$). Furthermore, these CBCs showed much larger $I_{t\text{-dep}}$ than that of CB3a. As a result, in contrast to CB3a, which showed larger $I_{t\text{-indep}}$ than $I_{t\text{-dep}}$, these CBCs showed larger $I_{t\text{-dep}}$ than $I_{t\text{-indep}}$. A representative recording is shown in Figure 4D. The I-V plots for the time-independent current, $I_{t\text{-indep}}$, measured at the beginning of the test steps (square) and the peak current, $I_{t\text{-indep}}$ plus $I_{t\text{-dep}}$, measured at the end of the test steps (circle) are shown in Figure 4E. The average peak currents of $I_{t\text{-indep}}$ and $I_{t\text{-dep}}$ evoked by the hyperpolarizing test pulse to -120 mV were 43.3 ± 13.5 pA and 87.7 ± 25.7 pA (mean $\pm$ SD; $n = 22$), respectively (Figures 4F and G). The results indicate that there are two subclasses of physiologically distinct CBCs that have similar terminal stratification at sublamina 3. To distinguish between these two groups of CBCs, we will refer to this latter group as CB3b.

Both CB3a and CB3b resemble type 5 CBCs described in rats (see Discussion). Type 5 CBCs are known to be ON-type CBCs (Wässle, 2004), but a previous study reported OFF light responses from type 5 CBCs (Euler *et al.* 1996; but see Hartveit, 1997). Therefore, we asked whether CB3a and CB3b could represent two subtypes of CBCs with opposite light response polarity. To address this question, we first examined their responses to L-AP4 (Slaughter & Miller 1981; Nawy & Jahr, 1990). Application of L-AP4 ($50 \mu\text{M}$) was found to reduce the steady inward current in both CB3a ($n = 4$) and CB3b ($n = 7$) (Figures 5A and B), suggesting that metabotropic glutamate receptors are present in both groups of CBCs. Furthermore, both CB3a ($n = 5$) and CB3b ($n = 7$) were found to respond to light with membrane depolarization (Figures 5C and D). These results confirm that both CB3a and CB3b are ON-type CBCs.

Voltage-gated Na^+ current and other membrane conductance in CB2

Voltage-gated Na^+ current judged based on the presence of a fast inward current were observed in the majority of CB2 cells (33 out of 39). Figure 6A shows a representative recording of CB2 that exhibited voltage-gated Na^+ current. A fast inward current was observed at the onset of the test pulses of > -50 mV. The presence of Na^+ current was confirmed by application of TTX ($1 \mu\text{M}$) as shown in Figure 6B ($n = 9$). Furthermore, no prominent inward calcium current was observed in these cells in the presence of TTX. The TTX-sensitive current is shown in Figure 6C ($n = 9$), and the I-V plot is shown in Figure 6D. The average peak Na^+ current was 165.2 ± 28.6 pA (mean $\pm$ SD; $n = 9$). The Na^+ current density was 79.5 ± 36 pA/pF (mean $\pm$ SD; $n = 9$). Both the peak Na^+ current and current density are larger than those of CB3a but are not statistically significant ($p > 0.05$).

Since no apparent Na^+ current was observed in some of the encountered CB2 cells, we probed whether there were two subclasses of CB2 as well by examining hyperpolarization-activated currents. All CB2 cells showed hyperpolarization-activated $I_{t\text{-indep}}$ and $I_{t\text{-dep}}$ (see Figures 7A

and B). The properties of $I_{t-indep}$ and I_{t-dep} between cells with and without Na^+ current, however, were not found to be different. The average peak amplitudes of $I_{t-indep}$ and I_{t-dep} evoked by test pulse to -120 mV were -38.4 ± 13.5 pA and -98.6 ± 36.5 pA (mean $\pm$ SD; $n = 9$), respectively (Figures 7C and D).

Na^+ -gated action potentials or spikes

Both CB3a and Na^+ channel-expressing CB2 were found to be capable of generating a rapid Na^+ -dependent action potential. A sample recording from CB3a is shown in Figure 8A. Under voltage-clamp, the cell exhibited Na^+ current that was blocked by TTX ($1 \mu M$; see top and middle panels in Figure 8A). During current-clamp, a prominent action potential or spike was evoked at the beginning of the membrane depolarization elicited by a current pulse (black trace in the bottom panel; $n = 22$ out of 22). Application of TTX mostly eliminated the action potential (red trace; $n = 7$). Similarly, a prominent action potential or spike was also observed in the Na^+ channel-expressing CB2 as a sample recording shown in Figure 8B ($n = 12$). Again, the action potential was mostly blocked by TTX (red trace in the bottom panel; $n = 3$). It should be noted here that for both CB3a and CB2, in the presence of TTX, they still showed a sign of regenerative potential. The regenerative potential was likely due to voltage-gated calcium currents.

Interestingly, however, we observed an apparent difference between CB3a and Na^+ channel-expressing CB2. That is, current injection was able to elicit multiple spike-like potentials in most of the CB3a cells (18/22) but only occasionally in CB2 cells (1/12). Figure 9A illustrates the recordings of a CB3a cell with regenerative potentials. In this cell, the presence of Na^+ current was confirmed under voltage-clamp (top panel in Figure 9A). Under current clamp, multiple spike-like potentials were evoked by current injection (bottom panel in Figure 9A). The spike-like potentials were mostly blocked by TTX as shown from another CB3a cell (Figure 9B). In addition, we recorded a CB3a cell that was capable of generating spontaneous spike firing (Figures 9C and D). The presence of Na^+ current in this cell is shown in Figure 9C. In current clamp with the membrane potential held around -70 mV, the cell displayed spontaneous membrane depolarization accompanied by robust spike firing (Figure 9D).

DISCUSSION

Two types of CBCs expressing voltage-gated Na^+ channels

We identified two groups of CBCs that express voltage-gated Na^+ channels in rats. The axon terminals of the first group, referred to as CB3a, ramified within sublamina 3 of the IPL or near the middle of the IPL, slightly distal to the ON-cholinergic band. Two types of CBCs, classified as type 5 and type 6, have been reported to have their axon terminals ramified around the middle of the IPL (Euler & Wässle, 1995; Hartveit, 1997). A single type of bipolar cell, referred to as CB3, was also described in mice and proposed to correspond to type 5 or type 6 CBC in rats (Pignatelli & Strettoi, 2004). The axon terminals of type 6 CBC, however, were reported to stratify proximal to the ON-cholinergic band (Euler & Wässle, 1995; Hartveit, 1997; Ivanova & Müller, 2006), which is clearly distinct from the stratification of CB3a reported in this study. Therefore, the first group of Na^+ channel-expressing CBCs, CB3a, should correspond to type 5 CBCs described by Euler and Wässle (1995).

In this study, we encountered another group of CBCs referred to as CB3b in this study. These cells had similar terminal stratification as CB3a but did not show any voltage-gated Na^+ current. In addition, they also differed from CB3a in the magnitude of voltage-gated calcium current and hyperpolarization-activated time-dependent current which has been reported to be I_h (Ma *et al.*, 2003; Müller *et al.*, 2003). For these reasons, the CB3b cells should be classified as type 5 CBCs. The existence of two subclasses of type 5 CBCs has recently been reported in the rat

based on the differential immunohistochemical staining of HCN channels (Fyk-Kolodziej & Pourcho, 2007). In the mouse, morphological variability in type 5 CBCs has also been previously reported (Ghosh *et al.*, 2004), suggesting the possibility of existing multiple subclasses of type 5 CBCs in the mouse. The results of this study, however, provide the first physiological evidence for the existence of two subclasses of type 5 CBCs in the rat, one of which expresses voltage-gated Na⁺ channels.

The second group of CBCs that exhibited voltage-gated Na⁺ current has axon terminals ramified within sublamina 2, proximal to and partially overlapping with the OFF-cholinergic band. These CBCs, referred to as CB2 in this study, should undoubtedly correspond to type 3 CBCs that have been described both in rats (Euler & Wässle, 1995; Hartveit, 1997) and in mice (Ghosh *et al.*, 2004; Pignatelli & Strettoi, 2004; Ivanova & Müller, 2006).

It is also noteworthy that Na⁺ current was not observed in all of the encountered CB2 cells in this study. This raises the possibility that more than one class of type 3 CBCs should be delineated. Consistent with such a possibility, two subclasses of type 3 CBCs have been recently reported based on immunohistochemical analysis (Mataruga *et al.*, 2007). However, except for the voltage-gated Na⁺ current, we did not notice any significant difference in the properties of other voltage-gated membrane currents, such as membrane hyperpolarization-activated currents, between CB2 with and without Na⁺ current. For this reason, we did not attempt to further subdivide the CB2 group.

In this study, voltage-gated Na⁺ currents were only observed in CB3a and in the majority of CB2 cells. Our results appear to suggest that these are the only bipolar cell types in the rat that express voltage-gated Na⁺ channels. However, it should be noted that type 9 CBCs were rarely encountered in our recordings. In addition, we did not encounter type 6 CBCs whose axon terminals should stratify proximal to the ON-cholinergic band (Euler & Wässle, 1995; Hartveit, 1997). The reason for not being able to encounter type 6 CBCs in our study is not clear (see below). Therefore, we cannot completely exclude the possibility of expressing voltage-gated Na⁺ channels in these or other rarely encountered bipolar cell types.

It should be mentioned here that two subclasses of type 6 CBCs, type 6a and 6b, were recently reported in the rat retina based on their distinct voltage-dependent membrane currents (Ivanova & Müller, 2006). Interestingly, based on the properties of the hyperpolarization-activated currents, type 6b CBCs reported by Ivanova and Müller (2006) resemble the CB3a cells of this current study. Furthermore, Ivanova and Müller (2006) mentioned the observation of a fast inward current in type 6b CBCs in response to membrane depolarization, although they did not investigate the nature of this current. The type 5 CBCs reported by Ivanova and Müller (2006) resemble the CB3b cells explored in this study, as both cell types exhibit distinct I_h with large current amplitude and fast activation kinetics. HCN1 channels are believed to underlie the major component of I_h in this cell type (Müller *et al.*, 2003; Ivanova & Müller, 2006). Accordingly, based on the immunohistochemical staining pattern of HCN1 channels, these cells are likely to be the designated type 5b cells reported by Fyk-Kolodziej and Pourcho (2007). Therefore, it is possible that the discrepancies regarding type 5 and 6 CBCs reported in the literature may arise from differences in the classification of these cell types.

Physiological properties of two Na⁺ channel-expressing CBCs

Our results demonstrate that the magnitude and density of voltage-gated Na⁺ currents are largely similar between CB3a and Na⁺ channel-expressing CB2 cells. On the other hand, CB3a and CB2 cells were found to differ across several properties. First, prominent voltage-gated calcium current was observed in CB3a but not in CB2. Second, the hyperpolarization-activated time-dependent currents, or I_h, was found to be much larger in CB2 than that in CB3a. In addition, we found that the recordings of the Na⁺ current in CB3a always displayed a sign of

escape from voltage control. This phenomenon was not observed in CB2. One possible explanation for this difference could be the different localization of Na⁺ channels in these two cell types. It is possible that the Na⁺ channels in CB3a are primarily localized to the axon terminals or distal dendrites. Finally, the most striking difference between CB3a and CB2 is the capability of CB3a of generating multiple spike-like action potentials, but this is seldom seen in CB2. The mechanisms underline the difference in the capability of generating multiple spike-like potentials between CB3a and CB2 remains to be determined.

Nevertheless, this study revealed for the first time that some mammalian retinal bipolar cells have the capacity of generating multiple spike-like action potentials or spike firing *in vitro*. Light evoked spike firing has been previously demonstrated in Mb1 bipolar cells in goldfish (Protti *et al.*, 2000). The spiking signal in bipolar cells of goldfish has been proposed to secure the synaptic transmission of dim and rapidly changing visual input (Protti *et al.*, 2000). Thus, it would be interesting for further studies to determine whether spike signaling could also play a role in information processing in mammalian retinal bipolar cells. However, it should be noted here that the spiking of Mb1 bipolar cells in goldfish is solely generated by L-type calcium channels (Zenisek & Matthews, 1998; Protti *et al.*, 2000). In contrast, our results indicate that the spiking of CBCs in rat retinas is primarily generated by Na⁺ channels, although other membrane currents, such as voltage-dependent calcium channels, may also contribute to the action potential and spike firing in these cells.

Functional implications

Our finding of Na⁺ channel expression in two distinct CBCs in the mammalian retina provides valuable insights for understanding the functional roles of voltage-gated Na⁺ channels in bipolar cells. First, the presence of Na⁺ channels both in ON-type and OFF-type CBCs implies the requirement of Na⁺ channels in visual processing in both ON and OFF pathways at the level of bipolar cells. Voltage-gated Na⁺ channels have also been found in both ON and OFF CBCs in goldfish (Zenisek *et al.*, 2001). However, Na⁺ current in tiger salamander and ground squirrel has only been reported in ON bipolar cells (Ichinose *et al.*, 2005; Saszik & DeVries, 2005). It remains to be seen if the expression of Na⁺ channels in both ON and OFF CBCs is a general property of the retina. Second, the axon terminals of the two types of Na⁺ channel-expressing CBCs were found to ramify in or near the middle of the IPL. Similar findings were also reported for the Na⁺ channel-expressing bipolar cells in the tiger salamander and in the ground squirrel (Ichinose *et al.*, 2005; Saszik & DeVries, 2005). It has been well established that the transient signal is processed in the middle of the IPL (Awatramani & Slaughter, 2000). Also, it is conceivable that Na⁺ current could be more likely to transmit fast and transient responses due to its fast activation and inactivation kinetics. Indeed, in the tiger salamander, Na⁺ current was observed in transient bipolar cells to enhance transient responses in ganglion cells (Ichinose *et al.*, 2005). Thus, the cell types of Na⁺ channel-expressing bipolar cells revealed in this study are consistent with the notion that voltage-gated Na⁺ channels may play a role in transmitting fast and transient signals in the retina.

Furthermore, this study revealed that the axon terminals of the ON and OFF Na⁺ channel-expressing CBCs are closely related to the processes of the ON and OFF starburst amacrine cells, respectively. In fact, the axon terminals of CB2 partially overlap with the processes of OFF starburst amacrine cells, whereas the axon terminals of CB3a ramify just slightly distal to the processes of ON starburst amacrine cells. Starburst amacrine cells are known to play a critical role in the generation of directional selectivity in the retina (Yoshida *et al.*, 2001). The directional selectivity of retinal ganglion cells has been reported to be formed in part by the direction-selective inhibitory inputs from starburst amacrine cells (Fried *et al.*, 2002; Euler *et al.*, 2002; Gavrikov *et al.*, 2003; Lee & Zhou, 2006). Direction-selective excitatory inputs from bipolar cells have also been proposed to be involved (Famiglietti, 2002; Fried *et al.*, 2005).

The cellular mechanism for the latter, however, remains unknown (Demb, 2007). Based on the proximity of their terminal stratifications to the processes of starburst amacrine cells, it is likely that CB2 and CB3a could directly or indirectly feed into the dendrites of the directional selective ON and OFF ganglion cells, respectively. Therefore, it would be of interest for future studies to investigate whether voltage-gated Na⁺ channels in bipolar cells play a role in the directional selectivity of the retina.

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Figure 1.

Morphology of two groups of cone bipolar cells that exhibit voltage-gated Na^+ current. A and C: Fluorescence images of the representative CB3a (A) and CB2 (C) in retinal slices. The recorded cells were filled with fluorescent dye (Alexa 488). The inner plexiform layer (IPL) is divided equally into five sublaminae, illustrating the terminal stratification of CB3a and CB2. B and D: the same cells shown in (A) and (C), respectively, were co-stained with antibodies against Alexa 488 (green) and choline acetyltransferase (ChAT; red). Scale bar: 25 μm in (A) and (C); 15 μm in (B) and (D).

Figure 2.

Membrane depolarization-evoked currents from CB3a. A: A representative recording of membrane depolarization-evoked currents from CB3a in control. The current traces were evoked by test pulses ranging from -60 to +10 mV from the holding potential of -80 mV. B: The recordings of the same cell shown in (A) in the presence of TTX (arrow points to voltage-gated calcium current). C: The TTX-sensitive current obtained by subtracting the current traces in the presence of TTX from those in the control. D: Current-voltage relationship of the TTX-sensitive current. The data are shown as mean $\pm$ SE ($n = 12$). E: Illustration of the presence of both TTX-sensitive Na^+ current (pointed to by an arrow) and voltage-gated calcium current. As illustrated, the amplitude of voltage-gated calcium current is measured based on the inward current.

Figure 3.

Membrane hyperpolarization-elicited currents from CB3a. A: A representative recording of the membrane hyperpolarization-activated currents. The currents were evoked by test pulses ranging from -70 to -120 mV from the holding potential of -60 mV. B: Current-voltage (I - V) relationships of $I_{t-indep}$ and $I_{t-indep} + I_{t-dep}$ for the recording shown in (A). C: Illustration of the measurement of $I_{t-indep}$ and I_{t-dep} from an individual trace evoked by a test pulse to -120 mV from the holding potential of -60 mV. D: The average amplitude of $I_{t-indep}$ and I_{t-dep} recorded from CB3a.

Figure 4.

Morphology and physiological properties of CB3b. A: The fluorescence image of a representative CB3b cell in retinal slices. B: The terminal stratification of the same cell shown in (A) as defined by co-staining with antibodies against Alexa 488 (green) and ChAT (red). C: The recordings of membrane depolarization-evoked currents from a representative CB3b cell in control (left panel) and in the presence of TTX (right panel). The current traces were evoked by test pulses ranging from -60 to -10 mV from the holding potential of -80 mV. D: Membrane hyperpolarization-elicited currents. The currents were evoked by test pulses ranging from -70 to -120 mV from the holding potential of -60 mV. E: The plot of current-voltage (I - V) relationships of $I_{t-indep}$ and $I_{t-indep} + I_{t-dep}$ for the cell shown in (D). F: Illustration of the

measurement of $I_{t-indep}$ and I_{t-dep} from an individual trace evoked by a test pulse to -120 mV from the holding potential of -60 mV. G: The average amplitude of $I_{t-indep}$ and I_{t-dep} recorded from CB3b.

Figure 5.

Response properties of CB3a and CB3b to L-AP4 and light. A and B: Representative responses of CB3a (A) and CB3b (B) to puffing L-AP4 (50 μ M) in retinal slices. In these recordings, electrode solution contained cyclic guanosine 3':5'-monophosphate (cGMP) (1 mM). Cells were held around -50 or -60 mV. The zero current level and the constantly activated currents are illustrated by dotted lines. C and D: Both CB3a (C) and CB3b (D) responded to light with membrane depolarization.

Figure 6.

Membrane depolarization-evoked currents from CB2. A: A representative recording of membrane depolarization-evoked currents from CB2 in the control case. The current traces were evoked by test pulses ranging from -60 to +10 mV from the holding potential of -80 mV. B: The recordings of the same cell shown in (A) in the presence of TTX. C: The TTX-sensitive current obtained by subtracting the current traces in the presence of TTX from those in the control. D: Current-voltage relationship of the TTX-sensitive current. The data are shown as mean $\pm$ SE ($n = 9$).

Figure 7.

Membrane hyperpolarization-evoked currents from CB2. A: A representative recording of the membrane hyperpolarization-activated currents. The currents were evoked by test pulses ranging from -70 to -120 mV from the holding potential of -60 mV. B: Current-voltage (I - V) relationships of $I_{t-indep}$ and $I_{t-indep} + I_{t-dep}$ for the recordings shown in (A). C: Illustration of the measurement of $I_{t-indep}$ and I_{t-dep} from an individual trace evoked by a test pulse to -120 mV from the holding potential of -60 mV. D: The average amplitude of $I_{t-indep}$ and I_{t-dep} recorded from CB2.

Figure 8.

Na⁺-dependent action potentials elicited by current injection. A and B: Representative recordings of Na⁺ current and Na⁺-dependent action potentials from CB3a (A) and CB2 (B). The presence of TTX-sensitive Na⁺ current was confirmed in voltage clamp recordings (top and middle panels). For the same cells, in current clamp recordings, a fast action potential was evoked by current injection. The action potential was mostly blocked by TTX (bottom panels). The action potentials in (A) and (B) were evoked by current steps to 10 and 0 pA from the holding current of -17 and -50 pA, respectively.

Figure 9.

Multiple action potentials observed in CB3a. A: A representative recording of multiple action potentials elicited by current injection from a CB3a cell. The presence of Na^+ current was confirmed under voltage-clamp (top panel). In the same cell, current injection elicited multiple action potentials (bottom panel). The voltage responses were evoked by current pulses in the range from -5 to $+20$ pA with increments of 5 pA from the holding current of -14.0 pA. B: A recording showing that the action potential was mostly blocked by TTX as demonstrated from another CB3a cell. The action potentials were evoked by current steps to 0 pA from the holding current of -32 pA. C and D: Spontaneous regenerative activity observed from a CB3a cell. The presence of voltage-dependent Na^+ current was shown in voltage clamp recordings. In current clamp at a holding current of -14.0 pA, this same cell exhibited spontaneous regenerative potentials accompanied by robust spike firing. The bottom panel in (D) illustrates one of the regenerative potentials on an expanded time scale.