EPINEPHRINE EFFECTS ON MAJOR CELL TYPES OF THE AQUEOUS OUTFLOW PATHWAY: IN VITRO STUDIES/CLINICAL IMPLICATIONS*

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INTRODUCTION

THE MECHANISM OF ACTION OF EPINEPHRINE (EPI) TO LOWER THE INTRAocular pressure (IOP) when used as a topical glaucoma medication remains unknown. This lack of progress is surprising in view of the facts that nearly 100 years ago the major catecholamine component of the adrenal gland was isolated and named epinephrine,[1] that 1 year later adrenal gland extracts were shown to lower IOP when injected subconjunctivally,[2] or when applied topically in other early experiments.[3] On the other hand, this situation is more understandable when one considers that EPI has been used clinically for the treatment of glaucoma for only 40 years.[4] However, much has been learned about EPI's site of action, adrenoreceptor types involved, the pharmacokinetics of its IOP response, and studies are underway to help uncover its mechanism of action.

When Goldman[4] introduced EPI as a glaucoma medication, he at once proposed that it acted on the ciliary body to reduce the rate of aqueous formation; although this notion was based on limited data, it prevailed for over a decade. Later Ballintine and Garner[5] and Kronfeld[6] found that EPI actually acted by increasing the facility of outflow and this concept has withstood intensive testing[7,8] and is widely accepted today. Other details

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about EPI's site of action remain unclear. For instance, it is not certain whether the outflow increase is due to effects on the major or trabecular pathway,[7] or on the minor or uveoscleral pathway[8] or on both. Furthermore, the nature of the adrenergic receptors mediating this response is equivocal. Some investigators believe that beta-2 receptors are involved because EPI's effect could be only partially blocked by "selective" beta-1 blockers but was completely blocked by "nonselective" beta blockers.[9-13] Other investigators have obtained different responses and have proposed involvement of alpha receptors,[10] even though such receptors have been shown to be absent in cells cultured from the human trabecular meshwork (HTM).[14]

Several details about the pharmacokinetics of EPI's action have been clarified over the years. Its IOP response is elicited when the anterior chamber *concentration* is maintained at 10^{-5} M to 10^{-6} M for a few hours.[15] The *time course* of this response is characterized by a rapid IOP drop which can be "recorded at the end of the first hour" and which reaches a maximum around 4 hours to be followed by a gradual return to baseline conditions within the next 12 to 24 hours.[5,6] This prolonged *duration* has led to the application of EPI drops every 12 hours. The *magnitude* of the response is modest as the pressure drops by 15% to 30%.[6,10,11]

We have considered that EPI and other adrenergic agonists may promote the exit of aqueous via direct cellular effects. We were originally encouraged to pursue this research avenue by a report of Neufeld et al[16] in which they showed that cultured corneal endothelial cells altered their shape and appearance when exposed to prostaglandin E-2. This particular prostaglandin as well as EPI, have been shown to raise cyclic-AMP levels in a variety of cell types, including human trabecular cells.[14,17,18] Therefore, we wondered whether such autocoids were also involved in altering the shape of cells in the outflow pathway to promote fluid flow through paracellular and other routes. We were also intrigued by our observations that eyes enucleated several hours postmortem do not respond to the application of EPI, while in freshly enucleated eyes, an IOP drop is sometimes observed.[19] The cells in the freshly obtained eyes must still be alive since pupillary dilatation occurs with EPI treatment. For these and other reasons, we decided to carry out experiments similar to those described by Neufeld and Sears.[7] Cultured HTM and Schlemm's canal endothelial (SCE) cells were used for our experiments. Concurrently, we also developed the appropriate methods to measure the rate of flow through a monolayer of cultured cells.[20,21] Profound changes in cell shape, widening of the intercellular space, and increases in the rate of

flow through the cells were observed following treatment with adrenergic agonists.[21,22]

Here we report on the effects of adrenergic agonists and antagonists on the hydraulic conductivity (flow/unit area) of monolayers of HTM and SCE cells. The experiments conducted were designed in consideration of the described major characteristics of EPI's IOP response. According to our findings, EPI's action on aqueous outflow is consistent with the described cellular and physiologic effects on fluid flow. In addition, the unique combination of a cell culture system with physiologic studies provides a valuable tool to study mechanisms of drug action and to evaluate currently used and new glaucoma medications.

MATERIALS AND METHODS

GROWTH OF CULTUED CELLS ON FILTERS

Millipore filters measuring 12 mm in diameter and premounted in polystyrene holders (Millipore Corp, Bedford, MA) serve as a support structure upon which cultured cells could be grown for these studies. HTM and SCE cells previously studied in our laboratory[14,20,23-25] were used to cover one surface of the filter supports. Frozen stocks of fourth passage cells from a normal 30-year-old donor were seeded first onto 10 cm plastic dishes, allowed to grow until the cells were about to reach confluence, and seeded (fifth passage) onto a millipore filter at a density of 10^4 cells/ml. Dulbecco's modified Eagle's medium (DME), to which we added 15% fetal calf serum (FBS), 2 mM glutamine, 50 μg/ml gentamycin, 2.5 μl/ml fungizone, and 250 ng/ml of fibroblast growth factor, was used to grow these cells from the frozen stocks. The growth factor was omitted after the cells were grown over the filter supports. According to methods we have previously described,[20] the prepared filters are kept in a standard incubator with 8% CO_2 at 37°C and have the growth media changed every other day. Typically, the seeded cells reach confluence and form a tightly packed monolayer by the end of the first week. At this time, the serum concentration is reduced from 15% to 10%. Most preparations can be used by the end of the second week at which time the hydraulic conductivity is tested.

MEASUREMENT OF HYDRAULIC CONDUCTIVITY

The device previously used in our laboratory[20] was replaced for these studies with a fully automated, computer-linked apparatus that measures flow by monitoring the pressure difference that develops as fluid flows across a known resistance. The flow producing this pressure difference is calculated by applying the equivalent of Ohm's Law: $P = QR$ or $Q = P/R$

where Q is flow, P the observed pressure difference, and R the known resistance. One pressure gauge is upstream from the known resistor and reads the pressure corresponding to the height of the fluid column provided by a reservoir filled with the same media used to grow the cells. The second pressure gauge is downstream from the known resistor and just in front of the filter supported monolayer. This gauge reads a reduced pressure that changes in response to increases or decreases in flow. Since the outer filter surface is at atmospheric or zero pressure, the pressure read by this second gauge is also the perfusion pressure for the monolayer.

Hydraulic conductivity (Lp) is calculated within the computer program by using the formula Lp (μl/min/mm Hg/cm^2) = Q/PA, when P is the perfusion pressure and A is the surface area of the monolayer. The calculations also correct for the contribution of the filter substrate to conductivity.[20] Initially, we implemented this system with four independent flow measurement stations housed in a single cell culture incubator. Recently, four additional stations in a second incubator were added enabling us to monitor the hydraulic conductivity (HC) of eight cell monolayers simultaneously.

ADRENERGIC AGENTS AND OTHER REAGENTS

Epinephrine and isoproterenol (ISO) (Sigma Biochemical, St. Louis, MO) were obtained in powder form and freshly prepared immediately before their use. A stock solution was first made in DME containing 5% FBS at a concentration of 10^{-2} M and diluted to the desired concentration (10^{-4} to 10^{-6} M) by adding the appropriate amount of DME + 5% FBS. All of these reagents were protected from the light to prevent loss of activity due to oxidation by keeping them covered with aluminum foil and carrying out the experiments in the darkened conditions of the tissue culture incubator.

Dibutyryl cyclic-AMP and timolol (Sigma Biochemical) as well as betaxolol (Alcon Labs, Ft. Worth, TX) are stable compounds. Nevertheless, they were also freshly prepared from their powder form into a solution of the appropriate concentration using DME + 5% FBS. Control solutions consisted of DME + 5% FBS.

EXPERIMENTAL PROTOCOLS

The millicell preparation can be perfused in two directions. In one direction, the perfusate flows through the cell monolayer first and then through the filter structure. In the second direction, the conditions are reversed and the perfusate flows through the filter first and then through

the cell monolayer. We used both of these conditions in our experiments because we believe that each of the two cell types studied faces aqueous flow differently. In the case of the trabecular cells lining the outermost aqueous channels, aqueous flow is through the lining cells first and then through the juxtacanalicular connective tissue. Thus, for the cultured HTM, the first direction of flow described above more closely resembles the condition in vivo. For the SCE cells, the situation is reversed, as aqueous flows first through the juxtacanalicular tissue and then through the lining cells. The cultured SCE cells were perfused using the direction of flow described in the second condition because it most closely approximates the in vivo situation.

The cell-filter preparations are first evaluated by perfusing them with the medium used to culture the cells (DME), but the content of FBS was reduced from 10% to 5%. Baseline conductivities are recorded for 30 to 40 minutes to determine the stability of the preparation before introducing drugs. Prior studies[20] using light and scanning electron microscopy which have shown that intact monolayers that cover the entire millipore surface have HC values between 0.3 and 2.0 μl/min/mm Hg/cm^2. For experiments reported here, we used preparations with mean conductivities ranging from 0.4 to 3.0 μl/min/mm Hg/cm^2.

To test the responses elicited by the application of adrenergic agents, we used three protocols. The first was a "pulse-chase" experiment, where the drug was introduced into the millicell housing and allowed to interact with the monolayer for 30 minutes to 2 hours; then, fresh DME with 5% FBS (control media) was used to monitor the HC for several hours. The second protocol was a prolonged or continuous treatment, where the millicell preparation was perfused with the drug throughout the experiment. In the third type of experiment, the cell monolayer was treated with drug for 1 hour, then perfused with the drug for an additional hour to measure the HC. In all experiments, controls underwent the same manipulations as the treated preparations.

RESULTS

1. "PULSE-CHASE" EXPERIMENTS

When the adrenergic agonists are introduced for only 30 minutes, followed by perfusion with the media alone, we observed a change in conductivity as shown in Fig 1. EPI and ISO were used at 10^{-5} M and both produced only a 30% increase in HC compared to a control preparation. This small change could be measured well-within the first hour,

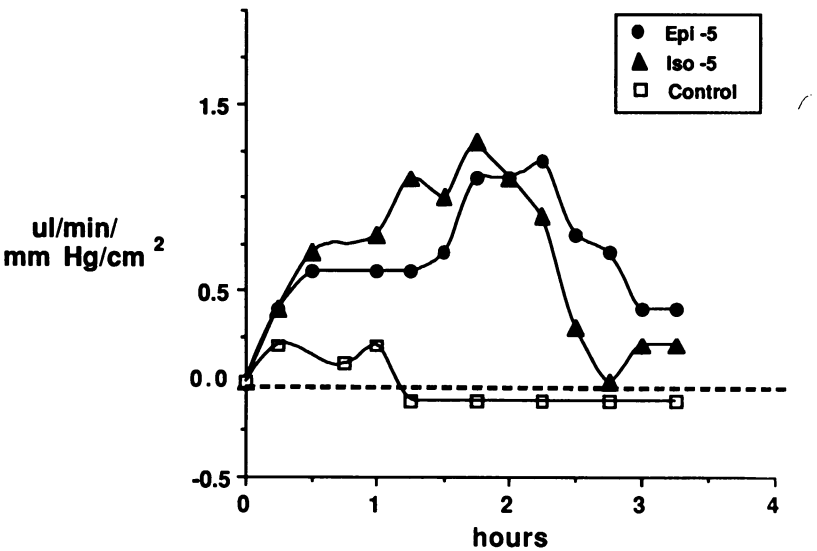


FIGURE 1
"Pulse-chase" experiment using SCE cells. Two cell-filter preparations exposed to 10^{-5} M epinephrine (EPI) or isoproterenol (ISO) for 30 minutes are compared to one preparation perfused with the culture media alone. The results are plotted as differences from baselines. The mean baseline hydraulic conductivity (HC) measured 1.96 μl/min/mm Hg/cm², with a range of 1.8 to 2.1 U. At zero time, the exposure to the adrenergic agonist ended and all three preparations were perfused with fresh media alone. Both adrenergic agonists produced a brief, 30% increase compared to the control-preparation. Some drift is noted for the control during the first hour.

reached a peak by the second hour and declined rapidly to return to baseline by the third hour. When the exposure-time was increased to 1 or 2 hours, as shown in Fig 2, EPI's effect (10^{-5} M) lasted considerably longer and while the rapid rise and peak are similar to those observed when only a brief exposure time was used, the magnitude of the induced change increased considerably (Figs 1 and 2). Such a prolonged effect would be more compatible with the use of this medication on a twice daily schedule.

2. CONTINUOUS DRUG EXPOSURE EXPERIMENTS

The millipore cell preparations were perfused continuously in the dark for over 12 hours in these experiments. The putative SCE cells were seeded over the filter support in such a way that the perfusate could flow

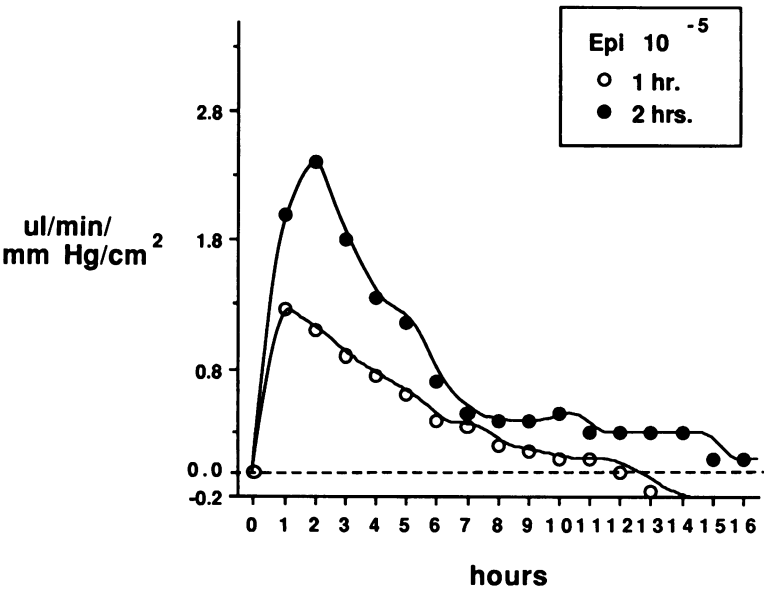


FIGURE 2
"Pulse-chase" experiment using HTM cells. Two cell-filter preparations were exposed to 10^{-5} M EPI for 1 or 2 hours to be chased with a perfusion of culture media alone at time zero. These were compared to two preparations exposed to the culture media alone. The results are plotted as differences from the control and from baseline values. The mean baseline HC measured 1.35 U with a range between 1.1 and 2.0 U. Close to a twofold increase was measured with the 2 hour exposure. An increase in the magnitude and duration of the HC change is observed with longer drug exposure times.

in the same manner as it occurs in vivo. That is, the fluid goes through the filter structure first, meets the base of the monolayer of cells, and next passes through the paracellular route and the transcellular giant vacuoles (see Methods). When fluid flows in this direction, the monolayer of SCE cells offers much less resistance than in the opposite direction.[20] A large change in HC is observed as shown in Fig 3. Since the mean HC is 1.0 μl/min/mm Hg/cm^2, this change represents an eightfold increase from baseline values. The maximal effect is reached by the 10th hour and the half maximal by 2.5 hours.

HTM cells were perfused so as to face flow of the perfusate in a similar manner as aqueous flow is faced in vivo. That is, the perfusate flows through the monolayer first and then through the supporting filter structure. When ISO at 10^{-4} M was added, a response such as shown in Fig 4 is observed. Again, very similar dynamics are noted as when SCE cells

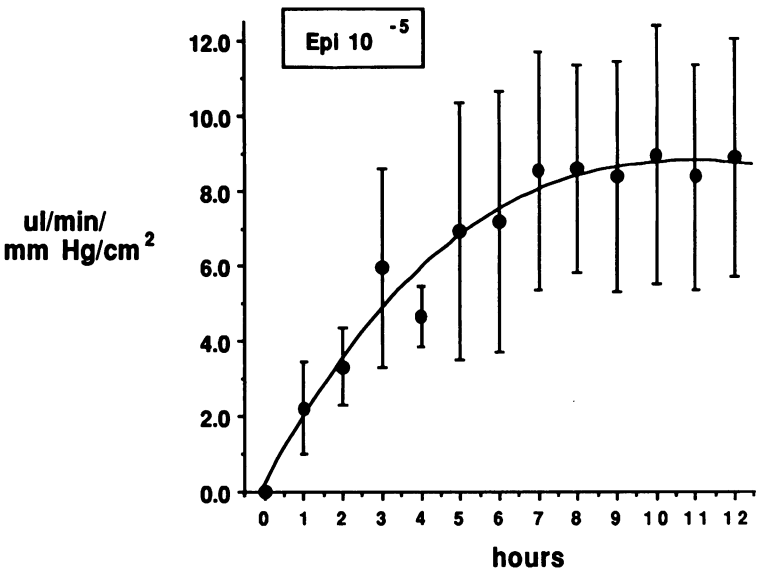


FIGURE 3

Prolonged or continuous treatment experiment using SCE cells. These cells were positioned to face flow in the same manner as occurs in vivo (see Methods). Four cell-filter preparations with EPI and one control were studied. The results are plotted as differences from control and from baseline values (means and standard deviations). Baseline HC had a mean value of 0.98 U with a spread between 0.7 and 1.5 U. EPI at 10^{-5} M produced an eightfold increase in HC. The effect persisted for over 12 hours and a maximal effect was reached by the 10th hour and the half-maximal effect by 2.5 hours.

are used. A near fourfold effect is measured by the 10th hour and the half maximal effect is reached by 2.5 hours.

3. RESPONSE TO c-AMP

The second messenger, c-AMP, has been shown to promote the exit of aqueous when it is given directly into the anterior chamber of experimental animals.[7] Therefore, it was important to learn whether the cell preparations would respond in the same manner. In Fig 5 the response to the application of 10^{-5} M c-AMP is compared to that of ISO used at a higher concentration (10^{-4} M). In this experiment, the millicell preparations were not allowed to go through a perfusion time prior to the addition of drugs and this accounts for the observed decline in conductivity in the control preparations (Fig 5). c-AMP induced a greater response than the beta-adrenergic agonist ISO.

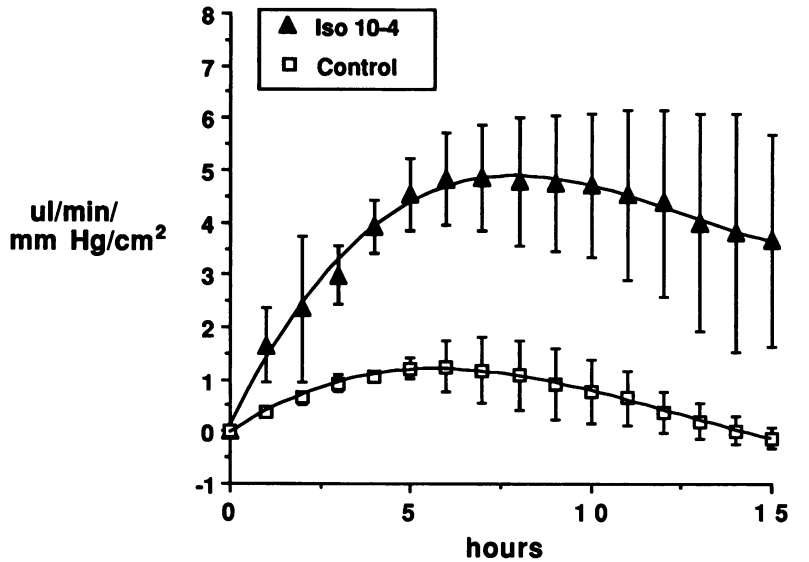
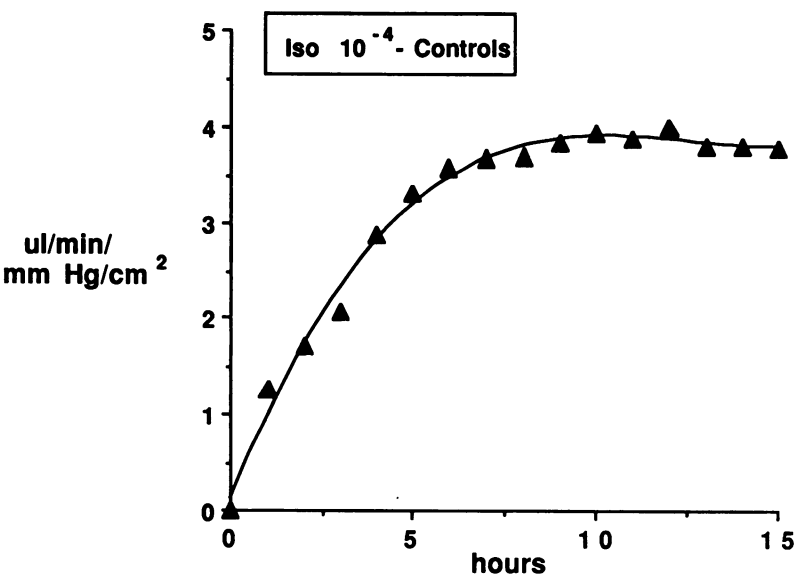


FIGURE 4

A: Prolonged treatment using HTM cells facing flow in the same manner as occurs in vivo (see Methods). Three preparations were treated with 10^{-4} M ISO and three served as controls. The mean baseline HC value was 1.5 μl/min/mm Hg/cm² with a range of 0.8 to 2.2 U. Means and standard deviations of differences from baselines are plotted. Note minor drift of control preparations. B: This graph from that shown in A by plotting the change in HC as a difference from the controls. Close to a fourfold increase is observed, with the maximal response obtained by the 10th hour and the half-maximal response by 2.5 hours.



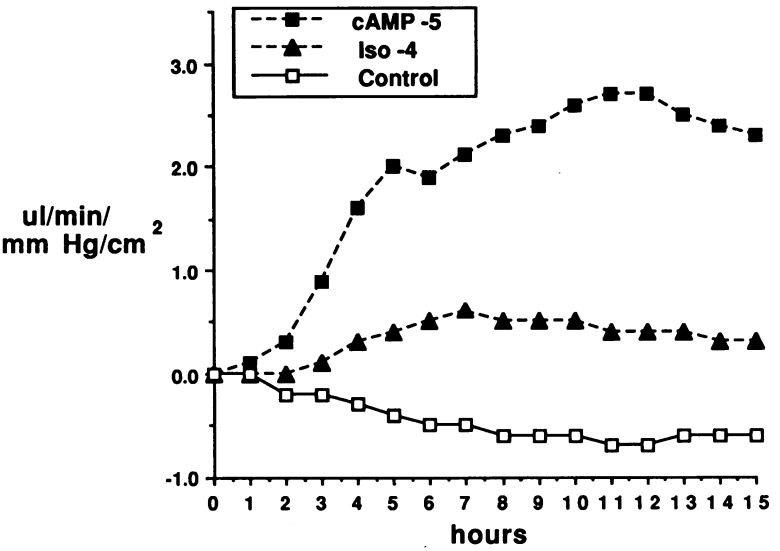


FIGURE 5
In this experiment using HTM cells, the period of stabilization prior to the addition of drugs was omitted, and a drift in baseline is observed for the control preparation (n = 2). There is a small ISO (10^{-4} M) response which amounts to approximately a 65% increase over baseline HC values (n = 3). c-AMP used at 10^{-5} M provided a greater response (n = 2). The baseline HC measured 1.26 U on the average and had a range of 0.6 to 1.6 U. Mean differences from baselines are plotted.

4. RESPONSE TO ADRENERGIC ANTAGONISTS

We evaluated the beta blockers in several experiments. In preliminary experiments, such as that shown in Fig 6, four millicell preparations were used. EPI at two concentrations (10^{-4} M and 10^{-5} M) was given to two millicells while one received timolol at 10^{-6} M for 1 hour prior to its exposure to both timolol at 10^{-6} M and EPI at 10^{-5} given together. A fourth millicell preparation was used as a control. Timolol (10^{-6} M) used in this manner appears to have blocked the agonist (10^{-5} M).

In a second experiment, we used a different protocol to increase the number of preparations studied. We could do this with the four separate stations available if the HC were measured for only 1 hour. This measurement was begun after the preparation had been exposed to the agents for 1 hour. Such an experiment is shown in Fig 7 where 20 millicell preparations were examined. Statistically significant differences (unpaired t-tests) were observed when the responses of 6 control preparations are compared with 7 preparations each treated either with timolol/EPI or EPI alone.

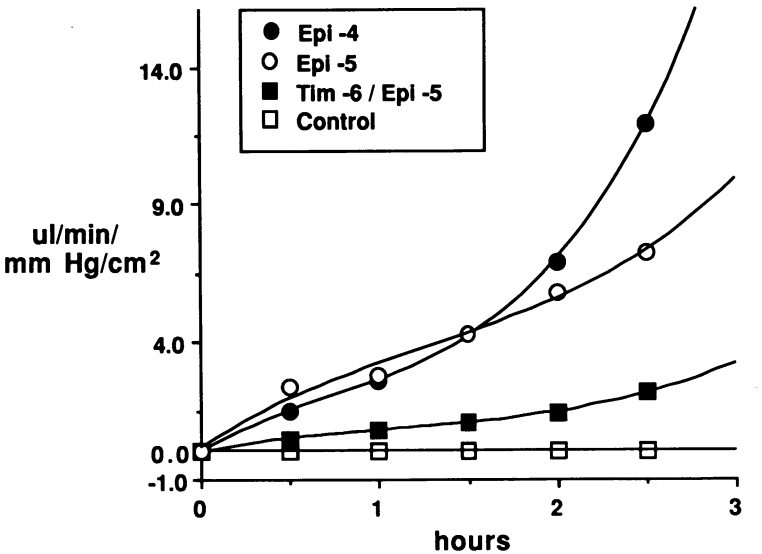


FIGURE 6

Preliminary experiments in which four millicell preparations of HTM cells were given either EPI at 10^{-4} M and 10^{-5} M, or timolol at 10^{-6} M for 1 hour followed by timolol (10^{-6} M) together with EPI (10^{-5} M). One preparation served as a control. Differences from baselines are plotted. The mean baseline HC measured 1.5 U with a range of 0.9 to 2.4 μl/min/mm Hg/cm².

A third experiment compared timolol and betaxolol in their ability to block ISO's effect when this beta-agonist was used at 10^{-5} M. As shown in Fig 8, ISO (n = 2) produced a large effect (two- to threefold) while timolol (n = 3) effectively held the HC at a steady level. In contrast, while betaxolol (n = 2) delayed the agonist response for a few hours, an increase in HC was ultimately observed. Thus, ISO was only partially blocked by betaxolol whereas it was completely blocked by timolol.

DISCUSSION

Our experiments provide a cellular basis and a physiologic mechanism whereby adrenergic agonists might be expected to increase the rate (or facility) of aqueous outflow. These findings lend further support to the pioneering studies of Ballintine and Garner[5] and Kronfeld.[6] However, more experimentation is required before we will know whether these in vitro studies are relevant to the in vivo clinical situation.

Previously we have reported that adrenergic agonists have direct cellular effects on cultured HTM and SCE cells; ie, a reduction in the cell size

and widening of the intercellular space. When those results are considered together with the ones reported here, a concept develops which suggests that such cellular effects may be involved in the mechanism of action of EPI. We used EPI at 10^{-5} M because in other experimental studies[15] it has been shown that EPI reaches an anterior chamber *concentration* between 10^{-5} M and 10^{-6} M for only a few hours. The *time course* of the IOP response elicited by EPI in patients has as its salient characteristics a rapid onset, early peak and gradual decline over the next 12 hours.[5,6] When we exposed our cell-filter preparations to EPI for 1 or 2 hours, the changes elicited came very close to reproducing the kinetics of EPI's response. Extrapolating from our reported findings, perhaps a 3- to 4-hour exposure would have yielded results which may have approximated the in vivo clinical situation even more closely. The *magnitude* of the measured effect is also modest in our system as it consists of a one- to twofold increase in HC. Again, such an effect is consistent with what is

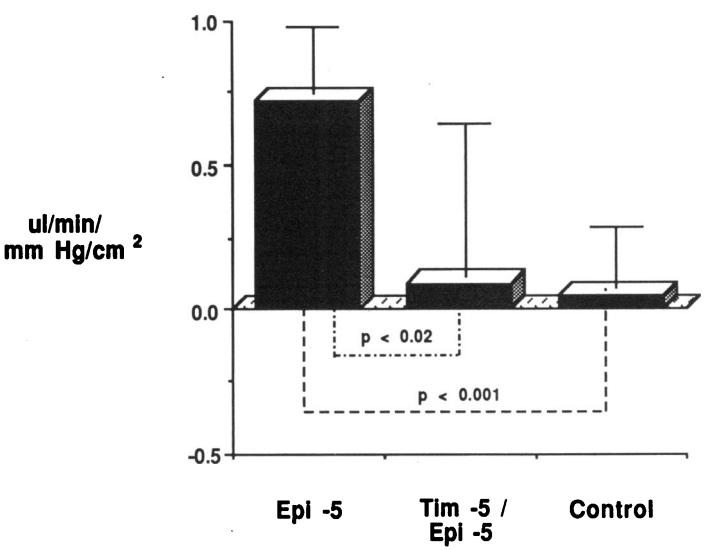


FIGURE 7
In this experiment, HC were measured for only 1 hour following exposure to drugs. Seven preparations of HTM cells were exposed to EPI at 10^{-5} M for 1 hour, seven other millicells were treated with timolol at 10^{-5} M for 1 hour and then given timolol together with 10^{-5} M EPI for a second hour, and there were six untreated controls. The mean baseline HC measured 1.05 μl/min/mm Hg/cm² with a range of 0.4 to 1.9 U. Both the control and timolol/EPI preparations had significantly lower HC values than did those treated with EPI alone, indicating that timolol blocked the response to the agonist. Mean differences from bselines (and standard deviations) are plotted.

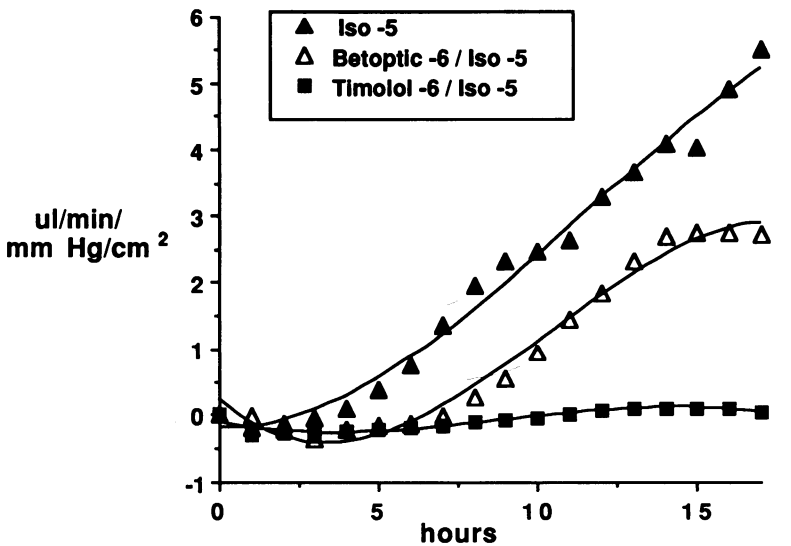


FIGURE 8

Seven millicell preparations of HTM cells were studied in this experiment. ISO (n = 2) at 10^{-5} M produced a large increase in HC while timolol (n = 3) added at 10^{-6} M before and subsequently given in the presence of ISO (10^{-5} M) completely blocked the adrenergic agonist effect. Betoptic (betaxolol) (n = 2), used in the same manner as timolol, blocked the agonist's effect only partially. The mean baseline HC measured 1.46 and the range was 0.9 to 3.0 μl/min/mm Hg/cm². Mean differences from baselines are plotted.

known about EPI's reduction of the IOP in glaucoma patients.[6,10,11] Thus, we may conclude that when the preparations of monolayers of HTM and SCE cells are exposed to EPI at a concentration of 10^{-5} M between 2 and 4 hours, the increase in HC produced closely resembles the time course of the EPI-IOP response in patients. For these reasons, we proposed that direct cellular effects may also be involved in the acute EPI effect to lower the IOP in vivo.

We would like to make two observations regarding the magnitude of the responses elicited. At a concentration of 10^{-5} M, the increase in HC produced by both EPI and ISO is rather small. Since higher concentrations of EPI are probably unlikely to be reached in the anterior chamber after its topical application, we believe that this finding of ours may have clinical relevance. It has been noted for some time that as many as 30% of glaucoma patients fail to respond when given EPI topically.[11] It seems possible to us that such a high failure rate may be related to the fact that in at least a portion of these patients, EPI does not reach the required anterior chamber concentration levels. Other clinical data support these

arguments as well. For instance, when patients who had failed to respond to propine (0.1% EPI) treatment were challenged with 2% Eppy, the majority were found to respond by undergoing an IOP decrease.[26] Another explanation is based on our prior studies in which we showed that in primary open angle glaucoma, the trabecular tissues have an abnormally lowered concentration of cells.[27,28] Since the present studies indicate that such cells are the potential targets of EPI, the existence of a diminished or altered population of cells in patients with glaucoma might also be expected to lead to a reduced response to the EPI treatment. Since the decline in cell density is progressive, EPI may additionally be expected to be less effective as time goes on because the number of target cells available is continuously decreasing.

The second observation is related to the experiments in which drug was maintained at a given level for a prolonged period and a greater as well as a more sustained response was observed. This finding suggests that if EPI could be continuously maintained at a certain level, the magnitude of its effect might increase considerably. While we must keep in mind the potential for cardiovascular side effects, taken at face value, these data provide a rationale for the assessment of alternate drug delivery approaches.

Our preliminary studies indicate that both EPI and ISO have nearly similar effects. EPI stimulates both alpha and beta receptors while ISO stimulates mostly beta receptors when used at low concentrations. We also found that in agreement with prior clinical studies,[12] the adrenergic agonist effect on HC was only partially blocked by betaxolol while timolol blocked it completely. This information, taken together with our unpublished observation that alpha agonists, such as phenylephrine, do not elicit a similar increase in HC, suggests that beta receptors, specifically beta-2 receptors, are involved. A clinical implication of such a finding might be to search for specific beta-2 agonists to increase the facility of outflow and beta-1 antagonists to decrease the rate of aqueous formation. Such a combination may be ideal and produce a maximal lowering of the IOP in glaucoma patients. Similarly, since the selective beta-1 blocker tested could only partially block the effect of the adrenergic agonist, these data are consistent with other clinical studies in which it has been shown that betaxolol and propine are synergistic.[12]

Although the studies reported here are preliminary in nature and more extensive evaluations are required before dose responses and a wide variety of adrenergic agonists and antagonists are fully assessed, we are encouraged by our early findings. While the responses studied are consis-

tent with previous biochemical evaluations,[13,14,17,18] the correlation of cell shape/intercellular space alterations with physiologic parameters (eg, increase in HC) is new. Moreover, the development of the automated/computer-run apparatus greatly facilitates the performance of such experiments. Future investigations may be directed toward the evaluation of potential and more effective adrenergic agonists (eg, beta-2 agonists), as well as toward the evaluation of the specific cellular mechanism(s) involved. Such studies may identify even newer pharmacologic approaches and agents to promote fluid flow through paracellular and other routes.

SUMMARY

We have investigated the possibility that direct cellular effects may mediate the action of EPI to lower the IOP. To do this, cultured HTM and SCE cells were grown as monolayers over a millipore-filter support structure. The monolayers were exposed to various adrenergic agonists and antagonists while flow of the perfusate (DME + 5% FBS) was measured using a specially-designed computer-linked apparatus. Exposure of the cells to 10^{-5} M EPI for around 2 hours led to a rapid twofold increase in HC which gradually declines over the next 12 hours. Continuous exposure of either cell type (ie, HTM or SCE cells) to EPI or ISO resulted in a four- to eightfold increase with a maximal effect measured around 10 hours and a half maximal effect at 2.5 hours. Administration of c-AMP alone gave similar responses. In agreement with clinical studies, timolol blocks EPI's effect completely while betaxolol acted as a partial antagonist. These findings suggest that the cellular changes and the increase in HC are mediated by a beta-2 receptor. There are many similarities between the responses observed using our in vitro system and the IOP-lowering response observed in vivo after the topical application of EPI (eg, concentration, time course, duration, and magnitude). The clinical implications of these preliminary results are discussed and it is proposed that the described in vitro system may be useful to select new adrenergic drugs for glaucoma therapy.

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DISCUSSION

DR MAX FORBES. In a series of in vitro experiments, the authors studied the influence of adrenergic agents on hydraulic conductivity (HC) of monolayers of cultured cells from one normal human trabeculocanalicular system. HC is the reciprocal of resistance to flow. The monolayer cell types, trabecular meshwork lining cells and putative Schlemm's canal inner wall endothelium, were selected as possible sites of aqueous humor outflow resistance which might respond to adrenergic stimulation. Since Doctor Alvarado is a preeminent authority on human trabecular meshwork (HTM) cell cultures, it is presumed that the cells were identified correctly and their differentiated characteristics retained through storage and processing.

For measurements of monolayer HC to be valid, the cells must be healthy, the monolayer must be confluent (rather than leaky) and the equipment should be capable of detecting minute volumes of flow. My colleague in the Department of Ophthalmology of Columbia University, Doctor Fischbarg, has been measuring corneal epithelial water transport for many years. His exquisitely sensitive apparatus was recently used to measure HC of cultured vascular endothelial monolayers. Mean conductivity for three types of vascular endothelial cells was 0.15 U (μl/min/mm Hg/cm^2) whereas the authors' mean control measurements were in the range of 1.5 U. That tenfold higher value for trabeculocanalicular cells is striking, but it may well represent a true biological property of cells that are specialized to facilitate bulk outflow of aqueous humor.

Whereas the effect of epinephrine (EPI) on outflow facility in vivo may be obscured by other factors, such as penetration, inflow, vasoconstriction, uveoscleral outflow, and episcleral venous pressure, its effect on monolayer HC in vitro appears to be sharply defined. The agonists, EPI, and isoproterenol (ISO), shrink the cells, enlarge intercellular spaces and increase conductivity. Those actions are totally blocked by timolol and partially blocked by betaxolol. Blockage by beta antagonists is the key; it indicates that the increase in conductivity is mediated by beta receptors.

Measurement of monolayer HC is a very interesting method of studying the cells. But how does the data apply to function of the considerably more complex trabeculocanalicular aqueous outflow pathway? It might be directly applicable if the monolayer cells constitute the critical site of outflow resistance. Anatomy points to the juxtacanalicular region, especially Schlemm's canal inner wall endothelium, which is a monolayer with tight junctions. The authors have demonstrated how adrenergic influence on monolayer HC simulates responses of the living outflow system. The model may be valid. More work is needed to establish the correspondence.

If a monolayer is maintaining its integrity, flow rate should be a linear function of pressure with the slope defined as HC. I would like to ask Doctor Alvarado if flow rate was measured at several pressure levels to verify the linear relationship? Is the response of aqueous outflow cell monolayers to beta adrenergic agonists unique and specific, or would other cell-type monolayers exhibit similar increases

in conductivity? Cyclic-AMP also increased conductivity. Was timolol tested against c-AMP, and if so, what happened?

I want to congratulate Doctor Alvarado and associates for this important contribution to glaucoma research. It could lead to a major breakthrough. I look forward to reports of their continuing investigations.

DR ROBERT DREWS. This was a brilliant paper and presentation, I must say. One question—considering the increasing interest in experimental studies on cataracts, can this technique be applied to lens epithelium as well?

DR JORGE A. ALVARADO. I have looked forward with much anticipation to the comments of Doctor Forbes. As expected, his questions are probing and astute and it will be my pleasure to answer them. I would like to thank him at the outset not only for his complimentary remarks but also for having made me feel comfortable and welcomed at this meeting. In providing my answers to you, I shall attempt to earn the right to wear this prestigious tie given to me as a new AOS member.

1. The first issued raised has to do with providing some evidence that the cells used for our studies have the appropriate "differentiated" properties.

This concern is frequently in our minds as well and one which my colleagues at UCSF have often raised. Several years ago, they wanted to know whether the cultured cells had adrenergic receptors. The data presented here today provides a convincing yes! to that query. We have used cultured cells from lines that had been established in cell culture over 10 years ago. To acquire the enormous supply of cells required to conduct experiments on a daily basis for such a long period of time, we have had to expand the original cells by passing them from one dish to many others. This process is known as a "cell passage" and each time that this is done the cell attachments must be broken by the use of proteolytic enzymes. This treatment also leads to the removal of many of the receptors from the cell surface. Since the cells used here had been "passaged" four or more times, this means that they must have had to synthesize receptors multiple times. Indeed, we have discovered that several days are required for the passaged cells to reconstitute the normal receptor population. Thus, we can say that these cells possess adrenergic receptors as a differentiated characteristic. They also have the ability to replenish themselves with receptors when challenged by the repeated removal of such proteins.

The morphological properties of the cultured cells are distinct and nearly identical to those present in intact and normal trabecular tissues (*Invest Ophthalmol Vis Sci* 1982; 23:464). The extracellular macromolecules (ECM) secreted by the cultured cells (*Invest Ophthalmol Vis Sci* 1989; 30:2012) are identical to those specifically found in the trabecular tissues of origin (*Am J Ophthalmol* 1987; 104:33). The cultured trabecular cells can be differentiated from cultured SCE cells by differences in the types of ECM synthesized under the influence of prolonged dexamethasone treatment (*Invest Ophthalmol Vis Sci* 1989; 30:2012), by HC differences (*Invest Ophthalmol Vis Sci* 1988; 29:1836-1846) and cell culture requirements as well as by their appearance in culture.

Incidentally, I was discussing the subject with Doctor Feeney-Burns at dinner last night. Doctor Feeney happens to be the individual who taught me electron microscopy, much ocular anatomy (along with Doctor Hogan) and who introduced me in 1965 to the intricate details of the outflow pathway and to many of the issues which we are still discussing today. She also did much of the pioneering work regarding the HTM and discovered together with Doctor Garron the so-called "giant vacuoles" of the endothelial cells of Schlemm's canal. I was mentioning to Doctor Feeney how I finally convinced myself that such vacuoles are real and not an artifact (as her PhD professor had once warned me they might be). Well, using the human cultured SCE cells, we have found that these cells make such giant vacuoles. On the other hand, the cultured trabecular cells do not. Moreover, these vacuoles are formed only when the SCE cells face flow in one direction (eg, along their base) and not in the other. This finding, in our opinion, is another example of how cultured cells can preserve many of their differentiated properties and be useful research subjects.

Finally, the most compelling reason I have to believe that the findings presented are indeed relevant to the mechanism of EPI's action, concerns some preliminary studies which I have conducted using either enucleated human eyes or experimental animals. I should mention as background that I have not been able to uncover a single publication in which EPI's effect could be shown to occur in human eyes obtained after death. We have also learned that eyes donated after death cannot be used to study EPI's action. Why? Because such eyes contain cells which are dying or dead or otherwise unresponsive. However, if freshly enucleated eyes, eyes obtained soon after death, or eyes which are fresh enough to demonstrate a pupillary response are used, then EPI can be shown to increase the facility of outflow when applied either topically or intracamerally. More important, when the kinetics of such a response are analyzed in terms of time of onset, magnitude and duration, we find that they are nearly identical to those shown here today when the cultured cells are used.

2. The second question is very much related to what I was just discussing and it has to do with the juxtacanalicular tissue (JXT). Since our preparations consist of cells, connective tissue effects were not studied, and the question raises the issue of relevancy.

Our position is that EPI's effect is mediated primarily by a cellular activity and while this does not exclude the possibility of a secondary connective tissue effect, to be relevant, such an effect must occur soon after the application of this glaucoma medication and be consistent with the kinetics of EPI's action.

The JXT is the albatross I wear about my neck! Nearly 15 years ago, I was reminded of it when my first glaucoma grant was evaluated. There, on the "pink sheet," it said: "Every experienced eye pathologist knows that the juxtacanalicular tissue is the major resistance site" for aqueous outflow. Three years later the reviewers became even more exasperated with me and the message was terse and clear: "pay supreme attention to the JXT!" No small wonder I refer to this position as the party line.

Well, the fact of the matter is that we have paid supreme attention to the JXT. I

am proud to claim that no one else in ophthalmology has looked longer or more carefully and fastidiously at the JXT. Unfortunately, I also have to say that "there's no there there" and that I have failed in my efforts to practice the mellowed California life style because of that thin strip of tissue known as the JXT. Yet, I also know that practically everybody in this audience believes that the JXT is the relevant and important tissue. Allow me to try to persuade you to modify your position.

While the JXT proper is the connective tissue found all along the canal wall, its most important portion is that which extends along the internal side of the canal. This portion comes in contact with the corneoscleral meshwork and the aqueous channels. For aqueous to reach the JXT, it must go through the layer of trabecular cells which lines these channels. To call attention to these ignored cells we like to refer to them as the penultimate layer. Following its passage through the penultimate layer, the aqueous then enters and passes through the extracellular environment of the JXT before it goes through another monolayer of cells, the endothelium of Schlemm's canal or the ultimate layer.

The permeability and resistance of the JXT in normals, POAG and pigmentary glaucoma has been calculated using porous media theory by Ethier et al (*Invest Ophthalmol Vis Sci* 1986; 27:1741) and by us. Similar findings were obtained in both of these studies. The JXT resistance in normals is around 0.03 mm Hg/μl/min which is about 100-fold less than the known resistance for the entire outflow pathway in vivo (≈ 3.3 mm Hg/μl/min). In POAG the resistance (R ≈ 0.21 mm Hg/μl/min) of the JXT increases by a factor of 6 and although this alteration is in the proper direction it is miniscule compared to the magnitude of the change needed (eg, R for the entire outflow pathway in POAG ≈ 14 mm Hg/μl/min in vivo). We also measured the content of plaque material and confirmed our original findings (*Arch Ophthalmol* 1986; 104:1517-1528) that such material is found in only slightly increased amounts in POAG to alter the JXT permeability. We then considered a different model of the JXT and measured the so-called gel fraction to find again only insignificant differences. Finally, we decided to look at specimens of patients with pigmentary glaucoma (PG) where melanin granules might be expected to be trapped and decrease the JXT permeability. Again, we found that in PG the JXT has essentially a normal resistance and contains less than 3% of the total amount of pigment present in the outflow pathway in that condition.

We have looked elsewhere for other tissues that may offer more resistance, such as the two cellular layers mentioned earlier. The resistance provided by the cells lining Schlemm's canal along its internal wall has been estimated using various approaches. We have used the HC measurements obtained from the cultured human SCE cells. The HC varies according to the direction of flow, being quite resistive in a direction as might exist in vivo during hypotony and aqueous reflux (HC ≈ 0.3 μl/min/mm Hg/cm^2) and quite leaky in the direction of aqueous outflow (HC ≈ 5 to 20 μl/min/mm Hg/cm^2). Assuming an HC of 20 and a surface area of 0.11 cm^2 for the canal inner wall, its facility (C) measures 2.2 μl/min/mm Hg and the corresponding resistance (R) is 0.46 mm Hg/μl/min. This value is very similar to that estimated by a different method by Bill and Svedbergh (*Acta*

Ophthalmol 1972; 50:295), and it is ten times greater than estimated for the JXT.

The resistance of the penultimate layer can be estimated using similar methods. We have found that cultured trabecular cells have an HC which varies between 1 and 10 μl/min/mm Hg/cm^2. Since the penultimate layer exists only at the sites where aqueous channels approach the JXT, it has an area which we have estimated to be equal to 38% that of the internal canal wall or 0.0423 cm^2. If we assume an HC of 10 for the lining cells, the facility of the penultimate layer would be 0.423 μl/min/mm Hg with a resistance of 2.36 mm Hg/μl/min. Recall that the total resistance for the entire outflow pathway actually is known to measure only 3.3 mm Hg/μl/min. Therefore, the HC of the penultimate layer in vivo may be higher (eg, leakier) than we have measured in our healthy cultured cells. If we assume an HC of 20, the resistance of the penultimate layer measures 1.2 mm Hg/μl/min which still would amount to a major portion of the total resistance.

Then, to summarize, we proposed that the cells in the vicinity of the JXT may be as important in the regulation of aqueous outflow as the JXT, which appears to account for only a minor portion of the total resistance. I hope you can appreciate the reasons we have for our exasperation with the JXT and the party line regarding aqueous outflow and the role of the various trabecular tissues.

3. The next question is related to the relationship between the transendothelial pressure and the HC measured.

We find a linear relationship at 2 to 8 mm Hg of transendothelial pressure. Below 2 mm Hg, we have technical difficulties measuring the minute flow volumes and above 8 mm Hg, the monolayer of cells begins to break down. In vivo, the transendothelial pressure across the endothelium of Schlemm's canal is around 5 mm Hg.

4. Doctor Forbes has asked whether leaks have produced a relatively high HC for our cultured cells in view of the fact that one of his associates has obtained values of 0.15 μl/min/mm Hg/cm^2 for a different cell type, a vascular endothelium. This value is ten times smaller than the one we measured for the trabecular cells.

We appreciate his concern as leaks can occur easily and it is one of the potential artifacts that one must guard against in using our system. We must recall that the trabecular cells, as we have pointed out before, are not typical endothelial cells as might be found in many capillary beds. They resemble instead the endothelial cells found in lymphatics. Furthermore, as pointed out in our reply to his second question the values we have measured are well within expectations. If anything, the cultured cells appear to be more resistive than is necessary to account for the resistance of the outflow pathway, which is actually quite low.

Regarding the value of 0.15, unless I have it incorrectly, I am surprised that it is so high. Doctor Perkins and I have written a paper in which we calculated the endothelial cells should be more like 1000 times iess leaky than trabecular cells. Anyway, we know that cultured corneal endothelial cells are far more resistive than 0.15 μl/min/mm Hg/cm^2. Doctor Forbes also asked about certain specific studies with timolol which we have not yet conducted.

Doctor Drew's kindness in making such complimentary remarks is also deeply appreciated. Regarding the use of a system similar to ours for cataract research, I am sure that it is likely that someone may be using it; however, I do not know about it. At our institution, Doctor Polansky and associates are studying lens epithelial cells challenged with superoxides and other highly reactive oxidative species. It is important to remember that lens epithelial cells tend to form lens fiber in culture and it is difficult to have them maintain the configuration of an epithelial monolayer. I also wish to thank this attentive audience. I have enjoyed the meeting very much.