Kv Channels Contribute to Nitric Oxide- and Atrial Natriuretic Peptide-Induced Relaxation of a Rat Conduit Artery

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ABSTRACT

The role of K⁺ channels in nitric oxide (NO)-induced vasorelaxation has been largely investigated in resistance vessels where iberiotoxin-sensitive MaxiK channels play a predominant role. However, the nature of the K⁺ channel(s) involved in the relaxation triggered by NO-releasing compounds [nitroglycerin, NTG; NOR 3 ((±)-(E)-4-ethyl-2-[(E)-hydroxymimo]-5-nitro-3-hexenamide)] or atrial natriuretic peptide (ANP) in the conduit vessel aorta has remained elusive. We now demonstrate that, in rat aorta, ANP in the conduit vessel aorta has predominance of iberiotoxin-resistant channels in this conduit vessel. Aortic relaxations were strongly diminished by 4-aminopyridine (4-AP) (≥5 × 10⁻³ M) or by tetraethylammonium (≥2 × 10⁻⁴ M) at concentrations known to inhibit voltage-dependent K⁺ (Kᵥ) 2-type channels but not by other K⁺ channel inhibitors, glibenclamide, apamin, charybotoxin, tertiapin, or E-4031 N-[4-[1-[2-(6-methyl-2-pyridinyl)ethyl]-4-piperidinyl]-carbonyl[phenyl][methanesulfonamide dihydrochloride]. Consistent with a role of Kᵥ2-type channels, Kᵥ currents in A7r5 aortic myocytes were stimulated by NTG and inhibited by ≥5 × 10⁻³ M 4-AP. Furthermore, immunocytochemistry, immunoblot, and real-time polymerase chain reaction analyses confirmed the presence of Kᵥ2.1 channels in aorta. Kᵥ2.1 transcripts were ~100-fold more abundant than Kᵥ2.2. Our results support low-affinity 4-AP-sensitive Kᵥ channels, assembled at least partially by Kᵥ2.1 subunit, as downstream effectors of NO/ANP-signaling cascade regulating aortic vasorelaxation and further demonstrate vessel-specific Kᵥ channel involvement in NO/ANP-induced relaxation.

Nitroglycerin (NTG), an organic nitrate, has been used for more than 100 years as a remedy for the treatment of cardiovascular diseases, including angina pectoris, myocardial infarction, and congestive heart failure. Nitrovasodilators, including NTG, release nitric oxide (NO) and increase cGMP levels via activation of soluble guanylyl cyclase. As a consequence, cGMP-dependent protein kinase (PKG) activity is enhanced causing modulation (phosphorylation) of various intracellular proteins and vascular relaxation (Waldman and Murad, 1987). Electrophysiological and pharmacomechanical studies in a variety of vascular tissues have pointed out to a pivotal role of plasmalemmal K⁺ channels in PKG-induced relaxation, mainly the large conductance voltage-dependent and Ca²⁺-dependent and Ca²⁺-activated K⁺ channel.

ABBREVIATIONS: NTG, nitroglycerin; NO, nitric oxide; PKG, cGMP-dependent protein kinase; RT, reverse transcription; ANP, atrial natriuretic peptide; 4-AP, 4-aminopyridine; KᵥATP, ATP-sensitive K⁺ channel; Kᵥ, voltage-dependent K⁺ channel; MaxiK or BK, large-conductance, voltage-dependent and Ca²⁺-activated K⁺ channel; nt, nucleotides; NOR 3, (±)-(E)-4-ethyl-2-[(E)-hydroxymimo]-5-nitro-3-hexenamide; 8-Br-cGMP, 8-bromo-cGMP; RA, rat aorta; RMA, rat mesenteric artery; E-4031, N-[4-[1-[2-(6-methyl-2-pyridinyl)ethyl]-4-piperidinyl][carbonyl[phenyl][methanesulfonamide dihydrochloride]; TEA, tetraethylammonium; ODQ, 1H-[1,2,4]-oxadiazolo-[4,3-a]-quinoxalin-1-one; TBS, Tris-buffered saline; PI, pixel intensity; HCA, human coronary artery.
activated K⁺ (MaxiK, BK) channel. MaxiK channels have been found to be downstream effectors of cGMP-PKG pathway in various nonconduit arteries like cerebral (Robertson et al., 1993; Price and Hellermann, 1997), coronary (Bylekhov et al., 1998; Khan et al., 1998), pulmonary (Archer et al., 1994; Bialecki and Stinson-Fisher, 1995, Zhao et al., 1997), and mesenteric arteries (Khan et al., 1993; Tanaka et al., 1998). In addition, Alioua et al. (1998) have demonstrated that activation of MaxiK channels by PKG in vivo involves a direct phosphorylation of the channel protein. Consistent with this idea, Nara et al. (2000) showed that mutations of serine residues of the MaxiK pore-forming α-subunit suppressed NO- or ANP-induced enhancement of the channel activity.

Other K⁺ channels found to play a role in NO-PKG relaxation pathway are the ATP-sensitive K⁺ channel (KATP) in pial arteries (Armstead, 1996) and voltage-dependent K⁺ channels (Kᵥ) in pulmonary arteries (Zhao et al., 1997). Increased K⁺ channel activity by PKG resulted in membrane hyperpolarization limiting sarcoplasmic Ca²⁺ influx through L-type Ca²⁺ channels, decreasing cytosolic Ca²⁺ concentration leading to vasorelaxation. Additional mechanisms found to contribute to NO-PKG relaxation are: 1) activation of the plasmalemmal Ca²⁺-extrusion pump; 2) stimulation of Ca²⁺-ATPase activity to produce enhanced loading of Ca²⁺ into intracellular Ca²⁺ stores; 3) inhibition of agonist-stimulated Ca²⁺ release from sarcoplasmic reticulum; and 4) decrease in the sensitivity of contractile elements to Ca²⁺ (for review, see Tanaka et al., 2004). The contribution of each of these mechanisms may be determined by the relative expression and/or colocalization of different proteins in each vascular bed.

Tissue-specific patterns of PKG-Kᵥ channel coupling have begun to emerge. For example, MaxiK but not KATP channels significantly contribute to the nitrocompound-induced vascular relaxation in rabbit mesenteric, canine coronary, and rat and guinea pig pulmonary arteries (Khan et al., 1993, 1998; Bialecki and Stinson-Fisher, 1995; Zhao et al., 1997). In contrast to smaller arteries, in conduit arteries, such as rabbit and guinea pig aorta (Bialecki and Stinson-Fisher, 1995; Ishibashi et al., 1995) and carotid artery (Bialecki and Stinson-Fisher, 1995; Plane et al., 1998), the role of MaxiK channel is absent or minimal as assessed by the channel blocker iberiotoxin (IbTX) (Galvez et al., 1990) and, when present, it seems to depend on the NO donor (Bialecki and Stinson-Fisher, 1995; Ishibashi et al., 1995). Moreover, the identification of K⁺ channels involved in NO/ANP-PKG-mediated vascular relaxation in conduit arteries has not been accomplished yet. In this regard, glibenclamide (blocker of KATP channels) (Ashcroft and Gribble, 2000), noxizotin (blocker of voltage-dependent K⁺ channels of the K₁ family K₁.2, 1.3, and 1.7) (Grismer et al., 1994; Kalman et al., 1998), and leuurotolin I (blocker of small conductance Kᵥ₉ channels, SK1 and SK2) (Strobaek et al., 2000) are ineffective blockers of NO donor-induced relaxation in aorta and carotid artery (Bialecki and Stinson-Fisher, 1995), discarding a possible role of KATP, K₁ family, and SK1/SK2 channels in these large vessels.

In this work, we investigated the nature of the K⁺ channel involved in NO/ANP-PKG relaxant pathway in a rat conduit artery. Our results show that activation of IbTX-sensitive MaxiK channels, which significantly contribute to the mesenteric artery relaxations, is not the main mechanism by which NO and ANP produce relaxation of rat aorta, although the channel α-subunit protein is expressed to similar levels in both arteries. The lack of IbTX effect in NO/ANP-induced relaxations cannot be explained by a MaxiK current resistant to IbTX but rather to the involvement of a K⁺ conductance sensitive to high concentrations of 4-aminopyridine (4-AP) (IC₅₀ 3.5 × 10⁻³ M) and to relatively high concentrations of tetraethylammonium (TEA) (IC₅₀ 3 × 10⁻³ M). This pharmacomechanical profile, together with electrophysiological, molecular biology, and immunochemical experiments, supports the view that, in rat aorta, a Kᵥ₂.1-type channel built at least partially by Kᵥ₂.1 subunit plays a significant role in NO/ANP-induced relaxations.

Materials and Methods

Male Wistar or Sprague-Dawley rats were used. Food and water were available ad libitum to all animals. This study was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Toho University School of Pharmaceutical Sciences, accredited by the Ministry of Education, Science, Sports and Culture, Japan. Procedures at UCLA (Los Angeles, CA) received institutional approval.

Tension Measurements. A section of the thoracic aorta between the aortic arch and the diaphragm was removed from male rats (200–300 g) and placed in normal Tyrode’s solution: 158.3 mM NaCl, 4.0 mM KCl, 2.0 mM CaCl₂, 1.06 mM MgCl₂, 0.42 mM NaH₂PO₄, 10.0 mM NaHCO₃, and 5.6 mM glucose. The aorta was cleaned of loosely adhering fat and connective tissue and cut into ring segments of approximately 2 mm in length. For preparation of mesenteric artery ring segments, a section of intestine between the pylorus and the colon and its arcade of supplying blood vessels were isolated. Superior mesenteric arteries with an average outer diameter of 500 to 700 μm were isolated. Arteries were cleared of connective tissue and cut into ring segments of approximately 1 mm in length. Endothelium of both thoracic aortic and mesenteric artery rings was removed by rubbing gently the intimal surface with cotton strings moistened with Tyrode’s solution.

Arterial rings were mounted using stainless steel hooks under a resting tension of 1.0 (aorta) and 0.75 g (mesenteric artery). A 5 ml organ bath was used (UC-5 or UC-5TD; UFER Medical Instrument, Kyoto, Japan). The bathing solution was normal Tyrode’s solution gassed with 95% O₂, 5% CO₂ mixture and maintained at 36.5 ± 0.5°C, pH 7.4. The changes in tension were isometrically recorded with a force-displacement transducer (UL-10GR (Minibeck, Tokyo, Japan) or T7-8-240 (Orientec, Tokyo, Japan)) connected to a miniploygraph (Signal Conditioner: model MSC-2; Labo Support, Co., Ltd., Suita-shi, Osaka, Japan). After a 40- to 60-min equilibration period, the rings were contracted with isonicotic high-KCl (80 × 10⁻² M) Tyrode’s solution of the following composition: 82.3 mM NaCl, 80.0 mM KCl, 2.0 mM CaCl₂, 1.06 mM MgCl₂, 0.42 mM NaH₂PO₄, 10.0 mM NaHCO₃, and 5.6 mM glucose. After replacing the high-KCl Tyrode’s solution with normal Tyrode’s solution, full recovery was obtained. A lack of acetylcholine-induced (10⁻⁵ M) relaxation confirmed the absence of functional endothelial cells. The bath solution was then exchanged with a fresh one, and the rings were left to re-equilibrate for another 30 min before starting the experiments. Arterial preparations were then contracted with phenylephrine (3 × 10⁻⁷ M for aorta or 3 × 10⁻⁶ M for mesenteric artery) or high-KCl (80 × 10⁻³ M).

Data were collected and analyzed using a MacLab/400 and Chart (version 3.5) software (ADInstruments Japan, Tokyo, Japan). The percentage of relaxation was calculated considering the maximal tension level obtained by phenylephrine or high-KCl just before administration of vasorelaxants as 0% relaxation and the full recov-
tery to basal tension before application to phenylephrine or high-KCl as 100% relaxation. Data were treated as a function of drug concentration and fitted to the equation,

\[ E = E_{\text{max}} \times A^{n/10 - EC_{50}^{n/10}} + A^{\text{no}} \]  

(1)

where \( E \) is percentage relaxation at a given drug concentration, \( E_{\text{max}} \) is the maximal relaxation, \( A \) is the concentration of the drug, \( n/10 \) is the slope function, and \( EC_{50}^{n/10} \) is the effective drug concentration that produces a 50% response. The curve fitting was carried out using Prism, version 2.01 (GraphPad Software Inc., San Diego, CA).

The IC50 (the drug concentration required to inhibit the tension development by 50%) values and the maximal relaxation responses were also determined from the concentration-response relationship of individual results. IC50 values were expressed as pIC50 (negative logarithm of IC50) for statistical analysis.

Evaluation of \( K^+ \) Channel Blockers and Inhibitors of Soluble Guanylyl Cyclase. Possible involvement of \( K^+ \) channels in the vascular relaxations was examined in the absence and presence of several \( K^+ \) channel blockers. IbTX (10 \( \mu M \)) (MaxiK channel blocker) and TEA (1–2 \( \times 10^{-3} \) M, MaxiK channel blocker; 5–10 \( \times 10^{-3} \) M, \( K \) channel blocker) were added to the bath solution 60 and 20 min before administration of the vasorelaxants. When examining the effect of higher concentrations (10–6 M) of IbTX, preparations were treated for 90 min before applying vasorelaxants. Pretreatment periods for other \( K^+ \) channel blockers before application of vasorelaxants are as follows: glibenclamide (10 \( \mu M \)), a blocker for \( K_{\text{ATP}} \) channel (Ashcroft and Gribble, 2000), 60 min; apamin (10 \( \mu M \)), a blocker for small \( Ca^{2+} \)-activated \( K^+ \) channels (SK2 and SKD) (Kohler et al., 1996), 20 min; charybdotoxin (10 \( \mu M \)), a blocker for MaxiK and voltage-dependent \( K^+ \) channels K1.2 and K1.3 (Grissem et al., 1994; Wallner et al., 1995), 20 min; and 4-AP (10–3–10–2 M), a blocker for \( K \) channels (Mathie et al., 1998), 20 min. In some experiments, the effect of 4-AP was examined by its cumulative applications after the vascular relaxation reached the maximal level. 

Membrane Preparations. All procedures were performed at 4°C, and all solutions contained protease inhibitors. Aorta and superior mesenteric artery were dissected from male rats and cleaned of loosely adhering fat and connective tissue. A section of the arteries was reserved for immunocytochemistry. Three to five arteries were opened longitudinally, and the internal surface was gently rubbed with a moistened filter paper to remove the endothelium. This procedure was performed as quickly as possible in 100% \( O_2 \)-gassed ice-cold Krebs’ solution: 126.9 mM NaCl, 5.9 mM KCl, 2.96 mM CaCl2, 1.18 mM MgCl2, 10.8 mM HEPES, and 11.8 mM glucose, pH 7.4, supplemented with protease inhibitors (100 \( \mu M \) phenylmethyl-sulfonyl fluoride, 1 \( \mu M \) pepstatin A, 1 \( \mu g/ml \) apronin, 1 \( \mu g/ml \) leupeptin, 8 \( \mu g/ml \) calpain inhibitor I, 8 \( \mu g/ml \) calpain inhibitor II, 0.2 \( \times 10^{-3} \) M Pefabloc SC, and 0.1 mg/ml benzamidine). Tissues were then quickly frozen in liquid nitrogen and stored at –80°C for later use. Frozen arteries were homogenized in 20 mM HEPES-KOH, 1 mM EDTA, and 250 mM sucrose, pH 7.4. The homogenate was centrifuged at 1000g for 15 min, and the supernatant was subsequently centrifuged at 100,000g for 30 min. Crude membranes were suspended in 10 mM HEPES-sucrose solution: 10 mM HEPES-KOH and 250 mM sucrose, pH 7.4. The protein content was determined using the Bradford method (Pierce Chemical, Rockford, IL). Membranes were then supplemented with Tris-SDS solution (62.5 \( \times 10^{-3} \) M Tris-HCl, 2% SDS, 10% glycerol, 0.01% bromphenol blue, and 42 mM DTT) and stored at –70°C until use.

Immunoblots. Arterial membrane proteins (2 \( \mu g \)) were separated by 7.5% SDS-polyacrylamide gels under reducing conditions and electrotransferred to nitrocellulose paper. Blots were blocked with Tris-buffered saline (TBS: 50 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100, and 0.1% Tween 20, pH 7.4) containing 5% nonfat dry milk for 1 h at room temperature. Blots were incubated with 1.2 \( \mu g/ml \) affinity-purified rabbit polyclonal anti-MaxiK channel \( \alpha_{683-886} \)-subunit antibody against residues 883 to 896 (VNDTNVQ-FLDQDDD) of the human MaxiK channel \( \alpha \)-subunit (hSiLo) (GenBank accession number U11058), with 3.3 \( \mu g/ml \) affinity-purified rabbit polyclonal anti-K-2.1 (Alomone Labs Ltd., Jerusalem, Israel) or with 0.33 \( \mu g/ml \) monoclonal antibody against smooth muscle \( \alpha \)-actin (Sigma, St. Louis, MO) in 1% nonfat milk/TBS for 12 h at 4°C, washed with TBS three times for 10 min each, and then incubated with horseradish peroxidase-conjugated anti-rabbit (0.125 \( \mu g/ml \)) for MaxiK or anti-mouse secondary antibody (0.125 \( \mu g/ml \)) for smooth muscle \( \alpha \)-actin (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) for 1 h. After washing, blots were treated for 1 min with Chemiluminescence Reagent Plus (PerkinElmer Life and Analytical Sciences, Inc., Wellesley, MA) and autoradiographed on Kodak BioMax film (Eastman Kodak, Rochester, NY). The specificity of the antibodies was tested by preadsorbing them with the antigenic peptides (80 \( \mu g \) of peptide/\( \mu g \) of anti-MaxiK channel \( \alpha_{683-886} \)-subunit antibody and 0.6 \( \mu g \) of antigen/\( \mu g \) of anti-K-2.1 antibody), which blocked the corresponding signals. Bands corresponding to the immunoreactive proteins were quantified using GS670 Imaging Densitometer (Bio-Rad, Hercules, CA). Results are expressed as arbitrary densitometric units.

Immunocytochemistry. Fresh artery segments were fixed by immersion for 2 h in 0.1 M phosphate-buffered saline, pH 7.4, supplemented with 4% paraformaldehyde and 2% picric acid. Transverse cryostat sections (10 \( \mu m \)) were incubated for 1 h at 4°C with 4.8 \( \mu g/ml \) affinity-purified anti-MaxiK \( \alpha_{683-886} \)-subunit antibody or with 3.3 \( \mu g/ml \) anti-K-2.1 antibody in 1% normal goat serum and 0.2% Triton X-100 in phosphate-buffered saline (Sigma). After washing, the tissue sections were incubated for 1 h at room temperature with Cy5 or fluorescein isothiocyanate-donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). In the control sections, antibody immunoreactivity was blocked by preadsorbing the antibody with an excess amount of the corresponding antigenic peptides. Concentrations were the same as for immunoblots. Immunofluorescence was measured in five to seven randomly selected visual fields from each tissue section using Image-Pro plus software (Media Cybernetics, Inc., Silver Spring, MD). Results are expressed as average pixel intensity.

Real-Time PCR. Approximately 20 ng of poly(A)+ RNA from rat aorta (endothelium-denuded) was converted to single-stranded cDNA by priming with an oligo(dT) primer. To quantify the relative abundance of each gene, we used real-time PCR (iCycler iQ Real-time PCR; Bio-Rad) with SYBR Green I (stock solution supplied at 1:8,000) as the fluorescent probe (Molecular Probes, Eugene, OR) and Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA). Gene-specific primers for K-2.1 (GenBank accession number NM_013186) were as follows: upstream primer corresponding to nucleotides 1282 to 1848 of 5′-AGTCTCTGCTGGCATCCTCTCC-T3′ and downstream primer corresponding to nucleotides 2093 to 2073 of 5′-CTAGAGGGATCTGTGATAGAA-3′. For K-2.2 (GenBank accession number NM_054000), the upstream primer corresponding to nucleotides 2313 to 2333 was 5′-AGGAGGAGCGCTACTGAGATT-3′, and the downstream primer corresponding to nucleotides 2564 to 2585 was 5′-TAGAAGAATGCTACTCATGTG-3′. As control, we used the reaction mixture without the cDNA. Reaction conditions were as follows: 5 min at 95°C to activate the “hot start” Platinum TaqDNA polymerase followed by 45 cycles at 95°C for 45 s, 58°C for 45 s, and 72°C for 45 s. As required for accurate measurements, melting curves showed the presence of a single peak. Standard curves for K-2.1 and K-2.2 were constructed using known amounts of the respective amplon subcloned into pcDNA3.

The quality of the cDNA was confirmed in separate RT-PCR reactions by the lack of detectable genomic DNA using primers flanking an intronic region of \( \beta \)-actin (GenBank accession number V01217). The upstream primer matches nucleotides 2383 to 2402 (5′-GGCTACAGCTTACACCACAC-3′), and the downstream primer
corresponds to nucleotides 3071 to 3091 (5'-TACTCCCTGCTGTGCT-GATCCAC-3'). β-Actin cDNA should give a product of 494 nt, and β-actin genomic DNA would give a product of 708 nt.

Cell Isolation and A7r5 Cell Culture. Freshly dissociated aortic myocytes were used to record MaxiK currents. Namely Ca\(^{2+}\)-free Krebs' solution (119 mM NaCl, 4.7 mM KCl, 1.18 mM K\(_2\)HPO\(_4\), 1.17 mM MgSO\(_4\), 22 mM NaHCO\(_3\), 8 mM HEPES, and 5.5 mM glucose) was used for cell isolation. After removing connective tissues, the dissected vessel was cut into small pieces. Tissue pieces were transferred to nominally Ca\(^{2+}\)-free Krebs' solution containing papain (1.5 mg/ml), dithiothreitol (10 mM), and bovine serum albumin (2 mg/ml) and incubated for 1 h at 4°C followed by 30 min at 37°C. Tissues then were immediately transferred to a solution containing collagenase F (1 mg/ml), collagenase H (0.3 mg/ml), and bovine serum albumin (2 mg/ml) and incubated for 10 min at 37°C. The digested tissues were subsequently washed with ice-cold Ca\(^{2+}\)-free solution and suspended with a wide-bore Pasteur pipette. The cell suspension was kept at 4°C until use.

The rat aortic smooth muscle cell line (passage 5) A7r5 (American Type Culture Collection, Manassas, VA) was used to record K\(_{\text{Ca}}\) currents. Cells were maintained at 37°C with 5% CO\(_2\) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were split usually once a week and cultured on glass coverslips for 1 to 2 days prior to electrophysiological recordings.

Electrophysiology. Whole-cell currents were measured at room temperature. Pipette resistances were 2 to 3 MΩ. Data were filtered at one-fifth the sampling frequency, which was 5 kHz. To record MaxiK currents, the bathing solution contained 135 mM sodium methanesulfonate, 5 mM potassium methanesulfonate, 2 mM MgCl\(_2\), 0.1 mM CaCl\(_2\), 10 mM HEPES, and 5.5 mM glucose, pH 7.4. The pipette solution contained 140 mM potassium methanesulfonate, 2 mM MgCl\(_2\), 0.1 mM CaCl\(_2\), 0.146 mM EGTA, 10 mM HEPES, and 10 mM glucose, pH 7.4 (calculated free Ca\(^{2+}\) concentration was 100 nM).

To record K\(_{\text{Ca}}\) currents, the bath solution contained 136 mM NaCl, 5.4 mM KCl, 2 mM MgCl\(_2\), 10 mM glucose, 10 mM HEPES, 0.02 mM glibenclamide, and 1 mM TEA, pH 7.4. The pipette solution was 135 mM KCl, 10 mM EGTA, 10 mM HEPES, 5 mM glucose, 1 mM MgATP, and 1 mM Na\(_2\)GTP, pH 7.2. To minimize MaxiK currents, the blocker 10\(^{-5}\) M TEA ± 10\(^{-7}\) M IbTX was included in the bath solution, Ca\(^{2+}\) was excluded in both bath and pipette solutions, and a high-Ca\(^{2+}\) buffer capacity, 10 mM EGTA, was used in the pipette solution. The calculated free Ca\(^{2+}\) of the pipette solution is <10\(^{-7}\) M (assuming a contaminant Ca\(^{2+}\) of 5 × 10\(^{-6}\) M); under these conditions, MaxiK α and β complexes are only activated at very high potencies of ~150 mM. Thus, K\(_{\text{Ca}}\) currents were elicited up to ±70 mV, and mean values were obtained at ±50 mV. Bath solutions containing 5 and 10 × 10\(^{-8}\) M 4-AP were readjusted to pH 7.4 before the experiment. These concentrations of 4-AP do not inhibit MaxiK channels (Wallner et al., 1995). Glibenclamide was used to abolish K\(_{\text{ATP}}\) currents. Data acquisition and analysis were performed using pCLAMP (Axon Instruments, Inc., Union City, CA) or a home-made acquisition system and software.

Drugs. The following drugs were used: l-phenylephrine hydrochloride (Wako Pure Chemical Industries, Osaka, Japan); NTG (Ni-hon Kayaku, Tokyo, Japan); NOR 3 (d(-)/L(+)4-ethyl-2(3H)-hydroxy-imino)-5-nitro-3-hexahemidine) (PK409; Doinjo, Kumamoto, Japan); IbTX, charybdoxin, apamin, and ANP (Peptide Institute, Minohshi, Osaka, Japan); ODQ (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA); methylene blue (Tokyo Kasei, Tokyo, Japan); glibenclamide (Sigma); acetylcholine (Daichi, Tokyo, Japan); and phentolamine mesylate (Ciba-Geigy, Takarazuka-shi, Hyogo, Japan). Cromakalim was kindly donated from Nissan Chemical Industries (Tokyo, Japan). All other chemicals used in the present study were commercially available and of reagent grade.

Cromakalim was dissolved in 70% ethanol at 10\(^{-2}\) M and diluted further with distilled water to the desired concentrations. Glibenclamide and ODQ were dissolved in dimethyl sulfoxide at 10\(^{-3}\) and 10\(^{-7}\) M. All other drugs were prepared as aqueous solutions and diluted with distilled water. Final ethanol and dimethyl sulfoxide concentrations in the bath medium did not exceed more than 1 and 0.1%, respectively, which did not affect the vascular responses. All drugs are expressed in molar concentrations in the bathing solution.

Statistical Analysis. The data are presented as mean value ± S.E.M., and n refers to the number of experiments. The significance of the difference between mean values was evaluated by paired or unpaired Student's t test, unpaired Student's t test with Welch's correction if necessary, and one-way analysis of variance followed by Tukey's multiple comparison test. A P value less than 0.05 was considered statistically significant.

Results

IbTX, a MaxiK Channel Blocker, Does Not Antagonize the Relaxation Induced by NO-Releasing Compounds in Rat Aorta but Does Inhibit It in Mesenteric Artery. We first determined whether NTG-induced relaxation could be prevented by IbTX, a MaxiK channel blocker, in rat aorta versus mesenteric artery. Figure 1 shows typical mechanical recordings in de-endothelialized aorta (A and B) and mesenteric artery (C and D) treated with NTG in the absence (−) or presence (+) of IbTX (10\(^{-7}\) M). Experiments were performed under the continuous presence of phenylephrine (3 × 10\(^{-7}\) M for aorta, 3 × 10\(^{-8}\) M for mesenteric artery) (line). Prior to treatment with any other drug, phenylephrine induced a sustained contraction in both arteries. Application of NTG (10\(^{-10}\)–10\(^{-5}\) M) produced a concentration-dependent relaxation in both types of blood vessels (Fig. 1, A, aorta, and C, mesenteric artery). However, IbTX appeared to only prevent NTG-induced relaxation in mesenteric artery.

In aortic smooth muscle, pretreatment with IbTX (10\(^{-7}\) M) caused no appreciable effects on NTG-induced relaxation (Fig. 1, B versus A). This lack of effect is also clear from the corresponding concentration-response relationships that were almost identical in the absence (open circles) and presence (filled circles) of IbTX (Fig. 2A; Table 1). In sharp contrast to the lack of effects in aorta, pretreatment of mesenteric artery with 10\(^{-7}\) M IbTX (applied at filled square) for 60 min profoundly attenuated NTG-induced relaxation (Fig. 1, D versus C). This effect is quantified in the concentration-response relationships in Figure 2B. The inhibitory effects of IbTX on pC\(_{\text{NO}}\) and E\(_{\text{max}}\) in mesenteric artery are shown in Table 1.

The distinct inhibitory action of IbTX in aorta versus mesenteric artery was also evident if the toxin was applied after vasorelaxation with NTG (Fig. 1, A and C); IbTX (10\(^{-7}\) M) counteracted the relaxant effect of NTG in mesenteric artery but did not in aorta. Maximal relaxation values attained with NTG were similar in the absence (102.3%, n = 2) or presence (98.3%, n = 2) of the toxin in aorta. In contrast, in mesenteric artery, 10\(^{-7}\) M IbTX applied after the complete relaxation with NTG (Fig. 1C, filled square) was able to produce contraction, inhibiting the NTG-induced relaxation by 44.3 ± 4.6% (n = 3; P < 0.01). As a control, this concentration of IbTX (10\(^{-7}\) M) did not affect the vascular relaxation induced by phentolamine, an α-adrenoceptor antagonist (Fig. 1, C and D). Relaxations by 3 × 10\(^{-6}\) M phentolamine achieved similar values with (103.3 ± 1.4%; n = 5) or without IbTX (108.1 ± 3.9%; n = 14) (P > 0.05).

Vascular relaxation induced by NTG is mediated via NO,
which is an intracellular byproduct of NTG (Waldman and Murad, 1987). Therefore, we decided to explore whether the differential inhibitory action of IbTX on NTG-induced relaxation in rat aorta versus mesenteric artery could be mimicked by an NO donor, NOR 3 (also named FK409). Similar to NTG, NOR 3 \((10^{-9} - 10^{-6} \text{M})\) produced a concentration-dependent relaxation in both endothelium-denuded aorta (Fig. 2C, open circles) and mesenteric artery (Fig. 2D, open circles). As in the case of NTG-induced relaxation, IbTX \((10^{-7} \text{M})\) did not significantly affect the NOR 3-induced relaxation in aorta but it significantly inhibited the relaxant response to NOR 3 \((<10^{-6} \text{M})\) in mesenteric artery (Fig. 2C versus D, filled circles). The effects of IbTX on \(pIC_{50}\) and \(E_{\text{max}}\) for NOR 3 in both arteries are shown in Table 1. These results demonstrate that rat aorta and mesenteric arteries use different mechanisms to relax in response to NO donors.

**ANP-Induced Relaxation Is Resistant to IbTX in Aorta but Not in Mesenteric Artery.** We have previously shown that, in rat mesenteric artery, IbTX substantially inhibits the relaxation induced by ANP (Tanaka et al., 1998); however, it is not known whether this also applies to aorta. In addition, NO-releasing agents and ANP use different guanylyl cyclases, soluble and membrane-bound, respectively; these cyclases may differentially deliver cGMP to its target(s) in aorta versus mesenteric artery. Thus, we tested whether ANP-induced relaxation could be inhibited by IbTX in aorta. In control experiments, ANP \((10^{-10} - 10^{-7} \text{M})\) produced a concentration-dependent relaxation in both aorta (Fig. 2E, open circles) and
confirming our previous work, Fig. 2F shows that, in mesenteric and NOR 3 (see Fig. 2, A and C) in this vessel. As control and relaxation to ANP (Fig. 2E; Table 1), as was the case for NTG.

Differences in Soluble Guanylyl Cyclase Activation.

in Aorta versus Mesenteric Artery Cannot Be Attributed to Differences in Soluble Guanylyl Cyclase Activity.

As expected, ANP-induced relaxations in both arteries were also significantly inhibited by methylene blue (10⁻⁵ M), another inhibitor of soluble guanylyl cyclase (Table 2). As expected, ANP-induced relaxations in both arteries were not affected by methylene blue (10⁻⁵ M) (Table 2), which was unable to prevent the activity of membrane-bound guanylyl cyclase. These results show that the lack of MaxiK channel role in the relaxation of aortic vessel cannot be explained by a lower activity of soluble guanylyl cyclase in this type of smooth muscle.

Neither High Concentrations of IbTX Nor TEA Pharmacology Support a Role for MaxiK in the Aortic Smooth Muscle Relaxations by NO-Releasing Compounds and ANP.

The access of IbTX to its binding site in the MaxiK channel pore-forming α subunit is greatly slowed down when channels are coassembled with the regulatory β₄ subunit, making the channels practically “insensitive” to nanomolar concentrations of IbTX within experimental times. To overcome the resistance to IbTX block of channels formed by α + β₄ subunits, IbTX concentrations need to be increased to the micromolar range; under these conditions, almost complete blockade is observed within ~10 min (Meera et al., 2000). Thus, to rule out the possibility that the lack of IbTX effect on the aortic vessel relaxations induced by NO and ANP is due to the expression of MaxiK channels assembled by α and β₄-like subunits, we examined the effects of 1 × 10⁻⁶ M IbTX. None of the aortic relaxations was affected by this high concentration of IbTX (Table 3). These observations are against the possibility that the resistance to IbTX blockade of rat aortic NO/ANP relaxations is due to the expression of an IbTX-inaccessible α + β₄ type of MaxiK channel.

To further test the contribution of MaxiK channel to aortic relaxations induced by NO-releasing compounds and ANP, we tested the effects of TEA. It is commonly accepted that blockade with 1 × 10⁻³ M TEA is sufficient to almost fully inhibit a MaxiK-related event since TEA blocks MaxiK channel from the external side of the channel with a IC₅₀ value of 2 to 3 × 10⁻⁴ M (Wallner et al., 1995), whereas Kᵣ and Kₐ₅₅ currents have a higher IC₅₀ (~10 mM) in smooth muscle (Nelson and Quayle, 1995). Although, 1 × 10⁻⁵ M TEA had a minor effect on aortic relaxations induced by NTG, NOR 3, and ANP (Table 3; see Fig. 6), this modest inhibitory action was clearly less effective than the one produced by 10⁻⁷ M IbTX in the mesenteric artery (Fig. 2, B, D, and F) and might reflect the partial blockade of another class of K⁺ channel. Taken together, the mechanical results obtained with IbTX and TEA support the view that in aorta the main effectors of these cGMP-elevating (and relaxant) agents are non-MaxiK of K⁺ channels. The results could also reflect a much less expression of MaxiK in rat aorta compared with mesenteric artery or that MaxiK channels in aorta need much less expression of MaxiK compared with mesenteric artery.
that MaxiK currents were significantly blocked by $2 \times 10^{-7}$ M IbTX ($n = 10$ cells) (data not shown). Therefore, we investigated whether MaxiK protein expression levels in aorta versus mesenteric artery differed. In addition, we examined whether high-K$^+$ or other K$^+$ channel blockers could inhibit the relaxation by NO-releasing compounds.

**Immunoblot Analysis and Immunocytochemistry**

**Show a Similar MaxiK Channel Expression in Rat Aorta and Mesenteric Artery.** Western blot analysis of smooth muscle membranes from both rat aorta and mesenteric artery showed a band at around 125 kDa corresponding to MaxiK channel $\alpha$-subunit protein (Fig. 3A). For comparison, its expression in human coronary artery (HCA) is also shown. No signal was obtained when the polyclonal antibody was preadsorbed with the antigenic peptide, showing the specificity of the antibody employed. As a control, we detected in the same blot the expression of smooth muscle $\alpha$-actin that has a molecular mass of $\sim 43$ kDa. Figure 3B shows the quantification of Western blots from several membrane preparations. The MaxiK channel signals were not

**TABLE 1**

$pIC_{50}$ and $E_{max}$ values for NTG, NOR 3, and ANP in rat aorta and mesenteric artery

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IbTX ($10^{-7}$ M)</th>
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<tbody>
<tr>
<td></td>
<td>pIC$_{50}$</td>
<td>$E_{max}$</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>NTG Aorta</td>
<td>7.96 ± 0.16 (5)</td>
<td>101.4 ± 0.6 (5)</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>7.69 ± 0.11 (8)</td>
<td>98.7 ± 1.3 (6)</td>
</tr>
<tr>
<td>NOR 3 Aorta</td>
<td>8.04 ± 0.10 (3)</td>
<td>106.8 ± 3.7 (3)</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>7.82 ± 0.15 (3)</td>
<td>104.2 ± 1.1 (3)</td>
</tr>
<tr>
<td>ANP Aorta</td>
<td>8.43 ± 0.10 (4)</td>
<td>102.6 ± 1.2 (4)</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>8.11 ± 0.12 (3)</td>
<td>94.4 ± 2.0 (3)</td>
</tr>
</tbody>
</table>

$pIC_{50}$, the negative logarithm of drug concentration required to induce 50% relaxation; $E_{max}$, the maximum relaxant responses (NTG at $10^{-8}$ M; NOR 3, $10^{-6}$ M; and ANP, $10^{-7}$ M). *$P < 0.05$, **$P < 0.01$, significant differences between two groups.

**TABLE 2**

Effects of ODQ and methylene blue on $pIC_{50}$ and $E_{max}$ values for NTG, NOR 3, and ANP in rat aorta and mesenteric artery

<table>
<thead>
<tr>
<th></th>
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<th>ODQ ($10^{-5}$ M)</th>
<th>Methylene Blue ($10^{-5}$ M)</th>
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<tr>
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<td>pIC$_{50}$</td>
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<td>pIC$_{50}$</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>NTG Aorta</td>
<td>7.75 ± 0.16 (5)</td>
<td>98.6 ± 0.8 (5)</td>
<td>&lt;5.0 (5)</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>7.69 ± 0.13 (4)</td>
<td>104.6 ± 1.2 (4)</td>
<td>&lt;5.0 (4)</td>
</tr>
<tr>
<td>NOR 3 Aorta</td>
<td>7.74 ± 0.13 (3)</td>
<td>99.6 ± 1.3 (3)</td>
<td>&lt;5.0 (4)</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>7.26 ± 0.08 (4)</td>
<td>98.1 ± 2.3 (4)</td>
<td>&lt;6.0 (4)</td>
</tr>
<tr>
<td>ANP Aorta</td>
<td>8.42 ± 0.08 (4)</td>
<td>102.5 ± 1.1 (4)</td>
<td>8.60 ± 0.10 (4)</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>8.01 ± 0.17 (3)</td>
<td>90.5 ± 5.0 (3)</td>
<td>8.60 ± 0.10 (4)</td>
</tr>
</tbody>
</table>

* $P < 0.05$, ** $P < 0.01$, significant differences between two groups.

**TABLE 3**

Effects of high-KCl, IbTX, and TEA on $pIC_{50}$ and $E_{max}$ values for NTG, NOR 3, and ANP in rat aorta

<table>
<thead>
<tr>
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<th>Control</th>
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<tr>
<td></td>
<td>pIC$_{50}$</td>
<td>$E_{max}$</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>NTG Aorta</td>
<td>High-KCl ($8 \times 10^{-3}$ M)</td>
<td>7.77 ± 0.13 (9)</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>IbTX ($10^{-6}$ M)</td>
<td>7.14 ± 0.27 (4)</td>
</tr>
<tr>
<td>NOR 3 Aorta</td>
<td>High-KCl ($8 \times 10^{-3}$ M)</td>
<td>7.85 ± 0.14 (6)</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>IbTX ($10^{-6}$ M)</td>
<td>7.69 ± 0.10 (13)</td>
</tr>
<tr>
<td>ANP Aorta</td>
<td>High-KCl ($8 \times 10^{-3}$ M)</td>
<td>7.53 ± 0.08 (6)</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>IbTX ($10^{-6}$ M)</td>
<td>7.91 ± 0.13 (6)</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>TEA ($10^{-5}$ M)</td>
<td>8.25 ± 0.08 (5)</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>TEA ($2 \times 10^{-5}$ M)</td>
<td>7.76 ± 0.09 (7)</td>
</tr>
</tbody>
</table>

* $P < 0.05$, ** $P < 0.01$, significant differences between two groups.
for each, the expression levels of MaxiK channel forming biochemical studies clearly indicate that MaxiK channel pore-

Fig. 3. Western blot analysis and immunocytochemistry of MaxiK channel α-subunit expression in rat aorta and mesenteric artery. A, Western blots of MaxiK channel α-subunit in rat aorta (RA) and rat mesenteric artery (RMA). Equal amounts of RA and RMA protein (2 μg) and 5 μg of human coronary artery (HCA) were separated by 6% SDS-polyacrylamide gels under reducing conditions and electrophoretically transferred to nitrocellulose paper. Western blots of smooth muscle α-actin in both arteries are also shown. B, mean densitometric values for MaxiK channel α-subunit and smooth muscle α-actin in aorta and mesenteric artery (left) and its ratio between these arteries (right). Results are expressed in arbitrary densitometric units (optical density) and show insignificant differences between aorta and mesenteric artery. Data are mean values ± S.E.M. of five experiments. C, MaxiK channel α-subunit immunolabeling in aorta and mesenteric artery smooth muscle cells. Rat aorta and mesenteric artery were labeled using a polyclonal anti-MaxiK channel α-subunit antibody. No staining of smooth muscle cells was detected when the antibody was preadsorbed with the corresponding antigen (control). Bar indicates 100 μm for all.

significantly different between rat aorta and mesenteric artery (aorta, 0.71 ± 0.14 O.D., n = 5; mesenteric artery, 0.53 ± 0.13 O.D., n = 5, P > 0.05) (Fig. 3B, left). In agreement, when the expression levels of MaxiK channel α-subunit were normalized to that of smooth muscle α-actin, the ratio values were not significantly different between both vessels (2.70 ± 0.76 for aorta versus 2.20 ± 0.81 for mesenteric artery, n = 5 for each, P > 0.05) (Fig. 3B, right).

MaxiK channel expression levels in aorta versus mesenteric artery were also examined in tissue sections with confocal microscopy (Fig. 3C). The specificity of the antibody was assessed by preadsorbing anti-α-skeletal muscle antibody with excess antigenic peptide, a maneuver that practically abolished the fluorescent signal, as shown in Fig. 3C (control). In agreement with Western blot analysis, MaxiK channel signals were similar in both aorta and mesenteric artery smooth muscle cells with pixel intensity (PI) values of 120.3 ± 23.8 PI (n = 4) for aorta and 137.9 ± 30.5 PI (n = 4) for mesenteric artery (P > 0.05). These biochemical studies clearly indicate that MaxiK channel pore-forming α-subunit is expressed at a similar level in both aortic and mesenteric artery smooth muscle cells. Thus, the lack of IbTX sensitivity of the aortic relaxations by NO-releasing compounds and ANP cannot be explained by a diminished expression of this channel α-subunit.

K+ Channel Activation as a Predominant Mechanism Responsible for Aortic Relaxation by NTG, NOR 3, and ANP. The contribution of K+ channels to drug-induced relaxations can be assessed by minimizing K+ efflux with high extracellular K+, which produces cell membrane depolarization and vessel contraction. Figure 4, A, B, and C, demonstrates that relaxations of rat aorta induced by NO donors (NTG and NOR 3) and ANP are strongly diminished when the muscles are contracted with high-KCl (80 × 10⁻³ M) (Table 3). Furthermore, the attenuation of the relaxant effects in high-KCl-contracted muscle was much more pronounced in aorta compared with mesenteric artery (Fig. 4, D and E, Table 3). These findings indicate that indeed K+ channel activation plays a predominant role in the relaxant effects of NO donors and ANP on rat aortic smooth muscle, although the contribution of MaxiK channel is apparently lacking. The nature of the K+ channel was established with the aid of a pharmacological screening supported with electrophysiological, molecular, and biochemical approaches.

The K+ Channel Inhibitors Glibenclamide, Apamin, Charybdotoxin, 10⁻³ M 4-AP, E-4031, and Tertiapin Are Ineffective on NTG-Induced Relaxations in the Aortic Vessel. To determine the possible contribution of ATP-sensitive K+ (KATP) channels in the NTG-induced relaxation, we examined the effect of its inhibitor, glibenclamide (Ashcroft and Gribble, 2000). In aortic vessels, glibenclamide (10⁻⁶ M) was unable to prevent NTG-induced relaxation (Fig. 5A; Table 4). As expected, when cromakalim, a KATP channel opener, was used to cause relaxation, glibenclamide (10⁻⁶ M) almost abolished its relaxant effect on aorta (Table 4). Glibenclamide (10⁻⁶ M) was also unable to affect NTG-induced relaxation of mesenteric artery (Table 4). Thus, glibenclamide-sensitive KATP channels do not contribute to the NTG-induced relaxation in aorta as well as in mesenteric artery. Furthermore, NTG-induced relaxation of rat aorta was not affected by 10⁻⁷ M apamin, an inhibitor of small conductance Ca²⁺-activated K+ channels SK2 and SK3 (Fig. 5B) (Köhler et al., 1996); 10⁻⁷ M charybdotoxin, a blocker of MaxiK, K, 1.2, and K, 1.3 channels (Fig. 5C) (Grissmer et al., 1994; Wallner et al., 1995); 10⁻⁷ M 4-AP, an inhibitor of K, 1 family (Grissmer et al., 1994; Yamane et al., 1995; Kalman et al., 1998; Mathie et al., 1998) and K, 3 family (Fig. 5D) (Kirsch and Drewes, 1993; Mathie et al., 1998); 10⁻⁶ M E-4031, a blocker of human ether-a-go-go-related gene and hEAG1 channels (human orthologues of rat Kcnh2 and Kcnh1, respectively) (Fig. 5E) (Zhou et al., 1998; Gessner and Heinemann, 2003); or by 10⁻⁶ M tertiapin, a blocker of K, 1.1 and K, 3,1/3.4 (Fig. 5F) (Jin and Lu, 1998). These pharmacological experiments rule out the possible contribution of KATP, SK2, SK3, K, 1, K, 3, Kcnh1, Kcnh2, Kir1.1, and Kir3.1/ 3.4 types of K+ channels to the aortic relaxation by NO donors. In addition, the lack of effect of 1 to 2 × 10⁻³ M TEA (Table 3) rules out a role of rKCNQ2 (Jow and Wang, 2000) and leaves K, 2.1, K, 2.2, K, 4, rEag2 (Kcnh5), and rElk (Kcnh3 and Kcnh4) K+ channels as potential candidates.

Higher Concentrations of 4-AP and TEA Counteract the Aortic Relaxations to NTG, NOR 3, and ANP. To test the possibility that K, 2.1, K, 2.2, K, 4, rEag2, or rElk K⁺
channels mediate the NO- and ANP-induced aortic relaxation, we tested the effect of higher concentrations (10^{-3}–10^{-2} M) of 4-AP and TEA. We found that the aortic relaxations induced by NTG, NOR 3, and ANP were not significantly inhibited by 10^{-3} M 4-AP (n = 3–6 for all relaxations). In contrast, 5 \times 10^{-3} and 10^{-2} M significantly

\[ \text{Fig. 4. Concentration-response relationships for the relaxations of rat aorta and mesenteric artery in response to nitroglycerin, NOR 3, or ANP in preparations contracted with phenylephrine or high-KCl. Tissue was precontracted with phenylephrine (aorta, 3 \times 10^{-7} M; mesenteric artery, 3 \times 10^{-6} M) or high-KCl (80 \times 10^{-3} M). Nitroglycerin (A and D), NOR 3 (B), and ANP (C and E) were applied cumulatively to the bath solution. Vascular relaxations are expressed as percentage inhibition of the tension development just before application of vasorelaxants and are plotted as a function of vasorelaxants concentration. Data are mean values \pm S.E.M. of three to nine experiments. \ast, P < 0.05; \ast\ast, P < 0.01, significant differences between two groups. Note that the extent of NTG- and ANP-induced relaxations in high-KCl-precontracted mesenteric artery (D and E) were close to those in the vessels treated with 10^{-7} Miberiotoxin (cf. Fig. 2, B and F; 19.5 versus 26.8% for NTG relaxation; 40.6 versus 41.8% for ANP relaxation).} \]

\[ \text{Fig. 5. Concentration-response relationships for the relaxations of rat aorta in response to nitroglycerin in the absence and presence of various types of K^+ channel blockers. A, glibenclamide. B, apamin. C, charybdotoxin. D, 4-AP. E, E-4031. F, tertiapin. Rat aorta was precontracted with phenylephrine (3 \times 10^{-7} M), and nitroglycerin was applied cumulatively to the bath solution. Vascular relaxations are expressed as percentage inhibition of the tension development just before application of nitroglycerin. Data are mean values \pm S.E.M. of three to four experiments.} \]

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TABLE 4
Effects of several K⁺ channel blockers on pIC5₀ and Eₘₐₓ values for NTG and cromakalim in rat aorta

<table>
<thead>
<tr>
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<th>Control</th>
<th>Treatment</th>
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<tbody>
<tr>
<td></td>
<td>pIC5₀</td>
<td>Eₘₐₓ (%)</td>
</tr>
<tr>
<td>Aorta</td>
<td>NTG</td>
<td>7.43 ± 0.17 (5)</td>
</tr>
<tr>
<td></td>
<td>Glibenclamide (10⁻⁶ M)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NTG</td>
<td>7.33 ± 0.18 (3)</td>
</tr>
<tr>
<td></td>
<td>Glibenclamide (10⁻⁶ M)</td>
<td></td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>NTG</td>
<td>6.60 ± 0.01 (4)</td>
</tr>
<tr>
<td></td>
<td>Glibenclamide (10⁻⁶ M)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NTG</td>
<td>6.20 ± 0.03 (4)</td>
</tr>
<tr>
<td></td>
<td>Glibenclamide (10⁻⁶ M)</td>
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</table>

Eₘₐₓ, the maximal relaxant responses (NTG at 10⁻⁶ M; cromakalim at 3 × 10⁻⁶ M) examined in the muscle contracted with phenylephrine (aorta, 3 × 10⁻⁷ M; mesenteric artery, 3 × 10⁻⁸ M);
⁺⁺ P < 0.01, significant differences between two groups.

Reduced the relaxations induced by all of the test compounds (Fig. 6, A–C). IC₅₀ values of 4-AP for aortic relaxations were around 3.5 × 10⁻³ M (2.9 × 10⁻³ M for NTG, 3.6 × 10⁻³ M for NOR 3, and 3.9 × 10⁻³ M for ANP). Likewise, intermediate concentrations of TEA (5 × 10⁻³ and 10⁻² M) reduced to a large extent the relaxations induced by all of the test compounds (Fig. 6, D–F). IC₅₀ values were around 3 × 10⁻³ M (4.1 × 10⁻³ M for NTG, 10⁻⁷ M; 2.2 × 10⁻³ M for NOR 3, 3 × 10⁻⁸ M; and 1.9 × 10⁻⁸ M for ANP, 10⁻⁸ M).

Because Kv4.3 and rElk1 (Kcnh4) channels are insensitive to external TEA (1 × 10⁻¹ M) (Engeland et al., 1998; Song et al., 2001) and NO relaxation in aorta is sensitive to intermediate concentrations of TEA (IC₅₀ ~ 3 × 10⁻³ M) (Fig. 6, D–F), it is unlikely that these classes of K⁺ channels are effectors of the NO cascade. Likewise, because rElag2 (Kcnh5) channels are insensitive to 10⁻² M 4-AP (Ludwig et al., 2000) and NO relaxation in aorta is prevented by 10⁻² M 4-AP (Fig. 6, A–C), this channel family can also be ruled out.

The reported IC₅₀ values for 4-AP (0.5–20 × 10⁻³ M) (Kirsch and Drewe, 1993; Mathie et al., 1998) and TEA (5 × 10⁻³ M) (Tagliaferla et al., 1991) against Kᵥ2.2 are in reasonable agreement with the ones found to inhibit NO-induced aortic relaxation in the present study (IC₅₀-4AP = 3.5 × 10⁻³ M and IC₅₀-TEA = 3 × 10⁻³ M). However, Kᵥ2.2 is also

Fig. 6. Inhibitory effects of higher concentrations of 4-AP and TEA on the relaxant responses of rat aorta to NO donors (nitroglycerin and NOR 3) and ANP. A, B, and C, when the relaxant response to a single administration of nitroglycerin (10⁻⁷ M) (A), NOR 3 (10⁻⁶ M) (B), and ANP (10⁻⁷ M) (C) reached a steady-state level, 4-AP (10⁻²–10⁻³ M) was applied cumulatively to the bath solution. Data are mean values ± S.E.M. of six to eight experiments. ++, P < 0.01, significant differences from the response before applying 4-AP. D, E, and F, concentration-response relationships for the relaxation of rat aorta in response to nitroglycerin (D), NOR 3 (E), and ANP (F) in the absence and presence of TEA (10⁻³–10⁻² M). Rat aorta was precontracted with phenylephrine (3 × 10⁻² M), and relaxants (nitroglycerin, NOR 3, and ANP) were applied cumulatively to the bath solution. Vascular relaxations are expressed as percentage inhibition of the tension development just before application of vasorelaxants. Data are mean values ± S.E.M. of four to 16 experiments. *, P < 0.05; ++, P < 0.01, significant differences from control responses.
sensitive to both 4-AP and TEA within similar ranges (IC_{50-4AP} = 1.5 × 10^{-3} M and IC_{50-TEA} = 3–8 × 10^{-3} M) (Yamane et al., 1995; Mathie et al., 1998). Thus, the pharmacomechanical profile, in particular the low-4-AP affinity, indicates that channels composed of K_{2.1} or K_{2.2} or both may be responsible for NO/ANP-induced aortic vessel relaxations. This prompted us to examine whether NO donors could stimulate K_{c} currents with low-4-AP affinity in aortic myocytes.

High Concentrations of 4-AP Inhibit K_{c} Currents following Stimulation by NTG in Aortic Myocytes. Whole-cell K_{c} currents from A7r5 aortic myocytes were recorded in the presence of MaxiK blockers (10^{-3} M TEA ± 10^{-2} M IbTX) in the bath solution and 10 mM EGTA in the patch pipette. Figure 7A shows typical K_{c} currents elicited from a holding potential of −70 mV with 200-ms test pulses from −80 to +70 mV in 10-mV increments. Resembling the mechanical results, currents were clearly stimulated by 4 × 10^{-7} M NTG and were insensitive to 10^{-3} M 4-AP but inhibited by 5 × 10^{-3} and 10^{-2} M 4-AP. Figure 7B shows the corresponding I-V curves, whereas Fig. 7C displays the mean values. At +50 mV, NTG stimulated K_{c} currents from 18.3 ± 3.7 to 29.5 ± 6.1 pA/pF (n = 8 of 10 cells, P < 0.01). Similar results were obtained with (n = 4) or without (n = 4) IbTX. 4-AP at 10^{-3} M had no appreciable effect on NTG-stimulated K_{c} currents (n = 5 cells, four in the continuous presence of NTG); in contrast, 5 × 10^{-3} M 4-AP inhibited K_{c} currents by ~40% from 29.3 ± 9.8 to 18.7 ± 7.3 pA/pF (n = 4 cells in the continuous presence of NTG, P < 0.01). Increasing concentrations of 4-AP (10^{-2} M) further decreased K_{c} currents (n = 2). Together the pharmacomechanical and electrophysiological studies support the view that one mechanism involved in NTG-induced relaxation in rat aorta is via activation of low-affinity 4-AP-sensitive K_{c} channels of the K_{2.2} class.

K_{2.1} Protein and Its mRNA Are Expressed in Rat Aorta. K_{2.1} mRNA has been detected in rat aorta using Northern blot hybridization (Robers and Tamkun, 1991). We now examined whether K_{2.1} protein is expressed as well. Western blot analysis of rat aorta and brain (positive control) membranes (10 μg of protein/lane) using a polyclonal anti-K_{2.1} α-subunit antibody showed a prominent band of the expected K_{2.1} molecular mass of ~100 kDa (n = 4 and 7 rats); this signal could be readily blocked by preincubation with the antigenic peptide showing the specificity of the antibody (Fig. 8A). Immunocytochemistry of aortic sections (Fig. 8B) using the same antibody showed a clear staining of smooth muscle cells (n = 6), which was practically absent when the antibody was preincubated with the antigenic peptide (control). Because antibodies against K_{2.2} were unavailable, we quantified the mRNA levels for K_{2.2} versus K_{2.1} using real-time PCR. mRNA levels were much higher for K_{2.1} than for K_{2.2} gene (Fig. 8, C and D); note that K_{2.1} had a lower mean threshold cycle number than K_{2.2} (Fig. 8C). The expression measured at threshold demonstrated that K_{2.1} transcripts are 97 ± 5-fold more abundant than K_{2.2} in rat aorta (n = 4 rats, two mRNA preparations) (Fig. 8D). Qualitatively similar results were obtained with RT-PCR (n = 4) (Fig. 8E). Assuming that mRNA levels correspond to expressed protein, these PCR results together with the pharmacomechanical and electrical analysis would support K_{2.1} subunit-containing channels as key downstream effectors that trigger rat aortic vessel relaxation after activation of cGMP cascades.

Discussion

In the present study, we identify for the first time the nature of the K_{c} channel involved in the endothelium-independent relaxations induced by NO-releasing compounds and ANP of a conduit artery. Our results show that, in aorta but not in mesenteric artery (small vessel control), the contribution of IbTX-sensitive MaxiK channels to the relaxations induced by NO-releasing compounds and ANP is apparently negligible or absent. Instead, pharmacomechanical, electrical, molecular, and biochemical evidence supports a major role of low-affinity 4-AP-sensitive K_{2.2} channel type typified as containing K_{2.1} subunit in the aortic smooth muscle relaxations induced by NO donors and ANP.

The lack or minimal contribution of IbTX-sensitive MaxiK channel to NO/ANP-induced relaxation of rat aorta is in agreement with previous findings in conduit vessels of other species (rabbit and guinea pig) like aorta and carotid artery (Bialecki and Stinson-Fisher, 1995; Ishibashi et al., 1995; Plane et al., 1998). In contrast, in smaller vessels where functional abnormalities can be closely related to circulatory dysfunctions, the role of MaxiK channels seems to be significant in NO-induced relaxation; for instance, in rat (this

Fig. 7. K_{c} currents are stimulated by NTG and inhibited by high concentrations of 4-AP in A7r5 rat aortic myocytes. A, original current traces showing the stimulation of K_{c} currents by NTG, lack of effect of 1 × 10^{-7} M 4-AP, and corresponding inhibition by 4-AP at ≥5 × 10^{-3} M. Test potentials were from −80 mV to +70 mV with a holding potential of −70 mV. B, corresponding I_{baseline}-V relationship of traces in A. I_{baseline} (pA/pF) was obtained by dividing each current value by the cell capacitance. C, mean values were obtained at +50 mV in each condition. Data are mean values ± S.E.M. of two to eight experiments. **, P < 0.01, significant differences between two groups.
work) and rabbit mesenteric arteries (Khan et al., 1993), canine coronary (Khan et al., 1998) and rat pulmonary (Zhao et al., 1997) arteries, the blockade of MaxiK channels, decrease NO-induced relaxation. Thus, the contribution of MaxiK channel activation to the relaxations in response to cGMP-elevating agents, including nitrates and ANP, is seemingly negligible or very small if it exists in the large conduit arteries but significant in smaller arteries. This inference needs to be confirmed in other animal species.

Our mechanical results indicate that activation of IbTX-sensitive MaxiK channel does not play a pivotal role in aortic relaxations by NO donors and ANP (Figs. 1 and 2). Nevertheless, these relaxations seem to be mainly mediated via the opening of K+ channels, since they were dramatically diminished in the preparations contracted with high-KCl (Fig. 4). Several explanations are possible to account for the apparent lack of IbTX sensitivity in the aortic relaxations.

1) Lowered Activity of Soluble Guanylyl Cyclase in the Aortic Vessel. This possibility can be ruled out because a) ODQ and methylene blue greatly diminished the relaxations induced by NO donors (NTG and NOR 3) in both arteries (Table 2) and b) selective inhibition by IbTX of the relaxation in mesenteric artery versus aorta was also observed in the relaxation due to particulate guanylyl cyclase activation by ANP (Fig. 2).

2) Lower Expression Level of MaxiK Channel Protein in Aortic Myocytes. MaxiK (α-subunit) protein expression levels were practically the same in both aortic and mesenteric myocytes, and thus, this possibility cannot account for the observed phenomenon (Fig. 3).

3) Slowdown of IbTX Association with MaxiK α-Subunit. β-Type subunit renders MaxiK α-subunit resistant to nanomolar IbTX and charybdotoxin, possibly via its extracellular loop. Therefore, high concentrations and/or long-exposure times are necessary to determine the role of the toxin-resistant MaxiK channel formed by α + β- subunits (Meera et al., 2000). Aortic relaxations induced by NO donors and ANP were not diminished by IbTX, even when the muscle was treated with 10−6 M IbTX for 90 min (Table 3). Therefore, the possible expression of β-type of subunit in rat aorta is not likely to account for the apparent lack of MaxiK role in the relaxations by NO donors and ANP in this vessel.

4) Possible Contribution of IbTX-Insensitive MaxiK Channel. Electrophysiological studies showed that MaxiK channel currents from rat aortic myocytes have a significant IbTX-sensitive component. If these IbTX-sensitive MaxiK channels could have been activated by NO donors and ANP and contributed to the resultant blood vessel relaxation, at least some portion of the relaxant response should have been diminished by IbTX (10−2−10−4 M). Because this was not the case, the role of IbTX-sensitive MaxiK in the relaxations to NO donors and ANP seems negligible or very minor if it exists. This view is also supported by the results with TEA. In particular, TEA blocks MaxiK from the external side with a C50 value of around 2 to 3 × 10−3 M (Wallner et al., 1995), and TEA at 1 to 2 × 10−3 M is expected to exhibit almost full blockade of MaxiK channel (−80%) (Braun et al., 2000). However, aortic relaxations induced by NTG, NOR 3, and ANP were not diminished by IbTX, even when the muscle was treated with 1 to 2 × 10−3 M TEA (Table 3). Therefore, NO donor- and ANP-induced aortic relaxations cannot be attributable to MaxiK activation. Although aortic muscle seems to contain all of the molecular components of the cGMP-MaxiK-signaling cascade, their subcellular microlocalization may not allow for a functional coupling to support relaxation as observed in the mesenteric artery.

The non-MaxiK type of K+ channel responsible for NO- and ANP-induced relaxations of rat aorta seems to be a...
low-affinity 4-AP-sensitive K\textsubscript{2} type. This is substantiated by an extensive pharmacological screening (Figs. 5 and 6) and by direct evidence in aortic myocytes of NTG-induced potentiation of a 4-AP-sensitive K\textsubscript{2} current (Fig. 7) whose low affinity resembled the concentration required to inhibit NTG-induced relaxations (Fig. 6). Because the degree of inhibition of NTG-induced relaxation by $5 \times 10^{-3}$ M 4-AP is larger than for NTG-potentiated K\textsubscript{2} currents, additional mechanisms may contribute to 4-AP effects (e.g., cell-cell coupling in tissue, regulation of other signaling pathways). The identification of K\textsubscript{2.1} protein in aortic myocytes by immunochemical methods and its predominant transcript expression over K\textsubscript{2.2} (Fig. 8) (assuming RNA to protein direct correlation) supports a key role of K\textsubscript{2.1} subunit as one of the molecular constituents of the low-affinity 4-AP-sensitive K\textsuperscript{+} channel triggering NO-mediated relaxation of rat aortic smooth muscle. Whether K\textsubscript{2.1} subunit protein forms heteromultimers with K\textsubscript{2.2} or with other subunits has yet to be determined. Because the relaxations of rat aorta in response to NTG and NOR 3 were almost abolished by the inhibitor of soluble guanylyl cyclase ODQ (Table 2), cGMP-dependent rather than cGMP-independent pathway (e.g., NO-direct action) (Yuan et al., 1996; Zhao et al., 1997) is likely to be the primary mechanism for the relaxant response in rat aorta.

Judging from the inhibition caused by IbTX in mesenteric arteries, the relative contribution of MaxiK channel activation to the NTG- and ANP-induced vasorelaxations is $\sim$20\% of the maximal response (Fig. 2). Mesenteric artery relaxations in response to NTG and ANP were also significantly suppressed by approximately 20\% when arterial preparations were precontracted with high-KCl (80 $\times$ 10$^{-3}$ M) instead of phenylephrine (Fig. 4). Based on the latter finding with high-KCl, plasma membrane K\textsuperscript{+} channels would mediate approximately 20\% of the NTG-induced relaxation of mesenteric artery. Thus, it is likely that the MaxiK channel is the main plasmalemmal K\textsuperscript{+} channel involved in cGMP-mediated relaxations of the rat mesenteric artery as opposed to other K\textsuperscript{+} channels like K\textsubscript{v}, inward rectifiers, and K\textsubscript{ATP} channels. Consistent with this view, NTG-induced relaxation of this artery preparation was not affected by glibenclamide (Table 4). Thus, the involvement of the K\textsubscript{ATP} channel activation can be ruled out as a major mechanism underlying cGMP-mediated relaxation of the rat mesenteric artery. Interestingly, K\textsubscript{2.1} protein has also been detected in mesenteric arteries (Xu et al., 1999), and its signals are similar as in aortic rings (data not shown). This underscores the possibility that the differential contribution of MaxiK versus K\textsubscript{v} channels to NO/ANP-induced relaxations in distinct vessels depends on their local distribution within the microdomain defining NO-relaxing signaling. In rat pulmonary arteries, both MaxiK and K\textsubscript{v} (of unknown nature) channels can mediate NO-induced relaxation (Yuan et al., 1996; Zhao et al., 1997), further highlighting the heterogeneity in vascular smooth muscle molecular architecture and its functional consequences.

Although several possibilities have been postulated as mechanisms responsible for the vascular relaxation induced by NO-releasing vasodilators and ANP, it is now widely recognized that the intracellular cGMP increase following the activation of soluble guanylyl cyclase by NO or of particulate guanylyl cyclase by ANP plays a central role in their vasorelaxant effects. This view would predict cGMP pathways as key components of the functional coupling between NTG (via NO) or ANP and MaxiK channel activation, leading to vascular relaxation of mesenteric artery. Results that support this view are as follows. 1) NTG- and NOR 3-induced relaxations were potently antagonized by ODQ or methylene blue, which inhibit soluble guanylyl cyclase, and 2) ANP-induced mesenteric relaxation was inhibited by IbTX (Fig. 2F), mimicked by a membrane-permeable cGMP analog, 8-Br-cGMP (Tanaka et al., 1998), and insensitive to methylene blue (Table 2). These findings indicate that NO- and ANP-induced relaxations of mesenteric arteries occur via an increase in cGMP contents, possibly activating PKG and enhancing MaxiK channel activity (Alioua et al., 1998).

In summary, we showed that the contribution of IbTX-sensitive MaxiK channel is irrelevant to NO donor- and ANP-induced relaxations in thoracic aorta but is significant in mesenteric artery isolated from the rat. NO aortic relaxations are largely mediated by a low-affinity 4-AP-sensitive K\textsuperscript{+} channel fingerprinted by K\textsubscript{2.1} subunit. Function, expression, and/or microlocalization of voltage-gated K\textsuperscript{+} channels, including MaxiK channels, with signaling proteins might largely differ in large conduit arteries compared with small arteries.

References


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