Mechanisms underlying epithelium-dependent relaxation in rat bronchioles: analogy to EDHF-type relaxation in rat pulmonary arteries

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Kroigaard C, Dalsgaard T, Simonsen U. Mechanisms underlying epithelium-dependent relaxation in rat bronchioles: analogy to EDHF-type relaxation in rat pulmonary arteries. Am J Physiol Lung Cell Mol Physiol 298: L531–L542, 2010. First published January 29, 2010; doi:10.1152/ajplung.00220.2009.—This study investigated the mechanisms underlying epithelium-derived hyperpolarizing factor (EpDHF)-type relaxation in rat bronchioles. Immunohistochemistry was performed, and rat bronchioles and pulmonary arteries were mounted in microvascular myographs for functional studies. An opener of small (SKCa) and intermediate (IKCa) conductance calcium-activated potassium channels, NS309 (6,7-dichloro-1H-indole-2,3-dione 3-oxime) was used to induce EpDHF-type relaxation. IKCa and SKCa opener, NS309 has been shown to induce endothelium-dependent relaxations to NS309 in (6,7-dichloro-1H-indole-2,3-dione 3-oxime) is a novel IKCa and SKCa opener with two to four times higher selectivity towards the IKCa channel than individual SKCa channels in human epithelial kidney cells, and is 30 times more potent than other known openers of IKCa channels, such as DC-EBIO (5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one), and 1,000 times more potent than 1-EBIO (1-ethyl-2-benzimidazol-2-one) (49). Moreover, in human umbilical vein endothelial cells, isolated rat mesenteric arteries, and guinea pig carotid arteries, NS309 has been shown to induce endothelium-dependent hyperpolarizations sensitive to blockers of SKCa and IKCa channels (30, 48).

We therefore hypothesized that: 1) IKCa and SKCa opener, NS309, salbutamol, potassium; endothelium; GEA 3175

THE RESPIRATORY EPITHELIUM releases factors that regulate bronchial smooth muscle relaxations (20), and removal of the bronchial epithelium in larger airways has been shown to enhance the effect of contractile agonists and reduce the relaxing response in bronchioles (50, 51). Nitric oxide (NO) and prostaglandin E2 (PGE2) are mediators of human bronchiolar smooth muscle-epithelium-dependent relaxations (22). In addition, a non-NO nonprostanoid epithelium-dependent hyperpolarizing factor (EpDHF) is also involved in epithelium-dependent relaxations. Epoxyeicosatrienoic acids (EETs) have been suggested to account for EpDHF-mediated relaxation in guinea pig airways (3). In comparison, opening of intermediate (IKCa) and endothelial subtype small (SKCa, SKCa3) conductance calcium-activated potassium channels account for the endothelium-derived hyperpolarizing factor (EDHF)-type mechanisms leading to dilation in arteries (6, 14, 19). EDHF-type relaxation induced by thapsigargin has been shown to involve cytochrome P450-dependent oxidases sensitive to thapsigargin in rat pulmonary arteries (37) and activation of the Na+–K+–ATPase in rat mesenteric small arteries (14, 15). Although studies in large canine bronchioles (4–7 mm in width) suggested that IKCa channels and the Na+–K+–ATPase are involved in the relaxation to salbutamol (53), the role of IKCa and SKCa channels, as well as the Na+–K+–ATPase in the relaxation of the small airways and their involvement in EpDHF-mediated relaxation, has not previously been addressed.

NS309 (6,7-dichloro-1H-indole-2,3-dione 3-oxime) has not previously been addressed.

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MATERIALS AND METHODS

Animals. Male Wistar rats (10–12 wk old) were killed by cervical dislocation and exsanguinated by decapitation in accordance with the Danish animal law and regulations, and the Danish animal care committee approved the study. The lungs were immediately removed and placed...
in a 4°C physiological saline solution (PSS). Bronchioles and pulmonary arteries of third and fourth order were carefully dissected under a microscope by removing the surrounding tissue as previously described (24).

**Immunohistochemistry of IKCa and SKCa3 proteins.** Evaluation of the IKCa and SKCa3 channel distribution was achieved by immunohistochemistry in rat pulmonary arteries and bronchioles. After myograph experiments, the segments were fixed in 4% formalin and embedded in paraffin. Sections of 3 μm were produced at a microtome and collected on glass slides. The sections were then deparaffinized and incubated with 0.3% H2O2 for 20 min. Sections were heated in the microwave for 2 × 7 min in TEG-buffer and transferred to 0.25% Triton X solution for 15 min. Blocking of nonspecific antibody binding was achieved by incubating the sections with 10% FCS in 1% BSA for 30 min. Incubation with the primary antibody, Von Willebrand factor (VWF; 1:100, Gene Tex), thyroid transcription factor-1 (TTF-1; 1:100, Diagnostic Biosystems, Pleasanton, CA), smooth muscle actin (SMA; 1:2,000, Dako, Glostrup, Denmark), IKCa (1:100; Cell Applications, Oberkochen, Germany), and SKCa3 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) channels was done overnight at 4°C. Negative controls without primary antibody and with rabbit and mouse IgG (Dako, Glostrup, Denmark) were also obtained. Sections were incubated for 1 h at room temperature with secondary antibody, goat anti-rabbit IgG coupled to Alexa 488, and/or goat anti-mouse IgG coupled to Alexa 633 (1:1,000; Invitrogen). Finally, antifade solution (Bio-Rad Laboratories, Herlev, Denmark) was added, and the sections were coverslipped. The preparations were analyzed by confocal microscopy (Zeiss LSM 510 Meta; Carl Zeiss, Oberkochen, Germany) equipped with a water-immersion objective (C-Apochromat ×40), which limited the aberration.

**Functional studies of rat pulmonary arteries and bronchioles.** Segments of ~2 mm were mounted on two 40-μm steel wires in microvascular myographs (Danish Myotechnology, Aarhus, Den-

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**Fig. 1.** Representative images showing the immunoreaction for IKCa in rat bronchioles (A and B) and pulmonary arteries (C and D). The arrowheads show positive immunoreaction for IKCa, thyroid transcription factor 1 (TTF-1), Von Willebrand factor (VWF), or smooth muscle actin (SMA), and the white line indicates 25 μm.
mark) for isometric tension recording. During mounting and normalization, bronchioles were in organ baths with Ca2+-free PSS to avoid development of spontaneous tension, whereas pulmonary arteries were in organ baths with PSS. The baths were heated to 37°C and equilibrated with 5% CO2 in bioair (21% O2, 74% N2) to maintain the desired pH of 7.4. The segments were then equilibrated for 10 min. For optimal measurements, bronchioles were stretched to 2.4 kPa, corresponding to a transmural pressure of 18 mmHg and pulmonary arteries to 3.9 kPa corresponding to 30 mmHg (17). After normalization, the organ baths were washed with PSS, and all experiments with pulmonary arteries and bronchioles were conducted in the presence of 1.6 mM Ca2+ (11, 47). After 10-min equilibration, potassium-rich PSS (KPSS) was added twice to test the viability of the segments. Bronchioles were contracted to 60 mM KPSS and pulmonary arteries to 124 mM KPSS. The pulmonary artery endothelial function was tested by contracting them to the thromboxane analog 9,11-dideoxy-9a,11a-epoxymethanoprostaglandin F2α (U46619, 0.1 μM) and then adding ACh (10 μM). Only segments with more than 50% relaxation to ACh were included in the study. There is currently no functional test for evaluating the presence of bronchiole epithelium; therefore, this was evaluated by histology. In a series of experiments, the endothelium/epithelium was removed by rubbing a human hair against the inner surface of the segment. The absence of endothelium was confirmed by lack of ACh relaxation and the absence of epithelium by histology. Thirty minutes before contraction and during the experiments, all preparations were incubated with the cyclooxygenase (COX) inhibitor indomethacin (3 μM). Moreover, 15 min before contraction of segments and during construction of the concentration relaxation curves, the segments were incubated with the following inhibitors/blockers: a NO synthase (NOS) inhibitor, Nω-nitro-L-arginine (L-NNA, 100 μM), an inhibitor of cytochrome 2C isoenzymes, sulfaphenazole (10 μM) (31, 32), a large (BKCa) conductance calcium-
activated potassium channel blocker, iberiotoxin (IBTX, 0.1 μM), an IKCaCa channel blocker, charybdotoxin (ChTX, 0.07 μM), a SKCaCa channel blocker, apamin (0.5 μM), and a Na+–K+-ATPase inhibitor, ouabain (0.1 and 100 μM). Pulmonary arteries were contracted with U46619 (0.03 μM) and bronchioles with serotonin [5-hydroxytryptamine (5-HT), 1 μM]. After obtaining a stable contraction, concentration relaxation curves (0.001–10 μM) were conducted for the IKCaCa channel opener NS309, the NO donor GEA 3175, and the β2-adrenoceptor agonist, salbutamol.

The following concentration-relaxation curves were obtained all in the presence of indomethacin, with and without: 1) epithelium or endothelium; 2) l-NNA with and without ChTX and apamin; 3) sulfaphenazole in the presence of l-NNA; 4) IBTX; 5) ChTX without endothelium or epithelium; 6) apamin with and without ChTX; 7) ouabain in the presence of l-NNA. As a control for cytochrome 2C isoenzyme inhibition, concentration-response curves were constructed with arachidonic acid (0.001–10 μM) with or without sulfaphenazole. Finally, in the presence of indomethacin and l-NNA, concentration-response curves with and without ouabain (100 μM) were constructed for KCl (0.5–20 mM) in PSS without K+. After finishing the experimental protocols, viability was again tested with relaxation curves (0.001–10 μM) for NS309 did not reach an asymptotic minimum. To avoid bias in the myograph experiments, concentration-response curves were performed in a random order. The results for isometric tension recordings are expressed as means ± SE. The responses with or without the appropriate inhibitor/blocker, endothelium or epithelium, were analyzed by two-way ANOVA followed by Bonferroni posttest. In all cases, P < 0.05 was considered significant.

Chemicals. The PSS used was of the following composition (mM): 119 NaCl, 4.7 KCl, 1.17 MgSO4, 25 NaHCO3, 1.18 KH2PO4, 5.5 glucose, 1.6 CaCl2, and 0.026 EDTA. The calcium-free solution had the same composition except that CaCl2 was absent. KPSS is a physiological saline solution with KCl exchanged for NaCl on an equimolar basis giving the final concentration of 123.7 mM KCl for experiments with arteries and 60 mM KCl for experiments with bronchioles. Potassium-free solution (PSS without K+) had the same composition as PSS except that KCl was exchanged for NaCl on an equimolar basis giving a final concentration of 123.7 mM NaCl.

The composition of the buffers were, for PBS, 150 mM NaCl and 50 mM NaH2PO4, and for triethylglycerol buffer (TEG), 10 mM Tris and 0.5 mM EGTA. FCS was from InVitrogen (Costa Mesa, CA). U46619, indomethacin, salbutamol, l-NNA, ouabain, and serotonin were purchased from Sigma Aldrich (St. Louis, MO). ChTX, apamin, and IBTX were from Latoxan (Valence, France), and acetylcholine hydrochloride was from ICN Biomedicals (Costa Mesa, CA). GEA 3175 was obtained from GEA (Copenhagen, Denmark), and NS309 was kindly donated by Dr. Søren Peter Olesen, Neurosearch (Denmark).

All drugs were dissolved in distilled water, except for U46619, which was dissolved in 50% ethanol; indomethacin and l-NNA, which were dissolved in Ca2+-free PSS; and ChTX and apamin.

![Fig. 3. The effect of NS309 and salbutamol in rat bronchioles contracted to 5-HT (1 μM) and rat pulmonary arterial segments contracted to U46619 (0.03 μM) in segments with (control) or without bronchial epithelium and arterial endothelium. Concentration-relaxation curves (10−9 M to 10−3 M) for NS309 (A and B), salbutamol (C and D), and GEA 3175 (E and F) in pulmonary arteries and bronchioles. The experiments were performed in the presence of indomethacin (3 μM). Data are means ± SE of n experiments, where n = 5–10. The results were analyzed by 2-way ANOVA, *P < 0.05 from control.](http://ajplung.physiology.org/)
which were dissolved in 0.9% NaCl. NS309 stock solutions were made in DMSO. Sulfaphenazole was dissolved in 96% ethanol. All further dilutions were made in distilled water. Preliminary experiments ascertained the lack of response to either vehicle in the concentrations assayed. All solutions were kept at \(-20^\circ\)C until use.

**RESULTS**

Localization of \(\text{IK}_{\text{Ca}}\) and \(\text{SK}_{\text{Ca}3}\) protein by use of immunofluorescence. In bronchioles, \(\text{IK}_{\text{Ca}}\) immunoreaction was observed in the epithelial cell layer (Fig. 1A). The merged image shows coimmunostaining of \(\text{IK}_{\text{Ca}}\) with the epithelial marker TTF-1 (Fig. 1A), and there was no colocalization with SMA and thus no immunoreaction for \(\text{IK}_{\text{Ca}}\) in the smooth muscle layer (Fig. 1B). In pulmonary arteries, \(\text{IK}_{\text{Ca}}\) immunoreaction was found mainly in the endothelial cell layer (Fig. 1C). The merged image shows coimmunostaining with VWF located in the endothelium (Fig. 1C). No colocalization of \(\text{IK}_{\text{Ca}}\) with SMA staining was found in the smooth muscle layer (Fig. 1D).

Mouse and rabbit IgG controls were negative (see Supplemental Fig. S1). TTF-1 and \(\text{IK}_{\text{Ca}}\) immunoreaction was absent in bronchioles without epithelium (Supplemental Fig. S2A), and there was no immunoreaction for VWF or \(\text{IK}_{\text{Ca}}\) in pulmonary arteries without endothelium (Supplemental Fig. S2B).

\(\text{SK}_{\text{Ca}3}\) immunoreaction was very marked in the bronchial epithelium (Fig. 2A). A weak positive immunoreaction was found for \(\text{SK}_{\text{Ca}3}\) in the smooth muscle layer, which was positive for SMA (Fig. 2B). In pulmonary arteries studied at confocal settings corresponding to those applied for the bronchioles, we did not observe \(\text{SK}_{\text{Ca}3}\) immunoreaction (Fig. 2C), and increasing gain only resulted in faint fluorescence from the endothelial cell layer, but marked autofluorescence from the smooth muscle layer, suggesting scanty expression of the \(\text{SK}_{\text{Ca}3}\) channel in the endothelium of rat pulmonary arteries (Fig. 2D).

No immunoreaction was found in mouse and rabbit IgG control stainings of bronchioles and pulmonary arteries (Supplemental Fig. S1).

Effect of removing the endothelium or epithelium on NS309- and salbutamol-evoked relaxations. Internal normalized diameters for the investigated bronchioles and pulmonary arteries were 828 ± 20 μm (n = 84) and 720 ± 24 μm (n = 68), respectively. In bronchioles, contraction to 5-HT averaged 0.76 ± 0.04 N/m (n = 84), whereas in pulmonary arteries, contraction to U46619 averaged 1.34 ± 0.10 N/m (n = 68). Removing the epithelium or endothelium did not alter the contraction levels.

In the presence of indomethacin, NS309 (0.001–10 M) evoked concentration-dependent relaxations in both bronchioles and pulmonary arteries (Fig. 3, A and B) with maximum relaxation levels in bronchioles of 92% (n = 5) and 89% (n = 5) in pulmonary arteries.

Fig. 4. The effect of a NO synthase inhibitor, L-NNA, on NS309-, salbutamol-, and GEA 3175-evoked (10−9 to 10−5 M) relaxations in rat bronchioles and pulmonary arteries. A: NS309 in bronchioles. B: NS309 in pulmonary arteries. C: salbutamol in bronchioles. D: salbutamol in pulmonary arteries. GEA 3175 in bronchioles (E) and GEA 3175 in pulmonary arteries (F). The experiments were conducted in the presence of indomethacin. Data are means ± SE of n experiments, where n = 5. The results were analyzed by 2-way ANOVA, *P < 0.05 from control and #P < 0.05 from L-NNA.
relaxations of 48 ± 6% (n = 10) compared with 53 ± 9% (n = 7), respectively. Removal of the epithelium or endothelium reduced NS309 relaxation (Fig. 3, A and B).

In the presence of indomethacin, salbutamol (0.001–10 μM) also evoked concentration-dependent relaxations in bronchioles and pulmonary arteries with maximum relaxations of 67 ± 7% and 83 ± 4%, and pD2 values of 6.82 ± 0.08 (n = 13) and 6.62 ± 0.16 (n = 8), respectively. Removal of the epithelium blunted salbutamol relaxation (Fig. 3C), whereas removal of the endothelium had no effect on salbutamol relaxation (Fig. 3D).

In the presence of indomethacin, the NO donor, GEA 3175, evoked concentration-dependent relaxations of both pulmonary arteries [pD2 values of 7.30 ± 0.12 (n = 8)] and bronchioles [pD2 values of 5.11 ± 0.51 (n = 8)] with a higher potency in pulmonary arteries. Maximum relaxation was 88 ± 5% in bronchioles and 84 ± 4% in pulmonary arteries. Removal of the epithelium or endothelium had no effect on GEA 3175 relaxations (Fig. 3, E and F).

Effect of inhibiting NOS and cytochrome 2C isoenzymes and blocking BKCa, IKCa, and SKCa on NS309-evoked relaxations. Incubation with the combination of L-NNA and the 1KCa and SKCa channel blockers ChTX (0.07 μM) and apamin (0.5 μM) markedly reduced NS309 relaxations in both bronchioles and pulmonary arteries (Fig. 4, A and B), whereas L-NNA did not change NS309 (Fig. 4, A and B) and salbutamol relaxation (Fig. 4, C and D). Inhibition of NOS with L-NNA (100 μM) only augmented the response to GEA 3175 in pulmonary arteries (Fig. 4F).

Arachidonic acid (0.001–10 μM) induced concentration-dependent relaxation in pulmonary arteries (Fig. 5A). There was no relaxation to arachidonic acid in bronchioles (n = 6, data not shown). Incubation with an inhibitor of cytochrome 2C isoenzyme, sulfaphenazole (10 μM), reduced arachidonic acid relaxation in pulmonary arteries but failed to alter NS309 relaxation in pulmonary arteries or bronchioles (Fig. 5, B and C).

Incubation with the BKCa blocker IBTX (0.1 μM) did not alter NS309 or salbutamol relaxation in either bronchioles or pulmonary arteries (n = 6, data not shown).

In both bronchioles and pulmonary arteries, incubation with an 1KCa and BKCa blocker, ChTX, reduced NS309 relaxation (Fig. 6, A and B). Epithelial or endothelial removal did not reduce this relaxation further (n = 5–10, data not shown). Incubation with ChTX only reduced the response to salbutamol in bronchiolar segments (Fig. 6, C and D). ChTX had no effect on GEA 3175 relaxation (n = 5–10, data not shown).

NS309-evoked relaxation of bronchioles was blunted in the presence of a SKCa channel blocker, apamin, and in the presence of apamin plus ChTX (Fig. 7A). In pulmonary arteries, NS309 relaxation was only reduced with a combination of apamin and ChTX (Fig. 7B). The presence of apamin, alone and in combination with ChTX, only reduced salbutamol relaxation in bronchioles (Fig. 7, C and D).

Effect of blocking Na+–K+–ATPase on NS309- and salbutamol-evoked relaxations. Inhibition of Na+–K+–ATPase with ouabain (100 μM) reduced NS309 and salbutamol relaxation in both bronchioles and pulmonary arteries, whereas a 1,000-fold lower concentration of ouabain (0.1 μM) had no effect (Fig. 8, A–D). In bronchioles, low concentrations of potassium evoked relaxations, which were completely inhibited in the presence of ouabain (Fig. 9) and independent of intact epithelium (data not shown). In pulmonary arteries, low concentrations of potassium (1–6 mM) evoked ouabain-sensitive concentration-dependent relaxations (Fig. 10).

DISCUSSION

The main findings of the present study are that in rat bronchioles, IKCa and SKCa3 channels are expressed in the epithelium, and an opener of IKCa and SKCa3 channels, NS309, induces relaxations by a non-NOS non-prostanoid epithelium-dependent mechanism that involves activation of the Na+–K+–ATPase. In rat pulmonary arteries, relaxation is evoked by opening of IKCa channels, compared with bronchioles, where opening of both IKCa and SKCa3 channels are involved in NS309 relaxation.

Localization of IKCa and SKCa3 protein in the epithelium and endothelium. In trachea and lung, real-time PCR, Northern blotting, and micro-RNA arrays have revealed IKCa mRNA.
expression (28, 33). In systemic arteries, IKCa protein is observed by immunohistochemistry in rat mesenteric artery endothelium (42), and there is a regional heterogeneity in mRNA expression of IKCa since higher expression is observed in fourth order compared with third-order mesenteric arteries (25). Furthermore, the IKCa channel protein is found in bronchial epithelium (55). In the present study, IKCa was specifically located, respectively, in the epithelium of third- and fourth-order rat bronchioles and in the endothelium of rat pulmonary arteries. SKCa3 mRNA was observed to be localized only in the endothelium and not in smooth muscle cells in rat mesenteric and porcine coronary arteries (5, 54). However, in another study, SKCa3 mRNA was found in the lung, and SKCa3 protein is present in human colonic, vascular, and

Fig. 6. The effect of IKCa channel blockade with charybdotoxin (ChTX). Results were evaluated on NS309- and salbutamol-evoked relaxations in rat bronchioles and pulmonary arteries in the presence of indomethacin. Bronchioles were contracted to 5-HT (1 μM) and pulmonary arteries to U46619 (0.05 μM). Average concentration-relaxation curves for NS309 in bronchioles (A), NS309 in pulmonary arteries (B), salbutamol in bronchioles (C), and salbutamol in pulmonary arteries (D). Data are means ± SE of n experiments, where n = 5–10. The results were analyzed by 2-way ANOVA, *P < 0.05 from control.

Fig. 7. The effect of apamin, a SKCa channel blocker, alone and in combination with ChTX on NS309- and salbutamol-evoked relaxations in rat bronchioles and pulmonary arteries. Bronchioles were contracted to 5-HT (1 μM) and pulmonary arteries to U46619 (0.05 μM) in the presence of indomethacin. Average concentration-relaxation curves for NS309 in bronchioles (A), NS309 in pulmonary arteries (B), salbutamol in bronchioles (C), and salbutamol in pulmonary arteries (D). Data are means ± SE of n experiments, where n = 6. The results were analyzed by 2-way ANOVA, *P < 0.05 from control.
cavernosal smooth muscle (33), suggesting SKCa3 is not only localized to vascular endothelial cells. We report here for the first time the localization of SKCa3 immunoreaction in rat bronchiole epithelium, while only scant SKCa3 immunoreactivity was observed in the rat pulmonary artery endothelium. Our findings suggest that IKCa and SKCa3 channels are mainly expressed in the epithelium of bronchioles.

**Epithelium-dependent relaxation in bronchioles and the role for IKCa and SKCa.** The epithelium possesses one or more factors that are important for the relaxation of the underlying bronchial smooth muscle layer (3, 51). Thus, removing the epithelium reduces isoproterenol relaxation in canine bronchi (51). Studies have shown that NO and PGE2 mediate these epithelium-dependent relaxations in canine bronchi (3, 51). Thus, removing the factors that are important for the relaxation of the underlying bronchial smooth muscle layer (3, 51). Therefore, epithelium-dependent relaxations in canine bronchi (3, 51) are not involved in the epithelium-dependent relaxations induced by NS309 and salbutamol in rat bronchioles.
Endothelium-dependent relaxation in pulmonary arteries and the role for \( \text{IK}_{\text{Ca}} \) and \( \text{SK}_{\text{Ca}} \). Mechanical removal of the endothelium abolishes the relaxation to, e.g., \( \text{ACh} \) in rabbit and rat arteries (23, 45), and to catecholamines and adenosine in canine coronary arteries (40). Moreover, a combination of apamin and ChTX inhibits \( \text{ACh} \) relaxation resistant to NO and COX inhibition (6, 14), and an EDHF-type relaxation is present in pulmonary arteries from pigs and rats (2, 37, 56). Experiments in \( \text{IK}_{\text{Ca}} \) knockout mice suggest that there is a considerable contribution of \( \text{IK}_{\text{Ca}} \) channels to endothelial hyperpolarization in EDHF-type relaxation in systemic arteries (46). In the present study, NS309 relaxes third- and fourth-order rat pulmonary arteries with endothelium, and, in contrast to salbutamol and GEA 3175, NS309 relaxation is markedly reduced by endothelial cell removal, suggesting it induces endothelium-dependent relaxations. Inhibiting COX and NO or inhibiting COX and blocking \( \text{SK}_{\text{Ca}} \) or \( \text{BK}_{\text{Ca}} \) channels did not alter the relaxation to NS309 or salbutamol, whereas inhibiting COX and blocking \( \text{IK}_{\text{Ca}} \) channels or inhibiting COX and NO and blocking \( \text{IK}_{\text{Ca}} \) completely abolished NS309 relaxation at concentrations below 10 \( \mu \text{M} \) and reduced salbutamol relaxation. As previously reported, NS309 at low concentrations is a specific opener of \( \text{SK}_{\text{Ca}} \) and \( \text{IK}_{\text{Ca}} \) channels (11), but might at high concentrations (\( >10 \mu \text{M} \)) operate through a different mechanism, e.g., blocking of \( L \)-type calcium channels (34). In bronchioles and pulmonary arteries without epithelium and endothelium, respectively, this could explain why relaxation to NS309 is not completely abolished. Together, these findings suggest that NS309 induces EDHF-type relaxation only through opening of \( \text{IK}_{\text{Ca}} \) channels located in endothelium and therefore play a role for regulation of pulmonary arteries.

Identity of EDHF: involvement of \( K^+ \) efflux and \( \text{Na}^+-\text{K}^+\text{-ATPase} \). The relaxation persisting in the presence of COX and NO inhibition is blunted by inhibitors of the EETs-producing enzyme cytochrome 2C isoenzymes in renal and coronary arteries (21), and several different EETs (5, 6, 8, 9, 11, 12, and 14, 15-EET) are suggested to contribute to EDHF-type relaxation (1, 7). In the present study, sulfaphenazole inhibited

exclude involvement of EETs in relaxation to other epithelium-dependent bronchodilators.

The \( \text{Na}^+-\text{K}^+\text{-ATPase} \) is found to contribute to \( \beta_2 \)-adrenoceptor relaxation of large canine bronchioles (53) and bovine bronchioles (16). In intact rat hepatic arteries, endothelium-derived \( K^+ \) efflux is thought to mediate EDHF-type relaxation by activation of smooth muscle \( \text{Na}^+-\text{K}^+\text{-ATPase} \) (15), and in this preparation endothelium-dependent relaxation is blocked by 0.5 \( \mu \text{M} \) but not 0.1 \( \mu \text{M} \) ouabain, suggesting participation of the \( \alpha_2 \)-subtype of \( \text{Na}^+-\text{K}^+\text{-ATPase} \). In the present study, inhibition of the \( \text{Na}^+-\text{K}^+\text{-ATPase} \) by 100 \( \mu \text{M} \) ouabain, but not 0.1 \( \mu \text{M} \) ouabain, abolished NS309 and reduced salbutamol relaxation in bronchioles, suggesting a role for the \( \text{Na}^+-\text{K}^+\text{-ATPase} \) \( \alpha_2 \)-subunit in bronchiolar relaxation. Furthermore, low concentrations of \( K^+ \) evoked epithelium-independent relaxations that were abolished in the presence of 100 \( \mu \text{M} \) ouabain. Although these findings require further substantiation by other approaches, e.g., measurement of smooth muscle membrane potentials, our findings suggest that the EpDHF-type relaxation is mediated by epithelial \( K^+ \) efflux through \( \text{IK}_{\text{Ca}} \) and \( \text{SK}_{\text{Ca}} \) channels followed by activation of the smooth muscle \( \text{Na}^+-\text{K}^+\text{-ATPase} \).
arachidonic acid relaxation, but it did not change NS309 relaxation. These findings suggest that EETs do not contribute to endothelium-dependent NS309 relaxation in rat pulmonary arteries. Recent studies have reported that cytochrome P450 epoxygenase derivatives are involved in hypoxic vasoconstriction (39), and the present results do not exclude EETs contributing to endothelium-dependent relaxations in arteries from animals with pulmonary hypertension (36).

K⁺ (5–22.5 mM) induces relaxation through activation of Na⁺-K⁺-ATPase and Kᵦ channels in the vascular smooth muscle (14), and it was suggested that K⁺ ions released from IKCa and SKCa channels in the endothelium accumulate in the intercellular space and thereby activate Kᵦ channels and Na⁺-K⁺-ATPase leading to EDHF-type relaxation in rat mesenteric arteries (14). Membrane potential was found to be changed from −57.7 ± 0.9 mV to −76.8 ± 1.3 mV by increasing extracellular K⁺ from 0 to 5 mM K⁺, suggesting K⁺ is a hyperpolarization factor (14), and a study by Dora and Garland (13) found that the magnitude of the hyperpolarization is around 15 mV. In rat hepatic arteries, EDHF-type relaxation is thought to be mediated through smooth muscle Na⁺-K⁺-ATPase containing the α₂ subtype, since these relaxations are blocked by low concentrations of ouabain, whereas the α₁ subtype only is blocked at ouabain concentrations above 300 μM (15). In endothelium-denuded rat mesenteric arteries, 30 μM Ba²⁺ was unable to affect relaxation, whereas 0.1 mM ouabain almost completely abolished relaxation to K⁺ (13). In agreement with these findings, we found in the present study that inhibition of Na⁺-K⁺-ATPase by 100 μM ouabain abolished NS309 relaxation and reduced salbutamol relaxation in pulmonary arteries. Furthermore, low concentrations of K⁺ evoked endothelium-independent relaxations that were abolished in the presence of ouabain. These findings suggest that the EDHF-type relaxation is mediated by K⁺ efflux through opening of IKCa channels leading to activation of the smooth muscle Na⁺-K⁺-ATPase α₂ subtype in pulmonary arteries.

Perspectives and conclusions. The present study shows that in bronchioles, opening of IKCa and SKCa channels leads to bronchodilation through K⁺ efflux and activation of the Na⁺-K⁺-ATPase in smooth muscle. IKCa and SKCa channels could therefore be important for epithelial regulation of the bronchial tone through smooth muscle hyperpolarization and hence decreased smooth muscle cell calcium as well as inhibition of nuclear factor of activated T cells, a calcium/calcineurin-sensitive transcription factor that among others is activated and thought to contribute to smooth muscle remodeling in patients with pulmonary arterial hypertension (4).

Chronic obstructive pulmonary disease (COPD) and asthma are diseases that involve increased bronchoconstriction. The current treatment is based on improving the lung function with bronchodilators, such as β-adrenoceptor agonists, that result in activation of the adenyl cyclase/protein kinase A pathway and smooth muscle relaxation (52). However, the treatment of these diseases is still poor and requires the development of new drugs that counteract the airway hyperresponsiveness and airway remodeling. Since patients with COPD and asthma have increased bronchoconstriction due to altered release of epithelium-dependent relaxing factors and therefore smooth muscle remodeling, drugs that open IKCa and SKCa channels may increase bronchodilation and reverse smooth muscle remodeling.

K⁺ channel openers have been investigated in the treatment of asthma and COPD, and, for instance, opening of K⁺ channels leading to K⁺ efflux and hyperpolarization contributes to the regulation of the bronchiolar contractility (38), and ATP-sensitive potassium (KATP) channel openers, cromakalim and levocromakalim, are able to inhibit experimentally induced bronchoconstrictions in vivo (38). Unfortunately, KATP openers have strong vasodilating effects leading to systemic hypotension (38), and the potency is less than for β-adrenoceptor agonists. Future in vivo studies may clarify whether this is also a limitation for application of SKCa₃ and IKCa channel openers in the treatment of airway disease.

In summary, IKCa and SKCa₃ protein were located in the rat bronchiole epithelium and pulmonary arterial endothelium. In rat bronchioles, a novel opener of IKCa and SKCa channels, NS309, induced EpDHF-type relaxation involving both IKCa and SKCa channels. This relaxation is probably mediated by K⁺ efflux leading to activation of the Na⁺-K⁺-ATPase. In rat pulmonary arteries, NS309 induced EDHF-type relaxation primarily by opening of IKCa channels, followed by K⁺ efflux and activation of Na⁺-K⁺-ATPase in the underlying smooth muscle layer.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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