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Effects of cardiotonic steroids on trabecular meshwork cells: Search for mediator of ouabain-enhanced outflow facility

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ABSTRACT

Lowering intraocular pressure (IOP) is currently the only strategy documented to slow the onset and progression of glaucomatous blindness. Ouabain, a cardiotonic glycoside inhibitor of Na^+, K^+-activated ATPase, was recently reported to enhance outflow facility in porcine anterior segments at concentrations as low as 30 nM for $\geq$4 h, suggesting a novel approach to lowering IOP. The underlying mechanism is unknown, but associated cytoskeletal changes were observed in porcine trabecular meshwork cells. We have previously found that changes in ATP release and subsequent ectoenzymatic conversion to adenosine may play a role in linking cytoskeletal remodeling with modulation of outflow resistance. We now tested whether altered ATP release might also be a mediator of ouabain's effect on outflow facility. ATP release from transformed human TM5 and explant-derived human trabecular meshwork cells was measured by the luciferin–luciferase reaction. Matrix metalloproteinases (MMPs) were studied by zymography, cell Na^+ concentration by SBFI fluorometry, gene expression of ATP-release pathways by real-time PCR, cell volume by electronic cell sorting and cell viability by the LDH and MTT methods. Actin was examined by confocal microscopy of phalloidin-stained cells. Contrary to expectation, ouabain at concentrations $\geq$10 nM inhibited swelling-triggered ATP release from TM5 cells after $\geq$4 h of exposure. Inhibition was enhanced by increasing ouabain concentration and exposure time. Similar effects were produced by the reversible cardiac aglycone strophanthidin. Ouabain also inhibited swelling-activated ATP release from explant-derived native human TM cells. Ouabain (4 h, 30 nM and 100 nM) did not alter gene expression of the ATP-release pathways, and cell viability was unchanged by exposure to ouabain (30 nM–1 μM). Preincubation with 30 nM ouabain for 4 h did not detectably change Na^+ level, the regulatory volume decrease (RVD) or the actin cytoskeleton of TM5 cells, but did inhibit hypotonicity-elicited ATP release. Moreover, even when N-methyl-D-glucosamine replaced Na^+ in the extracellular fluid, ouabain still inhibited swelling-initiated ATP release at 100 nM. In the absence of ouabain, extracellular ATP stimulated MMP secretion, which was largely blocked by inhibiting conversion of ATP to adenosine, as expected. In contrast, ouabain reduced ATP release, but did not alter secretion of MMP-2 and MMP-9 from cells pretreated for $\leq$4 h. The results suggest that: (1) ouabain can trigger enhancement of outflow facility independent of its transport and actin-restructuring effects exerted at higher concentration and longer duration; (2) ouabain exerts parallel independent effects on ATP release and outflow facility; and (3) these effects likely reflect ouabain-induced changes in the scaffolding and/or signaling functions of Na^+, K^+-activated ATPase.

1. Introduction

Reduction of intraocular pressure (IOP) is currently the only intervention demonstrated to delay the onset and slow the progression of irreversible blindness associated with glaucoma, even in patients without elevated tension (Collaborative Normal-

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Tension Glaucoma Study Group, 1998a,b; Kass et al., 2002; Leske et al., 2003; The AGIS investigators, 2000). IOP can be reduced by lowering the rate of aqueous humor formation, increasing outflow facility through the pressure-sensitive trabecular outflow pathway, or diverting part of the trabecular outflow to exit the relatively pressure-insensitive uveoscleral pathway. Recently, the cardiotonic steroid ouabain, an inhibitor of Na^+, K^+-activated ATPase, was found to increase outflow facility in perfused porcine anterior segments, suggesting a novel approach for lowering IOP (Dismuke et al., 2009). Ouabain was effective at relatively low concentrations ($\geq$30 nM) after perfusion for 4 h or more. The basis for the phenomenon is unknown, but parallel changes in the actin cytoskeleton were noted in ouabain-treated porcine trabecular meshwork (TM) cells.

Remodeling of the actin cytoskeleton is known to alter outflow facility, and cytoskeletal-disrupting drugs have been found to lower IOP in humans (Tanihara et al., 2008) and non-human primates (Tian et al., 2000). We have recently observed that changes in ATP release and subsequent ectoenzymatic conversion to adenosine may play a role in linking actin remodeling with modulation of outflow facility. In particular, cytoskeletal changes leading to increased ATP release might enhance ectoenzyme-mediated delivery of adenosine to A_1 adenosine receptors, thereby stimulating secretion of matrix metalloproteinases MMP-2 and MMP-9 by TM cells (Li et al., 2011). Enhanced MMP activity is known to increase outflow facility of human (Bradley et al., 2001) and bovine anterior segments (Crosson et al., 2005), and is thought to reflect changes in MMP secretion by TM cells.

In the present study, we have tested whether ouabain might alter outflow facility by modulating ATP release-initiated MMP secretion by trabecular meshwork cells. We have also examined whether ouabain's effects on TM cells are mediated solely by inhibiting the ion-exchange activity of the Na^+, K^+-activated ATPase or possibly by the scaffolding and signaling functions of the enzyme (Schoner and Scheiner-Bobis, 2007; Xie and Askari, 2002).

2. Materials and methods

2.1. Cellular model

Transformed normal human trabecular meshwork cell line TM5 (Alcon Research Inc., Fort Worth, TX) was maintained in DMEM high-glucose media supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 50 μg/mL of gentamicin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, as previously described (Li et al., 2010, 2011, 2012; Pang et al., 1994). Cells from passages 25 to 40 were used in the experiments. Primary human trabecular meshwork cells (HTM; Stamer et al., 1995) were maintained in DMEM low-glucose media containing the same supplements; cells from passages 3–5 were used in the experiments.

2.2. Solutions and pharmacological reagents

The isotonic solution (295–305 mosmol/kg) with 0.1 mM extracellular free Ca^{2+} contained (in mM): 110 NaCl, 4.7 KCl, 5.1 $CaCl_2$, 1.2 $MgCl_2$, 30 $NaHCO_3$, 1.2 KH_2PO_4, 15 HEPES, 5 EGTA and 10 glucose, as previously described (Li et al., 2010, 2011, 2012). Selectively omitting NaCl decreased osmolality to ~100 mosmol/kg (67% hypotonicity). Intermediate osmolalities were generated by appropriate mixing of the isotonic and hypotonic solutions. In some experiments, sodium and chloride were replaced with equimolar N-methyl-D-glucamine (NMDG) and gluconate, respectively, to maintain the desired ion strength and osmolality. The final osmolalities were verified and pH values adjusted to 7.4 before each experiment. Biochemical reagents were purchased from Sigma–Aldrich (St. Louis, MO). Chemicals and media for cell culture were obtained from GIBCO (Invitrogen, Carlsbad, CA). DMSO (<0.5%) was used to solubilize hydrophobic drugs, exposing controls to the same concentration of vehicle. Unless otherwise stated, all experiments were performed at room temperature.

2.3. ATP measurement

ATP release was determined by the bioluminescent luciferin–luciferase reaction with light emission recorded using the Synergy 2 microplate luminometer (BioTEK, Winooski, VT), as previously reported (Li et al., 2010, 2011, 2012). In brief, TM cells were seeded onto 96-well microplates (Corning Costar, Corning, NY) at 0.1 million per well, permitting confluence within 1–2 days. Drugs were added to the culture media at the final concentrations and for the periods specified. To minimize ATP release resulting from changing solutions, culture media were removed and replaced with 100 μL isotonic solutions with/without drugs 1 h before experiment. Thereafter, 75 μL of isotonic solutions were replaced by an equal volume of test solution to establish the final drug concentrations and osmolalities. Measurements were begun instantaneously after dispensing 10 μL of the ATP assay solution into each well through the internal dispenser system, and recorded for 2 h at 2-min intervals, with an integration time of 0.2 s/measurement. ATP levels were calculated at each time point from a standard curve converting arbitrary light units into ATP concentrations. Separate standard curves were utilized in experiments involving changes in ionic strength. No test substance interfered with the ATP assay at the specified concentration used in this study. Inhibition of the hypotonicity-triggered enhancement of ATP release was calculated from Eq. (1), as previously described (Li et al., 2010, 2011, 2012).

$$\text{Inhibition}\ (\%) = 100\% \cdot \left[(C_{max} - C_{exp}) / (C_{max} - C_{con}) \right] \quad (1)$$

C_{max} was the maximal ATP concentration after hypotonic treatment without inhibitor, C_{con} was the control ATP concentration in the isotonic bath at the same time point, and C_{exp} was the maximal ATP concentration after hypotonic treatment in the presence of inhibitor.

2.4. Gelatin zymography for matrix metalloproteinases (MMPs)

Using previously reported methods (Hawkes et al., 2010; Li et al., 2011; Sanka et al., 2007), the secretion of MMP-2 and MMP-9 into the external media was measured by gelatin zymography. Briefly, TM5 cells were plated onto 48-well plates at a density of 0.2 million per well, and allowed to grow to confluence, followed by serum starvation for 24 h. Thereafter, 140 μL of fresh DMEM media with or without drugs were added to each well to condition cells at 37 °C for the periods specified. The conditioned media were completely collected and cleared by centrifugation (10,000 g) for 20 min. The supernatants were then mixed with the Zymogram Sample Buffer (Bio-Rad, Hercules, CA), and 30 μL per sample were loaded onto each lane of the 10% Precast Zymogram Gels (Bio-Rad) for SDS-PAGE separation. After electrophoresis, gels were washed sequentially with the Zymogram Renaturing Buffer for 3 h, Zymography Developing Buffer for 24 h (at 37 °C), and Coomassie Brilliant Blue R-250 Staining Solution for 8 h (all from Bio-Rad). Gels were destained in the Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad) until clear bands were visible against the blue background. The gels were subsequently scanned (Scanjet 3570c, Hewlett–Packard, Palo Alto, CA), and band density was analyzed by Image J Software (Ver. 1.45, National Institutes of Health, Bethesda, MD).