学位論文

Beraprost Sodium, a Stable Prostacyclin Analogue, Elicits Dilation of Isolated Retinal Arterioles: Roles of eNOS and Potassium Channels

（ベラプロストナトリウムは網膜細動脈を拡張させる）

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Beraprost Sodium, a Stable Prostacyclin Analogue, Elicits Dilation of Isolated Porcine Retinal Arterioles: Roles of eNOS and Potassium Channels

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Running title: BPS-Induced Vasodilation in Retinal Arterioles

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Supported by a Grant-in-Aid for Scientific Research (B) 25293352 and Challenging Exploratory Research 25670724 from the Ministry of Education, Science, and Culture, Tokyo. (TN)

Word count: 3,267 words

The authors have no financial/conflicting interests to disclose.

ABSTRACT

PURPOSE. Prostacyclin (PGI_2) is usually described as an endothelium-derived relaxing factor, but the vasoreactivity to PGI_2 in the retinal arterioles and the underlying mechanisms are not fully understood. We examined the effects of PGI_2 on the retinal microcirculation using beraprost sodium (BPS), a stable PGI_2 analogue, and the signaling mechanisms involved in this vasomotor activity.

METHODS. Porcine retinal arterioles were isolated, cannulated, and pressurized without flow in vitro. Video microscopic techniques recorded the diametric responses to BPS.

RESULTS. Beraprost sodium elicited dose-dependent (0.1 pM-0.1 μM) vasodilation of the retinal arterioles that was abolished by the PGI_2 receptor (IP) antagonist CAY10441. Beraprost sodium-induced vasodilation decreased by 50% after the endothelium was removed and was inhibited by the nitric oxide (NO) synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) comparable with denudation. Inhibition of soluble guanylyl cyclase by 1H-1,2,4-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and blockage of protein kinase A (PKA) by Rp-8-Br-cAMPS were comparable to L-NAME. Beraprost sodium-induced vasodilation was also inhibited by the nonselective potassium channel inhibitor, tetraethylammonium, and the adenosine triphosphate-sensitive potassium (K_{ATP}) channel blocker, glibenclamide. Residual vasodilation in the presence of glibenclamide decreased further with subsequent application of ODQ.

CONCLUSIONS. Beraprost sodium, a stable PGI_2 analogue, causes vasodilation of the retinal

arterioles mediated via the IP receptor. The current findings suggest that BPS elicits endothelium-dependent and -independent dilation of the retinal arterioles mediated by NO induced by activation of PKA in the endothelium and the K_{ATP} channel activation in the vascular smooth muscle, respectively. (240 words)

Diabetes mellitus is a multifactorial condition characterized by hyperglycemia, leading to both macro- and microvascular complications such as atherosclerosis, nephropathy, neuropathy, and retinopathy.[1] Several reports have shown that impaired endothelial function could play an important role in development of diabetic retinopathy (DR) in patients with type 2 diabetes.[2-4] Indeed, the vascular endothelium regulates vascular tone by producing endothelium-derived relaxing factors (EDRFs)—in other words, nitric oxide (NO), prostacyclin (PGI_2), and endothelium-derived hyperpolarizing factor (EDHF).[5] Moreover, production of PGI_2 and NO, which are known to be powerful retinal vasodilators generated from the endothelium, decreases in patients with diabetes.[6-9] We previously reported that the retinal blood flow (RBF) and endothelial function are impaired in patients with type 2 diabetes mellitus with no and mild DR,[10,11] suggesting that the impaired RBF caused by reduction of EDRFs may contribute to the pathogenesis of DR. Therefore, there is a possibility that a drug enhancing the effects of EDRFs on retinal circulation may be a novel therapeutic agent for DR.

Prostacyclin is a major product of arachidonic acid metabolism and exhibits various physiologic effects such as vasodilation,[12,13] protection of endothelial function,[14] and anti-aggregation.[12,13] Prostacyclin-induced vasodilation may be mediated by activation of the PGI_2 receptor (IP receptor), which leads to elevation of cyclic adenosine monophosphate (cAMP) levels in the vascular smooth muscle cells,[12,15] whereas previous studies have reported that PGI_2 also can promote vasoconstriction mediated by activation of thromboxane A_2 receptor (TP receptor) in rat pulmonary arteries[16] and prostaglandin E_2 receptor subtype (EP_1

receptor) in rat mesenteric arteries.[17] Thus, vasomotor activity in response to PGI_2 varies by vascular beds of various tissues and vessel size depending on distribution of prostaglandin receptors. Although previous studies have reported that PGI_2 induced vasodilation of retinal arterioles,[18,19] the underlying mechanisms of the response to PGI_2 are not fully understood. It is worth noting that PGI_2 is degraded rapidly in a few minutes and is unsuitable as a clinical drug; therefore, a number of PGI_2 analogues have been developed.[20] Among the stable PGI_2 analogues, it has been reported that beraprost sodium (BPS) has a higher affinity for the IP receptor than PGI_2 per se, owing to its chemical characteristics.[21] Indeed, there were some clinical studies to report that BPS is beneficial for treating various vascular disorders.[22,23] Taken together, it is reasonable to consider that BPS may be more suitable for investigating the effect of PGI_2 to retinal microcirculation. Herein, we examined the effect of a stable PGI_2 analogue BPS on the retinal microvessels and the signaling mechanisms involved in this vasomotor activity using a technique to isolate retinal arterioles.

MATERIALS AND METHODS

Animal Preparation

The Animal Care Committee of Asahikawa Medical University approved all animal procedures, which were performed according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The eyes were enucleated immediately from pigs of either sex (age, 16-24 weeks; weight, 25-35

kg) after the animals were killed in a local abattoir and transported to the laboratory in a moist chamber on ice.

Isolation and Cannulation of Microvessels

The techniques used to identify, isolate, cannulate, pressurize, and visualize the retinal microvessels have been described previously.[24-27] Briefly, single second-order retinal arterioles (90-110 μm in situ) were dissected with microdissection forceps and the isolated retinal arterioles were cannulated with a pair of glass micropipettes and pressurized to 55 cm H_2O intraluminal pressure without flow using two independent pressure reservoir systems.[28] The internal diameter of the isolated vessels was recorded continuously using video microscopic techniques throughout the experiments.[24]

Control Experiment

Cannulated and pressurized arterioles were bathed in physiologic saline solution (PSS) with albumin (0.1%) at 36°C to 37°C to allow development of basal tone. After the vessels developed a stable basal tone (~30-40 minutes), dose-dependent vasodilation to various concentrations of BPS (dose range, 0.1 pM-0.1 μM) was evaluated. The vessels were exposed to each concentration of agonists for 3 to 5 minutes until a stable diameter was established. After the control responses were completed, the vessels were washed with PSS to allow redevelopment of basal tone. The vasodilation elicited by BPS was reexamined after 30

minutes to confirm the response reproducibility.

Role of Prostaglandin Receptors in BPS-Induced Dilation

To study the involvement of the prostaglandin receptors (i.e., IP, TP, EP_1, and EP_3) on BPS-induced dilation, we assessed the arterioles preincubated with the IP antagonist CAY10441 (0.1 μM),[29] TP antagonist SQ29548 (10 μM),[30] EP_1 antagonist SC19220 (10 μM),[31] and EP_3 antagonist L-798106 (1 μM),[32] respectively.

Mechanistic Studies of BPS-Induced Dilation

In the first series of studies, we examined the role of the endothelium in BPS-induced dilation by comparing the responses before and after removal of the endothelium by intraluminal perfusion of the nonionic detergent CHAPS (0.4%) as described previously.[26,27,33] We also assessed the involvement of endothelium-derived vasodilators (i.e., NO and cytochrome P450 metabolites), in mediating the vascular response in the presence of known effective concentrations of specific enzyme inhibitors N^G-nitro-L-arginine methyl ester (L-NAME, 10 μM),[24,25] and sulfaphenazole (10 μM),[34] respectively. We also assessed the effects of EDHF using the large- and intermediate-conductance Ca^{2+}-activated K channel (BK_{Ca} and IK_{Ca}) blocker charybdotoxin (ChTx, 0.1 μM) plus the small-conductance Ca^{2+}-activated K channel (SK_{Ca}) blocker apamin (0.1 μM) because these potassium channels are required to activate EDHF-type relaxation.[25,35,36] We assessed the role of guanylyl cyclase/cyclic guanosine

monophosphate (cGMP) signaling by treating vessels with the soluble guanylyl cyclase inhibitor 1H-1,2,4-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 0.1 μM).[25,26]

In the second series of studies, to examine the involvement of protein kinase A (PKA), we studied the BPS-induced response after incubation with the PKA inhibitor, Rp-8-Br-cAMPS (100 μM).[37]

In the third series of studies, to elucidate the involvement of the K channels, we examined this pathway by treating the vessels with various potassium channel inhibitors: the nonselective potassium channel blocker tetraethylammonium (TEA, 10 mM),[38] BK_{Ca} channel blocker iberiotoxin (0.1 μM),[38,39] IK_{Ca} channel blocker TRAM34 (1 μM),[40] SK_{Ca} channel blocker apamin (0.1 μM),[41] voltage-gated K^+ channel blocker 4-AP (0.1 mM),[42] adenosine triphosphate-sensitive potassium (K_{ATP}) channel blocker glibenclamide (5 μM),[25] and the inward rectifier K^+ channel blocker barium chloride ($BaCl_2$, 30 μM).[43]

Response to Sodium Nitroprusside

Sodium nitroprusside (SNP, 0.1-100 μM) was used to probe endothelium-independent vasodilation. The vascular response to SNP was examined in the presence of various interventions, as mentioned previously.

All drugs were administered extraluminally unless otherwise stated. The vessels were incubated with each pharmacologic inhibitor for a minimum of 30 minutes.

Immunohistochemistry

The immunohistochemical detection of the vascular IP receptor was performed after preparation of cryomicrotome sections of the retinal arterioles. We previously described the techniques for immunohistochemical staining of the isolated retinal arterioles.[27] We used the following specific primary antibodies: an anti-IP receptor antibody, an anti-endothelial NO synthase (eNOS) antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and an anti-α-smooth muscle actin antibody (Sigma-Aldrich Corp., St. Louis, MO, USA). The slides then were incubated with fluorescein isothiocyanate (FITC)-conjugated antibody (Santa Cruz Biotechnology, Inc.), Alexa Fluor 647-conjugated antibody (Invitrogen, Carlsbad, CA, USA), and Cy3-conjugated antibody (GE Healthcare Life Sciences, Piscataway, NJ, USA) and observed for green (FITC), blue (Alexa Fluor 647), and red (Cy3) staining and analyzed with a confocal microscope (FluoView FV1000; Olympus, Tokyo, Japan). Merged images were created using Java-based imaging software (ImageJ, http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Measurement of Nitrite/Nitrate

The stable NO end products nitrite and nitrate, collectively NOx, were measured by high-performance liquid chromatography (ENO-20; Eicom, Kyoto, Japan). We collected samples from the chamber 5 minutes after administration of BPS 0.1 μM and measured NOx

production using the Griess method.[44]

Chemicals

Beraprost sodium was obtained from Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). CAY10441, SQ 29548, and SC19220 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Other drugs were purchased from Sigma-Aldrich Corp. CAY10441, SQ29548, SC19220, L-798106, TRAM34, and glibenclamide were dissolved in dimethyl sulfoxide (DMSO). Sulfaphenazole and ODQ were dissolved in ethanol; other drugs were dissolved in PSS. All subsequent dilutions of these drugs were prepared in PSS. The final concentrations of DMSO and ethanol in the vessel bath were less than 0.1%. Vehicle controlled studies indicated that these final solvent concentrations did not affect the arteriolar diameter.[25]

Data Analysis

At the end of each experiment, the vessels were relaxed in ethylenediaminetetraacetic acid (1 mM) calcium-free PSS to obtain the maximal diameter at 55 cm H_2O intraluminal pressure.[24,26] All diametric changes in response to agonists were normalized to this maximal vasodilation and expressed as a percentage of the maximal dilation.[24,26] Data are reported as the mean ± SEM; *n* represents the number of vessels studied. Statistical comparisons of the changes in resting tone by antagonists were performed with the Student's *t*-test. Two-way ANOVA, followed by the Bonferroni multiple-range test, was used to determine the significance of the

difference between the control and the experimental interventions. One-way ANOVA followed by Dunnett's post hoc comparison was used to determine the significance of changes in the baseline diameter using different concentrations of agonists. Statistical differences in NOx production between agonists and vehicle treatment were examined using the Mann-Whitney *U* test. $P < 0.05$ was considered significant.

RESULTS

Dilation of Retinal Arterioles Induced by BPS

The basal tone in all vessels ($n = 88$) ranged from 54% to 68% (average, 62% ± 1%) of the maximal diameter. The average resting and maximal vessel diameters were 60 ± 1 μm and 97 ± 1 μm, respectively. Beraprost sodium induced dose-dependent dilation of the retinal arterioles within 3 to 5 minutes. The threshold concentration for vasodilation was 10 pM, and the highest concentration (0.1 μM) of BPS caused approximately 70% of the maximal dilation (Fig. 1). Further study showed that BPS-induced dilation was reproducible and did not deteriorate after repeated applications (Fig. 1).

Role of Prostaglandin Receptors

Inhibition of the TP, EP_1, and EP_3 by SQ29548, SC19220, and L-798106, respectively, did not affect the vasodilatory response to BPS (Fig. 2). Blockage of the IP receptor by CAY10441 abolished the BPS-induced vasodilation. These agents did not alter the basal tone.

Role of the Endothelium and Endothelium-Derived Factors

In the denuded vessels, the BPS-induced dilation decreased partly and the response to the highest BPS concentration significantly ($P < 0.01$) decreased from 70% to 40% (Fig. 3A). The NOS inhibitor L-NAME significantly ($P < 0.001$; Fig. 3A) reduced BPS-induced vasodilation, which was comparable to that produced by denudation (L-NAME versus denudation, $P > 0.05$). In addition, the NOx levels in the vessel chamber significantly ($P < 0.001$) increased after application of BPS compared with vehicle (Fig. 3B). Inhibition of cytochrome P450 epoxygenase and the combination of BK_{Ca}, IK_{Ca}, and SK_{Ca} by sulfaphenazole and apamin plus ChTx did not affect the vasodilatory response to BPS (Fig. 3A). The vasodilatory response to BPS was significantly ($P < 0.01$) reduced by ODQ in a manner similar to L-NAME. Any pretreatment did not significantly alter the basal tone.

Localization of the IP Receptor in the Retinal Arterioles

In the retinal arterioles, the IP receptor was expressed in the vascular endothelium and the smooth muscle (Fig. 4).

Role of PKA

The PKA inhibitor Rp-8-Br-cAMPS significantly ($P < 0.01$) inhibited BPS-induced vasodilation (Fig. 5) comparable with that produced by L-NAME. The combination of

Rp-8-Br-cAMPS and L-NAME did not further reduce the vasodilatory response to BPS compared with Rp-8-Br-cAMPS alone. Incubation with Rp-8-Br-cAMPS also inhibited elevation of NOx levels in the vessel chamber induced by BPS (Fig. 3B). Rp-8-Br-cAMPS did not affect the basal tone.

Role of Potassium Channels

Tetraethylammonium significantly ($P < 0.05$) inhibited BPS-induced vasodilation of the retinal arterioles (Fig. 6). In addition, glibenclamide attenuated BPS-induced dilation of the retinal arterioles in a manner similar to that of TEA, but 4-AP, iberiotoxin, TRAM34, apamin, and $BaCl_2$ were ineffective (Fig. 6). These agents did not affect the basal tone. Residual vasodilation in the presence of glibenclamide significantly ($P < 0.01$) decreased further after coincubation with the soluble guanylyl cyclase inhibitor ODQ.

Response to SNP

Various interventions did not affect the SNP-induced dilation of the retinal arterioles (Table), suggesting that the vascular smooth muscle function was unaffected by these interventions.

DISCUSSION

In the current study, we showed for the first time that BPS induced concentration-dependent vasodilation of the retinal arterioles with approximately 70% dilation at high concentrations

(Fig. 1). Because the plasma BPS concentration reaches 0.1 to 1 nM within 1 hour after oral administration of 40 μg in healthy men,[45] the current data showed that BPS might have clinical potential to elicit 10% to 20% vasodilation of the retinal arterioles at these concentrations. Although no study has examined the effects of BPS on RBF, a previous animal study reported that BPS improved not only the b-wave of the electroretinogram but also the sciatic nerve blood flow in streptozotocin (STZ)-induced diabetic rats,[46] which seems to support this possibility. Since the RBF is impaired in early-stage DR in patients with type 2 diabetes mellitus,[10] our findings indicate that BPS may be a new therapeutic agent for treating DR due to improvement of impaired RBF.

Although PGI_2 and PGI_2 analogue are generally considered to be vasodilators, some studies have reported that these agents induced vasoconstriction mediated by activation of the TP, EP_1, and EP_3 receptors in various vascular beds.[12,16,17] The decrease in RBF can be attenuated especially by the TP receptor antagonist vapiprost in STZ-induced diabetic mice,[47] suggesting that the density of these receptors may be changed in diabetic animal models. If BPS has some effects not only on IP receptor but also other receptors including TP receptor, the effect of BPS on RBF may be blunted in patients with diabetes. In the current study, the IP receptor antagonist CAY10441 abolished the BPS-induced vasodilation, whereas the TP antagonist SQ29548, EP_1 antagonist SC19220, and EP_3 antagonist L-798106 did not change this response (Fig. 2), suggesting that BPS-induced vasodilation is mediated by the IP receptor alone in the retinal arterioles.

Previous studies have shown that BPS has beneficial effects on the endothelium such as vascular endothelial cell protection[48,49] and an anti-inflammatory effect[50] in large vessels. In the current study, we for the first time examined the effect of BPS on the retinal arterioles and found that removing the endothelium with CHAPS significantly attenuated, but not abolished, the BPS-induced vasodilation (Fig. 3A), suggesting that BPS elicits both endothelium-dependent and -independent vasodilation of the retinal arterioles.

Although several studies have examined IP receptor expression in various vascular beds,[51-54] there are no histologic data regarding the distribution of the IP receptor in the retinal arterioles. The current study for the first time confirmed the expression of the IP receptor in the retinal arterioles immunohistologically (Fig. 4). Our data showed that IP receptor was expressed in the endothelium and the smooth muscle of the retinal arterioles, which supports our functional data that both endothelium-dependent and -independent pathways may be involved with BPS-induced vasodilation in the retinal arterioles.

We observed that NOS blockage by L-NAME inhibited BPS-induced vasodilation comparable to that of denudation (Fig. 3A) and the levels of the NO metabolites (nitrite and nitrate) were elevated in the chamber after BPS administration (Fig. 3B), suggesting that BPS causes vasodilation via NO production from the endothelium in the retinal arterioles. Our finding was consistent with that of a previous study that showed that BPS increased the expression of the eNOS gene and protein level in murine aorta and cultured bovine aortic endothelial cells.[21] In contrast to L-NAME, the vasodilatory response to BPS was unaffected

by pretreatment with the cytochrome P450 metabolite inhibitor sulfaphenazole and the specific K channel blockers, BK_{Ca} and IK_{Ca} blocker ChTx plus SK_{Ca} blocker apamin (Fig. 3A), indicating that EDHF might not be involved in BPS-induced vasodilation in the retinal arterioles. Taken together, we speculate that NO mainly contributes to the endothelium-dependent component of BPS-induced vasodilation of the retinal arterioles.

Beraprost sodium-induced vasodilation is believed to be mediated by activation of adenylate cyclase and increasing intracellular cAMP levels,[12] but no previous reports have confirmed if BPS increases intracellular cGMP levels. In the current study, BPS-induced vasodilation was inhibited partly by the soluble guanylyl cyclase inhibitor ODQ in a manner identical to that produced by denudation and L-NAME (Fig. 3A), suggesting that vasodilation of the retinal arterioles induced by BPS occurs via the NO/cGMP pathway.

The current study showed that inhibition of PKA, which contributes to phosphorylation of eNOS and stimulation of NO production,[55] reduced the BPS-induced vasodilation of the retinal arterioles (Fig. 5) and suppressed elevation of NOx levels in the vessel chamber (Fig. 3B). Our results agreed with another study that BPS induced PKA-dependent eNOS phosphorylation and NO release in bovine aortic endothelial cells.[21] Moreover, the combination of Rp-8-Br-cAMPS and L-NAME did not further reduce the BPS response in the retinal arterioles (Fig. 5). Taking these findings together, it is likely that BPS induces vasodilation of the retinal arterioles by NO production from the retinal vascular endothelium via activation of PKA and phosphorylation of eNOS in the retinal vascular endothelial cells.

Previous studies have reported that various potassium channels are involved in the vasodilatory response of the retinal arterioles.[24,26,28,39,56-58] In the current study, BPS-induced vasodilation was inhibited significantly by the nonselective potassium channel inhibitor TEA, indicating the involvement of the potassium channel in this vasodilatory response in the retinal arterioles (Fig. 6). Beraprost sodium-induced vasodilation is mediated by activation of the BK_{Ca} channel in guinea pig aorta[59] and porcine retinal pericytes.[60] However, we found that the BK_{Ca} selective inhibitor iberiotoxin did not affect the vasodilatory response, whereas the K_{ATP} channel blocker glibenclamide inhibited BPS-induced vasodilation (Fig. 6) in the same manner as the nonselective potassium channel inhibitor TEA, suggesting that activation of the K_{ATP} channel may be involved in BPS-induced vasodilation of the retinal arterioles. Although it has been reported that increased cGMP may lead to activation of the K_{ATP} channel in retinal arterioles,[25] the current finding showed that the combination of ODQ and glibenclamide further reduced vasodilation in response to BPS comparable with glibenclamide alone. Moreover, in our preliminary study ($n = 4$), K_{ATP} channel activator pinacidil-induced vasodilation[24] was unaffected by incubation with the NOS blocker L-NAME (pinacidil 10 μM versus pinacidil 10 μM with L-NAME 10 μM; 81.7 ± 2.8% vs. 81.8 ± 3.2%; $P = 0.30$). We believe that the K_{ATP} channel may be involved in the endothelium-independent pathway in the retinal arterioles in response to BPS.

Because the current study was designed specifically to evaluate the effects of BPS, a stable PGI_2 analogue, on the retinal microcirculation due to the short life of PGI_2, we could not

exclude the possibility that nonspecific vasodilatory effects of BPS, independent of the PGI_2/IP receptor pathway, were involved in the current findings. Our preliminary study also found that PGI_2 per se induced concentration-dependent vasodilation of the retinal arterioles comparable with BPS (data not shown). Moreover, PGI_2-induced vasodilation was mediated by the IP receptor alone in the retinal arterioles and did not involve the other prostaglandin receptors (data not shown). Therefore, it is reasonable that PGI_2 may have the same effect as BPS on vasodilation of the retinal arterioles.

In summary, the current study showed that BPS, a stable PGI_2 analogue, elicits potent dilation of the retinal arterioles, which has two components of endothelium-dependent and -independent pathways. The endothelium-dependent dilation is mediated through the PKA/eNOS/NO pathway. The endothelium-independent pathway is related mainly to activation of the K_{ATP} channel in the smooth muscle (Fig. 7). Because RBF and endothelial function are impaired in early-stage DR in patients with type 2 diabetes,[10,11] BPS may be a novel potential drug for treating DR by compensating for the reduced EDRFs (i.e., PGI_2 and NO), in the retinal arterioles. Further clinical study is needed to determine if BPS can improve impaired RBF and endothelial function in patients with diabetes.

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Legends

FIGURE 1. Response of isolated retinal arterioles to BPS. There is no significant difference between the two repeated trials ($n = 8$).

FIGURE 2. The role of prostaglandin receptors in retinal arteriolar dilation in response to BPS (0.1 μM). Incubation with IP antagonist CAY10441 (0.1 μM, $n = 5$) but not TP antagonist SQ29548 (10 μM, $n = 5$), EP_1 antagonist SC19220 (10 μM, $n = 5$), or EP_3 antagonist L-798106 (10 μM, $n = 5$) significantly reduces vasodilation in response to BPS. $^{*}P < 0.05$ versus control.

FIGURE 3. (A) The role of endothelium in the retinal arteriolar dilation in response to BPS (0.1 μM). Endothelium removal by perfusion with 0.4% CHAPS ($n = 4$), incubation with the NOS inhibitor L-NAME (10 μM, $n = 6$) or soluble guanylyl cyclase inhibitor ODQ (0.1 μM, $n = 4$) but not cytochrome P450 epoxygenase inhibitor sulfaphenazole (10 μM, $n = 4$) or EDHF blocker apamin 0.1 μM plus ChTx 0.1 μM ($n = 4$) significantly reduces vasodilation in response to BPS. $^{*}P < 0.05$ versus control. **(B)** The NOx production response to vehicle ($n = 12$), BPS (0.1 μM, $n = 6$) or BPS (0.1 μM) after incubation with the PKA inhibitor Rp-8-Br-cAMPS (100 μM, $n = 6$) is examined 5 minutes after injection of vehicle or BPS into the vessel chamber. Beraprost sodium increases the NOx levels in the vessel chamber, whereas incubation with Rp-8-Br-cAMPS inhibits elevation of NOx levels in response to BPS. $^{*}P <$

0.05 versus vehicle.

FIGURE 4. Immunohistochemical localization of IP in the retinal arterioles. Staining with anti-IP (*green*), anti-α-smooth muscle actin (SMA, *red*), and anti-eNOS (*blue*) antibodies shows expression of IP, SMA, and eNOS. The merged image shows overlapping staining (*yellow*) of IP with SMA and eNOS. The images are representative of three separate experiments.

FIGURE 5. The role of PKA in the retinal arteriolar dilation in response to BPS (0.1 μM). Incubation with the PKA inhibitor Rp-8-Br-cAMPS (100 μM, $n = 5$) reduces BPS-induced vasodilation to a similar extent to L-NAME (10 μM, $n = 4$). Residual vasodilation in the presence of Rp-8-Br-cAMPS does not decrease further after coincubation with L-NAME 10 μM ($n = 4$). $^{*}P < 0.05$ versus control.

FIGURE 6. The role of potassium channels in retinal arteriolar dilation in response to BPS (0.1 μM). Incubation with the nonselective potassium channel blocker TEA (10 mM, $n = 5$) and the K_{ATP} channel blocker glibenclamide (5 μM, $n = 4$)—but not 4-AP (0.1 mM, $n = 5$), iberiotoxin (0.1 μM, $n = 5$), TRAM34 (1 μM, $n = 4$), apamin (0.1 μM, $n = 6$), or $BaCl_2$ (30 μM, $n = 4$)—reduces vasodilation in response to BPS. Residual vasodilation in the presence of glibenclamide decreases further after coincubation with the soluble guanylyl cyclase inhibitor

ODQ (0.1 μM, $n = 4$). $^{*}P < 0.05$ versus control, †$P < 0.05$ versus glibenclamide.

FIGURE 7. Schematic illustration of proposed signaling mechanisms involved in retinal arteriolar dilation in response to BPS. Inhibition of these signaling pathways by their respective inhibitors is indicated by the vertical lines in reference to the direction of the straight line.

Table. Resting Diameters and Diameter Responses of Retinal Arterioles to SNP

			SNP, μM			
	n	**Resting Diameter**	**0.1**	**1**	**10**	**100**
Control	8	60.2 ± 1.2	7.1 ± 1.5	25.5 ± 2.5	55.3 ± 3.4	83.6 ± 3.3
CAY10441	5	61.4 ± 1.5	6.1 ± 1.5	21.8 ± 1.3	53.3 ± 8.3	83.8 ± 2.1
SQ29548	5	59.4 ± 2.6	7.9 ± 0.6	22.1 ± 0.5	51.6 ± 3.8	81.4 ± 2.3
SC19220	5	59.4 ± 1.6	9.0 ± 1.8	21.1 ± 3.3	51.3 ± 6.1	84.6 ± 1.5
L-798106	5	60.0 ± 2.0	5.2 ± 0.3	25.5 ± 1.5	49.1 ± 4.6	81.3 ± 2.5
Denudation	4	60.8 ± 3.2	6.0 ± 1.4	29.0 ± 3.0	55.1 ± 9.4	82.5 ± 3.8
L-NAME	6	57.5 ± 1.9	7.2 ± 0.2	24.1 ± 4.9	56.4 ± 6.5	81.9 ± 4.6
Sulfaphenazole	4	60.5 ± 2.8	4.3 ± 1.0	29.7 ± 7.4	56.7 ± 9.3	85.7 ± 2.8
Apamin + ChTx	4	58.8 ± 2.8	7.1 ± 1.0	20.2 ± 1.8	56.6 ± 9.6	87.8 ± 1.7
Rp-8-Br-cAMPS	5	58.0 ± 2.2	7.4 ± 3.0	22.0 ± 2.6	57.8 ± 7.4	85.5 ± 1.9
Rp-8-Br-cAMPS + L-NAME	4	57.5 ± 3.4	3.3 ± 0.3	23.3 ± 2.1	48.3 ± 7.8	86.3 ± 4.7
4-AP	5	60.6 ± 3.2	3.4 ± 0.5	21.2 ± 1.7	53.9 ± 6.3	89.4 ± 1.9
Iberiotoxin	5	57.0 ± 4.5	4.4 ± 1.1	23.5 ± 2.6	51.7 ± 3.0	83.8 ± 4.4
TRAM34	4	59.3 ± 1.8	6.4 ± 2.0	28.5 ± 2.1	48.8 ± 4.5	85.2 ± 2.4
Apamin	6	59.8 ± 2.4	4.1 ± 0.7	27.0 ± 5.9	51.4 ± 4.1	86.2 ± 4.2
$BaCl_2$	4	58.3 ± 1.0	5.9 ± 2.3	20.9 ± 2.1	49.2 ± 1.1	83.3 ± 2.2
Glibenclamide	4	61.6 ± 2.9	3.1 ± 0.7	24.3 ± 4.3	53.9 ± 6.5	88.1 ± 4.5
TEA	5	56.6 ± 2.3	6.2 ± 1.4	31.0 ± 3.1	58.6 ± 2.2	82.4 ± 3.3

Data are expressed as the mean ± SEM. Based on two-way ANOVA, compared with control, the response to SNP are unaffected by any perturbation.

Figure 1.

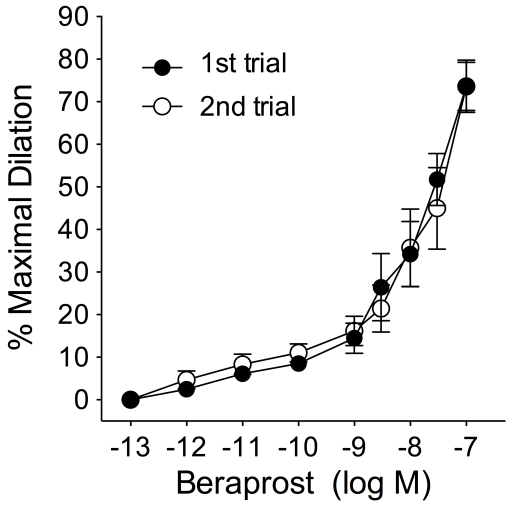

90
80
70
60
50
40
30
20
10
0
% Maximal Dilation
1st trial
2nd trial
-13
-12
-11
-10
-9
-8
-7
Beraprost (log M)

Figure 2.

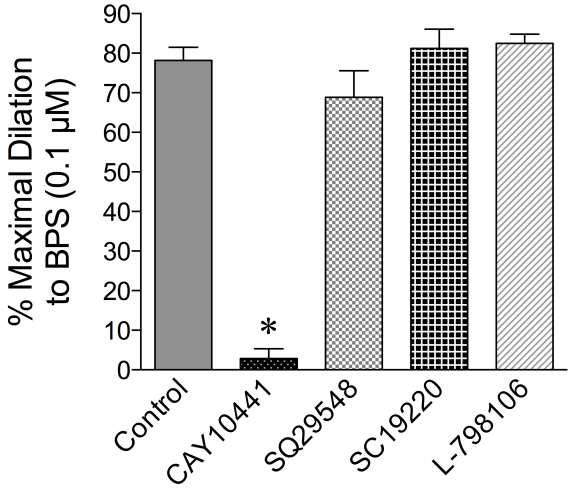

% Maximal Dilation to BPS (0.1 μM)
90
80
70
60
50
40
30
20
10
0
*
Control
CAY10441
SQ29548
SC19220
L-798106

Figure 3.

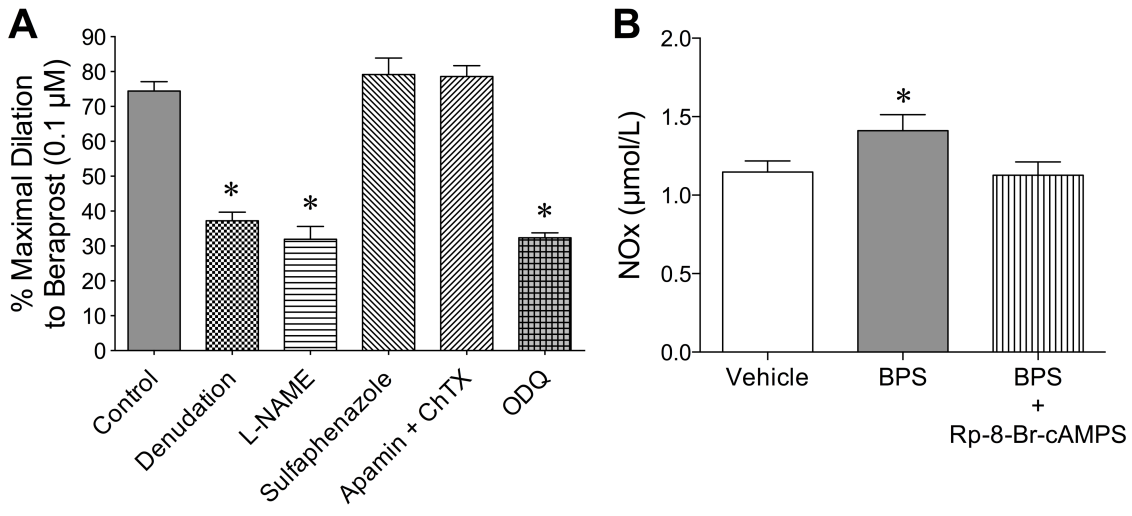

A
% Maximal Dilation to Beraprost (0.1 μM)
90
80
70
60
50
40
30
20
10
0
*
*
*
Control
Denudation
L-NAME
Sulfaphenazole
Apamin + ChTX
ODQ
B
NOx (μmol/L)
2.0
1.5
1.0
0.5
0.0
*
Vehicle
BPS
BPS + Rp-8-Br-cAMPS

Figure 4.

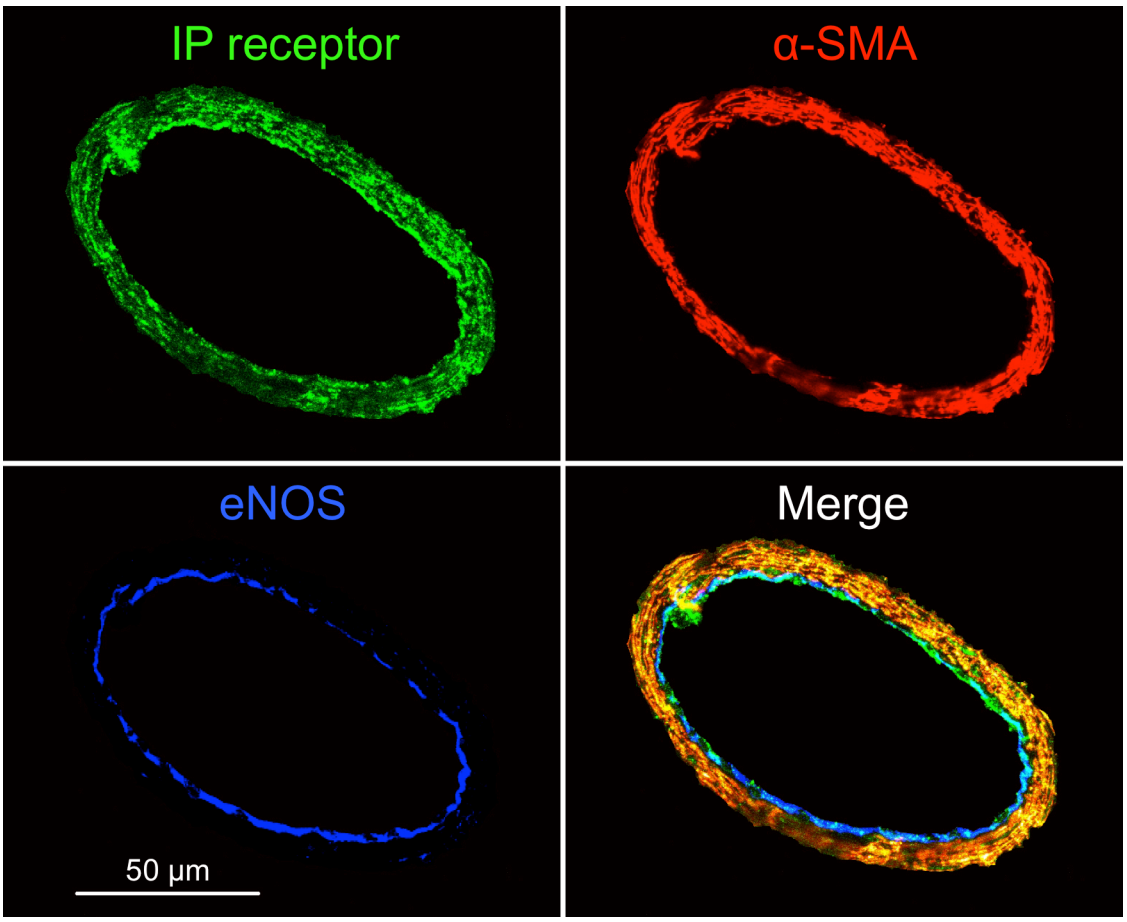


Figure 5.

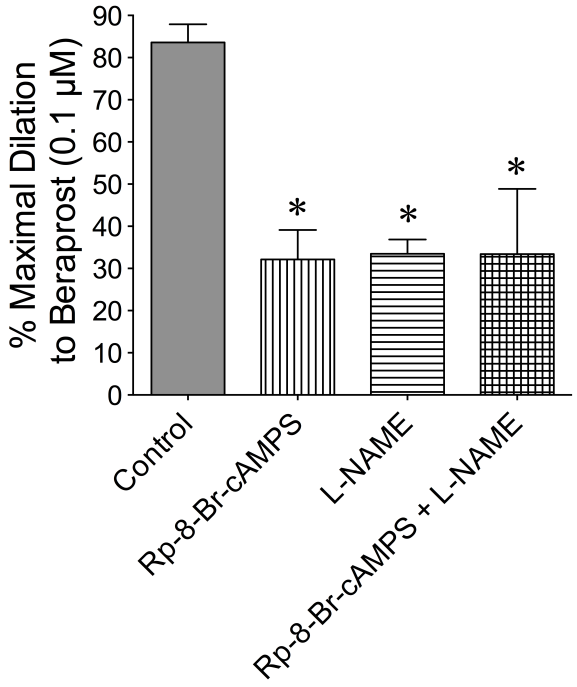
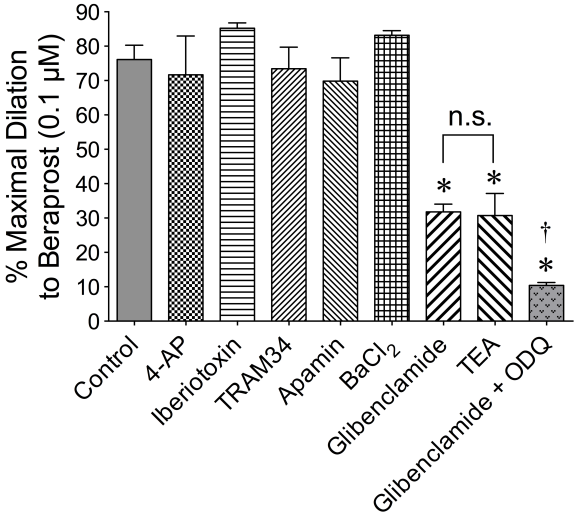
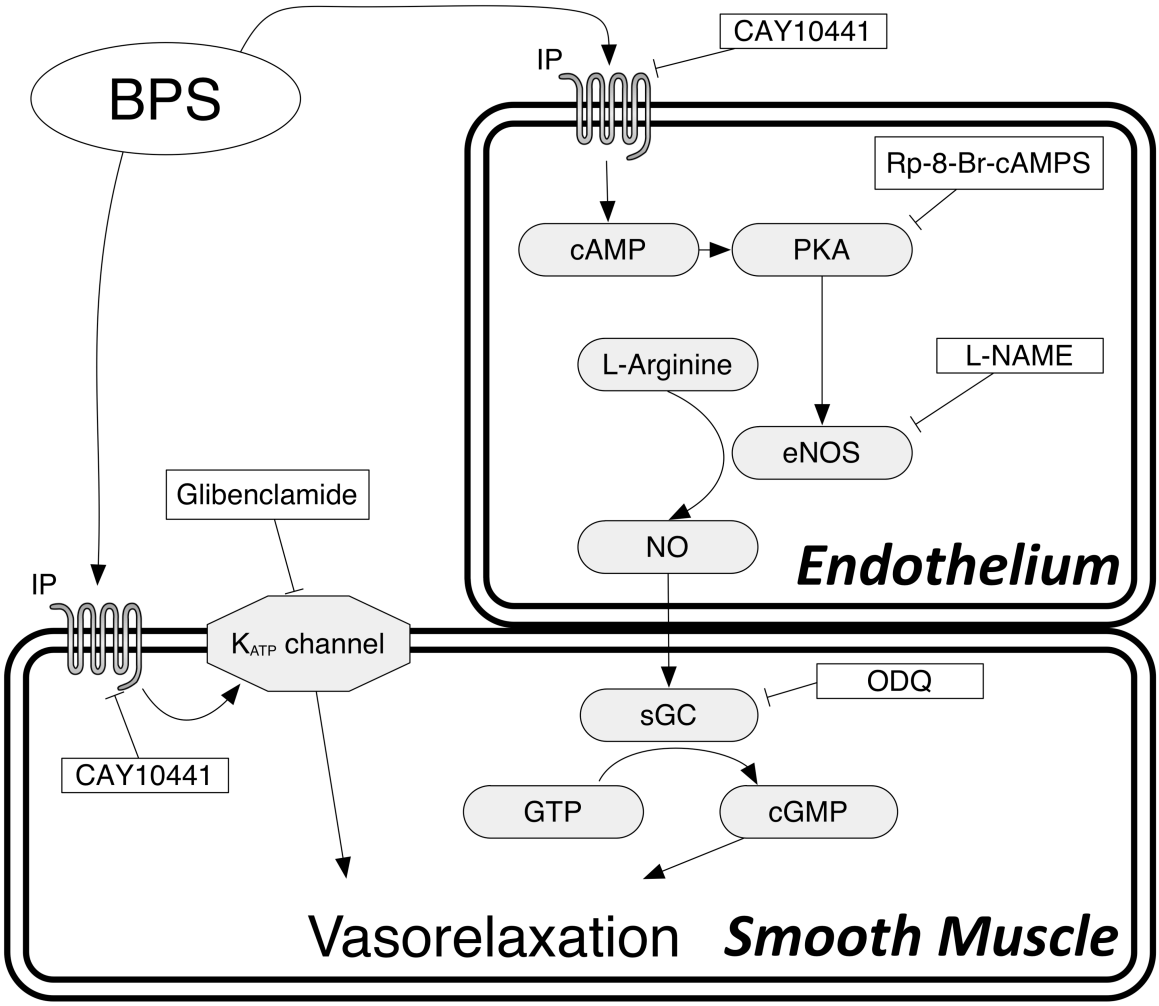

% Maximal Dilation to Beraprost (0.1 μM)
90
80
70
60
50
40
30
20
10
0
*
*
*
Control
Rp-8-Br-cAMPS
L-NAME
Rp-8-Br-cAMPS + L-NAME

Figure 6.


% Maximal Dilation to Beraprost (0.1 μM)
90
80
70
60
50
40
30
20
10
0
n.s.
*
*
†
*
Control
4-AP
Iberiotoxin
TRAM34
Apamin
$BaCl_2$
Glibenclamide
TEA
Glibenclamide + ODQ

1 **Figure 7.**


BPS
IP
CAY10441
Rp-8-Br-cAMPS
cAMP
PKA
L-Arginine
L-NAME
eNOS
NO
Endothelium
Glibenclamide
IP
K_{ATP} channel
ODQ
sGC
CAY10441
GTP
cGMP
Vasorelaxation
Smooth Muscle

2