Identification of Kir2.1 Channel Activity in Cultured Trabecular Meshwork Cells

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PURPOSE. To study the presence of inwardly rectifying K⁺ (Kir) channels in cultured bovine (BTM) and human (HTM) trabecular meshwork cells.

METHODS. Cultures of BTM and HTM cells were obtained by an extracellular matrix digestion technique. Whole-cell patch-clamp recordings of BTM cells were performed with the appropriate solutions to detect K⁺ currents. Also, Western blot analysis of Kir2.1 protein expression was performed on both cultured BTM and HTM cells.

RESULTS. A strong inwardly rectifying current at negative potentials to the equilibrium potential for K⁺ (Eₘ₉) and highly selective for K⁺ was detected in 60% of cultured BTM cells. The slope conductance of the inward rectification was more pronounced when the extracellular [K⁺] was increased and was proportional to [K⁺]₀.45. The current was blocked by Ba²⁺ and Cs⁺ in a voltage- and concentration-dependent manner, with K₆ at 0 mV, of 74.7 μM and 45.6 mM, respectively. Current amplitude was reduced by increasing extracellular [Ca²⁺]. The current was insensitive to 10 μM glibenclamide and 10 nM tertiapin. The application of 100 μM 8-Br-cAMP reduced the current by 50%. Kir2.1 channel expression was detected in confluent monolayers of BTM and HTM cells by Western blot analysis.

CONCLUSIONS. A population of cultured BTM cells expressed an inwardly rectifying K⁺ current that illustrates the biophysical and pharmacologic characteristics of the detected Kir2.1 channel protein. Kir2.1 channels are also thought to be present in HTM cells. Kir2.1 channels could be related to TM physiology, because they are involved in contractile and cell volume regulatory responses, two mechanisms that modify TM permeability. (Invest Ophthal Vis Sci. 2001;42:2371-2379)

The trabecular meshwork (TM) works as a filter that provides resistance to aqueous humor outflow, and it is a determinant of the intraocular pressure.¹ It is well accepted that morphologic alterations of the TM that increase its resistance are related to development of glaucoma.² It has generally been accepted that the TM by itself cannot modulate its permeability. It is the tone of the ciliary muscle that does this by pulling or relaxing its tendons inserted in the TM.³ However, recent studies have pointed out that regulatory volume responses⁴,⁵ or contractile properties⁶ of the TM can also modulate the resistance of the conventional outflow pathway, independently from the ciliary muscle action. Taking into account that the TM can show an active role in aqueous humor outflow regulation, a further knowledge of the properties from the cells that form this tissue is required.

The TM is a heterogeneous tissue with different and well-characterized morphologic regions.³ In fact, TM cell cultures from different species display heterogeneity in the morphology and protein expression (i.e., α-smooth muscle actin)⁷ as observed in the intact tissue.⁸,⁹ A correlation between morphology and cell function was suggested by Coroneo et al.¹⁰ after their observation that two morphologically distinct cell types in bovine trabecular meshwork (BTM) cultures had high and low resting membrane potentials (Eₘ₉). The Eₘ₉ is crucial for cell function, because it is involved in many cell responses (e.g., secretion, contraction). It is possible to hypothesize that the basis of the different Eₘ₉ found in cultured TM cells is attributable to differential expression of ion channels, especially of those contributing to the establishment of Eₘ₉.

The channels of the inwardly rectifying potassium (Kir) channel family conduct inward currents at potentials negative to the equilibrium potential for K⁺ (Eₘ₉). They also permit smaller outward currents at potentials positive to the Eₘ₉ and participate in the establishment of Eₘ₉.¹² Up to the present, members of the Kir family have been found in different cell types, and their function is related to cell excitability and K⁺ transport.¹³ The main objective of the present work was to study the presence and physiological conditions of Kir channels in cultured TM cells under physiological conditions (normal culture conditions), to obtain a better understanding of TM function. In this study, the results showed that a Kir2.1 channel was functionally expressed in more than half of cultured bovine trabecular meshwork (BTM) cells. The expression of this channel was also shown in cultured human trabecular meshwork (HTM) cells.

MATERIALS AND METHODS

Cell Culture

BTM and HTM cells were cultured by an extracellular matrix digestion procedure described elsewhere.¹⁴,¹⁵ Strip cultures were obtained from anterior segment tissues under a dissecting microscope in sterile conditions. Tissue strips were enzymatically digested for 40 to 60 minutes in a PBS solution containing 1.5 to 2.5 mg/ml collagenase (Sigma, Madrid, Spain), followed by a mechanical trituration using siliconized, fire-polished Pasteur pipettes. Collagenase action was stopped by adding 10 mg/ml bovine serum albumin (Sigma) and the solution was centrifuged at 100g for 10 minutes. The pellet containing the cells was resuspended in DMEM culture medium (Bio-Whitaker, Barcelona, Spain) supplemented with 10–12% fetal bovine serum (Sigma), 100 U/ml penicillin, 10 mg/ml streptomycin, and 2.5 μg/ml amphotericin-B (Bio-Whitaker, Barcelona, Spain) and seeded on 25 cm² culture flasks. Cells were passaged using trypsin-EDTA (Bio-Whitaker). Experiments were performed on first- and second-passage BTM cells and first- to fourth-passage HTM cells.

Donor eyes, generously provided by the eye bank of Institut Universitari Barraquer (Barcelona, Spain), were used for the HTM cell...
cultures. In all cases (n = 4) the TM was dissected after the cornea was removed for transplantation purposes. The average of the donors was 42 ± 5 years, and the time lapse between the death and dissection was always less than 20 hours. Cells displayed typical HTM appearance.\textsuperscript{16} Immunochemistry for desmin and α-smooth muscle actin was performed using a previously described technique.\textsuperscript{9} The primary antibody for desmin was a polyclonal raised in rabbit (Sigma) and for α-smooth muscle actin was a monoclonal antibody raised in mouse (clone no. 1A4; Sigma). A negative staining for desmin and a positive staining for α-smooth muscle actin in 8% of the cells were observed. These properties are characteristic of HTM cell cultures\textsuperscript{8} and thus they were considered successful.

**Electrophysiological Recording and Analysis**

Ionic currents were recorded at 36.5°C in a thermostated chamber (PDM1-2; Harvard Apparatus, S. Natick, MA) using the whole-cell mode of the patch-clamp technique.\textsuperscript{17} Patch pipettes were made from borosilicate glass (1B150-5; World Precision Instruments, Sarasota, FL), pulled (CA P-97; Sutter Instrument, San Rafael, CA) and heat polished (MF-830; Narishige Scientific Laboratory, Tokyo, Japan) to give resistances of 2 to 4 MΩ when filled with electrode solution.

Recordings were made with a patch-clamp amplifier (EPC-7; Heka Electronics, Lambrecht, Germany) and a data-acquisition interface (CED 1401; Cambridge Electronic Design, Cambridge, UK), driven by a software program (WCP for Windows ver. 2.1–2.4; Strathclyde University, Glasgow, Scotland, UK) that was kindly provided by John Dempster (Strathclyde University). Membrane voltages (V_m) are reported without correction for the liquid junction potential between the pipette and bath solutions, because its value was small (1–4 mV). Cell capacitance was measured with the patch-clamp amplifier cancellation circuitry. The mean cell capacitance was 46 ± 2 pF (n = 112). There was no electrical coupling between cells, because all experiments were performed in cultures in pre confluent states. Series resistance (R_s) was measured at random intervals during the experiment by analyzing the capacitive current transients, as previously described.\textsuperscript{18} R_s was typically in the range of 4 to 15 MΩ and was compensated when it was higher than 8 to 10 MΩ.

Data are reported as mean ± SEM. Curve fitting was performed on computer (Origin 5.0 software; Microcal Software, Northampton, MA).

**Solutions and Drugs**

Cells were dialyzed with electrode solution containing (mM) 107 KCl, 25 KOH, 1 MgCl_2, 2.5 Na_2 adenosine triphosphate (ATP), 10 EGTA, and 10 HEPES (pH 7.2, adjusted with KOH). The standard extracellular solution (physiological solution), contained (mM) 130 NaCl, 2.7 KCl, 2.5 CaCl_2, 1 MgCl_2, 10 HEPES, and 10 glucose. In the solutions in which KCl was increased to 10, 50, and 150 mM, the equivalent amount of NaCl was removed. All extracellular solutions were adjusted to pH 7.42 with NaOH and had an average osmolality of 295 ± 5 mOsm/kg.

Glibenclamide and all the salts used for preparing the solutions were obtained from Sigma. Tertiapin was obtained from Alomone Laboratories (Jerusalem, Israel).

**Western Blot Analysis**

Cell homogenates were prepared from confluent BTM and HTM cell cultures grown in 25-cm\textsuperscript{2} culture flasks. Cell cultures were suspended in TH buffer containing 25 mM Tris (pH 7.4); 150 mM NaCl; 5 mM EDTA; 1 mM phenylmethylsulfonyl fluoride (PMSF); 5 μg/ml each aprotinin, leupeptin, and pepstatin; 0.1% sodium dodecyl sulfate (SDS); and 1% deoxycholate. Cell homogenates were obtained by adding 300 μl TH and gently scratching the bottom of the flask with a pipette. Further homogenization was achieved by mildly stirring the sample. Protein concentration was determined using a BCA kit (Pierce, Rockford, IL).

BTM and HTM proteins were fractionated by SDS-PAGE. Each lane contained 25 μg protein resuspended in loading buffer (0.125 mM Tris [pH 6.8], 20% glycerol, 10% mercaptoethanol, 4% SDS, and 0.002% bromophenol blue). Protein analyzed by electrophoresis was transferred to nitrocellulose membranes (Amersham, Madrid, Spain) using a semidry transfer system (Bio-Rad, Madrid, Spain). After that, the membranes were blocked with T-TBS buffer containing Tris (pH 7.4), 150 mM NaCl, 0.1% Tween-20, and 5% skimmed milk. The primary antibody against Kir2.1 (Alomone Laboratories) was used at a dilution of 1:400 in T-TBS and incubated overnight at 4°C. The Kir2.1 polyclonal antibody was raised in rabbit against a highly purified peptide corresponding to amino acid residues 392-410 of human Kir2.1 channel. The epitope is specific for Kir2.1, and it is identical in bovine species. After washing, the membranes were incubated with the secondary anti-rabbit antibody labeled with horse radish peroxidase (HRP) at a dilution of 1:1000 for 1 hour at room temperature. The membranes were washed and then developed with a chemiluminescent Western blot system (ECL; Amersham, Madrid, Spain). The specificity of the primary antibody was tested by incubating 25 μg of the Kir2.1 antibody with the corresponding antigenic peptide for 1 hour at room temperature before incubating it with the membrane.

**Results**

**Ionic Selectivity and Conduction Properties**

To identify the presence of Kir channels in BTM cells, the V_m was changed in a 2-second ramp from −150 mV to +60 mV. In all the experiments cells were dialyzed with 130 mM KCl. Because the only K\textsuperscript{+} channel described in BTM cells has been the high-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel (BK_{Ca}),\textsuperscript{19} 10 mM EGTA was introduced in the electrode solution to prevent the channel’s opening. Moreover, the V_m was held at 0 mV to inactivate possible voltage-dependent K\textsuperscript{+} currents. The voltage ramps showed an inwardly rectifying current at potentials negative to the E_{K+}, in 77 (60%) of 129 cells. The current showed an increase in the slope conductance of the inward rectification when the extracellular [K\textsuperscript{+}] was increased (Fig. 1A). To test K\textsuperscript{+} selectivity, the reversal potentials of the inwardly rectifying current were calculated at different extracellular [K\textsuperscript{+}]. When the [K\textsuperscript{+}] was changed from 2.7 mM to 10, 50, and 130 mM, the reversal potentials were −98.2 ± 3 mV (n = 10), −67.0 ± 2 mV (n = 7), −26.0 ± 2 mV (n = 10), and −1.0 ± 0.5 mV (n = 9), respectively. The plot of those values against the logarithmic [K\textsuperscript{+}] provided a linear relationship (r > 0.999) with a slope of −59 mV, close to the theoretical level of −61 mV predicted by the Nernst equation in the experimental conditions used (Fig. 1B).

The slope conductance of the inwardly rectifying current was calculated 10 mV below the E_{K+}, and normalized to the cell capacitance to correct changes in cell size. When the extracellular [K\textsuperscript{+}] was 10, 50, and 130 mM, the slope conductance was 0.12 ± 0.03 (n = 8), 0.17 ± 0.03 (n = 10), and 0.29 ± 0.04 nS/pF (n = 11), respectively. The relation between conductance and [K\textsuperscript{+}] was proportional to [K\textsuperscript{+}] raised to a power of 0.45 (K\textsuperscript{+})\textsuperscript{0.45; Fig. 1C}.

The inward current showed a fast activation that could be fitted to a single exponential equation. In conditions in which 130 mM [K\textsuperscript{+}] was present on both sides of the membrane, the time constant (τ) obtained from the exponential fitting was shorter when hyperpolarization increased. As an example, at −20, −60, and −100 mV, τ was 2.28 ± 0.3 (n = 10), 1.81 ± 0.2 (n = 10), and 1.72 ± 0.2 msec (n = 10), respectively. Also, the current showed very little inactivation across the whole range of potentials tested, even at the more hyperpolarized ones (−130 and −140 mV; Fig. 1D).
Effect of Extracellular Cations: Block by Ba$^{2+}$, Cs$^+$, and Ca$^{2+}$

Kir channels show a high sensitivity to extracellular Ba$^{2+}$ application in different cell types. The effect of Ba$^{2+}$ concentrations ranging from 1 μM to 1 mM on the identified inwardly rectifying K$^+$ current was studied in BTM cells. Voltage pulses (250 msec) were applied between $-60$ mV and $+130$ mV in 10-mV steps from a holding voltage of 0 mV. A tail current was recorded at $-60$ mV. [Ba$^{2+}$] at greater than 10 μM elicited a voltage- and concentration-dependent block of the current (Fig. 2A). Ba$^{2+}$ blockade was especially marked at the more hyperpolarized potentials and consisted of a decrease in amplitude and an increase in the inactivation rate of the K$^+$ current. For any given cell, the tail current was always the same when Ba$^{2+}$ was not present. However, at [Ba$^{2+}$] greater than 10 μM the tail current showed a different profile. It reached a maximum when $V_m$ was previously set to depolarized levels (e.g., $+60$ mV) and a minimum when the previous $V_m$ was very hyperpolarized (e.g., $-130$ mV; Fig. 2A).

The fractional inhibition of the steady state current by Ba$^{2+}$ ($I_{Ba^{2+}}/I_0$) was calculated at the end of voltage pulses. The data obtained were fitted to the Hill equation (Fig. 3A):

$$I_{Ba^{2+}}/I_0 = 1 / (1 + ([Ba^{2+}]/K_d))$$

(1)

where $K_d$ is the apparent dissociation constant for Ba$^{2+}$. $K_d$ obtained from the fittings at $-30$, $-50$, $-70$, $-90$, and $-110$ mV was 40, 30, 20, 10, and 8 μM, respectively. To find the $K_d$ for the Ba$^{2+}$ block at 0 mV $K_d$ was plotted against $V_m$ and fitted to the Boltzmann equation (Fig. 3C):

$$K_d = K_{d(0)} \cdot \exp[zF\mu V/RT]$$

(2)

where $K_{d(0)}$ is the dissociation constant at 0 mV, $z$ is the valency of the ion (e.g., for Ba$^{2+}$ ions, it is 2), $F$ is the Faraday constant, $\mu$ is the constant that indicates the sensitivity of $K_d$ to $V_m$ changes, $R$ is the gas constant, and $T$ is the temperature in Kelvin. The fitting showed a $K_{d(0)}$ of 74.7 μM and a $\mu$ of 0.27.
Kir channels previously identified in cell types such as smooth muscle, astrocytes, or Müller cells are sensitive to Cs\(^+\).\(^{21-23}\) Thus, the effect of extracellular application of [Cs\(^+\)] ranging from 1 \(\mu\)M to 10 mM was tested in BTM cells. Again, 250-msec voltage pulses were applied between +60 and −130 mV in 10-mV steps, and a tail current was recorded at −60 mV. [Cs\(^+\)] greater than 10 \(\mu\)M elicited a voltage- and concentration-dependent block of the inwardly rectifying K\(^+\) current that was especially marked at the more hyperpolarized potentials (Fig. 2B). In contrast to the Ba\(^{2+}\) effect, the tail current remained constant for a stimulus protocol performed at a given level of [Cs\(^+\)] (Fig. 2B).

The blocking affinity of Cs\(^+\) was always lower than Ba\(^{2+}\) (Figs. 2, 3). To quantify the Cs\(^+\) blockade, the fractional inhibition of the steady state current in the presence of Cs\(^+\) (\(I_{Cs+}/I_0\)) was calculated at the end of the voltage pulses. The data obtained were fitted to the Hill equation (equation 1) and the apparent \(K_d\) for Cs\(^+\) was obtained (Fig. 3B). At −30, −50, −70, −90, and −110 mV, \(K_d\) was 12, 6, 1.29, 0.43, and 0.32 mM, respectively. \(K_d\) was plotted against \(V_m\) and fitted to the Boltzmann equation (equation 2; Fig. 3D). The fitting provided a \(K_{d50}\) of 45.6 mM and a \(\mu\) of 1.05. It is remarkable that both the Cs\(^+\) and the Ba\(^{2+}\) block were completely reversible and the control level of the current was fully recovered once any of these cations were removed.

An increase in the concentration of external divalent cations such as Ca\(^{2+}\) or Mg\(^{2+}\) blocks the Kir currents of smooth muscle cells from coronary arteries.\(^{21}\) To test the effect of extracellular Ca\(^{2+}\) in cultured BTM cells, [Ca\(^{2+}\)] was increased from 1 to 5 mM (\(n = 5\)) or to 10 mM (\(n = 4\)), while [Mg\(^{2+}\)] was kept constant at 100 \(\mu\)M. [K\(^+\)] was 130 mM. The increase of extracellular [Ca\(^{2+}\)] elicited a concentration dependent inhibition of the inwardly rectifying K\(^+\) current (Fig. 4A). As plotted in Figure 4B, the inhibition of the Kir current was constant at a given extracellular [Ca\(^{2+}\)] for the whole range of voltages tested. For example, the inhibition of the control current observed at −110 and −30 mV when Ca\(^{2+}\) was increased from 1 to 5 mM, was 17% and 18%, respectively. At the same voltages with external [Ca\(^{2+}\)] raised to 10 mM, the inhibition was 27% and 30%, respectively.

**Effect of Glibenclamide and Tertiapin**

There are few selective pharmacologic blockers available for the Kir channel family. Glibenclamide is a sulfonylurea that blocks K\(_{ATP}\) channels, which are heteromultimers formed by a Kir 6.x channel and a sulfonylurea receptor.\(^{24}\) Tertiapin is a toxin from the honey bee (Apis mellifera), and it blocks Kir1.1 and Kir3.1 channels.\(^{25,26}\)

The inwardly rectifying current of BTM cells was insensitive to 10 \(\mu\)M glibenclamide. No differences were found between control conditions or in the presence of glibenclamide, either in the voltage ramps or in the pulse protocols applied. As an example, the inwardly rectifying current change in presence of glibenclamide compared with the control levels at −110 mV was 1.04 ± 0.04 (\(n = 6\)). The inwardly rectifying current of BTM cells was also insensitive to 10 nM tertiapin. No differences were found between control conditions or in the presence of tertiapin, either in the voltage ramps or in the pulse protocols applied. For example, the inwardly rectifying current change in presence of tertiapin compared with the control levels at −110 mV was 1.04 ± 0.03 (\(n = 3\)).

**Kir2.1 Channel Protein Expression**

Western blot analysis was used to investigate whether the Kir2.1 channel is expressed in cultured BTM and HTM cells. The analysis of the extracts from three different cultures of HTM cells (HTMA, -B, and -C) revealed a doublet band at 54 to 55 kDa (Fig. 5) in all the samples. Extracts of BTM cells showed a band at 55 kDa. The specificity of the antibody was tested by preincubating the Kir2.1 antibody with the antigenic peptide, as indicated in the Materials and Methods section. In this case, the antibody was unable to identify the 54- to 55-kDa doublet (Fig. 5).
Effect of cAMP

cAMP is involved in many cell responses and can modulate the activity of ion channels. In fact, stimulation of cAMP production increases aqueous humor outflow. The effect of cAMP on the inwardly rectifying K\textsubscript{1} current was tested with the permeable analogue 8-Br-cAMP. \(V_m\) was held at -240 mV, and the Kir current was studied in 2-second voltage ramps from -2120 to -160 mV. Kir current presence was identified by the change in the \(E_K\) when \([K^+]_o\) was increased from 10 to 50 mM.

In 50 mM \([K^+]_o\), the cAMP analogue decreased the Kir current amplitude in four of the five cells studied, especially at very hyperpolarized potentials (Fig. 6A). The effect of 100 \(\mu\)M 8-Br-cAMP was maximal 1 to 2 minutes after its application (Fig. 6B). The mean inhibition at -2100 mV was 47\% \pm 14\% compared with the average current found at the same \(V_m\) during the control 5 minutes. Current control levels were immediately recovered after 8-Br-cAMP removal (Fig. 6B). Finally, 1 mM Ba\textsuperscript{2+} application suppressed the inwardly rectifying component of the current (Fig. 6A). It could be considered that in the experimental conditions used, the Ba\textsuperscript{2+}-insensitive current may not represent the Kir channel conductance. If this component is then subtracted, the average blockade of the Ba\textsuperscript{2+}-sensitive current by 100 \(\mu\)M 8-Br-cAMP at -100 mV should be even greater, more specifically 57\% \pm 17\%.

DISCUSSION

The Kir family is composed of 15 different channels, distributed in 7 subfamilies according to their amino acid sequence. Kir channels contribute to \(E_m\) establishment and modulate cell excitability. However, the different Kir subfamilies and members show differences in the magnitude of inward rectification, single-channel conductance, and modulation by intracellular mediators (e.g., nucleotides, second messengers, G-proteins) all of which are determinants of their physiological function.
negligible. The inwardly rectifying K\textsuperscript{+} current of BTM cells displayed the typical characteristics of the Kir2.x channel subfamily\textsuperscript{12,13,32}: strong inward rectification, rapid activation and a small amount of inactivation at very negative potentials, an inward slope conductance almost proportional to the square root of [K\textsubscript{o}], and sensitivity to external Ba\textsuperscript{2+} and Cs\textsuperscript{+}. The highest concentration of Ba\textsuperscript{2+} used (5 mM) did not completely block the inwardly rectifying K\textsuperscript{+} current, and the remaining current did not show an inward rectification. Although this component may not be carried by a Kir channel, this small value (\textsim 8% of the total current) was not subtracted.

Up to the present, four members of the Kir2.x subfamily have been described,\textsuperscript{30} and they display different sensitivities to Ba\textsuperscript{2+} and Cs\textsuperscript{+} block.\textsuperscript{33–36} The inwardly rectifying K\textsuperscript{+} current of BTM cells was more sensitive to Ba\textsuperscript{2+} than Cs\textsuperscript{+}, as described by the \( K_{d(0)} \) of 74.7 mM and 45.6 mM. The \( K_{d(0)} \) is similar to that found for the Kir2.1 channel, where the \( K_{d(0)} \) for Ba\textsuperscript{2+} ranges from 21 to 62 mM and the \( K_{d(0)} \) for Cs\textsuperscript{+} ranges between 19 and 54 mM.\textsuperscript{21,32,37} The Kir channel of BTM cells was also sensitive to extracellular [Ca\textsuperscript{2+}] increases, a characteristic feature of Kir2.1 channels.\textsuperscript{21,36} Moreover, the inwardly rectifying K\textsuperscript{+} current was insensitive to glibenclamide, a blocker of the K\textsubscript{ATP} channel and tertiapin, a blocker of Kir1.1 and Kir3.1 channels. Thus, the participation of these channel types was discarded. Taking all these data together, a functional presence of Kir2.1 channels in cultured BTM cells can be proposed (Table 1). In addition, Western blot analysis specifically revealed a band at 55 kDa, which confirmed the expression of Kir2.1 channel in BTM cells.

Functional expression of a Kir channel was also studied in cultured HTM cells with the patch-clamp technique. Very few HTM cells showed acceptable input resistances, which makes it difficult to report significant data on patch-clamp studies. Nevertheless, Western blot for HTM cell protein extracts identified a 54- to 55-kDa doublet specifically immunoreactive to the Kir2.1 antibody. These results suggest that the Kir2.1 channel is also expressed in HTM cells.

The TM is a heterogeneous structure that shares some properties with other tissues. The TM is able to contract or to relax\textsuperscript{6} and in fact 10% to 19% of the cultured TM cells from some species stain for α-smooth muscle actin,\textsuperscript{7,8,11} a specific marker of smooth muscle cells. Also, cultured TM cells show properties typical of vascular endothelial cells (e.g., a prominent Na-K-2Cl cotransport).\textsuperscript{38} Moreover, different pharmacologic agents that modify aqueous humor outflow, such as...
reduced the inward rectification. The current was almost completely like cells with a high inward rectifying K
morphologic heterogeneity. In the present work, the inwardly E
allowed us to argue that there may be at least a population of
adrenergics or neuropeptides, increase [Ca2+], and other cations and also on the specific type of TM cell in question. In other cells, Kir channels perform a number of different roles. For example, those in Müller cells regulate the composition and concentration of intracellular polyamines which may affect Kir2.1 channel activity. Also, Kir2.1 channel activity can be modulated by different intracellular mediators such as cAMP. The effects of blocking the Kir current. The experiments were performed with a high concentration of the Ca2+ buffer EGTA in the electrode solution, which indicates the participation of a [Ca2+]-independent mechanism. Because cloned Kir2.1 channels are inhibited by a PKA-mediated phosphorylation, the involvement of a cAMP-dependent protein kinase can be suggested.

It is not immediately clear what the physiological significance would be of blocking the Kir.1 channel in TM cells with intracellular mediators such as cAMP. The effects of blocking depend on the E
of the cell, the extracellular concentrations of K
and other cations and also on the specific type of TM cell in question. In other cells, Kir channels perform a number of different roles. For example, those in Müller cells regulate the [K]
, in the retina by the process of K
siphoning, whereas in vascular smooth muscle cells Kir channels participate in the establishment of the vascular tone. Therefore, to determine whether there is indeed a relationship in the TM between the effects of cAMP on outflow facility and the Kir2.1 channel, it is necessary to know the distribution of this channel in the intact tissue and the morphologic characteristics of the Kir2.1-positive cells.

In conclusion, Kir2.1 channels are functionally expressed in cultured BTM cells, and our results suggest that they are also present in HTM cells. It is likely that channel activity is in-

adrenergics or neuropeptides, increase [Ca2+], in only part of the cultured TM cells. According to these previous studies, it can be concluded that TM cultures display functional and morphologic heterogeneity. In the present work, the inwardly rectifying K
current was found in 60% of the cells. This fact allows us to argue that there may be at least a population of cells in the TM in which the Kir2.1 channel is involved in the establishment and modulation of their E
.

The BTM cultures used for the study, when confluent, showed a majority of epithelial-like cells and a minor proportion of spindle-like cells, in agreement with previous studies. Patch-clamp studies were performed in preconfluent states, and all the cells showed a similar epithelial-like appearance. In their study, Coroneo et al. found a population of epithelial-like cells with a high E
(approximately −50 mV) and a population of spindle-like cells with a low E
(approximately −70 mV) in BTM cell cultures. Although, a possible association of Kir2.1 channel expression to the epithelial-like population could be suggested, the development of a suitable immunohistochemical technique is required to explore this point.

In the present study, the E
was studied at the beginning of 24 experiments and showed a mean of −26 ± 2 mV. This is higher than the average E
of −55 mV reported for BTM cell cultures, by using intracellular micro-electrodes. The reason for the discrepancy may be attributable to the different stages of confluence (isolated cells versus confluent monolayers), the presence of small leak currents in the patch, or the dialysis of the cytoplasm. In physiological conditions the [K+]i in the aqueous humor is approximately 4 mM19 and assuming a [K+]i of 130 mM, the calculated E
, obtained with the Nernst equation is −92 mV. Therefore, the differences between the theoretical E
and the described E
for BTM cells show the participation of other ion channels in the establishment of the E
besides the Kir2.1 channel.

Extracellular Ba2+ application in BTM and HTM cells induces a depolarization of the plasma membrane. The present results suggest that Kir2.1 can be involved in this effect. As it can be observed in Figure 1A, for a [K+]o of 2.7 mM there is a small outward current (leak current) for the channel for a theoretical physiological range of E
between −20 and −70 mV. Application of Ba2+ in the millimolar range may block the outward component and depolarize the membrane as has been described in rat corticotropes and canine colonic smooth muscle cells.

The Kir2.1 channel is blocked intracellularly by Mg2+ and polyamines (putrescine, spermidine, and spermine), which mediate its physiological action. Therefore, it has to be taken into account that any metabolic activity that alters the composition and concentration of intracellular polyamines may affect Kir2.1 channel activity. Also, Kir2.1 channel activity can be modulated by different intracellular mediators such as protein kinase A (PKA), tyrosine kinases, or phospholipids.

It is well established that the maneuvers that raise the intracellular Ca2+ drive an increase of outflow facility in bovine and human anterior segments as well as in monkey eyes. In the present study the Kir2.1 channel expressed in BTM cells was sensitive to increases in intracellular cAMP, because the permeant analogue 8-Br-CAMP elicited a ~50% blockade of the Kir current. The experiments were performed with a high concentration of the Ca2+ buffer EGTA in the electrode solution, which indicates the participation of a [Ca2+]-independent mechanism. Because cloned Kir2.1 channels are inhibited by a PKA-mediated phosphorylation, the involvement of a cAMP-dependent protein kinase can be suggested.

In conclusion, Kir2.1 channels are functionally expressed in cultured BTM cells, and our results suggest that they are also present in HTM cells. It is likely that channel activity is in-

FIGURE 6. Application of the permeable cAMP analogue, 8-Br-cAMP, blocked the inwardly rectifying current of BTM cells. Two-second voltage ramps from −120 to +60 mV were applied. Holding voltage was −40 mV. Dashed line: zero current level. (A) The presence of the inwardly rectifying current selective for K
was confirmed by the right shift of E
(arrows) seen when the [K+]o was changed from 10 (dotted line) to 50 mM (solid line). Application of 100 μM 8-Br-cAMP reduced the inward rectification. The current was almost completely blocked by 1 mM Ba2+. (B) Time-course of the 8-Br-cAMP blockade of the inwardly rectifying K
current measured at −100 mV. Every point represents the mean ± SEM of three to four measurements. The cAMP analogue decreased the magnitude of the current, reaching a maximum 1 to 2 minutes after its application. When 8-Br-CAMP was removed, the current recovered to control levels. After application of 1 mM Ba2+ the total current was immediately reduced to less than 10% of its control level.
volved in the establishment of ionic gradients across the plasma membrane. Because this process can modulate cell contractility or intracellular volume, two actions that modify aqueous humor outflow, we propose that the Kir2.1 channel may play a significant role in TM physiology.

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References


Table 1. Summary of the Similarities between the Kir Channel Found in BTM Cells and the Kir2.1 Channel

<table>
<thead>
<tr>
<th>Characteristics of Kir Channel</th>
<th>BTM Cells</th>
<th>Kir2.1 Channel</th>
<th>Reference Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selectivity for K⁺</td>
<td>High</td>
<td>High</td>
<td>12</td>
</tr>
<tr>
<td>Type of inward of rectification Conductance</td>
<td>Strong</td>
<td>Strong</td>
<td>12</td>
</tr>
<tr>
<td>Inactivated at hyperpolarized V m</td>
<td>Little</td>
<td>Little</td>
<td>21, 32, 36</td>
</tr>
<tr>
<td>Blocked by Ba²⁺</td>
<td>K d(0) = 74.7 μM</td>
<td>μ = 0.27</td>
<td>21, 36, 37</td>
</tr>
<tr>
<td>Blocked by Cs⁺</td>
<td>K d(0) = 45.6 mM</td>
<td>μ = 1.05</td>
<td>21, 36, 37</td>
</tr>
<tr>
<td>Blocked by extracellular Ca²⁺ increase</td>
<td>Yes</td>
<td>Yes</td>
<td>21, 36</td>
</tr>
<tr>
<td>Sensitive to sulfonylureas</td>
<td>No</td>
<td>No</td>
<td>13</td>
</tr>
<tr>
<td>Sensitive to tertiapin</td>
<td>No</td>
<td>No</td>
<td>25</td>
</tr>
<tr>
<td>Inhibited by cAMP</td>
<td>Yes</td>
<td>Yes</td>
<td>46</td>
</tr>
</tbody>
</table>

Comparison between the properties of the inwardly rectifying K⁺ current found in cultured BTM cells and the properties of cloned Kir2.1 channels or Kir2.1 channels described in vascular smooth muscle cells. The K d(0) and μ values were obtained with the Boltzmann equation.