INTRODUCTION

Nitric oxide (NO), prostaglandin I2, and endothelium-derived hyperpolarizing factors (EDHFs) are the major endogenous vasodilators in retinal circulation.1) In addition to transferable EDHFs, the hyperpolarization initiated in endothelial cells, which transmits to adjacent smooth muscle cells via gap junctions, induces endothelium-dependent hyperpolarization (EDH)-related vasodilation.2) Depending on the size and location of blood vessels and animal species, the vascular gap junctions comprise different connexin proteins (Cx): Cx37, Cx40, Cx43, and Cx45. Moreover, pharmacological blockade of gap junctions attenuates the EDH-related vasodilation.5–8) Thus, an electrical communication between endothelial and smooth muscle cells via gap junctions serve as a mechanism for regulating the vascular tone.

We have previously demonstrated that NO and EDHF contribute to the dilation of retinal arteriole to acetylcholine (ACh) in rats. In the rat retinal vasculature, NO causes retinal vasodilator response via stimulation of prostaglandin I2 production9) and activation of 4-aminopyridine-sensitive K+ channels.10) With regard to EDHF-mediated vasodilation, we have demonstrated the importance of large-conductance, Ca2+-activated K+ channels (BKCa) channels in rat retinal arterioles.11) More recently, we found that CYP epoxygenase-derived epoxyeicosatrienoic acids (EETs) may function as an EDHF in the rat retinal vasculature.12) However, the role of gap junctions in the EDH-related dilation of retinal blood vessels remains to be elucidated. Therefore, in the present study, we aimed to determine whether gap junctions contribute to ACh-induced, EDH-type dilation of rat retinal arterioles.

MATERIALS AND METHODS

Animals

In total, 42 male Wistar rats (8–10 weeks old) were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). The rats were housed in a temperature (22 ± 2°C)- and humidity (55 ± 5%)-controlled animal room, subjected to a 12-h light–dark cycle (lights on at 8.00 a.m. and off at 8.00 p.m.), supplied with a standard diet (Japan SLC, Inc., Hamamatsu, Japan), and were given access to tap water ad libitum. All animal experiments described in this study were approved by the Institutional Animal Care and Use Committee for Kitasato University (Approval No. T04-1). All animals care techniques and experimental procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animal at Kitasato University and the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research.

Reagents

The following reagents were used: ACh chloride, carbamoxonolone, 18β-glycyrrhetinic acid, indomethacin, methoxamine hydrochloride, N⁴-nitro-1-arginine methyl ester (L-NAME) (Sigma-Aldrich, St. Louis, MO, U.S.A.), pentobarbital sodium, tetrodotoxin (Nacalai Tesque, Kyoto, Japan), 18β-glycyrrhetinic acid, ibero

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toxin (Peptide Institute, Osaka, Japan). 18β-glycyrrhetinic acid and carbenoxolone were dissolved in dimethyl sulfoxide (DMSO) and further diluted with saline (final concentration of DMSO, 1%). Indomethacin was dissolved in 0.24% sodium carbonate. All other drugs were dissolved in saline.

**General Preparations** Surgical procedures were performed as described previously.9–12 Briefly, rats were intraperitoneally anesthetized with pentobarbital sodium (50 mg/kg). The trachea, left femoral artery, and jugular and femoral veins were cannulated for adequate ventilation, measurement of blood pressure and heart rate, and drug administration, respectively. Additional pentobarbital sodium (10 mg/kg) was injected as needed.

The rats were mechanically ventilated and the eye movement was prevented by a single intravenous (i.v.) injection of tetrodotoxin (50 µg/kg) to capture fundus images at the same angle throughout the experiment. After tetrodotoxin injection, the systemic blood pressure decreased. To maintain adequate systemic blood pressure, the rats were intraperitoneally injected with pentobarbital sodium (50 mg/kg). The systemic blood pressure decreased. To maintain adequate systemic blood pressure.

**Measurement of Retinal Arteriolar Diameters** Fundus images were captured before and during intravenous infusion of ACh or NOR3 as described in our previous reports.9–12 A region (138 × 276 µm) of the fundus image (3456 × 5184 µm) containing a retinal arteriole was selected, and the diameter of the vessel was measured throughout the experiment.

**Data Analysis** The diameter of retinal arteriole, mean arterial pressure, and heart rate were expressed as a percentage (%) of the values obtained at steady state just before commencing infusion of ACh or NOR3 (i.e., mean values of the data obtained at the time from ~2 to 0 min).

All values are presented as mean ± standard error of the mean (S.E.M.). The baseline values between the multiple groups were analyzed by one-way ANOVA, and the responses to ACh or NOR3 between the multiple groups were analyzed by linear mixed models, followed by Tukey–Kramer’s honestly significant difference (HSD)-test (JMP Pro 15.1.0, SAS Institute Inc., Cary, NC, U.S.A.). A p-value < 0.05 was considered statistically significant.

**RESULTS**

Table 1 summarizes the parameters measured just before commencing infusion of ACh or NOR3 in each protocol. No significant differences were observed in baseline values of retinal arteriolar diameter and heart rate among the experimental groups in each protocol. However, baseline values of mean arterial pressure were higher in the presence of l-NAME and indomethacin (Protocol 2) than in those in the absence of the inhibitors (Protocols 1, 3).

Figure 1 illustrates the representative images of the fundus captured at baseline and 0.5 min after commencing infusion of ACh (10 µg/kg/min, i.v.). ACh induced a remarkable increase

| Groups | Retinal arteriolar diameter (µm) | Mean arterial pressure (mmHg) | Heart rate (beats/min) |
|--------|---------------------------------|------------------------------|------------------------|
| Protocols 1&3 | Vehicle (n = 9) | 43.4 ± 0.7 | 120 ± 2 | 327 ± 7 |
| | 18β-Glycyrrhetinic acid (n = 8) | 43.7 ± 1.0 | 118 ± 3 | 342 ± 6 |
| | Carbenoxolone (n = 5) | 41.8 ± 1.4 | 121 ± 3 | 332 ± 12 |
| Protocol 2 | l-NAME + indomethacin + vehicle (n = 5) | 43.3 ± 2.2 | 137 ± 3 | 349 ± 6 |
| | l-NAME + indomethacin + 18β-glycyrrhetinic acid (n = 6) | 42.3 ± 2.3 | 142 ± 5 | 333 ± 11 |
| | l-NAME + indomethacin + iberiotoxin (n = 5) | 40.1 ± 1.4 | 141 ± 4 | 325 ± 5 |
| | l-NAME + indomethacin + 18β-glycyrrhetinic acid + iberiotoxin (n = 4) | 38.7 ± 2.4 | 145 ± 4 | 331 ± 10 |

The values were measured just before commencing the infusion of ACh or NOR3. Values are means ± S.E.M.
In accordance with our previous reports, ACh (0.3–10 µg/kg/min, i.v.) caused a dose-dependent increase in the diameter of retinal arterioles (Fig. 2A). The retinal vasodilator responses to ACh (1–10 µg/kg/min, i.v.) were composed of an initial, phasic component (within 1 min after increasing the dose) and a sustained component. Both components were diminished by intravitreal injection of 18β-glycyrrhetinic acid (2 nmol/eye) or carbenoxolone (2 nmol/eye). ACh decreased the mean arterial pressure in a dose-dependent manner, whereas the gap junction blockers had no significant effect on the depressor response (Fig. 2B). No significant changes were observed in the heart rate (Fig. 2C).

The responses to ACh under treatment with L-NAME and indomethacin are illustrated in Fig. 3. Inhibition of NO synthase and cyclooxygenase attenuated the initial, phasic component of ACh-induced dilation of retinal arterioles. For example, the retinal arteriolar diameter in the presence of L-NAME and indomethacin at 0.5 min after increasing the infusion rate from 3 to 5 µg/kg/min (110.2 ± 2.6%, n = 5, Fig. 3A) was smaller than that in the absence of the inhibitors (126.8 ± 1.6%, n = 5, see Fig. 2A). On the other hand, there was no significant difference in the ACh-induced depressor responses between the absence and presence of L-NAME and indomethacin (vehicle group in Fig. 2B vs. L-NAME + indomethacin + vehicle group in Fig. 3B). The L-NAME and indomethacin-resistant component of dilation of retinal arterioles was remarkably smaller in 18β-glycyrrhetinic acid-injected eyes than that in vehicle-injected eyes (Fig. 3A); however, intravitreal injection of 18β-glycyrrhetinic acid plus ibeberiotoxin, or the vehicle was injected intravitreally into the rat eye. Both 18β-glycyrrhetinic acid and ibeberiotoxin showed the preventive effect on the ACh-induced, EDH-type dilation of the retinal arterioles. The effects were additive. n = 4–6 *p < 0.05.

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NOR3 (0.5–10 µg/kg/min, i.v.), like ACh, increased the diameter of retinal arterioles and reduced the mean arterial pressure in a dose-dependent manner (Figs. 4A, B). The NOR3-induced responses remained unaffected by intravitreal
injection of 18β-glycyrrhetinic acid (2 nmol/eye) (Figs. 4A, B). Heart rate showed no significant changes during infusion of NOR3 (Fig. 4C). Thus, it is unlikely that 18β-glycyrrhetinic acid impairs NO-induced dilation of retinal arterioles under our experimental conditions.

DISCUSSION

The present study demonstrates that gap junction blockers, namely 18β-glycyrrhetinic acid and carbenoxolone, prevented the ACh-induced dilation of retinal arterioles in rats in vivo. The retinal vasodilator response to ACh was attenuated by 18β-glycyrrhetinic acid under a combined treatment with L-NAME and indomethacin. These findings indicate that gap junction-mediated cell-cell interaction may play a pivotal role in the ACh-induced, EDH-type dilation of rat retinal arterioles.

Under our experimental conditions, ACh (1–10 µg/kg/min, i.v.) induced an initial increase in diameter of retinal arterioles (phasic component) followed by a decline to a sustained level (sustained component). The combined treatment with L-NAME and indomethacin preferentially suppressed the initial, phasic component of retinal vasodilator responses rather than their sustained components. On the other hand, gap junction blockers attenuated both phasic and sustained components. Thus, the relative importance of NO and prostaglandin I₂ may differ between the phasic and sustained component of ACh-induced dilation of retinal arterioles.

The NO- and prostaglandin-independent vasodilator response is generally associated with EDH. Two pathways have
been proposed to explain EDH: 1) endothelium-derived diffusible substances (EDHFs) hyperpolarize vascular smooth muscle cells by stimulating K<sub>Ca</sub> channels, and 2) the transfer of hyperpolarization initiated in the endothelial cells to the adjacent smooth muscle cells via myoendothelial gap junctions. We recently found that CYP epoxidegenase-derived EETs may function as an EDHF in the rat retinal vasculature and dilate the retinal arterioles by activating BK<sub>Ca</sub> channels. Indeed, iberiotoxin attenuated the ACh-induced retinal vasodilator response seen in the presence of l-NAME plus indomethacin. Interestingly, the combined treatment with 18β-glycyrrhetinic acid and iberiotoxin produced a marked effect on ACh-induced response compared to that by the individual treatment. These results suggest that the release of EDHFs and the transfer of endothelial hyperpolarization via myoendothelial gap junctions separately contribute to the ACh-induced, EDH-type dilation of retinal arterioles (Fig. 5).

In addition to myoendothelial gap junctions, interendothelial gap junctions and gap junctional communication between smooth muscle cells can also modulate vascular tone. The expression pattern of Cxs varies depending on vascular beds and animal species; however, in general, Cx37, Cx40, and Cx43 are present between endothelial cells, and Cx43 and either Cx40 or Cx37 form myoendothelial gap junctions. Arteriolar smooth muscle cells express predominantly Cx43 and Cx45 and these Cxs can form smooth muscle cell-smooth muscle cell connections. In retinal arterioles, 18β-glycyrrhetinic acid and carbeneoxolone are broad-spectrum connexin channel and gap junction blockers. Therefore, the gap junctions formed by multiple Cxs could be interrupted by these blockers. An immunohistochemical study revealed that in the rat retina Cx37 and Cx40 were detected in endothelial cells, but not smooth muscle cells, in arterioles. Cx43 was absent from arterioles, whereas it was detected in astrocytes. Although there is a possibility that myoendothelial gap junctions could not be identified by immunostaining, it remains unclear at present how myoendothelial gap junctions are formed in the rat retinal arteriole.

In endothelial cells expressing the muscarinic M3 receptors, ACh-induced Ca<sup>2+</sup> increases can spread adjacent endothelial cells via carbeneoxolone-sensitive gap junctions. The increased Ca<sup>2+</sup> elicits multiple Ca<sup>2+</sup>-dependent responses, including formations of NO, prostaglandins, and EETs, in endothelial cells. Thus, blockade of interendothelial gap junctions may contribute to the attenuation of retinal vasodilator response to ACh. Interendothelial gap junctional communication can be positively regulated by EETs, and CYP epoxidegenase-derived EETs contribute to the ACh-induced, EDH-type dilation of retinal arterioles. Therefore, it would be interesting to investigate how gap junction blockers affect EET-related retinal vasodilator response.

The mechanism underlying the initiation of endothelial hyperpolarization in rat retinal arterioles, remains to be elucidated. In several vascular beds, endothelial hyperpolarization is activated via intermediate-conductance K<sub>Ca</sub> (IK<sub>Ca</sub>) channels and small-conductance K<sub>Ca</sub> (SK<sub>Ca</sub>) channels, however, in rat retinal arterioles, iberiotoxin reduced the dilator response to ACh, whereas neither TRAM-34, a blocker of IK<sub>Ca</sub> channels, nor apamin, a blocker of SK<sub>Ca</sub> channels, altered the response. Thus, the BK<sub>Ca</sub> channel, and not the IK<sub>Ca</sub> and SK<sub>Ca</sub> channels, appeared to be primarily responsible for the ACh-induced, EDH-type dilation. It remains unclear whether the functional BK<sub>Ca</sub> channels are present in endothelial cells in rat retinal arterioles. Therefore, further research is needed to clarify the detailed mechanism of EDH-related dilation of retinal arterioles.

It has been demonstrated that NO affects the gap junction function, particularly when NO production is stimulated. ACh stimulates NO production via endothelial NO synthase (eNOS) in vascular endothelial cells. Nevertheless, 18β-glycyrrhetinic acid did not affect the NOR3-induced dilation of retinal arterioles. Under same experimental conditions, iberiotoxin had no significant effect on the retinal vasodilator response to NOR3. Thus, NO produced in the endothelial cells may play a minor role in modulating the gap junction function in rat retinal arterioles. The stimulation of NO production in endothelial cells and the activation of BK<sub>Ca</sub> channels in smooth muscle cells could independently contribute to ACh-induced dilation of rat retinal arterioles.

In summary, the present study is the first to provide pharmacological evidence suggesting the contribution of gap junctions to the NO- and prostaglandin-independent, EDH-type dilation of retinal arterioles in rats in vivo.

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Conflict of Interest The authors declare no conflict of interest.

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