Mammalian Retinal Bipolar Cells Express Inwardly Rectifying $K^+$ Currents ($I_{\text{Kir}}$) With a Different Distribution Than That of $I_h$

Yu-Ping Ma, Jinjuan Cui, Hui-Juan Hu, and Zhuo-Hua Pan
Department of Anatomy and Cell Biology, Wayne State University School of Medicine, Detroit, Michigan 48201

Submitted 5 May 2003; accepted in final form 23 June 2003

INTRODUCTION

Hyperpolarization-activated membrane currents have been proposed to contribute to a variety of neuronal functions in the CNS. Two types of hyperpolarization-activated currents have been extensively studied: the pure inwardly rectifying $K^+$ (Kir) current ($I_{\text{Kir}}$), which is believed to be responsible for setting resting membrane potential and regulating cellular excitability (Hille 1992; Isomoto et al. 1997), and the hyperpolarization-activated cationic current, also called $h$-current ($I_h$), which is characterized by low Na$^+$/K$^+$ permeation selectivity and slow time-dependent activation (DiFrancesco 1993). $I_h$ is well known for its role in pacemaker activity, but it has also been proposed to regulate other functions, such as resting membrane potential, cell conductance, dendritic integration, and synaptic transmission (Rape 1996; Robinson and Siegelbaum 2003).

The potential importance of these membrane currents in determining the diversity of neuronal physiological properties and functions is implied by their heterogeneous expression among similar neuronal populations (Cathala and Paupardin-Tritsch 1999; Holt and Eatock 1995; Travaglì and Gillis 1994; Yahou et al. 1998).

Ma, Yu-Ping, Jinjuan Cui, Hui-Juan Hu, and Zhuo-Hua Pan. Mammalian retinal bipolar cells express inwardly rectifying $K^+$ currents ($I_{\text{Kir}}$) with a different distribution than that if $I_h$. J Neurophysiol 90: 3479–3489, 2003; 10.1152/jn.00426.2003. Retinal bipolar cells comprise multiple subtypes that are well known for the diversity of their physiological properties. We investigated the properties and functional roles of the hyperpolarization-activated currents in mammalian retinal bipolar cells using whole cell patch-clamp recording techniques. We report that bipolar cells express inwardly rectifying $K^+$ currents ($I_{\text{Kir}}$) in addition to the hyperpolarization-activated cationic currents ($I_h$) previously reported. Furthermore, these two currents are differentially expressed among different subtypes of bipolar cells. One group of cone bipolar cells in particular displayed mainly $I_{\text{Kir}}$. A second group of cone bipolar cells displayed both currents but with a much larger $I_h$. Rod bipolar cells, on the other hand, showed primarily $I_{\text{Kir}}$. Moreover, we showed that $I_{\text{Kir}}$ and $I_h$ differentially influence the voltage responses of bipolar cells: $I_{\text{Kir}}$ facilitates and/or accelerates the membrane potential rebound, whereas $I_{\text{Kir}}$ counteracts or prevents such rebound. The findings of the expression of $I_{\text{Kir}}$ and the differential expression of $I_h$ and $I_{\text{Kir}}$ in bipolar cells may provide new insights into an understanding of the physiological properties of bipolar cells.

METHODS

Dissociation of bipolar cells

Bipolar cells were isolated from approximately 6- to 8-wk-old Long Evans rats by dissociation methods previously described (Pan 2000). All animal handling procedures were approved by the Institutional

Address for reprint requests and other correspondence: Z.-H. Pan, Dept. of Anatomy and Cell Biology, Wayne State Univ. School of Medicine, 540 E. Canfield Ave., Detroit, MI 48201 (E-mail: zhp@med.wayne.edu).

Retinal bipolar cells are second-order neurons that relay visual signals from photoreceptors to third-order neurons, amacrines, and ganglion cells. Bipolar cells have been classified into multiple functional subtypes (Kaneko 1970; Werblin and Dowling 1969). Mammalian bipolar cells are particularly well characterized morphologically; they consist of a single type of rod bipolar cells (RBCs) and multiple subtypes of on- and off-cone bipolar cells (CBCs) (Boycott and Dowling 1969; Boycott and Kolb 1973; Dacheux and Raviola 1986; Euler and Wässle 1995; Famiglietti 1981; Greferath et al. 1990; Pourcho and Goebel 1987). Bipolar cells of different types are known for the diversity of their physiological properties, such as resting membrane potential, voltage operation range, response waveform, and kinetics (Awatramani and Slaughter 2000; Euler and Masland 2000; Wu et al. 2000). Such diversity is believed to be important in overall retinal processing. Increasing evidence suggests that intrinsic membrane conductances may contribute to the diversity of physiological properties observed among bipolar cells (Burrone and Lagnotto 1997; Ma and Pan 2003; Mao et al. 1998; Protti et al. 2000; Zenisek and Matthews 1998). A detailed knowledge of the intrinsic membrane conductances among different bipolar cell types would therefore be important for understanding their physiological functions.

The expression of $I_h$ in bipolar cells has been described in a variety of species (Connoughton and Maguire 1998; Kaneko and Tachibana 1985; Karschin and Wässle 1990; Lasater 1988; Tessier-Lavigne et al. 1988). However, expression of $I_{\text{Kir}}$ in bipolar cells has not been reported previously, although hyperpolarization-activated currents with properties different from those of $I_h$ have been observed in fish bipolar cells (Lasater 1988). In this study, we report that bipolar cells in the mammalian retina express $I_{\text{Kir}}$ in addition to $I_h$. We examined the possible roles of these hyperpolarization-activated currents in the response properties of bipolar cells. Part of this work has appeared in abstract form (Hu and Pan 2000; Ma et al. 2003).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Animal Care Committee at Wayne State University and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. In brief, animals were deeply anesthetized with CO2 and killed by decapitation. Retinas were removed and placed in a Hanks’ solution (in mM): 138 NaCl, 1 NaHCO3, 0.3 Na2HPO4, 5 KCl, 0.3 KH2PO4, 1.25 CaCl2, 0.5 MgSO4, 0.5 MgCl2, 5 HEPES, and 22.2 glucose, with phenol red, 0.001% vol/vol; adjusted to pH 7.2. The retinas were incubated for 40–50 min at 34–37°C in an enzymatic solution that consisted of the normal Hanks’ solution described above supplemented with d-cysteine, 0.2 mg/ml; bovine serum albumin, 0.2 mg/ml; and papain, approximately 2.0 U/ml, and mechanically dissociated by gentle trituration. The resulting cell suspension was plated onto culture dishes. RBCs and CBCs were identified based on their characteristic morphology (Karschin and Wässle 1990; Pan 2000).

Preparation of retinal slices

Retinal slices were prepared by established procedures (Werblin 1978). In brief, retinal slices about 150 μm thick were mounted in a glass-bottomed recording chamber. The recording chamber was mounted on the stage of an upright microscope equipped with a 40X water immersion objective and differential interference contrast and epifluorescence optics. The recording chamber was continuously superfused with oxygenated Hanks’ solution at the rate of approximately 2 ml/min. Bipolar cells were identified on the basis of their characteristic morphology, as described by Euler and Wässle (1995).

Electrophysiological recordings

Recordings were made in whole cell patch mode at room temperature (approximately 22°C) with an EPC-9 amplifier and PULSE software (Heka Electronik, Lambrecht, Germany). Some recordings were also made in perforated patch mode. Electrodes were coated with SYLGARD (Dow Corning, Midland, MI) and fire-polished. The resistance of the electrodes ranged from 7 to 14 MΩ. The series resistance was usually <40 MΩ in whole cell patch mode and >50 MΩ in perforated patch mode. Series resistance was not routinely compensated. Cell capacitance was canceled, and the value was recorded using an automatic feature of the EPC-9 amplifier. Leak subtraction was not performed. Substantial leak-like currents were frequently observed in bipolar cells after prolonged recordings, which might be due to the deterioration of the recordings and/or the development of other conductances. Such recordings were excluded in the analysis. To reduce the recording period, the studies of the pharmacological and biophysical properties of hyperpolarization-activated currents were performed primarily on currents evoked by a single test step to −120 mV. All the recordings on isolated RBCs were made on cells that retained their axon terminals. The axon terminals of isolated CBCs were usually small or were lost during the dissociation. Extracellular solutions contained (in mM) 136 NaCl, 5 KCl, 1 MgCl2, 2.5 CaCl2, 5 HEPES, and 22.2 glucose, pH 7.2. The electrode solution contained (in mM) 128 K-glucocinate, 12 KCl, 1 MgCl2, 0.5 CaCl2, 5 EGTA, 10 HEPES, 0.5 Na-GTP, and 2 Na-ATP, pH 7.4. For perforated-patch recordings, the electrodes were tip-filled with the normal electrode solution and then backfilled with electrode solution containing nystatin (200 μg/ml). Recordings were usually made after the access resistance reached <100 MΩ. Liquid junction potentials were corrected. In slice recordings, the fluorescent dye Alexa 488 was added to the electrode solution at a concentration of 100 μM. Fluorescence images for some of the recorded cells in the slices were taken after the recording.

Chemicals and data analysis

For isolated cell recordings, chemical agents were applied to the cells by gravity-driven superfusion pipettes placed about 200–300 μm away from the cell. The effect of ZD 7288 on Ih was time-dependent. Its effect was assessed after application of >46 s. The effects of other chemicals were assessed after the application of >23 s but were not found to be time-dependent. ZD 7288 was purchased from Tocris (Ellisville, MO). All other chemicals were purchased from Sigma (St. Louis, MO). The activation course for the hyperpolarization-activated current was fitted with a single or double-exponential: I(t) = I1 × [exp(−t/τ1)] + I2 × [exp(−t/τ2)] + I0, where τ1 (1 or 2) represents the activation time constant, I1 is the corresponding current amplitude, and I0 is the nonactivating component. Data were analyzed off-line using ORIGIN programs (Microcal Software, Northampton, MA). Data are presented as means ± SD.

RESULTS

The properties of the hyperpolarization-activated currents in bipolar cells were investigated by use of whole cell patch-recording techniques. Two components of these with distinct activation kinetics were observed. One component showed instantaneous or time-independent activation, and the second component showed time-dependent activation. The latter resembles the characteristic of Ih (DiFrancesco 1993), which has previously been reported in bipolar cells (Connaughton and Maguire 1998; Kaneko and Tachibana 1985; Karschin and Wässle 1990; Lasater 1988). The expression of these two currents was observed in both isolated bipolar cells and bipolar cells in retinal slice preparations. Similar results were also observed by perforated-patch recordings from isolated cells. For simplicity, the instantaneous current and the time-dependent current will be referred to as I0h and Ih, respectively. We will demonstrate in the later part of this study that the instantaneous current is IKr and confirm that the time-dependent current is Ih. The characterizations of these currents presented in this study were all made by whole cell patch recordings. In addition, all the data except those shown in Fig. 1 were obtained from isolated cells.

Differential expression of two types of hyperpolarization-activated inward currents

The properties of the hyperpolarization-activated currents among CBCs were heterogeneous. In contrast, the currents of RBCs were largely homogeneous. Based on the expression pattern of the hyperpolarization-activated currents, two distinct groups of CBCs were predominant, both in retinal slices (Fig. 1) and in acutely isolated cells (Fig. 2). Representative recordings of these two groups of CBCs and of RBCs obtained from retinal slices are shown in Fig. 1, A–C. One group of CBCs (n = 9) expressed primarily I0h, although a small time-dependent current was also evident in this group, especially in retinal slice recordings (Fig. 1A). The second group of CBCs (n = 13) expressed both I0h and Ih (Fig. 1B), but unlike the first group, Ih was much larger than I0h. The axon terminals of both groups of CBCs were found to ramify near or slightly below the middle of the inner plexiform layer (IPL). Sample fluorescence images of the two groups of CBCs are shown in Fig. 1, D and E. In addition, some less frequently encountered CBCs, especially in retinal slices, were found to display no or only a minuscule inward current (<10 pA evoked by the test step to −120 mV; see Fig. 2). RBCs, on the other hand, showed predominantly Ih (n > 15; Fig. 1C). A sample fluorescence image of RBCs is shown in Fig. 1F. The expression of Ih in rat
RBCs has been reported previously (Karschin and Wässle 1990). It should be noted here that although large tail currents generally were found to be closely associated with $I_h$, in some cells other voltage-dependent currents, such as voltage-dependent Ca$^{2+}$ and Na$^{+}$ currents, were activated after the termination of the testing potential. For example, some of the "tail current" seen in Fig. 1A is likely due to the activation of these other currents.

The magnitude of the inward currents differed among the groups of bipolar cells that are described above. To quantify this property, we compared $I_{Kir}$ and/or $I_h$ evoked by a 2-s hyperpolarizing step to $-120$ mV in isolated cells. $I_{Kir}$ was measured at the beginning of the test step, and $I_h$ was obtained by the difference between the peak current measured at the end of the test step and $I_{Kir}$ (Fig. 2A). The average amplitudes of $I_{Kir}$ and/or $I_h$ are shown in Fig. 2B. The average whole cell capacitance and the average current density for each group are shown in Fig. 2, C and D, respectively. For the CBCs that express both $I_{Kir}$ and $I_h$, the amplitude of $I_h$ is about five times that of $I_{Kir}$. In addition, the $I_h$ in these CBCs is also significantly larger, especially the current density, than the $I_h$ in RBCs (1-way ANOVA test, $P < 0.01$). Furthermore, a subset of CBCs displayed almost no hyperpolarization-activated currents. The CBCs that express mainly $I_{Kir}$ appeared to have a smaller somas and longer axons than those of CBCs that expressed both $I_{Kir}$ and $I_h$. Consistent with this observation, the average whole cell capacitance of CBCs that expressed $I_{Kir}$ was $2.1 \pm 0.3$ pF ($n = 26$). In contrast, the average whole cell capacitance of CBCs that expressed both $I_{Kir}$ and $I_h$ was $2.7 \pm 0.4$ pF ($n = 24$). These two values are significantly different (1-way ANOVA test, $P < 0.01$).

The activation kinetics of $I_h$ in RBCs and CBCs also differed. Although the activation kinetics of $I_h$ were voltage-dependent (Ishii et al. 2001; Santoro et al. 2000), the difference
in the activation kinetics of $I_h$ between RBCs and CBCs could be clearly revealed by fitting the initial portion of the current time-course evoked by the test step to $-120$ mV from the holding potential of $-60$ mV. The time course of $I_h$ in the RBCs for times of $\leq 1,200$ ms can be well fitted by a single exponential with average time constants of $379 \pm 46$ ms ($n = 10$; Fig. 3, A and C). In contrast, the time course of $I_h$ for the CBCs under the same conditions has to be fitted by the sum of two exponentials with the average time constant of $128 \pm 28$ and $994 \pm 455$ ms ($n = 10$), respectively (Fig. 3, B and C). It

FIG. 2. Comparison of the current amplitude and density of $I_{K_S}$ and/or $I_h$. All data were obtained from acutely isolated cells. Currents were elicited by a 2-s test step to $-120$ mV from the holding potential of $-60$ mV. A: representative current traces for 3 groups of CBCs as well as RBCs. $I_{K_S}$ is measured at the beginning of the test step, and $I_h$ is the difference between the peak measured at the end of the test step and the instantaneous current. B: average amplitude of $I_{K_S}$ and $I_h$ for each group of bipolar cells. C: average whole cell capacitance. Note that the capacitances between the group of CBCs showing $I_{K_S}$ and the groups of CBCs showing both $I_{K_S}$ and $I_h$ are significantly different. D: average current densities for $I_{K_S}$ and $I_h$. Data are mean $\pm$ SD from indicated number of cells. *$P < 0.01; 1$-way ANOVA test.
sensitive to Ba$^{2+}$ instantaneous current. First, the current was found to be highly classical property of Kir channels (Hille 1994).

The time-independent activation of the inward current by hyperpolarizations resembles the property of the inwardly rectifying K⁺, or Kir current ($I_{Kir}$) (Hille 1992). The expression of this current by retinal bipolar cells, however, has not been previously reported. Thus we investigated the biophysical and pharmacological properties of the $I_{Kir}$. For this purpose, recordings were made from the group of CBCs that expressed mainly $I_{Kir}$. First, we examined the ionic permeation of the inward current by retinal bipolar cells, however, has not been previously reported. Thus we investigated the biophysical and pharmacological properties of the $I_{Kir}$. For this purpose, recordings were made from the group of CBCs that expressed mainly $I_{Kir}$. First, we examined the ionic permeation of the $I_{Kir}$ for the CBCs and RBCs. Data are mean ± SD.

**Instantaneous current is $I_{Kir}$**

The time-independent activation of the inward current by hyperpolarizations resembles the property of the inwardly rectifying K⁺, or Kir current ($I_{Kir}$) (Hille 1992). The expression of this current by retinal bipolar cells, however, has not been previously reported. Thus we investigated the biophysical and pharmacological properties of the $I_{Kir}$. For this purpose, recordings were made from the group of CBCs that expressed mainly $I_{Kir}$. First, we examined the ionic permeation of the current. Increasing the extracellular K⁺ from 5 (in control) to 20 mM almost tripled the amplitude of the current (281 ± 48% of control, $n = 8$; Fig. 4, A and F). In contrast, replacing extracellular Na⁺ (136 mM) with N-methyl-D-glucamine (NMDG) resulted in only a slight decrease in the current (90 ± 9% of control, $n = 11$; Fig. 4, A and F). These results indicate that the channels are mainly permeable to K⁺ but not significantly to Na⁺. The voltage dependency of the current was also examined with voltage-ramp stimulation (Fig. 4B). In control, the $I-V$ relation exhibited an inward rectification. Increasing the extracellular K⁺ (from 5 to 20 mM) resulted in a largely parallel shift of the $I-V$ curve ($n = 5$). The latter represents a classical property of Kir channels (Hille 1994).

Next, we examined the pharmacological properties of the instantaneous current. First, the current was found to be highly sensitive to Ba$^{2+}$. Application of Ba$^{2+}$ (50 μM) blocked most of the inward current (to 9 ± 9% of the control amplitude, $n = 13$), as evidenced by the inability to produce further blockade by 1 mM Cs⁺ (to 7 ± 6% of the control amplitude, $n = 13$), a nonselective blocker of hyperpolarization-activated currents (Fig. 4, C and D). In contrast, ZD 7288 (50 μM), a selective blocker of $I_{Kir}$ (BoSmith et al. 1993; Harris and Constanti 1995), did not produce any significant blockade of the current (to 94 ± 5% of the control amplitude, $n = 9$; Fig. 4, D and F). In addition, a significant tail current was absent in most of these recordings (see Fig. 4, C and D), a finding consistent with the fast deactivation property of Kir channels.

We then examined the reversal potential of the Ba$^{2+}$-sensitive current. For the cell shown in Fig. 4E, the Ba$^{2+}$-sensitive

![Image](http://jn.physiology.org/)
The distinct ionic and pharmacological profiles of $I_h$ and $I_{Kir}$ described above enabled us to demonstrate that the instantaneous and time-dependent currents observed in a subset of CBCs are $I_{Kir}$ and $I_h$, respectively. First, as shown in a sample recording in Fig. 6A from a CBC showing both these two currents, increasing extracellular K+ markedly enhanced the currents of both components, whereas replacing extracellular Na+ with NMDG only decreased the time-dependent component. Similar results were observed in eight other cells. These results suggest that both components are permeant to K+, but that only the time-dependent component is significantly permeant to Na+, consistent with the presence of $I_{Kir}$ and $I_h$ in these cells. This was further confirmed pharmacologically. In a sample recording shown in Fig. 6B from another such a CBC, application of Ba2+ (50 µM) primarily blocked the time-independent component, as evidenced by a largely parallel decrease in the inward current (Fig. 6B). Application of ZD 7288 (50 µM), on the other hand, blocked the time-dependent component. The presence of the ZD 7288-resistant $I_{Kir}$ was evidenced by the further application of Cs+ (1 mM), which blocked most of the remaining inward currents. Similar results were observed in seven other cells.

**Roles of $I_{Kir}$ and $I_h$ in voltage responses**

We next investigated the possible roles for the differential expression of $I_{Kir}$ and $I_h$ in bipolar cells. For this purpose, we examined the effects of the blockade of $I_h$ or $I_{Kir}$ on voltage

![A](image)

**Fig. 5.** Ionic and pharmacological properties of $I_h$. Recordings were made from RBCs. Currents were evoked by a test step to −120 mV from the holding potential of −60 mV. A: replacing extracellular Na+ (136 mM) with NMDG markedly decreased the current. Increasing the extracellular K+ from 5 mM (in control) to 20 mM increased the current. B: current was insensitive to 50 µM Ba2+. Application of 50 µM ZD 7288 largely blocked the current. Application of 1 mM Cs+ blocked almost all the inward current. C: average effects of 20 mM K+, NMDG, Ba2+, ZD 7288, and Cs+ on $I_h$. Effect of ZD 7288 was assessed after application for 46 s. Data are mean ± SD. Dotted line indicates the control level.

Current reversed around −87 mV. The average value was −82 ± 7 mV (n = 14). This value is virtually identical to the expected reversal potential of K+ ions under our recording conditions (−83 mV). Taken together, our results strongly indicate that the instantaneous current is an inwardly rectifying K+ current, or $I_{Kir}$.

**Time-dependent inward current is $I_h$**

Retinal bipolar cells, including mammalian RBCs, have previously been reported to express $I_h$, which shows time-dependent activation (Connaughton and Maguire 1998; Kaneko and Tachibana 1985; Karshin and Wässle 1990; Laster 1988). As further evidence for the expression of $I_{Kir}$ and the differential expression of $I_{Kir}$ and $I_h$ in bipolar cells, we went on to confirm that the time-dependent current in bipolar cells is $I_h$. For this purpose, we examined the properties of the current in RBCs because they express mainly the time-dependent current. Consistent with the idea that the current is carried by both Na+ and K+ ions, replacing extracellular Na+ (136 mM) with NMDG markedly reduced the current (20 ± 14% of control, n = 4), whereas increasing extracellular K+ (from 5 to 20 mM) almost doubled the current (182 ± 36% of control, n = 11; Fig. 5, A and C). Furthermore, application of ZD 7288 (50 µM) blocked most of the inward current (Fig. 5, B and C). The blockade of ZD 7288 was found to be irreversible and time-dependent, consistent with the reports in other systems (Hogg et al. 2001; Larkman and Kelly 2001; Satoh and Yamada 2000). After application of ZD 7288 (50 µM) for 23, 46, and 69 s, the peak current was reduced to 42 ± 19% (n = 19), 24 ± 12% (n = 9), and 15 ± 3% (n = 5) of control, respectively. As expected, the current also was mostly blocked by Cs+ (1 mM) (10 ± 6% of control, n = 12; Fig. 5, B and C). On the other hand, Ba2+ (50 µM) had no effect on the current (98 ± 4% of control, n = 14; Fig. 5, B and C). Taken together, these results confirm that the time-dependent inward current is $I_h$.

![B](image)

**Fig. 6.** Ionic and pharmacological properties for the hyperpolarization-activated currents in CBCs that displaying both $I_{Kir}$ and $I_h$. Currents were evoked by a test step to −120 mV from the holding potential of −60 mV. A: replacing extracellular Na+ (145 mM) with NMDG decreased the component of $I_h$ but not $I_{Kir}$. Increasing the extracellular K+ (from 5 to 20 mM) markedly increased both currents. B: application of Ba2+ (50 µM) resulted in a parallel shift of the current, indicating the blockade of $I_{Kir}$. Application of ZD 7288 blocked $I_h$. Application of 1 mM Cs+ blocked almost all the inward currents.
responses for RBCs and two groups of CBCs that express $I_{\text{Kir}}$ and/or $I_{\text{h}}$. In this set of experiments, after having examined the hyperpolarization-activated currents in voltage clamp, we recorded cells in current clamp and applied a negative current pulse (step to $-15 \text{ pA}$ for 400 ms) that elicited membrane hyperpolarization during the pulse as well as rebound depolarization after termination of the pulse (Fig. 7).

For RBCs (Fig. 7A) the current pulse produced a large membrane hyperpolarization followed by rectification, or sag (black traces). The voltage sag is a characteristic effect of $I_{\text{h}}$ (Robinson and Siegelbaum 2003). After the termination of the pulse, the membrane potential rebound always overshoots the initial membrane potential. The overshoot is produced, at least in part, by a Ca$^{2+}$-dependent regenerative mechanism, because it could be blocked by Co$^{2+}$ (data not shown). As expected, application of ZD 7288 (50 $\mu$M) blocked the voltage sag during the current injection (gray traces). In addition, for most cells the application of ZD 7288 markedly slowed the voltage rebound and overshoot. The average time from the termination of the current to the peak of the overshoot was 160 ± 22 ms in control and 284 ± 48 ms in ZD 7288 ($n = 9$). For some cells, the overshoot was totally abolished by the application of ZD 7288 (Fig. 7, right; $n = 4$). These results indicate that the presence of $I_{\text{h}}$ counteracts the membrane hyperpolarization, albeit with a delay, and accelerates and/or facilitates membrane potential rebound or depolarization. Similar effects of $I_{\text{h}}$ on voltage responses have been described in retinal ganglion cells (Tabata and Ishida 1996).

For the group of CBCs that expressed mainly $I_{\text{Kir}}$ (Fig. 7B), the current injection produced a smaller membrane hyperpolarization than that observed in RBCs (black traces). In addition, the membrane potential did not change during the course of the current pulse. Both properties would be expected based on the instantaneous activation and nonactivation properties of $I_{\text{Kir}}$. After the termination of the current, for the majority of the cells (12 of 18), the membrane potential also showed rebound and overshoot but with a prolonged delay (Fig. 7B, left). For a smaller number of the cells ($n = 6$), the overshoot of the membrane potential was absent (Fig. 7B, right). In addition, the overshoot of the membrane potential was frequently accompanied with a spike or action potential. The expression of voltage-dependent Na$^+$ channels has been previously reported in a subset of CBCs in the rat retina (Pan and Hu 2000). After application of Ba$^{2+}$ (50 $\mu$M), the negative current pulse produced a larger hyperpolarization (gray traces). In addition, the application of Ba$^{2+}$ accelerated the membrane potential rebound and overshoot (Fig. 7B, left). The average time from the termination of the current to the peak of overshoot was 308 ± 15 ms in controls and 175 ± 64 ms in Ba$^{2+}$ ($n = 7$). For those cells in which the overshoot was absent in control, the application of Ba$^{2+}$ restored the membrane potential overshoot (Fig. 7B, right). These results indicate that the presence of $I_{\text{Kir}}$ counteracts the membrane potential hyperpolarization as well as depolarization, consistent with its role in stabilizing the membrane potential around the K$^+$ reversal potential (Nichols and Lopatin 1991; Reimann and Ashcroft 1999; Ruppersberg 2000).

Furthermore, for the groups of CBCs that expressed both $I_{\text{Kir}}$ and $I_{\text{h}}$ (Fig. 7C), the current injection evoked a small membrane hyperpolarization that was similar to that of CBCs that expressed mainly $I_{\text{Kir}}$ (black traces). This is consistent with the expression of $I_{\text{Kir}}$ in these cells. In addition, after the termination of the current, membrane potential rebound and overshoot were observed in all recorded cells in this group ($n = 7$). The rebound, however, was found to be much faster, as to be expected by cells expressing relatively large $I_{\text{h}}$. Consistently, after application of Ba$^{2+}$ (50 $\mu$M; gray trace in Fig. 7C, left), the current pulse produced a larger hyperpolarization followed by a sag. Again, application of Ba$^{2+}$ accelerated the membrane potential rebound and overshoot. The average time from the termination of the current to the peak of overshoot was 125 ± 17 ms in controls and 88 ± 24 ms in Ba$^{2+}$ ($n = 4$). On the other hand, application of ZD 7288 (50 $\mu$M) eliminated the overshoot in all recorded cells ($n = 3$), further supporting the role of $I_{\text{h}}$ in the membrane potential rebound and overshoot.

Finally, these roles of hyperpolarization-activated currents in bipolar cells were further demonstrated in the group of CBCs that expressed almost no amount of these current. Figure 7D shows two typical recordings. For these CBCs, a negative current injection was found to produce a huge membrane hyperpolarization. After termination of the current, the majority of the cells did not show the overshoot of the membrane potential (6 of 9; Fig. 7D, left). For a smaller number of cells ($n = 3$), a small overshoot of membrane potential was observed (Fig. 7D, right).

**DISCUSSION**

We report in this study for the first time that mammalian retinal bipolar cells express $I_{\text{Kir}}$ in addition to the previously reported $I_{\text{h}}$. Furthermore, we found differential expression of $I_{\text{Kir}}$ and $I_{\text{h}}$ in RBCs and CBCs and among different CBCs. Moreover, our results suggest that the differential expression of $I_{\text{Kir}}$ and $I_{\text{h}}$ could distinctively influence the voltage-response properties of bipolar cells.

**Expression of $I_{\text{Kir}}$ in bipolar cells**

The expression of $I_{\text{Kir}}$ in bipolar cells is supported by several lines of evidence: 1) the hyperpolarization-activated current showed time-independent activation, a typical property of $I_{\text{Kir}}$; 2) the current was carried mainly by K$^+$ and reversed around the predicted K$^+$ reversal potential; 3) the I-V relation of the current shift paralleled the change of extracellular K$^+$, a characteristic of inwardly rectifying channels (Hille 1994); and 4) the current was selectively blocked by a low concentration of Ba$^{2+}$, a potent blocker of $I_{\text{Kir}}$ (Totpert et al. 1998). From these results, we conclude that bipolar cell express $I_{\text{Kir}}$.

Multiple gene subfamilies have been known to encode $I_{\text{Kir}}$ (Isomoto et al. 1997; Krapivinsky et al. 1998). Mammalian retinas have been reported to express the mRNAs of a number of Kir subunits, although most of these mRNA signals have been reported to be located in glial cells (Hughes et al. 2000; Raap et al. 2002). Further studies will be necessary to determine the molecular identity of $I_{\text{Kir}}$ in bipolar cells.

Consistent with previous reports (Connahington and Maguire 1998; Kaneko and Tachibana 1985; Karschin and Wässe 1990; Lasater 1988), we confirmed the presence of $I_{\text{h}}$ in bipolar cells. First, we showed in this study that $I_{\text{h}}$ is carried by both K$^+$ and Na$^+$. Second, the current was blocked by ZD 7288, a selective blocker of $I_{\text{h}}$ (BoSmith et al. 1993; Harris and Constanti 1995). Furthermore, Ba$^{2+}$ had no effect on this current,
FIG. 7. Properties of the current-evoked voltage response and the effects of blockade of $I_{\text{h}}$ and $I_{\text{Kir}}$. Bipolar cells were first established in voltage clamp at the holding potential of $-70$ mV. Recordings were switched to current clamp. This usually resulted in a small negative holding current as indicated. Membrane hyperpolarization was elicited by a 400-ms current pulse to $-15$ pA (indicated by a solid line).

A: 2 typical voltage responses from RBCs to the negative current injection in control (black traces) and after application of $50 \mu M$ ZD 7288 (gray traces).

B: 2 typical voltage responses recorded from the CBCs that expressed mainly $I_{\text{Kir}}$ to the negative current injection in control (black traces) and after application of $50 \mu M$ Ba$^{2+}$ (gray traces).

C: Voltage responses recorded from the CBCs that expressed both $I_{\text{h}}$ and $I_{\text{Kir}}$ to the negative current injection in control (black traces) and after application of $50 \mu M$ Ba$^{2+}$ (gray traces, left) or $50 \mu M$ ZD 7288 (gray traces, right).

D: 2 typical voltage responses recorded from the CBCs that expressed almost no hyperpolarization-activated currents.
consistent with the property of \( I_h \) reported in many other systems (Khakh and Henderson 1998; Lamas 1998; Tabata and Isida 1996). Although the expression of \( I_h \) in mammalian bipolar cells was expected since \( I_h \) has been previously reported in rat RBCs (Karschin and Wässle 1990), the distinct biophysical and pharmacological properties of the two components of the hyperpolarization-activated currents shown in this study clearly demonstrate that bipolar cells of different subtypes differentially express two types of hyperpolarization-activated currents (see Discussion below). In addition, the high selectivity of \( \text{Ba}^{2+} \) and \( \text{ZD 7288} \) on antagonizing \( I_{\text{Kir}} \) and \( I_h \), respectively, assessed by this study provides a pharmacological basis for investigating the possible roles of these conductances in bipolar cell functions. It is worth noting here that a previous study reported a huge hyperpolarization-activated \( \text{Cl}^- \) current in rat RBCs (Enz et al. 1999). We did not observe such current under our recording conditions (see Methods). In fact, the magnitude of the hyperpolarization-activated inward current observed in RBCs in this study are comparable to that reported by Karschin and Wässle (1990).

**Differential expression of \( I_{\text{Kir}} \) and \( I_h \) in bipolar cells**

Results of this study indicate a differential expression of \( I_{\text{Kir}} \) and \( I_h \) among different subtypes of bipolar cell. First, the properties of the hyperpolarization-activated currents are different between RBCs and CBCs. Second, the currents observed among CBCs are heterogeneous. We observed two groups of CBCs that expressed substantial hyperpolarization-activated currents, one that expresses mainly \( I_{\text{Kir}} \) and the other that expresses both \( I_{\text{Kir}} \) and \( I_h \). In addition, we observed a subset of CBCs that displayed no or only small hyperpolarization-activated currents. At least nine subtypes of CBCs have been described in the rat retina (Euler and Wässle 1995; Hartveit 1997). Further study will be required to determine the correspondence between these subtypes and their expression of \( I_{\text{Kir}} \) and \( I_h \).

This study indicates that there are differences, not only in the expression pattern of the two currents, but also in the expression level and biophysical properties among bipolar cells. In the group of CBCs that express both currents, \( I_h \) is much larger than \( I_{\text{Kir}} \). In addition, \( I_h \) of this group of CBCs is different from the \( I_h \) of RBCs in two major aspects. First, the current density of \( I_h \) in this group of CBCs is significantly larger than that in RBCs. Second, the activation kinetics of the \( I_h \) in these CBCs is different from that of RBCs. The time course of the \( I_h \) evoked by a test step to \(-120 \text{ mV}\) in RBCs can be well fitted by a single exponential with an average time constant around 380 ms, whereas that of the \( I_h \) in these CBCs under the same conditions has to be fitted by the sum of two exponentials with time constants of approximately 130 ms and 1 s, respectively. Four types of genes, termed HCN1–4, encoding \( I_h \) have been cloned (Robinson and Siegelbaum 2003). The recombinant channels have been reported to show different activation kinetics ranging from tens of milliseconds to several seconds (Ishii et al. 2001; Santoro et al. 2000). Furthermore, we observed an additional extremely slow component of the current in RBCs. The kinetics of this slow component does not appear to match the kinetics of any of the known HCN subunits (Robinson and Siegelbaum 2003). Nevertheless, our results suggest that \( I_h \) in RBCs and CBCs differs not only in the expression level but also likely in the molecular composition. Consistent with this finding is the preliminary report a differential expression of HCN channels among mammalian bipolar cells (Müller et al. 2001).

The magnitude and the expression pattern of hyperpolarization-activated currents among RBCs and two groups of CBCs observed in isolated cells and retinal slices were largely similar. However, a slight difference was noted regarding the group of CBCs that express mainly \( I_{\text{Kir}} \), i.e., a small time-dependent current was observed in retinal slice recordings (see Fig. 1A) but was usually absent in isolated cell recordings. This discrepancy may be due to the localization of \( I_h \) in the axon terminals of these CBCs. Thus the current would not be detected in the majority of the isolated cell recordings because most of the axon terminals of CBCs were lost during the dissociation (Pan 2000; also see Methods). This raises the possibility that hyperpolarization-activated currents could be localized in different cellular regions for targeting specific functions (Robinson and Siegelbaum 2003).

Taken together, our findings provide evidence for the heterogeneous expression of two types of hyperpolarization-activated currents, \( I_{\text{Kir}} \) and \( I_h \), among different bipolar cells. Heterogeneous properties of hyperpolarization-activated currents have been observed previously in lower vertebrates (Connaughton and Maguire 1998; Lasater 1988). In particular, differential expression of \( I_h \) and a time-independent current has been reported in two morphologically different bipolar cells in fish, although the latter did not appear to be \( I_{\text{Kir}} \) (Lasater 1988). Thus the differential expression of multiple types of hyperpolarization-activated currents among different bipolar cell subtypes may be a general property of the vertebrate retina.

**Functional implications**

The differential expression of \( I_{\text{Kir}} \) and \( I_h \) among different subtypes of bipolar cells implies a potential functional significance. Bipolar cells of different types in lower vertebrates as well as in mammals have been shown to display diverse light response properties (Awatramani and Slaughter 2000; Berntson and Taylor 2000; Euler and Masland 2000). Intrinsic membrane conductances have been shown to contribute to the light response properties of bipolar cells (Pretti et al. 2000). In this study, we investigated the possible roles of these two currents in bipolar cell response properties. Our results indicate that \( I_h \) and \( I_{\text{Kir}} \) can influence the voltage response of bipolar cells. First, although both \( I_h \) and \( I_{\text{Kir}} \) counteracted excessive membrane hyperpolarization, they exhibited the effect with distinct kinetics. Bipolar cells that express \( I_h \) exhibit a delayed reaction to membrane hyperpolarization. Thus a transient hyperpolarization overshoot was observed in these cells with negative current injection. Such a property has been described in retinal bipolar cells and ganglion cells (kaneko and Tachibana 1985; Tabata and Ishida 1996). However, we showed in this study that such an overshoot was not observed in bipolar cells that express \( I_{\text{Kir}} \). It is interesting to note that light-evoked hyperpolarizing overshoot has been reported in some bipolar cells, although the underlying mechanism remains unknown (Matsui et al. 2001). Second, these two currents exhibit distinct effects on membrane potential depolarization. As demonstrated previously in retinal ganglion cells (Tabata and Ishida 1996), the presence of \( I_h \) facilitates or accelerates the membrane response.
potential rebound from the hyperpolarization. In contrast, the presence of $I_{\text{Kir}}$ counteracts or prevents such rebound depolarization. Thus the differential expression of $I_h$ and $I_{\text{Kir}}$ in bipolar cells could affect the rising time of light response, albeit in opposite manners. In addition, these results support the idea that these two currents serve to regulate the membrane potential at two different levels, as has been reported in other systems (Doan and Kunze 1999; Lamas 1998).

However, further studies in more physiological conditions would be required to examine the functional significance of these currents in bipolar cells. First, the differential expression of $I_{\text{Kir}}$ and $I_h$ is demonstrated in this study mostly using a test potential to $-120$ mV. The significance for the effect of these currents on the voltage response would be expected to be dependent on the physiological potential range of bipolar cells. Particularly, our results showed that significant $I_{\text{Kir}}$ was observed only at membrane potentials below the $K^+$ reversal potential. Although membrane pumps and transporters could drive the membrane potential below the $K^+$ reversal potential or an alteration in extracellular $K^+$ concentration could shift the activation voltage range of $I_{\text{Kir}}$, the question remains as to what extent these currents are activated in bipolar cells in vivo. The dark or resting membrane potentials of mammalian bipolar cells have been reported to range from $-60$ to $-25$ mV (Berntson and Taylor 2000; Euler and Masland 2000; Gillette and Dacheux 1995; Kaneko et al. 1989; Karschin and Wässle 1990), although a wider range has been reported in lower vertebrates (Connaughton and Maguire 1998; Tessier-Lavigne et al. 1988). Thus the hyperpolarization-activated currents, especially $I_{\text{Kir}}$, are likely to be small in the physiological potential range of bipolar cells. However, it is important to point out that, although $I_{\text{Kir}}$ appeared to display only a tiny outward rectification (see Fig. 4E), the presence of this current noticeably affected the overshoot of the voltage response (Fig. 7, B and C). Likely, this is because the input resistance of bipolar cells is high (Tessier-Lavigne et al. 1988) so that even a tiny current could significantly influence their voltage response properties. Furthermore, the hyperpolarization-activated currents have been reported to be regulated by many endogenous factors as well as by temperature (Cuevas et al. 1997; Isomoto et al. 1997; Rape 1996). Therefore it is possible that the properties of these currents may be altered by our recording at room temperature in vitro.

In summary, this study demonstrated the expression of $I_{\text{Kir}}$ and the differential expression of $I_h$ and $I_{\text{Kir}}$ in mammalian bipolar cells. The results of this study suggest that the differential expression of $I_h$ and $I_{\text{Kir}}$ may contribute to the diversity of the response properties of bipolar cells. Consistently, $I_h$ has been reported to affect the response properties of other retinal neurons such as photoreceptors (Demontis et al. 1999; Kawai et al. 2002), horizontal cells (Dong and Wherlin 1995), and ganglion cells (Tabata and Ishida 1996). Moreover, $I_h$ and $I_{\text{Kir}}$ have been proposed to play roles in a variety of other neuronal functions, such as in the regulation of ionic homeostasis, resting membrane potential, cell conductance, dendritic integration, and synaptic transmission (Hille 1992; Rape 1996; Robinson and Siegelbaum 2003). Therefore further studies will be important to examine their possible roles in these other physiological properties of bipolar cells.

We thank Drs. R. Pourcho and D. Pieper for comments on the manuscript.

**DISCLOSURES**

This research was supported by National Institutes of Health Grant EY-12180 and Core Grant EY-04068.

**REFERENCES**


**HOLT JR and Eatock RA. Inwardly rectifying currents of sacular hair cells from the leopard frog. *J Neurophysiol* 73: 1484–1520, 1995.**
Differential expression of $I_{\text{Kir}}$ and $I_{\text{H}}$ in retinal bipolar cells


