Intracellular ATP activates inwardly rectifying K^+ channels in human and monkey retinal Müller (glial) cells

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1. In the vertebrate retina, the inwardly rectifying K^+ (K_{IR}) channels of the Müller (glial) cells are pathways for the redistribution of excess extracellular K^+. Due to this role in K^+ homeostasis, the activity of Müller cell K_{IR} channels is likely to have significant functional consequences for the retina. In this study we asked whether intracellular ATP regulates the function of K_{IR} channels expressed by Müller cells, the principal glia of the retina.

2. Freshly dissociated Müller cells from the human and monkey (*Macaca fascicularis*) retina were studied with various configurations of the patch-clamp technique.

3. Whole-cell recordings from Müller cells revealed that a run-down of the inwardly rectifying K^+ current ($I_{K(IR)}$) was prevented if the pipette solution contained Mg-ATP. Chemical ischaemia induced by inhibitors of glycolysis and oxidative phosphorylation caused a nearly 10-fold reduction in the $I_{K(IR)}$ that was fully restored when metabolically inhibited Müller cells were internally perfused with ATP.

4. In recordings from membrane patches of fresh primate Müller cells, we found that inward-rectifying channels with a conductance of 20 pS in 100 mM K_o^+ were the predominant type of K_{IR} channel. In excised patches these 20 pS K_{IR} channels were activated when Mg-ATP was at the cytoplasmic surface. Experiments with inside-out patches indicated that the activity of the 20 pS K_{IR} channels can be maintained by ATP synthesized at sites located close to the channel.

5 The inability of the non-hydrolysable ATP analogue 5′-adenylylimidodiphosphate (AMP-PNP) to prevent the run-down of $I_{K(IR)}$ and the Mg^{2+} dependence of the ATP effect on K_{IR} channels are consistent with a mechanism of activation requiring the hydrolysis of ATP.

6. These observations suggest that the metabolic state of a Müller cell regulates the activity of its 20 pS K_{IR} channels and thus influences the function of the glial cell in maintaining K^+ homeostasis in the retina.

Glial cells are essential functional elements of the CNS. One fundamental task of the glia is to regulate the extracellular K^+ concentration ($[K^+]_o$). This is vital since wide fluctuations in $[K^+]_o$ alter neuronal excitability. Glial cells reduce excess K_o^+ by several mechanisms (Newman, 1995). One likely mechanism is the intracellular accumulation of K^+. Another involves a redistribution via glial cells of K^+ from regions of high $[K^+]_o$ to areas where $[K^+]_o$ is lower (Orkand, Nicholls & Kuffler, 1966). The relative significance of K^+ accumulation *versus* redistribution by a spatial buffering mechanism is unclear and may vary at different sites within the nervous system.

In the brain, where approximately 90% of the cells are glia, the intracellular accumulation of K^+ by glial cells may remove a majority of the excess K_o^+ (Dietzel, Heinemann & Lux, 1989). However, in the primate retina, where neurons outnumber the glial cells by more than 10 to 1 and account for ~90% of the retinal volume (Reichenbach & Robinson, 1995), other mechanisms may be particularly important. In agreement with this, there is evidence that the Müller cells, the principal glia of the vertebrate retina, are capable of redistributing excess K_o^+ (Newman, Frambach & Odette, 1984; Karwoski, Lu & Newman, 1989).

Müller cells are morphologically suited as pathways for moving K^+ through the retina. They are radially oriented and span the entire depth of the neural retina from the interface of the retina and vitreous to the subretinal space adjacent to the photoreceptors. While a redistribution of K^+

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through the depth of the retina is enhanced by the radial shape of these glia, the lateral spread of excess K_o^+ via Müller cells is minimal in the mammalian retina due to the lack of gap junctions between these cells.

When depolarized retinal neurons release K^+ at a localized site, e.g. the inner synaptic layer, the Müller cells provide a pathway for the redistribution of excess K^+ toward the vitreous and/or the subretinal space (Newman & Reichenbach, 1996). This redistribution is likely to involve a specialized form of spatial buffering, termed K^+ siphoning (Newman *et al.* 1984). An initial step in this mechanism is a shift in the K^+ equilibrium potential to a less negative potential at the site where the Müller cell is exposed to locally increased $[K^+]_o$. At the localized site of elevated $[K^+]_o$, the equilibrium potential for K^+ ($E_{K,high}$) may become more positive than the resting membrane potential. As a result, K^+ moves down its electrochemical gradient and enters the Müller cell via K^+-permeable ion channels. This localized influx of K^+ causes a partial depolarization of much of the Müller cell. Since only a localized area of the cell is exposed to elevated $[K^+]_o$, the membrane potential depolarizes to a voltage more positive than $E_{K,low}$ (i.e. the K^+ equilibrium potential where $[K^+]_o$ is lower) and more negative than $E_{K,high}$. At sites distant from the localized increase in $[K^+]_o$, the depolarization of the membrane potential (V_m) enhances the electronic driving force ($E_{K,low} - V_m$) for the efflux of K^+ through ion channels. Thus, K^+ enters the Müller cell where $[K^+]_o$ is high and exits where $[K^+]_o$ is lower. From modelling studies of K^+ dynamics, such a spatial buffering mechanism is estimated to clear excess K^+ from the retina several times faster than extracellular diffusion (Newman, 1993).

Potassium-permeable ion channels provide the pathways for the redistribution of K^+ by glial cells. In the Müller cells, the predominant K^+ channels active near the normal resting membrane potential are of the inwardly rectifying type (Brew, Gray, Mobbs & Attwell, 1986; Newman, 1993; Chao, Henke, Reichelt, Eberhardt, Reinhardt-Maelicke & Reichenbach, 1994; Puro & Stuenkel, 1995). Since most of the K^+ channels in the amphibian retina are localized to the endfoot (Newman, 1985), the K^+ spatial buffering current exits preferentially from the endfoot into the vitreous humor (Karwoski *et al.* 1989). In contrast, functional K^+ channels in the vascularized mammalian retinas are distributed more evenly across the surface of the Müller cells (Newman, 1987), and excess K^+ from the inner retina is predominantly redistributed towards the outer (photoreceptor) portion of the retina (Frishman & Steinberg, 1989).

Since the inward-rectifying K^+ (K_{IR}) channels in Müller cells are likely to have an important role in the maintenance of K^+ homeostasis in the retina, the regulation of the activity of these channels should have significant functional consequences for the retina. It is now evident that there are multiple pathways regulating the activity of the K_{IR} channels in Müller cells. For example, activation of glutamate or dopaminergic receptors inhibits these channels in Müller cells of various species (Schwartz, 1993; Akopian & Witkovsky, 1995; Biedermann, Frohlich, Grosche, Wagner & Reichenbach, 1995; Puro, Yuan & Sucher, 1996). In addition thrombin, which may enter the retina with a breakdown of the blood–retina barrier, acts at its receptors to reduce the activity of K_{IR} channels in human Müller cells (Puro & Stuenkel, 1995). Thus, the function of K_{IR} channels in the Müller cell is influenced significantly by a variety of extracellular molecules.

In this study, we asked whether intracellular ATP (ATP_i) also plays a role in the regulation of the activity of the K_{IR} channels expressed by human and monkey Müller cells. We examined the effect of ATP for several reasons. One reason is that intracellular ATP is known to regulate the activity of a number of K_{IR} channels cloned from various cell types (Ho *et al.* 1993; Fakler, Brandle, Glowatzki, Zenner & Ruppersberg, 1994; Takumi *et al.* 1995), although an effect of ATP_i on glial channels has not been reported previously. Another reason for studying ATP is that the concentration of this nucleotide is an important indicator of the metabolic state of the cell. Since ischaemia is associated with an increase in $[K^+]_o$ and a decrease in $[ATP]_i$, an understanding of how Müller cells respond to this pathophysiological state requires characterization of the possible effects of ATP on the ion channels which serve as pathways for the redistribution of excess K_o^+.

Based on whole-cell recordings of freshly dissociated human and monkey Müller cells, we report here that intracellular ATP activates the inwardly rectifying K^+ current ($I_{K(IR)}$) of these glial cells. Experiments with excised, inside-out patches revealed that the activity of the 20 pS K_{IR} channel, which we find is the predominant K_{IR} channel expressed by primate Müller cells, can be maintained by ATP synthesized at sites located close to the channel. In addition, chemical ischaemia induced by inhibitors of glycolysis and oxidative phosphorylation causes a dramatic decrease in the $I_{K(IR)}$ which is fully restored when the metabolically inhibited Müller cell is internally perfused with ATP.

METHODS

Fresh Müller cells

Freshly dissociated human and monkey (cynomolgus macaque, *Macaca fascicularis*) Müller cells were prepared as detailed previously (Kusaka, Dabin, Barnstable & Puro, 1996). In brief, approximately 0·25 cm × 0·25 cm pieces of retina were incubated in Earle's balanced salt solution (Gibco) supplemented with 0·5 mM EDTA, 1·5 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM glucose, 26 mM sodium bicarbonate, 15 u papain (Worthington), 0·04% DNase, 2 mM cysteine and 12% chicken serum (Gibco) for 40 min at 30 °C while 95% oxygen–5% CO_2 was bubbled through to maintain pH and oxygenation. The piece of retina was then washed with the appropriate bathing solution, drawn up into a glass pipette and gently ejected back into a microcentrifuge tube. A suspension of cells (~0·1 ml) was placed in a recording chamber and allowed to settle for ~15 min prior to the addition of bathing solution to the

chamber. Cells were examined at ×400 magnification with an inverted microscope equipped with phase-contrast optics. Müller cells were identified by their characteristic morphology (Puro *et al.* 1996).

Electrophysiology

Electrophysiological experiments were made at room temperature (22–24 °C). Patch-clamp recordings of fresh Müller cells were made using the whole-cell, cell-attached patch and inside-out patch configurations. Unless noted otherwise, the bathing solution for whole-cell and cell-attached voltage-clamp recordings 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 ~310 mosmol l^{-1} and the bathing solution for inside-out patch recordings contained (mM): 10 NaCl, 125 KCl, 2 $MgCl_2$ and 10 K-Hepes with the pH at 7·4 and the osmolarity adjusted to ~310 mosmol l^{-1}.

Patch pipettes of Corning No. 7052 glass (Garner Glass, Claremont, CA, USA) were pulled using a multistage programmable puller (Sutter Instruments, San Rafael, CA, USA), coated with Sylgard 184 (Dow Corning) to within 100 μm of their tips and heat polished to tip diameters of 2–3 μm. The resistances of the pipettes used were 2–5 MΩ in the bathing solution. Unless noted otherwise, pipettes for whole-cell recordings were filled with (mM): 120 potassium aspartate, 10 KCl, 10 NaCl, 1·5 $CaCl_2$, 3 EGTA, 2 $MgCl_2$ and 10 K-Hepes at pH 7·4 with the osmolarity adjusted to ~280 mosmol l^{-1}. For cell-attached patch and inside-out patch recordings, the pipette solution contained (mM): 100 KCl, 23 NaCl, 1·8 $CaCl_2$, 0·8 $MgCl_2$, 20 tetraethylammonium and 10 Na-Hepes with the pH at 7·4 and the osmolarity at ~280 mosmol l^{-1}.

The pipettes were mounted in the holder of a Dagan 3900 patch-clamp amplifier (Dagan Corp., Minneapolis, MN, USA). The pipette potential was adjusted to zero current flow before establishment of a seal to a Müller cell. Seals of >10 GΩ usually formed over a period of 1–30 s. 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 of < 20 MΩ were used. Whole-cell recordings were used after the ratio of cell resistance to series resistance was >10. Since such a ratio would introduce a <10% error in the true voltage step, series resistance compensation was not used in whole-cell recordings. The series resistance was monitored frequently during recordings; if the series resistance increased by >10%, recording was terminated. We did not detect a population of primate Müller cells with cell resistances < 25 MΩ and resting membrane potentials near the K^+ equlibrium potential. Rather, lower resistances were associated invariably with depolarized membrane potentials, suggesting cellular injury.

For the construction of continuous current–voltage plots, whole-cell currents were evoked by ramping membrane voltage (~60 mV s^{-1}) from negative to positive membrane potentials. Command pulse protocols were used to vary the potential across cell-attached and inside-out excised patches. Currents were filtered at 1 kHz and digitally sampled (at 1 ms for ramps and 250 μs for cell-attached and excised patches) using a Lab Master DMA acquisition system (Axon Instruments), an IBM-compatible microcomputer (Gateway 2000) and pCLAMP software (version 6, Axon Instruments), which also controlled voltage protocols and helped with data analysis. A scientific plotting program (ORIGIN version 4, MicroCal, Northhampton, MA, USA) was used for data exported from pCLAMP.

The recorded membrane potentials were corrected for liquid junction potentials, which were calculated using a computer program (Barry, 1993). The potentials across cell-attached patches (e.g. as shown in Fig. 4) were determined from the potential applied to the pipette, the calculated liquid junction potential (+4 mV) and the mean membrane potential (−68 mV, S.D. = 7, $n = 22$) of human Müller cells in the bathing solution used. For current records from cell-attached patches and inside-out patches, leak currents, which were calculated at voltage steps in which no channel activity occurred, were subtracted after data collection.

The conductances of the inward currents in Müller cells were determined between the K^+ equilibrium potential (−66 mV for Figs 2 and 7; −74 mV for Fig. 6) and −90 to −110 mV, depending on the ramp stimulus protocol. The conductances of the inwardly rectifying channels plotted in Fig. 3 were calculated between −60 and −164 mV. The open probability of channels in a patch was calculated from the formula, $P_o = X/(iN)$. The mean current (X) at a holding potential was calculated using a pCLAMP program; the amplitude of single channel currents (i) was measured manually from digitized records, and the maximal number of simultaneously opened channels was used as N, the number of channels in the patch.

Solutions could be miniperfused 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 tip of a recording pipette containing an inside-out excised patch. Less than 7 kPa (1 p.s.i.) of pressure was used for miniperfusion.

Chemicals and ocular tissue

ATPγS (adenosine-5′-*O*-(3-thiotriphosphate)) was purchased from Boehringer Mannheim. Staurosporine was obtained from Alexis Corporation (San Diego, CA, USA). Unless noted otherwise, the other chemicals used in this study were from Sigma.

Donor adult human eyes were supplied within 24 h of death by the Michigan Eye and Transplantation Centre (Ann Arbor, MI, USA), which obtained proper consent from relatives. This study protocol was approved by the Institutional Review Board of the University of Michigan. Monkey eyes were obtained from Bio-Whittaker (Walkersville, MD, USA) or the New Iberia Research Centre (New Iberia, LA, USA). The animals were sedated with intramuscular ketamine (10 mg kg^{-1}) prior to intravenous administration of pentobarbitone (up to 25 mg kg^{-1}) to induce cardiac arrest; the eyes were enucleated promptly and kept cold (4–8 °C) during overnight shipment for receipt within 24 h. All tissue was obtained and used in accordance with applicable laws and regulations.

Data are given as means ± S.D. unless otherwise stated, and probability was evaluated using Student's t test.

RESULTS

Run-down of Müller cell currents

Whole-cell recordings of freshly dissociated Müller cells from the human retina revealed voltage-dependent currents including an inwardly rectifying current as well as outward currents (Fig. 1*A*). The current–voltage relations of fresh human Müller cells are similar to those observed in freshly dissociated or cultured Müller cells from a variety of species (Newman, 1993; Chao *et al.* 1994; Puro & Stuenkel, 1995; Kusaka *et al.* 1996).

We found that currents in fresh human Müller cells are not stable when monitored with standard whole-cell recordings.

A progressive decrease in the recorded currents was observed when the pipette solution contained (mM): 120 potassium aspartate, 10 KCl, 10 NaCl, 1·5 $CaCl_2$, 3 EGTA 2 $MgCl_2$ and 10 K-Hepes (Figs 1*A* and 2*A*). Much of the current which runs down under these recording conditions shows steep inward rectification and a reversal potential near the equilibrium potential for potassium (Fig. 1*B*). Thus, a significant component of the run-down is likely to be due to a decrease in the inwardly rectifying potassium current ($I_{K(IR)}$), the predominant current active near the resting membrane potential of Müller cells (Newman, 1993; Chao *et al.* 1994; Puro & Stuenkel, 1995). In addition to the $I_{K(IR)}$, there appears to be a loss of outwardly rectifying current at potentials depolarized to −40 mV (Fig. 1*B*). In this study, attention focused on the run-down of the $I_{K(IR)}$, which reflects the activity of ion channels that are likely to be pathways for the redistribution of K^+ from regions in the retina with higher $[K^+]_o$ to areas where $[K^+]_o$ is lower (Newman *et al.* 1984; Brew *et al.* 1986; Newman, 1993).

ATP-dependent regulation of $I_{K(IR)}$

It seemed likely that the run-down of current during our whole-cell recordings was due to a washout of intracellular molecules necessary for the function of ion channels. Since some of the K_{IR} channels which have been cloned are known to be activated by ATP (Ho *et al.* 1993; Fakler *et al.* 1994; Takumi *et al.* 1995), we asked whether the $I_{K(IR)}$ of human Müller cells is affected by this nucleotide. As demonstrated in Fig. 2*A*, the addition of 4 mM Mg-ATP to the recording pipette solution prevented the run-down of inward currents in human Müller cells.

To help examine the nature of the ATP-dependent maintenance of Müller cell currents, we tested various analogues of this nucleotide. When the pipette solution contained a non-hydrolysable ATP analogue, 5′-adenylyl-imidodiphosphate (AMP-PNP), there was a run-down of this current (Fig. 2*B*). This observation suggests that maintenance of $I_{K(IR)}$ in human Müller cells requires ATP hydrolysis. This hydrolysis is likely to involve either a protein kinase or ATPase reaction. To begin to differentiate between these possibilities, whole-cell recordings (Fig. 2*C*) were made using pipettes with solutions containing ATPγS, which is a substrate for most protein kinases, but not most ATPases (Eckstein, 1985). The inability of ATPγS to prevent the run-down of the current is consistent with the idea that the hydrolysis of ATP by an ATPase-like mechanism plays a role in the activation of K_{IR} channels in Müller cells.

K_{IR} channels in human Müller cells

In addition to assessing the influence of ATP_i on the $I_{K(IR)}$, we wished to examine the effect of this nucleotide on the activity of K_{IR} channels in patches of human Müller cells. In a series of cell-attached patch recordings, we found that the mean conductance of the inwardly rectifying channels sampled in fresh human Müller cells was 20·1 ± 3·9 pS ($n = 76$) when the pipette solution contained 100 mM K^+. No significant ($P = 0·63$) difference in the mean conductance was detected in channels recorded from the soma (20·4 ± 3·9 pS, $n = 40$) *versus* those sampled from the endfoot (19·9 ± 5·1 pS, $n = 36$). As shown in Fig. 3, the unitary conductance of most of the sampled channels

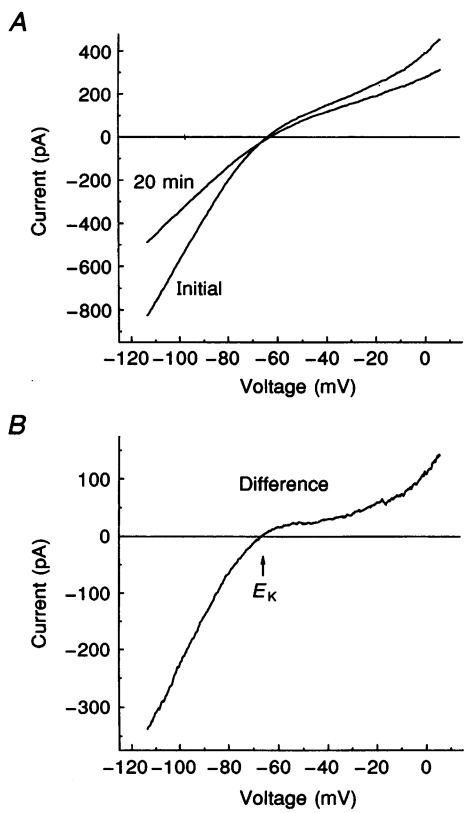

Figure 1. Run-down of the whole-cell currents of a fresh human Müller cell monitored with the whole-cell recording configuration of the patch-clamp technique

A, current–voltage relations initially after going whole-cell and 20 min later. *B*, plot of the difference between the *I–V* curves shown in *A*. E_K indicates the equilibrium potential of K^+. A steeply inwardly rectifying current, which has a reversal potential near E_K, runs down during standard whole-cell recordings of human Müller cells.

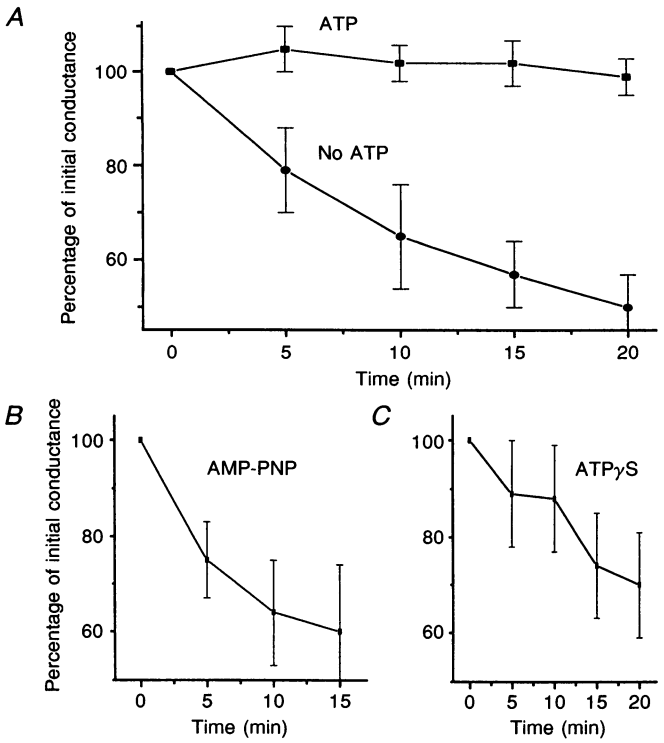

Figure 2. Effect of ATP, AMP-PNP and ATPγS on the run-down of current in human Müller cells

A, time course of the normalized inward conductances of Müller cells monitored with whole-cell recording pipettes containing solutions with or without 4 mM Mg-ATP. Time was measured from the onset of going whole-cell. Six cells were in the ATP group; 5 cells were in the control group. The means and S.E.M. values are shown. At times equal to or greater than 5 min, the values for the ATP group were significantly ($P < 0{\cdot}025$) greater than those for the controls. *B*, time course of the means of the normalized inward conductances of 4 Müller cells monitored with whole-cell recording pipettes containing 4 mM AMP-PNP. At times equal to or greater than 5 min, the values were significantly ($P < 0{\cdot}02$) less than the comparable values for the ATP group shown in *A*. *C*, time course of the means of the normalized inward conductance of 6 Müller cells monitored with whole-cell recording pipettes containing 4 mM ATPγS. At times equal to or greater than 5 min, values were significantly ($P < 0{\cdot}02$) less than the comparable values for the ATP group shown in *A*. Internal perfusion of ATP, but not AMP-PNP or ATPγS, prevents the run-down of Müller cell currents.

appears to fall within a distribution around 20 pS. However, the histogram in Fig. 3 also suggests that other K_{IR} channels with larger unitary conductances (e.g. 28 pS) may also be expressed by these glia.

The 20 pS channels have functional characteristics which are similar to those of the $I_{K(IR)}$ of Müller cells. One similarity is that the 20 pS channels show steep inward rectification (Fig. 4*A* and *B*); in fact, outward currents greater than the noise level (~0·2 pA) were not detected. Consistent with their being potassium channels, the 20 pS inwardly rectifying channels have apparent reversal potentials (e.g. Fig. 4*B*) close to the K^+ equilibrium potential (−7 mV). Another similarity between the $I_{K(IR)}$ and the 20 pS K_{IR} channels is time-dependent gating. In Müller cells, whole-cell currents elicited with large hyperpolarizing steps peak rapidly and then decline to a sustained level (Newman, 1993; Puro & Stuenkel, 1995). As illustrated in the upper panel of

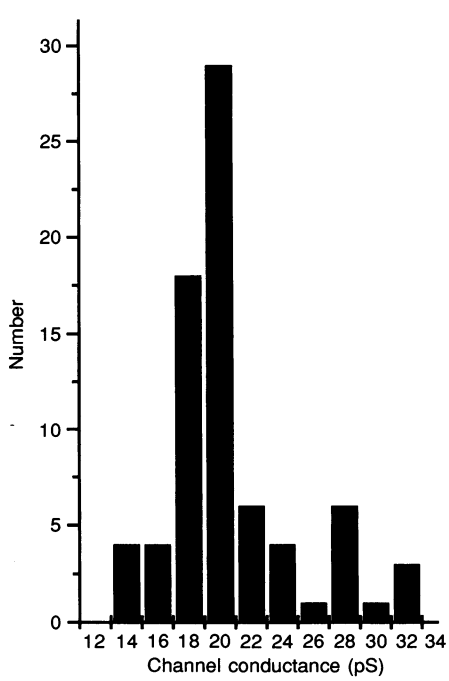

Figure 3

Unitary conductances of inwardly rectifying channels sampled in cell-attached patches of fresh human Müller cells.

Fig. 4C, which shows thirty superimposed traces, a multiple number of 20 pS K_{IR} channels in a cell-attached patch of a human Müller cell opened initially after a hyperpolarizing step to −164 mV. Most of these channels then closed rapidly as the hyperpolarized potential was maintained. The time course of the mean current, calculated from the thirty cycles of hyperpolarizing steps, is shown in the lower part of Fig. 4C and further documents the time-dependent change in channel activity at extreme hyperpolarization. Figure 4D also shows time-dependent changes with a step to −114 mV although the decrease in current with time is less dramatic than at −164 mV.

In addition to having time-dependent gating, the 20 pS K_{IR} channels of human Müller cells are modulated by voltage. In a series of cell-attached patches, the open probability increased from 0·04 ± 0·01 ($n = 4$) at a holding potential of −164 mV to 0·40 ± 0·04 ($n = 4$) at −114 mV. Thus, with depolarization the open probability of the 20 pS K_{IR} channel increases.

Activation of K_{IR} channels by ATP

We examined the effect of ATP on the activity of the 20 pS K_{IR} channels in excised, inside-out patches. Soon after the excision, the open probability of the 20 pS K_{IR} channels decreased markedly in some, but not all (see below), inside-out patches. For the channels in the inside-out patch illustrated in Fig. 5, the open probability decreased from approximately 0·3 to less than 0·1 within 40 s after excision of the patch; the bathing solution lacked ATP. Channel activity remained low until 4 mM Mg-ATP was added to the perfusate bathing the cytoplasmic surface of the patch. Within 30 s, the open probability increased markedly. Similar observations were made in three inside-out patches containing 20 pS K_{IR} channels.

Metabolic regulation of $I_{K(IR)}$

The finding that ATP activates the $I_{K(IR)}$ of Müller cells led us to predict that metabolic inhibitors which block ATP synthesis would be associated with a decrease in $I_{K(IR)}$. To test this prediction, we exposed Müller cells to iodoacetate, a blocker of glycolysis, and antimycin A, a blocker of oxidative phosphorylation. These inhibitors were used since exposure of cultured Müller cells to these chemicals causes a profound reduction in the intracellular concentration of ATP (Winkler & Puro, 1996). In this series of experiments, we studied the effect of these metabolic inhibitors on fresh

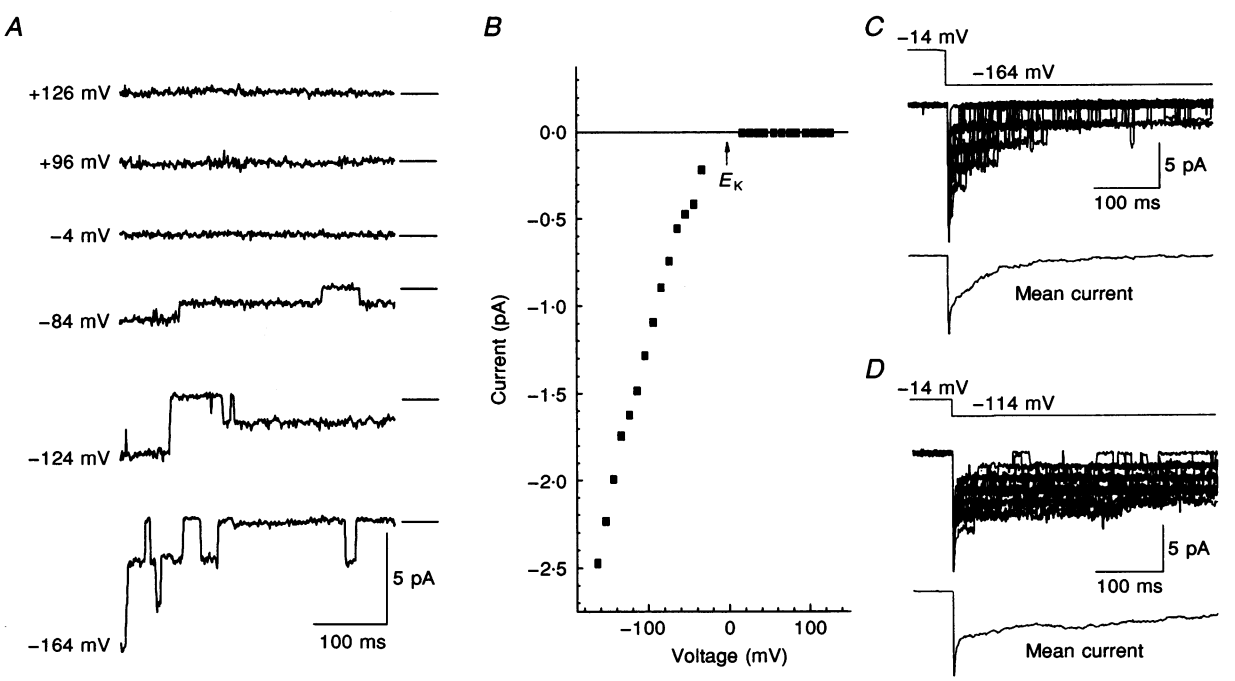

Figure 4. Characteristics of K_{IR} channels in a cell-attached patch from the soma of a human Müller cell

A, voltage-clamp records of inwardly rectifying channels during the first 400 ms after a change in holding potential from −14 mV to the holding potentials listed at the left of each trace. Horizontal line segments at the right indicate the closed-state current level. *B*, plot of the amplitude of the single channel current *versus* the holding potential. E_K shows the equilibrium potential of K^+. No channel currents above the noise level (0·2 pA) were detected positive to E_K, and values in that range were plotted arbitrarily at 0 pA. The unitary channel conductance was 19 pS. *C*, channel currents evoked by a step change in the holding potential from −14 to −164 mV. The upper panel shows 30 superimposed current traces. The bottom panel shows the mean current of the 30 traces averaged together. *D*, similar to *C*, but with a step change from −14 to −114 mV. These channels show steep inward rectification and a time dependence of their open probability.

monkey Müller cells, which we find have an $I_{K(IR)}$ and 20 pS K_{IR} channels that are virtually identical to those of human Müller cells. When these Müller cells were exposed to 1 mM iodoacetate and 10 μM antimycin A for approximately 1–2 h, the conductance of the inward current was reduced by nearly 10-fold (Fig. 6).

We investigated whether perfusion of Mg-ATP into metabolically inhibited Müller cells could restore the $I_{K(IR)}$. Figure 7*A* shows an example of a fresh Müller cell that was exposed to iodoacetate and antimycin A for 56 min prior to whole-cell recording. Minimal $I_{K(IR)}$ was detected initially after going whole-cell with a recording pipette containing 4 mM Mg-ATP. However, after 20 min of internal perfusion of Mg-ATP via the recording pipette, the $I_{K(IR)}$ had increased markedly despite the continued presence of the metabolic inhibitors in the bathing solution. As shown in Fig. 7*B*, the ATP-induced current had a reversal potential near the equilibrium potential of K^+ and showed steep inward rectification, i.e. characteristics of the $I_{K(IR)}$.

Experiments were performed to detail the time course for the ATP-induced reactivation of the $I_{K(IR)}$ in Müller cells pre-exposed to iodoacetate and antimycin A. As shown in Fig. 7*C*, Müller cells monitored with pipettes containing a solution lacking ATP failed to restore their $I_{K(IR)}$. In contrast, the inward conductances increased in the cells internally perfused with 4 mM Mg-ATP. After 10 min of whole-cell recording, the mean conductance of the cells internally perfused with Mg-ATP was significantly ($P = 0{\cdot}002$) greater than the mean conductance in the control group. By 15 min, the mean conductance of the cells receiving ATP was essentially the same ($P = 0{\cdot}733$) as that of Müller cells (Fig. 6, Control) which had never been exposed to the metabolic inhibitors.

In similar experiments, we lowered the concentration of Mg-ATP in the pipette solution to 100 μM. Internal perfusion of this concentration of ATP for 15 min did not significantly ($P = 0{\cdot}406$, $n = 4$) increase the $I_{K(IR)}$ in the metabolically inhibited Müller cells. Specifically, the inward conductance initially after going whole-cell in Müller cells pre-exposed to 1 mM iodoacetate and 10 μM antimycin A for 64–100 min was $0{\cdot}8 \pm 0{\cdot}2$ nS ($n = 4$); 15 min later the conductance was $0{\cdot}9 \pm 0{\cdot}1$ nS ($n = 4$). These observations indicate that > 100 μM ATP in the recording pipette is needed to activate the K_{IR} channels in Müller cells. However, the actual concentration of internally perfused ATP located adjacent to the K_{IR} channels is uncertain. Future studies using excised patches should allow a more precise determination of the dose–response relation for the ATP-induced activation of the 20 pS K_{IR} channels.

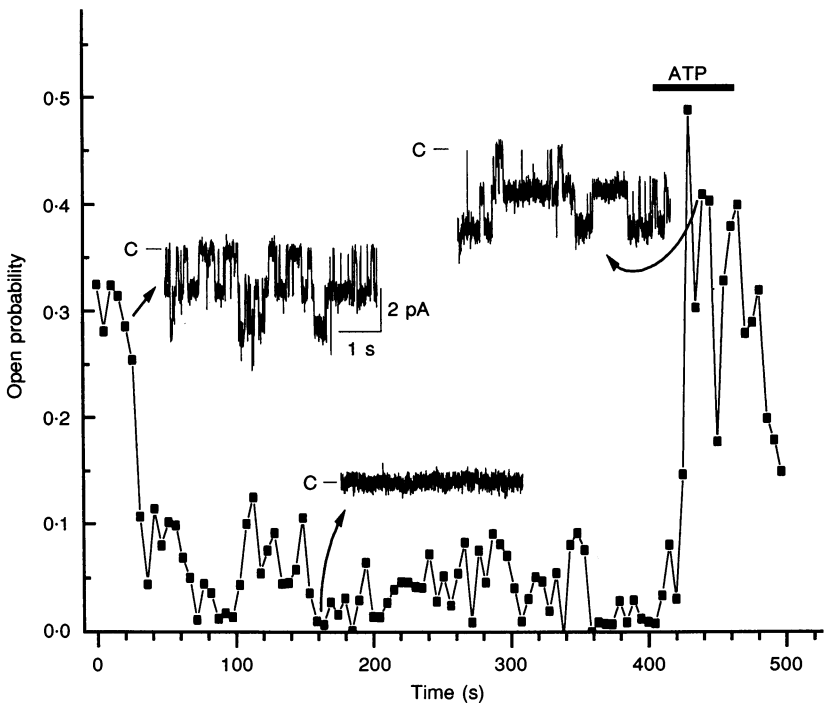

Figure 5. Channel open probability *versus* time after excision of an inside-out patch from the soma of a human Müller cell

During the time indicated by the bar, the perfusate contained Mg-ATP. Insets, examples of channel activity at the points indicated by the arrows. The line segments to the left of the current traces indicate the closed-state current levels (C). The time and current scales apply to all traces. The holding potential was −99 mV. Prior to excision, channels in this patch were found to have a unitary conductance of 18 pS, steep inward rectification and an extrapolated reversal potential near E_K. ATP at the cytoplasmic surface of an inside-out patch reactivates K_{IR} channel activity.

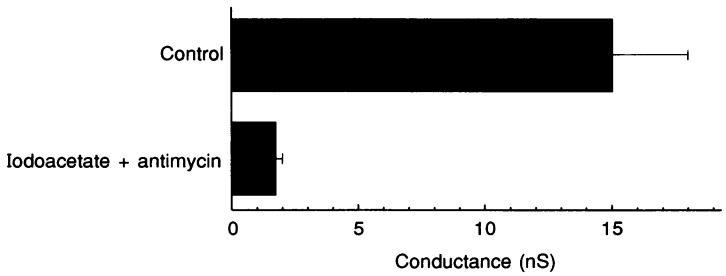

Figure 6. Effect of inhibitors of ATP synthesis on the inward currents of fresh monkey Müller cells

This bar graph compares the mean conductances, determined between −68 and −100 mV, for a control group ($n = 4$) and an experimental group ($n = 5$) in which cells were exposed to 1 mM iodoacetate and 10 μM antimycin A for 61–105 min prior to being assayed by whole-cell recording. S.E.M. values are shown. Exposure to iodoacetate and antimycin A markedly reduces the inward current of Müller cells.

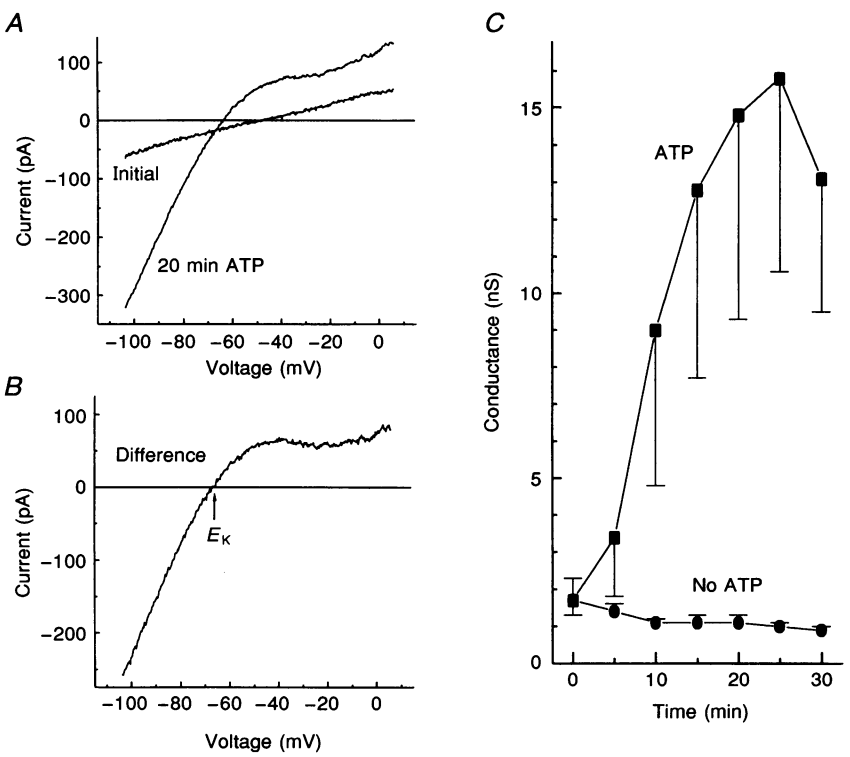

Figure 7. Effect of internal perfusion of Mg-ATP on currents in fresh monkey Müller cells exposed to inhibitors of ATP synthesis

A, current–voltage relations initially and 20 min after going whole-cell in a Müller cell that was exposed to 1 mM iodoacetate and 10 μM antimycin A for 56 min. The recording pipette solution contained 4 mM Mg-ATP; the metabolic inhibitors remained in the bathing solution during the electrophysiological recordings. *B*, plot of the difference between the *I–V* curves shown in *A*. E_K indicates the equilibrium potential for K^+. *C*, plot of the mean inward conductance at various times after going whole-cell. The Müller cells were exposed to the inhibitors of ATP synthesis for 53–105 min prior to recording. Recordings were made with pipettes containing solutions with ($n = 4$) or without ($n = 5$) 4 mM Mg-ATP. Iodoacetate (1 mM) and antimycin A (10 μM) remained in the bathing solution during the whole-cell recordings. The bars show S.E.M. values. Internal perfusion of ATP restores the $I_{K(IR)}$ in Müller cells exposed to inhibitors of ATP synthesis.

Mg^{2+} dependence

In other experiments using Müller cells exposed to the inhibitors of ATP synthesis, we found that Mg^{2+} was required for the reactivation of the $I_{K(IR)}$ by ATP. When the solution in the whole-cell recording pipette lacked Mg^{2+} and contained 4 mM Na-ATP, rather than Mg-ATP, no increase in the $I_{K(IR)}$ was detected during 15 min of internal perfusion. The mean conductances at 1, 5, 10 and 15 min after going whole-cell were 0·9 ± 0·3, 0·7 ± 0·3, 0·8 ± 0·3 and 0·8 ± 0·3 nS, respectively ($n = 6$), in Müller cells pre-exposed to iodoacetate (1 mM) and antimycin A (10 μM) for 62–112 min prior to electrophysiological recordings. This requirement for Mg^{2+} lends further support to the idea that the ATP-induced activation of Müller cell K_{IR} channels involves hydrolysis of this nucleotide.

Effect of staurosporine and ATPγS

The protocol of monitoring the recovery of the inward current in Müller cells exposed to inhibitors of ATP synthesis was used to assess whether protein kinases may be involved in the ATP-mediated activation of K_{IR} channels. We found that 100 μM staurosporine, which at this concentration is a non-specific inhibitor of protein kinases, did not block the increase in inward current induced by the internal perfusion of ATP (4 mM) in the presence of Mg^{2+}. Specifically, soon after going whole-cell, the mean inward conductance of primate Müller cells exposed to iodoacetate (1 mM) and antimycin A (10 μM) for 38–62 min was 0·8 ± 0·3 nS ($n = 3$); within 10 min, the mean conductance increased to 11·7 ± 1·5 nS.

Also consistent with protein kinases not playing a significant role, ATPγS, a substrate for most protein kinases, failed to mimic the ATP-induced increase in inward current during whole-cell recordings from Müller cells exposed to iodoactetate (1 mM) and antimycin A (10 μM). In these experiments, the mean conductance was initially 1·0 ± 0·2 nS ($n = 5$) and, after 10 min of whole-cell recording with 4 mM ATPγS in the pipette solution, the mean conductance was only 0·8 ± 0·2 nS. This observation complements the findings in Fig. 2*C* showing that ATPγS failed to prevent the run-down of the $I_{K(IR)}$.

Locally synthesized ATP regulates K_{IR} channels

Although excision of membrane patches could be associated with a prompt run-down of the 20 pS K_{IR} channels (Fig. 5), we were surprised to find that most often this was not the case. In nine of twelve inside-out patches, the K_{IR} channel activity was maintained with minimal, if any, decrease in the open probability for more than 10 min. To account for the sustained activity of K_{IR} channels in a majority of the excised patches bathed in an ATP-free solution, we hypothesized that this nucleotide may be synthesized in the cytoplasm adherent to inside-out patches. This idea seems plausible since Ruknudin, Song & Sachs (1991) observed variable amounts of cytoplasm adherent to patches excised from various types of cells.

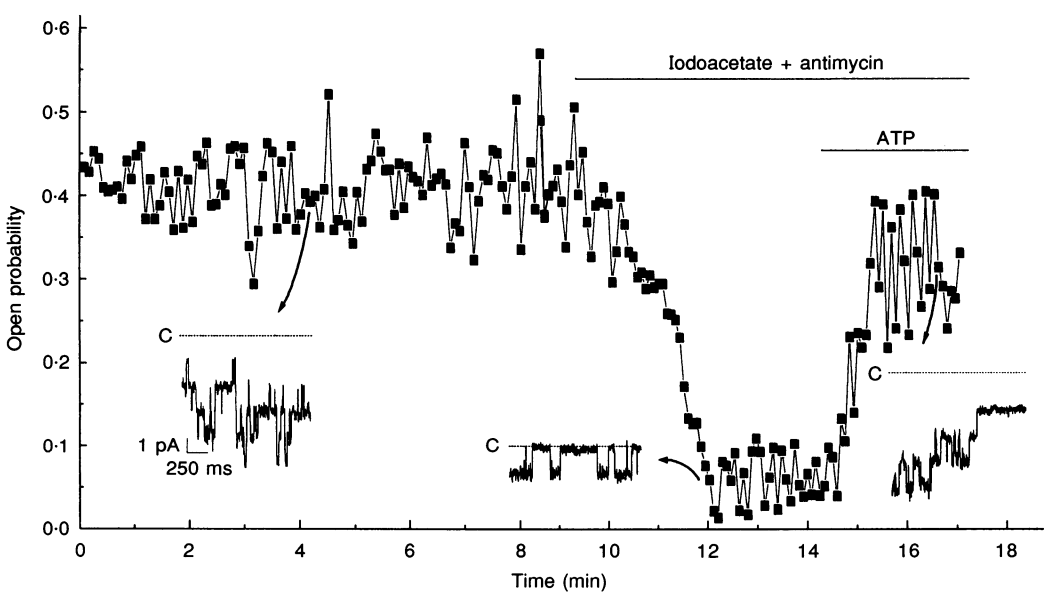

Figure 8. Open probability of channels in an inside-out patch from a fresh human Müller cell

At time 0, the patch was excised. The periods during which the bathing solution contained 1 mM iodoacetate + 10 μM antimycin A and 4 mM Mg-ATP are indicated above the data points. Insets, examples of channel activity at the points indicated by the arrows. In the insets, the dotted lines show the closed-state current levels (C). The time and current scales in the left-hand inset apply to all traces. The holding potential was −99 mV. Prior to excision, channels in the patch showed steep inward rectification, a unitary conductance of 19 pS and a reversal potential near E_K. Persistent K_{IR} channel activity in inside-out patches is reduced by inhibitors of ATP synthesis and is restored when ATP is at the cytoplasmic surface.

In Fig. 8 we tested the possibility that the sustained activity of 20 pS K_{IR} channels in inside-out patches is due to ATP generated in the cytoplasm attached to the patch. This figure shows an example of 20 pS K_{IR} channels that did not spontaneously run down after formation of an inside-out patch from a fresh human Müller cell. For approximately 10 min, the open probability remained stable at ~0·4 ($V_h = -99$ mV). However, when the cytoplasmic surface of the excised patch was exposed to iodoacetate (1 mM) and antimycin A (10 μM), the open probability decreased to less than 0·1 within 2 min. With the addition of 4 mM Mg-ATP to the bathing solution, the K_{IR} channel activity increased markedly. Similar findings were observed in three inside-out patches. These observations suggest that the 20 pS K_{IR} channels of Müller cells can be regulated by ATP synthesized at sites located adjacent to the channels.

DISCUSSION

The results show that intracellular ATP activates K_{IR} channels of Müller cells freshly dissociated from primate retinas. We found that the run-down of the Müller cell $I_{K(IR)}$ during whole-cell recording is prevented if the pipette solution contains ATP. In other experiments, the internal perfusion of ATP via a whole-cell recording pipette restored the $I_{K(IR)}$ of Müller cells exposed to inhibitors of ATP synthesis. In addition, closed 20 pS K_{IR} channels are reactivated by ATP at the cytoplasmic surface of inside-out patches excised from human Müller cells.

There are a number of mechanisms by which ATP may regulate K_{IR} channels of primate Müller cells. One possibility is the pure binding of ATP to a nucleotide-binding site. However, this mechanism seems unlikely since a high concentration (4 mM) of the non-hydrolysable ATP analogue AMP-PNP failed to maintain $I_{K(IR)}$. Rather, the failure of AMP-PNP to prevent run-down of $I_{K(IR)}$ points to a mechanism involving the hydrolysis of ATP. Further evidence for hydrolysis of the nucleotide is our observation that this ATP-induced activation is dependent on the presence of Mg^{2+}.

Since the effect of ATP on this channel is likely to require hydrolysis of the nucleotide, possible molecular mechanisms for K_{IR} activation include a kinase-mediated phosphorylation or an ATPase-like reaction (Yount, Ojala & Babcock, 1971). While a number of types of K^+ channels appear to be activated by phosphorylation involving kinases (Findlay & Dunne, 1986; Rehm *et al.* 1989; Chung, Reinhart, Martin, Brautigan & Levitan, 1991), other K^+ channels, including K_{IR}2.1 (Fakler *et al.* 1994), appear to be regulated by an ATPase mechanism. In our experiments, the inability of ATPγS to mimic the effect of ATP suggests an ATPase- rather than a kinase-mediated process. Consistent with this, our whole-cell recordings suggest that > 100 μM ATP is required to activate the Müller cell $I_{K(IR)}$; this concentration is markedly greater than the K_D of ~3 μM for nucleotide binding proteins such as protein kinase A (Flockhart, Freist, Hoppe, Lincoln & Corbin, 1984). In addition, a high concentration of the protein kinase inhibitor staurosporine failed to block the ATP effect. Overall, our observations suggest that an ATPase-like, rather than protein kinase-mediated, mechanism regulates the activity of the 20 pS K_{IR} channels in primate Müller cells.

The identities of the K_{IR} channels expressed in primate Müller cells remain uncertain. The steep inward rectification, ATP dependence and 20 pS conductance (in 100 mM external K^+) of the predominant K_{IR} channel of the primate retina are features shared with the cloned K_{IR}2.1 (Kubo, Baldwin, Jan & Jan, 1993) and $K_{AB\text{-}2}$ (Takumi *et al.* 1995) channels. *In situ* hybridization studies by Kurachi and associates (Takumi *et al.* 1995; Horio, Morishige, Takahashi & Kurachi, 1996) indicate that in the brain, K_{IR}2.1 is expressed chiefly in neurons and $K_{AB\text{-}2}$ mainly in glia. In a brief report, Horio *et al.* (1996) recently cited strong evidence that Müller cells of the rat and rabbit retina express the $K_{AB\text{-}2}$ channel. Thus, evidence is accumulating that the $K_{AB\text{-}2}$ (also named K_{IR}4.1) subtype of channel may be an important K_{IR} channel in Müller cells.

The 20 pS K_{IR} channel may not be the only K_{IR} channel expressed by primate Müller cells. We occasionally found channels with significantly larger unitary conductances (e.g. 28 pS). Although we did not systematically examine the 28 pS channels, they may be similar to the 27·8 pS channel (in 98 mM external K^+) which Newman (1993) found to be the predominant K_{IR} channels sampled in salamander Müller cells. In human and monkey Müller cells, we did not detect inwardly rectifying channels with conductances of 60, 105 or 360 pS as found in rabbit Müller cells by Nilius & Reichenbach (1988) or of 68 pS as Le Dain, Anderton, Martin & Millar (1994) observed in Müller cells of the turtle retina. Perhaps there are species differences which may account for some of the diversity of K_{IR} channels observed in Müller cells from various animals.

Our finding that intracellular ATP activates the predominant K_{IR} channels of human and monkey Müller cells raises the question of the role of this regulation in the function of these glia. Since our experiments indicate that 20 pS K_{IR} channels are activated by ATP synthesized at sites adjacent to the channel, we suspect that the topographical distribution of functional K_{IR} channels across the surface of a Müller cell is regulated by local metabolic conditions within the retina. At present, it is unclear whether the $[ATP]_i$ near the Müller cell K_{IR} channels fluctuates enough under physiological conditions to influence the channels. However, it does seem likely that ATP levels would fall sufficiently with profound ischaemia. Consistent with this, Winkler & Puro (1996) found in an *in vitro* model of ischaemia that the concentration of ATP drops by > 99% in cultured human Müller cells bathed for 4 h in a glucose-free solution containing antimycin A (10 μM), a blocker of oxidative phosphorylation. Perhaps less severe ischaemia also may influence the activity of the 20 pS K_{IR} channels if

ATP generated by local glycolysis, rather than by oxidative phosphorylation, preferentially regulates this Müller cell channel as has been suggested for ATP-sensitive K^+ channels in myocytes (Weiss & Lamp, 1987); future studies are needed to address this possibility. In addition, the possibility that the ratio of $[ATP]_i$ to $[ADP]_i$ plays a role in regulating the K_{IR} channels of Müller cells has yet to be evaluated.

An unexpected finding was that the activity of K_{IR} channels in inside-out membrane patches often did not run down although the $I_{K(IR)}$ monitored by whole-cell recordings always decreased when the pipette solution lacked ATP. These observations seem paradoxical since washout should be more complete for excised patches than for whole-cell recordings. The mechanism for the persistence of channel function is not known. One possibility is that, in addition to being activated by ATP_i, K_{IR} channels can be inhibited by other molecules, which remain to be identified. We speculate that in excised patches these postulated inhibitors are washed out extensively and the ATP synthesized in the cytoplasm adherent to the patch can be sufficient to maintain channel activity. Further experimental work will be necessary to examine the interactions of activators and inhibitors on the functioning of the K_{IR} channels expressed by Müller cells of the primate retina.

The regulation of Müller cell K_{IR} channels by intracellular ATP may have significant functional consequences for the retina since these channels appear to be important pathways for the redistribution of K^+ from regions of the retina high in $[K^+]_o$ to areas where $[K^+]_o$ is lower (Newman, 1985, 1993; Brew *et al.* 1986). A marked reduction in the activity of K_{IR} channels in Müller cells may limit the movement into these glia of excess K^+ released by neurons. Limiting the influx of excess K^+ may compromise retinal function as increased $[K^+]_o$ depolarizes nerve cells and blocks the glial uptake of glutamate (Sarantis & Attwell, 1990), which is neurotoxic in the retina (Lucas & Newhouse, 1957).

Alternatively, Puro & Stuenkel (1995) have theorized that a partial inhibition of K_{IR} channels may be beneficial. Since the vitreal endfoot of a Müller cell in the vascularized mammalian retina appears to be relatively isolated electrically from the soma (Reichelt *et al.* 1993), the predominant movement of K^+ is normally to the outer retina and not toward the vitreous (Frishman & Steinberg, 1989). However, when K_{IR} channel activity is reduced, there is an increase in the membrane resistance and, consequently, a lengthening of the distance for electrotonic conduction (λ) within the Müller cell. As a result, a modest decrease in K_{IR} channel activity and the resulting increase in an initially short λ value could theoretically enhance the flow of K^+ from the soma of a Müller cell to its endfoot (Puro & Stuenkel, 1995). Under pathophysiological conditions, an enhancement of the soma-to-endfoot pathway may be an adaptive response allowing excess K^+ to be siphoned away from the retina to the vitreous.

Our experiments show that intracellular ATP activates the 20 pS K_{IR} channels expressed by primate Müller cells. However, ATP is not the only regulatory molecule for these ion channels. Recent studies reveal that the function of K_{IR} channels in Müller cells is modulated by a variety of extracellular molecules. For example, the neurotransmitter glutamate inhibits the $I_{K(IR)}$ of salamander and human Müller cells via activation of specific glutamate receptors (Schwartz, 1993; Puro & Stuenkel, 1995). Also, the activation of D_2 dopamine receptors on Müller cells of the guinea-pig and amphibian retinas reduce the $I_{K(IR)}$ (Biedermann *et al.* 1995; Akopian & Witkovsky, 1995). In addition to neurotransmitters, extracellular thrombin can inhibit these channels (Puro & Stuenkel, 1995). Based on these observations, it seems evident that the activity of the K_{IR} channels in a Müller cell, and thus the function of the cell, is dynamic and reflects the status of the retinal microenvironment and the metabolic state of the Müller cell.

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