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Effects of Adrenergic Agents and Phosphodiesterase Inhibitors on Outflow Facility and Cell Shape of Bovine Trabecular Meshwork

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Abstract: Changes in the outflow facility of perfused calf eyes and in the shape of cells in cultured trabecular meshwork (TM) have been studied, following exposure to adrenergic agents and phosphodiesterase inhibitors (PDE). Dobutamine caused confluent TM cells to change their usual polygonal shape to a characteristic stellate shape. Salbutamol had no effect, but PDE inhibitors, isobutylmethylxanthine (IBMX), theophylline, and caffeine were very effective in producing this shape change. Epinephrine, isoproterenol, dobutamine, and salbutamol did not increase the outflow facility, either at 22°C or 36°C, while theophylline, caffeine, and IBMX did increase it in a dose-dependent manner. On the other hand, the high concentrations of β-adrenergic agents required to produce even a small change in outflow facility and cell shape argue against the involvement of adrenergic-receptor mediation and may suggest another mechanism; on the other, the enhancement of epinephrine effects by PDE inhibitors and the similar effect produced by cyclic adenosine 3′,5′-cyclic phosphate (cAMP) and purines suggest that changes in the cell shape are produced by β-receptor activation. The β-adrenergic agents were not effective in changing outflow facility, but the PDE inhibitors were remarkably effective both in changing the shape and in increasing facility. **Jpn J Ophthalmol 1997;41:31–37** © 1997 Japanese Ophthalmological Society

Key Words: Adrenergic agents, cGMP, cyclic AMP, cytoskeleton, epinephrine, glaucoma, outflow facility, phosphodiesterase inhibitors, sulfhydryl agent, trabecular meshwork.

Introduction

Epinephrine has long been used topically for treatment of glaucoma.[1–3] Epinephrine effectively lowers the intraocular pressure (IOP) in the majority of patients, apparently by increasing the facility of the aqueous outflow. It is also effective in lowering IOP in the normal human eye and in the eyes of various experimental animals.[2,4] Erickson-Lamy[3] demonstrated an epinephrine effect on outflow facility in human eyes in organ culture,[5] but there has as yet been no similar success with eyes in vitro.

Human trabecular meshwork (TM) cells in tissue culture have β-receptors.[6–8] Epinephrine at 10^{-5} mol caused contraction of human TM cells in tissue culture and severe damage to the cells within 4~5 days; at 10^{-6} mol, contraction and cell damage developed more slowly.[6] Epinephrine-caused contraction may affect the aqueous outflow facility. Sulfhydryl agents affect the shape of TM cells in culture,[9–11] and shape change may be the principal mechanism by which these agents facilitate aqueous outflow.[3,10,11]

At this time, there have been no reports on the in vitro effects of dobutamine (β_1-agonist), salbutamol (β_2-agonist), cyclic adenosine-3′,5′-monophosphate (cAMP) derivatives, and phosphodiesterase (PDE) inhibitors on the shape of TM cells in culture and on the facility of outflow. In the present study, we investigated the effects of these compounds on cell shape and possible influence on the facility of aqueous out-

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flow. Since the change of outflow facility of calf eyes is similar to that of human eyes when perfused,[12] we examined these effects using calf eyes.

Materials and Methods

Calf Eyes

Freshly enucleated calf eyes were obtained from a local slaughterhouse, packed in mixed liquid/frozen saline. Eyes for perfusion were trimmed of extraocular muscle and other loose tissue and kept cool until used.

Tissue Culture

Calf TM tissues were isolated and cultured as described by Crean et al.[13] Cells were monitored by phase-contrast microscopy. These cells were then grown on glass cover slips and actin-stained for visualization with antihuman actin monoclonal antibody, using a commercially available kit (du Pont, Wilmington, DE, USA). The cells were then photographed on a Zeiss phase-contrast microscope with a fluorescence attachment.[10,14] After exposure to experimental treatment, cells in each dish changing completely to the dendritic shape were counted and the dish was classified: 5–24%, +; 25–70%, ++; > 70%, +++.

Eye Perfusion

A device for perfusing 10 calf eyes in parallel was made from a set of 10 narrow-tipped 2 mL pipettes held horizontally in a wooden frame. Fluid flow from a reservoir to the pipettes, or from the pipettes to the eyes, was controlled by a 3-way stopcock. Volume readings from the pipettes were recorded on a personal computer programmed to time the interval between entries for each pipette. Facilities were calculated, displayed, printed, and saved on computer disk for later analysis. Outflow facility was evaluated using a base norm of equal inflow and outflow.[9,15,16]

All perfusion was done at a pressure of 15 mm Hg at 34–35°C, unless otherwise indicated. The inflow needle was placed in the posterior chamber to prevent chamber-deepening effects on facility; a second needle was placed in the anterior chamber to facilitate the exchange of medium. Experimental agents were administered continuously after the time indicated. Other procedures followed previously reported methods.[16,17]

Drugs

Epinephrine, timolol, (±)-isoproterenol HCl, cyclic adenosine-3′,5′-cyclic phosphate Na (cAMP), N^6,2′-0-dibutyryl cyclic AMP (dB-cAMP), theophylline, isobutylmethylxanthine (IBMX), caffeine (PDE inhibitors), and dobutamine were obtained from Sigma (St. Louis, MO, USA). Salbutamol (Lilly, Indianapolis, IN, USA) and cytoskeletal staining kits (Biotechnology Systems) were also used.

Results

Effects on Cell Shape

Normal cells typically had polygonal cell bodies with occasional narrow processes, which were lost as they approached confluence. With prolonged incubation, overlapping of the cell processes was observed, but the cells did not overlie each other.

Epinephrine, applied to confluent cells in culture, produced a characteristic shape change in some, but not all, cells, in a dose- and time-dependent manner. Affected cells changed from the normal polygonal shape to a highly contracted, dendritic form with the cell body condensed around the nucleus, leaving long radiating processes to points of attachment on the substrate (Figure 1).

Theophylline produced this same shape change, but much more rapidly than dobutamine or epinephrine. The change was accompanied by actin condensation so that no filament structures were visible (Figure 2). Electrophoresis of extracted cytoskeletal protein, as described by Mirabelli et al,[18] showed that this condensation was not accompanied by disulfide cross-linking of the actin (data not shown).

Dose–Time Relationships

Changes produced by adrenergic agonists are shown in Figure 3. At 10^{-3} mol, dobutamine produced the full effect in 2 hours and isoproterenol in 3–6 hours, but epinephrine took 12–15 hours. At 10^{-4} mol, epinephrine took 18–24 hours; at 10^{-5} mol, 3 days. At all concentrations, the effect was: dobutamine > isoproterenol > epinephrine (Figure 3a).

Salbutamol was also tested over the concentration range 10^{-7}–10^{-3} mol and found to have no effect. Timolol, even when present at a 10-fold excess over agonist, could not completely block the agonist effect.

PDE inhibitors were much more effective in producing the change in cell shape, even in the absence of an adrenergic agonist. IBMX was the most effective, followed by theophylline and caffeine. Most of the effect occurs in the first 3 hours, with only slight change continuing for up to 12 hours. Theophylline at concentrations < 1 mmol was ineffective in causing shape change, even after 12 hours (Figure 3b). However, ad-

Figure 1. Cell-shape change caused by 10^{-3} mol epinephrine at 3 hours.

dition of theophylline at 0.3 mmol or 0.6 mmol to each of the adrenergic agents enhanced the effect, which then appeared earlier and at lower concentrations.

At relatively high concentrations of 5–10 mmol, cAMP produced a similar cell shape change. The effect seemed to reverse by 12–18 hours, probably because affected cells were lysing with such high concentrations rather than recovering. Dibutyryl cAMP, which is permeable through the cell membrane, produced the same effect at lower concentrations (Figure 3c). Adenosine behaved like cAMP, whereas ATP, ADP, AMP, impermeable peptides, and butyrate at similar concentrations were without effect. Overall, PDE inhibitors had the most potent effect (Figure 3b vs Figures 3a and c).

Temperature Effects on Facility of Outflow

Effects of β-adrenergic agents and PDE inhibitors on outflow facility in the perfused calf eye were examined at 22°C and 36°C. At 22°C, epinephrine, isoproterenol, and dobutamine had no effect on outflow facility, even at concentrations as high as 10^{-3} mol (Figure 4a). IBMX, theophylline, and caffeine caused a remarkable increase in outflow facility compared with controls at 22°C; at 3 mmol, IBMX had almost doubled the facility at 60 minutes; all three agents produced a doubling within 90 minutes (Figure 4b).

At 35–36°C, epinephrine, isoproterenol, and salbutamol had only a slight effect on outflow facility, even at concentrations as high as 10^{-3} mol and at

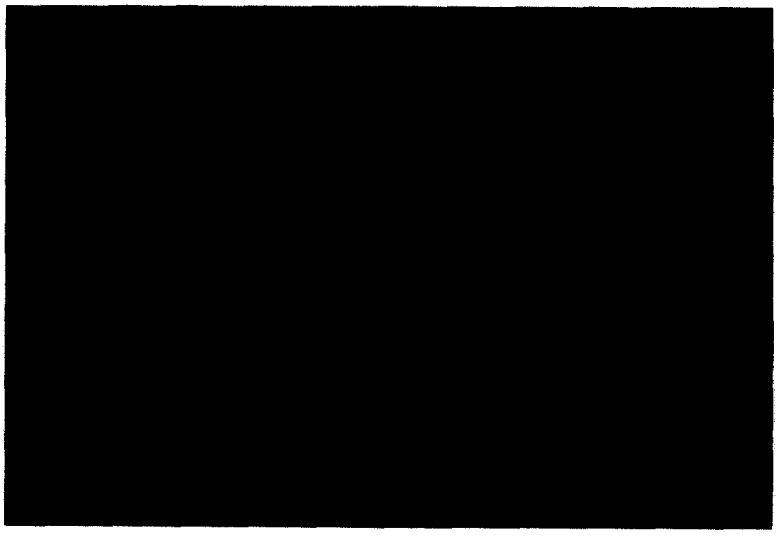

Figure 2. Cell-shape change caused by theophylline: (**A**: left) phase-contrast photograph; (**B**: right) actin staining.

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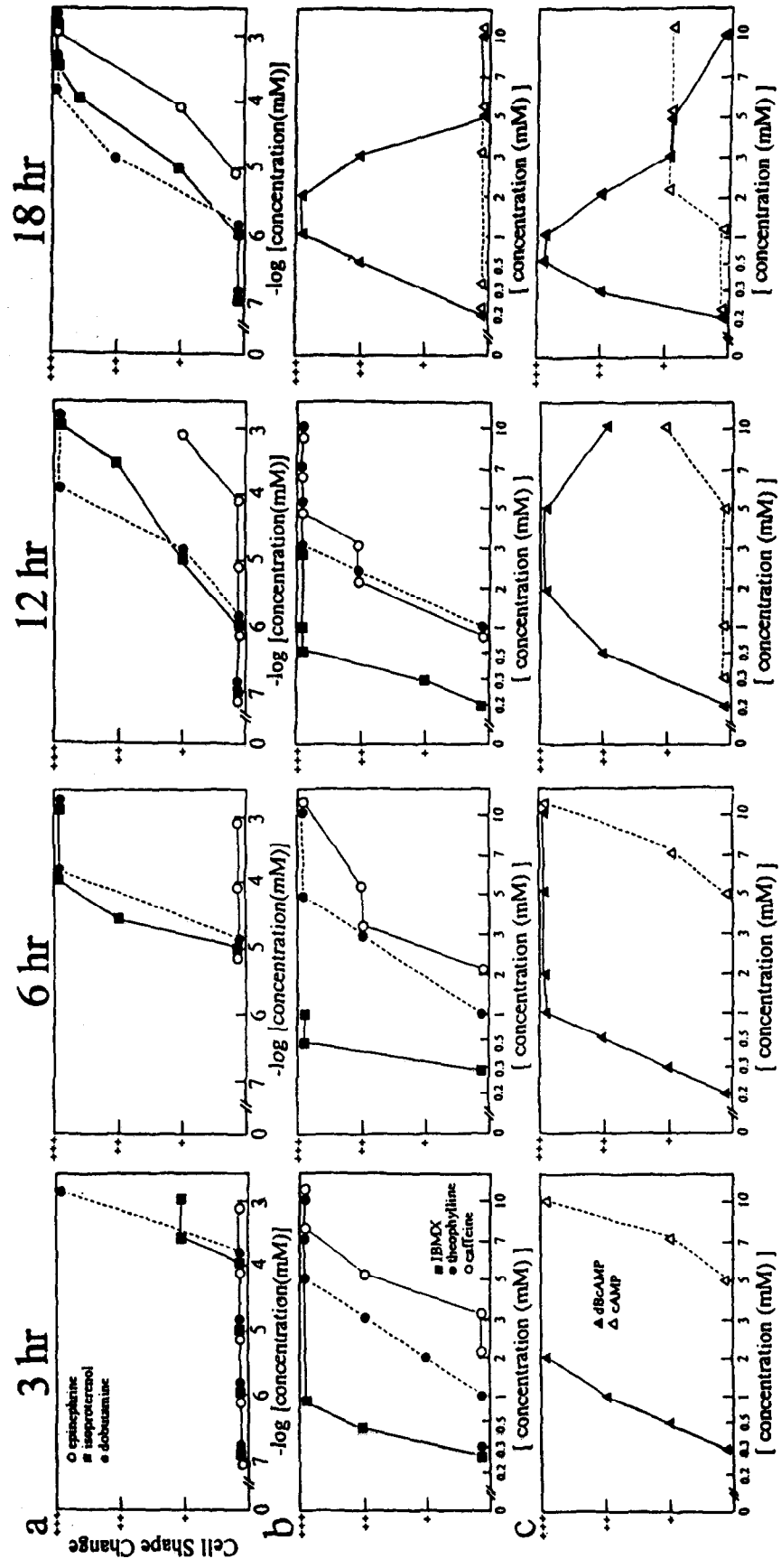

Figure 3. Time course of cell shape change from (**A**) adrenergic β agonists, (**B**) *PDE inhibitors, and* (**C**) cAMP. (*Each dish was sampled by observing different* fields.) The proportion of cells undergoing complete change to dendritic shape was counted in each dish and dish was classified as +: 5–24%, ++: 25–70%, and +++: > 70%. *Each group represents the mean of results from three to five dishes.* Cell shape change effectiveness: dobutamine > isoproterenol > epinephrine; IBMX > theophylline > caffeine; dBcAMP > cAMP. PDE: phosphodiesterase. dBcAMP: dibutyryl cyclic adenosine monophosphate.

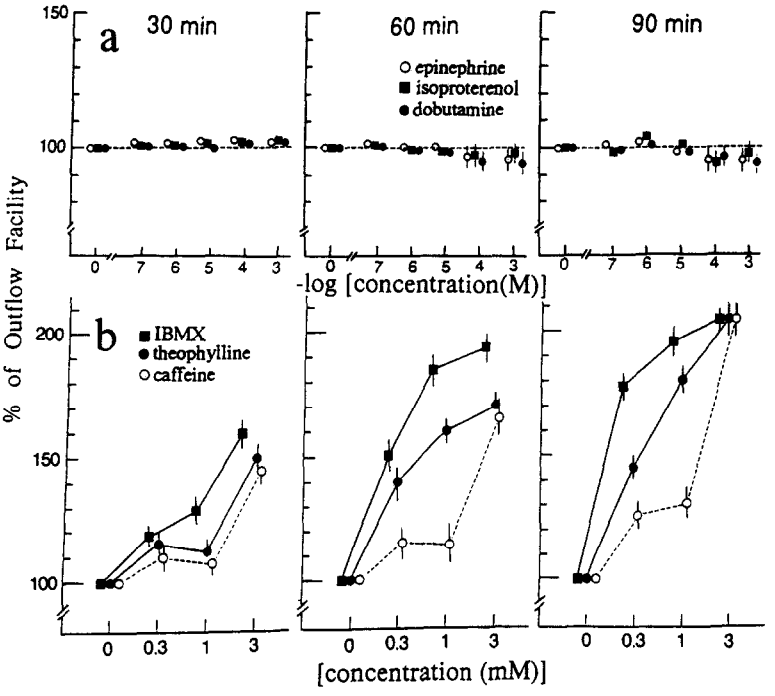

Figure 4. Effects of β-adrenergic agonists and PDE inhibitors on outflow facility of enucleated calf eyes tested at 22°C. All eyes were perfused, with continuous recording, for a baseline period of 30 min; then the agent was added as indicated. A placebo exchange was done on control eyes. Recording was continued and facilities at 30, 60, and 90 min were determined and expressed as percent of control. All points are means of results of 4 to 6 perfusions. Error bars represent SEM. PDE: phosphodiesterase. IBMX: isobutylmethylxanthine.

times as long as 4 hours. Dobutamine (10^{-5}, 10^{-4}, 10^{-3} mol) produced a small but significant reduction in facility to 87.3 ± 6.8, 83.5 ± 5.1, and 80.8 ± 5.9% (mean ± SEM) of control, respectively (Figure 5a). The facility continued to decline with longer times; further increases in concentration up to 10^{-3} mol produced little further reduction. PDE inhibitors, however, caused a marked increase in facility at 22°C with IBMX again being the most effective and caffeine the least (Figure 5b).

In contrast to the effect of the PDE inhibitors (Figures 4b, 5b), adrenergic agents caused no increase in outflow facility and much slower change in cell shape. High concentrations of β-adrenergic agents reduced the outflow facility instead. The relative reduction in facility seen at 35–36°C, compared

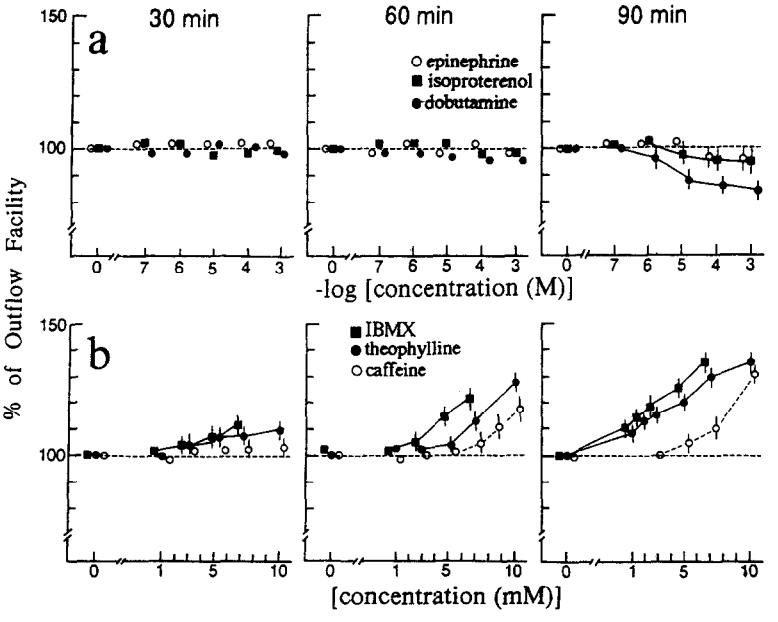

Figure 5. Effects of adrenergic β-agonists and PDE inhibitors on outflow facility of enucleated calf eyes tested at 35°C. All experiments as described for Figure 4.

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with that at 22°C, may be caused by greater washout in the controls at the higher temperature.

Discussion

The effects of epinephrine on the cell shape of calf trabecular endothelial cells in culture were similar to those which have been reported.[6,14,19] Isoproterenol, which acts on both β_1-receptors and β_2-receptors had a similar effect, but at lower concentrations. Kawa et al[20] observed "cell elongation" with addition of epinephrine to cultured bovine TM cells; these agents also inhibited cell division. High concentrations of timolol and levobunolol caused rounding of the cells.

In our study, dobutamine, a selective β_1-agonist, was most potent; salbutamol, a selective β_2-agonist, had no effect, even at the high concentration of 10^{-3} mol. At either 22°C or 36°C, these agents did not change the outflow facility of the enucleated perfused eye except for a slight *reduction* by dobutamine.

Similar cell-shape changes in tissue culture were caused by the PDE inhibitors theophylline, caffeine, and IBMX; dB cAMP and adenosine were also effective. ATP, ADP, AMP, and butyrate had no effect when tested at similar concentrations. Although dB cAMP and adenosine are permeable through the cell membrane, ATP, ADP, and AMP are only minimally permeable. These observations, therefore, do not prove, but at least are consistent with a β-mediated process. In contrast to the action of the adrenergic agents, the effects of these PDE inhibitors occurred much more rapidly. Another possibility exists that such responses are mediated by cGMP, because zaprinast,[21] another selective PDE inhibitor that increases cGMP content, had remarkable effects on the cultured cells, outflow facility and isolated ciliary muscles (not shown).

In cultured human TM cells, the predominant type of β-receptor is β_2.[7,8,19] A number of physiologic studies reviewed by Mittag[22] indicate a predominant role for β_2-agonists in lowering IOP in rabbit, human, and nonhuman primates. Our data on cell shape and actin change contrast with this general understanding. Further work is required to specifically describe the type of receptor and its function.

Care is needed in attempting to correlate effects of agents observed in tissue culture of TM cells with effects of the same agent on the facility of aqueous outflow in vivo. The cells that grow in tissue culture may not be the same as those controlling resistance in the intact tissue. Even if they are identical, they are not in the same microenvironment and therefore are not likely to be in the same metabolic state.[16,22] Yet, there is evidence that trabecular cells in culture possess many of the same properties as those observed in vivo: glycosaminoglycan synthesis,[8,24,25] β-receptors,[6,7] steroid responsiveness,[5,26] tissue plasminogen activator synthesis,[26,27] and the capacity to carry out phagocytosis.[6,28,29] Therefore, it is also possible that the important resistance-controlling properties are common across changes in metabolic state or even across variations in cell type.

In the present study, it is unlikely that pure β-receptor activation alone increases outflow facility, at least from the test data of the first several hours. Tested at 22°C or 36°C, epinephrine and isoproterenol did not increase the facility of outflow and at higher concentrations, they decreased outflow facility. PDE inhibitors, however, remarkably and acutely increased the facility earlier and with usual concentrations. N-ethylmaleimide,[9] iodoacetic acid,[15] and ethacrynic acid[9,10,30] increase outflow facility; the mechanism may be explained by the chemical properties of sulfhydryl agents.[9,11] These are all PDE inhibitors and all cause the characteristic stellate-shape change in the cells.[10,11] Ciliary muscles respond only slightly to β-adrenergic agents, but relax greatly with PDE inhibitors.[16,17,21]

The most striking finding is the speed and magnitude of the increase in facility brought about by the PDE inhibitors, especially IBMX and zaprinast[21] (not shown), and the contrasting failure of the adrenergic agents to do so. In fact, the only effect of the adrenergic agents was a small *reduction* in facility. If the effect on facility is mediated by the same kind of change in cell shape as seen in vitro, then the absence of an adrenergic effect may be caused by the difference in timing of the shape change produced by the adrenergic agents or the PDE inhibitors.

In principle, perfusions could be prolonged up to 10 hours in order to see if the adrenergic agents could produce appreciable effects. However, there are serious doubts about the viability of the perfused enucleated eye during such a long experiment, and the facility was reduced by high concentrations of β agonists. The synergistic effect on shape change noted in theophylline combined with adrenergic agents suggests that PDE may be highly active in TM in the resting state. It is interesting that PDE inhibition had a much greater influence on and around the outflow channels than β-adrenergic agonists. We cannot deny that perfusing the eye with a subthreshold level of PDE inhibitor may well promote an adrenergic effect on facility within an acceptable span of time. However, because zaprinast[21] had a stronger ef-

fect on the outflow facility, cell shape change, and ciliary muscle contractions, the involvement of GMP should also be taken into account.

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