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I_h without K_{ir} in Adult Rat Retinal Ganglion Cells

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Abstract

Antisera directed against hyperpolarization-activated mixed-cation (“ I_h ”) and K^+ (“ K_{ir} ”) 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 due to activation of I_h . However, patch-clamp studies have reported that rat ganglion cells lack inward rectification, or present an inwardly rectifying K^+ current. We therefore tested whether hyperpolarization activates I_h in dissociated, adult rat retinal ganglion cell somata. We report here that while we found no inward rectification in some cells, and a K_{ir} -like current in a few cells, hyperpolarization activated I_h 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 Ba^{2+} , that the current amplitude and reversal potential are sensitive to extracellular Na^+ and K^+ , and that we found no evidence of K_{ir} in cells presenting I_h . 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 I_h 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.

Keywords

I_h ; inward rectification; voltage-gated current

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 Fettiplace 1977; Meister and Berry 1999; Wiesel 1959; Zaghloul 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 following 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 (I_h) 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 I_h and inward rectifier K^+ (K_{ir}) channels (Chen et al. 2004; Ishii et al. 2003; Ivanova and Müller 2006; Müller et al. 2003; Tian et al. 2003). While these studies suggest that more than one channel type may be activated by hyperpolarization, it is unknown whether individual ganglion cells possess both I_h and K_{ir} channels, or if I_h and K_{ir} only occur 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, Ba^{2+} -sensitive K_{ir} -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 I_h in adult rat retinal ganglion cells. We report here that (i) in perforated-patch whole-cell recordings, I_h can be activated in some of these cells during moderate hyperpolarizations at a physiological extracellular K^+ concentration (Ames and Nesbett 1981), (ii) we found no evidence of K_{ir} in cells that possessed I_h , (iii) a K_{ir} -like current can be activated in other cells under identical recording conditions, and (iv) still other cells displayed neither I_h nor K_{ir} . 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 I_h 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 Müller 2006; Müller 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 $\sim 23^\circ C$ on a 12-hr/12-hr 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 $30^\circ C$ 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 NaH_2PO_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 i.d.) 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 hr at 30 °C. After three PBS rinses, the dishes were incubated with anti-Thy1 antibody for an additional 2 hr 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, non-adherent 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 adjusted to 7.2-7.3 with HCl). The cells were stored at 30 °C for 12-16 hr, and the culture medium replaced once more prior to 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 have 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 approximately 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 below 100 MΩ. The recording electrode solution contained (in mM): 115 K-D-gluconic acid, 15 KCl, 15 NaOH, 2.6 MgCl₂, 0.34 CaCl₂, 1 EGTA, and 10 HEPES; the pH of this solution was adjusted with methanesulfonic acid to 7.4. The extracellular solution contained (in mM): 145 NaCl, 3.5 KCl, 2.5 CaCl₂, 1.0 MgCl₂, 10 D-glucose, and 5 HEPES; the pH of this solution was adjusted with NaOH to 7.4. The osmolality of the extracellular and recording electrode solutions were 290-310 mmol/kg and 260-280 mmol/kg, respectively. To apply reagents under uniform conditions, saline was continuously superfused over each cell recorded from, through a hole (~500 μm i.d.) 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 via 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 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. 8.2.0.224, 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 4-pole Bessel filter ($f_c = 0.5$ 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:

$$A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} + C$$

where A_x is the maximum amplitude of component x , t is time, τ_x is the time constant for the change in A_x , and C is an offset constant. 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 equation:

$$E_r = (RT/F) \ln \{ (P_K [K]_o + P_{Na} [Na]_o) / (P_K [K]_i + P_{Na} [Na]_i) \}$$

where E_r is the reversal potential; R , T , and F have their usual thermodynamic meanings; P_x denotes the membrane permeability to X ; and the concentration of X in the recording bath and recording pipette solutions are used as $[X]_o$ and $[X]_i$, respectively. All means are presented as $\pm$ SE.

Reagents

Reagents were obtained from the following sources: Abbott (Chicago, IL): sodium pentobarbital (#0074-378-05); Aldrich Chemical (Milwaukee, WI): D-gluconic acid, potassium salt (#86,037-9), methanesulfonic acid (#47,135-6); BDH Laboratory Supplies (Poole, 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 (#99-0254DG), L-15 (#41300-039); ICN (Aurora, OH): CsCl (#813061); Jackson Immuno Research (West Grove, PA): goat anti-mouse IgM (#115-005-020); Molecular Probes (Eugene, OR): Pluronic F-127 (#P6867); Sigma Chemical (St. Louis, MO): amphotericin B (#A-4888), bovine serum albumin (#A7284), DMSO (#317275), DNase I (#D4527), HEPES (#H-4034), ovomucoid trypsin inhibitor type 3-0 (#T2011); Tocris Bioscience (Ellisville, MO): 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 cation current known as " I_h " and the inwardly rectifying, K^+ current known as " K_{ir} " 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 have been 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 Nesbett 1981; Newman and Bartosch 1999; Weinstein et al. 1967) and because the gating kinetics of I_h (and the amplitude of I_h and K_{ir}) have been 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 Müller 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 wished 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 following 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. 1A₁). 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, but the slope conductance increased markedly as the test potential was made more negative than -93 mV (Fig. 1B₁). Low concentrations of Ba^{2+} (50-100 μ M) blocked this rectification (Fig. 1B₂), indicating it is likely to be K_{ir} (Hagiwara et al. 1978). Aside from finding only an ohmic current (viz., a voltage- and time- independent conductance) during this block, we could not study this conductance further because we encountered it in only 2 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, as described below, to I_h .

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

I_h can be reduced to negligibly small amplitudes by Cs^+ (1-5 mM) and ZD7288 (4-ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride; 1-100 μ 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+} block K_{ir} (see above), because some neurons possess both I_h and K_{ir} (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 mV to -123 mV in 10-mV decrements, during superfusion of control saline (Fig. 1C₁), saline supplemented with 1 mM Ba^{2+} (Fig. 1C₂), saline supplemented with 1 mM Ba^{2+} plus 2 mM Cs^+ (Fig. 1C₃), and thereafter, control saline again (Fig. 1C₄). 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 (viz., to test potentials less negative than $\sim$ -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 remained at that amplitude for the remainder of the hyperpolarization. The current in Figs. 1C₁, 2, 4 thus activates more slowly than K_{ir} currents seen in other retinal neurons (Chen et al. 2004; Ma et al. 2003; Tachibana 1983). After repolarizing to the holding potential, the whole-cell current partially decreased instantaneously and then continued to decrease over the next several seconds. In most somata, and as described below in more detail, the current returned to the holding level within 5-10 s. In a few cells, this deactivation appeared to be incomplete even after 10 s; in these cases, at least 25 s were allowed to elapse between successive test pulses. Slowly gating, hyperpolarization-activated currents with these general properties were observed in about 75% of the cells we recorded from (n≈120).

At 1-2 mM, Ba²⁺ reduced the total current when the test hyperpolarizations activated a slow component. However, the Ba²⁺-sensitive portion did not have the same fast onset and voltage-dependence as K_{ir} (not shown), and the reduction was slight (Fig. 1C₂) unlike the suppression of rapidly gating, inward rectifier current noted above (Fig. 1B₂). For example, during hyperpolarizations from -83 to -123 mV, 1 mM Ba²⁺ reduced the total current amplitude by 13±3% (mean±SE, n=4), and the remaining (Ba²⁺-resistant) current gated slowly and rectified inwardly like the control current (Fig. 1C₂). By contrast, subsequent addition of 2 mM Cs⁺ markedly reduced the remaining current (Fig. 1C₃), especially by suppressing the slowly activating and deactivating current (see below). The ability of Cs⁺ to block the slowly gating current did not require previous or concurrent exposure to Ba²⁺, and Ba²⁺ produced little or no reduction of the total current when its application started only after current was blocked by Cs⁺ (not shown), as if Ba²⁺ 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}-sparing I_h antagonist, after describing the effects of Cs⁺.

Cs⁺ reduced the whole-cell current in four ways. First, it reduced the holding current (compare Figs. 1C₁, C₂ and C₃). Digital subtraction of the current recorded before and during the Cs⁺ application (viz., subtraction of the current recorded in Ba²⁺ and Cs⁺ from that recorded in Ba²⁺) 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 Figs. 1C₂ and C₃). This is shown by the instantaneous current steps at the onset and termination of the test hyperpolarizations in the Cs⁺-blocked current traces (Fig. 1C₅). Third, it abolished the slowly activating current during the test hyperpolarizations, leaving only a time-independent current (Fig. 1C₃). Consistent with this, we found no effect of 1 mM Cs⁺ on currents that were ohmic in control solution; see Fig. 1A₂.) Fourth, Cs⁺ abolished the slowly deactivating “tail” current seen after terminating each test hyperpolarization, leaving a time-independent tail current (Fig. 1C₃). 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. 1C₅).

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 below), and at a test potential of -103 mV to check for voltage-dependence. At 2 mM, Cs⁺ reduced the total current by 79±4% at -123 mV and by 75±5% at -103 mV (mean±SE, n=6). A similar level of block was achieved with 1 mM Cs⁺ (81±7% at -123 mV; 79±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 ± 1.3 pA/pF (mean $\pm$ SE, for the $n=5$ cells used below for kinetics measurements; range in density: 4.2 - 12.3 pA/pF; range in capacitance: 5.8 - 11.2 pF; mean capacitance: 8.5 pF). 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 (following 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 (Figs. 2C₁, 2C₂). Both the faster and slower time constants of the fitted functions decreased exponentially with voltage (closed symbols, Fig. 2E), ranging from 459 ± 82 and 2718 ± 783 ms at -93 mV to 105 ± 10 and 726 ± 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 3 of the cells we recorded from; monoexponential fits were adequate in the other 2 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 ± 23 ms (mean $\pm$ SE, $n=3$); after repolarizing from -123 mV, it was 328 ± 6 ms. The slower time constant of these fits was 2122 ± 228 ms after the repolarizations from -93 mV, and 2113 ± 385 ms after the repolarizations from -123 mV. The fast deactivating component was $51 \pm 4\%$ of the biexponential fits, and did not differ markedly over the voltage range we examined. The average monoexponential decay constant (in the 2 cells noted above) was 1163 ± 114 ms.

The range of membrane potentials that activated current (viz., 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 Figs. 2B and 2D). The tail current amplitude (I_V , 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 maximum conductance ($V_{1/2}$) and the slope factor values obtained this way were -86 ± 3 mV (mean $\pm$ SE, $n=5$) and 7.9 ± 0.9 , respectively. Figure 2F displays these measurements, from all of the cells recorded under this condition, after normalizing the current amplitudes for each cell to its respective $I_{\max}$ (measured during the repolarization from -123 mV).

ZD7288 and high [K⁺]_o effects are consistent with I_h

ZD7288 is a bradycardiac agent that can block current through all of the cloned I_h channel types studied to date (Santoro and Tibbs 1999; Stieber et al. 2005), and abolish native I_h without blocking K_{ir} (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 I_h in physiological concentrations of Na⁺ and K⁺, and after raising the extracellular K⁺ concentration from 3.5 mM to 38 mM (Fig. 3B) to double-check for the presence of K_{ir} (by using conditions that increase its amplitude; Chen et al. 2004; Dixon and Copenhagen 1997;

Hagiwara et al. 1978; Tachibana 1983). Fig. 3A shows the current activated by voltage steps from a holding potential of -73 mV to test potentials between -78 mV and -98 mV during superfusion of control saline (Fig. 3A₁), and then control saline supplemented with 100 μ M 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 mV to test potentials between -68 mV and -98 mV during superfusion of control saline (Fig. 3B₁), high-K⁺ saline (Fig. 3B₂), and high-K⁺ saline supplemented with 100 μ M 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. 3B₁ and 3B₂) while the amplitude of the slowly activating component increased as much as 8-fold during test hyperpolarizations [e.g., compare the slow increase in current at -98 mV in Fig. 3B₁ (58 pA) versus that in Fig. 3B₂ (368 pA)].

Since 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 I_h 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 minutes of continuous 100 μ M ZD7288 application in all of the cells we tested (n=23, viz., 14 in control saline; 5 in high-K⁺ saline; 4 in low-Na⁺ saline, as shown below). The fraction of total current suppressed by 10 μ M ZD7288 was nearly equivalent (n=7), with slower onset; 2 μ M ZD7288 had only a small effect (n=3) and 5 μ M 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 hr) if at all (e.g., Berger et al. 1994; Harris and Constanti 1995), and we found only very slight reversal of 100 μ M ZD7288 after washing continuously with control saline for as long as 25 min.

Tail current reversal at low [Na⁺]_o is consistent with I_h

The pharmacological properties, kinetics, and activation range described above suggest that I_h can be activated in rat retinal ganglion cell somata. To test further whether our protocols activated I_h, we measured the reversal potential of ZD7288-sensitive current. Because I_h 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 defies suppression by K⁺ channel blockers (Lukasiewicz and Werblin 1988; Sucher and Lipton 1992), we looked for reversal of I_h tail currents at voltages more negative than -70 mV. To do so, we measured ZD7288-blocked current after replacing 95% of the Na⁺ in the superfusate solution with an impermeant organic cation (*N*-methyl-D-glucamine; cf. Tabata and Ishida 1996; Wollmuth and Hille 1992).

Figure 3C shows the whole-cell current activated by 3-s hyperpolarizations from a holding potential of -80 mV to a conditioning potential of -120 mV, followed by depolarizations to test potentials of -90, -80, and -70 mV, during superfusion of low Na⁺ saline (Fig. 3C₁), and low-Na⁺ saline supplemented with 100 μ M ZD7288 (Fig. 3C₂). The difference between the currents recorded before and during application of ZD7288 (Figs. 3C₃, 3C₄) shows that the tail current was inward at -90 mV, null near -80 mV, and outward at -70 mV. The “shoulder” on the outward current (immediately following the voltage shift from -120 to -70 mV; see bracket in Fig. 3C₄) resembles that recorded after long conditioning pulses in other preparations (Maruoka et al. 1994). The outward current subsequently declines to a steady plateau as expected from partial deactivation of current at this voltage and the activation range in Fig. 2F. Similar results were obtained from all 3 cells tested, and linear regressions on plots of the currents measured at these test potentials yielded a reversal potential of -78 ± 0.6 mV (mean $\pm$ SE, $n=3$; Fig. 3C₅). Assuming that only Na⁺ and K⁺ carried these currents and that the relative permeability to Na⁺ and K⁺ ions are described by the Goldman-Hodgkin-Katz equation (see Methods), the ratio of Na⁺ permeability to K⁺ permeability (“ P_{Na}/P_K ”) 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 mV, 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 (at -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 I_h can elicit rebound spiking

Following activation, the slow deactivation of I_h produces depolarizations that drive various cells to spike threshold. To determine if I_h could serve a similar role in mammalian retinal ganglion cells, we employed a combination of voltage and current clamp on individual cells. Two examples of this are shown in Fig. 4 (A-C and D-F). Figs. 4A₁ and D₁ show 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 (Figs. 4C₁ and F₁). We then injected constant current steps that hyperpolarized this cell, at some point, to the voltages used in Figs. 4A₁ 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 (Figs. 4B₁ 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 μ M to the first cell (Figs. 4A-C) and 5 μ M to the second (Figs. 4D-F). This antagonist of I_h produced four characteristic changes: (i) it caused the cell to hyperpolarize, suggesting that there is a small component of I_h active at our holding potential; (ii) it removed the membrane potential sag observed during injections of hyperpolarizing current; (iii) 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 (iv) it eliminated the rebound spikes (Figs. 4B₂ and E₂). All of this occurred without significant effect on spikes elicited by depolarizing current steps (Figs. 4C₂ and F₂). We then switched back to voltage clamp to show directly the block of I_h (Figs. 4A₂ 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 $25\pm 5\%$ ($n=4$) during application of 5 or 10 μM ZD7288. With 100 μM ZD7288, cells hyperpolarized 14 ± 4 mV and membrane resistance increased $52\pm 19\%$ ($n=4$).

Discussion

This study provides the first measurements of I_h in mammalian retinal ganglion cells. 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 . Lastly, several cells presented no detectable inward rectification. As discussed below, 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. 3A-C, 4), and a $P_{\text{Na}}/P_{\text{K}}$ 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 markedly 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 (less than 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 dependence 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 approximately 75% of the cells we recorded from. We do not yet know if 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 we know of, shears the dendrites and axons off of ganglion cells (see Hayashida et al. 2004). While 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 comparison 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. Secondly, the immunostaining data published to date have not shown whether the cells stained are ganglion cells as opposed to displaced amacrine cells (cf., Perry 1981).

A second group of cells possesses K_{ir} . 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 non-selective 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_{ir} 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 Thy1 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). While our results do not exclude the possibility that I_h and K_{ir} 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_{ir} in different cell populations might explain why I_h isoforms have been 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- $\text{K}_{\text{ir}}3.2$ antibody in one study (see Fig. 3C of Chen et al. 2004), while 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 “ α ” 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 ohmic currents we found in some cells (Fig. 1A). While rat “I” cells may be anatomical homologs of cat α 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 α ganglion cells possess I_h . Recording *in situ* was not a viable alternative because α 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 markedly 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.

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Figure 1. Leak, Ba²⁺-blocked, and Cs⁺-blocked currents

Whole-cell current in three different cells (A, B, C) at the voltages shown schematically at the top of A, while the following solutions were superfused over them. Control (A₁), then control supplemented with 1 mM CsCl (A₂). Control (B₁), then control supplemented with 100 μM BaCl₂ (B₂). Control (C₁), control supplemented with 1 mM BaCl₂ (C₂), control supplemented with 1 mM BaCl₂ and 2 mM CsCl (C₃), control (C₄). In panels A₁ and A₂, the holding potential was -78 mV; in all other panels, the holding potential was -73 mV. The test potentials were -83 to -123 mV in 10-mV decrements for all cells; in C, the current at -73 mV is also shown. No leak subtraction was used. Time and amplitude are calibrated by the scale bars to the right of A, B, and C. Currents from each cell plotted relative to same zero current level; arrowhead in B marks -20 pA for A and B; that in C marks -60 pA. C₅ plots the Cs⁺-blocked current from digitally subtracting the traces in C₃ from those in C₂ (see Fig. 2 for calibrations). Note in B₂ that Ba²⁺ blocked current that is both inwardly rectifying and rapidly gating in B₁, Cs⁺ blocked the inwardly rectifying and slowly gating current in C₂ (C₃), and pre-Cs⁺ amplitude and kinetics were recovered almost completely by washing with control solution (C₄).

Figure 2. Time constants of activation and deactivation

A₁ shows the Cs⁺-blocked current (red) during the 3 s spent at each test potential (“E_t”, ranging from -73 to -123 mV) in Fig. 1C₅, with best fits of a biexponential time function superimposed over each (black; see text). B₁ shows the Cs⁺-blocked current (red) during the first 1.00 s after each test potential terminated in Fig. 1C₅. Best biexponential fits to each 6-s epoch of deactivation at the holding potential in Fig. 1C₅ are superimposed (in black). C₁ and D₁ plot the same current segments as A₁ and B₁ (red), with best fits of monoexponential time functions to the same 3-s activation and 6-s deactivation epochs. For each panel (A₁–D₁), times shown on abscissa match those of currents in Fig 1C, and residuals between the current traces and fits at -123 mV are plotted in A₂, B₂, C₂, and D₂, respectively. E plots time constants of activation and deactivation versus test potential. Means from biexponential fits to the gradual (as opposed to instantaneous) increase (filled symbols) and decrease (open symbols) of Cs⁺-blocked current amplitude from all cells (n=5 for biexponential activation, 3 for biexponential deactivation; see text). Lines drawn through each set of points are linear regressions. Fast (●) and slow (▼) activation time constants change exponentially with voltage. Fast (○) and slow (▽) deactivation time constants are roughly constant over the same voltage range. F plots mean of the amplitudes of tail component of Cs⁺-blocked current from each cell in E, when they were repolarized from test potential (abscissa) to the holding potential (-73 mV). Amplitudes back-extrapolated to the moment of repolarization by best fit of mono- or bi-exponential function to deactivation (see text), and normalized to

the largest amplitude recorded from each cell. The error bars not visible in E and F are within the symbols.

Figure 3. I_h in normal saline, high $[K^+]_o$, and low $[Na^+]_o$

Whole-cell current in three different cells (A, B, C) while the following solutions were superfused over them. A_1 : normal (3.5 K^+ mM); A_2 : normal supplemented with 100 μ M ZD7288. B_1 : 3.5 mM K^+ ; B_2 : high K^+ (38 mM); B_3 : high K^+ supplemented with 100 μ M ZD7288. C_1 : low Na^+ (7.4 mM Na^+ , normal K^+), C_2 : low Na^+ supplemented with 100 μ M ZD7288. Voltage protocols shown above each set of currents. Time and amplitude calibrations for each cell shown at right side of A_2 , B_3 , and C_1 , respectively. Holding potential is -73 mV in all panels of A and B. Test potentials are -78, -88, and -98 mV in A; -68, -78, -88, and -98 mV in B. For comparison, dotted line positioned at the control holding current and extended across other currents traces in A (-58 pA) and B (-35 pA). Note that ZD7288 blocks time-dependent current, and reduces time-independent current, in normal and high $[K^+]_o$. A_3 : I_h in normal saline obtained by subtracting the currents in A_2 from those in A_1 , and plotted at higher gain than A_1 . Panels C_1 and C_2 plot the total current recorded at three test potentials (-90, -80, then -70 mV) after hyperpolarizing from -80 mV to -120 mV (protocol at top of C_1). C_3 plots I_h in low $[Na^+]_o$, obtained by subtracting the currents in C_2 from those in C_1 . The arrowheads in C_1 , C_2 , and C_3 mark the 0 pA level. C_4 superimposes biexponential fits (black) over the I_h tail currents (grey) from C_3 . The times on the abscissa are those in C_3 (where each sweep begins at $t=0$). The tail current amplitude is the maximum of these fits for outward tails (at -70 mV), and the minimum for inward tails (at -90 and -80 mV). C_5 plots the mean $\pm$ SE of the tail current amplitudes from all cells tested ($n=3$). The

linear regression on these points crosses the abscissa at -78 mV. Low $[\text{Na}^+]_o$ experiments were done in the presence of 1 μM TTX to block activation of voltage-gated Na^+ current.

Figure 4. Rebound spikes elicited by activation of I_h and block by ZD7288

Whole-cell voltage- and current-clamp in two different cells (A-C and D-F). In all cases, panels with subscript 1 are control, and panels with subscript 2 are in the presence of 100 μ M (A-C) or 5 μ M (D-F) ZD7288. In voltage-clamp, the voltage protocol shown schematically at the top of A and D (steps from holding potential -73 mV to -68, -83, -93, -103, -113 and -123 mV) was used to activate I_h (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 (B₁, E₁). Blocking I_h 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, while -28 pA was sufficient to induce a similar hyperpolarization in B₂ after I_h 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 μ M ZD7288 does not produce 100% inhibition of I_h . Arrowheads in A and D indicate 0 current. Initial membrane potential for each cell in current clamp was ca. -76 mV (B₁, C₁) and -74 mV (E₁, F₁).