Properties of the inwardly rectifying K^+ conductance in the toad retinal pigment epithelium

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1. An inwardly rectifying K^+ current was analysed in isolated toad retinal pigment epithelial (RPE) cells using the perforated-patch clamp technique.

2. The zero-current potential (V_0) of RPE cells averaged −71 mV when the extracellular K^+ concentration ($[K^+]_o$) was 2 mM. Increasing $[K^+]_o$ from 0·5 to 5 mM shifted V_0 by +43 mV, indicating a relative K^+ conductance (T_K) of 0·74. At $[K^+]_o$ greater than 5 mM, T_K decreased to 0·53.

3. Currents were larger in response to hyperpolarizing voltage pulses than depolarizing pulses, indicating an inwardly rectifying conductance. Currents were time independent except in response to voltage pulses to potentials positive to 0 mV, where the outward current decayed with an exponential time course.

4. Both the inwardly rectifying current and the transient outward current were eliminated by the addition of 0·5 mM Ba^{2+}, 5 mM Cs^+ or 2 mM Rb^+ to the extracellular solution. The current blocked by these ions reversed near the K^+ equilibrium potential (E_K) over a wide range of $[K^+]_o$, indicating a highly selective K^+ channel.

5. The current–voltage relationship of the isolated K^+ current exhibited mild inward rectification at voltages negative to −20 mV and a negative slope conductance at voltages positive to −20 mV.

6. The Cs^+- and Ba^{2+}-induced blocks of the K^+ current were concentration dependent but voltage independent. The apparent dissociation constants were 0·8 mM for Cs^+ and 40 μM for Ba^{2+}. The K^+ conductance decreased when extracellular Na^+ was removed.

7. Increasing $[K^+]_o$ decreased the K^+ chord conductance (g_K) at negative membrane potentials. In the physiological voltage range, increasing $[K^+]_o$ from 2 to 5 mM caused g_K to decrease by approximately 25 %.

8. We conclude that the inwardly rectifying K^+ conductance represents the resting K^+ conductance of the toad RPE apical membrane. The unusual properties of this conductance may enhance the ability of the RPE to buffer $[K^+]_o$ changes that take place in the subretinal space at the transition between dark and light.

In the vertebrate eye, the retinal pigment epithelium (RPE) separates the photoreceptor cells from their main blood supply in the choroid. Previous studies on the RPE of a variety of species have shown that the apical membrane, which faces the photoreceptor outer segments across the subretinal space, has a high K^+ conductance (Lasansky & De Fisch, 1966; Miller & Steinberg, 1977; Griff, Shirao & Steinberg, 1985; Joseph & Miller, 1991; Quinn & Miller, 1992). This conductance is thought to participate in several aspects of RPE transport. First, it mediates the return to the subretinal space of K^+ that enters the cell across the apical membrane via the Na^+–K^+ pump and Na^+–K^+–Cl^- cotransporter (la Cour, Lund-Andersen & Zeuthen, 1986; Miller & Edelman, 1990). Second, it is a primary determinant of the apical membrane potential (Miller & Steinberg, 1977), which influences the transport of other ions whose transmembrane movements are driven by voltage (Hughes, Adorante, Miller & Lin, 1989; Fujii, Gallemore, Hughes & Steinberg, 1992). Third, it mediates a component of the c-wave of the direct-current electro-retinogram, which arises from a voltage response of the RPE apical membrane to changes in subretinal K^+ concentration that are generated by changes in photo-receptor cell activity at the transition between dark and light (Oakley, 1977; Steinberg, Linsenmeier & Griff, 1985).

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Previous patch-clamp studies of freshly isolated cells from bullfrog (Hughes & Steinberg, 1990) and primate RPE (Wen, Lui & Steinberg, 1993) have identified two types of voltage-dependent K^+ conductances: a delayed rectifier-type K^+ conductance and an inwardly rectifying K^+ conductance. The function of the delayed rectifier in the RPE is unknown, since it only activates at potentials positive to the normal resting potentials of the apical and basolateral membranes. The inwardly rectifying K^+ conductance, however, is active in the physiological voltage range and hence is a likely candidate for the resting K^+ conductance of the apical membrane. This conductance was shown to be selective for K^+ and was blocked by the addition of either Ba^{2+} or Cs^+ to the bath (Hughes & Steinberg, 1990; Wen *et al.* 1993).

In the present study, we investigated the properties of the inwardly rectifying K^+ conductance in isolated RPE cells from the marine toad. This species was chosen because its RPE cells have a large inwardly rectifying K^+ current but little or no delayed-rectifier current. Currents were measured using the perforated-patch variant of the patch-clamp method (Horn & Marty, 1988; Rae & Cooper, 1990). This approach prevented the time-dependent decline in the inwardly rectifying K^+ current that had been observed previously in the whole-cell recording mode (Hughes & Steinberg, 1990) and allowed the current to be studied for several hours within a single cell.

We show that the inwardly rectifying K^+ current of the toad RPE has several unique properties that distinguish it from inwardly rectifying K^+ currents in excitable cells. Some of this work has been presented previously in preliminary form (Hughes & Segawa, 1992; Segawa & Hughes, 1993).

METHODS

Solutions

The normal external solution had the following composition (mM): 110 NaCl, 2 KCl, 5 Hepes–NaOH, 1·8 $CaCl_2$, 1 $MgCl_2$, 10 glucose, at pH 7·4. The concentrations of K^+ and tetraethylammonium (TEA) were varied by replacing NaCl with the appropriate chloride salt. Cs^+, Rb^+ and Ba^{2+} were added to the external solution as chloride salts. Diphenylamine-2-carboxylate (DPC) was added as a 0·5 M stock solution in DMSO. The internal solution in the patch pipette consisted of (mM): 72 potassium gluconate, 25 KCl, 10 Hepes–KOH, 5·5 EGTA, 0·5 $CaCl_2$, 2 $MgCl_2$, and 120 mg ml^{-1} amphotericin B, at pH 7·2. The osmolalities of the external and internal solutions were 230 ± 5 and 205 ± 5 mosmol kg^{-1}, respectively. All solutions were equilibrated with room air.

The cell isolation medium had the following composition (mM): 110 *N*-methyl-D-glucamine (NMDG)-Cl, 2 KCl, 5 Hepes–NaOH, 3 EDTA–KOH, 10 glucose, 3 cysteine, 100 mannitol, and 0·2 mg ml^{-1} papain (type III). DPC was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Cell isolation

Medium-sized marine toads (*Bufo marinus*) were kept for 1 week or longer in tap water on a cycle of 12 h light–12 h dark and fed live crickets once a week. Toads were dark adapted for at least 3 h before being killed by decapitation and pithed. The RPE–choroid was dissected from the sensory retina and sclera under dim red light as previously described (Miller & Steinberg, 1977).

RPE cells were dispersed enzymatically by incubating pieces of RPE–choroid for 5–10 min in the cell isolation medium. The tissue was transferred to normal external solution containing 0·1 % bovine serum albumin for 5 min, and then to normal external solution for another 10 min. This series of incubations was repeated 3 or 4 times before dissociating cells by drawing the tissue several times through the opening of a fire-polished Pasteur pipette. Viable cells could also be isolated from pieces of RPE–choroid that had been stored at 4 °C overnight in normal external solution.

Perforated-patch recording

Isolated RPE cells were transferred to a Lucite recording chamber with a glass coverslip bottom (total volume, 400–500 μl) and allowed to settle for several minutes before starting perfusion. Solutions were delivered to one end of the chamber by gravity feed at a rate of 0·5–1 ml min^{-1} and removed at the opposite end by suction. Solutions were changed using two 6-way distribution valves connected to a 4-way slider valve (Hodgkin, McNaughton, Nunn & Yau, 1984). Cells were rejected for recording if they were rounded or if the cytoplasm appeared vesicularized. All experiments were conducted at room temperature (20–25 °C) within 4 h of cell dissociation.

Membrane currents were recorded by the perforated-patch recording technique (Horn & Marty, 1988; Rae & Cooper, 1990). Pipettes were pulled from 7052 glass tubing (Garner Glass, Claremont, CA, USA) using a multistage programmable puller (Sutter Instruments, San Rafael, CA, USA) and coated with Sylgard (Dow Corning, Midland, MI, USA). Prior to use, pipette tips were heat-polished to a bubble test number between 6·0 and 6·5 ml using a 10 ml syringe (Corey & Stevens, 1983), which corresponded to a resistance of 1 to 5 MΩ when filled with internal solution and placed in normal external solution. Patch pipettes were front-filled with a 200–300 μm column of internal solution without amphotericin B and then back-filled with internal solution containing 120 mg ml^{-1} amphotericin B. Series resistance commonly decreased to 25 MΩ or less within 5–15 min after gigaseal formation on the basolateral membrane.

Currents were recorded with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA, USA) with the built-in low-pass filter set to 1 kHz unless noted otherwise. Recordings were referenced to a Ag–AgCl electrode separated from the bath by a short column of 150 mM KCl set in 4 % agar. Command potentials were generated by software control (pCLAMP, Axon Instruments). Signals were digitized on-line and stored on disk in a microcomputer for subsequent analysis. Series resistance (R_s) was determined by dividing the voltage pulse amplitude by the peak of the resulting change in capacitative current measured in the absence of capacitance compensation. Membrane capacitance (C) was then determined by fitting the relaxation of the capacitative transient to

a single exponential to find the time constant τ, and then calculating C from the relationship

$$C = \tau(1 + R_s/R_{in})/R_s,$$

where R_{in} is the input resistance of the cell. In most experiments, R_s was compensated by 50–70 %. Pipette and membrane capacitances were compensated by built-in circuits. Pipette tip potentials and liquid junction potentials at the interface between the bathing solution and the agar bridge were measured against a flowing KCl bridge and were used to correct the apparent membrane potential as described previously (Hughes & Steinberg, 1990). Statistical data are presented as means ± S.D., except where noted otherwise.

RESULTS

Isolated toad RPE cells were morphologically similar to solitary bullfrog RPE cells described previously (Hughes & Steinberg, 1990). Cell bodies were approximately 20–30 μm in length, 10–20 μm in diameter, and often narrowed at the expected location of the tight junction. The apical pole of the cell contained numerous pigment granules, whereas the basal membrane generally had a bright appearance under phase contrast microscopy. Most cells retained long apical processes 20–40 μm in length. The membrane capacitance of isolated toad RPE cells averaged 71 ± 25 pF ($n = 138$), which is somewhat larger than the mean capacitance of bullfrog RPE cells reported in a previous study (Hughes & Steinberg, 1990).

Previous studies with intracellular microelectrodes showed that in intact toad RPE explants bathed in 2 mM K^+ Ringer solution, the apical membrane potential is in the range −73 to −83 mV (Griff, 1990, 1991). We measured the zero-current potential (V_0) of isolated RPE cells in the zero current-clamp condition after the series resistance of the perforated membrane patch had fallen to less than 50 MΩ. This value might be somewhat different from the resting membrane potential measured with microelectrodes if the ion activities of the solution in the pipette, which equilibrates with the cell interior, differed from the normal intracellular ion activities. V_0 averaged −71 ± 8 mV ($n = 148$), and remained stable for several hours of recording. These results indicate that the resting K^+ conductance was present in isolated toad RPE cells and that its 'washout' was negligible in the perforated-patch configuration.

Dependence of the zero-current potential on $[K^+]_o$

Electrophysiological studies in RPE explants from toad (Lasansky & De Fisch, 1966; Griff, 1991), bullfrog (Miller & Steinberg, 1977; Griff, Shirao & Steinberg, 1985), cattle (Joseph & Miller, 1991), and man (Quinn & Miller, 1992) have shown that the apical membrane has a high K^+ conductance. To determine the relative K^+ conductance of isolated toad RPE cells, we measured V_0 while bathing the cell with various concentrations of extracellular K^+ ($[K^+]_o$). Figure 1 plots V_0 as a function of log $[K^+]_o$. Consistent with the presence of a K^+ conductance, V_0 shifted in the positive direction as $[K^+]_o$ was increased. In the concentration range between 0·5 and 5 mM, V_0 changed +43 mV per decade change in $[K^+]_o$, indicating a relative K^+ conductance (T_K) of 0·74. The slope of this relationship decreased at higher $[K^+]_o$: between 5 and 112 mM K^+, V_0 changed only +31 mV per decade change in $[K^+]_o$, corresponding to a T_K of about 0·53. These results indicate that at low $[K^+]_o$, the membrane properties of toad RPE cells are dominated by a K^+ conductance. As shown below, the decrease in T_K at higher concentrations of K^+ results from two factors: an inverse dependence of this conductance on $[K^+]_o$, and its dependence on extracellular Na^+.

Whole-cell currents

As shown previously, the resting membrane properties of isolated toad RPE cells are dominated by an inwardly rectifying K^+ conductance (Hughes & Segawa, 1993). The upper panel of Fig. 2*A* shows an example of superimposed whole-cell currents generated by a series of 100 ms voltage pulses ranging from −160 to +40 mV in 20 mV increments. Between voltage pulses, the membrane potential was held at −70 mV, which was close to the resting membrane potential of this cell. In the voltage range −160 to −20 mV, currents

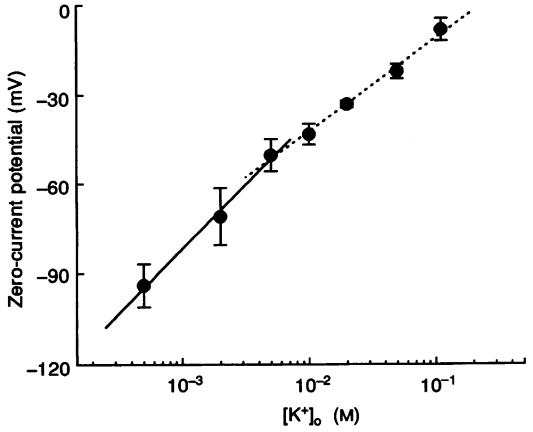

Figure 1. Relationship between $[K^+]_o$ and the zero-current potential (V_0) of isolated toad RPE cells
$[K^+]_o$ was varied by substituting KCl for NaCl. Each point represents the mean value of V_0 and vertical lines indicate S.D. The mean values of V_0 at the various values of $[K^+]_o$ were as follows: 0·5 mM, −94 ± 7·1 mV ($n = 5$); 2 mM, −71 ± 9·5 mV ($n = 34$); 5 mM, −50 ± 5·5 mV ($n = 10$); 10 mM, −43 ± 3·5 mV ($n = 5$); 20 mM, −33 ± 1·1 mV ($n = 3$); 50 mM, −22 ± 2·4 mV ($n = 4$); and 112 mM, −8·5 ± 3·8 mV ($n = 5$). The continuous line represents the least-squares regression line for the data points between 0·5 and 5 mM (slope = 43 mV per decade change in $[K^+]_o$) and the dashed line represents the regression line for points between 5 and 112 mM (slope = 31 mV per decade change in $[K^+]_o$).

were essentially time independent, activating with approximately the same time constant as that of the voltage clamp. In contrast, voltage pulses positive to −20 mV produced a decay in outward current, which could be fitted by a single exponential (not shown). The amplitude of the steady-state current was larger in response to hyperpolarization than to depolarization, indicating inward rectification. This pattern of whole-cell currents is similar to that described previously for isolated bullfrog RPE cells in which the delayed rectifier K^+ current was inactivated by holding the membrane potential at a depolarized level (Hughes & Steinberg, 1990). In contrast to the frog RPE, toad cells exhibited little, if any, delayed rectifier current.

The inwardly rectifying current–voltage (I–V) relationship of the toad RPE can be better appreciated by inspecting the trace labelled 'control' in the upper panel of Fig. 2*B*, which plots the steady-state current evoked by a 1 s voltage ramp. In thirty-four cells, the average inward conductance (measured as the slope conductance between −100 and −80 mV) was 6·3 ± 2·9 nS, compared to an average outward conductance (measured between −60 and −40 mV) of 4·2 ± 2·0 nS (mean ± S.D.). In some cells, a negative slope conductance region was observed in the voltage range between +20 and +50 mV (see Fig. 6*A*). This negative slope conductance arose from the decay in outward current that took place in response to strong depolarizations (Figs 2*A* and 7).

Effects of K^+ channel blockers

Barium

Previous studies in both intact epithelial sheets (Griff *et al.* 1985) and isolated cells (Hughes & Steinberg, 1990) of the bullfrog RPE showed that extracellular Ba^{2+} blocks the resting K^+ conductance. The lower panel in Fig. 2*A* shows the effect of 0·5 mM Ba^{2+} on whole-cell currents in an isolated toad RPE cell. Ba^{2+} blocked the fast-activating inwardly rectifying current as well as the time-resolved decay of outward current, suggesting that the decay in outward current represents a decrease in outward K^+ current rather than the activation of an inward current. Figure 2*B* (upper panel) shows that the addition of Ba^{2+} to the bath produced a linear I–V relationship and shifted V_0 in the positive direction. In fourteen cells, the application of 0·5 mM Ba^{2+} depolarized V_0 from −71·4 ± 10·8 to −36·4 ± 6·3 mV. This latter value of V_0 is nearly identical to the Cl^- equilibrium potential (−34 mV), consistent with the possibility that the residual conductance was a Cl^- conductance, which has been shown to be present on the basolateral membrane (Fujii *et al.* 1992). Alternatively, it could reflect the presence of a non-selective cation conductance (Hughes & Segawa, 1992).

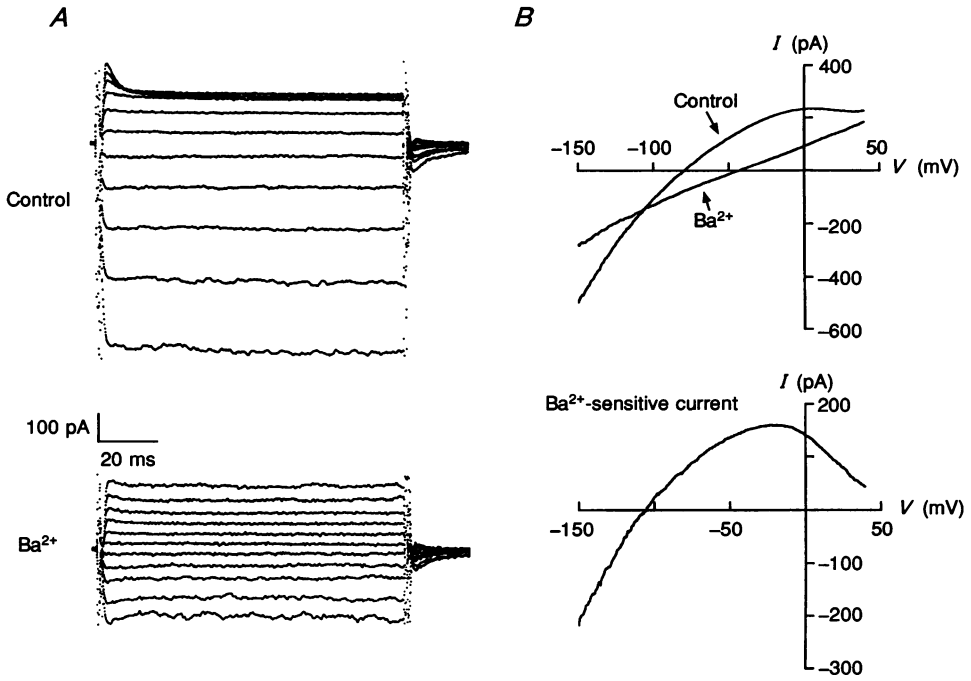

Figure 2. Whole-cell currents and the effect of Ba^{2+}

A, families of whole-cell currents measured in the absence (top) and presence (bottom) of 0·5 mM Ba^{2+}. $[K^+]_o$ was 2 mM. Currents were generated by a series of 100 ms voltage pulses ranging from −160 to +40 mV in 20 mV increments from a holding potential of −70 mV. *B*, steady-state I–V relationships of the same cell as in *A* using a 1 s voltage ramp ranging from −150 to +40 mV. The upper panel shows I–V curves measured in control external solution and in the presence of 0·5 mM Ba^{2+} and the lower panel displays the difference between these two currents. The Ba^{2+}-sensitive current reversed at −104 mV, near E_K.

The I–V relationship of the Ba^{2+}-sensitive current (Fig. 2*B*, lower panel), obtained by taking the difference between currents measured in the absence and presence of Ba^{2+}, exhibited mild inward rectification and substantial outward current flow in the physiological voltage range (−90 to −60 mV). At voltages positive to about −10 mV, the outward current decreased sharply, giving rise to a negative slope conductance. The reversal potential (V_r) of the Ba^{2+}-sensitive current averaged $-104{\cdot}9 \pm 8{\cdot}8$ mV ($n = 14$). This value is not significantly different from the K^+ equilibrium potential ($E_K = -103$ mV), indicating that the inwardly rectifying current is carried by K^+.

To determine the dose–response relationship of the Ba^{2+}-induced block of the inwardly rectifying K^+ current, we measured the effect of various Ba^{2+} concentrations on the whole-cell current at −35 mV, which is near the reversal potential of the residual conductance (see above). At this voltage, residual currents should be negligible, thus isolating the inwardly rectifying K^+ current. Figure 3*A* plots the fraction of K^+ current blocked by Ba^{2+} as a function of the logarithm of the Ba^{2+} concentration. The filled circles represent the mean values of measurements in three to twelve cells and the vertical bars indicate S.D. The continuous trace is the non-linear least-squares fit of the data to the first order equation:

$$I_B/I_{control} = 1 - \{[B]/(K_d + [B])\}, \quad (1)$$

where I_B and $I_{control}$ are the currents measured in the presence and absence of blocker, [B] is the concentration of blocking ion and K_d is the apparent dissociation constant, which is equal to the concentration at which the block is half-maximal. The apparent K_d for Ba^{2+} was 40 μM.

Caesium

As shown previously in isolated RPE cells of the bullfrog (Hughes & Steinberg, 1990) and primate (Wen *et al.* 1993), the inwardly rectifying K^+ current in toad RPE cells was also blocked by extracellular Cs^+. The addition of 5 mM Cs^+ to the bath eliminated the time-dependent decay of outward current, blocked the inwardly rectifying current and shifted V_0 in the positive direction (Fig. 6). In twenty-four cells bathed in 2 mM K^+, the application of 5 mM Cs^+ depolarized V_0 from -68 ± 11 to -34 ± 7 mV. The reversal potential of the Cs^+-sensitive current averaged -106 ± 5 mV, near the K^+ equilibrium potential.

Figure 3*B* shows concentration dependence of the Cs^+-induced block of the K^+ current measured at −35 mV. The filled circles represent the mean values of measurements in four to fourteen cells and the continuous line is the non-linear least squares fit of the data to eqn (1). The apparent K_d for Cs^+ was 790 μM.

Other blockers

The inwardly rectifying current and transient outward current were also blocked by extracellular Rb^+ (2 mM) and diphenylamine-2-carboxylate (DPC, 1 mM), which has recently been shown to block an inwardly rectifying K^+ channel in turtle colon epithelial cells (Richards & Dawson, 1993). The block by DPC developed slowly over a period of 4–10 min and was only partly reversible. Other K^+ channel

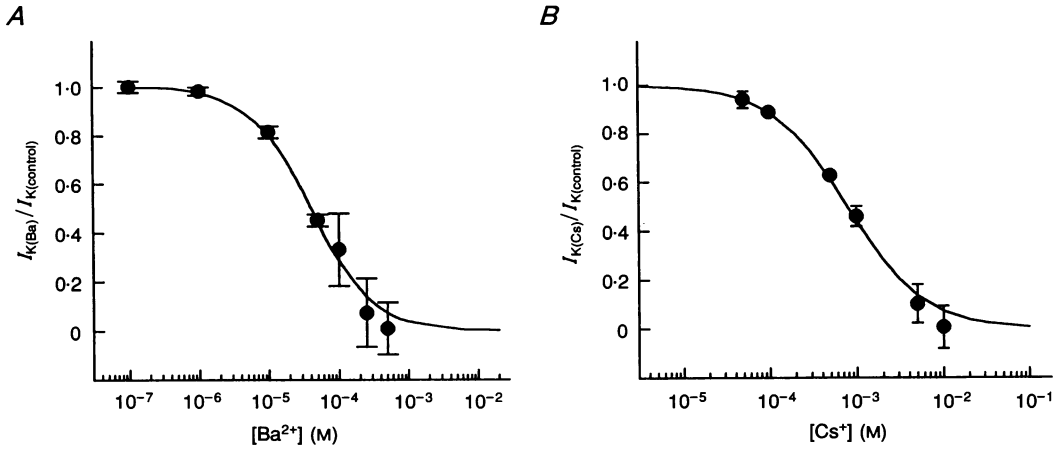

Figure 3. Concentration dependence of the Ba^{2+}- and Cs^+-induced blocks of the inwardly rectifying K^+ current

A, Ba^{2+}. The fraction of K^+ current blocked was estimated by taking the ratio of currents measured at −35 mV in the presence and absence of Ba^{2+}. Each point represents the average of the ratio in 3–12 cells and vertical lines indicate S.D. The continuous curve is the best fit of the data to eqn (1) by non-linear regression analysis, with $K_d = 40$ μM. *B*, Cs^+. The fraction of K^+ current blocked was estimated by taking the ratio of currents measured at −35 mV in the presence and absence of Cs^+. Each point represents the average of measurements made in 4–14 cells and vertical lines indicate S.D. The continuous curve is the best fit of the data to eqn (1) by non-linear regression analysis, with $K_d = 790$ μM.

blockers such as quinidine (100 μM), tolbutamide (1 mM, a specific blocker of ATP-sensitive K^+ channels; Cook & Quast, 1990), 4-aminopyridine (4-AP; 5 mM), and tetraethylammonium (TEA; 20 mM) were ineffective at the concentrations tested.

The insensitivity of the transient outward current to extracellular TEA and 4-AP, and its sensitivity to the same blockers that affected the inward rectifying K^+ current, suggest that it is mediated by the inwardly rectifying channel rather than a transient A-type channel.

Voltage dependence of the Cs^+- and Ba^{2+}-induced block

Inwardly rectifying K^+ currents in starfish oocytes (Hagiwara, Miyazaki & Rosenthal, 1976; Hagiwara, Miyazaki, Moody & Patlak, 1978), skeletal muscle fibres (Gay & Stanfield, 1977; Standen & Stanfield, 1978) and cardiac myocytes (Harvey & Ten Eick, 1989*a*) are blocked by Cs^+ and Ba^{2+} in a time- and voltage-dependent manner. In these channels, external Ba^{2+} and Cs^+ block a larger fraction of the K^+ current at hyperpolarized potentials than they do at depolarized potentials, and this voltage-dependent block leads to a negative slope conductance at negative potentials in *I–V* plots of steady-state currents. To determine whether the Cs^+- and Ba^{2+}-induced blocks of the RPE inwardly rectifying K^+ current were voltage-dependent, we estimated the chord conductance of the inwardly rectifying K^+ channel at various potentials from the equation:

$$g_K = \frac{I_T - g_L(V_m - E_L)}{(V_m - E_K)}, \quad (2)$$

where I_T is the whole-cell current at membrane voltage V_m, E_L is the reversal potential of the leak or residual current (−35 mV), and g_L is the leak conductance estimated from amplitude of whole-cell current measured at E_K divided by the difference between E_K and E_L. Figure 4*A* plots the fraction of g_K blocked by Ba^{2+} as a function of the logarithm of the extracellular Ba^{2+} concentration. The symbols represent mean values of the normalized K^+ conductance at 0 (○), −35 (●) and −60 mV (▼), and the continuous curve is the least squares fit of the data at −35 mV to a first-order equation with the same form as eqn (1). As can be seen, the curve also fits the data at 0 and −60 mV, suggesting that the Ba^{2+}-induced block was voltage independent. Figure 4*B* shows that in a like manner, the dose–response relationship for the Cs^+ block of the inwardly rectifying K^+ conductance was voltage independent.

The calculation of K^+ conductance in the above analysis assumed that the residual current had a linear *I–V* relationship. This assumption is not entirely justified because in some cells the current remaining after Cs^+ or Ba^{2+} addition was mildly inwardly rectifying (see Fig. 6*B*). As a further test of whether the Cs^+- and Ba^{2+}-induced blocks of the inwardly rectifying K^+ current were voltage dependent, we compared the *I–V* relationships of the currents blocked by different concentrations of the blocking ion. We reasoned that if the block was voltage dependent, then the shape of the *I–V* curve should change with different blocker concentrations: at high concentrations of blocking ion, the channel might be blocked at all voltages, but at low concentrations, the block should be greater at negative potentials than at positive potentials. Figure 5*A* and *B* compare *I–V* plots of Ba^{2+}- and Cs^+-sensitive currents corresponding to different blocker concentrations, and these same data are shown in Fig. 5*C* and *D* scaled to

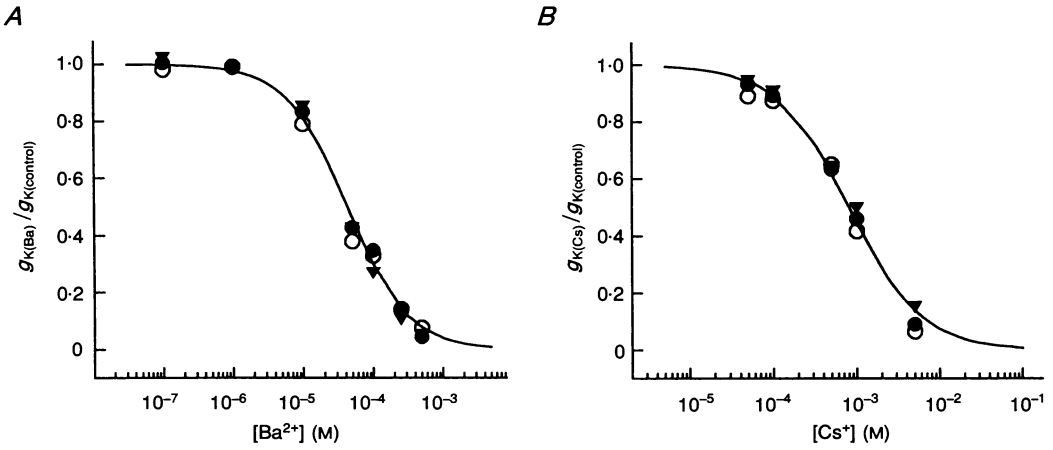

Figure 4. Voltage independence of the Ba^{2+}- and Cs^+-induced blocks of the inwardly rectifying K^+ conductance (g_K)

g_K was calculated according to eqn (2) at V_m values of 0 (○), −35 (●) and −60 mV (▼). The ratio of g_K measured in presence and absence of Ba^{2+} (*A*) or Cs^+ (*B*) is plotted as a function of the logarithm of the concentration of blocking ion. Each point represents the mean value of measurements in 3–12 cells. The continuous curve is the non-linear least squares fit of the data measured at $V_m = -35$ mV by an equation with the same form as eqn (1). Data corresponding to 0 and −60 mV also fit to this curve. K_d was 42 μM for Ba^{2+} and 800 μM for Cs^+.

superimpose on $I-V$ curve of the current blocked by the highest blocker concentration. The shape of the $I-V$ curves and their position along the x-axis are nearly identical over a wide range of blocker concentrations, providing further evidence that Cs^+- and Ba^{2+}-induced blocks of the inwardly rectifying K^+ current are voltage independent.

Selectivity of the inwardly rectifying K^+ conductance

To determine the selectivity of the inwardly rectifying K^+ current, we measured its reversal potential (V_r) as a function of extracellular K^+ concentration ($[K^+]_o$). Cells were superfused with solutions containing 0·5, 2, 5, 10, 20, or 50 mM K^+ and V_r was estimated from V_0 of the whole-cell current component blocked by either 0·5 mM Ba^{2+}, 2 mM Rb^+, or 5 mM Cs^+. Each measurement was made in at least three cells. Over this range of $[K^+]_o$, the mean value of V_r was not significantly different from E_K (data not shown), indicating that the inwardly rectifying K^+ conductance of the toad RPE is highly selective for K^+.

Dependence of the inwardly rectifying K^+ conductance on $[K^+]_o$

In the intact eye, the concentration of K^+ outside the RPE apical membrane decreases during the transition between dark and light from approximately 5 to 2 mM due to changes in photoreceptor activity (Oakley, 1977). This decrease in extracellular K^+ concentration, in turn, hyperpolarizes the apical membrane owing to its relatively large K^+ conductance, generating part of the c-wave component of the direct current electroretinogram (Steinberg *et al.* 1985). We were therefore interested in determining how the properties of inwardly rectifying K^+ conductance might be affected by changes in $[K^+]_o$.

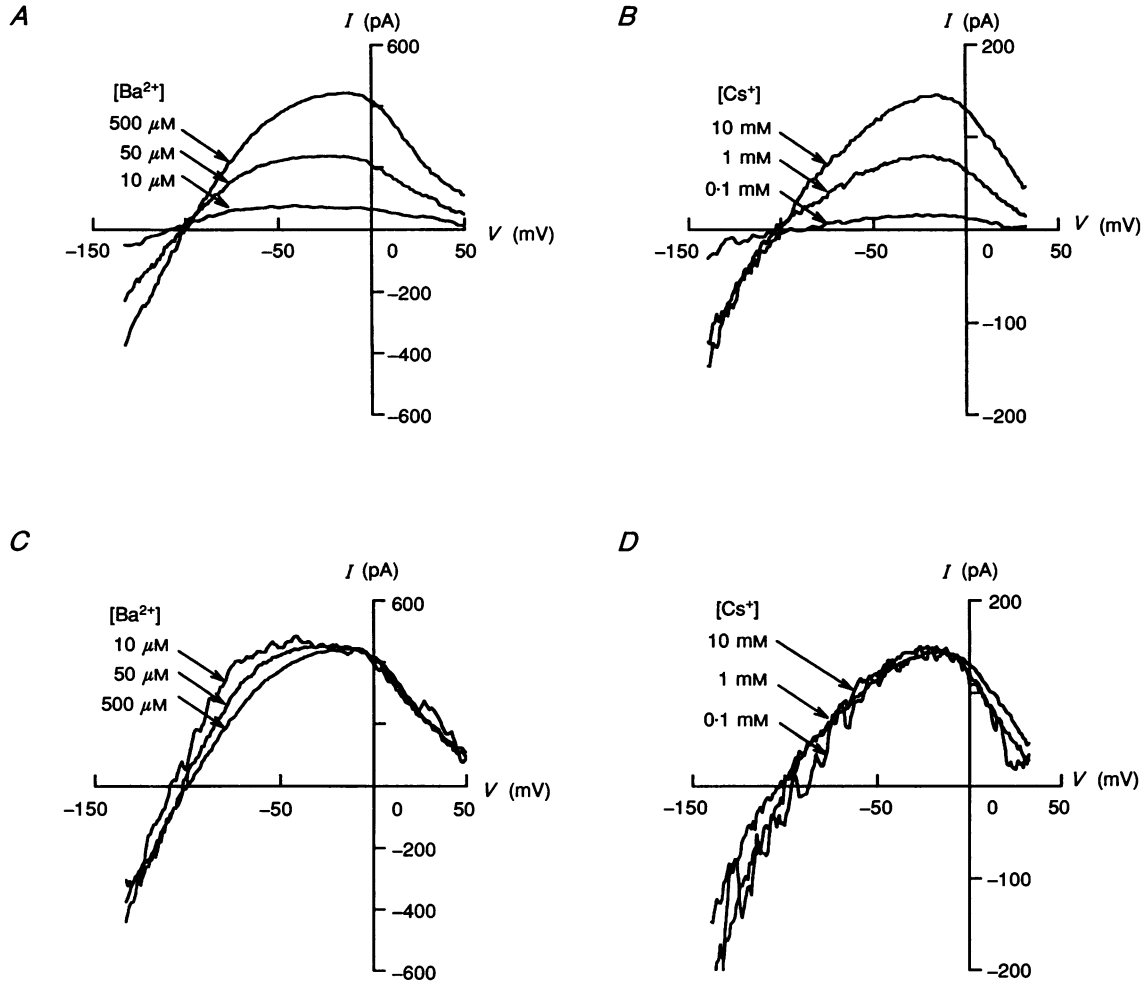

Figure 5. Voltage independence of the Ba^{2+}- and Cs^+-induced blocks of whole-cell currents
A, $I-V$ relationships of the whole-cell current component blocked by 10, 50 and 500 μM Ba^{2+}. *B*, $I-V$ relationships of the whole-cell current component blocked by 0·1, 1 and 10 mM Cs^+. *C*, same data as *A* scaled to superimpose on the $I-V$ curve of the current blocked by 500 μM Ba^{2+}. Scaling factors were 6·3 for current blocked by 10 μM Ba^{2+} and 1·9 for current blocked by 50 μM Ba^{2+}. *D*, same data as in *B* scaled to superimpose on the $I-V$ curve of the current blocked by 10 mM Cs^+. Scaling factors were 9·3 for current blocked by 0·1 mM Cs^+ and 1·9 for current blocked by 1 mM Cs^+.

Figure 6*A* compares the steady-state *I–V* relationships of whole-cell currents in a single isolated RPE cell bathed in 0·5, 2 and 5 mM K^+. Increasing $[K^+]_o$ decreased the rectification of the *I–V* curve and shifted its position along the voltage axis in the positive direction. These effects can be attributed to changes in K^+ current, since in the presence of 5 mM Cs^+, the *I–V* curve was nearly the same in all K^+ concentrations (Fig. 6*B*). Similar results were obtained when 0·5 mM Ba^{2+} was used instead of Cs^+. Figure 6*C* plots *I–V* relationships of the isolated K^+ currents. Outward currents rectified most strongly in 0·5 mM K^+, resulting in a peak in the *I–V* curve and a pronounced negative slope conductance at potentials positive to about −25 mV. With increasing $[K^+]_o$, there were decreases both in the slope conductance near the reversal potential and in the negative slope conductance at positive potentials. The *I–V* curve for 0·5 mM K^+ crossed over the curves for 2 and 5 mM K^+ at around −5 mV and 0 mV respectively, leading to larger current amplitudes in high $[K^+]_o$ than in low $[K^+]_o$.

To view these changes in the *I–V* curve in terms of the K^+ conductance, we calculated the chord conductance, g_K, according to the equation:

$$g_K = I_K/(V_m - E_K). \quad (4)$$

Figure 6*D* summarizes the results of measurements in eleven cells bathed in 0·5, 2 and 5 mM K^+ and plots the normalized conductance as a function of voltage. At voltages negative to about −30 mV, the normalized conductance was largest in 0·5 mM K^+ and decreased with increasing $[K^+]_o$. The K^+ conductance decreased even further when $[K^+]_o$ was increased to 10, 20 or 50 mM (not shown), but part of this effect might have been due to the reduced Na^+ concentration in these solutions (see below).

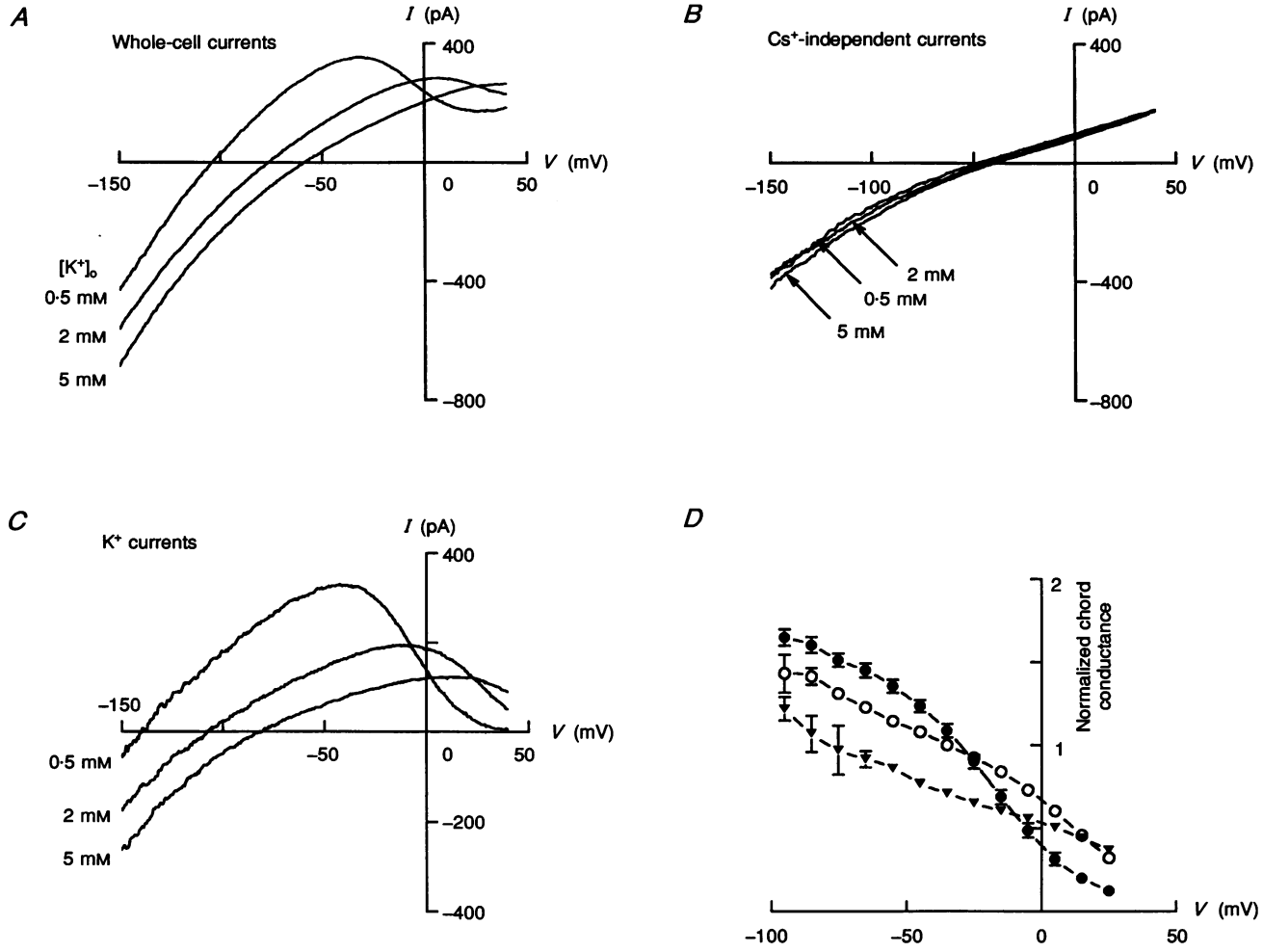

Figure 6. Dependence of K^+ current and conductance on $[K^+]_o$
A, *I–V* relationships of whole-cell currents measured in a single cell bathed in 0·5, 2 and 5 mM $[K^+]_o$. *B*, *I–V* relationships of currents measured in the same cell after applying 5 mM Cs^+. *C*, K^+ currents isolated by taking the difference between the currents shown in *A* and *B*. *D*, normalized conductance–voltage relationship in extracellular K^+ concentrations of 0·5 (●), 2 (○), and 5 (▼) mM. For each cell, conductances were normalized to that measured at −35 mV in 2 mM K^+. Each point represents the mean of measurements in eleven cells and vertical lines indicate S.E.M.

For the physiologically relevant K^+ concentrations at −75 mV, the conductance was approximately 25 % smaller in 5 mM K^+ than it was in 2 mM K^+. These results suggest that at negative membrane potentials, extracellular K^+ may block the inwardly rectifying K^+ channel.

At all K^+ concentrations, g_K decreased with membrane depolarization, but the shape of the conductance–voltage curve was concentration dependent. In 5 mM K^+, g_K decreased monotonically with depolarization. Although the conductance–voltage curve for 0·5 mM K^+ was essentially parallel to the other curves in the voltage range between −100 and −50 mV, it became steeper at more positive potentials, resulting in a smaller conductance at +25 mV in 0·5 mM K^+ than in either 2 or 5 mM K^+. The conductance–voltage curve for 2 mM K^+ exhibited a qualitatively similar voltage dependence, but the roll-off was smaller and it occurred at more positive potentials.

Since the inward rectification of the K^+ conductance at positive potentials was due in large part to the time-resolved decay in outward K^+ current (Fig. 2), we examined the time course of whole-cell currents as a function of $[K^+]_o$. Figure 7 compares families of whole-cell currents measured in the same cell in four different concentrations of extracellular K^+. Currents were evoked by a series of 100 ms voltage pulses ranging from −160 to +60 mV in 20 mV increments, with the membrane potential held at −70 mV between voltage pulses. Decreasing $[K^+]_o$ from 2 to 0·5 mM increased the size of the time-dependent decay of outward K^+ currents, resulting in steady-state outward currents at +20, +40 and +60 mV that were smaller than the current at −40 mV. In contrast, increasing $[K^+]_o$ to 5 or 10 mM dramatically decreased the size of the outward current relaxations. Although the driving force for outward K^+ movement decreased with increasing $[K^+]_o$, this alone cannot account for the differences in the amplitudes of the current relaxations. For example, at +60 mV, the outward driving force was approximately 30 % smaller in 5 mM K^+ than in 0·5 mM K^+ and yet the amplitude of the current relaxation was more than 75 % smaller in 5 mM than in 0·5 mM K^+. This result implies that the conductance decrease underlying the relaxation in K^+ current was considerably smaller in 5 mM K^+ than in 0·5 mM K^+. Although a smaller absolute conductance change is expected from the K^+ dependence of the K^+ conductance at negative potentials, the cross-over of the conductance–voltage curves indicates that the decrease in K^+ conductance during the relaxation was proportionally smaller in high $[K^+]_o$ than in low $[K^+]_o$.

Dependence on extracellular Na^+

A common feature of inwardly rectifying K^+ currents in other cell types is a voltage-dependent block by extracellular Na^+ (Standen & Stanfield, 1979; Harvey & Ten Eick, 1989*b*; Cooper, Rae & Dewey, 1991). This phenomenon is usually manifested by a relaxation of inward currents at large, negative potentials, leading to a negative slope conductance in *I–V* plots of steady-state currents. Although relaxations of inward currents were rarely observed in the toad RPE (1 out of 138 cells), it is possible that a Na^+ block of the K^+ channel occurred on a faster

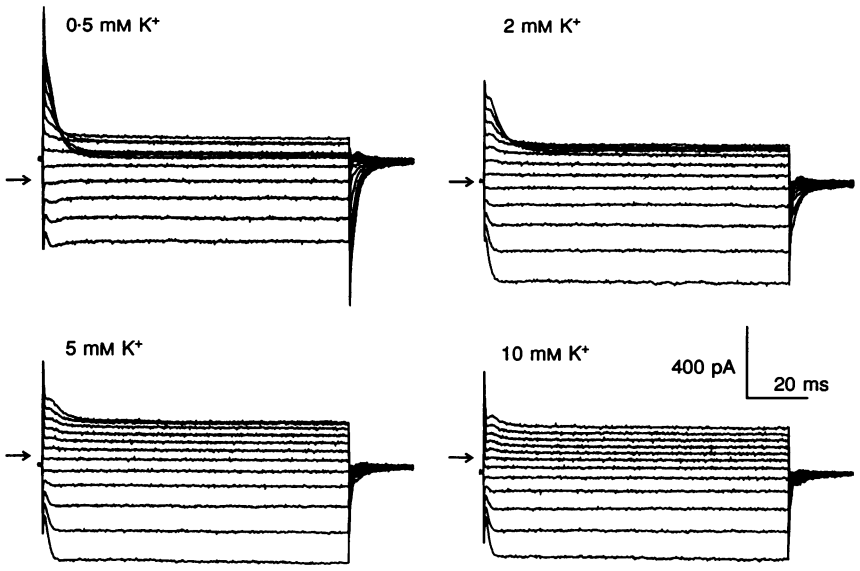

Figure 7. Dependence of outward K^+ current relaxation on $[K^+]_o$
Families of whole-cell currents were measured in the same cell bathed in 0·5, 2, 5 and 10 mM K^+ external solution. Currents were evoked by 100 ms voltage pulses from a holding potential of −70 mV to potentials ranging from −160 to +60 mV in 20 mV steps. Horizontal arrows to the left of each family of currents indicate the zero-current level.

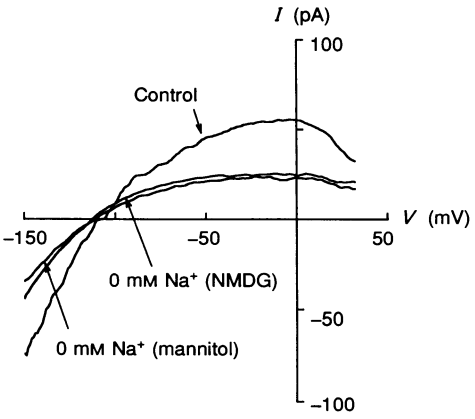

Figure 8. Na^+ dependence of the inwardly rectifying K^+ current
I–V relationships of the K^+ current was measured in a cell bathed with control external solution (110 mM Na^+) and then in two Na^+-free solutions, one in which Na^+ was replaced with NMDG and the other in which NaCl was replaced with 220 mM mannitol. K^+ currents were isolated by taking the difference between the currents in the absence and the presence of 0·5 mM Ba^{2+} in each condition. Na^+ removal by either manoeuvre appeared to block the same fraction of I_K.

time scale than we could resolve. Hence, we tested the possibility of an interaction between extracellular Na^+ and the inwardly rectifying K^+ conductance by examining the effect of removing Na^+ from the external solution. Figure 8 compares steady-state *I–V* curves for the isolated K^+ (Ba^{2+}-sensitive) current measured in control external solution and then in an external solution in which Na^+ was completely replaced by *N*-methyl-D-glucamine (NMDG). The replacement of Na^+ with NMDG reduced the size of the inwardly rectifying current at −60 mV by about 50 %. Similar results were obtained in three additional cells.

The reduction in the amplitude of the inwardly rectifying current by Na^+ replacement with NMDG could indicate a dependence of the K^+ conductance on extracellular Na^+, or a block of the channel by the replacement ion. To test this latter possibility, we superfused the same cell with a solution in which NaCl had been replaced with mannitol to keep the osmolality constant and the results of this experiment are also shown in Fig. 8. Again, the outward K^+ current was reduced by almost 50 % by removing external Na^+. Together, these results suggest that inwardly rectifying K^+ conductance is regulated, directly or indirectly, by external Na^+.

DISCUSSION

The present study shows that the membrane properties of freshly isolated toad RPE cells are dominated by an inwardly rectifying K^+ conductance. These results differ from previous observations in isolated RPE cells of the bullfrog (Hughes & Steinberg, 1990) and primate (Wen *et al.* 1993), which, in addition to an inwardly rectifying K^+ current, also contain a delayed outwardly rectifying K^+ current. The absence of an outwardly rectifying K^+ current in the toad RPE made it possible to measure outward K^+ flow through the inwardly rectifying K^+ conductance without contamination by other time-dependent currents.

Since the RPE inwardly rectifying K^+ conductance allows outward K^+ flow at the zero-current potential, it is a major determinant of the resting membrane potential. This is evident from the magnitude of the change in the zero-current potential (V_0) that occurred when $[K^+]_o$ was increased from 0·5 to 5 mM, which indicated that the relative K^+ conductance was approximately 0·74. This resting K^+ conductance can be ascribed to the inwardly rectifying K^+ conductance, since blockade of the inward rectifier by either Ba^{2+} or Cs^+ depolarized the zero-current potential (V_0) from approximately −70 to −35 mV and made membrane currents insensitive to changes of $[K^+]_o$.

Membrane location

Studies in the bullfrog RPE–choroid have established the presence of relatively large K^+ conductances on both apical and basolateral membranes (Miller & Steinberg, 1977). Although the apical membrane of the toad RPE is also known to have a relatively large K^+ conductance (Griff, 1991), nothing is known about the relative K^+ conductance of the basolateral membrane. Considerations of the ratio of apical-to-basal membrane resistances, however, suggest that the inwardly rectifying K^+ conductance lies mainly in the apical membrane. Intracellular recordings in the toad RPE–choroid preparation have shown that the ratio of apical-to-basolateral membrane resistances is 0·6–0·8 (Griff, 1991; Fujii *et al.* 1992), indicating that 37–44 % of the total membrane conductance lies in the basolateral membrane. Since the relative Cl^- conductance of the basolateral membrane is approximately 0·45 (Fujii *et al.* 1992), and the inwardly rectifying K^+ conductance accounts for about 75 % of the total membrane conductance, at most 28–29 % of the inwardly rectifying K^+ conductance could reside in the basolateral membrane.

There is pharmacological evidence in the bullfrog RPE to support the notion that the inwardly rectifying K^+ conductance represents the resting K^+ conductance of the apical membrane. Previous studies in bullfrog RPE–choroid showed that the apical membrane K^+ conductance was blocked by 200 μM Ba^{2+}, whereas a concentration of 5 mM was required to block the basolateral membrane K^+ conductance (Griff *et al.* 1985). In the present study, we found that the Ba^{2+}-induced block of the inwardly rectifying K^+ conductance had an apparent K_d of 43 μM, and that at 200 μM the block was nearly complete. Preliminary studies indicate that in millimolar concentrations, Ba^{2+} also blocks a non-selective cation conductance (Hughes & Segawa, 1992). Assuming the inwardly rectifying K^+ conductances of the bullfrog and toad RPE have a similar sensitivity to Ba^{2+}, these results suggest that the inwardly rectifying K^+

conductance represents the resting K^+ conductance of the apical membrane.

Comparison to other inwardly rectifying K^+ currents

Inwardly rectifying K^+ currents have been identified in a variety of cell types. Classic inwardly rectifying K^+ channels present in frog skeletal muscle (Leech & Stanfield, 1981) and starfish oocytes (Hagiwara & Takahashi, 1974) open with a steep voltage dependence on membrane hyperpolarization, and the voltage dependence of their gating depends on the extracellular $[K^+]$, shifting along the voltage axis with the quantity $RT\ln[K^+]_o$. A number of other inwardly rectifying K^+ channels are more weakly voltage dependent and allow measurable outward current flow; these include cardiac muscarinic K^+ channels and ATP-sensitive K^+ channels (Noma, 1983; Soejima & Noma, 1984) and certain epithelial K^+ channels (Parent, Cardinal & Sauve, 1988; Giebisch, Hunter & Kawahara, 1990; Hunter, 1991; Mieno & Kajiyama, 1991; Richards & Dawson, 1993). The toad RPE K^+ conductance likewise shows moderate inward rectification.

Inward rectification of K^+ currents in other cells has been shown to result from a voltage dependence of the channel's intrinsic gating (Silver & DeCoursey, 1990) or from a voltage-dependent block by some intracellular ion such as Na^+ and Mg^{2+} (Yellen, 1984; Matsuda, Saigusa & Irasawa, 1987). In the present study, we found evidence for two possible mechanisms causing inward rectification: a fast process active in the physiological voltage range that resulted in rectification with a time constant less than 1 ms, and a time-resolved process active at depolarized potentials that produced negative slope conductances. This latter mechanism was enhanced in low $[K^+]_o$ and decreased in high $[K^+]_o$, suggesting the possibility that extracellular K^+ relieves the voltage-dependent block of the inwardly rectifying K^+ channel by some intracellular ion, as has been shown for other K^+ channels (Yellen, 1984; Matsuda, 1991). Experiments in the whole-cell recording mode, which allows internal dialysis with divalent cations, will be required to confirm this possibility and to identify the blocking ion.

Several properties distinguish the RPE inwardly rectifying K^+ conductance from inward rectifiers in other cell types. The conductance of inward rectifiers generally increases with the square root of $[K^+]_o$ (Hagiwara *et al.* 1976; Standen & Stanfield, 1978; Cooper *et al.* 1991). In contrast, at negative membrane potentials the RPE inwardly rectifying K^+ conductance decreased with increases in $[K^+]_o$. This effect was apparent even for relatively small concentration changes in the physiological range from 2 to 5 mM. The mechanism for the reduction in K^+ conductance by increases in extracellular $[K^+]$ is not known, but it could be explained by a two-site multi-ion channel model in which the conductance decreases at higher concentrations of permeant ion as the probability of the channel being doubly occupied increases (Hille & Schwartz, 1978).

The blocker sensitivity of the RPE inwardly rectifying K^+ conductance is qualitatively similar to that of other inward rectifiers in that Ba^{2+} and Cs^+ are effective blockers, but 4-AP and TEA are not (Cook & Quast, 1990). However, a common feature of inwardly rectifying K^+ channels in other tissues is that the blocks by external Cs^+ and Ba^{2+} are voltage dependent (Hagiwara *et al.* 1976; Gay & Stanfield, 1977; Standen & Stanfield, 1978; Harvey & Ten Eick, 1989*a*). This voltage dependence is commonly understood in terms of a model in which the blocking ion is driven under the influence of the transmembrane electric field part way into the channel, where it binds to a site and blocks the channel. For an external blocking cation, the block is enhanced by membrane hyperpolarization and relieved by depolarization. The present results provide evidence that, in the case of the RPE inwardly rectifying K^+ conductance, the Ba^{2+}- and Cs^+-induced blocks are voltage independent (see Figs 4 and 5). Hence, it seems possible that these ions block the conduction to K^+ by binding to a site that lies near the external mouth of the channel but outside the electric field that drops across the membrane.

Inwardly rectifying K^+ channels in some other tissues are also blocked by external Na^+ in a voltage-dependent manner (Standen & Stanfield, 1979; Harvey & Ten Eick, 1989*b*; Cooper *et al.* 1991), which causes a time-dependent relaxation of inward currents during sustained hyperpolarization. In the toad RPE, relaxations of inward K^+ currents were rarely observed in the presence of high concentrations of external Na^+, indicating that this cation does not block the K^+ current. Similar results have been reported for freshly dissociated human and monkey RPE cells, although cultured primate RPE cells apparently contain a different type of inwardly rectifying K^+ current that exhibits a voltage-dependent block by external Na^+ (Wen *et al.* 1993). Nonetheless, the inwardly rectifying K^+ conductance appeared to be regulated by Na^+, since it was decreased by the removal of extracellular Na^+ (Fig. 8). This result is reminiscent of the decrease in the inwardly rectifying K^+ conductance in cardiac myocytes produced by the removal of extracellular Na^+ (Harvey & Ten Eick, 1989*b*), which has been attributed to a block by intracellular acidification that results from the suppression of Na^+–H^+ exchange. Additional experiments are required to ascertain whether the Na^+ effect on the inwardly rectifying K^+ conductance in RPE occurs by this or some other mechanism.

Physiological significance

The frog RPE actively transports K^+ in the retina-to-choroid direction (Miller & Steinberg, 1977). Active K^+ transport across the RPE begins with K^+ transport into the cell across the apical membrane by the Na^+–K^+ pump (Miller, Steinberg & Oakley, 1978; la Cour *et al.* 1986) and Na^+–K^+–Cl^- cotransporter (Adorante & Miller, 1989; Miller & Edelman, 1990), where it accumulates above electrochemical equilibrium. Since K^+ conductances are

present at both membranes of bullfrog RPE, some of this K^+ diffuses back across the apical membrane, while the remainder flows out across the basolateral membrane to generate net K^+ absorption (la Cour *et al.* 1986). Since the inwardly rectifying K^+ conductance allows outward K^+ flow, it is well suited to serve as an efflux pathway.

In the intact eye, concentration of K^+ in the subretinal space outside the RPE apical membrane transiently decreases at the transition between dark and light from about 5 to 2 mM due to changes in photoreceptor activity (Oakley, 1977). Assuming that the inwardly rectifying K^+ conductance is located on the apical membrane (see above), its K^+ dependence has important implications regarding K^+ movements between the subretinal space and the RPE. In a previous study on the isolated frog RPE–choroid preparation, la Cour *et al.* (1986) presented indirect evidence that the apical K^+ permeability is decreased following increases in apical $[K^+]_o$. The cause of this apparent permeability change was not elucidated, but the present data suggest that it could result from a direct effect of $[K^+]_o$ on the apical K^+ conductance. Since the inwardly rectifying K^+ conductance is inversely dependent on $[K^+]_o$ in the physiological voltage range, the rate of K^+ efflux from the RPE into the subretinal space will tend to be higher in the face of a subretinal $[K^+]$ decrease than it would be if the conductance displayed Goldman rectification. This mechanism, together with the inhibition of the Na^+–K^+ pump (Griff *et al.* 1985) and Na^+–K^+–Cl^- cotransporter (Joseph & Miller, 1991) by the decrease in $[K^+]_o$, will reduce K^+ absorption across the RPE, thus buffering the photoreceptor-induced decrease in subretinal K^+ concentration.

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