Endothelium-derived NO inhibits the relaxation of the porcine coronary artery to natriuretic peptides by desensitizing BKCa channels of vascular smooth muscle

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Abbreviations:

ANP, atrial natriuretic peptide; BK_{Ca}, big conductance calcium-activated potassium channel; CNP, C-type natriuretic peptide; cyclic AMP, cyclic adenosine monophosphate; cyclic GMP, cyclic guanosine-3’, 5’-monophosphate; EDCF, endothelium-derived contracting factors; EDHF, endothelium-derived hyperpolarizing factors.
factors; EDRF, endothelium-derived relaxing factors; eNOS, endothelial nitric oxide synthase; IBMX, 3-isobutyl-1-methyl-xanthine; IK_{Ca}, intermediate conductance calcium-activated potassium channel; iNOS, inducible nitric oxide synthase; L-NAME, N\textsuperscript{ω}-nitro-L-arginine methyl ester; NO, nitric oxide; NPR, natriuretic peptide receptor; NS1619, 1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one; NS2028, 4H-8-bromo-1,2,4-oxadiazolo[3,4-d]benz[b][1,4]oxazin-1-one; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PGE\textsubscript{2α}, prostaglandin F\textsubscript{2α}; SK_{Ca}, small conductance calcium-activated potassium channel; TRAM-34, 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole; UCL-1684, 6,12,19,20,25,26-hexahydro-5,27:13,18:21,24-Trietheno-11,7-metheno-7H-dibenzo[b,m][1,5,12,16]tetraazacyclotricosine-5,13-diium difluoroacetate hydrate;

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Abstract:

The present experiments investigated whether or not endothelium-derived mediators modulate the effect of natriuretic peptides in porcine coronary arteries. Rings with and without endothelium were suspended in organ chambers for isometric tension recording. Concentration-relaxation curves to CNP and ANP were obtained during contractions to endothelin-1. Removal of the endothelium potentiated relaxations to both CNP and ANP. L-NAME potentiated relaxations to natriuretic peptides only in arteries with endothelium. SNP inhibited the response to the natriuretic peptides only in the absence of the endothelium. In rings with endothelium, ODQ and NS2028 potentiated CNP-mediated relaxations. Iberiotoxin reduced the response only in rings without endothelium. Glybenclamide inhibited the relaxations both in the presence and absence of endothelium. CNP-induced relaxations were reduced by 8-bromo-cyclicGMP to the same extent in rings with and without endothelium. There was no significant difference between the increased cyclic GMP content caused by CNP in porcine coronary arteries with or without endothelium. In patch clamp studies in porcine coronary arterial smooth muscle cells, the natriuretic peptides-mediated enhancement of the iberiotoxin-sensitive BK$_{Ca}$ amplitude was reversed by sodium nitroprusside and 8-bromo-cyclicGMP. These findings demonstrate that, in the porcine coronary artery, the opening of BK$_{Ca}$ and ATP-dependent potassium channels...
of the vascular smooth muscle contributes to CNP-mediated relaxations.

Endothelium-derived and exogenous NO inhibit the direct relaxing effect of natriuretic peptides by desensitizing the response of the BKCa channels of the vascular smooth muscle to the generation of cyclic GMP.
Introduction:

Nitric oxide (NO) and natriuretic peptides contribute to the regulation of vascular tone (Moncada et al., 1991, Woodard and Rosado, 2008). The mammalian natriuretic peptide family includes atrial (ANP), brain (BNP), and C-type (CNP) natriuretic peptide as well as urodilatin (Levin et al., 1998). ANP and BNP are produced in the atria and ventricles of the heart (Woodard and Rosado, 2008). CNP is produced by endothelial cells (Stingo et al., 1992).

Three subtypes of natriuretic peptide receptor (NPR) have been cloned and characterized: NPR-A, NPR-B, and NPR-C. NPR-A binds ANP and BNP with equal affinity, however, CNP seems to be the endogenous ligand for NPR-B (Woodard and Rosado, 2008). Both NPR-A and NPR-B are coupled to particulate guanylyl cyclase which stimulates the production of the intracellular second messenger cyclic guanosine-3’, 5’-monophosphate (cyclic GMP) (Koller et al., 1991). By contrast to NPR-A/B, NPR-C functions as a clearance receptor for ANP, BNP and CNP, although it may also contribute to the CNP-induced relaxation (Sandow et al., 2007).

NO is one of the main endothelium-derived relaxing factors (EDRF) (Palmer et al., 1987; Moncada et al., 1991) and causes relaxation in arteries in part by stimulating soluble guanylyl cyclase and thus inducing the synthesis of cyclic GMP (Ignarro et al., 1987). Prostaglandins and NO contribute to the relaxation of renal afferent arterioles.
to CNP (Amin et al., 1995). Relaxations to CNP are larger in rings of canine coronary arteries without than in those with endothelium (Wright et al., 1996). In the aorta of eNOS knock-out mouse, CNP causes larger relaxations than in that of wild type controls (Madhani et al., 2003). These earlier findings suggest an interaction between CNP and NO. The common link between these two vasodilators is that both stimulate the production of cyclic GMP (Ignarro et al., 1987; Moncada et al., 1991, Koller et al., 1991; Woodard and Rosado, 2008).

In addition to activating the production of cyclic GMP, CNP can cause hyperpolarization of vascular smooth muscle cells in the porcine coronary artery, an effect which may involve the opening of potassium channels (Barber et al., 1998). Actually, the natriuretic peptide has been proposed as an endothelium-derived hyperpolarizing factor (EDHF) (Chauhan et al., 2004). It is unknown whether or not EDHF-mediated responses (Féléto and Vanhoutte, 2006; Ko et al., 2008) affect the relaxing effect of natriuretic peptides. Likewise, it is unknown whether or not in intact blood vessels the release of endothelium-derived contracting factors (EDCF) (Lüscher and Vanhoutte, 1986) attenuates the relaxations to the natriuretic peptides.

The present experiments were designed to determine how NO and other endothelium-derived mediators affect relaxations to natriuretic peptides in the porcine coronary artery, and to determine the involvement of potassium channels in the dilator response.
of that artery to these peptides.
Material and Methods

Tissue Preparation. The experiments were performed on isolated coronary arteries which were dissected free from porcine hearts obtained at the local slaughterhouse. Briefly, the arteries were placed in ice-cold Krebs-Ringer bicarbonate buffer of the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, and 11.1 mM glucose (control solution). The adherent fat and connective tissue were removed, and the arteries were cut into rings (approximately 3-4 mm in length). In certain preparations, the endothelium was removed by the infusion of Triton-X 100 (0.5%) at a rate of 1 ml/min for 30 seconds before the arteries were cut into rings. Removal of endothelium was confirmed by the loss of relaxation in response to bradykinin (10⁻⁵ M).

Isometric Tension. The rings were suspended in conventional organ chambers (5 ml), filled with control solution aerated with 95% O₂-5% CO₂ and maintained at 37°C. The rings were subjected to 5 g tension, which in preliminary experiments (data not shown) was the optimal tension for rings of porcine right coronaries obtained from the same source. Isometric tension was measured by means of force transducers (AD Instruments Pty Ltd., Sydney, Australia) coupled to an amplifier and a personal computer for data collection (PowerLab, ADInstruments Pty Ltd.). The rings were
exposed to 60 mM potassium chloride twice before the actual experiment.

Certain rings were incubated for 40 minutes with the following agents alone or in combination: Nω-nitro-L-arginine methyl ester (NO synthase inhibitor; 10⁻⁴ M) (Tang et al., 2005); indomethacin (non-selective cyclooxygenase inhibitor; 10⁻⁵ M) (Tang et al., 2008); S18886 (TP receptor blocker; 10⁻⁷ M) (Tang et al., 2008); iberiotoxin (selective inhibitor of big conductance calcium-activated potassium channels (BKCa channels; 10⁻⁷ M) (Leuranguer et al., 2008); charybdotoxin (nonselective IKCa and BKCa inhibitor; 5x10⁻⁷ M) (Leuranguer et al., 2008); TRAM-34 (IKCa inhibitor; 5x10⁻⁷ M) (Leuranguer et al., 2008); UCL-1684 (non-peptide blocker of SKCa; 5x10⁻⁷ M) (Leuranguer et al., 2008); apamin (selective SKCa inhibitor; 10⁻⁶ M) (Keung et al., 2005); sodium nitroprusside (NO-donor; 10⁻⁹ M); oxadiazoloquinoxaline (ODQ, selective inhibitor of soluble guanylyl cyclase; 10⁻⁹ M) (Tang et al., 2005); 4H-8-bromo-1,2,4-oxadiazolo[3,4-d]benz[b][1,4]oxazin-1-one (NS2028, specific inhibitor of soluble guanylyl cyclase; 10⁻⁶ M) (Yang et al., 2004); 8-bromo-cyclicGMP (cell permeable analog of cyclic GMP; 10⁻⁵ M) (Leuranguer et al., 2008) and glybenclamide (blocker of ATP-sensitive K⁺ channels; 10⁻⁶ M) (Hedayati et al., 2009). After the incubation period, sustained contractions were obtained with the ED₅₀ concentration of either prostaglandin F₂α (10⁻⁶ M) or endothelin-1 (10⁻⁹ M). There
were no statistically significant differences in pre-contraction levels between the different experimental groups. The rings were then exposed to progressively increasing concentrations of CNP (10^{-10} M to 10^{-6} M), ANP (10^{-10} M to 10^{-7} M), isoproterenol (10^{-10} M to 10^{-6} M) or NS1619 (Selective BK\text{Ca} channels activator, 10^{-10} M to 10^{-6} M) (Edwards et al., 1994). Decreases in tension are expressed as percentage of the maximal reference relaxation to isoproterenol (10^{-5} M) or sodium nitroprusside (10^{-4} M) (O'Rourke et al., 2003) obtained at the end of the experiment.

**Cyclic GMP.** Rings with or without endothelium, studied in the organ chambers, were incubated for 45 minutes with the nonselective phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX; 10^{-4} M) before exposing them to endothelin-1 (10^{-8} M), followed by a single, maximal concentration of CNP (10^{-6} M). The rings were flash-frozen in liquid nitrogen either immediately before (time 0) or five minutes after the addition of CNP. The frozen rings were homogenized in 1 ml 6% trichloroacetic acid and the homogenate centrifuged at 10,000 rpm for 20 minutes. The supernatant was extracted four times with four volumes of water-saturated ether and lyophilized. The levels of cyclic GMP were determined by radioimmunoassay (guanosine 3', 5'-cyclic mononucleotide EIA kit (Cayman Chemical, Ann Arbor,
Michigan, U.S.A]) and normalized to the protein content determined using a protein assay reagent.

Patch-clamp Experiments

Pig left anterior descending coronary artery smooth muscle cells were dissociated enzymatically, as reported (Au et al., 2003) for single-cell, patch-clamp electrophysiology experiments. Whole-cell, membrane-rupture recording of the macroscopic iberiotoxin-sensitive Ca\(^{2+}\)-activated K\(^+\) (BK\(_{Ca}\)) channels gating of single coronary artery smooth muscle cells were recorded (Au et al., 2003). The external physiological solutions for recording the BK\(_{Ca}\) channel amplitude contained (in mM):

NaCl 130, KCl 5, MgCl\(_2\) 1.2, CaCl\(_2\) 1.5, glucose 10 and HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid) 10 (pH 7.4 with NaOH). The internal pipette solution had the following composition (in mM): NaCl 10, KCl 110, MgCl\(_2\) 5, CaCl\(_2\) 2, ethylene glycol-bis [\(\beta\)-aminoethylether] N,N’,N’-tetraacetic acid 10, K\(_2\)ATP 5 and HEPES 10 (pH 7.2 with KOH). For a free Ca\(^{2+}\) concentration of approximately 444 nM (estimated using the computer programme: Maxchelator, Stanford University, Stanford, CA USA), the pipette solution contained 7 mM instead
of 2 mM CaCl$_2$, and 4 mM instead of 5 mM MgCl$_2$, as described (Seto et al., 2007).

The range of free intracellular Ca$^{2+}$ concentration chosen in this study was similar to the previously measured global levels observed in agonist-stimulated single porcine coronary arterial myocytes (Ndiaye et al., 2003). To allow for an equilibration of the pipette solution with the cell interior, all recordings were started five minutes after the establishment of the whole-cell configuration. Most experiments were performed within 15 minutes of gaining access, during which time the macroscopic BK$_{Ca}$ current amplitude remained stable.

To measure the effects of drugs on BK$_{Ca}$ channels, the BK$_{Ca}$ current was elicited with a test potential to +80 mV (500-ms duration) from a holding potential of −60 mV and stimulated at 0.1 Hz. Effects of drugs (e.g. ANP, CNP) on BK$_{Ca}$ amplitude were examined/compared after a stable BK$_{Ca}$ amplitude was achieved (i.e. control). Cell membrane capacitance was estimated, as described (Au et al., 2003), and averaged 19.2±2.3 pF (n=30). The amplitude of the BK$_{Ca}$ current was recorded with an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA, USA), before (control), during treatment and after washout. Only one concentration of a particular drug was tested in each cell. The external solution was delivered through gravity, and controlled by solenoid valves coupled to a four-channel valve driver (General Valve, Brookshire, TX, USA). A solution change (approximately 5 ml,
corresponding to ten times the volume of the recording chamber) could be completed in 15 to 20 seconds. Drugs (dissolved in the external recording solution) were applied to the external cell surface.

**Drugs.** C-type natriuretic peptide, atrial natriuretic peptide, 4-[1-hydroxy-2-(isopropylamino)ethyl]benzene-1,2-diol (isoproterenol), endothelin-1, (Z)-7-[(1R,2R,3R,5S)-3,5-dihydroxy-2-[(E,3S)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoic acid (prostaglandin F2α), No-Nitro-L-arginine methyl ester (L-NAME), 2-[(4-chlorophenyl)carbonyl]-5-methoxy-2-methyl-1H-indol-3-yl]acetic acid (indomethacin), sodium pentacyanonsitrosylferrate(III) (sodium nitroprusside), 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 4H-8-Bromo-1,2,4-oxadiazolo[3,4-d]benz[b][1,4]oxazin-1-one (NS2028), charybdotoxin, 1-[(2-Chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34), ditrifluoroacetate hydrate (UCL-1684), apamin, iberiotoxin, N-p-[(2-(5-Chloro-2-methoxybenzamido)ethyl]benzenesulfonyl-N’-cyclohexylurea, 5-Chloro-N-[4-(cyclohexylureidosulfonyl)phenethyl]-2-methoxybenzamide (glybenclamide), 3-Isobutyl-1-methylxanthine (IBMX), 7-Methoxycoumarin-4-acetyl [Ala7-(2,4-Dinitrophenyl)Lys9]-Bradykinin trifluoroacetate salt (bradykinin) and 1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one
(NS1619) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 8-Bromoguanosine \(3',5'\)-cyclic monophosphate sodium salt (8-Bromo-cyclicGMP) was obtained from Biolog (Bremen, Germany). Sodium 3-((6R)-6-[[4-chlorophenyl)sulfonyl]amido]-2-methyl-5,6,7,8-tetrahydronaphthalen-1-yl)propanoate (S18886, Terutroban) was a kind gift from the Institut de Recherches Servier (Suresnes, France). Triton-X100 was purchased from Pharmacia Biotech (Uppsala, Sweden). Stock solutions of indomethacin, ODQ and NS2028 were prepared in sodium bicarbonate (1mM). NS1619 was dissolved in pure ethanol. All other compounds were prepared in deionized water.

**Data Analysis.** The results are shown as means ± S.E.M. with n being the number of individual observations on rings from different pigs. Data were analyzed using the statistical program Prism version 5 (GraphPad Software Inc., San Diego, CA). For the sake of clarity, most of the results are represented as area under the concentration-relaxation curve, using a computer package [Prism version 5 (GraphPad Software)]. Student's t-test for paired observations was used for comparison of two groups. One-way analysis of variance with repeated measures followed by the Bonferroni *post hoc* test was carried out for multiple comparisons. A difference was accepted as statistically significant when the P value was less than 0.05. In the whole-cell, patch-
clamp electrophysiology experiments, $n$ refers to the number of single vascular smooth muscle cells used. The results are expressed as means ± S.E.M.
Results

1. Relaxations:

1.1. Endothelium-removal

CNP evoked concentration-dependent relaxations in rings, with or without endothelium, during contractions to prostaglandin F$_{2\alpha}$ (10$^{-6}$ M) (Supplemental figure. S-1) and endothelin-1 (10$^{-9}$ M; Fig.1). The relaxations were significantly larger in rings without than in those with endothelium (Fig. 2, upper left). ANP had a comparable effect to that of CNP in rings with and without endothelium (Fig. 2, upper right).

1.2. Endothelium-derived vasoactive factors

In rings with endothelium, contracted with either prostaglandin F$_{2\alpha}$ (Supplemental figure. S-2) or endothelin-1, (Figs. 2, lower left and Figs. 3) L-NAME (10$^{-4}$ M) significantly potentiated the relaxations to CNP. The degree of relaxation observed after incubation with L-NAME was comparable to that achieved by the removal of the endothelium. L-NAME potentiated the relaxations of rings with endothelium to ANP to a similar extent as those to CNP (Fig. 2, lower right). Indomethacin (10$^{-5}$ M) and S18886 (10$^{-7}$ M) did not significantly affect the relaxations to CNP in arteries with and without endothelium (Supplemental figure. S-3 and S-4).

1.3. Interaction with NO
In rings with endothelium, ODQ (10^-6 M) and NS2028 (10^-6 M) significantly potentiated the relaxations to CNP (Fig. 3). Previous incubation with sodium nitroprusside (10^-9 M) did not significantly affect the response while that with 8-bromo-cyclicGMP (10^-5 M) slightly but significantly inhibited it (Fig. 3).

In ring without endothelium, L-NAME did not significantly alter the relaxations to CNP (Fig.4) and ANP (Supplemental figure S-5) during contractions to endothelin-1 (Fig. 4). Incubation with sodium nitroprusside (10^-9 M) significantly inhibited the relaxations to CNP (Fig. 4) and ANP (Fig. 5, left). By contrast, sodium nitroprusside did not alter significantly the relaxations to isoproterenol (Fig. 5, right). Incubation with ODQ or NS 2028 did not significantly affect relaxations to CNP in rings without endothelium, while 8-bromo-cyclicGMP significantly reduced them (Fig. 4).

The relaxations to increasing concentrations of sodium nitroprusside and 8-bromo-cyclicGMP were significantly smaller in rings with than in those without endothelium (Fig. 6).

1.4. Potassium channel inhibitors

In rings with endothelium, TRAM-34, UCL-1684, apamin, charybdotoxin, and iberiotoxin did not significantly affect the relaxations to CNP, while glybenclamide significantly reduced them (Fig. 7).

In rings without endothelium, apamin, TRAM-34 or UCL-1684 did not significantly
affect the CNP-induced relaxations (Fig. 8). By contrast, charybdotoxin (5×10^{-7} M), iberiotoxin (10^{-7} M), and glybenclamide (10^{-6} M) significantly reduced the response to the natriuretic peptide (Fig. 8). The inhibitory effects of iberiotoxin and sodium nitroprusside were not additive (Fig. 8). Combined incubation with sodium nitroprusside, iberiotoxin and glybenclamide nearly abolished the response (Fig. 8). NS1619-mediated relaxation was potentiated in the absence of endothelium. (Fig. 9)

2. Cyclic GMP levels:

In the presence of 3-isobutyl-1-methyl-xanthine (IBMX; 10^{-4} M), the level of cyclic GMP was significantly larger in rings with than in those without endothelium (with endothelium, 1745.25±32.07; without endothelium, 232.25±20.87 pmol/mL/g wet weight). CNP (10^{-6} M) significantly increased the level of cyclic GMP in both rings with and without endothelium (Fig. 10). The absolute increases in cyclic GMP level in response to CNP were not different in the presence and absence of endothelium. (Fig. 10). ODQ and NS2028 did not significantly affect the increase in cyclic GMP caused by CNP in the absence of endothelium. (Fig. 10)

3. BKCa channel gating

ANP (10^{-8} M and 10^{-7} M) and CNP (10^{-7} M and 10^{-6} M) elicited a concentration-dependent enhancement of iberiotoxin (10^{-7} M) -sensitive BK_Ca amplitude (Figure 11A and D). Incubation (for 5 to 10 min before the recording of BK_Ca amplitude) with
sodium nitroprusside ($10^{-9}$ M) and 8-bromo-cyclicGMP ($10^{-5}$ M) augmented the basal noise level of the current recordings. In the presence of sodium nitroprusside and 8-bromo-cyclicGMP, the ANP ($10^{-8}$ M) and CNP ($10^{-7}$ M)-mediated effects on the $\text{BK}_{\text{Ca}}$ amplitude were eradicated (Figure 11B-C and E-F), whereas the enhancement effects of the higher concentrations of ANP ($10^{-7}$ M) and CNP ($10^{-6}$ M) on $\text{BK}_{\text{Ca}}$ channels were attenuated significantly.

**Discussion**

The present data confirm that CNP and ANP relax the porcine coronary artery with or without endothelium during contractions to endothelin-1 (Kedia et al., 2006) and prostaglandin $F_{2\alpha}$ (Barber et al., 1998), demonstrating the non-selectivity of this inhibitory effect. They also confirm that accumulation of cyclic GMP is involved in the response to CNP (Barber et al., 1998). The present observations further confirm that, under control conditions, the relaxations to the natriuretic peptides are attenuated by the presence of endothelial cells (Wright et al., 1996). In the present study, incubation with the non-selective cyclooxygenase inhibitor, indomethacin, did not affect the blunting by the presence of the endothelium of the relaxation to CNP, ruling out a contribution of vasodilator endothelium-derived prostaglandins, in particular prostacyclin (Moncada and Vane, 1979). The similar lack of effect of TRAM-34 plus
UCL-1684 permits to rule out a contribution to, or an interference with the effect of CNP by EDHF-mediated responses (Feletou and Vanhoutte, 2006). Likewise, the absence of effect of the cyclooxygenase inhibitor and of the TP receptor antagonist S18886 excludes a confounding role for endothelium-derived vasoconstrictor prostaglandins activating TP receptors on the vascular smooth muscle (Vanhoutte and Tang, 2008). Thus, it seems logical to conclude that the inhibitory effect of the presence of endothelial cells is due solely to endothelium-derived NO. This conclusion is supported by the experiments showing the potentiating effect of the inhibitor of NO synthase L-NAME on CNP- and ANP-mediated relaxations in arteries with endothelium. These present findings are in agreement with the results obtained in the aorta of eNOS knockout mice in which the CNP-mediated relaxation is greater than in the aorta of wild-type animals (Madhani et al., 2003).

The potentiating effect of L-NAME on the relaxation to CNP and ANP was not observed in the absence of endothelial cells, implying that indeed it is endothelium-derived NO, produced by e-NOS, that exerts an inhibitory effect on the response to the peptides. This conclusion is supported by the experiments with the NO donor sodium nitroprusside which inhibited the CNP-induced relaxations in the coronary artery, as it does in the aorta of eNOS knockout mice (Madhani et al., 2003).

Moreover, the potentiating effect of the two inhibitors of soluble guanylyl cyclase
tested, ODQ and NS2028, on the relaxations to CNP in the presence of endothelium is consistent with the effect of L-NAME. Also in line with the inhibitory effect of endothelium-derived NO on the response to the natriuretic peptides, the cell permeable analog of cyclic GMP inhibited the effect of CNP in rings without endothelial cells, an effect comparable to that obtained with the NO donor sodium nitroprusside. Taken in conjunction, the present findings thus suggest that endothelium-derived NO does not affect the relaxations to CNP by interfering with the binding to, or the activation of NPR-B receptors (Woodard and Rosado, 2008), a conclusion supported by the comparable increases in cyclic GMP evoked by CNP in rings with and without endothelium. The basal levels of cyclic GMP were higher in rings with than in those without endothelium, illustrating the basal release of NO and the subsequent activation of soluble guanylyl cyclase. This basal production of cyclic GMP in preparations with endothelium must result in a desensitization of the cellular response to the relatively modest amounts of cyclic nucleotide generated by the natriuretic peptides, presumably resulting from activation of the cyclic GMP-degrading phosphodiesterase 5 (Friebe and Koesling, 2003; Yang, et al., 2004). This interpretation is consistent with earlier findings that the inhibitory effect of the endothelium on the relaxations to CNP of the canine coronary artery is no longer observed in preparations treated with a selective inhibitor of particulate guanylyl
cyclase (Wright et al., 1996). It is also consistent with the present findings that the inhibitory effect of the presence of the endothelium is also observed for relaxations to both a NO-donor (sodium nitroprusside) and a cell permeable analog of cyclic GMP (8-bromo-cyclicGMP), but not for those to isoproterenol, which are mediated by an increase in cyclic adenosine monophosphate (Ehlerta et al., 1997). Earlier work has demonstrated that actually the presence of endothelial cells enhances relaxations to the beta-adrenergic agonist in coronary arteries (Rubanyi and Vanhoutte, 1985).

The opening of potassium channels contributes to the direct relaxations of vascular smooth muscle to natriuretic peptides (Barber et al., 1998). In the present study, TRAM-34 and UCL-1684 did not affect the relaxations to CNP in rings with or without endothelium ruling out a contribution of SKCa and IKCa (Feletou and Vanhoutte, 2006) channels to the response. By contrast, both charybdotoxin and iberiotoxin, known inhibitors of BKCa channels (Cox et al., 2005; Feletou and Vanhoutte, 2006) inhibited the relaxations to CNP in preparations without endothelium. In the vascular wall, BKCa channels are located mainly in vascular smooth muscle cells (Cox et al., 2005), and the present results supports their involvement in the endothelium-independent relaxing effect of CNP. The fact that the effects of sodium nitroprusside and iberiotoxin are not additive suggests that the
desensitization caused by endothelium-derived NO involves a reduced opening of

\( \text{BK}_{\text{Ca}} \) channels. This interpretation is supported by the observation that the inhibitory
effect of charybdotoxin and iberiotoxin on the response to CNP is not observed in
rings with endothelium. Furthermore, the potentiated NS1619-mediated relaxation in
the absence of the endothelium is strong evidence that NO causes desensitization of

\( \text{BK}_{\text{Ca}} \) channels. These conclusions are supported fully by the patch clamp experiments.

Indeed, ANP and CNP caused a concentration-dependent enhancement in \( \text{BK}_{\text{Ca}} \)
channel gating which could be eradicated by iberiotoxin. In line with the organ
chamber experiments, this enhancement was abolished or attenuated after incubation
with sodium nitroprusside or 8-bromo-cyclicGMP, which strongly suggests a
desensitization process. The latter desensitization presumably explains why the
prolonged incubation with either sodium nitroprusside or 8-bromo-cyclicGMP did not
result in an enhancement of the \( \text{BK}_{\text{Ca}} \) current as this effect is transient (Miyoshi and
Nakaya, 1994). Alternatively, the present experiments may have used a threshold
concentration of sodium nitroprusside and 8-bromo-cyclicGMP, insufficient to cause
overt activation of the channel.

In addition, ATP-dependent potassium channels contribute to the direct relaxing effect
of CNP as demonstrated by the inhibition of the response by glybenclamide. However,
the ATP-dependent potassium channels appear not to be involved in the inhibitory
effect of NO. This conclusion is based on the observations that glybenclamide inhibits
the relaxations to CNP in rings with endothelium and that in rings without
endothelium the combination of glybenclamide with iberiotoxin and sodium
nitroprusside caused a further inhibition of the response to CNP. The opening of such
ATP-dependent potassium channels presumably explains the remaining relaxations
caused by CNP in the presence of an inhibitor of particulate guanylyl cyclase (Wright
et al., 1996).

The present study does not permit to conclude whether CNP opens potassium
channels directly or indirectly as a result of changes in cyclic GMP. NO can activate
calcium-activated potassium channels dependently of cyclic GMP in arteries of
porcine (Miyoshi and Nakaya, 1994), rabbit (Robertson et al., 1993) and rat (Peral de
Bruno et al., 1999). Activation of protein kinases by cyclic GMP can result in
phosphorylation of potassium channels (Robertson et al., 1993). However, NO can
also activate calcium-activated potassium channels directly in rabbit (Bolotina et al.,
1994) and rat (Mistry and Gerland, 1998) arteries. To judge from the increased noise
level observed in the patch clamp experiments of the present study low concentrations
of sodium nitroprusside (and 8-bromo-cyclicGMP) may activate calcium-activated
potassium channels slightly in porcine coronary arterial smooth muscle, an
interpretation which is in line with previous findings (Miyoshi and Nakaya, 1994,).
Possibly, this transient effect leaded to desensitization of calcium-activated potassium channels, which in turn affects the relaxation to CNP.

The present findings confirm that CNP and ANP are endothelium-independent vasodilators in the porcine coronary artery. Accumulation of cyclic GMP and opening of big conductance calcium-activated potassium channels and ATP-dependent potassium channels contribute to the direct effect of the natriuretic peptides. Basally released endothelium-derived NO inhibits the response of the vascular smooth muscle of that artery to the natriuretic peptides, at least partially by desensitizing the response of the $\text{BK}_{\text{Ca}}$ channels of the vascular smooth muscle to the generation of cyclic GMP.


channel modulation by NS 1619, the putative BKCa channel opener, in vascular smooth muscle. Br J Pharmacol. 113(4):1538-47.


peptide receptor-linked particulate guanylate cyclases are modulated by nitric oxide-cyclic GMP signalling. *Br J Pharmacol* **139**: 1289-96.


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Legends for figures

Fig. 1. Isometric tension recordings in rings with (upper) and without (lower) endothelium of the same porcine coronary artery. Concentration-relaxation curve to cumulative concentrations of CNP during contractions to endothelin-1. At the end of the experiment full relaxation is obtained with isoproterenol. M = mol/liter;

Fig. 2. Relaxations to CNP (left) and ANP (right) during contractions to endothelin-1 (10^{-9} M) in isolated porcine coronary arteries. Upper: Effect of removal of the endothelium. +EC, with endothelium; -EC, without endothelium; Lower: Effect of L-NAME (10^{-4} M) in rings with endothelium. Data expressed as percentage of the maximal relaxation to isoproterenol (10^{-5} M) obtained at the end of the experiments; n = 6-8; *, P < 0.05 versus control.

Fig. 3. Effect of L-NAME (10^{-4} M; data from Fig.2, lower left), sodium nitroprusside (SNP, 10^{-9} M), ODQ (10^{-6} M), NS2028 (10^{-6} M) and 8-bromo-cyclicGMP(10^{-5} M), on CNP (10^{-10} M to 10^{-6} M)-induced relaxations [expressed as area under the curve (AUC)] during contractions to endothelin-1(10^{-9} M) in isolated porcine coronary arteries with endothelium. n = 6-8; *, P < 0.05 versus control.
Fig. 4. Effect of L-NAME (10^{-4} M), sodium nitroprusside (SNP; 10^{-9} M), ODQ (10^{-6} M), NS2028 (10^{-6} M), and 8-bromo-cyclicGMP (10^{-5} M) on CNP (10^{-10} M to 10^{-6} M)-induced relaxations [expressed as area under the curve (AUC)] during contractions to endothelin-1 (10^{-9} M) in isolated porcine coronary arteries without endothelium. n = 6; *, P < 0.05 versus control.

Fig. 5. Effect of sodium nitroprusside (SNP) on relaxations to ANP (10^{-10} M to 10^{-7} M) (left) and isoproterenol (10^{-10} M to 10^{-4} M, right) [expressed as area under the curve (AUC)] during contractions to endothelin-1 (10^{-9} M) in isolated porcine coronary arteries without endothelium (-EC). n = 6 left: *, P < 0.05 versus control. right: There were no significant differences between the two curves.

Fig. 6. Relaxations [expressed as area under the curve (AUC)] to the cumulative addition of sodium nitroprusside (SNP, 10^{-10} M to 10^{-4} M) and 8-bromo-cyclicGMP (10^{-10} M to 10^{-4} M) during contractions to endothelin-1 (10^{-9} M) in porcine coronary arteries with (+EC) and without (-EC) endothelium; n = 6; *, P < 0.05 versus control.

Fig. 7. Effect of TRAM-34, UCL1684, charybdotoxin, apamin, iberiotoxin and glybenclamide (Gly) on relaxations [expressed as area under the curve (AUC)] to increasing concentrations of CNP (10^{-10} to 10^{-6} M) during contractions to endothelin-
1(10^{-9} \text{ M})$ in porcine coronary arteries with endothelium. $n = 8$; *, $P < 0.05$ versus control.

Fig. 8 Effect of TRAM-34, UCL1684, charybdotoxin, apamin, iberiotoxin (IBTX), glybenclamide (Gly), sodium nitroprusside (SNP), the combination of SNP and iberiotoxin, and the combination of SNP, iberiotoxin and glybenclamide on relaxations [expressed as area under the curve (AUC)] to increasing concentrations of CNP ($10^{-10}$ to $10^{-6}$ M) during contractions to endothelin-1 ($10^{-9}$ M) in porcine coronary arteries without endothelium. $n = 8$; *, $P < 0.05$ versus control.

Fig. 9 Relaxations to NS1619 during contractions to endothelin-1 ($10^{-9}$ M) in isolated porcine coronary arteries with or without endothelium. Data expressed as percentage of the maximal relaxation to isoproterenol ($10^{-5}$ M) obtained at the end of the experiments; $n = 5$; *, $P < 0.05$ versus control.

Fig. 10 Effect of five minutes of exposure to CNP ($10^{-6}$ M) on the accumulation of cyclic GMP in porcine coronary arteries with (+EC) and without (-EC) endothelium, and after incubation with ODQ ($10^{-6}$ M) or NS2028 ($10^{-6}$ M) in rings of coronary arteries without endothelium, studied in the presence of IBMX ($10^{-4}$ M). Data shown
in absolute values; n=6.

Fig. 11 Effects of Atrial natriuretic peptide (ANP) on the BK_{Ca} amplitude of single porcine coronary artery smooth muscle cells. The extent of block of BK_{Ca} amplitude by ANP (10^{-8}M and 10^{-7}M, A-C) (with and without SNP/8-Br-cGMP-pretreatment) was evaluated using a train-pulse protocols (holding potential = -60 mV, stimulating test potential = 80 mV for 500 ms, stimulated at 0.033 Hz). Insets illustrated the representative steady-state BK_{Ca} traces recorded in controls, ANP/CNP and ANP/CNP plus iberiotoxin (100 nM) (with and without SNP/8-bromo-cGMP pretreatment). The lower panel summarized the steady-state maximum effects of drugs on the macroscopic BK_{Ca} amplitude recorded (expressed as pA/pF). Results are expressed as mean ± S.E.M., and the number of experiments is indicated in parentheses. * P < 0.05, ** P < 0.01 and *** P < 0.001.

Fig. 12 Effects of C-type natriuretic peptide (CNP) on the BK_{Ca} amplitude of single porcine coronary artery smooth muscle cells. The extent of block of BK_{Ca} amplitude by C-type natriuretic peptide (CNP, 10^{-7}M and 10^{-6}M, A-C) (with and without SNP/8-bromo-cGMP-pretreatment) was evaluated using a train-pulse protocols (holding potential = -60 mV, stimulating test potential = 80 mV for 500 ms, stimulated at 0.033 Hz).
Hz). Insets illustrated the representative steady-state BKCa traces recorded in controls, CNP and CNP plus iberiotoxin (100 nM) (with and without SNP/8-bromo-cGMP pretreatment). The lower panel summarized the steady-state maximum effects of drugs on the macroscopic BKCa amplitude recorded (expressed as pA/pF). Results are expressed as mean ± S.E.M., and the number of experiments is indicated in parentheses. * P < 0.05, ** P < 0.01 and *** P < 0.001.
Fig. 1

CNP, -Log M

With endothelium

Without endothelium

Endothelin-1 (10^-9 M)

Isoproterenol (10^-5 M)

10 9 8 7 6

1 g

5 minutes
Fig. 2
Fig. 3
Fig. 4
**Fig. 5**

![Graphs showing AUC (Arbitrary units) for Relaxation to ANP and isoproterenol.](image-url)
Fig. 7
Fig. 8
Fig. 9
Fig. 10
Fig. 11
Fig. 12