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Regulation of inwardly rectifying K^+ channels in retinal pigment epithelial cells by intracellular pH

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Inwardly rectifying K^+ (Kir) channels in the apical membrane of the retinal pigment epithelium (RPE) play a key role in the transport of K^+ into and out of the subretinal space (SRS), a small extracellular compartment surrounding photoreceptor outer segments. Recent molecular and functional evidence indicates that these channels comprise Kir7.1 channel subunits. The purpose of this study was to determine whether Kir channels in the RPE are modulated by extracellular (pH_o) or intracellular pH (pH_i), both of which change upon illumination of the dark-adapted retina. The Kir current (I_{Kir}) in acutely dissociated bovine RPE cells was recorded in the whole-cell configuration while altering pH_o or pH_i. In cells dialysed with pipette solution buffered to pH 7.2, step changes in pH_o from 7.4 to 8.0, 7.0 or 6.5 had little effect on I_{Kir}. Acidification to pH_o 6.0, however, caused a transient activation of I_{Kir} followed by a slower inhibition. To determine the dependence of I_{Kir} on pH_i, we altered pH_i within individual RPE cells at constant pH_o by imposing transmembrane acetate concentration gradients. These experiments revealed a biphasic relationship between I_{Kir} and pH_i: I_{Kir} was maximal at about pH_i 7.1, but decreased sharply at more acidic or alkaline levels. To evaluate the role of Kir7.1 channels in the pH_i-dependent changes in I_{Kir}, we tested the effect of transmembrane acetate concentration gradients on Rb^+ currents, which are 10-fold larger than K^+ currents for this channel subtype. Inwardly rectifying Rb^+ currents were maximal at about pH_i 7.0 and were inhibited by intracellular alkalinization or acidification. We conclude that the Kir conductance in the RPE is modulated by intracellular pH in the physiological range and that this reflects the behaviour of Kir7.1 channels. This sensitivity to pH_i may provide an important mechanism linking photoreceptor activity and RPE function.

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Inwardly rectifying K^+ (Kir) channels are a widely distributed class of membrane proteins that are involved in a broad range of cell functions, including generation of the resting membrane potential, the regulation of action potential duration and membrane excitability, and the secretion and absorption of K^+ (Lagrutta *et al.* 1996; Isomoto *et al.* 1997; Reimann & Ashcroft 1999). Members of this gene family share the common property of conducting K^+ more readily in the inward than the outward direction, but exhibit considerable diversity in terms of their regulation by intracellular molecules such as ATP, phosphatidylinositol-4,5-bisphosphate (PIP_2), G proteins, Na^+ and protons.

In the distal retina, the composition and volume of the extracellular fluid in the subretinal space (SRS) surrounding the photoreceptor outer segments are controlled in large part by the transport activity of the adjacent retinal pigment epithelium (RPE), an epithelial monolayer that separates the photoreceptors from their major blood supply in the choroid. The apical membrane of the RPE is highly elaborated with long processes that interdigitate with the photoreceptor outer segments, giving rise to an enormous surface area for interactions between the two cell types. Illumination of the dark-adapted retina produces a rapid decrease in SRS K^+ concentration ($[K^+]$) due to a change in photoreceptor activity (Oakley & Green, 1976; Steinberg *et al.* 1980), and this is followed by a partial recovery as a result of K^+ efflux from the RPE and Müller cells (Karwoski *et al.* 1989). A major component of the K^+ efflux from the RPE is mediated by the large K^+ conductance in its apical membrane (Miller & Steinberg, 1977; Bialek & Miller, 1994; Segawa & Hughes, 1994; Hughes *et al.* 1995). Recent molecular and functional evidence from studies on bovine RPE indicates that this conductance comprises the novel Kir channel Kir7.1 (Shimura *et al.* 2001).

In addition to changes in SRS $[K^+]$, illumination of the dark-adapted retina also produces changes in the pH of the SRS as well as the RPE cytosol (Yamamoto *et al.* 1992;

Keynon *et al.* 1997). Extracellular and intracellular pH modulate the activity of native Kir channels in other cells (Takahashi & Copenhagen, 1995; Rich *et al.* 1997) as well as some cloned Kir channels (Coulter *et al.* 1995; Fakler *et al.* 1996; Sabirov *et al.* 1997; McNicholas *et al.* 1998; Qu *et al.* 1999; Hughes *et al.* 2000), but little is known about the pH sensitivity of Kir channels in the RPE. In an intracellular microelectrode study on cultured bovine RPE cells, Keller *et al.* (1986) described a K^+ conductance that was decreased by extracellular or intracellular acidification and increased by extracellular or intracellular alkalinization. The significance of these findings to the function of the RPE *in situ* is unclear, however, because the properties of Kir channels in cultured RPE differ substantially from those present in native RPE cells (Hughes & Takahira, 1996). Thus, the aim of this study was to determine the sensitivity of the Kir conductance in native RPE cells to changes in extracellular and intracellular pH.

METHODS

Materials

The free acid form of 2′,7′-bis(caboxyethyl)–5(6)-carboxyfluorescein (BCECF) and nigericin were purchased from Molecular Probes (Eugene, OR, USA). Papain (type III) was obtained from Worthington Biochemical (Lakewood, NJ, USA). All other chemicals were obtained from Sigma (St Louis, MO, USA).

Cell isolation

Fresh bovine eyes were obtained from local slaughterhouses within 30 min of death and transported to the laboratory in cold Hepes-buffered Ringer solution (see below). RPE cells were isolated by enzymatic dispersion as described previously (Shimura *et al.* 2001). Briefly, 5–10 mm square pieces of RPE-choroid were dissected from bovine eyecups and then incubated in the cell isolation solution at 37 °C for 30 min. The cell isolation solution consisted of (mM): 135 *N*-methyl-D-glucamine (NMDG)-Cl, 5 KCl, 10 Hepes, 3 EDTA-KOH, 10 glucose and 3 cysteine, and was titrated to pH 7.4 with NMDG (free base). Papain (0.2 mg ml^{-1}) and 4 μg ml^{-1} DNase were added in the isolation solution just before use. Cells were then dissociated by vortexing the RPE sheets in Ringer solution containing 0.03 % glutathione and 0.005 % taurine (pH 7.4). Isolated cells were stored in this solution at 4 °C for up to 8 h before use.

Solutions

The standard Ringer solution consisted of (mM): 135 NaCl, 5 KCl, 10 Hepes, 10 glucose, 1.8 $CaCl_2$ and 1.0 $MgCl_2$, and was titrated to the desired pH with NaOH or HCl. In experiments testing the effects of changes in extracellular pH (pH_o), the pipette solution contained (mM): 30 KCl, 83 potassium gluconate, 10 Hepes, 5.5 EGTA-KOH, 0.5 $CaCl_2$, 2 $MgCl_2$ and 4 K_2ATP, titrated to pH 7.2 with KOH.

pH_i clamping

Intracellular pH (pH_i) was manipulated in individual RPE cells in the whole-cell configuration using a modification of the method described by Grinstein *et al.* (1994). Briefly, pH_i was altered by changing the transmembrane concentration gradient of the weak electrolyte acetate (Ac^-). Assuming that the uncharged species (HAc) is highly permeable and rapidly equilibrates across the membrane and that the charged species Ac^- and H^+ do not, then pH_i will change in a predictable fashion when the extracellular concentration of acetate ($[Ac^-]_o$) is altered according to the relationship:

$$pH_i = pH_o - \log([Ac^-]_o/[Ac^-]_i), \quad (1)$$

where $[Ac^-]_o$ and $[Ac^-]_i$ are the extracellular and intracellular concentrations of Ac^-, respectively. In practice, the actual pH_i can differ significantly from that predicted because of the transport of H^+, base equivalents or Ac^- across the membrane. To validate the technique, we made direct measurements of pH_i (see below) while changing the transmembrane Ac^- concentration gradient (Fig. 2). In order to achieve a wide pH_i range, we found it necessary to use two separate pipette solutions, one containing 5 mM Ac^- and the other 50 mM Ac^-. The 5 mM Ac^- pipette solution consisted of (mM): 69 potassium gluconate, 5 potassium acetate, 30 KCl, 5 Hepes, 10 K_2BAPTA, 0.6 $CaCl_2$, 4 $MgCl_2$ and 4 K_2ATP, titrated to pH 7.4 with KOH. The 50 mM Ac^- pipette solution was identical except that potassium acetate was increased to 50 mM and potassium gluconate was reduced to 24 mM. In these experiments, BAPTA was used to buffer Ca^{2+} because its binding to ATP and Ca^{2+} is less pH sensitive than EGTA. The free Ca^{2+} concentration calculated from dissociation constants was ~40 nM. The external solution used in these experiments contained (mM): *X* NaCl, *Y* sodium acetate (where $X + Y = 126$), 5 KCl, 20 Hepes, 10 glucose, 1.8 $CaCl_2$ and 1 $MgCl_2$, and was titrated to pH 7.4 with NaOH. In a series of experiments testing the effect of pH_i changes on Rb^+ currents, NaCl and KCl in the external solution were replaced by equimolar RbCl and sodium acetate was replaced by equimolar rubidium acetate. Gd^{3+} (100 μM) was added routinely to the external solutions to block a non-specific cation current that was activated in some cells by membrane hyperpolarization. The results were qualitatively similar in the absence and presence of Gd^{3+}.

pH_i measurement

Non-pigmented bovine RPE cells were transferred to a recording chamber on the stage of an inverted microscope (Nikon TE300, Melville, NY, USA) and brought into focus through a × 40 CFI Super Fluor oil objective lens (NA 1.25). Epifluorescence measurements were made after dialysing cells with pipette solution containing 300 μM BCECF free acid in the whole-cell configuration. The excitation source was a xenon lamp and excitation wavelengths were alternated between 440 and 490 nm using an Optoscan Monochromator from Cairn Research (Faversham, UK) with a bandwidth of 30 nm. Emission fluorescence was passed through a 520 nm long-pass filter (Omega Optical, Battleboro, VT, USA) and imaged on a cooled CCD camera (Sensicam, Kelheim, Germany). Average fluorescence within a region of interest (ROI) encompassing the apical or basolateral pole of the cell was sampled at the two wavelengths every 5 s using Axon Imaging Workbench 2.2 software (Axon Instruments, Union City, CA, USA). Background fluorescence in the ROI was measured at the start of each experiment before the membrane patch was ruptured. This was necessary because BCECF free acid in the patch pipette produced significant background fluorescence.

pH_i was calculated in each cell at the end of the experiment using the nigericin calibration technique (Thomas *et al.* 1979). Briefly, BCECF-loaded cells were superfused with 140 mM K^+ solutions containing 10 μM nigericin and buffered to different pH values while monitoring the fluorescence intensities in the ROI at the two excitation wavelengths (see Fig. 2). The Teflon perfusion lines were flushed with ethanol following each calibration to remove residual nigericin (Bevensee *et al.* 1999). After correcting

fluorescence intensities for background fluorescence, ratios were calculated and fitted to the following equation using SigmaPlot version 4 or SigmaPlot 2001 software (SPSS Science, Chicago, IL, USA):

$$\mathrm{pH} = \mathrm{p}K_A + \log((R - R_{min})/(R_{max} - R)), \quad (2)$$

where, R is the ratio of fluorescence intensities measured at 490 nm and 440 nm and R_{min} and R_{max} are the basic and acidic endpoints of the titration curve.

Electrophysiological recording

Membrane currents were recorded from isolated pigmented and non-pigmented RPE cells using the conventional whole-cell voltage-clamp technique. Patch electrodes were pulled from 7052 glass tubing (Garner Glass, Claremont, CA, USA) using a multistage programmable microelectrode puller (model P-87, Sutter Instruments, San Rafael, CA, USA) and had an impedance of 2–5 MΩ after fire polishing. Signals were amplified with an Axopatch 200 amplifier (Axon Instruments) and analysed using pCLAMP 8 software (Axon Instruments). Series resistance (R_s) was normally less then 10 MΩ and was uncompensated except when Rb^+ was in the bath, in which case R_s was compensated by 80 %.

The steady-state current–voltage (I–V) relationship of the cell was measured every 15 s by ramping the membrane voltage from +40 to −160 mV (−110 mV for Rb^+ currents) at a rate of 50 mV s^{-1}, with the voltage held at −10 mV between ramps. This voltage-clamp protocol inactivated a delayed rectifier K^+ current (Takahira & Hughes, 1997) and elicited currents through Kir channels as well as Cl^-- and Na^+-selective channels. Inwardly rectifying K^+ currents (I_{Kir}) were isolated by taking the difference between currents recorded in the absence and presence of 2 mM Ba^{2+}. Offset potentials resulting from liquid junction potentials between the pipette tip and bath solution were calculated using the Junction Potential Calculator tool in pCLAMP 8, and ranged from −10 to −13 mV. Consequently, all voltages were corrected for an offset potential of −10 mV.

Statistics

Results are presented as mean ± S.E.M.

RESULTS

Dependence of Kir conductance on pH_o

To determine whether pH_o modulates the Kir conductance of the RPE, we tested the effects of step changes in pH_o on I_{Kir} in isolated bovine RPE cells recorded in the whole-cell configuration. At each pH_o, currents were measured in the absence and presence of 2 mM Ba^{2+} and the I_{Kir} was calculated as the Ba^{2+}-sensitive component of whole-cell current (Shimura *et al.* 2001). Decreasing pH_o

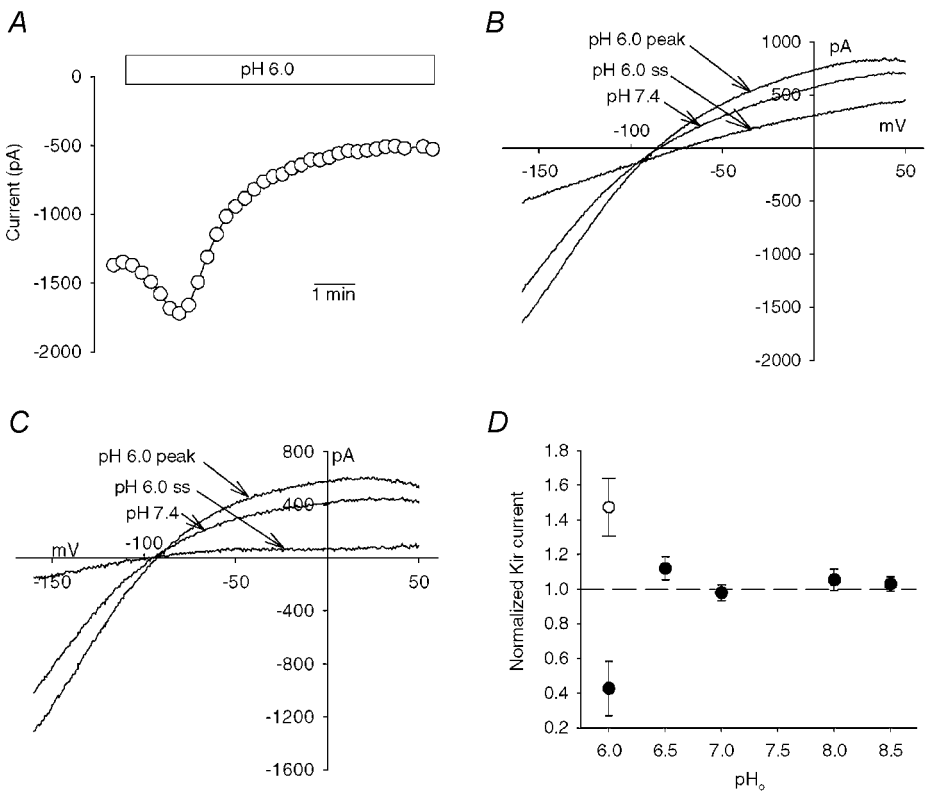

Figure 1. Effect of changes in extracellular pH (pH_o) on inwardly rectifying K^+ (Kir) currents

A, time course of whole-cell current at −160 mV in response to a change in pH_o from 7.4 to 6.0. *B*, current–voltage (I–V) relationships for the cell depicted in *A* measured in pH 7.4 Ringer solution, at the peak of activation in pH 6.0 Ringer solution (pH 6.0 peak), and in pH 6.0 Ringer solution at steady state (pH 6.0 ss). *C*, I–V relationships of Ba^{2+}-sensitive currents for the cell depicted in *A* and *B* in pH 7.4 and pH 6.0 Ringer solution. *D*, relationship between Kir current and pH_o. In each cell, current at −160 mV was measured in each test pH_o and normalized to the current obtained in pH_o 7.4. Symbols depict mean values from four to nine cells and error bars are S.E.M. ●, steady-state currents; ○, peak current at pH_o 6.0.

from 7.4 to 7.0 or 6.5 had little or no effect, but stronger extracellular acidification caused a biphasic change in I_{Kir}. Figure 1*A* shows the time course of a typical response to a decrease in pH_o from 7.4 to 6.0. After a brief delay due to clearance of dead space in the valve and perfusion line, the inward current at −160 mV began to increase, reaching a peak about 1.5 min later. This was followed by a slow decrease in current over the next 6 min. Figure 1*B* plots the *I–V* relationships from the same cell measured in pH 7.4 Ringer solution, at the peak of activation in pH 6.0 Ringer solution and after inhibition had reached steady state. The *I–V* curves for both the activation and inhibition phases of the response intersect the pH 7.4 curve near E_K (K^+ equilibrium potential), indicating that the changes in current were mediated by Kir channels. Similar results were obtained in four other cells, but in a sixth cell, only the slower inhibition phase was apparent. For the five cells exhibiting transient activation, the inward current rose with a half-time of 1.6 ± 0.34 min to a peak value that was 47 ± 17 % larger than control. For a total of six cells, the half-time for the inhibitory phase was 10.6 ± 2.2 min and the inward current decreased to a steady state that was 43 ± 16 % of control values (Fig. 1*D*). Figure 1*C* plots the *I–V* relationships of the Ba^{2+}-sensitive current and shows that both inward and outward currents through Kir channels were modulated by a reduction in pH_o. Thus, the activation and inhibition of Kir conductance induced by exposure to pH_o 6.0 appear to be voltage-independent processes. In contrast to acidification, increasing pH_o to 8.0 or 8.5 had no significant effect on I_{Kir} (Fig. 1*D*).

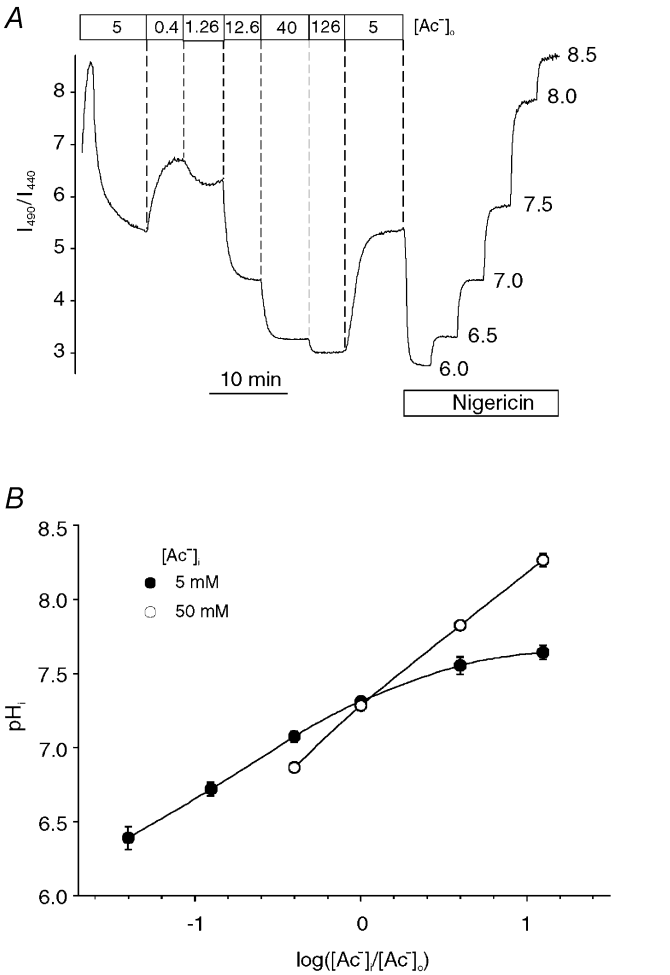

Figure 2. Effect of transmembrane acetate concentration gradients on intracellular pH (pH_i)

A, time course of changes in fluorescence intensity ratio of the pH-sensitive dye BCECF (I_{490}/I_{440}) in a single bovine cell dialysed with 5 mM acetate and exposed to various extracellular concentrations of acetate ($[Ac^-]_o$). At the end of the experiment, pH_i was calibrated by the nigericin technique. The numbers to the right of the trace indicate pH. *B*, relationship between transmembrane acetate concentration gradients and pH_i. ●, mean for seven cells dialysed with 5 mM acetate; ○, mean for five cells dialysed with 50 mM acetate. $[Ac^-]_i$ = intracellular acetate concentration.

These results indicate that Kir channels in the RPE are relatively insensitive to extracellular alkalinization, but are activated by acidification with a threshold of about pH 6.5. Further acidification to pH_o 6.0 appears to trigger a secondary inhibitory process. The slow time course of the inhibitory phase suggests that it may involve an allosteric mechanism. Alternatively, extracellular acidification may lead to intracellular acidification, which modulates the channel in a biphasic manner.

Dependence of Kir conductance on pH_i

In the whole-cell configuration, pH_i can be controlled by heavily buffering the pipette solution. This approach, however, is limited because pH_i cannot be readily changed unless the patch pipette is perfused with different solutions, which is a slow and technically demanding procedure. As an alternative, we utilized a method first described by Grinstein *et al.* (1994) that employs alterations in the transmembrane concentration gradient of a weak base or weak acid to manipulate pH_i to various levels (see Methods). In an initial series of experiments, we used the weak electrolyte NH_4^+, which has been employed extensively to modulate pH_i in a variety of cell types, including the RPE (Keller *et al.* 1986; Lin & Miller, 1991; Kenyon *et al.* 1997). We found, however, that NH_4^+ had multiple effects that were consistent with this cation behaving as a permeant blocker of RPE Kir channels (results not shown), thus invalidating its use in the study of the dependence of Kir channels on pH_i.

As an alternative to NH_4^+, we turned to the weak electrolyte Ac^-. To test the effectiveness of transmembrane Ac^- concentration gradients in controlling pH_i, we dialysed bovine RPE cells with pipette solution containing Ac^- and BCECF free acid and measured by epifluorescence microspectroscopy the changes in pH_i resulting from alterations in the extracellular Ac^- concentration ($[Ac^-]_o$). Figure 2*A* shows the time course of changes in the fluorescence excitation ratio (I_{490}/I_{440}) in a cell dialysed with 5 mM Ac^- pipette solution and exposed to various concentrations of Ac^- in the bath. After the membrane

patch was ruptured, I_{490}/I_{440} rose and then fell to a steady value as the contents of the patch pipette and cytoplasm equilibrated and the transmembrane Ac^- concentration gradient was established. At the time indicated, $[Ac^-]_o$ was decreased from 5 to 0.4 mM, resulting in an increase in I_{490}/I_{440} due to intracellular alkalinization. Subsequently, $[Ac^-]_o$ was increased in a stepwise fashion to 1.26, 12.6, 40 and 126 mM, which caused progressive decreases in I_{490}/I_{440} as the cell acidified. After $[Ac^-]_o$ was returned to the control value of 5 mM, the cell was exposed to a series of nigericin solutions buffered to different levels to calibrate pH_i. The results from this and six other experiments are summarized in Fig. 2*B* (filled circles). Although increases in $[Ac^-]_o$ were effective in acidifying RPE cells, the degree of acidification was somewhat less than what might be expected. For example, with 126 mM Ac^- in the bath, eqn (1) predicts a pH_i of 6.0, and yet the measured pH_i was 6.39 ± 0.08; this higher-than-predicted pH could have been the result of pH regulatory mechanisms (Kenyon *et al.* 1997). Decreases in $[Ac^-]_o$ were relatively ineffective in producing intracellular alkalinization, probably because the intracellular concentration of acetic acid was too low. In view of this, we carried out another series of experiments with 50 mM Ac^- in the pipette and measured I_{490}/I_{440} while superfusing cells with solutions containing 126, 50, 12.6 and 4 mM Ac^- (data not shown). Again, pH_i was calibrated in each cell by superfusion with a series of nigericin solutions buffered to different levels. The results of five experiments are summarized in Fig. 2*B* (open circles). With 50 mM Ac^- in the pipette, decreases in $[Ac^-]_o$ produced alkalinization of pH_i to values close to those predicted by eqn (1).

We next carried out a separate series of patch-clamp experiments to determine whether intracellular or extracellular Ac^- concentration *per se* affected I_{Kir} because studies on other cell types have shown that the activity of K^+ channels can be modulated by anions (Rae *et al.* 1990; Yuan *et al.* 2000). To test this, we compared I_{Kir} recorded in cells with either 5 mM or 50 mM Ac^- in both the pipette solution and bath. Figure 3 plots the *I–V* relationships of the Ba^{2+}-sensitive current measured under these two conditions and shows no significant difference. We conclude from these results that acetate *per se* does not significantly affect I_{Kir} in bovine RPE cells.

Figure 4 shows the effects of various Ac^- concentration gradients on currents in an RPE cell dialysed with 5 mM Ac^-. At each $[Ac^-]_o$, currents were recorded in the absence and presence of 2 mM Ba^{2+} to allow isolation of the I_{Kir}. Figure 4*A* shows the time courses of changes in inward and outward currents (measured at −60 mV and −110 mV, respectively). At the start of the record, the bath contained 5 mM Ac^- and the addition of 2 mM Ba^{2+} blocked Kir channels, resulting in a substantial reduction in both inward and outward currents. Following the washout of Ba^{2+} and the recovery of currents to their initial levels, the extracellular concentration of Ac^- was increased to 12.6 mM. This manoeuvre, which decreases pH_i from ~7.38 to ~7.0 (Fig. 2), caused increases in inward and

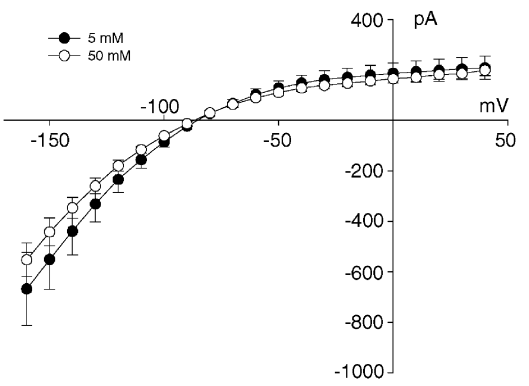

Figure 3. Lack of effect of acetate *per se* on Kir currents

The *I–V* relationships of Ba^{2+}-sensitive currents were measured in cells with equal acetate concentrations in the pipette solution and bath. Symbols represent the mean of measurements in nine cells recorded with 5 mM acetate (●) and 13 cells recorded with 50 mM acetate (○).

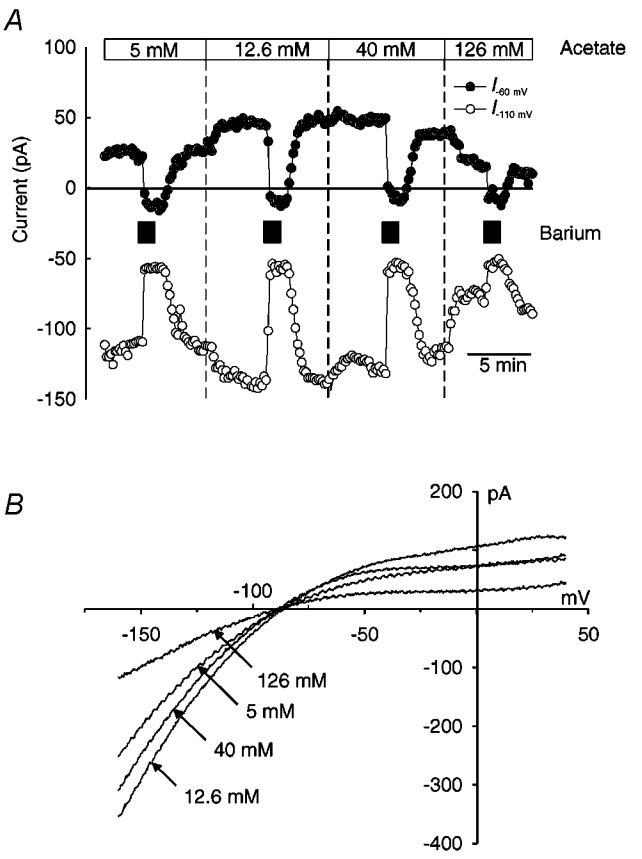

Figure 4. Effect of intracellular acidification on Kir currents

A, time courses of currents at −110 mV and −60 mV recorded in a cell with 5 mM acetate in the pipette and exposed various acetate concentrations in the bath. *B*, *I–V* relationships of Ba^{2+}-sensitive currents in the presence of various acetate concentration gradients.

outward currents that were largely blocked by the addition of Ba^{2+}, indicating that intracellular acidification had increased I_{Kir}. This increase in I_{Kir} is illustrated in Fig. 4*B*, which shows the *I–V* relationships of Ba^{2+}-sensitive currents in the presence of various Ac^- concentration gradients. When pH_i was acidified further to ~6.8 by increasing $[Ac^-]_o$ to 40 mM, the Ba^{2+}-sensitive current decreased slightly, and when $[Ac^-]_o$ was increased to 126 mM, thereby lowering pH_i to ~6.4, the current was strongly inhibited. The inhibition produced by exposure to 126 mM acetate was partially reversible (not shown).

Figure 5 shows the results of a different experiment carried out with 50 mM instead of 5 mM Ac^- in the pipette solution. In the first part of the experiment, the cell was acidified to ~pH 6.9 by increasing $[Ac^-]_o$ from 50 mM to 126 mM; this produced large increases in inward and outward currents due to stimulation of the Ba^{2+}-sensitive current. In contrast, when pH_i was alkalinized to ~7.8 and 8.3 by decreasing $[Ac^-]_o$ to 12.6 mM and 4 mM, respectively, inward and outward currents decreased dramatically and Ba^{2+}-sensitive currents were small and difficult to quantify. Ba^{2+}-insensitive currents were largely unaffected by these manipulations in pH_i (not shown), indicating that pH_i affects neither the effectiveness of the Ba^{2+} block of Kir channels, nor the activity of other channels present.

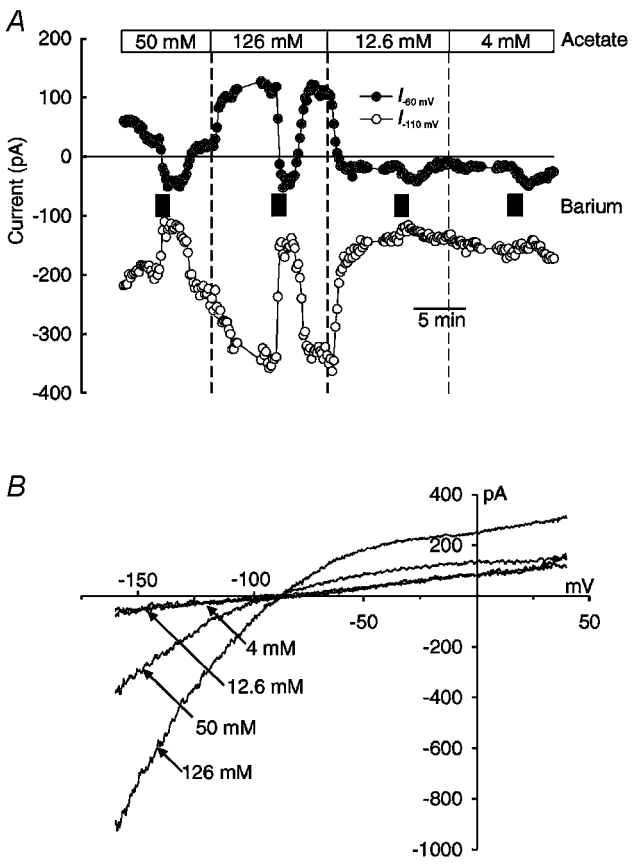

Figure 5. Effect of intracellular alkalinization on Kir currents

A, time courses of currents at −110 mV and −60 mV recorded in a cell with 50 mM acetate in the pipette and exposed various acetate concentrations in the bath. *B*, *I–V* relationships of Ba^{2+}-sensitive currents in the presence of various acetate concentration gradients.

The results of these and similar experiments carried out on both pigmented and non-pigmented RPE cells are summarized in Fig. 6, which plots normalized I_{Kir} as a function of pH_i. The relationship between I_{Kir} and pH_i is biphasic, with an optimal pH of ~7.1 and inhibition at more alkaline or acidic pH values. The relationship is particularly steep in the physiological pH_i range and indicates a 60% increase in I_{Kir} for intracellular acidification from pH 7.31 to pH 7.07.

Previously, we presented molecular and functional evidence that Kir7.1 channels are a major component of the Kir conductance in bovine RPE cells (Shimura *et al.* 2001). Hence, it seemed likely that the pH_i-dependent changes in I_{Kir} reflected the modulation of Kir7.1 channels. To test this idea, we next investigated the effects of transmembrane acetate gradients on inwardly rectifying Rb^+ currents. This strategy takes advantage of a distinguishing property of the Kir7.1 channel, namely a 10-fold higher macroscopic conductance when Rb^+ is the charge carrier instead of K^+ (Wischmeyer *et al.* 2000; Shimura *et al.* 2001). Figure 7 shows the results of an experiment carried out with 50 mM Ac^- in the pipette solution. Initially, the cell was bathed with 5 mM K^+, 50 mM Ac^- solution, and inward currents were relatively small (Fig. 7*A*). When external K^+ and Na^+ were replaced with Rb^+, inward currents grew dramatically and had a strongly inwardly rectifying *I–V* relationship (Fig. 7*B*), a characteristic of Rb^+ currents in cloned Kir7.1 channels.

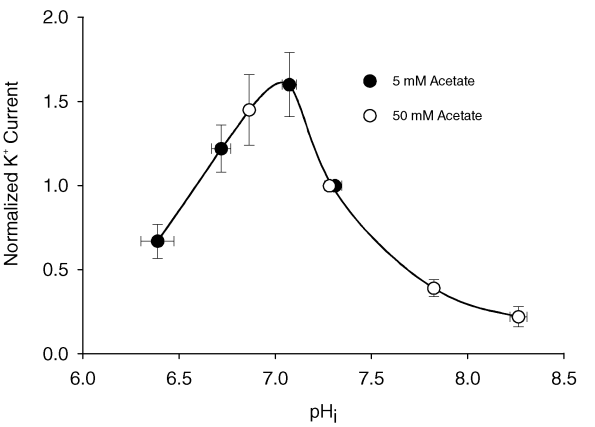

Figure 6. Relationship between Kir current and pH_i determined from transmembrane acetate concentration gradient experiments

In each cell, currents were measured at −110 mV and normalized to the current obtained in the absence of a transmembrane acetate concentration gradient. ●, mean ± S.E.M. for nine cells dialysed with 5 mM acetate; ○, mean ± S.E.M. for 11 cells dialysed with 50 mM acetate. The pH_i data are the same as those presented in Fig. 2*B*.

After currents had stabilized, the cell interior was acidified by increasing $[Ac^-]_o$ from 50 mM to 126 mM, leading to a > 25 % increase in inward Rb^+ current. In contrast, when pH_i was alkalinized by decreasing $[Ac^-]_o$ to 12.6 mM, inward Rb^+ currents decreased substantially, and currents decreased even further when $[Ac^-]_o$ was lowered to 4 mM. In other experiments (not shown), we tested the effects of transmembrane Ac^- concentration gradients on Rb^+ currents with 5 mM Ac^- in the pipette. We found that increasing $[Ac^-]_o$ from 5 to 12.6 mM, which was expected to produce mild intracellular acidification, caused small increases in inward Rb^+ current but that further acidification by increasing $[Ac^-]_o$ to 40 or 126 mM had the opposite effect and produced strong inhibition.

Figure 8 summarizes the results obtained in six cells dialysed with 5 mM Ac^- and eight cells dialysed with 50 mM Ac^- and plots normalized Rb^+ current as a function of $\log([Ac^-]_i/[Ac^-]_o)$. The inwardly rectifying Rb^+ current had a pH_i-sensitivity profile that was similar to that observed for the I_{Kir}; it appeared to be maximal near pH_i 7.0 and decreased with either acidification or alkalinization of the cell. These results indicate that Kir7.1 channels in bovine RPE cells are sensitive to changes in pH_i and strongly support the idea that Kir7.1 channels mediate that pH_i-dependent modulation of I_{Kir} that is seen when RPE cells are bathed in a physiological concentration of K^+.

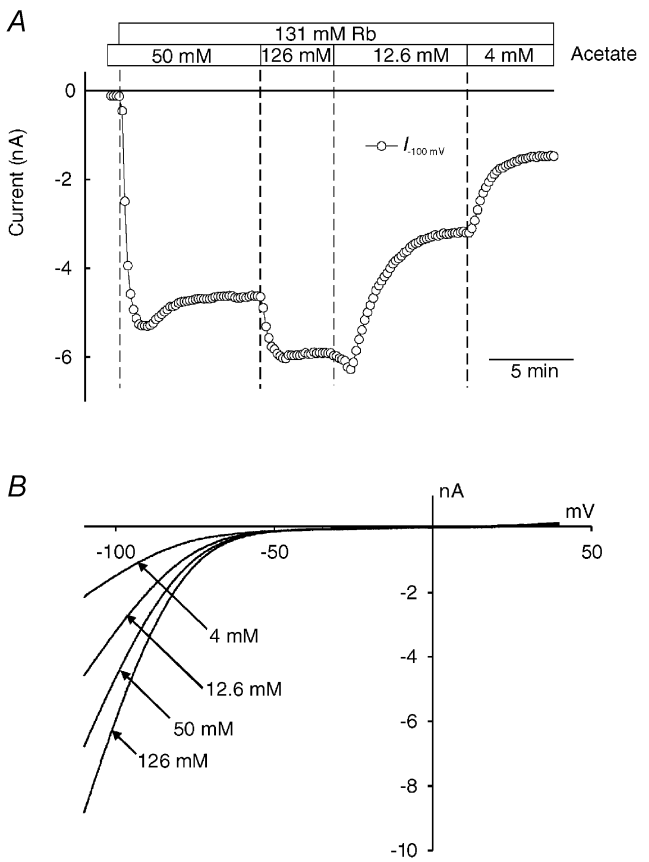

Figure 7. Effect of pH_i changes on Rb^+ currents

A, time course of currents at −100 mV recorded in a cell with 50 mM acetate in the pipette and exposed various acetate concentrations in the bath. This cell was initially bathed in 5 mM K^+, 50 mM acetate solution. *B*, *I–V* relationships of whole-cell currents in the presence of 131 mM Rb^+ various acetate concentration gradients.

DISCUSSION

The main finding of this study is that the Kir conductance in freshly isolated bovine RPE cells is exquisitely sensitive to changes in pH_i. The relationship between Kir conductance and pH_i is biphasic, with mild intracellular acidification enhancing Kir conductance and stronger acidification or alkalinization inhibiting it. Kir conductance is extremely sensitive to pH_i changes in the physiological range, such that acidification from pH 7.3 to 7.07 gives rise to a 60 % increase. We also found that the Kir conductance is relatively insensitive to pH_o except when pH_o is decreased to 6.0, which causes a transient stimulation followed by steady-state inhibition.

We altered pH_i in RPE cells recorded in the whole-cell configuration using a variation of the method of Grinstein *et al.* (1994), who used transmembrane NH_4^+ concentration gradients to modulate pH_i in macrophages. Our results indicate that NH_4^+ is a permeant blocker of the Kir conductance in the RPE, making this weak electrolyte unsuitable for our experiments. As an alternative, we used Ac^- ($pK_a = 4.75$), which readily permeates the plasma membrane in its undissociated form. Because the effectiveness of transmembrane acetate gradients to alter pH_i could be affected by the activity of pH regulatory

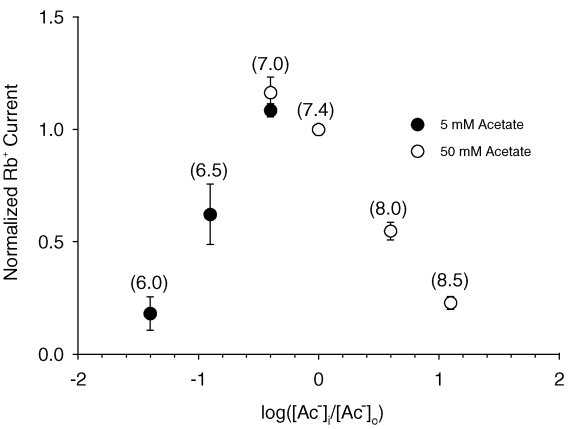

Figure 8. Relationship between Rb^+ current and $\log([Ac^-]_i/[Ac^-]_o)$

Cells were dialysed with 5 mM or 50 mM acetate and bathed with solutions containing 131 mM Rb^+ and various concentrations of acetate. In each cell, currents were measured at −100 mV and normalized to the current obtained when $[Ac]_o = [Ac]_i$. Closed symbols represent mean ± S.E.M. for six cells dialysed with 5 mM acetate and open symbols represent the mean ± S.E.M. for eight cells dialysed with 50 mM acetate. Numbers in parentheses above symbols indicate approximate pH_i as calculated using eqn (1).

mechanisms or acetate transport, we validated this approach by measuring pH_i with the pH-sensitive dye BCECF. We found that the cytoplasm of RPE cells could be rapidly and reproducibly acidified or alkalinized by altering the transmembrane Ac^- concentration gradient.

Previous studies have shown that pH_o can modulate the activity of a variety of Kir channels. Kir2.3 (Coulter *et al.* 1995) and Kir2.4 (Hughes *et al.* 2000), which share in common a pH-sensing domain that is exposed to the extracellular environment, are extremely sensitive to pH_o changes in the physiological range ($pK_a = 7.4$) and are activated by alkalinization and inhibited by acidification. Kir2.1, a structurally related channel that lacks the pH-sensing domain, exhibits voltage-dependent inhibition by protons, but only at very low pH_o ($pK_a = 4.6$; Sabirov *et al.* 1997). In the present study, step changes from pH_o 7.4 to a pH_o in the range 6.5–8.5 had little or no effect on the Kir conductance in the RPE, but acidification to pH_o 6.0 caused a transient activation followed by a slow inhibition to a level that was ~43 % of control. Activation and inhibition were voltage independent, indicating that protons may bind to a low-affinity site outside the channel pore. Alternatively, the effects of extracellular acidification may be secondary to intracellular acidification.

Intracellular acidification inhibits several members of the Kir channel family, including Kir1.1 (Fakler *et al.* 1996; McNicholas, *et al.* 1998), Kir2.3 (Qu *et al.* 1999; Zhu *et al.* 1999), Kir4.1 (Shuck *et al.* 1997; Yang & Jiang, 1999) and heteromeric Kir4.1-Kir5.1 channels (Tanemoto *et al.* 2000; Xu *et al.* 2000). A more complex pattern of pH_i sensitivity is seen in the ATP-sensitive K^+ channel, which in excised patches exhibits activation by mild acidification and inhibition by strong acidification (Lederer & Nichols, 1989; Koyano *et al.* 1993). Studies on Kir6.2 have shown that activation is the direct result of proton binding to a histidine residue (H175) conserved in all K_{ATP} channels, whereas inhibition appears to be the result of rundown and is absent in intact cells (Xu *et al.* 2001). We demonstrate here that the Kir conductance in bovine RPE has a pH_i sensitivity profile that is qualitatively similar to that of Kir6.2, with activation by mild acidification and inhibition by stronger acidification. Unlike Kir6.2, however, the Kir conductance in the RPE is inhibited by alkalinization and acid-induced inhibition takes place in intact cells and is at least partially reversible.

In an intracellular microelectrode study of confluent monolayers of cultured bovine RPE cells, Keller *et al.* (1986) found that the membrane potential is sensitive to changes in pH, with extracellular or intracellular acidification causing depolarization and extracellular or intracellular alkalinization causing hyperpolarization. These pH-dependent voltage responses were greatly diminished in the presence of Ba^{2+} and were associated with changes in the K^+ transference number, suggesting that they were mediated by a K^+ conductance that increases with alkalinization and decreases with acidification. This behaviour appears to be inconsistent with our present findings in native bovine RPE cells and suggests that native and cultured bovine RPE cells express different subtypes of Kir channel. This possibility is supported by our previous observation that cultured human RPE cells express a 'classical' Kir channel whose properties are distinct from those of the Kir channel present in freshly isolated human RPE cells (Hughes & Takahira, 1996). Alternatively, these differences might be explained if cultured bovine RPE cells differed from native cells in terms of resting pH_i or the expression of pH_i-sensitive K^+ channel regulatory molecules. Direct measurements of K^+ conductance in cultured bovine RPE cells as a function of pH_i will be necessary to distinguish between these possibilities.

In a recent report, we showed that Kir7.1 is highly expressed in native bovine RPE (Shimura *et al.* 2001). Moreover, we established that this channel subunit is a major component of the Kir conductance by demonstrating that the native Kir channel and cloned Kir7.1 channel share several unusual permeation properties including a low dependence of macroscopic conductance on extracellular $[K^+]$, a 10-fold higher conductance for Rb^+ than K^+, and a single-channel K^+ conductance in the order of 0.2 pS. In rat RPE, there is evidence for the expression of Kir7.1 (Kusaka *et al.* 2001) as well as Kir4.1 (Kusaka *et al.* 1999) and Kir6.2/SUR1 (Ettaiche *et al.* 2001). There is, however, no functional evidence for either Kir4.1 or Kir6.2 channels in bovine RPE (Hughes & Takahira, 1998; Shimura *et al.* 2001). Hence, it seems likely that the pH_i-sensitive Kir channel in bovine RPE cells is Kir7.1.

To obtain more direct evidence for this idea, we examined the effect of Ac^--induced pH_i changes on Rb^+ currents. Cloned Kir7.1 channels exhibit an 8- to 10-fold larger macroscopic conductance when Rb^+ is the charge carrier instead of K^+ (Wischmeyer *et al.* 2000; Shimura *et al.* 2001), which is one of several unusual permeation properties that distinguish it from other Kir channel subtypes. As shown previously (Shimura *et al.* 2001), superfusion of bovine RPE cells with high Rb^+ solution resulted in a large inward current with an 'activation potential' near −50 mV, consistent with the properties of cloned Kir7.1. We found that Rb^+ currents were enhanced by mild acidification but inhibited by strong acidification or alkalinization, giving rise to a pH_i-sensitivity profile that was qualitatively similar to that observed for I_{Kir}. These results provide strong evidence that Kir7.1 channels underlie the pH_i-sensitive Kir conductance in RPE. It remains to be determined whether the pH_i sensitivity is an inherent property of the Kir7.1 channel or is mediated by a pH-dependent interaction with some other molecule.

Physiological significance

The RPE has an intimate anatomical and functional relationship with the adjacent photoreceptor cells. Microvilli project from the apical surface of the RPE and interdigitate with the photoreceptor outer segments, separated by a small extracellular compartment, the SRS. The RPE provides crucial support to the photoreceptors by regulating this microenvironment through the vectorial transport of fluid, ions and metabolites (Hughes *et al.* 1998). K^+ channels in the apical membrane function in SRS K^+ homeostasis by controlling the direction and magnitude of net K^+ transport across the RPE (Miller & Edelman, 1990; Joseph & Miller, 1991). In addition, apical K^+ channels play an important role in RPE-photoreceptor interactions.

At light onset, SRS $[K^+]$ decreases by several millimoles due to changes in photoreceptor activity (Oakley & Green, 1976; Steinberg *et al.* 1980). By virtue of the apical membrane K^+ channels, this extracellular $[K^+]$ decrease hyperpolarizes the RPE and alters the driving force on a variety of transport processes (Hughes *et al.* 1998). Step changes in $[K^+]$ from 5 to 2 mM outside the apical membrane of bovine RPE explants, which mimic the dark-light transition in SRS $[K^+]$, acidify the RPE cell by ~0.35 pH units (Kenyon *et al.* 1997) due to the slowing or reversal of the apical electrogenic $NaHCO_3$ cotransporter (Hughes *et al.* 1989). Our present findings suggest that this acidification would produce a significant increase in apical K^+ conductance, which would increase K^+ efflux and enhance the capacity of the RPE to buffer SRS $[K^+]$.

pH_o in the retina varies with retinal depth, with it being most acidic at the outer nuclear layer (ONL) where the photoreceptor inner segments lie, consistent with the high metabolic activity of these cells (Borgula *et al.* 1989; Yamamoto *et al.* 1992). Illumination causes alkalinization of pH_o in the ONL, and even larger changes take place under control of a circadian clock (Dmitriev & Mangel, 2001). Although pH_o changes also occur in the SRS outside the RPE apical membrane, they are typically small and vary among species. Given that the Kir conductance in the RPE is insensitive to changes in pH_o except at $pH_o < 6.5$, it is unlikely that Kir channels are modulated by pH_o *in situ*, except perhaps under extreme conditions such as ischaemia, hypoxia or hypercapnia (Linsenmeier *et al.* 1983; Yamamoto & Steinberg, 1992).

In summary, we have demonstrated that the Kir conductance in bovine RPE cells, which evidence suggests is comprised of Kir7.1 channels, is exquisitely pH_i-sensitive, being activated by acidification in the physiological pH_i range. Modulation of Kir channels in the RPE by pH_i may be an important regulatory mechanism involved in SRS K^+ homeostasis.

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