LABORATORY SCIENCE

Renin-angiotensin system expression and secretory function in cultured human ciliary body non-pigmented epithelium

A B Cullinane, P S Leung, J Ortego, M Coca-Prados, B J Harvey

Background: Renin-angiotensin system (RAS) components have been identified in human ciliary body and aqueous humour, pointing to a role for the RAS in the regulation of aqueous humour dynamics. Here, the authors examine the functional expression of a RAS and the effects of angiotensin II (AII) on a signal transduction pathway and ion secretion mechanism in cultured human ciliary body non-pigmented epithelium (HNPE).

Methods: RAS expression was examined in cultured HNPE cells using polymerase chain reaction (PCR) analysis. Secretory function was determined using spectrofluorescence imaging microscopy to measure cell calcium ($Ca^{2+}$) and volume responses. Single channel patch clamp techniques were employed to investigate ion channel activity.

Results: PCR analysis demonstrated the expression of angiotensinogen and the AT$_1$ receptor in HNPE cells. A large conductance potassium (BK) channel (mean 190 (SEM 5.6) $pS, n = 22$ cells), was observed in plasma membrane patches. This channel was calcium sensitive, with a calcium open probability ($P_o$) increasing with increasing $Ca^{2+}$; ($K_+$, 10.79 (0.44) $\mu$M $Ca^{2+}$; Hill coefficient of 1.04 (0.04)). All (100 $nM$) increased the number ($N$) of active BK channels in HNPE cells and also the probability of channel opening ($P_o$). $N/P_o$ increased from 0.008 (0.002) to 1.38 (0.4) following the addition of AII (p=0.0064). AII also increased in calcium, from resting values of 112 (14) $nM$ to a peak of 992 (106) $nM$ ($p<10^{-4}$). A simultaneous cell volume reduction of 24.70% (3.34%) ($p<10^{-4}$) occurred during this calcium signal. Losartan (1 $\mu$M) significantly blocked the AII induced BK channel activation ($p=0.0131$), the $Ca^{2+}$ response ($p<10^{-4}$), and the AII induced volume effect ($p=0.0046$).

Conclusion: It was demonstrated that AII activates a $Ca^{2+}$ signalling system which subsequently increases potassium ion channel activity. These effects are accompanied simultaneously by cell volume loss, indicating that AII acts as receptor operated secretagogue in HNPE cells. The ability of an AT$_1$ receptor antagonist to inhibit these processes may thus offer a new family of pharmaceutical agents to the current armamentarium in the treatment of glaucoma.

METHODS

Cell culture

The cell clone (ODM Cl-2) was established from a primary culture of human non-pigmented ciliary epithelium. The cells were grown in polystyrene culture flasks (25 cm$^2$, Falcon 3801 Primary) containing Dulbecco’s modified Eagle’s medium (DMEM catalogue No 320–1965, Gibco Laboratories, Life Technologies, Inc) with 10% fetal calf serum (FCS) and 50 mg per ml gentamicin sulphate (garamycin, Schering corporation). The medium contained 4500 mg per litre, D-glucose and L-glutamine. NaHCO$_3$ was added to the medium to adjust the pH to between 7.3 and 7.4 and the final osmolality was 328 mOsm. The medium was sterilised by membrane filtration with 0.2 $\mu$m Corning pore filters. The cells were grown at 37°C in 5% carbon dioxide in incubators. Cells were passaged every 6–7 days at a split ratio of 1:2 or 1:3 and the medium changed three times a week. All of the cells studied experimentally were grown on filter cups (Millipore, area 0.6 cm$^2$), Corning, Bedford, MA, USA), with a base composed of collagen (types I, III, and IV). Experiments were performed on single cells isolated from monolayers between passages 6 to 16. HNPE

Abbreviations: ACE, angiotensin converting enzyme; AII, angiotensin II; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; HNPE, human ciliary body non-pigmented epithelium; PCR, polymerase chain reaction; RAS, renin-angiotensin system
Intracellular calcium imaging

The isolated cells were placed in glass bottomed perfusion chambers containing DMEM. The cells were loaded with Fura-2/AM (Molecular Probes) in DMSO (final concentration <0.01%) at a concentration of 5 μM and incubated in 5% carbon dioxide and 95% air at 35°C for 35 minutes. Cells were washed to remove the medium and Fura-2 completely, in the following solution containing (mM): 138 NaCl, 4.7 KCl, 1.2 MgCl₂, 1.2 KH₂PO₄, 6 HEPES, 10 glucose, and 2.5 CaCl₂ pH 7.4 with NaOH, pCa 3, and 297 mM. All experiments were performed at room temperature 23–25°C to minimise dye leakage. Microfluorometric measurements of intracellular calcium were made using an inverted epifluorescence microscope (Diaphot 200, Nikon) equipped with ×40 and ×100 oil immersion lens. The light from a 100 W xenon lamp (Nikon) was filtered through alternating 340 nm and 380 nm interference filters (10 nm band width, Nikon). A custom built chopper circuit controlled the motorised filter wheel, and allowed an electronic adjustment of the duration of the exposure at each wavelength. The resultant fluorescence was passed through a 400 nm dichroic mirror, filtered at 510 nm, and then collected using an intensified CCD camera system (Darkstar, Photonic Science). Video fluorescence images were digitised and analysed using the Starwise, Fluo system (Imstar, Paris, France). Fluorescence signals were calibrated in vivo over a range of calcium concentrations in the presence of the calcium ionophore 4-bromo-A23187 as previously described in detail.³

Cell volume measurements

Fluorometric analysis

An estimate of cell volume was obtained by analysing the fluorescent intensity of Fura-2 at the “isosbestic” wavelength. At this wavelength the fluorescence intensity is independent of intracellular calcium levels and dependent on dye concentration. Thus relative changes in cell volume can be determined from changes in fluorescent intensity at the isosbestic wavelength according to the following equation: volume/volume = (380° × –Bk) + (340° × –Bk))/2, where 380° represents the fluorescent intensity at 380 nm, 340° is the value of the signal at 340 nm, and Bk is the value of the background fluorescence signal. Three dimensional images of cell volume calculated by this method were generated by the computer and the ratiometric cell calcium level could be superimposed on this volume image, yielding simultaneous spatiotemporal measurements of cell calcium and volume.

Morphometric analysis

Isolated HNPE cells were seeded on glass bottomed petri dishes in NaCl Ringer solution and placed on the inverted stage of a microscope equipped with Nomarski optics. (Nikon Diaphot TMD). Video images of the cells were captured at ×100 magnification with a camera (WAT 902, Watex, Japan) connected to a video frame grabber (model LG3, Scion Corporation). An image of the cell was stored on a computer each second and later analysed with edge detection software on a Macintosh computer using the public domain NIH Image program (v1.57pcc), developed at the US National Institutes of Health (available on floppy disk from the National Technical Information Service, Springfield, Virginia, part number PB95-500195GE1). Assuming a spherical shape of isolated cells, the cell volume was calculated as 4πr³/3.

Single channel patch clamp recording

The cell attached and inside out configurations of the patch clamp technique were used for single channel studies.³ Patch pipettes (tip resistance 4–10 MW) were prepared from borosilicate glass capillaries (Vitrex, Modulohm, Herlev, Denmark), pulled and polished on a programmable puller (DMZ, Zeitz Instrumente, Augsburg, Germany). The patch pipettes were filled with either standard NaCl Ringer or an intracellular-like K⁺ solution (“Kint” composition given in Table 1). All solutions were filtered through 0.2 μm Millipore cellulose discs. Current and voltage recordings from patch clamp electrodes were amplified (Biologic RK300 patch clamp amplifier, Echirolles, France), passed through a 30 KHz filter, and stored on video tape (Betamax Sony, Tokyo, Japan) after pulse code modulation (PCM model 501ES, Sony, Tokyo, Japan). The single channel current records were digitised and displayed in real time using a digital oscilloscope (Model 310 Nicolet, Madison, WI, USA). Single channel current records for illustrations were filtered at 1 KHz using a Tchebycheff filter and replayed on a chart recorder (Model RS3200 Gould Instruments, Valley View, OH, USA). Current and voltage signals were digitised using a 16 bit A/D converter (CED 1401, Cambridge Electronic Design, Cambridge) after low pass (~3dB) filtering at 5 KHz (8 pole Bessel filter, Model 902, Frequency Devices Inc, Haverhill, MA, USA) and analysed with an 80486–50 MHz Dell PC. Steady state current voltage relations were run and analysed using VGEN and PAT programs (J Dempster, Strathclyde Electrophysiology Software version 6.1, University of Strathclyde, Scotland) and instantaneous I-V trials were generated using PULSETIME and VCLAMP programs (Cambridge Electronic Design, release EPC 5.0). Single channel current amplitude histograms, open probability-individual open and closed dwell times were analysed from data segments of 20–60 second duration using the PAT program. In cell attached configuration cation movement across the membrane from the cytoplasm to the external side is defined as outward current and shown as upward deflections in single channel records. Channel activity was determined from the product of the number of channels active in the patch (N) and the channel open probability (Po).

Whole cell current voltage analysis

The whole cell current voltage configuration was obtained from the cell attached mode after breaking the membrane patch by applying a brief negative pressure pulse (~200 mbar) in the pipette. Whole cell currents were amplified (RK300 Biologic), digitised at 20 KHz (CED 1401), and sampled in real time on hard disk at an acquisition rate of 5 KHz. Current voltage relations were analysed using CED VCLAMP software. The “outside out” configuration was obtained from whole cell patches by withdrawing the electrode from the cell, which resulted in the formation of a membrane patch at the tip of the electrode with the extracellular surface facing the bathing solution.

PCR analysis of angiotensinogen

The set of oligonucleotide primers to amplify the human angiotensinogen gene by the polymerase chain reaction (PCR) was selected based on the published cDNA nucleotide sequence (GenBank accession number X15324) and with the aid of a primer select program of Dnastar (Dnastar Inc, Madison, WI, USA). The forward primer: 5'-GGGGAGAGGCAACCACCCATGC-3' (location 28 to 48); and the reverse primer: 5'-GTTCCAAAGGCTCCACAGTAAG-3' (location 482 to 462) were synthesised in the DNA Synthesis Facility at Yale University. PCR conditions were determined according to the primer select program (Dnastar Inc, Madison, WI, USA). The PCR method of Saiki et al. was used to anneal the above primers to DNA from 108 pfu of a cDNA library of the human non-pigmented ciliary epithelial cell line ODM-2 NPE³ and from a cDNA library of the human ciliary body.⁴ Reaction mixtures in 100 μl in MicroAmp tubes (Perkin Elmer, Emeryville, CA, USA) contained, 10 mM TRIS-HCl (pH 9), 50 mM

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Table 1 Composition (in mmol/l) of saline solutions used in bath and patch pipette

<table>
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<tr>
<th>Component</th>
<th>NaCl</th>
<th>KCl*</th>
<th>MgCl₂</th>
<th>CaCl₂</th>
<th>HEPES</th>
<th>EGTA</th>
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<td>pCa8</td>
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<td>10</td>
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* pH of Kₑ solutions was adjusted with KOH which increased the K⁺ concentration to 120 mmol/l.

Kgluc = potassium gluconate, NaAc = sodium acetate, NaPyr = sodium pyruvate, Asp = aspartic acid.

Note: In potassium gluconate solutions Ca²⁺ activity is reduced by 90% due to a chelating effect of gluconate (measured with a Ca²⁺ selective macroelectrode). To compensate for this, free calcium was present at 10-fold higher concentrations.

RESULTS

BK channels

A large conductance K⁺ channel (BK channel) was observed in “cell attached” plasma membrane patches when the pipette contained Kₑ solution and the cells bathed in NaCl Ringer solution. Out of 100 patches from single HNPE cells analysed in this study, the BK channel was found in 77 membrane patches and the number of active BK channels per patch varied between one and five. The channel activity is voltage dependent. Single channel conductance (γ) and probability of opening (Pₒ) were increased at more negative pipette voltages (membrane depolarisation). Only outward currents could be recorded through the BK channel and the channel thus behaves as a pure “outward rectifier.” This channel property is apparent from the single channel current voltage (I/Vₜ) relation (Fig 1A). In the cell attached patch configuration the mean conductance for outward current through the channel was 190 (5.6) pS (n=12) with a reversal potential of −35.66 (6.02) mV (n=7). This should correspond to the membrane potential of the HNPE cell since the chemical potential for K⁺ across the cell membrane patch is close to zero. Voltage clamping the cell plasma membrane to potentials which should drive K⁺ into the cell failed to activate the BK channel. In addition, the open probability of the channel increases when the pipette voltage (Vₜ) is clamped at negative values favouring K⁺ efflux (Fig 1B). Thus, in physiological terms, the BK channel acts a secretory ion channel. High conductance BK channels are typically inhibited by quaternary ammonium compounds such as tetraptethylammonium (TPEA), which are known to evoke a drastic reduction in the BK channel conductance and open state probability when acting from outside of the cell membrane. This drug is useful for identifying BK channels as other K⁺ channels are insensitive to TPEA concentrations less than 10 µM. The effect of TPEA on BK channel activity in the “outside out” patch configuration is shown in Figure 1D. The addition of TPEA...
Whole cell current recording

Whole cell K⁺ currents showed outward rectification when the pipette contained a K⁺ rich solution at pCa 3 and the cells exposed to a similar K⁺ (Kₒ) solution (Fig 1C). The reversal potential was 0 mV in agreement with the Nernst equilibrium potential for K⁺ under these symmetrical K⁺ solutions. Whole cell conductance (G) for outward current at (Vₒ +100 mV) was 19 nS and for inward G was 5.5 nS. Thus the activity of a large number of channels favours K⁺ efflux over K⁺ influx in the whole cell configuration as predicted from the single BK channel I/Vₒ relation. The channel underlying the whole cell membrane conductance was inhibited by tetrapentylammonium (TPeA), which significantly reduces whole cell conductance (G) and the number of BK channels active in the membrane at all membrane voltages tested between −100 to +100 mV (open circles). (D) Upper trace: Single channel current recording of BK channels from an excised (outside out) membrane patch with Kₒ in the bath and Vₒ of +80 mV. The open state (outward current) is seen as upward deflections on the trace and the closed channel state is indicated by the broken line. (Lower trace) Demonstrates the significant reduction in channel activity when (1 µM) TPeA is added to the bathing solution.

Ca²⁺ sensitivity of BK channels

Large conductance K⁺ channels are often found to be Ca²⁺ sensitive—that is, an increase in Ca²⁺ on the cytosolic side of the ion channel increases the probability and duration of channel opening. In order to examine if this was a property of BK channels in HNPE cells, active patches containing the channel were excised from the cell into an “inside out” configuration where the cytosolic side of the channel faces the bathing solution. The bath was then perfused with solutions of increasing levels of Ca²⁺ from pCa 9 to pCa 3 (10⁻⁹ to 10⁻⁵ M) and the corresponding changes in BK channel activity recorded. An increase in the level of Ca²⁺ in the bathing solution facing the cytosolic side of the cell membrane increased the number of BK channels active in the patch membrane and the open probability of these channels (Fig 2D). The relation N.Pₒ versus Ca²⁺ was fitted by the Hill equation:

\[ N.Pₒ = N.Pₒ₊₋ + \left(\frac{N.Pₒ₋₋ - N.Pₒ₊₊}{1 + \left(\frac{[Ca^{2+}]}{[Ca^{2+}]_h}\right)^{n}}\right) \]

Open probability (Pₒ) increased with increasing Ca²⁺, and the best curve fit gave a half maximal activation (Kₒ) of the channel at cytosolic Ca²⁺ = 10.79 (0.44) µM ([Ca²⁺]ₒ) at Vₒ = −50 mV. Hill coefficient of 1.04 (0.04), reaching a maximum Pₒ of 0.99 for Ca²⁺, at 100 µM. Increasing Ca²⁺, further to 1 mM had little additional effect on Pₒ.

Angiotensin II activation of BK channels

We have demonstrated that the BK channel is a predominant ion channel in the HNPE cell with high conductance for K⁺ secretion. This potassium channel may therefore play an important part in the formation of aqueous humour. Inhibitors of renin and angiotensin converting enzyme (ACE) have been shown to reduce intraocular pressure in humans. If AII is indeed a secretagogue, then a likely ion transport target is the BK channel. Figure 2 demonstrates the effect of AII (100 nM) on single BK channels in HNPE cells. The addition of AII to the control bathing solution (Fig 2A) increased the number of active BK channels in the membrane.
Angiotensin II effects on intracellular calcium and cell volume

The BK channel is activated by calcium, a known intracellular mediator of secretion. Angiotensin II activation of these channels may be mediated via intracellular calcium signalling. This hypothesis was tested in HNPE cells loaded with the calcium fluoroprobe, Fura-2. The extracellular addition of AII (100 nM) to single HNPE cells induced a rapid and significant rise in intracellular free calcium from mean basal values of 112 (14) nM to a peak of 992 (106) nM (p<0.01, n=7) which was followed by an oscillatory decline back to basal levels (Fig 3) A normal Ca$^{2+}$ response to AII was also elicited when the peptide was added following the reduction of extracellular calcium. Under conditions of low external calcium, AII (100 nM) produced oscillations in Ca$^{2+}$ of similar magnitude and pattern as in the control experiments. The mean basal and peak Ca$^{2+}$ response to AII from HNPE cells exposed acutely (<20 seconds) to 1 mM extracellular calcium was 100 (8) nM and 966 (168) nM, respectively (n=6). Under these conditions, the Ca$^{2+}$ response to AII was not significantly different from that observed in normal extracellular calcium (p=0.97). These results demonstrate that AII can activate release of calcium from intracellular stores and the initiation of the calcium
response to AII does not require entry of calcium across the plasma membrane.

The process of secretion involves the net efflux of osmotically active ions with a concomitant reduction in cell volume. Secretion in HNPE cells may therefore be examined by monitoring acute fluctuations in cell volume. Using spectrophotometry imaging single HNPE cell volume was measured in parallel with the calcium signal (Fig 3). The addition of AII (100 nM) to HNPE cells induced a significant peak volume loss of 24.70% (3.34%) (n=7, p<0.0001). Figure 3 demonstrates that changes in cell volume coincide with alterations in the Ca\(^{2+}\) signal; however, these volume changes lag slightly out of phase. The oscillatory cycles in decreasing cell volume, or secretory events, are observed to follow several seconds after the cycles in increasing Ca\(^{2+}\). In order to confirm the role of AII receptors in this process the AT\(_1\) receptor antagonist, losartan, was used. Exposure of HNPE cells to losartan (1 µM) had no effect on basal intracellular calcium (p=0.8, n=7). In the presence of losartan (1 µM) the addition of AII (100 nM) to the bathing solution failed to produce a significant increase in Ca\(^{2+}\), (Control-Ca\(^{2+}\); 103 (8) nM, AII+losartan-Ca\(^{2+}\); 141 (23) nM, n=7, p=0.1446). In addition, under these conditions cell volume did not significantly change from a mean resting cell volume of 1.0 (0.024) to 0.959 (0.043) in the presence of AII (p=0.42, n=7). This response differed significantly from the volume response in the absence of losartan (p=0.0046). Taken together, these results provide evidence for AII induced secretion in HNPE cells via calcium activation of BK channels. These findings were also supported by direct morphometric measurement of cell volume. The addition of AII (100 nM) to the bathing solution failed to produce a significant increase in Ca\(^{2+}\), (Control-Ca\(^{2+}\); 103 (8) nM, AII+losartan-Ca\(^{2+}\); 141 (23) nM, n=7, p=0.017). However, AII (100 nM) in the presence of losartan (1 µM) failed to produce a reduction in cell volume (resting volume: 100% (4%), AII+losartan; 98% (4.2%), n=6, p=0.74) and this differed significantly from the control AII cell volume effect (p=0.04).

The expression of AII receptor subtypes and angiotensinogen in HNPE cells

RT-PCR coupled with specific primers based on the corresponding AII receptor subtypes—namely, AT\(_{1a}\) and AT\(_{1b}\)—was performed to study the mRNA expression of their respective genes in the HNPE cells. Results showed that AII receptor subtypes in the cells appeared to be differentially expressed. RT-PCR analysis showed that mRNA from AT\(_{1a}\) (Fig 4) was not detected, whereas mRNA from AT\(_{1b}\) was found to be expressed in the cells. On the other hand, AT\(_{1a}\) and AT\(_{1b}\) mRNAs were detected consistently in the rat tissues of kidney and adrenal gland, which were known to express these receptor subtypes and were employed as positive controls for the experiments, in parallel by PCR using oligonucleotide primers complementary to the human angiotensinogen gene, as described in Methods. The predicted 455 nt fragment amplified from the libraries was resolved on a 1% agarose gel with ethidium bromide stain, and its migration position was marked at right. Lane M = 1 kb ladder DNA marker; CB = human ciliary body library; ODM-2 = human NPE cell line cDNA library.

DISCUSSION

Evidence now suggests the presence of a local renin-angiotensin system in the human eye\(^{7–11}\) and, specifically, RAS components have been identified in human ciliary body and aqueous humour.\(^{5,11}\) In addition, corneal application of...
ACE and renin inhibitors has been shown to lower intraocular pressure in patients with ocular hypertension and primary open angle glaucoma. These findings point to a role for the RAS in the regulation of aqueous humour dynamics. It has previously been hypothesised that the effect of the RAS is mediated predominantly by its ability to alter vascular tone at this site. However, in this report we demonstrate direct effects of AII on a receptor activated signal transduction pathway and ion secretion mechanism in human ciliary body non-pigmented epithelium.

Our data support previous work by Barros et al., who first identified the large conductance potassium channel (BK or "Maxi-K" channel) in this cell type. BK channels have been well characterised over the past decade in fluid secreting epithelia and have been confirmed to be a regulatory driving force for secretion in many cell types. In this study, we have demonstrated, from single channel and whole cell current analysis, that the BK channel dominates the overall conductance of the HNPE cell membrane. In addition, we have confirmed the sensitivity of this channel to small changes in the level of cytosolic free Ca\(^{2+}\), a known ubiquitous mediator of secretion. The Ca\(^{2+}\) dependency of the HNPE large conductance potassium channel was found to be similar to that of BK channels in other cell types. When activated by an increase in intracellular Ca\(^{2+}\), BK channels respond with increased open probability and concomitantly increased ion flux. These potassium channels therefore can serve as a link between cellular processes which involve intracellular Ca\(^{2+}\) elevation and those which involve membrane excitability and thus ion transport or solute secretion.

Angiotensin II, through AT1 receptor associated G\(_{i}\) protein activation, is known to release intracellular store calcium via the phosphoinositid/phospholipase C pathway. Activation of these channels, therefore, may result in increased open probability and concomitantly increased ion flux. These potassium channels therefore can serve as a link between cellular processes which involve intracellular Ca\(^{2+}\) elevation and those which involve membrane excitability and thus ion transport or solute secretion.

In order to further examine the role of the AII receptor in this process the specific AT1 receptor surmountable antagonist, losartan, was used. In the presence of losartan, the addition of AII failed to significantly increase Ca\(^{2+}\) or reduce cell volume. On a molecular level, losartan also significantly blocked the AII induced increase in K\(^+\) channel activity normally observed under control conditions. As the cell attached pipette prevents exposure of the channel to the drug, this effect must be mediated via AT1 receptor antagonism rather than by direct interaction with the potassium channel.

In conclusion, we have established that a calcium sensitive BK channel dominates the overall characteristics of the HNPE cell membrane. We have confirmed using RT-PCR, the presence of the AII receptor on the HNPE cell. We have shown that external AII activates an intracellular calcium signalling system which subsequently increases potassium channel activity in the plasma membrane. These effects are accompanied simultaneously by cell volume loss, indicating that AII acts as receptor operated secretagogue in HNPE cells. The ability of an AT1 receptor antagonist to inhibit these processes may thus offer a new family of pharmaceutical agents to the current armamentarium in the treatment of glaucoma.

ACKNOWLEDGEMENTS
This work was supported by the Health Research Board of Ireland and the National Institute of Health National Eye Institute (EY04873).

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