Characterization With Barium of Potassium Currents in Turtle Retinal Müller Cells

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Solessio, Eduardo, David M. Linn, Ido Perlman, and Eric M. Lasater. Characterization with barium of potassium currents in turtle retinal Müller cells. *J. Neurophysiol.* 83: 418–430, 2000. Müller cells are highly permeable to potassium ions and play a crucial role in maintaining potassium homeostasis in the vertebrate retina. The potassium current found in turtle Müller cells consists of two components: an inwardly rectifying component and a linear, passive component. These currents are insensitive to broadband potassium channel blockers, tetraethylammonium (TEA) and 4-aminopyridine (4-AP) and well blocked by barium. Differential block of the polyamine spermine suggests that these currents flow through different channels. In this study, we used barium ions as a probe to investigate the properties of these currents by whole cell, voltage-clamp recordings from isolated cells. Current-voltage (I-V) relationships generated from current responses to short (35 ms) and long (3.5 s) voltage pulses were fit with the Hill equation. With extracellular barium, the time course of block and unblock was voltage and concentration dependent and could be fit with single exponential functions and time constants larger than 100 ms. Blocking effects by extracellular barium on the two types of currents were indistinguishable. The decrease of the outward current originates in part due to charge effects. We also found that intracellular barium was an effective blocker of the potassium currents. The relative block of the inward rectifier by intracellular barium suggests the existence of two “apparent” binding sites available for barium within the channel. Under depolarizing conditions favoring the block by internal polyamines, the Hill coefficient for barium binding was 1, indicating a single apparent binding site for barium within the pore of the passive linear conductance. The steepness of the blocking functions suggests that the potassium currents flow through two different types of channels, an inward rectifier and a linear passive conductance. Last, we consider the use of barium as an intracellular K\(^+\) channel blocker for voltage-clamp experiments.

**INTRODUCTION**

To maintain potassium homeostasis of the extracellular space, Müller cells are highly permeable to potassium ions (Newman 1996; Reichenbach et al. 1993). The potassium channels are unevenly distributed along the length of the cell and are found at higher density in regions close to large potassium sources and sinks such as the plexiform layers, the vitreous humor, and blood vessels (Brew et al. 1986; Newman 1987, 1988; Skatchkov et al. 1995). In some species, only inwardly rectifying channels have been found (Newman 1993), whereas in others, a delayed rectifier, a transient outward current (\(I_o\)) and a voltage-independent potassium conductance have been identified (Chao et al. 1994; Reichenbach et al. 1993).

There are two types of potassium currents in turtle Müller cells: a weak inward rectifying component (Conner et al. 1985; Le Dain et al. 1994; Linn et al. 1998) gated by intracellular polyamines and a passive component that remains linear within the physiological voltage range (E. Solessio, K. Rapp, I. Perlman, and E. M. Lasater, unpublished observations). The relative magnitude of each current remained constant from cell to cell, even across different preparations, indicative of some degree of pairing between the two channel types, as in coupled or tandem channels (Chavez et al. 1999; Ketchum et al. 1995). However, the possibility remains that currents could flow through a single type of channel as would be the case if polyamines cannot completely block the inward rectifiers of turtle Müller cells, thus allowing outward currents under depolarized conditions (Ishihara 1997).

Despite the differential block by polyamines, it is otherwise difficult to separate these currents because they possess similar pharmacological characteristics. Like their counterparts in frog (Skatchkov et al. 1995), the potassium channels in turtle are insensitive to the nonspecific potassium channel blockers tetraethylammonium (TEA) and 4-aminopyridine (4-AP) but are effectively blocked by low extracellular concentrations of barium or cesium (Le Dain et al. 1994; Linn et al. 1998). Barium is an effective blocker of potassium channels in nerve cells (Armstrong et al. 1982) and has been effectively used to probe the properties of these channels (reviewed by Latorre et al. 1997). Barium ions are also effective in blocking potassium channels in Müller cells of other species (Newman 1987, 1989; Reichelt and Pannicke 1993) and has been applied extensively to determine the contribution of Müller cells to the electroretinogram (ERG) (Xu and Karwoski 1994) and siphoning of potassium away from the inner retina (Karwoski et al. 1989). However, no in-depth characterization of the voltage- and concentration-dependent properties of the block by barium of the potassium channels in Müller cells has been carried out. In this study we investigated the effects of intracellular and extracellular barium ions on isolated turtle Müller cells with the premise that structural differences in the two types of channels would be reflected in blocking characteristics such as the affinity of block, steepness of the blocking function, or voltage dependency. We found, from the perspective of extracellular barium block, that the channels appeared structurally

**REFERENCES**


similar. However, the characteristics of block by intracellular barium differed radically from that of extracellular block suggesting that two “apparent” binding sites are available for barium to block the inward rectifying currents, whereas only one is required to block the passive linear currents. The simplest explanation of these dissimilar properties of block by intracellular barium would be that the currents flow through two different types of channels differing primarily in their structure on the cytoplasmic face of the membrane.

In addition we extended our results to a mathematical model of Müller cells and explored the effects that the block of the potassium channels by intracellular barium block has on its electrotonic properties under different conditions and clamping configurations.

**MET HODS**

**Preparation**

The study was conducted on Müller cells isolated from the retina of the fresh water turtle *Pseudemys scripta elegans*. Turtles were killed in accordance with American Association of Laboratory Animal Care guidelines and the guidelines set forth by the Association for Research in Vision and Ophthalmology for the use of animals in research. The retinas from the two eyes were isolated and placed in a calcium/magnesium-free Ringer for 15 min. The isolated retinas were then incubated for 30 min in papain (300 U/20 ml) dissolved in L-15 (GIBCO BRL, Gaithersburg, MD). The retinal pieces were then gently triturated onto glass coverslips. All recordings were performed within 12 h after dissociation.

In the present experiments it was important to obtain a high quality voltage clamp of the cell. We have previously shown (Linn et al. 1998) that the same cohort of channels exist on all parts of the cell but that their spatial distribution varied. Therefore to obtain the best space clamp possible for the present study, all recordings were done on cells lacking the long, thin proximal processes that characterize these cells (see Linn et al. 1998). Cells missing these processes showed no difference in the nature of the currents we recorded compared with their intact counterparts (Linn et al. 1998).

**Experimental solutions**

The normal turtle Ringer’s consisted of the following (in mM): 110 NaCl, 2.0 CaCl₂, 2.6 KCl, 2.0 MgCl₂, 8.4 HEPES, and 10 D-glucose. All chemicals were purchased from Sigma Chemicals (St. Louis, MO). Solutions were delivered via a 12-channel pressure ejection system controlled by a personal computer (DAD-12, ALA Scientific, MO). The patch pipette solution consisted of 130 mM NaCl, 2.0 CaCl₂, 2.6 KCl, 2.0 MgCl₂, 8.4 HEPES, and 10 D-glucose. All chemicals were purchased from Sigma Chemicals (St. Louis, MO). Solutions were delivered via a 12-channel pressure ejection system controlled by a personal computer (DAD-12, ALA Scientific, MO). The patch pipette solution was composed of 130 mM potassium gluconate, 4 mM NaCl, 0.1 mM EGTA, 1 mM MgCl₂, 8.4 mM HEPES, and 7.5 μM CaCl₂. Barium chloride at different concentrations was added to the normal Ringer solution or to the patch pipette solution.

**Recording procedures**

Whole cell, patch-clamp recordings (Hamill et al. 1981) were performed from Müller cells lacking most processes and endfeet (lost during the dissociation procedure) to avoid complications arising from less than ideal clamping conditions (Solessio et al., submitted for publication). Micropipettes were pulled on a two-stage puller (model PP-83, Narishige Instruments, Tokyo) and coated with silicone elastomer (Sylgard). The electrode tip resistance was 2–4 MΩ when measured in the bath solution. Recordings were obtained using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Series resistance and capacitance were compensated for electronically with the amplifier. Data collection was performed by a personal computer interfaced to a Digidata 1200 data acquisition board (Axon Instruments, Foster City, CA) driven by Axon’s pClamp software.

**Data analysis**

Responses were measured at the end of short (35 ms) and/or long (3.5 s) voltage pulses. The holding potential was 80 mV unless stated otherwise. Currents were normalized (I/Iₒ) with respect to control in the absence of extracellular barium. Or, in the case of intracellular barium, with respect to a control series recorded immediately after the whole cell configuration was established, but before barium block occurred. Normalized data (I/Iₒ) were averaged and displayed as a function of voltage step or barium concentration. Data points represent the average normalized responses and standard deviation from three or more barium concentrations. To quantify the degree of block, the data were plotted with barium as the independent variable, and fit with the modified Hill equation

\[ \frac{I}{I_o} = 1 - \frac{[\text{Ba}^{2+}]}{[\text{Ba}_c^{2+}] + [EC_{50}]} \]

where in these studies the EC₅₀ is the “apparent” binding affinity for barium and n the apparent cooperativity for binding. Modeling and computation of noise power spectra (see Noise analysis) were performed with Mathematica (Wolfram Research, Champaign, IL).

**Noise analysis**

Noise analysis can be applied for the determination of the membrane resistivity (Rₑ) without application of current pulses (DeFelice 1981; Hille 1992). Briefly, for this, the recordings were done with 10 mM barium chloride in the pipette solution. The cell was clamped at a holding potential (HP) = −40 mV, the currents were low-pass filtered (cutoff frequency was 1 kHz), and the sampled 3-s intervals at 10 kHz. The power density was calculated using Mathematica software. Curve fitting and plotting was performed with SigmaPlot. Three power spectra were averaged to obtain an average power spectra. In turn, the average power spectra was averaged in the frequency domain, five or more adjacent frequency points (Leibrock et al. 1994), except at the lower frequencies (<2 Hz). The resulting power spectrum was then fit with a Lorentzian (Fig. 10)

\[ S(f) = S(0)(f/f_c)^2 + 1 \]

where f_c is the cutoff frequency and

\[ \tau_m = R_m C_m = \frac{1}{\nu R_m} \]

where \( \tau_m \) is the time constant, \( R_m \) the membrane resistivity, and \( C_m \) the membrane specific capacitance.

**RESULTS**

**Block by extracellular barium: short pulses**

Figure 1 shows typical effects of extracellular barium ions on the whole cell responses from an isolated turtle Müller cell body. The cell was clamped to −80 mV, and voltage steps of 35 ms duration were applied from −120 to +40 mV in 20-mV increments. Disregarding the capacitive artifacts that lasted ~2 ms [difficult to completely eliminate electronically because of the large size of the cell (see Linn et al. 1998; Sollessio et al., unpublished observations)], the current responses reached a sustained level of response to both depolarizing and hyperpolarizing pulses. Barium ions attenuated both inward and outward currents by a similar degree that increased, as the concentration of extracellular barium ions was raised (Fig. 1, B and C).

To quantitatively evaluate the degree of block for both
inward and outward currents, current-voltage (I-V) curves were constructed using current values measured at the end of each voltage pulse in the presence of different extracellular concentrations of barium (Fig. 1D). All the I-V curves pivot around a single voltage at about 280 mV (approximately the Nernst potential for K⁺ as calculated from the potassium concentrations in the pipette and extracellular solutions). Almost complete block can be attained with extracellular barium concentrations over 30 μM. This supports our suggestion that the I-V curve of turtle Müller cells, studied using the whole cell configuration, reflects currents carried by potassium ions through potassium channels that are effectively blocked by barium ions (Solessio et al., submitted for publication). To test for voltage dependency of the barium block, I/Iₒ plots were constructed from the I-V curves. Again, for these plots, Iₒ is the current measured at each particular voltage immediately after breaking into the cell before barium can take affect (i.e., it is not I_max). I is the current measured after barium block has taken affect. These I/Iₒ plots, shown in Fig. 3A, indicate that the barium block remains relatively constant at hyperpolarized levels, but decreases with depolarization, particularly at the higher barium concentrations.

Block by extracellular barium: long pulses

Although the blocking affects of barium seems to be instantaneous, application of long voltage pulses revealed a second, slow component to the effect of extracellular barium ions. A representative experiment is shown in Fig. 2. The cell was voltage clamped at −80 mV and stepped for 3.5 s to different voltages ranging from −120 to +40 mV in 20-mV steps. The interval between voltage steps was 10 s to allow complete recovery from the effect of the preceding voltage step. The current responses to these pulses are shown for control turtle Ringer (Fig. 2A) and during superfusion with a Ringer solution containing 1.8 μM (Fig. 2B) and 15 μM (Fig. 2C) barium chloride. These responses indicate that the effect of barium on turtle Müller cells is not only voltage and dose dependent as was shown in Fig. 1 but also time dependent. At low extracellular barium concentrations (Fig. 2B), the block of the inward current slowly develops with time (τ ~ 1.5 s, at −120 mV), whereas the outward current is only slightly affected. At high barium concentrations (Fig. 2C), the inward current is blocked significantly, whereas the outward current, which is partially blocked at onset, slowly recovers with time (τ ~ 0.5 s, at 40 mV) before reaching a plateau level.

The I-V curves for the long (3.5 s) voltage pulses are shown in Fig. 2D. Like the currents in response to short pulses (Fig. 1D), the I-V curves measured in the presence of different extracellular concentrations of barium ions pivot about −80 mV, indicating that potassium ions carried the majority of the measured current. However, the blocking effect of barium differed significantly between short- and long-duration pulses.
The inward currents elicited by hyperpolarizing pulses were more effectively blocked during long duration pulses (compare effects of 1.8 μM barium in Figs. 1D and 2D). In contrast, the outward currents in response to depolarizing pulses exhibit the opposite behavior. The degree of barium block decreases with time, and the I-V characteristics for depolarizing pulses remain parallel to each other. These effects and the differences between short-duration and long-duration measurements are readily illustrated by the normalized I/Io plots shown in Fig. 3. Unlike the relatively mild voltage-dependent block measured with short voltage pulses (Fig. 3A), the barium block exhibits strong voltage dependency with pulses of long duration (Fig. 3C). This voltage dependency, together with the slow time course of development of barium block and unblock (see Time course of block by extracellular barium) may explain the block by extracellular barium in terms of a binding site deep within the channels.

To obtain a more quantitative measure of the voltage-dependent characteristics of the block, the IIo curves (Fig. 3, A and C) were replotted with barium concentration as the independent variable for three different voltage levels (Fig. 3, B and D). These curves were then fit with the Hill equation (continuous curves through the data points). Clearly, the effectiveness of the block decreases with depolarization for both short- and long-duration pulses (Fig. 3, B and D, respectively). In response to the short (35 ms) pulses, the EC50 values remained relatively constant at 2.1, 2.3, and 3.9 μM at −120, −40, and +20 mV, respectively. The Hill coefficients decreased from 1.2 to 1.0 and 0.8 for the same voltages. However, the effect is more pronounced in the response to long (3.5 s) voltage pulses. The EC50 values shifted from 1.1 to 9.6 and 65 μM at −120, −40, and +20 mV, respectively. The Hill coefficients decreased from 1.1 to 0.68 and 0.57 for the same voltages as the curves flattened with depolarization.

Time course of block by extracellular barium

The block of the inward current by low extracellular concentrations of Ba2+ ions develops slowly with time during a hyperpolarizing voltage pulse (Fig. 2B). Similarly, with prolonged depolarizing pulses the Ba2+ blocking effect is slowly reduced (Fig. 2C). To characterize the temporal properties of barium block and unblock, the current responses to long duration (4.5 s) voltage steps were fit to an exponential function. To avoid complications due to the transient outward currents observed with application of depolarizing potentials, the fits were started 30 ms following the onset of the voltage pulses. Figure 4A shows the average values of the time constants of the single exponential functions fitting the block (hyperpolarized potentials) and unblock (depolarizing potentials) of the currents.
during prolonged (4.5 s) voltage pulses. The time constants are strongly voltage dependent, typically lasting three to five times longer than the duration of the short pulses used here (35 ms). These relatively large time constants explain the mild voltage dependency of barium block that was observed during application of short pulses (Fig. 3A). With short voltage pulses, barium has no time to move in or out of the channels, and the block remains close to the value preset by the concentration of barium and the holding potential.

Examining the $I-V$ curves for long durations more closely, we observed that the apparent binding coefficient has a value of one at hyperpolarized levels. Within this range the rate of blocking (inverse of the time constant for block) is linearly dependent on the barium concentration (Fig. 4B), in line with

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**FIG. 3.** Average $I/I_o$ curves, computed as the ratio of each $I-V$ curve to the control ($n = 3$). A: in response to short, 35-ms voltage pulses. B: plotted as a function of extracellular barium concentration at the indicated voltages. Continuous lines represent fits using the Hill equation (see text). C: $I/I_o$ response to long, 3.5-s voltage pulses at given barium concentrations. D: same as in B but plotted from the data in C, in response to 3.5-s pulses.

**FIG. 4.** A: time constants of the exponential function fitting the time course of block and unblock at the indicated barium concentrations (in $\mu$M). B: mean values of the rate of blocking (inverse of the time constant for block) of the inward currents as a function of barium concentration at indicated voltages.
a binding process involving a single bimolecular interaction (Woodhull 1973).

**Screening effects by extracellular barium**

At depolarized levels beyond 0 mV, where the inward rectifier is blocked by intracellular polyamines (Solessio et al., unpublished observations) and only the passive conductance remains active, the I-V relationships remain parallel to each other (slope 22 pA/mV in the figure) shifting in the depolarizing direction as the barium concentration is increased (Fig. 5A; also Fig. 2D). This feature of the I-V curves suggests a charge or screening effect (Hille 1992) by barium that modifies the local potential around the channel. Consistent with this, the effects of barium can be partially relieved by reducing the concentration of divalent ions in the extracellular solution (Piccolino and Pignatelli 1996). The I-V curve shifts back in the hyperpolarizing direction when the concentration of calcium and magnesium ions in the extracellular solution is decreased (Fig. 5A). We evaluated the shift of the I-V relations in the depolarizing direction as a function of barium concentration. For this we extrapolated the parallel segment of the I-Vs (beginning at voltages beyond 0 mV) and determined the corresponding “extrapolated reversal potential” at the point where it crossed the current axis (Fig. 5A). The shift of the extrapolated reversal potential with increasing barium concentration is well fit by an exponential function with a constant of 4.5 μM of barium as it reaches a sustained value of −39 mV (Fig. 5B). In this context it would be valid to suggest that within the range of barium concentrations tested, as the block of the linear, passive currents by barium decreases with depolarization, the screening effect becomes more prominent.

**Block by intracellular barium**

Intracellularly applied barium is known to block potassium currents in neurons (Armstrong et al. 1982). We tested the ability of intracellular barium to block potassium currents in turtle Müller cells by adding barium chloride to the pipette solution. Immediately after establishing a whole cell configuration with a pipette containing a solution with 1 mM barium chloride, control responses to short (35 ms) pulses were recorded (Fig. 6A). After allowing 4 min for the barium to diffuse into the cell and to spread throughout the intracellular compartments, its blocking effects reached a steady state. The degree of intracellular barium-induced block was found to depend on the holding potential. With a holding potential of −80 mV, a slight diminution of the inward current was seen with a slightly more pronounced reduction in the outward currents (Fig. 6B). This can be better appreciated from the I-V curve measured at the end of the voltage pulses (Fig. 6D, ∇). In contrast, when the holding potential was set at −40 mV, the outward currents were almost completely blocked with a slightly smaller effect on the inward currents as shown in Fig. 6C. This is clearly evident in the I-V relationship shown in Fig. 6D (■). Regardless of the degree of barium block, all the I-V curves intersect at one point that corresponds to the equilibrium potential for potassium (Fig. 6D), indicative of a selective block of the potassium channels by intracellular barium.

The relationships between holding potential, intracellular barium concentration, and the degree of barium-induced block were studied in detail with short-duration (35 ms) voltage pulses to elucidate the properties of the channels. Normalized $I/\text{i}_n$ curves ($n = 3$) are plotted for HP = −80 mV and HP = −40 mV in Fig. 7A and B, respectively. Different concentrations of barium ions were used to construct these curves. There are considerable differences in the voltage- and dose-dependent characteristics of the block at each holding potential. With a holding potential HP = −80 mV, the degree of block is graded with barium concentration, and a smooth increase in block can be detected with depolarization (Fig. 7A). Even with the largest barium concentration tested (3 mM), the degree of block was considerably more pronounced for outward currents than for inward currents (Fig. 7A, ∇). With a HP = −40 mV, there is a more pronounced block than for any barium concentration tested, even at hyperpolarized levels (Fig. 7B). A discontinuity is seen at −80 mV where the block increases rather abruptly in the depolarizing direction. A mild relief of block is observed at low barium concentrations and large depolarizing voltage levels. The latter effect is somewhat unexpected given the cationic nature of the barium block of the K$^+$ channel.

The same data were plotted using barium concentration as the independent variable and voltage as the free parameter (Fig. 7, C and D). We found that both the data obtained with a HP = −80 mV and with a HP = −40 mV could be well fit with the Hill function. When the holding potential was set at

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**Fig. 5.** Effects of barium in magnesium-free solution. A: long-duration I-Vs under control, in 10 μM barium, and 10 μM barium in calcium and magnesium-free Ringers (divalent free (DVF)). Arrows indicate extrapolation from the parallel segments of the I-Vs to the “extrapolated” reversal potentials. B: plot of the extrapolated reversal potentials as a function of extracellular barium concentration. Fit with a single exponential function.
−80 mV, the Hill function had a similar shape for all potentials indicating a similar value for the Hill coefficient. At −120, −60, −20, and +20 mV the Hill coefficient was 2.0, 2.2, 2.1, and 1.9, respectively. The EC_{50} was relatively constant for hyperpolarizing potentials and decreased with depolarizing pulses. It was 2.1 mM for −120 mV and fell to 1.9, 1.5, and to 1.1 mM at −60, −20, and +20 mV, respectively. These findings indicate that with HP = −80 mV, depolarizing voltages induced a shift of the function toward lower barium concentrations without changing its general shape, as expected in the case of a simple voltage-dependent block (increased affinity). The EC_{50} was relatively constant for hyperpolarizing potentials and decreased with depolarizing pulses. It was 2.1 mM for −120 mV and fell to 1.9, 1.5, and to 1.1 mM at −60, −20, and +20 mV, respectively.

Analogous to the block by extracellular barium, an explanation for the effects of the holding potential on the degree of intracellular barium-induced block probably lies in the slow kinetics of barium block and unblock. This can be determined by using voltage pulses of long duration to allow equilibration of the barium action. One such experiment, using voltage pulses of 3.5 s in duration is shown in Fig. 8. The holding potential was set at −80 mV (Fig. 8A) or −40 mV (Fig. 8B). The pipette solution contained 1 mM barium chloride, and enough time (4 min) was allowed for barium to diffuse into the cell before the first series of voltage pulses was applied. The difference in the response time course between the two different holding potentials is evident. With a HP = −80 mV, inward currents elicited by hyperpolarizing voltage pulses readily reach a plateau level. Responses to depolarizing pulses are characterized by a slow transient before a steady block was reached (Fig. 8A). Conversely, with a HP = −40 mV, the inward currents in response to hyperpolarizing voltage steps are initially small but then develop gradually. However, even with 3.5 s pulse duration, a steady state is not reached (Fig. 8B). When depolarizing voltage pulses are applied, the outward currents exhibit a very fast, small transient that slowly builds toward a steady state (Fig. 8B). We computed the I-V relationships at two different time intervals after onset of the voltage pulses: 35 ms and 3.5 s. Plots at these intervals are shown in Fig. 8, C and D. Note in Fig. 8C that the responses to hyperpolarizing pulses applied from a HP = −40 mV tend to converge with time and match those applied from a HP = −80 mV (compare curves 4 and 2 to curve 1), which are time invariant. It seems that if longer voltage pulses were applied, the inward portion of the I-V curve measured with a HP = −40 mV would match the one elicited with HP = −80 mV. Conversely, the curves in Fig. 8D indicate that with HP = −80 mV the responses to depolarizing pulses (3 and 1) change with time.
and tend to converge and match the depolarizing responses originating from HP = −80 mV, which are time invariant.

A simple explanation for these observations is that at hyperpolarized holding potentials, such as −80 mV, a minimal degree of channel block is maintained by intracellular barium ions (1 mM) because the magnitude of the electrical field is too small to drive barium ions into the channels. A block develops with time when depolarizing pulses are applied as barium and other intracellular constituents (i.e., polyamines) (Solessio et al., unpublished observations) flow into the channels. At depolarized holding potentials, such as −40 mV, a sustained block is maintained. Under these conditions, the initial current responses to hyperpolarizing pulses are small but slowly develop as barium clears out of the channels. With depolarizing pulses, the block is already close to maximal and therefore relatively time-invariant current responses are seen. Thus the holding potential primes the state of block or unblock and the time invariant (or limiting) I-V relationship represents the steady state voltage-dependent block. This is illustrated in Fig. 8, C and D. Responses at long intervals into long pulses (3.5 s; curve 1) or at short intervals (35 ms, curve 3) into long hyperpolarizing pulses originating from HP = −80 mV closely match. At depolarized voltage levels, the curves for long (3.5 s, curve 2) and for short (35 ms, curve 4) pulses applied from HP = −40 mV, matched closely, whereas only long pulses delivered from HP = −80 mV (curve 1) exhibited a similar degree of block (Fig. 7D). That is to say the hyperpolarizing I-V curve for short pulses obtained from a HP = −80 mV (curve 3) and the depolarizing I-V curve for short pulses originating from HP = −40 mV (curve 4) match the long duration I-V relationships and thus can be combined to reproduce the plateau or “limiting” I-V currents.

Computation of the normalized IIIo curves in response to long voltage pulses is technically complicated to do. It takes over 2 min to record a complete control series, enough time for substantial barium to diffuse from the pipette into the cell. However, we can take advantage of the “priming” effect of the holding potentials on the barium block and what we call the “limiting” I-V relationships obtained with short pulses to construct the IIIo curves for “pseudo” long-term barium block. As was shown above, for hyperpolarizing pulses, the currents obtained with short pulses originating from a holding potential HP = −80 mV (curve 3 in Fig. 8D) closely match the long duration I-V curves (the limiting I-V relationships for hyperpolarizing pulses). Similarly, that portion of the I-V curves obtained with short depolarizing pulses originating from HP = −40 mV (curve 4 in Fig. 8D) closely matches the long duration I-V (the limiting I-V for depolarizing pulses). Thus we constructed normalized IIIo curves for long-term block by intracellular barium as a combination of the portions of the IIIo curves corresponding to the limiting I-Vs. The resulting IIIo curves are shown in Fig. 9 using as the independent variable

![Graphs showing normalized I/Io curves with intracellular barium](http://jn.physiology.org/DownloadedFromhttp://jn.physiology.org/)

**Fig. 7.** Normalized IIIo curves with intracellular barium: responses to short 35-ms voltage pulses. Barium concentrations are noted in the figure. A: with HP = −80 mV, there is an increase in block with depolarizing voltage pulses. B: with HP = −40 mV, the degree of block has intensified for the same barium concentrations. C: normalized IIIo curves as a function of intracellular barium. HP = −80 mV results in rather steep curves that are well fit by Hill functions with coefficient n = 2. Depolarization promotes blocking (or affinity of barium to binding site) as the curves shift to lower barium concentrations with increased voltages. Applied voltage steps are indicated in the figure. D: IIIo curves derived from a HP = −40 mV, the Hill coefficient that fits the curves is n = 1. The degree of block, as reflected by the shift of the curves and the computed EC50 values, first increases with small depolarizations, but then unexpectedly drops with larger depolarizations.
either voltage (Fig. 9A) or barium concentration (Fig. 9B). The curves in Fig. 9A are characterized by a striking discontinuity in the block at −80 mV and little voltage dependency at either depolarized or hyperpolarized levels as illustrated by the relatively flat curves.

Replotting the data with barium concentration as the independent variable (Fig. 9B) shows significantly different blocking characteristics with voltage. Block at hyperpolarized levels can be fit with a Hill coefficient of $n = 2$. At depolarized levels the Hill coefficient is 1; however, the $EC_{50}$ values under both conditions remained close to 2 mM. These results imply different binding conditions for barium that is dependent on the polarization conditions of the Müller cells. Given that the inward rectifier channels are blocked by internal spermine during depolarization and that at long durations the block is saturating, it is likely that under such conditions barium can only bind and block the linear, passive conductance. On the other hand, during hyperpolarizing pulses, the polyamines are driven out of the inward rectifying channels (Solessio et al., unpublished observations), and the barium ions can now find binding sites in both the passive linear conductance and the inward rectifier. The different Hill coefficients imply that the binding conditions for barium in the two channels are significantly different.

**FIG. 9.** Block by intracellular barium: normalized $I/I_0$ curves for “pseudo” long pulses. A: average $I/I_0$ curves constructed as described in the text. Hyperpolarized levels correspond to the $I/I_0$ curves obtained from short (35 ms) pulses and a HP = −80 mV. Depolarized levels correspond to the $I/I_0$ curves obtained from short pulses and a HP = −40 mV. B: normalized $I/I_0$ curves as a function of intracellular barium concentration.
Our results are consistent with potassium currents in turtle Müller cells flowing through an inward rectifier and a linear, passive conductance. Barium ions applied extracellularly or intracellularly block both of these currents. The block by extracellular barium is strongly voltage dependent, suggesting that the binding site is located deep within the channel(s). This is confirmed by the slow time course of block and unblock (Hurst et al. 1995). In this regard, the currents in response to short-duration voltage pulses (35 ms), often used for biophysical characterization of ionic conductances in Müller cells, are shorter than the time constant of block and unblock. Therefore the measured current responses only reflect the block by barium at the holding potential. That barium binds within the channel pore is reflected in the degree of block that tends to change as barium ions flow into or out of the channels according to the electrical driving force and the source of barium ions. Thus with extracellular barium, the degree of block is high at the onset of a depolarizing voltage step but decreases with time as barium ions are driven out of the channels (Fig. 2C). When a hyperpolarizing voltage step is applied, the degree of barium block is initially small but increases with time as barium ions flow into the channel (Fig. 2B). The opposite occurs when barium ions are present inside the cell. The degree of block increases with time during depolarizing voltage pulses (Fig. 8A) and decreases with time during hyperpolarizing voltage pulses (Fig. 8B).

At hyperpolarized levels, extracellular barium appears to block the two conductances through similar, indistinguishable processes as follows from the dose-response and the time course of block. At depolarized levels the inward rectifier is blocked by intracellular polyamines (Solessio et al., unpublished observations), and the outward currents flow primarily through the passive conductance. Extracellular barium shifts the I-V curves consistent with a neutralization (or screening) of membrane surface charges rather than a block of the channel by barium (Green and Andersen 1991; Hille 1992; Piccolino and Pignatelli 1996). Our experiments cannot differentiate between screening arising from a distributed planar charge or from charged groups on the channel itself. But the results suggest that increasing the concentration of barium makes the surface potential less negative and the voltage across the channel more negative resulting in a local decrease of the driving force (Zhou and Jones 1995) for the potassium ions flowing into the channels. Unscreening of the surface charges by lowering the concentration of calcium and magnesium had the opposite effect; the I-V relation shifted in the hyperpolarizing direction without a change in slope. A competitive block of the channel by divalent cations would have brought about an increased block by barium under these circumstances (Piccolino and Pignatelli 1996).

Thus our results indicate that within the concentration range tested, the effect of extracellular barium is to block both the potassium channel types for inward currents induced at hyperpolarized levels. Whereas at depolarized levels the effect on the outward currents partially reflects screening of charge on the passive, linear conductance. More importantly, at hyperpolarized levels (the voltage range in which the two types of channels are active in vivo) our results suggest a one-to-one binding process by barium in the two channels, such that we cannot distinguish a differential effect of barium from which to infer different structural properties between the two channel types.

Intracellular barium also blocks the potassium channels, although the underlying mechanisms differ considerably from those observed with extracellular use. As seen in the curves for steady-state block derived above, the slope of the dose-response curves varied dramatically with polarization. When the data were fit with the Hill function, a Hill coefficient of 1 best fit the data when the cells were depolarized, whereas a value of 2 fit the data best when the cells were hyperpolarized. If we assume that barium does not interfere with the depolarizing block of the inward rectifier by polyamines, this suggests one apparent binding site for barium available within the pore of the passive conductance and two apparent binding sites for the inward rectifier. The biophysical interpretation of these “apparent binding coefficients” is limited given the multi-ion channel properties of potassium channels (Hille 1992; Hille and Schwarz 1978). In this regard, an Eyring rate theory model supported by kinetic studies would be necessary to completely describe the block. However, our results provide further evidence that the currents in turtle Müller cells do not flow through a single type of channel but rather through two different types. This and the effect of charge screening have not been considered in past studies of Müller cells.

Our findings are also indicative of a structural difference in the intracellular side of the channels that correlates with the differential affinity of the channels to bind polyamines and

**TABLE 1. Müller cell cable parameters**

<table>
<thead>
<tr>
<th></th>
<th>Cell A</th>
<th>Cell B</th>
<th>Cell C</th>
</tr>
</thead>
<tbody>
<tr>
<td>( f_c ), Hz</td>
<td>1.76</td>
<td>0.8</td>
<td>1.34</td>
</tr>
<tr>
<td>( \tau_{m, s} ), s</td>
<td>0.09</td>
<td>0.194</td>
<td>0.117</td>
</tr>
<tr>
<td>( R_m ), ( \Omega ) cm²</td>
<td>90,000</td>
<td>198,000</td>
<td>117,000</td>
</tr>
<tr>
<td>( x_{Ba} )</td>
<td>52</td>
<td>116</td>
<td>68</td>
</tr>
<tr>
<td>( R_m (m) ), ( \Omega )</td>
<td>3.3</td>
<td>9</td>
<td>4.5</td>
</tr>
<tr>
<td>( R_m (c) ), ( \Omega )</td>
<td>4.1</td>
<td>7.7</td>
<td>5</td>
</tr>
</tbody>
</table>

\( f_c \), cut off; \( \tau_{m, s} \), time constant; \( R_m \), resistivity; \( x_{Ba} \), blocking factor; \( R_m (m) \), input resistance (measured); \( R_m (c) \), input resistance (computed).
their resulting rectifying properties. In fact, it has been suggested that the blocking process in inward rectifiers involves at least two binding sites (Lopatin et al. 1995; Yang et al. 1995) and more than one blocking particle. Our results suggest that the pores of the inward rectifying channels, which have the steeper blocking function and can apparently accommodate more than one barium ion arriving from the intracellular side, are also suitable for block by polyamines (Solessio et al., unpublished observations) and that the intrinsic concentration of spermine is enough for a saturating block at long durations. On the other hand, channels binding a single barium ion are not blocked by polyamines.

Nonetheless, the similarity in the apparent affinity values for block by barium ions in the two channels argues against the idea of large structural differences between the channels. This probably translates into the observed lack of differential sensitivity to extracellular barium block. This is not without precedent. Mutation of a single amino acid residue can convert a weakly rectifying channel into a strong one (Lu and MacKinnon 1994). Or, conversely, a single amino acid change turns a strong rectifier into a weak rectifier (Yang et al. 1995) or can even alter the sensitivity to barium block (Krapivinsky et al. 1998; Zhou et al. 1996). This suggests that changes in binding affinities and rectifying properties do not involve dramatic structural changes in the ion channel protein. Two channels with different rectifying properties may be structurally quite similar. In this context, we found that the linear, passive conductance and the inward rectifying channels maintain the same constant ratio between cells and between different preparations (Solessio et al., unpublished observations). This is consistent with the idea that the inward rectifier channels in turtle Müller cells may be coupled to the linear channel and be a type of tandem pore potassium channel (Chavez et al. 1999; Ketchum et al. 1995).

FIG. 11. Intracellular barium improves the quality of voltage clamp under various clamping configurations. Hypothetical cells were voltage clamped at either the cell body or end process as shown at bottom. Four graphs (A–D) illustrate calculated voltage drops observed when moving away from the electrode at the distances shown. A and C: effects in isolated cells. B and D: effects in the retinal slice. Continuous lines represent the voltages computed along the cell body of a Müller cell as illustrated in the bottom of the figure. Although only 4 processes are shown for illustrative purposes in the cartoon at bottom, calculations were made on a hypothetical cell with 6 processes. The location of the recording pipette on the theoretical cell is indicated by an asterisk. In A and B the cell is clamped at the soma, and the voltage drop measured out to the end of all 6 processes; each process is assumed to be identical. Dashed line represents the voltage drop along the 6 processes under control conditions. Dotted line indicates the voltage drop when barium is present within the cell. C and D: the cell is being voltage clamped at the end of one process, and the voltage drop toward the cell body along that process and the other 5 processes is plotted. Dashed line represents the voltage drop along process 6 when it is voltage clamped under control and barium conditions in an isolated cell (C) and in the slice (D). Dotted line represents the voltage drop along hypothetical process 1–5 under control and barium conditions.
Voltage clamping in the presence of barium block

An important observation we made is that beyond a certain concentration and for short pulses at depolarized potentials, the block by intracellular barium is no longer voltage dependent. This is a general consideration that should be taken into account for voltage-clamp studies when using blocking agents, which bind within channel pores. This results in a more effective, voltage-independent block and more efficient clamping of the cell, which can be easily demonstrated and understood with application of a simple mathematical model of the turtle Müller cell.

The standard method used to determine the membrane resistivity is based on cable analysis. Briefly, the membrane resistivity is determined from

$$\tau_m = R_m C_m$$

where $\tau_m$ is the time constant fitting the exponential response to a current pulse, $R_m$ the membrane resistivity, and $C_m$ the membrane-specific capacitance. The average value of the time constant of the exponential function fitting the responses to depolarizing pulses was $82 \pm 27$ ms (mean $\pm$ SD, n = 3). To verify these results we also employed noise analysis (see METHODS; Fig. 10), which does not involve application of voltage steps. The results from three cells are summarized in Table 1.

With 10 mM intracellular barium the membrane resistivity increased by a factor (xBa) of $\sim 50$–100 with respect to an average control value of 1,700 $\Omega$ cm$^2$. To validate these results we compared the measured input resistances to input resistances computed applying cable equations and the new value of $R_m$. The values matched quite well. It is important to point out that the experiments were performed on isolated cell somas without end processes. Given that we previously showed similar conductances along all cell parts (Linn et al., 1998), with a higher density in the processes, we will assume the blocking factor xBa = 80 to equally affect all membrane compartment resistivities.

We next applied the $R_m$ values in the presence of barium to a compartmental model of the turtle Müller. We evaluated the effects that the increased block had on the quality of the clamp, for cells both in isolation and in the slice. Under control conditions, cells in isolation have larger processes; $\sim 2$ $\mu$m diam compared with 0.5 $\mu$m in the slice (Solessio et al., submitted for publication). Figure 11 demonstrates how the addition of barium into the Müller cell improves the clamping conditions even in the slice preparation. When whole cell recording is done at the cell soma, the voltage drop toward the endfeet is negligible in the presence of intracellular barium ions (Fig. 11, A and B, dotted line). This is in contrast to a 20 and 40% drop in the control conditions of isolated cells or in the intact retina respectively (Fig. 11, A and B, dashed line). When a whole cell recording is made at the endfoot, the voltage drop toward the cell soma is significant. It can be 60% in isolated cells (Fig. 11C, bottom traces) and close to 90% in the slice preparation (Fig. 11D, bottom traces). This space clamp is significantly improved when barium ions are added to the intracellular space (Fig. 11, C and D, top traces). The quantitative analysis shown in Fig. 11 indicates that using a pipette solution containing barium chloride in a concentration of 3–10 mM is advantageous when the biophysical properties of Müller cells are studied in the intact retina.