# Effects of Antiglaucoma Medications on Bovine Trabecular Meshwork Cells In Vitro

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Using an in vitro culture system, we investigated the effects of five antiglaucoma drugs on growth and morphologic characteristics of bovine trabecular meshwork cells. Epinephrine hydrochloride (55–550 $\mu$M) and pilocarpine hydrochloride (0·8–16 mM), when added to the cultures for 3 days, inhibited trabecular cell growth in a dose-dependent manner. The lowest concentration at which the inhibitory effect was observed was 109 $\mu$M and 0·8 mM, respectively, for epinephrine and pilocarpine. Dipivefrin hydrochloride (26–260 $\mu$M), timolol maleate (116–1160 $\mu$M), and levobunolol hydrochloride (150–1500 $\mu$M) were also added to the cells for 3 days. These drugs caused a reduction in cell density, respectively, at concentrations higher than 103, 460, and 616 $\mu$M. Cell elongation was seen in cultures treated with epinephrine and dipivefrin, whereas levobunolol and timolol induced the cells to adopt a rounded appearance. Cells that had been exposed to pilocarpine were enlarged with numerous vacuoles.

By scanning electron microscopic techniques, epinephrine, timolol, and levobunolol were found to retard the phagocytosis of latex beads by trabecular meshwork cells. Immunostaining with the use of antibodies to vimentin and actin revealed disorganization and condensation of cytoskeletal fibers in trabecular meshwork cells after treatment with epinephrine and dipivefrin. Little change was seen with comparable concentrations of a preservative, benzalkonium chloride, and a vehicle, Liquifilm tears. These results showed that antiglaucoma drugs, depending on their concentrations, may profoundly influence the growth and activity of trabecular meshwork cells.

Key words: antiglaucoma medications; trabecular meshwork; tissue culture; growth; morphology; phagocytosis; cytoskeletal structure.

## 1. Introduction

Antiglaucoma medications such as timolol maleate and epinephrine hydrochloride are extensively prescribed for the treatment of elevated intraocular pressure. Patients are often expected to use these drugs on a long-term basis. After each application, portions of the drugs diffuse into the aqueous humor or reach the trabecular meshwork from the ocular surface. The continuous use of these agents may thus exert an effect on the cells in the trabecular meshwork.

The direct (canalicular) aqueous outflow system is composed of trabecular meshwork cells, interwoven collagenous beams, extracellular matrix, Schlemm's canal, and collector channels. Trabecular meshwork cells are crucial to the normal functioning of the aqueous outflow system. These cells have been shown to phagocytose (Rohen and Van der Zypen, 1968; Van Buskirk and Leure-DuPree, 1978; Epstein et al., 1986; Grierson et al., 1986; Barak, Weinreb and Ryder, 1988; Sherwood, Richardson and Epstein, 1988), to elaborate enzymes (Anderson et al., 1984; Park et al., 1987; Yue et al. 1987; Shuman et al., 1988; Jeng, Weinreb and Miller, 1990; Alexander et al., 1991),

and to produce extracellular matrix materials (Acott et al., 1985; Kurosawa et al., 1987; Hernandez et al., 1987; Yun et al., 1989; Yue, Higginbotham and Chang, 1990). Alvarado, Murphy and Juster (1984) demonstrated a loss of trabecular meshwork cells in trabeculectomy specimens derived from patients with glaucoma. There was also a decreased number of cells with increasing age. Loss of cells leads to denuded beams, which eventually collapse and obliterate intertrabecular spaces.

Following administration of eye drops, the drugs can be achieved in the aqueous humor and in the trabecular meshwork. The level of the drugs that reach the tissue is unknown. In the aqueous humor, it has been reported that 1 hr after topical application of Epifrin drops, the concentration of epinephrine was approximately 5 $\mu$M in eyes of cynomolgus monkeys (Kaufman and Rentzhog, 1981). The pilocarpine concentration was estimated to be 15–20 $\mu$M in the aqueous humor of rabbit eyes 30 min after the topical application of 2 or 4% solution (Ellis, Matsumra and Rendi, 1985; Ellis, Wu and Riegel, 1991). Also, 30 min after applying 0·5% Timoptic eyedrops in rabbits, the timolol level in the aqueous humor reached 1–4 $\mu$M (Polansky and Alvarado, 1985; Ellis et al., 1991). Since trabecular meshwork cells are constantly bathed by the aqueous humor, long-term

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use of antiglaucoma medications may cause long-term exposure of cells to the drugs and therefore instigate extra injury to the cells already in diseased states. Investigators have previously reported deleterious effects of epinephrine on human trabecular meshwork cells in vitro (Tripathi and Tripathi, 1984a; Samples, Binder and Nayak, 1989).

In the present study, we extended the previous studies and investigated systematically the effects of five antiglaucoma medications on growth, morphologic features, and phagocytosis of bovine trabecular meshwork cells in vitro. The five drugs included epinephrine, dipivefrin hydrochloride, pilocarpine hydrochloride, timolol, and levobunolol hydrochloride. Since a large number of cells were needed for all the experiments, bovine rather than human trabecular meshwork cells were used. Bovine tissues are readily available from the slaughterhouse. These cultures can be established in large quantity and bovine cells also grow much faster than the human cells. Our results revealed that the effects of drugs such as epinephrine on bovine cells were consistent with those reported for the human cells (Tripathi and Tripathi, 1984a; Samples et al., 1989), suggesting that bovine trabecular meshwork cells are valid in establishing at least the baseline information regarding the drug effects.

Tripathi, Tripathi and Millard (1989) in their study have suggested that the epinephrine-induced toxicity may be mediated through damage to cellular contractile proteins. Alvarado et al. (1990) in addition have shown that epinephrine could induce a decrease in trabecular cell size and an increase in hydraulic conductivity accompanied by a retraction of the trabecular cells and widening of the intercellular spaces. Since both of these studies implied that the cytoskeletal architecture was involved, we decided to include additional investigations to evaluate the possible modulation of cytoskeletal structure in trabecular meshwork cells by the various drugs. Specifically, actin and vimentin, two prominent cytoskeletal proteins found in cultured trabecular meshwork cells (Ryder et al., 1988; Tamura et al., 1989), were examined.

## 2. Materials and Methods

### Cell Culture

Fresh bovine eyes were obtained from a local slaughterhouse. The trabecular meshwork cell cultures were established as previously described (Higginbotham et al., 1988). The medium consisted of Eagle's minimum essential medium, 10% fetal bovine serum, 5% calf serum, glutamine, essential and nonessential amino acids, gentamicin (10 $\mu$g ml$^{-1}$), and amphotericin B (1·2 $\mu$g ml$^{-1}$). When cultures reached confluency, the cells were trypsinized with 0·25% trypsin and were subcultured with a 1:2 splitting ratio

(Higginbotham et al., 1988). The cells used for our experiments were in either the first or the second passage.

### Drug Solutions

Epinephrine hydrochloride, 1% with 0·01% benzalkonium chloride (Epifrin); dipivefrin hydrochloride, 0·1% with 0·004% benzalkonium chloride (Propine); levobunolol hydrochloride, 0·5% with 1·4% Liquifilm tears and 0·004% benzalkonium chloride (Betagan); and Liquifilm tears were obtained from Allergan Pharmaceuticals, Inc., Irvine, CA, U.S.A. Timolol maleate, 0·5% with 0·01% benzalkonium chloride (Timoptic), and pure timolol maleate solution without preservatives were obtained from Merck Sharp & Dohme Co., West Point, PA, U.S.A. Pilocarpine hydrochloride, 4% with 0·01% benzalkonium chloride (Pilocar) was obtained from IOLAB Pharmaceuticals, Claremont, CA, U.S.A. Pure epinephrine, pilocarpine and benzalkonium chloride (reagent grade) were purchased from Sigma Chemical Co., St Louis, MO, U.S.A.

To test their effects on bovine trabecular meshwork cells, dipivefrin, timolol (Timoptic), levobunolol, Liquifilm tears, and 0·01% benzalkonium chloride were diluted from 1:10 to 1:100 with culture medium. Pilocarpine (Pilocar) was diluted from 1:10 to 1:200 and epinephrine (Epifrin) was diluted from 1:100 to 1:1000. Pure epinephrine, timolol, and pilocarpine solutions were made and diluted to the same concentration range as the respective ophthalmic solutions. The concentration ranges tested for epinephrine, dipivefrin, pilocarpine, timolol, and levobunolol were 55–550 $\mu$M, 26–260 $\mu$M, 0·6–16 mM, 116–1160 $\mu$M, and 150–1500 $\mu$M respectively.

### Drug Effects on Cell Density

Forty thousand bovine trabecular meshwork cells were plated onto each well of 12-well Corning tissue culture plates. After 24 hr, growth medium containing a specific antiglaucoma drug in various concentrations was added to quadruplicate cultures in randomized fashion. Cultures receiving no drugs served as controls. The preservative benzalkonium chloride (0·01%) and Liquifilm tears in different concentrations were added to other cultures to evaluate their effects. After 3 days, cells were washed and harvested with trypsin. The resultant cell density in each well was determined with a ZBI Coulter cell counter. All experiments were repeated at least three times.

### Drug Effects on Cell Morphology

Bovine trabecular meshwork cells were set up as described above. At the end of the 3-day incubation with the drugs, the morphologic features of drug-treated cells were monitored by phase contrast

microscopy. Changes in cell appearance were documented and recorded photographically.

## Assay of Phagocytic Activity

Forty thousand bovine trabecular meshwork cells were plated on Thermonox coverslips. On the next day, growth medium containing a specific concentration of the five drugs was added in duplicate. Those serving as controls received growth medium only. After a 2-day incubation, cells were fed latex beads ($1 \cdot 0$ $\mu$M, 300 $\mu$g ml$^{-1}$) with or without drugs for 24 hr at 37°C. The free beads were removed by rinsing coverslips in phosphate-buffered saline (PBS). Cells were then fixed in McDowell and Trump's fixative for 30 min at 4°C, postfixed and dehydrated. After critical-point drying with $CO_2$, coverslips were sputter coated with gold–palladium and examined on a JEOL 3C scanning electron microscope. Cells from at least ten separate fields of each coverslip were examined, total number of cells were counted, and the percentage of cells with ingested particles was determined. The percentage of cells that phagocytosed latex beads in these fields was averaged and the difference between control and drug-treated cells was evaluated by means of a two-tailed Student's $t$-test. These experiments were repeated twice.

## Immunohistochemical Staining of Vimentin and Actin

Bovine trabecular meshwork cells were plated on coverslips and treated with a specific concentration of drugs for 3 days as described above. Before staining, one half of the coverslips received 5 $\mu$M of colchicine for 18 hr at 37°C as described previously (SundarRaj, Anderson and Barbacci-Tobin, 1988) to demonstrate the specificity of the antibodies used. The coverslips were fixed in paraformaldehyde–lysine–periodate fixative (pH $6 \cdot 2$), rinsed in PBS, incubated for 4 min in $0 \cdot 1$ M sodium phosphate buffer (pH $7 \cdot 0$) containing $0 \cdot 1$% bovine serum albumin and $0 \cdot 2$% Triton X-100, and rinsed again in PBS.

The cells were incubated with normal goat blocking serum and then with mouse anti-vimentin antibody (1:200; BioGenex Laboratories, Dublin, CA, U.S.A.) or rabbit anti-actin antibody (1:150; BioGenex). Those serving as negative controls received normal mouse IgG (1:200) or normal rabbit IgG (1:150). The cells on coverslips were incubated with either biotinylated goat anti-mouse IgG (1:500 for vimentin) or biotinylated goat anti-rabbit IgG (1:250 for actin), followed by incubation with avidin–biotin horseradish peroxidase complex. The color was developed in $0 \cdot 01$% $H_2O_2$, $0 \cdot 05$% 3,3-diaminobenzidine tetrahydrochloride as previously described (Kurosawa et al., 1987; Yue et al., 1990). The coverslips were examined under a light microscope. Immunostaining experiments were performed four times.

## 3. Results

The effects of antiglaucoma medications on the trabecular cell density are summarized in Figs 1–3. Epinephrine [Fig. 1(A)] and pilocarpine (Fig. 2) both reduced cell number in a dose-dependent manner. Inhibition of trabecular cell growth was seen even at
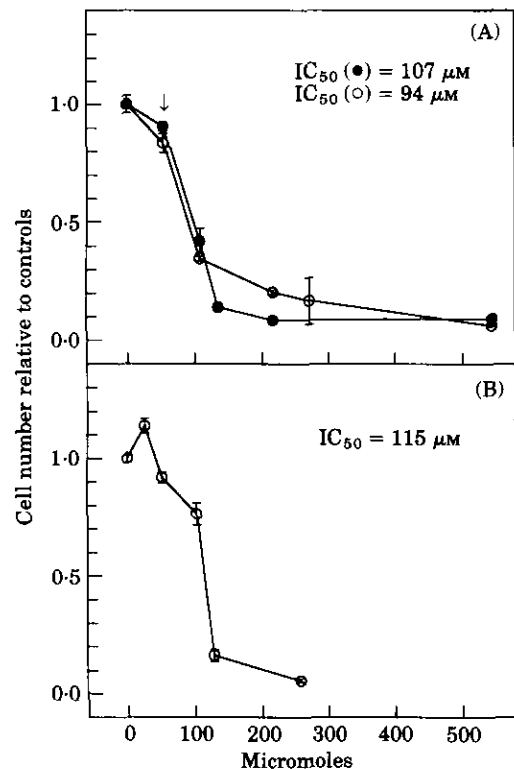


Fig. 1. Effects of epinephrine hydrochloride (A) and dipivefrin hydrochloride (B) on cell density of bovine trabecular meshwork cells in culture. Epinephrine was prepared either from pure powder (●) or from Epifrin ophthalmic solution (○). Arrow indicates one epinephrine data point that is not significantly different from controls. All the rest of the epinephrine data are significantly ($P < 0 \cdot 0085$) lower than the controls. Experiments were repeated three to five times, yielding similar results.
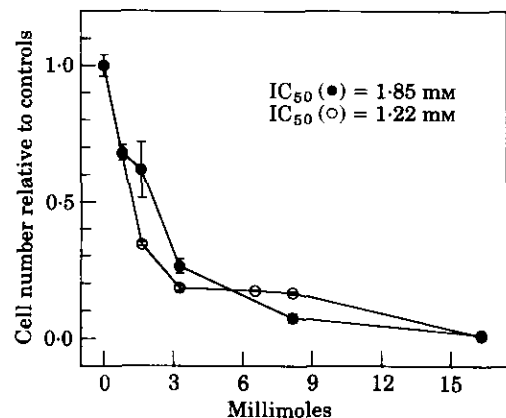


Fig. 2. Effects of pilocarpine hydrochloride on cell density of bovine trabecular meshwork cells in culture. Pilocarpine was prepared either from pure powder (●) or from Pilocar ophthalmic solution (○). All pilocarpine data points are significantly ($P < 0 \cdot 015$) different from controls.
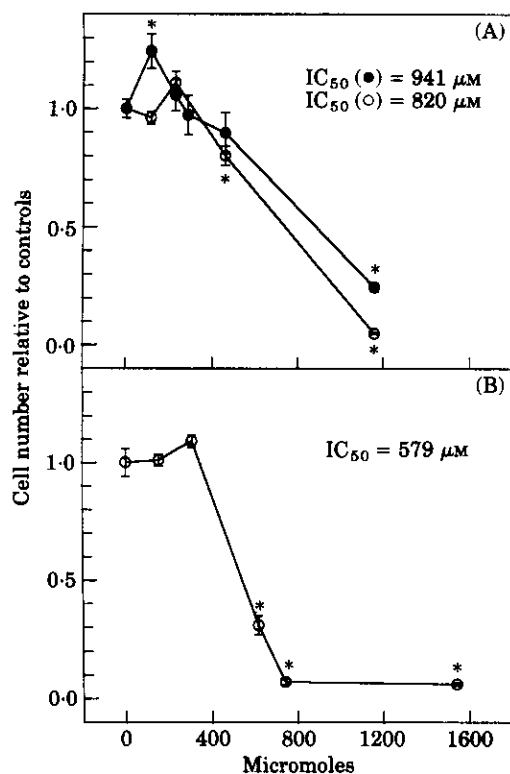
Fig. 3. Effects of timolol maleate (A) and levobunolol hydrochloride (B) on cell density of bovine trabecular meshwork cells in culture. Timolol was prepared either from pure solution (●) or from Timoptic ophthalmic solution (○). Asterisks indicate all experimental data points that are significantly ($P < 0.002$) different from controls.



Fig. 4. Effects of 0·01% benzalkonium chloride (A) and Liquifilm tears (B) on cell density of bovine trabecular meshwork cells in culture. The solutions were diluted from 1:10 to 1:100 and tested for their effects. Only data (asterisks) from the highest concentration (1:10 dilution) are significantly ($P < 0.035$) different from controls.

low concentrations. Dipivefrin [Fig. 1(B)], timolol [Fig. 3(A)], and levobunolol [Fig. 3(B)] also adversely affected the trabecular cell growth in culture, but only at concentrations higher than 103, 460, and 616 μM respectively. Timolol in concentrations less than 200 μM in fact mildly stimulated cell growth [Fig. 3(A)]. Epinephrine prepared from the pure powder yielded data similar to those for epinephrine diluted from the Epifrin solution. Pilocarpine and timolol seemed to be slightly less toxic in the pure form than in ophthalmic solutions. The slight differences were probably due to the presence or absence of preservatives. Accordingly, a reduction of trabecular cell number by approximately 20% was observed with 0·001% benzalkonium chloride [Fig. 4(A)] and 1:10 dilution of Liquifilm tears [Fig. 4(B)]. At lower concentrations, no toxicity was noted with either benzalkonium chloride or Liquifilm tears. The concentration at which cell number was reduced by 50% ($IC_{50}$) was estimated for each of the five antiglaucoma drugs (Figs 1–3). Drugs with higher $IC_{50}$ are less toxic than those with lower ones.

Morphologically, cells treated with epinephrine [Fig. 5(B)] or dipivefrin [Fig. 5(C)] became somewhat elongated or fibroblast-like, whereas cells exposed to 3·3 mM of pilocarpine [Fig. 5(D)] were enlarged, with numerous vacuoles. Timolol [Fig. 5(E)] and levobunolol [Fig. 5(F)] caused cells to adopt a rounder appearance with an accumulation of pigmented
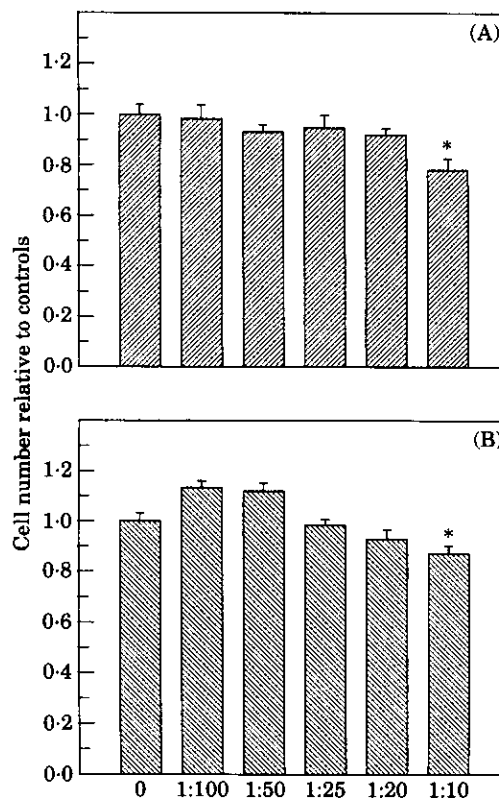
granules surrounding the nucleus. Liquifilm tears, and low concentrations of benzalkonium chloride produced little change in cell morphology. At high concentrations (1:10 and 1:20 dilutions of the 0·01% solution), benzalkonium chloride induced vacuole formation in trabecular meshwork cells.

Table I shows the percentage of cells that phagocytosed latex beads in cultures with or without the treatment of antiglaucoma drugs. A small, but statistically significant decrease of phagocytosis was seen in cells treated with epinephrine, timolol, and levobunolol. No changes were observed in cells treated with dipivefrin or pilocarpine.

Staining for the cytoskeletal proteins vimentin (Fig. 6) and actin revealed disorganization and condensation of the fibers in trabecular meshwork cells after treatment with epinephrine [Fig. 6(B)] and dipivefrin [Fig. 6(C)]. Epinephrine induced a very dramatic effect; almost all the stress fibers and filaments were disassembled and condensed. This effect was similar to those reported by Tripathi and Tripathi (1984a). Colchicine, as expected, induced disruption and striking alteration in the organization of cytoskeletal fibers in all cultures. Pilocarpine [Fig. 6(D)] and the vehicle, Liquifilm tears, and the preservative, benzalkonium chloride, did not elicit much cytoskeletal change. Cells treated with timolol [Fig. 6(E)] and levobunolol [Fig. 6(F)] seemed to have lost some fiber definition.
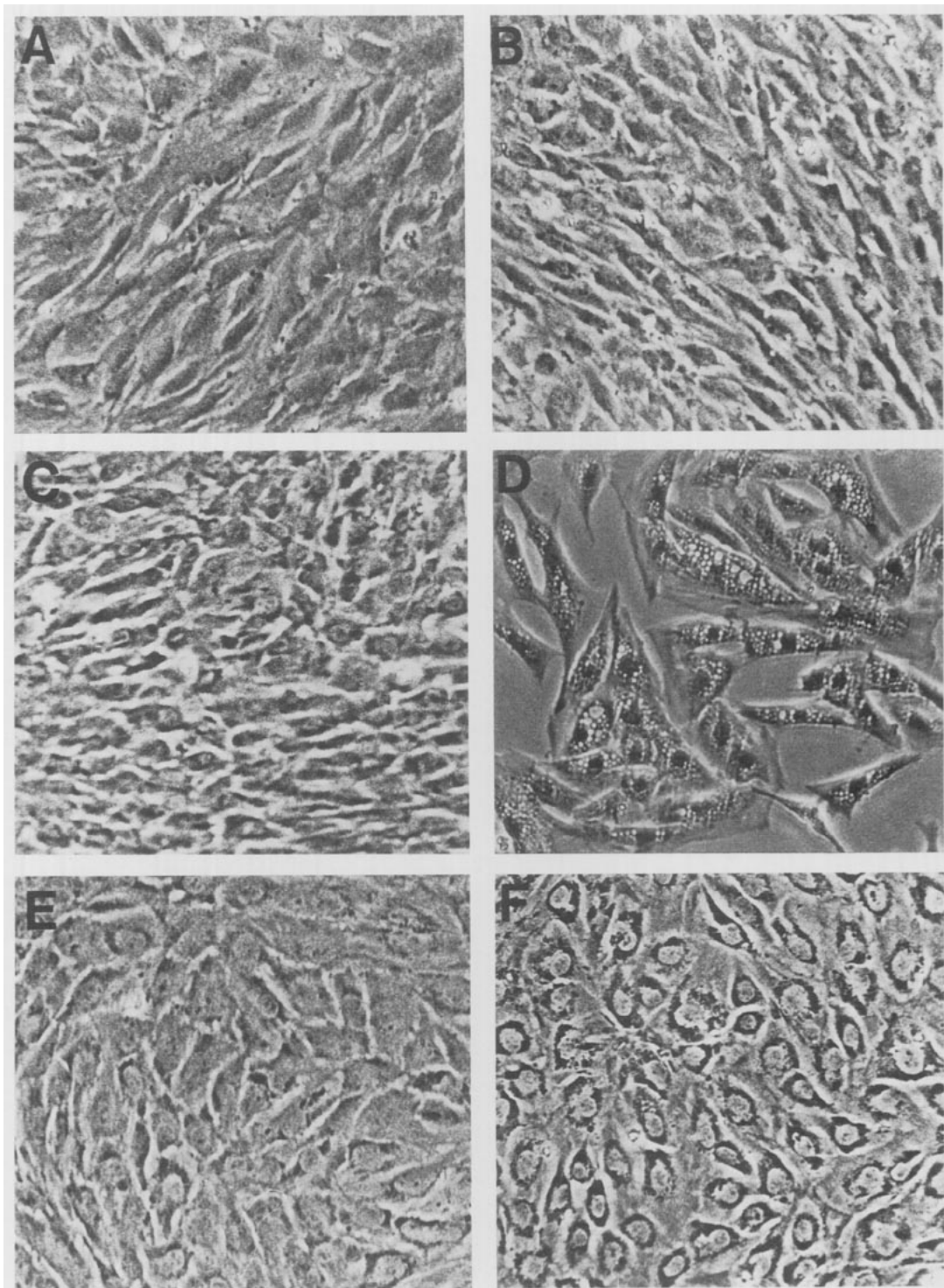
Fig. 5. Phase contrast micrographs of bovine trabecular meshwork cells after incubation for 3 days with culture medium alone (control) (A), and with culture medium containing 109 $\mu$M of epinephrine (B), 103 $\mu$M of dipivefrin (C), 3·3 mM of pilocarpine (D), 462 $\mu$M of timolol (E), and 300 $\mu$M of levobunolol (F). × 10.

## 4. Discussion

This study demonstrated that antiglaucoma drugs, depending on their concentrations, may profoundly influence the growth and activity of bovine trabecular meshwork cells. Some of the drugs studied have been examined previously, but only epinephrine has been studied extensively. The results available from pre-

vious studies were confirmed by the current findings. For instance, the alterations induced by epinephrine in our bovine cells paralleled those found in cultures of human trabecular meshwork cells (Tripathi and Tripathi, 1984a; Samples et al., 1989). Condensation of cytoskeletal structures by epinephrine also corroborated the theory that toxic effects of this drug involve damage to contractile proteins (Tripathi et al.,

TABLE I

*Percentage of trabecular meshwork cells that phagocytosed latex beads*

| Drug used | Percentage of cells that phagocytosed latex beads |
|---|---|
| Control | $47 \pm 10$ |
| Epinephrine (109 $\mu$M) | $38 \pm 6^*$ |
| Dipivefrin (103 $\mu$M) | $50 \pm 8\dagger$ |
| Pilocarpine (3·3 mM) | $40 \pm 13\dagger$ |
| Timolol (462 $\mu$M) | $36 \pm 7^*$ |
| Levobunolol (300 $\mu$M) | $38 \pm 6^*$ |

$^*P < 0.04$ compared with controls.

$\dagger$ Not significantly different from the controls.

1989). In addition, we found that timolol enhanced trabecular cell growth at low concentrations, as was also seen previously by Tripathi and Tripathi (1984b). The effects of pilocarpine and levobunolol on trabecular meshwork cells have not been described previously. It is, however, of interest that the pilocarpine-induced vacuoles have been observed in the treated rabbit corneal epithelial and endothelial cells (Liu, Trope and Basu, 1989).

Among all the drugs examined, epinephrine has the lowest $IC_{50}$ concentration and is probably the most toxic one toward trabecular meshwork cells. Epinephrine has been speculated to act through both $\alpha$- and $\beta$-adrenergic receptors, causing a net increase in cyclic AMP level and affecting indirectly the contractile protein system of trabecular meshwork cells (Neufeld and Bartel, 1982; Tripathi et al., 1989; Robinson and Kaufman, 1991). The involvement of more than one subtype of receptors is supported by the fact that the epinephrine-induced cyclic AMP elevation and changes in mitotic and phagocytic activities of cells can only be partially blocked by $\beta$ blockers such as timolol (Tripathi and Tripathi, 1984a; Polansky and Alvarado, 1985). The $\beta$-adrenergic receptors involved are probably of the $\beta_2$ type, which has been characterized in the human trabecular meshwork both in vivo and in culture (Wax et al., 1989).

As demonstrated in the current study, epinephrine dramatically condenses cytoskeletal elements of trabecular meshwork cells. The cytoskeletal architecture is believed to be important for the maintenance of cell–cell and cell–substratum attachments (Tsukita et al., 1992). Alterations in the cytoskeletal structure may directly influence the cell shape and the mobility functions of cells including cytokinesis and phagocytosis (Lazarides, 1980; Ishikawa, 1987). It has been shown that sulfhydryl blocking agents such as iodoacetamide (Epstein et al., 1981, 1987) and actin-disrupting agents such as cytochalasin B (Kaufman and Barany, 1977) can disrupt the outflow system and increase the outflow facility, through a common mechanism of cytoskeletal changes (Erickson-Lamy,

Schroeder and Epstein, 1992). The condensation of the cytoskeletal elements by epinephrine may therefore have additional impact, furthering the adverse effects. Dipivefrin, a prodrug that can be hydrolysed by ocular esterases to epinephrine, has an $IC_{50}$ similar to that of epinephrine. However, dipivefrin does not induce as notable the cytoskeletal changes as epinephrine. This drug may thus be deemed as a more favorable substitute of epinephrine.

Pilocarpine is a naturally occurring alkaloid with dominant muscarinic action. This agent lowers the intraocular pressure primarily by increasing the outflow of aqueous humor. Pilocarpine inhibits trabecular cell growth. It does not have much effect on the cytoskeletal structure but induces vacuole formation in the trabecular meshwork cells. Timolol and levobunolol are two nonselective $\beta$-adrenergic antagonists that reduce the inflow. These two drugs, especially timolol, have a much milder influence than epinephrine and dipivefrin on either the cell growth or on the cytoskeletal structure of cultured trabecular meshwork cells. Tripathi and Tripathi (1984a) have previously noted that cells pretreated with timolol evidence fewer toxic changes when exposed to epinephrine. It would be interesting in futures studies to examine whether the cytoskeletal changes induced by epinephrine can be eliminated by pretreatment of timolol.

Benzalkonium chloride is a preservative and an antiseptic commonly used in ophthalmic solutions in concentrations ranging from 0·004 to 0·01%. Although its aqueous humor uptake is minimized (Champeau and Edelhauser, 1986), this agent has been shown to cause damage to corneal epithelial and endothelial cells in animal studies (Gasset et al., 1974; Pfister and Burnstein, 1976) as well as in tissue culture systems (Neville et al., 1986; Samples et al., 1989). For human trabecular meshwork cells, benzalkonium chloride at a concentration as low as 0·00002% induced a significant reduction in final cell density (Samples et al., 1989). In the current study with bovine trabecular cells, however, growth inhibition by benzalkonium chloride was not seen until its concentration reached a much higher level, 0·001%. The disparity in the benzalkonium chloride effects in the two studies may be related to differences in the state of the cells and/or the species from which they were derived.

The concentration ranges of drugs that we tested in our study (55–550 $\mu$M for epinephrine, 0·8–16 mM for pilocarpine, and 116–1160 $\mu$M for timolol) are much higher than those reported in the aqueous humor following administration of eyedrops. For example, the concentration of epinephrine in the aqueous humor in cynomolgus monkeys (Kaufman and Rentzhog, 1981) was estimated to be 5 $\mu$M 1 hr after topical application of Epifrin drops. The pilocarpine concentration was approximately 15–20 $\mu$M in the aqueous humor of rabbit eyes 30 min after instillation of 2 or 4%
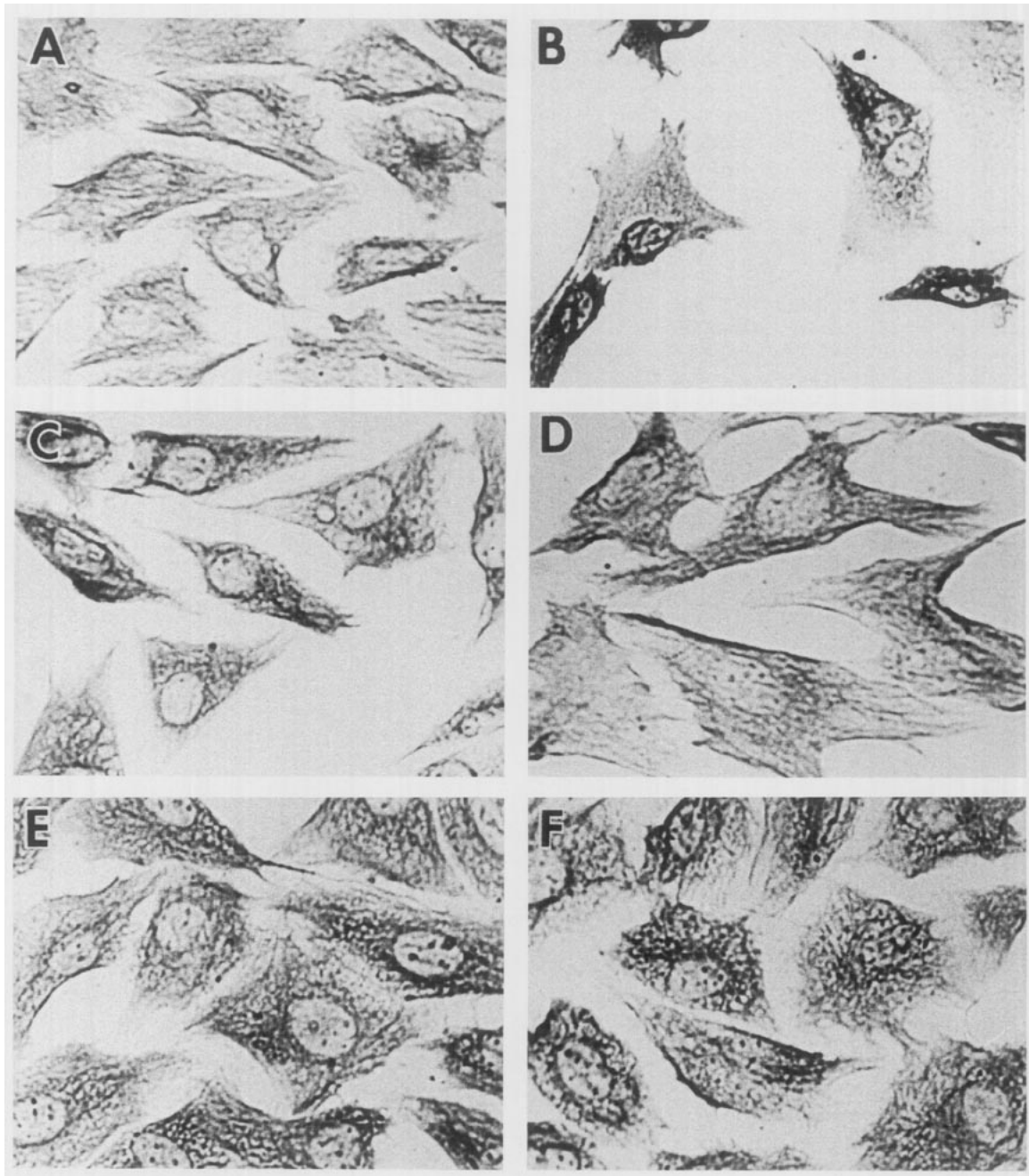
FIG. 6. Immunostaining of bovine trabecular meshwork cells with anti-vimentin. Before the staining, cells were incubated for 3 days with culture medium alone (control) (A), and with culture medium containing 109 $\mu$M of epinephrine (B), 103 $\mu$M of dipivefrin (C), 3·3 mM of pilocarpine (D), 462 $\mu$M of timolol (E), and 300 $\mu$M of levobunolol (F). ×25.

solution (Ellis et al., 1985, 1991). At such low concentrations, no effect was seen with any of these drugs in our culture systems. However, the drugs may also diffuse into the trabecular meshwork from the ocular surface and thus, antiglaucoma drugs can reach the trabecular meshwork cells directly. The combined concentration may be higher. While the drugs in the aqueous humor may turn over within 24 hr, those in the trabecular meshwork tissue may retain for a longer period of time. Pigment within the trabecular meshwork may also act as a depot for ocular medications. With repeated topical applications over years, the drugs may accumulate in the tissue

and may escalate changes in the trabecular meshwork cells. In any event, results obtained from our in vitro study may not extrapolate directly to in vivo situations, particularly considering the accelerated time course and high concentrations of medications used in vitro. Nevertheless, results of the present study indicate the need for caution because of potential toxicity and side effects of antiglaucoma drugs, especially for long-term usage and for those patients who may be more sensitive to the drug effects.

Our demonstration also underscores the importance of evaluating drug effects while conducting studies with glaucomatous tissues or cells. Patients with

glaucoma are often prescribed medications for an extended period, and changes in the trabecular meshwork noted in end stage disease may result from not only the disease process but also long-term drug treatment. Only through the careful sorting of the various factors can relevant information be obtained for the eventual elucidation of glaucomatous conditions.

## Acknowledgements

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