Report


Ih Without Kir in Adult Rat Retinal Ganglion Cells

Sherwin C. Lee1 and Andrew T. Ishida1,2
1Section of Neurobiology, Physiology, and Behavior and 2Department of Ophthalmology and Vision Science, University of California, Davis, California

Submitted 27 November 2006; accepted in final form 14 February 2007

Lee SC, Ishida AT. Ih without Kir in adult rat retinal ganglion cells. J Neurophysiol 97: 3790–3799, 2007; doi:10.1152/jn.01241.2006. Antisera directed against hyperpolarization-activated mixed-cation (“Ih”) and K+ (“Kir”) channels bind to some somata in the ganglion cell layer of rat and rabbit retina. Additionally, the termination of hyperpolarizing current injections can trigger spikes in some cat retinal ganglion cells, suggesting a rebound depolarization arising from activation of Ih. However, patch-clamp studies showed that rat ganglion cells lack inward rectification or present an inwardly rectifying K+ current. We therefore tested whether hyperpolarization activates Ih in dissociated, adult rat retinal ganglion cell somata. We report here that, although we found no inward rectification in some cells, and a Kir-like current in a few cells, hyperpolarization activated Ih in roughly 75% of the cells we recorded from in voltage clamp. We show that this current is blocked by Cs+ or ZD7288 and only slightly reduced by Ba2+, that the current amplitude and reversal potential are sensitive to extracellular Na+ and K+, and that we found no evidence of Kir in cells presenting Ih. In current clamp, injecting hyperpolarizing current induced a slowly relaxing membrane hyperpolarization that rebounded to a few action potentials when the hyperpolarizing current was stopped; both the membrane potential relaxation and rebound spikes were blocked by ZD7288. These results provide the first measurement of Ih in mammalian retinal ganglion cells and indicate that the ion channels of rat retinal ganglion cells may vary in ways not expected from previous voltage and current recordings.

INTRODUCTION

Increases and decreases in the spiking of retinal ganglion cells signal variations in visible light. Over the range of voltages traversed by these spikes (Baylor and Fettiple 1977; Meister and Berry 1999; Wiesel 1959; Zaghoul et al. 2003), changes in the gating of several ion currents have been found and the kinetics of at least some of these changes are suitable for rendering spiking a malleable rather than static process. Among these changes are fast and slow inactivation of high-threshold current after depolarizations and priming of low-threshold currents by hyperpolarizations (Berry and Meister 1998; Eng et al. 1990; Hayashida and Ishida 2004; Hidaka and Ishida 1998; Kaneda and Kaneko 1991; Karschin and Lipton 1989; Kim and Rieke 2001; Lee et al. 2003). Other effects of hyperpolarization on ganglion cells are known, but descriptions of the ion channels gated by these voltage changes have been unclear in a number of ways. Initial recordings showed that moderate hyperpolarizations activate inward voltage rectification in axons (Eng et al. 1990) and a mixed-cation current (Ih) in somata (Tabata and Ishida 1996) of rat and goldfish retinal ganglion cells, respectively. Anatomical studies subsequently found that somata in the ganglion cell layer of rat retina bind antisera directed against Ih and inward rectifier K+ (Kir) channels (Chen et al. 2004; Ishii et al. 2003; Ivanova and Muller 2006; Muller et al. 2003; Tian et al. 2003). Although these studies suggest that more than one channel type may be activated by hyperpolarization, it is unknown whether individual ganglion cells possess both Ih and Kir channels or whether Ih and Kir occur only in different cells. Interestingly, no inward rectification was seen in large rat retinal ganglion cell somata (Reiff and Guenther 1999; see also Akamine et al. 2002; O’Brien et al. 2002) and the only inwardly rectifying current characterized in any mammalian retinal ganglion cell to date is a rapidly activating, Ba2+-sensitive Kir-like current observed during large hyperpolarizations or in elevated extracellular K+ levels (Chen et al. 2004).

Because these observations differ from the slowly activating, Cs+-sensitive mixed-cation current originally inferred on the basis of voltage recordings from rat optic nerve (Eng et al. 1990), we tested for the presence of Ih in adult rat retinal ganglion cells. We report here that 1) in perforated-patch whole cell recordings, Ih can be activated in some of these cells during moderate hyperpolarizations at a physiological extracellular K+ concentration (Ames and Nesbett 1981); 2) we found no evidence of Kir in cells that possessed Ih; 3) a Kir-like current can be activated in other cells under identical recording conditions; and 4) still other cells displayed neither Ih nor Kir. These results provide the first identification of different ion currents activated by hyperpolarization in retinal ganglion cells of a single species and the first measurement of Ih in mammalian retinal ganglion cells.

METHODS

Animals

Adult rat retinas were used for the experiments reported here to compare our results with those of previous anatomical and electrophysiological studies (Chen et al. 2004; Eng et al. 1990; Ishii et al. 2003; Ivanova and Muller 2006; Muller et al. 2003; Reiff and Guenther 1999; Tian et al. 2003). Long–Evans rats (female, P60–P120, 150–250 g) were obtained from a commercial supplier (Harlan Bioproducts, San Diego, CA) and housed in standard cages at about 23°C on a 12-h/12-h light/dark cycle. All animal care and experimental protocols were approved by the Animal Use and Care Administrative Advisory Committee of the University of California, Davis.

Cell dissociation and panning

The retinal ganglion cells used in this study were dissociated by a combination of standard protocols and methods developed in our laboratory (e.g., Hayashida et al. 2004; Vaquero et al. 2001). Each rat
used was killed by an overdose of pentobarbital. Sources of the reagents used are listed below. Briefly, retinas were isolated from two freshly enucleated eyes and cells on the distal side of these retinas (mostly photoreceptors) were manually sliced off with a razor. The remaining retinal tissue was incubated for 5–10 min at 30°C in a 5-ml plastic tube containing a papain solution [16 U/ml papain in low-Ca\(^{2+}\) solution mixed 1:1 with L-15 culture medium that was supplemented with 1 μM tetrodotoxin (TTX)]. The low-Ca\(^{2+}\) solution contained (in mM): 140 sucrose, 2.5 KCl, 70 CsOH, 20 NaOH, 1 NaHPO\(_4\), 15 CaCl\(_2\), 20 EDTA, 11 d-glucose, 15 HEPES, 0.1 glutathione, 1 kynurenic acid, 0.001 TTX, and 0.025 mg/ml DNase I. The estimated free Ca\(^{2+}\) concentration was 100–200 nM. To inhibit the papain activity, the enzyme solution was replaced with ovomucoid solution (0.5 mg/ml ovomucoid in low-Ca\(^{2+}\) solution mixed 1:1 with L-15 supplemented with 1 μM TTX and 0.025 mg/ml DNase I) for 5 min at room temperature. The retinal tissue was rinsed a few times with fresh L-15 medium (supplemented with 0.025 mg/ml DNase I, pH 7.2–7.3) and triturated. Supernatant was layered over fresh L-15 medium in a 5-ml plastic tube (10 mm ID) and allowed to sit for 20 min. The top 1–2 cm of this solution was then discarded and the remaining solution, except for undissociated retinal pieces at the tube bottom, was transferred to an empty 5-ml plastic tube.

We isolated retinal ganglion cells from the final cell suspension by a “panning” method based on the expression of Thy1 (Barres et al. 1988) and nearly all cells were checked for the presence of voltage-gated Na\(^+\) current and/or large spikes to verify that they were ganglion cells (Barres et al. 1988; Boos et al. 1993). We prepared panning dishes by cutting a 13-mm hole in the bottom of 35-mm plastic tissue culture dishes and attaching a glass coverslip with Sylgard 184. The upper side of each coverslip was coated with goat anti-mouse IgM (diluted 1:200 in 0.1 M Tris, pH 9.5) for 2 h at 30°C. After three PBS rinses, the dishes were incubated with anti-Thy1 antibody for an additional 2 h at room temperature, rinsed again with PBS, followed by one final rinse with L-15.

Retinal ganglion cells were panned by placing several drops of the final cell suspension onto the prepared glass area of these culture dishes. After allowing cells to settle for 30 min at 30°C, nonadherent cells were removed from the dishes by rinsing each dish three times with L-15. The dishes were then filled with culture medium (L-15 medium; supplemented with 1% B-27; pH was adjusted to 7.2–7.3 with HCl). The cells were stored at 30°C for 12–16 h and the culture medium was replaced once more before electrophysiological recordings.

**Recording configuration and solutions**

The recordings presented here were performed in perforated-patch whole cell mode at room temperature (21–23°C) using methods we described previously (e.g., Hayashida and Ishida 2004; Hidaka and Ishida 1998; Lee et al. 2003; Tabata and Ishida 1996; Vaquero et al. 2001). Patch electrodes were pulled from borosilicate glass capillaries (#64–0786; Warner Instruments, Hamden, CT) to tip resistances of about 2–4 MΩ. Amphotericin B was included in the recording electrode-filling solution as the perforating agent (200 μg/ml, with 100 or 300 μg/ml Pluronic F-127) and data were collected after the series resistance fell to <100 MΩ. The recording electrode solution contained (in mM): 115 K-d-glucuronate acid, 15 KCl, 15 NaOH, 2.6 MgCl\(_2\), 0.34 CaCl\(_2\), 1 EGTA, and 10 HEPES; the pH of this solution was adjusted with methanesulfonic acid to 7.4. The extracellular solution mixed 1:1 with L-15 culture medium that was supplemented with 1 μM tetrodotoxin (TTX), the low-Ca\(^{2+}\) solution contained (in mM): 145 NaCl, 3.5 KCl, 2.5 CaCl\(_2\), 1.0 MgCl\(_2\), 10 d-glucose, and 5 HEPES; the pH of this solution was adjusted with NaOH to 7.4. Osmolalities of the extracellular and recording electrode solutions were 290–310 and 260–280 mmol/kg, respectively. To apply reagents under uniform conditions, saline was continuously superfused over each cell recorded from, through a hole (ID = 500 μm) at the bottom of a U-shaped Teflon tube. By changing the solution reservoir feeding the top of this U-tube, effects of pharmacological agents or of altered extracellular ion concentrations could be measured at the control flow rate (200 μl/min). The extracellular solution was grounded by an agar bridge. The membrane potentials reported here were corrected for liquid junction potentials due to measured differences between the extracellular and patch electrode-filling solutions. For recordings in control bath solution, the correction was made by adding −13 mV to the voltages in the current- and voltage-clamp protocols. For those initiated in low-Na\(^{+}\) solutions, the correction was −20 mV. No correction was made for the 1-mV difference between the bath solutions containing different K\(^{+}\) concentrations.

An Axopatch-1D patch-clamp amplifier (Axon Instruments, Union City, CA) and pCLAMP software (v. 8.20.224; Axon Instruments) were used to generate voltage jumps, inject constant current, and acquire data. In voltage-clamp mode, the current monitor output of the amplifier was analog-filtered by the built-in four-pole Bessel filter (fc = 0.5 or 1 kHz) and digitally sampled (usually at 2 kHz). Current traces are displayed without leak subtraction, signal averaging, or post hoc filtering and series resistance compensation was used to check for voltage errors in current amplitudes. When recording spikes, the voltage monitor output was analog-filtered at either 2 or 5 kHz and digitally sampled at 10 kHz.

Data were analyzed in pCLAMP. Three standard equations were routinely used in these analyses. First, the sum of two exponential time functions for fitting current traces

\[
A_x e^{-t/v_1} + A_y e^{-t/v_2} + C
\]

where \(A_x\) is the maximum amplitude of component \(x\), \(t\) is time, \(v_x\) is the time constant for the change in \(A_x\), and \(C\) is an offset constant. Second, a Boltzmann equation

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

where \(I_{max}\) is the maximum current amplitude, \(V\) is the test potential (in mV), \(V_{1/2}\) is the test potential that activates half of the maximum conductance, and \(s\) is a slope factor; Boltzmann fits were made using a least-squares nonlinear curve-fitting routine in SigmaPlot (v. 8.02a, SYSTAT Software, Richmond, CA). Third, a Goldman–Hodgkin–Katz (GHK) equation

\[
E_i = (RT/F)ln(P_{Na}[K] + P_{K}[Na]/P_{Na}[K] + P_{K}[Na])
\]

where \(E_i\) is the reversal potential; \(R\), \(T\), and \(F\) have their usual thermodynamic meanings; \(P_j\) denotes the membrane permeability to \(X\); and the concentrations of \(X\) in the recording bath and recording pipette solutions are used as \([X]\), and \([X]_r\), respectively. All means are presented as ± SE.

**Reagents**

Reagents were obtained from the following sources: Abbott Laboratories (North Chicago, IL): sodium pentobarbital (#0074-378-05); Aldrich Chemical (Milwaukee, WI): d-glucuronate acid, potassium salt (#86037–9), methanesulfonic acid (#47135–6); BDH Laboratory Supplies (Poule, UK): CaCl\(_2\); Calbiochem (San Diego, CA): tetrodotoxin (#584411); Dow Corning (Midland, MI): Sylgard 184; Fluka (Milwaukee, WI): EGTA (#03777); Invitrogen (Carlsbad, CA): B-27 (#280–0954), TTX (#584411); Meta Bioscience (Milwaukee, WI): DNase I (#D4527), HEPES (#H-4034), ovomucoid trypsin inhibitor (#O2841), 0.5% Pluronic F-127 (#P6867); Sigma Chemical (St. Louis, MO): alamethicin B (#A-4888), bovine serum albumin (#A7284), DMSO (#317275), RNase I (#R0425), HEPES (#H-4034), ovomucoid trypsin inhibitor (#584411); Dow Corning (Midland, MI): Sylgard 184; Fluka (Milwaukee, WI): EGTA (#03777); Invitrogen (Carlsbad, CA): B-27 (#280–0954), TTX (#584411); Meta Bioscience (Milwaukee, WI): DNase I (#D4527), HEPES (#H-4034), ovomucoid trypsin inhibitor (#584411); Tocris Bioscience (Ellisville, MO): 4-(N-ethyl-N-phenylaminio)-1,2-dimethyl-6-(methylamino) pyrimidinium chloride (ZD7288, #1000); Worthington, (Freehold, NJ): papain (#3126). The salts (NaCl, etc.) used for electrophysiological recordings and
buffers were all reagent grade and obtained from Sigma unless otherwise specified.

RESULTS

This study tests whether the inwardly rectifying, mixed-conduction current known as $I_{h}$ and the inwardly rectifying, K\(^+\) current known as $I_{K_{r}}$ can be activated in mammalian retinal ganglion cells. For this purpose, we characterized pharmacological and biophysical properties of the ion current activated by hyperpolarization in adult rat retinal ganglion cell somata, and we did so under conditions constrained in four ways. First, we used adult rats as in recent immunostaining studies (e.g., Ivanova and Müller 2006; Müller et al. 2003) because inwardly rectifying current types and densities were previously found to change with age in other preparations (e.g., Bayliss et al. 1994; Hogg et al. 2001; Tanaka et al. 2003). Second, the control external K\(^+\) concentration was set to 3.5 mM, to match the K\(^+\) concentration in solutions used to perfuse retinas during electrophysiological studies (Ames and Nesbitt 1981; Newman and Bartosch 1999; Weinstein et al. 1967) and because the gating kinetics of $I_{h}$ (and the amplitude of $I_{h}$ and $I_{K_{r}}$) were found to differ at higher K\(^+\) concentrations (Halliwell and Adams 1982). Third, all recordings were made in perforated-patch mode (see METHODS) because second messengers modulate inwardly rectifying currents in various preparations including retinal photoreceptor, horizontal, and Müllor cells (Akopian and Witkovsky 1996; Demontis et al. 2002; Dixon and Copenhagen 1997; Kusaka and Puro 1997; Puro et al. 1996; Solessio et al. 2001) and we sought to minimize perturbation of the intracellular milieu. Fourth, currents were recorded at room temperature to compare with results of previous studies (Chen et al. 2004; Ma et al. 2003; Tabata and Ishida 1996).

Different conductances were detectable

Under these conditions, we observed three conductances when cells were hyperpolarized from a holding value of $-73$ mV to test potentials between $-83$ and $-123$ mV. In several cells, the clamp current changed in amplitude without delay after the voltage steps; the amplitude did not change during voltage steps as long as 4 s; and the amplitude increased linearly with test potential (Fig. 1A). These resemble time-independent currents in previous recordings from postnatal rat retinal ganglion cells (Akamine et al. 2002; Reiff and Guenther 1999) and were not routinely examined here. In a very small number of other cells, the current amplitude also showed no time dependence after either the onset or termination of voltage steps, although the slope conductance increased considerably as the test potential was made more negative than $-93$ mV (Fig. 1B). Low concentrations of Ba\(^{2+}\) (50–100 \(\mu\)M) blocked this rectification (Fig. 1B), indicating it is likely to be $I_{K_{r}}$ (Hagihara et al. 1978). Aside from finding only an ohmic current (that is, a voltage- and time-independent conductance) during this block, we could not study this conductance further because we encountered it in only two of the cells we recorded from over the course of several months. In the remainder of the cells we recorded from, the whole cell current presented a slowly gating component (Fig. 1C) due to $I_{h}$ as described below.

Ba\(^{2+}\)-resistant, Cs\(^+\)-sensitive current

$I_{h}$ can be reduced to negligibly small amplitudes by Cs\(^+\) (1–5 mM) and ZD7288 (1–100 \(\mu\)M) (Dickson et al. 2000; Santoro and Tibbs 1999) and it resists blockade by 2 mM Ba\(^{2+}\) (Ludwig et al. 1998). Because even smaller amounts of Ba\(^{2+}\) (1–5 mM) and ZD7288 (1–100 \(\mu\)M) blocked $I_{K_{r}}$ (see above), because some neurons possess both $I_{h}$ and $I_{K_{r}}$ (Dickson et al. 2000; Hogg et al. 2001; Ma et al. 2003; Scroggs et al. 1994), and given the results of previous ganglion cell studies (Chen et al. 2004; Eng et al. 1990; O’Brien et al. 2002; Tabata and Ishida 1996), we first characterized the slowly gating current by sequentially applying Ba\(^{2+}\) and Cs\(^+\) to individual cells. Figure 1C shows the whole cell current activated by voltage steps from a holding potential of $-73$ mV to test potentials ranging from $-83$ to $-123$ mV in 10-mV decrements, during superfusion of control saline (Fig. 1C), saline supplemented with 1 mM Ba\(^{2+}\) (Fig. 1C), saline supplemented with 1 mM Ba\(^{2+}\) and 2 mM Cs\(^+\) (Fig. 1C), control saline (Fig. 1C), and pre-Cs\(^+\) blocked current from A,B,C, respectively. Note in B,C that $I_{h}$ blocked current that is both inwardly rectifying and rapidly gating in B, $I_{h}$ blocked the inwardly rectifying and slowly gating current in C (C), and pre-Cs\(^+\) amplitude and kinetics were recovered almost completely by washing with control solution (C).
plemented with 1 mM Ba\(^{2+}\) plus 2 mM Cs\(^+\) (Fig. 1C\(_{1}\)), and thereafter, control saline again (Fig. 1C\(_{4}\)). In control saline, after the onset of each hyperpolarizing step, the whole cell current increased in amplitude instantaneously and then continued to increase gradually. During small hyperpolarizations (i.e., to test potentials less negative than about \(-100\) mV), the current did not stop increasing in amplitude even if the test pulse duration was as long as 4 s. During larger hyperpolarizations (e.g., to \(-113\) and \(-123\) mV), the current increased instantaneously, rose to a peak amplitude within 1 s, and pulse duration was as long as 4 s. During larger hyperpolarizations the current did not stop increasing in amplitude even if the test hyperpolarization took place. The onset and termination of the test hyperpolarizations in the holding level within 5–10 s. In a few cells, this deactivation appeared to be incomplete even after 10 s; in these cases, \(\geq 25\) s were allowed to elapse between successive test pulses. Slowly gating, hyperpolarization-activated currents with these general properties were observed in roughly 75% of the cells from which we recorded (\(n = 120\)).

At 1–2 mM Ba\(^{2+}\) reduced the total current when the test hyperpolarizations activated a slow component. However, the Ba\(^{2+}\)-sensitive portion did not have the same fast onset and voltage dependence as K\(_{ir}\) (not shown) and the reduction was slight (Fig. 1C\(_{2}\)), unlike the suppression of rapidly gating, inward rectifier current noted earlier (Fig. 1B\(_{2}\)). For example, during hyperpolarizations from \(\sim 83\) to \(-123\) mV, 1 mM Ba\(^{2+}\) reduced the total current amplitude by 13 \(\pm\) 3% (mean \(\pm\) SE, \(n = 4\)) and the remaining (Ba\(^{2+}\)-resistant) current gated slowly and rectified inwardly like the control current (Fig. 1C\(_{2}\)). By contrast, subsequent addition of 2 mM Cs\(^+\) markedly reduced the remaining current (Fig. 1C\(_{3}\)), especially by suppressing the slowly activating and deactivating current (see following text). The ability of Cs\(^+\) to block the slowly gating current did not require previous or concurrent exposure to Ba\(^{2+}\), and Ba\(^{2+}\) produced little or no reduction of the total current when its application started only after current was blocked by Cs\(^+\) (not shown), as if Ba\(^{2+}\) was affecting a small portion of the Cs\(^+\)-sensitive current (see Ludwig et al. 1998). We address this possibility further, by use of a K\(_{ir}\)-sparking antagonist, after describing the effects of Cs\(^+\).

Cs\(^+\) reduced the whole cell current in four ways. First, it reduced the holding current (compare Fig. 1, C\(_{1}\), C\(_{2}\), and C\(_{3}\)). Digital subtraction of the current recorded before and during the Cs\(^+\) application (i.e., subtraction of the current recorded in Ba\(^{2+}\) and Cs\(^+\) from that recorded in Ba\(^{2+}\)) therefore yielded a net inward “Cs\(^+\)-blocked” current at the holding potential (see current trace at \(-73\) mV, Fig. 2A). Second, Cs\(^+\) reduced the amplitude of the instantaneous current steps seen at the onset and termination of the test hyperpolarizations (compare Fig. 1, C\(_{2}\) and C\(_{3}\)). This is shown by the instantaneous current steps at the onset and termination of the test hyperpolarizations in the Cs\(^+\)-blocked current traces (Fig. 1C\(_{3}\)). Third, it abolished the slowly activating current during the test hyperpolarizations, leaving only a time-independent current (Fig. 1C\(_{3}\)). Consistent with this, we found no effect of 1 mM Cs\(^+\) on currents that were ohmic in control solution; see Fig. 1A\(_{2}\). Fourth, Cs\(^+\) abolished the slowly deactivating “tail” current seen after terminating each test hyperpolarization, leaving a time-independent tail current (Fig. 1C\(_{3}\)). Thus the Cs\(^+\)-blocked current consisted of a standing inward current at the holding potential, an instantaneous component at the onset of the test hyperpolarization, a slowly activating component during the test hyperpolarization, and a slowly deactivating tail after shifting the voltage back to the holding potential (Fig. 1C\(_{3}\)).

The fraction of the whole cell current blocked by Cs\(^+\) was estimated by the ratio of the Cs\(^+\)-blocked current amplitude to the amplitude of the hyperpolarization-activated current in the control solution, with each of these amplitudes taken to be the difference between the mean current at the holding potential and the maximum steady-state current at the test potential. The block was calculated at a test potential of \(-123\) mV because the conductance was maximally activated at \(-123\) mV (see following text) and at a test potential of \(-103\) mV to check for voltage dependence. At 2 mM, Cs\(^+\) reduced the total current by \(79 \pm 4%\) at \(-123\) mV and by \(75 \pm 5%\) at \(-103\) mV (mean \(\pm\) SE, \(n = 6\)). A similar level of block was achieved with 1 mM Cs\(^+\) (81 \(\pm\) 7% at \(-123\) mV; 79 \(\pm\) 6% at \(-103\) mV; \(n = 3\)). Dividing the maximum amplitude of the Cs\(^+\)-blocked tail current by the cell capacitance yielded a current density of \(9.2 \pm 1.3\) pA/\(\mu F\) (mean \(\pm\) SE, for the \(n = 5\) cells used later for kinetics measurements; range in density: 4.2–12.3 pA/\(\mu F\); range in capacitance: 5.8–11.2 \(\mu F\); mean capacitance: 8.5 \(\mu F\)). There was no correlation between cell size and current density (not shown).

Current activation was gauged from the rate at which Cs\(^+\)-blocked current rose in amplitude when cells were hyperpolarized. The gradual increases in current (after the instantaneous increases) at test potentials between \(-93\) and \(-123\) mV were fitted by sums of two exponential time functions (see METHODS; Fig. 2A). These fits were not significantly improved by using the sum of three exponentials, but they were clearly poorer with one exponential (Fig. 2, C\(_{1}\) and C\(_{2}\)). Both the faster and slower time constants of the fitted functions decreased exponentially with voltage (closed symbols, Fig. 2E), ranging from 459 \(\pm\) 82 and 2,718 \(\pm\) 783 ms at \(-93\) mV to 105 \(\pm\) 10 and 726 \(\pm\) 122 ms at \(-123\) mV, respectively (mean \(\pm\) SE, \(n = 5\)); the faster component comprised 70 \(\pm\) 3% of these fits at \(-123\) mV and less at more positive test potentials (e.g., 50 \(\pm\) 13% at \(-93\) mV; mean \(\pm\) SE, \(n = 5\)). The decrease in the faster time constant is readily apparent from the increased rate of current rise as cells were hyperpolarized to more negative test potentials (Fig. 2A). The rate at which Cs\(^+\)-blocked current deactivated at the holding potential could also be fitted better by the sum of two exponential time functions in three of the cells from which we recorded; monoexponential fits were adequate in the other two cells (Maricq and Korenbrot 1990; Mayer and Westbrook 1983). In either case, we observed no dependence of the tail current time constants on the membrane potential at which the current was initially activated (open symbols, Fig. 2E). For example, after repolarizing to the holding potential (\(-73\) mV) from the test jump to \(-93\) mV, the faster time constant of the biexponential fits was 317 \(\pm\) 23 ms (mean \(\pm\) SE, \(n = 3\)); after repolarizing from \(-123\) mV, it was 328 \(\pm\) 6 ms. The slower time constant of these fits was 2,122 \(\pm\) 228 ms after the repolarizations from \(-93\) mV and 2,113 \(\pm\) 228 ms.
385 ms after the repolarizations from −123 mV. The fast deactivating component was 51 ± 4% of the biexponential fits and did not appreciably differ over the voltage range we examined. The average monoexponential decay constant (in the two cells noted above) was 1,163 ± 114 ms.

The range of membrane potentials that activated current (i.e., the “activation range”) was estimated from the amplitude of Cs⁺-blocked current immediately after each repolarization to the holding potential (i.e., after terminating each test hyperpolarization). These “tail” current amplitudes were back-extrapolated to the moment of repolarization, based on the time functions fitted to them and provided that the currents did not noticeably diverge from these functions near the moment of repolarization (compare Fig. 2, B and D). The tail current amplitude (Iₜᵢₙ, in pA) from each cell was then plotted against test potential and fitted with a Boltzmann equation (see Methods). The means of the test potential that activates half of the test current amplitude (V<textarea>max</textarea> (measured during the repolarization from −123 mV). ZD7288 and high [K⁺]<sub>ᵢₙ</sub> effects are consistent with Iₚ

ZD7288 is a bradycardiac agent that can block current through all of the cloned Iₚ channel types studied to date (Santoro and Tibbs 1999; Stieber et al. 2005) and abolish native Iₚ without blocking Kᵢₙ (e.g., Dickson et al. 2000; Hogg et al. 2001; Ma et al. 2003). Given this specificity, we tested whether ZD7288 blocked the slowly gating and inwardly rectifying type of current shown in Figs. 1C and 2. We did so in control saline (Fig. 3A) to assay for Iₚ in physiological concentrations of Na⁺ and K⁺, and after raising the extracellular K⁺ concentration from 3.5 to 38 mM (Fig. 3B) to double-check for the presence of Kₚ (by using conditions that increase its amplitude; Chen et al. 2004; Dixon and Copenhagen 1997; Hagiwara et al. 1978; Tachibana 1983). Figure 3A shows the current activated by voltage steps from a holding potential of −73 mM to test potentials between −78 and −98 mV during superfusion of control saline (Fig. 3A₁) and then control saline supplemented with 100 µM ZD7288 (Fig. 3A₂); the ZD7288-blocked current obtained by digital subtraction of these currents is shown at the right (Fig. 3A₃).

Figure 3B shows the current activated by voltage steps from a holding potential of −73 mM to test potentials between −68
and −98 mV during superfusion of control saline (Fig. 3B₁), high-K⁺ saline (Fig. 3B₂), and high-K⁺ saline supplemented with 100 μM ZD7288 (Fig. 3B₃). These panels show that ZD7288 blocks current with instantaneously and slowly gating components and that the whole cell current in the presence of ZD7288 is time independent both during and after the test voltage steps. Thus the ZD7288-sensitive current kinetically resembles the Cs⁺-sensitive current shown above. Moreover, Fig. 3B shows that when the extracellular K⁺ concentration was raised from 3.5 to 38 mM, the amplitude of the instantaneously rising component changed by relatively small amounts (compare amplitudes at the brackets in Fig. 3, B₁ and B₂), whereas the amplitude of the slowly activating component increased as much as eightfold during test hyperpolarizations [e.g., compare the slow increase in current at −98 mV in Fig. 3B₁ (58 pA) vs. that in Fig. 3B₂ (368 pA)].

Because we were able to measure current kinetics and voltage sensitivities in saline containing a physiological K⁺ concentration, we did not analyze the currents recorded in high-K⁺ medium. However, the tail currents in both normal and high-K⁺ medium were inward at the holding potential (−73 mV), even though the K⁺ equilibrium potential (calculated from the bath and electrode solutions) moved from −91 mV in normal saline (Fig. 3B₁) to −31 mV when the bath contained 38 mM K⁺ (Fig. 3B₂). This is consistent with previous findings that the activation range of Iₜ is unaltered by increases in extracellular K⁺ concentration (Hogg et al. 2001; Tabata and Ishida 1996; Wollmuth and Hille 1992) and this differs from the change in tail current polarity that would be expected for a K⁺-selective current under the same conditions.

As in other tissues (Harris and Constanti 1995; Stieber et al. 2005), current amplitude declined over the course of 5–10 min of continuous 100 μM ZD7288 application in all of the cells we tested (n = 23, i.e., n = 14 in control saline; n = 5 in high-K⁺ saline; n = 4 in low-Na⁺ saline, as shown below). The fraction of total current suppressed by 10 μM ZD7288 was nearly equivalent (n = 7), with slower onset; 2 μM ZD7288 had only a slight effect (n = 3) and 5 μM was intermediate (n = 3; e.g., Fig. 4). We did not routinely test for reversibility of this block because other studies found the block to reverse slowly (>1 h), if at all (e.g., Berger et al. 1994; Harris and Constanti 1995), and we found only very slight reversal of 100 μM ZD7288 after washing continuously with control saline for as long as 25 min.

**Tail current reversal at low [Na⁺]₀ is consistent with Iₜ**

The pharmacological properties, kinetics, and activation range described earlier suggest that Iₜ can be activated in rat retinal ganglion cell somata. To test further whether our protocols activated Iₜ, we measured the reversal potential of ZD7288-sensitive current. Because Iₜ is carried slightly better by K⁺ than by Na⁺ in solutions containing physiological concentrations of Na⁺ and K⁺, the reversal potential in control
saline would be expected to be more negative than 0 mV, but more positive than our holding potential. However, various K+ currents activate in ganglion cells at voltages more positive than −70 mV (unpublished observations; see also Lipton and Tauck 1987). Because part of this current defines suppression by K+ channel blockers (Lukasiewicz and Werblin 1988; Sucher and Lipton 1992), we looked for reversal of Ih by interchange of Na+ saline, it appears to have shifted even more in the positive direction. From this result and because the K+ permeability (“PNa/PK”) is estimated from these data to be 0.37 ± 0.02. In extracellular solution containing 10% of the normal Na+, the ZD7288-sensitive tail current was inward at −90 and −80 mV and nulled at around −70 mV (n = 2; result not illustrated). In normal saline, the ZD7288-sensitive tail current was inward at −83, −73, and −63 mV (n = 2; result not illustrated). Thus the null potential was more positive when the extracellular solution contained 10% of the control Na+ than when it contained 5% of the control Na+ and, in normal saline, it appears to have shifted even more in the positive direction. From this result and because the K+ equilibrium potential was unchanged (−91 mV) during all of these recordings, the ZD7288-sensitive current appeared to be carried by a mixture of Na+ and K+ ions, and not by K+ alone.

**Activation of Ih can elicit rebound spiking**

After activation, the slow deactivation of Ih produces depolarizations that drive various cells to spike threshold. To determine whether Ih could serve a similar role in mammalian retinal ganglion cells, we used a combination of voltage and current clamp on individual cells. Two examples of this are shown in Fig. 4 (A–C and D–F). Figure 4, A1 and D1 shows the total current during voltage steps like those used in Figs. 1 and 2. Switching to current clamp, we injected a small amount of current to hold the membrane potential at about the same level as we used in voltage clamp and verified we had a cell capable of firing action potentials by injecting a series of depolarizing current steps (Fig. 4, C1 and F1). We then injected constant-current steps that hyperpolarized this cell, at some point, to the

---

**FIG. 4.** Rebound spikes elicited by activation of Ih and block by ZD7288. Whole cell voltage and current clamp in 2 different cells (A–C and D–F). In all cases, panels with subscript 1 are control; panels with subscript 2 are in the presence of 100 μM (A–C) or 5 μM (D–F) ZD7288. In voltage clamp, the voltage protocol shown schematically at the top of A and D (steps from holding potential −73 to −68, −83, −93, −103, −113, and −123 mV) was used to activate Ih (A1, D1). After switching to current clamp, spiking was elicited by 0.5-s injections of depolarizing current (21 pA in C, 30 pA in F). Then, each cell received 0.5-s injections of hyperpolarizing current that produced characteristic activation of inward current and a depolarizing sag of the membrane potential (B1, E1). When the hyperpolarizing current step was turned off, membrane potential rebounded and elicited a short series of spikes (B2, E2). Blocking Ih by the introduction of ZD7288 removed the depolarizing sag and prevented rebound spikes (B2, E2). ZD7288 also produced an apparent increase in membrane resistance, leading to a negative shift in membrane potential at constant holding current and larger voltage deflections in response to the injection of hyperpolarizing current. For the 1st cell, a −60-pA step produced the voltage pattern in B1, whereas −28 pA was sufficient to induce a similar hyperpolarization in B2 after Ih was blocked. For the 2nd cell, the same −35-pA step was used in both E1 and E2, but hyperpolarization was much greater in the presence of ZD7288. Membrane resistance in the depolarizing direction was less affected and ZD7288 had minimal effect on spiking induced by depolarizing current steps (compare C1, C2 and F1, F2). Small residual hyperpolarization-activated current persists in D2 and E2 because 5 μM ZD7288 does not produce 100% inhibition of Ih. Arrowheads in A and D indicate 0 current. Initial membrane potential for each cell in current clamp was about −76 mV (B1, C1) and −74 mV (E1, F1).
voltages used in Fig. 4, A1 and D1. As is typical of \( I_h \), prolonged injection of hyperpolarizing current led to activation of inward current and a depolarizing sag of the membrane potential (Fig. 4, B1 and E1). When the hyperpolarizing current was turned off, the membrane potential rebounded and elicited a short series of spikes.

To demonstrate that the membrane potential sag and rebound spikes were a consequence of \( I_h \) activation, we applied ZD7288, at 100 μM to the first cell (Fig. 4, A–C) and 5 μM to the second (Fig. 4, D–F). This antagonist of \( I_h \) produced four characteristic changes: 1) it caused the membrane to hyperpolarize, suggesting that there is a small component of \( I_h \) active at our holding potential; 2) it removed the membrane potential sag observed during injections of hyperpolarizing current; 3) it increased the size of the membrane potential excursion elicited by the same hyperpolarizing current, suggesting that the membrane resistance is increased in this voltage range; and 4) it eliminated the rebound spikes (Fig. 4, B2 and E2). All of this occurred without significant effect on spikes elicited by depolarizing current steps (Fig. 4, C2 and F2). We then switched back to voltage clamp to show directly the block of \( I_h \) (Fig. 4, A2 and D2). Both voltage rectification and rebound spikes were blocked when ZD7288 blocked the slowly activating and deactivating current, as seen here \( (n = 8) \). Although block of \( I_h \) by 5 μM ZD7288 was not complete (note current activating during strong hyperpolarization in Fig. 4D2 and persisting voltage sag in Fig. 4E2), cells hyperpolarized 10 ± 1 mV and membrane resistance to hyperpolarizing current increased 25 ± 5% \( (n = 4) \) during application of 5 or 10 μM ZD7288. With 100 μM ZD7288, cells hyperpolarized 14 ± 4 mV and membrane resistance increased 52 ± 19% \( (n = 4) \).

**Discussion**

This study provides explicit measurements of \( I_h \) in mammalian retinal ganglion cell somata. None of our protocols activated \( K_{ir} \) in cells with measurable \( I_h \). Conversely, we were able to activate a \( K_{ir} \)-like conductance in a small number of cells and, after blocking this current with low concentrations of Ba\(^{2+}\), did not find \( I_h \). Last, several cells presented no detectable inward rectification. As discussed in the following text, these results begin to reconcile differences among previously published whole cell currents, somatic and axonal voltage recordings, and immunostaining patterns. The presence and properties of inwardly rectifying current also suggest a heterogeneity among adult rat retinal ganglion cell somata that is similar in some, but not all, respects to that reported for another widely studied mammalian species.

**General features of \( I_h \)**

Our finding of \( I_h \) fundamentally agrees with the inference that an \( I_h \)-like conductance produces voltage rectification in recordings from rat optic nerve (Eng et al. 1990) and with the finding that Cs\(^{+}\) blocks the slowly developing voltage rectification and hyperpolarization-activated, Ba\(^{2+}\)-resistant, inwardly rectifying current in goldfish retinal ganglion cell somata (Tabata and Ishida 1996). The properties we found—block by 2 mM Cs\(^{+}\) (Fig. 1), dose-dependent block by 2–100 μM ZD7288 (Figs. 3, A–C and 4), and a \( P_{SC}/P_{K} \) estimate of 0.37 in low-Na\(^{+}\) solution (Fig. 3C)—resemble those of \( I_h \) in other preparations (e.g., photoreceptors: Wollmuth and Hille 1992; brain: Dickson et al. 2000; Harris and Constanti 1995; peripheral neurons: Hogg et al. 2001; HCN1–4: Santoro and Tibbs 1999; Steiber et al. 2005). Furthermore, the activation kinetics and activation range of \( I_h \) did not differ significantly from those found in other retinal cells under similar conditions (e.g., Fig. 1 of Tabata and Ishida 1996; Fig. 3 of Demontis et al. 2002; Table 3 of Ivanova and Müller 2006).

Our current measurements show that the activation and deactivation time constants are larger than the membrane time constants reported for mammalian retinal ganglion cells (<50 ms in nearly all cell types; O’Brien et al. 2002; Robinson and Chalupa 1997). This suggests that \( I_h \) gates slowly enough to contribute to the delayed sag during inward voltage rectification seen in mammalian retinal ganglion cells and also to rebound depolarizations seen at the termination of even moderate hyperpolarizations (Eng et al. 1990; O’Brien et al. 2002). Our measurements from individual cells under both voltage and current clamp confirmed these possibilities (and our preliminary measurements of the temperature dependency of \( I_h \) gating indicates that this could also occur at body temperature). \( I_h \) differs from \( K_{ir} \) in this respect because the activation threshold of \( I_h \) is less negative and \( K_{ir} \) gates so rapidly that the voltage rectification it produces would not include either a slowly developing or decaying component (e.g., Dickson et al. 2000; Hogg et al. 2001; Ma et al. 2003). However, because marked inactivation has not yet been found in either conductance, both \( I_h \) and \( K_{ir} \) are likely to contribute to membrane resistance in the cells that express them. Our current and voltage measurements show that \( I_h \) could do so at voltages near typical resting potentials and at more negative voltages; \( K_{ir} \) would probably do so around the K\(^{+}\) equilibrium potential and at more negative voltages.

\( I_h \) in some, but not all, ganglion cells

All retinal ganglion cells are known to have high-threshold Na\(^{+}\), K\(^{+}\), and Ca\(^{2+}\) currents. Our results suggest that \( I_h \) differs from these depolarization-activated currents, in mammalian retinal ganglion cells, because we detected \( I_h \) in roughly 75% of the cells from which we recorded. We do not yet know whether only specific subtypes of rat retinal ganglion cells possess \( I_h \)—and, if so, which ones—because our dissociation protocol, like those used in all previous studies of which we are aware, shears the dendrites and axons off of ganglion cells (see Hayashida et al. 2004). Although it was consequently impossible for us to identify anatomical types of ganglion cells in our recordings, three electrophysiologically distinct cell types could account for the results we have presented here along with those of other laboratories.

One of these possesses \( I_h \). This would be consistent with the presence of \( I_h \) channel-like immunoreactivity in some somata in the ganglion cell layer of rat (Müller et al. 2003) and rabbit (Kim et al. 2003) retinas. A consistent feature of the cells possessing \( I_h \) is that, both in normal and high-K\(^{+}\) extracellular solutions, the whole cell current did not noticeably rectify at the voltages we tested in the presence of ZD7288. Also, the reversal potential of this current was sensitive to extracellular Na\(^{+}\) and, after reducing the total current by Cs\(^{+}\), we found no further effect of Ba\(^{2+}\). We conclude that we found no evidence of \( K_{ir} \) in any of the cells that displayed \( I_h \). Although compar-
ison of cells with \( I_h \) versus cells that are stained by antibodies directed against \( I_h \) channels might reveal further properties of \( I_h \), we refrained from attempting this with the data at hand for two reasons. First, we have no way of knowing whether the cells we recorded from included all morphological cell types in the rat retina. Second, the immunostaining data published to date do not show whether the cells stained are ganglion cells as opposed to displaced amacrine cells (cf. Perry 1981).

A second group of cells possesses \( K_r \). The absence of slow gating at the voltages that activate inwardly rectifying current (Fig. 1B), the agreement between the whole cell current reversal potential and the calculated \( K^+ \) equilibrium potential at various \( K^+ \) concentrations (see Fig. 5F of Chen et al. 2004), and the nonselective suppression of \( Ba^{2+} \)-resistant outwardly rectifying and inwardly rectifying current by \( Cs^+ \) (see Fig. 5D of Chen et al. 2004) are all consistent with the possibility that these cells lack \( I_h \). The absence of cells with detectable \( I_h \) in the latter study and the near absence of cells with \( K_r \) in the present study are consistent with the possibility that we recorded from subtypes of ganglion cells that, in almost all cases, differ from those studied by Chen et al. (2004). Whereas Thy-1 panning as used here could introduce a selection bias in some circumstances (Barres et al. 1988), it is unlikely to be the sole explanation of the dichotomy of our results, given that we observed a broader range of current types than Chen et al. (2004). Although our results do not exclude the possibility that \( I_h \) and \( K_r \) colocalize in some ganglion cells (Chen et al. 2004), we also do not have results that support this possibility. Moreover, the expression of \( I_h \) and \( K_r \) in different cell populations might explain why \( I_h \) isoforms were found in only some somata in the ganglion cell layer of rat and rabbit retina (Kim et al. 2003; Müller et al. 2003) and why several adjacent, or nearly adjacent, ganglion cell layer somata were stained by an anti-\( K_{ir} \) antibody in one study (see Fig. 3C of Chen et al. 2004), whereas no ganglion cell somata were stained in another study (see Fig. 1D of Tian et al. 2003).

A third set of cells lacks \( I_h \) and, more generally, inward rectification altogether. This would be consistent with the absence of time-dependent voltage rectification in intact cat retinal ganglion cells morphologically identified as the “a” type (O’Brien et al. 2002) and the absence of hyperpolarization-activated current in large-diameter, type “1” rat retinal ganglion cells (Reiff and Guenther 1999). At the same time, it remains to be seen to what extent this explains the exclusively ommatic currents we found in some cells (Fig. 1A). Although rat “1” cells may be anatomical homologs of cat “a” cells (e.g., Huxlin and Goodchild 1997), the largest ganglion cells in rat are thought to be comprised of at least two distinct subtypes (Peichl 1989). Because no methods are available to unequivocally distinguish these cell subtypes after cell isolations, we could not test whether rat “a” ganglion cells possess \( I_h \). Recording in situ was not a viable alternative because “a” ganglion cells are coupled by gap junctions to amacrine and other ganglion cells (e.g., Hidaka et al. 2004; Vaney 1991) and this would obscure the origin and detailed properties of inward rectification.

Recent recordings from intact cat retina show a similarly small number of substantial electrophysiological differences among a large number of anatomically distinct ganglion cell subtypes (O’Brien et al. 2002). These recordings resemble ours in that constant-current injections elicited no inward rectification in some retinal ganglion cells and a slowly developing, inward rectification as other cells were hyperpolarized to voltages more negative than −70 mV. However, a similarly delayed inward rectification was found in still other cells only if they were hyperpolarized beyond −90 mV. This differs from our results in suggesting that a slowly activating, inwardly rectifying current may have appreciably different activation thresholds in different cell types. Identifying the channel isoforms and properties that contribute to these differences and understanding why inward rectification is detectable in some cell types but not others remain to be determined by future studies.

Acknowledgments

We thank G. J. Partida for preparing the cell dissociates used in this study and A. Felipe Colado for preparing the figures.

Grants

This work was supported by National Eye Institute (NEI) Grant EY-08120 to A. T. Ishida and NEI Core Grant P30 EY-12576.

References


Dopamine receptor activation can reduce voltage-gated Na\(^+\) current by modulating both entry into and recovery from inactivation. *J Neurophysiol* 92: 3134–3141, 2004.


Variation in \(I_h\), \(I_{KA}\), and \(I_{LEAK}\) between acutely isolated adult rat dorsal root ganglion neurons of different size. *J Neurophysiol* 71: 271–279, 1994.


Transient and sustained depolarization of retinal ganglion cells by \(I_{h}\), \(I_{KOH}\), and \(I_{LEAK}\). *Vis Neurosci* 8: 171–176, 1992.


Intrinsic physiological Report

\(I_h\) OF RAT RETINAL GANGLION CELL SOMATA

3799


Tabata T, Ishida AT. Transient and sustained depolarization of retinal ganglion cells by \(I_{h}\), \(I_{KOH}\), and \(I_{LEAK}\). *Vis Neurosci* 8: 171–176, 1992.


