Opening of Small and Intermediate Calcium-Activated Potassium Channels Induces Relaxation Mainly Mediated by Nitric-Oxide Release in Large Arteries and Endothelium-Derived Hyperpolarizing Factor in Small Arteries from Rat

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ABSTRACT

This study was designed to investigate whether calcium-activated potassium channels of small (SK_{Ca} or $K_{Ca}2$) and intermediate (IK_{Ca} or $K_{Ca}3.1$) conductance activated by 6,7-dichloro-1H-indole-2,3-dione 3-oxime (NS309) are involved in both nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF)-type relaxation in large and small rat mesenteric arteries. Segments of rat superior and small mesenteric arteries were mounted in myographs for functional studies. NO was recorded using NO microsensors. SK_{Ca} and IK_{Ca} channel currents and mRNA expression were investigated in human umbilical vein endothelial cells (HUVECs), and calcium concentrations were investigated in both HUVECs and mesenteric arterial endothelial cells. In both superior ($\sim$1093 μm) and small mesenteric ($\sim$300 μm) arteries, NS309 evoked endothelium- and concentration-dependent relaxations. In superior mesenteric arteries, NS309 relaxations and NO release were inhibited by both N^G,N^G-asymmetric dimethyl-L-arginine (ADMA) (300 μM), an inhibitor of NO synthase, and apamin (0.5 μM) plus 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34) (1 μM), blockers of SK_{Ca} and IK_{Ca} channels, respectively. In small mesenteric arteries, NS309 relaxations were reduced slightly by ADMA, whereas apamin plus an IK_{Ca} channel blocker almost abolished relaxation. Iberiotoxin did not change NS309 relaxation. HUVECs expressed mRNA for SK_{Ca} and IK_{Ca} channels, and NS309 induced increases in calcium, outward current, and NO release that were blocked by apamin and TRAM-34 or charybdotoxin. These findings suggest that opening of SK_{Ca} and IK_{Ca} channels leads to endothelium-dependent relaxation that is mediated mainly by NO in large mesenteric arteries and by EDHF-type relaxation in small mesenteric arteries. NS309-induced calcium influx appears to contribute to the formation of NO.

Introduction

The vascular endothelium plays a major role in the regulation of blood flow. Through the release of vasoactive agents, the endothelium may decrease the contraction level of vascular smooth muscle and hence increase blood flow. The vasoactive

ABBREVIATIONS: NO, nitric oxide; ADMA, N^G,N^G-asymmetric dimethyl-L-arginine; Ct, cycle threshold; ANOVA, analysis of variance; BK_{Ca} channel, large conductance calcium-activated potassium channel; ChTX, charybdotoxin; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; EDHF, endothelium-derived hyperpolarizing factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cell; IbTX, iberiotoxin; IK_{Ca} channel, intermediate conductance calcium-activated potassium channel; NS309, 6,7-dichloro-1H-indole-2,3-dione 3-oxime; PSS, physiological saline solution; QPCR, quantitative polymerase chain reaction; SK_{Ca} channel, small conductance calcium-activated potassium channel; SNAP, S-nitroso-N-acetylpenicillamine; TRAM-34, 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole; U46619, 9,11-dideoxy-9a,11a-epoxymethanoprostaglandin $F_{2\alpha}$; BAPTA-1-AM, 1,2-bis(2-aminophenoxy)ethane-$N,N,',N'$-tetraacetic acid-acetomethyl ester; DAF-FM, 4-amino-5-methylamino-2',7'-difluorofluorescein; CyPPA, N-Cyclohexyl-N-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-4-pyrimidinamine.

agents released by the endothelium include nitric oxide (NO), prostacyclin, and factors involved in endothelium-dependent hyperpolarizing factor (EDHF)-type relaxation (Félétou and Vanhoutte, 1988, 2009). The production of endothelium-dependent relaxing factors generally involves an increase in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. An increase in endothelial $[Ca^{2+}]_i$ will open calcium-activated potassium channels of small (SK_{Ca} or $K_{Ca}2$) and intermediate (IK_{Ca} or $K_{Ca}3.1$) conductance, thereby hyperpolarizing the endothelial cell. These channels have been acknowledged as a required component for the activation of EDHF-type relaxation (Burnham et al., 2002; Bychkov et al., 2002; Eichler et al., 2003). In vivo studies in mice show that suppression of the $SK_{Ca}3$ channel (Taylor et al., 2003), knockout of the IK_{Ca} channel (Si et al., 2006), and deficit of both $SK_{Ca}3$ and IK_{Ca} channels (Brähler et al., 2009) lead to elevated blood pressure. In addition, opening SK_{Ca} and IK_{Ca} channels decreases myogenic tone, increases acetylcholine (ACh)-induced relaxation in rat cremaster arterioles (Sheng et al., 2009), and restores attenuated EDHF-type relaxation in mesenteric small arteries from Zucker diabetic fatty rats (Brøndum et al., 2010). Moreover, opening IK_{Ca} channels decreases mean arterial blood pressure in angiotensin II-induced hypertensive mice (Sankaranarayanan et al., 2009) and conscious dogs (Damkjaer et al., 2011). These results support findings that SK_{Ca} and IK_{Ca} channels are involved in controlling blood pressure and organ blood flow, and this often is attributed to EDHF-type relaxation. However, recent studies suggest that SK_{Ca} and IK_{Ca} channel opening also is associated with the activation of NO synthase and NO production (Stankevicius et al., 2006; Sheng and Braun, 2007; Brähler et al., 2009; Sheng et al., 2009; Dalsgaard et al., 2010a). In addition, mechanisms involved in SK_{Ca} and IK_{Ca} channel activation leading to vasodilation are largely unknown.

Therefore, the present study investigated the effects of opening endothelial SK_{Ca} and IK_{Ca} channels by the use of 6,7-dichloro-1H-indole-2,3-dione 3-oxime (NS309) in a large artery and small artery and hypothesized that opening of endothelial SK_{Ca} and IK_{Ca} channels would lead to NO-induced relaxation in large arteries and EDHF-type relaxation in small arteries. NS309 is an activator of SK_{Ca} and IK_{Ca} channels in human epithelial kidney cells, possibly acting through increasing the channel sensitivity for Ca^{2+} (Strøbaek et al., 2004). Therefore, we chose to characterize the effects of the compound in human umbilical vein endothelial cells (HUVECs) by patch-clamp studies and to address how the compound may lead to NO release by measuring NS309-induced changes in $[Ca^{2+}]_i$ in HUVECs and freshly isolated mesenteric arterial endothelial cells.

Materials and Methods

Rat Mesenteric Arteries. Adult male Wistar rats (12 weeks old) were killed in accordance with a protocol from the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996). The protocol was approved by The Danish Ministry of Justice (permission 2005/561-964). The mesenteric vascular bed was isolated, and the superior mesenteric artery and third-order small mesenteric arteries were dissected, isolated, and transferred into cold physiological saline solution (PSS) of the following composition: 119 mM NaCl, 4.7 mM KCl, 5.5 mM glucose, 1.17 mM $MgSO_4$, 25 mM $NaHCO$, 1.18 mM KH_2PO_4, 1.6 mM $CaCl_2$, and 0.026 mM EDTA. The solution was equilibrated with bioair of the composition 5% CO_2, 21% O_2, and 74% N_2 to maintain pH at 7.4.

Isometric Tension Recordings. Superior mesenteric arteries (internal diameter, 1093 ± 9 μm; $n = 39$) were mounted on two 100-μm wires in a wire myograph (model 310A; DMT, Aarhus, Denmark) for isometric tension recordings and normalized as described previously (Simonsen et al., 1999). In brief, arterial segments were stretched to their optimal lumen diameters for active tension development (i.e., to an internal circumference of 90% of that achieved when the vessels were exposed to a passive tension yielding a transmural pressure of 100 mm Hg) (Simonsen et al., 1999). Segments were discarded if noradrenalin-induced (5 μM) contraction was <10 kPa or if ACh-induced (10 μM) relaxation on noradrenalin-induced (5 μM) contraction was <75%.

To investigate the role of opening SK_{Ca} and IK_{Ca} channels in tension, arterial segments were incubated with N^G,N^G-asymmetric dimethyl-L-arginine (ADMA) (300 μM), the SK_{Ca} channel blocker apamin (0.5 μM), and the IK_{Ca} channel blocker 1-[(2-chlorophenyl) diphenylmethyl]-1H-pyrazole (TRAM-34) (1 μM). Unless otherwise stated, arterial segments always were incubated with the cyclooxygenase inhibitor indomethacin (3 μM). After 30 min of incubation with the inhibitor and/or blocker, segments were contracted with norepinephrine (0.5 μM) to constrict the vessel to 60 to 70% of the maximal response. When contraction was stable, cumulative concentration-response curves for NS309 (0.01–10 μM) were constructed, and changes in tension were recorded.

Isobaric Tension Recordings. Third-order mesenteric small arteries (internal diameter, 300 ± 30 μm; $n = 34$) were mounted in a myograph (model 110P; DMT), where pressure and flow could be adjusted as described previously (Thorsgaard et al., 2003). In brief, the arterial segment was mounted on two micropipettes and viewed through an inverted microscope equipped with a charge-coupled device video camera linked to a personal computer via a frame grabber. Hydrostatic pressures of both inlet and outlet reservoirs were measured by pressure transducers connected to the perfusion line on the inlet and outlet sides, respectively, and the segment was pressurized to 50 mm Hg. The inclusion criteria for the arterial segments were as follows: 1) the contractile response induced by 11-dideoxy-9a,11a-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619) (10 nM) should reduce vessel diameter by at least 25%, 2) relaxation evoked by ACh (10 μM) should be >50% of U46619-induced constriction, and 3) if an air bubble passed through the arterial segment, then the experiment was excluded. Unless otherwise stated, arterial segments always were incubated with indomethacin (3 μM).

To investigate the role of opening SK_{Ca} and IK_{Ca} channels in vessel diameter, arterial segments were incubated with ADMA (300 μM), apamin (0.5 μM), the IK_{Ca} channel blockers TRAM-34 or charybdotoxin (ChTX) (0.1 μM), and the large conductance calcium-activated potassium channel (BK_{Ca} or $K_{Ca}1.1$) blocker iberiotoxin (IbTX) (0.1 μM). After 30 min of incubation with inhibitor and/or blocker, segments were contracted with U46619 (10–40 nM) to constrict the vessel to 60% of its resting diameter. When contraction was stable, cumulative concentration-response curves for NS309 (0.001–1 μM) were constructed, and changes in diameter were recorded.

Simultaneous Measurements of Force and NO. Superior and third-order small mesenteric arteries (internal diameter, 238 ± 26 μm; $n = 20$) were mounted on two 40-μm wires in a wire myograph (model 310A; DMT) for isometric tension recordings with the normalization procedure and inclusion criteria as described above. For simultaneous measurement of NO concentration and force, a NO-sensitive microsensor, either ISONOP3020 (diameter of 30 μm) or ISONOP007 (diameter of 7 μm) (World Precision Instruments, Inc., Sarasota, FL), first was calibrated by the use of NO gas in solution, and selectivity was confirmed by the absence of a response to sodium nitrite (10 μM) and noradrenaline (1 μM). Changes in NO concentration were recorded using a NO meter (ISO-NO Mark II; World Precision Instruments, Inc.). The sensor then was introduced into the lumen of the artery mounted in the myograph. Unless otherwise

stated, arterial segments always were incubated with indomethacin (3 μM).

To investigate the role of opening SK_{Ca} and IK_{Ca} channels in NO production and relaxation, segments were incubated with ADMA (300 μM), the NO scavenger oxyhemoglobin (OxHb) (10 μM), apamin (0.5 μM), and TRAM-34 (1 μM). After 30 min of incubation with the inhibitors and/or blockers, segments were contracted with noradrenalin (0.5 μM). When contraction was stable, NS309 (1 μM) was added to the organ bath, and NO concentration was recorded simultaneously with force.

Membrane Potential Measurements. Segments from small mesenteric arteries were mounted on 40-μm wires in a myograph (model 410A; DMT) with the normalization procedure and inclusion criteria as described above. Microelectrodes (glass, AS100F; World Precision Instruments, Inc.) were prepared on a horizontal puller (P-97; Sutter Instrument Company, Novato, CA), filled with 3 M KCl, and connected to an amplifier (Intra 767; World Precision Instruments, Inc.). Electrodes with stable resistances (>30 MΩ) were used to measure membrane potentials as described previously (Mulvany et al., 1982). Recordings from smooth muscle cells in small mesenteric arterial segments were made by advancing the electrode through the adventitia into the media. Unless otherwise stated, the segments always were incubated with indomethacin (3 μM). The effect of NS309 (1 μM) on membrane potential was investigated in the presence of ADMA (300 μM) alone and in combination with ChTX (0.1 μM) and apamin (0.5 μM).

Studies in Isolated Endothelial Cells . HUVECs were isolated and cultured as described previously (Ostergaard et al., 2007). In brief, pregnant women attending routine antenatal care at the Department of Obstetrics and Gynecology, Aarhus University Hospital, Skejby, Denmark, were invited to participate in the study. Written consent was obtained from those who agreed to participate (*n* = 20). The investigation conformed to the principles outlined in the Declaration of Helsinki and was approved by the local ethics committee of Aarhus University (Reference Number 20040154). The umbilical cords were obtained immediately after delivery. HUVECs were isolated by rinsing the veins with Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) to remove as much blood as possible. Then, 10 ml of 0.1% collagenase solution was flushed through the vein, and the umbilical cord was closed at both ends. After the incubation for 1 h at 37°C, the umbilical cord was washed with 20 ml of DMEM with 10% fetal calf serum. The cells were spun, and the pellet was resuspended in growth medium containing 2% serum, epidermal growth factor, hydrocortisone, vascular endothelial growth factor, fibroblast growth factor, insulin growth factor, ascorbic acid, heparin, amphotericin B, and gentamicin (Endothelial Cell Growth Medium 2 with SupplementPack; PromoCell, Heidelberg, Germany). Cells were then plated on gelatin-coated flasks or dishes and allowed to adhere overnight. Growth medium was changed every second day, and the cells were passaged with 0.25% trypsin when confluent. In all of the experiments, cells were used at passage one or two. The endothelial phenotype was confirmed using phase-contrast microscopy (i.e., cuboidal, cobblestone-appearing monolayer of cells) and positive immunofluorescence staining for anti-von Willebrand factor (1:4000; Dako Denmark A/S, Glostrup, Denmark).

Isolation of endothelial cells from the superior mesenteric artery was carried out using protocols described previously (Brähler et al., 2009). In brief, a 1- to 2-cm segment of the superior mesenteric artery was dissected from the mesenteric bed and cleaned of fat and connective tissue. The segment was mounted on a small glass capillary and filled with a trypsin (0.25%)/EDTA buffer (Biochrom, Berlin, Germany). After being filled, the superior mesenteric artery was sutured and incubated for 45 min at 37°C. Thereafter, the vessel was cut open, and the luminal surface was scraped gently with a 10-μl pipette tip. Detached single endothelial cells and endothelial cell clusters were aspirated and transferred to a culture dish containing DMEM supplemented with 10% fetal calf serum and penicillin/strep-

tomycin (Biochrom) and cover slips. Cells were allowed to settle down for 2-4 h and used for calcium measurements within 24 h.

Quantitative Polymerase Chain Reaction. RNA extraction from HUVECs was performed using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA then was treated with DNase I (QIAGEN Nordic, Copenhagen, Denmark). The concentration of the RNA was estimated by optical density measurements at 260 nm. Total RNA (100 μg/ml) then was reverse-transcribed using oligo(dT) primer and Superscript III reverse transcriptase (Invitrogen).

Expression of SK_{Ca}, IK_{Ca}, and BK_{Ca} channels was assessed by TaqMan quantitative polymerase chain reaction (QPCR). Two-hundred nanograms of RNA was used in a 25-μl reaction, and QPCR was conducted using Ex Taq polymerase (Takara Bio Inc., Shiga, Japan). The following cycles were run: 1 cycle at 95°C for 2 min, 40 cycles at 95°C for 15 s, 1 cycle at 55°C for 1 min, and 1 cycle at 70°C for 15 s on a Stratagene Mx3000P machine (Agilent Technologies, Santa Clara, CA). Primer and fluorogenic probes were designed using PerlPrimer (Eurofins MWG Operon, Huntsville, AL). The probe contained the reporter dye 6-carboxyfluorescein at the 5′ end and the Black Hole Quencher 1 dye at the 3′ end. The following primers and probes were used: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank accession no. BC_023632), forward (237–259), 5′-AAA TCC CAT CTT CCA GG-3′, reverse, (338–355), 5′-AGC CCC AGC CTT CTC CA-3′, probe (290–317), 5′-ATG CTG GCG CTG AGT ACG TCG TGG AGT-3′; BK_{Ca} (GenBank accession no. U13913), forward (3010–3030), 5′-TTC CTC AGC AAT CAG AGC CTC-3′, reverse (3121–3141), 5′-ACA GCA TTT GCC GTC AGT GTC-3′, probe (3067–3099), 5′-AAT ATC CTC ACC CTG ATA CGG ACC CTG GTG ACC-3′; IK_{Ca} (GenBank accession no. NM_002250), forward (1368–1390), 5′-GTT CTA CAA ACA TAC TCG CAG GA-3′, reverse (1572–1592), 5′-GCG TGT CAA TCT GTT TCT CAA-3′, probe (1439–1467), 5′-TCA ACG CGT TCC GCC AGG TGC GGC TGA AA-3′; $SK_{Ca}2.3$ (GenBank accession no. NM_002249), forward (2087–2107), 5′-GAT TGA CCA TGC CAA AGT GAG-3′, reverse (2210–2230), 5′-ACA TGA CAT TCT GCA TCT TGG-3′, probe (2125–2149), 5′-TCC TCC AAG CTA TCC ACC AGT TGA G-3′.

Patch Clamp. HUVECs were dispersed using 0.25% trypsin or nonenzymatic cell dissociation solution (Sigma-Aldrich) and seeded onto glass coverslips ~1 h before the start of the experiments. Once cells had attached, patch-clamp experiments were performed at room temperature (~20°C). Patch pipettes were fabricated from borosilicate glass and had a resistance of 3 to 6 MΩ after being fire polished. Membrane currents were measured using the conventional whole-cell patch-clamp configuration with an EPC 10 patch-clamp amplifier controlled by Pulse Trace 8.65 (HEKA, Lambrecht, Germany) connected to a computer. Currents were recorded using an extracellular solution containing: 105 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 10 mM HEPES, 0.026 mM EGTA, 5.5 mM glucose, and 1.6 mM $CaCl_2$ (pH 7.4). In some experiments, $CaCl_2$ was omitted from the solution (Ca-free extracellular solution). The pipette solution contained: 30 mM KCl, 100 mM potassium aspartate, 1 mM $MgCl_2$, 10 mM EGTA, 3 mM Na_2ATP, 5 mM HEPES, and 8.5 mM $CaCl_2$ (pH 7.2). Free $[Ca^{2+}]$ in the pipette solution was estimated to be 1 μM, and free $[Mg^{2+}]$ was 50 μM (calculated by Winmaxc32, version 2.50, http://www.stanford.edu/~cpatton/maxc.html, using the constants in CMC0204E.TCM). In some experiments, $CaCl_2$ was omitted from the solution (Ca-free pipette solution). Currents were sampled at 2 kHz and analyzed using PatchMaster software (HEKA). Series resistances were <5 MΩ and were not compensated for. Current-voltage (*I/V*) relationships were constructed using a holding membrane potential of −60 mV and by stepping from −120 to 120 mV in 40-mV increments for 200 ms. Currents measured at a step holding potential of 80 mV were used to compare the effects of NS309 (0.1 and 1 μM) in the absence and presence of apamin (0.3 μM), ChTX (0.1 μM), TRAM-34 (1 μM), IbTX (0.1 μM), and glibenclamide (0.1 μM).

Measurements of NO Concentration in HUVECs. HUVECs were seeded onto glass coverslips coated with gelatin (0.5%). After cells reached a confluence of 50-70%, the coverslips were transferred into an organ bath, a NO-sensitive microsensor with a diameter of 30 μm (ISONOP30; World Precision Instruments, Inc.) was placed above the HUVECs, and changes in NO concentration were recorded as described above.

Measurements of Intracellular Ca^{2+}. For measurements of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$, HUVECs were seeded onto gelatin-coated glass coverslips (0.5%) and loaded with FURA-2-AM (4 μM) for 30 min. After being loaded, cells were transferred into a perfusion chamber (Warner Instruments, Hamden, CT). Ratio images of 340/380 nm were used for the measurement of increases in $[Ca^{2+}]_i$ as described previously (Ostergaard et al., 2007). To investigate the effects of opening of SK_{Ca} and IK_{Ca} channels on $[Ca^{2+}]_i$, NS309 was applied in three different concentrations (0.01, 0.1, and 1 μM) in the absence and the presence of TRAM-34 (1 μM) and compared with the effect of histamine (1 μM). Maximum $[Ca^{2+}]_i$ was estimated by the addition of ionomycin (1 μM).

For calcium measurements, the mesenteric endothelial cells were loaded with a buffer consisting of the visible light excitable calcium indicator Oregon Green BAPTA-1-AM (7.24 mM), the nonionic surfactants Cremophor EL [0.066% (w/v)] and Pluronic F-127 (10.66 mM), and dimethylsulfoxide (DMSO) [0.32% (w/v)] at 37°C for 20 min in PSS. The solutions were equilibrated with bioair of the following composition: 5% CO_2, 21% O_2, and 74% N_2. Image acquisition of endothelial cell calcium was performed with an inverted confocal microscope (model LSM 510 Exciter; Carl Zeiss GmbH, Jena, Germany) equipped with a 63×, numerical aperture 0.75 long working distance objective excited at a wavelength of 488 nm using the Zeiss LSM Image Browser software program (Carl Zeiss GmbH). To investigate the effects of opening of SK_{Ca} and IK_{Ca} channels on $[Ca^{2+}]_i$, NS309 (1 μM) was applied in the absence and the presence of TRAM-34 (1 μM) and extracellular Ca^{2+} and compared with the effect of ACh (10 μM). Maximum $[Ca^{2+}]_i$ was estimated by the addition of ionomycin (1 μM).

Drugs. ACh, ADMA, IbTX, glibenclamide, indomethacin, noradrenalin, TRAM-34, Cremophor EL, Pluronic F-127, DMSO, and U46619 were purchased from Sigma-Aldrich. Apamin and ChTX were purchased from Latoxan (Valence, France). FURA-2-AM and Oregon Green BAPTA-1-AM were obtained from Invitrogen. NS309 was kindly donated by Dr. Joachim Demnitz (NeuroSearch A/S, Ballerup, Denmark). Indomethacin was prepared in PSS without $CaCl_2$ (PSS0.0). NS309 and glibenclamide stock solutions were dissolved in DMSO and further diluted in distilled water. The total amount of DMSO never exceeded 1% in organ baths and did not affect any of the results. All of the other drugs were prepared in distilled water.

Data and Statistical Analysis. For the isometric tension recordings, the stable tension after each addition of NS309 was expressed as a percentage of the active tension, where the active tone was defined as the level of contraction after the addition of noradrenalin minus the level of contraction after the normalization procedure. For NO measurements, NO concentrations and relaxation were measured 3 min after exposure to *S*-nitroso-*N*-acetylpenicillamine (SNAP), ACh, or NS309. For calcium measurements, the relative increases in fluorescence intensity ($\Delta F/F_0$) of FURA-2-AM or Oregon Green BAPTA-1-AM were used for the measurement of the increases in $[Ca^{2+}]_i$. For QPCR measurements, the amount of cDNA was normalized to the reference gene by using the threshold Ct values, $\Delta Ct = Ct(target) - Ct(GAPDH)$, and the results were analyzed as a ratio to the GAPDH expression, ratio = $2^{-\Delta Ct}$. For patch-clamp studies, the changes in current were expressed relative to the capacitance of the cell. Results are expressed as mean ± S.E.M. and analyzed by a Student's t test or a two-way analysis of variance (ANOVA) as appropriate. $P < 0.05$ was considered significant.

Results

Effects of an Opener of SK_{Ca} and IK_{Ca} Channels, NS309, in Rat Superior Mesenteric Arteries. To investigate the effect of opening SK_{Ca} and IK_{Ca} channels on relaxation and the relative contributions from NO and EDHF-type relaxation in a larger sized artery, NS309-induced NO release and relaxation in superior mesenteric arterial segments were investigated. NS309 (0.01–10 μM) induced concentration-dependent relaxations (EC_{50}, 1.4 ± 1.2 μM; $n = 7$) that were endothelium dependent. Inhibition of NO synthase with ADMA (300 μM) and blocking IK_{Ca} channels with TRAM-34 (1 μM) reduced NS309-induced relaxation (EC_{50}, 5.6 ± 1.4 and 15.3 ± 3.1 μM, respectively; $n = 7$), whereas blocking SK_{Ca} channels with apamin (0.3 μM) had no effect on NS309-induced relaxation (EC_{50}, 1.7 ± 1.4 μM; $n = 5$). NS309-induced relaxation was not decreased further when both SK_{Ca} and IK_{Ca} channels were blocked simultaneously (EC_{50}, 17.4 ± 4.8 μM; $n = 7$) compared with blocking only the IK_{Ca} channels. Inhibition of NO synthase and blocking both IK_{Ca} and SK_{Ca} channels reduced NS309-induced relaxation to the same level as removal of the endothelium (Fig. 1A). Blocking BK_{Ca} channels reduced NS309 relaxation in rat superior mesenteric artery (Fig. 2A) and shifted concentration-response curves rightward for the NO donor SNAP (U. Simonsen, unpublished observations).

Simultaneous measurements of NO release and relaxation in superior mesenteric arteries showed that ACh (10 μM) increased NO release to 22.9 ± 2.1 nM ($n = 5$), which was accompanied by relaxation of 87.9 ± 5.2% ($n = 5$). Inhibition of NO synthase by ADMA (300 μM) reduced ACh-induced increases in NO and relaxation to 3.1 ± 0.5 nM and 65 ± 7% ($n = 5$), respectively. Likewise, NS309 (1 μM) increased NO release to 20.1 ± 1.7 nM, resulting in 22.9 ± 4% relaxation ($n = 6$). ADMA reduced NS309-induced NO release to 4.1 ± 1.6 nM and abolished NS309-induced relaxation ($n = 6$). In the presence of apamin (0.5 μM) and TRAM-34 (1 μM), NS309-induced release of NO and relaxation were reduced to 4.4 ± 1.3 nM and 4.3 ± 2.5%, respectively ($n = 5$). Finally, in the presence of the NO scavenger OxHb (10 μM), NS309 failed to release NO and cause relaxation (Fig. 1, B and C; $n = 6$).

Effects of an Opener of SK_{Ca} and IK_{Ca} Channels, NS309, in Small Mesenteric Arteries. To investigate the effect of opening SK_{Ca} and IK_{Ca} channels on relaxation and the relative contributions from NO and EDHF-type relaxation in a smaller sized artery, NS309-induced release of NO and relaxation in small mesenteric arterial segments were investigated. NS309 (0.001–1 μM) induced potent concentration-dependent relaxations (EC_{50}, 39 ± 12 nM; $n = 7$). Inhibition of NO synthase with ADMA (300 μM) reduced NS309-induced relaxations only at the highest concentration (1 μM) (EC_{50}, 72 ± 16 nM; $n = 7$), whereas blocking SK_{Ca} channels with apamin (0.5 μM) (EC_{50}, 0.62 ± 0.13 μM; $n = 7$) or blocking IK_{Ca} channels with TRAM-34 or ChTX (0.1 μM) (EC_{50}, not available; $n = 4$ and $n = 7$, respectively) led to a much higher reduction of NS309 relaxation. Inhibition of NO synthase and blocking both SK_{Ca} and IK_{Ca} channels abolished NS309-induced relaxations ($n = 7$) (Fig. 3A). Blocking BK_{Ca} channels with IbTX (0.1 μM) had no effect on NS309-induced relaxations in mesenteric small arteries (Fig. 2B).

In small mesenteric arteries, ACh (10 μM) hyperpolarized
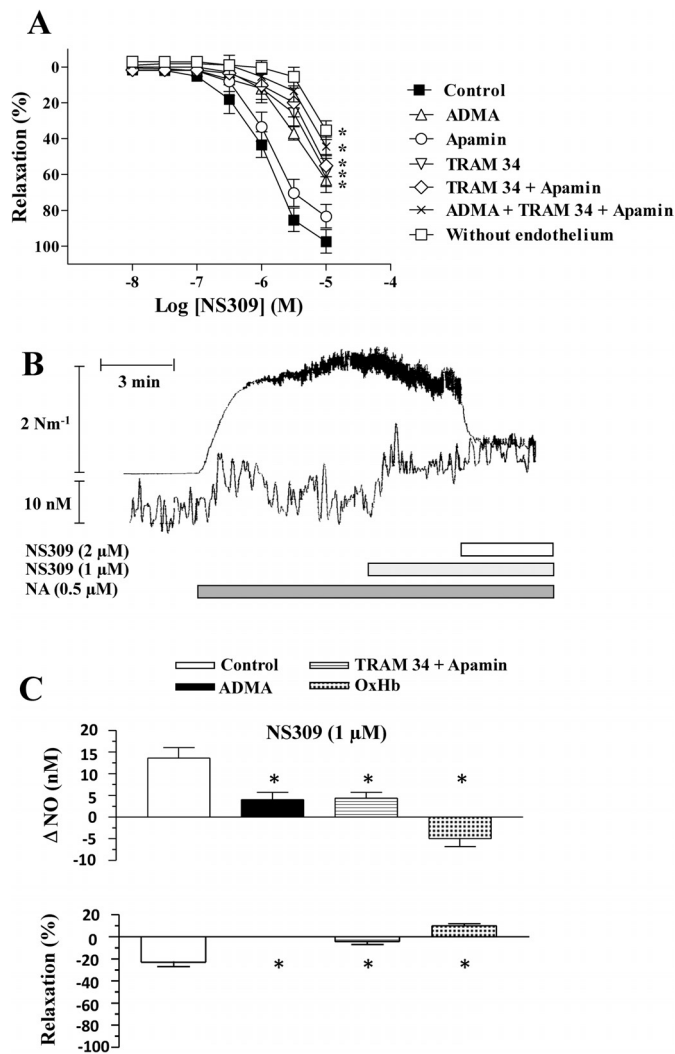
Fig. 1. Effect of blocking NO synthase and SK_{Ca} and IK_{Ca} channels on NS309-induced release of NO and relaxation in rat superior mesenteric arteries. A, isometric tension recordings showing the relaxing effect of NS309 in the absence and presence of ADMA (300 μM), apamin (0.5 μM), and TRAM-34 (1 μM). All of the experiments were performed in the presence of indomethacin (3 μM). Results are mean ± S.E.M. n = 5–7. Two-way ANOVA. ∗, P < 0.05 from control. B, original trace showing NS309-induced NO release and relaxation in a rat superior mesenteric artery contracted with noradrenaline (0.5 μM). C, average increase in NO concentration evoked by NS309 in the absence and presence of apamin (0.5 μM), TRAM-34 (1 μM), ADMA (300 μM), and OxHb (10 μM). All of the experiments were performed in the presence of indomethacin (3 μM). Results are mean ± S.E.M. n = 5–6. Student's t test. ∗, P < 0.05 from control.

the smooth muscle cell membrane (ΔV_m, −25.3 ± 2.5 mV; n = 5). ACh-induced hyperpolarizations were reduced in the presence of ADMA (300 μM) (ΔV_m, −17.3 ± 2.0 mV; n = 5) and abolished by simultaneous inhibition of NO synthase with ADMA (300 μM), blocking SK_{Ca} channels with apamin (0.5 μM), and blocking IK_{Ca} channels with ChTX (0.1 μM) (ΔV_m, 0.6 ± 1.5 mV; n = 4). NS309 (1 μM) also hyperpolarized smooth muscle cell membrane (ΔV_m, −17.7 ± 2.3 mV; n = 6). Treatment with ADMA alone had no effect on the membrane potential (ΔV_m, −11.7 ± 2.3 mV; n = 4), whereas the combination of ADMA, apamin, and ChTX abolished NS309-induced hyperpolarization (ΔV_m, −0.4 ± 1.9 mV; n = 4) (Fig. 3B).

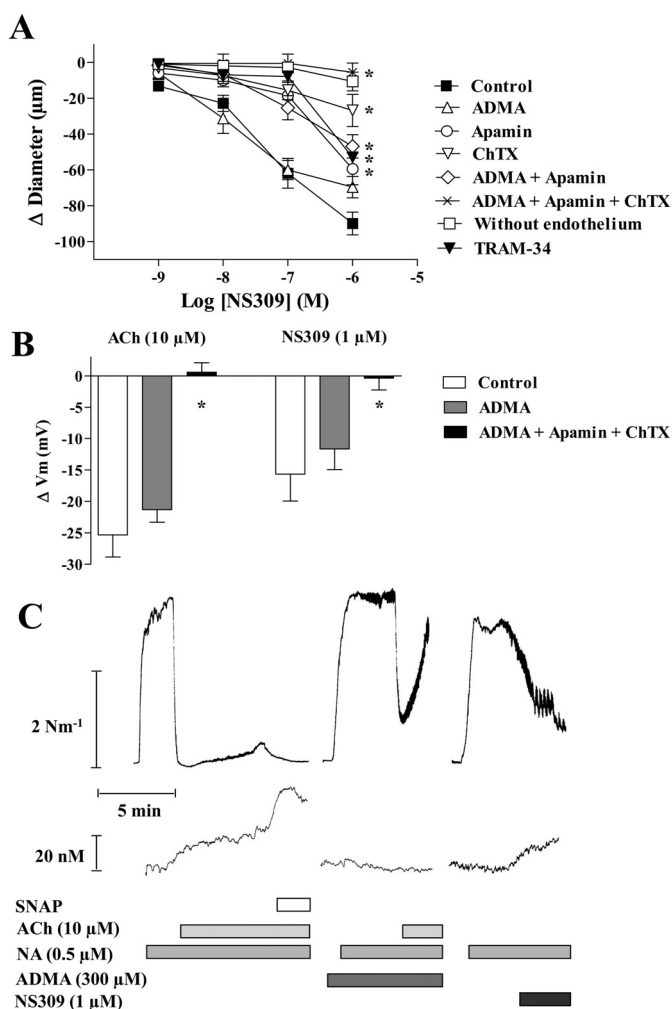Simultaneous measurement of NO release and relaxation
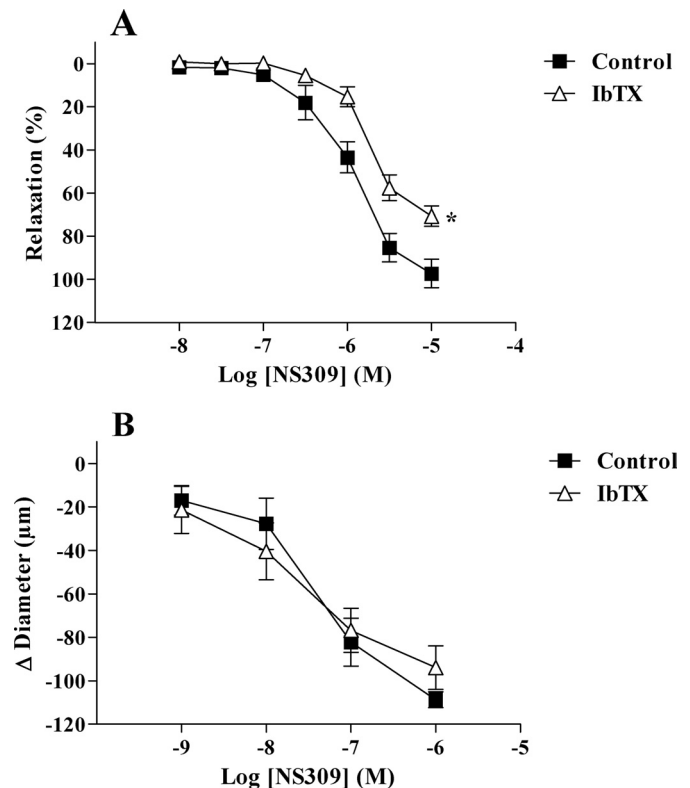
Fig. 2. Effect of blocking BK_{Ca} channels on NS309 relaxation in large and small rat mesenteric arteries. Isometric tension recordings in rat superior mesenteric artery (A) and isobaric diameter recordings in small mesenteric arteries (B) showing the relaxing effect of NS309 in the absence and presence of a blocker of BK_{Ca} channels (0.1 μM). All of the experiments were performed in the presence of indomethacin (3 μM). Results are mean ± S.E.M. n = 5–6. Two-way ANOVA. ∗, P < 0.05 from control.

in small mesenteric arteries showed that ACh (10 μM) increased NO concentration to 8.8 ± 2.0 nM and relaxed the arteries by 100 ± 1% (n = 4). Inhibition of NO synthase by ADMA (300 μM) markedly reduced NO release (Fig. 3C) but only reduced the peak relaxation induced by ACh to 91 ± 5% (P < 0.05; n = 3). Addition of NS309 (1 μM) increased the luminal NO concentration to 10.1 ± 2.0 nM and relaxed the arteries by 57 ± 10% (Fig. 3C).

Effects of an Opener of SK_{Ca} and IK_{Ca} Channels, NS309, in Isolated Endothelial Cells. In HUVECs, $SK_{Ca}3$ mRNA showed ~30 times higher expression than IK_{Ca} mRNA, whereas no expression of BK_{Ca} mRNA was found (Fig. 3A). NS309 (0.1 and 1 μM) increased the outward current in HUVECs. The NS309-induced increase in outward current was abolished in the absence of intra- and extracellular Ca^{2+} (Fig. 3B). When the involvement of different potassium channels in the NS309-induced increase in current was investigated, ChTX (0.1 μM) or TRAM-34 (1 μM) abolished the increase in current, and apamin (0.3 μM) reduced the increase in outward current, whereas IbTX (0.1 μM) or glibenclamide (0.1 μM) had no effect on the increase in current (Fig. 4, C and D).

In HUVECs, histamine (1 μM) increased the release of NO to 8.95 ± 0.2 nM (n = 6). In the presence of apamin (0.5 μM) and TRAM-34 (1 μM), the response to histamine was reduced to 6.36 ± 0.1 nM (n = 7) (Fig. 5, A and B). When TRAM-34 was replaced with ChTX (0.1 μM), the histamine-induced release of NO was reduced to 1.9 ± 2.1 nM (n = 6). In the

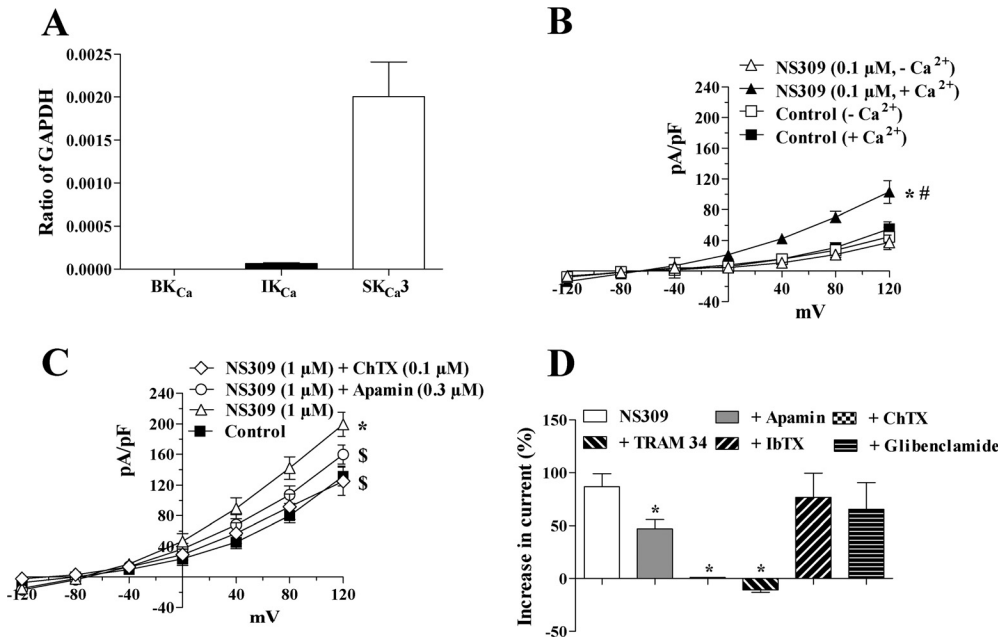0.26 ± 0.03 ($n = 6$), and abolished the NS309-induced increase in $[Ca^{2+}]_i$ (Fig. 5C).

In mesenteric arterial endothelial cells loaded with Oregon Green BAPTA-1-AM, both ACh (10 μM) and NS309 (1 μM) increased whole-cell $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} (0.27 ± 0.04 and 0.10 ± 0.01, respectively; $n = 5–6$). In contrast, in the absence of extracellular Ca^{2+}, NS309 (1 μM) failed to change $[Ca^{2+}]_i$ (0.02 ± 0.02; $n = 3$), whereas ACh (10 μM) induced a transient increase (0.07 ± 0.03; $n = 3$) (Fig. 5D). In the presence of TRAM-34 (1 μM), ACh and NS309 induced less increase in $[Ca^{2+}]_i$ (0.16 ± 0.01 and 0.04 ± 0.01, respectively; $n = 4$), and ionomycin-induced increases in calcium were unaltered (0.66 ± 0.08 and 0.68 ± 0.16, respectively; $n = 5$) (Fig. 5D).

Discussion

The main findings of the present study are that NS309 induces endothelium-dependent relaxations that are mediated largely by the release of NO in superior mesenteric arteries, whereas NS309 induces hyperpolarization and EDHF-type relaxation in small mesenteric arteries. In HUVECs, NS309 increased current sensitive to SK_{Ca} and IK_{Ca} channel blockers, but the current remained unaltered in the presence of a blocker of BK_{Ca} channels, IbTX. Moreover, NS309-induced calcium influx appears to contribute to the formation of NO.

Contributions of NO and EDHF to NS309-Induced Relaxation. NS309 induced more potent relaxations in small arterial segments compared with those in superior mesenteric arterial segments in the present study. Compared with isometric preparations, the membrane potential of pressurized arteries is more depolarized (Schubert et al., 1996), and together with the observations that smooth muscle hyperpolarization induced by an endothelium-dependent vasodilator (e.g., ACh) is attributable to SK_{Ca} channels, whereas IK_{Ca} channels play an important role during the ACh-induced repolarization phase after the depolarization (Crane et al., 2003), we cannot exclude the possibility that this may contribute to the different potency of NS309 observed in large versus small mesenteric arteries in the present study. However, calcium-activated potassium channels were suggested to play a role in the regional heterogeneity in ACh-induced relaxation in the rat mesenteric vascular bed (Hilgers et al., 2006), and that also may contribute to the different potency of NS309 in large versus small arteries. In both the superior and the small mesenteric arteries, NS309 relaxation was markedly inhibited in the presence of blockers of IK_{Ca} channels and further reduced by the combined inhibition of IK_{Ca} and SK_{Ca} channels, suggesting that the endothelium-dependent relaxations induced by NS309 involve IK_{Ca} and SK_{Ca} channels. In contrast to the small mesenteric arteries, the BK_{Ca}-selective blocker IbTX also reduced NS309 relaxation, but it also reduced relaxation in response to the NO donor SNAP, suggesting that the effect of IbTX on NS309 relaxation probably can be attributed to smooth muscle BK_{Ca} channels activated by NO in rat superior mesenteric artery.

We have found in previous studies that endothelial SK_{Ca} and IK_{Ca} channels contribute to ACh-induced endothelial hyperpolarization and NO-mediated relaxation in large arteries (Stankevicius et al., 2006). Moreover, in an endothelial cell line (EA.hy926) derived from human umbilical vein, in-

Fig. 3. Effect of blocking NO synthase and SK_{Ca} and IK_{Ca} channels on NS309-induced release of NO and relaxation in small rat mesenteric arteries. A, isobaric diameter recordings showing the relaxing effect of NS309 in the absence and presence of ADMA (300 μM), apamin (0.5 μM), ChTX (0.1 μM), TRAM-34 (1 μM), and apamin plus ChTX. All of the experiments were performed in the presence of indomethacin (3 μM). Results are mean ± S.E.M. $n = 7$. Two-way ANOVA. *, $P < 0.05$ from control. B, smooth muscle cell hyperpolarizations induced by ACh (10 μM) and NS309 (1 μM) in the absence and presence of apamin (0.5 μM), ChTX (0.1 μM), and ADMA (300 μM). All of the experiments were performed in the presence of indomethacin (3 μM). Results are mean ± S.E.M. $n = 4–6$. Student's t test: *, $P < 0.05$ from control. C, average trace ($n = 3$) showing SNAP- (10 μM), ACh- (10 μM), and NS309-induced (1 μM) NO release and relaxation in the absence and presence of indomethacin (3 μM) and ADMA (300 μM) in small mesenteric arteries contracted with noradrenaline (0.5 μM).

presence of ADMA (300 μM), apamin, and ChTX, the histamine-induced release of NO was reduced to 0.7 ± 0.4 nM ($n = 6$). NS309 (1 μM) increased the NO concentration to 12.9 ± 1.8 nM ($n = 5$), which was reduced to 6.4 ± 2.0 nM ($n = 5$) in the presence of apamin (0.5 μM) and TRAM-34 (1 μM) (Fig. 5, A and B).

In HUVECs loaded with FURA-2-AM, histamine (1 μM) increased whole-cell $[Ca^{2+}]_i$ by 0.36 ± 0.03 ($n = 6$), whereas stimulation with NS309 (0.01 μM) increased $[Ca^{2+}]_i$ by 0.06 ± 0.02 ($n = 6$). Increasing the concentration of NS309 to 0.1 and 1 μM had no further effect on whole-cell $[Ca^{2+}]_i$ (Fig. 5C). TRAM-34 (1 μM) decreased basal $[Ca^{2+}]_i$ by 0.27 ± 0.03 ($n = 6$), reduced the histamine-induced increase in $[Ca^{2+}]_i$ to

Fig. 4. SK$_{Ca}$ and IK$_{Ca}$ channel currents and expression in HUVECs. A, QPCR showing the expression of SK$_{Ca}$2.3, IK$_{Ca}$, and BK$_{Ca}$ RNA. B and C, current-voltage relationships derived from whole-cell patch-clamp experiments showing the effect of NS309 on whole-cell currents in the presence and absence of intracellular Ca^{2+}, apamin (0.3 μM), and ChTX (0.1 μM). Results are mean ± S.E.M. $n = 4$–7. Two-way ANOVA. *, $P < 0.05$ from control with calcium. #, $P < 0.05$ from NS309 (0.1 μM, $-$Ca^{2+}). $, $P < 0.05$ from NS309 (1 μM). D, relative NS309-induced increase in current at -80 mV after the addition of various potassium channel blockers: apamin (0.3 μM), ChTX (0.1 μM), TRAM-34 (1 μM), IbTX (0.1 μM), and glibenclamide (0.1 μM). Results are mean ± S.E.M. $n = 4$–7. Student's t test. *, $P < 0.05$ from NS309.
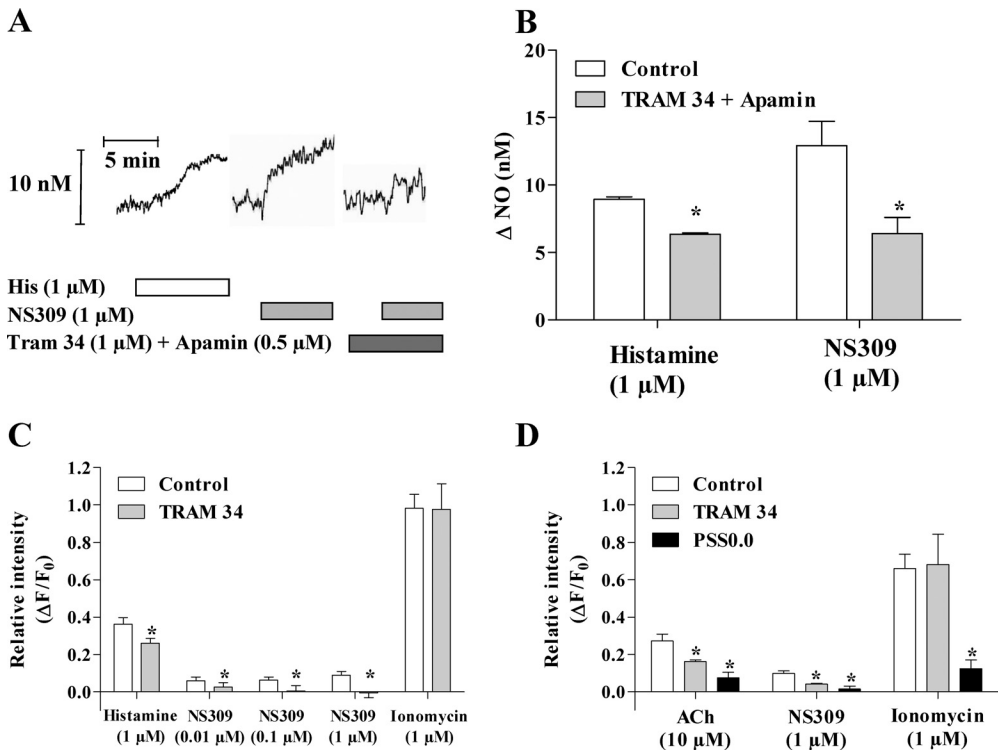
Fig. 5. NS309-induced release of NO and increase in intracellular Ca^{2+} in isolated endothelial cells. A, original recording showing NS309-induced (1 μM) NO release in HUVECs. B, average increase in NO concentration induced by NS309 (1 μM) in the absence and presence of apamin (0.5 μM) and TRAM-34 (1 μM). Results are mean ± S.E.M. $n = 5$–7. Student's t test. *, $P < 0.05$ from control. C, histamine-(1 μM) and NS309-induced (1 μM) increase in intracellular Ca^{2+} concentration in HUVECs in the absence and presence of TRAM-34 (1 μM). Results are mean ± S.E.M. $n = 6$. Student's t test. *, $P < 0.05$ from histamine control. Two-way ANOVA. *, $P < 0.05$ from NS309 controls. D, relative increase in rat mesenteric endothelial cell calcium after the addition of ACh (10 μM), NS309 (1 μM), and ionomycin (1 μM) in the absence and presence of TRAM-34, an IK$_{Ca}$ channel blocker, and of ACh (10 μM) and NS309 (1 μM) in the absence of extracellular calcium. Results are mean ± S.E.M. ($n = 3$–5). Student's t test. *, $P < 0.05$ from control.

hibition of SK$_{Ca}$ and IK$_{Ca}$ channels decreased, whereas openers of SK$_{Ca}$ and IK$_{Ca}$ channels were found to increase NO-sensitive DAF-FM fluorescence (Sheng et al., 2009). In the present study, an opener of SK$_{Ca}$ and IK$_{Ca}$ channels, NS309, increased the NO concentration in superior mesenteric arteries and primary cultured HUVECs, providing direct evidence that opening of SK$_{Ca}$ and IK$_{Ca}$ channels is involved in the release of NO in large arteries.

Opening endothelial SK$_{Ca}$ and IK$_{Ca}$ channels is thought to be pivotal for EDHF-type relaxation, and inhibition of these channels by the combination of apamin and ChTX has been considered as a unique characteristic of non-NO, nonprostan-

oid-type relaxations (Edwards et al., 1998; Buus et al., 2000) and hyperpolarization evoked by agonists in small arteries (Zygmunt and Högestätt, 1996; Edwards et al., 1998; Yamamoto et al., 1999). Confirming these previous studies, this study found that ACh-induced hyperpolarizations and relaxations also were blocked when apamin and ChTX were added together with an inhibitor of NO synthase in rat mesenteric small arteries (Fig. 3). This also was the case for NS309-induced hyperpolarizations and relaxations in rat mesenteric small arteries, suggesting that, in this preparation, an EDHF is the main contributor to NS309-induced hyperpolarizations and relaxations. However, NO has been

suggested previously to contribute to EDHF-type relaxation and hyperpolarization induced by ACh in rat mesenteric arteries (Chauhan et al., 2003). In the present study, ADMA also inhibited ACh-induced hyperpolarizations and abolished increases in NO concentration in rat small mesenteric arteries, hence providing further support that NO contributes to EDHF-type vasodilation in this preparation. Therefore, in addition to contributing to the main EDHF component, NO also contributes to the sustained ACh-induced relaxation in rat small mesenteric arteries.

In porcine retinal arteries, we found that NS309 as well as a selective opener of SK_{Ca} channels, CyPPA, induced apamin-sensitive, NO-mediated relaxations and potentiation of brady-kinin relaxations, suggesting that SK_{Ca} channels are coupled to the release of NO from the endothelial cell layer (Dalsgaard et al., 2009, 2010a). Moreover, down-regulation of the $SK_{Ca}3$ channel in mice appears to inhibit N^{ω}-nitro-L-arginine methyl ester-sensitive bradykinin relaxation in carotid arteries (Brähler et al., 2009). However, in the present study, TRAM-34 both reduced release of NO and abolished endothelium-dependent relaxation induced by NS309 in rat superior mesenteric artery, suggesting that mainly IK_{Ca} channels are coupled to the release of NO in this preparation. Thus, depending on the vascular bed and/or the specific endothelial cell compartmentalization, either SK_{Ca} or IK_{Ca} channels may be coupled to the release of NO.

Cellular Mechanisms Involved in Responses to NS309. In the present study, we found that in primary HUVECs both $SK_{Ca}3$ and IK_{Ca} channels were expressed, whereas BK_{Ca} channel expression was undetectable. These findings agree with previous work in early passage HUVECs (Kestler et al., 1998) and our previous observations that an opener of BK_{Ca} channels, 1-(3,5-bis-trifluoromethyl-phenyl)-3-[4-bromo-2-(1*H*-tetrazol-5-yl)-phenyl]-thiourea) (NS11021), does not change the current-voltage relationship in early passage HUVECs (Kun et al., 2009). Furthermore, our patch-clamp studies in HUVECs indicated that the increased potassium conductance induced by NS309 was dependent on the presence of $[Ca^{2+}]_i$ and that it was inhibited partially by apamin and abolished by ChTX and TRAM-34, whereas it remained unaltered in the presence of IbTX. In conjunction with the more marked inhibition of vasodilation in response to NS309 by blockers of IK_{Ca} channels (Fig. 1), these data suggest that IK_{Ca} channels may play a more prominent role in the response to NS309 in HUVECs and isolated arteries.

Opening of potassium channels will draw the membrane potential toward the equilibrium potential for potassium and lead to hyperpolarization. In endothelial cells, hyperpolarization is thought to increase the driving force for the influx of Ca^{2+} via cation channels belonging to transient receptor potential ion channels (e.g., transient receptor potential cation channels and transient receptor potential cation channel subfamily V member 4) and thereby prolongs and strengthens the activating Ca^{2+} signal (Nilius and Droogmans, 2001; Earley and Brayden, 2010). Inhibition of calcium-activated potassium channels is associated with a decrease in $[Ca^{2+}]_i$ (Nilius and Droogmans, 2001; Sheng and Braun, 2007). Moreover, studies in an endothelial cell line (EA.hy926) derived from human umbilical veins suggested that opening SK_{Ca} channels was associated with an increase in Ca^{2+} and NO formation (Sheng and Braun, 2007; Sheng et al., 2009). In the present study, both histamine and NS309 increased $[Ca^{2+}]_i$ and NO release in HUVECs, and in both cases, the increase in NO was reduced by incubation with TRAM-34 and apamin. These results suggest that opening of SK_{Ca} and IK_{Ca} channels is involved in the effects of histamine and NS309 and is therefore consistent with the view that membrane hyperpolarization by opening of SK_{Ca} and IK_{Ca} channels leads to increased influx of extracellular Ca^{2+}. Moreover, the findings that in freshly isolated mesenteric arterial cells the increase in $[Ca^{2+}]_i$ induced by NS309 was reduced markedly by TRAM-34 and completely abolished in the absence of extracellular calcium suggest that opening of IK_{Ca} channels is coupled to calcium influx.

In contrast to isolated endothelial cell studies, openers of SK_{Ca} and IK_{Ca} channels (e.g., NS309) fail to change Ca^{2+} in intact segments of retinal (Dalsgaard et al., 2010a) and rat small mesenteric arteries (Brøndum et al., 2010), and a combination of apamin plus ChTX fails to change agonist-induced increases in endothelial cell Ca^{2+} in rat superior mesenteric artery and small mesenteric arteries (Ghisdal and Morel, 2001; McSherry et al., 2005; Stankevicius et al., 2006). Despite comparable increases in NO concentration, the increase in $[Ca^{2+}]_i$ was much more marked with histamine compared with that with NS309 in HUVECs and with ACh compared with that with NS309 in mesenteric arterial endothelial cells in the present study. These findings suggest that, also in isolated endothelial cells, it is questionable whether the increase in $[Ca^{2+}]_i$ can account completely for the increase in NO seen with NS309. Thus, these findings suggest that Ca^{2+}-independent events also may couple SK_{Ca} and IK_{Ca} channel opening to changes in the release of NO as proposed either through membrane potential regulation of superoxide production and/or L-arginine uptake (Dalsgaard et al., 2010b). Moreover, it was suggested recently that potassium efflux resulting in an acute increase of potassium in the physiological range swells endothelial cells and hence may increase the release of NO (Oberleithner et al., 2009). However, further studies are required to clarify how activation of these mechanisms is coupled to increased formation of NO in endothelial cells.

Conclusion and Perspectives

In conclusion, in superior mesenteric arteries, NS309-induced relaxation was mediated mainly by NO, whereas in small mesenteric arteries, NS309-induced relaxation was mediated mainly by EDHF. NS309-induced calcium influx appears to contribute to the formation of NO. These results suggest that treatment with SK_{Ca} and IK_{Ca} channel openers in atherosclerotic or hypertensive disease involving a reduction in blood flow will not only improve blood flow in vascular beds, where the relative contribution from EDHF-type vasodilation is high, such as in coronary vasodilation from enhanced perfusion pulsatility (Paolocci et al., 2001), but also may improve blood flow in vascular beds, where the relative contribution from NO to vasodilation is high.

Authorship Contributions

Participated in research design: Hughes and Simonsen.

Conducted experiments: Stankevičius, Dalsgaard, Kroigaard, Beck, Boedtkjer, Misfeldt, Nielsen, and Schjorring.

Performed data analysis: Stankevičius and Dalsgaard.

Wrote or contributed to the writing of the manuscript: Stankevičius, Dalsgaard, Hughes, and Simonsen.

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