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The Inhibitory Effect of Nilvadipine on Calcium Channels in Retinal Ganglion Cells in Goldfish

TSUGIHISA SASAKI, YUUSUKE NAKATANI, and KAZUHISA SUGIYAMA

ABSTRACT

Purpose: **Our aim was to examine the inhibitory effect of nilvadipine on voltage-gated calcium (Ca) channels in solitary ganglion cells.**

Methods: **Eyes were excised from goldfish. Ganglion cells were enzymatically dissociated from isolated retina. Whole-cell currents were recorded with the perforated-patch clamp technique.**

Results: **Depolarizing step pulses to more than −48 mV evoked a slowly inactivating inward Ca current. The current-voltage relation for the nilvadipine-sensitive current was bell-shaped, and the peak current reached a maximum at −8 mV in the presence and absence of nilvadipine. Nilvadipine block of voltage-gated Ca current was dose-dependent between 1 and 100 μM. The half-maximum inhibitory dose was 35 μM.**

Conclusions: **The inhibitory effect of orally administered nilvadipine on Ca channels had a mild influence in ganglion cells.**

INTRODUCTION

REDUCTION OF INTRAOCULAR PRESSURE is the first choice to treat primary open-angle glaucoma (POAG). Clearly, an excessive increase in intracellular calcium (Ca^{2+}) concentration can activate cytotoxic mechanisms associated with injury in various cell systems and organs.[1] Some studies have suggested that the favorable effect of organic Ca^{2+} antagonists on ischemic brain damage may result partly from their inhibitory action on the voltage-gated Ca^{2+} channels of the neurons.[2–4]

Nilvadipine, a dihydropyridine derivative, is an L-type Ca^{2+} channel blocker. It has selective and long-lasting effects on cerebral arteries, compared with other Ca^{2+} antagonists, such as nifedipine and diltiazem.[5] Nilvadipine is used predominantly as an antihypertensive agent and as a potent vasodilator for increasing cerebral blood flow circulation after a stroke. Its effectiveness in protecting the visual field and improving retrobulbar perfusion has been reported.[6] There is evidence for an effect of nilvadipine on voltage-gated Ca^{2+} channels in freshly isolated pyramidal neurons, which are known to be highly sensitive to ischemia.[7] Fish retinal ganglion cells and other neurons express multiple types of pharmacologically distinct, voltage-gated Ca^{2+} channels[8,9] involved in neuron excitability, intracellular Ca^{2+} regulation, and neurotransmitter release. However, the direct effect of nilvadipine on retinal ganglion cells is still unclear. Therefore, in this study, we used an amphotericin B-perforated patch recording technique to investigate the inhibitory effects of nil-

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vadipine on voltage-gated Ca^{2+} channels in freshly isolated retinal ganglion cells under voltage-clamp conditions.

METHODS

The protocol was approved by the University Animal Care Committee, and the care of the animals was in accordance with guidelines for the Care and Use of Laboratory Animals, Kanazawa University School of Medicine (Kanazawa, Japan).

Dissociation and identification of the goldfish retinal ganglion cells and the liquid junction potential correction technique have been described previously.[10,11] Briefly, 8- to 10-cm dark-adapted goldfish were pithed under tricaine methasulfonate anesthesia (1% in solution bath), their enucleated eyes were hemisected, and the retina was separated from the retinal pigment epithelium. The isolated retina was incubated in well-oxygenated standard external solution (135 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM N-2-hydroxyethylpiperazine-N'-2,4-ethanesulfonic acid [HEPES], 10 mM glucose, and 0.0003 mM tetrodotoxin [TTX; Sankyo Co., Tokyo, Japan], pH 7.4 adjusted with NaOH), containing 10 U/mL papain (Worthington; Lakewood, NJ) and 0.1 mg/mL cysteine (Sigma; St Louis, MO) at 28°C for 30 min. The ganglion cells were then dispersed mechanically with fire-polished glass pipettes. Dissociated cells were plated onto the coverslip of the recording chamber. Recordings were made at room temperature (19–22°C).

Dissociated cells were identified as ganglion cells by morphological features described previously[12] or by the presence of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Invitrogen, Carlsbad, CA) transported retrogradely through the optic nerve. The electrophysiological recordings were made in the amphotericin B-perforated patch recording configuration under voltage-clamp conditions.[13]

To minimize contamination by voltage-dependent potassium and sodium currents, cells were superfused with a solution containing Cs, tetraethylammonium (TEA), and TTX. The experimental solution for Ca^{2+} current (I_{Ca}) recording (4 mM Ca^{2+} solution) was: 79 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 4 mM $CaCl_2$, 10 mM CsCl, 25 mM TEA-Cl, 10 mM HEPES, 16 mM glucose, and 0.0003 mM TTX, pH 7.4 adjusted with NaOH. Pipettes were filled with a solution containing 120 mM CsOH, 30 mM TEA-Cl, 3 mM $CaCl_2$, 3 mM $MgCl_2$, 10 mM 1,2-bis(2 aminophenoxy) ethane-N,N,N',N',-tetraacetic acid (BAPTA), and 5 mM HEPES, pH 7.5 adjusted with methanesulfonic acid. The shanks of these pipettes were filled with this solution after addition of a 1:100 dilution of a stock solution of amphotericin B (0.5% wt/vol in dimethyl sulfoxide [DMSO]; Sigma). Nilvadipine (100 mM in DMSO; Astellas Pharma Inc, Tokyo, Japan), Nifedipine (10 mM in ethanol; Sigma) and ω-conotoxin GVIA (ω-CTX GVIA Nakalai Tesque, Kyoto, Japan) was diluted with experimental solution. The pipette resistance in the bath solution was approximately 7 MΩ when filled with pipette solution. The recording pipette was connected to a low-noise current-voltage converter (EPC-8; List-Medical, Darmstadt, Germany). The bath solution was connected to the Ag/AgCl bath electrode by a 1% agar bridge. Data were sampled at 10 kHz after passing through a low-pass filter with a cut-off frequency of 5 kHz. Stored data were analyzed offline on a personal computer (ibook; Apple Computer, Cupertino, CA). Stimulus generation, data acquisition, and plotting were controlled by the Pulse and Pulse-fit programs (List-Medical; Darmstadt). All currents were corrected for linear leak and capacitative currents using scaled currents elicited by depolarization or hyperpolarization from a holding potential (Vh). Perforated-patch access occurred usually in 5 min, and series resistance was compensated. The perforated-patch technique prevents the run-down of voltage-dependent calcium current and allows stable current recording for over 1 h. Solution was perfused by rapid superfusion with a U-tube system.[14]

RESULTS

The blocking effect of 30 μM of nilvadipine reached a plateau within 2 min and declined partially within 5 min after washout of nilvadipine (Fig. 1). To obtain steady-state inhibition of I_{Ca}, recordings were started 2 min after the start of drug application, except for ω-CTX GVIA application. To examine the effect of ω-CTX GVIA, I_{Ca} recordings were started 3 min after the start of drug application because the increase in blocking effect was more gradual.[8] Superfusion with nilvadipine (10 μM) caused decrease in the I_{Ca} amplitude, but only partial recovery was observed over a 5-min period following washout of the drug. Cells were held at a potential of −88 mV and then stepped in 10-mV increments from −88

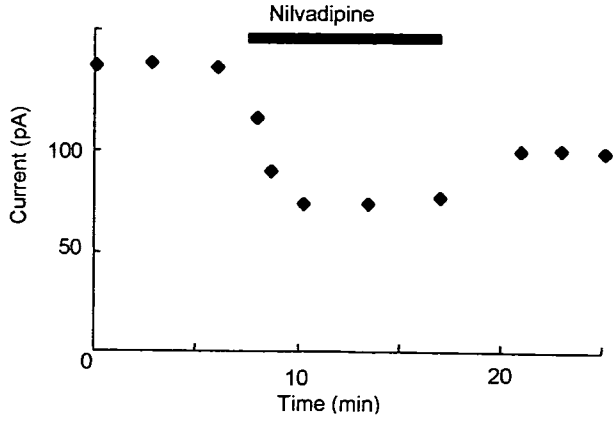

FIG. 1. Inhibition of calcium current (I_{Ca}) by nilvadipine. Time course of current block by 30 μM nilvadipine. Peak inward current was elicited at −8 mV and plotted against time. Full recovery from nilvadipine is not observed. Bar: period of nilvadipine application.

mV to +52 mV in the absence and presence of nilvadipine to study the current-voltage relations of I_{Ca}. When cells were held at −88 mV and a step pulse to −48 mV for 200 msec was applied, inward current started to flow without detectable delay and inactivated slowly (Fig. 2, inset). Current amplitude at the peak (peak current) and at the end of the command pulse (steady current) were measured and plotted against the step pulses (Fig. 2). At approximately −8 mV, the current reached a maximum. At more positive voltages than −8 mV, the current amplitude decreased gradually. For the steady current, the threshold voltage and the voltage at which the peak amplitude was recorded were nearly identical to those of the peak current. In the presence of nilvadipine, current amplitude was reduced at all voltages (Fig. 2, inset), but the threshold voltages and voltages at the amplitude of the peak and steady current did not change. At more positive voltages, the peak and steady current amplitude were reduced.

The inhibitory actions of nilvadipine on the peak I_{Ca} were normalized to I_{Ca} recorded without nilvadipine, and the pooled data were averaged and plotted against the nilvadipine concentration (Fig. 3). The lowest nilvadipine concentration giving a detectable effect was 1 μM. Further increases in the concentration of nilvadipine reduced I_{Ca} amplitude in a concentration-dependent manner. Data points were fitted by least square method to a Hill equation with a half-maximum inhibitory dose (IC_{50}) of approximately 35 μM. To determine which subtypes of Ca^{2+} channels were blocked by nilvadipine, the following experiments were performed. To avoid the contamination of low-voltage-activated (LVA) I_{Ca}, ganglion cells were voltage-clamped at a Vh of −48 mV (the potential at which LVA Ca^{2+} channels are inactivated).[7,15] The application of 1 μM ω-CTX GVIA inhibited the high-voltage-activated (HVA) I_{Ca} by 72.3% ± 7.1% (n = 4). After exposure to 1 μM ω-CTX GVIA, exposure to 1 μM nifedipine inhibited HVA I_{Ca} by 92.0% ± 8.7% (n = 4). After complete blockage of L- and N-type current by 2 pharmacological agents (1 μM nifedipine and 1 μM ω-CTX GVIA), 1 μM nilvadipine did not block the residual currents (n = 4), which include P/Q- and R-type I_{Ca} (Fig. 4). These results suggest that low-concentration nilvadip-

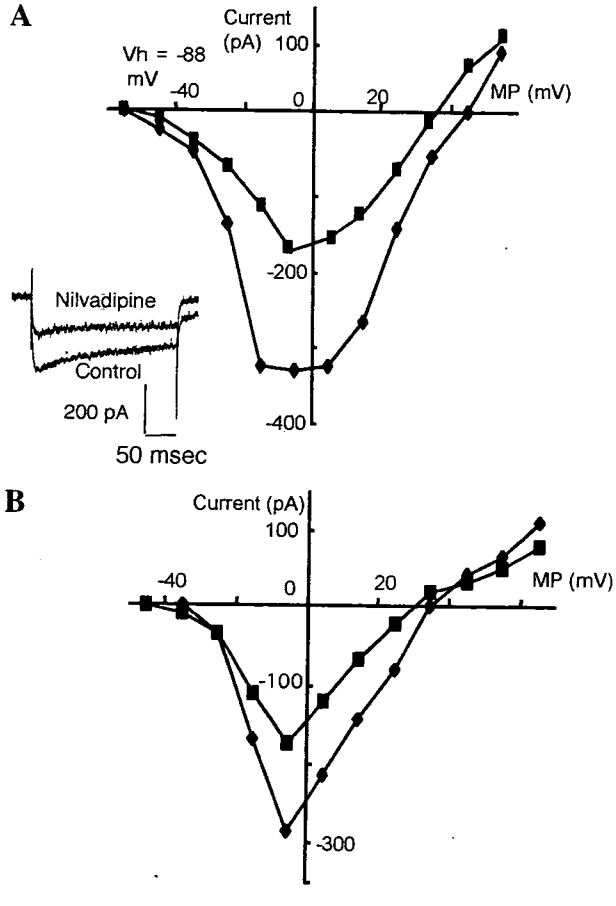

FIG. 2. The perforated-patch clamp technique was used to monitor current-voltage (I-V) relations for I_{Ca} in isolated goldfish ganglion cells in control solution (◆) and in control solution with 10 μM nilvadipine (■). I_{Ca} was recorded at a holding potential (Vh) of −88 mV, which was then stepped up in 10-mV increments from −88 to +52 mV. (**A**) I-V relation recorded at the peak current amplitude. (**B**) I-V relation recorded at the end of the command pulse. The inset shows representative current traces recorded at +2 mV in the control and in 10-μM nilvadipine solutions.

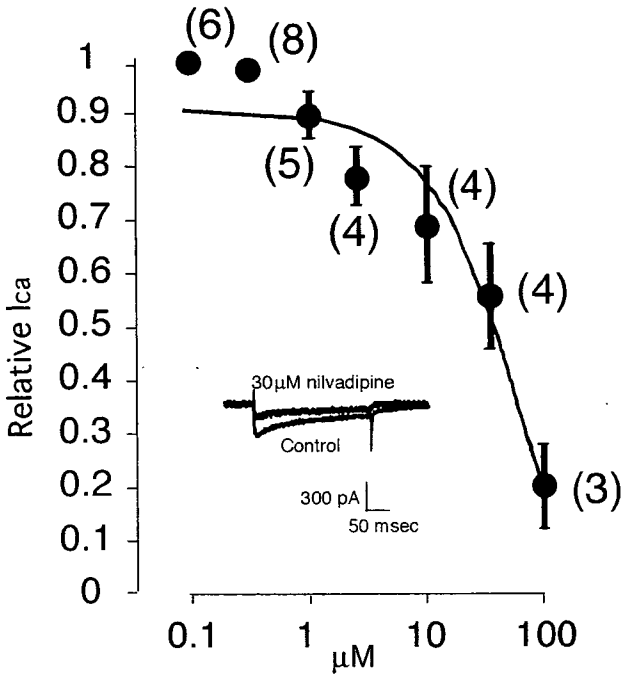

FIG. 3. Dose-response relations for nilvadipine. Cells were depolarized to −8 mV for 200 msec from a Vh of −88 mV. The response of I_{Ca} to a given nilvadipine concentration is expressed as the value of I_{Ca} relative to that evoked without nilvadipine. The inset provides representative current traces recorded at −8 mV in the control and 30 μM nilvadipine solutions. Each point is the average value from a group of cells (number of cells in parentheses), and vertical lines show standard deviation. Data points were fitted by least square method to a Hill equation.

ine blocked the L-type Ca^{2+} channels selectively in goldfish ganglion cells. High-dose nilvadipine (> 10 μM) blocked other types of I_{Ca}.

DISCUSSION

This study shows that nilvadipine at more than 1 μM inhibits voltage-gated calcium channels in acutely dissociated goldfish retinal ganglion cells.

Nilvadipine (100 μM) reduced I_{Ca} amplitude by 81%, whereas low-dose nilvadipine (< 1 μM) reduced I_{Ca} amplitude, at most, 10%. ω-CTX GVIA, an N-type specific blocker, suppressed 72% of HVA I_{Ca}. These results indicate that N- and L-type I_{Ca} constitute at least 72% and 20% of total I_{Ca} in ganglion cells, respectively, and that a low dose of nilvadipine blocked L-type Ca^{2+} channels. Cyprinid retinal ganglion cells possess as many as three types of I_{Ca}. One, which is responsible for at least three fourths of the total I_{Ca}, is blocked by ω-CTX GVIA.[8] This finding coincides with our results. It is thought that a large portion of I_{Ca} blockage by high-dose nilvadipine (> 10 μM) and nifedipine is a result of nonspecific effects on L- and N-type Ca channels.[16–19]

The IC_{50} of nilvadipine for inhibition of I_{Ca} is 35 μM. The reported concentration in rat cerebrum after intravenous or oral application of nilvadipine was 1.1–2.6 μM.[20] The L-type Ca channel current constitutes one fifth of the total I_{Ca} in ganglion cells. Therefore, orally administered nilvadipine might show a mild suppressive effect on voltage-gated calcium channels. The effect of nilvadipine on ganglion cells may be mild and, therefore, provide only mild protection from progressive deterioration of the visual field in POAG patients. On the other hand, nimodipine and nicardipine do protect the brain against ischemic damage.[21] The ganglion cells in this study had no distal dendrites. Nilvadipine might affect other neurons and glia, as these cells have L-type Ca^{2+} channels.[22,23] New drugs that prevent ischemia-induced neuronal damage by inhibiting N- and L-type Ca^{2+} channels should be investigated.

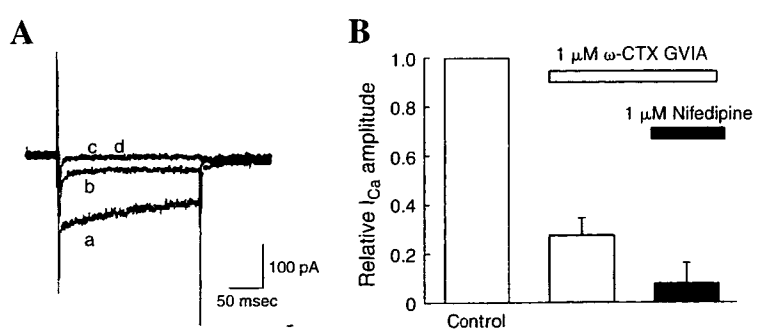

FIG. 4. Effects of nilvadipine on the ω-conotoxin GVIA (ω-CTX GVIA) and nifedipine-insensitive high-voltage-activated (HVA) I_{Ca} components. HVA I_{Ca} was evoked by a depolarizing step from Vh of −48 mV to +2 mV. (A) Superimposed current traces in control conditions and in the presence of these Ca^{2+} antagonists applied cumulatively. Current traces (a–d) were obtained in a: control solution, or in the presence of b: ω-CTX GVIA, c: ω-CTX GVIA + nifedipine, or d: ω-CTX GVIA + nifedipine + nilvadipine. All concentrations of the antagonists were 1 μM. Trace d is drawn faintly for clarity. (B) The residual HVA I_{Ca} after application of various Ca^{2+} antagonists. The amplitude of I_{Ca} was measured at the peak. Each point is the average value from a group of cells (n = 4) and vertical lines show standard deviations. Bar: application of Ca^{2+} antagonists.

CONCLUSION

The inhibitory effect of orally administered nilvadipine on Ca channels had a mild influence in ganglion cells.

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REFERENCES

1. Choi, D.W. Calcium-mediated neurotoxicity: Relationship to specific channel types and role in ischemic damage. *Trends Neurosci.* 11:465–469, 1988.
2. Alps, B.J., Calder, C., Hass, W.K., et al. Comparative protective effects of nicardipine, flunarizine, lidoflazine, and nimodipine against ischaemic injury in the hippocampus of the Mongolian gerbil. *Br. J. Pharmacol.* 93:877–883, 1988.
3. Beck, T., Nuglisch, J., Sauer, D., et al. Effects of flunarizine on postischemic blood flow, energy metabolism, and neuronal damage in the rat brain. *Eur. J. Pharmacol.* 158:271–274, 1988.
4. Kawamura, S., Yasui, N., Shirasawa, M., et al. Effects of a Ca^{2+} entry blocker (nilvadipine) on acute focal cerebral ischemia in rats. *Exp. Brain Res.* 83:434–438, 1991.
5. Tokuma, Y., Fujiwara, T., and Noguchi, H. Absorption, distribution, and excretion of nilvadipine, a new dihydropyridine calcium antagonist, in rats and dogs. *Xenobiotica* 17:1341–1349, 1987.
6. Harris, A., Evans, D.W., Cantor, L.B., et al. Hemodynamic and visual function effects of oral nifedipine in patients with normal-tension glaucoma. *Am. J. Ophthalmol.* 124:296–302, 1997.
7. Ishibashi, H., Murai, Y. and Akaike, N. Effect of nilvadipine on the voltage-dependent Ca^{2+} channels in rat hippocampal CA1 pyramidal neurons. *Brain Res.* 813:121–127, 1998.
8. Bindokas, V.P., and Ishida, A.T. Conotoxin-sensitive and conotoxin-resistant Ca^{2+} currents in fish retinal ganglion cells. *J. Neurobiol.* 29:429–444, 1996.
9. Randall, A., and Tsien, R.W. Pharmacological dissection of multiple types of Ca^{2+} channel currents in rat cerebellar granule neurons. *J. Neurosci.* 15:2995–3012, 1995.
10. Kaneko, A., and Tachibana, M. Effects of gamma-aminobutyric acid on isolated cone photoreceptors of the turtle retina. *J. Physiol.* 373:443–461, 1986.
11. Tabata, T., and Ishida, A.T. Transient and sustained depolarization of retinal ganglion cells by Ih. *J. Neurophysiol.* 75:1932–1943, 1996.
12. Ishida, A.T., and Cohen, B.N. GABA-activated whole-cell currents in isolated retinal ganglion cells. *J. Neurophysiol.* 60:381–396, 1988.
13. Horn, R., and Marty, A. Muscarine activation of ionic currents measured by a new whole-cell recording method. *J. Gen. Physiol.* 92:145–159, 1988.
14. Suzuki, S., Tachibana, M., and Kaneko, A. Effects of glycine and GABA on isolated bipolar cells of the mouse retina. *J. Physiol.* 421:645–662, 1990.
15. Murai, Y., Uneyama, H., Ishibashi, H., et al. Preferential inhibition of L- and N-type calcium channels in the rat hippocampal neurons by cilnidipine. *Brain Res.* 31:6–10, 2000.
16. Ozawa, Y., Hayashi, K., Nagahama, T., et al. Effect of T-type selective calcium antagonist on renal microcirculation: Studies in the isolated perfused hydronephrotic kidney. *Hypertension* 38:343–347, 2001.
17. Furukawa, T., Yamakawa, T., Midera, T., et al. Selectivities of dihydropyridine derivatives in blocking Ca^{2+} channel subtypes expressed in Xenopus oocytes. *J. Pharmacol. Exp. Ther.* 291:464–473, 1999.
18. Jones, S.W., and Jacobs, L.S. Dihydropyridine actions on calcium currents of frog sympathetic neurons. *J. Neurosci.* 10:2261–2267, 1990.
19. Kaneda, M., and Kaneko, A. Voltage-gated calcium currents in isolated retinal ganglion cells of the cat. *Jpn. J. Physiol.* 41:35–48, 1991.
20. Takakura, S., Sogabe, K., Satoh, H., et al. Nilvadipine as a neuroprotective calcium entry blocker in a rat model of global cerebral ischemia. A comparative study with nicardipine hydrochloride. *Neurosci. Lett.* 20:199–202, 1992.
21. Izumiya, K., and Kogure, K. Prevention of delayed neuronal death in gerbil hippocampus by ion channel blockers. *Stroke* 19:1003–1007, 1988.
22. Kaneko, A., Suzuki, S., Pinto, L.H., et al. Membrane currents and pharmacology of retinal bipolar cells: A comparative study on goldfish and mouse. *Comp. Biochem. Physiol. C.* 98:115–127, 1991.
23. Puro, D.G., Hwang, J.J., Kwon, O.J., et al. Characterization of an L-type calcium channel expressed by human retinal Muller (glial) cells. *Brain Res. Mol. Brain Res.* 37:41–48, 1996.

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Action of biologically active peptides on monkey iris sphincter and dilator muscles

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Abstract

Biologically active peptides modulate pupillary responsiveness in many non-primate mammals. We examined the action of seven different peptides on iris sphincter and dilator muscles of rhesus monkey. Iris sphincter and dilator muscle preparations from rhesus monkeys were mounted in an organ bath, and tension changes were recorded by an isometric transducer. Electrical field stimulation (100 Hz, 0·3 msec, 10 V) was applied through a pair of platinum plate electrodes. Monkey iris sphincter and dilator muscles produced simple cholinergic and adrenergic excitatory responses respectively to electrical field stimulation. Strong field stimulation did not elicit slow Substance P (SP) mediated contractions like those in rabbit iris sphincter. Exogenously applied pituitary adenylate cyclase-activating peptide (PACAP) enhanced in a concentration-dependent manner (0·3 nM–0·1 μM) the sphincter response to field stimulation, while neuropeptide Y (NPY) and somatostatin (SRIF) attenuated it. These three peptides did not affect sphincter contractions induced by acetylcholine, and therefore were acting at presynaptically. SP, calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP) and galanin (GAL) had no effect (at 0·1 μM) on iris sphincter. None of seven exogenously applied peptides had an effect on monkey iris dilator muscle. The innervation of primate irises may be relatively simple compared to non-primates because each of the peptides in this study can modulate miosis or mydriasis in non-primate mammals. Future studies will be expected on the functional significance of species differences in iridial innervation.

Keywords: iris sphincter; iris dilator; rhesus monkey; peptide; isometric contraction

1. Introduction

The innervation of the ocular pupillary control system differs considerably among species. Universally, the iris sphincter and dilator muscles are innervated by cholinergic and adrenergic excitatory nerve fibers respectively. While these fibers induce muscle contraction, adrenergic and cholinergic inhibitory innervations produce muscle relaxation when iris sphincter and dilator muscles of dogs (Yoshitomi and Ito, 1986), cats (Schaeppi and Koella, 1964a,b), rats (Narita and Watanabe, 1981, 1982) and cattle (Suzuki et al., 1983) are contracted by electrical field stimulation. Although exogenously applied noradrenaline relaxes the monkey iris sphincter (van Alphen, 1976), little is known about the inhibitory response to field stimulation in this species. Understanding these responses can provide new insights to the physiological control of the pupillary reflex in vivo.

As with autonomic agents, iris muscle responsiveness varies among species with respect to peptidergic agents. The rabbit iris muscles are affected by substance P (SP) (Ueda et al., 1981), vasoactive intestinal polypeptide (VIP) (Hayashi et al., 1982, 1983), neuropeptide Y (NPY) (Piccone et al., 1988), calcitonin gene-related peptide (CGRP) (Haruno et al., 1996), pituitary adenylate cyclase-activating peptide (PACAP) (Yoshitomi et al., 2002), somatostatin (SRIF) (Yamaji et al., 2003b), and galanin (GAL) (Yamaji et al., 2003b). Almegård et al. (1992) showed that cholecystokinin and vasopressin contract

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the monkey iris sphincter muscle directly but SP, VIP, CGRP, SRIF and GAL have no action on it. The possibility of muscle relaxation induced by these agents still remains untested. Moreover, Firth et al. (2002) recently demonstrated rich innervation of SRIF-like immunoreactive axons within the iris sphincter muscle in monkey, necessitating further physiological studies. Therefore, this study utilized electrical field stimulation of rhesus iris sphincter and dilator muscles and direct stimulation with autonomic and peptidergic agents to determine which agents may modulate miosis and/or mydriasis at pre- or post-synaptic sites.

2. Materials and methods

2.1. Isolation and incubation of muscle specimens

The experiments were performed on seven male rhesus monkeys weighing 6·1–6·4 kg. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The monkeys were sacrificed by pentobarbital sodium (Abbott Laboratories, North Chicago, IL). The eyes were immediately enucleated and placed in Krebs solution composed of (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 and gassed with 95% O_2 and 5% CO_2. After removal of the cornea, a ring-shaped iris sphincter muscle specimen (1 mm in width) or radial-shaped dilator muscle specimen (45 degrees in sector) was prepared according to the method previously reported (Yamaji et al., 2003a). The ends of each specimen were tied with 8–0 braided silk thread and mounted vertically in an organ bath (1·5 ml) by suspending one end to the isometric tension transducer (Nihon Kohden Co., TB-612T) and securing the other end to bottom of the bath. Oxygenated Krebs solution warmed to 37°C was perfused continuously (4·2 ml/min) throughout the experiment. The sphincter and dilator muscle specimens were stretched to 10 and 4 mm respectively. These lengths were defined from the length-tension relationship of each specimen obtained in the same manner as described previously for rabbits (Yamaji et al., 2003a). These lengths produce the maximum response to each stimulus. For approximately 1 hr after stretching, the sphincter and dilator muscle tones gradually declined, reaching steady-states at $13·4 \pm 5·1$ and $35·4 \pm 10·5$ mg, respectively.

2.2. Electrical field stimulation experiments

Electrical field stimulation was applied through a pair of platinum electrodes with 11 mm separation and placed in the organ bath so that the current pulse passed transversely across the tissue. The stimulation consisted of 10 pulses delivered at 0·3 msec/pulse, 100 Hz, ·10 V, and applied every 1·5 min (Yamaji et al., 2003b). After starting the electrical stimulation, the amplitude of response gradually increased and then stabilized within 1 hr. When stable responses were attained, the following experiments were performed.

The specificity of cholinergic and adrenergic responses to field stimulation was determined by addition of 10 μM atropine and 10 μM phentolamine to the incubation media of sphincter and dilator muscles respectively (Yoshitomi et al., 1985, 1986). In rabbit sphincter muscle, strong electrical stimulation consisting of 100 pulses produces slow contractions mediated by SP in addition to fast cholinergic contractions (Yoshitomi et al., 2002). This phenomenon was examined in monkey iris sphincter by comparing the responses evoked by both 10 and 100 pulses.

The effects of seven peptides known to alter contractions of rabbit iris sphincter and dilator muscles were tested in this study ($n=4$). To determine if the peptide solutions were active, they were first applied to rabbit iris smooth muscles before executing the monkey experiments. In the monkey experiments, prior to each peptide application, the muscle was stimulated for 20 min with 10 pulses to achieve normal contractions. The peptide was then added to the perfusion medium, and the contractions were recorded for another 20 min with the same stimulation. Since the effect of each peptide reached steady-state in the first 10 min, stable effect was recorded for the last 10 min of incubation period. A peptide washout period of at least 1 hr, during which electrical stimulation was maintained, followed each test period. Normal contractions were reestablished for 20 min prior to the next test period. The concentration of each peptide was 0·1 μM, which was the highest concentration used in the rabbit experiments (Haruno et al., 1996; Yoshitomi et al., 2002; Yamaji et al., 2003b). The effect of each peptide was quantified by calculating the average ratio of 3 responses taken just before peptide application and 3 responses just before peptide washout. For controls ratios were taken from the responses of the first and last 20 min in the placebo trial. Concentration-response curves ranging from 1 nM to 0·1 μM were also obtained ($n=4$). Each specimen produced the curves for all peptides which were found to be effective at 0·1 μM.

2.3. Direct contraction experiments

These experiments were performed to determine if the peptides effective in the electrical field stimulation experiments were acting on pre- or postsynaptic sites. For many species, acetylcholine has low potency on the iris sphincter muscle (Harris et al., 1974; Hasegawa et al., 1987; Patil et al., 2003; Suzuki and Kobayashi, 1989; Yoshitomi et al., 2001). Therefore the concentration-response relationship of acetylcholine on the monkey iris sphincter was generated by applying this agonist cumulatively (1 μM–10 mM). Based upon these results, 0·3 mM acetylcholine was chosen to elicit a submaximal contraction to determine the effect of exogenously applied peptides. At this concentration, it took about 15 min to reach a steady contraction, whereupon

0·1 μM ($n=4$) of each peptide was added to the solution for 20 min. Then the peptide was washed out for at least 1 hr prior to the next peptide trial.

2.4. Data analysis

We applied one way ANOVA followed by Dunnet's test as a post hoc test, with $\alpha=0·01$ as the acceptable level of significance, to compare the control and peptide-applied contractions. Concentration-response curves were calculated by fitting a sigmoidal function to the individual experimental data. The parameters of sigmoidal function were estimated using BFGS, a nonlinear optimization method (Gill et al., 1981). The median effective concentration (EC_{50}) was estimated from the function as that which produced 50% of contractile amplitude. All other results are expressed as means ± SD.

2.5. Chemicals

The following drugs and chemicals were used in this study: NPY, SRIF, GAL, CGRP, SP, VIP and PACAP 27 (all from Peptide Institute, Inc., Osaka, Japan), and phentolamine, atropine and acetylcholine (all from Wako Chemical Inc., Osaka, Japan). Peptides were prepared in aliquots and stored at −30°C.

3. Results

3.1. Electrical field stimulation experiments

Electrical field stimulation evoked a simple monophasic excitatory response in both sphincter and dilator muscles (Fig. 1). Inhibitory responses were not observed in monkey. The sphincter and dilator responses were blocked by application of 10 μM atropine and 10 μM phentolamine respectively (Fig. 1). The results indicate that the monkey sphincter and dilator muscles are innervated mainly by excitatory cholinergic and adrenergic nerve fibers, respectively. In response to strong field stimulation of 100 pulses, the sphincter muscles had only fast contractions (Fig. 2) in contrast to rabbit sphincter where both fast cholinergic and slow SP-mediated contractions occur (Yoshitomi et al., 2002).

In the experiment where seven peptides were applied, 0·1 μM PACAP significantly enhanced the response about 28·0±9·2%, while 0·1 μM NPY and SRIF significantly attenuated it about 35·5±3·4 and 12·3±7·4% respectively ($n=4$, $P<0·01$, Fig. 3, Fig. 4 left). The concentration-response relationships (1 nM–0·1 μM) for these peptides were monotonic and well fitted by a sigmoidal function (Fig. 5). CGRP, SP, VIP and GAL had no significant effect on the sphincter response at 0·1 μM (Fig. 4 left). The dilator muscle contractile response to field stimulation was not affected by any peptides we examined (Fig. 4 right).

3.2. Direct contraction experiments

The effect of NPY, SRIF and PACAP on acetylcholine-induced contractions was examined. A concentration-response curve for acetylcholine was generated for sphincter muscle (Fig. 6). The EC50 estimated from the sigmoidal function was 0·54 mM (±0·15, 95% confidence interval). Since acetylcholine contraction was continuous for 35 min in each trial (see detail in Methods), partial contraction by 0·3 mM was chosen to avoid desensitization and fatigue of the muscle. After the application of NPY, SRIF and PACAP, acetylcholine contractions were 99·1±4·7%, 100·4±7·1% and 96·2±7·6% ($n=4$) respectively. Thus, none of the three peptides affected the response amplitude induced by acetylcholine ($P>0·05$). This suggests that NPY, SRIF and PACAP act on presynaptic sites.

4. Discussion

Electrical stimulation evoked cholinergic excitatory contraction in sphincter muscle and adrenergic excitatory contraction in dilator muscle. Histological investigations have revealed that the iris sphincter muscle is innervated by both adrenergic and cholinergic neurons in the monkey (Nomura and Smelser, 1974) and human (Ehinger, 1966). Electric stimulation of human iris sphincter muscle, however, does not produce the adrenergic inhibitory response (Yoshitomi et al., 1988). This observation is consistent with our finding in monkey iris sphincter muscle. Moreover, similar electric stimulation developed similar amplitudes of cholinergic contractions (about 50 mg) in human (Yoshitomi et al., 1988) and monkey iris sphincter muscles. Thus, the iris sphincter muscle component of the pupillary reflex in humans and monkeys may be controlled principally by cholinergic innervation.

Monkey iris dilator muscle produced only an adrenergic excitatory response that was considerably stronger than that generated by human dilator, which produced only a few milligrams tension (Yoshitomi et al., 1985). This may relate to the fact that human iris dilator muscle also produced a cholinergic inhibitory response in addition to adrenergic excitatory response (Yoshitomi et al., 1985). Some of these differences may be attributed to different experimental techniques. In any case, the differences will need to be resolved by direct comparisons using iris dilator muscle from a variety of primates. In addition, to confirm the involvement of cholinergic and adrenergic receptors in monkey iris sphincter and dilator muscles, carbachol- and phenylephrine-induced muscle contraction should be performed in the presence and absence of atropine and phentolamine.

In previous reports, the effects of peptides on iris muscles were examined mainly in rabbit tissue, and each of the seven peptides used in this study had significant effects on the iris

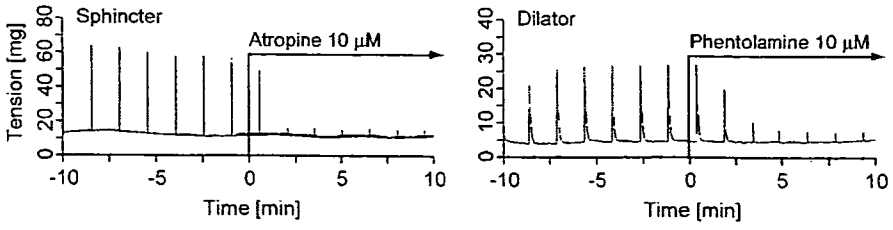

Fig. 1. Response and specificity of iris sphincter and dilator muscles to field stimulation. The sphincter and dilator muscles responded to 10-pulse field stimulations with sharp monophasic contractions. The contractions were abolished by 10 μM atropine applied to the sphincter (left) and 10 μM phentolamine applied to the dilator (right). The antagonists were present continuously after $t=0$ min.

sphincter and/or dilator muscles (Ueda et al., 1981; Hayashi et al., 1982, 1983; Piccone et al., 1988; Haruno et al., 1996; Yoshitomi et al., 2002; Yamaji et al., 2003b). SP mediates irritative ocular responses (Bill et al., 1979), and in rabbit induces strong miosis by sphincter muscle contraction (Ueda et al., 1981). Rabbit iris sphincter muscle is richly innervated with SP-like immunoreactive nerves; however, this innervation in monkey is far less dense than in rabbit (Stone et al., 1982). Consistent with these observations, monkey iris sphincter did not produce a SP-mediated response to relatively strong electrical stimulation like that present in rabbit (Ueda et al., 1981). Isolated iris sphincter muscles from baboon and human also do not respond to exogenously applied SP up to 5 μM (Unger and Tighe, 1984), and this is also consistent with our results here. CGRP coexists with SP in trigeminal nerve fibers (Kuwayama and Stone, 1987). Although CGRP inhibits iris dilator muscle contraction in rabbit (Haruno et al., 1996), this effect was not observed in monkey at the concentration we examined. This is consistent with the absence of specific binding sites for CGRP in monkey iris (Heino et al., 1995). VIP, which relaxes the iris sphincter and dilator muscles in rabbit (Hayashi et al., 1982, 1983) where there is a high density of binding sites (Denis et al., 1991), was ineffective in our experiments. This is consistent with the low levels of immunoreactivity for VIP in monkey iris muscles (Stone, 1986). For each peptide that did not elicit a detectable effect at 0·1 μM, it may be useful to determine if other concentrations do.

PACAP enhanced the field stimulation-mediated contraction of the monkey sphincter. PACAP-like immunoreactive nerves are localized in primate trigeminal ganglion (Tajti et al., 1999) along with cholecystokinin (Ghilardi et al., 1992), CGRP (Tajti et al., 1999), and SP (Tajti et al., 1999). Thus, PACAP, and possibly cholecystokinin, which causes contraction of human iris sphincter muscle (Almegård et al., 1992), may act in concert with several peptides of trigeminal origin to induce or modulate miosis in primates.

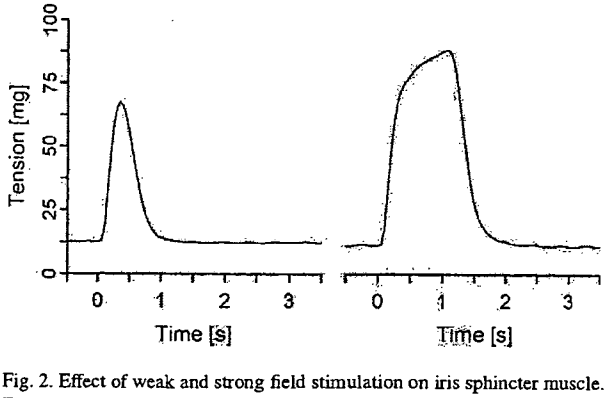

Fig. 2. Effect of weak and strong field stimulation on iris sphincter muscle. The responses to weak field stimulation (left) (10 pulses) and strong stimulation (right) (100 pulses) were both rapid and monophasic, lacking a slow component typical of the SP-mediated response. Each figure is the averaged curve of 5 responses. The gray bar at the bottom of each figure indicates the duration of electrical stimulation.

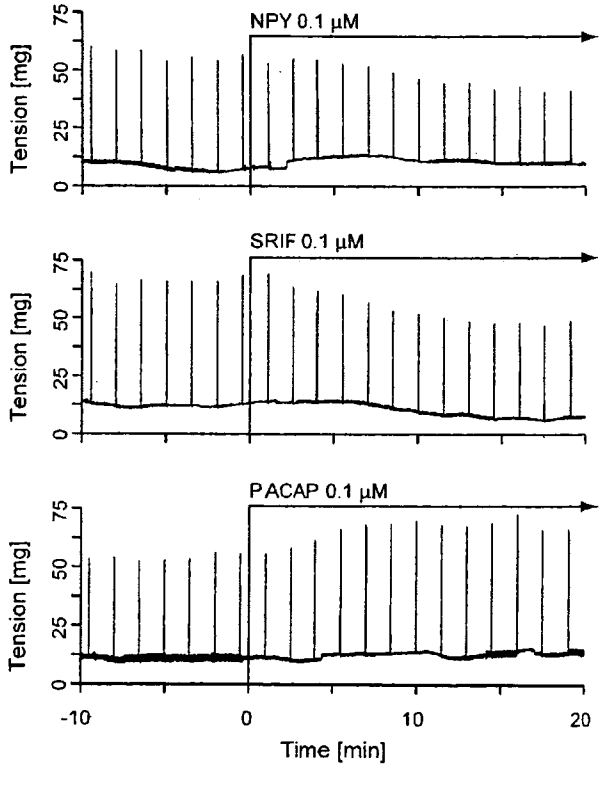

Fig. 3. Effect of NPY, SRIF and PACAP (0·1 μM) on field stimulation-induced contractions of sphincter muscles. Stimulation (10 pulses) was applied every 1·5 min. The peptide was present continuously after $t=0$ min. NPY and SRIF attenuated the sphincter response, while PACAP enhanced it without effect on the basal tone. Each peptide was applied to the same specimen after at least a 1 hr washout period.

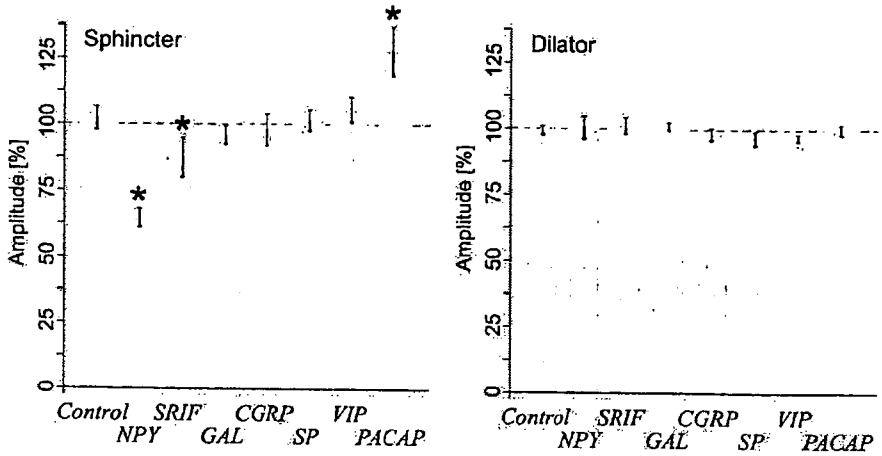

Fig. 4. Effect of seven peptides on sphincter and dilator muscles. The amplitude [%] in each specimen was calculated as the ratio of the response before (100%) and after drug application. The attenuation by 0·1 μM NPY and SRIF were 35·5 and 12·3% respectively, while the enhancement by PACAP was 28·0%. CGRP, SP, VIP and GAL had no significant effect on the sphincter. None of seven exogenously applied peptides had an effect on dilator muscle. $n=4$, $^*P<0.01$.

Based upon the prominent NPY-like immunoreactive innervation of monkey dilator muscle (Stone et al., 1986), we expected a significant response to NPY. To our surprise, this was not evident. In isolated rabbit iris dilator muscle, NPY enhances phenylephrine-induced contractions, but has no direct effect on basal tone and does not alter the electrically induced contraction (Piccone et al., 1988). In the current experiments, we did not determine the effects of phenylephrine on monkey dilator muscle or test the possibility that NPY modulates the response to it. Not only NPY but also other peptides should be examined for effect on sphincter and dilator muscles after acetylcholine or phenylephrine treatment. These will be the subjects of future experiments. Compared to the dilator, the distribution of NPY-like immunoreactive nerve fibers in monkey sphincter muscle is modest (Stone et al., 1986). Our physiological results showed that NPY inhibits the sphincter muscle contraction. This suggests that functionally NPY-like containing fibers may modulate miosis or enhance mydriasis.

In rabbit, SRIF inhibits cholinergic transmission at pre-synaptic sites less than GAL does (Yamaji et al., 2003b). Here we showed that SRIF also inhibits pre-synaptic cholinergic transmission in monkey sphincter muscle and could attenuate miosis, while GAL was not effective in either muscle. These results are consistent with immunohistological observations (Firth et al., 2002) that showed SRIF-like immunoreactive axons are abundant in sphincter muscle and less so in dilator muscle, and GAL-like immunoreactive axons are only occasionally observed in either muscle in monkey.

In conclusion, we investigated the autonomic responsiveness of monkey iris muscles to electrical field stimulation and the ability of peptides to modulate the responses. There are significant species differences in peptidergic innervation of monkey and rabbit irises. The effects of histamine (Yoshitomi et al., 1995) and bradykinin (El Sayah and Calixto, 2003) may also be important. Examining the reasons for the differences may lead to

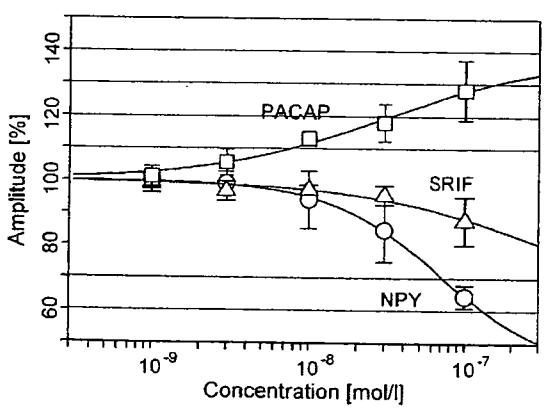

Fig. 5. Concentration-response relationship of NPY, SRIF and PACAP on the iris sphincter muscle response to electrical field stimulation. Each curve was fitted by a sigmoidal function to the data using a nonlinear optimization method. $n=4$ for each peptide.

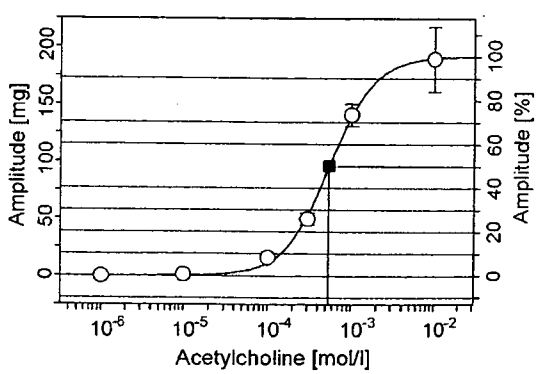

Fig. 6. Concentration-response relationship of acetylcholine on the iris sphincter muscle. Acetylcholine was applied cumulatively. Amplitude [%], indicated on the right hand axis, was normalized to the estimated sigmoidal function shown by the solid curve. The filled square indicates the median EC_{50}, 0·54 mM of which 95% confidence intervals are ±0·15 mM. $n=4$.

a better understanding of the innervation in pupillary control systems.

References

Almegård, B., Stjernschantz, J., Bill, A., 1992. Cholecystokinin contracts isolated human and monkey iris sphincters; a study with CCK receptor antagonists. Eur. J. Pharmacol. 211, 183–187.

Bill, A., Stjernschantz, J., Mandahl, A., Brodin, E., Nilsson, G., 1979. Substance P: release on trigeminal nerve stimulation, effects in the eye. Acta Physiol. Scand. 106, 371–373.

Denis, P., Dussaillant, M., Nordmann, J.P., Elena, P.P., Saraux, H., Rostene, W., 1991. Autoradiographic characterization and localization of vasoactive intestinal peptide binding sites in albino rat and rabbit eyes. Exp. Eye Res. 52, 357–366.

Ehinger, B., 1966. Adrenergic nerves to the eye and to related structures in man and in the cynomolgus monkey (*Macaca irus*). Invest. Ophthalmol. 5, 42–52.

El Sayah, M., Calixto, J.B., 2003. Study of the mechanisms involved in the bradykinin-induced contraction of the pig iris sphincter muscle in vitro. Eur. J. Pharmacol. 458, 175–181.

Firth, S.I., Kaufman, P.L., De Jean, B.J., Byers, J.M., Marshak, D.W., 2002. Innervation of the uvea by galanin and somatostatin immunoreactive axons in macaques and baboons. Exp. Eye Res. 75, 49–60.

Ghilardi, J.R., Allen, C.J., Vigna, S.R., McVey, D.C., Mantyh, P.W., 1992. Trigeminal and dorsal root ganglion neurons express CCK receptor binding sites in the rat, rabbit, and monkey: possible site of opiate-CCK analgesic interactions. J. Neurosci. 12, 4854–4866.

Gill, P.E., Murray, W., Wright, M.H., 1981. Practical Optimization. Academic Press, London.

Harris, L.S., Shimmyo, M., Hughes, J., 1974. Dose–response of cholinergic agonists on cat irides. Arch. Ophthalmol. 91, 299–302.

Haruno, I., Yoshitomi, T., Harada, Y., Katori, M., Ishikawa, S., 1996. Calcitonin gene-related peptide induced relaxation of the rabbit iris dilator muscle. Curr. Eye Res. 15, 105–110.

Hasegawa, N., Imaizumi, Y., Watanabe, M., 1987. Parasympathetic denervation supersensitivity in the rat iris sphincter muscle: an in vitro study. Jpn. J. Pharmacol. 43, 143–151.

Hayashi, K., Masuda, K., 1982. Effects of vasoactive intestinal polypeptide (VIP) and cyclic-AMP on the isolated sphincter pupillae muscles of the albino rabbit. Jpn. J. Ophthalmol. 26, 437–442.

Hayashi, K., Mochizuki, M., Masuda, K., 1983. Effects of vasoactive intestinal polypeptide (VIP) and cyclic AMP on isolated dilator pupillae muscle of albino rabbit eye. Jpn. J. Ophthalmol. 27, 647–654.

Heino, P., Oksala, O., Luhtala, J., Uusitalo, H., 1995. Localization of calcitonin gene-related peptide binding sites in the eye of different species. Curr Eye Res. 14, 783–790.

Kuwayama, Y., Stone, R.A., 1987. Distinct substance P and calcitonin gene-related peptide immunoreactive nerves in the guinea pig eye. Invest. Ophthalmol. Vis. Sci. 28, 1947–1954.

Narita, S., Watanabe, M., 1981. Response of the isolated rat iris sphincter to cholinergic and adrenergic agents and electrical stimulation. Life Sci. 29, 285–292.

Narita, S., Watanabe, M., 1982. Response of isolated rat iris dilator to adrenergic and cholinergic agents and electrical stimulation. Life Sci. 30, 1211–1218.

Nomura, T., Smelser, G.K., 1974. The identification of adrenergic and cholinergic nerve endings in the trabecular meshwork. Invest. Ophthalmol. 13, 525–532.

Patil, K.D., Buerki, R.A., Patil, P.N., 2003. Potentiation of acetylcholine action by huperzine-A and physostigmine on some vertebrate effectors, including human iris sphincter muscle. J. Ocul. Pharmacol. Ther. 19, 135–143.

Piccone, M., Littzi, J., Krupin, T., Stone, R.A., Davis, M., Wax, M.B., 1988. Effects of neuropeptide Y on the isolated rabbit iris dilator muscle. Invest. Ophthalmol. Vis. Sci. 29, 330–332.

Schaeppi, U., Koella, W.P., 1964a. Adrenergic innervation of cat iris sphincter. Am. J. Physiol. 207, 273–278.

Schaeppi, U., Koella, W.P., 1964b. Innervation of cat iris dilator. Am. J. Physiol. 207, 1411–1416.

Stone, R.A., 1986. Vasoactive intestinal polypeptide and the ocular innervation. Invest. Ophthalmol. Vis. Sci. 27, 951–957.

Stone, R.A., Laties, A.M., Brecha, N.C., 1982. Substance P-like immunoreactive nerves in the anterior segment of the rabbit, cat and monkey eye. Neuroscience 7, 2459–2468.

Stone, R.A., Laties, A.M., Emson, P.C., 1986. Neuropeptide Y and the ocular innervation of rat, guinea pig, cat and monkey. Neuroscience 17, 1207–1216.

Suzuki, R., Kobayashi, S., 1989. Possible mechanisms related to contraction of the bovine iris sphincter in the presence of acetylcholine and carbachol. Doc. Ophthalmol. 70, 293–300.

Suzuki, R., Oso, T., Kobayashi, S., 1983. Cholinergic inhibitory response in the bovine iris dilator muscle. Invest. Ophthalmol. Vis. Sci. 24, 760–765.

Tajti, J., Uddman, R., Moller, S., Sundler, F., Edvinsson, L., 1999. Messenger molecules and receptor mRNA in the human trigeminal ganglion. J. Auton. Nerv. Syst. 76, 176–183.

Ueda, N., Muramatsu, I., Sakakibara, Y., Fujiwara, M., 1981. Noncholinergic, nonadrenergic contraction and substance P in rabbit iris sphincter muscle. Jpn. J. Pharmacol. 31, 1071–1079.

Unger, W.G., Tighe, J., 1984. The response of the isolated iris sphincter muscle of various mammalian species to substance P. Exp. Eye Res. 39, 677–684.

van Alphen, G.W., 1976. The adrenergic receptors of the intraocular muscles of the human eye. Invest. Ophthalmol. 15, 502–505.

Yamaji, K., Yoshitomi, T., Usui, S., Ohnishi, Y., 2003a. Mechanical properties of the rabbit iris smooth muscles. Vision Res. 43, 479–487.

Yamaji, K., Yoshitomi, T., Usui, S., 2003b. Effect of somatostatin and galanin on isolated rabbit iris sphincter and dilator muscles. Exp. Eye Res. 77, 609–614.

Yoshitomi, T., Ito, Y., 1986. Double reciprocal innervations in dog iris sphincter and dilator muscles. Invest. Ophthalmol. Vis. Sci. 27, 83–91.

Yoshitomi, T., Itom, Y., Inomata, H., 1985. Adrenergic excitatory and cholinergic inhibitory innervations in the human iris dilator. Exp. Eye Res. 40, 453–459.

Yoshitomi, T., Ito, Y., Inomata, H., 1988. Functional innervation and contractile properties of the human iris sphincter muscle. Exp. Eye Res. 46, 979–986.

Yoshitomi, T., Ishikawa, H., Haruno, I., Ishikawa, S., 1995. Effect of histamine and substance P on the rabbit and human iris sphincter muscle. Graefes Arch. Clin. Exp. Ophthalmol. 233, 181–185.

Yoshitomi, T., Sakamoto, T., Ohnishi, Y., 2001. Gene transfer by adenovirus in rabbit iris sphincter muscle. Ophthalmic. Res. 33, 292–297.

Yoshitomi, T., Yamaji, K., Ishikawa, H., Ohnishi, Y., 2002. Effect of pituitary adenylate cyclase-activating peptide on isolated rabbit iris sphincter and dilator muscles. Invest. Ophthalmol. Vis. Sci. 43, 780–783.

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Prostaglandins E_1 and E_2, but not $F_{2\alpha}$ or Latanoprost, Inhibit Monkey Ciliary Muscle Contraction

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ABSTRACT *Purpose*: To investigate the effects of prostaglandin (PG) E_1, E_2, $F_{2\alpha}$, and latanoprost acid on the electrically evoked contractile response of isolated rhesus monkey ciliary muscle. *Methods*: Longitudinal ciliary muscle preparations from rhesus monkeys were mounted in an organ bath, and tension changes were recorded by an isometric transducer. Electrical field stimulation (100 Hz, 0.3 ms, 10 V) was applied through a pair of platinum plate electrodes. *Results*: The ciliary muscle produced atropine-sensitive excitatory contraction in response to field stimulation. PGE_1 and PGE_2 (1 μM) attenuated the contraction to levels that were 68% and 65.1%, respectively, of the normal amplitude. However, $PGF_{2\alpha}$ and latanoprost acid (1 μM) did not significantly change the response amplitude. *Conclusions*: Our results indicate that $PGF_{2\alpha}$ and latanoprost acid do not interact with the prostanoid receptor involved at the pre- and/or postsynaptic site. Therefore, it is unlikely that the hypotensive action by these agents is due to relaxation of the ciliary muscle.

KEYWORDS ciliary muscle; intraocular pressure; latanoprost; prostaglandin; uveoscleral outflow

INTRODUCTION

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and its analogue latanoprost lower intraocular pressure (IOP) by increasing the uveoscleral outflow.[1–3] These agents increase production of matrix metalloproteinases,[4,5] thereby reducing collagen types I, III, and IV, which are key elements in the extracellular matrix between ciliary muscle bundles.[6,7] This structural change causes the enlargement of space between bundles, thus enhancing uveoscleral outflow.[8,9] These morphological changes occur after topical application of $PGF_{2\alpha}$ for four days.[8] In contrast to these long-term actions, a hypotensive effect by $PGF_{2\alpha}$ and latanoprost can be seen within a couple of hours after a topical application.[10,11] It is reasonable, therefore, to assume that different mechanisms underlie the long-term actions and the acute hypotensive action caused by $PGF_{2\alpha}$ and latanoprost.

Pilocarpine-induced contraction of the ciliary muscle nearly blocks the uveoscleral outflow.[12] The hypotensive action by $PGF_{2\alpha}$ is also blocked by pilocarpine,[1,13] suggesting that uveoscleral outflow is dependent on the degree

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of the ciliary muscle tone. $PGF_{2\alpha}$ induces relaxation in monkey ciliary muscle, although the maximum reduction was less than 20% of the contractile force induced by a near maximal carbachol dose.[14] Latanoprost might be expected to relax the ciliary muscle because it binds much more selectively to the FP receptor than $PGF_{2\alpha}$ does.[3] However, there are no published data regarding the effects of latanoprost on ciliary muscle. Also, little is known about the effect of PGs on the ciliary muscle response to electrical field stimulation. Electrical field stimulation excites the intrinsic nerves and is a suitable method for examining the physiological effects of different treatments on the ciliary muscle. Here we have used the field stimulation method to clarify the effects of PGs, including latanoprost acid, on isolated monkey ciliary muscle.

MATERIALS AND METHODS

Isolation and Incubation of Muscle Specimens

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male rhesus monkeys weighting 4.6–6.4 kg were sacrificed by pentobarbital sodium (Abbott Laboratories, North Chicago, IL, USA). The eyes were immediately enucleated and placed in Krebs solution composed of (in 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) and gassed with 95% O_2 and 5% CO_2. The ciliary muscles were dissected in the same manner as previously described for the bovine eye.[15] Briefly, we prepared longitudinal ciliary muscle specimens of approximately 2 mm in width and 5 mm in length. Previous reports showed that circular and longitudinal ciliary muscle have the same contractile properties and action in response to $PGF_{2\alpha}$ treatment.[14] The increase of uveoscleral outflow occurs primarily by morphological changes in the anterior portion of the longitudinal muscle.[8,9] Thus, we chose the longitudinal muscle for these experiments. Each end of the muscle specimen was tied with braided silk thread and vertically mounted in an organ bath (1.5 ml). One end was suspended from the isometric tension transducer (Nihon Kohden Co., TB-612T; Tokyo, Japan) and the other end was secured to the bottom of the bath. Initial tension was set at about 150 mg,[14] which gradually relaxed to a steady state of about 70 mg after 0.5–1 hr. Oxygenated Krebs solution warmed to 37°C was perfused continuously (4.2 ml/min) throughout the experiment.

Electrical Field Stimulation Experiments

Electrical field stimulation was applied through a pair of platinum electrodes placed 11 mm apart in the organ bath so that the current pulse passed transversely across the tissue. The stimulation, consisting of 100 pulses delivered at 0.3 ms/pulse, 100 Hz, and 10 V, was applied every 1.5 min. Prior to each PG application, the muscle was stimulated for 20 min to achieve normal contractions. The PG was then added to the perfusion medium, and the contractions were recorded for another 20 min using the same stimulation. The control data were obtained by recording the responses for 40 min in order to compare the responses in the first 20 min with those in the last 20 min. A washout period of at least 1 hr followed each PG application. Then, the normal contractions were repeated, and a different PG was applied. In addition to $PGF_{2\alpha}$ and latanoprost acid, PGE_1 and PGE_2 were also tested as agonists for different prostaglandin receptor subtypes. To produce the maximum relaxation, we used a concentration of 1 μM for each reagent.[14] Different concentrations ranging from 1 nM to 1 μM was also examined in case of latanoprost acid.

Chemicals

The following drugs and chemicals were used in this study: atropine (Wako Chemical Inc., Osaka, Japan), PGE_1, PGE_2, $PGF_{2\alpha}$, and latanoprost acid (all from Sigma Chemical Co., St. Louis, MO, USA).

Results

The ciliary muscle produced a simple monotonic excitatory response to the field stimulation. This response was abolished by the application of 1 μM atropine, suggesting that the response was cholinergic in origin (Fig. 1). PGE_1 and PGE_2 (1 μM) inhibited the response to field stimulation. However, inhibition was not observed after application of $PGF_{2\alpha}$ or latanoprost acid (1 μM). None of the agent had an effect on the basal tone (Fig. 2). Because the responses to PGE_1 and PGE_2 became stable within 10 min after the application, the averaged amplitude response from 10 min to 20 min (about 7 responses) was compared with the

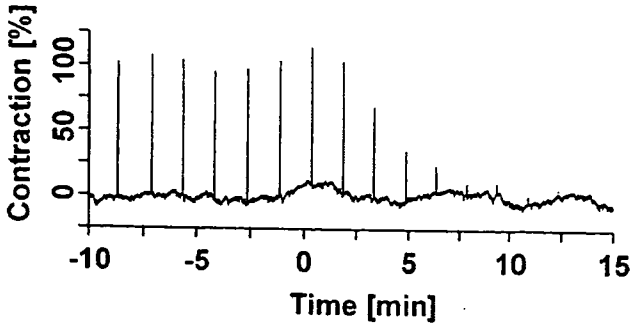

FIGURE 1 Parasympathetic innervation of ciliary muscles. Response of ciliary muscle to electrical field stimulation was abolished by 1 μM atropine. The antagonist was present continuously after $t = 0$ min. Stimulation consisting of 100 pulses was applied every 1.5 min.

control (Fig. 3). PGE_1 and PGE_2 significantly attenuated the ciliary muscle response to 68% and 65.1%, respectively, of the normal contraction amplitude. Although $PGF_{2\alpha}$ also tended to inhibit the response, its influence was not statistically significant. Latanoprost acid had no significant effect in concentrations ranging from 1 nM to 1 μM (Table 1).

DISCUSSION

This study demonstrates that whereas PGE_1 and PGE_2 attenuate the monkey ciliary muscle contraction evoked by electrical field stimulation, $PGF_{2\alpha}$ and latanoprost acid do not significantly affect the muscle response. A previous report showed that $PGF_{2\alpha}$ has the potential to induce relaxation of carbachol precontracted ciliary muscle in rhesus monkeys.[14] The maximum effect occurred when the dose was higher than 100 nM $PGF_{2\alpha}$ and the reduction was less than 20% of the near maximal contraction of 1 μM carbachol. Although the effect of $PGF_{2\alpha}$ in this study was not significant, the same trend from the previous study[14] can be observed. Latanoprost acid is a much more selective PG F (FP) receptor agonist than $PGF_{2\alpha}$.[3] If the muscle relaxation is mediated by the FP receptor, then latanoprost acid should have a greater relaxation effect at lower doses than $PGF_{2\alpha}$. However, the difference between the control and 1 μM latanoprost acid treatment data was less than 0.5%. Thus, latanoprost acid was not active in the ciliary muscle. A previous immunohistochemical study reported a low abundance of FP receptor in monkey ciliary muscle.[16] In human tissue, FP receptor is expressed more in the circular muscle of the ciliary body than in the longitudinal muscle,[17] although both vectors of ciliary muscles have the same responses to $PGF_{2\alpha}$ in monkey.[14] $PGF_{2\alpha}$ is active not only at FP receptors, but also at PG E (EP) and PG T (TP) receptors.[3,18] All factors considered, it is likely that the relaxation by $PGF_{2\alpha}$ in monkey ciliary muscle was mediated by EP receptors, as shown in cat ciliary muscle.[21] In fact, in contrast to $PGF_{2\alpha}$ and latanoprost acid, PGE_1 and PGE_2 clearly reduced the muscle contraction, suggesting that the EP receptor is functionally active in this tissue. EP_2 and EP_4 receptors are coupled to G_s and increase cyclic AMP concentration,[19] and the presynaptic EP_3 receptor inhibits the response to field stimulation.[20] Thus, these receptors may mediate the relaxant effect of PGE_1 and PGE_2, although this study did not identify which subtype is involved.

The relatively long-term hypotensive effect of $PGF_{2\alpha}$ and latanoprost on IOP is thought to result

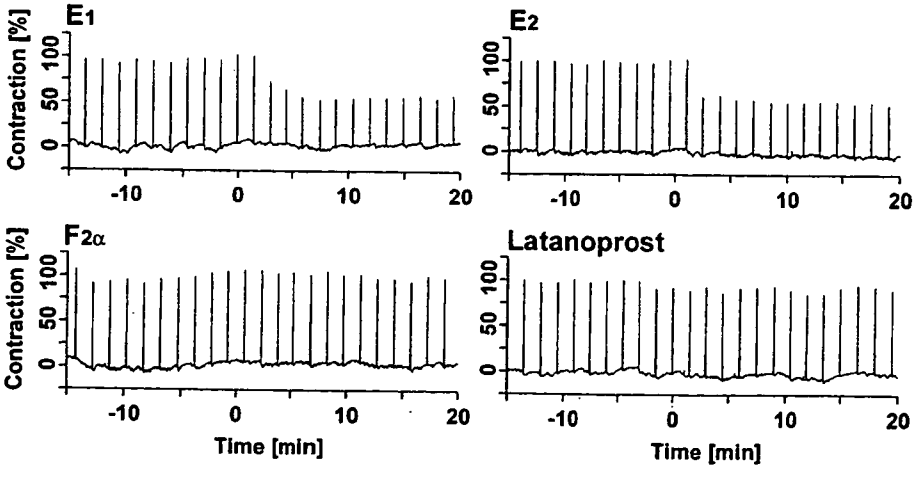

FIGURE 2 Responses of ciliary muscle before and after application of prostaglandins. Stimulation (100 pulses) was applied every 1.5 min. The prostaglandin was present continuously after $t = 0$ min. Each prostaglandin was applied to the same specimen after a washout period of at least 1 hr. Prostaglandins E_1 and E_2 clearly inhibit the ciliary muscle response to field stimulation.

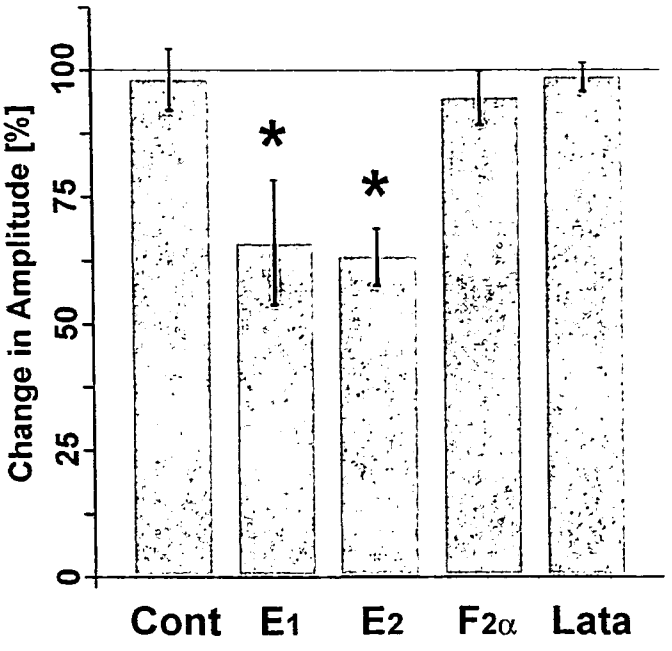

FIGURE 3 **Effect of prostaglandins on ciliary muscle. Prostaglandins E_1 and E_2 significantly attenuated the ciliary muscle response to 68% and 65.1%, respectively, of the normal contraction amplitude (*$p < 0.01$). Cont = control; Lata = latanoprost acid. Data are given as mean ± SD, n = 6.**

from the widening of space between ciliary muscle bundles.[8,9] However, short-term hypotensive action occurs within a couple of hours after treatment with these reagents.[10] Several papers hypothesized that the acute hypotensive action originates from the relaxation of the ciliary muscle.[1,13,14,21] Topical application of 10 μg $PGF_{2\alpha}$ isopropylester is enough to reduce IOP in monkeys.[11] This dosage corresponds to about 2.5 μM[14] by assuming that 1% of a topical dose penetrates[22] into 100 μl of the anterior chamber.[23] That is, the concentration adopted in this study can induce IOP reduction *in vivo*. However, $PGF_{2\alpha}$ and latanoprost acid have no significant effect on ciliary muscle contraction, suggesting that ciliary muscle relaxation cannot be a major cause of the acute hypotensive action. We currently do not have an alternative hypothesis to explain the acute hypotension. There still is a possibility that the lytic enzymes are responsible for the acute hypotension. In fact, mRNA of metalloproteinase increased at 4 or 6 hr after exposure to latanoprost acid.[24] To our knowledge, there is no published data for shorter periods of time that exactly correspond to the acute action. Further investigation will be needed to determine the mechanism of IOP reduction via $PGF_{2\alpha}$ or latanoprost.

TABLE 1 **Effect of Different Concentrations of Latanoprost Acid on Ciliary Muscle Contraction**

Latanoprost acid (mol/L)			
10^{-9}	10^{-8}	10^{-7}	10^{-6}
95.6 ± 3.2%	100.0 ± 2.7%	99.1 ± 3.8%	98.6 ± 2.9%

There is no significant difference between the control and each of latanoprost acid concentration ($P > 0.05$). Data are given as mean ± SD, n = 6.

REFERENCES

[1] Crawford K, Kaufman PL. Pilocarpine antagonizes prostaglandin $F_{2\alpha}$-induced ocular hypotension in monkeys. Evidence for enhancement of Uveoscleral outflow by prostaglandin $F_{2\alpha}$. *Arch Ophthalmol.* 1987;105:1112–1116.

[2] Gabelt BT, Kaufman PL. Prostaglandin $F_{2\alpha}$ increases uveoscleral outflow in the cynomolgus monkey. *Exp Eye Res.* 1989;49:389–402.

[3] Stjernschantz J, Selen G, Sjoquist B, Resul B. Preclinical pharmacology of latanoprost, a phenyl-substituted $PGF_{2\alpha}$ analogue. *Adv Prostaglandin Thromboxane Leukot Res.* 1995;23:513–518.

[4] Lindsey JD, Kashiwagi K, Boyle D, Kashiwagi F, Firestein GS, Weinreb RN. Prostaglandins increase proMMP-1 and proMMP-3 secretion by human ciliary smooth muscle cells. *Curr Eye Res.* 1996;15:869–875.

[5] Schachtschabel U, Lindsey JD, Weinreb RN. The mechanism of action of prostaglandins on uveoscleral outflow. *Curr Opin Ophthalmol.* 2000;11:112–115.

[6] Ocklind A. Effect of latanoprost on the extracellular matrix of the ciliary muscle. A study on cultured cells and tissue sections. *Exp Eye Res.* 1998;67:179–191.

[7] Sagara T, Gaton DD, Lindsey JD, Gabelt BT, Kaufman PL, Weinreb RN. Topical prostaglandin $F_{2\alpha}$ treatment reduces collagen types I, III, and IV in the monkey uveoscleral outflow pathway. *Arch Ophthalmol.* 1999;117:794–801.

[8] Lütjen-Drecoll E, Tamm E. Morphological study of the anterior segment of cynomolgus monkey eyes following treatment with prostaglandin $F_{2\alpha}$. *Exp Eye Res.* 1988;47:761–769.

[9] Richter M, Krauss AH, Woodward DF, Lütjen-Drecoll E. Morphological changes in the anterior eye segment after long-term treatment with different receptor selective prostaglandin agonists and a prostamide. *Invest Ophthalmol Vis Sci.* 2003;44:4419–4426.

[10] Lee PY, Podos SM, Severin C. Effect of prostaglandin $F_{2\alpha}$ on aqueous humor dynamics of rabbit, cat, and monkey. *Invest Ophthalmol Vis Sci.* 1984;25:1087–1093.

[11] Crawford KS, Kaufman PL. Dose-related effects of prostaglandin $F_{2\alpha}$ isopropylester on intraocular pressure, refraction, and pupil diameter in monkeys. *Invest Ophthalmol Vis Sci.* 1991;32:510–519.

[12] Bill A. Effects of atropine and pilocarpine on aqueous humour dynamics in cynomolgus monkeys (Macaca irus). *Exp Eye Res.* 1967;6:120–125.

[13] Nilsson SF, Samuelsson M, Bill A, Stjernschantz J. Increased uveoscleral outflow as a possible mechanism of ocular hypotension caused by prostaglandin $F_{2\alpha}$-1-isopropylester in the cynomolgus monkey. *Exp Eye Res.* 1989;48:707–716.

[14] Poyer JF, Millar C, Kaufman PL. Prostaglandin F2 alpha effects on isolated rhesus monkey ciliary muscle. *Invest Ophthalmol Vis Sci.* 1995;36:2461–2465.

[15] Yoshitomi T, Yamaji K, Ishikawa H, Ohnishi Y. Effect of latanoprost, prostaglandin $F_{2\alpha}$ and nipradilol on isolated bovine ciliary muscle. *Jpn J Ophthalmol.* 2002;46:401–405.

[16] Ocklind A, Lake S, Wentzel P, Nister M, Stjernschantz J. Localization of the prostaglandin $F_{2\alpha}$ receptor messenger RNA and protein in the cynomolgus monkey eye. *Invest Ophthalmol Vis Sci.* 1996;37:716–726.

[17] Schlotzer-Schrehardt U, Zenkel M, Nusing RM. Expression and localization of FP and EP prostanoid receptor subtypes in

human ocular tissues. *Invest Ophthalmol. Vis Sci.* 2002;43:1475–1487.
[18] Dong YJ, Jones RL, Wilson NH. Prostaglandin E receptor subtypes in smooth muscle: agonist activities of stable prostacyclin analogues. *Br J Pharmacol.* 1986;87:97–107.
[19] Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. *Physiol Rev.* 1999;79:1193–1226.
[20] Tam FS, Chan K, Borreau JP, Jones RL. The mechanisms of enhancement and inhibition of field stimulation responses of guinea-pig vas deferens by prostacyclin analogues. *Br J Pharmacol.* 1997;121:1413–1421.
[21] Chen J, Woodward DF. Prostanoid-induced relaxation of precontracted cat ciliary muscle is mediated by EP_2 and DP receptors. *Invest Ophthalmol Vis Sci.* 1992;33:3195–3201.
[22] Lee VH, Robinson JR. Topical ocular drug delivery: recent developments and future challenges. *J Ocul Pharmacol.* 1986;2:67–108.
[23] Erickson-Lamy KA, Kaufman PL, McDermott ML, France NK. Comparative anesthetic effects on aqueous humor dynamics in the cynomolgus monkey. *Arch Ophthalmol.* 1984;102:1815–1820.
[24] Weinreb RN, Lindsey JD. Metalloproteinase gene transcription in human ciliary muscle cells with latanoprost. *Invest Ophthalmol Vis Sci.* 2002;43:716–722.

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Pharmacological vascular reactivity in isolated diabetic rabbit ciliary artery

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Abstract

Impairment of the ocular circulation induced by diabetes mellitus has not been fully defined, but is thought to be related to hemodynamic changes in the ocular circulation. The purpose of the present study is to investigate the functional and morphological changes occurring in the ciliary artery wall of rabbits with alloxan-induced diabetes mellitus. A single intravenous bolus injection of alloxan (100 mg/kg) was given to each of 26 10-week-old rabbits and 16 sham-injected control rabbits. Twenty weeks later, control rabbits and diabetic rabbits were sacrificed, and their ciliary arteries were mounted in a myograph system. The responses of these arteries to high K^+ solution (K-Krebs solution), phenylephrine and carbachol were investigated using isometric tension recording. L-NAME (*NG*-nitro-L-arginine methyl ester; 100 μM) and indomethacin (1 μM) were also used to test the mechanism causing the carbachol induced relaxation. The arteries were also examined morphologically. The maximum tensions induced by K-Krebs solution in this tissue were not significantly different: 17.2 ± 0.8 mN ($n = 16$) in the control rabbits and 17.6 ± 0.8 mN ($n = 23$) in the diabetic rabbits ($P = 0.36$). Phenylephrine caused dose-dependent contraction with EC_{50} values of 1.3 ± 0.4 μM ($n = 6$) in the control and 5.1 ± 2.3 μM ($n = 6$) in the diabetic rabbits, but there was no significant difference between the two ($P = 0.36$). Carbachol induced dose-dependent relaxations in segments precontracted with K-Krebs solution. These relaxations were significantly reduced in the diabetic rabbits. The maximum relaxation induced by carbachol was 77.0 ± 2.4% (10 μM) and 66.4 ± 2.5% (100 μM) in the control and diabetic rabbits, respectively. These values were significantly different ($P = 0.0076$). The IC_{50} value for carbachol was 396.3 ± 58.4 nM ($n = 16$) in the control, and 443.6 ± 141.1 nM ($n = 23$) in the diabetic rabbit ($P = 0.87$). Application of a 100 μM nitric oxide synthase inhibitor, L-NAME, significantly inhibited the amplitude of relaxations evoked by carbachol ($P = 0.0066$). However, these relaxations were not inhibited by pretreatment with 1 μM indomethacin ($P = 0.60$). Histologically, the frequency of invaginations was less in the diabetic arterioles with a flattening of the lamina in the diabetic rabbits than in the controls. The cytoplasm of endothelial cells contained large vacuoles, indicating weak adhesion to the lamina. Some endothelial cells even showed vacuolar degeneration due to breakdown of the cell membranes. However, the smooth muscle cells were well preserved in the diabetic rabbit. These results suggest that the mechanism of impairment of ocular circulation induced by diabetes mellitus is mainly the reduction of NO synthase due to endothelial cell dysfunction. Furthermore, the characteristics of rabbits with alloxan-induced diabetes mellitus probably make them a useful model for investigating ocular complications induced by diabetic mellitus.

Keywords: rabbit; ciliary artery; alloxan; diabetes mellitus; ocular circulation; vasodilatation; vasoconstriction

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1. Introduction

There are many factors that influence the ocular circulation, for example, diabetes mellitus, hypercholesterolemia and hypertension. One of the major concerns in the long-term management of diabetes is the development of chronic complications. Diabetes mellitus is a very commonly seen risk factor in the development of non-arteritic anterior ischemic optic neuropathy (Kelman, 1998). Impairment of vascular reactivity has been demonstrated in diabetic animals and humans, and has been studied using streptozotocin-induced hyperglycemic rats and alloxan-induced hyperglycemic rabbits as diabetic models. (Taylor et al., 1992; Tesfamariam et al., 1989; Yu et al., 1998, 2001; Masuda et al., 1999).

Since the original observations of Furchgott and Zawadzki (1980), it has been demonstrated that the vascular endothelium plays an important role in the regulation of vascular tonus. Various agents are reported to generate relaxation through the activation of endothelium-derived relaxing factor (EDRF), which is now recognized to be nitric oxide (NO) (Ignarro et al., 1987; Palmer et al., 1987). The majority of studies have focused on the role of NO. However, it is becoming clear that other factors, such as endothelium-derived hyperpolarizing factor (EDHF), may play a role. The importance of this lies in the fact that, whereas in large vessels NO is the predominant endothelial vasodilator, in many smaller arteries EDHF assumes a prominent role in endothelium-dependent vasodilatation (Shimokawa et al., 1996; Busse et al., 2002; Fitzgerald et al., 2005).

Many investigators have reported functional alterations of the vascular endothelial cells that are related to hypertension (Luscher and Vanhoutte, 1986), hyperlipidemia (Ishikawa et al., 2004; Verbeuren et al., 1986), atherosclerosis (Jayakody et al., 1987), and diabetes (Kamata et al., 1989; Abiru et al., 1990). Previous work on endothelium-dependent relaxation in diabetic animals has demonstrated controversial responses in different vascular beds (Wakabayashi et al., 1987; Kamata et al., 1989; Abiru et al., 1990; Forti and Fonteles, 1998). Yu et al. (2001) reported that the acetylcholine-induced vasodilatation response is impaired in the ocular microvasculature of rats with streptozotocin-induced diabetes. Moreover, exogenous tetrahydrobiopterin reversed the endothelium impairment in this model.

The present study was initiated to investigate the functional and morphological changes occurring in the vascular wall with alloxan-induced diabetes in the isolated rabbit ciliary artery. The data obtained from the diabetic rabbits was compared with age-matched control rabbit data.

2. Materials and methods

2.1. General

Experiments were conducted in accordance with the ARVO Resolution on the Use of Animals in Research. Forty-two male Japanese White rabbits were used. They were purchased at 8 weeks of age and were housed in a temperature- and humidity-controlled room (24–25 °C, 55–60%). They were fed regular chow (120 g/day of CR-1, Clea, Tokyo, Japan) throughout the experimental period. A single intravenous bolus injection of alloxan (100 mg/kg) in 0.9% w/v saline was given via the marginal ear vein to 26 rabbits aged 10 weeks. Age-matched control rabbits ($n = 16$) were injected with a similar volume of saline. We measured blood glucose and weight once a week.

Twenty weeks after the alloxan injection, age-matched control rabbits and diabetic rabbits were sacrificed by administration of an overdose of intravenous pentobarbital sodium (Abbot, North Chicago, IL, USA).

2.2. Ring segment preparation and mounting

The eyes were immediately enucleated with care to remove a maximum length of optic nerve. The specimens were placed in oxygenated Krebs solution that contained the following (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 ciliary artery with its connective tissue was carefully dissected free from the optic nerve. Vascular segments (200–300 μm in diameter, 1–2 mm in length) were cut from the distal section of the ciliary artery and mounted in a double myograph system (JP Trading, Denmark) under microscopic observation (Nyborg et al., 1990). An arterial specimen was prepared from one animal only.

This myograph system allows direct determination of the isometric tension of the vessels while the internal circumference is controlled. The vessels were equilibrated for 30 min in oxygenated Krebs solution with 5% CO_2 and 95% O_2 at 37 °C. Detailed methods for isometric tension recording with a myograph system have been described by Mulvany and Halpern (1977).

2.3. Functional examinations

After the mounting and equilibration of the artery specimen, K-Krebs solution prepared by isotonically replacing equimolar NaCl with KCl ($K^+ = 100.7$ mM) was introduced three times into the chamber, causing three sequential contractions in the specimen with replacement of normal Na-Krebs solution in between each contraction. The isometric tensions of each of these contractions were measured at 90-min intervals to establish the viability and stability of the preparation. During the contractions, it was confirmed that 10 μM carbachol induced relaxation in all preparations, which indicated that the endothelium in each preparation was intact.

Phenylephrine (PE) was added cumulatively into the organ-bath. Indomethacin (1 μM) and *NG*-nitro-L-arginine methyl ester (L-NAME) (100 μM) were added to the bath 20 min before the start of administration of carbachol.

2.4. Morphological examinations

Segments of the ciliary specimens not used for the functional studies were fixed by immersion in 10% phosphate-buffered

formalin and were then embedded in paraffin. From each specimen, serial sections were cut and then stained with hematoxylin and eosin. For transmission electron microscopy, the extracted arterial specimens were fixed overnight with 4% glutaraldehyde buffered with 0.075 M phosphate solution. After being washed in buffer, these specimens were post-fixed overnight with 1% OsO_4 in the same buffer solution, dehydrated in an ethanol series, and embedded in Quetol 812. After polymerization, the specimens were cut with a Reichert-Nissei Ultratome (Ultracut N, Reichert Optische Werke AG, Vienna, Austria). These sections were stained with lead citrate and uranyl acetate and examined with an electron microscope (HU-12A, Hitachi, Tokyo). Some of the specimens were intended for scanning electron microscopy and so were transferred to isoamyl acetate. After critical-point drying, they were sputtered with gold, and examined in a scanning electron microscope.

2.5. Data analysis

The EC_{50} and IC_{50} values were calculated using nonlinear regression fits performed with Prism (GraphPad, San Diego, CA. USA) and were presented as means ± S.E. Statistical differences between points were determined by the Mann–Whitney *U*-test, and between the concentration–response curves by repeated-measures ANOVA with a post hoc test. $P < 0.05$ was considered significant.

2.6. Chemicals

The following drugs were used: carbachol hydrochloride and alloxan HCl (Sigma–Aldrich Co., St Louis, MO, USA), phenylephrine (PE; Wako Chemical Inc., Osaka, Japan), *NG*-nitro--arginine methyl ester (L-NAME; Wako Chemical, Osaka, Japan), indomethacin (Banyu Pharmaceutical Co. Ltd., Tokyo, Japan). All chemicals were of reagent grade.

3. Results

3.1. Baseline data

We administered alloxan to 26 rabbits. Two out of the 26 died of a hypoglycemic attack the following day. One of the alloxan treated rabbits did not develop hyperglycemia. Table 1 shows the body weight change rate and plasma blood glucose levels in experimental rabbits. Diabetic rabbits, especially those with glucose levels more than 600 mg/dl, did not increased their body weights compared with age-matched control rabbits (Table 1).

3.2. Contractile activity

When specimens of the ciliary artery were mounted in the organ bath, the tissue gradually relaxed to a steady tone after 0.5–1 h superfusion with Krebs solution. No spontaneous contractions occurred during the experiment. The maximum tensions developed by K-Krebs solution in this tissue were 17.2 ± 0.8 mN ($n = 16$) in the control rabbits and 17.6 ± 0.8 mN ($n = 23$) in the diabetic rabbits, showing no significant difference between the two ($P = 0.36$). PE that was exogenously applied to six rabbits caused dose-dependent contraction in this tissue (Fig. 1). The EC_{50} values were 1.3 ± 0.4 μM ($n = 6$) in the control rabbits and 5.1 ± 2.3 μM ($n = 6$) in the diabetic rabbits, showing no significant difference ($P = 0.37$).

Table 1
Plasma blood glucose levels and weight change rate

Plasma glucose	Weight change (%)
Control: 85–134 mg/dl ($n = 16$)	146.1 ± 4.2
<200 mg/dl[a] ($n = 1$)	159.5
200–600 mg/dl ($n = 12$)	135.3 ± 3.2
>600 mg/dl ($n = 11$)	104.5 ± 3.4
Total ($n = 24$[b])	

Blood glucose values were at the maximum level during follow-up. They were non-fasting values. Weight change rate (%) = weight at time of sacrifice (kg)/weight before alloxan administration (kg) × 100.

[a] Data on plasma blood glucose levels less than 200 mg/dl were excluded from this study.

[b] We have injected alloxan in 26 rabbits. Two rabbits died of hypoglycemia.

3.2.1. Mechanical responses to carbachol

After pre-contraction by K-Krebs solution, carbachol provoked relaxation in a dose-dependent manner in both control and diabetic rabbits (Fig. 2). Fig. 2 shows the dose–response relationship of carbachol in the rabbit ciliary artery pre-contracted with K-Krebs solution, where the amplitude of contraction that was evoked by such solutions was defined as the relative amplitude of 100%. The relaxation response was significantly reduced in diabetic segments. The maximum relaxation induced by carbachol was 77.0 ± 2.4% (10 μM) and 66.4 ± 2.5% (100 μM) in the control and diabetic rabbits, respectively. These values were significantly different ($P = 0.0076$). The IC_{50} value was 396.3 ± 58.4 nM ($n = 16$) in the control, and 443.6 ± 141.1 nM ($n = 23$) in the diabetic rabbit ($P = 0.87$).

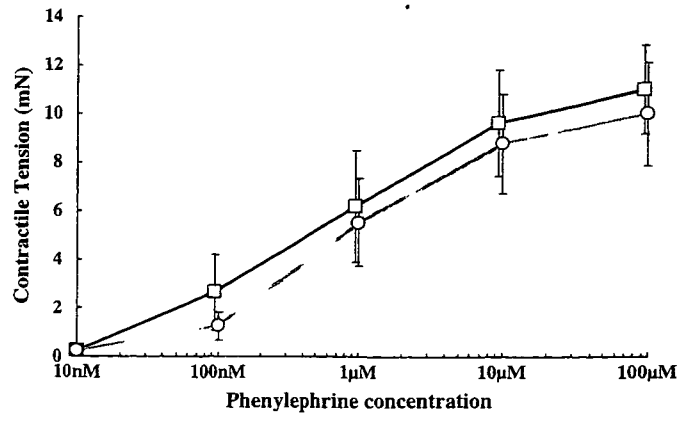

Fig. 1. Dose–response curves in the diabetic rabbit (open squares, $n = 6$), age-matched control (open circles, $n = 6$) ciliary arteries for phenylephrine. The bars show S.E.M. The maximum contraction induced by phenylephrine was 10.0 ± 2.1 mN and 11.3 ± 1.8 mN in the control and diabetic rabbits, respectively. There was no significant difference between the two ($P = 0.30$).

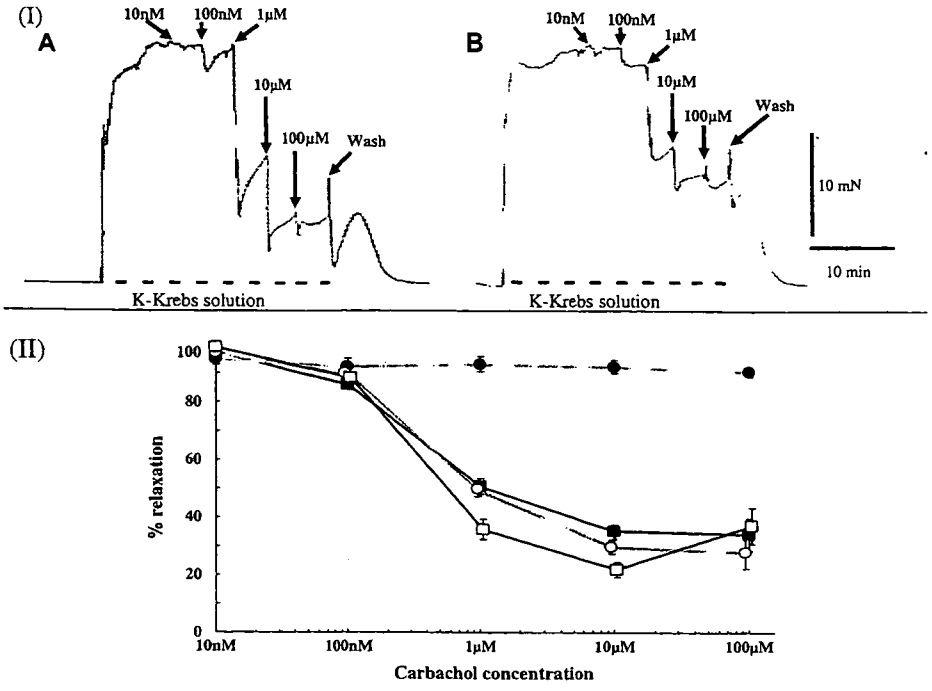

Fig. 2. (I) Typical tracing showing effects of cumulative application of carbachol to the rabbit ciliary artery (diameter 200–300 μM) pre-contracted with K-Krebs solution. (A) Control; (B) diabetes. (II) Dose–response relationships of carbachol applied to diabetes (closed squares, carbachol alone ($n = 23$); open circles, carbachol with 1 μM of indomethacin ($n = 6$); closed circles, carbachol with 100 μM of L-NAME ($n = 6$)) and age-matched control (open squares, $n = 16$) rabbit ciliary artery pre-contracted with K-Krebs. The bars show S.E.M.

Carbachol induced relaxation only in preparations with an intact endothelium, and had no effect on preparations lacking the endothelium (data not shown). Application of a nitric oxide synthase inhibitor (L-NAME, 100 μM) inhibited the amplitude of relaxation evoked by carbachol ($P = 0.0066$, Fig. 2). However, relaxation evoked by carbachol was not inhibited by pretreatment with 1 μM indomethacin ($P = 0.60$, Fig. 2).

3.3. Morphologic changes

Histologically, the cross-section of the rabbit ciliary artery in the age-matched control showed relatively regular invaginations of internal elastic lamina, whereas in the diabetic rabbit ciliary artery, invaginations of the internal elastic lamina clearly decreased. However, the size of the lumen was almost the same in both arteries (Fig. 3).

Electron-microscopically, deep invaginations of the internal elastic lamina in the control group were confirmed. The frequency of invaginations in the arteries of diabetic rabbits was relatively low, and the lamina was flattened. Furthermore, the cytoplasms of endothelial cells contain large vacuoles, indicating weak adhesion to the lamina. However, the smooth muscle cells were well preserved (Fig. 4). Some endothelial cells even showed vacuolar degeneration due to breakdown of their cell membranes (Fig. 5). Scanning electron-microscopic observations showed that the internal surface of the artery in the control group consisted of endothelial cells of a constant width, while that of the diabetic rabbits comprised irregularly arranged cells of varying sizes (Fig. 6). In the diabetic group, a network, considered to consist of fibrin, was

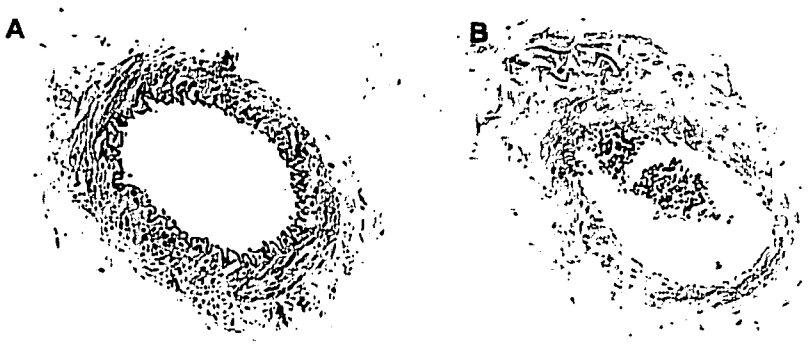

Fig. 3. Morphological cross-section (×200) of representative age-matched control (A) and diabetic (B) rabbit ciliary arteries.