Spermine Mediates Inward Rectification in Potassium Channels of Turtle Retinal Müller Cells

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Retinal Müller cells are highly permeable to potassium as a consequence of their intrinsic membrane properties. Therefore these cells are able to play an important role in maintaining potassium homeostasis in the vertebrate retina during light-induced neuronal activity. Polyamines and other factors present in Müller cells have the potential to modulate the rectifying properties of potassium channels and alter the Müller cells capacity to siphon potassium from the extracellular space. In this study, the properties of potassium currents in turtle Müller cells were investigated using whole cell voltage-clamp recordings from isolated cells. Overall, the currents were inwardly rectifying. Depolarization elicited an outward current characterized by a fast transient that slowly recovered to a steady level along a double exponential time course. On hyperpolarization the evoked inward current was characterized by an instantaneous onset (or step) followed by a slowly developing sustained inward current. The kinetics of the time-dependent components (block of the transient outward current and slowly developing inward current) were dependent on holding potential and changes in the intracellular levels of magnesium ions and polyamines. In contrast, the instantaneous inward and the sustained outward currents were ohmic in character and remained relatively unaltered with changes in holding potential and concentration of applied spermine (0.5–2 mM). Our data suggest that cellular regulation in vivo of polyamine levels can differentially alter specific aspects of potassium siphoning by Müller cells in the turtle retina by modulating potassium channel function.

INTRODUCTION

Müller cells play an important role in retinal physiology. They play a role in maintaining retinal cell metabolism (Newman 1996; Reichenbach et al. 1993) and are essential for maintaining potassium homeostasis of the extracellular space during neuronal activity (Karwoski et al. 1989; Newman 1987; Newman et al. 1984). To maintain potassium homeostasis of the extracellular space, Müller cells are highly permeable to potassium ions, predominantly through inward rectifier potassium channels (Brew et al. 1986; Chao et al. 1994, 1997; Newman 1987, 1988, 1993; Nilius and Reichenbach 1988). Calcium-activated potassium channels (Bringmann et al. 1997; Newman 1985; Puro et al. 1989) and voltage-activated potassium channels (Chao et al. 1994; Reichelt and Pannicke 1993) have also been described but probably play only a minor role in potassium homeostasis.

The biophysical properties of potassium channels in Müller cells determine the efficacy with which these cells can maintain potassium homeostasis. Recent studies demonstrated that Müller cells in rabbit, rat, and mouse retina express Kir4.1 inward rectifier channels (Ishii et al. 1997; Kofuji et al. 2000). Similar to other inward rectifier channels (Fakler et al. 1994; Ficker et al. 1994; Lopatin et al. 1994), spermine plays a dominant role in conferring potassium channels in rabbit Müller cells with voltage-dependent rectification (Biedermann et al. 1998). Müller cells have the capacity to produce polyamines such as spermine, which are postulated to regulate potassium currents and modulate neuronal function in vivo (Biedermann et al. 1998). Thus the degree of rectification may be modified by the intracellular milieu of Müller cells and by intracellular metabolic pathways.

Previous studies (Conner et al. 1985; LeDain et al. 1994; Linn et al. 1998) demonstrated that turtle Müller cells are highly permeable to potassium ions and that potassium fluxes could be induced in an inward or outward direction dependent on the driving force. The outward currents consisted of a fast onset transient followed by a sustained component. At potentials more negative than the resting potential of the cell, the currents had properties of inward rectifying channels. Like the potassium channels in the endfeet of Müller cells in frog (Skatchkov et al. 1995), barium ions blocked both inward and outward currents, cesium ions blocked only the inward current (Linn et al. 1998; Solessio et al. 2000), while tetraethylammonium (TEA) had no effect on either current (Le Dain et al. 1994; Linn et al. 1998). The stoichiometry of block by intracellular barium ions differed with the state of polarization of the cells. The inward currents induced by hyperpolarizations required two “apparent” binding sites for block by barium ions, whereas a single apparent binding site was available for block of the outward current during prolonged depolarizations. A parsimonious interpretation of these findings suggested that on depolarization, divalent ions and polyamines, known to block inward rectifier channels, were driven into the channel pore, thereby competing with the barium ions for binding sites.
(Solessio et al. 2000). It follows then, that, if the concentration of intracellular polyamines is sufficient to completely block the rectifying component, the inward and sustained outward currents most likely flow through two different types of channels. Thus the nature of the K⁺ channels present, their regulation by polyamines, and their exact role in the siphoning of K⁺ in turtle Müller cells remains unclear. This is of particular importance in view of the complex structure of turtle Müller cells (Conner et al. 1985; Linn et al. 1998) that raises concerns about their ability to siphon K⁺. To better understand the physiology of turtle Müller cells, we undertook an investigation of the mechanisms underlying the rectifying properties of the potassium channels in turtle Müller cells and their role in potassium siphoning. While spermine, and other polyamines, are effective modulators of Müller cell potassium currents (Biedermann et al. 1998), we have limited knowledge of their role in the overall physiology of the Müller cells. Our purpose here was to characterize the different types of potassium currents, determine their functional contribution to the overall membrane properties of the cells, and evaluate the effects that up or down-regulation of polyamines may have on the potassium current (and its particular components). Applying whole cell techniques to freshly dissociated cells, we recorded the membrane currents while controlling the intracellular levels of divalent ions and polyamines such as spermine.

Our results demonstrate that two components contribute to the potassium currents in turtle Müller cells. A rectifying, time-dependent component that is blocked by spermine; and a second, approximately “ohmic” component, with little sensitivity to added spermine (0.5–2 mM). The findings described here and elsewhere are compatible with two alternatives: 1) the two current components are mediated by two different types of channels or 2) one type of inwardly rectifying potassium channel exists with a wide inner pore that can simultaneously accommodate more than one blocking ion and through which ions do not necessarily flow in single file.

METHODS

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 the guidelines set out by the Association for Research in Vision and Ophthalmology for the use of animals in research. The eyes were enucleated and the globes then hemisectioned. The retinas from the two eyes were isolated from the eyecups and placed in a calcium/magnesium–free Ringer solution for 15 min. The isolated retinas were then incubated for ½ h in papain (100 U/20 ml) dissolved in L-15 (GIBCO BRL, Gaithersburg, MD). Then the retinal pieces were gently triturated onto glass coverslips. Recordings were performed within 4–10 h following dissociation.

Turtle Müller cells have five to six thin processes extending from the cell soma, each ending in an endfoot (Conner et al. 1985; Linn et al. 1998). This complex structure prevents adequate space clamp when the whole cell recording configuration is established in the soma of the cell (Perlman et al. 2001). We have found that the cell soma and the processes of turtle Müller cells express potassium channels with similar biophysical properties if at different densities (Perlman et al. 2001). So we limited all recordings in this study to Müller cells that had lost their processes during the isolation procedures. Application of cable equations predicts that for cells under these conditions, the space clamp is complete and allows accurate determination of the biophysical properties of the different channels underlying the currents measured (Perlman et al. 2001).

Experimental solutions

The normal turtle Ringer consisted of the following (in mM): 110 NaCl, 2.0 CaCl₂, 2.6 KCl, 2.0 MgCl₂, 8.4 HEPES, and 10 d-glucose with pH 7.4. All chemicals were purchased from Sigma (St. Louis, MO). Solutions were delivered via a 12-reservoir pressure ejection system controlled by a personal computer (DAD-12, Adams, NY). The patch pipette solution consisted of 130 mM potassium gluconate, 4 mM NaCl, 0.2 mM EGTA, 2 mM MgCl₂, 10 mM HEPES, and 7.8 µM CaCl₂ with pH 7.4. In some of the experiments the pipette solution was supplemented with 500 µM spermine. In other experiments we buffered spermine and other polyamines by adding 5 mM tris-ATP (Watanabe et al. 1991) to a divalent-free solution. For the divalent-(Ca²⁺ and Mg²⁺) free solution, both MgCl₂ and CaCl₂ were omitted, and EDTA and EGTA were added at 5 mM each. Supplementing the intracellular solutions with EGTA, EDTA, and/or ATP introduced less than a 5% change in osmolarity of the intracellular medium.

Recording procedures

Whole cell recordings (Hamill et al. 1981) were made with micropipettes that had been pulled on a two-stage puller (model PP-83, Narishige Instruments, Tokyo, Japan) and were used unpolished and coated with silicone elastomer (Sylgard). The electrode tip resistance was 2–3 MΩ when measured in the bath solution. Series resistance and capacitance were compensated for electronically. Electrical potentials were recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Data collection was controlled by a personal computer interfaced to a Digidata 1200 data acquisition system driven by the pClamp suite of programs (Axon Instruments). After establishing a whole cell configuration, the cells were held at a potential (HP) of −80 mV unless indicated otherwise. The stimuli, short (60 ms) or long (1 s or longer) incremental and decremental voltage pulses, were preceded by a brief prepulse to −100 mV to preactivate the channels.

Data analysis

Current–voltage functions (*I*-*V*s) were measured from the responses to incremental and decremental voltage pulses. The chord conductances were computed according to

\[
g_i = I(V_i - V_m) 
\]

where \(I_i\) is the current measured at the voltage \(V_i\), \(V_{rev}\) is the reversal potential determined from the respective \(I-V\); typical values ranged from −75 to −85 mV. The conductances were normalized with respect to their maximal value, and fit with a Boltzmann equation with a pedestal (or standing conductance)

\[
g_i = (1 - S)/(1 + \exp[-\delta(V_i - V_{sk})/V_T])] + S
\]

where \(S\) is the pedestal level, \(V_{sk}\) is the blocking voltage or the voltage required to decrease the conductance to half of its variable range \((1 - S)\), \(\delta\) is the charge factor and a measure of the slope of the function, and \(V_T\) is given the typical value of 25 mV.

Fits were performed with Sigma Plot (Chicago, IL), which applies the Marquardt-Levenberg algorithm to minimize the sum of the squared differences between the Boltzmann equation and the conductance data. Tests for statistical significance were performed using ANOVA. Conductance plots show average values measured at respective potentials, bars are standard deviations, whereas number of observations are indicated in the text. The time-dependent currents were fit with two exponential curves

\[
f(t) = A_1 \exp[-(t - k_1)t] + A_2 \exp[-(t - k_2)t] + A_3
\]

using the Simplex method (Axon Instruments).
RESULTS

Figure 1A shows the current responses of an isolated cell soma to a series of 60-ms-long voltage steps, applied from a holding potential of −80 mV. A prepulse to −100 mV preceded each voltage step. Inward currents readily reach a sustained level, as is characteristic of inward rectifiers (note that the prepulse to −100 mV fully preactivated this current). The outward currents showed a fast initial transient followed by a slower inactivation. Similar recordings were done from the same cell exposed to different concentrations of extracellular potassium. The corresponding I-V curves evaluated 25 ms following onset of the voltage pulses (dashed line in Fig. 1A) showed only mild rectification (Fig. 1B). A strong dependency on extracellular potassium was revealed by the depolarizing shift of the resting potential (46.2 ± 3.3 mV/decade, mean ± SD, n = 4), an increase in the slope conductance (fit with a power function with coefficient 0.7 ± 0.06, n = 4), and a crossover of the curves with increased concentrations of extracellular potassium ions. The latter effects imply a rather weak blocking mechanism underlying inward rectification that can be overcome by an increase in the outward flow of potassium ions through the channels (Hille and Schwarz 1978).

Application of longer voltage pulses revealed an additional block that developed at a slower rate (Fig. 1C). The current responses to long (1.6 s), depolarizing voltage pulses exhibited a decay of the outward currents that could be described by the sum of two exponentials. A fast component characterized by a time constant ranging between 2 and 5 ms and a second slower component with a time constant of about 100 ms (see Fig. 4D). The I-V curves were generated from data measured 1 s into the pulses (dashed vertical line in Fig. 1C) and are shown in Fig. 1D. As observed in response to the short (25 ms) pulses, these curves showed a strong dependency on extracellular potassium concentration. Raising extracellular potassium induced a depolarization of the resting potential that was approximately Nernstian in nature (47.5 ± 1.9 mV/decade, n = 4), suggesting a selective conductance for potassium. This study replicated the work of Newman (1993) in salamander Müller cells, and these results are similar to his findings including the slope conductance, which followed a power function with a power coefficient of 0.68 ± 0.07 (n = 4). However, in contrast to the I-Vs obtained when measured at 25 ms (Fig. 1B), these I-V curves showed strong rectification and did not cross over as potassium levels were increased. This implies an augmentation of the block with time that cannot be overcome in spite of an increased potassium gradient (Hille and Schwarz 1978).

To study the temporal progression of the block, we compared in Fig. 2A the I-V curves computed from the same cell, at 4, 25, and 1,000 ms following the onset of the voltage pulses. The curves overlapped at potential levels that were hyperpolarized from the resting potential, indicating that the inward currents developed within 4 ms or less and stabilized. At depolarized levels the I-V curves departed considerably from each other with rectification becoming more apparent with time. However, even at the long time period (1 s) when a steady state was achieved, the currents increased linearly with voltage, thus falling short of complete rectification. A quantitative measure of the rectifying properties of the channels in the Müller cells was obtained by fitting the chord conductances with a Boltzmann function as shown in Fig. 2B (Lopatin et al. 1995; Newman 1993). As rectification increased with time, the conductance curve sharpened. The Boltzmann coefficients needed to fit the curves increased significantly (P < 0.01) from 1.25 ± 0.05 (n = 7) at 4 ms, to 1.5 ± 0.08 (n = 8) at 25 ms.
Transient and sustained currents do not flow through typical potassium channels. However, these currents are strongly affected by holding potential (Fig. 3). Holding the cells at a holding potential levels that were depolarized with respect to the reversal potential for potassium (in turtle Müller cells this value is about \(-85\) mV), promoted the voltage-dependent block of the potassium channels by divalent ions and polyamines.

When Müller cells were studied for prolonged periods of time (4 min or more), we noticed that the degree of rectification and the rate of its development changed with time, as shown in Fig. 4A. To investigate the outward current, a depolarizing pulse to 0 mV was applied after the inward rectifying channels were activated by a prepulse to \(-100\) mV. The outward current rose to a transient peak and then recovered gradually to a steady state. Four minutes after establishing the whole cell configuration, the rate of recovery slowed down, but the steady-state current remained the same. Similar effects were seen in five other Müller cells. This observation can be accounted for by time-dependent changes in the intracellular milieu of the cell due to equilibration between the intracellular space and the pipette filling solution. It was likely that we diluted or washed out some blocking factor in the cell.

Probable candidates for intracellular blocking agents are divalent cations, in particular magnesium ions (Matsuda et al. 1987; Vandenberg 1987) and polyamines such as spermine or spermidine (Biedermann et al. 1998; Watanabe et al. 1995). To investigate these possibilities, we omitted calcium and/or magnesium from the pipette solution or added ATP to buffer intracellular polyamines (Fakler et al. 1995; Lopatin et al. 1995). To investigate these possibilities, we omitted calcium and/or magnesium from the pipette solution or added ATP to buffer intracellular polyamines (Fakler et al. 1995; Watanabe et al. 1991). Given that ATP also binds magnesium, we added ATP to a divalent-free solution. In each experiment, the outward current was recorded immediately after establishing the whole cell configuration and 4 min later to compare the effects of our experimental manipulations to the control conditions. The results of representative experiments are shown in Fig. 4. When a calcium-free intracellular solution with normal levels of magnesium was used, the time course of the response did not differ greatly from that obtained under control conditions (Fig. 4, A vs. B). However, combined removal of calcium and magnesium produced a decrease in the extent of block in the current recorded 4 min after establishing the whole cell configuration (Fig. 4C). The decrease in block was further

and 2.23 \(\pm\) 0.19 (\(n = 9\)) at 1,000 ms. These values are typical of weak rectifiers (Hille 1992). Furthermore, there is a concurrent decrease in the value of the pedestal needed to generate a fit of the Boltzmann function. The standing conductance values progressively decreased significantly (\(P < 0.01\)) from 0.53 \(\pm\) 0.045 (\(n = 7\)), to 0.43 \(\pm\) 0.01 (\(n = 8\)) and 0.28 \(\pm\) 0.017 (\(n = 9\)) for 4, 25, and 1,000 ms, respectively. A significantly more depolarized blocking voltage (\(P < 0.01\)) was observed at 4 ms (\(-57 \pm 6\) mV, \(n = 7\)), compared with those determined at 25 (\(-74 \pm 4\) mV, \(n = 8\)) and 1,000 ms (\(-75.5 \pm 5.7\) mV, \(n = 9\)).

Three distinct current features are apparent from the responses of turtle Müller cells to voltage pulses (Fig. 1): an inwardly rectifying current, a transient outward current, and a sustained outward current. Although all three features are effectively blocked by barium, none is sensitive to TEA or 4-aminopyridine (4-AP) (Le Dain et al. 1994; Linn et al. 1998; Solessio et al. 2000), which suggests that the outward currents are effectively blocked by barium but reach a standing or pedestal level as the cell becomes even more depolarized. As the duration of the pulses increased, the curves sharpened, and the amplitude of the pedestal level decreased.

FIG. 2. Rectification intensifies with duration of voltage pulses. A: representative \(I-V\) curves computed at indicated times following onset of the voltage pulses for a single cell. B: average (\(\pm\)SD) of the normalized cord conductance computed from the \(I-Vs\) of 7 or more cells. The chord conductances decrease as the cell is depolarized but reach a standing or pedestal level as the cell becomes even more depolarized. As the duration of the pulses increased, the curves sharpened, and the amplitude of the pedestal level decreased.

![Graph](http://jn.physiology.org/)

There is also a reduction in the pedestal level that has a value of 0.43 \(\pm\) 0.05 (\(P < 0.01, n = 5\)) compared with 0.53 \(\pm\) 0.045 (\(n = 7\)) at \(-80\) mV; although the slope of the function as inferred from the Boltzmann coefficients has not changed significantly (1.16 \(\pm\) 0.32, \(n = 5\)). Given that the inward currents slowly relax to their standing levels, the effects of holding potential are less pronounced with time after onset of the test pulses. When measured at 25 ms (Fig. 3D), the blocking voltage recovered to a hyperpolarized value of \(-96 \pm 5.3\) mV (\(P < 0.01, n = 5\)), compared with \(-74 \pm 4\) mV (\(n = 8\)) at a holding potential of \(-80\) mV. There was no significant change in the pedestal level (0.39 \(\pm\) 0.03, \(n = 5\)), and the estimated Boltzmann coefficient of 1.94 \(\pm\) 0.25 (\(P < 0.01, n = 5\)) reflects a sharpening of the block compared with 1.5 \(\pm\) 0.08 (\(n = 8\)) obtained with a holding potential of \(-80\) mV. The effects of holding potential lasted <1 s as no significant changes were detected when currents were measured 1 s after the onset of test pulses. The voltage-dependent changes that we observed in the currents of turtle Müller cells were similar to those that had been observed in recombinant Kir channels expressed in murine fibroblast cells (Ishihara 1997). Here the holding potential levels that were depolarized with respect to the reversal potential for potassium (in turtle Müller cells this value is about \(-85\) mV), promoted the voltage-dependent block of the potassium channels by divalent ions and polyamines.

![Graph](http://jn.physiology.org/)

FIG. 3. Voltage-dependent changes affect the time course of the response and polyamines. Probable candidates for intracellular blocking agents are divalent cations, in particular magnesium ions (Matsuda et al. 1987; Vandenberg 1987) and polyamines such as spermine or spermidine (Biedermann et al. 1998; Lopatin et al. 1995). To investigate these possibilities, we omitted calcium and/or magnesium from the pipette solution or added ATP to buffer intracellular polyamines (Fakler et al. 1995; Watanabe et al. 1991). Given that ATP also binds magnesium, we added ATP to a divalent-free solution. In each experiment, the outward current was recorded immediately after establishing the whole cell configuration and 4 min later to compare the effects of our experimental manipulations to the control conditions. The results of representative experiments are shown in Fig. 4. When a calcium-free intracellular solution with normal levels of magnesium was used, the time course of the response did not differ greatly from that obtained under control conditions (Fig. 4, A vs. B). However, combined removal of calcium and magnesium produced a decrease in the extent of block in the current recorded 4 min after establishing the whole cell configuration (Fig. 4C). The decrease in block was further
enhanced when ATP was added to the divalent-free solution as shown in Fig. 4D. Under these conditions, the increase in the amplitude of the transient was accompanied by a significant slowdown in the rate of decay from the transient peak to the sustained level.

We next assessed the effects of the different manipulations on the time-dependent changes in the outward currents (Fig. 4E). When the time constants of the exponential functions fitting the transient currents were calculated for different voltage pulses, we found that under control conditions the responses were well fit by two components. A fast component ($\tau_1$) ranging between 2 and 3 ms, and a second, slower component ($\tau_2$), ranging between 70 and 180 ms ($n = 5$). The data shown in Fig. 4E were computed from the responses to long 1-s depolarizing pulses in Müller cells studied after 8 min of whole cell recording with the divalent-free and 5 mM ATP pipette solution. The time constant of the fast blocking component increased from about 2 ms to about 10 ms, while the time constant of the slow blocking mechanism did not change significantly. When comparing $\tau_1$ with $\tau_2$ for the control and ATP situations, they were significantly different (ANOVA test, at $P < 0.01$) when tested at $-40$, 0, and $+40$ (mV) membrane potentials. Likewise, control versus ATP for $\tau_1$ at these membrane potential levels were significantly different ($P < 0.01$), while $\tau_2$ control versus ATP were not different.

Differential blocking effects by divalent ions and intracellular polyamines are reflected in the I-V curves evaluated 25 ms following onset of the voltage pulses. Figure 5A (left) shows the effects that buffering both magnesium and calcium ions has on the currents. There is an increase in the slope of the curve for outward currents with minimal effect on the inward currents. When spermine and any other polyamines present are buffered by adding ATP to the divalent-free solution, the outward currents grow even further (Fig. 5A, right). Under these conditions, the relationship between the outward current and the voltage is nonlinear as indicated in Fig. 5A (right) by the deviation of the I-V data (○) from linearity (—). The effects of this solution are particularly apparent in the physiological range of $-60$ to $+10$ mV and is reduced for more depolarized levels. The inward currents remained relatively unchanged.

The voltage-dependent changes brought about by divalent-free solutions with and without ATP are illustrated and quantified by plotting the corresponding chord conductances (Lopatin et al. 1995) in Fig. 5B. These data were calculated from the I-V curves measured 4 min after establishing whole cell configuration and allowing the intracellular space to equilibrate with the pipette contents. With a pipette solution with no added calcium (Calcium-free in Fig. 5B), we observed a depolarizing change in the blocking voltage, from $-74$ to $-65$ mV (SD, 6.4 mV; $P < 0.01$, $n = 5$). With a divalent-free solution, the corresponding pedestal level rose significantly ($P < 0.01$) from $0.43 \pm 0.01$ ($n = 8$) to $0.52 \pm 0.03$ ($n = 6$) without a major
change in the blocking voltage, which, similar to control values, averaged $-72 \pm 2.9$ mV ($n = 6$). This last observation is apparently in conflict with the depolarizing shift of the conductance observed in the figure, but probably arises as a result of fitting data of unequal pedestal levels with Boltzmann functions. In the figure, the conductance obtained under divalent-free conditions does not overlap the control conductance, but rather its voltage-dependent portion traces over the conductance obtained under Ca$^{2+}$-free conditions, until it diverges due to the unequal pedestal levels. As a consequence of this “truncation” of the voltage-dependent portion of the conductance, the Boltzmann function tends to underestimate the blocking voltage. The overlap of the Ca$^{2+}$-free and divalent-free conductances suggests that omitting intracellular calcium shifts the blocking potential in the depolarizing direction by approximately 10 mV while magnesium ions contribute to the pedestal level under these conditions.

We next studied the effects resulting from a reduced concentration of polyamines. Buffering the intracellular levels of free polyamines with ATP in a divalent-free solution significantly increased the blocking voltage to $-37 \pm 7.0$ mV ($P < 0.01, n = 5$) and the pedestal conductance level to $0.60 \pm 0.1$ ($P < 0.01, n = 5$). The value of the pedestal level does not differ significantly from that obtained using divalent-free solutions, nor is there a significant change in the Boltzmann coefficients ($1.34 \pm 0.16, n = 5$) in ATP solutions. This parallel shift of the conductance is consistent, to a first approximation, with the decrease in block expected when the concentration of the blocking agent is reduced (Lopatin et al. 1995).

In summary, our results indicate that calcium ions had a minor effect on the blocking voltage, magnesium ions had their major impact on the pedestal level (vertical arrow in Fig. 5B), whereas polyamine concentration determined the blocking voltage (horizontal arrow in Fig. 5B). It appears that changes in magnesium ions and polyamines alter the $I-V$ curves and the chord conductances differentially. It is possible that the difference arises from the fact that we may be more effectively buffering the divalent ions than the polyamines. This would shift the blocking voltage to levels beyond $+40$ mV, and we might then only observe a change in the pedestal conductance. The changes in intracellular concentration of divalent ions or polyamines affected primarily the initial portion of the responses and did not significantly change the $I-V$s or chord conductances evaluated at the end of long (1 s) duration pulses (data not shown).

If the reduction in the concentration of free intracellular polyamines (by buffering with ATP) promotes a decrease in the block of outward currents and a slow-down in the voltage-dependent block of the K$^+$ channels, then raising the level of intracellular polyamines should induce the opposite effects. To test this prediction, we added spermine to the pipette filling solution (Bianchi et al. 1996). Only marginal reductions of the
initial transient currents were observed with concentrations under 100 μM. However, with 500 μM spermine in the pipette, significant changes were observed in the current responses to depolarizing and hyperpolarizing voltage pulses as shown by representative recordings in Fig. 6. With spermine added, the initial transient in the outward current response disappeared, and only sustained currents remained (Fig. 6A), as is evident by comparing responses to depolarizing voltage pulses (Fig. 6B). This effect is consistent with a speeding up of the development of the block.

In addition to its effects on outward currents, added spermine also reduced the magnitude of the inward currents (Fig. 6A). As a consequence, the regular spacing between the outward currents extends to the smaller inward currents elicited by voltage pulses to −90 and −100 mV, as rectification is reduced and the currents are reminiscent of an ohmic conductance. These effects were more clearly demonstrated by applying hyperpolarizing pulses from a holding potential of −40 mV. Under these conditions the currents are characterized by an instantaneous onset followed by a slow relaxation (Fig. 6, C and D). Raising intracellular spermine affected primarily the slow phase, which decreased in amplitude in a voltage-dependent manner. For a hyperpolarizing step to −100 mV, the relaxation phase almost completely disappeared due to the blocking action of spermine (Fig. 6C), and only a sustained current with amplitude similar to the instantaneous component seen under normal conditions remained. With a step to a more hyperpolarized level (−120 mV), the block by added spermine

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**Fig. 5.** I-V relationship measured 25 ms after onset of the command pulse under conditions of different intracellular solutions. A: in a divalent-free intracellular solution, the slope of the outward current increased after 4 min of equilibration as compared with controls (● and •, left). In a divalent-free solution supplemented with 5 mM ATP, the currents grew nonlinearily with time (○ compared with ⋅, right). B: average chord conductances in control solution (CTL, ●, n = 8), in a calcium-free solution (○, n = 5), a divalent-free solution (▼, n = 6), and 5 mM ATP in a divalent-free solution (rists, n = 5) evaluated 25 ms into the command pulse (n = 5).

**Fig. 6.** A series of representative current responses with 500 μM spermine added to the intracellular solution. A: control currents recorded immediately after establishing whole cell (left) and after allowing 4 min for spermine to diffuse from the pipette into the cell (right). There is a block of the transient outward currents and only sustained currents remain. There is also a reduction of the inward currents so that the rectifying properties are less pronounced and the currents take on an "ohmic" appearance, as apparent from their regular spacing, particularly for voltages depolarized from −100 mV. Bottom: representative time course of test pulses. B: the outward currents induced by a voltage pulse to 0 mV immediately after establishing whole cell (solid line) and 4 min later (gray line). The initial transient is greatly attenuated under the influence of spermine. There is also a reduction of the inward currents (star). C: stepping the membrane potential from a depolarized level (HP = −40 mV) to −100 mV resulted in an instantaneous current followed by a slow relaxation. Addition of spermine reduced the standing current at HP = −40 mV (arrows) and the amplitude of the relaxation (gray line). D: same as C, but voltage step is from HP = −40 to −120 mV.
is partly overcome, and a small relaxation of the inward current ensues (Fig. 6D). The $I-V$ curves measured at 25 ms clearly indicate a reduction of the outward currents compared with the normal conditions (Fig. 7A). Note that with added spermine the remaining currents are no longer dependent on holding potential as the respective $I-V$s overlap with each other at the two different holding potentials.

The instantaneous onset of the inward currents is marginally affected by the added spermine, certainly to a lesser degree than the relaxation phase (Fig. 6, C and D). Evaluation of the $I-V$ curve 4 ms after onset of the voltage pulses, early during the relaxation phase, close to the so-called “pseudo instantaneous phase” (Ishihara et al. 1989), reveals that rectification is much less marked (Fig. 7B). In fact, the $I-V$ can be well fit by a single straight line fitting the outward currents and extending to the inward currents, suggesting that both inward and outward currents remaining after the block by spermine may flow through the same, linear channel. In other experiments, the spermine level was raised to 1 mM and even 2 mM. The higher concentrations of spermine were not well tolerated by the cells, but the results were basically similar to those shown in Figs. 6 and 7. There is the possibility that the instantaneous current arises from the fast unblock by magnesium ions (Ishihara et al. 1989). However, in our experiments, this component remained unaltered when a divalent-free intracellular solution was used (results not shown).

The corresponding plots of the chord conductances as shown in Fig. 7, C and D, illustrate the voltage-dependent changes brought about by exogenous spermine. Addition of 500 \mu M spermine to the pipette solution results in a hyperpolarizing shift of the conductances. Despite a holding membrane potential of $-80$ mV, the blocking voltages have shifted significantly to $-140 \pm 4.2$ mV ($P < 0.01, n = 5$) at 4 ms, $-113 \pm 6.9$ mV ($P < 0.01, n = 5$) at 25 ms (Fig. 7C), and $-119 \pm 15$ mV ($P < 0.01, n = 5$) for the conductance computed at 1 s

(Fig. 7D). This is indicative of a fascilitatory effect on the block (Lopatin et al. 1995). The Boltzmann coefficient has similar values when computed at 25 ms ($1.63 \pm 0.24, n = 5$) and at 1 s ($1.86 \pm 0.63, n = 5$) but is shallower ($0.68 \pm 0.26, n = 5$) when computed at 4 ms. It is not clear whether this is due to an artifact arising from the shift of the voltage-sensitive portion of the conductance beyond the range of voltages applied in these experiments. The pedestal levels measured at 4 ms ($0.33 \pm 0.04, n = 5$), 25 ms ($0.33 \pm 0.05, n = 5$), or 1 s ($0.31 \pm 0.03, n = 5$) are similar in value and overlap (Fig. 7C).

These values are not significantly different from the pedestal value obtained under control conditions in response to long voltage pulses (1 s; Fig. 7D). This suggests that the contribution of the added spermine (0.5–2 mM) to the sustained block of the outward currents is only marginal and primarily speeds up the rate of block of the transient currents. Its action on the inward currents is different, affecting primarily the development and magnitude of the time-dependent component. The compound effects of spermine on the inward and outward currents translated primarily into a hyperpolarizing shift of the blocking voltage without a change in the pedestal level, thus extending the range of linear operation (Fig. 7D), as is implied by the extended range of the pedestal value. This range is further extended when the conductances are computed at 4 ms (Fig. 7C), when the conditions for block by the added spermine are maximized.

**DISCUSSION**

Turtle Müller cells are highly and almost exclusively permeable to potassium ions (Conner et al. 1985; Linn et al. 1998). On membrane polarization, several components of the potassium currents are easily discerned. There is a moderate rectification of inward currents, and for depolarizations an initial transient, and then a later, sustained component to the

![Figure 7](http://jn.physiology.org/).

**FIG. 7.** $I-V$ curves generated with 500 \mu M spermine added to the intracellular solution. A: a representative set of curves showing that spermine in the solution results in a decrease of the inward and outward currents. Spermine blocked the outward currents to the same extent, regardless of holding potential. B: representative $I-V$ curves evaluated 4 and 25 ms after onset of the voltage pulses. Holding potential was $-40$ mV. Rectification is reduced at 4 ms. C: average chord conductances with 500 \mu M intracellular spermine evaluated 25 ms ($n = 5$) and 4 ms ($n = 5$) into the command pulse. The conductances shifted in the hyperpolarizing direction (compare with control at 25 ms). The effects are more pronounced at 4 ms. The pedestal levels are reduced and overlapping. D: average chord conductances evaluated 1 s into the command pulse, under control conditions (CTL, $n = 9$) and with 500 \mu M spermine (SPM, $n = 5$). The pedestal levels are not altered, although spermine induces a significant hyperpolarizing shift of the conductance and thereby causes an extension of the range covered by the pedestal.
outward currents. These features do not arise as a result of intrinsic properties of the membrane ion channels but rather reflect the blocking action of intracellular polyamines and divalent ions.

Time-dependent and ohmic components

The transient outward current that was observed on depolarization of turtle Müller cells was not due to a voltage-activated current, such as the $I_h$ current found in Müller cells of salamander (Newman 1985) and rabbit (Nilius and Reichenbach 1988). In turtle, the outward current is insensitive to TEA and 4-AP (Linn et al. 1998) but is strongly dependent on holding potential (Fig. 3), and on the level of intracellular divalent cations and polyamines. Removing calcium ions induced a modest effect on the current (Fig. 4, A and B); but, when both calcium and magnesium ions were removed from the intracellular space, the outward currents greatly increased in magnitude while the inward currents were hardly affected (Fig. 4C). This increased the slope of the $I-V$ relationship (Fig. 5A), which translated into a change in the pedestal levels of the chord conductance. This suggests a blocking action by magnesium ions on the potassium channels. However, we did not observe significant changes in the steady-state currents measured in response to long 1-s pulses (data not shown). The role for magnesium ions in the short-term block and its diminishing effect with time is suggestive of a competitive block between magnesium and polyamines (Ishihara 1997). Without divalent ions and with ATP chelating intracellular polyamines, the outward transient currents increased in amplitude, and their rate of decay slowed down considerably, particularly affecting the fast component of the block (Fig. 4). These observations indicate that the transient outward current may arise from the voltage- and time-dependent block of the potassium channels by magnesium and polyamines. When expressed in terms of the chord conductance, these changes in the block of the potassium currents translated primarily into a shift in the blocking voltages (Fig. 5B). Lower concentrations of intracellular spermine require increasingly depolarized voltages to achieve similar degrees of block. This is similar to findings in expressed HRK1 channels (Lopatin et al. 1995), confirming that the effect of ATP is to lower the concentration of free intracellular spermine. Note that ATP also binds spermidine, another blocker of inward rectifier channels (Lopatin et al. 1994), although with lesser efficacy than spermine (Watanabe et al. 1991). Given that Müller cells also produce spermidine (Biedermann et al. 1997), the effects we describe here resulting from the buffering action of ATP do not necessarily imply that we are observing the blocking action of spermine exclusively (Ishihara et al. 1996).

When spermine, which was found to be a highly effective blocker of the $K_n$ channels in rabbit Müller cells (Biedermann et al. 1998), was added to the pipette solution at a concentration of 500 μM, the rate at which the block of the outward current developed was sped up considerably, so much so that the initial current transient disappeared (Fig. 6), and the block appeared to be instantaneous. Under these conditions, we observed very little change in the sustained current levels. Thus under normal conditions, the intracellular constituents that block outward potassium currents (divalent ions, polyamines) are at a concentration sufficient to produce maximal block; adding spermine only sped up the development of the block.

Addition of spermine also affected the inward currents. There was little effect on the instantaneous, onset portion of the current. But there was a reduction of the time-dependent, slowly developing portion of the inward current, with consequent reduction of the rectifying properties (Figs. 6 and 7). The compound effect of the added spermine on the inward and outward currents produced a parallel shift of the conductance toward hyperpolarizing voltages, extending the range of the pedestal level (Fig. 7, C and D), and thereby of the linear or ohmic conductance to voltages hyperpolarized to $-100$ mV. In summary, we can distinguish two kinetically distinct components to the Müller cell potassium conductance: a time-dependent, rectifying component sensitive to spermine, and an ohmic component that is marginally sensitive to spermine.

One or two potassium channel types

The question remains as to whether the time-dependent and the ohmic current components that were observed here flow through the same channel or through physically distinct potassium channel types (Solessio et al. 2000). If turtle Müller cells are similar to those isolated from the guinea pig, then spermine would be expected to abolish outward currents through the inward rectifier channels (Biedermann et al. 1998) (note, however, that those experiments were performed with symmetric concentrations of high potassium, a condition that leads to the enhancement of inward currents relative to the outward currents, see our Fig. 1). Based on this assumption, the spermine-sensitive current represents the current carried through inwardly rectifying potassium channels, blocked by polyamines by way of a plugging mechanism (Lopatin et al. 1995), and that are characterized by two apparent binding sites for block by intracellular barium ions under hyperpolarized conditions (Solessio et al. 2000). In such a case, occupancy of the channels and block by spermine (or other intracellular polyamines) during depolarization would interfere with binding of the barium ions. The ohmic current could be carried through a different type of potassium channel that was blocked by intracellular barium ions occupying a single site in the channel pore (Solessio et al. 2000). This current is not sensitive to other known blockers of potassium channels (TEA and 4-AP) or changes in intracellular calcium, suggesting that it is not a typical delayed rectifier and not a calcium-dependent potassium current (Bringmann et al. 1997). These considerations give credence to the idea that potassium currents in turtle Müller cells flow through two types of channels: an inward rectifier and an ohmic conductance.

However, other properties of the currents are not readily reconcilable with the two-channel model, and we cannot discard the possibility that all the potassium currents flow through weakly rectifying potassium channels (Fakler et al. 1994; Kravivinsky et al. 1998; Kubo et al. 1996; Takumi et al. 1995). For example, the ratio of the linear, or ohmic, component to the rectifying component was the same from cell to cell and from preparation to preparation, as indicated by the constancy of the pedestal level. We also found that the same currents were observed in different parts of the cells, such as the soma and the processes (Perlman et al. 2001). This indicates that if the linear and rectifying components flow through separate chan-
nels, then these channels are linked or coupled as is the case for a tandem pore potassium channel (Chavez et al. 1999). Evidence from Müller cells in rat, rabbit, and mouse (Ishii et al. 1997; Kofuji et al. 2000; Tada et al. 1998) indicates that Müller cells express Kir4.1 inward rectifiers. In fact, the properties of the whole cell currents in turtle Müller cells bear close resemblance to those recorded from HEK293T cells expressing Kir4.1 channels (Tada et al. 1998), suggesting that the potassium currents in turtle Müller cells flow through a single type of channel, probably belonging to a subfamily of Kir4.1 channels. This is also compatible with the observed sensitivity of these currents to block by extracellular barium (10- to 100 mM). This is also compatible with the observed sensitivity of the potassium currents in turtle Müller cells with a biochemical mechanism with which to maintain potassium homeostasis by siphoning potassium in the cell as well. One can imagine metabolic states (light or dark adaptation), where synthesis and/or transport are up and down regulated (Morgan 1999). Such changes on a relatively rapid time scale have been described in the mouse retina (Macone et al. 1993). This regulation in turn would facilitate a decrease or increase in potassium influx, respectively. If this is closely coupled to increases or decreases in extracellular potassium levels, then a mechanism exists for fine tuning the process of siphoning.

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REFERENCES


SPERMINE MODULATES POTASSIUM CHANNELS IN MÜLLER CELLS


