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RESEARCH PAPER

Openers of small conductance calcium-activated potassium channels selectively enhance NO-mediated bradykinin vasodilatation in porcine retinal arterioles

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Background and purpose: Small (SK_{Ca} or $K_{Ca}2$) and intermediate (IK_{Ca} or $K_{Ca}3.1$) conductance calcium-activated potassium channels are involved in regulation of vascular tone and blood pressure. The present study investigated whether NS309 (6,7-dichloro-1H-indole-2,3-dione 3-oxime) and CyPPA (cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine), which are selective openers of SK_{Ca} and IK_{Ca} channels and of $SK_{Ca}2$ and $SK_{Ca}3$ channels, respectively, enhance endothelium-dependent vasodilatation in porcine retinal arterioles.
Experimental approach: In porcine retinal arterioles, $SK_{Ca}3$ and IK_{Ca} protein localization was examined by immunolabelling. Endothelial cell calcium was measured by fluorescence imaging. For functional studies, arterioles with internal diameters of 116 ± 2 μm ($n = 276$) were mounted in microvascular myographs for isometric tension recordings.
Key results: $SK_{Ca}3$ and IK_{Ca} protein was localized in the endothelium. Bradykinin, but not NS309 or CyPPA increased endothelial cell calcium. Pre-incubation with NS309 or CyPPA enhanced bradykinin relaxation without changing endothelial cell calcium. This enhanced relaxation was abolished by blocking SK_{Ca} channels with apamin. In the presence of NS309 or CyPPA, mainly inhibition of NO synthase with asymmetric dimethylarginine, but also inhibition of cyclooxygenase with indomethacin, reduced bradykinin relaxation. Bradykinin relaxation was completely abolished by NO synthase and cyclooxygenase inhibition together with a NO scavenger, oxyhaemoglobin.
Conclusions and implications: In porcine retinal arterioles, bradykinin increases endothelial cell calcium leading to activation of SK_{Ca} and IK_{Ca} channels. Without altering endothelial cell calcium, NS309 and CyPPA open SK_{Ca} channels that enhance NO-mediated bradykinin relaxations. These results imply that opening SK_{Ca} channels improves endothelium-dependent relaxation and makes this channel a potential target for treatments aimed at restoring retinal blood flow.
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Keywords: bradykinin; CyPPA; H_2S; HOE-140; IK_{Ca}; NO; NS309; prostaglandins; retinal arterioles; SK_{Ca}; SNP; vasodilatation

Abbreviations: 1-EBIO, 1-ethyl-2-benzimidazolinone; ADMA, asymmetric dimethylarginine; ChTX, charybdotoxin; CyPPA, cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine; DC-EBIO, 5,6-dichloro-1-ethyl-1,3dihydro-2H-benzimidazol-2-one; DMSO, dimethylsulphoxide; EDHF, endothelium-derived hyperpolarizing factor; HOE-140, icatibant acetate; IK_{Ca} ($K_{Ca}3.1$), intermediate conductance calcium-activated potassium channel; KPSS, 125 mM K^+ physiological saline solution; NO synthase, nitric oxide synthase; NS309, 6,7-dichloro-1H-indole-2,3-dione 3-oxime; PPG, D,L-propargylglycine; PSS, physiological saline solution; PSS0.0, physiological saline solution without $CaCl_2$; SKA-31, naphtho[1,2-d]thiazol-2-ylamine; SK_{Ca} ($K_{Ca}2.3$), small conductance calcium-activated potassium channel; SNP, sodium nitroprusside; U46619, 9,11-dideoxy-9a,11a-epoxymethanoprostaglandin $F_{2\alpha}$

Introduction

Disturbance in the blood supply to the retina is a common finding in retinal diseases leading to blindness. A disturbed blood supply affects the delivery of oxygen and metabolic

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substrates necessary for the maintenance of retinal structure and function. The vascular endothelium modulates the blood flow by smooth muscle relaxation through activation of the vasodilating pathways involving endothelial nitric oxide (NO) synthase, cyclooxygenase, and the calcium-activated potassium channels of small (SK_{Ca} or $K_{Ca}2$) and intermediate (IK_{Ca} or $K_{Ca}3.1$) conductance (Feletou and Vanhoutte, 2006; channel nomenclature follows Alexander *et al*., 2009). Activation of SK_{Ca} and IK_{Ca} channels is generally acknowledged as a required component for the activation of the relaxation mediated by endothelium-dependent hyperpolarizing factor (EDHF) (Burnham *et al*., 2002; Bychkov *et al*., 2002; Eichler *et al*., 2003), but SK_{Ca} and IK_{Ca} channel activation has also been associated with activation of NO synthase (Stankevicius *et al*., 2006; Sheng and Braun, 2007; Brahler *et al*., 2009; Sheng *et al*., 2009). Supporting these findings, *in vivo* studies in mice show that doxycycline-induced suppression of the $SK_{Ca}3$ channel ($SK_{Ca}3^{T/T}$ + Dox) (Taylor *et al*., 2003), knockout of the IK_{Ca} channel ($IK_{Ca}^{-/-}$) (Si *et al*., 2006) and deficit of both $SK_{Ca}3^{T/T}$ + Dox and $IK_{Ca}^{-/-}$ (Brahler *et al*., 2009) leads to elevated blood pressure. In addition, opening SK_{Ca} and IK_{Ca} channels decreases myogenic tone, increases acetylcholine-induced relaxation in rat cremaster arterioles (Sheng *et al*., 2009), and restores the attenuated EDHF-type relaxation in mesenteric small arteries from Zucker diabetic fatty (ZDF) rats (Brondum *et al*., 2009). Moreover, opening of IK_{Ca} channels decreases mean arterial blood pressure in angiotensin II-induced hypertensive mice (Sankaranarayanan *et al*., 2009). These results suggest that SK_{Ca} and/or IK_{Ca} channels are very important in the regulation of endothelium-dependent vasodilatation, and hence in controlling blood pressure and organ blood flow.

Patients with retinal diseases, such as glaucoma and diabetes, have a reduced ocular blood flow that in part is caused by endothelial dysfunction and a reduced release of vasodilating factors (Schmetterer *et al*., 1997; Delles *et al*., 2004; Resch *et al*., 2009). We have previously found that in retinal arterioles, the endothelium-dependent relaxation induced by the endogenous agonist, bradykinin, is mainly mediated by NO (Jeppesen *et al*., 2002; Dalsgaard *et al*., 2009), and that blocking SK_{Ca} and IK_{Ca} channels reduces this relaxation (Dalsgaard *et al*., 2009). Thus, opening of SK_{Ca} and/or IK_{Ca} channels may enhance endothelium-dependent relaxation and increase retinal blood flow, which may be beneficial for these patients.

The present study hypothesized that opening of endothelial cell SK_{Ca} and IK_{Ca} channels would enhance endothelium-dependent relaxation in retinal arterioles. To address this hypothesis, localization of $SK_{Ca}3$ and IK_{Ca} protein was investigated in porcine retina. Moreover, porcine retinal arterioles were isolated and bradykinin-induced relaxation was evaluated in the presence of NS309 (6,7-dichloro-1H-indole-2,3-dione 3-oxime) (Strobaek *et al*., 2004), an opener of SK_{Ca} and IK_{Ca} channels, and CyPPA (cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine), an opener of $SK_{Ca}2$ and $SK_{Ca}3$ channels (Hougaard *et al*., 2007). To address the mechanisms involved in the enhanced relaxation, we measured the effect on endothelial cell calcium of bradykinin, NS309, and CyPPA. We also determined whether NS309 and CyPPA directly affect the bradykinin receptor and whether increased formation of NO, prostaglandins or an EDHF-type response are involved in the enhanced bradykinin relaxation in the presence of NS309 and CyPPA. As we found no evidence for involvement of EDHF-type relaxation in the enhanced bradykinin relaxation, we investigated the role of applying different preconstrictors on bradykinin relaxation, and whether hydrogen sulphide (H_2S) contributes to bradykinin relaxation.

Methods

Tissue preparation

Eyes from pigs of approximately 6 months of age and a weight of 85–90 kg were obtained from a local abattoir. From each animal, one eye was removed immediately after the pig had been exposed to carbon dioxide (CO_2) and killed by exsanguination. The eyes were transferred to a container with cold (4°C) physiological saline solution (PSS) [composition (mM): 4.8 KCl, 1.14 $MgSO_4$, 118 NaCl, 25 $NaHCO_3$, 5 HEPES, 5.5 glucose and 1.6 $CaCl_2$] and transported to the laboratory, where they were dissected and the retinas isolated as previously described (Jeppesen *et al*., 2002). In brief, the eyes were bisected by a frontal section, the vitreous was removed and the retina was detached from the underlying pigment epithelium. An arteriolar segment located approximately 1–2 mm from the optic disk with a length of ~ 2 mm was dissected from the retina.

Histology of arteriolar segments and immunohistochemistry of $SK_{Ca}3$ and IK_{Ca} protein

For histological and immunohistochemical processing, arteriolar segments with surrounding retinal tissue were fixed in cold (4°C) 4% paraformaldehyde, pH 7.0 for 1 h and placed in 8% agar prior to embedding in paraffin. Longitudinal sections of 3 µm were cut on a microtome, placed on glass slides, heated at 80°C for 1 h, and deparaffinized in xylene and decreasing concentrations of ethanol. For histology, sections were stained with haematoxylin and 0.1% w/v eosin, and dehydrated in increasing concentrations of ethanol. For evaluation of $SK_{Ca}3$ and IK_{Ca} protein distribution, sections were incubated with 0.3% H_2O_2 for 20 min, rinsed with phosphate buffered saline (PBS: 1.5 M NaCl, 0.5 M NaH_2PO_4), transferred to TEG-buffer (10 mM Tris, 0.5 mM EGTA, pH 9.0), heated in the microwave for 2×7 min and rinsed with PBS. Thereafter, sections were transferred to 0.25% Triton X solution for 15 min and rinsed with PBS afterwards. Sections were incubated with 10% fetal calf serum in PBS with 1% bovine serum albumin (BSA) for 30 min in order to block non-specific antibody binding. Incubation with the primary antibodies for $SK_{Ca}3$ protein (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and IK_{Ca} protein (1:400, Alomone, Jerusalem, Israel) in PBS containing 1% BSA was performed overnight in a humidified chamber at 4°C. Negative controls for each sample section were without primary antibody. Sections were rinsed with PBS and incubated for 1 h at room temperature with secondary antibody, goat anti-rabbit IgG coupled to Alexa 488 (1:1000, Molecular Probes Inc., Eugene, OR, USA) with PBS containing 1% BSA for 1 h followed by rinsing with PBS. Finally, antifade solution (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) was added and a cover slip

was mounted. Preparations were analysed on an inverted confocal microscopy (LSM 510 Meta, Carl Zeiss Inc, Oberkochen, Germany) equipped with an oil immersion objective (C-Aprochromat 63 × NA = 0.75) excited at a wavelength of 488 nm using the Zeiss LSM Image Browser software program (Zeiss Inc, Oberkochen, Germany).

Calcium measurements

Mounting, loading and normalization. Arteriolar segments were mounted on two stainless steel wires 20 μm in diameter in a confocal myograph (model 120CW, Danish Myo Technology, Aarhus, Denmark) and loaded with a buffer consisting of the visible light excitable calcium indicator; Oregon Green BAPTA 1-AM (7.24 μM), the nonoinic surfactants; Cremophor EL [0.066% (w/v)], and Pluronic F-127 (10.66 μM), and dimethylsulphoxide [DMSO, 0.32% (w/v)] at room temperature (21°C). The loading buffer was introduced to the luminal side of the segments and loading time was 45 min. After loading, each segment was allowed to equilibrate for 30 min at 37°C in PSS. The solutions were equilibrated with bioair of the following composition: 5% CO_2, 21% O_2 and 74% N_2. Subsequently, they were normalized in PSS without $CaCl_2$ (PSS0.0) to an internal circumference corresponding to 94% of the tone at a transmural pressure of 70 mmHg (Hessellund *et al.*, 2003). After normalization, PSS0.0 was replaced with PSS, and the segments were allowed to equilibrate for another 30 min. Image acquisition of endothelial cell calcium was performed with an inverted confocal microscope (model LSM 510 Exciter, Carl Zeiss Inc, Oberkochen, Germany) equipped with a 63 × NA = 0.75 long working distance objective excited at a wavelength of 488 nm using the Zeiss LSM Image Browser software program (Zeiss Inc, Oberkochen, Germany). A fluorescent signal in the first cell layer towards the luminal side, the longitudinally orientation of this cell layer in the arteriolar segment, and an increased fluorescence upon addition of bradykinin was used to confirm successful loading of endothelial cells. In contrast, abluminal loading of smooth muscle cells showed perpendicular orientation in the segment, an increase in calcium upon addition of the thromboxane analogue, U46619 (9,11-dideoxy-11a,9a-epoxymethanoprostaglandin F_{2a}) and a decrease in signal upon addition of bradykinin (data not shown).

Experimental protocol. After equilibration, the calcium signal in endothelial cells was recorded before and after addition of bradykinin (0.1, 1, 3 and 10 nM), NS309 (1 μM), NS309 (1 μM) + bradykinin (3 nM), CyPPA (6 μM) or CyPPA (6 μM) + bradykinin (3 nM). Bradykinin (3 nM), NS309 (1 μM) and CyPPA (6 μM) were the concentrations leading to approximately 50% relaxation in U46619 (0.1 μM)-contracted segments.

Functional studies

Mounting and normalization. An isolated arteriolar segment was transferred to a chamber of a dual wire myograph system (model 410A, Danish Myo Technology, Aarhus, Denmark) for isometric tension recording using the Chart5 software programme (ADInstruments Ltd., Oxfordshire, UK). Each arteriole was mounted in the wire myograph using two tungsten wires 25 μm in diameter and was allowed to equilibrate for 30 min at 37°C after which it was normalized as described above. A total of 276 arteriolar segments were used for the functional studies, where the internal diameter of the arterioles averaged 116 ± 2 μm.

Control of viability. After equilibration, smooth muscle cell viability was tested by addition of PSS, where equimolar concentration of NaCl was replaced with KCl giving a concentration of 60 mM K^+ (KPSS), and U46619 (0.1 μM) to test contraction induced by depolarization and by activation of the thromboxane A_2 receptor, followed by exposure to U46619 (0.1 μM) alone. Endothelium-dependent relaxation was tested by addition of bradykinin (0.03 μM) in U46619 (0.1 μM)-contracted arterioles. Arterioles were discarded, if U46619 contraction was less than 0.25 Nm^{-1}, or if bradykinin relaxation was less than 50% (Dalsgaard *et al.*, 2009).

Experimental protocol. After control of viability, segments were contracted with U46619 (0.1 μM) and concentration–response curves were obtained by cumulative additions of bradykinin (0.01 nM–300 nM) alone or in the presence of NS309 (1 μM), CyPPA (6 μM) or a NO donor, sodium nitroprusside (SNP, 1 μM). Moreover, concentration–response curves for NS309 (0.1–10 μM), CyPPA (1–100 μM), NO solution (0.01–3 μM), hydrogen sulphide (H_2S, 0.01–10 mM) and sodium nitroprusside (SNP, 0.01–100 μM) were obtained. To investigate the contribution of different enzymes and channels in the concentration–response experiments, 30 min prior to contraction, segments were incubated with the NO synthase inhibitor, asymmetric dimethylarginine (ADMA, 300 μM) (Stankevicius *et al.*, 2006), the cyclooxygenase inhibitor, indomethacin (3 μM) (Stankevicius *et al.*, 2006), the NO-scavenger, oxyhaemoglobin (25 μM) (Simonsen *et al.*, 1999), the IK_{Ca} channel blocker, charybdotoxin (ChTx, 0.1 μM) (Alexander *et al.*, 2009), the SK_{Ca} channel blocker, apamin (0.5 μM) (Alexander *et al.*, 2009), the bradykinin receptor B_2 antagonist, icatibant acetate (HOE-140, 0.1, 1, 10 and 100 nM) (Tirapelli *et al.*, 2007), the cystathionine γ-lyase inhibitor, D,L-propargylglycine (PPG, 5 mM) (Zhao *et al.*, 2003; Yang *et al.*, 2008) or a combination of these inhibitors or blockers. In a series of experiments, the arterial segments were contracted with KPSS (125 mM) or endothelin-1 (0.03 μM) instead of U46619 (0.1 μM). In another series of experiments, the endothelium was mechanically removed by introducing tungsten wires 25 μm in diameter into the lumen of the mounted segment. Removal of the endothelium was considered successful, if U46619 (0.1 μM) induced a contraction of more than 0.25 Nm^{-1}, and bradykinin (0.03 μM) subsequently induced a relaxation of less than 10%.

Data analysis

For calcium measurements, 5–10 endothelial cells were selected on each isolated arterial segment and marked with a region of interest. For each region of interest, the Oregon Green fluorescent signal was determined after each treatment, minus the Oregon Green fluorescent signal before treatment.

In the functional studies, the tension in the peak response after each addition of bradykinin or NO solution, and the

stable tension after each addition of NS309, CyPPA, H_2S or SNP were expressed as percentage of the active tension, where the active tone was defined as the level of contraction after addition of U46619, minus the level of contraction after the normalization procedure. For bradykinin curves obtained in the presence of NS309, CyPPA or SNP, the active tone was defined as the level of contraction after addition of U46619 and NS309, CyPPA or SNP, minus the level of contraction after the normalization procedure.

Myogenic tone was calculated as the level of contraction 30 min after addition of blocker/inhibitor minus the level of contraction after the normalization procedure for bradykinin concentration–response curves. EC_{50} values were calculated by nonlinear regression for a variable slope, where minimum and maximum relaxation was set to 0% and 100%, respectively, using the GraphPad Prism 5.02 program (GraphPad software Inc., La Jolla, CA, USA) for bradykinin, NO solution, H_2S, NS309 and CyPPA concentration–response curves. Maximum relaxation was determined for bradykinin, NO solution, H_2S, NS309 and CyPPA concentration–response curves.

Statistical analysis

All data are presented as mean ± SEM with a significance level of $P < 0.05$, where n represents the number of segments from individual animals.

A Wilcoxon matched pair test was used to test for differences in the intracellular concentration of calcium in endothelial cells. A two-way ANOVA was used to test for differences in concentration–response curves and a Student's *t*-test was used to test for differences in myogenic tone, U46619 contraction, EC_{50} and maximum relaxation. All data were analysed using GraphPad Prism 5.02 software (GraphPad software Inc., La Jolla, CA, USA).

Materials

For histological and immunohistochemical preparations, xylene, Meyer's haematoxylin and eosin were purchased at Bie & Berntsen, Herlev, Denmark. Fetal calf serum was purchased at Biochrom, Berlin, Germany. Triton X and BSA were purchased at Sigma Aldrich, St. Louis, MO, USA.

For calcium measurements, DMSO, Chremophore EL and Pluronic F-127 were purchased from Sigma Aldrich, St. Louis, MO, USA, while Oregon Green Bapta 1-AM was obtained from Invitrogen, Costa Mesa, CA, USA.

For the functional studies, U46619 (9,11-dideoxy-11a,9a-epoxymethano-prostaglandin F_{2a}), endothelin-1, HOE-140 (icatibant acetate), PPG (D,L-propargylglycine), ADMA (asymmetric dimethylarginine), indomethacin, haemoglobin (bovine), bradykinin, SNP (sodium nitroprusside) and NaSH (sodium hydrosulfide hydrate) were purchased from Sigma Aldrich, St. Louis, MO, USA. Apamin and charybdotoxin were purchased from Latoxan, Valence, France. NS309 (6,7-dichloro-1H-indole-2,3-dione 3-oxime) and CyPPA (cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine), were kindly donated by Dr Søren-Peter Olesen, Neurosearch A/S, Ballerup, Denmark.

U46619 was dissolved in ethanol, NS309 and CyPPA in DMSO (99%), apamin and bradykinin in distilled water, charybdotoxin and indomethacin in PSS0.0. Apamin, bradykinin and charybdotoxin were prepared in 2% albumin-coated Eppendorf tubes.

Oxyhaemoglobin was prepared from commercial haemoglobin by addition of 10 mM sodium dithionite ($Na_2S_2O_4$, Sigma Aldrich, St. Louis, MO, USA). The reducing agent converting methaemoglobin to oxyhaemoglobin was removed by 2 h of dialysis against 100 volumes of PSS0.0 equilibrated with N_2 at 4°C. The purity of the oxyhaemoglobin solution was determined spectrophotometrically and gave a final concentration of 1 mM (Simonsen *et al*., 1995).

Nitric oxide solution was prepared from deoxygenated (argon-bubbled) distilled water, contained in glass vials with a septum closure. The argon gas was led through a 10 mM pyrogallol (1,2,3-trihydroxybenzene, Sigma Aldrich, St. Louis, MO, USA) solution to remove traces of oxygen. Pure NO gas, bubbled through sodium hydroxide (NaOH, 10 mM) to remove higher nitrogen oxides, was then bubbled through the deoxygenated water for 10 min at room temperature (20°C), giving a concentration of 1.9 mM, which was then diluted to 0.19 and 0.019 mM. The dissolved NO gas in solution was withdrawn from the stock solution using gas-tight Hamilton microlitre syringes (Simonsen *et al*., 1999). H_2S was prepared by dissolving NaSH in PSS0.0 and was titrated to pH ~7.4 with HCl shortly before experimentation (Olson *et al*., 2008).

Results

Localization of $SK_{Ca}3$ and IK_{Ca} protein

In arteriolar segments fixed with surrounding retinal tissue, $SK_{Ca}3$ and IK_{Ca} immunoreactions were observed in the vascular endothelium, but not in the vascular smooth muscle layer (Figure 1). Moreover, in the retina, $SK_{Ca}3$ immunoreaction was observed in a few single cells, whereas IK_{Ca} immunoreaction was observed in the majority of all retinal cells (data not shown).

Mechanisms involved in NS309 and CyPPA relaxation

In order to evaluate the selectivity of NS309 and CyPPA for SK_{Ca} and IK_{Ca} channels, concentration–response curves for NS309 and CyPPA were performed. Both NS309 (0.1–10 μM) and CyPPA (1–100 μM) induced concentration-dependent relaxations (EC_{50}: 1.1 ± 0.5 μM and 7.5 ± 2.9 μM, maximum relaxation: 93 ± 3% and 96 ± 2%, $n = 7$ respectively) (Figure 2A,B). Removal of the endothelium or blocking SK_{Ca} channels with apamin (0.5 μM, $n = 7$) reduced NS309 and CyPPA relaxation ($n = 7$) (Figure 2A,B), and we have previously shown that blocking IK_{Ca} channels with charybdotoxin (0.1 μM, $n = 7$) has no effect on NS309 relaxation (Dalsgaard *et al*., 2009). To investigate whether NS309 and CyPPA in addition to opening potassium channels also have other effects, segments were contracted with 125 mM KPSS. In KPSS-contracted segments only high concentrations of NS309 (>6 μM) and CyPPA (>20 μM) induced relaxations ($n = 7$) (Figure 2A,B). Moreover, blocking SK_{Ca} channels ($n = 7$), removal of the endothelium ($n = 7$) or contraction with KPSS (125 mM) ($n = 7$) reduced CyPPA relaxations to similar levels

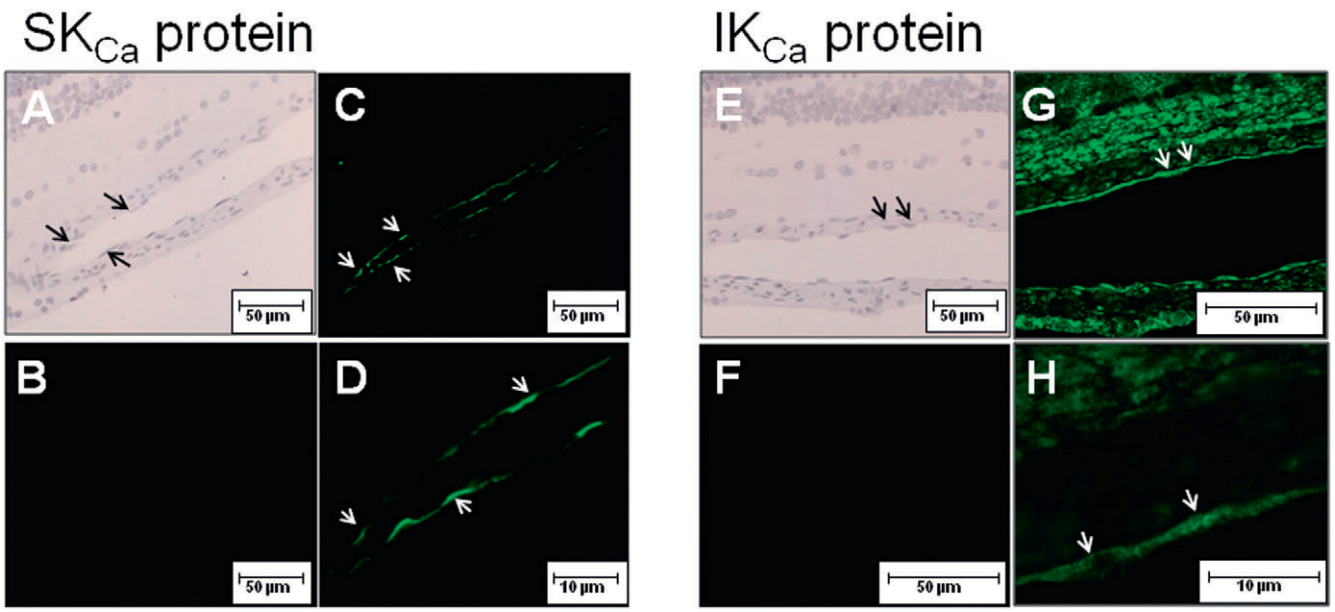


Figure 1 H&E staining (A, E) and localization of $SK_{Ca}3$ protein (C, D) and IK_{Ca} protein (G, H) by immunoreaction (green fluorescence, arrows) in retinal arterioles. B and F are negative controls without primary antibody. D and H are enlargements of C and G. The scale bars in A-C, E-F are 50 µm, and in D and H 10 µm.

(Figure 2B). NS309 relaxation in KPSS-contracted segments was reduced more than in segments where the SK_{Ca} channels were blocked ($n = 7$) or where the endothelium was removed ($n = 7$) (Figure 2A).

To address the relative contribution from NO and prostaglandins to CyPPA relaxation, segments were incubated with ADMA (300 µM) to inhibit NO synthesis, indomethacin (3 µM) to inhibit prostaglandin synthesis, and oxyhaemoglobin (25 µM), to scavenge NO. ADMA alone ($n = 7$) or in combination with indomethacin ($n = 7$) and/or oxyhaemoglobin ($n = 7$) reduced CyPPA relaxation. Indomethacin alone had no effect on CyPPA relaxation ($n = 7$) (Figure 3). We have previously found that NS309 relaxation is mainly mediated by NO (Dalsgaard *et al*., 2009).

To address whether NS309 or CyPPA activated the bradykinin B_2 receptor, segments were incubated with the bradykinin B_2 receptor antagonist, HOE-140. This antagonist induced concentration-dependent reductions in bradykinin relaxation ($n = 6$–8) (Figure 4A). In contrast, HOE-140 (10 nM) failed to change NS309 ($n = 7$) or CyPPA relaxation ($n = 7$) (Figure 4B,C).

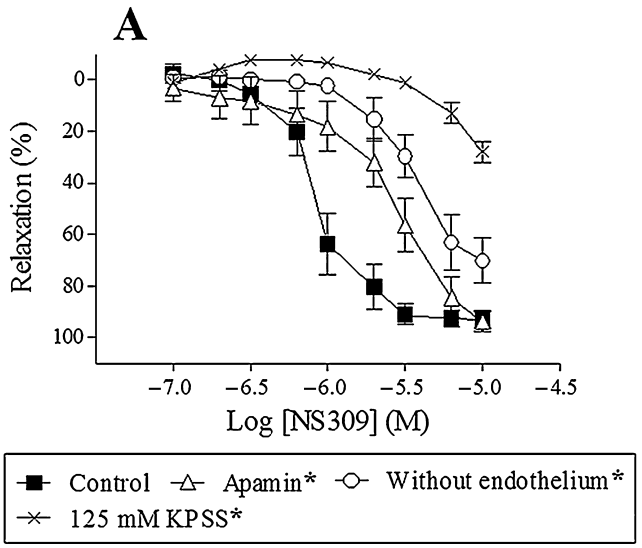
Effect of NS309 and CyPPA on bradykinin relaxation

In U46619 (0.1 µM)-contracted segments, bradykinin (0.01 nM–300 nM) induced concentration-dependent relaxations (Figure 5A). To investigate the effect of opening SK_{Ca} and IK_{Ca} channels or opening SK_{Ca} channels alone on bradykinin relaxation, NS309 (1 µM) or CyPPA (6 µM) was added to the organ bath after the segments were contracted with U46619 (0.1 µM) and then concentration–response curves for bradykinin were performed. Addition of a single concentration of NS309 (1 µM) or CyPPA (6 µM) induced a relaxation of 14 ± 6% ($n = 8$) and 18 ± 8% ($n = 7$) respectively (Figure 5C,D). To exclude the effect of the reduced contraction level after addition of NS309 or CyPPA on bradykinin relaxation, a single concentration of the NO donor, SNP (1 µM), was used to reduce the contraction to the same level as with NS309 or CyPPA without activating the SK_{Ca} and IK_{Ca} channels. Addition of a single concentration of SNP (1 µM) induced a relaxation of 15 ± 8% ($n = 7$) (Figure 5B), and had no effect on bradykinin relaxation ($n = 7$) (Figure 6A). In contrast, both NS309 and CyPPA enhanced bradykinin relaxation ($n = 7$–8) (Figure 6A). Incubation with NS309 (1 µM) or CyPPA (6 µM) did not affect relaxation to SNP ($n = 7$) (Figure 6B). Blocking IK_{Ca} channels with charybdotoxin (0.1 µM, $n = 8$) had no effect on the enhanced bradykinin relaxation induced by NS309 (Figure 6C). In contrast, blocking SK_{Ca} channels with apamin (0.5 µM, $n = 7$–8) abolished the enhanced bradykinin relaxation induced by NS309 or CyPPA (Figure 6C,D). Myogenic tone after addition of apamin and/or charybdotoxin, U46619 (0.1 µM) contraction, EC_{50}-values and maximum relaxation for bradykinin after addition of a single concentration of SNP (1 µM), NS309 (1 µM) and CyPPA (6 µM) are reported in Table 1. No increase in myogenic tone was found after treatment with apamin and/or charybdotoxin.

Mechanisms involved in the enhanced bradykinin relaxation induced by NS309 and CyPPA

Investigating the calcium signalling in endothelial cells, concentrations of bradykinin above 1 nM increased the intracellular calcium concentration in endothelial cells ($n = 5$), whereas neither NS309 (1 µM) nor CyPPA (6 µM) changed the intracellular calcium concentration ($n = 5$). Furthermore, simultaneous addition of bradykinin (3 nM) and NS309 (1 µM) or CyPPA (6 µM) did not increase the intracellular calcium concentration further (Figure 7).

To address the relative contribution from NO and prostaglandins to bradykinin relaxation in the presence of NS309 or



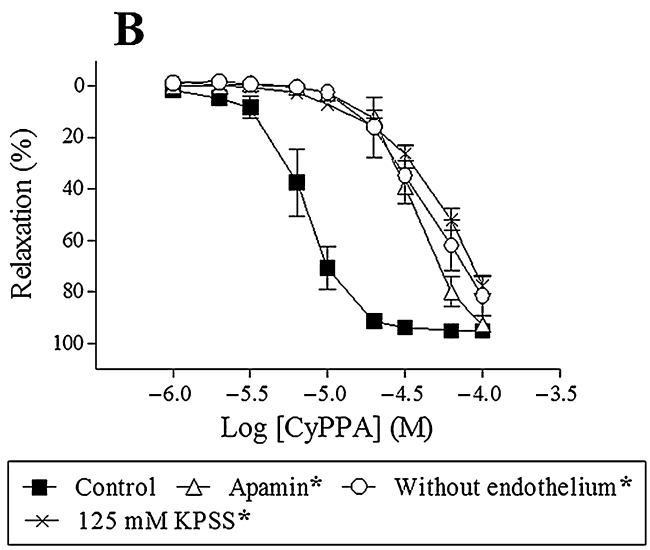


Figure 2 NS309 relaxation (A) or CyPPA relaxation (B) in the absence or presence of apamin (0.5 μM), in segments without endothelium, or contracted with 125 mM KPSS. Results are mean ± SEM ($n = 7$). Two-way ANOVA. *$P < 0.05$ from control.

CyPPA, segments were incubated with ADMA (300 μM) to inhibit NO synthesis, indomethacin (3 μM) to inhibit prostaglandin synthesis, and oxyhaemoglobin (25 μM) to scavenge NO. ADMA ($n = 7$) and/or indomethacin ($n = 8$) reduced, but did not eliminate bradykinin relaxation, whereas ADMA, indomethacin and oxyhaemoglobin ($n = 8$) abolished bradykinin relaxation (Figure 8A,B). Myogenic tone after addition of ADMA and/or indomethacin, EC_{50} values and maximum relaxation for bradykinin are found in Table 2. Only those treatments involving ADMA increased myogenic tone.

Investigating the effect of the constrictor on bradykinin relaxation, segments were contracted with U46619 (0.1 μM) or endothelin-1 (0.03 μM). No difference in bradykinin relaxations were observed using U46619 or endothelin-1 (EC_{50}: 2.9 ± 0.8 nM vs. 3.6 ± 0.6 nM, maximum relaxation: 96 ± 2% vs. 94 ± 2%, $n = 7$). Furthermore, in segments contracted with either U46619 or endothelin-1, bradykinin relaxation was abolished by the combination of ADMA (300 μM), indomethacin (3 μM) and oxyhaemoglobin (25 μM) ($n = 7$) (Figure 9).

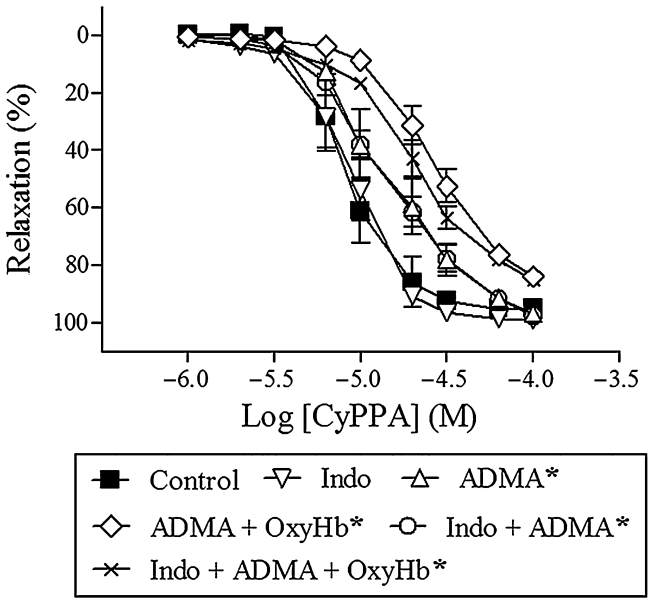


Figure 3 CyPPA relaxation in the presence of asymmetric dimethylarginine (300 μM, ADMA), ADMA + oxyhaemoglobin (25 μM, OxyHb), indomethacin (3 μM, Indo), Indo + ADMA and Indo + ADMA + OxyHb in porcine retinal arterioles contracted with U46619 (0.1 μM). Results are mean ± SEM ($n = 7$). Two-way ANOVA. *$P < 0.05$ from control.

To investigate if oxyhaemoglobin is a scavenger of only NO, or if it is also a scavenger of H_2S that may be involved in EDHF-type relaxations (Feletou and Vanhoutte, 2009), relaxations to NO solution and H_2S in the absence and the presence of oxyhaemoglobin were investigated. Both NO solution and H_2S induced concentration-dependent relaxations (EC_{50}: 0.090 ± 0.02 μM and 170 ± 30 μM, maximum relaxation: 95 ± 4% and 99 ± 1%, respectively $n = 7$–8) (Figure 10A,B). Oxyhaemoglobin (25 μM) completely abolished NO relaxation ($n = 6$) (Figure 10A), whereas H_2S relaxation remained unchanged in the presence of oxyhaemoglobin (EC_{50}: 300 ± 70 μM, maximum relaxation: 98 ± 2%, $n = 7$) (analysed by a two-way ANOVA) (Figure 10B). However, a subanalysis (Student's *t*-test) showed that oxyhaemoglobin reduced the relaxation to 0.1 mM H_2S. Investigating the contribution of H_2S to bradykinin relaxation, inhibiting cystathionine γ-lyase with D,L-propargylglycine (PPG, 5 mM) in the presence of ADMA and indomethacin failed to change bradykinin relaxation ($n = 7$, data not shown).

Discussion

In a previous study, we found that SK_{Ca} and IK_{Ca} channels were involved in endothelium-dependent bradykinin relaxation of retinal arterioles (Dalsgaard *et al.*, 2009). The present study shows that $SK_{Ca}3$ and IK_{Ca} protein is localized in the vascular endothelium in porcine retinal arterioles, and that opening of

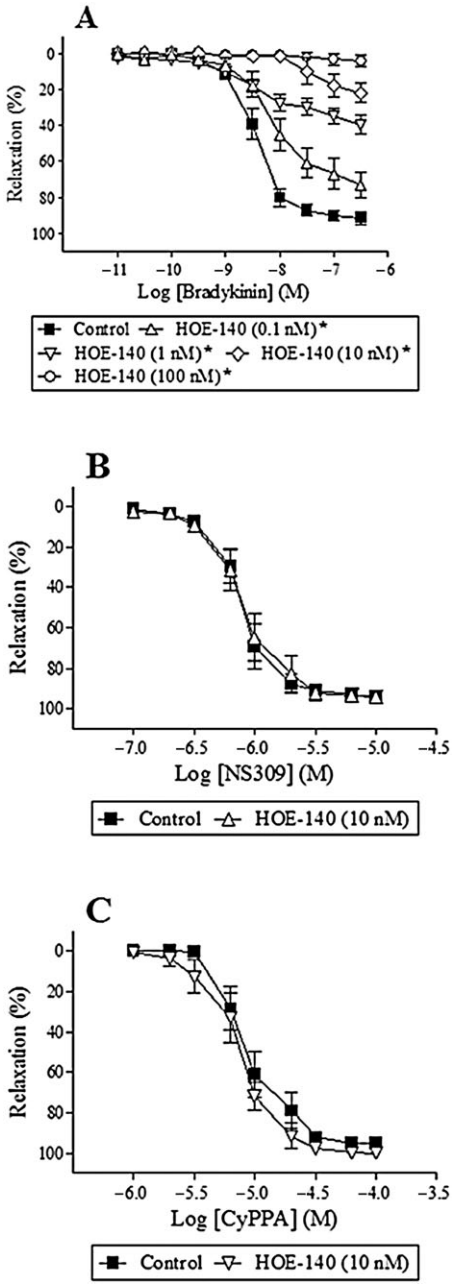


Figure 4 Effect of the bradykinin-receptor B_2 antagonist, HOE-140 (0.1, 1.0, 10 and 100 nM), on bradykinin relaxation (A), HOE-140 (10 nM) on NS309 relaxation (B) and HOE-140 (10 nM) on CyPPA relaxation (C) in porcine retinal arterioles contracted with U46619 (0.1 μM). Results aremean ± SEM ($n = 6$–8). Two-way ANOVA. *$P < 0.05$ from control.

SK_{Ca} channels with either NS309 or CyPPA, without causing changes in endothelial cell calcium, selectively enhance bradykinin relaxation in retinal arterioles. Moreover, our findings suggest that the enhanced bradykinin relaxation in the presence of SK_{Ca} openers can primarily be ascribed to increased formation of NO, as bradykinin relaxation, independent of contractile agonist applied, was abolished in the presence of a combination of inhibitors of cyclooxygenase and NO synthase, and the NO scavenger, oxyhaemoglobin.

In porcine coronary arteries, RT-PCR, Western blot and immunofluorescent labelling have shown that $SK_{Ca}3$ protein is expressed in endothelial cells and not in smooth muscle cells (Burnham *et al*., 2002). Single channel recordings from outside-out patches on isolated porcine coronary artery endothelial cells also confirm the existence of IK_{Ca} channels, and IK_{Ca} mRNA has been detected in intact porcine coronary artery endothelium (Bychkov *et al*., 2002). In agreement with these findings, $SK_{Ca}3$ and IK_{Ca} protein was expressed in the endothelium and not in the smooth muscle of the vascular segments, suggesting that $SK_{Ca}3$ and IK_{Ca} channels are important for endothelial function in porcine retinal arterioles.

In the present study, NS309 was applied to open SK_{Ca} and IK_{Ca} channels. NS309 has a slight selectivity for IK_{Ca} over SK_{Ca}, and no effect on large conductance calcium-activated potassium channels (BK_{Ca} or $K_{Ca}1.1$) (Strobaek *et al*., 2004). Previously, we have found that relaxation to NS309 was reduced upon inhibiting NO synthase and blocking SK_{Ca} channels in porcine retinal arterioles (Dalsgaard *et al*., 2009), suggesting that SK_{Ca} channels are involved in regulation of NO synthesis. These observations are further supported by other studies showing that blocking SK_{Ca} and IK_{Ca} channels inhibits ATP, histamine and NS309-induced NO synthesis in human umbilical vein endothelial cells (HUVECs) (Sheng and Braun, 2007), and noradrenaline and acetylcholine-induced NO formation in rat superior mesenteric artery (Stankevicius *et al*., 2006). Using a novel selective opener of $SK_{Ca}2$ and $SK_{Ca}3$ channels, CyPPA (Hougaard *et al*., 2007), the present study found that CyPPA induced endothelium-dependent relaxations. These relaxations were reduced by inhibition of NO synthase and a NO scavenger, but not by inhibiting cyclooxygenase, suggesting that opening SK_{Ca} channels indeed induce NO-mediated relaxation, and for the first time links SK_{Ca} channels to formation of NO in small arterioles.

In addition to opening of SK_{Ca} and IK_{Ca} channels, NS309 has been reported to block voltage-dependent Ca^{2+} (Ca_V) channels in bladder smooth muscle (Morimura *et al*., 2006) and voltage-dependent K^+ ($K_V11.1$) channels (Strobaek *et al*., 2004) at high concentrations, while CyPPA in HEK293 cells was reported also to block BK_{Ca} and voltage-dependent Na^+ channels (Na_V) channels in high concentrations (Hougaard *et al*., 2007). Therefore, to address the specificity of the channel openers in porcine retinal arterioles, we investigated their effects in segments contracted with high extracellular K^+, which blocked the response to NS309 at concentrations below 6 μM and to CyPPA at concentrations below 20 μM. In the case of CyPPA no difference in relaxation was observed between blocking SK_{Ca} channels, removing the endothelium or contracting with high K^+, suggesting that CyPPA is specific for $SK_{Ca}2$ and $SK_{Ca}3$ channels, but we cannot exclude that CyPPA may block other channels at high concentrations. However, NS309 relaxation was less pronounced in high K^+ contracted segments compared with endothelium-denuded segments. Although we carefully removed adhering tissue in the present study, IK_{Ca} immunoreaction was observed in the majority of all retinal cells, and we cannot exclude that IK_{Ca} channels in residual perivascular tissue affected NS309 relaxation, explaining the difference in NS309 relaxation between high K^+ contracted and endothelium-denuded segments.

Bradykinin binds to G-protein coupled B_2 receptors in the endothelial cell and activates endothelium-dependent pathways leading to vasodilatation (Adams *et al*., 1989; Mombouli

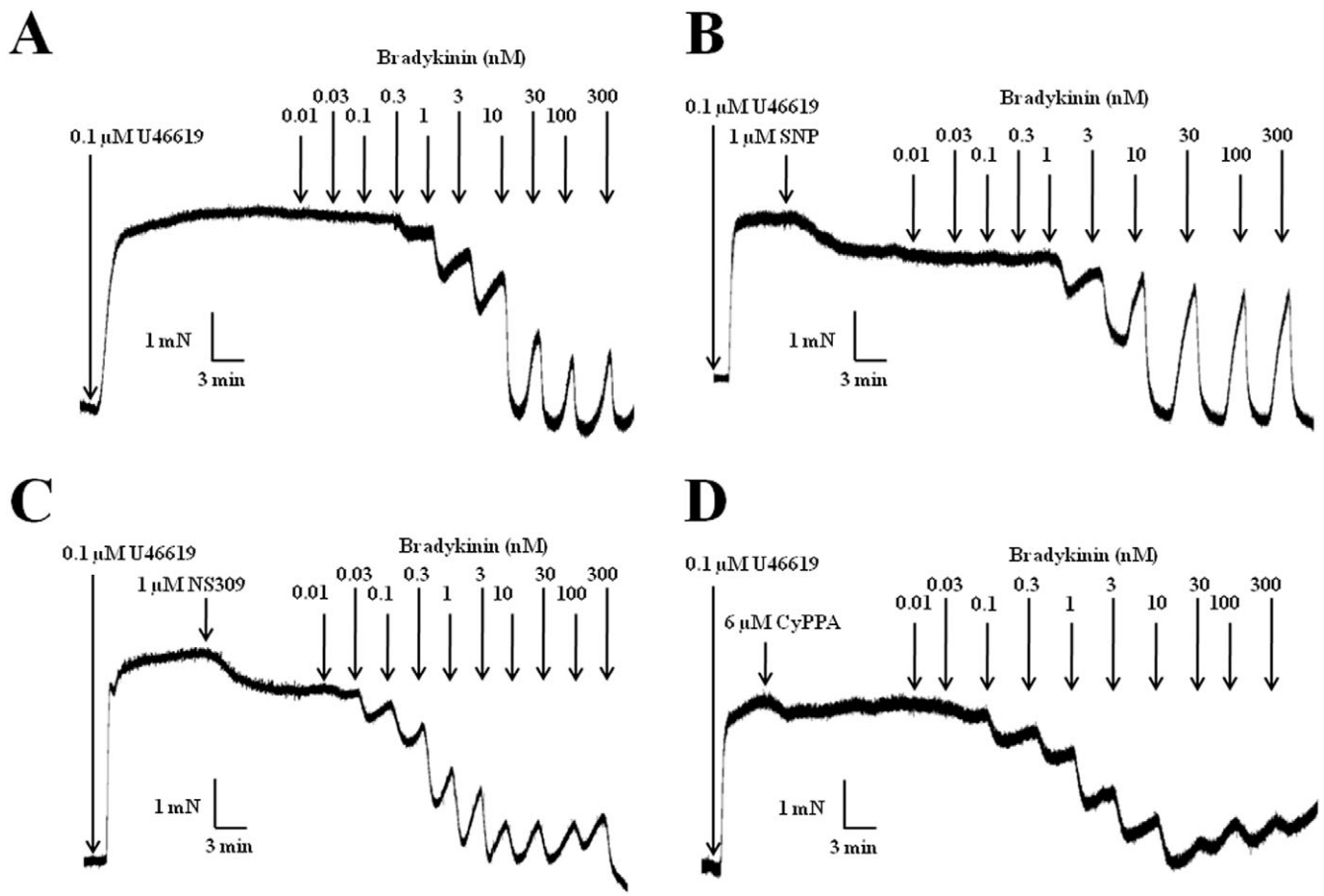


Figure 5 Original traces showing the response to increasing concentrations of bradykinin (A), bradykinin in the presence of sodium nitroprusside (SNP, 1 μM) (B), bradykinin in the presence of NS309 (1 μM) (C) and bradykinin in the presence of CyPPA (6 μM) (D), in porcine retinal arterioles contracted with U46619 (0.1 μM).

and Vanhoutte, 1995; Mombouli *et al*., 1996). To exclude that NS309 and CyPPA cause vasodilatation by activation of the bradykinin receptors, we investigated the effect of the bradykinin B_2 receptor antagonist, HOE-140, and found that it concentration-dependently reduced bradykinin relaxation without changing NS309 and CyPPA relaxation, hence suggesting that NS309 and CyPPA induce relaxation independent of the bradykinin B_2 receptor.

Previous studies have reported that opening of SK_{Ca} and IK_{Ca} channels with NS309 is associated with increased endothelium-dependent relaxation in mesenteric small arteries from Zucker lean (ZL) and Zucker diabetic fatty (ZDF) rats (Brondum *et al*., 2009), and also enhances acetylcholine relaxation in rat cremaster arterioles (Sheng *et al*., 2009). Moreover, another SK_{Ca} and IK_{Ca} opener, naphtho[1,2-d]thiazol-2-ylamine (SKA-31) enhanced EDHF-type relaxation in mouse carotid artery (Sankaranarayanan *et al*., 2009). In the present study, opening of SK_{Ca} and IK_{Ca} channels with NS309 or opening of only SK_{Ca} channels with CyPPA enhanced bradykinin relaxation. Bradykinin relaxation was not changed when a NO donor, SNP, was used to reduce the U46619 contraction to a similar level as caused by NS309 or CyPPA. Moreover, NS309 and CyPPA did not change SNP-induced relaxation. These results suggest that the reduction in U46619 contraction has no implications for the NS309- and CyPPA-enhanced bradykinin relaxation, and that NS309 or CyPPA do not change the sensitivity to NO. In addition, the pronounced leftward shifts of the concentration–response curves for bradykinin, induced by NS309 and CyPPA, were abolished by blocking SK_{Ca} channels with apamin, but not by blocking IK_{Ca} channels with charybdotoxin, suggesting that selective opening of SK_{Ca} channels is sufficient to enhance endothelium-dependent vasodilatation in porcine retinal arterioles.

Activation of the bradykinin B_2 receptor leads to activation of phospholipase C, and thereby increase the intracellular calcium concentration in the endothelial cell through inositol 1,4,5-trisphosphate and diacylglycerol (Adams *et al*., 1989; Mombouli and Vanhoutte, 1995; Mombouli *et al*., 1996). NS309 and DC-EBIO have been reported to increase calcium in isolated HUVECs stimulated with ATP (Sheng *et al*., 2009). In contrast, endothelial cell calcium was not increased in intact rat cerebral arteries upon relaxation induced by the SK_{Ca} and IK_{Ca} channel opener, 1-EBIO (Marrelli *et al*., 2003). We have recently found that combined blockade of SK_{Ca} and IK_{Ca} channels fails to change the increase in endothelial cell calcium evoked by acetylcholine in rat superior mesenteric artery (Stankevicius *et al*., 2006), and that incubation with NS309 increases acetylcholine relaxation without changes in endothelial cell calcium from mesenteric small arteries from

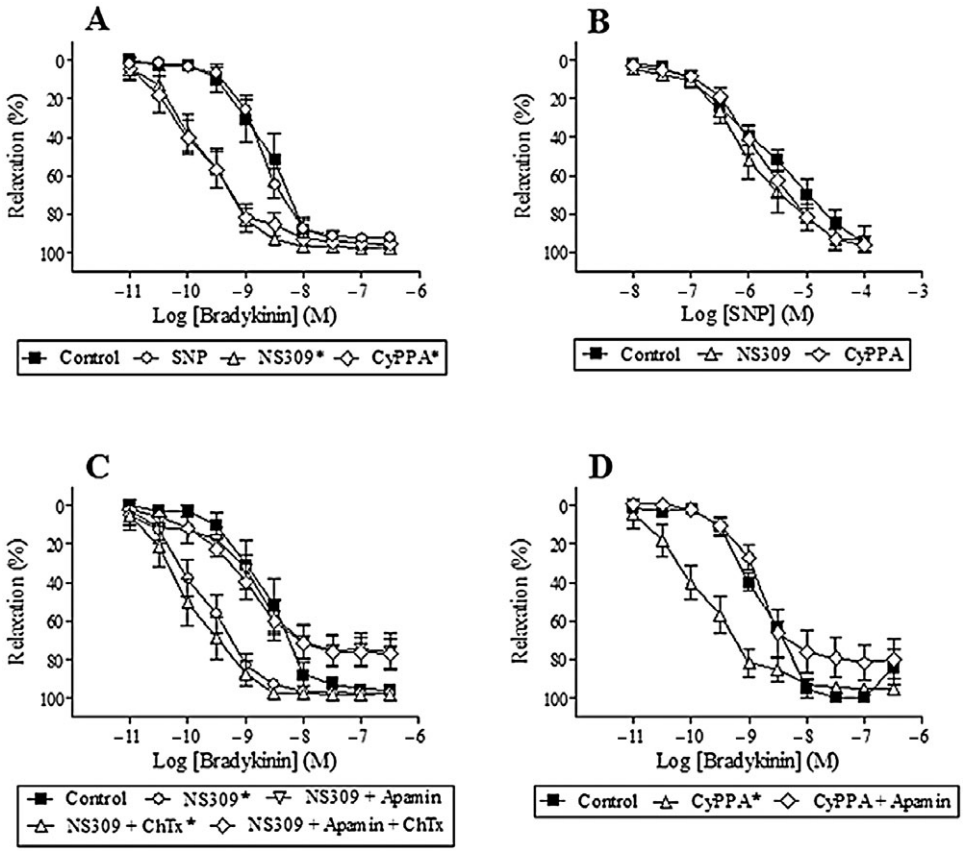


Figure 6 Effect of sodium nitroprusside (SNP, 1 μM), NS309 (1 μM) and CyPPA (6 μM) on bradykinin relaxation (A). Effect of NS309 (1 μM) and CyPPA (6 μM) on SNP relaxation (B). Effect of NS309 (1 μM) on bradykinin relaxation in the absence and presence of apamin (0.5 μM), and/or charybdotoxin (ChTx, 0.1 μM) (C). Effect of CyPPA (6 μM) on bradykinin relaxation in the absence and presence of apamin (0.5 μM) (D). Results are mean ± SEM ($n = 6–8$). Two-way ANOVA. *$P < 0.05$ from control.

Table 1 Myogenic tone after addition of blockers; EC_{50}-values and maximum relaxation for bradykinin in the presence of SNP (1 μM), NS309 (1 μM) and CyPPA (6 μM)

Treatment	n	*Myogenic tone Contraction (Nm^{-1})*	*U46619 (0.1 μM) Contraction (Nm^{-1})*	*Bradykinin*	
				EC_{50} (nM)	*Maximum relaxation (%)*
Control	8	0.28 ± 0.06	1.0 ± 0.1	3.1 ± 1.1	96.3 ± 2.9
SNP	7	0.31 ± 0.04	1.0 ± 0.1	2.2 ± 1.4	92.2 ± 2.8
Control	8	0.30 ± 0.04	1.0 ± 0.1	3.9 ± 1.2	95.7 ± 1.9
NS309	8	0.26 ± 0.05	1.0 ± 0.1	0.4 ± 0.2*	97.4 ± 1.1
NS309/charybdotoxin	8	0.38 ± 0.10	1.0 ± 0.1	0.3 ± 0.2*	98.3 ± 1.1
NS309/apamin	8	0.48 ± 0.12	0.9 ± 0.1	3.8 ± 1.4	75.8 ± 9.4*
NS309/charybdotoxin/apamin	8	0.55 ± 0.13	1.0 ± 0.1	3.7 ± 1.5	76.8 ± 7.5*
Control	7	0.26 ± 0.07	1.0 ± 0.1	2.6 ± 0.5	95.4 ± 4.6
CyPPA	7	0.31 ± 0.06	0.9 ± 0.1	0.3 ± 0.2*	95.1 ± 1.9
CyPPA/apamin	7	0.45 ± 0.09	1.0 ± 0.1	3.2 ± 1.4	81.8 ± 9.3

Mean ± SEM Student's *t*-test.
*$P < 0.05$ from their respective controls.
CyPPA, cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4- yl]-amine; NS309, 6,7-dichloro-1H-indole-2,3-dione 3-oxime; SNP, sodium nitroprusside; U46619, 9,11-dideoxy-9a,11a-epoxymethanoprostaglandin $F_{2\alpha}$.

ZL and ZDF rats (Brondum *et al*., 2009). In the present study, bradykinin induced concentration-dependent increases in endothelial cell calcium, whereas neither NS309 nor CyPPA alone or in the presence of bradykinin increased endothelial cell calcium. Thus, the present study provides further support to the observation that the effect on endothelial cell calcium of SK_{Ca} and IK_{Ca} channel modulation is different in cultured isolated endothelial cells (Sheng and Braun, 2007; Sheng *et al*., 2009) versus intact arteries (Marrelli *et al*., 2003; McSherry *et al*., 2005; Stankevicius *et al*., 2006; Brondum *et al*.,

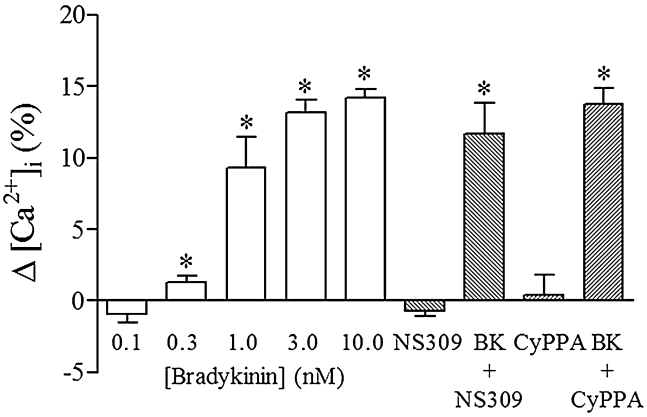


Figure 7 Relative change in intracellular calcium ($\Delta[Ca^{2+}]_i$) for bradykinin (0.1 nM, 1 nM, 3 nM and 10 nM), NS309 (1 μM), NS309 (1 μM) + bradykinin (BK, 3 nM), CyPPA (6 μM) and CyPPA (6 μM) + BK (3 nM) in endothelial cells from porcine retinal arterioles. Results are mean ± SEM (n = 4–6). Wilcoxon matched pair test, *P < 0.05 from zero $[Ca^{2+}]_i$.

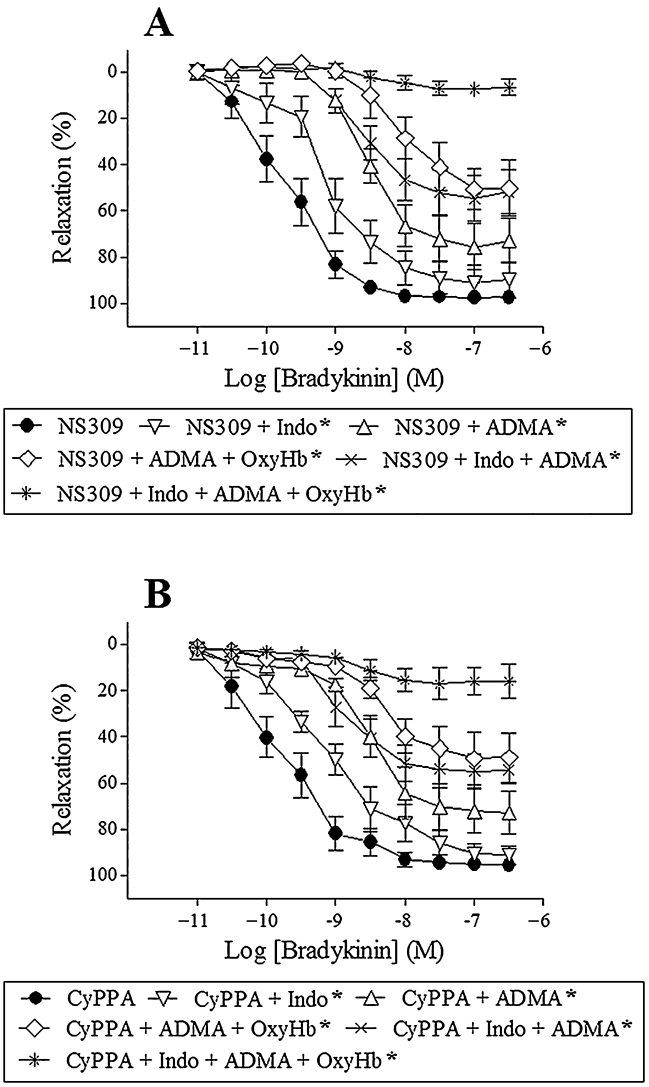


Figure 8 Bradykinin relaxation in the presence of NS309 (1 μM) (A) or CyPPA (6 μM) (B) and asymmetric dimethylarginine (300 μM, ADMA), ADMA + oxyhaemoglobin (25 μM, OxyHb), indomethacin (3 μM, indo), indo + ADMA and indo + ADMA + OxyHb in porcine retinal arterioles contracted with U46619 (0.1 μM). Results are mean ± SEM (n = 6–8). Two-way ANOVA. *P < 0.05 from their respective controls.

2009). In arterial segments, calcium has been shown to be transported through gap junctions (Dora *et al*., 1997; Yashiro and Duling, 2000), and the adjacent endothelial cells may therefore rapidly buffer local calcium events in isolated arterial segments. Although, we cannot exclude that other approaches such as high velocity scanning or total internal reflection fluorescence (Navedo *et al*., 2006) developed for intact arteries may reveal local changes in endothelial cell calcium upon addition of SK_{Ca} and IK_{Ca} openers, the present results suggest that in intact arteries, NS309 and CyPPA rather than increasing endothelial cell calcium, sensitize the SK_{Ca} and IK_{Ca} channels for calcium, and hence enhance bradykinin relaxation. As contractile agonists may also increase endothelial cell calcium and lead to formation of endothelium-derived factors (Dora *et al*., 1997; Stankevicius *et al*., 2006), sensitization of the SK_{Ca} and IK_{Ca} channels may also explain the direct vasodilatatory effect of NS309 and CyPPA in porcine retinal arterioles.

Previous studies show that opening of IK_{Ca} and SK_{Ca} channels with either NS309 or SKA-31 enhances endothelium-dependent vasodilatation and ascribes this to an enhanced EDHF-type relaxation (Brondum *et al*., 2009; Sankaranarayanan *et al*., 2009; Sheng *et al*., 2009). In porcine retinal arterioles, we have previously found that NO and prostaglandins mediate bradykinin relaxation in porcine arterioles (Jeppesen *et al*., 2002; Dalsgaard *et al*., 2009). In agreement with these latter studies, this study found that in the presence of NS309 or CyPPA, bradykinin relaxation was still abolished by inhibiting cyclooxygenase, NO synthase and NO scavenging in porcine retinal arterioles. These findings suggest that NS309 and CyPPA through opening of SK_{Ca} channels enhance bradykinin relaxation by increasing mainly the formation of NO rather than increasing EDHF-type relaxations in porcine retinal arterioles.

In rat mesenteric arteries, it has been reported that stimulation of the thromboxane A_2 receptor with U46619 leads to progressive loss of K_{Ca} channel activity, where the SK_{Ca} channel subtype is most sensitive, and ultimately loss of EDHF-linked changes in smooth muscle membrane potential and tension (Plane and Garland, 1996; Crane and Garland, 2004). However, in the present study the vascular segments were only contracted twice with U46619, and we have previously reported that SK_{Ca} channel blockade reduces bradykinin relaxation in U46619-contracted retinal arterioles (Dalsgaard *et al*., 2009). Moreover, in segments contracted with endothelin-1, bradykinin relaxation was not different to bradykinin relaxation in U46619-contracted segments, and relaxation was still abolished by inhibition of NO synthase, cyclooxygenase and in the presence of a NO scavenger. These results suggest that bradykinin relaxation is not affected by U46619 in porcine retinal arterioles.

Methaemoglobin, the product of oxidized haemoglobin, has been reported to scavenge H_2S (Beauchamp, Jr *et al*., 1984), and as H_2S has been suggested to be involved in EDHF-

Table 2 Myogenic tone after addition of inhibitors; EC_{50}-values and maximum relaxation for bradykinin in the presence of NS309 (1 μM) and CyPPA (6 μM)

Treatment	n	*Myogenic tone*	*U46619 (0.1 μM)*	*Bradykinin*	
		Contraction (Nm^{-1})	*Contraction* (Nm^{-1})	EC_{50} (*nM*)	*Maximum relaxation* (%)
NS309	8	0.25 ± 0.06	0.9 ± 0.1	0.4 ± 0.2	97.4 ± 1.1
NS309/ADMA	7	0.63 ± 0.14*	1.0 ± 0.1	8.4 ± 1.3*	75.7 ± 10.0*
NS309/ADMA/oxyhaemoglobin	8	0.56 ± 0.12*	1.0 ± 0.1	–	50.5 ± 8.8*
NS309/indomethacin	8	0.39 ± 0.09	1.0 ± 0.1	1.6 ± 0.7*	91.0 ± 7.3
NS309/indomethacin/ADMA	8	0.78 ± 0.10*	0.9 ± 0.1	–	54.7 ± 10.0*
NS309/indomethacin/ADMA/oxyhaemoglobin	8	0.85 ± 0.12*	0.9 ± 0.1	–	7.3 ± 2.5*
CyPPA	8	0.30 ± 0.04	1.0 ± 0.1	0.5 ± 0.3	95.1 ± 2.0
CyPPA/ADMA	8	0.58 ± 0.12*	1.0 ± 0.1	8.7 ± 0.9*	72.7 ± 9.4*
CyPPA/ADMA/oxyhaemoglobin	8	0.66 ± 0.16*	1.0 ± 0.1	–	48.9 ± 10.6*
CyPPA/indomethacin	8	0.45 ± 0.09	1.0 ± 0.1	1.6 ± 0.8*	91.2 ± 4.0
CyPPA/indomethacin/ADMA	8	0.86 ± 0.12*	1.0 ± 0.1	–	54.4 ± 5.8*
CyPPA/indomethacin/ADMA/oxyhaemoglobin	8	0.80 ± 0.13*	0.9 ± 0.1	–	16.0 ± 7.4*

Mean ± SEM Student's *t*-test. *$P < 0.05$ from NS309 or CyPPA – indicates that EC_{50} values could not be calculated, as concentration-response experiments did not reach an asymptotic minimum.
ADMA, asymmetric dimethylarginine; CyPPA, cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4- yl]-amine; NS309, 6,7-dichloro-1H-indole-2,3-dione 3-oxime; U46619, 9,11-dideoxy-9a,11a-epoxymethanoprostaglandin $F_{2\alpha}$.

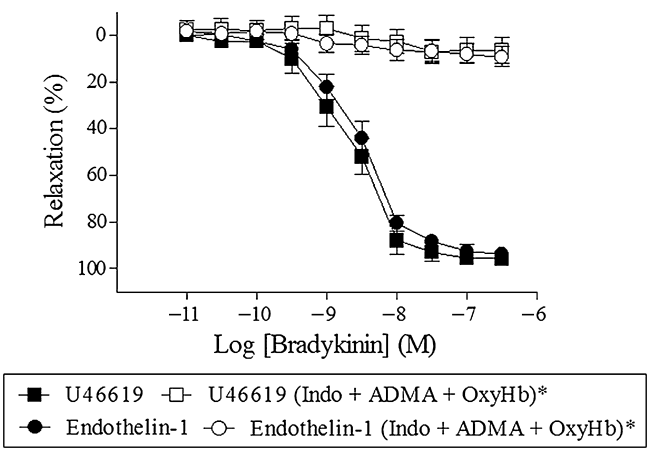


Figure 9 Bradykinin relaxation in the absence or presence of indomethacin (3 μM, Indo) + asymmetric dimethylarginine (ADMA; 300 μM) + oxyhaemoglobin (OxyHb; 25 μM) in porcine retinal arterioles contracted with U46619 (0.1 μM) or endothelin-1 (0.03 μM) (C). Results are mean ± SEM (n = 6–8). Two-way ANOVA. *$P < 0.05$ from their respective controls.

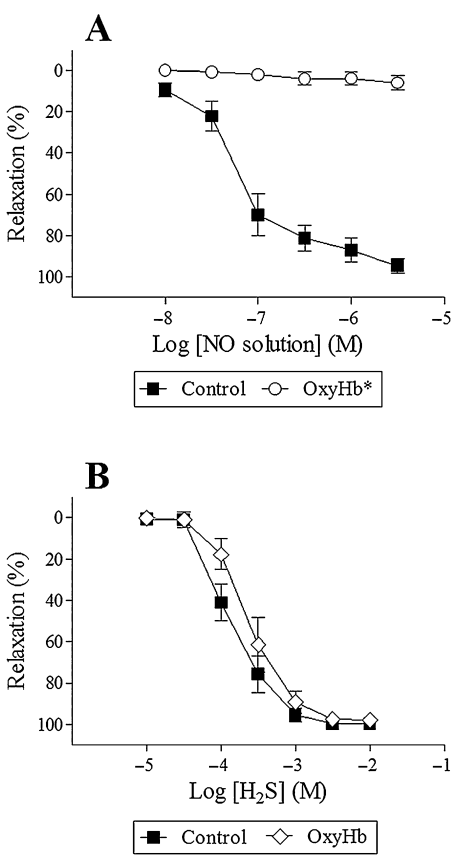


Figure 10 Relaxation to NO solution (A) and H_2S (B) in the absence and presence of oxyhaemoglobin (25 μM, OxyHb) in porcine retinal arterioles contracted with U46619 (0.1 μM). Results are mean ± SEM (n = 6–8). Two-way ANOVA. *$P < 0.05$ from control.

type relaxation (Feletou and Vanhoutte, 2009), the use of oxyhaemoglobin as a NO scavenger could block a potential relaxing effect of H_2S in the arterioles. Therefore, the observed reduction in bradykinin relaxation upon addition of oxyhaemoglobin might also be related to the scavenging of not only NO, but also H_2S. However, this study found that oxyhaemoglobin was a very poor scavenger of H_2S compared with NO. Furthermore, inhibition of cystathionine γ-lyase failed to change bradykinin relaxation during inhibition of NO synthase and cyclooxygenase. Taken together these results support the findings that in porcine retinal arterioles, bradykinin relaxation does not involve H_2S and is mainly mediated by NO.

In conclusion, in porcine retinal arterioles, $SK_{Ca}3$ and IK_{Ca} protein was localized to the vascular endothelium. Bradykinin increased endothelial cell calcium leading to opening of SK_{Ca} and IK_{Ca} channels. Without altering endothelial cell calcium, NS309 and CyPPA opened SK_{Ca} channels and enhanced the NO-mediated bradykinin relaxation. These findings imply

that in retinal arterioles, opening of SK_{Ca} channels improves endothelium-dependent relaxation mediated by NO release. As a decreased endothelium-dependent relaxation is found in patients with glaucoma (Henry *et al*., 1999; Flammer *et al*., 2002; Galassi *et al*., 2004; Polak *et al*., 2007; Mozaffarieh *et al*., 2008) and diabetic retinopathy (Pemp and Schmetterer, 2008) treatment with specific SK_{Ca} channel openers may improve endothelium-dependent vasodilatation and restore retinal blood flow in these patients.

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Conflicts of interest

None.

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