# Thrombin-induced inhibition of potassium currents in human retinal glial (Müller) cells

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1. Glial cells are known to play a role in regulating the microenvironment of the nervous system. While earlier considerations of glial function assumed a passive, static physiology for these cells, this is not likely to be the case. In this study, we begin to examine how the physiology of Müller glial cells changes in response to molecules in the microenvironment.

2. Perforated-patch recordings and intracellular calcium measurements were performed on human retinal Müller cells *in vitro*.

3. Analysis of whole-cell currents revealed that the human Müller glial cells have an inwardly rectifying $K^+$ current ($I_{K(IR)}$) which is active near the resting membrane potential. This $I_{K(IR)}$ is significantly inhibited when the Müller cell is exposed to thrombin, a molecule that is likely to enter the retina with a breakdown of the blood–retinal barrier and may be endogenous to the nervous system.

4. A variety of experiments point to a role for $Ca^{2+}$ as a second messenger mediating the inhibitory effect of thrombin on the $I_{K(IR)}$ of Müller cells. Specifically, thrombin evokes an increase in intracellular [$Ca^{2+}$] in the Müller cells; the $Ca^{2+}$ chelator BAPTA blocks the effects of thrombin on both the inhibition of $I_{K(IR)}$ and the rise in intracellular [$Ca^{2+}$]; exposure to ionomycin, a calcium ionophore, induces a reduction in the $I_{K(IR)}$ of Müller cells.

5. A thrombin-induced inhibition in the $I_{K(IR)}$ of Müller cells is likely to have significant functional consequences for the retina since these ion channels are involved in $K^+$ homeostasis.

6. Our experiments support the idea that the physiology of Müller glial cells is dynamic and can be markedly affected by molecules in the microenvironment.

Glial cells are active participants in the normal functioning of the nervous system and in the response of the central nervous system (CNS) to pathophysiological conditions. This concept replaces the notion that glia have a passive role. In fact, glial cells are more complex than previously thought. For example, they express a surprising number of receptors for neurotransmitters, growth factors and cytokines (Benveniste, 1992; Bevan, 1990). In addition, the patch-clamp technique has revealed that glial cells have a variety of voltage-sensitive and ligand-gated ion channels (Barres, Chun & Corey, 1990; Bevan, 1990; Ritchie, 1992). At present, the functional implications of this complexity are not fully understood. However, the presence of multiple types of receptors and ion channels suggest that glia are responsive to many molecular components of their microenvironment and are capable of altering their physiology in diverse ways.

To understand better the interactions between molecules in the extracellular space and glial physiology, we examined the effect of thrombin on the predominant $K^+$ current of retinal Müller glial cells. Although long known for its enzymatic role in the blood clotting cascade, thrombin also activates specific receptors (Vu, Hung, Wheaton & Coughlin, 1991) on the surface of a variety of cell types such as platelets, endothelial cells, neurons and glia, including Müller cells of the human retina (Puro, Mano, Chan, Fukuda & Shimada, 1990). Cells of the nervous system are likely to be exposed to plasma-derived thrombin when there is a breakdown of the barrier between the circulatory and nervous systems, a common occurrence in many pathophysiological conditions. In addition, the recent demonstration that cells in many parts of the CNS express mRNA for prothrombin (Dihanich, Kaser, Reinhard, Cunningham & Monard, 1991), the precursor of thrombin, suggests that this molecule may be endogenous to the nervous system. Despite the possibility that thrombin is likely to enter the CNS after a breakdown of the blood–brain barrier and may normally play a role in cellular interactions in the nervous system, little is known

about the effects of thrombin on the function of the neurons or glia.

In this study we searched for an effect of thrombin on $K^+$ currents of human retinal Müller glial cells. The function of $K^+$ channels in these glial cells is of interest since they are thought to be pathways for the redistribution of $K^+$ in the retina. Localized changes in $[K^+]_o$, such as those occurring with neural depolarization, result in $K^+$ entering Müller glial cells in regions of high $[K^+]_o$ and leaving these cells where $[K^+]_o$ is lower (Newman, Framback & Odette, 1984; Karwoski, Lu & Newman, 1989). This redistribution of $K^+$ serves to limit wide swings in the $[K^+]_o$ which could alter neuronal excitability.

Using the perforated-patch configuration of the patch-clamp technique to examine the whole-cell currents, we found that Müller cells have an inwardly rectifying $K^+$ current ($I_{K(IR)}$) that is functional near the resting membrane potential and is inhibited when the glial cell is exposed to thrombin. Calcium imaging studies, as well as experiments using a calcium ionophore and a calcium chelator, suggest that calcium may serve as a second messenger for the thrombin-induced inhibition of the Müller cell $I_{K(IR)}$.

## METHODS

Müller glial cells were isolated from human retinas using a modification of methods developed by Aotaki-Keen, Harvey, de Juan & Hjelmeland (1991). As detailed elsewhere (Puro, 1994*a*), retinas from postmortem adult eyes were removed, exposed to a calcium- and magnesium-free phosphate buffer supplemented with 0·1% trypsin (3× crystallized), 0·2% hyaluronidase and 4% chicken serum for 45 min at 37 °C and then dissociated mechanically in medium A, which contained 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, some of which contained 22 mm diameter glass coverslips. Cells were kept for 5 days to 8 weeks in a humidified environment of 96·5% air and 3·5% $CO_2$ at 37 °C and fed with medium A twice per week.

Electrophysiological recordings were made at room temperature. Unless noted otherwise, the bathing solution contained 138 mM NaCl, 5 mM KCl, 1·8 mM $CaCl_2$, 0·8 mM $MgCl_2$, 10 mM Na-Hepes, 20 mM glucose and 10 $\mu$M nimodipine at pH 7·4 with the osmolarity adjusted to 315 mosmol (l solution)$^{-1}$. When the concentration of KCl was altered, compensatory changes in the NaCl concentration were made. Cells were examined at ×400 magnification with an inverted microscope equipped with phase contrast optics. Müller cells could be identified by their characteristic shape, long (> 60 $\mu$m) and slender (< 10 $\mu$m). Immunocytochemical studies using a monoclonal antibody (Chan *et al.* 1984) specific in the retina for Müller cells confirmed that all cells of this morphology are Müller cells.

Whole-cell currents were monitored using the perforated-patch configuration of the patch-clamp technique (Rae, Cooper, Gates & Wesky, 1991). Patch pipettes of Corning no. 7052 glass were pulled in four stages using a Sutter model P80/PC (Sutter Instrument Co., Novato, CA, USA), coated with Sylgard no. 184 (Dow Corning, Midland, MI, USA) to within 100 $\mu$m of their tips and heat polished to tip diameters of 2–3 $\mu$m. A pipette tip was filled to approximately 400 $\mu$m by applying negative pressure to the back end of the pipette while briefly dipping the tip into the pipette solution, which consisted of 40 mM KCl, 60 mM $K_2SO_4$, 10 mM K-Hepes and 6 mM $MgCl_2$ at pH 7·4 with the osmolarity adjusted to 285 mosmol (l solution)$^{-1}$. The remainder of the pipette was back-filled with the pipette solution supplemented with freshly mixed amphotericin B (120 $\mu$g ml$^{-1}$). The resistances of the pipettes used were 2–5 MΩ. 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 generally formed over a period of 1–30 s and reached resistances of greater than 1 GΩ. Measurements of seal resistances were likely to be underestimated because perforation of the patches usually began within 30 s. As amphotericin perforated the patch, the series resistance of the studied cells decreased to < 15 MΩ within 20 min. Recordings were used when the ratio of cell resistance to series resistance was greater than 10. Series resistance compensation was not used. The space clamp of the cells studies appeared to be satisfactory, in part, since markedly inwardly rectifying whole-cell currents were detected; poor space clamp would have been expected to linearize the current–voltage relation (e.g. Newman, 1993). The delivery of voltage step protocols and the sampling of currents filtered at 1–5 kHz and digitized at 4 kHz were performed using a Lab Master DMA acquisition system (Axon Instruments) and pCLAMP software (version 5.5, Axon Instruments), which was also used for data analysis. Leak subtraction was not used. Cell capacitance was estimated by using circuitry of the Dagan 3910 expander module. Solutions could be miniperfused in the area of a Müller cell by applying < 3·5 kPa (0·5 lbf in$^{-2}$) of pressure from a pressure ejection system (Medical Systems, Greenvale, NY) to the back end of a pipette which had a tip size of approximately 3 $\mu$m and was positioned ~50 $\mu$m from the cell being recorded. Miniperfusion of the bathing solution alone or with the buffer (91 $\mu$M sodium citrate) used in the commercially available preparations of thrombin altered the amplitude of Müller cell currents by < 10%.

For measurements of intracellular calcium, cells plated on glass coverslips were incubated for 20–30 min at 37 °C with 1 $\mu$M fura-2 AM. After fura-2 loading the cells were given a further incubation period of approximately 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 (Stuenkel & Nordmann, 1993). Briefly, the coverglass containing the cells was placed into a holder that formed the base of a chamber (65 $\mu$l volume) before beginning rapid exchange of the superfusing physiological saline (1·5 ml min$^{-1}$). With the exception of the digital imaging experiments, monitoring of fura-2 fluorescence was as follows. Selection of a single cell was performed and masked from surrounding regions by a pinhole diaphragm. Alternating excitation wavelengths of 340 and 380 nm and monitoring of emitted light at 500 nm was performed by a photomultiplier-based SPEX Industries (Edison, NJ, USA) AR-CM system. The calculated $[Ca^{2+}]_i$ represents a spatially averaged value as the emitted signal at each wavelength was collected from the entire cell. Digital imaging of fura-2 fluorescence was performed using an attofluor (Zeiss, Thornwood, NY, USA) imaging system. The fluorescence ratio (340 : 380 nm) in both cases 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.

BAPTA AM and fura-2 AM were purchased from Molecular Probes; ionomycin was from LC Laboratories (Woburn, MA, USA); trypsin was from Worthington Biochemical (Freehold, NJ, USA); sera and growth media were from Life Technologies/Gibco (Grand Island, NY, USA); pertussis toxin was from Sigma and Research Biochemicals Inc. (Natick, MA, USA); human thrombin (~4500 units $mg^{-1}$) and the other chemicals were from Sigma. Donor eyes were supplied by the Michigan Eye Bank and Transplantation Center (Ann Arbor, MI, USA); tissue was used in accordance with all applicable laws and regulations.

## RESULTS

Perforated-patch recordings of human Müller cells *in vitro* revealed a number of voltage-dependent currents (Fig. 1). These cells typically have an inwardly rectifying current, as well as transient and sustained outward currents. This profile of ionic currents appears to be stable *in vitro*, being found in Müller cells maintained *in vitro* for up to 8 weeks.

The inwardly rectifying current of the Müller cell was of particular interest since it is the predominant current near the resting membrane potential (Fig. 1). To characterize better this current, the effect of varying $[K^+]_o$ on the current–voltage relationship was examined. Increasing $[K^+]_o$ shifted the reversal potential in a depolarizing direction (Fig. 2*A* and *B*). This change in the reversal potential was close to that predicted by the Nernst equation (Fig. 2*B*). Also, as predicted by the Goldman–Hodgkin–Katz current equation (Hille, 1992), decreasing $[K^+]_o$ reduced the slope of the current–voltage plot (i.e. conductance) of the potassium current (Fig. 2*A*). As found for inwardly rectifying $K^+$ currents in a variety of cell types (Hagiwara & Takahashi, 1974; Sakmann & Trube, 1984), the conductance of the inward current was approximately proportional to the square root of the $[K^+]_o$.

To characterize further the inwardly rectifying current, the effect of barium was examined. Inwardly rectifying potassium channels are known to be blocked by this divalent cation (Hille, 1992). The current–voltage relations in Fig. 3 demonstrate that 100 $\mu$M $Ba^{2+}$ markedly reduced

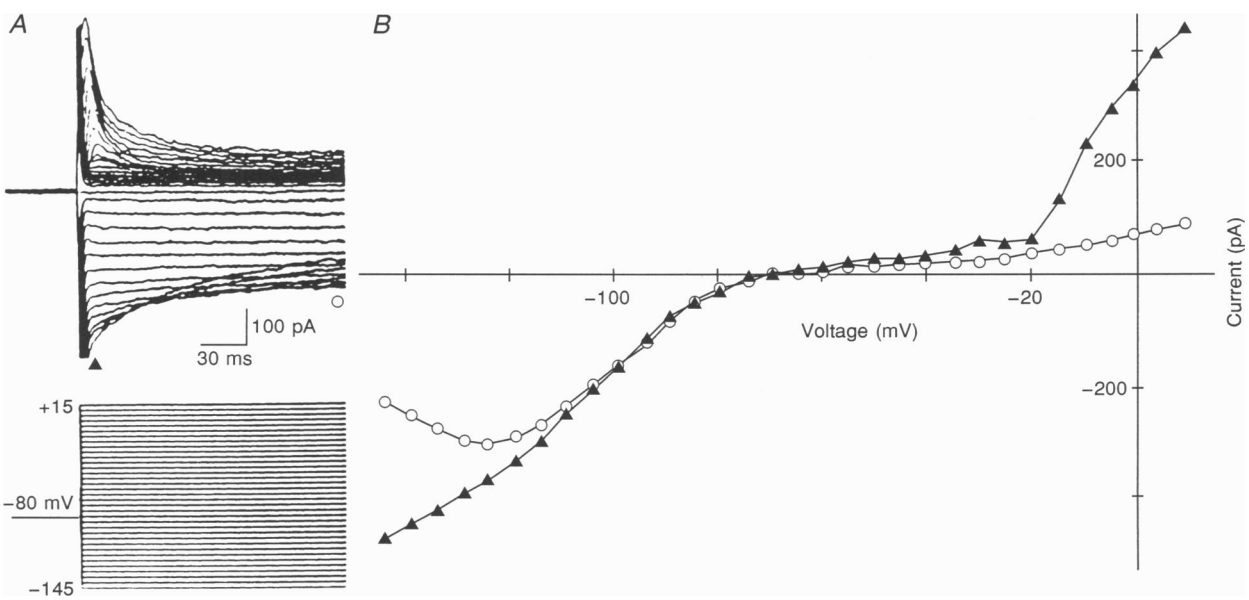


**Figure 1. Perforated-patch recordings from a human Müller cell *in vitro***

*A*, currents evoked with a clamp protocol, illustrated below the traces, consisting of voltage steps to a series of potentials between −145 and +15 mV from a holding potential of −80 mV. *B*, current–voltage relations from the voltage-clamp records in *A*. ▲, peak current amplitudes; ○, current amplitudes at the end of the command pulse. The plots show inactivation with extreme hyperpolarizing steps, inward rectification, and both transient and sustained outward currents.

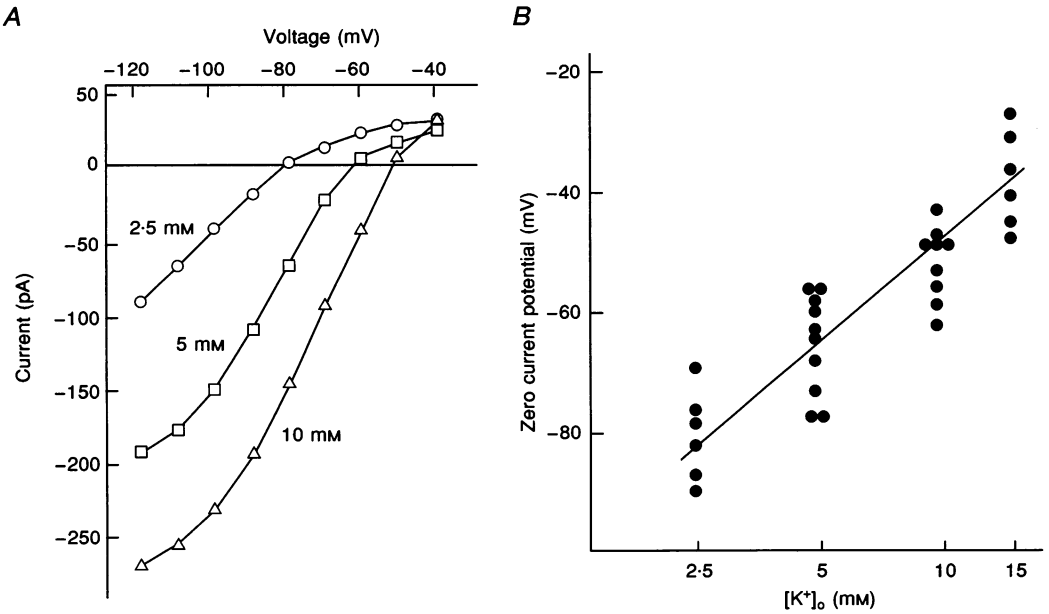


**Figure 2. Potassium dependence of the inwardly rectifying current**

*A*, the *I–V* relations as the $[K^+]_o$ was raised from 2·5 to 10 mM. Current amplitudes at the end of the 180 ms command pulse were used. *B*, a plot of the zero current potential (reversal potential) as a function of $[K^+]_o$ for successfully sampled Müller cells. The line has a slope of 57 mV per tenfold change in $[K^+]_o$, the Nernstian value. The reversal potential shifts towards 0 mV and the slope of the *I–V* plot (conductance) of the inward current increases as $[K^+]_o$ rises.

the inwardly rectifying current. The sensitivity to $Ba^{2+}$ and the effects of varying $[K^+]_o$ indicate that human Müller cells *in vitro* express an inwardly rectifying potassium current ($I_{K(IR)}$).

The effect of thrombin on the $I_{K(IR)}$ of human Müller cells is shown in Fig. 4. Exposure of a Müller cell to 100 ng $ml^{-1}$ thrombin was associated with a reversible reduction in the current elicited by voltage steps from −80 mV. The

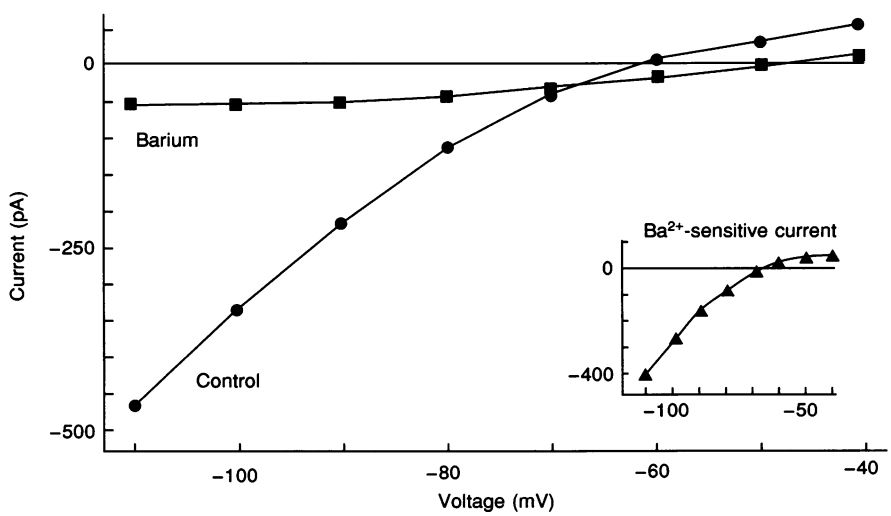


**Figure 3. Effect of barium on the inwardly rectifying current**

*I–V* plots before and during miniperfusion of the bathing solution supplemented with 100 μM barium. Inset, *I–V* relations of the barium-sensitive current obtained by subtracting current traces during exposure to barium from those in the control period. Current amplitudes at the end of the command pulses were used for the *I–V* plots. The bathing solution contained 10 mM KCl. Barium blocks the inwardly rectifying current.

current–voltage relationship of the thrombin-sensitive current (Fig. 4*B*) demonstrates that the inwardly rectifying current was inhibited. In a series of experiments, the inward current elicited with a voltage shift from −60 to −100 mV was reduced by a mean of 53% (S.D. = 18, $n = 18$) with exposure of Müller cells to a perfusate containing 100 ng ml$^{-1}$ thrombin. A similar magnitude of inhibition occurred with 5–1100 ng ml$^{-1}$ thrombin. A concentration of 0·5 ng ml$^{-1}$ thrombin did not significantly affect $I_{K(IR)}$.

To help understand the mechanism by which thrombin induces inhibition of the $I_{K(IR)}$, the time course for onset of the effect was examined. A relatively slow onset would be consistent with a second messenger system. Figure 5 shows the time course for the onset of the inhibitory effect of thrombin when it was miniperfused near a Müller cell. This time course is compared with the effect of the miniperfusion of barium, which is known to directly block the $I_{K(IR)}$ (Hille, 1992). For a series of cells exposed to thrombin, it took 6·7 s (S.D. = 1·7, $n = 8$) to reach the half-maximal inhibition. The comparable time for the barium block was 1·5 s (S.D. = 0·4, $n = 4$). The time course for the inhibitory effect of thrombin was significantly ($P < 0·001$, Student's $t$ test) slower than that of barium.

The relatively slow onset of the inhibitory effect of thrombin on the Müller cell $I_{K(IR)}$ is consistent with a mechanism of action involving metabolic changes in the glial cell. Since thrombin is known to elicit elevations in intracellular calcium in a number of cell types (Adams, Barakeh, Laskey & Van Breemen, 1989; Siess, 1989; Crook, Lui & Polansky, 1992), we used the calcium indicator fura-2 to measure [$Ca^{2+}$] in human Müller cells. Exposure of Müller cells to thrombin, in concentrations ranging from 5 to 1100 ng ml$^{-1}$, was consistently (22/23 sampled cells) associated with elevations in $[Ca^{2+}]_i$. For thrombin concentrations $\geqslant 5$ ng ml$^{-1}$, the evoked increases in $[Ca^{2+}]_i$ were of a similar magnitude; $[Ca^{2+}]_i$ increased from a mean basal level of 77 nM (S.D. = 23, $n = 18$) to a mean peak of 483 nM (S.D. = 307, $n = 18$).

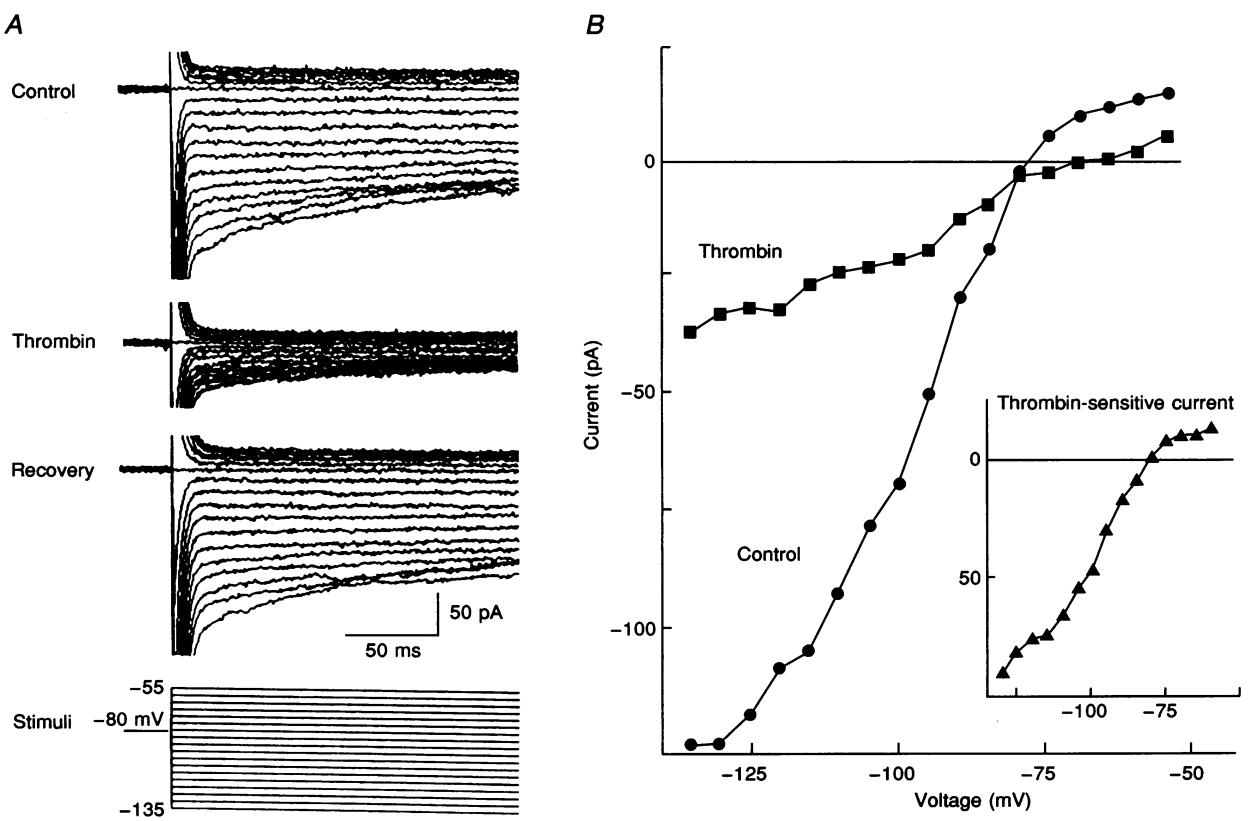


**Figure 4. The effect of thrombin on the $I_{K(IR)}$ of human Müller cells**

*A*, currents evoked before, during and after miniperfusion of the bathing solution supplemented with 100 ng ml$^{-1}$ human thrombin. The clamp protocol is shown below. *B*, *I–V* relations before and during exposure to thrombin. The inset shows the *I–V* relations for the thrombin-sensitive current, which was calculated by subtracting currents in the presence of thrombin from those under control conditions. Current amplitudes at the end of the command pulse were used for the *I–V* plots. With exposure to thrombin, the Müller cell $I_{K(IR)}$ is reduced.

To determine whether the increase in $[Ca^{2+}]_i$ evoked by thrombin was dependent on an influx of calcium from outside the cell, some experiments were performed using a $Ca^{2+}$-free bathing solution. Figure 6 shows that a substantial increase in $[Ca^{2+}]_i$ could be evoked in the absence of extracellular $Ca^{2+}$. Thus thrombin induces a release of intracellularly sequestered $Ca^{2+}$ in human Müller cells. Often an increase in $[Ca^{2+}]_i$ was first evident in a localized area of the Müller cell, as was the case for the cell illustrated in Fig. 6. Further studies are needed to examine systematically the topography of thrombin-evoked increases in $[Ca^{2+}]_i$.

The observation that thrombin induced an increase in $[Ca^{2+}]_i$ raised the possibility that $Ca^{2+}$ may be an intracellular messenger mediating the inhibitory effect of thrombin on the Müller cell $I_{K(IR)}$. This possibility was examined by using BAPTA AM, a cell-permeable calcium chelator. Experiments with fura-2 established that pre-exposure to 50 $\mu$M BAPTA AM for 20 min prevented a thrombin-induced rise in $[Ca^{2+}]_i$ (Fig. 7*A*, lower record). In addition, electrophysiological recordings showed that treatment with BAPTA AM profoundly reduced ($P < 0{\cdot}001$, Student's $t$ test) the inhibition of $I_{K(IR)}$ induced by thrombin (Fig. 7*B*). These findings are consistent with a role for calcium in mediating the thrombin-induced inhibition of $I_{K(IR)}$ in human Müller cells.

The calcium ionophore ionomycin was also used to help test the hypothesis that an increase in $[Ca^{2+}]_i$ can lead to the inhibition of the Müller cell $I_{K(IR)}$. An example is shown in Fig. 8; a reduction in the inward current of a Müller cell was detected within 1–2 min of exposure to 3 $\mu$M ionomycin. Much of the interval between initial exposure and onset of effect is likely to be due to the time for incorporation of the ionophore into the cell membrane. The experiments using BAPTA AM and ionomycin strongly suggest that the thrombin-induced inhibition of $I_{K(IR)}$ in Müller cells is mediated, at least in part, by an elevation of $[Ca^{2+}]_i$.

To help examine the mechanism(s) by which thrombin increases $[Ca^{2+}]_i$ and inhibits $I_{K(IR)}$, we tested the effect of pertussis toxin (PTX). PTX, which inhibits some G proteins, is reported to block the thrombin-evoked

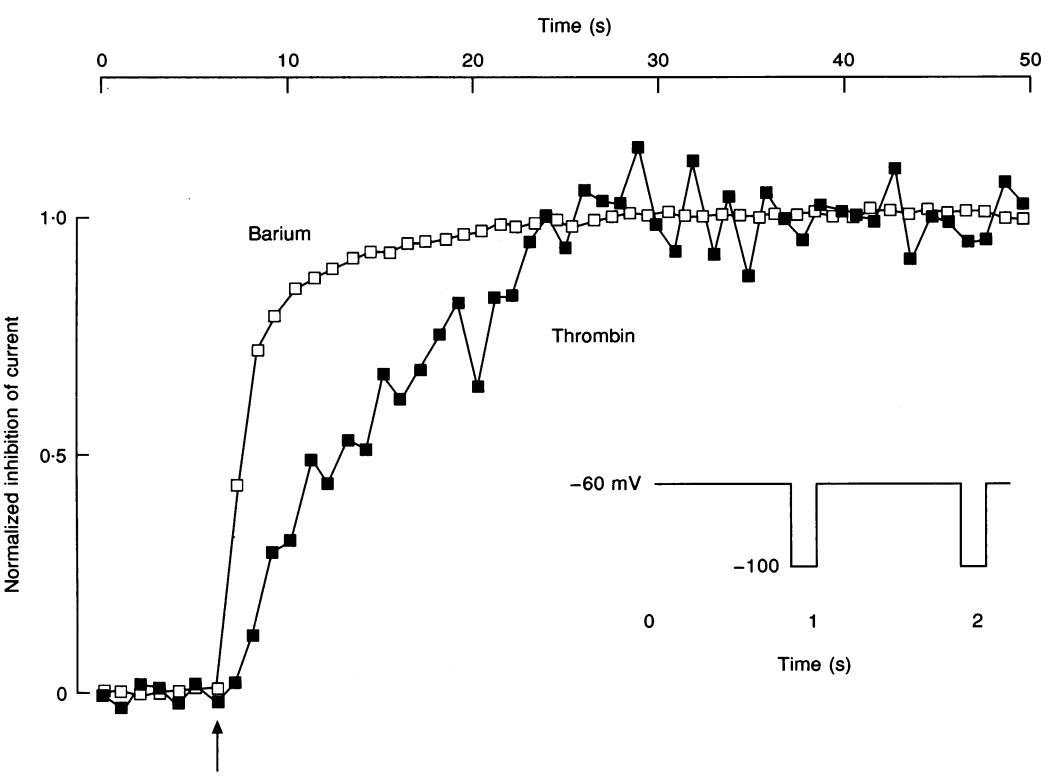


**Figure 5. Time courses for the inhibition of $I_{K(IR)}$ by barium and thrombin**

The stimulus protocol is shown in the inset. At 1 s intervals, the potential was stepped from −60 to −100 mV for 180 ms. Current amplitudes at the end of each step were normalized to the mean amount of inihibition at 30–45 s after the onset of exposure to barium or thrombin. Normalized currents are plotted as a function of time. The arrow shows the onset of miniperfusion of the bathing solution supplemented with 100 $\mu$M barium or 100 ng ml$^{-1}$ thrombin. The bathing solution contained 10 mM KCl. The Müller cell $I_{K(IR)}$ is inihibited more slowly by thrombin than by barium.

increases in $[Ca^{2+}]_i$ in some cell types (Brass, 1992), but not in others (Gupta, Diez, Heasley, Osawa & Johnson, 1990; Hung, Wong, Vu & Coughlin, 1992). An 18 h exposure to 400 ng ml$^{-1}$ PTX did not prevent a thrombin-mediated increase in $[Ca^{2+}]_i$ in 3/3 Müller cells tested; an example is shown in Fig. 7*A* (upper record). PTX exposure also did not significantly ($P = 0{\cdot}206$, Student's $t$ test) block the inhibitory effect of thrombin on $I_{K(IR)}$ (Fig. 7*B*). These findings indicate that both the thrombin-evoked increase in $[Ca^{2+}]_i$ and the thrombin-mediated inhibition of $I_{K(IR)}$ in Müller cells are mediated via PTX-insensitive pathways.

To explore further the relationship of $[Ca^{2+}]_i$ and the thrombin-induced inhibition of $I_{K(IR)}$, we compared the duration of the thrombin-induced increase in $[Ca^{2+}]_i$ with the duration of the thrombin-induced inhibition of $I_{K(IR)}$. As illustrated in Fig. 9, a 5 min exposure to 100 ng ml$^{-1}$ thrombin elicits an elevation in $[Ca^{2+}]_i$ that peaks in approximately 1 min and returns to near basal levels within 4 min; a transient increase in $[Ca^{2+}]_i$ was observed in 4/4 cells tested. Since the thrombin-evoked increase in $[Ca^{2+}]_i$ is transient, we asked whether the inhibitory effect on $I_{K(IR)}$ was also transient. However, this was not the case

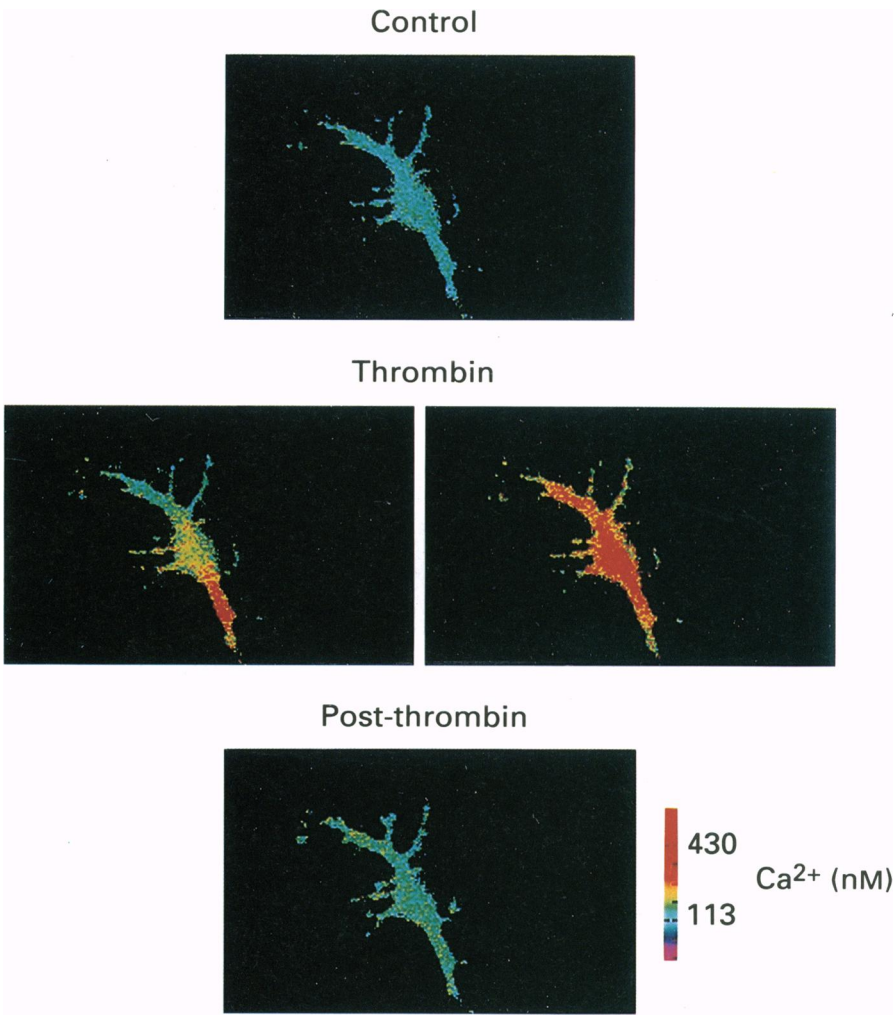


**Figure 6. Fura-2 imaging of $[Ca^{2+}]_i$ in a Müller cell before, during and after exposure to thrombin**

At each time, the perfusate contained 2 mM EGTA and no added calcium. The top image shows the $[Ca^{2+}]_i$ approximately 30 s prior to exposure to thrombin. The image on the left side of the middle row occurred within a few seconds after the onset of an increase in $[Ca^{2+}]_i$ associated with exposure to 100 ng ml$^{-1}$ thrombin. The image on the right is from 15 s later. The bottom image occurred 2 min after a return to the thrombin-free perfusate. A thrombin-induced increase in $[Ca^{2+}]_i$ in Müller cells does not require extracellular $Ca^{2+}$.

(Fig. 10). A significant inhibitory effect of thrombin on the $I_{K(IR)}$ persisted for more than 10 min in 6/6 Müller cells tested. Thus it appears that the continued inhibition of $I_{K(IR)}$ is not dependent upon a sustained high level of $[Ca^{2+}]_i$.

## DISCUSSION

Our findings show that the physiology of human Müller glial cells can be regulated by thrombin via a second messenger system. Several observations suggest that a rise in $[Ca^{2+}]_i$ plays a role in mediating the inhibitory effect of thrombin on the $I_{K(IR)}$ of human Müller cells. One finding is that thrombin evokes a release of intracellular calcium in these glial cells. Another observation is that the calcium chelator BAPTA almost completely prevents the thrombin-induced inhibition of the $I_{K(IR)}$. In addition, exposure of the Müller cells to a calcium ionophore, ionomycin, also results in a reduction in the $I_{K(IR)}$. Although this evidence strongly supports a role for increased $[Ca^{2+}]_i$ in initiating $I_{K(IR)}$ inhibition, the mechanism for sustaining the inhibition is less clear. We found that while the thrombin-induced elevation in $[Ca^{2+}]_i$ is transient (less than 4 min), the inhibition of $I_{K(IR)}$ during a continuous exposure to thrombin is long lasting (greater than 10 min). Thus it appears that a sustained high $[Ca^{2+}]_i$ is not the intracellular signal for the continued presence of thrombin. However, the possibility that the persistent inhibition of $I_{K(IR)}$ is mediated by a small (~5 nM) elevation of $[Ca^{2+}]_i$, which may occur after a large transient increase (Fig. 9), is not excluded. Alternatively, rapid calcium cycling across the plasma membrane in response to thrombin may maintain a localized elevation of calcium near inwardly rectifying $K^+$ ($K_{IR}$) channels. Such a localized increase may not be detected when the calcium-imaging technique measures spatially averaged responses. Future studies are needed to definitively identify the intracellular messengers mediating the long-term inhibition by thrombin of the Müller cell $I_{K(IR)}$.

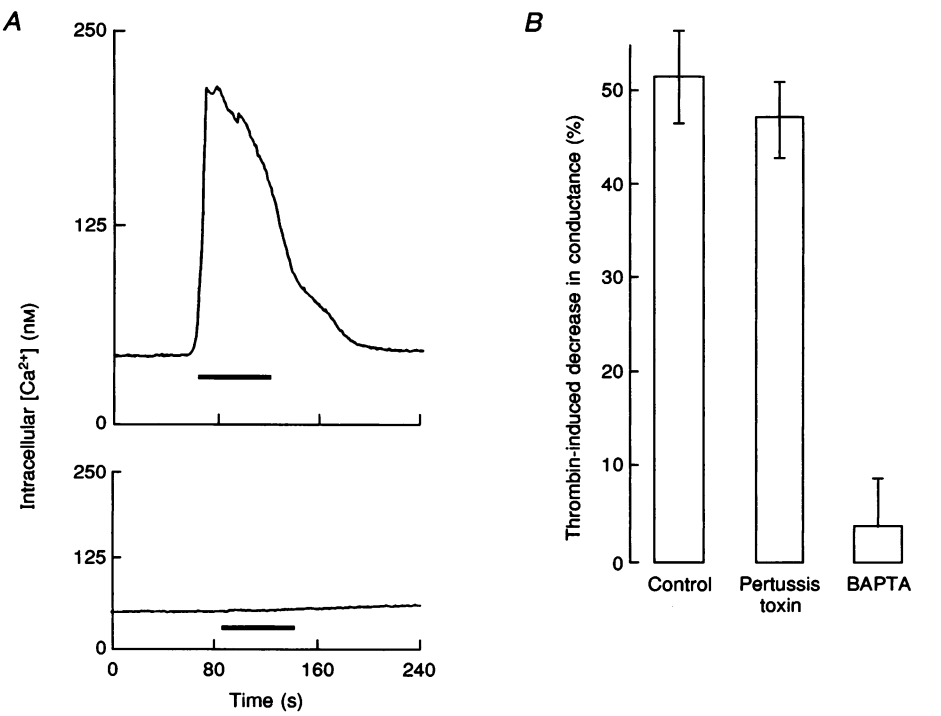


**Figure 7. Effects of pertussis toxin and BAPTA AM on the responses of Müller cells to thrombin**

*A*, upper record: thrombin-induced increase in $[Ca^{2+}]_i$ in a Müller cell exposed to 400 ng $ml^{-1}$ pertussis toxin for 18 h. The bar shows the period of perfusion of the bathing solution supplemented with 100 ng $ml^{-1}$ thrombin. Lower record: lack of an effect of thrombin on $[Ca^{2+}]_i$ in a Müller cell pre-treated for 20 min with 50 $\mu$M BAPTA AM. The bar shows the period of perfusion of the thrombin-containing solution. *B*, effects of pertussis toxin and BAPTA on the thrombin-induced inhibition of $I_{K(IR)}$. Müller cells were exposed to 400 ng $ml^{-1}$ pertussis toxin for 18 h, 50 $\mu$M BAPTA AM for 20 min or control conditions prior to perforated-patch recordings. Currents at the end of a voltage step from −50 to −100 mV were compared before and during a 2–3 min miniperfusion of the bathing solution supplemented with 100 ng $ml^{-1}$ thrombin. Nineteen, ten and seven Müller cells were sampled in control, pertussis toxin and BAPTA groups, respectively. The bathing solution contained 10 mM KCl. Both the inhibition of $I_{K(IR)}$ and the increase in $[Ca^{2+}]_i$ evoked by thrombin are insensitive to pertussis toxin, but are blocked by BAPTA.

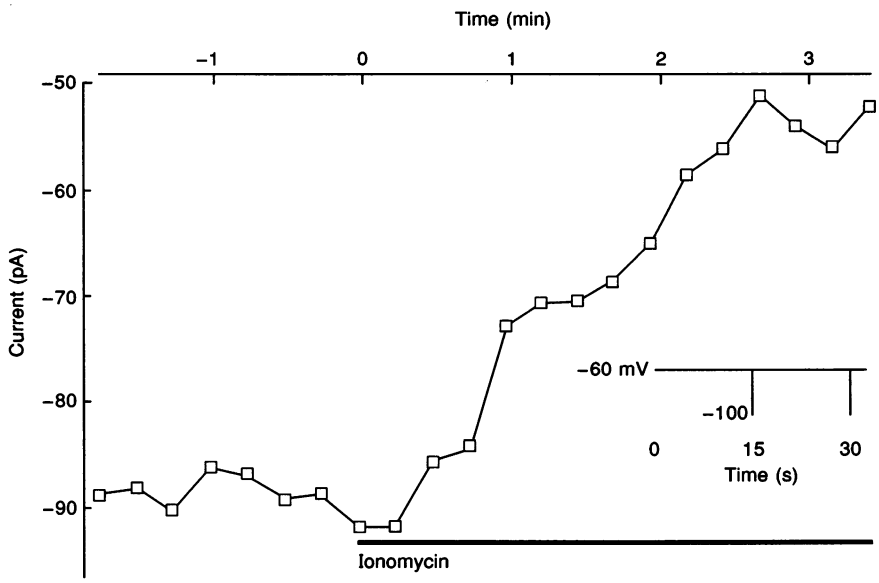


**Figure 8. Time course for the inhibition of $I_{K(IR)}$ by ionomycin**
As illustrated in the inset, the potential was stepped from −60 to −100 mV for 180 ms at 15 s intervals. Current amplitudes at the end of the command pulses were measured and plotted against time. During the time indicated by the bar, the Müller cell was miniperfused with the bathing solution supplemented with 3 $\mu$M ionomycin. The bathing solution contained 10 mM KCl. Exposure to ionomycin is associated with a reduction in the Müller cell $I_{K(IR)}$.

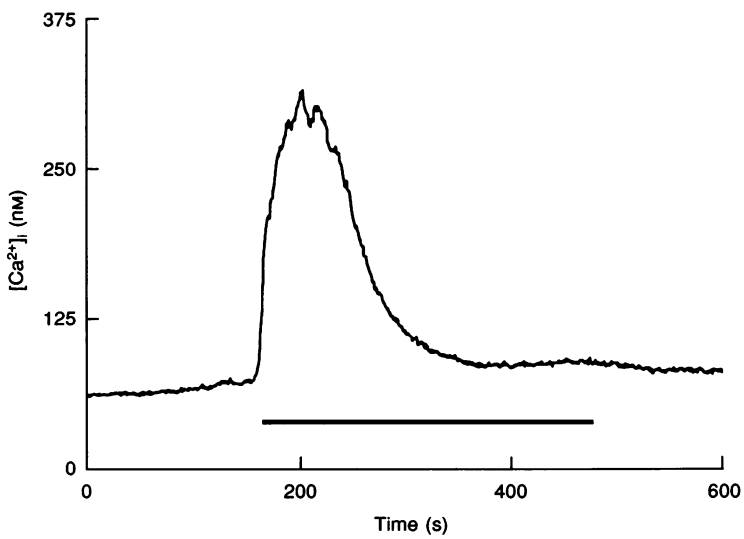


**Figure 9. Effect of a 5 min exposure to thrombin on the intracellular $Ca^{2+}$ concentration of a Müller cell**
The bar shows the period of perfusion with the bathing solution supplemented with 100 ng ml$^{-1}$ thrombin. A transient increase in $[Ca^{2+}]_i$ is evoked by thrombin.

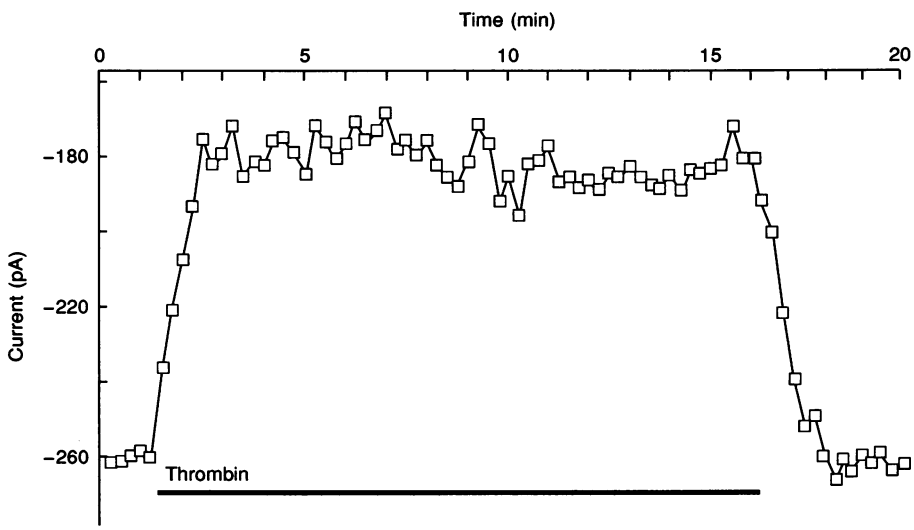


**Figure 10. Long-term effect of thrombin on the $I_{K(IR)}$ of a Müller cell**
Current amplitudes at the end of command pulses, which stepped from −50 to −100 mV for 180 ms at 15 s intervals, are plotted against time. The bar shows the period of miniperfusion of the bathing solution supplemented with 100 ng ml$^{-1}$ thrombin. The bathing solution contained 10 mM KCl. Thrombin induces prolonged inhibition of the $I_{K(IR)}$.

The $I_{K(IR)}$ of the human Müller cells is similar to that described for Müller cells of the salamander and rabbit retinas (Newman, 1993; Chao, Henke, Reichelt, Eberhardt, Reinhardt-Maelicke & Reichenbach, 1994), which have also been analysed in some detail. For example, the inwardly rectifying current is the predominant current at the resting membrane potential for Müller cells of these three species. Also, for each of these species this current is blocked by extracellular barium, shows inactivation with large hyperpolarizing voltage steps and increases its conductance approximately in proportion to the square root of $[K^+]_o$. Although the inwardly rectifying $K^+$ currents of the Müller cells from various species are qualitatively similar, there are likely to be differences in the number and topographical distribution (Newman, 1987) of $K_{IR}$ channels. Also, it remains to be determined whether thrombin regulates the $K_{IR}$ channels in Müller cells from non-human species.

Our experiments indicate that thrombin can regulate the physiology of human Müller cells. The question arises as to when Müller cells *in vivo* are exposed to thrombin. One likely circumstance for this is with a breakdown of the blood–retinal barrier, a common occurrence in many retinal disorders (Jampol, 1994). Since human serum contains approximately 2 $\mu$M prothrombin, cleavage of this precursor molecule by tissue thromplastin could yield a concentration of thrombin that is about 1000-fold greater than used in this study. Thus a leakage of serum associated with the breakdown of the blood–retinal barrier is likely to produce effective concentrations of thrombin in the retina. Another possible source of thrombin may be the retinal cells themselves. Dihanich *et al.* (1991) have raised the possibility that cells of the CNS might be a physiological source of thrombin, which they suggest may play a role in the normal functioning of the nervous system. Their molecular studies indicate the synthesis of prothrombin in many regions of the CNS although, unfortunately, the retina has yet to be examined.

A thrombin-induced inhibition of the $I_{K(IR)}$ in Müller cells would be expected to have significant functional consequences for the retina, since these ion channels appear to be important pathways for the redistribution of $K^+$ from regions of the retina of high $[K^+]_o$ to areas where $[K^+]_o$ is lower (Newman, 1985, 1993; Brew, Gray, Mobbs & Attwell, 1986). If thrombin is endogenous to the retina, perhaps it may play a physiological role along with various neurotransmitters (Schwartz, 1993; Puro, 1994*b*) in optimizing glial function under different lighting conditions. In pathophysiological conditions involving a breakdown of the blood–retinal barrier, a reduction in $K_{IR}$ activity in Müller cells exposed to serum-derived thrombin may limit the movement into Müller cells of excess $K^+$ released by damaged retinal neurons. Limiting the influx of excess $K^+$ may compromise retinal function since increased $[K^+]_o$ depolarizes neurons and blocks the uptake of glutamate by Müller cells (Barbour, Brew & Attwell, 1988; Sarantis & Attwell, 1990). Reduced glutamate uptake may be a significant problem in the inner retina where neurons are particularly vulnerable to glutamate toxicity (Lucas & Newhouse, 1957; Olney, 1988). From this perspective, the inhibition of $K_{IR}$ channels of Müller cells may have detrimental effects.

On the other hand, an inhibition of Müller cell $K_{IR}$ channels may be a beneficial adaptive response. For example, a reduction in the $I_{K(IR)}$ would increase the membrane resistance and, consequently, lengthen the distance for electrotonic conduction within the Müller cell. Under certain conditions, this may enhance the flow of current through a Müller cell to its proximal (vitreal side) endfoot.

As a first approximation, current flow at the endfoot may be estimated using the formula $i = gE_o e^{-x/\lambda}$, based on passive cable properties, where $i$ is the current flowing from the end of a cable-like process, $g$ is the membrane conductance, $E_o$ is the electrotonic driving force of $K^+$ (i.e. the difference between the reversal potential of $K^+$ and the membrane potential) at the origin of the process, $x$ is the length of the process and $\lambda$ is the electrotonic length constant which is proportional to $g^{-\frac{1}{2}}$. In this formula, the relationship between $g$ and $i$ is biphasic. As $g$ decreases from $\infty$ to 0, $i$ initially increases and then decreases. The phase in which $i$ increases as $g$ is decreasing occurs when $\lambda$ is short, i.e. $< x/2$. Hence decreasing the membrane conductance by inhibiting ion channels could enhance current flow from the endfoot of a Müller cell if $\lambda$ is short. At present, this remains a theoretical consideration because the length constant for mammalian Müller cells *in vivo* is unknown. However, the length constant may normally be short since recent experiments by Reichelt *et al.* (1993) indicate that the Müller cell endfoot in the mouse retina is relatively isolated electrically from the rest of the cell. This contrasts with the situation for amphibian Müller cells (Newman, 1985), which have thick processes, and is consistent with morphological studies of Müller cells in the vascularized mammalian retina showing extremely slender processes, connecting the cell soma and endfoot (Reichelt *et al.* 1993).

Thus in the vascularized mammalian retina (e.g. that of mouse, cat and human), a thrombin-induced inhibition of the Müller cell $I_{K(IR)}$ may significantly alter the pathway for the redistribution of $K^+$. Normally, the redistribution of $K^+$ in the vascularized retina is chiefly between the inner and outer parts of the retina (Frishman & Steinberg, 1989); the $K^+$ remains in the retina. However, if an inhibition of $K_{IR}$ channels and the resulting increase in the electronic length constant enhance the flow of $K^+$ to the Müller cell endfoot, then some excess $K^+$ may be siphoned to the vitreous humour and away from the retina, a strategy that lower vertebrates use normally (Newman *et al.* 1984; Karwoski *et al.* 1989).

The effect of thrombin on human Müller cells is not limited to a release of intracellularly stored $Ca^{2+}$ and an inhibition of $I_{K(IR)}$. This molecule is also a potent mitogen for these glial cells (Puro *et al.* 1990). The existence of multiple

effects of thrombin raises the question of whether the same signal transduction pathway mediates the various responses of Müller cells to thrombin. This seems unlikely since the proliferative effect of thrombin is blocked by pertussis toxin (Puro *et al.* 1990), but the inhibition of $I_{K(IR)}$ and the increase in $[Ca^{2+}]_i$ are not (Fig. 7). Thus it appears that thrombin activates at least two pathways. In agreement with this possibility, Hung *et al.* (1992) found that the thrombin receptor cloned from platelets is coupled to multiple G proteins with the phosphoinositide hydrolysis response being insensitive to pertussis toxin, and the adenylate cyclase response blocked by pertussis toxin. Further studies into the signal transduction mechanisms for the thrombin receptor of human Müller glial cells will be necessary.

Thrombin does not appear to be the only extracellular molecule that regulates the $I_{K(IR)}$ of Müller cells. Recently, Schwartz (1993) reported that glutamate inhibits the $I_{K(IR)}$ of salamander Müller cells. In contrast to our findings with thrombin, the glutamate-mediated inhibition does not appear to involve changes in $[Ca^{2+}]_i$. Rather, a pertussis toxin-sensitive cascade of second messengers involving cyclic AMP induces a very slowly developing (half-time of minutes) inhibition of the $I_{K(IR)}$. Also, unknown second messengers mediate a more rapid glutamate-induced reduction in the $I_{K(IR)}$ of the amphibian Müller cell (Schwartz, 1993). In addition, we have recently found that certain retinal neurotransmitters inhibit the $I_{K(IR)}$ of human Müller cells (Puro, 1994*b*). Thus evidence is accumulating that multiple extracellular signals and intracellular messengers may regulate the function of ion channels in Müller cells (Puro & Mano, 1991). Appreciation of the dynamic physiology of Müller glial cells is likely to be necessary to more fully understand the responses of the retina to both physiological and pathophysiological changes in the microenvironment.


Adams, D. J., Barakeh, J., Laskey, R. & Van Breemen, C. (1989). Ion channels and regulation of intracellular calcium in vascular endothelial cells. *FASEB Journal* **3**, 2389–2400.

Aotaki-Keen, A. E., Harvey, A. K., de Juan, E. & Hjelmeland, L. M. (1991). Primary culture of human retinal glia. *Investigative Ophthalmology and Visual Science* **32**, 1733–1738.

Barbour, B., Brew, H. & Attwell, D. (1988). Electrogenic glutamate uptake in glial cells is activated by intracellular potassium. *Nature* **335**, 433–435.

Barres, B. A., Chun, L. L. Y. & Corey, D. P. (1990). Ion channels in vertebrate glia. *Annual Review of Neuroscience* **13**, 441–474.

Benveniste, E. N. (1992). Inflammatory cytokines within the central nervous system: sources, function and mechanism of action. *American Journal of Physiology* **263**, C1–16.

Bevan, S. (1990). Ion channels and neurotransmitter receptors in glia. *Seminars in the Neurosciences* **2**, 467–481.

Brass, L. F. (1992). Homologous desensitization of HEL cell thrombin receptors. *Journal of Biological Chemistry* **267**, 6044–6050.

Brew, H., Gray, P. T. A., Mobbs, P. & Attwell, D. (1986). End feet of retinal glial cells have higher densities of ion channels that mediate $K^+$ buffering. *Nature* **324**, 466–468.

Chan, C.-C., Rosenszajn, L. A., Nussenblatt, R. B., Mullenberg-Coulombre, C., Hsu, S.-M., Palestine, A. G., Lando, Z. & Benezra, D. (1984). Monoclonal antibodies to Müller cells of the retina. *Investigative Ophthalmology and Visual Science* **25**, 1008–1012.

Chao, T. I., Henke, A., Reichelt, W., Eberhardt, W., Reinhardt-Maelicke, S. & Reichenbach, A. (1994). Three distinct types of voltage-dependent $K^+$ channels are expressed by Müller (glial) cells of the rabbit retina. *Pflügers Archiv* **426**, 51–60.

Crook, R. B., Lui, G. M. & Polansky, J. R. (1992). Thrombin stimulates inositol phosphate formation, intracellular calcium fluxes and DNA synthesis in cultured fetal human non-pigmented ciliary epithelial cells. *Experimental Eye Research* **55**, 785–795.

Dihanich, M., Kaser, M., Reinhard, E., Cunningham, D. & Monard, D. (1991). Prothrombin mRNA is expressed by cells of the nervous system. *Neuron* **6**, 575–581.

Frishman, L. J. & Steinberg, R. H. (1989). Light-evoked increases in $[K^+]_o$ in proximal portion of the dark-adapted cat retina. *Journal of Neurophysiology* **61**, 1233–1243.

Graniano, M. P. & Gilman, A. G. (1987). Guanine nucleotide-binding regulating proteins: mediators of transmembrane signalling. *Trends in Pharmacological Sciences* **8**, 478–481.

Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985). A new generation of calcium indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**, 3440–3454.

Gupta, S. K., Diez, E., Heasley, L. E., Osawa, S. & Johnson, G. L. (1990). A G-protein mutant that inhibits thrombin and purinergic receptor activation of phospholipase $A_2$. *Science* **249**, 662–666.

Hagiwara, J. & Takahaski, K. (1974). The anomalous rectification and cation selectivity of the membrane of a starfish egg cell. *Journal of Membrane Biology* **18**, 61–80.

Hille, B. (1992). *Ionic Channels of Excitable Membranes.* Sinnauer, Sunderland, MA, USA.

Hung, D. T., Wong, Y. M., Vu, T.-K. H. & Coughlin, S. R. (1992). The cloned platelet thrombin receptor couples to at least two distinct effectors to stimulate phosphoinositide hydrolysis and inhibit adenylyl cyclase. *Journal of Biological Chemistry* **267**, 20831–20834.

Jampol, L. M. (1994). Macular edema. In *Retina*, ed. Ryan, S. J., pp. 999–1008. C. V. Mosby Co., St Louis, MO, USA.

Karwoski, C. J., Lu, H.-K. & Newman, E. A., (1989). Spatial buffering of light-evoked potassium increases by retinal Müller (glial) cells. *Science* **244**, 578–580.

Lucas, D. R. & Newhouse, J. P. (1957). The toxic effect of sodium L-glutamate on the inner layers of the retina. *Archives of Ophthalmology* **58**, 193–201.

Newman, E. A. (1985). Membrane physiology of retinal glial (Müller) cells. *Journal of Neuroscience* **5**, 2225–2239.

Newman, E. A. (1987). Distribution of potassium conductance in mammalian Müller (glial) cells: a comparative study. *Journal of Neuroscience* **7**, 2423–2432.

Newman, E. A. (1993). Inward-rectifying potassium channels in retinal glial (Müller) cells. *Journal of Neuroscience* **13**, 3333–3345.

NEWMAN, E. A., FRAMBACK, D. A. & ODETTE, L. L. (1984). Control of extracellular potassium levels by retinal glial cells $K^+$ siphoning. *Science* **225**, 1174–1175.

OLNEY, J. W. (1988). Endogenous excitotoxins and neuropathological disorders. In *Excitatory Amino Acids in Health and Disease*, ed. LODGE, D., pp. 337–351. John Wiley & Son, New York.

PURO, D. G. (1994*a*). Calcium channels of human retinal glial cells. In *Methods in Neurosciences*, vol. 19, ed. NARAHASHI, T., pp. 68–81, Academic Press, Orlando, FL, USA.

PURO, D. G. (1994*b*). Neurotransmitters modulate the function of human Müller glial cells. *Investigative Ophthalmology and Visual Science* (suppl.) 35, 524.

PURO, D. G. & MANO, T. (1991). Modulation of calcium channels in human retinal glial cells by basic fibroblast growth factor: a possible role in retinal pathobiology. *Journal of Neuroscience* **11**, 1873–1880.

PURO, D. G., MANO, T., CHAN, C.-C., FUKUDA, M. & SHIMADA, H. (1990). Thrombin stimulates the proliferation of human retinal glial cells. *Graefe's Archive for Clinical and Experimental Ophthalmology* **228**, 169–173.

RAE, J., COOPER, K., GATES, P. & WESKY, M. (1991). Low access resistance perforated patch recordings using amphotericin B. *Journal of Neuroscience Methods* **37**, 15–26.

REICHELT, W., MÜLLER, T., PASTOR, A., PANNICKE, T., ORKAND, P. M., KETTENMANN, H. & SCHNITZER, J. (1993). Patch-clamp recordings from Müller (glial) cell endfeet in the intact isolated retina and acutely isolated Müller cells of mouse and guinea-pig. *Neuroscience* **57**, 599–613.

RITCHIE, J. M. (1992). Voltage-gated ion channels in Schwann cells and glia. *Trends in Neurosciences* **15**, 345–351.

SAKMANN, B. & TRUBE, G. (1984). Conductance properties of single inwardly rectifying potassium channels in ventricular cells from guinea-pig heart. *Journal of Physiology* **347**, 641–657.

SARANTIS, M. & ATTWELL, D. (1990). Glutamate uptake in mammalian retinal glia is voltage- and potassium-dependent. *Brain Research* **516**, 322–325.

SCHWARTZ, E. A. (1993). L-Glutamate conditionally modulates the $K^+$ current of Müller glial cells. *Neuron* **10**, 1141–1149.

SIESS, W. (1989). Molecular mechanisms of platelet activation. *Physiological Reviews* **69**, 58–178.

STUENKEL, E. L. & NORDMANN, J. J. (1993). Intracellular calcium and vasopressin release from rat isolated neurohypophysial nerve endings. *Journal of Physiology* **468**, 335–365.

VU, T.-K. H., HUNG, D. T., WHEATON, V. I. & COUGHLIN, S. R. (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64**, 1057–1068.

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