

EFFECTS OF ANTIGLAUCOMA DRUGS ON CALCIUM MOBILITY IN CULTURED CORNEAL ENDOTHELIAL CELLS

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The aim of this study was to estimate the effects of various antiglaucoma drugs including betaxolol, timolol, levobunolol, brimonidine, carteolol, dipivefrin, dorzolamide, brinzolamide, latanoprost, unoprostone, and pilocarpine on intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) mobility in cultured bovine corneal endothelial cells. Various antiglaucoma drugs were diluted from original concentrations to 1/100, 1/1,000, and 1/10,000. The $[\text{Ca}^{2+}]_i$ mobility was studied by spectrofluorophotometry after loading with the ester of fura-2 (fura-2/AM). It was found that timolol (58 μM and 5.8 μM), levobunolol (171 μM , 17.1 μM , and 1.71 μM), betaxolol (162 μM , 16.2 μM , and 1.62 μM), carteolol (680 μM and 68 μM), dipivefrin (28 μM and 2.8 μM), dorzolamide (616 μM and 61.6 μM), brinzolamide (260 μM), latanoprost (1.1 μM), unoprostone (28.2 μM , 2.82 μM , and 0.282 μM), and pilocarpine (408 μM and 40.8 μM) induced a significant increase in $[\text{Ca}^{2+}]_i$. Nevertheless, only brimonidine (68 μM and 6.8 μM) decreased $[\text{Ca}^{2+}]_i$ concentration significantly. Benzalkonium chloride preservative did not affect $[\text{Ca}^{2+}]_i$ after addition of 0.001, 0.0001 and 0.00001 mg/mL to cells. These results indicate that all antiglaucoma drugs may affect the physiologic function of corneal endothelial cells through change of $[\text{Ca}^{2+}]_i$ mobility.

Key Words: antiglaucoma drugs, calcium mobility, corneal endothelial cells
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Corneal endothelial cells play a crucial role in maintaining corneal transparency. Corneal clarity requires a net movement of fluid from the corneal stroma to the aqueous humor. This flux depends on corneal endothelial ion transports and regulation [1,2]. Recent studies have reported that an absence of extracellular calcium can lead to rounding of endothelial cells, their losing of the apical junction, and increasing paracellular calcium permeability. These changes result in rapid corneal swelling [3]. After calcium is added to the solution, however, normal endothelial configuration

is restored [4]. In the corneal endothelial cell membrane, the Na^+/K^+ cation-sensitive channel is activated by intracellular calcium [5]. Adenosine triphosphate (ATP), purinergic receptor agonists, and endoplasmic reticulum (ER) Ca^{2+} -pump inhibitor are reported to enhance HCO_3^- permeability in corneal endothelium via increase in intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) [6]. The swelling rate of corneas increased in the presence of the calcium channel antagonist verapamil [7].

Many glaucoma patients require the administration of antiglaucoma drugs for a long period of time unless the intraocular pressure is controlled by other alternatives such as laser therapy or surgery. Under such circumstances, the corneal endothelium is continuously soaked in antiglaucoma drugs for a protracted period. The physiologic function of corneal endothelial cells may change gradually during the

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process of drug administration. Our previous study shows that the $[Ca^{2+}]_i$ in corneal endothelial cells is inhibited by isoproterenol and norepinephrine but enhanced by propranolol [8]. Until now, the effects of antiglaucoma drugs on the change of calcium signal transduction of corneal endothelial cells have had little investigation. In the present study, we evaluated the effects of various commercial antiglaucoma drugs including timolol, levobunolol, betaxolol, carteolol, dipivefrin, brimonidine, dorzolamide, brinzolamide, latanoprost, unoprostone, and pilocarpine on the $[Ca^{2+}]_i$ in cultured bovine corneal endothelial (BCE) cells.

MATERIALS AND METHODS

Materials

Culture materials, including trypsin, minimal essential medium (MEM), glutamine, gentamicin, and fetal bovine serum (FBS), were obtained from GIBCO (Grand Island, NY, USA). Benzalkonium chloride, ester of fura-2 (fura-2/AM), and bovine serum albumin (BSA) were purchased from Sigma Chemical (St. Louis, MO, USA). Commercially available preparations of antiglaucoma drugs, namely 0.5% (16.2 mM) betaxolol (Betoptic, Alcon, containing 0.1 mg/mL benzalkonium chloride), 0.25% (5.8 mM) timolol (Timoptol, Merck Sharp & Dohme-Chibret, containing 0.048 mg/mL benzalkonium chloride), 0.5% (17.1 mM) levobunolol (Bunolgan, Allergan, containing 0.04 mg/mL benzalkonium chloride), 1% (68 mM) carteolol (Arteoptic, Otsuka, containing 0.005 mg/mL benzalkonium chloride), 0.2% (6.8 mM) brimonidine (Alphagan, Allergan, containing 0.01 mg/mL benzalkonium chloride), 0.1% (2.8 mM) dipivefrin (Propine, Allergan, containing 0.04 mg/mL benzalkonium chloride), 2% (61.6 mM) dorzolamide (Trusopt, Merck Sharp & Dohme-Chibret, containing 0.075 mg/mL benzalkonium chloride), 1% (26 mM) brinzolamide (Azopt, Alcon, containing 0.1 mg/mL benzalkonium chloride), 0.005% (0.11 mM) latanoprost (Xalatan, Pharmacia & Upjohn, containing 0.08 mg/mL benzalkonium chloride), 0.12% (2.82 mM) unoprostone (Rescula, Fujisawa, containing 0.015 mg/mL benzalkonium chloride) and 1% (40.8 mM) pilocarpine (Spersacarpine, Dispersa, containing 0.1 mg/mL benzalkonium chloride), were diluted with control buffer containing 129 mM NaCl, 2 mM $CaCl_2$, 4.7 mM KCl, 1.25 mM $MgSO_4$, and 10 mM Na_2PO_4 at pH 7.4 to make three dilutions of these drugs, from 1/100, 1/1,000, to 1/10,000, for the calcium experiment. All other chemicals were obtained from Merck (Darmstadt, Germany).

Culture of bovine corneal endothelial cells

BCE C/D-1b cells, a stable cell line derived from bovine corneal endothelium, were obtained from ATCC (American Type Culture Collection). Cells were grown in MEM containing 20% FBS, 3.8 mM L-glutamine, and 50 μ g/mL gentamicin according to our previously published method [9]. Culture conditions were maintained in a humidified chamber with 5% CO_2 at 37°C, and the medium was changed every 2 or 3 days.

Detection of intracellular calcium by spectrofluorometry

To determine the effects of antiglaucoma drugs on $[Ca^{2+}]_i$, various concentrations of drugs were diluted with control buffer containing 129 mM NaCl, 2 mM $CaCl_2$, 4.7 mM KCl, 1.25 mM $MgSO_4$, and 10 mM Na_2PO_4 at pH 7.4 to make three dilutions of these drugs from their original concentrations to 1/100, 1/1,000, and 1/10,000. $[Ca^{2+}]_i$ was measured according to our previously published method [10]. Approximately 10^6 cells/mL were loaded with 2 μ M fura-2/AM and 0.25% BSA for 60 minutes at 37°C in control buffer containing 129 mM NaCl, 2 mM $CaCl_2$, 4.7 mM KCl, 1.25 mM $MgSO_4$, and 10 mM Na_2PO_4 at pH 7.4. The cells were then washed and resuspended in control buffer. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette containing 1 mL of control buffer and 1 mL of cells. Fluorescence was monitored with a fluorescent spectrophotometer (Shimadzu RF-5301PC, Japan) by continuously recording excitation signals at 340 and 380 nm, and emission signals at 510 nm at 1-second intervals at 25°C. After equilibration with control buffer, various drugs were added to the cells for specific experiments. $[Ca^{2+}]_i$ was estimated and calculated according to the equation, $[Ca^{2+}]_i = K_d\beta(R - R_{min}) / (R_{max} - R)$, where the value of β is equivalent to 380 nm at R_{min} /380 nm at R_{max} , and a K_d (dissociation constant) value of 155 nM is assumed. Maximal (R_{max}) and minimal (R_{min}) fluorescence values were obtained by adding Triton X-100 (0.1%) and EGTA (20 mM) sequentially at the end of experiments. Autofluorescence was determined by quenching fura-2/AM fluorescence with 2 mM $MnCl_2$, and 340 nm/380 nm quenching values were subtracted before calculating the data.

Calculation and statistics

The net area under the response curve (AUC) of $[Ca^{2+}]_i$ increase with baseline subtracted was integrated with six experiments. The values were presented as the mean \pm SEM of six different experiments. All data were analyzed with analysis of variance followed by comparison with a Dunnett test. The values were considered significantly different from the control group at $p < 0.05$.

RESULTS

After treatment of cells with 58 μM and 5.8 μM timolol for 200 seconds, the drug-induced $[\text{Ca}^{2+}]_i$ AUCs were significantly increased to $136 \pm 4\%$ and $119 \pm 3\%$ when compared with the AUCs for control cells, which was assumed as a 100% response (Figure 1A). Levobunolol concentrations of 171 μM , 17.1 μM , and 1.71 μM all induced significant increases in the AUCs for $[\text{Ca}^{2+}]_i$ to $409 \pm 4\%$, $353 \pm 3\%$, and $136 \pm 5\%$ (Figure 1B). Treatment of cells with betaxolol at 162 μM , 16.2 μM , and 1.62 μM also resulted in significant AUC increases for $[\text{Ca}^{2+}]_i$ to $123 \pm 5\%$, $118 \pm 4\%$, and $114 \pm 4\%$, when compared with the AUC for control cells (Figure 2A). When carteolol

was added at concentrations of 680 μM and 68 μM for 200 seconds, the amount of drug-induced $[\text{Ca}^{2+}]_i$ was increased to $168 \pm 5\%$ and $125 \pm 4\%$ when compared with the AUC for control cells (Figure 2B). After addition of 28 μM and 2.8 μM dipivefrin for 200 seconds, the drug-induced $[\text{Ca}^{2+}]_i$ increased to $131 \pm 5\%$ and $117 \pm 3\%$ (Figure 3A). When cells were exposed to 68 μM and 6.8 μM brimonidine, the drug-induced $[\text{Ca}^{2+}]_i$ was significantly decreased to $44 \pm 3\%$ and $76 \pm 4\%$ (Figure 3B). Two carbonic anhydrase inhibitors, dorzolamide and brinzolamide, were also evaluated for their effects on drug-induced $[\text{Ca}^{2+}]_i$ change. It was found that dorzolamide at 616 μM and 61.6 μM increased $[\text{Ca}^{2+}]_i$ to $123 \pm 3\%$ and $116 \pm 5\%$ (Figure 4A). However, only at 260 μM

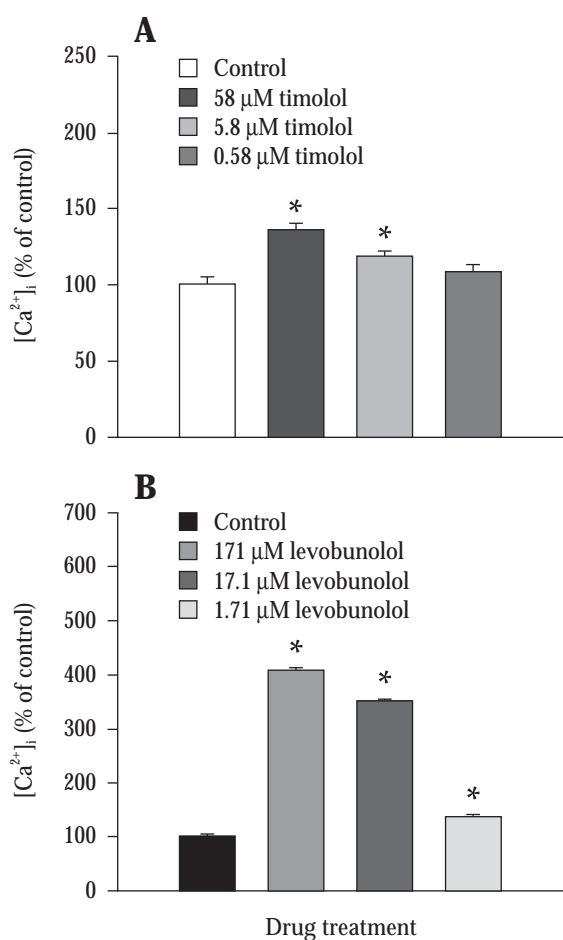


Figure 1. Dose-dependent effects of timolol (A) and levobunolol (B) on $[\text{Ca}^{2+}]_i$ mobility in cultured bovine corneal endothelial cells. The y-axis is the area under the curve (AUC) of $[\text{Ca}^{2+}]_i$ change in cells with baseline subtracted, where the AUC in the control group is assumed to be a 100% response. The x-axis presents various concentrations of timolol or levobunolol for 200 seconds. Data are presented as mean \pm SEM ($n = 6$). * $p < 0.05$ compared with the AUC of control cells.

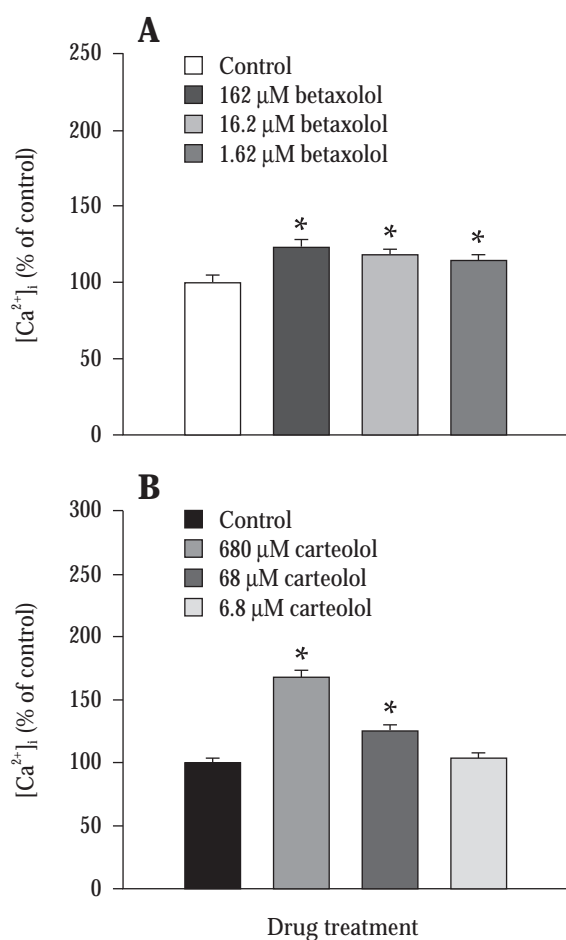


Figure 2. Dose-dependent effects of betaxolol (A) and carteolol (B) on $[\text{Ca}^{2+}]_i$ mobility in cultured bovine corneal endothelial cells. The y-axis is the AUC of $[\text{Ca}^{2+}]_i$ change in cells with baseline subtracted, where the AUC in the control group is assumed to be a 100% response. The x-axis presents various concentrations of betaxolol or carteolol for 200 seconds. Data are presented as mean \pm SEM ($n = 6$). * $p < 0.05$ compared with the AUC of control cells.

did brinzolamide increase the $[Ca^{2+}]_i$ concentration to 119% brinzolamide 5% (Figure 4B). In the case of latanoprost, only the 1.1 μM concentration induced a $[Ca^{2+}]_i$ increase to $123 \pm 5\%$ (Figure 5A). However, unoprostone concentrations of 28.2 μM , 2.82 μM , and 0.282 μM all significantly induced $[Ca^{2+}]_i$ increases to $214 \pm 5\%$, $159 \pm 3\%$, and $123 \pm 4\%$ (Figure 5B). In the presence of the parasympathomimetic drug pilocarpine for 200 seconds, the $[Ca^{2+}]_i$ was induced by 408 μM and 40.8 μM concentrations to $135 \pm 3\%$ and $115 \pm 4\%$ (Figure 6A). In the case of the preservative benzalkonium chloride, the $[Ca^{2+}]_i$ area was not affected after addition of drugs with 0.001, 0.000,1, and 0.000,01 mg/mL for 200 seconds (Figure 6B).

DISCUSSION

Our data show that most ocular hypotensive agents, including betaxolol, timolol, levobunolol, carteolol, dipivefrin, dorzolamide, brinzolamide, latanoprost, unoprostone, and pilocarpine, have potential to increase the basal cellular $[Ca^{2+}]_i$, whereas brimonidine inhibits the $[Ca^{2+}]_i$ concentration in cultured BCE cells.

It is known that calcium plays an important role in the regulation of physiologic functions and pharmacologic responses such as cellular pump function [6] and cellular migration [11]. Responses mediated by endothelin-1 [9], ATP [12], and thrombin [13] all involve the increase of $[Ca^{2+}]_i$.

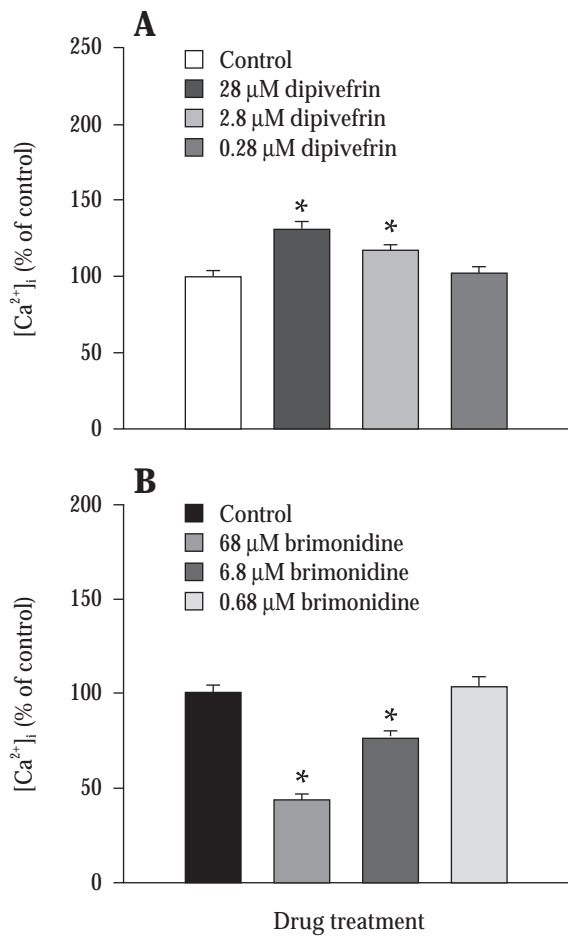


Figure 3. Dose-dependent effects of dipivefrin (A) and brimonidine (B) on $[Ca^{2+}]_i$ mobility in cultured bovine corneal endothelial cells. The y-axis is the AUC of $[Ca^{2+}]_i$ change in cells with baseline subtracted, where the AUC in the control group is assumed to be a 100% response. The x-axis presents various concentrations of dipivefrin or brimonidine for 200 seconds. Data are presented as mean \pm SEM (n = 6). *p < 0.05 compared with the AUC of control cells.

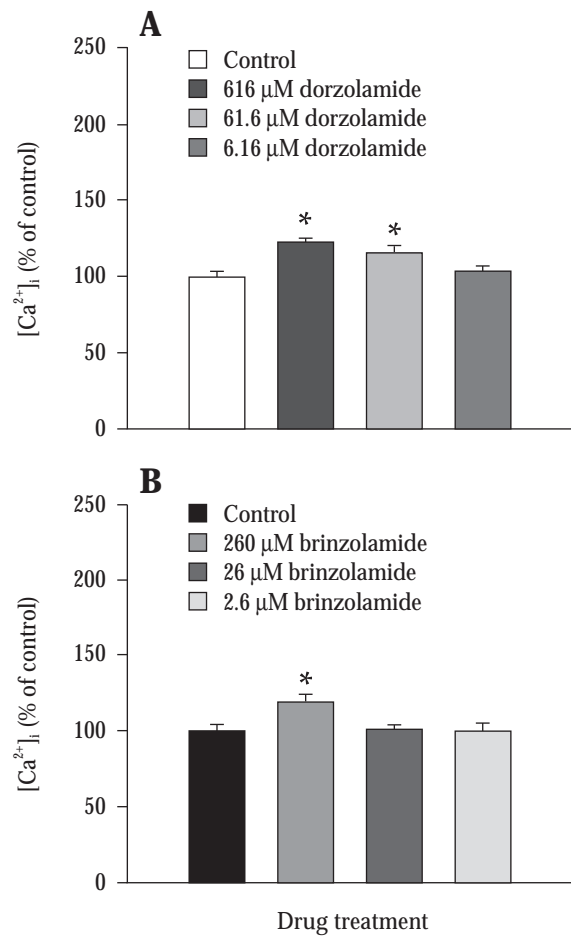


Figure 4. Dose-dependent effects of dorzolamide (A) and brinzolamide (B) on $[Ca^{2+}]_i$ mobility in cultured bovine corneal endothelial cells. The y-axis is the AUC of $[Ca^{2+}]_i$ change in cells with baseline subtracted, where the AUC in the control group is assumed to be a 100% response. The x-axis presents various concentrations of dorzolamide or brinzolamide for 200 seconds. Data are presented as mean \pm SEM (n = 6). *p < 0.05 compared with the AUC of control cells.

mobility in corneal endothelial cells. Various calcium channel antagonists have also been found to inhibit intracellular Ca^{2+} mobilization of the corneal endothelial cells and cause corneal swelling [7]. According to our previously published data, the $[Ca^{2+}]_i$ in cultured porcine corneal endothelial cells was inhibited by isoproterenol and norepinephrine, yet enhanced by propranolol [8]. In the present study, we found that many β -adrenergic antagonists, including betaxolol, timolol, levobunolol, and carteolol, all increased $[Ca^{2+}]_i$ significantly with 1/100 and 1/1,000 concentrations. Thus, the $[Ca^{2+}]_i$ mobility in corneal endothelial cells may be increased by β -adrenergic antagonists and suppressed by agonists.

In human corneas, endothelial wound healing is predominantly by stimulation of cell migration. The decrease of $[Ca^{2+}]_i$ in endothelial cells may reduce the contraction of actin filaments after wound healing migration [14]. Based upon the response of calcium channel blockers in the cornea, it has been demonstrated that calcium channels are presented in corneal endothelial cells. Verapamil, a phenylalkylamine calcium channel antagonist, blocks the L-type calcium channel to a far greater extent than the T-type calcium channel, increasing the swelling rate of the cornea. In contrast, nifedipine and diltiazem, which also inhibit L-type channel activity in other tissues [15,16], exerted no effect on the corneal swelling rate [7]. This

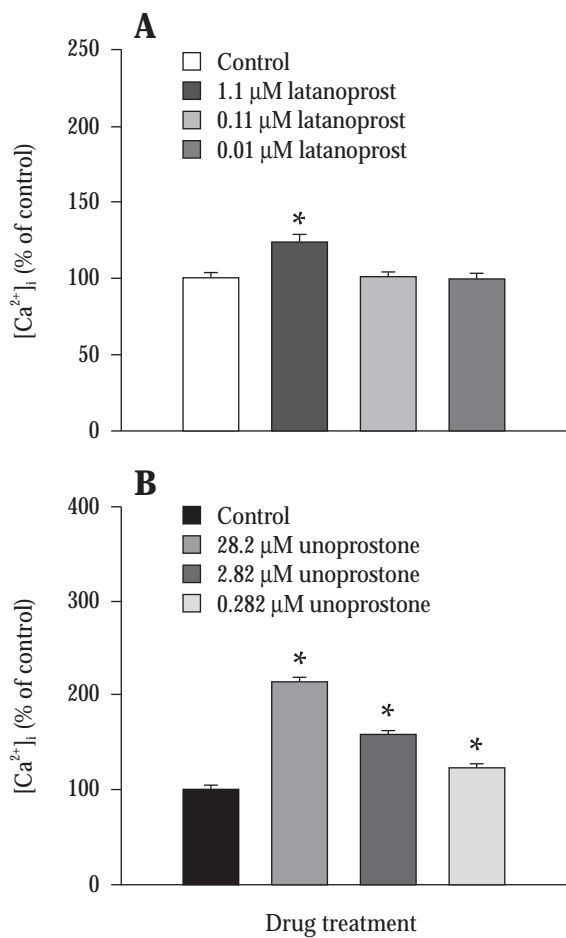


Figure 5. Dose-dependent effects of latanoprost (A) and unoprostone (B) on $[Ca^{2+}]_i$ mobility in cultured bovine corneal endothelial cells. The y-axis is the AUC of $[Ca^{2+}]_i$ change in cells with baseline subtracted, where the AUC in the control group is assumed to be a 100% response. The x-axis presents various concentrations of latanoprost or unoprostone for 200 seconds. Data are presented as mean \pm SEM (n = 6). *p < 0.05 compared with the AUC of control cells.

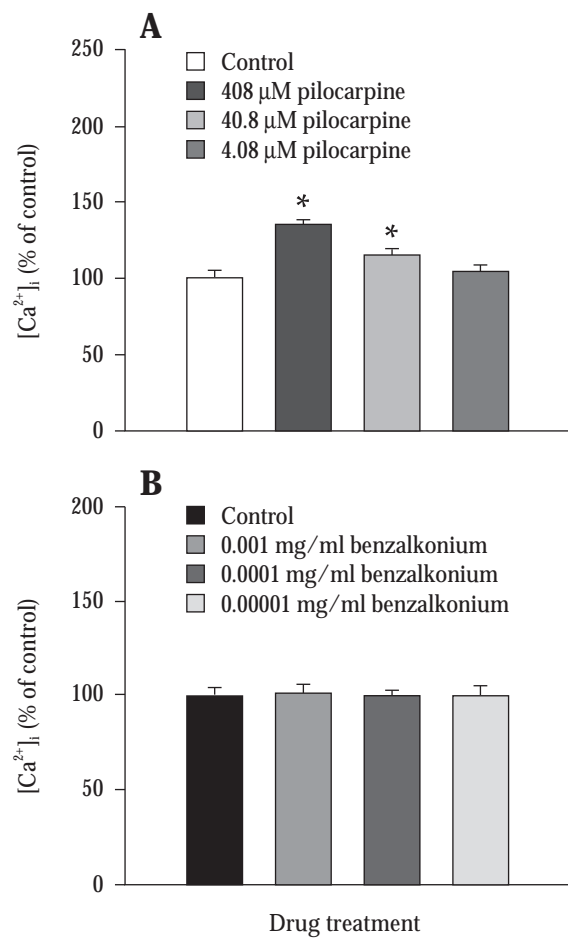


Figure 6. Dose-dependent effects of pilocarpine (A) and benzalkonium chloride (B) on $[Ca^{2+}]_i$ mobility in cultured bovine corneal endothelial cells. The y-axis is the AUC of $[Ca^{2+}]_i$ change in cells with baseline subtracted, where the AUC in the control group is assumed to be a 100% response. The x-axis presents various concentrations of pilocarpine or benzalkonium chloride for 200 seconds. Data are presented as mean \pm SEM (n = 6). *p < 0.05 compared with the AUC of control cells.

indicates that corneal endothelial calcium channels exhibit a different sensitivity to different L-type calcium channel blockers. The intracellular calcium blocker of TMB-8, which blocks calcium release from intracellular stores including ER and interruption of calcium metabolism [17], also causes an increase in corneal swelling rate [7]. Thus, any agent that inhibits $[Ca^{2+}]_i$ in corneal endothelium may alter the corneal physiologic function (e.g. decreasing endothelial pumping activity, then increasing the corneal swelling rate).

Brimonidine is an $\alpha 2$ -adrenergic agent currently used to lower intraocular pressure in glaucomatous patients [18]. One report showed that the $\alpha 2$ -adrenoceptor agonist decreased the intracellular Cl^- concentration in cultured rabbit corneal endothelium [19]. It is accepted that the maintenance of stromal hydration by the corneal endothelium relies on active transendothelial anion transporters such as HCO_3^- and Cl^- with the anions carrying the current [20]. Although the mechanism for brimonidine-induced $[Ca^{2+}]_i$ decrease in corneal endothelial cells is still unknown, this decrease may be involved in the regulation of pump function in corneal endothelial cells.

Dipivefrin is a dipivalyl-epinephrine drug. It is hydrolyzed to the active drug, epinephrine, by esterases in the cornea [21]. It is difficult to interpret the mechanism of dipivefrin-induced $[Ca^{2+}]_i$ increase in corneal endothelial cells, because dipivefrin may affect the physiologic response in corneal endothelial cells by activation of adrenergic β and/or α receptors.

In the case of dorzolamide, brinzolamide, latanoprost, unoprostone, and pilocarpine, however, few studies have been reported on these drugs with respect to the changes and regulative mechanisms of $[Ca^{2+}]_i$ mobility in corneal endothelial cells.

In summary, $[Ca^{2+}]_i$ mobility in corneal endothelial cells was significantly changed by all commercial antiglaucoma drugs; this change may affect the physiologic function in the cornea. Thus, long-term use of antiglaucoma drugs in patients must be carefully monitored with respect to the corneal endothelial changes.

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抗青光眼藥對角膜內皮細胞 內鈣離子流動性的影響

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本實驗主要是探討不同的抗青光眼藥，包括 betaxolol，timolol，levobunolol，brimonidine，carteolol，dipivefrin，dorzolamide，brinzolamide，latanoprost，unoprostone，pilocarpine 對牛角膜內皮細胞內鈣離子流動性的影響。不同的青光眼藥由原液稀釋成 1/100，1/1,000 及 1/10,000 三種濃度。細胞經 fura-2-AM 吸收後以螢光光度計測量細胞內鈣離子濃度的變化。結果發現 timolol (58 μM 、5.8 μM)，levobunolol (171 μM 、17.1 μM 及 1.71 μM)，betaxolol (162 μM 、16.2 μM 及 1.62 μM)，carteolol (680 μM 及 68 μM)，dipivefrin (28 μM 及 2.8 μM)，dorzolamide (616 μM 及 61.6 μM)，brinzolamide (260 μM)，latanoprost (1.1 μM)，unoprostone (28.2 μM 、2.82 μM 及 0.282 μM) 以及 pilocarpine (408 μM 、40.8 μM) 都會明顯增加角膜內皮細胞內鈣離子的濃度，只有 brimonidine (68 μM 6.8 μM) 會明顯降低細胞內鈣離子的濃度，而 Benzalkonium chloride 防腐劑對細胞內鈣離子的流動性沒有影響。綜合以上的結果顯示所有的抗青光眼藥可能會改變角膜內皮細胞內鈣離子濃度而影響細胞的生理功能。

關鍵詞：抗青光眼藥，鈣離子流動性，角膜內皮細胞

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