cGMP-mediated effects on the physiology of bovine and human retinal Müller (glial) cells

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1. Whole-cell currents of freshly dissociated or cultured Müller cells from human and bovine retinas were studied using the perforated-patch and standard whole-cell recording techniques.

2. We found that internal perfusion of cGMP or external exposure to 8-bromo-cGMP activated a calcium permeable, non-selective cation current in Müller cells, the principal glial cells of the retina. In addition, the activity of calcium-activated potassium channels increased markedly. These currents were minimally affected by cAMP.

3. Molecular studies using the reverse transcription–polymerase chain reaction demonstrated that human Müller cells in culture contain transcripts closely related to the rod cyclic nucleotide-gated (CNG) channel.

4. Since guanylate cyclase is a known target for nitric oxide (NO), we tested the effect of NO donors on Müller cell currents. These agents induced currents that were qualitatively similar to those activated by cGMP.

5. Our experiments support the idea that the NO–cGMP pathway regulates the physiology of Müller cells and may play a role in integrating neuron–glia interactions in the retina.

There is a growing consensus that glial cells are active participants in the normal functioning of the nervous system and in the response of the CNS to pathological conditions. This idea is supported by the discovery in recent years that glia are responsive to numerous neurotransmitters, growth factors and cytokines (Puro, 1995). Further, the expression by glial cells of a diversity of ion channels (Sontheimer, 1994) suggests a complexity of function not fully considered even a decade ago. While it is now evident that glial cells are affected by molecular changes in the extracellular space, the second messengers linking molecules in the microenvironment with changes in glial function are not well characterized.

In this report, we consider the possibility that cGMP serves as a second messenger regulating the activity of ion channels in Müller cells, the predominant glia of the vertebrate retina. In neuronal cells, it is well established that cGMP can influence ion channel activity indirectly via the activation of protein kinases (White, Lee, Shcherbatko, Lincoln, Schonbrunn & Armstrong, 1993) and directly by binding to cyclic nucleotide-gated non-selective cation (CNG) channels (Fesenko, Kolesnikow & Lyubarsky, 1985; Nakamura & Gold, 1987; Leinders-Zufall, Rosenboom, Barnstable, Shepherd & Zufall, 1995). In the retina, CNG channels are essential for the functioning of photoreceptors (Yau & Baylor, 1989) and are likely to play a role in bipolar cells (Shiells & Falk, 1992) and ganglion cells (Ahmad, Leinders-Zufall, Kocsis, Shepherd, Zufall & Barnstable, 1994). However, although CNG channels are present in many regions of the nervous system, little is known about their expression and function in glial cells.

Interest in cGMP as a second messenger in the nervous system is heightened by finding that the soluble form of guanylate cyclase is a target for nitric oxide (NO; Knowles, Palacios, Palmer & Moncada, 1989). This gas appears to be an important intercellular messenger in numerous areas of the CNS (Garthwaithe & Boulton, 1995). In the retina, the NO–cGMP pathway may be functional. Soluble guanylate cyclase is expressed in relatively high concentrations in the inner retina (Ahmad & Barnstable, 1993), and immunochemical studies (Yamamoto, Bredt, Snyder & Stone, 1993) indicate that a subpopulation of amacrine cells contain NO synthase, the enzyme which catalyses the synthesis of NO from L-arginine. Our working hypothesis is that NO serves to integrate neuronal and glial responses and acts, in part, as a signal regulating the physiology of the retinal Müller cells by activating cGMP-sensitive ion channels.

We now report that Müller cells express a cGMP-activated non-specific cation channel that is similar to the CNG channel of rod photoreceptors. In addition to activating this

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non-specific cation current, exposure of Müller cells to a membrane-permeable cGMP analogue markedly increases the activity of calcium-activated potassium (K_{Ca}) channels. Similar effects on the physiology of Müller cells are induced by NO donors. These observations lead us to postulate that the NO–cGMP mechanisms for activating CNG and K_{Ca} channels serve to help regulate the function of Müller cells in the redistribution of excess extracellular potassium.

METHODS

Dissociated Müller cells

Freshly dissociated human and bovine Müller cells were derived from approximately 0·25 cm × 0·25 cm pieces of retina incubated in scintillation vials containing 2·5 ml of Earle's balanced salt solution supplemented with 0·5 mM EDTA, 1·5 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM glucose, 26 mM sodium bicarbonate, 2 mM cysteine, 15 u papain, 0·04% DNase (2500 u mg^{-1}, Type II from bovine pancreas) and 12% chicken serum for 40 min at 30 °C while 95% O_2–5% CO_2 was bubbled through the solution to maintain pH and oxygenation. The piece of retina was then gently transferred to a 1·5 ml microcentrifuge tube and washed with the bathing solution to be used in the experiment. The retina was then drawn up into a glass pipette with a fire-polished tip (inner diameter, 1 mm) and gently ejected back into the microcentrifuge tube. The piece of retina was allowed to settle to the bottom of the tube, and ~0·1 ml of the suspension of cells was placed in a recording chamber which had either a glass or plastic bottom. After allowing 10–15 min for the cells to settle, the bathing solution was added to the chamber. Müller cells were identified by their characteristic morphology (Puro, Yuan & Sucher, 1996*b*).

Cultured Müller cells

Cultures of human retinal glial cells were prepared as detailed elsewhere (Puro, 1994). Retinas were removed, exposed to a calcium- and magnesium-free phosphate buffer supplemented with 0·1% trypsin (3 × crystallized), 0·2% hyaluronidase (~400 u mg^{-1}, Type I-S from bovine testes) and 4% chicken serum for 45 min at 37 °C and then dissociated mechanically in medium A (40% Dulbecco's modified Eagle's medium, 40% Ham's F12 medium and 20% fetal bovine serum). Dissociated cells from one retina were added to three 35 mm Petri dishes, kept in a humidified environment of 96·5% air–3·5% CO_2 at 37 °C and fed medium A twice per week. Cultures were split three or four times during 7–11 weeks *in vitro*. As reviewed elsewhere (Puro, 1994), it appears that the cells in these cultures are Müller cells since virtually all (> 99%) of the cells prepared and maintained by this method are immunoreactive to a monoclonal antibody specific for retinal Müller glial cells and to polyclonal antibodies against glutamine synthetase, which is localized in the retina of the Müller glial cells. (Lewis, Erickson, Kaska & Fisher, 1988; Dabin & Barnstable, 1995).

Electrophysiology

Electrophysiological recordings were made at room temperature (22–24 °C). Cells were examined at ×400 magnification with an inverted microscope equipped with phase contrast optics. Unless noted otherwise, the bathing solution consisted of (mM): 133 NaCl, 10 KCl, 1·8 $CaCl_2$, 0·8 $MgCl_2$, 10 Na-Hepes and 20 glucose, at pH 7·4 and 305–310 mosmol l^{-1}. Whole-cell currents were monitored using the perforated-patch (Rae, Cooper, Gates & Wesky, 1991) or standard whole-cell (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) configurations of the patch-clamp technique. Patch pipettes were pulled from glass tubing (no. 7052, Corning) using a multistage programmable puller (Sutter Instruments, San Rafael, CA, USA), coated with Sylgard (no. 184, Dow Corning) to within 100 μm of their tips and heat polished to tip diameters of 2–3 μm. For standard whole-cell recordings, the pipettes were filled with 140 mM CsCl, 0·661 mM $CaCl_2$, 3 mM EGTA and 20 mM Cs-Hepes at pH 7·2 with the osmolarity adjusted to 300 mosmol l^{-1}. In the case of pipettes used for perforated-patch recordings, a pipette tip was filled to approximately 400 μm from the tip by applying negative pressure to the back end of the pipette while briefly dipping the tip into the pipette solution. The pipette solution, unless stated otherwise, consisted of (mM): 45 KCl, 60 K_2SO_4, 10 K-Hepes and 6 $MgCl_2$, at pH 7·4 and 285 mosmol l^{-1}. The remainder of the pipette was then backfilled with this solution supplemented with freshly mixed amphotericin B (240 μg ml^{-1}) and nystatin (240 μg ml^{-1}). The resistances of the pipettes used were 2–5 MΩ when tested in the bathing solution. The pipettes were mounted in the holder of a Dagan 3900 patch-clamp amplifier (Dagan Corp., Minneapolis, MN, USA) and sealed to Müller cells. Seals were generally formed over a period of 1–30 s and reached resistances of greater than 1 GΩ.

The standard whole-cell configuration was achieved by applying suction to the pipette with a pneumatic transducer (Bio-Tek, Winooski, VT, USA); recordings with series resistances <15 MΩ were used. For the perforated-patch configuration, as amphotericin B/nystatin perforated the patch, the access resistance to the cell usually decreased to less than 20 MΩ within a few minutes for the cultured cells studied and within 20 min for the fresh Müller cells analysed. Recordings were used after the ratio of cell membrane to series resistance was greater than 10. This ratio was monitored periodically; if the ratio decreased to below 10, the analysis of the cell was terminated. Since a ratio of cell resistance to series resistance of >10 would introduce a <10% error in the true voltage step, series resistance compensation was not used. Currents were monitored continuously at selected holding potentials or evoked by ramping membrane voltage (140 mV s^{-1}) from negative to positive membrane potentials for construction of continuous current–voltage (I–V) plots. Currents were filtered at 1 kHz and digitally sampled (at 1 ms for ramps and 250 μs for continuous records) using a Lab Master DMA acquisition system (Axon Instruments) and pCLAMP software (v. 6.0, Axon Instruments), which also controlled voltage protocols and helped with data analysis. This study is based on recordings from 175 cells.

The recording chamber (0·5 ml volume) could be continuously perfused (1–2 ml min^{-1}) with various solutions with a gravity-fed system using multiple reservoirs. In some experiments (e.g. Fig. 7*A*), 5 μl of an agent was added directly to the stationary bath. Solutions also could be miniperfused in the area of the Müller cell by attaching a pressure ejection system (Medical Systems, Greenvale, NY, USA) to the back end of a pipette which had a tip size of approximately 3 μm and was positioned ~50 μm from the cell being recorded. Less than 3·5 kPa (0·5 p.s.i.) of pressure was used for miniperfusion.

Intracellular calcium measurements

For measurements of intracellular calcium, freshly dissociated Müller cells plated on glass coverslips were incubated for 30 min at 37 °C with 6 μM of the acetoxymethyl ester form of fura-2 (fura-2 AM). After fura-2 loading, the cells were given a further incubation period of 10–20 min at 37 °C to allow for cleavage of intracellularly accumulated fura-2 AM. Monitoring of $[Ca^{2+}]_i$ in individual glial cells was performed by dual-wavelength microspectrofluorometry similar to that described previously by Puro & Stuenkel (1995). Briefly, the suspension of cells was placed on a glass coverslip which formed the base of a chamber (65 μl volume).

After allowing 10–20 min for Müller cells to adhere to the coverslip, the perfusion (~1–2 ml min^{-1}) began of a solution of composition (mM): 138 NaCl, 5 KCl, 1·6 $CaCl_2$, 0·8 $MgCl_2$, 10 Na-Hepes and 24 glucose, at pH 7·4 and 310 mosmol l^{-1}. A gravity-fed mechanism of reservoirs allowed the changing of perfusion solutions.

Digital imaging of fura-2 fluorescence was performed using an Attofluor (Zeiss, Thornwood, NY, USA) imaging system. The fluorescence ratio (340 : 380 nm) was converted to $[Ca^{2+}]_i$ using the equation of Grynkiewicz, Poenie & Tsien (1985). An external standard calibration approach was used to determine values of R_{min}, R_{max}, and F_o/F_s where R_{min} and R_{max} are the values of R at limiting and saturating Ca^{2+} concentrations, respectively, and F_o/F_s represents the ratio of emitted fluorescence intensity at 380 nm excitation at the limiting (F_o) and saturating (F_s) Ca^{2+} conditions. A K_d value for fura-2 of 224 nM was taken from the literature (Grynkiewicz *et al.* 1985). Autofluorescence, determined on unloaded cells, was found to be less than 10% of the emitted signal and was not subtracted. Loading the cells with fura-2 AM at 20–25 °C, which is reported to reduce incorporation into endocytotic vesicles, resulted in no apparent differences in the $[Ca^{2+}]_i$ measurements. No punctate fluorescence was observed.

Reverse transcriptase–polymerase chain reaction (RT–PCR) analysis

From cultures of Müller cells, total RNA was prepared using tRNA as carrier and dissolved in 1 × PCR amplification buffer (10 mM Tris–HCl (pH 8·0), 50 mM KCl, 1·5 mM $MgCl_2$). First-strand cDNA was synthesized using Moloney murine leukaemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD, USA) and then aliquots were amplified by a step cycle (94 °C, 1 min; 55 °C, 1 min; 72 °C, 1·5 min) for thirty-five cycles. Controls included pretreatment of RNA samples with RNase-free DNase and omitting reverse transcriptase from the cDNA synthesis reactions to ensure that results were not due to amplification of any DNA contaminants. PCR products were resolved on a 1% agarose gels, transferred to nylon membranes (Schleicher and Schuell, Keene, NH, USA) and cross-linked by UV irradiation. Membranes were probed with ^{32}P-labelled fragments of CNG channels using standard methods (Sambrook, Fritsch & Maniatis, 1989).

Primers for a 608 bp sequence in the human rod CNG channel gene (bases 1429–2037) (forward, 5′-GCTGATTGTGAAGCTGGT CTGTTGGTG-3′ and reverse, 5′-TTCCGTCCAGGT CCCTCAATACTTGA-3′) were based on the published sequence (Dhallan *et al.* 1992). The reverse primer corresponds to a sequence close to the 3′ end of the coding region that shows extensive variation between species and between the related olfactory and cone types of CNG channels. Primers were also chosen to amplify a 548 bp fragment from the β-actin gene (forward, 5′-GTGGGGCGCCCCAGGCACCA-3′ and reverse, 5′-CTC CTTAATGTCACGCACGATTTC-3′) as previously described (Ahmad *et al.* 1994).

Chemicals

Sodium nitroprusside (SNP) was dissolved in the appropriate bathing solution. A 100 mM stock of *S*-nitrosocysteine (SNC) was prepared by combining L-cysteine hydrochloride and sodium nitrite in equimolar amounts then adding 10 N HCl to a final concentration of 0·5 N (Lei *et al.* 1992); this stock was diluted in the appropriate bathing solution. SNP and SNC were used within 1 h of preparation.

Fura-2 AM was purchased from Molecular Probes; trypsin and papain were from Worthington Biochemical (Freehold, NJ, USA); sera and growth media were from Life Technologies/Gibco (Grand Island, NY, USA); charybdotoxin was from Research Biochemicals International; the other chemicals were from Sigma.

Ocular tissue

Bovine eyes were obtained soon after a near-instantaneous death induced at a local licensed abattoir. Donor adult human eyes were supplied within 24 h of postmortem by the Michigan Eye Bank and Transplantation Centre (Ann Arbor, MI, USA). All tissue was obtained and used in accordance with applicable laws and regulations.

Presentation of results

Unless otherwise stated, results are given as means ± S.D.

RESULTS

Effect of 8-bromo-cGMP on Müller cell currents

Perforated-patch recordings of freshly dissociated bovine Müller cells revealed a number of voltage-dependent currents including an inwardly rectifying current as well as outward currents (Fig. 1*A*, control). These *I–V* relations are similar to those observed in Müller cells from a variety of species (Newman, 1985; Chao, Henke, Reichelt, Eberhardt, Reinhardt-Maelicke & Reichenbach, 1994), including human (Puro & Stuenkel, 1995). An initial question was whether a membrane-permeable analogue of cGMP, 8-bromo-cGMP (50 μM), affected the currents of Müller cells. As seen from the *I–V* plot in Fig. 1*A*, exposure to 8-bromo-cGMP was associated with a depolarization of the resting membrane (zero current) potential, an increase in outward current at depolarized potentials and an increase in inward current at hyperpolarized potentials. Similar results were observed in seven cells. The membrane potential depolarized by a mean of 15 mV from −49 ± 1 to −34 ± 1 mV (means ± S.E.M). These effects of 8-bromo-cGMP were reversible.

Finding that 8-bromo-cGMP can induce changes in the currents of Müller cells led us to ask how these effects compared with those of 8-bromo-cAMP. In Fig. 2, a fresh bovine Müller cell was exposed initially to 8-bromo-cAMP. Changes in the currents were minimal. In contrast, when the perfusate subsequently contained 8-bromo-cGMP, significant changes were observed. Similar findings were made in four other fresh bovine Müller cells. These findings indicate that certain ion channels in Müller cells are more potently affected by cGMP than cAMP.

Currents activated by cGMP

To begin to characterize the Müller cell currents affected by cGMP, the *I–V* relations of the net induced current were determined. This was achieved by subtracting the *I–V* curve generated under control conditions from that obtained in the presence of 8-bromo-cGMP. As shown in Fig. 1*B*, a net outward current was induced at potentials more depolarized than ~−25 mV. At hyperpolarized potentials, there was a net increase in inward current. Similar results were observed in seven cells; the mean increase in inward current at a holding potential of −70 mV (the reversal potential of K^+) was 64 ± 19 pA, and the mean increase in outward current at 0 mV was 131 ± 29 pA (means ± S.E.M).

Since Müller cells are known to express calcium-activated potassium (K_{Ca}) channels that contribute to the outward current (Newman, 1985; Puro, Hwang, Kwon & Chin, 1996*a*), we postulated that a component of the outward current induced by 8-bromo-cGMP could be due to an increase in activity of these channels. To test this possibility, charybdotoxin (ChTX, a blocker of K_{Ca} channels; Hille, 1992) was added to the bathing solution while Müller cells continued to be exposed to 8-bromo-cGMP. Subtraction of ramp-evoked currents monitored in the presence of 8-bromo-cGMP from those subsequently detected during exposure to both the cGMP analogue plus ChTX revealed a significant ChTX-sensitive component at potentials more depolarized than ~−40 mV (Fig. 1*C*). At 0 mV, virtually all of the outwardly rectifying current was blocked by 5 nM ChTX. Similar findings were observed in three cells. These findings indicate that the activity of K_{Ca} channels is increased when Müller cells are exposed to 8-bromo-cGMP.

In addition to K_{Ca} channels, at least one other current is affected by this cGMP analogue. Evidence for this is the presence of induced inward current at potentials more hyperpolarized than the threshold for activation of the K_{Ca} channels (Fig. 1*B*). Continuous current records ($n = 5$) revealed that development of this inward current was accompanied by an increase in the noise level (Fig. 1*D*), consistent with greater channel activity. To characterize this current better, we analysed Müller cells under recording conditions in which K^+ channel activity was near totally eliminated by having Ba^{2+} and Cs^+ in the bathing solution and Cs^+ in the pipette solution. (It was necessary to use internal Cs^+ in addition to external Ba^{2+} since Ba^{2+} does not block completely all of the voltage-dependent K^+ channels of these cells (Reichelt & Pannicke, 1993).) Under these recording conditions, 8-bromo-cGMP induced a current having some outward rectification and a reversal potential near 0 mV (Fig. 1*E*). Similar results were observed in seven cells.

A reversal potential near 0 mV is consistent with a non-specific current induced by the cGMP analogue. To assess the cation and/or anion selectivity of this current, the change in reversal potential was determined with differing concentrations of external anions and cations. As was done in the experiment illustrated in Fig. 1*E*, the solution in the perforated-patch pipette contained Cs^+ to help block K^+

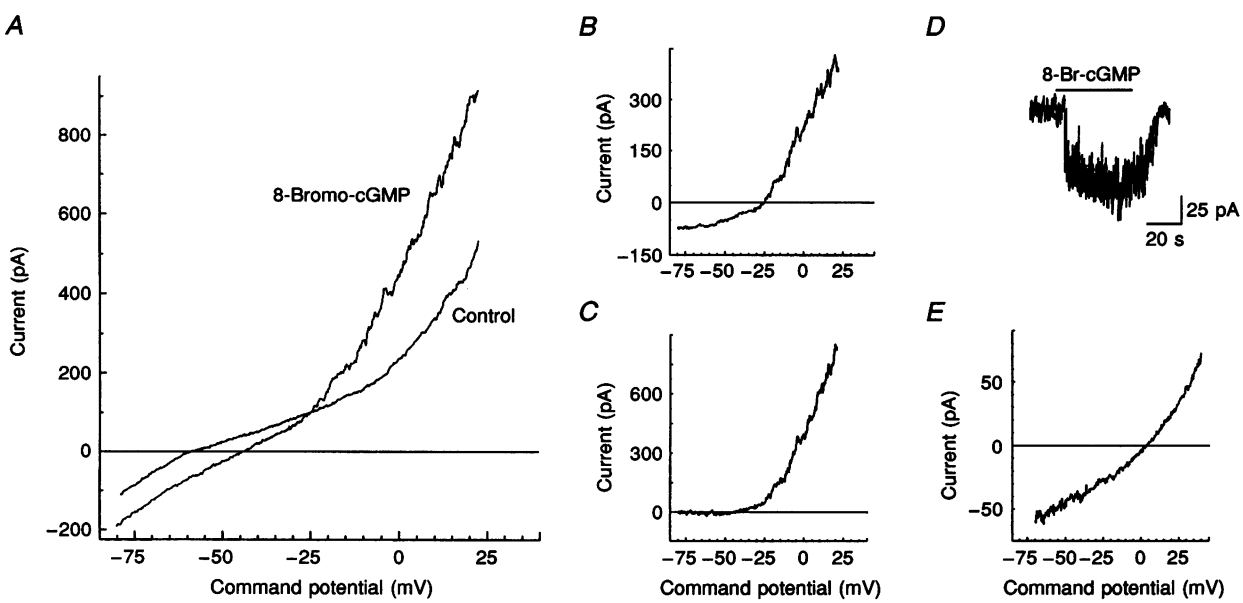

Figure 1. Effect of 8-bromo-cGMP on whole-cell currents of freshly dissociated bovine Müller cells monitored with the perforated-patch configuration

A, *I–V* relations in the absence (Control) and presence of 8-bromo-cGMP. The predominant cation in the pipette solution was K^+; Na^+ was the predominant cation in the bathing solution, which did not contain any ion channel blockers. *B*, plot of the difference between the *I–V* curves shown in *A*. *C*, for the same cell as in *A* and *B*, the plot of the difference between the *I–V* curves in the presence of 8-bromo-cGMP and in the presence of this cGMP analogue plus charybdotoxin (5 nM). *D*, continuous current recording of a Müller cell held at a holding potential of −80 mV. The bar indicates the period of miniperfusion of the bathing solution supplemented with 8-bromo-cGMP (1 mM). There is a 10 s gap in the record after cessation of miniperfusion. *E*, *I–V* plot of the difference between *I–V* curves in the presence and absence of 8-bromo-cGMP with the bathing solution containing 143 mM CsCl, 0·8 mM $MgCl_2$, 1·8 mM $BaCl_2$, 10 mM Cs-Hepes and 20 mM glucose (pH 7·4 and 310 mosmol l^{-1}) and with the pipette solution consisting of 135 mM CsCl, 6 mM $MgCl_2$ and 10 mM Cs-Hepes (pH 7·4 and 285 mosmol l^{-1}). For *A*, *B*, *C* and *E*, the concentration of 8-bromo-cGMP was 50 μM. Exposure to 8-bromo-cGMP activates a ChTX-sensitive current and a non-specific current in Müller cells.

channels and, thus, reveal the non-specific current. When the bathing solution contained 143 mM CsCl plus 0·8 mM $MgCl_2$, 1·8 mM $BaCl_2$, 10 mM Cs-Hepes and 20 mM glucose, the reversal potential (i.e. zero current potential) was $+5 \pm 6$ mV ($n = 4$). Reducing the extracellular CsCl concentration from 143 to 30 mM (with mannitol added to maintain osmolarity) shifted the reversal potential to -22 ± 4 mV ($n = 4$). This 27 mV hyperpolarization of the reversal potential is close to the calculated hyperpolarization of 32 mV for an ideally cation-selective channel. Further, replacing the CsCl and Cs-Hepes in the bathing solution with 143 mM NaCl and 10 mM Na-Hepes resulted in a reversal potential of $+11 \pm 5$ mV ($n = 3$) for the current induced by 8-bromo-cGMP. These experiments indicate that this channel is permeable to small monovalent cations. These findings also are consistent with the channel having a greater permeability for Na^+ than Cs^+. However, to calculate accurately the relative inonic permeabilities, future studies will need to use excised patches which allow precise control of the solutions at both sides of the channel.

The effect of external calcium on the reversal potential of the cGMP-activated current was also assessed. With the addition of 20 mM $CaCl_2$ to the bathing solution containing 30 mM CsCl and 10 mM Cs-Hepes (the concentration of mannitol was adjusted to maintain osmolarity), the mean reversal potential of the cGMP-activated current was $+5 \pm 4$ mV ($n = 6$) in whole-cell recordings of fresh bovine Müller cells. Since this reversal potential was markedly depolarized relative to the reversal potential in the calcium-free solution, it seems likely that the CNG channel of Müller cells is also permeable to calcium. Taken together, these observations and those illustrated in Fig. 1 suggest that exposure of fresh Müller cells to 8-bromo-cGMP activates a non-specific cation channel and a ChTX-sensitive K^+ channel.

Internal perfusion of cGMP

Internal perfusion of cGMP via a conventional whole-cell recording pipette induced an inward current at hyperpolarized holding potentials in ~90% (29 of 33) of the bovine Müller cells sampled. As shown in Fig. 3*A*, the increase in current was associated with an increase in the noise level, which is consistent with an increase in channel activity. The current reached a peak in approximately 10–90 s. The mean maximal amplitude of the induced inward current measured at a holding potential of −70 mV within the first 2 min of going whole-cell was 169 ± 146 pA (range, 40–450 pA; $n = 9$). In whole-cell recordings with the pipette solution lacking the cyclic nucleotide, the maximal inward current within 2 min was 10 ± 10 pA ($n = 6$) at −70 mV. The inward current in Müller cells with cGMP was significantly ($P = 0{\cdot}021$, Student's t test) greater than in the central group. Also, the changes in current detected under control conditions were associated with a decrease, not an increase, in the noise level. The record shown in the inset of Fig. 3*A* demonstrates that the increase in current was relatively long lasting suggesting minimal desensitization or inactivation under these recording conditions.

In addition to studying Müller cells from the bovine retina, we also have had the opportunity to examine cells from the human retina. In ~90% (11 of 12) of the fresh human Müller cells sampled, internal perfusion of cGMP resulted in an inward current associated with an increase in noise level

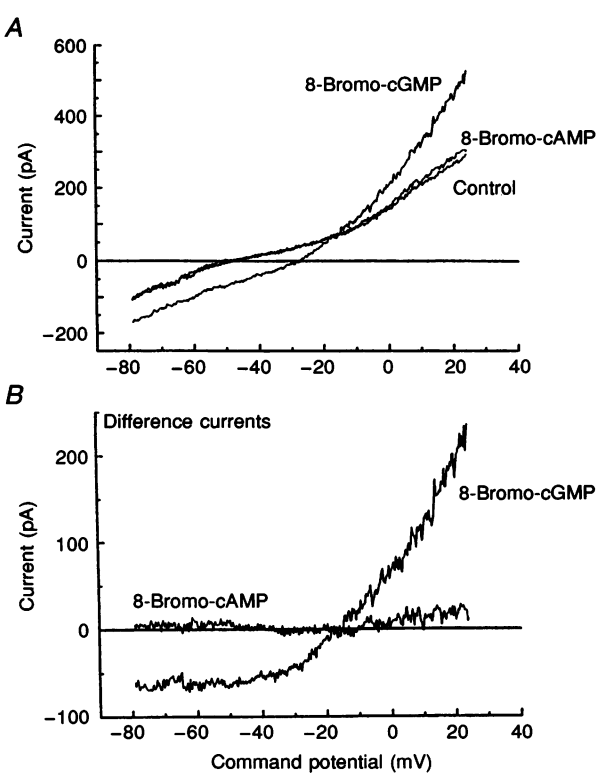

Figure 2. Relative effects of 8-bromo-cAMP and 8-bromo-cGMP

A, *I–V* relations of a freshly dissociated bovine Müller cell recorded with the perforated-patch configuration during perfusion of the chamber with the control bathing solution (Control) and with this solution supplemented with 500 μM 8-bromo-cAMP or 500 μM 8-bromo-cGMP. *B*, plots of the difference between the *I–V* curves during the control period and during exposure to 8-bromo-cAMP or 8-bromo-cGMP. The effects of this cGMP analogue on Müller cell currents were markedly greater than those of 8-bromo-cAMP.

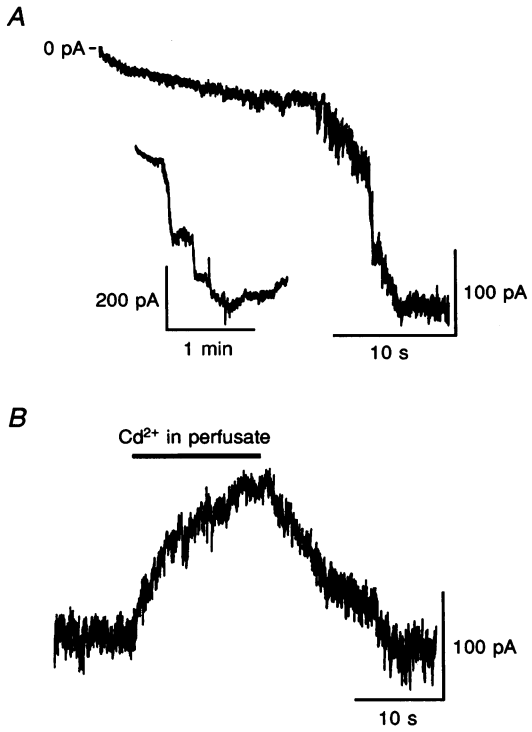

Figure 3. Internal perfusion of cGMP in fresh bovine Müller cells

A, onset of an inward current activated by the internal perfusion of cGMP (1 mM) from a standard whole-cell recording pipette sealed to the soma of a Müller cell. The increased noise level probably reflects greater channel activity. The holding potential was −70 mV. The inset shows the time course of this recording over a longer period. *B*, effect of the miniperfusion of the bathing solution supplemented with 3 mM $CdCl_2$ on the inward current, which was activated during standard whole-cell recording with cGMP (1 mM) in the pipette solution. As detailed in the Methods, the pipette solution for whole-cell recordings contained caesium, which blocks K^+ channels (Hille, 1992). The holding potential was −70 mV. The recording pipette was sealed to the soma. cGMP activates a Cd^{2+}-sensitive current in Müller cells.

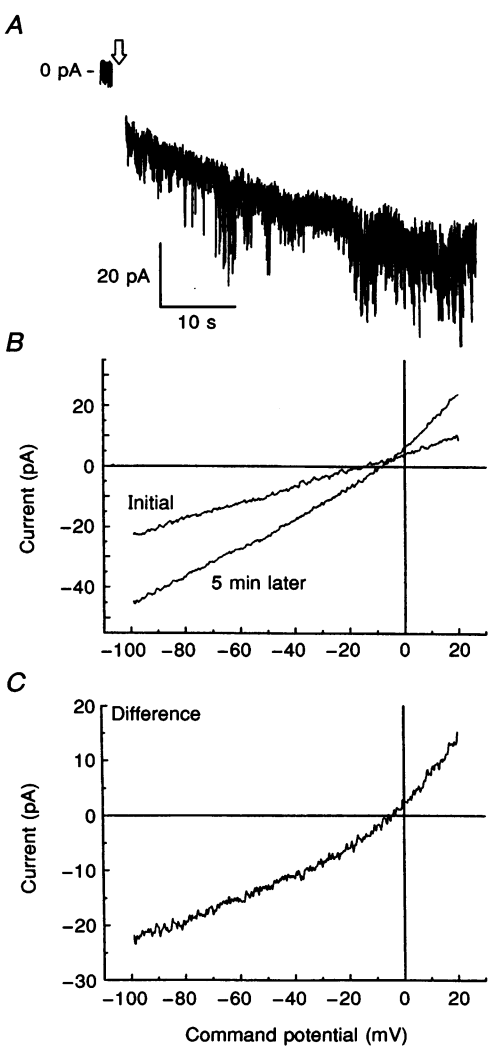

Figure 4. Human Müller cells internally perfused with cGMP

A, onset of an inward current activated by internal perfusion of cGMP (1 mM) from a standard whole-cell recording pipette sealed to the soma of a freshly dissociated human Müller cell. The arrow indicates the approximate time of going whole-cell; artifacts generated while going whole-cell were blanked. The increase in noise level occurring as the inward current was induced is consistent with fluctuations in the underlying channel activity. The holding potential was −70 mV. *B*, *I–V* relations of a cultured human Müller cell within a few seconds and 5 min after going whole-cell with a standard whole-cell recording pipette containing cGMP (1 mM). *C*, *I–V* curves of the difference between the plots shown in *B*. cGMP activates a non-specific current in human Müller cells.

(Fig. 4A). The mean peak current induced at a holding potential of −70 mV was 46 ± 11 pA ($n = 11$) in the cells with detected responses. In recordings lacking cGMP in the pipette, the inward current at −70 mV was 15 ± 19 pA ($n = 5$), which was significantly ($P = 0{\cdot}001$, Student's t test) less than in the experimental group. Thus, Müller cells from the human retina also have a cGMP-activated current although, for reasons not yet identified, the amplitude of the induced currents are less than those detected in bovine Müller cells.

Cadmium block

Since cyclic nucleotide-gated non-selective cation channels in other cell types are blocked by cadmium (Yau & Baylor, 1989; Zufall & Firestone, 1993; Ahmad *et al.* 1994), we tested the effect of this divalent cation. Figure 3B shows the current at a holding potential of −70 mV in a Müller cell monitored by a standard whole-cell recording pipette containing 1 mM cGMP. Similar to the cell in Fig. 3A, internal perfusion of cGMP induced an inward current. During the time indicated by the bar in Fig. 3B, the bathing solution contained 3 mM Cd^{2+}, which reversibly decreased the inward current to near zero. In a series of experiments with recording pipettes containing cGMP, Cd^{2+} reduced the current at −70 mV by 116 ± 78 pA ($n = 6$) or 84 ± 20%. In contrast, Müller cells monitored by whole-cell recordings lacking cGMP showed a decrease of 7 ± 12 pA ($n = 4$) or 15 ± 10%. The Cd^{2+} block in the experimental group was significantly ($P = 0{\cdot}026$ for the change in current and $P < 0{\cdot}001$ for the percentage reduction) greater than the effect of this divalent cation on cells in the control group.

Molecular detection of a cGMP-gated cation channel

Cultures of human Müller cells were used to help determine the molecular basis for the cGMP-activated non-specific cation current expressed by these glia. Our culture system provides a population of multipassaged cells in which virtually all have immunocytochemical characteristics of Müller cells (Puro, 1994). As shown in Fig. 4, the internal perfusion of cultured human Müller cells with cGMP could be associated with the induction of an outwardly rectifying current having a reversal potential near 0 mV. Since these experiments were designed to detect a CNG current, the pipette solution contained caesium to block K^+channels. Four of six sampled human Müller cells in culture had detectable cGMP-activated current. Of those with responses, the maximal inward current within 2 min of going whole-cell was 42 ± 31 pA. In recordings lacking cGMP in the pipette, the inward current at −70 mV was 3 ± 4 pA ($n = 4$), which is significantly ($P = 0{\cdot}047$) less than in the experimental group.

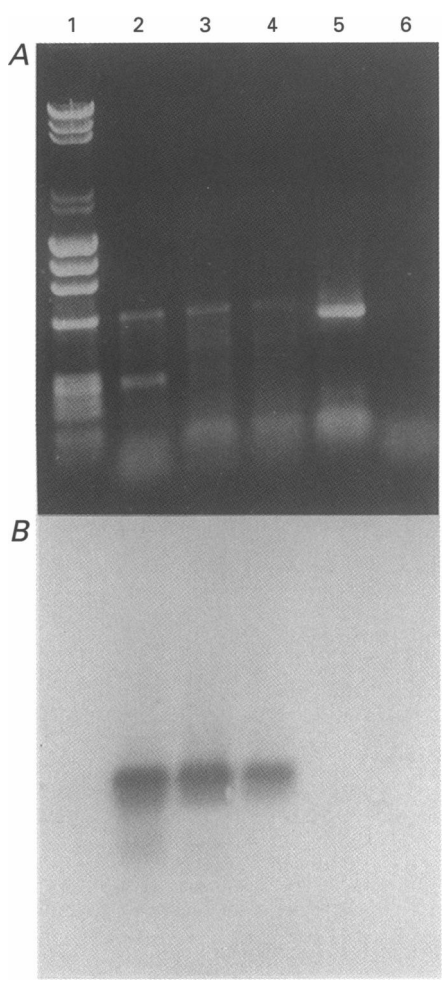

Figure 5. Amplification of cGMP-gated channel transcripts from cultured human Müller cells

A, ethidium bromide-stained gel of amplified products. Lane 1, DNA markers consisting of *Hind*III digested l and *Hae*III digested θX174 DNAs (Life Technologies); lane 2, Müller cell cDNA amplified with CNG primers with 1·5 mM $MgCl_2$; lane 3, Müller cell cDNA amplified with CNG primers with 2·0 mM $MgCl_2$; lane 4, Müller cell cDNA amplified with CNG primers with 3·0 mM $MgCl_2$; lane 5, Müller cell cDNA amplified with β-actin primers; lane 6, no cDNA control. *B*, Southern blot of the same gel hybridized with a probe corresponding to the 3′-portion of the rod CNG channel. At all magnesium concentrations a 608 bp band is detected that specifically hybridizes with a CNG channel probe.

Demonstrating that human Müller cells in culture express a cGMP-activated current allowed us to carry out RT–PCR analysis on RNA from these cells. Control amplification with primers for β-actin gave a band at the expected size of 548 bp (Fig. 5*A*). Since the primers correspond to sequences located in different exons, this control provides further evidence that amplification occurred from mature channel RNA. PCR detection of cGMP-gated channel transcripts used primers based on a region in the α-subunit of the rod CNG channel that included the proposed cGMP-binding site (Barnstable, 1993). Amplification using these primers gave a band of the expected size (608 bp) that could be detected by Southern blot analysis (Fig. 5*B*). These PCR results indicate that human Müller cells in culture contain transcripts closely related to the α-subunit of the cGMP-gated channel found in rod photoreceptors.

Effect of 8-bromo-cGMP on $[Ca^{2+}]_i$

Since rod CNG channels are known to be permeable to calcium (Yau & Baylor, 1989) and our electrophysiological exerpiments suggested a calcium permeability of the Müller cell CNG channel, we asked whether exposure of fresh human Müller cells to 8-bromo-cGMP is associated with an increase in intracellular Ca^{2+}. Using the calcium indicator fura-2 to measure $[Ca^{2+}]_i$, we detected 8-bromo-cGMP-evoked increases in five of eight sampled Müller cells. In the responding cells, $[Ca^{2+}]_i$ increased from a mean of 129 ± 23 nM to a mean peak of 224 ± 63 nM ($P = 0{\cdot}013$, Student's t test).

To determine whether the increase in $[Ca^{2+}]_i$ evoked by 8-bromo-cGMP was dependent on an influx of Ca^{2+} from outside the cell, fresh Müller cells were tested for responsiveness to the cGMP analogue in the absence and presence of extracellular calcium (Fig. 6). Removal of extracellular calcium eliminated the increase in $[Ca^{2+}]_i$ mediated by 8-bromo-cGMP ($n = 4$). Thus, exposure to 8-bromo-cGMP induces a rise in $[Ca^{2+}]_i$ by a mechanism dependent on extracellular calcium. Since these measurements of $[Ca^{2+}]_i$ were not made under voltage-clamped conditions, a role for voltage-gated calcium channels is not excluded. However, in our perforated-patch recordings (e.g. Figs 1*A* and 2) the depolarization induced by 8-bromo-cGMP did not reach the threshold (−25 mV) for activation of the L-type calcium channels expressed by Müller cells (Puro & Mano, 1991; Puro *et al.* 1996*a*). One of a number of possible pathways for a cGMP-induced influx of calcium is via cyclic nucleotide-gated channels.

Effect of NO on Müller cell current

Next we asked whether the cGMP-regulated currents in Müller cells are affected by nitric oxide (NO), which is likely to be synthesized by a subpopulation of retinal amacrine cells (Yamamoto *et al.* 1993) and is known to activate guanylate cyclase in many cell types (Knowles *et al.* 1989). Figure 7 shows the effect on the I–V relations of the NO donor, sodium nitroprusside (SNP). External application of SNP (Fig. 7*A*) induced changes qualitatively similar to those seen with 8-bromo-cGMP (Fig. 1*A* and *B*). Similar findings were observed in four cells. When K^+ channels were blocked, the presence of SNP in the bathing solution was associated ($n = 4$) with the development of an outwardly rectifying current with a reversal potential near 0 mV (Fig. 7*B*). A similar effect was observed in four Müller cells locally perfused with a bathing solution containing the more specific NO-generating agent, *S*-nitrosocysteine (Fig. 7*C*).

One difference between the effects of the NO donors and 8-bromo-cGMP on the currents of Müller cells was a longer delay with SNP or SNC. While the cGMP analogue induced responses within 1–10 s (Fig. 1*D*), changes in Müller cell currents were detected only after 1–5 min of exposure to the NO-generating agents. The longer delay is likely to reflect the time required for NO activation of guanylate cyclase and for synthesis of sufficient cGMP. These experiments are consistent with the idea that exposure to NO activates the cGMP-sensitive currents of Müller cells.

DISCUSSION

The results show that the physiology of retinal Müller cells can be regulated by cGMP. Both electrophysiological and molecular analyses indicate that Müller cells express a cGMP-gated cation channel. In addition, cGMP also appears to play a role in regulating the activity of the Müller cell K_{Ca} channels. Our experiments with NO donors further

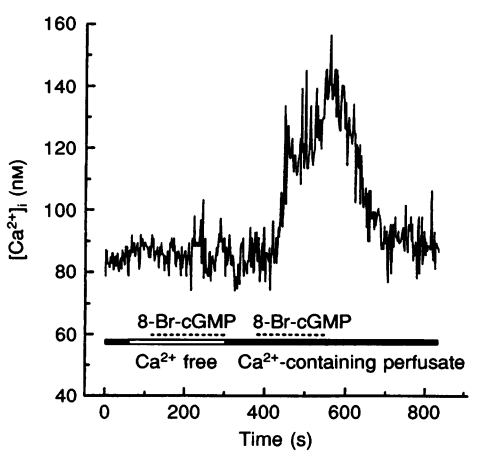

Figure 6. Effect of 8-bromo-cGMP on $[Ca^{2+}]_i$ in a fresh human Müller cell

The horizontal bar shows the periods of perfusion with calcium-containing (filled bar) or calcium-free (no $CaCl_2$, 2 mM EGTA; open bar) bathing solutions. The dashed lines indicate the periods in which the perfusate contained 500 μM 8-bromo-cGMP. The increase in $[Ca^{2+}]_i$ evoked by the cGMP analogue is dependent on external calcium.

suggest that NO may be one of the intercellular signals initiating a cascade of events regulating the activity of the cGMP-sensitive ion channels in Müller cells.

A CNG channel in Müller cells

The RT–PCR technique provided molecular evidence for the expression by human Müller cells of transcripts for a cGMP-gated channel. These channels must be similar to those found in rod photoreceptors since we used a set of primers corresponding to the H2 to H5 putative transmembrane domain of the α-subunit of the rod photoreceptor CNG channel. However, determination of whether this glial channel has precisely the same sequence and subunit structure as the photoreceptor channel will require more detailed molecular analysis.

Our electrophysiological study of cGMP-gated cation currents in cultured as well as freshly dissociated Müller cells indicates significant physiological similarities with the CNG channels of rod photoreceptors. The similarities include outward rectification of the I–V relations, a reversal potential near 0 mV, a blockade by Cd^{2+} and a markedly greater sensitivity to cGMP than cAMP. Further, the CNG channels of Müller cells appear to have a non-specific cation permeability as do rod CNG channels. The sensitivity of the reversal potential to changes in extracellcular calcium indicates a permeability of the Müller cell CNG channel to divalent as well as monovalent cations. In addition, our finding that 8-bromo-cGMP induces an influx of Ca^{2+} is consistent with the Müller cell CNG channels being permeable to this divalent cation although other pathways for Ca^{2+} influx are not excluded. More detailed characterization of the ion permeation of the cGMP-gated channels in Müller cells will require the use of excised membrane patches. Also, analysis at the single channel level will be needed in order to demonstrate rigorously that cGMP directly activates non-specific cation channels in Müller cells.

It appears that most (~90%) Müller cells from the human or bovine retina express CNG channels as detected physiologically with the internal perfusion of cGMP. However, although our electrophysiological and molecular findings indicate that Müller cells have functional CNG channels, an antibody against the rod CNG channel is reported to label

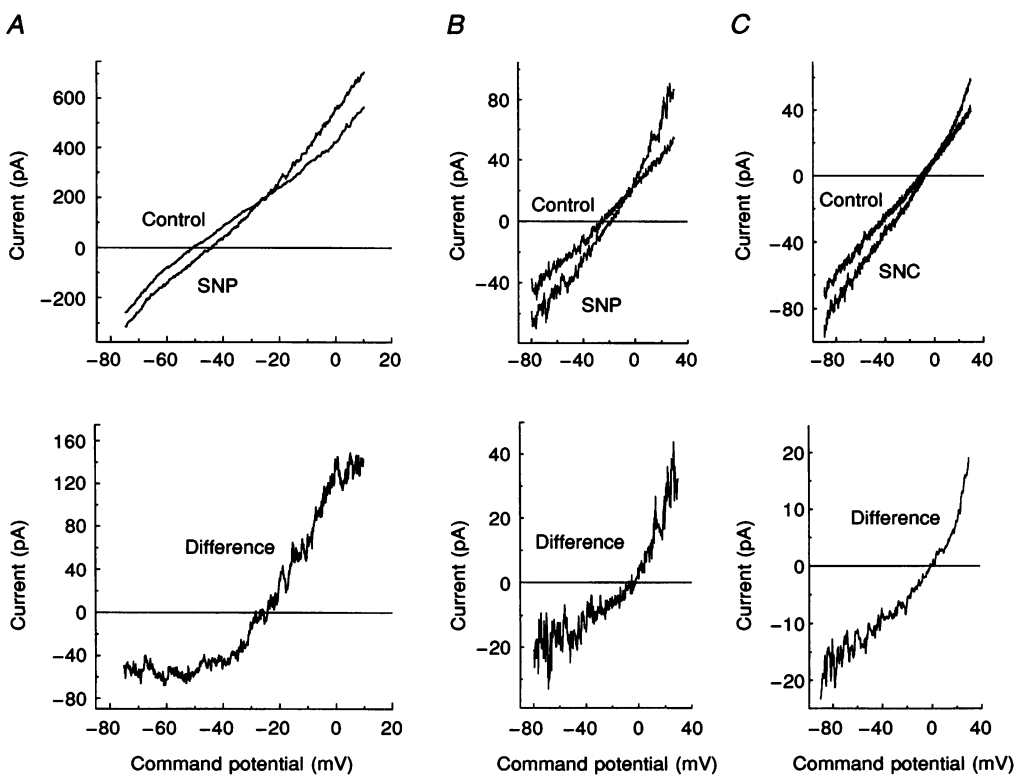

Figure 7. Effect of NO donors on currents in fresh bovine Müller cells monitored with the perforated-patch configuration

A, top panel, I–V relations before (Control) and during miniperfusion of the bathing solution supplemented with 100 μM SNP. Bottom panel, I–V plot of the difference between the curves in the upper panel. *B*, top panel, I–V relations before (Control) and during exposure to 100 μM SNP under recording conditions designed to block K^+ channels. The bathing and pipette solutions were as in Fig. 1*E*. Bottom panel, plot of the difference between the curves in the upper panel. *C*, I–V relations before (Control) and during exposure to 100 μM SNC under recording conditions as in *B*. Bottom panel, plot of the difference between the curves in the upper panel. Exposure to NO-generating agents induces changes in Müller cell currents qualitatively similar to those observed with cGMP and its membrane-permeable analogue.

only rod outer segments in rat and cat retinas (Wässle, Grünert, Cook & Molday, 1992; Vardi, Matesic, Manning, Leibman & Sterling, 1993). Possible explanations for not finding Müller cells to be labelled include species differences, a relatively low density of Müller cell channels or variant subunits not detected with the antibody used. Details of the topographical distribution of CNG channels in Müller cells await future analysis.

cGMP-induced activation of K_{Ca} channels

In addition to activating CNG channels, exposure of Müller cells to 8-bromo-cGMP is associated with an increase in the K_{Ca} current, which was identified by its sensitivity to caesium and charybdotoxin. It seems likely that one mechanism by which cGMP activates the Müller cell K_{Ca} channels involves an increase in $[Ca^{2+}]_i$. Previously, we demonstrated a calcium sensitivity of the large conductance K_{Ca} (BK)

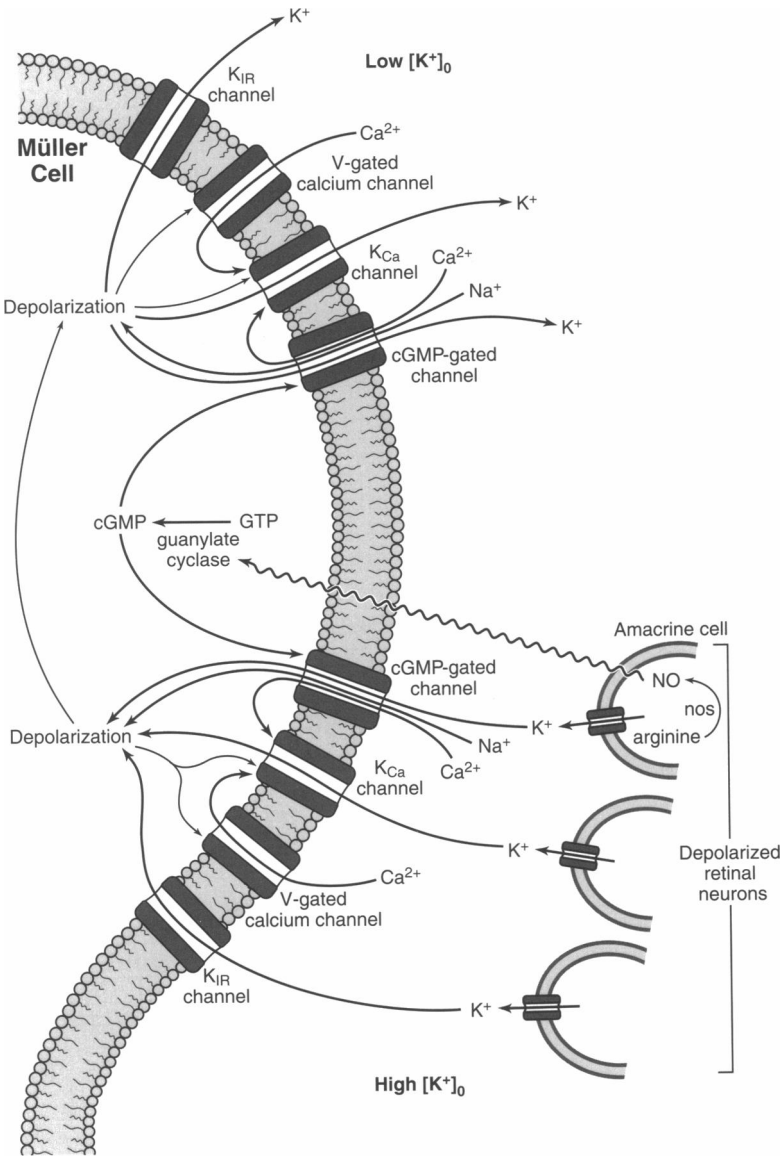

Figure 8. Schematic representation of the putative role of various ion channels in the redistribution of excess extracellular K^+ by human Müller cells

The efflux of K^+ from depolarized neurons causes a localized increase in $[K^+]_o$. At sites where $[K^+]_o$ is elevated, this ion enters Müller cells. A localized influx of K^+ causes a depolarization of much of the Müller cell and, thus, enhances the electrotonic driving force for the efflux of K^+ at sites distant from the localized increase in $[K^+]_o$. Earlier studies (Brew *et al.* 1986; Newman, 1993; Puro & Stuenkel, 1995) indicate that K_{IR} channels are important pathways for the movement of K^+ across the Müller cell membrane. As discussed in the text, we postulate that ion channels activated directly or indirectly by cGMP may also play a role in the regulation of $[K^+]_o$ by Müller cells. Abbreviations: NO, nitric oxide; nos, NO synthase; K_{Ca} channel, calcium-activated K^+ channel; K_{IR} channel, inwardly rectifying K^+ channel; V-gated, voltage-gated; GTP, guanosine 5′-triphosphate; cGMP, guanosine 3′:5′-cyclic monophosphate.

channels of Müller cells (Puro, 1991*a*). In this report, we document that $[Ca^{2+}]_i$ increases when Müller cells are exposed to 8-bromo-cGMP. The cGMP-induced rise in $[Ca^{2+}]_i$ is dependent on extracellular calcium and may be mediated by an influx of this cation via CNG channels and quite possibly other pathways. In addition to providing a putative pathway for an influx of calcium, CNG channels cause a depolarization of the membrane potential due to their non-specific cation permeability. Depolarization would contribute to the activation of K_{Ca} channels, which are known to be voltage dependent (Hille, 1992). Another mechanism by which cGMP may activate K_{Ca} channels is via cGMP-dependent protein kinases as has been shown for BK channels in other cell types (White *et al.* 1993). Although the actions of these kinases are not well studied in Müller cells, recent experiments have documented the presence of both isoforms of the soluble or type I cGMP-dependent protein kinase in cultured rat Müller cells (I. Dabin & C. J. Barnstable, unpublished observations).

NO as an intercellular signal

NO is of interest since it is known to activate guanylate cyclase (Knowles *et al.* 1989) and is likely to be synthesized by a specific population of amacrine cells (Yamamoto *et al.* 1993). A role in retinal function is suggested by recent experiments showing that NO donors can lead to the activation of a cGMP-gated conductance in cultured retinal ganglion cells (Ahmad *et al.* 1994). In this report, we investigated the possibility that NO donors can induce changes in the Müller cell currents similar to those detected with cGMP or its analogue. Striking similarities were observed although evidence demonstrating that NO increases the activity of guanylate cyclase in Müller cell is not available. Also, the possibility that NO directly interacts with Müller cell channels is not excluded. The fact that NO-generating agents induce significant changes in Müller cell currents raises the possibility that NO serves as a neuron-to-glial signal regulating the physiology of Müller cells.

Functional implications

Evidence is good that Müller cells play a role in maintaining K^+ homeostasis in the retina (Karwoski, Lu & Newman, 1989). Potassium-permeable ion channels in these glia are one of the important pathways for the redistribution of K^+ from regions of the retina with higher $[K^+]_o$ to areas where $[K^+]_o$ is lower (Newman, 1985, 1993; Brew, Gray, Mobbs & Attwell, 1986). Under usual assay conditions, the predominant potassium-permeable ion channels detected in Müller cells are of the inwardly rectifying type (Brew *et al.* 1986; Newman, 1993; Chao *et al.* 1994; Puro & Stuenkel, 1995). However, with an elevation of cGMP levels, additional pathways for the movement of K^+ may be activated. The CNG channels would provide one of the additional K^+-permeable pathways. Perhaps of greater significance, the effect of cGMP on K^+ movement may be amplified as Ca^{2+} entering via the CNG channels activates K_{Ca} channels and other calcium-activated cation channels expressed by Müller cells (Puro, 1991*b*). Also, the depolarization induced by an influx of cations through CNG channels may help activate K_{Ca} channels directly as well as indirectly with the opening of the L-type voltage-gated calcium channels present in these glia (Puro *et al.* 1996*a*).

Based on these considerations, we postulate that direct and indirect effects of cGMP on multiple types of ion channels may, under certain conditions, regulate the role of Müller cells in maintaining K^+ homeostasis in the retina (Fig. 8). While CNG channels are relatively voltage- independent and function at all membrane potentials, the role of K_{Ca} channels is probably limited to pathophysiological conditions since their threshold for activation is significantly depolarized relative to the normal resting membrane potential (Newman, 1985; Puro *et al.* 1996*a*; Fig. 1*D*). The fact that NO-generating agents can induce significant changes in the currents of Müller cells as well as retinal ganglion cells (Ahmad *et al.* 1994) raises the possibility that NO functions as an intercellular messenger integrating the responses of both glia and neurons to physiological and pathophysiological changes in the retina.

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Acknowledgements

We thank Dr B. Hughes for comments on the manuscript. This work was supported by grants from the NIH, EY06931 (D.G.P), NS20483 (C.J.B.), EY07003 (Core) EY00785 (Core), the Ziegler Foundation and Mr James M. Kemper Jr. C.J.B. is a Jules and Doris Stein Research to Prevent Blindness Professor. D.G.P. is a Research to Prevent Blindness Senior Scientific Investigator.

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Received 25 March 1996; accepted 11 September 1996.