Regular Article

4-Aminopyridine, a Voltage-Gated K⁺ Channel Inhibitor, Attenuates Nitric Oxide-Mediated Vasodilation of Retinal Arterioles in RatsAsami Mori,[†] Ryo Namekawa, Kenji Sakamoto,[†] Kunio Ishii, and Tsutomu Nakahara**Department of Molecular Pharmacology, Kitasato University School of Pharmaceutical Sciences; 5–9–1 Shirokane, Minato-ku, Tokyo 108–8641, Japan.*

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Nitric oxide (NO) is an important regulator of the retinal blood flow. The present study aimed to determine the role of voltage-gated K⁺ (K_V) channels and ATP-sensitive K⁺ (K_{ATP}) channels in NO-mediated vasodilation of retinal arterioles in rats. *In vivo*, the retinal vasodilator responses were assessed by measuring changes in the diameter of retinal arterioles from ocular fundus images. Intravitreal injection of 4-aminopyridine (a K_V channel inhibitor), but not glibenclamide (a K_{ATP} channel blocker), significantly attenuated the retinal vasodilator response to the NO donor (±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (NOR3). Intravitreal injection of indomethacin (a non-selective cyclooxygenase inhibitor) also reduced the NOR3-induced retinal vasodilator response. The combination of 4-aminopyridine and indomethacin produced a greater reduction in the NOR3-induced response than either agent alone. 4-Aminopyridine had no significant effect on pinacidil (a K_{ATP} channel opener)-induced response. These results suggest that the vasodilatory effects of NO are mediated, at least in part, through the activation of 4-aminopyridine-sensitive K_V channels in the retinal arterioles of rats. NO exerts its dilatory effect on the retinal vasculature of rats through at least two mechanisms, activation of the K_V channels and enhancement of prostaglandin production.

Key words ATP-sensitive K⁺ channel; cyclooxygenase; nitric oxide; retinal blood vessel; voltage-gated K⁺ channel

INTRODUCTION

Nitric oxide (NO) is a potent vasodilator and plays an important role in regulating blood flow.¹⁾ In many cases, NO-mediated vasodilator responses are primarily mediated by guanosine 3',5'-cyclic monophosphate (cGMP), which is synthesized by NO-activated soluble guanylyl cyclases.¹⁾ However, in the retinal circulation, cGMP plays a minor role in NO-mediated vasodilator responses.^{2–5)} In rats, the vasodilatory effects of NO on the retinal blood vessels are largely mediated by the cyclooxygenase-1/cAMP-mediated pathway.^{4,5)} However, a notable residual NO-induced vasodilation observed in animals treated with indomethacin, a non-selective cyclooxygenase inhibitor, and SC-560, a selective cyclooxygenase-1 inhibitor, suggests the cAMP- and cGMP-independent vasodilatory mechanisms.

It has been reported that NO modulates multiple K⁺ channels, such as large-conductance Ca²⁺-dependent K⁺ (BK_{Ca}) channels, voltage-gated K⁺ (K_V) channels, and ATP-sensitive K⁺ (K_{ATP}) channels, in the vascular smooth muscles.^{6–11)} We previously demonstrated that the BK_{Ca} channel inhibitor, iberiotoxin, had no significant preventive effects on the retinal vasodilator responses to NO donors in rats.¹²⁾ Therefore, in the present study, we investigated the roles of K_V channels and K_{ATP} channels in NO-mediated vasodilation of retinal arterioles of rats *in vivo*.

MATERIALS AND METHODS

All procedures in this study were approved by the Institutional Animal Care and Use Committee for Kitasato University (Approval No. T04-1, 17-28) and are in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research published by the US National Institutes of Health.

Experimental Procedures Thirty-five male Wistar rats (8–10 week-old) were maintained in a room with constant temperature (22 ± 2°C), humidity (55 ± 5%), and a 12-h light/dark cycle and were allowed free access to standard rat chow and tap water.

The experimental procedures used in this study were described in our previous reports.^{5,12,13)} Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally; Nacalai Tesque, Kyoto, Japan). After the disappearance of the corneal reflex, tracheotomy was performed for artificial ventilation. Catheters were inserted into the femoral and jugular veins and the left femoral artery for the administration of drugs and measurement of arterial pressure, respectively. The heart rate was measured using a cardi tachometer triggered by the blood pressure pulse. The mean arterial pressure and heart rate were digitized at 1 Hz (15BXW-H4, Dacs Giken, Okayama, Japan) and stored on the hard disk of a personal computer. Additional pentobarbital sodium (10 mg/kg) was administered as required.

The anesthetized rats were treated with tetrodotoxin (50 µg/kg, intravenously (i.v.); Nacalai Tesque) under artificial ventilation with room air (stroke volume, 10 mL/kg; frequency, 80 strokes/min) to prevent eye movements for capturing

[†]Present address: Laboratory of Medical Pharmacology, Department of Clinical & Pharmaceutical Sciences, Faculty of Pharma-Sciences, Teikyo University; 2–11–1 Kaga, Itabashi-ku, Tokyo 173–8605, Japan.

*To whom correspondence should be addressed. e-mail: nakaharat@pharm.kitasato-u.ac.jp

fundus images at the same angle throughout the experiment. Treatment with tetrodotoxin reduced systemic blood pressure. Therefore, methoxamine hydrochloride (approx. $30\mu\text{g/kg/min}$; Sigma-Aldrich, St. Louis, MO, U.S.A.) was infused into the jugular vein using a syringe pump (Model 1140-001, Harvard Apparatus, South Natick, MA, U.S.A.) to compensate for the drop in blood pressure.

The fundus photography and retinal arteriolar diameter measurement were performed as described in our previous studies.^{5,12,13} Briefly, hydroxyethylcellulose drops (Scopisol 15[®]; Senju Pharmaceutical, Osaka, Japan) were administered to the cornea to prevent drying of the eye; the optic disc was centered and focused in the field of view. Sodium fluorescein (10%, 0.8mL/kg ; Sigma-Aldrich) and pontamine sky blue 6B (5%, 0.8mL/kg ; Tokyo-Kasei Kogyo, Tokyo, Japan) solutions were injected into the femoral vein to enhance the blood vessel contrast in the retina. Fundus images were captured before and during intravenous infusion of the vasodilators using a digital camera (EOS7D, Canon, Tokyo, Japan) equipped with a bore scope-type objective lens for small animals (Model 01; Scalar, Tokyo, Japan). A region of the fundus image (5184×3456 pixels, pixel size = $1\mu\text{m}$) containing a retinal arteriole was selected (138×276 pixels, $138 \times 276\mu\text{m}$), and the vessel diameter in the same region was measured throughout the experiment. The retinal vasodilator response was evaluated by measuring the changes in the diameter.

Protocols

Protocol 1: We examined the effects of 4-aminopyridine, indomethacin, and the combination of 4-aminopyridine and indomethacin on ($\pm$)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR3; Dojindo, Kumamoto, Japan)-induced vasodilation of retinal arterioles. 4-Aminopyridine (660nmol ; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), indomethacin (10nmol ; Sigma-Aldrich), or 4-aminopyridine (660nmol) plus indomethacin (10nmol), in a total volume of $10\mu\text{L}$, was injected into the vitreous cavity of the left eye before surgical procedures and tetrodotoxin treatment. The solutions for intravitreal injection were prepared with 0.12% Na_2CO_3 in saline. The control rats were similarly administered with the vehicle. Approximately 60 min after the intravitreal injection, intravenous infusion of NOR3 (0.5 – $10\mu\text{g/kg/min}$) was administered using a syringe pump

(Model 1140-001, Harvard Apparatus). We also examined the effects of 4-aminopyridine on the K_{ATP} channel opener pinacidil (1 – $30\mu\text{g/kg/min}$; Sigma-Aldrich)-induced responses to determine whether 4-aminopyridine alters the vasodilator responses induced by the activation of other K^+ channels.

Protocol 2: We examined the effects of the K_{ATP} channel blocker glibenclamide (20mg/kg , i.v.; Nacalai Tesque) on NOR3 (0.5 – $10\mu\text{g/kg/min}$, i.v.)-induced vasodilation of the retinal arterioles. Glibenclamide was dissolved in 0.1N NaOH and further diluted in 5% D-glucose solution (0.1N NaOH: 5% D-glucose solution = $1:4$). Glibenclamide, at the selected dose, markedly attenuated the retinal vasodilator responses induced by the doses of pinacidil.³⁾

Statistical Analyses An unpaired *t*-test was used to compare the values between the groups and the data obtained from the multiple groups were analyzed by ANOVA followed by Tukey's *post-hoc* test. A two-way ANOVA was used to compare the responses between the groups (PRISM6, GraphPad Software, San Diego, CA, U.S.A.). A *p*-value of <0.05 was considered statistically significant. All values are presented as the mean $\pm$ standard error of the mean (S.E.M.).

RESULTS

The baseline values of the retinal arteriolar diameter, mean arterial pressure, and heart rate could be adjusted to the same ranges (retinal arteriolar diameter, approx. $46\mu\text{m}$; mean arterial pressure, approx. 115mmHg ; and heart rate, approx. 350 beats/min) in the experimental groups, by changing the methoxamine infusion rates.

Figure 1 shows the representative fundus images captured at baseline and 10 min after initiation of the intravenous infusion of NOR3 ($10\mu\text{g/kg/min}$), which clearly dilated the retinal arterioles. The data are summarized in Fig. 2.

NOR3 (0.5 – $10\mu\text{g/kg/min}$, i.v.) increased the diameter of the retinal arterioles and reduced the MAP in a dose-dependent manner (Figs. 2A, B). However, it had no significant effect on the heart rate (Fig. 2C). Intravitreal injection of 4-aminopyridine (660nmol/eye) significantly prevented NOR3-induced vasodilation of the retinal arterioles without affecting the depressor responses (Figs. 2A, B). Similarly, indomethacin (10nmol/eye) prevented retinal vasodilation,

Fig. 1. Representative Fundus Images at Baseline and 10 min after Initiating Intravenous Infusion of NOR3 ($10\mu\text{g/kg/min}$) in Rats

The increase in diameter of the retinal arteriole in the selected region was 34%.

Fig. 2. Involvement of K_V Channels and Prostaglandins in the NO-Mediated Retinal Vasodilation

A–C: Changes in retinal arteriolar diameter (A, AD), mean arterial pressure (B, MAP) and heart rate (C, HR) induced by intravenous infusion of NOR3 (0.5–10 $\mu\text{g/kg/min}$) in rats following intravitreal injection of vehicle (0.12% Na_2CO_3 in saline, $n=5$), 4-aminopyridine (4-AP; 660 nmol, $n=5$), indomethacin (10 nmol, $n=5$), or 4-AP (660 nmol) plus indomethacin (10 nmol) ($n=5$). Data are expressed as percentage of the baseline level (values measured just before initiating the NOR3 infusion). * $p<0.05$.

Fig. 3. Effects of 4-Aminopyridine on the Pinacidil-Induced Retinal Vasodilation

A–C: Changes in retinal arteriolar diameter (A, AD), mean arterial pressure (B, MAP) and heart rate (C, HR) induced by intravenous infusion of pinacidil (1–30 $\mu\text{g/kg/min}$) in rats following intravitreal injection of vehicle (0.12% Na_2CO_3 in saline, $n=4$), or 4-aminopyridine (4-AP; 660 nmol, $n=4$). Data are expressed as percentage of the baseline level (values measured just before initiating the pinacidil infusion).

Fig. 4. Role of K_{ATP} Channels in the NO-Mediated Retinal Vasodilation

A–C: Changes in retinal arteriolar diameter (A, AD), mean arterial pressure (B, MAP) and heart rate (C, HR) induced by intravenous infusion of NOR3 (0.5–10 $\mu\text{g/kg/min}$) in rats following intravenous injection of glibenclamide (20 mg/kg, $n=4$) of the vehicle ($n=3$). Data are expressed as percentage of the baseline level (values measured just before initiating the NOR3 infusion).

but not the depressor responses to NOR3. The combination of 4-aminopyridine and indomethacin reduced the retinal vasodilator response more than either drug alone.

Pinacidil (1–30 $\mu\text{g/kg/min}$, i.v.) also increased the diameter of the retinal arterioles and reduced the MAP in a dose-dependent manner (Figs. 3A, B). However, intravitreal injection of 4-aminopyridine did not affect pinacidil-induced vasodilation of the retinal arterioles (Fig. 3A). Thus, it is unlikely that 4-aminopyridine affects K_{ATP} channels under our experimental conditions.

Glibenclamide (20 mg/kg, i.v.) had no significant effects on NOR3-induced responses (Fig. 4).

DISCUSSION

Our previous studies have shown that the NO-induced vasodilator response is mediated through the cyclooxygenase-1/prostaglandin I_2 /prostanoid IP receptor/cAMP signaling pathway in the retinal arterioles of rats.^{4,5} In the present study, we found that 4-aminopyridine, as well as indomethacin, significantly prevented the retinal vasodilator response to NOR3. The combination of 4-aminopyridine and indomethacin showed a further reduction of the NOR3-induced response. These results suggest that, in addition to the enhancement of prostaglandin production, activation of 4-aminopyridine-sensitive K_V channels likely contributes to the NO-induced vasodilator response in the retinal arterioles of rats.

Multiple K^+ channels contribute to the regulation of blood flow in various vascular beds. NO is one of the modulators of the activities of these multiple K^+ channels in vascular smooth muscles.^{6–11} Our present results suggest that NO activates 4-aminopyridine-sensitive K_V channels, thereby dilating retinal arterioles of rats. However, it is unlikely that NO affects

the activity of BK_{Ca} and K_{ATP} channels in the retinal arterioles because neither the BK_{Ca} channel inhibitor iberiotoxin¹²⁾ nor the K_{ATP} channel blocker glibenclamide (current study) attenuated NOR3-induced vasodilation of the retinal arterioles.

NO could activate the K⁺ channels directly or through a cGMP-dependent mechanism.^{14–16)} Our previous studies demonstrated that a soluble guanylyl cyclase inhibitor failed to prevent retinal vasodilator responses to NO donors.⁴⁾ Therefore, the involvement of cGMP-dependent mechanism in the activation of 4-aminopyridine-sensitive K_V channels may be unlikely in the retinal arterioles of rats. Vasodilators that act through the Gs-coupled receptors and the cAMP signaling pathway also activate the 4-aminopyridine-sensitive K_V channels.¹⁷⁾ To test the possibility that NO activates 4-aminopyridine-sensitive K_V channels through the cAMP-dependent mechanism, we examined the effects of 4-aminopyridine on NOR3-induced retinal vasodilation following blockade of the cyclooxygenase-1/prostaglandin I₂/prostanoid IP receptor/cAMP signaling pathway by indomethacin. The results suggest that NO activates the 4-aminopyridine-sensitive K_V channels independently of cAMP in the retinal arterioles of rats. Thus, NO may activate 4-aminopyridine-sensitive K_V channels in cAMP- or cGMP-independent mechanisms in the retinal vascular bed. With regard to the direct action, NO modifies the K⁺ channel or a closely associated regulatory protein, by S-nitrosylation.^{18,19)} In human cardiac fibroblasts, the effects of NO on K_V channels are not mediated by S-nitrosylation.²⁰⁾ However, 4-aminopyridine inhibits K_{V1–4} channel subtypes,¹⁷⁾ and it remains unclear which subtypes are involved NO-induced vasodilation of retinal arterioles. Therefore, NO may directly affect activity of 4-aminopyridine-sensitive K_V channels expressed in rat retinal arterioles.

The mechanism by which NO preferentially stimulates the cGMP-independent pathway remains to be elucidated. Previous immunohistochemical studies revealed that vascular smooth muscle cells of the retinal blood vessels showed a low expression of soluble guanylyl cyclase.^{4,21)} Because of the low expression level of soluble guanylyl cyclase in the retinal blood vessels, NO may preferentially interact with cyclooxygenase^{22–25)} and 4-aminopyridine-sensitive K_V channels, but not with soluble guanylyl cyclase.

Our present results suggest that 4-aminopyridine-sensitive K_V channels (K_{V1–4}) contribute, at least in part, to the vasodilatory effects of NO on rat retinal arterioles. However, vascular smooth muscle cells have been reported to express members of the K_{V1–4}, K_{V6}, K_{V7}, K_{V9}, and K_{V11} families of K_V channels.¹⁷⁾ Other families of K_V channels (e.g., K_{V7}) might contribute to the regulation of retinal vascular tone. Therefore, further studies are needed to elucidate the expression of K_V channel subtypes in rat retinal arterioles and to examine the involvement of other families of K_V channels in NO-induced vasodilation of rat retinal arterioles.

In summary, we found that 4-aminopyridine-sensitive K_V channels contribute, at least in part, to the vasodilatory effects of NO on the retinal arterioles of rats. In the rat retinal circulation, the vasodilator action of NO is mediated through at least two mechanisms, opening of 4-aminopyridine-sensitive K_V channels and enhancement of prostaglandin production.

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Conflict of Interest The authors declare no conflict of interest.

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