Developmental Switch in Excitability, Ca\textsuperscript{2+} and K\textsuperscript{+} Currents of Retinal Ganglion Cells and Their Dendritic Structure

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Olson, Andrew J., Arturo Picones, and Juan I. Korenbrot. Developmental switch in excitability, Ca\textsuperscript{2+} and K\textsuperscript{+} currents of retinal ganglion cells and their dendritic structure. J Neurophysiol 84: 2063–2077, 2000. In the retina of teleost fish, continuous neuronal development occurs at the margin, in the peripheral growth zone (PGZ). We prepared tissue slices from the retina of rainbow trout that include the PGZ and that comprise a time line of retinal development, in which cells at progressive stages of differentiation are present side by side. We studied the changes in dendritic structure and voltage-dependent Ca\textsuperscript{2+}, Na\textsuperscript{+}, and K\textsuperscript{+} currents that occur as ganglion cells mature. The youngest ganglion cells form a distinct bulge. Cells in the bulge have sparse and short dendritic trees. Only half express Ca\textsuperscript{2+} currents and then only high-voltage-activated currents with slow inactivation (HVASlow). Bulge cells are rarely electrically excitable. They express a mixture of rapidly inactivating and noninactivating K\textsuperscript{+} currents (IKsa and IKdr). The ganglion cells next organize into a transition zone, consisting of a layered structure two to three nuclei thick, before forming the single layered structure characteristic of the mature retina. In the transition zone, the dendritic arbor is elaborately branched and extends over multiple laminae in the inner plexiform layer, without apparent stratification. The arbor of the mature cells is stratified, and the span of the dendritic arbor is well over five times the cell body’s diameter. The electrical properties of cells in the transition and mature zones differ significantly from those in the bulge cells. Correlated with the more elaborate dendritic structures are the expression of both rapidly inactivating HVA (HVAFast) and of low-voltage-activated (LVA) Ca\textsuperscript{2+} currents and of a high density of Na\textsuperscript{+} currents that renders the cells electrically excitable. The older ganglion cells also express a slowly activating K\textsuperscript{+} current (IKsa).

INTRODUCTION

In the course of development the dendritic structure and functional connectivity of individual neurons frequently transform from an initial, immature pattern to a final, mature one through activity-dependent rearrangements (reviews in Fields and Nelson 1992; Goodman and Shatz 1993; Purves 1994). Ganglion cells in the vertebrate retina provide remarkable examples of activity-dependent developmental changes. The afferent activity of these cells is critical for the development of the structure and connectivity of the cells’ postsynaptic partners (review in Katz and Shatz 1996; Shatz 1996). In both lower (Olson and Meyer 1991; Reh and Constantine-Paton 1985; Schmidt 1985, 1990) and higher vertebrates (Casagrande and Condo 1988; Galli-Resta et al. 1993; Stryker and Harris 1986), blocking action potentials in the ganglion cells disrupts the orderly development of their axon terminals and their connectivity to next order neurons. This block does not affect the dendritic structure of the ganglion cells themselves (Campbell et al. 1997; Wong et al. 1991). The input connectivity of ganglion cells also matures with development. In the rabbit’s retina, for example, ganglion cells respond robustly to light by postnatal day 10 (P10), but only by P21 do they exhibit the adult pattern of visual field organization (Bowe-Anders et al. 1975; Masland 1975). Developmental maturation of ganglion cell field organization also occurs in lower vertebrates (Sernagor and Grzywacz 1995). The development of the ganglion cells’ dendritic structure depends on incoming electrical activity. Laminar stratification and receptive fields of ganglion cells fail to mature when synaptic activity of cells in the pathway between photoreceptors and ganglion cells is blocked (in higher vertebrates: Bodnarenko et al. 1995; in lower vertebrates: Sernagor and Grzywacz 1996). The mechanism of this phenomenon is unclear (Tagawa et al. 1999).

To investigate the mechanisms of activity-dependent dendritic restructuring in ganglion cells, it must first be established that developmental maturation occurs both in the electrical properties of the cells and in their structure. Elegant studies of ganglion cells in retinas isolated from animals at various developmental stages have demonstrated that the electrophysiological properties of the cells change with development. Cells both dissociated and in tissue pieces have been studied (Rorig and Grantyn 1994; Rothe and Grantyn 1994; Schmid and Guenther 1996, 1999; Skaliara et al. 1995; Wang et al. 1997). In studies of dissociated cells, however, it is not possible to correlate electrical and structural events. In studies of retinal pieces, whether slices or whole-mount, developmental changes can only be established with relatively limited precision, by comparing tissues isolated from different animals. To overcome some of these limitations we have developed a new experimental preparation amenable to electrophysiological and anatomical investigation, a retinal slice from teleost fish that includes the peripheral growth zone (Olson et al. 1999).

Teleost fish are unique among vertebrates because their eyes continue to grow throughout the life of the animal (Grun 1975; Lyall 1957; Muller 1952). Addition and differentiation of new retinal tissue occurs continually in a narrow ring at the periphery of the mature retina and in a fissure that extends along the...
TABLE 1.

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4-AP, 4-aminopyridine; DIDS, 4,4-diisothio-cyanostilbene-2,2-disulfonic acid; ICS, intracellular solution. * Minimum essential medium (MEM) total amino acids and vitamins, GIBCO-BRL (Grand Island, NY).
Our Golgi staining procedure is based on the method developed for goldfish retina by Stell (1975). The extent of staining obtained with the Golgi method varied considerably, despite every effort to repeat methodology exactly. In addition to reporting the concentrations of reagents that typically gave us good staining, we also give the ranges within which we obtained useful results. A section of retina containing the PGZ and attached to a piece of Millipore was held on a glass slide with small dabs of silicone grease. The retinal piece and filter membrane were then covered with a small square of the perforated plastic film from a Tefla brand bandage, which was secured onto the glass slide with cyanoacrylate glue applied at its corners (Loctite, Hartford, CT). The “sandwiched” retinas were fixed in 1% glutaraldehyde plus four parts 2.5% K2Cr2O7 (we found that here, and in the next step, up to 3.5% K2Cr2O7 can be used). The tissue was treated for 2 days (this time can be increased to as much as 14 days, if necessary) with 2.5% K2Cr2O7. The slides were drained and placed in 1.4% AgNO3 solution and maintained in darkness for 1 day (we as detailed elsewhere (Miller and Korenbrot 1994).

Following a brief rinse in water, we removed the plastic film and lens (Zeiss, Oberkochen, Germany) with an overall magnification of ×400. The external solution, normal Ringer (Table 1), was saturated with 100% oxygen and continuously superfused at a flow rate of 10 ml/min.

We measured whole cell membrane currents under voltage clamp using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA). A Ag/AgCl reference electrode was connected to the bath through a 1 M KCl agar bridge. Analog signals were filtered at either 1 or 5 kHz with an eight-pole low-pass Bessel filter (Frequency Devices, Haverhill, MA) and digitized on-line with 12-bit accuracy at a sampling rate of either 2.5 or 12.5 kHz (Indec Systems, Sunnyvale, CA). We used tight-seal electrodes produced from aluminosilicate glass (Corning 1724, 1.5 × 1.0 mm, OD × ID), Whole cell mode was achieved either by mechanical disruption or by chemical perforation. For perforation, the electrode filling solution contained nystatin (100 μg/ml) or amphotericin B (112.5 μg/ml) prepared as detailed elsewhere (Miller and Korenbrot 1994).

### Electrical recordings

For electrophysiological studies, three to four retinal slices cut from the nastro-temporal region were held in the recording chamber on the fixed stage of an upright microscope equipped with DIC optics. We observed them using a ×40, 0.75 N.A. water-immersion objective lens (Zeiss, Oberkochen, Germany) with an overall magnification of ×400. The external solution, normal Ringer (Table 1), was saturated with 100% oxygen and continuously superfused at a flow rate of ~0.5 ml/min.

We measured whole cell membrane currents under voltage clamp using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA). A Ag/AgCl reference electrode was connected to the bath through a 1 M KCl agar bridge. Analog signals were filtered at either 1 or 5 kHz with an eight-pole low-pass Bessel filter (Frequency Devices, Haverhill, MA) and digitized on-line with 12-bit accuracy at a sampling rate of either 2.5 or 12.5 kHz (Indec Systems, Sunnyvale, CA). We used tight-seal electrodes produced from aluminosilicate glass (Corning 1724, 1.5 × 1.0 mm, OD × ID). Whole cell mode was achieved either by mechanical disruption or by chemical perforation. For perforation, the electrode filling solution contained nystatin (100 μg/ml) or amphotericin B (112.5 μg/ml) prepared as detailed elsewhere (Miller and Korenbrot 1994).

### Ionic solutions

In whole cell mode, whether attained by disruption or perforation, the tight seal electrode was filled with normal intracellular solution (Normal ICS, Table 1), pH adjusted to 7.3 with KOH, osmotic pressure 303 mosM. The normal extracellular Ringer was modified in various experimental protocols, as detailed in Table 1.

### Data analysis

Capacitance was measured under the ionic conditions that isolated Ca2+ currents (see Voltage-dependent Ca2+ currents). We integrated the capacitative current transient generated by either −20- or +10-mV steps from a holding voltage of −100 mV and divided by the voltage pulse amplitude. To calculate membrane capacitance, the electrode capacitance was measured immediately after forming a gigaseal and this value subtracted from the capacitance measured under whole cell mode. In the analysis of K+ currents, we did not determine a normalized K+ current in each cell (pA/PF), as we did in the analysis of Ca2+ currents, because the acquisition bandwidth that we used to record the long epochs necessary to study K+ currents (up to 4 s) was not sufficiently fast to accurately determine capacitance in the same cell.

Selected functions were fit to experimental data using nonlinear, least-square minimization algorithms (Origin, Microcal software, Northampton, MA). Statistical errors throughout are given as means ± SE. To compare means among more than two populations, it is common to execute ANOVA. However, ANOVA simply tests the null hypothesis and determines whether any of the means differ from each other, with some confidence interval. If the means are not the same, other tests of significance must be executed to determine which two means are not the same. This paired comparison of means is valid as long as the total number of comparison is less than or equal to six (Snedecor and Cochran 1967). Since we only compared three populations at a time, we report here the significance of difference between means calculated by pairing any two of the three populations at a time and applying Student’s two-tailed t-test.

### Results

#### Ganglion cell morphology

On the basis of gross histological criteria, we identified three distinct regions in the ganglion cell (GC) layer of the PGZ in the retina of trout. The typical features of these regions are illustrated in Fig. 1 (for a more detailed description, see Olson et al. 1999). Just beneath the initial portion of the inner plexiform layer (IPL) is a multi-layered region of GC somas that stands out as an oval-shaped bulge of cells (Fig. 1, top left). This region consists of the youngest GCs, and we refer to it as the bulge. In the morphologically mature retina, the GC somas form a single layer, so we refer to this region as the monolayer (Fig. 1, top right). In between the bulge and monolayer regions, the GC somas form a bilayer; we call this the transition zone (the initial part of the transition region can be seen in Fig. 1, top right).

Individual cells in each of the three regions have different dendritic morphologies. Figure 2 illustrates tracings of typical neurons in each of the three regions in the GC layer. The tracings are projections onto a single plane of the cellular structures. These tracings were created with either of two methods: 1) hand tracing of biotin-filled single cells using a camera lucida attachment and observing fixed tissue after development with an immunomarker linked to avidin and 2) computer-assisted image reconstructions of Lucifer yellow–filled cells using a confocal microscope. Results with both methods are indistinguishable and pooled. In addition, we also traced with the camera lucida single cells stained with the Golgi method. This method was, in our hands, of very limited success. The cells we selected for this illustration reflect the typical features of cells found in each of the three regions. We studied the anatomical details of 12 cells in the bulge, 19 cells in the transition, and 15 cells in the mature region. Our sampling size is sufficient to identify the major gross features of the dendritic tree in the developing ganglion cells. It is not sufficient to define fine features comparably to previous developmental anatomical studies of...
others (Hitchcock and Easter 1986). Such detailed studies, moreover, would be unwise in tissue slices, since dendritic trees are likely truncated.

Within the restrictions of our anatomical universe, changes in dendritic structure as ganglion cells matured from bulge to mature regions are so dramatic as to be readily definable. GC in all three regions had dendrites, identified as the structures connected to the cell bodies and located in the IPL. We scored dendritic trees by 1) their length and branching, 2) their extension into the IPL and, most especially, 3) their stratification within the IPL. A feature common to mature ganglion cells of all species, and first described in fish retina by Famiglietti et al. (1977) is the selective distribution and termination of dendrites in distinct lamina of the IPL. Dendrites are stratified either in a single lamina or in two laminae (mono- and bistratified ganglion cells). Sublamina a, nearest the inner nuclear layer, is the layer in which the dendrites of the hyperpolarizing (OFF) ganglion cells distribute (Fig. 2, mature GC on the left). Sublamina b, nearest the GC cell bodies, is the layer in which the dendrites of the depolarizing (ON) ganglion cells distribute (Fig. 2, mature GC on the right). Bulge cells have extremely short and sparse dendritic trees. On average, the longest single dendrite was 1.35 ± 0.72 times the longest cell body axis. The average long body axis was 17.2 ± 6.6 μm, and the short body axis was 12.6 ± 5.2 μm. Dendrites in bulge cells did not end in a specific lamina and, on average, extended 25.5 ± 8.3% of the width of the IPL. In the first third of the transition zone, the dendritic trees are long, on average, 3.45 ± 1.3 times the longest cell body axis. The average long body axis was 12 ± 3.3 μm, and the short body axis was 10.5 ± 1.8 μm. The dendrites are elaborately branched, and they extend across the full width of the IPL but are not stratified within the IPL (Fig. 2, transition cells, left and middle). Stratification of dendritic trees first appears in the central two-thirds of the transition zone (Fig. 2, transition cell on the right). We defined a stratified dendritic tree as one that 1) does not reach across the full width of the IPL and 2) terminates in a well-defined laminae of the IPL. Within that lamina, the dendrites extend parallel with the GC layer. The dendritic trees of all cells in the monolayer region are stratified (Fig. 2). Along with the growth of bipolar and amacrine cell synaptic terminals, these changes in individual GC dendritic morphology are responsible for the rapid increase in the thickness of the IPL as retinal maturation proceeds (compare top panels of Fig. 1) (see also Olson et al. 1999).

**Voltage-dependent ionic currents in ganglion cells**

We systematically explored the features of voltage-dependent ionic currents in the cells in the three developmental regions of the GC layer. In fish, <1% of the cells in the ganglion cell layer are not ganglion cells (probably displaced amacrine) (Hitchcock and Easter 1986). The distinct anatomical regions in the PGZ of rainbow trout described above are correlated with specific changes in the electrophysiological properties of the cells. We identified the principal voltage-dependent Ca2+ and K+ membrane currents in GC using...
established biophysical and pharmacological criteria. For each class of currents, we first describe their defining features and then detail their developmental changes.

Voltage-dependent Ca$^{2+}$ currents

We investigated the properties of Ca$^{2+}$ currents in slices continuously bathed with Ringer containing tetrodotoxin to block voltage-dependent Na$^{+}$ currents, tetraethyl ammonium (TEA) and 4-aminopyridine (4-AP) to block all identified K$^{+}$ currents (see below) and diisothiocyanatostilbene-2,2-disulfonic acid (DIDS) to block Cl$^{-}$ currents (Table 1, Ca$^{2+}$ Ringer). Whole cell mode was attained either by membrane disruption or by perforation. In the conventional whole cell mode, the tight-seal electrode was filled with a solution containing Cs$^{+}$ and EGTA (Table 1, Cs, EGTA ICS) to further reduce K$^{+}$ currents and to attenuate possible Ca$^{2+}$-dependent currents, such as Ca$^{2+}$-dependent K$^{+}$ and Ca$^{2+}$-dependent Cl$^{-}$ currents. Results with both methods were indistinguishable and are presented together. Under these experimental conditions, depolarization to between −60 and +10 mV from a conditioning voltage of −100 mV, activated inward currents that were blocked by external Co$^{2+}$ and that therefore we identify as Ca$^{2+}$ currents. There was also a small and variable outward current that is unidentified. We recognized three distinct Ca$^{2+}$ currents by their voltage dependence of activation, and their voltage dependence and time course of inactivation: 1) high voltage activated and rapidly inactivating (HVAfast), 2) high voltage activated and slowly inactivating (HVAslow), and 3) low voltage activated (LVA). Identification of these current types is an operational definition based on and consistent with commonly identified Ca$^{2+}$ current types (Hille 1992). We do not hold that these current types necessarily correspond to a single molecular ion channel type. The identification of particular molecular type(s) of Ca$^{2+}$ channel requires investigation of the pharmacological action of a collection of specific toxins (Birnbaumer et al. 1994), a task now left for future work.
Voltage dependence of activation and inactivation

Typical features of HVA Ca\(^{2+}\) currents are illustrated in Fig. 3. Illustrated is a current with slow inactivation. Holding membrane voltage was \(-70\) mV. To investigate the activation voltage, membrane voltage was first held at \(-100\) mV for 500 ms to remove any steady-state inactivation and was then successively stepped up to \(+40\) mV in 10-mV increments. This voltage protocol activated inward currents that reached a peak within 20 ms and then inactivated slowly and only partially (Fig. 3, top left). The current-voltage (I-V) curve of these currents (Fig. 3, bottom left) showed an activation threshold between \(-50\) and \(-40\) mV and a maximum amplitude at about \(-20\) mV. For \(n = 25\) cells, on average, threshold voltage was \(-40.8 \pm 1.6\) mV, and peak current occurred at \(-9.2 \pm 1.8\) mV.

HVA currents either inactivated rapidly or very slowly (some not at all). For rapidly inactivating currents, HVAfast (see below for further definition), we determined the voltage dependence of the inactivation by successively holding the membrane voltage for 500 ms at values between \(-120\) and \(+10\) mV, in 10-mV increments, and then stepping the voltage to \(-20\) mV (Fig. 3, top right). The inward currents activated at \(-20\) mV were large only when the conditioning pulse was more negative than \(-60\) mV. Conditioning pulses more positive than \(-60\) mV reduced the peak amplitude of the current activated at \(-20\) mV, indicating that the current was inactivated. We analyzed the voltage dependence of steady-state inactivation by determining the dependence on conditioning voltage of the normalized current activated at \(-20\) mV. We normalized the current activated at \(-20\) mV by dividing it by the maximum value of the current and is plotted as a function of the conditioning voltage in the bottom right panel. This analysis reveals voltage dependence of the steady-state inactivation of the currents shown in the top right panel. The continuous line depicts a Boltzmann function (Eq. 1) optimally fit to the data points. For the curve shown, \(V_{1/2} = -76.0\) mV and \(k = 3.5\) mV.

Voltage dependence of activation and inactivation

Figure 4. Low-voltage–activated Ca\(^{2+}\) currents (LVA) in mature ganglion cells. Top left: currents activated by step depolarization to the voltage values indicated by the labels following a 500-ms step to \(-100\) mV to remove inactivation. Leakage currents are subtracted from the records shown. The dashed lines indicate the current at the holding voltage of \(-70\) mV. Command voltage pulses were presented at 4-s intervals. The complete I-V curve for the current peak is illustrated in the bottom left panel. Peak current amplitude was normalized by dividing it by cell capacitance. For the cell shown, \(C_m = 9.3\) pF. Top right: currents recorded from the same cell in response to voltage steps to \(-50\) mV presented at the end of a 500-ms conditioning pulse to the voltages indicated by the labels. Holding voltage was \(-70\) mV, and pulse protocols were presented at 5-s interval. The peak amplitude of the current activated at \(-50\) mV was normalized by the maximum value of the current and is plotted as a function of the conditioning voltage in the bottom right panel. This analysis reveals voltage dependence of the steady-state inactivation of the currents shown in the top right panel. The continuous line depicts a Boltzmann function (Eq. 1) optimally fit to the data points. For the curve shown, \(V_{1/2} = -76.0\) mV and \(k = 3.5\) mV.

\[ I(V) = \frac{I_{max}}{1 + \exp\left(\frac{V_{1/2} - V}{k}\right)} \]
that reflects the steepness of the voltage dependence. For six cells, on average, $V_{1/2}$ was $-48.2 \pm 4.8 \text{ mV}$ and $k$ was $10.1 \pm 2.0 \text{ mV}$ (Fig. 3, bottom right).

Typical features of LVA currents are illustrated in Fig. 4. Holding membrane voltage was $-70 \text{ mV}$. To investigate the activation voltage of these currents, membrane voltage was first held at $-100 \text{ mV}$ for 500 ms to remove any steady-state inactivation and was then successively stepped in 10-mV increments up to $-40 \text{ mV}$. This voltage protocol activated inward currents that reached a peak within 10 ms and then inactivated rapidly and completely (Fig. 4, top left). For four cells the average time-to-peak of the maximum current was $9.8 \pm 3.1 \text{ ms}$. The $I-V$ curve of these currents (Fig. 4, bottom left) showed an activation threshold at $-70 \text{ mV}$ and a maximum amplitude at about $-50 \text{ mV}$. For four cells, on average, threshold voltage was $-70 \pm 0 \text{ mV}$, and peak current occurred at $-50 \pm 8.2 \text{ mV}$.

To investigate the steady-state inactivation of the LVA currents, we successively held the membrane voltage for 500 ms at voltages between $-120$ and $-10 \text{ mV}$, in 5-mV increments, and then stepped the voltage to $-50 \text{ mV}$. The inward current activated at $-50 \text{ mV}$ was large only when the conditioning pulse equal to or more negative than $-100 \text{ mV}$. Pulses more
Agents used to isolate the Ca$^{2+}$ outward currents not 100% blocked by the pharmacological  

can also arise from the persistence of small amplitude, this pool is likely a reflection of cellular subtypes, but techni-

average, $V_{peak}$ described by the Boltzmann function (Eq. 1). For four cells, on average, $V_{1/2}$ was $-68.4 \pm 5.8$ mV and $k$ was $4.0 \pm 0.9$ mV.

**Kinetics of inactivation**

The typical inactivation features of HVA and LVA Ca$^{2+}$ currents are illustrated in Fig. 5. Based on kinetics, we identi-
fied HVA currents as HVAFast or HVASlow. For HVAFast, 90% of the inactivating component exhibited a time course  

t described by a single exponential

$$I(t) = I_{peak} \exp\left(\frac{-t}{\tau}\right) + I_{SS}$$

where $I_{peak}$ is the maximum current amplitude, $I_{SS}$ is the steady-state current amplitude at the end of inactivation, and $\tau$ is the  
time constant of inactivation. For HVAFast in seven cells, the average value of $\tau$ at the voltage that generated the maximum  
peak amplitude was $12.2 \pm 2.1$ ms. Outside of this cluster of rapid inactivation, other HVA currents inactivated slowly or  

not at all. Accordingly, all HVA currents that inactivated with a time constant $>50$ ms and up to $\infty$ were classified as  
HVASlow (Fig. 5, top). The large range of inactivation rates in this pool is likely a reflection of cellular subtypes, but techni-
cially it can also arise from the persistence of small amplitude, outward currents not 100% blocked by the pharmacological  
agents used to isolate the Ca$^{2+}$ currents.

All LVA current inactivated rapidly at a rate that was voltage dependent (Fig. 5, middle). At any given voltage, the  
inactivation is well described by a single exponential function

$$I(t) = I_{peak} \exp\left(\frac{-t}{\tau}\right) + I_{SS}$$  

(Eq. 2; Fig. 5, bottom). For four cells, the average value of $\tau$ at the voltage that generated the maximum peak amplitude was  
$8.0 \pm 2.9$ ms.

**Developmental changes in voltage-dependent Ca$^{2+}$ currents**

Over all our sample of cells that expressed Ca$^{2+}$ currents ($n = 29$), in all but two we observed only one type of Ca$^{2+}$  
current, as defined above. Of these, both were mature cells that coexpressed LVA and HVASlow.

In the bulge zone, Ca$^{2+}$ currents of any type were expressed in only 46% of the cells (6 of 13), but about 90% of the cells  
in the transition and monolayer zones expressed Ca$^{2+}$ currents (23 of 26; Fig. 6, left). There was no statistically significant  
difference in the fraction of cells expressing these currents between transition and monolayer zones.

**HVASlow currents.** About half of the bulge cells studied (6 of 13) expressed any voltage-dependent Ca$^{2+}$ currents, and these were exclusively HVASlow currents (Fig. 6).

To compare current amplitudes in the various cell stages, we normalized the Ca$^{2+}$ current amplitude measured in each cell  
by the capacitance of the same cell. We measured cell capacitance through an analysis of the capacitative transient in the  
voltage-clamped current, as detailed under Methods. Assuming that specific membrane capacitance ($1 \mu F/cm^2$) does not  

change with development, dividing current amplitude by the cell’s capacitance is a reliable method to assess membrane  
current density. The mean capacitance was as follows: bulge cells, 11.1 $\pm$ 3.7 pF; transition cells, 6.7 $\pm$ 1.2 pF; and  

monolayer cells, 7.6 $\pm$ 1.6 pF. The values are not significantly different.

In the bulge cells that expressed HVASlow, the current density tended to be smaller in amplitude than in transition or  

monolayer cells (Fig. 7, top). This difference in amplitude must now be stated as a tendency, rather than a statistical difference,  
because the universe of cells is small and the dispersion in the data significant (Fig. 7). There are no statistically significant  
differences between the HVA currents in transition and monolayer cells with respect to their amplitude and voltage depend-
ence of activation (Fig. 7).

**HVAFast currents.** Bulge cells did not express HVAFast currents (0 of 13). Of the cells that expressed Ca$^{2+}$ currents in  

transition and monolayer stages, about 35% of the cells expressed HVAFast currents (8 of 23, Fig. 6, right). There is no  

statistically significant difference in the fraction of cells expressing this current in transition and monolayer stages. There  

were no statistically significant differences between the HVAFast currents in transition and monolayer cells with respect to  

their amplitude, voltage dependence of activation (Fig. 7, middle), voltage dependence of inactivation, or inactivation rates.

**LVA currents.** Bulge cells did not express LVA currents (0 of 13). These currents were observed only in transition and monolayer  

cells, in 17% of those cells with Ca$^{2+}$ currents (4 of 23, Fig. 6, right). The biophysical features of LVA were essentially  

the same in all cells that expressed them. Cells expressing LVA currents did not simultaneously express any other type of Ca$^{2+}$  
current. Figure 7 (bottom) illustrates average $I$-$V$ curves of normalized LVA currents measured in transition and monolayer GC.  

There are no statistically significant differences between the cur-
rents in their amplitude, voltage dependence of activation, voltage
dependence of inactivation, or inactivation rates. Thus only the
more mature transition and monolayer cells express HVAfast and
LVA currents, the youngest (bulge) cells exclusively express
HVAslow. Once the currents are expressed, however, their bio-
physical features are essentially invariant, all that changes are the
levels of expression.

Development of electrical excitability

Mature ganglion cells are electrically excitable and sus-
tain Na\(^+\)-dependent action potentials (Kaneda and Kaneko
1991a; Lipton and Tauck 1987). In the course of develop-
ment, spiking activity in GC is first spontaneous, then cor-
related among neighboring cells (Meister et al. 1991; Wong
et al. 1993), and, finally, light driven (Bowe-Anders et al.
1975; Masland 1975). We wondered whether at their earliest
developmental stage, GC that express voltage-dependent
Ca\(^{2+}\) currents might also sustain Ca\(^{2+}\)-dependent action
potentials. In other excitable cells, a developmental switch
from Ca\(^{2+}\) to Na\(^+\)-dependent spikes has been documented
(Moody 1995; Spitzer and Ribera 1998). We investigated
electrical excitability by measuring voltage under current clamp
using the whole cell mode and a patch-clamp ampli-
fier. While this method is a technical compromise that
underrepresents the true time course and amplitude of the
action potentials (Magistretti et al. 1998), it provides, none-
theless, an effective means to test cell excitability.

In normal Ringer, we recorded from each GC under voltage
clamp. If the cell exhibited inward current between
−250 and −10 mV, we then switched to current clamp. The mean zero
membrane voltage was −70 mV. The dashed lines indicate the zero current level in every panel. In each column, the
data are from the same cell. Left: data from a cell that exhibited a rapidly inactivating outward current. Middle: data from a cell with
moderately slow inactivation kinetics. Right: data from a cell that inactivated very slowly. For each cell, the top panels are currents
recorded under the control condition (1 μM TTX and 5 mM Co added to the normal Ringer solution). The middle panels are currents
recorded in the presence of 5 mM 4-aminopyridine (4-AP) added to the control solution. The bottom panels are the difference currents
computed by subtracting the current measured in the presence of added 4-AP from those recorded in its absence. These difference currents
represent IKA, and their inactivation kinetics were well described by a single exponential transient with time constants, \(\tau\) (in ms), of 75,
590, and 696 for the left, middle, and right bottom panels, respectively. Thus the top panels are the total currents, the sum of the
inactivating currents (IKA, bottom), and the noninactivating currents (IKdr, middle). The left bottom panel includes an inset with the same
current at higher time resolution.

![Fig. 9. Coexistence of transient K\(^+\) current (IKA) and delayed rectifier K\(^+\) current (IKdr) in ganglion cells of the mature zone. Data from 3 different cells are shown. The data span the range of inactivation kinetics of IKA observed. Shown in each panel are currents activated by 3.8 s duration steps to −90, 0, and +50 mV that followed a period of 500 ms at −90 mV. Leakage currents are subtracted from the records shown. Holding voltage was −70 mV. The dashed lines indicate the zero current level in every panel. In each column, the data are from the same cell. Left: data from a cell that exhibited a rapidly inactivating outward current. Middle: data from a cell with moderately slow inactivation kinetics. Right: data from a cell that inactivated very slowly. For each cell, the top panels are currents recorded under the control condition (1 μM TTX and 5 mM Co added to the normal Ringer solution). The middle panels are currents recorded in the presence of 5 mM 4-aminopyridine (4-AP) added to the control solution. The bottom panels are the difference currents computed by subtracting the current measured in the presence of added 4-AP from those recorded in its absence. These difference currents represent IKA, and their inactivation kinetics were well described by a single exponential transient with time constants, \(\tau\) (in ms), of 75, 590, and 696 for the left, middle, and right bottom panels, respectively. Thus the top panels are the total currents, the sum of the inactivating currents (IKA, bottom), and the noninactivating currents (IKdr, middle). The left bottom panel includes an inset with the same current at higher time resolution.]}
of 6 cells), but action potentials could not be elicited in bulge cells even at voltages near zero mV (5 of 6 cells; Fig. 8). The action potentials were reversibly blocked with 1 mM TTX (Table 1, TTX Ringer, data not shown). Thus in agreement with all previous findings (review in Robinson and Wang 1998), mature GC sustain Na\textsuperscript{+}-dependent action potentials, and the youngest GC are simply not excitable, even if they express voltage-dependent Ca\textsuperscript{2+} currents.

We do not report here on the features of voltage-dependent Na\textsuperscript{+} currents of developing GC, although we observed them under voltage clamp, as is obviously expected. Inadequate clamp of Na\textsuperscript{+} currents was apparent by the absence of a graded, voltage-dependent increase in time-to-peak of these currents (data not shown). The issue of quality of space clamp in ganglion cells studied in retinal slices is thorny. In general, experimental and theoretical arguments suggest that space clamp is adequate for only relatively slow events (slower than a few ms) (Taylor et al. 1996; Velte and Miller 1996). Note that Ca\textsuperscript{2+} currents (see above) are sufficiently slow to be well clamped, as evidenced by the fact that the activation threshold, I-V curves and voltage dependence of inactivation of these currents are similar to those of dissociated neurons (Hille 1992).

Voltage-dependent K\textsuperscript{+} currents

We investigated the properties of K\textsuperscript{+} currents of GC recorded in conventional whole cell mode using electrodes filled with normal intracellular solution. Under these experimental conditions, depolarization to +50 mV, from a holding voltage of −100 mV, activated outward currents that were attenuated by 5 mM external TEA (data not shown), and therefore we identify them as K\textsuperscript{+} currents. We recognized three distinct K\textsuperscript{+} currents by the kinetics and voltage dependence of activation, the kinetics of inactivation, and the sensitivity to specific K\textsuperscript{+} channel blockers: 1) a noninactivating delayed rectifier (IKdr), 2) a slowly activating delayed rectifier (IKsa), and 3) a rapidly inactivating, “A” type current (IKA).

**Kinetics of inactivation**

Typical features of K\textsuperscript{+} currents that demonstrate an inactivating component are illustrated in Fig. 9. Three different cells are illustrated that represent the range of kinetic behavior we observed. To investigate the inactivation kinetics, cells were bathed in Ringer containing TTX and Co\textsuperscript{2+} to block Na\textsuperscript{+} and Ca\textsuperscript{2+} currents (Table 1, Co\textsuperscript{2+}, TTX Ringer). The membrane voltage, held at −70 mV, was first stepped to −100 mV for 750 ms to remove any steady-state inactivation and was then successively stepped for 3 s in 10-mV increments up to +50 mV. Pulses were repeated at 4-s intervals. At depolarizing voltages (≥0 mV), the current rapidly reached peak (≤30 ms) and then inactivated. In every instance tested (6 of 6), the inactivating component reversibly disappeared in the presence of 5 mM 4-AP, a specific blocker of IKA type of inactivating current (Table 1, Co\textsuperscript{2+}, TTX, 4-AP Ringer). The residual currents (those measured with the same voltage protocols in the continuous presence of 4-AP) are illustrated for each cell in the
middle panels of Fig. 9. These currents are voltage dependent and essentially of constant amplitude for the duration of the voltage pulse. The bottom panels illustrate the 4-AP–sensitive current, computed from the difference between the initial and the residual currents. In Fig. 10 we illustrate the I-V curves measured at the maximum amplitude of the residual (top left) and the peak of the 4-AP–sensitive (top right) currents in each of the three cells shown. Residual and 4-AP–sensitive currents have nearly identical I-V curves. Since these currents 1) were measured under conditions that block Na\(^+\) and Ca\(^{2+}\) currents, 2) are both attenuated by external TEA, and 3) have the same voltage dependence of activation, we identify them as K\(^+\) currents of two types: inactivating (IKA) and noninactivating (IKdr).

The kinetics of IKA inactivation indicate that about 90% of the inactivating component declined with a single exponential time course with a time constant that is voltage dependent (Fig. 10, bottom). At +50 mV, this time constant ranged in value between 80 and 700 ms over all our cell population. We did not average these values because they did not distribute uniformly nor did they cluster at particular values. We did not discern a pattern to the distribution of time constant values. The large range of values likely reflects a mixture of the expression level of the many molecular forms of the members of the Shaker family of inactivating K\(^+\) channels (Panyi and Deutsch 1996).

**Activation kinetics**

IKdr and IKA activated rapidly; for all cells measured time to maximum or peak currents was ≤20 ms at +50 mV. We distinguished a third type of K\(^+\) current by its kinetics of activation. Typical features of these currents are illustrated in Fig. 10. Membrane voltage was held at −100 mV for 1 s and was then successively stepped in 10-mV increments up to +50 mV for 900 ms. Pulses were repeated at 4-s intervals. The outward currents activated at large depolarizing voltages activated instantaneously and then continued to grow in amplitude. This behavior was observed for all voltages greater than +10 mV. The extent and speed of activation of the slow component was voltage dependent, but the value of the rate of activation varied from cell to cell. The cells illustrated in Fig. 11 present the range of kinetic behavior we observed for IKsa. At ±50 mV the time to reach midway between the starting and final values ranged from 38 to 258 ms. We did not average data because these values were widely dispersed. The I-V curves of the currents measured for the cells shown either instantaneously or at the end of the voltage pulse are shown in Fig. 11. The curves have the same shape, they simply differ in amplitude. Since the instantaneous and slowly activating currents were attenuated by external TEA and since they have the same voltage dependence of activation, we identify them as K\(^+\) currents of two types: slowly activating (IKsa) and rapidly activating, noninactivating (IKdr, delayed rectifier type). IKsa currents are reminiscent of slow K\(^+\) currents described in other tissues (Hille 1992).

**Developmental changes in voltage-dependent K\(^+\) currents**

Every cell that we investigated expressed K\(^+\) currents (68 of 68). Some expressed only one type of current, invariably IKdr.

**FIG. 11.** Coexistence of slow activating K\(^+\) currents (IKsa) and delayed rectifier K\(^+\) currents (IKdr). Shown are data from 2 different cells that span the range of observed activation kinetics of IKsa. Leakage currents are subtracted from the records shown. **Top left:** currents activated by 1-s voltage steps from a holding potential of −70 mV to −90–40, 0, 10, 20, 30, 40, and 50 mV. **Top right:** currents activated by 1-s voltage steps from a holding potential of −70 mV to −80–40, 0, 10, 20, 30, 40, and 50 mV. **Bottom:** the I-V curves measured at 10 ms (open circles) and at 1 s (filled circles) in the data shown in the top panels. Smooth lines connecting data points are B-splines.
(27 of 68, 39%) while others expressed a mixture of only two currents (41 of 68, 61%), one of which was always IKdr. Developmental changes were reflected in two parameters: the level of expression of IKA and IKsa and the amplitude of the total peak K⁺ current.

The expression of IKA and IKsa currents changed with development. Every bulge cell we studied expressed both IKA and IKdr (9 of 9), but none expressed IKsa (0 of 9). In the transition zone all cells expressed IKdr (25 of 25, 100%), and of these about half (13 of 25, 52%) expressed IKdr alone. The combination IKdr + IKA was observed in 40% of the cells (10 of 25) and the combination of IKdr + IKsa in only 8% (2 of 25). In the monolayer, all cells expressed IKdr (34 of 34), and of these, again, nearly half (14 of 34, 41%) expressed IKdr alone. The combination IKdr + IKA was observed in 50% of the cells (17 of 34) and the combination of IKdr + IKsa in only 9% (3 of 34). At all stages of development, in cells with mixed current we could not discern any pattern in the ratio of the two currents present. Thus the expression of IKA and IKsa appear to be developmentally regulated: IKA is found in all bulge cells, but only in about 40–50% of the transition and mature cells. Expression of IKsa is relatively infrequent, about 8 to 9%, and only occurs in transition and mature cells.

We measured the total K⁺ current amplitude as the peak of the outward current activated by a voltage step to +50 mV from a −70-mV holding voltage. Figure 12 illustrates the changes in total current amplitude with cell maturation. The average peak currents were as follows: bulge cells, 628 ± 180 pA (n = 15); transition cells, 1,494 ± 227 pA (n = 14); and monolayer cells, 1,296 ± 219 (n = 21). The mean of the bulge cells differs significantly (P < 0.05) from those of either transition or monolayer cells, which do not differ from each other. Since the average cell capacitance does not change with maturation (see above), the data indicate that the surface density of K⁺ channels increases with development and the changes in current amplitude do not simply reflect changes in cell dimension.

**DISCUSSION**

**Developmental changes in dendritic structure**

In the ganglion cell layer of the PGZ, three different regions can be discerned by gross histological criteria: bulge, transition, and monolayer zones. Cells in the bulge have sparse and short dendritic trees that extend, at most, about 25% of the width of the IPL. As the cells mature, their dendritic structure changes. In the cells in the most peripheral third of the transition zone, the dendrites are elaborately branched and extend over the full width of the IPL, but do not stratify or terminate in specific sublaminae of the IPL. Over the central two-thirds of the transition zone and in the monolayer, dendrites of the GC stratify in the IPL into distinct sublaminae a and b. Expression and biophysical features of voltage-dependent currents in the young GC of the bulge are different from those in transition and monolayer GC, which do not differ from each other. That is, voltage-dependent ionic currents change dramatically between cells that have a sparse and minimal dendritic structure and those with an elaborate dendritic arbor, but do not change with the stratification of the dendrites.

Anatomical studies in 250-μm-thick retinal slices likely underrepresent the magnitude of changes in dendritic arbor, yet facilitate the identification of changes in stratification. In elegant studies, Hitchcock and Easter (1986) comprehensively documented the developmental changes in arbor size of GC labeled by retrograde transport of horseradish peroxidase (HRP) added to the optic nerve. Our results, while in general agreement with their findings, extend information in two respects: 1) we have mapped the extent of dendritic stratification, a feature that can be readily analyzed in slices, but not in the whole-mounts studied by Hitchcock and Easter (1986); and 2) we explored the dendritic structure of bulge cells, cells that cannot be filled by retrograde transport of HRP, because their axons are not sufficiently long to emerge from the eyeball in the optic nerve.

As in other vertebrates, mature ganglion cells in fish have been recognized to be of distinct anatomical subtypes. Specific classification schemes have been proposed by Naka and Carraway (1975), Murakami and Shimoda (1977), Kock and Reuter (1978), Dunn-Meynell and Sharma (1986), Hitchcock and Easter (1986), Cook et al. (1992), and Cook and Sharma (1995). In our studies we did not attempt to classify the developing GC into specific categories because we could not discern systematic patterns of change in the population we sampled.

**Developmental changes in voltage-dependent ionic currents**


In brief summary, we have found that about half of the youngest identified GC (bulge cells) do not express Ca²⁺ currents, and those that do only express HVA slow. Bulge cells are not electrically excitable. All bulge cells express K⁺ currents, invariably a mixture of IKA and IKdr.

Expression and biophysical features of all currents in transition and monolayer cells are essentially the same, but they differ significantly from those in the bulge cells. Ca²⁺ currents
are expressed in the vast majority of the cells; LVA currents and rapidly inactivating HVA currents are first switched on. Transition and monolayer cells are electrically excitable, and their action potentials are blocked by TTX. This indicates that they express voltage-gated Na\(^+\) currents. In the bulge, we found that 67% of the cells (10 of 15) expressed voltage-gated Na\(^+\) currents; their density, however, is evidently not sufficient to sustain action potentials. All transition and monolayer GC express K\(^-\) currents, but IKA is switched off in about half the cells. About half of the cells express only IKdr, while the other half express a mixture of two currents, one of which is always IKdr and the other is, commonly, IKA and, infrequently, IKsa.

Developmental changes in voltage-dependent currents, then, are of two principal types: 1) switching currents on or off and 2) changing the current density. On the other hand, we did not observe large changes in the biophysical features, e.g., voltage dependence of activation and inactivation, of the currents expressed.

Developmental changes in currents have previously been investigated in GC dissociated from fetal cat retinas. LVA, HVAslow, and HVAfast Ca\(^{2+}\) currents have been identified. All are expressed at every developmental stage investigated, with a continuous increase in the current density and in the fraction of cells expressing LVA and HVAfast (Huang and Robinson 1998). The fact that a significant fraction of the youngest GC we sampled lack Ca\(^{2+}\) currents suggests that our sample included GC at an earlier developmental stage than those in the cat. Na\(^+\) currents are expressed in the youngest cells examined and increase in density as time progresses (Skaliola et al. 1993). K\(^-\) currents of both the IKA and IKdr type are expressed and young cells generally express both, but as they mature it is more frequent to find cells expressing IKdr alone (Skaliola et al. 1995). These important reports demonstrate that changes in the electrical properties occur, but their correlation with specific changes in structure or network function cannot be assessed in dissociated cells.

The limitations of cell dissociation have been addressed in studies of the electrical properties of GC in isolated retinas or retinal slices prepared from rats or mice at various developmental stages (Röig and Grantyn 1994; Schmid and Guenther 1996, 1999). In both rats and mice, there is an early onset of both Na\(^+\) and Ca\(^{2+}\) currents, with a significant fraction of the cells failing to express either current. There is a progressive increase in density of both Ca\(^{2+}\) and Na\(^+\) currents, reaching mature values slightly earlier for Na\(^+\) than Ca\(^{2+}\) currents (in rats at around P6 for Na\(^+\) and P18 for Ca\(^{2+}\)). Among Ca\(^{2+}\) currents, LVA are expressed in the youngest cells and are entirely absent in mature ones. In the cells expressing HVA currents, the fraction of cells expressing inactivating versus noninactivating HVA was, essentially, constant (Schmid and Guenther 1996). The absence of LVA currents in mature GC of retinal slices is surprising because dissociated, mature rat GC express a complex mixture Ca\(^{2+}\) current types, including a prominent LVA component (Guenther et al. 1994). This discrepancy is unexplained; it could be a sampling problem or a true effect of dissociation.

GC in all species evidence a developmental regulation of Ca\(^{2+}\) currents, a phenomenon observed in other neurons as well (Desmadryl et al. 1998; Hilaire et al. 1996; Tarasenko et al. 1998), but there are striking species-specific differences. In rodents, only LVA currents are expressed in the youngest GC cells, and the fraction of cells expressing these currents decreases as development progresses, just as the expression level of HVA currents increases. In fish, in contrast, the youngest cells do not express LVA currents or inactivating HVA currents, and the fraction of cells expressing these currents increases with maturation. GC in cats have not been explored at nearly as early an age as in fish and rats, nonetheless their developmental pattern is more like that of fish than of rats. Younger cells express LVA and inactivating HVA currents at low levels, and the fraction of cells expressing these currents increases with development. The differences in developmental changes between GC of rats and those of fish and cats may reflect the need to build functionally distinct neuronal networks in the retina. Rodent retinas are nearly free of cone photoreceptors and the animals are essentially scotopic; their visual system is driven almost exclusively by rod inputs. Fish and cats, in contrast, are animals in which rod- and cone-driven pathways coexist and the intensity of the background light determines which of the two pathways dominates the ganglion cell signal.

Functional significance

Elevated extracellular K\(^-\) promotes the expression of neuronal phenotypes in dissociated cultures of developing rat retinal cells, specifically favoring the differentiation and maturation of ganglion cells (Araki et al. 1995). Elevated K\(^-\) is also a necessary condition for neurotrophic factors to promote survival of dissociated rat GC (Meyer-Franke et al. 1995). In both of these reports, it is argued that the effect of K\(^-\) is not direct, but depends on its ability to depolarize the cells. Changes in the density and identity of K\(^-\) currents, relative to other currents in the cell, must unavoidably affect the value of resting membrane potential. Indeed, we observed a systematic difference in the density and identity of K\(^-\) currents and of resting potential of GC in the bulge versus the more mature cells. If the effects of membrane voltage observed in vitro indeed occur in vivo, then the changes we observe in K\(^-\) currents and resting potential may be important factors in the normal progression of GC development.

It is plausible that changes in Ca\(^{2+}\) current correlated with developmental processes are causally related (e.g., Desmadryl et al. 1998; Hilaire et al. 1996; Schmid and Guenther 1999), but proving a causative role is difficult. We must await direct experimental tests of this hypothesis, for which the fish model system is well suited. As a matter of speculation, however, it is interesting to consider that the most dramatic effect we have observed are switches in the expression of Ca\(^{2+}\) current types. A different set of Ca\(^{2+}\) channels and a different resting potential as cells develop inevitably implies that the resting cytoplasmic Ca\(^{2+}\) concentration changes with development. Since young GC are not excitable, there is no reason to suspect large changes in membrane voltage, although changing synaptic input may be of consequence. Thus in the youngest fish GC, the higher resting membrane potential and the expression of HVAslow current suggests that a steady inward flux of Ca\(^{2+}\) exists at all times. In the youngest rat GC, in contrast, at comparable resting potentials and given the exclusive expression of LVA currents, a continuous inward flux of Ca\(^{2+}\) is unlikely. Most Ca\(^{2+}\)-dependent regulatory processes in neurons, whether cytoplasmic or nuclear, respond only slowly to
Ca$^{2+}$ concentration changes and their functional performance, therefore depends on the standing Ca$^{2+}$ concentration. Significant differences may exist between younger and older GC in the standing cytoplasmic Ca$^{2+}$ concentration that may be an important biochemical regulator of cellular maturation.

By identifying changes in expression of voltage-dependent currents of retinal ganglion cells and correlating them with the developmental state of the cells, our studies provide a basic framework for further investigation of the role of electrical activity in GC maturation. Our results suggest several potentially fruitful lines of inquiry. Simultaneous anatomical and electrophysiological studies should reveal whether specific patterns of current switching correlate with anatomical classes. Such a study would also provide a developmental map in the PGZ with finer spatial resolution, which might reveal tighter correlations between expression of particular currents and dendritic stratification. Finally, for future studies of the development of neurotransmitter-gated currents, these results have provided us with an understanding of the maturational changes in intrinsic membrane properties on which the developing synaptic currents are overlaid.

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