

Fig. 4. Transmission electron micrographs comparing age-matched control (A, C) and diabetic (B, D) rabbit ciliary arteries. (A, B: $\times 1000$; C, D: $\times 2500$).

seen on the internal surface of the arteries, adhering to the endothelial cells (Fig. 6).

4. Discussion

4.1. Alloxan-induced DM

Dysfunction of vascular control is a common complication of diabetes mellitus. In the present study, an intravenous bolus injection of alloxan (100 mg/kg) induced hyperglycemia in 24 rabbits out of 26. Two rabbits died of hypoglycemia. The remaining rabbit showed normoglycemia, indicating variability in the susceptibility of rabbits to alloxan. Alloxan injections are known to induce pancreatic damage, the changes consisting mainly of selective necrosis and disappearance of the beta cells with consequent destruction of the architecture of the islets (Lazarow and Palay, 1946; Lukens, 1948). Diabetes mellitus was induced by this mechanism. Body weight loss was reported in many cases in alloxan-induced animal models, and our data of body weight changes supported these previous studies (Abiru et al., 1990; Tesfamariam and Cohen, 1995; Masuda et al., 1999). Humans are similar in that most type 1 diabetic patients are ill and symptomatic, most commonly presenting with polyuria, polydipsia, polyphagia, and weight loss (Sherwin, 2004).

4.2. Functional findings

In the present study, there was no significant difference in the average maximum tension developed by K-Krebs solution, between the control and diabetic rabbits. Moreover, PE caused dose-dependent contraction, the EC_{50} values and maximum

contraction induced by PE were not significantly different between control and diabetic rabbit. The lack of any effect of diabetes on PE-induced contraction suggests that physiological antagonism of this response by endothelium-derived NO is not observed in rabbit ciliary arteries in a static system, but only in a perfused system. Similar observations have been made in bovine ciliary arteries (Buckley et al., 1998).

Sullivan and Sparks (1979) found decreased vascular response to norepinephrine in isolated diabetic rabbit aortas, while Christlieb (1974) found normal vascular sensitivity in diabetic rats. Moreover, the responses to endogenous noradrenalin released from adrenergic nerves by electrical stimulation did not differ between arteries from normal and diabetic

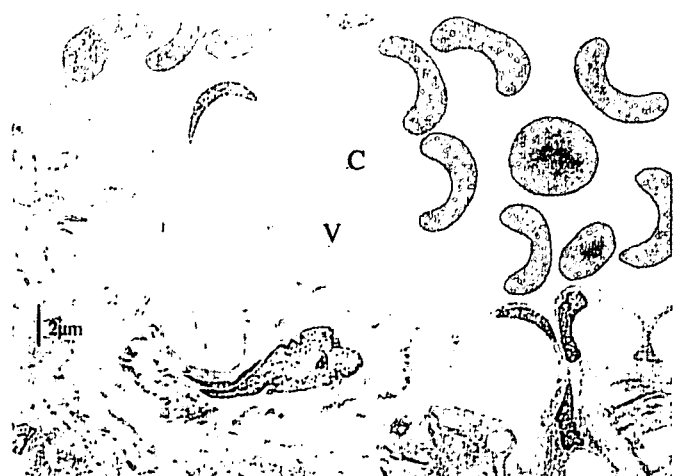


Fig. 5. Transmission electron micrographs ($\times 3000$). Loss of endothelial cells (C) in contact with vacuolar degeneration (V) in diabetic rabbit ciliary artery.

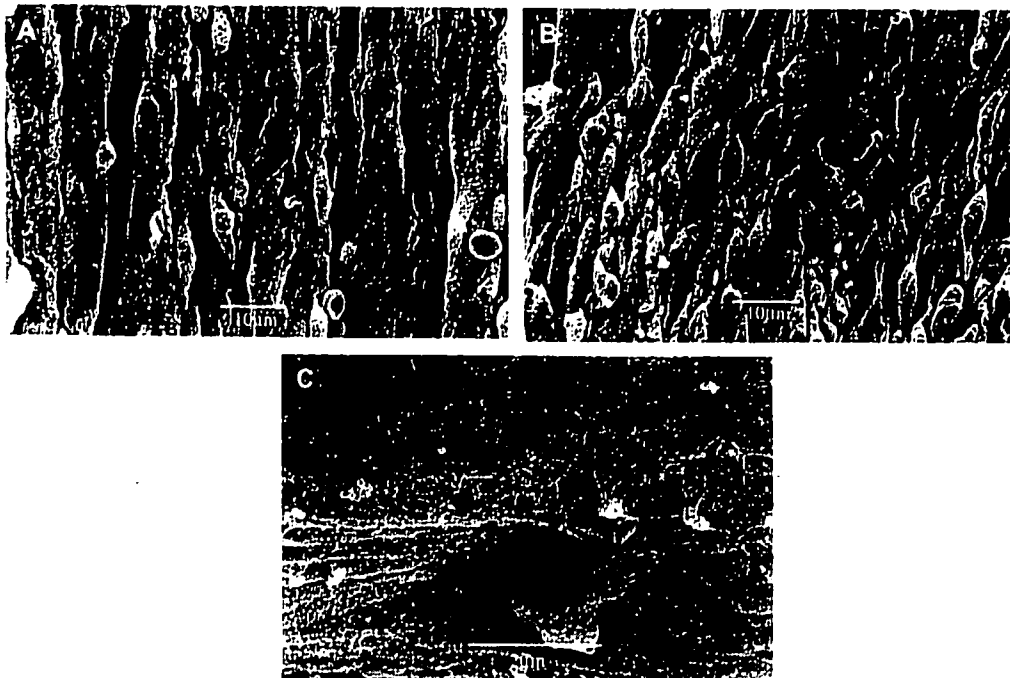


Fig. 6. Scanning electron micrographs ($\times 1500$) comparing age-matched control (A) and diabetic (B) rabbit ciliary arteries. (C) Apparently a fibrin-like network adheres to endothelial cells in diabetic rabbit ciliary artery.

rabbit carotid arteries (Tesfamariam and Cohen, 1995). However, it was reported that a contractile response to KCl was diminished in the diabetic rat aortas (Sullivan and Sparks, 1979; Pfaffman et al., 1980; Turlapaty et al., 1980). These differences may be due to differences in experimental conditions such as the preparation of diabetic animals, the duration and severity of the diabetic condition, the animal species and the method of blood vessel preparation as well as the particular artery tested.

Carbachol could relax the smooth muscle of the rabbit ciliary artery. Our previous study demonstrated that rabbit ciliary artery is innervated by at least two different types of nerves, which evoke contraction and cause an endothelium-independent release of NO. Moreover, pilocarpine and carbachol relaxation of this muscle was dependent on the endothelium and NO synthesis (Yoshitomi et al., 2000). In the present study, denudation of endothelium and pretreatment of L-NAME inhibited the carbachol-induced relaxations. However, indomethacin had no effect on carbachol-induced relaxation, indicating endogenous prostaglandins had little effect on this relaxation. It is well known that the endothelium synthesizes and releases vasodilator substances, including vasodilator prostaglandins, NO, and EDHF.

In the present study, potassium was used for the precontraction for the experimental stability; however, it is suspected that precontraction with potassium will block any EDHF-mediated relaxation (McNeish et al., 2001). It has been demonstrated that vasodilatation to acetylcholine or bradykinin was virtually abolished following treatment with a high concentration of KCl in the bovine isolated perfused eye. Blockade by high concentrations of KCl strongly suggests that vasodilatation in this tissue was mediated by an EDHF (McNeish et al.,

2001). Moreover, in the porcine ciliary artery endothelium-dependent relaxations to bradykinin are primarily mediated by NO and can be partially inhibited by K^+ -channel blocker (Zhu et al., 1997).

McNeish et al. (2002) also have shown that EDHF-mediated vasodilatation in the bovine perfused ciliary circulation induced by bradykinin or acetylcholine (ACh) is sensitive to inhibition by ascorbate. However, ascorbate failed to affect bradykinin- or acetylcholine-induced, EDHF-mediated vasodilatation in rings of bovine ciliary and coronary artery (McNeish et al., 2003; Nelli et al., 2004). Although the nature of EDHF has not been fully elucidated, EDHF assumes a prominent role in endothelium-dependent vasodilatation in many smaller arteries (Shimokawa et al., 1996; Busse et al., 2002; Fitzgerald et al., 2005). Further studies are required to elucidate the physiological and pathological roles of EDHF.

In the diabetic rabbit kidney, a vasodilator response to (ACh) and bradykinin was decreased in comparison with the controls (Forti and Fonteles, 1998). Also, ACh-induced relaxation was significantly attenuated in the aortic specimens of diabetic rabbits with basal levels of cyclic GMP and with ACh-induced decrease of cyclic GMP (Abiru et al., 1990). It has been shown in diabetic rats that the relaxation response of blood vessels to ACh is inconsistent: both decreases (Oyama et al., 1986; Durante et al., 1988) and increases (White and Carrier, 1986) in endothelium-dependent relaxation have been obtained.

4.3. Interaction of morphological and functional findings

The cross-section of the diabetic rabbit ciliary artery, and the invaginations of the internal elastic lamina clearly

decreased. Moreover, the cytoplasm of endothelial cells contained large vacuoles, indicating weak adhesion to the lamina, and some endothelial cells even showed vacuolar degeneration due to breakdown of their cell membranes. However, the smooth muscle cells were well preserved. Scanning electron microscopic observations showed that the internal surface of the artery in the control group consisted of endothelial cells that had a constant width, while that of the hyperglycemia comprised irregularly arranged cells of varying sizes. Therefore, major changes existed in the intima of the diabetic rabbit ciliary artery. This is in good agreement with our pharmacological functional result that the contraction induced by KCl and PE were preserved; however, carbachol-induced relaxation was reduced in the diabetic rabbit ciliary artery.

Ocular circulation is mainly influenced by hypercholesterolemia, diabetes mellitus, intraocular pressure, blood pressure, and so on. Our previous data demonstrated that the vasoconstricting function was reduced but that vasodilatation was well preserved in the hypercholesterolemic rabbit ciliary artery (Ishikawa et al., 2004). The morphological findings of hypercholesterolemic rabbit ciliary artery revealed a deformation of the shape of the medial smooth muscle cells with irregularity in size and widening of the intercellular spaces. However, the endothelial cells were well preserved (Ishikawa et al., 2004). These morphological findings supported pharmacological functional results in hypercholesterolemic rabbit ciliary artery. These results indicated that the mechanism of dysfunction of the vascular control in hyperglycemia and hypercholesterolemia may be different.

5. Conclusions

The relaxation induced by carbachol in the ciliary artery of rabbits with alloxan-induced diabetes was attenuated, although the contractile activity was preserved. These functional results correspond with morphological findings that the endothelial cells were mainly damaged. Both functional and morphological examinations were needed to investigate the ocular circulation disorder. Alloxan may be one of the agents that induce diabetes mellitus and the alloxan-induced diabetic rabbit appeared to make it a useful model for investigating the ocular complications induced by diabetes. In humans, the higher the blood glucose level, and also the longer the duration of the illness, the more easily side effects appear, but in future, in this research it is considered necessary to carry out histological and pharmacological study for longer periods.

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LABORATORY INVESTIGATION

Effect and Mechanism of Betaxolol and Timolol on Vascular Relaxation in Isolated Rabbit Ciliary Artery

Yaru Dong^{1,3}, Hitoshi Ishikawa², Yazhen Wu³, Kimiya Shimizu¹,
Toshiaki Goseki¹, and Takeshi Yoshitomi⁴

¹Department of Ophthalmology, School of Medicine, Kitasato University, Kanagawa, Japan;

²Department of Orthoptics and Visual Science, School of Allied Health, Kitasato University, Kanagawa, Japan; ³Ophthalmology Department, the Second Clinical Hospital of Jilin University, Jilin, China; ⁴Department of Ophthalmology, Akita University, Akita, Japan

Abstract

Purpose: In order to clarify the vasodilatory mechanism of betaxolol and timolol, we studied the effects of these drugs in isolated rabbit ciliary arteries.

Methods: Rabbit ciliary artery specimens were mounted in a double myograph system, and betaxolol, timolol, or another agent was introduced into the organ chamber. The mechanical response of the arteries was studied using an isometric tension recording method. The intracellular free calcium concentration $[Ca^{2+}]_i$ was also measured using fluorescence photometry.

Results: Betaxolol and timolol induced dose-dependent relaxation in the rabbit ciliary arteries precontracted by high- K^+ Krebs solution. The minimum concentrations required to cause relaxation were $10\mu M$ of betaxolol, and $30\mu M$ of timolol. At the maximum concentration of 1 mM, betaxolol induced almost complete relaxation of the ciliary arteries, whereas timolol induced approximately 70% relaxation. These actions were not inhibited by pretreatment with $100\mu M$ N^G -nitro-L-arginine methylester (L-NAME), a nitric oxide synthase inhibitor, or by denudation of the vascular endothelium. However, $300\mu M$ of betaxolol or timolol decreased the $[Ca^{2+}]_i$ of the vascular smooth muscle, an action similar to that of diltiazem, a typical L-type voltage calcium-channel blocker.

Conclusions: Betaxolol, a selective β_1 -adrenoceptor antagonist, and timolol, a nonselective β -adrenoceptor antagonist, both frequently used in the medical management of glaucoma, decrease $[Ca^{2+}]_i$ by acting as Ca^{2+} channel blockers, thus causing relaxation of isolated rabbit ciliary artery. **Jpn J Ophthalmol** 2006;50:504-508 © Japanese Ophthalmological Society 2006

Key Words: betaxolol, Ca^{2+} blocking, fura-2, rabbit ciliary artery, timolol

Introduction

Betaxolol, a selective β_1 -adrenergic receptor antagonist, and timolol, a nonselective β -adrenergic antagonist, are widely used as clinical antiglaucomatous agents. β -adrenergic antagonists exert their intraocular pressure

(IOP)-lowering effects by suppressing aqueous humor production in the ciliary epithelium. Although it is generally accepted that increased intraocular pressure is a major risk factor in glaucoma, ocular vascular perfusion is also important in the pathogenesis of optic nerve damage and visual field loss, especially in patients with normal-tension glaucoma.¹⁻³ It has been reported that betaxolol also induced relaxation in isolated rat aorta, in an action resembling that of calcium antagonists.⁴ Patch-clamp methods have confirmed that the relaxing action of betaxolol is due to inhibition of voltage-dependent calcium channels in the vascular smooth muscle cells of guinea pig mesenteric artery.⁵

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Correspondence and reprint requests to: Hitoshi Ishikawa, Department of Orthoptics and Visual Science, School of Allied Health, Kitasato University, 1-15-1 Kitasato, Sagami-hara, Kanagawa 228-8555, Japan
e-mail: hitoshi@kitasato-u.ac.jp

We have previously reported that betaxolol and timolol could directly relax rabbit ciliary artery *in vitro* at relatively high concentrations, and the relaxing action with betaxolol was more potent than that with timolol at equal concentrations.⁶ Although the pattern of relaxation induced by the β_1 -adrenergic antagonists was similar to that of calcium-channel blockers, the exact mechanism of this relaxation was not clear. Recently, fluorescent dyes have become available for measuring the change in cytosolic Ca^{2+} concentrations in various biological preparations.⁷ Therefore, to investigate the mechanisms of relaxation induced by betaxolol and timolol, we have recorded calcium signals using the fluorescent dye, fura-2, in isolated rabbit ciliary artery.

Materials and Methods

General

All experiments were performed according to the guiding principles for animal experimentation of Kitasato University School of Medicine. Male albino rabbits weighing 2 to 3 kg were killed with an overdose of intravenous pentobarbital sodium (Abbott, North Chicago, IL, USA). The eyes were immediately enucleated, ensuring that a maximum length of optic nerve was removed, then placed in oxygenated Krebs solution of the following composition (mM): NaCl 94.8, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0, and glucose 11.7. The K-Krebs solution was prepared by replacing equimolar NaCl with KCl, isotonicity ($K^+ = 100.7$ mM).

Functional Experiments

The ciliary artery, with connective tissue, was carefully dissected free from the optic nerve. Vascular ring segments (diameter, 150–300 μ m; length, 1–2 mm) were cut from the distal section of the ciliary artery and mounted in the double myograph system (JP Trading, Copenhagen, Denmark) under microscopic observation.⁸ The myograph system allowed direct determination of vessel isometric tension while the internal circumference was controlled. The vessels were equilibrated for 30 min in oxygenated Krebs solution with 5% CO_2 and 95% O_2 , maintained at 37°C, and then stretched to their optimal lumen diameter for active tension development, I_0 , corresponding to 0.9 I_{100} , where I_{100} is the diameter.⁹ After the mounting and equilibration period, the amplitudes of three successive contractions evoked by K-Krebs solution were measured at 90-min intervals to establish the viability and stability of the preparation. During the K-Krebs-induced contractions, it was confirmed that 1 μ M carbachol induced relaxation in all preparations, indicating that the preparations had intact endothelium. In certain studies, the endothelium was removed by gentle rubbing; the absence of endothelium was confirmed by the lack of relaxation with carbachol (1 μ M) during contractions with K-Krebs solution.

Spectrofluorometry

Segments of the ciliary artery specimens not used for functional studies were employed (length, 6 mm; width, 1.5 mm). The vascular tissue was then incubated for 3 h at room temperature in a dark room in a mixed solution containing 5 μ M fura-2-AM (Dojindo, Kumamoto, Japan) and 250 ppm Pluronic F-127 (Sigma, St. Louis, MO, USA) with 5% CO_2 and 95% O_2 . The muscle strip was then kept in an organ bath (7 ml). Fura-2 fluorescence measurements were performed with a fluorimeter (CAF-110, Japan Spectroscopic, Tokyo, Japan). Fluorescence was monitored by recording excitation signals at wavelengths of 340 nm and 380 nm at room temperature in a dark room. The fluorescence ratio of 340:380 nm was used to indicate changes in the intracellular free calcium concentration $[Ca^{2+}]_i$. The details of the method for determining $[Ca^{2+}]_i$ have been reported by Ozaki et al.⁷ and Sato et al.¹⁰

Data Analysis

Statistical differences between points were determined by the unpaired two-tailed Student *t* test, and those between the concentration-response curves, by two-way analysis of variance (ANOVA). $P < 0.05$ was considered significant.

Chemicals

The following drugs were used: carbachol hydrochloride, timolol maleate (Sigma), betaxolol hydrochloride (Alcon, Ft. Worth, TX, USA), N^G -nitro-L-arginine methylester, and diltiazem (Wako Chemical, Osaka, Japan).

Results

Contractile Activity

When specimens of the ciliary artery were mounted in the organ bath, the tissue gradually relaxed to a steady tone after 0.5 to 1 h of superfusion with Krebs solution. No spontaneous contractions occurred during the experiment. Figures 1 and 2 show the dose-dependent relaxing trace of betaxolol and timolol on isolated ciliary artery precontracted by K-Krebs solution. Each drug was introduced into the organ chamber in a cumulative manner. The minimum concentration of drug required to induce relaxation was 10 μ M of betaxolol, and 30 μ M of timolol. At its maximum concentration of 1 mM, betaxolol induced nearly complete relaxation of the ciliary arteries, while timolol induced approximately 70% relaxation. Thus, the relaxing potency of betaxolol was significantly stronger than that of timolol.

N^G -nitro-L-arginine methylester (L-NAME) is a nitric oxide synthase inhibitor. Incubation with 100 μ M L-NAME 30 min before vascular contraction abolished the carbachol (1 μ M)-induced relaxation (data not shown). Denudation of

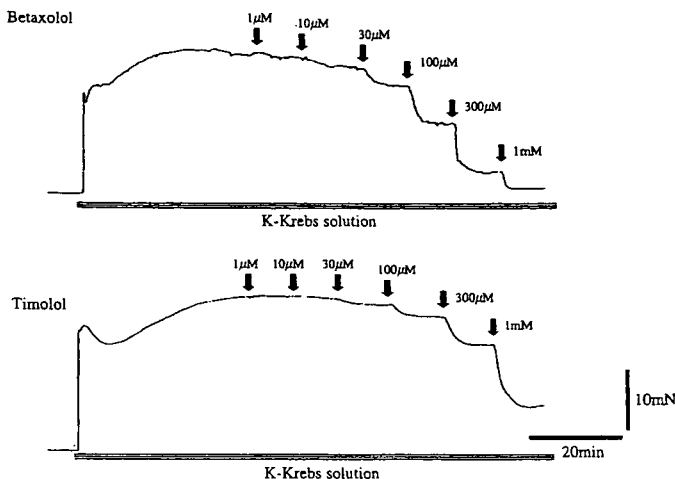


Figure 1. Effects of cumulatively applied betaxolol and timolol on rabbit ciliary artery precontracted with K-Krebs solution.

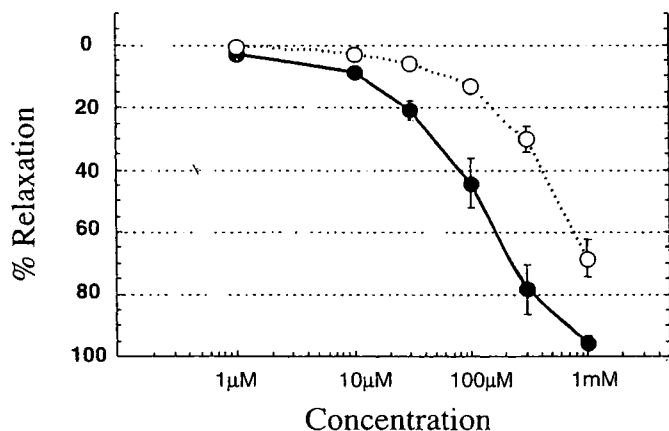


Figure 2. Dose-response curves for relaxation induced by betaxolol (●: *n* = 12) and timolol (○: *n* = 12). The amplitude of contraction evoked by K-Krebs solution was expressed as a percentage of the maximum amplitude for each drug. The bars indicate SE.

vascular endothelium also abolished 1 μM carbachol-induced relaxation (data not shown). However, neither pretreatment of 100 μM L-NAME nor denudation of the vascular endothelium affected the relaxing effect of betaxolol on preparations precontracted with K-Krebs solution (Fig. 3).

Intracellular Calcium Changes

In vascular smooth muscle, the ratio 340/380 nm increased, meaning that $[Ca^{2+}]_i$ was elevated by the perfusion of K-Krebs solution. When the curve of the ratio became stable, 10 μM diltiazem, a typical L-type voltage-dependent Ca^{2+} channel blocker, was applied. Diltiazem rapidly decreased the $[Ca^{2+}]_i$. After an ample washout period, 300 μM betaxo-

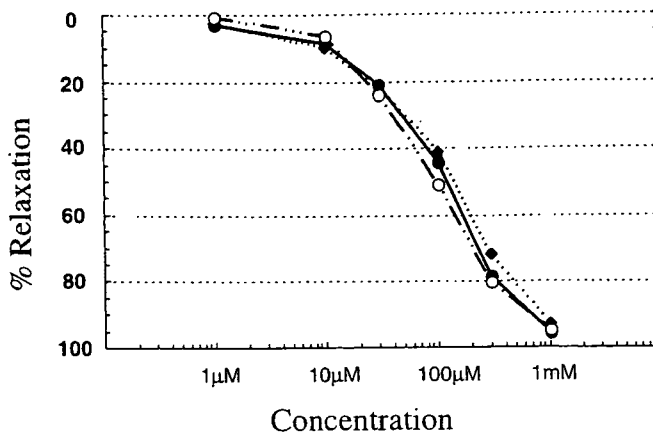


Figure 3. Dose-response curves for relaxation induced by betaxolol alone (●: *n* = 12). Incubation of 100 μM N^G -nitro-L-arginine methyl ester did not change betaxolol-induced dose-dependent relaxation (◆: *n* = 12). Denudation of vascular endothelium also did not significantly change betaxolol-induced relaxation on K-Krebs solution precontracted preparations (○: *n* = 12).

lol or timolol was administered when the elevated $[Ca^{2+}]_i$ curve became steady in response to the perfusion of K-Krebs solution. The addition of betaxolol or timolol decreased $[Ca^{2+}]_i$, as did the addition of diltiazem (Fig. 4).

Discussion

We have previously reported that some antiglaucoma agents cause relaxation in the rabbit ciliary artery.^{6,11-14} The rabbit ciliary artery is innervated by at least two types of nerves: adrenergic nerves, which evoke contraction, and nerve fibers that cause an endothelium-independent release of nitric oxide (NO). Pilocarpine relaxation of this muscle is dependent on the endothelium and NO synthesis.¹¹ In the present study, betaxolol and timolol caused relaxation in the isolated rabbit ciliary artery, which was precontracted with K-Krebs solution. The relaxation induced by betaxolol was not affected by L-NAME or by mechanical endothelium denudation. These results were very similar to those of our previous data, reported by Morimoto et al.⁶ However, the precise mechanism of the relaxation induced by betaxolol was not clear.

Recently, fura-2 has been used to measure the $[Ca^{2+}]_i$ in smooth muscle simultaneously with the tension development.^{7,10} In the fura-2-loaded smooth muscle strips, an increase in F340, a decrease in F380 (increase in R340/380) indicated an increase in $[Ca^{2+}]_i$. In order to improve our understanding of the mechanisms of betaxolol- and timolol-induced relaxation, we have employed this method to directly measure the $[Ca^{2+}]_i$. In the isolated rabbit ciliary artery, in the presence of a high concentration of K^+ , the fluorescence ratio R340/380 increased, and diltiazem (10 mM), betaxolol, and timolol (300 mM) decreased the ratio, which may indicate an increase and decrease of $[Ca^{2+}]_i$, respectively.

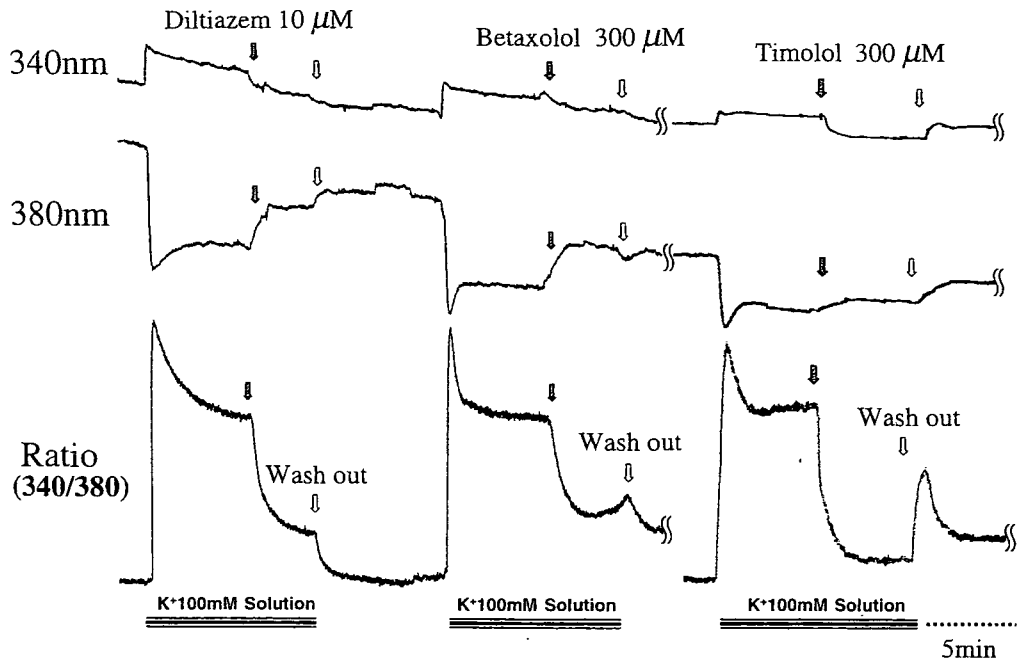


Figure 4. The fluorescence ratio R340/380 decreased after diltiazem (10 μM), betaxolol (300 μM), or timolol (300 μM) was added in the pretreatment of K-Krebs solution.

It has been reported that betaxolol also induces relaxation in isolated rat aorta; its action is similar to that of calcium antagonists.⁴ Patch-clamp methods have confirmed that the relaxing action of betaxolol is due to inhibition of voltage-dependent calcium channels in vascular smooth muscle cells of guinea pig mesenteric artery.⁵ It has been suggested that the neuroprotective actions of betaxolol occur as a result of the Ca²⁺ influx decrease that follows ischemia and injury-induced overactivation of glutamatergic receptors.¹⁵ Moreover, Melena et al.¹⁶ reported that betaxolol, carteolol and timolol significantly interacted with radioligand binding to L-type voltage-dependent Ca²⁺ channels, with betaxolol displaying the highest potency.

These results taken together suggest that betaxolol and timolol are capable of reducing K⁺-induced contraction in a dose-dependent manner, possibly by inhibiting voltage-gated Ca²⁺ entry into vascular smooth muscle. In this regard, betaxolol caused relaxation in the rabbit ciliary artery.

In summary, betaxolol and timolol, frequently used in the medical management of glaucoma, decrease intraocular pressure by acting at sites in the ciliary processes, and also by acting as Ca²⁺ channel blockers, thus reducing any excessive cellular influx of Ca²⁺.

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Vasodilatory mechanism of levobunolol on vascular smooth muscle cells

Yaru Dong^{a,c}, Hitoshi Ishikawa^b, Yazhen Wu^c, Takeshi Yoshitomi^{a,*}^a Department of Ophthalmology, Akita University School of Medicine, 1-1-1 Hondo, Akita, Akita 010-8543, Japan^b Department of Orthoptics and Visual Sciences, School of Allied Health, Kitasato University, Kanagawa 228-8555, Japan^c Department of Ophthalmology, The Second Hospital of Jilin University, Jilin 130042, P.R. China

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Abstract

Topical application of levobunolol hydrochloride, a β -adrenergic antagonist used for treatment of glaucoma, is reported to increase ocular blood flow. We studied the mechanism of levobunolol-induced vasodilation in arterial smooth muscle. The effects of levobunolol or other agents on isolated, pre-contracted rabbit ciliary artery were investigated using an isometric tension recording method. The effects of the same agents on intracellular free calcium ($[Ca^{2+}]_i$) in cultured human aortic smooth muscle cells were also studied by fluorophotometry. Levobunolol relaxed ciliary artery rings that were pre-contracted with either high-K solution, 1 μ M histamine, 10 μ M phenylephrine, or 100 nM endothelin-1. The relaxation induced by levobunolol was concentration-dependent over the range of 10 μ M to 0.3 mM. Inhibition of endothelial nitric oxide synthase or denudation of the endothelium did not affect this relaxation. Histamine-induced contractions were inhibited by the histamine H_1 antagonist pyrilamine. Radioligand binding experiments showed that levobunolol did not bind to the H_1 receptor. Further, histamine induced transient contraction in Ca^{2+} -free solution, and levobunolol inhibited this contraction by $74.6 \pm 11.0\%$. In cultured smooth muscle cells in the presence of extracellular Ca^{2+} , levobunolol significantly inhibited the histamine-induced elevation of $[Ca^{2+}]_i$. However, it did not inhibit the increase of $[Ca^{2+}]_i$ in histamine-stimulated cells incubated in Ca^{2+} -free solution. These results indicate that levobunolol may relax rabbit ciliary artery by two different mechanisms. First, the relaxation could be due to the blockade of Ca^{2+} entry through non-voltage-dependent Ca^{2+} channels. Second, levobunolol may change the Ca^{2+} sensitivity of vascular smooth muscle cells.

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Keywords: Ca^{2+} channel; Ca^{2+} sensitivity; fura-2; levobunolol; relaxation; vascular smooth muscle

1. Introduction

Though it is generally accepted that increased intraocular pressure (IOP) is a major risk factor in glaucoma, vascular perfusion is also considered to be important in the pathogenesis of optic nerve damage and visual field loss, especially in patients with normal tension glaucoma (Collignon-Brach, 1994; Drance, 1998; Flammer, 1994). Therefore, a better understanding of the effect of antiglaucoma agents on ocular

circulation is important for optimizing their clinical use. We have reported that timolol (Hayashi-Morimoto et al., 1999), betaxolol (Hayashi-Morimoto et al., 1999), pilocarpine (Yoshitomi et al., 2000), unoprostone isopropyl (Yoshitomi et al., 2004), latanoprost (Ishikawa et al., 2002) and nipradilol (Yoshitomi et al., 2002) induced relaxation of the isolated rabbit ciliary artery. Each of these drugs acts through different mechanisms. For example, betaxolol (Hayashi-Morimoto et al., 1999) and nipradilol (Yoshitomi et al., 2002), both β -adrenergic antagonists, relaxed rabbit ciliary artery. Betaxolol can inhibit voltage depended calcium (Ca^{2+}) channel in this tissue (Hayashi-Morimoto et al., 1999) which also were seen in other tissue such as guinea-pig mesenteric artery and portal

* Corresponding author. Tel.: +81 18 884 6164; fax: +81 18 836 2621.

E-mail address: yoshitom@med.akita-u.ac.jp (T. Yoshitomi).

115 vein smooth muscle cells (Setoguchi et al., 1995). On the other
 116 hand, nipradilol relaxed rabbit ciliary artery by inducing nitric
 117 oxide (NO) production and blocking α -adrenergic receptor
 118 (Yoshitomi et al., 2002). We have reported that isoproterenol,
 119 a β -adrenergic agonist, has no effect on mechanical properties
 120 of rabbit ciliary artery (Hayashi-Morimoto et al., 1999). So,
 121 vasodilatory action of these β -adrenergic antagonists seems
 122 to be unrelated to the β -adrenergic receptor and have different
 123 mechanism in each agent. Various antiglaucoma agents includ-
 124 ing β -adrenergic antagonists also increase ocular blood flow in
 125 vivo (Arend et al., 1998; Georgopoulos et al., 2002; Grunwald,
 126 1990; Kimura et al., 2005; Shaikh and Mars, 2001), but this
 127 effect seem not to be generated by decreasing IOP. Chiou
 128 et al. (1990) have reported that D-timolol which is less potent
 129 than L-timolol to lower IOP, is more potent than L-timolol to
 130 improve blood flow in retina and choroid of rabbit. There
 131 are also other reports that betaxolol may increase significantly
 132 ciliary body blood flow, choroidal blood flow, and retinal
 133 blood flow in rabbits' eyes in vivo (Sato et al., 2001). Levobu-
 134 nolol, a non-selective β -adrenergic antagonist, is widely used
 135 in antiglaucoma treatment. A number of clinical trials have
 136 suggested that levobunolol also can improve ocular circulation
 137 (Arend et al., 1998; Boses et al., 1992; Morsman et al., 1995;
 138 Ogasawara et al., 1999). Morsman et al. (1995) reported that
 139 pulsatile ocular blood flow increased by 22% after 1 week's
 140 administration of levobunolol. Arend et al. (1998) indicated
 141 that levobunolol produced a decrease in arteriovenous passage
 142 time of approximately 25%, and it increased macular capillary
 143 blood velocity by approximately 20%. Concerning the vasodi-
 144 latory mechanism of levobunolol, Wu et al. (2004), using
 145 a spectrofluorometry method, indicated that levobunolol in-
 146 hibits the intracellular Ca^{2+} increases by blocking the L-type
 147 voltage-dependent Ca^{2+} channel. However, the underlying
 148 mechanisms of its effects on ocular circulation are not yet
 149 clear.

150 In an attempt to clarify the vasodilatory mechanism of
 151 levobunolol, we have investigated the effect of this drug using
 152 an isometric tension recording method on isolated rabbit cili-
 153 ary artery. Additionally, we used fluorescence photometry to
 154 measure the effect of levobunolol on intracellular Ca^{2+} levels
 155 in cultured human aortic artery smooth muscle cells.

157 2. Materials and methods

159 2.1. Isometric tension recording method in isolated 160 ciliary arteries

161
 162 All animals were treated in accordance with the ARVO
 163 Statement for the Use of Animals in Ophthalmic and Vision
 164 Research and in approval by Animal Experiment Committee
 165 of Akita University. Male albino rabbits weighing 2–3 kg
 166 were killed with an overdose of intravenous pentobarbital so-
 167 dium (Abbott, North Chicago, IL, USA). The eyes were imme-
 168 diately enucleated, ensuring that a maximum length of optic
 169 nerve was removed, and then placed in oxygenated Krebs so-
 170 lution of the following composition (mM): NaCl 94.8, KCl
 171 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$

25.0, glucose 11.7 and aerated with 95% O_2 and 5% CO_2 .
 With the aid of a dissecting microscope, the ciliary artery
 and surrounding connective tissue were carefully isolated
 from the optic nerve. Vascular ring segments (150–300 μm
 in diameter, 1–2 mm in length) were cut from the distal
 section of the ciliary artery and mounted in a chamber of
 the double Myograph System[®] (JP Trading, Denmark) with
 Krebs solution. After a process of the temperature rose to
 37 °C in the chamber, the vessels were stretched to each opti-
 mal lumen diameter for active tension development, i.e. nor-
 malization. Then the vessels were equilibrated for 30 min.
 The Myograph System[®] allowed direct determination of ves-
 sel isometric tension while the internal circumference was
 controlled. Detailed methods for isometric tension recordings
 by the Myograph System[®] have been described by Mulvany
 and Halpern (1976, 1977).

188 After the equilibration period, the amplitudes of the con-
 189 tractions were evoked by the high-K solution and were mea-
 190 sured at 20-min intervals to establish preparation viability
 191 and stability. High-K solutions were prepared by replacing
 192 NaCl with isotonic, equimolar KCl to give a final K^+ con-
 193 centration of 100.7 mM. Cholinergic agonists, acting on re-
 194 ceptors in the endothelium, were applied at 20-min
 195 intervals of the contraction and induce relaxation of vascular
 196 smooth muscle (Keef and Bowen, 1989). Thus, we tested the
 197 susceptibility of contracted ciliary arteries to cholinergic re-
 198 laxation with 1 μM carbachol. All preparations entirely un-
 199 derwent the contraction and relaxation described above,
 200 before the separate experiments. In certain experiments,
 201 the vascular rings in baseline tone were incubated with in-
 202 hibitor 30 min before the separate experiment. In other ex-
 203 periments, we gently denuded the endothelium by rubbing
 204 the inside of the vascular ring with a scalp hair before the
 205 normalization.

206 Additionally, the ability of levobunolol to relax ciliary ar-
 207 tery rings pre-contracted with other agents, including hista-
 208 mine, phenylephrine, and endothelin-1, and in Ca^{2+} -free
 209 media was also determined. Generally, the pre-contraction
 210 was maintained for 20 min, then levobunolol was applied ev-
 211 ery 10 min in a cumulative manner. The Ca^{2+} -free solutions
 212 were prepared by replacing $CaCl_2$ with isotonic, equimolar
 213 $MgCl_2$ and adding 1 mM EGTA, a chelating agent for Ca^{2+}
 214 in the presence of magnesium. The ability of the H_1 histamine
 215 antagonist pyrilamine, the H_2 histamine antagonist cimetidine,
 216 and the L-type voltage-dependent Ca^{2+} channel blocker diltiaz-
 217 em to induce relaxation of contracted ciliary artery rings was
 218 also tested.

220 2.2. Smooth muscle cell cultures

221
 222 Human aortic smooth muscle cells at passage 3 were pur-
 223 chased from Cambrex (Walkersville, MD, USA). The cells
 224 were grown in the culture medium (SmGM-2, Sanko Junyaku
 225 Co. Ltd., Tokyo, Japan) in 5% CO_2 -containing air at 37 °C.
 226 The culture medium was exchanged every 48 h until a subcon-
 227 fluent growth stage was obtained. The cells were detached by
 228 exposure for approximately 3 min to 0.025% trypsin in

a Ca²⁺- and Mg²⁺-free solution containing 0.01% EDTA. The detached cells were diluted in the culture medium, and then reseeded with a cell density of approximately 2500 cells/cm² on coverslips (9 × 9 mm) coated with fibronectin (Biomedical technologies, Stoughton, MA, USA). The cells were maintained in culture for 1–4 days before use.

2.3. Intracellular Ca²⁺ determination by fluorescence photometry

To determine intracellular Ca²⁺ levels, cultured smooth muscle cells were loaded with 10 μM fura-2 acetoxymethyl ester at 37 °C for 30 min in HEPES-buffered saline (HBS). HBS containing 5 mM CaCl₂ instead of 1 mM CaCl₂ was used as a standard bath solution. Thereafter, cells were rinsed several times with HBS to remove extracellular fura-2 and used for experiments within 3 h. Changes of intracellular free calcium concentration [Ca²⁺]_i in individual cells were measured using an Aquacosmos System (Hamamatsu Photonics kk, Shizuoka, Japan) equipped with a Nikon epifluorescence microscope (TE2000-U; Nikon, Tokyo, Japan) and band-pass filters for wavelengths of 340 and 380 nm. After correction for the individual background fluorescence, the ratio of the fluorescence at both excitation wavelengths (F_{340}/F_{380}) was monitored simultaneously to determine the [Ca²⁺]_i. In the present experiments, the amplitude of [Ca²⁺]_i induced by the first application of 1 μM histamine was defined as 100%. Values were expressed as percents of [Ca²⁺]_i in the first application.

2.4. Radioligand binding assays to human recombinant receptors

Cloned human recombinant histamine receptors were commercially obtained (H₁: Cat. No. ES-390-M; H₂: Cat. No. ES-391-M, Euroscreen SA, Gosselies, Belgium). Radioligand binding studies to H₁ and H₂ receptors were carried out with [³H]pyrilamine (Cat. No. TRK608; GE Healthcare Bio-Sciences) and [³H]tiotidine (Cat. No. NET688; PerkinElmer Life & Analytical Sciences), respectively. Detailed methods for radioligand binding assays have been described by Nguyen et al. (2001) with minor modifications. Affinity of the competing ligand was expressed as the percent inhibition of radioligand binding, $[(B - N)/(B_0 - N)] \times 100$, where B is the total bound radioactivity in the presence of competing ligand, B_0 is the mean total bound radioactivity in the absence of competing ligand, and N is the mean non-specifically bound radioactivity.

2.5. Drugs

The following drugs and chemicals were used: carbachol hydrochloride, histamine, ethylene glycol-bis (2-aminoethyl ether)-*N,N,N,N*-tetraacetic acid (EGTA), endothelin-1, cimetidine, pyrilamine (all from Sigma Chemical Co., St. Louis, USA), phenylephrine hydrochloride, *N*^G-nitro-L-arginine methylester (L-NAME), diltiazem (all from Wako Chemical,

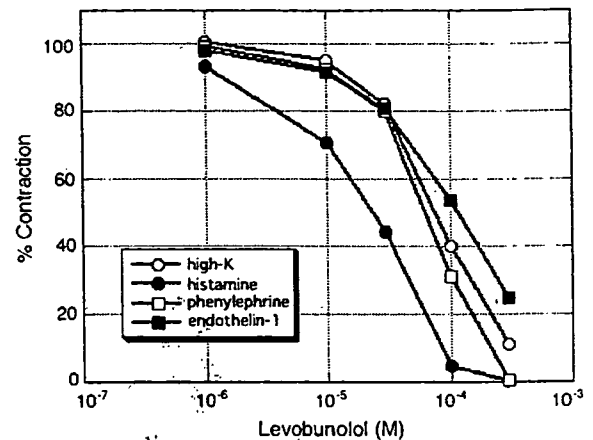


Fig. 1. Levobunolol-induced relaxation of pre-contracted rabbit ciliary arteries. Levobunolol induced concentration-dependent relaxation in the arterial rings that were pre-contracted with high-K solution ($n = 8$), 1 μM histamine ($n = 8$), 10 μM phenylephrine ($n = 11$) or 100 nM endothelin-1 ($n = 10$). The amplitude of contraction induced by High-K solution was defined as 100%.

Osaka, Japan), and levobunolol hydrochloride (Kaken Pharm, Tokyo, Japan). Concentrations were expressed as final molar concentrations in the organ chambers.

2.6. Statistical analysis

Results were expressed as means ± S.D. with n representing the number of vessels or cells studied. The concentrations of drugs causing 50% of the maximum relaxation (EC₅₀) were expressed as the log M concentration. Statistical differences between values were determined by the unpaired two-tailed Student's *t*-test. Differences between the concentration–response curves were analyzed by two-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3. Results

Stimulation with 1 μM histamine, 10 μM phenylephrine or 100 nM endothelin-1 elicited nearly the same level of contraction in this preparation as that induced by the high-K solution. Levobunolol caused a concentration-dependent relaxation of the vascular rings that were pre-contracted by each of these agents (Fig. 1). The minimum concentration of levobunolol that induced relaxation was 10 μM. Contractions induced by histamine, phenylephrine, high-K solution, and endothelin-1 were relaxed by 99.7%, 99.6%, 88.9% and 75.1%, respectively

Table 1
Inhibitory effect of levobunolol on radioligand binding to histamine receptors

Receptor (³ H-ligand)	Inhibition (%)	
	Levobunolol	Receptor-specific ligand
Histamine H ₁ ([³ H]pyrilamine)	0.0	99.8 (pyrilamine)
Histamine H ₂ ([³ H]tiotidine)	28.8	99.2 (cimetidine)

Competing ligand concentration: 10 μM.

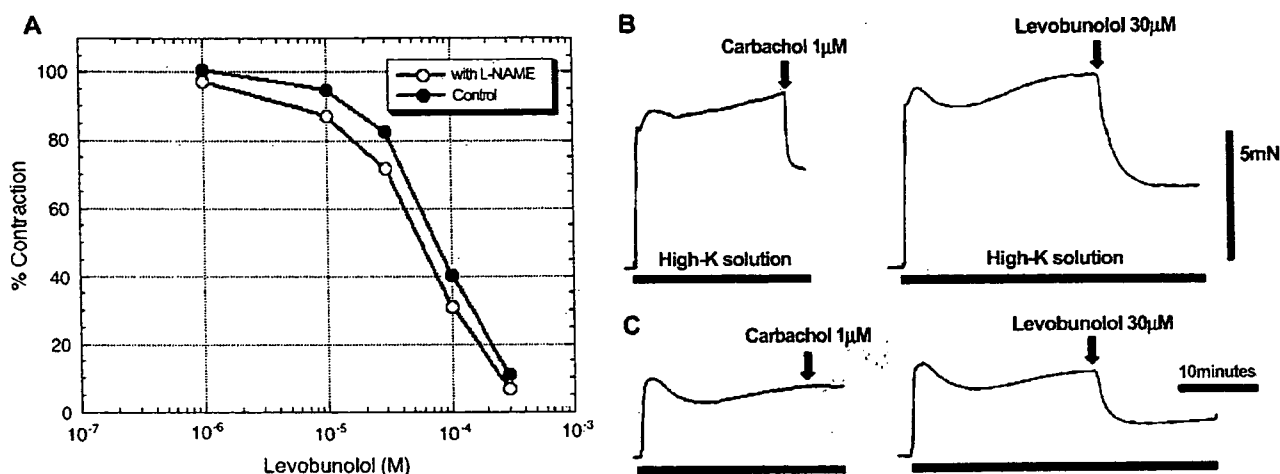


Fig. 2. Effects of L-NAME or denudation of endothelium on levobunolol-induced relaxation of rabbit ciliary arteries. (A) L-NAME (300 μM; *n* = 6) did not significantly alter (*P* > 0.05) the concentration-response curve for relaxation of high-K contracted ciliary arteries. The amplitude of contraction induced by high-K solution was defined as 100%. (B) Both carbachol (1 μM) and levobunolol (30 μM) induced relaxation in high-K pre-contracted ciliary artery. (C) In ciliary arteries denuded of the endothelium, carbachol (1 μM) did not induce relaxation. Denudation did not affect the response to levobunolol. The vertical bar with 5mN represents the actual isometric tension of rabbit ciliary arteries in myograph system.

with 30 mM levobunolol. Levobunolol was more effective in relaxing histamine-induced contractions ($EC_{50} = 20.7 \pm 16.3 \mu\text{M}$, *n* = 8) compared to phenylephrine, high-K or endothelin-1 induced contractions ($EC_{50} = 60.8 \pm 15.3 \mu\text{M}$, *n* = 11; $EC_{50} = 80.3 \pm 12.6 \mu\text{M}$, *n* = 8; $EC_{50} = 106.7 \pm 23.2 \mu\text{M}$, *n* = 10, respectively; Fig. 1).

Cimetidine (100 μM), a histamine H₂ receptor antagonist, did not relax the ciliary arteries pre-contracted with histamine (data not shown). On the other hand, 10 nM pyrilamine, a histamine H₁ antagonist, completely relaxed the histamine-contracted ciliary arteries (data not shown). These results indicated that the H₁ receptor was responsible for histamine induced vascular contraction. To determine if the relaxation effect of levobunolol could be mediated through either of

the histamine receptors, we measured the displacement of receptor-specific radiolabeled ligands by levobunolol. Levobunolol had no effect on [³H]pyrilamine binding to the histamine H₁ receptor (Table 1), but it did inhibit 28.8% of [³H]-tiotidine binding to the histamine H₂ receptor.

Pretreatment with 300 μM L-NAME did not change the levobunolol induced concentration-dependent relaxation of the ciliary artery (Fig. 2A). Denudation of the vascular endothelium also had no effect on the 30 μM levobunolol-induced relaxation although it completely abolished the carbachol-induced relaxation (Fig. 2B,C).

Phenylephrine provoked concentration-dependent contractions in this preparation (Fig. 3). Pretreatment of levobunolol depressed the contractions. However, it did not shift the phenylephrine concentration-response curve to the right.

We compared the relaxing effect of 0.1 mM levobunolol and 1 μM diltiazem in the same preparations that were pre-contracted with either high-K solution or 1 μM histamine (Fig. 4). These concentrations were chosen because they produced approximately 50% relaxation in the high-K pre-contracted arterial rings. Levobunolol induced $59.2 \pm 3.6\%$ relaxation of the high-K pre-contracted preparations (*n* = 6), and $89.7 \pm 4.2\%$ of the 1 μM histamine (*n* = 6) pre-contracted preparations (Fig. 4A,B). In contrast, diltiazem induced $63.4 \pm 7.6\%$ relaxation of the high-K pre-contracted preparations (*n* = 6), but only $40.0 \pm 7.2\%$ of the 1 μM histamine (*n* = 6) pre-contracted preparations (Fig. 4C,D).

In Ca²⁺-free solutions, 1 μM histamine induced a transient contraction of the ciliary artery (Fig. 5). Pretreatment of levobunolol (0.1 mM) markedly inhibited this contraction by $74.6 \pm 11.0\%$ (Fig. 5A,B). After a 30 min washout of the levobunolol, the transient contractions were almost completely restored.

In Ca²⁺-containing Krebs solution, [Ca²⁺]_i of cultured human aortic smooth muscle cells was elevated by 1 μM

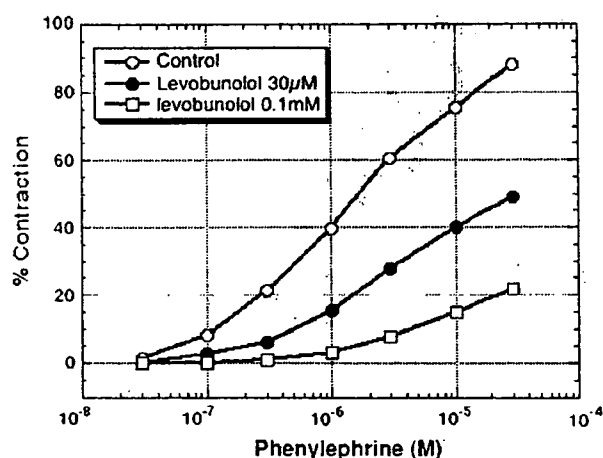


Fig. 3. Effects of levobunolol on phenylephrine concentration-dependent contractions of rabbit ciliary arteries. Levobunolol at 30 μM (*n* = 10) and at 100 μM (*n* = 10) significantly reduced the contractile response to phenylephrine (*P* < 0.05, *n* = 14). The amplitude of contraction induced by high-K solution was defined as 100%.

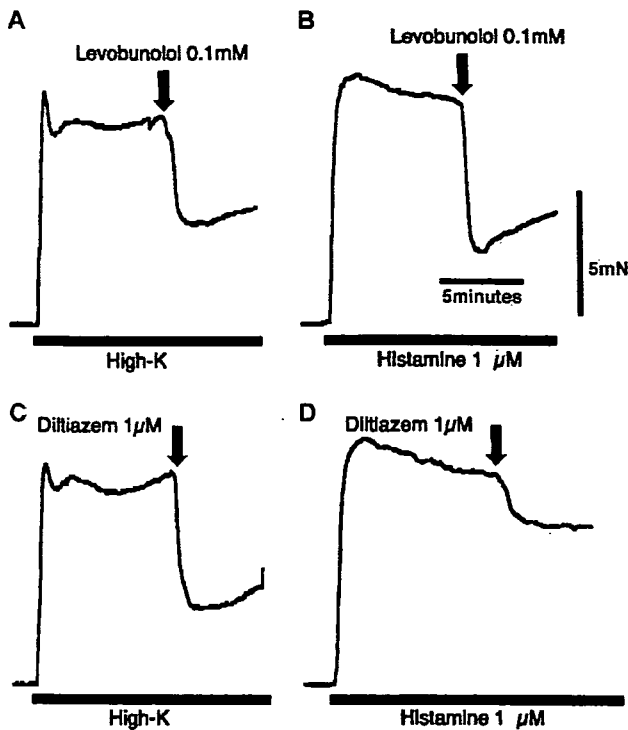


Fig. 4. Effects of levobunolol and diltiazem on high-K or histamine pre-contracted rabbit ciliary arteries. (A) Levobunolol (0.1 mM) induced less relaxation in preparations pre-contracted with high-K ($n = 6$) than with (B) histamine (1 μM , $n = 6$). (C) On the other hand, diltiazem (1 μM) induced a greater relaxation in high-K contracted arteries ($n = 6$) compared to histamine (1 μM) contracted arteries ($n = 6$). The vertical bar with 5mN represents the actual isometric tension of rabbit ciliary arteries in myograph system.

histamine. After a 7 min washout, the amplitude of the $[\text{Ca}^{2+}]_i$ response to a second application of 1 μM histamine decreased by $27.6 \pm 6.9\%$ (Fig. 6A). To determine the effect of levobunolol on the histamine induced $[\text{Ca}^{2+}]_i$ increase, 3 μM levobunolol was added to the incubation medium just after washing out the first application of histamine. The amplitude of the $[\text{Ca}^{2+}]_i$ due to the second application of histamine in the presence of 3 μM levobunolol was decreased $45.9 \pm 18.9\%$, a significantly greater decrease than the control (Fig. 6B, Table 2); whereas in Ca^{2+} -free solution, 3 μM levobunolol had no effect on $[\text{Ca}^{2+}]_i$ increase with 1 μM histamine (Fig. 6C,D, Table 2). Pyrilamine (10 nM) completely inhibited the elevation of $[\text{Ca}^{2+}]_i$ by 1 μM histamine in both the Ca^{2+} -containing Krebs solution and in the Ca^{2+} -free solution (Fig. 6E,F).

4. Discussion

The present studies demonstrated that levobunolol, a β -adrenergic antagonist, relaxed rabbit ciliary arteries that were pre-contracted with high-K solution, histamine, phenylephrine, or endothelin-1. These agents contract vascular smooth muscle by variety of mechanisms. The high-K solution induces contractions that depend on the passage of extracellular Ca^{2+} through voltage-dependent Ca^{2+} channels (Gustafsson, 1993). Histamine provokes contractions using Ca^{2+} that enters through voltage-dependent and receptor-dependent Ca^{2+} channels, as well as from intracellular Ca^{2+} stores (Laporte and Laher, 1997). Phenylephrine induces contraction through activation of α_1 -adrenergic receptors that open voltage-dependent Ca^{2+} channels and induce Ca^{2+} release from intracellular Ca^{2+} stores (Eckert et al., 2000). Endothelin-1 is one of the most potent vasoconstrictors (Highsmith et al., 1989; Murakawa et al., 1990). When it binds to either G-protein-linked endothelin

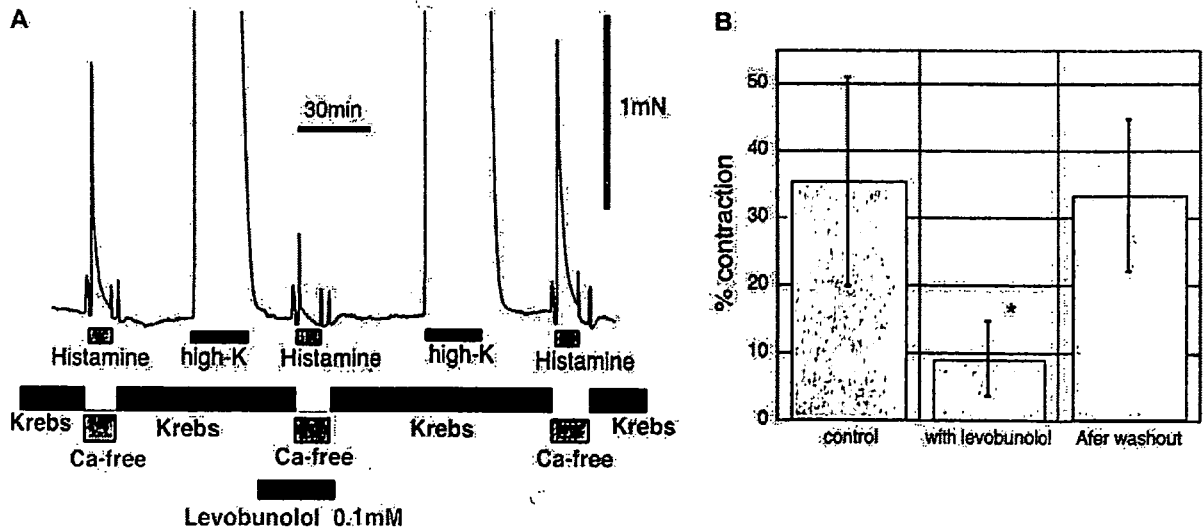


Fig. 5. Effects of levobunolol on histamine-induced transient contraction of rabbit ciliary arteries in Ca^{2+} -free solution. (A) Histamine (1 μM) was applied 6 min after incubation in Ca^{2+} -free solution. This was followed by high-K solution-induced contraction. Afterwards, the preparation was washed out with Krebs solution containing levobunolol (0.1 mM). (B) The amplitude of histamine-induced contraction in Ca^{2+} -free solution was then compared to the contractions in the presence or absence of levobunolol. Levobunolol significantly reduced the histamine-induced contraction in Ca^{2+} -free medium ($*P < 0.05$, $n = 22$). The amplitude of contraction induced by high-K solution was defined as 100%. The vertical bar with 1mN represents the actual isometric tension of rabbit ciliary arteries in myograph system.

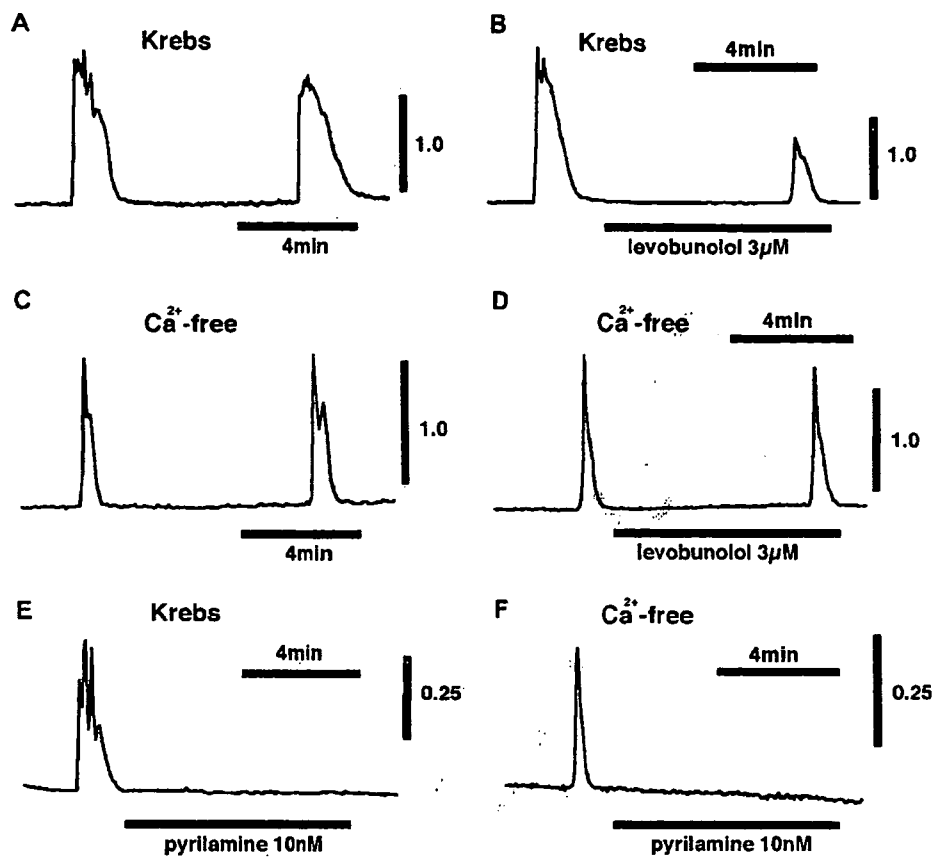


Fig. 6. Effects of levobunolol and pyrilamine on the histamine-induced increase of $[Ca^{2+}]_i$ in human aortic smooth muscle cells. Histamine ($1 \mu M$) was applied twice with a 7 min interval between application. Levobunolol ($3 \mu M$) or pyrilamine ($10 nM$) were pre-incubated before the second application of histamine. (A, B) In Krebs medium, levobunolol reduced the $[Ca^{2+}]_i$ in response to histamine. (C, D) In Ca^{2+} -free medium, levobunolol did not reduced the $[Ca^{2+}]_i$ in response to histamine. (E, F) Pyrilamine ($10 nM$) reduced the $[Ca^{2+}]_i$ in response to histamine in both Krebs medium and in Ca^{2+} -free medium. The vertical bar with either 1.0 or 0.25 represents the ratio of F_{340}/F_{380} which was monitored to determine the $[Ca^{2+}]_i$ in Aquacosmos System.

A or endothelin B receptors, it induces smooth muscle contraction by releasing Ca^{2+} from intracellular stores and promoting entry from the extracellular medium (Masaki, 2004).

Levobunolol was more effective on histamine-induced contractions compared to others. However, its relaxation effect is unlikely to be mediated through the H_1 receptor because levobunolol does not displace $[^3H]$ pyrilamine binding to that receptor. In the absence of any affinity for the H_1 receptor, levobunolol must antagonize the histamine-induced contraction through some other mechanism.

NO is formed from L-arginine by NO synthase in vascular endothelial cells and mediates the endothelium-dependent relaxation of vascular smooth muscle (Palmer et al., 1988). L-NAME, a NO synthase inhibitor, had no effect on the levobunolol-induced relaxation. Further, to investigate whether any endothelium-derived factor was involved in the relaxation mechanism, we denuded the vascular endothelium. This also had no effect on levobunolol-induced relaxation. These results suggested that NO or other endothelium-derived factors are not involved in the levobunolol relaxing mechanism.

In the present studies, phenylephrine, an α_1 -adrenergic agonist, induced concentration-dependent contractions in rabbit ciliary artery. Levobunolol may have some α_1 -antagonistic

action in addition to the well known β -antagonistic action (Mitsuoka et al., 1997). However, levobunolol only depressed the phenylephrine concentration-response curve and did not shift this curve to the right. Therefore, it seems that the α_1 -adrenergic receptor does not participate in the relaxant effect of levobunolol.

It has been reported that levobunolol inhibits the intracellular Ca^{2+} increases by blocking the L-type voltage-dependent Ca^{2+} channel (Wu et al., 2004). In present studies, we found that levobunolol was more effective in relaxing the histamine-induced contraction compared to the high-K-induced contraction. On the other hand, diltiazem, a typical L-type voltage-dependent Ca^{2+} channel blocker (Gunthorpe and Lummis, 1999), had a greater effect on the high-K-induced contraction. Diltiazem blocks L-type voltage-dependent Ca^{2+} channels, but these channels were not blocked by levobunolol in our cultured human aortic smooth muscle cells, though at higher concentrations they may do so. These differences between levobunolol and diltiazem suggest that the relaxing mechanism of levobunolol is different from that of diltiazem.

Our fluorescence photometry studies using cultured human aortic smooth muscle cells indicated that levobunolol

Table 2
Inhibitory effects of levobunolol or pyrilamine on histamine-induced increase of $[Ca^{2+}]_i$

Incubation medium	Control	3 μ M Levobunolol	10 nM Pyrilamine
Krebs solution	72.5 \pm 6.9 (35)	45.9 \pm 18.9* (41)	20.8 \pm 30.3* (27)
Ca ²⁺ -free solution	83.5 \pm 9.2 (34)	99.8 \pm 37.0 (35)	15.3 \pm 23.8* (28)

The amplitude of $[Ca^{2+}]_i$ induced by the first application of 1 μ M histamine was defined as 100%. Values were expressed as per cents of $[Ca^{2+}]_i$ in the first application. * $P < 0.05$ compared to control.

decreased the $[Ca^{2+}]_i$ that was elevated by histamine in Ca²⁺-containing Krebs solution, but it had no effect in Ca²⁺-free solution. Thus, we conclude that the relaxation mechanism of levobunolol is partly due to the blocking of extracellular Ca²⁺ entry through non-voltage-dependent Ca²⁺ channels. These data also suggest that levobunolol does not inhibit Ca²⁺ release from intracellular stores. However, levobunolol did inhibit the rabbit ciliary artery contraction induced by histamine in Ca²⁺-free solution. These apparent discrepancies may be due to species-specific differences in smooth muscle physiology. They may also be due to differences in the responsiveness of cultured cells compared to in situ cells.

Pyrilamine, a histamine H₁ antagonist, relaxed histamine pre-contracted preparations, and it also strongly inhibited the histamine-induced increase of $[Ca^{2+}]_i$ in the cultured human aortic smooth muscle cells in both Ca²⁺-containing Krebs and in Ca²⁺-free solution. This suggests that levobunolol, which did not affect $[Ca^{2+}]_i$ in Ca²⁺-free media, induces relaxation by changing Ca²⁺ sensitivity or by other intracellular Ca²⁺ independent mechanisms.

In summary, the vasodilatory mechanism of levobunolol was independent of NO or vascular endothelium, and it was not dependent on α_1 -adrenergic receptor antagonism. Moreover, levobunolol did not have histamine receptor antagonistic action. We conclude that levobunolol relaxes rabbit ciliary artery by two different mechanisms. First, the relaxation may be due to the blockade of Ca²⁺ entry through non-voltage-dependent Ca²⁺ channels. Second, levobunolol may induce relaxation by intracellular Ca²⁺-independent mechanisms. These actions of levobunolol may explain the mechanisms of increased ocular blood flow in vivo.

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Vasodilatory mechanism of levobunolol on vascular smooth muscle cells

Yaru Dong^{a,c}, Hitoshi Ishikawa^b, Yazhen Wu^c, Takeshi Yoshitomi^{a,*}

^a Department of Ophthalmology, Akita University School of Medicine, 1-1-1 Hondo, Akita, Akita 010-8543, Japan

^b Department of Orthoptics and Visual Sciences, School of Allied Health, Kitasato University, Kanagawa 228-8555, Japan

^c Department of Ophthalmology, The Second Hospital of Jilin University, Jilin 130042, P.R. China

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Abstract

Topical application of levobunolol hydrochloride, a β -adrenergic antagonist used for treatment of glaucoma, is reported to increase ocular blood flow. We studied the mechanism of levobunolol-induced vasodilation in arterial smooth muscle. The effects of levobunolol or other agents on isolated, pre-contracted rabbit ciliary artery were investigated using an isometric tension recording method. The effects of the same agents on intracellular free calcium ($[Ca^{2+}]_i$) in cultured human aortic smooth muscle cells were also studied by fluorophotometry. Levobunolol relaxed ciliary artery rings that were pre-contracted with either high-K solution, $1 \mu M$ histamine, $10 \mu M$ phenylephrine, or 100 nM endothelin-1. The relaxation induced by levobunolol was concentration-dependent over the range of $10 \mu M$ to 0.3 mM . Inhibition of endothelial nitric oxide synthase or denudation of the endothelium did not affect this relaxation. Histamine-induced contractions were inhibited by the histamine H_1 antagonist pyrilamine. Radioligand binding experiments showed that levobunolol did not bind to the H_1 receptor. Further, histamine induced transient contraction in Ca^{2+} -free solution, and levobunolol inhibited this contraction by $74.6 \pm 11.0\%$. In cultured smooth muscle cells in the presence of extracellular Ca^{2+} , levobunolol significantly inhibited the histamine-induced elevation of $[Ca^{2+}]_i$. However, it did not inhibit the increase of $[Ca^{2+}]_i$ in histamine-stimulated cells incubated in Ca^{2+} -free solution. These results indicate that levobunolol may relax rabbit ciliary artery by two different mechanisms. First, the relaxation could be due to the blockade of Ca^{2+} entry through non-voltage-dependent Ca^{2+} channels. Second, levobunolol may change the Ca^{2+} sensitivity of vascular smooth muscle cells.

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Keywords: Ca^{2+} channel; Ca^{2+} sensitivity; fura-2; levobunolol; relaxation; vascular smooth muscle

1. Introduction

Though it is generally accepted that increased intraocular pressure (IOP) is a major risk factor in glaucoma, vascular perfusion is also considered to be important in the pathogenesis of optic nerve damage and visual field loss, especially in patients with normal tension glaucoma (Collignon-Brach, 1994; Drance, 1998; Flammer, 1994). Therefore, a better understanding of the effect of antiglaucoma agents on ocular

circulation is important for optimizing their clinical use. We have reported that timolol (Hayashi-Morimoto et al., 1999), betaxolol (Hayashi-Morimoto et al., 1999), pilocarpine (Yoshitomi et al., 2000), unoprostone isopropyl (Yoshitomi et al., 2004), latanoprost (Ishikawa et al., 2002) and nipradilol (Yoshitomi et al., 2002) induced relaxation of the isolated rabbit ciliary artery. Each of these drugs acts through different mechanisms. For example, betaxolol (Hayashi-Morimoto et al., 1999) and nipradilol (Yoshitomi et al., 2002), both β -adrenergic antagonists, relaxed rabbit ciliary artery. Betaxolol can inhibit voltage depended calcium (Ca^{2+}) channel in this tissue (Hayashi-Morimoto et al., 1999) which also were seen in other tissue such as guinea-pig mesenteric artery and portal

* Corresponding author. Tel.: +81 18 884 6164; fax: +81 18 836 2621.
E-mail address: yoshitom@med.akita-u.ac.jp (T. Yoshitomi).

vein smooth muscle cells (Setoguchi et al., 1995). On the other hand, nipradilol relaxed rabbit ciliary artery by inducing nitric oxide (NO) production and blocking α -adrenergic receptor (Yoshitomi et al., 2002). We have reported that isoproterenol, a β -adrenergic agonist, has no effect on mechanical properties of rabbit ciliary artery (Hayashi-Morimoto et al., 1999). So, vasodilatory action of these β -adrenergic antagonists seems to be unrelated to the β -adrenergic receptor and have different mechanism in each agent. Various antiglaucoma agents including β -adrenergic antagonists also increase ocular blood flow in vivo (Arend et al., 1998; Georgopoulos et al., 2002; Grunwald, 1990; Kimura et al., 2005; Shaikh and Mars, 2001), but this effect seem not to be generated by decreasing IOP. Chiou et al. (1990) have reported that D-timolol which is less potent than L-timolol to lower IOP, is more potent than L-timolol to improve blood flow in retina and choroid of rabbit. There are also other reports that betaxolol may increase significantly ciliary body blood flow, choroidal blood flow, and retinal blood flow in rabbits' eyes in vivo (Sato et al., 2001). Levobunolol, a non-selective β -adrenergic antagonist, is widely used in antiglaucoma treatment. A number of clinical trials have suggested that levobunolol also can improve ocular circulation (Arend et al., 1998; Bosem et al., 1992; Morsman et al., 1995; Ogasawara et al., 1999). Morsman et al. (1995) reported that pulsatile ocular blood flow increased by 22% after 1 week's administration of levobunolol. Arend et al. (1998) indicated that levobunolol produced a decrease in arteriovenous passage time of approximately 25%, and it increased macular capillary blood velocity by approximately 20%. Concerning the vasodilatory mechanism of levobunolol, Wu et al. (2004), using a spectrofluorometry method, indicated that levobunolol inhibits the intracellular Ca^{2+} increases by blocking the L-type voltage-dependent Ca^{2+} channel. However, the underlying mechanisms of its effects on ocular circulation are not yet clear.

In an attempt to clarify the vasodilatory mechanism of levobunolol, we have investigated the effect of this drug using an isometric tension recording method on isolated rabbit ciliary artery. Additionally, we used fluorescence photometry to measure the effect of levobunolol on intracellular Ca^{2+} levels in cultured human aortic artery smooth muscle cells.

2. Materials and methods

2.1. Isometric tension recording method in isolated ciliary arteries

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in approval by Animal Experiment Committee of Akita University. Male albino rabbits weighing 2–3 kg were killed with an overdose of intravenous pentobarbital sodium (Abbott, North Chicago, IL, USA). The eyes were immediately enucleated, ensuring that a maximum length of optic nerve was removed, and then placed in oxygenated Krebs solution of the following composition (mM): NaCl 94.8, KCl 4.7, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2, CaCl_2 2.5, KH_2PO_4 1.2, NaHCO_3

25.0, glucose 11.7 and aerated with 95% O_2 and 5% CO_2 . With the aid of a dissecting microscope, the ciliary artery and surrounding connective tissue were carefully isolated from the optic nerve. Vascular ring segments (150–300 μm in diameter, 1–2 mm in length) were cut from the distal section of the ciliary artery and mounted in a chamber of the double Myograph System[®] (JP Trading, Denmark) with Krebs solution. After a process of the temperature rose to 37 °C in the chamber, the vessels were stretched to each optimal lumen diameter for active tension development, i.e. normalization. Then the vessels were equilibrated for 30 min. The Myograph System[®] allowed direct determination of vessel isometric tension while the internal circumference was controlled. Detailed methods for isometric tension recordings by the Myograph System[®] have been described by Mulvany and Halpern (1976, 1977).

After the equilibration period, the amplitudes of the contractions were evoked by the high-K solution and were measured at 20-min intervals to establish preparation viability and stability. High-K solutions were prepared by replacing NaCl with isotonic, equimolar KCl to give a final K^+ concentration of 100.7 mM. Cholinergic agonists, acting on receptors in the endothelium, were applied at 20-min intervals of the contraction and induce relaxation of vascular smooth muscle (Keef and Bowen, 1989). Thus, we tested the susceptibility of contracted ciliary arteries to cholinergic relaxation with 1 μM carbachol. All preparations entirely underwent the contraction and relaxation described above, before the separate experiments. In certain experiments, the vascular rings in baseline tone were incubated with inhibitor 30 min before the separate experiment. In other experiments, we gently denuded the endothelium by rubbing the inside of the vascular ring with a scalp hair before the normalization.

Additionally, the ability of levobunolol to relax ciliary artery rings pre-contracted with other agents, including histamine, phenylephrine, and endothelin-1, and in Ca^{2+} -free media was also determined. Generally, the pre-contraction was maintained for 20 min, then levobunolol was applied every 10 min in a cumulative manner. The Ca^{2+} -free solutions were prepared by replacing CaCl_2 with isotonic, equimolar MgCl_2 and adding 1 mM EGTA, a chelating agent for Ca^{2+} in the presence of magnesium. The ability of the H_1 histamine antagonist pyrilamine, the H_2 histamine antagonist cimetidine, and the L-type voltage-dependent Ca^{2+} channel blocker diltiazem to induce relaxation of contracted ciliary artery rings was also tested.

2.2. Smooth muscle cell cultures

Human aortic smooth muscle cells at passage 3 were purchased from Cambrex (Walkersville, MD, USA). The cells were grown in the culture medium (SmGM-2, Sanko Junyaku Co. Ltd., Tokyo, Japan) in 5% CO_2 -containing air at 37 °C. The culture medium was exchanged every 48 h until a subconfluent growth stage was obtained. The cells were detached by exposure for approximately 3 min to 0.025% trypsin in