Differential P1-purinergic modulation of human Schlemm’s canal inner-wall cells

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Intraocular pressure; aqueous humor outflow; ion transport; adenosine receptors in SC cells.

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A2B, but not consistently affected by A3, stimulation. A1, A2A, and A3 specifically inner-wall SC cells. Those currents were also increased by 19104-6085 (E-mail: civan@mail.med.upenn.edu).

SC cells isolated by a novel enzyme-assisted technique and with stimulation. Parallel studies were conducted with human inner-wall SC cells isolated by a novel enzyme-assisted technique and with cannula-derived mixed inner- and outer-wall SC cells. A1 agonists increased whole cell currents of both inner-wall and cannula-derived SC cells. An A2A agonist reduced currents most consistently in specifically inner-wall SC cells. Those currents were also increased by A2B, but not consistently affected by A3, stimulation. A1, A2A, and A3 agonists all increased SC-cell intracellular Ca2+. The electrophysiological results are consistent with the possibility that inner-wall SC cells may mediate the previously reported modulatory effects of adenosine on outflow resistance. The results are also consistent with the presence of functional A2B, as well as A1, A2A, and A3 adenosine receptors in SC cells.

intraocular pressure; aqueous humor outflow; ion transport; adenosine agonists

**INTRAOCULAR PRESSURE** depends directly on the rate of inflow and the resistance to outflow of aqueous humor. The ciliary epithelium secretes aqueous humor, transferring solute and water from the stroma of the ciliary process into the posterior chamber. Fluid then flows between the lens and iris into the anterior chamber. The primary aim of the present work was to test whether selective A1 and A2A AR agonists exert opposing effects on human inner-wall SC cells in vitro, corresponding to their opposing effects on intraocular pressure in vivo. For this purpose, we developed a new enzyme-assisted technique to isolate and grow human inner-wall SC cells for study at an early passage number. The current study also examined the responses of SC cells to selective stimulation of A3ARs, progress in understanding the cellular physiology of this region of the eye.

Resistance to aqueous humor outflow is likely regulated in vivo, but the sensory and target cells of this regulation are poorly understood (14, 32). Cells in the outflow pathway likely play a role in regulating outflow resistance because maneuvers that swell cells in this pathway increase resistance, and those that shrink cells reduce resistance in human, nonhuman pri- mate, and calf eyes (1, 21, 48). The site of the cell-mediated regulation is unknown, but one likely potential site of outflow regulation is the cells of the inner wall of SC, which, together with their tight intercellular junctions, form an anatomically continuous barrier to flow. Fluid may cross through pores within and/or between these cells (14, 32) and through the unusually leaky “tight junctions” linking the cells (47). The structure and dynamics of tight junctions are regulated by a broad range of signaling cascades (8, 57). Mechanical stress can also modulate junctional permeability because increasing the intraocular pressure reduces the number of tight junctional strands between SC cells of perfusion-fixed human eyes (59). Whether stresses induced by volume changes of inner-wall SC cells modulate the tight junctions is unknown.

We have tried to identify the site of cell regulation of outflow resistance by taking advantage of the known effects of adenosine agonists on outflow and intraocular pressure. Aden- osine (P1 purinergic) receptors (ARs) are likely important in modulating pressure, both physiologically (5) and pathophysiological (49) (see DISCUSSION). A1 and A2A AR agonists lower and raise intraocular pressure, respectively, in rabbits (9, 10), mice (3, 4) and monkeys (54). The ocular hypertensive effect has been ascribed in monkeys entirely to outflow alteration (54). The ocular hypertensive effect of A2A AR agonists is also likely mediated by altered outflow because it cannot be ascribed to other potential mechanisms, such as increased inflow (54) or breakdown of the blood-aqueous barrier, at least at low doses of agonist (11, 54).

The primary aim of the present work was to test whether selective A1 and A2A AR agonists exert opposing effects on human inner-wall SC cells in vitro, corresponding to their opposing effects on intraocular pressure in vivo. For this purpose, we developed a new enzyme-assisted technique to isolate and grow human inner-wall SC cells for study at an early passage number. The current study also examined the responses of SC cells to selective stimulation of A3ARs,

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receptors thought to regulate aqueous humor secretion (see Discussion). In addition, we tested whether the fourth known AR, A\textsubscript{2B} AR, is functionally present in inner-wall SC cells.

METHODS

Enzyme-Assisted Technique for Preparing Human Inner-Wall SC Cells

To isolate and culture inner-wall SC cells, we adapted a method used for isolating and culturing microvascular endothelial cells (26), modified to accommodate the tissue complexity of SC and the limited number of cells provided per donor eye (Fig. 1A). We also exploited the observation that SC cells, unlike TM cells, express the marker CD31 (platelet endothelial cell adhesion molecule-1; PECAM-1) (Fig. 1B) (24, 55). We isolated SC cells from both nonglaucomatous donor eyes stored in moist chambers and corneoscleral buttons stored in solution (Optisol-GS, Bausch and Lomb Surgical, Irvine, CA) at 4°C provided by the Lions Eye Bank of Delaware Valley (Philadelphia, PA) within 48 and 96 h after death, respectively. Fibroblasts may potentially migrate into the tissue during prolonged periods of storage; these and other potential contaminants were excluded from the final cell harvest and culture by the selective isolation procedure described below.

Fig. 1. Isolation and culture of human inner-wall Schlemm’s canal (SC) cells. A: principal outflow pathway of human aqueous humor. Fluid leaves the eye sequentially through the trabecular meshwork (TM), SC, and collector channels (CC). In addition to a cribiform or juxtacanalicular tissue region, the TM comprises uveal (UTM) and corneoscleral (CSTM) regions extending from the ciliary muscle (CM) and scleral spur (SS) posteriorly to Schwalbe’s line (SL) delimiting the corneal endothelium (CE) anteriorly. B–D: light micrographs of fixed and stained sagittal cryosections through the angle of a human anterior chamber. B: as indicated by the stain, platelet endothelial cell adhesion molecule-1 (PECAM-1)/CD31 is immunolocalized specifically to SC endothelial cells within the human aqueous humor outflow region. C: the position of a gelatin-coated 6-0 nylon monofilament suture used as a matrix for proliferation of cannula-derived SC cells (52) is indicated by the asterisk. D: new method for inner-wall SC cell isolation entails removal of most TM tissue, preserving a thin SC inner-wall cell membrane (arrow). Note that most of the tissue overlying SC before the dissection step (Fig. 1C) had been removed in D. B and D appear different because of the background nuclear stain in B and the difference in magnification indicated by the bars. E: phase-contrast photomicrograph of individual endothelial inner-wall SC cells recognized by, and decorated with, a cell-specific antibody precoated with immumagnetic beads. Inner-wall SC cells were purified by antibody to the endothelial-cell specific PECAM-1/CD31 antigen. F: phase-contrast microscopy of cultured human inner-wall SC cells isolated by antibody to PECAM-1/CD31. A confluent monolayer at passage 2 displayed convex polygonal cells with close cell-to-cell apposition for the most part. Elongated and spindle-shaped cells at preconfluent state can also be noted.

Corneal buttons, either dissected from whole eyes or received as such, were placed endothelial side up and divided into quarters. With the use of a scalpel blade (no. 10), the corneal endothelium and underlying Descemet’s membrane with Schwalbe’s line and some anterior TM were gently peeled away. The scleral spur was incised radially using a sapphire knife and stripped along its course with forceps together with all uveal TM and a major portion of the outermost Descemet’s membrane. A 6-0 nylon monofilament suture was transferred to a petri dish and incubated in Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-free physiological buffered saline (PBS; Invitrogen, Carlsbad, CA) containing 1.0 mg/ml collagenase IV (232 U/mg protein; Worthington Biochemicals, Lakewood, NJ, IUB 3.4.24.3), 0.2 mg/ml elastase (Worthington, 3.94 U/mg protein, IUB 3.4.24.2), 0.2 mg/ml hyaluronidase (Worthington, 499 U/mg dry wt, IUB 3.2.1.35), 0.05 mg/ml DNase type 1 (2,000 U/mg protein, Roche Diagnostics, Indianapolis, IN, E.C.3.1.21.1) and 1.0 mg/ml bovine serum albumin (Sigma, St. Louis, MO). Over the next 60–90 min, cells dissociated into single cells and/or into clumps. Depending on the degree of cell clumping, a further 5 min in 0.25% trypsin solution (Invitrogen) with 1.0 mg/ml DNase was optionally applied.
After detachment, cells were twice suspended in F99 medium, supplemented with 10% certified fetal bovine serum (FBS), 2.5 μg/ml amphotericin B and 50 μg/ml gentamicin, and centrifuged. Cells were incubated in four gelatin-coated four-well cell culture multi-dishes (1.9 cm² area per well, Nalgene Nunc, Rochester, NY) with supplemented F99 medium as described above plus 20 ng/ml human recombinant basic fibroblast growth factor (Roche Diagnostics) and 10 ng/ml human recombinant epidermal growth factor (Roche Diagnostics) to induce proliferation. Media were changed after 24 h for the first time and thereafter three times a week without subsequent growth factor supplementation.

After growth to confluence, cells were trypsinized (0.25%) until suspensions of single cells were obtained. A cell strainer (Falcon, 40 μm) was used if cell clumping was still observable before the cells were seeded onto antibody-coated wells. The wells were prepared by coating cell culture 4-well plates with the SC-cell specific CD31 antibody (Fig. 1E) (24, 55), following the approach of Matteson and Deutsch (39). Plates were incubated with mouse anti-human CD31 antibody (DAKO Cytomation, Carpinteria, CA) diluted 1:20 (22.5 μg/ml) in a 0.2 M TRIZMA-HCl buffer (pH 9.5; Sigma) for 1 h at 4°C. The wells were rinsed three times with cold PBS containing 1% FBS before the cells were seeded. After being seeded, the cells were incubated for ≥2 h at 4°C. Nonadherent cells were carefully rinsed off three times with cold PBS containing 1% FBS. After nonadherent cells were removed, culture medium containing 20 μg/ml bovine fibronectin (Invitrogen) was added once, and the SC cells were grown after separation with the antibody (passage 1) (Fig. 1F). Upon reaching confluence, the cells were used for experiments and/or split for passage 2. All measurements were conducted at passages 1–3.

Cannula-Derived Mixed Human Inner- and Outer-Wall SC Cells

For purposes of comparison, we also isolated and grew SC cells by an established cannula-derived technique (52). A gelatin-coated suture serving as growth substrate was inserted into the intact SC. After 3 wk, cells were transferred from the suture to 6-well culture plates containing Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate, incubated at 37°C in humidified air with 7% CO₂ and further passaged at the split ratio 1:4 in T25 culture flasks. Cells strains used in this study were from three different donors (SC3, SC20, and SC26) and studied at passages 2 and 3.

SC cells cultured by the cannula-derived method were distinguished from cultures of fibroblasts by morphology and growth characteristics. Cultures of TM cells grew in a monolayer and had broad, flat cell bodies with many processes, whereas cultures of fibroblasts grew in multiple layers, were thin and elongated, and displayed thin multidigited processes. Cultures of SC cells were distinguished from cultures of fibroblasts by morphology and growth factor supplementation.

Cells were trypsinized, resuspended, and permitted to settle on glass coverslips. Whole cell patch-clamp currents were recorded in the ruptured-patch mode using an Axopatch 1B or 1D patch-clamp amplifier (Axon Instruments, Foster City, CA) connected to an external 8-pole Bessel filter (model 990C, Frequency Devices, Haverhill, MA). Micropipettes were pulled from Corning no. 7052 glass (World Precision Instruments, Sarasota, FL) with a Flaming/Brown micropette puller (model P-97, Sutter Instruments, Novato, CA), coated with Sylgard (World Precision Instruments) and fire polished with a microforge (model MF-830, Narishige). Micropipettes displayed resistances of 2–4 MΩ when filled with micropipette solution (Table 1) and several gigohms after forming successful seals. Potential was measured in a perfusion chamber connected to an Ag/AgCl pellet in 3 M KCl solution via a 3 M KCl agar bridge. The potential was stepped for 30 ms from a holding potential of −80 mV to test values from −100 to +80 mV in 20-mV increments at 2-s intervals. Applied voltages were not corrected for the small junction potential (about −2.8 mV; Ref. 7) arising from the present micropipette filling and external solution (Table 1). Data were acquired at 2–5 kHz and filtered at 500 Hz using an Axon Digidata interface coupled with pCLAMP version 8.2 software. The results were analyzed with Clampfit 8.2 software (all from Axon Instruments). Current activations were quantified at the peak responses and current inhibitions at the smallest values following the perturbation;
where possible, 2–3 points were averaged to estimate baseline and perturbed values. In analyzing data from multiple experiments, we have followed our standard practice of averaging currents measured at +80 mV because the values were largest at that test potential.

**Drugs and Experimental Solutions**

Unless otherwise stated, all drugs were purchased from Sigma. The AR agonists and antagonists used are presented in Table 2. Of these purinergic drugs, the A2A antagonist 4-[(2-[7-amino-2-(2-furyl)](1,2,4)triazolo[2,3-a]1,3,5 triazin-5-ylamino)ethyl]phenol (ZM-241385) was obtained from Tocris (Ballwin, MO).

The Cl−-channel and K+-channel blockers used were 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) (56) and tetraethylammonium (TEA), respectively. Compositions of solutions for patch clamping are entered in Table 1.

**Data Analysis**

Means ± SE were calculated from analysis of n experiments. The SE rather than SD was chosen as the index of uncertainty because we are focusing on the significance of averaged responses and not on the uncertainty associated with a single measurement. Uncertainties are focusing on the significance of averaged responses and not on the uncertainty associated with a single measurement. Uncertainties are estimated with Student’s paired t-test applied to results satisfying normality. Where normality was not met by the raw data, exponentially transformed data did conform to normality. Results were considered significant if P was <0.05.

**RESULTS**

**Inner-Wall SC Cells: Whole Cell Currents**

**Baseline properties.** Confluent cultures of cells derived from SC inner wall were largely polygonal with close cell-to-cell apposition. Subconfluent cultures in passage 1 displayed a range of morphologies, for the most part elongated to spindle shaped; this was especially true of cells in migration or establishing contact with one another (Fig. 1). A similar range of morphologies has been reported for various endothelial cells in culture (25, 51). Inner-wall SC cells at around passage 4 also tended to start displaying increased cell size and change in shape to a more elongated morphology, as observed in the cannula-derived cultures. The latter SC cells were fusiform, forming nonoverlapping linearly oriented monolayers, as previously reported (52).

We patch clamped round cells (21.8 ± 0.8 μm in diameter, measured in 51 cells). TEA (7.5 mM) inhibited inner-wall SC currents by 57 ± 4% from baseline values of 984 ± 319 pA (39 pA/Pf, n = 28, P < 0.001) at +80 mV. This indicated that K+ channels contributed much of the baseline membrane conductance, as found with TM cells (17, 53).

**Selective agonists of A1, A2A, and A3 ARs.** We tested whether the baseline currents were differentially affected by subtype-selective adenosine receptor agonists. The A1AR agonists S-ENBA and N6-cyclopentyladenosine (CPA) both activated whole cell currents (Figs. 2 and 3, Table 3). At 100 nM, S-ENBA increased currents by 587 ± 199% (n = 22, P < 0.001). Details concerning the fraction of applications and cells that responded significantly to this and other agonists and the statistical test used for each data set are provided in Table 3. Unless otherwise indicated, the entries in Table 3 are means ± SE averaged for all agonist and antagonist applications. The S-ENBA-activated difference currents displayed delayed outward rectification (Fig. 2, B and C) and were inhibited by 7.5 mM TEA by 53 ± 7% (n = 6, P = 0.002, Fig. 2A), comparable to the effect of TEA on baseline currents cited above. As illustrated by Fig. 2A, the magnitude of the S-ENBA-triggered activation was variable, even in repetitive applications to the same cell. In that figure, the initial response was small and transient, whereas the two subsequent responses were more sustained and an order of magnitude larger. CPA also activated whole cell currents, albeit less consistently. The frequencies and magnitudes of CPA-triggered activations at concentrations of 15, 50, and 100 nM are presented in Fig. 3.

**Table 2. Adenosine receptor agonists and antagonists used in present study**

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<th>agonists</th>
<th>potent agonists</th>
<th>Potency, nM</th>
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<th> </th>
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<tr>
<td><strong>Agonists</strong></td>
<td></td>
<td>A1</td>
<td>A2A</td>
<td>A2B</td>
<td>A3</td>
<td></td>
<td></td>
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<td>Nonselective</td>
<td>Adenosine</td>
<td>300–3,000*</td>
<td>700*</td>
<td>24,000*</td>
<td>290*</td>
<td>Human (20)</td>
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<td>Nonselective</td>
<td>NECA</td>
<td>14 (6.4–29)</td>
<td>20 (12–35)</td>
<td>330±60</td>
<td>6.2 (3.5–11)</td>
<td>Human (19)</td>
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<tr>
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<td>CPA</td>
<td>2.3</td>
<td>790</td>
<td>34,400±11,100</td>
<td>43</td>
<td>Human (19, 33)</td>
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<tr>
<td>A1</td>
<td>(S)-ENBA</td>
<td>0.3</td>
<td>1,400</td>
<td>Unknown</td>
<td>915</td>
<td>Rat (19)</td>
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<td>A1</td>
<td>ADAC</td>
<td>0.85</td>
<td>210</td>
<td>Unknown</td>
<td>281</td>
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<tr>
<td>A2A</td>
<td>CGS 21680</td>
<td>290 (230–360)</td>
<td>27 (12–59)</td>
<td>361,000±21,000</td>
<td>67 (50–90)</td>
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<td>A3</td>
<td>CI-IB-MECA</td>
<td>115 (114–116)</td>
<td>2,100 (1,700–2,500)</td>
<td>Unknown</td>
<td>11 (9.4–13)</td>
<td>Human (19, 33)</td>
<td></td>
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<tr>
<td>A3</td>
<td>CI-IB-MECA</td>
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<td></td>
<td>Human (13)</td>
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<tr>
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<td>A2A</td>
<td>A2B</td>
<td>A3</td>
<td></td>
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<tr>
<td>A1</td>
<td>DPCPX</td>
<td>3.9 (3.5–4.2)</td>
<td>129 (35–260)</td>
<td>50±37</td>
<td>4,000 (2,600–6,000)</td>
<td>Human (19, 33)</td>
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<tr>
<td>A2A</td>
<td>ZM-241385</td>
<td>260 (190–390)</td>
<td>0.8 (0.7–1.0)</td>
<td>31.6±6</td>
<td>&gt;10,000</td>
<td>Human (19)</td>
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<tr>
<td>A3</td>
<td>ZM-241385</td>
<td>536±63</td>
<td>1.4±0.6</td>
<td>31±1</td>
<td>269±91</td>
<td>Human (33)</td>
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<tr>
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<td>MRS-1754</td>
<td>400±190</td>
<td>500±11</td>
<td>2±0.31</td>
<td>570±180</td>
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<tr>
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<td>MRS-1191</td>
<td>40,000*</td>
<td>10,000*</td>
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NECA, 5′-(N-ethylcarboxamido)adenosine; S-ENBA, (25)-N6-[2-endo-norbornyl]adenosine; CPA, N6-cyclopentyladenosine; ADAC, adenosine amine generator; CGS-21680, 2-p-(2-carboxyethyl)-phenethylamino-5′-N-ethylcarbox-amidoadenosine; CI-IB-MECA, 2-chloro-N6-(3-iodobenzyl)-adenosine-5′-N-methyluracil-uronamide; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; ZM-241385, 4-[(2-amino-2-(2-furyl)](1,2,4)triazolo[2,3-a]1,3,5 triazin-5-ylamino)ethyl]phenol; MRS-1754, 8-((4-(4-chlorophenyl)-carbamoylmethyl)oxy]phenyl)-1,3 di(n-propyl) xanthine; and MRS-1191, 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropridine-3,5-dicarboxylate. *Potency (adenylate cyclase assay).

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The bar graphs of Figs. 3 and 6 present means ± SE separately for stimulatory (upward) and inhibitory (downward) responses. In contrast to subtype-selective A1 AR agonists, the A2A AR agonist CGS-21680 (30 nM) blocked whole cell currents by 17 ± 7% (n = 7, P = 0.03) (Figs. 3 and 4, Table 3). The A3 AR agonist 2-chloro-N6-(3-iodobenzyl)-9-[5-(methylcarbamoyl)-D-ribofurosanyl]adenosine (Cl-IB-MECA) produced inconsistent effects at 30 and 100 nM (Fig. 3), most commonly producing small reductions in current at 100 nM but also stimulating whole cell currents by ~400% in 2/8 applications.

NECA-based stimulation of A2A and A2B ARs. The magnitude of the response to the selective A2A AR agonist was much smaller than that to the selective A1 agonists. We also probed for the putative A2A ARs by a different strategy. We applied a high enough concentration (10 μM) of the nonselective, non-metabolizable AR agonist NECA to stimulate all four known ARs, but in the presence of selective antagonists to A1 (20 nM DPCPX), A2B (100 nM MRS-1754; Refs. 31, 36), and A3 ARs (100 nM MRS-1191). We first perfused the cells with the antagonists. During the initial period, most cells displayed either little change or only transient increases or decreases in whole cell currents. After a steady-state response was attained, the bar graphs of Figs. 3 and 6 present means ± SE separately for stimulatory (upward) and inhibitory (downward) responses. In contrast to subtype-selective A1 AR agonists, the A2A AR agonist CGS-21680 (30 nM) blocked whole cell currents by 17 ± 7% (n = 7, P = 0.03) (Figs. 3 and 4, Table 3). The A3 AR agonist 2-chloro-N6-(3-iodobenzyl)-9-[5-(methylcarbamoyl)-D-ribofurosanyl]adenosine (Cl-IB-MECA) produced inconsistent effects at 30 and 100 nM (Fig. 3), most commonly producing small reductions in current at 100 nM but also stimulating whole cell currents by ~400% in 2/8 applications.

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Fig. 3. Effects of subtype-selective AR agonists on whole cell currents of IW SC cells. The heights of the bars above and below the x-axis display the means ± SE of current activations and inhibitions, respectively. The numbers adjacent to the x-axis represent the numbers of activations and inhibitions, with the total number of applications of each agonist presented at the bottom. The potencies of the agonists are provided in Table 2. CPA, N6-cyclopentyladenosine; CI-IB-MECA, 2-chloro-N6-(3-iodobenzyl)-9-[5-(methylcarbamoyl)-D-ribofurosanyl]adenosine.
usually after 5 min, 10 μM NECA was added to the same perfusate to stimulate A2A ARs (Fig. 5B). With this approach, activation of A2A ARs inhibited whole cell currents by 17 ± 4% (n = 11, P = 0.001, Table 3), the same percentage inhibition produced by directly applying the A2A AR-selective agonist CGS-21680.

We used the same NECA-based strategy to probe for A2B AR function because selective A2B AR agonists are unavailable. We first perfused cells with antagonists to A1 (20 nM DPCPX), A2A (10 nM ZM-241385), and A3 ARs (100 nM MRS 1191) until a steady state was reached. At that point, NECA was added (Fig. 5A, Table 3). Under these conditions and excluding an uniquely large increase of 1,376%, NECA activated whole cell currents by 50 ± 16% (n = 25, P < 0.001).

Summary of AR agonist and antagonist effects on inner-wall SC currents. The results obtained with subtype-selective AR agonists and antagonists indicated that stimulation of A1 and A2B ARs significantly increased, whereas A2A AR stimulation significantly decreased, inner-wall SC whole cell currents (Figs. 3 and 5, Table 3). A3AR agonists exerted inconsistent effects. Given these results with subtype-selective drugs, it was not surprising that application of the physiological, nonselective agonist adenosine produced a wide range of responses at a concentration of 10 μM, which stimulates all four known adenosine receptors. Adenosine increased whole cell currents in 13 applications (by 297 ± 196%), decreased currents in 13 applications (33 ± 5%) and exerted biphasic effects in 4 of 30 applications to 16 cells. The overall mean response to adenosine was an increase of 132 ± 100% (P = 0.3).

Cannula-Derived SC Cells: Whole Cell Currents

For purposes of comparison, the electrophysiological effects of subtype-selective AR agonists were also measured in a smaller series of cannula-derived SC cells, likely arising from both inner and outer wall. On average, these cells were larger than pure inner-wall SC cells and were not sensitive to the A1 AR agonist S-ENBA. As was the case for pure inner-wall SC cells, CGS-21680 significantly inhibited inner-wall SC currents (17 ± 4% inhibition). A2B AR agonists also significantly increased, whereas A2A AR stimulation produced a significant decrease.

Table 3. Summary of significant current responses to selective AR stimulation of SC cells

<table>
<thead>
<tr>
<th>Agonist</th>
<th>AR Target</th>
<th>±Antag. (To Other ARs)</th>
<th>Means ± SE (%) (All Applications)</th>
<th>P Value Test</th>
<th>Responders/Total Applications Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure inner-wall SC cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-ENBA (100 nM)</td>
<td>A1</td>
<td></td>
<td>↑ 587±199 &lt;0.001</td>
<td>B</td>
<td>18/22 9/11</td>
</tr>
<tr>
<td>CGS-21680 (30 nM)</td>
<td>A2A</td>
<td></td>
<td>↓ 17±7 =0.03</td>
<td>B</td>
<td>4/7 3/4</td>
</tr>
<tr>
<td>NECA (10 μM)</td>
<td>A2A</td>
<td>DPCPX, MRS-1754, MRS-1191</td>
<td>↓ 17±4 =0.001</td>
<td>A</td>
<td>8/11 4/4</td>
</tr>
<tr>
<td>NECA (10 μM)</td>
<td>A2B</td>
<td>DPCPX, ZM-241385, MRS-1191</td>
<td>↑ 50±16 &lt;0.001</td>
<td>B</td>
<td>19/25 8/9</td>
</tr>
<tr>
<td>Mixed inner- and outer-wall SC cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPA (100 nM)</td>
<td>A1</td>
<td></td>
<td>↑ 13±2 =0.01</td>
<td>A</td>
<td>4/4 3/3</td>
</tr>
<tr>
<td>CGS-21680 (100 nM)</td>
<td>A2A</td>
<td></td>
<td>↓ 8±6 =0.1</td>
<td>A</td>
<td>5/5 5/5</td>
</tr>
<tr>
<td>Inhib Alone</td>
<td>A2A</td>
<td></td>
<td>↓ 17±3 =0.002</td>
<td>A</td>
<td>5/5 5/5</td>
</tr>
<tr>
<td>Stim Alone</td>
<td>A2A</td>
<td></td>
<td>↑ 4±2 =0.075</td>
<td>A</td>
<td>3/5 3/5</td>
</tr>
</tbody>
</table>

Values are means ± SE. AR, adenosine receptor. The probability (P) of the null hypothesis was calculated by applying Student’s t-test to data sets which satisfied normality; A, normality satisfied by the untransformed data; B, normality satisfied upon exponential transformation. For CGS-21860 (100 nM) applied to the mixed SC cells, all 5 cells displayed an inhibitory response; 2/5 responses were purely inhibitory and 3/5 biphasic. The stimulation noted in 3/5 applications appeared either before (n = 1) or after (n = 2) the inhibition. The mean calculated by averaging the net stimulation + inhibition for each cell was insignificant. Separate analysis of the stimulatory and inhibitory responses indicated that CGS-21680 produced a significant decrease.

Fig. 4. Effects of the A2A AR agonist CGS-21680 on whole cell currents of an IW SC cell. The A2A AR agonist produced slight decreases in IW SC whole cell currents reversibly and repetitively. The baseline currents were reversibly inhibited by 7.5 mM TEA.
(34 ± 4 μm in diameter) than the inner-wall SC cells at the time point studied. As with inner-wall SC cells, 7.5 mM TEA inhibited baseline whole cell currents (by 40 ± 7%, n = 7, P = 0.002).

As in the case of the purely inner-wall SC cells, selective A1 and A2A AR agonists exerted different effects on whole cell currents of cannula-derived SC cells. Although the magnitude of the effect was smaller (13 ± 2%, n = 4, P = 0.01, Table 3, Fig. 6), the A1 AR agonist CPA (100 nM) also increased whole cell currents. In contrast, the A2A AR agonist CGS-21680 (100 nM) initially appeared to have no significant effect, reducing currents by 8 ± 6% (n = 5, P = 0.1, Table 3). However, we noted that the A2A AR agonist solely inhibited currents in two cells, and produced a biphasic response (both inhibition and stimulation) in three others. When the inhibitions and stimulations were averaged separately (Table 3), the A2A AR agonist inhibited currents by 17 ± 3% (n = 5, P = 0.002) and produced an insignificant stimulation (P > 0.05). Therefore, the CGS-21680 seemed to produce two different effects on the cannuma-derived cells, a significant decrease and a less consistent stimulation. The A3 AR agonist CI-IB-MECA (100 nM) produced mixed responses leading to an overall decrease of 10 ± 5% (n = 6, P = 0.07). The frequency distributions and magnitudes of the responses are summarized in Fig. 6 and Table 3.

Thus although the responses of the cannula-derived SC cells to subtype-selective AR agonists (Fig. 6) were qualitatively similar to those of inner-wall SC cells (Fig. 3), several notable differences were observed. In addition to the smaller A1-triggered stimulation and biphasic A2A-triggered responses (Table 3), the responses to the nonselective agonist adenosine (10 μM) were more consistently inhibitions. The overall effect was a mean inhibition of 36 ± 3% (n = 16, P < 0.001). Adenosine activated currents in 1, inhibited currents in 10, exerted biphasic effects in 4, and had no effect in 1 of 16 applications to 10 cells.
Effects of AR agonists on Intracellular Ca\(^{2+}\) of SC Cells

AR agonists can trigger changes in intracellular Ca\(^{2+}\) concentration in addition to the more commonly addressed alterations in cAMP levels (19, 35, 36, 42). We measured the AR-agonist effects on SC-cell Ca\(^{2+}\) to examine whether the differential effects on whole cell currents might be mediated by differential effects on Ca\(^{2+}\). The nonselective agonist adenosine elicited concentration-dependent increases in intracellular Ca\(^{2+}\) of SC cells, as illustrated in Fig. 7A by the responses, first to 3 μM, and then to 10 μM adenosine. However, 100 μM adenosine subsequently produced a smaller stimulation, possibly because of AR desensitization. ATP (10 μM) later triggered a larger response, suggesting that the small response to 100 μM adenosine had not been limited by intracellular Ca\(^{2+}\) depletion.

The effects of AR agonists on inner-wall SC cells derived by enzymatically assisted isolation and cannula-derived SC cells are presented in the lefthand and righthand columns, respectively, of Fig. 7. The inner-wall SC cells were studied as individual cells with a CCD camera because of the difficulty in obtaining large numbers of confluent sheets of these cells without further passaging. The cannula-derived cells were studied in confluent monolayers with a photomultiplier tube; study of the cells as confluent sheets provided a more favorable signal-to-noise ratio. The baseline Ca\(^{2+}\) levels were 69 ± 8 nM (n = 21) and 169 ± 33 nM (n = 19) for the inner-wall SC cells and cannula-derived SC cells, respectively. For purposes of comparison, baseline Ca\(^{2+}\) concentrations of TM cells have been found to be 136 ± 15 nM (17) and 106 ± 0.5 nM (27). The responses to subtype-selective AR agonists were qualitatively similar for the two preparations of SC cells. A\(_1\), A\(_{2A}\), and A\(_3\) AR agonists all increased the intracellular Ca\(^{2+}\) concentrations of the cannula-derived and inner-wall SC cells. Further details are provided in the legend to Fig. 7.

DISCUSSION

The salient findings of the present work were the following: 1) populations of purely inner-wall SC cells could be isolated and cultured after an enzymatic treatment of human SC, and 2) A\(_1\) and A\(_{2A}\) AR agonists exert differential effects on the inner-wall SC cells.

Isolation and Culture of Inner-Wall SC Cells

The new technique for isolating and growing inner-wall SC cells was adapted from a technique for culturing microvascular endothelial cells (26). Following the preliminary dissection and enzymatic incubation, we separated inner-wall SC cells selectively from the harvested mixed-cell population with antibody to CD31 (PECAM-1), a highly reliable marker of cells of endothelial origin (2, 43, 50) expressed in human SC, but not in TM or other cells within the outflow region (24, 55). The inner, rather than outer, wall of SC may be a/the major site of outflow regulation (14, 32). The inner wall differs from the outer wall in several respects. First, it is usually flatter (14) with different-shaped cells about twice as small in surface size (38). Second, there are no areas of desmin-positive cells, in contrast to the outer wall (23). Third, reactivity to Factor VIII-related antigen is very weak, whereas the reactivity of outer-wall SC is similar to that of the collector channels and aqueous veins (22). Fourth, the inner wall of adult human and monkey SC rarely displays Weibel-Palade bodies, which can, however, be consistently found in sections of outer-wall SC (22). The latter two observations (3–4) have been interpreted to suggest that the inner wall retains a less vascular character than the outer wall (22). To the best of our knowledge, the present study reports the first isolation and study of SC cells selectively from the inner wall.

Differential Effects of Subtype-Selective AR Agonists on Inner-Wall SC Cells

The electrophysiological (Figs. 2–4) and intracellular Ca\(^{2+}\) (Fig. 7) results documented that human inner-wall SC cells respond to subtype-selective A\(_1\), A\(_{2A}\), and A\(_3\) adenosine agonists. In the absence of known selective, efficacious agonists of A\(_{2B}\)ARs, we successfully detected evidence for functional A\(_{2B}\) receptors by applying a nonselective agonist (NECA) in the presence of antagonists to the other three receptors. The data are consistent with the functional presence of all four known AR subtypes in inner-wall SC cells. Strategies to stimulate A\(_1\) and A\(_{2B}\) ARs increased, and maneuvers designed to stimulate A\(_{2A}\), ARs decreased, whole cell currents, largely through TEA-sensitive channels (Figs. 2–5). A selective agonist of A\(_3\) ARs stimulated mixed responses.

Together, these results suggest that A\(_1\) and A\(_{2A}\) ARs might modulate aqueous humor outflow at the level of SC cells and that these opposing membrane-transport effects may be involved in the cascade of events leading to the opposing effects of A\(_1\) and A\(_{2A}\) ARs on aqueous humor dynamics. In contrast, we previously observed that selective A\(_1\), A\(_{2A}\), and A\(_3\) AR agonists all increased whole cell currents of cultured, nontransformed human TM cells, another potential site of outflow regulation (17). The observation that A\(_1\) and A\(_{2A}\) AR agonists exert opposite effects on inner-wall SC whole cell currents is analogous to their opposing effects on intraocular pressure.

Fig. 6. Effects of subtype-selective AR agonists on whole cell currents of cannula-derived SC cells. The responses to the subtype-selective agonists for A\(_1\) (CPA), A\(_{2A}\) (CGS-21680), and A\(_3\) (Cl-IB-MECA) ARs were qualitatively similar to those displayed by IW SC cells (Fig. 3). Of the responses to CGS-21680, only the two purely inhibitory effects are averaged here; three additional responses were biphasic, and are included in the data reduction presented in Table 3.
The mechanisms by which SC cells might mediate the opposing effects of A1 and A2A AR agonists on outflow resistance are unclear. Among other possible sequences of events, A1AR activation of K+/H+ channels is expected to hyperpolarize the SC-cell membrane, increasing the electrical driving force favoring Cl-/H+ release, enhancing transfer of K+/H+ and Cl-/H+ and secondarily water to the extracellular space. Cell shrinkage might possibly lower outflow resistance by increasing the probability of transmembrane pore formation (14, 32) or by placing stress on the intercellular tight junctions, reducing the number and complexity of the junctional strands (32, 59). A2AAR inhibition of K+/H+ channels is expected to exert the opposite effects, which might contribute to cell swelling and enhanced outflow resistance.

Effects of AR Agonists on Ca2+

Selective A1, A2A, and A3 AR agonists all increased Ca2+ concentration in inner-wall SC and cannula-derived SC cells (Fig. 7). Adenosine-selective (P1) receptors also act partly by increasing (A2A, A2B) or decreasing (A1, A3) intracellular cAMP levels (18), but the full signaling cascades are complex and incompletely understood (37). In view of the similarity of the Ca2+ responses to the different AR agonists, it is uncertain...
whether their differential outflow effects are mediated by changes in intracellular Ca\(^{2+}\). Nevertheless, signaling can be conducted in local cellular areas where hormone receptors and downstream pathways and effector channels may be clustered (28, 44, 58). Thus measurements of changes in second-messenger concentrations averaged over the entire cell may be poor indicators of the changes produced at the cell membrane. For example, increased Ca\(^{2+}\) levels arising from ATP-stimulated apical, but not basolateral, P2Y receptors have been reported to activate Cl\(^{-}\) channels in equine sweat gland E9/2/3 cells (58). Therefore, the increases in total cell Ca\(^{2+}\) measured in the current study may not accurately reflect changes at the target site. In addition, more than additive increases can be produced by co-delivery of adenosine and other biologically active molecules (16).

**Multiple Potential Roles of ARs in Regulating Intraocular Pressure**

The present work used adenosine agonists to probe the cellular site of aqueous humor outflow regulation. The data are also relevant to the complex regulation of intraocular pressure by adenosine receptors.

The physiological source of adenosine in the anterior segment in vivo derives from ATP, which can be released by the ciliary epithelium (15, 40), the site of aqueous humor secretion, and by TM cells in the outflow pathway (17). ATP is metabolized to adenosine by ecto-enzymes (40). Adenosine receptors are of particular physiological importance because knockout of A\(_2\) ARs lowers intraocular pressure in mice (5) and antagonists to A\(_1\) ARs lower pressure in both mice (4, 5) and nonhuman primates (45). The potential pathophysiological importance of ARs is suggested by very recent observations that A\(_2\) ARs are markedly upregulated in nonpigmented ciliary epithelial cells in the pseudoexfoliation syndrome, a major cause of open-angle glaucoma (49). Interestingly, aqueous humor concentrations of adenosine are increased in patients with primary open-angle glaucoma and aqueous adenosine levels are correlated with pressure in ocular hypertensive patients (12), but whether the changes are a cause of, or response to, the elevated intraocular pressure is unclear.

The current work demonstrates that A\(_1\) and A\(_2\)A AR agonists exert opposite effects on whole cell currents in these cells, a difference that might be related to their opposite effects on aqueous humor outflow resistance. The data also suggest that stimulation of A\(_2\)B ARs increased SC-cell currents, but the physiological importance of this stimulation is unclear. These receptors are stimulated by adenosine only at concentrations of magnitude higher than those for activating A\(_1\), A\(_2\)A, or A\(_2\)B receptors (Table 2). In contrast to A\(_1\) and A\(_2\)A ARs, A\(_3\) receptors have been thought to increase intraocular pressure by enhancing inflow, an effect mediated by activating Cl\(^{-}\) channels of the nonpigmented cells of the ciliary epithelium (6, 7, 42). In agreement with this concept, stimulation of A\(_3\) receptors produced no consistent change in SC-cell currents. Taken together with published data, the current results lead us to suggest that A\(_3\) ARs increase intraocular pressure largely by stimulating inflow of aqueous humor and that A\(_1\), A\(_2\)A, and A\(_2\)B ARs can modulate pressure by altering outflow resistance at the SC-cell level.

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