NO-induced regulation of human trabecular meshwork cell volume and aqueous humor outflow facility involve the BK$_{Ca}$ ion channel

William M. Dismuke, Chigozirim C. Mbadugha, and Dorette Z. Ellis

University of Florida, College of Pharmacy, Department of Pharmacodynamics, Gainesville, Florida

Submitted 13 August 2007; accepted in final form 1 April 2008

Dismuke WM, Mbadugha CC, Ellis DZ. NO-induced regulation of human trabecular meshwork cell volume and aqueous humor outflow facility involve the BK$_{Ca}$ ion channel. Am J Physiol Cell Physiol 294: C1378–C1386, 2008. First published April 2, 2008; doi:10.1152/ajpcell.00363.2007.—Nitric oxide (NO) donors decrease intraocular pressure (IOP) by increasing aqueous outflow facility in the trabecular meshwork (TM) and/or Schlemm’s canal. However, the cellular mechanisms are unknown. Cellular mechanisms known to regulate outflow facility include changes in cell volume and cellular contractility. In this study, we investigated the effects of NO donors on outflow facility and NO-induced effects on TM cell volume. We tested the involvement of soluble guanylate cyclase (sGC), cGMP, PKG, and the large-conductance Ca$^{2+}$-activated K$^+$ (BK$_{Ca}$) channel using inhibitors and activators. Cell volume was measured using calcein AM fluorescent dye, detected by confocal microscopy, and quantified using NIH ImageJ software. An anterior segment organ perfusion system measured outflow facility. NO increased outflow facility in porcine eye anterior segments (0.4884–1.3956 mm Hg $\cdot$ min$^{-1}$ $\cdot$ mm Hg$^{-1}$) over baseline (0.2373–0.5220 mm Hg $\cdot$ min$^{-1}$ $\cdot$ mm Hg$^{-1}$) within 10 min of drug application. These NO-induced increases in outflow facility were inhibited by the the BK$_{Ca}$ channel inhibitor IBTX. Exposure of TM cells to NO resulted in a 10% decrease in cell volume, and these decreases were abolished by the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one and IBTX, suggesting the involvement of sGC and K$^+$ efflux, respectively. NO-induced decreases in cell volume were mimicked by 8-Br-cGMP and abolished by the PKG inhibitor (RP)-8-Br-PET-cGMP-S, suggesting the involvement cGMP and PKG. Additionally, the time course for NO-induced decreases in TM cell volume correlated with NO-induced increases in outflow facility, suggesting that the NO-induced alterations in cell volume may influence outflow facility.

NO synthase (NOS), particularly endothelial NOS (NOS III) (39) and nitrinergic nerve terminals (48). NO donors reduced IOP in normal rabbit eyes without systemic effects (38). Additionally, the intracameral injection of NO donors decreased IOP by increasing outflow of aqueous humor (29). Similarly, intravitreal and intracameral injections of NO donors in rabbits caused drastic decreases in IOP, concurrent with nitrite production, the measurable result of NO production (6). Another study (47) has shown that NO donors reduce IOP in monkeys through an action on outflow resistance. While NO donors effectively reduce IOP, the signaling cascade mediating the cellular response in this tissue is unknown. The present study was designed to elucidate the mechanism of response of the TM to NO.

We tested the hypothesis that NO donors regulate TM cell function by decreasing TM cell volume. We tested the involvement of a regulatory pathway by which NO acting via activation of soluble guanylate cyclase (sGC), cGMP, and PKG decrease TM cell volume. Because BK$_{Ca}$ channels are activated by NO (44, 45), we also examined the role of BK$_{Ca}$ channels in NO-induced decreases in cell volume and NO-induced increases in outflow facility. Additionally, we determined the time course for NO-induced decreases in TM cell volume correlated with NO-induced increases in outflow facility.

MATERIALS AND METHODS

Tissue. Because morphological and biochemical studies have suggested that the porcine anterior chamber perfusion model can be correlated with the human perfusion system (4), perfusion experiments were performed using porcine eyes. Cellular experiments were performed using porcine anterior chamber segments. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
performed in human primary TM cells, and parallel experiments were performed in primary porcine TM cells. Tissues used were approved by Institutional Review Board and Institutional Animal Care and Use Committee, University of Florida.

**Cell culture.** Eyes from human donors with no history of ocular disease or surgery were obtained from Lions Eye Institute (Tampa, FL) within 24–30 h postmortem. Primary human TM cell lines (HTM44, a generous gift of Dr. D. Stamer; HTM26, HTM71, HTM36, HTM80, and HTM86; where numbers represent ages of the donors) were developed. For our experimental protocols, cells from early passages (passages 3–5) were used. Human TM explants were obtained from either whole eyes that were stored in a moist environment at 4°C or corneal scleral rims stored in Optisol (Dexol, Chiron Ophthalmics, Irvine, CA) at 4°C. Porcine eyes were obtained from the local abattoir within 1 h postmortem and maintained on ice. We used standard ophthalmic microsurgery instruments to bisect the eyes and remove the cornea, iris, lens, and ciliary body. TM cells were isolated after collagenase digestion of TM explants (52). Collagenase-treated cells remove the cornea, iris, lens, and ciliary body. TM cells were isolated on ice following enucleation, and bisected within 2 h postmortem. Eyes were bisected at the equator, and the iris, lens, and ciliary processes were removed. Anterior segments were cultured at 37°C in 100% humidity at 5% CO₂ atmosphere and perfused at constant pressure of 14 mmHg (15, 41). Outflow rates were determined gravimetrically as the changes in weight of the medium as the eyes were perfused over time. Data were captured at 1-min intervals by WinWedge software attached to the balance and recorded in an Excel spreadsheet; outflow facility was expressed as perfusion pressure (in μl·min⁻¹·mmHg⁻¹). Organ perfusions were performed with isotonic DMEM (~309 mosM/kg) to establish baseline facility followed by perfusion under several experimental conditions: hypotonic and hypertonic media (to establish that the volume-regulatory mechanisms were functional) and the NO donor diethylenetriamine (DETA)-NO (100 μM). Hypotonic and hypertonic media were made as described above. After a stable baseline had been established, drugs were delivered via a drug exchange system, which supplied a bolus of drug. Specifically, a 10-ml syringe, held at a height above the mounted anterior segment and containing DMEM plus drugs, was attached to one of the perfusion chamber’s ports. Another 20-ml waste syringe, held below the mounted anterior segment, was attached to a chamber port, and this line was clamped. By slightly opening the clamp to the waste syringe, the anterior chamber medium was slowly exchanged with the medium in the drug supply syringe in a manner that prevented any changes in the perfusion pressure. After medium containing drugs was in the chamber, entry to this port was clamped, and the flow of medium without drugs was restarted through a third port.

The viability of the tissue was evaluated using live-dead stain (Molecular Probes, Carlsbad, CA), which stained for the total number of nuclei in the TM following perfusion. Specifically, flatmount intact TM tissue was treated with live-dead stain and visualized using confocal microscopy. Live cells were stained with green fluorescence, and dead cells were stained with red fluorescence. Cellular viability was determined to be good, and data were considered usable when 85–90% of the total cells stained with green fluorescence.

**Materials and reagents.** Routine reagents and IBTX were purchased from Sigma (St. Louis, MO). Other reagents were obtained as follows: 8-Br-cGMP sodium salt, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), and DETA-NO were from Sigma-RBI (Natick, MA) and (RP)-8-Br-PET-cGMP-S was from Calbiochem (La Jolla, CA).

**Statistics.** Statistical analysis was performed using ANOVA followed by the Holm-Sidak method for comparison of significant difference among different means.

**RESULTS**

**NO donors increase outflow facility.** Outflow facility was measured in porcine anterior eye segments as previously described. Freshly dissected eye anterior segments were allowed to adapt to their new environment, during which time outflow facility increased for the first 3–8 h, a phenomenon referred to as “washout” (16, 41), after which outflow facility remained stable (4). Basal outflow facility (predrug treatment) was 0.2373–0.5220 μl·min⁻¹·mmHg⁻¹ among experiments, was stable for several hours prior to drug treatment, and remained stable after the drug effect. Because of the stability of the
outflow facility baseline after the initial washout period, it was not necessary to correct for nondrug-related changes in outflow facility in our experimental protocol.

After a stable baseline had been established, DETA-NO (100 μM) was added to the perfusate and resulted in a significant increase in outflow facility (Fig. 1A). Outflow facility was increased at 10 min and reached its maximal level at 20 min following the application of DETA-NO. The maximal effect of the drug was sustained for 1.5 h, after which outflow facility returned to values similar to baseline outflow facility between 5 and 6 h postdrug application. We observed significant increase in outflow facility (0.4884 – 1.3956 μl·min⁻¹·mmHg⁻¹; mean ± SE: 0.8635 ± 0.1029 μl·min⁻¹·mmHg⁻¹) over baseline values in response to DETA-NO in eight separate experiments.

We tested the ability of the outflow pathway to respond to changes in osmolarity preceding NO treatment. This allowed us to determine that the perfused eye segments were healthy and responded with expected changes in outflow facility when challenged with changes in osmolarity. Figure 1B shows that exposure of porcine eye anterior segments to perfusion with hypertonic media resulted in a significant increase in outflow facility (40% above baseline), after which outflow facility returned to baseline. Eyes were perfused for 19 h with DMEM only, and baseline values decreased slightly but remained constant for several hours. Subsequently, a bolus of DETA-NO was added to the porcine anterior segment and allowed to perfuse. Figure 1B shows that, as with the results shown in Fig. 1A, DETA-NO increased outflow facility after the eye had previously responded to hyperosmotic challenge.

NO donors are known to activate BK₉Ca channels, resulting in the efflux of K⁺ from the cell. We wanted to determine the involvement of BK₉Ca channels in the NO-induced regulation of outflow facility. After baseline outflow facility had been determined, anterior segments were perfused with DETA-NO as previously described. IBTX (50 nM) (49, 53) was added to the perfusate after the NO-induced increase in outflow facility had been observed. The addition of IBTX resulted in a significant decrease in the NO-induced response in outflow facility within 10 min after IBTX had been added (Fig. 1C).

**NO decreases TM cell volume.** To quantitatively measure changes in cell volume, human TM cells (HTM26, HTM44, and HTM86) were incubated in calcein AM dye, imaged at the 0-min time point (control), subsequently exposed to the NO donor DETA-NO (100 μM), and then imaged at 20 min. z-Stack images demonstrated that DETA-NO decreases TM cell volume (Fig. 2A). To determine the concentration needed to decrease cell volume, early-passage human TM cells were exposed to varying concentrations of DETA-NO (1–300 μM). Images were taken without drug treatment (the 0-min time point), which served as a control for the treatment groups. Drugs were then added to the culture and images were taken of the same cells at 20 min. Figure 2B shows that DETA-NO elicited a dose-dependent decrease in human TM cell volume with maximal effects produced by 100 and 300 μM.

We also tested the reversibility of DETA-NO-induced decreases in TM cell volume. In our hands, calcein AM was stable for up to 1 h after calcein AM incorporation into cells and exposure to laser treatments. Therefore, all experimental treatments needed to be done within a 1-h time period. Because in our preliminary experiments we had observed significant

**Fig. 1. Nitric oxide (NO) donors increase outflow facility in a porcine anterior organ culture perfusion.** A: a stable baseline was achieved, after which the anterior chamber perfusate was replaced with an acute treatment of diethylenetriamine (DETA)-NO (100 μM) dissolved in DMEM. The drug entry port was clamped, and perfusion continued with DMEM alone. Data shown are representative of 8 experiments. *Significantly different from baseline values (P < 0.05 by ANOVA and the Holm-Sidak method). B: a stable baseline was achieved, after which the isotonic medium was exchanged with hypertonic DMEM. Following the effects of hypertonic DMEM, a stable baseline was reestablished, and DETA-NO (100 μM) was then added to the perfusate. Data shown are representative of 3 experiments. *Significantly different from baseline values; #significantly different from DETA-NO-treated samples (P < 0.05 by ANOVA and the Holm-Sidak method).
Fig. 2. NO decreases trabecular meshwork (TM) cell volume. A: thresholded z-stack images of a TM cell. At 0 min (without drug), thresholded voxels were qualitatively and quantitatively greater than at 20 min after DETA-NO (100 μM) treatment. Voxel counts for cells were 27,650 at 0 min and 23,342 at 20 min. Scale bar = 20 μm. B: NO-induced decreases in cell volume were concentration dependent. Confocal images of the same cells were acquired with a ×20 objective lens at 1-μm z-step intervals to a depth of 15 μm. Human TM cells were exposed to varying concentrations of DETA-NO (1–300 μM). Images were captured at 0- and 20-min time points. Data shown for 1, 10, 30, 50, 100, and 300 μM DETA-NO represent means ± SE for 23, 31, 19, 22, 44, and 23 cells, respectively, and are expressed as percentages of initial volume at the 0-min time point without drugs. The average voxel count was 25,449 ± 4,398. *Significantly different from control (P < 0.05); #significantly different from 30 and 50 μM DETA-NO (P < 0.05 by ANOVA and the Holm-Sidak method). C: decreases in human TM cell volume were reversible. Data are expressed as percentages of the initial volume at the 0-min time point and are means ± SE; n = 23 cells. The voxel count for the 0-min time point was 11,606 ± 1,155. *Significantly different from control (0-min time point; P < 0.001); #significantly different from DETA-NO-treated cells (P < 0.05).

Changes in cell volume in response to changes in osmolarity. To assess the function of volume-regulatory mechanisms in TM cells, we exposed both early-passage human (HTM80 and HTM86) and porcine TM cells to hypo- and hypertonic DMEM, which would be expected to cause swelling and shrinkage of the cells, respectively. TM cells were incubated with calcein AM as previously described, and stable baselines were established. Images were captured at the 0-min time point in isotonic medium (control), after which the osmolarity of the medium was altered. Porcine TM cell volume was altered in response to hypotonic as well as hypertonic media (Fig. 3A). There was a 12.4% increase in TM cell volume after the medium was changed from isotonic to hypertonic medium. Thereafter, TM cell volume gradually decreased without changes in osmolarity of the medium. Exposure of TM cells to hypertonic medium resulted in a 16.2% decrease in cell volume postisotonic changes. Cell volume increased after hypertonic medium was exchanged with isotonic medium 4 min postosmotic challenge at 37°C in 5% CO2 (Fig. 3A).

Figure 3B demonstrates that exposure of human TM cells to hypertonic medium resulted in a 11% increase in cell volume. Images were captured of cells treated with isotonic medium, after which cells were exposed to hypotonic medium, and images were captured within 1 min of exposure of cells to anisosmotic medium. There was an increase in TM cell volume that peaked within 3 min after the medium had been changed from isotonic to hypertonic medium and returned to baseline. We then tested the involvement of the BKCa channel in the TM volume-regulatory mechanism. Calcein AM-loaded cells were exposed to hypertonic medium in the presence or absence of IBTX (50 nM). Figure 3B shows that the maximum cell volume increase was observed in the presence of IBTX. Additionally, IBTX inhibited the regulatory volume decrease and allowed for a sustained increase in cell volume in response to hypotonicity compared with cells that were not treated with IBTX.

Exposure of TM cells to hypertonic medium resulted in a 31% decrease in cell volume. At 4 min, hypertonic medium was exchanged with isotonic medium at 37°C in 5% CO2, after which cell volume increased (Fig. 3B).

NO-induced decreases in cell volume involve the activation of sGC and cGMP. To test the involvement of sGC in NO-induced decreases in cell volume, primary human TM cells (HTM44 and HTM86) were incubated with calcein AM as described above. Images were taken at the 0-min time point, without drug treatment; DETA-NO (100 μM) was added to cells in the presence or absence of ODQ (1 μM) (22), the specific sGC inhibitor; and images were taken at 5-, 10-, 15-, and 20-min time points. Figure 4, A and B, shows that ODQ...
DETA-NO, 8-Br-cGMP significantly reduced TM cell volume, suggesting the involvement of cGMP and the possible involvement of PKG in the regulation of TM cell volume. To further determine if PKG is involved in NO-induced decreases in TM cell volume, human TM cells (HTM26 and HTM86) were incubated with DETA-NO (100 μM) or 8-Br-cGMP (2 mM) (46) with or without the specific inhibitor of PKG (RP)-8-Br-PET-cGMP-S (50 μM) (10). Figure 5 shows that (RP)-8-Br-PET-cGMP-S partially inhibited DETA-NO- and 8-Br-cGMP-induced decreases in TM cell volume, suggesting a role for PKG in the regulation of cell volume.

**BKCa channels are involved in NO-induced decreases in TM cell volume.** To test whether or not activation of the BKCa channel was involved in NO-induced decreases in cell volume, human TM cells (HTM26 and HTM80) were preincubated

![Image](https://example.com/image-url)
Fig. 5. Effects of PKG inhibitor (RP)-8-Br-PET-cGMP-S (PKGi) on 8-Br-cGMP- and DETA-NO-induced decreases in TM cell volume. Cells were incubated with 8-Br-cGMP (2 mM) or DETA-NO (100 µM) in the presence or absence of PKGi (50 µM). Images were taken, and the cell volume was measured. Data are expressed as percentages of the initial volume at the 0-min time point and are means ± SEM; n = 100 cells for the 8-Br-cGMP-treated group, n = 86 cells for the 8-Br-cGMP + PKGi-treated group, n = 97 cells for the DETA-NO + PKGi-treated group. Voxel counts for the 0-min time point were 2,771 ± 102 for the 8-Br-cGMP group, 4,742 ± 155 for the 8-Br-cGMP + PKGi-treated group, 4,125 ± 123 for the DETA-NO + PKGi-treated group, and 3,422 ± 131 for the DETA-NO + PKGi-treated group. *Significantly different from the 0-min time point; #significantly different from the 8-Br-cGMP-treated group (P < 0.001); ##significantly different from the 8-Br-cGMP-treated group (P < 0.001).

with IBTX (100 nM), and images were captured at the 0-min time point. DETA-NO (100 µM) was then added to the cells, and images were captured at the 5-, 10-, 15-, and 20-min time periods after DETA-NO exposure. Figure 6 (open diamonds) demonstrates that NO was unable to cause decreases in TM cell volume in cells that were pretreated with IBTX. We next wanted to determine the effects of IBTX on cells that had experienced decreased cell volume in response to NO treatment. Images were captured without drugs at the 0-min time point. Cells were then treated with DETA-NO (100 µM), and images were captured at 5, 10, and 15 min. IBTX (100 nM) was then added at 15 min after DETA-NO treatment, and images were captured at the 20-min time point. Figure 6 shows that decreases in cell volume were time dependent, with significant decreases observed at 10 and 15 min after drug incubation. The addition of IBTX at 15 min after incubation with DETA-NO reduced NO-induced decreases in cell volume compared with only DETA-NO-treated cells. Additionally, IBTX alone had no significant effect on human TM cell volume (Fig. 6).

DISCUSSION

In this study, we provide evidence that NO decreases TM cell volume by the activation of the sGC/cGMP/PKG pathway in a manner dependent on BKCa channels (Fig. 7). We also show that the time course for increased outflow facility in response to NO correlates with changes in cell volume in response to NO.

Outflow facility. In our experimental protocol, an acute application of DETA-NO increased outflow facility in porcine eye anterior segments. Other studies in monkeys (28) and rabbits (29) have demonstrated the involvement of the NO/sGC and cGMP pathway in increasing outflow facility. The NO effect was immediate and transient, and the degree of increases

Fig. 6. Large-conductance Ca
2+
-activated K+
(BKCa)
 channels mediate NO-induced decreases in TM cell volume. For IBTX (100 nM) only, data are expressed as percentages of the initial volume at the 0-min time point and represent means ± SE; n = 58 cells. For DETA-NO + IBTX, images were captured at the 0-min time point without drugs. DETA-NO (100 µM) was added, and images were then captured at 5, 10, and 15 min. IBTX (100 nM) was added at 15 min, and images were taken at 20 min. For DETA-NO, cells were incubated with DETA-NO only, and data at the 20-min time point were compared with data obtained at the 20-min time point for DETA-NO + IBTX-treated cells. Data are expressed as percentages of the initial volume at the 0-min time point and represent means ± SE; n = 46 cells. *DETA-NO + IBTX-treated cells were significantly different from DETA-NO-treated cells (P < 0.05). For IBTX + DETA-NO, TM cells were preincubated with IBTX, and then images were captured. Cells were then exposed to DETA-NO (100 µM), and images were captured at 5, 10, 15, and 20 min. Data are expressed as percentages of the initial volume at the 0-min time point and represent means ± SE; n = 51 cells. Voxel counts for the 0-min time point were 13,147 ± 865 for the IBTX-treated group, 9,202 ± 625 for the DETA-NO + IBTX-treated group, and 19,202 ± 925 for the IBTX + DETA-NO-treated group.

Fig. 7. Summary diagram of the pathway of NO regulation of TM cell volume. NO donors cause the formation of NO, which then binds to and activates sGC, the synthetic enzyme of cGMP. cGMP and its analog 8-Br-cGMP activate PKG, which may, directly or indirectly, phosphorylate BKCa channels, with subsequent K+ efflux and decreases in cell volume.
in outflow facility varied among experiments. We do not know the reason for this variability in response to NO. The possibility exists that lower flow rates allow for increased time for medium to be held in the anterior segment of the eye, thus bathing the tissue with the drug for a longer period of time and hence producing a more sustained drug effect. The ability of NO to increase outflow facility is corroborated by a report from Kotikoski et al. (29) demonstrating the ability of NO donors to increase outflow facility in rabbit eyes.

Additionally, we demonstrated the ability of IBTX to inhibit NO-induced increases in outflow facility. The rapid reversal of NO-induced increases in outflow facility by IBTX would suggest that inhibition of the BKCa channel would possibly stop cell shrinkage and allow for a rapid regulatory volume increase. Another study (54) has demonstrated that BKCa channels are downstream effectors of NO and are involved in mediating the NO/cGMP-induced smooth muscle relaxation. In the TM, the BKCa channel is expressed and is involved in the NO-induced relaxation of precontracted bovine TM muscle strips (53). These data suggest that in addition to its ability to regulate NO-induced decreases in cell volume, the BKCa channel may be involved in regulating the contractile states of cells in the outflow pathway. In physiological states, NO could cause previously contracted cells to relax, thus increasing outflow facility. Blockade of the BKCa channel would potentially cause the cells to contract, thus causing outflow facility to return to baseline values. Taken together, these data suggest the possible involvement of both NO-induced decreases in cell volume and/or changes in the cells’ contractile states as cellular functions by which aqueous outflow is altered.

The proposed role of NO in regulating IOP is not without controversy, however. One report by Krupin et al. (31) demonstrated an increase in IOP in rabbits in response to the NO donor sodium nitroprusside. While we do not understand the reason for this discrepancy, it can possibly explained by dose-dependent effects of NO on IOP. Higher doses of NO donors result in increases in IOP, whereas lower doses result in decreases in IOP (17, 38). Additionally, repeated use of the organic nitrate nitroglycerin resulted in tolerance, whereas chronic usage of the nucleophile hydralazine did not result in tolerance (38).

Increases in the osmolarity of the perfusion medium resulted in increases in outflow facility that mimicked the effects of DETA-NO. Consistent with the literature, anterior eye segments perfused with hypertonic medium (2, 24) resulted in increases in outflow facility, whereas hypertonic medium (2, 24, 49) resulted in decreases in outflow facility.

Cell volume experiments. Our protocol allowed us to quantify changes in cell volume in hundreds of intact, adherent cells in their native states, and each cell was able to serve as its own control. While we allowed for calcein AM to achieve a stable baseline, after which cell volume was measured in response to drug treatment in isometric medium, we also imaged cells that were not treated with drugs to assess any changes in fluorescence in response to laser exposure. Additionally, because during the experimental protocol these cells were in their native state and were not harvested, we did not experience the movement of cells from the region of study or observe the rapid contraction and relaxation phenomenon as previously described (12, 36).

DETA-NO decreased TM cell volume in a concentration-dependent manner. Whereas 30 and 50 μM DETA-NO decreased cell volume, maximal decreases occurred at 100 and 300 μM DETA-NO, suggesting that 100 μM was saturating. It has been observed that the TM cell culture contains two distinct cell populations (1, 13), which is consistent with the identified regions of the TM: the cribriform or juxtacanalicular region, the uveal region, and the corneoscleral regions (20, 30). In our hands, we were able to visually identify the different cell types; however, as with a report by Mitchell et al. (36), all cells that were analyzed did not experience decreases in cell volume in response to drug treatment. The juxtacanalicular region (and hence juxtacanalicular cells) is a region of high resistance to aqueous humor outflow and may constitute the area where changes in cell volume may affect outflow resistance. Together, these data would suggest that modulation of the volume of TM cells may modify outflow resistance of aqueous humor and may alter IOP.

We tested the validity of our protocol with agents and conditions known to alter the osmolarity of the medium and subsequently alter TM cell volume. Both swelling and shrinkage of TM cell after exposure to hypotonic or hypertonic media, respectively, demonstrated that TM cells have the ability to respond to osmotic changes in their extracellular environment. Our initial experiments were performed in porcine cells, where we observed that increases in cell volume were detectable at 1 min after osmotic changes and that cell volume decreased within 3 min after hypotonic exposure.

Porcine TM cell volume in response to hypertonic changes decreased gradually over a 7.5-min period and remained constant for the duration of the experiment. Because of these observations, we performed similar experiments in human TM cells. Cell volume changes in response to hypertonic treatment mimicked the hypotonic effects observed in porcine TM cells.

Exposure of human TM cells to hypotonic medium resulted in a 11% increase in cell volume. These results mimic similarly treated TM cells (36, 50). To date, the correlation between the amount of changes in cell volume in response to changes in osmolarity or drugs and the physiological relevance of these changes to cell function has not been ascertained. As with this study, another study by Mitchell et al. (36) demonstrated that human TM cells exposed to hypotonic solution experienced a regulatory volume decrease within 4 min of treatment with hypotonic medium. In our experimental protocols, neither porcine nor human cells treated with mannitol or NaCl experienced a regulatory volume increase in the presence of hypertonic medium. An immediate regulatory volume increase was observed when hypertonic medium was exchanged with isotonic medium at 37°C (40), with cell volume restored to baseline values within 6 min of medium exchange.

In our hands, TM cells exposed to hypotonic medium experienced a peak cell volume increase when treated with IBTX. This suggests that in physiological states, the BKCa channel may be involved in the regulatory volume decrease mechanism, inhibition of which may potentiate cell swelling. IBTX abolished the regulatory volume decrease that occurred spontaneously in hypotonic medium-treated cells, suggesting a role for the BKCa channel in the cell regulatory volume decrease mechanism.

NO donors can activate sGC in a number of tissues, presumably through the release of NO. The ability of the specific
sGC inhibitor ODQ to antagonize the actions of DETA-NO on TM cell volume would suggest that a direct consequence of NO stimulation is the activation of sGC. Technical criticisms did not allow us to correlate changes in endogenous cGMP levels with changes in TM cell volume. However, 8-Br-cGMP mimicked the actions of DETA-NO, suggesting that cGMP is involved in TM cell volume regulation. In fact, our experiments involving 8-Br-cGMP corroborated previous results published by O’Donnell et al. (40). In this study (40), exposure of bovine TM cells to 8-Br-cGMP (50 µM) resulted in an 8% decrease in TM cell volume. These experiments were performed using electronic cell sizing of suspended TM cells. Similar changes in cell volume were observed in human TM cells in our experiments using fluorescent probes, validating our protocol.

In other experiments, exposure of TM cells to 8-Br-cGMP (50 µM) resulted in inhibition of the bumetanide-sensitive K⁺ influx, demonstrating the involvement of cGMP in the Na⁺-K⁺-2Cl⁻ cotransport regulation (40). In our hands, decreases in TM cell volume in response to DETA-NO were similar to decreases in cell volume in response to 8-Br-cGMP, suggesting that cGMP maybe the second messenger mediating the effects of NO on cell volume.

Our study demonstrated that IBTX inhibited NO-induced decreases in TM cell volume, suggesting the involvement of the BKCa channel and K⁺ efflux in regulating NO-induced decreases in TM cell volume. We also demonstrated that the BKCa channel is necessary for the NO-induced response in TM cells as DETA-NO was unable to decrease cell volume in TM cells that were preincubated with IBTX. We do not know the mechanism by which BKCa channels mediate the NO-induced response. The possibility exists that the NO-induced activation of PKG would result in the phosphorylation of BKCa channels and subsequent decreases in TM cell volume, as studies (3, 44) in cerebral artery smooth muscle cells and X. oocyte have demonstrated that PKG phosphorylation of the α-subunit of the BKCa channel results in its activation.

Another study (11) has demonstrated that cGMP generated by the activation of the atrial natriuretic peptide receptor and by the NO donor sodium nitroprusside decreased cardiac cell volume by inhibiting ion uptake by the Na⁺-K⁺-2Cl⁻ cotransporter. These observations suggest that NO regulation of K⁺ transport and cell volume are bidirectional, facilitating both K⁺ efflux via the BKCa channel and K⁺ influx via the bumetanide-sensitive K⁺ cotransporter.

PKG inhibitors were able to inhibit NO- and 8-Br-cGMP-induced changes, demonstrating the role of PKG and protein phosphorylation events in regulating TM cell volume. Our study, however, does not preclude the involvement of other second messengers, including cAMP (42, 50) or PKC (32) or the involvement of other ion transporters and cotransporters in the modulation of cell volume (36).

We were not able to demonstrate that NO-induced increases in outflow facility occurred as a result of changes in TM cell volume. However, we demonstrated that the time course for DETA-NO-induced increases in outflow facility correlate with the time course for DETA-NO-induced decreases in cell volume. Changes in TM cell volume induced by changes in tonicity correlated with tonicity-induced changes in outflow facility. Previous studies (2, 49) have demonstrated that drugs known to cause cell swelling reduce outflow facility, whereas drugs known to shrink cells increase outflow facility in human and bovine eyes. While the results of a study by Gabelt et al. (21) demonstrated that bumetanide, an inhibitor of the Na⁺-K⁺-2Cl⁻ cotransporter, had no effect on outflow facility in living primate eyes (suggesting that alterations in cell volume had no effect on outflow facility), a preponderance of electrophysiological, biochemical, and pharmacological studies have demonstrated a correlation between cell volume changes and outflow facility. Together, these studies suggest that there might be multiple transporters involved in cell volume regulation and the subsequent regulation of outflow facility.

We conclude that NO decreases TM cell volume; that the cellular response to NO is mediated by sGC, cGMP, PKG, and BKCa channels; and that changes in cellular volume are correlated with changes in outflow facility. We are mindful that there might not be a direct cause-and-effect relationship between TM cell size and outflow facility because we have not accounted for the possible involvement of TM contractile mechanisms (25, 55, 58).

ACKNOWLEDGMENTS

We thank Drs. T. Acott and D. Stamer for teaching the anterior segment organ perfusion protocol and TM cell culture techniques, Douglas Smith for technical assistance with the confocal microscope, and Drs. Charles Wood and Elaine Sumner for helpful discussion of the manuscript.

GRANTS

This work was supported by a grant from the American Health Assistance Foundation, National Glaucome Research.

REFERENCES


