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Relaxation of isolated arteries by C-type natriuretic peptide; a model for developing hyperpolarizing vasodilators

University Doctoral Dissertation

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Inter-species differences and extracellular calcium dependence in the vasorelaxing effect of cromakalim in isolated human, porcine and canine coronary arteries.
Vascular Pharmacology. (Manuscript No. VPH-D-07-00008) Submitted

1. INTRODUCTION

1.1 The C-type natriuretic peptide as an endogenous regulator

C-type natriuretic peptide (CNP), an endogenous substance, plays an important role in the control of circulation through the regulation of vascular tone. This endogenous peptide belongs to the natriuretic peptide (NP) family besides atrial natriuretic peptide (ANP) [1], brain natriuretic peptide (BNP) [2], urodilatin [3] and dendroaspis natriuretic peptide (DNP);[4]. Natriuretic peptides and their receptors have been identified in a wide range of vertebrate species from elasmobranchs to mammals [5]. NPs act more specifically on Na^+ regulation in lower species, in mammals their key role is to maintain the physiologic body fluid homeosthasis and blood pressure. [6]. CNP was firstly identified in 1990 by Sudoh et al. [2] from porcine brain extract as a peptide having 22 amino acid residues and showing sequence homology to ANP and BNP within the ring structure formed by an intramolecular disulfid linkage. Facing this structural relation CNP is distinct from ANP and BNP both genetically and functionally. Unlike ANP and BNP it acts as a paracrine, local modulator and not as a systemic hormone. Furthermore, facing it's name C-type natriuretic peptide, it has no significant effect on renal fluid or electrolyte excretion [7].

Besides its presence in the central nervous system [8] and kidney [9], the CNP is an important regulator of cartilage homeostasis and endochondral bone growth [10]. It is produced by the vascular endothelium in a constitutive manner [11,12]. Although CNP was discovered as a neuromodulator, soon the vascular effects of the peptide came into prominence: 1/ it is an effective smooth muscle relaxant [13,14] and 2/ a potent anti-proliferative and anti-migratory substance on vascular smooth muscle cells [15,16].

1.2. Effector systems of CNP

CNP exerts its biological effects by binding to cell surface receptors denoted NP receptor B and C (NPR-B, NPR-C, respectively).

The activation of the particulate guanylyl cyclase linked NPR-B receptor increases the intracellular cGMP level that provokes the relaxation of smooth muscle cells [17,18].

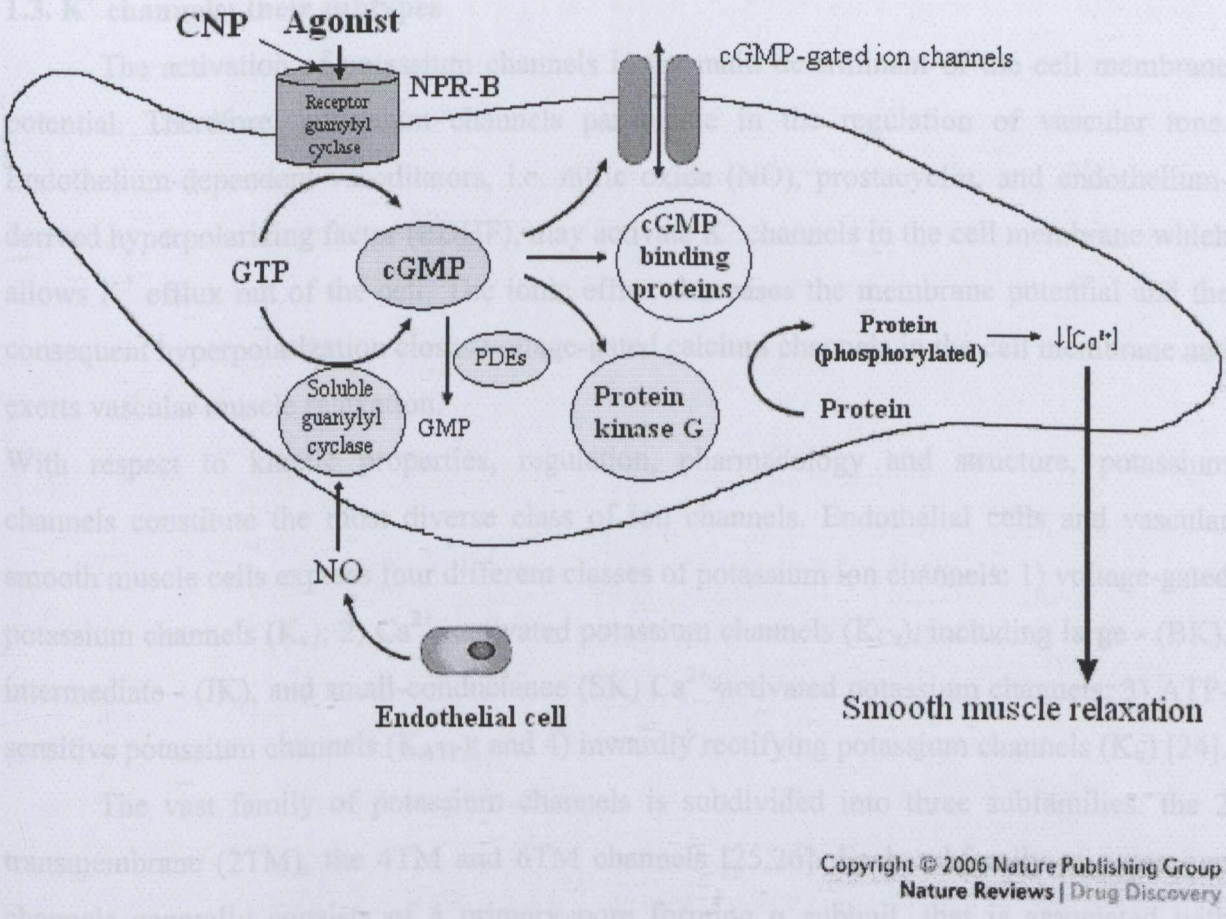

Figure 1.
The role of C-type natriuretic peptide in cGMP-linked vasodilation.
The figure is pointing at the receptorial effect of C-type natriuretic peptide. The peptide binds to the NPR-B receptor that results in activation of guanylyl cyclase. The elevated production of cGMP leads to vasodilation through protein kinase G activation and through cGMP-gated ion channels by the reduction of intracellular Ca^{2+} concentration. NO exerts vasorelaxation through the same mechanism albeit the source of cGMP is the soluble guanylyl cyclase.

In contrast to NPR-B, NPR-C does not contain guanylyl cyclase domain and has no direct effect on cGMP levels [19].

The mechanism of CNP induced vasorelaxation through cGMP-gated ion channels is not exactly clear at present. However, it is supposed to exert a regulatory effect on Ca^{2+} -activated K^+ channels (K_{Ca} -s) of the smooth muscle cell membrane and results in its hyperpolarization [20;21;22;23].

1.3. K^+ channels; their subtypes

The activation of potassium channels is the main determinant of the cell membrane potential. Therefore, potassium channels participate in the regulation of vascular tone. Endothelium-dependent vasodilators, i.e. nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF), may activate K^+ channels in the cell membrane which allows K^+ efflux out of the cell. The ionic efflux decreases the membrane potential and the consequent hyperpolarization closes voltage-gated calcium channels in the cell membrane and exerts vascular muscle relaxation.

With respect to kinetic properties, regulation, pharmacology and structure, potassium channels constitute the most diverse class of ion channels. Endothelial cells and vascular smooth muscle cells express four different classes of potassium ion channels: 1) voltage-gated potassium channels (K_v); 2) Ca^{2+} -activated potassium channels (K_{Ca}), including large - (BK), intermediate - (IK), and small-conductance (SK) Ca^{2+}-activated potassium channels; 3) ATP-sensitive potassium channels (K_{ATP}); and 4) inwardly rectifying potassium channels (K_{ir}) [24].

The vast family of potassium channels is subdivided into three subfamilies: the 2 transmembrane (2TM), the 4TM and 6TM channels [25,26]. Each subfamily of potassium channels generally consists of a primary pore forming α subunit, that is associated with several regulatory subunits [27].

Channels of 2 TM and 4TM family were not the subject of our study.

The 6TM family includes the voltage-gated potassium (K_v) channels, the KCNQ channels, the EAG channels (also including HERG channels), and the calcium –activated potassium channels BK (Slo) and SK.

On the basis of α subunit differences K_v channels are divided to the following groups: K_v 1.x (Shaker), K_v 2.x (Shab), K_v 3.x (Shaw) and K_v 4.x (Shal); the presence of Shaker and Shab type channels has been identified in smooth muscle and mesenteric arteries. These channels are sensitive to the blocker 4-aminopyridin (4-AP).

KCNQ channels (KCNQ1-5), also known as K_v 7.1 -7.5 have significant role in the repolarization of cardial action potential, but they are also found in kidney, brain and lungs.

Further, possibly 4-aminopyridin sensitive voltage dependent channels are the EAG (K_v10.x, K_v11.x, K_v12.x) channels that have crucial role in cardiac repolarization.

Structurally, the calcium-activated potassium channels can be divided into two groups: the small or intermediate conductance potassium channels (SK/IK), and the high conductance potassium channels (BK, or Maxi-K). The open probability of these channels is increased by the elevation of intracellular calcium, leading to membrane hyperpolarization. BK channels consisting of a pore forming α and a regulatory β subunit affect the regulatory mechanisms of contraction in smooth muscle cells [24,26,27]. The channels - SK and IK – responsible for hyperpolarization have also been found in smooth muscle.

Table 1. Calcium activated potassium channel subtypes of 6TM family

α Subunit Name	Alternative Names	Distribution	Function
BK	KCNMA1, Slo, Maxi-K^+, K_{ca} 1.1	Brain, skeletal muscle, smooth muscle, adrenal cortex, kidney	Smooth muscle: regulation of contraction (Heppner, 1997) Neurons: hyperpolarization of plasma membrane
SK	SK1-3 (K_{Ca} 2.1-2.3) SK4 (IK_{Ca} 1, K_{Ca} 3.1)	Brain, aorta, lung, skeletal muscle, smooth muscle, erythrocytes	Involved in the afterhyperpolarization in vertebrate neurons; volume regulation in erythrocytes

1.4. Elimination of CNP

CNP is eliminated through the above mentioned NPR-C receptors, but another very significant way of elimination, the enzymatic metabolism is also present. The main enzymatic elimination pathway of CNP is neutral endopeptidase (EC. 3.4.24.11, NEP). The NEP is a membrane bound zinc-containing metallopeptidase that cleaves various vasoactive peptides exerting various effects on the blood vessel tone [28]. Some of these peptides have vasodilating activity such as ANP, bradykinin or substance P, while others are vasoconstrictors i.e. endothelin [29-32].

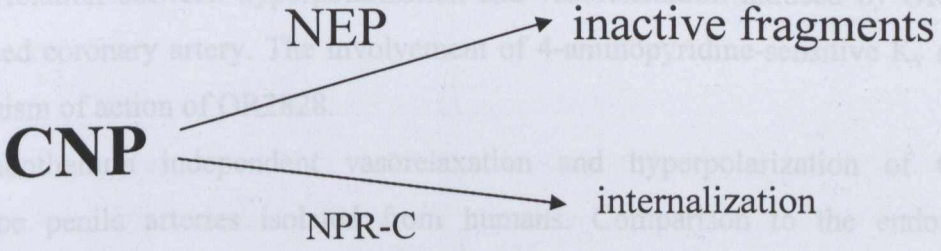

Figure 2.
Elimination pathways of CNP
Neutral endopeptidase EC.3.4.24.11. (NEP) enzyme degrades C-type natriuretic peptide to inactive fragments representing its main elimination pathway. The NPR-C receptor mediates the cellular internalization and subsequent lysosomal degradation of the peptide.

2. AIMS

The main aim of the present work was to study the vasorelaxing capacity of CNP on conductance type of isolated rat systemic and porcine coronary arteries as well as on the resistance type of human penile arteries. The involvement of the vascular endothelium in the mechanism of vasorelaxation and an elimination pathway of the peptide were investigated by using pharmacological inhibitors. The effect of CNP as well as that of two potent vasodilators on the hyperpolarization of the smooth muscle membrane as a possible cause of vasorelaxation were studied in porcine coronary artery. K_v and BK_{Ca} channels, as mediators of hyperpolarization were studied in porcine coronary and human penile arteries, respectively. Our studies were focused on the following:

1/ Determination of the efficacy of CNP on rat isolated carotid, mesenteric and femoral arteries.

2/ The possible involvement of the vascular endothelium in the vasorelaxing mechanism of CNP in porcine isolated coronary artery. The effect of the endothelin inhibitor, PD142893.

3/ The role of NEP-enzyme, as an important elimination pathway of CNP in porcine isolated coronary artery. The effect of the inhibitors of NEP-enzyme, phosphoramidon and thiorphan, on CNP-induced relaxation.

4/ Hyperpolarizing effect of CNP and its comparison to the calcium sensitezer levosimendan and to a new vasodilator, OR2828, in porcine isolated coronary artery.

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5/ Correlation between hyperpolarization and vasorelaxation induced by OR2828 in porcine isolated coronary artery. The involvement of 4-aminopyridine-sensitive K_v channels in the mechanism of action of OR2828.

6/ Endothelium independent vasorelaxation and hyperpolarization of CNP in resistance type penile arteries isolated from humans. Comparison to the endothelium-dependent relaxant, acetylcholine.

7/ Involvement of BK_{Ca} channels in the non-nitrergic/non prostaglandin type of CNP-induced vasorelaxation in human resistance type of penile arteries.

3. MATERIALS AND METHODS

3.1. Effect of C-type NP on isolated femoral, mesenteric and carotid arteries of the rat

3.1.1. Tissue preparation and measurement of isometric tension

The peripherial blood vessels of the rat were investigated on three different types of isolated arteries, such as a. femoralis, a. mesenterica superior and a. carotis externa. Rats were anesthetized by aether, and following exsanguination, arterial samples were obtained for the studies. 2 mm long ring segments were cleaned from the surrounding tissues and longitudinally two stainless-steel wires were inserted to their lumen. The wires were fixed to the headstage of a Mulvany-type myograph for isometric measurement of tension. The water-jacketed baths contained 2 ml KH solution. The KH solution had the following composition (in mM): NaCl 120, KCl 4.2, $CaCl_2$ 1.5, $NaHCO_3$ 20, $MgCl_2$ 1.2, KH_2PO_4 1.2, and glucose 11, pH=7.4. The rings were stretched up to an optimalized force according to Mulvany et al. [33] in order to achieve maximum active contractions. Two ring segments were investigated simultaneously. In every 15 min, during 45 min stabilization period, the arterial preparations were washed with fresh KH solution and then the initial passive tone was readjusted.

3.1.2. Experimental protocol

Active contractions were induced by KCl (80 mM). After the stabilization of contractile tone CNP was administered cumulatively (1 nM – 1 μM) or was given in one single dose (1 μM).

3.2. Effect of CNP on the vascular tone and membrane potential in isolated porcine coronary arteries

3.2.1. Tissue preparation and measurement of isometric tension

Fresh porcine hearts, obtained from the local slaughterhouse, were placed in a container filled with ice-cold KH solution and transported to the laboratory within half an hour. Upon receipt of the heart, epicardial coronary arteries of the circumflex branch were carefully dissected. The vessels were cleaned of fat and the surrounding connective tissue, and cut into cylindrical rings of 5 mm length with a pair of blades set to fixed distance.

Two coronary rings of each porcine heart were mounted separately in isolated tissue baths containing 2 ml of KH solution bubbled with 95% O_2 and 5% CO_2 at 37 °C. The rings were suspended between a force-displacement transducer (Hugo-Sachs Elektronic, Type F30, Germany) and an anchor, and output data were recorded on a polygraph (KUTESZ, Hungary). The preparations were streched up to 30 mN and allowed to equilibrate for 90 min. Within this equilibration period the incubation medium was changed in every 15 min and responses to 30 mM potassium chloride (KCl) were produced in order to stabilize the contractile activity of the coronary artery rings.

3.2.2. Tissue preparation for membrane potential measurements

The source and preparation of porcine epicardial coronary arteries were the same as described before in 2.1.1. Coronary artery rings were cut open along the longitudinal axis and pinned to the bottom of an organ chamber (0.5 ml in volume) with the intimal surface upward.

3.2.3. Measurement of the membrane potential

The isolated vascular segments were superfused at a constant flow (2 ml min^{-1}) with KH solution (37C°, aerated with 95% O_2/5% CO_2 gas mixture, pH 7.4). The transmembrane potential of the smooth muscle cells was measured using a conventional glass microelectrode technique. Glass electrodes were produced by Flaming/Brown micropipette puller (Model-97, Shutter Instruments Co. USA) from standard borosilicate glass capillaries (single-barrel, D=1.2 mm; World Precision Instruments Inc., Sarasota, FL, USA). The resistance of the tip filled with KCl solution (3M) was 30 to 90 MΩ. The microelectrode was advanced using a pipette holder containing Ag/AgCl pellet in contact with the bathing solution and was directly

connected to the headstage of a recording amplifier (Intra 767, World Precision Instruments Inc., Sarasota, FL, USA) with capacitance neutralization. The Ag/AgCl reference electrode was placed directly in the organ chamber. The holder was mounted on a three-dimensional mechanical micromanipulator (Wetzlar, Leitz, Germany) placed on a vibration-free table. The signal was continuously monitored and recorded both on 80807-00 Cole Palmer Solid State Paperless Data Recorder at 4 Hz and Radelkis OH 814/1 type Potentiometric Recorder.
The microelectrode was inserted into a single muscle cell from the endothelial side. The following criteria were used to assess the validity of a successful impalement: (1) a sudden negative drop in potential from the baseline (zero potential reference) followed by (2) a stable negative voltage for more than 3 min; and (3) an instantaneous return to the previous voltage level on dislodgement of the microelectrode. Hyperpolarizing effect of 5 μM pinacidil proved the position of the electrodes within the smooth muscle cell and not in the endothelial cells.

3.2.4. Experimental protocol for the measurement of isometric tension

In order to observe the role of endothelium, experiments were performed on endothelium intact and endothelium deprived ring segments. In the case of endothelium deprived arterial rings the endothelium was mechanically removed from the intimal surface of the blood vessel by rubbing the intimal surface with a stainless steel rod covered with a cotton swab.

In experiments designed to determine the presence or absence of functional endothelium, after equilibration of the pairs of ring segments, contractions to the prostaglandin analogue, 0.75 μM U46619 were induced. When the steady-state contraction amplitude produced by U46619 had developed, both the endothelium-intact and the endothelium-deprived rings were exposed to bradykinin (1 μM) or A23187 (0.5 μM).
In another series of experiments the capacity of the combined NEP/ECE enzyme inhibitor, phosphoramidon, to influence relaxations caused by CNP was assessed. In each experiment two parallel coronary artery rings were mounted in separate organ baths: both were either endothelium intact or endothelium deprived preparations. During the equilibration period, 30 min prior to the addition of the contractile agent, 0.75 μM U46619, phosphoramidon (10 μM) to one bath and its solvent (20 μl of 96% ethanol) to the other were added. Then at the steady-

state contraction amplitude of U46619 CNP was applied in a cumulative manner (0.006-1.4 μM) to both coronary artery preparations.

In a third series of experiments the ability of the specific NEP-enzyme inhibitor, thiorphan to influence the coronary relaxations by CNP was investigated. Thiorphan was administered to one of the coronary rings in a concentration of 10 μM, while the other one was preincubated with its solvent (20 μl of 96% ethanol). Experiments were conducted with either two endothelium intact or two endothelium denuded coronary rings. Contractions to U46619 and concentration-response curves for CNP were established as described above with phosphoramidon.

The effect of endothelin receptor inhibition was also studied on CNP-induced coronary artery relaxation by using the non-specific endothelin receptor antagonist, PD142893. The agent (10 μM) and its solvent (20 μl of 96% ethanol) were added to the organ bath 30 min prior to U46619 and then a single concentration of CNP (0.02 μM) was applied to the coronary rings with and without endothelium. The experiments were perfomed with paired arterial rings as described above.

The vasorelaxant effect of OR-2828 (0.38 μM – 230.6 μM) was assumed on KCl (80mM) precontracted porcine isolated coronary arteries as described before in case of CNP. In a separate group of experiments the effect of the solvent used for the drug was investigated. The possible capacity of the Kv channel inhibitor, 4-aminoyridine (4-AP), to influence the relaxation evoked by OR-2828 has also been investigated. In each experiment two endothelium intact rings were mounted parallel in seperate organ baths. One of the rings was pretreated with 4-aminopyridine (5 mM), while the other one was preincubated with the corresponding volume of the 4-aminopyridine solvent (20 μl distilled water). After 10 minutes both rings were precontracted with 30 mM KCl, and then OR-2828 (41.6-230.6 μM) was administered in a cumulative manner. Because the solvent of OR-2828 exerted significant relaxing effect at and above the volumes corresponding to 117.2 and 230.6 μM OR-2828 (90 and 180 μl solvent, respectively), the mean effects of the solvents were subtracted from the effects of the vasodilator.

3.2.5. Experimental protocol for the measurement of membrane potential

Arterial strips were equilibrated for 45 min at 37±0.1 °C before the microelectrode was inserted. Solutions of the tested substances were given directly into the organ chamber in single doses. CNP was dissolved in distilled water, and the following concentrations were applied: 0.7 μM, 1.4 μM, 2.4 μM. OR-2828 was added in concentrations of 60 μM, 120 μM and 180 μM; its solvent had the following composition: $NaHCO_3$ 50 mM, NaOH 100 mM, glucose 25 mM and ethanol in 1/18 ratio. Levosimendan was administered in 3.7 μM concentration in a solution containing 692 mM Na_2PO_4 and 6 mM NaOH.

3.3. Investigation of the effect of CNP on isolated human penile arteries

3.3.1. Preparation of human penile arteries

Penile erectile tissue was obtained in connection with transsexual operations. All patients had been cross dressing, living as women (mean age 29, range 18–49, n=14). Before surgery, each underwent a complete psychiatric and medical evaluation. The penis was excised and submerged immediately in ice-cold (4 °C) physiological salt solution (PSS). The PSS solution had the following composition (in mM): NaCl 120.0, $NaHCO_3$ 20.0, KCl 4.1, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.5 and glucose 11.0; pH: 7.4. The penile artery was carefully dissected and cleaned from the adherent connective tissue. Ring segments (ca. 2 mm long) were mounted on two 40 μm wires of isometric double myograph (Danish Myotechnology, Aarhus, Denmark) according to Mulvany as described in 3.1.1. The preparations were allowed to equilibrate in PSS solution at 37°C and pH 7.4, for 30 minutes.

3.3.2. Experimental protocol for the measurement of isometric tension

To test the contractility of the preparations, they were exposed twice to phenylephrine (PE, 10 μM). The presence of intact endothelium was evaluated by inducing a stable contraction with phenylephrine (10 μM) followed by addition of Ach (10 μM). Relaxation greater than 50% was taken as evidence of endothelial integrity. Arteries were incubated with LNA (100 μM) and indomethacin (10 μM) for 20 minutes before being contracted with phenylephrine (3 μM), and cumulative concentration–response curves were constructed for CNP (0.01-1 μM). When the effects of inhibition of K^+ channels were tested, the arteries

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were incubated for 20 minutes with either the combination of charybdotoxin (0.1 μM) plus apamin (0.5 μM), charybdotoxin (0.1 μM), or iberiotoxin (0.1 μM).

3.3.3. Experimental protocol for the measurement of membrane potential

Membrane potential measurements were conducted according to 3.2.3. Drugs (CNP (1.4 μM), Ach (10 μM)) were added directly to the organ bath.

3.4. Drugs

The components of the KH solution and barium-chloride were obtained from Reanal (Budapest, Hungary). The prostaglandin analogue, U46619 (9,11-dideoxy-11$_\alpha$,9$_\alpha$,-epoxymethano-prostaglandin $F_{2\alpha}$), acetylcholine chloride, bradykinin-acetate, L-ω-nitro arginine, indomethacin, phosphoramidon [N-(α-Rhamnopyranosylphosphono)-L-leucyl-L-triptophan disodium salt], thiorphan [(DL-3-mercapto-2-benzylpropanoyl)-glycine], PD142893 (N-Acetyl-β-Phenyl-D-Phe-Leu-Asp-Ile-Ile-Trp), natriuretic peptide, C-type (Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys), cromakalim, charybdotoxin, apamin, iberiotoxin and ouabain were purchased from Sigma-Aldrich (St. Louis, MO, USA). Calcium ionophore A23187 (Calcimycin; $C_{29}H_{37}N_3O_6$) was purchased from Fluka Chemie AG (Buchs, Switzerland). The source of levosimendan and OR-2828 was the ORION PHARMA (Espoo, Finland).

U46619 was dissolved in 96 % ethanol in order to obtain a stock solution of 0.4 mM. Acetylcholine chloride, charybdotoxin and apamin were dissolved in double-distilled water to give 1 mM, 23 μM and 50 μM stock solutions, respectively. Bradykinin was dissolved in distilled water, phosphoramidon, thiorphan, PD 142893 and U46619 were dissolved in 96% ethanol. A23187 was dissolved in 1% dimethyl sulfoxide. Cromakalim was dissolved in distilled water containing 70% ethanol.

All stock solutions were stored frozen at –20 °C. The appropriate dilutions were obtained by freshly diluting the stocks with KH solution.

3.5. Data analysis & data handling

3.5.1. Data analysis of isometric force studies

Degree of the induced vasoconstriction was expressed in millinewton (mN). Any further change of the vascular tone resulted by the application of the observed substances was calculated as percent of the induced steady-state contraction amplitude of the same preparation. Data are shown as mean ± standard error of the mean (s.e.m.). The effective concentration, which caused 50% of maximum relaxation, was defined as EC_{50}. For the calculation of EC_{50} values the (a*x)/(x+b) logistic equation was fitted to the individual concentration-response curves, in some experiments to the individual curves, in order to calculate differences between them. Statistical significance was tested with Student's two tailed paired t-test and with one-way analysis of variance (ANOVA). Values of P less than 0.05 were considered statistically significant. Correlation between hyperpolarizing and vasodilatator effect was calculated by the linear regressional analysis of the paired data.

3.5.2. Data analysis of membrane potential measurements

In each experiment the stable membrane potential value measured after the successful impalement was considered as baseline. The hyperpolarizing effect was calculated as a difference between the baseline value and the measured most negative potential after adding the substance. Data are shown as mean ± s.e.m. for n observations, where n equals the number of cells from different coronary arteries in which membrane potential was recorded. Statistical analysis was performed by one-way ANOVA and the non-parametric Wilcoxon-test. Values of P less than 0.05 were considered statistically significant.

3.5.3. Data handling

Calculations were performed by the assistance of the following programs: Microsoft® Excel 2002, GraphPad® Prism 3.0, Nfit® 1.0, Cole-Parmer® Companion 3.61.

3.6. Ethics

All animals received human care, the procedure of handling all the animals, their care met the requirements of Guide for the Care and Use of Laboratory Animals and the experimental protocols were approved by the Ethical Review Board for Animal Experiments of the

University of Szeged, Hungary (I 74-3/2002.MÁB.sz). The procedures with human penile tissues were in accordance with the permission granted by the Ethical Committee of the University of Szeged (No.1988/Szeged Tudományegyetem [SZTE]).

4. RESULTS

4.1. Conductance arteries

4.1.1. Vasorelaxant effect of CNP on rat isolated arteries

Vasorelaxant effect of CNP was examined on three different types of rat isolated conductance type arteries *in vitro*. CNP exerted dose dependent relaxations in rat femoral, carotid and superior mesenteric arteries. (Fig. 3.). CNP relaxed the phenylephrine contracted carotid and mesenteric arteries to a larger extent than the femoral arteries. The measured maximal vasorelaxant effects of the peptide were the following: arteria carotis: 86.4±2.9% (n=5); arteria mesenterica superior: 72.6±4.1% (n=9); arteria femoralis: 22.1±4.6% (n=7). The calculated EC_{50} values were in the same magnitude: arteria carotis: $7.9 * 10^{-7}$ M; arteria mesenterica superior: $7.0 * 10^{-7}$ M; arteria femoralis: $1.8 * 10^{-7}$ M. Using the equation of $y= a*x/(x+b)$, 1 μM CNP can cause 81.0% relaxation in the carotid artery, 74.5% in mesenteric artery and 31.0% in femoral artery. These calculated values are close to the measured maximal values, as described above. Consequently, 1 μM concentration of CNP is able to exert submaximal relaxations of the systemic blood vessels studied.

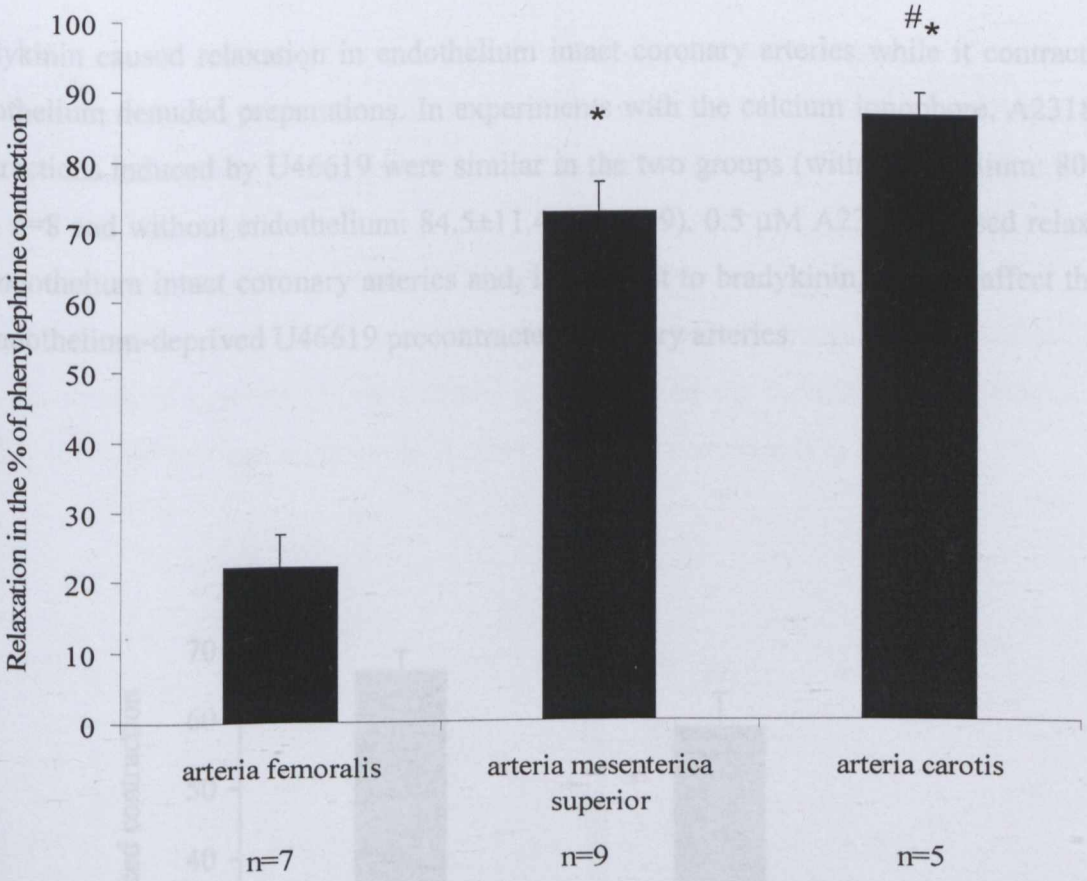

Figure 3.
Vasorelaxant effect of C-type natriuretic peptide (CNP) in three different types of arteries isolated from rat
The mean±s.e.m. values represent the maximal vasodilating effects of 1 μM CNP. Results are expressed as percentage of the contractions induced by phenylephrine. $^{*}p<0.05$ show significant differences in CNP induced relaxations compared to arteria femoralis and, $^{\#}p<0.05$ to arteria mesenterica superior.

Based on these results we tested the vasodilator capacity of the substance on a specialized and highlighted type of vessels: coronary arteries.

4.1.2. Effect of CNP on porcine isolated coronary arteries

4.1.2.1.Characterization of the endothelial function in porcine coronary artery

Fig. 4. shows the effect of endothelial stimulants, bradykinin and A23187, on the tone of coronary ring preparations precontracted with the prostaglandin analogue, U46619. Experiments were conducted in isolated arteries in the presence (■) and absence (□) of endothelium (n=8 and 9, respectively). When bradykinin was used as the endothelial stimulant, the steady-state tone induced by U46619 was comparable in the presence and absence of endothelium (84.5±7.2 mN, n=8 and 87.6±16.3 mN, n=9, respectively). 1 μM

bradykinin caused relaxation in endothelium intact coronary arteries while it contracted the endothelium denuded preparations. In experiments with the calcium ionophore, A23187, the contractions induced by U46619 were similar in the two groups (with endothelium: 80.8±6.9 mN, n=8 and without endothelium: 84.5±11.4 mN, n=9). 0.5 μM A23187 caused relaxations of endothelium intact coronary arteries and, in contrast to bradykinin, did not affect the tone of endothelium-deprived U46619 precontracted coronary arteries.

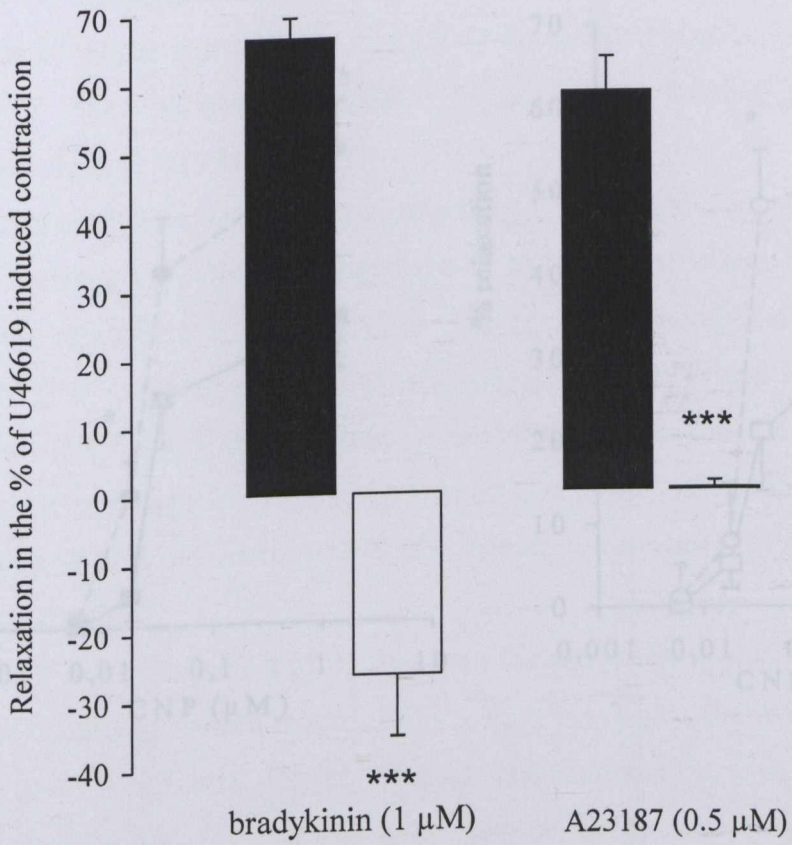

Figure 4.
Effect of bradykinin (1 μM) and the calcium ionophore, A23187 (0.5 μM), on the tone of porcine isolated coronary arteries. Endothelium intact (■) and endothelium deprived (□) arterial rings were investigated (n=8 and 9, respectively). Data are shown as percent changes in tension induced by U46619 and presented as means ±s.e.m. *** $p<0.001$

Since endothelial function is vulnerable and determined by many independent factors, in order to retrieve comparable data, we tested the endothelial function by bradykinin (1 μM) before each measurment to evaluate the presence and absence of functional endothelium.

4.1.2.2. Vasorelaxant effect of CNP on porcine coronary arteries

4.1.2.2.1. Effect of phosphoramidon on the C-type natriuretic peptide-induced relaxation

Another series of experiments were aimed at determining the capacity of phosphoramidon to modify the CNP induced relaxations in porcine coronary arteries (Fig. 5).

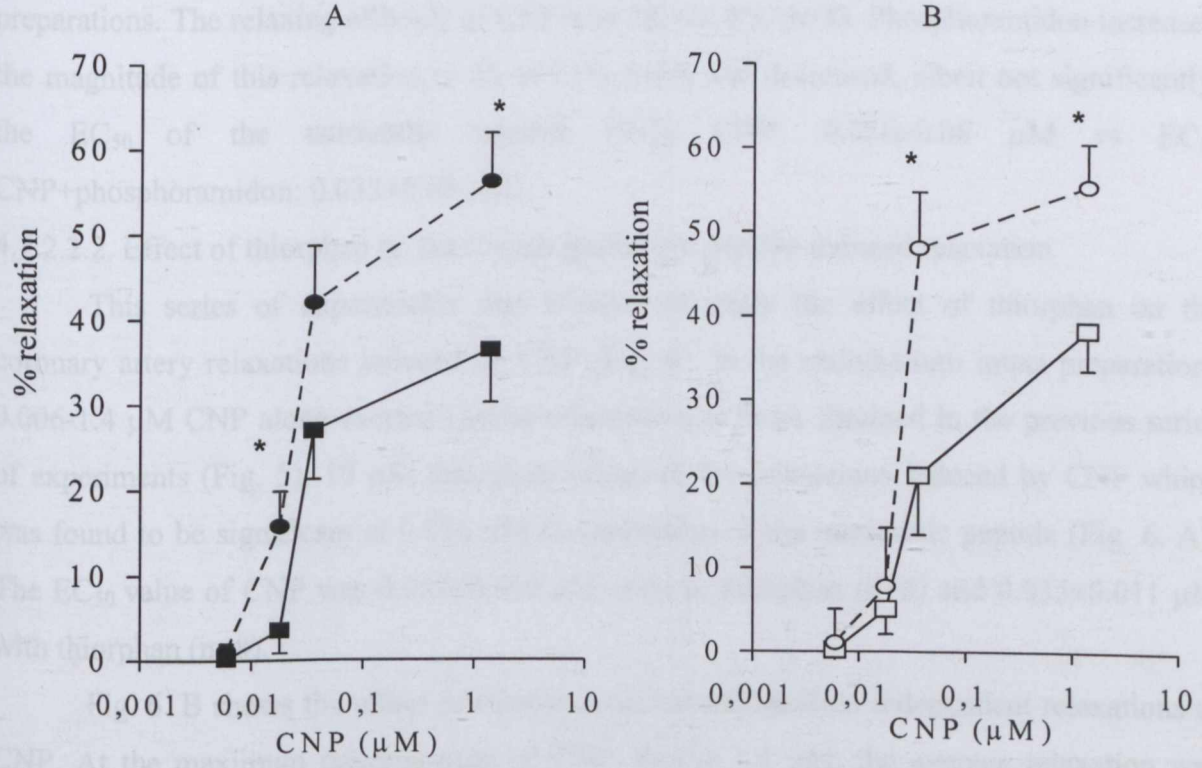

Figure 5.
Effect of phosphoramidon (10 μM) on the relaxations induced by C-type natriuretic peptide (CNP) in porcine isolated coronary arteries.
Experiments were performed in the presence (A) and absence (B) of endothelium (n=8 and 8, respectively). Data are shown as percent changes in tension induced by U46619 and presented as means ± s.e.m. Statistical significance in the magnitude of CNP exerted relaxations is marked (*) between phosphoramidon pretreated (●,○) and solvent control (■,□) groups. * $p<0.05$

In the presence of functional endothelium CNP exerted concentration-dependent relaxations with a maximum of 36.4±4.5% at 1.4 μM of the peptide against U46619-induced contractions (Fig. 5 A). 10 μM phosphoramidon significantly enhanced the effect of CNP at as low as 0.018 μM concentration of the natriuretic peptide and increased the maximal effect of CNP (from 36.4±4.5% to 56.2±6.2%, n=8, $p<0.05$). The calculated EC_{50} value of CNP was 0.042±0.008 μM and this value was not changed significantly by phosphoramidon (0.031±0.012 μM).

Fig. 5 B depicts that, in the absence of functional endothelium, the relaxing capacity of CNP virtually did not differ from that obtained with endothelium intact coronary artery preparations. The relaxing efficacy of CNP was 38.3±4.8% (n=8). Phosphoramidon increased the magnitude of this relaxation to 55.4±5.1% (n=8) and decreased, albeit not significantly, the EC_{50} of the natriuretic peptide (EC_{50} CNP: 0.056±0.06 μM vs EC_{50} CNP+phosphoramidon: 0.033±0.09 μM).

4.1.2.2.2. Effect of thiorphan on the C-type natriuretic peptide-induced relaxation

This series of experiments was devoted to study the effect of thiorphan on the coronary artery relaxations induced by CNP (Fig. 6). In the endothelium intact preparations 0.006-1.4 μM CNP alone exerted similar relaxations to those obtained in the previous series of experiments (Fig. 5). 10 μM thiorphan enhanced the relaxations induced by CNP which was found to be significant at 0.036 μM concentration of the natriuretic peptide (Fig. 6. A). The EC_{50} value of CNP was 0.053±0.010 μM without thiorphan (n=8) and 0.035±0.011 μM with thiorphan (n=8).

Fig. 6. B shows the effect of thiorphan on the endothelium independent relaxations to CNP. At the maximum concentration of CNP, that is 1.4 μM, the average relaxation was 33.0±4.7 % (n=8) which was smaller, although not significantly, than that obtained in the presence of endothelium (41.4±5.1%, n=8). Similar to the endothelium intact coronary arteries, the endothelium independent relaxation by CNP was enhanced by tiorphan (to 47.1±5.4%, n=8). Thiorphan did not change the EC_{50} of the natriuretic peptide (EC_{50} CNP: 0.023±0.05 μM vs EC_{50} CNP+thiorphan: 0.031±0.08 μM, not significant).

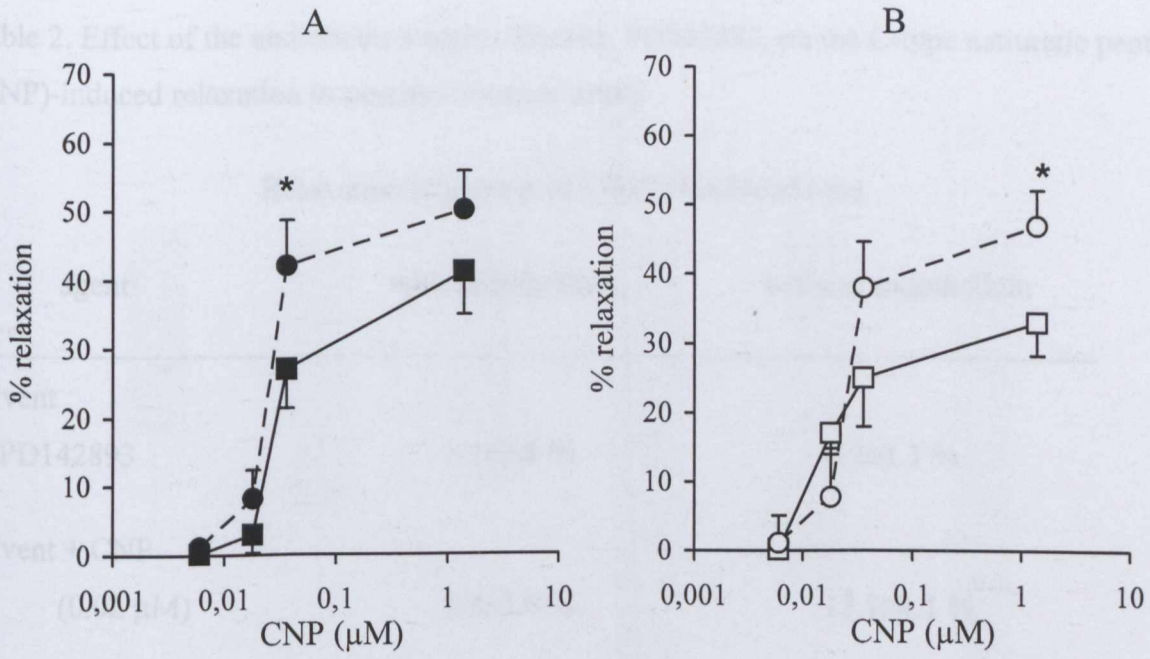

Figure 6.
Effect of thiorphan (10 μM) on the relaxations induced by C-type natriuretic peptide (CNP) in porcine isolated coronary arteries. Experiments were performed in the presence (A) and absence (B) of endothelium (n=8 and 8, respectively). Data are shown as percent changes in tension induced by U46619 and presented as means ± s.e.m. Statistical significance in the magnitude of CNP exerted relaxations is marked (*) between thiorphan pretreated (●,○) and solvent control (■,□) groups. *$p<0.05$

4.1.2.3. Effect of endothelin receptor inhibition on phosphoramidon-induced potentiation of coronary artery relaxation

In order to explore the possible role of endothelin under our experimental conditions the effect of the endothelin receptor antagonist, PD142893, on CNP-induced relaxation was also investigated. Incubation of the coronary artery rings with 10 μM endothelin antagonist did not change the U46619-induced contraction with and without endothelium (74.6±6.4 mN, n=9 and 83.1±9.9 mN, n=8, respectively). As shown in Table 2. PD142893 alone caused a small, insignificant increase in the basal tone compared to its solvent suggesting the lack of basal endothelin release in this artery. It is conceivable, however, that the vasorelaxing effect of low concentration of CNP was potentiated by the inhibition of endothelin receptors in the presence but not in the absence of endothelium.

Table 2. Effect of the endothelin receptor blocker, PD142893, on the C-type natiuretic peptide (CNP)-induced relaxation in porcine coronary artery

	Relaxation in percent of U46619-induced tone	
agent	with endothelium	without endothelium
solvent of PD142893	1.3±0.8 %	0.7±1.1 %
solvent + CNP (0.02 μM)	5.4±3.6 %	13.1±4.1 %
PD142893 (10 μM)	3.5±1.6 %	2.7±4.4 %
PD142893 (10 μM) + CNP (0.02 μM)	16.7±4.0 % *‡	10.3±5.2 %

Values are means ± s.e.m., n=8-9 per group. *$p<0.05$ statistical significance vs. solvent+CNP with endothelium; ‡$p<0.01$ statistical significance vs. PD142893 with endothelium

4.1.2.4. Hyperpolarizing effect of CNP in porcine coronary arteries

CNP is considered as a candidate for endothelium-derived hyperpolarizing factor (EDHF) [22,34] resulting in hyperpolarization and consequent relaxation of the vascular smooth muscle cells. In order to prove the hyperpolarizing capacity of the substance we performed electrophysiological measurements on porcine isolated coronary arteries. Resting membrane potential of smooth muscle cells was established before each mesasurement and was considered as baseline. Before the appication of CNP in 1.4 μM concentration baseline of the resting-tone membrane potentials of the smooth muscle cells was -49.79±1.4 mV (n=15), that was reduced to -53.12±1.2 mV, n=15 (Fig. 7.). This significant decrease apparently prove the hyperpolarizing ability of this endogenous peptide on isolated porcine coronary arteries.

CNP achieved detectable, but insignificant hyperpolarization in 0.7 and 2.1 μM concentration.

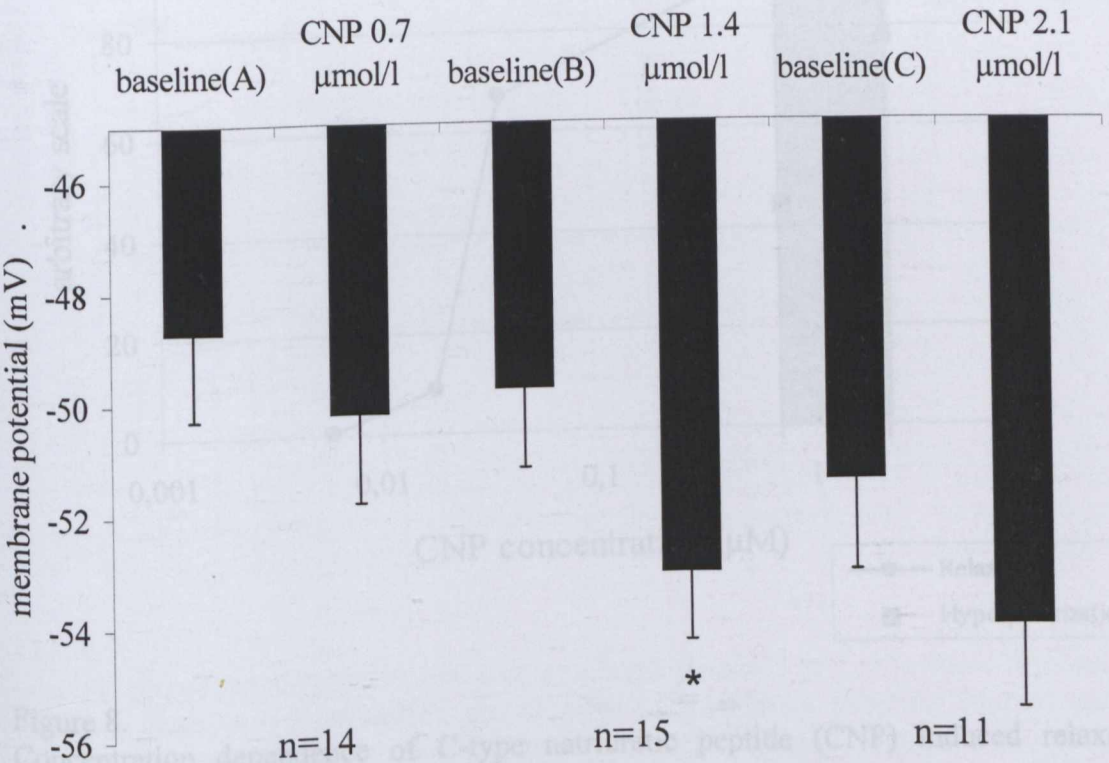

Figure 7.
Hyperpolarizing effect of C-type natriuretic peptide (CNP) on porcine coronary arterial smooth muscle cells
Membrane potentials are shown as mean±s.e.m. values before (baseline (A-C)) and after administration of CNP (0.7-2.1 μM). *$p<0.05$ show significant difference compared to baseline(B) in CNP induced hyperpolarization.

4.1.2.5. Comparison of relaxation and hyperpolarization elicited by differrent doses of CNP

Relaxation and hyperpolarization as responses to concentrations of CNP were plotted in the same coordinate system, to reveal if any correspondence is present between their dose-dependence. (Fig. 8.). The dose-response curve of hyperpolarization covers a well defined segment of the relaxation curve, showing that hyperpolarization can be a considerable component of CNP induced relaxation, at least, in the large concentrations of the peptide.

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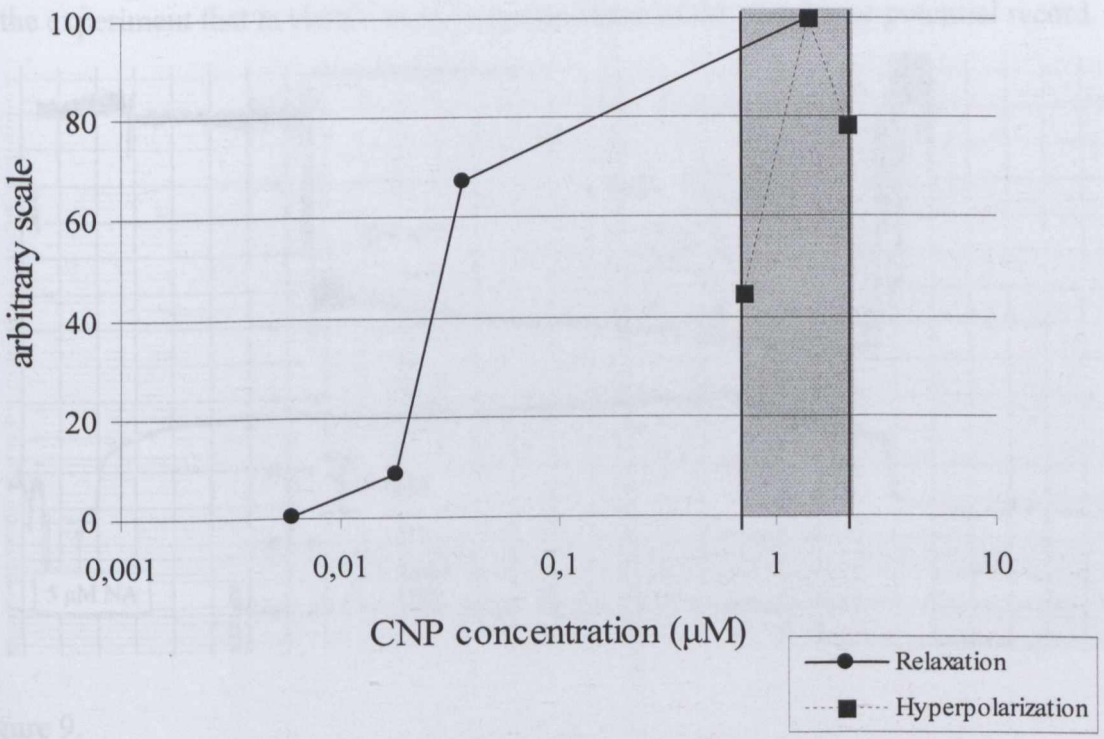


Figure 8.
Concentration dependence of C-type natriuretic peptide (CNP) induced relaxation and hyperpolarization
Arbitrary units (0-100) were applied in order to obtain comparable data. The maximal hyperpolarization obtained from Fig. 7. and maximal relaxation from Fig. 5.A. were considered as 100 arbitrary units.

4.1.3. Correlation between CNP induced relaxation and hyperpolarization

In order to prove the connection between CNP induced relaxation and hyperpolarization a simultaneous measurement of the two parameters has been recorded (Fig. 9.). The mounted rat mesenteric artery was precontracted by noradrenaline (NA) and, after the insertion of the intracellular microelectrode, effects of three doses of CNP (166 nM, 500 nM, 1.6 μM) and a single dose of Ach (10 μM) were investigated. In this experiment 500 nM and 1.6 μM CNP decreased the vacular tone of the NA precontracted vessels. 1.6 μM CNP produced a hyperpolarizing effect on the impaled smooth mucle cell (-3.7 mV). Lower doses of CNP did not influence the development of NA induced depolarization. These data are in agreement with our previous calculations on porcine coronary arteries. Application of Ach (10 μM) resulted in further decrease of the vascular tone and caused a fast and marked

hyperpolarization (-12.7 mV). Dislocation of the electrode prevented us from the continuance of the experiment that is visible as an irregular shake of the membrane potential record.

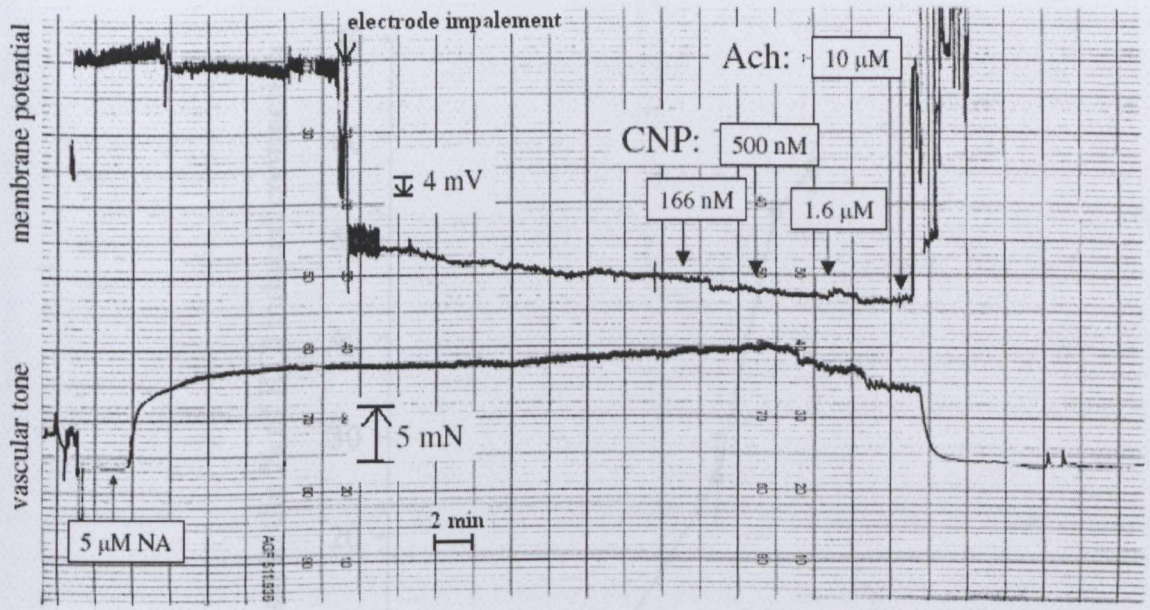


Figure 9.
Original recording of vascular tone and membrane potential in a simultaneous measurement
The two charts are representing the changes of membrane potential (upper) and vascular changes (lower). Units of tone (mN) and membrane potential changes (mV) as well as the time scale (min) are shown. After the development of NA (5 µM) induced contraction a smooth muscle cell was impaled resulting in a sudden drop of the membrane potential. Hyperpolarization induced by 1.6 µM CNP preceeds the relaxation in time. 500 nM CNP caused relaxation without a preliminary hyperpolarizing effect of the peptide.

C-type natriuretic peptide as an EDHF-like endogenous vasodilator can be applied as a reference substance in the development of new vasodilator molecules. In the next series of our experiments we compared the vasoactive capacity of CNP with levosimendan and OR-2828.

4.1.4. Investigation of the possible inodilator capacity of OR-2828

4.1.4.1. Effect of OR-2828 on isometric tone of porcine coronary artery

OR-2828 elicited concentration-dependent relaxation (0.38 µM – 230.6 µM) in the porcine isolated coronary artery. Fig. 10. shows the magnitude of this relaxation in the percentage of the applied KCl (30 mM) precontraction. As the solvent for OR-2828 itself evoked relexation, its effect was deducted from that obtained with the corresponding concentrations of OR-2828 and the curve represents the net effect of the compound. The

calculated maximum relaxation (a) of OR-2828 was 78.1 % and the EC_{50} value (b) was found to be 72.2 μM.

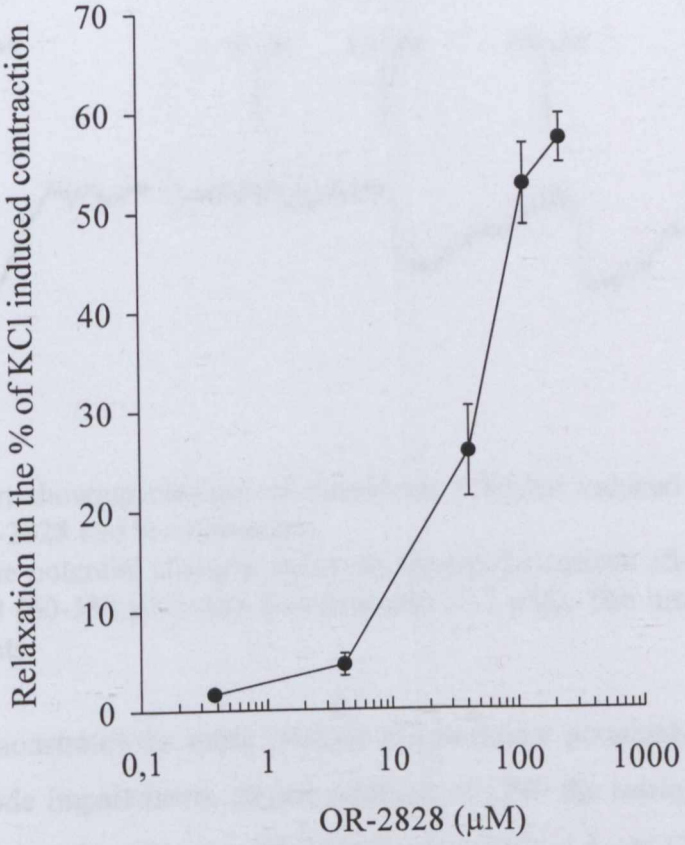


Fig. 10.
Effect of OR-2828 on precontracted porcine coronary artery
The vasodilating effects of OR-2828 are shown as percentage of KCl (30 mM) induced contractions. Data are shown as mean±s.e.m. values. The application of the substance resulted in a dose-dependent relaxation of the coronary artery.

4.1.4.2. Effect of OR-2828 in comparison to C-type natriuretic peptide and levosimendan on membrane potential of the coronary artery smooth muscle

Fig. 11. (representative record) and Fig. 12. demonstrate the hyperpolarizing effect evoked by CNP and the two coronary artery dilators (OR-2828 and levosimendan) on isolated porcine coronary arterial smooth muscle cells.

Fig. 11. shows the magnitude of hyperpolarization induced by the three compounds. The basal membrane potential values are obtained from three different experiments. The hyperpolarizing effect of the reference substance, CNP (1.4 μM), was -3,7 mV. OR-2828

concentration-dependently decreased the membrane potential with a maximum of -2.8 mV at 120 μM. Levosimendan (3,7 μM) caused -2.2 mV hyperpolarization at 3.7 μM.

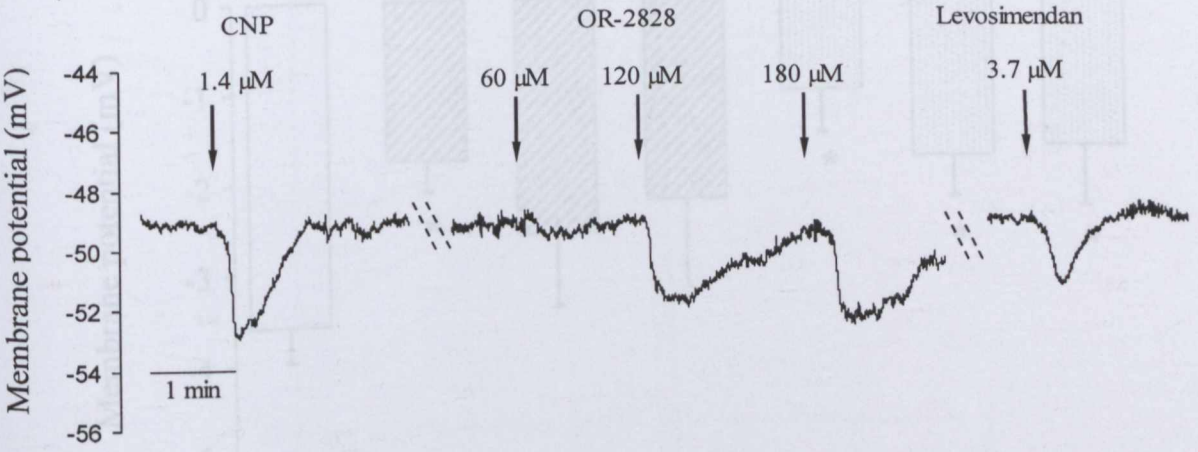


Figure 11.
Representative chart showing changes of membrane potential induced by C-type natriuretic peptide (CNP), OR-2828 and levosimendan
Temporal membrane potential changes represent hyperpolarizations after application of CNP (1.4 μM), OR-2828 (60-180 μM) and levosimendan (3.7 μM). The breaks in the chart show separate impalements.

Fig. 12. demonstrates the mean changes in membrane potentials obtained from 10-22 independent electrode impalements. Before addition of CNP the resting membrane potential of coronary smooth muscle cells were -49.9±0.92 mV (n=17). 1.4 μM CNP caused an average of -3.6±0.38 mV hyperpolarization. Resting membrane potentials were determined before each concentration of OR-2828 (60, 120 and 180 μM). These membrane potentials were: -48.8±1.15 mV (n=14); –48.8±0.88 mV (n=10) and -50.3±0.41 mV (n=12). The magnitude of changes in hyperpolarization induced by 60, 120 and 180 μM OR-2828 were found to be -1.8±0.35 mV, -2.6±0.81 mV and -2.3±0.99 mV, respectively. The hyperpolarizing effect of 60 μM OR-2828 was calculated to be significantly less than that obtained with 1.4 μM CNP. Resting membrane potentials before 1.8, 3.7 and 5.5 μM levosimendan were -49.7±0.79 mV (n=18); -50.8±0.96 mV (n=22) and -50.9±1.18 mV (n=15), respectively. Maximum hyperpolarization by this inodilator was obtained at 3.7 μM (-1.82±0.44 mV). All the concentrations of levosimendan produced significantly less hyperpolarizations than that obtained with the reference compound, CNP.

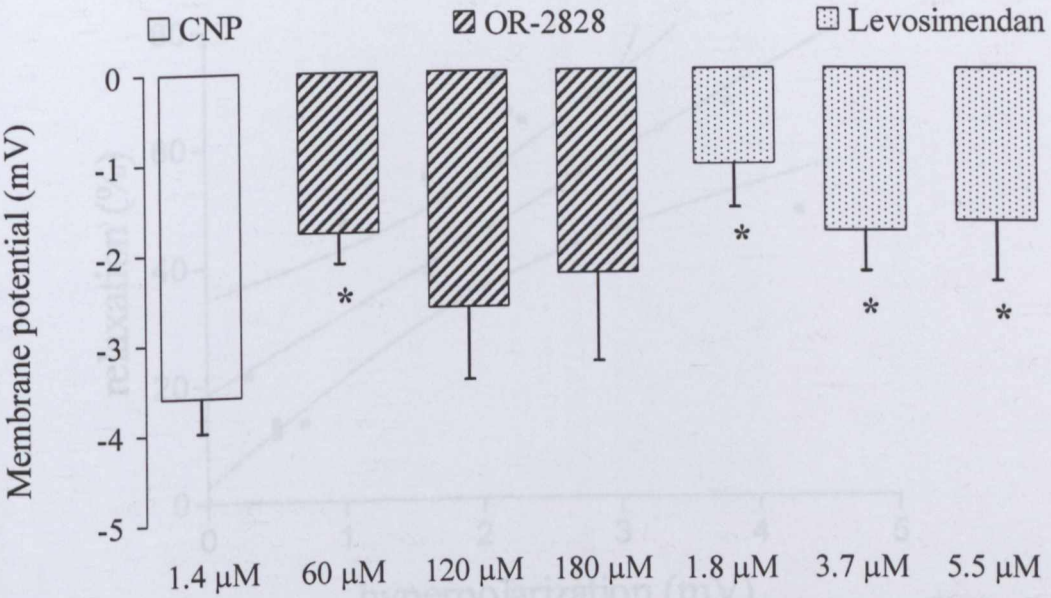


Figure 12.
Changes of membrane potential induced by C-type natriuretic peptide (CNP), OR-2828 and levosimendan
CNP (1.4 µM), OR-2828 (60-180 µM) and levosimendan (1.8-5.5 µM) induced membrane potential changes are shown as mean±s.e.m. values. The data were retrieved from different preparations (n=10-22). All the applied substances hyperpolarized the smooth muscle cells. The magnitude of hyperpolarization was compared to the effect of CNP; statistical significance is shown. *$p<0.05$.

4.1.4.3. Correlation between hyperpolarization and relaxation induced by OR-2828

Fig 13. demonstrates that the magnitude of OR-2828-induced hyperpolarization, expressed as mV values of changes in membrane potential, showed correlation with relaxation. Individual values of both hyperpolarization and relaxation represent experiments with 60 µM OR-2828. As the effect of this concentration fits to the slope of the concentration-response curve of the compound (see Fig. 10.), linear correlation of the individual relaxing values with hyperpolarization was carried out. Equation of the correlation was $y=13.4*x+18.7$ ($r=0.75$, $p<0.01$).

The slope value (13.4) divided by 3.2 shows that 1 mV hyperpolarization is responsible for 4.2 % relaxation. The y intercept reveals that this calculation is valid only above 18.7 % relaxation by OR-2828.

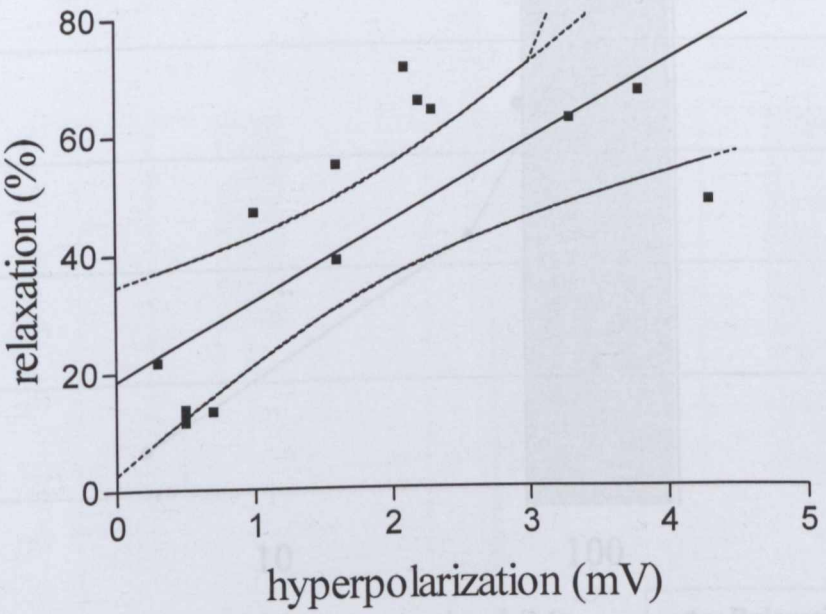


Figure 13.
Correlation between OR-2828 induced relaxation and hyperpolarization
Effect of 60 μM OR-2828 on hyperpolarization (y axis) and relaxation (x axis) are shown. The values of OR-2828 exerted hyperpolarization (y axis) and relaxation (x axise) are shown. Linear regression analysis revealed an acceptable correlation between hyperpolarization and relaxation (r=0.75), the best fitting line is drawn with two lines of the standard error (dotted lines).

4.1.4.4. Comparison of relaxation and hyperpolarization elicited by differrent doses of OR-2828

The concentration dependence of OR-2828 induced vasodilation and hyperpolarization was plotted in the same coordinate system. (Fig. 14.). The curve of relaxation overlaps with the curve of hyperpolarization at the investigated doses. The synthetized substance, OR-2828 is able to hyperpolarize the smooth muscle cell membrane in a wider range of concentrations compared to CNP (Fig. 8). Lack of data does not allow us to state that OR-2828 is able to achieve any hyperpolarization at concentrations lower than 60 μM, but it is important to note that, facing to CNP, the synthetic substance is able to decrease the value of resting membrane potential at concentration lower than the EC_{50} value of relaxation (72.2 μM).

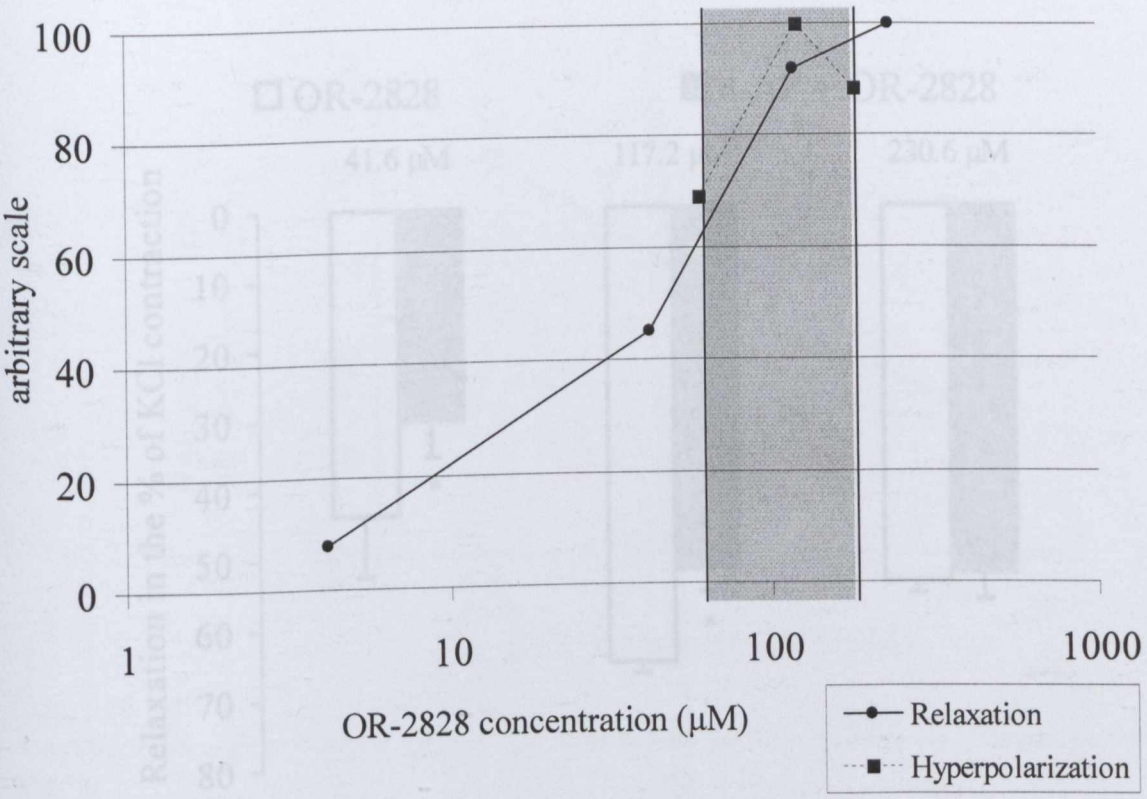


Figure 14.
Concentration dependence of OR-2828 induced relaxation and hyperpolarization
Arbitrary units (0-100) were applied in order to obtain comparable data. The maximal hyperpolarization obtained from Fig. 7. (2.6 mV) and maximal relaxation (see Fig. 10.: 57.2%) were considered as 100 arbitrary units.

4.1.4.5. Effect of the voltage dependent potassium channel inhibitor, 4-aminopyridine on OR-2828-induced relaxation

Pretreatment of the coronary preparations with 5 mM 4-AP for 10 min resulted in a moderate but not significant enhancement of the KCl-induced tone (control: 48.6±9.83 mN; 4-AP: 73.4±10.29 mN, n=5). 4-aminopyridine (5 mM) decreased the coronary artery relaxation induced by 41.6 and 117.2 μM OR-2828. Mean relaxing values of OR-2828 in this figure were obtained after deducting the effect of the corresponding volumes of the solvent. 4-AP had no effect on the relaxations induced by the solvent of OR-2828 (n=5). The blocker of the K_v channels significantly decreased the OR-2828-induced relaxation from 44.0±8.52% to 30.3±5.17% at 41.6 μM ($p<0.05$, n=5). At 117.2 μM concentration of OR-2828 the relaxation was also significantly reduced by 4-AP from 65.6±1.34% to 52.3±3.15% ($p<0.05$, n=5). At the largest concentration of OR-2828 4-AP did not influence the relaxation.

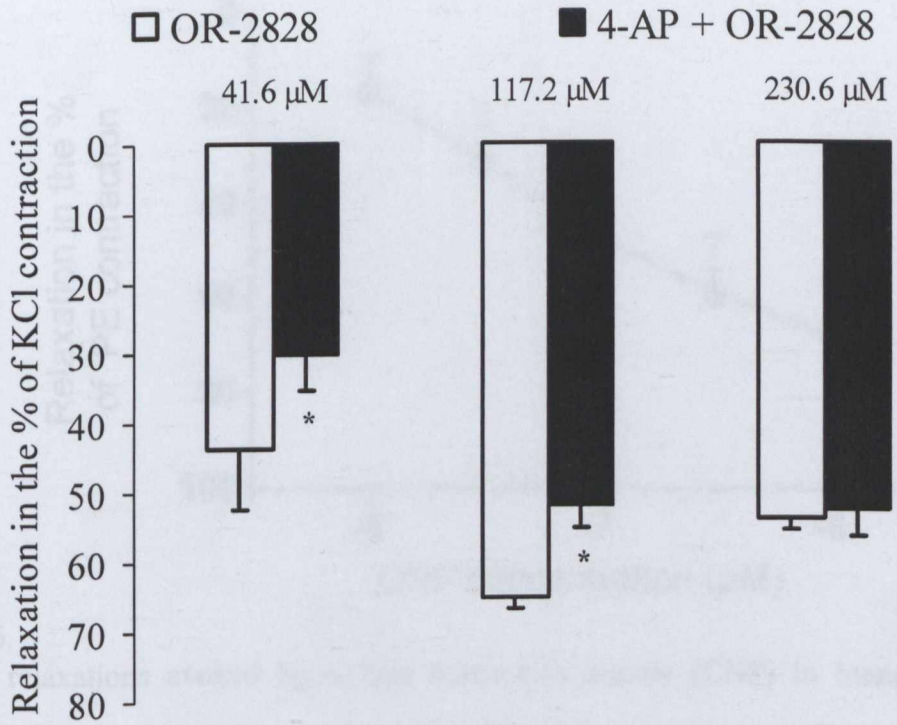


Figure 15.
Effect of 4-AP pretreatment on the vasorelaxant effect of OR-2828
Vasorelaxant effects of OR-2828 (41.6-230.6 μM) are shown (□) as mean ± s.e.m. values in the percentage of KCl induced contractions. 4-AP significantly (* $p<0.05$) decreased the effect of OR-2828 (■) at concentcentrations of 41.6 μM and 117.2 μM.

4.2. Resistance type vessel - human penile artery

4.2.1. Vasorelaxant effect of CNP on human penile arteries

In arteries with endothelium and contracted with phenylephrine in the presence of NG-nitro-L-arginine (LNA) and indomethacin, CNP (0.01–1 mM) evoked concentration-dependent relaxations (Figure 16.). The concentration–response curves for CNP were reproducible up to three times (n=4) and were unaltered in preparations without endothelium (n=2, results not shown).

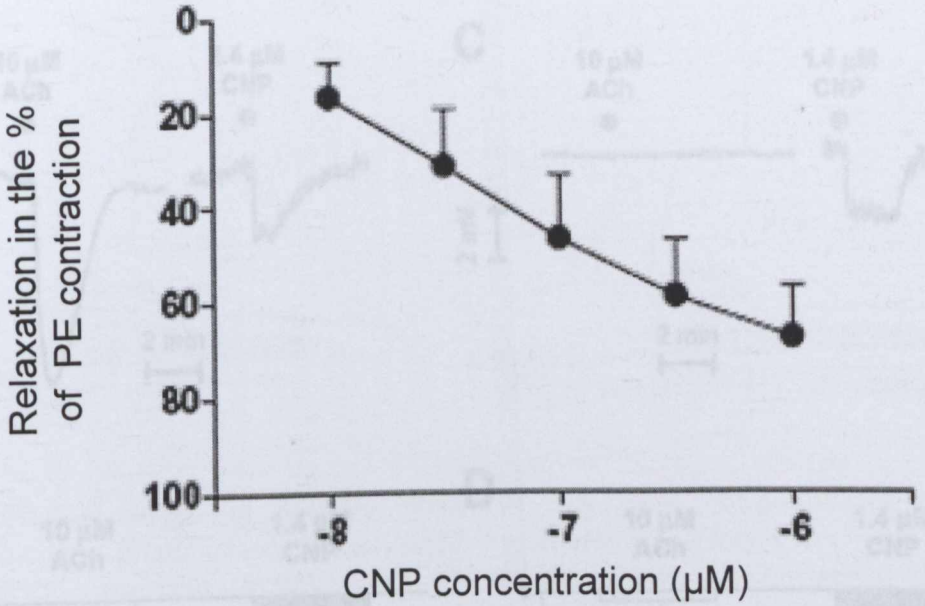
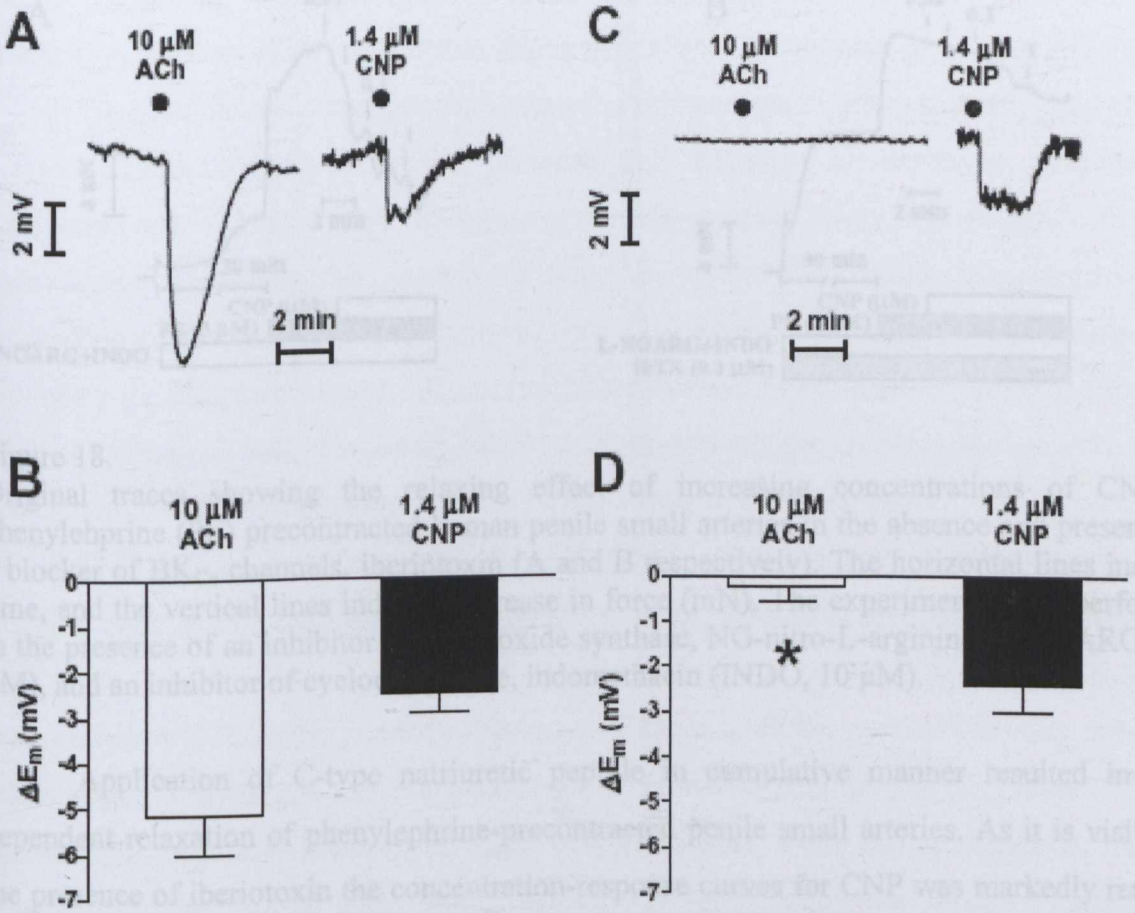


Figure 16.
Average relaxations evoked by C-type natriuretic peptide (CNP) in human penile small arteries.
The mean of five experiments obtained from four patients are shown in the presence of NG-nitro-L-arginine (10^{-4} M) and indomethacin (10^{-5} M). CNP resulted in dose dependent relaxation of the arteries. Data are shown as percent changes in tension induced by phenylephrine (PE) and presented as means ± s.e.m. The measured maximal relaxation was 68±11% at 1 μM concentration of the peptide.

4.2.2. Hyperpolarizing effect of CNP on human isolated penile arteries

Smooth muscle membrane potential in resting, untreated arterial preparations was -44.8±1.3 mV (n=12). In endothelium intact preparations, infusion of Ach (10 μM) hyperpolarized membrane potential by -5.2±0.9 mV (n=6), while infusion of an equieffective relaxant concentration of CNP (1.4 μM) hyperpolarized smooth muscle membrane potential by -2.5±0.5 mV (n= 10; Figure 18.A, B). Mechanical removal of the endothelium abolished the Ach-evoked hyperpolarization (0.2±0.3 mV, n= 7), while the CNP evoked hyperpolarization was unchanged (2.4±0.6 mV, n=10; Figure 18.C,D).



Figue 17.
Acetylcholine (ACh) and C-type natriuretic peptide (CNP) hyperpolarizes human penile arteries. Original recordings (panels A, C) and averages (panels B, D) of smooth muscle cell membrane potential changes (ΔE_m) induced by Ach (10 µM) and CNP (1.4 µM) in penile arterial segments with endothelium (A, B) and without endothelium (B, D). Significant differences were evaluated by paired t-test. $^{*}p< 0.05$ vs. arterial segment without endothelium.

4.2.3. Investigation of the involvement of BK_{Ca} channels in CNP-evoked relaxation

To test whether BK_{Ca} channels were involved in CNP-evoked relaxation, the blood vessels were incubated with iberiotoxin (0.1 µM).

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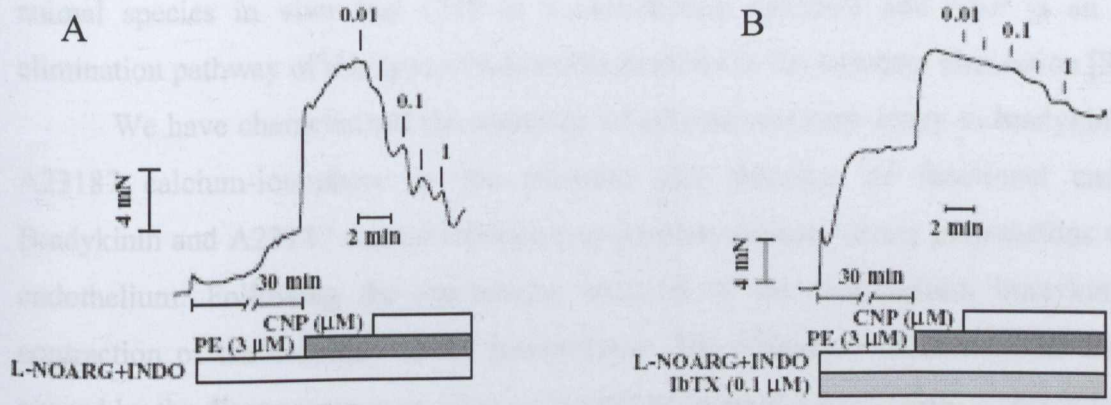

Figure 18.
Original traces showing the relaxing effect of increasing concentrations of CNP in phenylehprine (PE) precontracted human penile small arteries in the absence and presence of a blocker of BK_{Ca} channels, iberiotoxin (A and B respectively). The horizontal lines indicate time, and the vertical lines indicate increase in force (mN). The experiments were performed in the presence of an inhibitor of nitric oxide synthase, NG-nitro-L-arginine (L-NOARG, 100 μM), and an inhibitor of cyclooxygenase, indomethacin (INDO, 10 μM).

Application of C-type natriuretic peptide in cumulative manner resulted in dose dependent relaxation of phenylephrine-precontracted penile small arteries. As it is visible in the presence of iberiotoxin the concentration-response curves for CNP was markedly reduced (Figure18.B) compared to parallel control experiment (Figure 18.A).
This result unequivocally shows that large-conductance Ca^{2+}-activated K (BK_{Ca}) channels are involved in C-type natriuretic peptide (CNP)-evoked relaxation.

5. DISCUSSION

5.1. Conductance arteries

In the first part of our experiments we have demonstrated the vasorelaxant potency of CNP on rat isolated femoral, mesenterial and carotid arteries. CNP had unequivocal vasorelaxant effect on all the tree types of arteries, nevertheless the most outstanding impact was observed in carotid arteries.

In the second series of our experiments we have demonstrated the functional importance of the inhibition of neutral endopeptidase 24.11. activity in the augmentation of the coronary artery relaxation induced by C-type natriuretic peptide. Two known inhibitors of NEP, phosphoramidon and thiorphan, potentiated the effect of CNP both in the presence and absence of functional endothelium. Our results are in agreement with those obtained in other

animal species in vivo that CNP is a vasodilating mediator and NEP is an important elimination pathway of this type of natriuretic peptides in the coronary circulation [35,36].

We have characterized the reactivity of porcine coronary artery to bradykinin and the A23187 calcium-ionophore in the presence and abscence of functional endothelium. Bradykinin and A23187 caused relaxation in porcine coronary artery preparations with intact endothelium. Following the mechanical removal of the endothelium bradykinin caused contraction of the coronary artery preparations. The efficiency of endothelial damage was proved by the disappearance of calcium ionophore induced vasodilation, representing the lack of all calcium-mediated, receptorial independent functions of the endothelial cells, such as the production of nitric oxide and endothelium-derived hyperpolarizing factor.

CNP relaxed both intact and deendothelized porcine isolated coronary artery rings showing endothelium-independent vasodilator potency. Therefore in contrast to bradykinin, CNP does not require the mediation of nitric oxide or prostaglandins for producing vasorelaxation [37]. In the present experiments CNP relaxed the porcine coronary artery, an effect which was almost the same in magnitudes and similar in potency values both in endothelium intact and endothelium deprived preparations. These results match the findings obtained by others in the same blood vessel [38]. Our experimental conditions simulate a selective damage of the endothelial 'vasodilator reserve' in that CNP is superior to bradykinin for preventing local vasoconstriction. The relevance of this superiority to *in vivo* conditions is supported by the presence of CNP in those parts of large coronary arteries known to have dysfunctional endothelium, i.e. the atherosclerotic plaque. Moreover, the CNP gene has been found, besides the endothelium, in smooth muscle cells of the human coronary arteries including samples of early and advanced atherosclerotic lesions [39]. The adenovirus mediated gene transfer to the damaged area of large coronary arteries has been demonstrated to prevent coronary restenosis after balloon injury in pigs [40].

Inhibitors of neutral endopeptidase enzyme (EC. 3.4.24.11.), phosphoramidon and thiorphan, potentiated the vasorelaxing effect of CNP both on intact and endothelium deprived coronary arteries. NEP is the primary enzymatic elimination pathway for CNP limiting the available concentration of the peptide in the vascular wall. The enzyme is known to be located on the surface of the endothelial cells and as an ectoenzyme degrades and inactivates several other vasoactive peptides in the blood circulation [16,41,42]. Recent

studies have demonstrated that the enzyme is constitutively expressed in the rat and human vascular smooth muscle cells [43-45]. Phosphoramidon was able to potentiate the effect of bradykinin in the presence of damaged endothelial function of the porcine coronary artery [46].

In the presence of endothelium the production of endothelin, known to be a strong vasoconstrictor mediator, may counteract the vasorelaxing effect of CNP. In a series of our experiments, in which thiorphan was used to modulate the effect of CNP, 0.018 μM peptide alone caused a virtually smaller relaxation in the presence of endothelium compared to those obtained in the endothelium deprived preparations (see Fig. 5.). In the presence of 0.018 μM CNP, phosphoramidon, known to exhibit an inhibitory effect on endothelin converting enzyme, resulted in a significant increase of the vasorelaxation in endothelium intact coronary artery. This effect was not observed in the endothelium denuded preparations (see Fig. 4.). Inhibition of the endothelin receptors by PD142893 increased the 0.02 μM CNP-induced coronary artery relaxation to similar extent as obtained with phosphoramidon. The effect was found only in the presence of endothelium. These results together are indicative of a functional antagonism between CNP and endothelin in this artery and demonstrate the sensitivity of the porcine large coronary arteries to endothelin converting enzyme inhibition as was also showed in some human studies [47,48].

Recent studies have supported that hyperpolarization is an efficient vasodilating mechanism regulating the tension of the conduit type of coronary arteries [49]. In addition to nitric oxide and vasodilator prostaglandins, the EDHF is an endogenous mediator released from the vascular endothelium. Although nitric oxide by itself is able to hyperpolarize the smooth muscle membrane, a nitric oxide independent EDHF has also been demonstrated in the regulation of porcine, canine and human coronary artery tone [49-52]. An independent EDHF was shown to play roles in relaxation of coronary arteries in experimental heart failure and coronary angioplasty, under pathological conditions in which nitric oxide production was impaired [53-55]. These findings serve the basis for developing hyperpolarizing coronary artery vasodilators.

The results of membrane potential measurements on porcine coronary artery smooth muscle cells indicate that the hyperpolarization of the smooth muscle membrane plays an important role in the regulation of coronary arterial tone. CNP was able to decrease the resting

membrane potential of the smooth muscle cells isolated from porcine coronary arteries. The measured hyperpolarization was in accordance with the results of Barton et al. [38], who demonstrated -4.4 mV maximal change in the resing membrane potential in similar conditions.

The hyperpolarizing effect of CNP was pronounced at micromolar concentrations of the peptide, where the maximal vasorelaxation was previously measured. This fact denotes that CNP induced hyperpolarization is an important component of the vasorelaxation. Although different simultanous mechanisms are supposed to generate the complex effect of the peptide (NPR-B, NPR-C, K^+ channels), in physiologic conditions CNP induced hyperpolarization seems to be an accessory, subsidiary factor in the development of its vasorelaxant effect, as CNP is proved to be efficient vasorelaxant factor even in those lower concentrations, where membrane potential change was not detected. According to recent findings hyperpolarization comes to prominence as a major vasorelaxant factor when vasorelaxant capacity is decreased due to pathologic changes. This is indicative of the importance of such a mechanism as a possible therapeutic pathway in the augmentation of endogenous vasorelaxation. The limitation of the method necessitates further investigation of the observed phenomenon.

In the next series of our experiments we have demonstrated the coronary artery dilating and hyperpolarizing effects of OR-2828 and levosimendan in comparison to C-type natriuretic peptide, as a reference substance. Maximum increase of the resting membrane potential induced by OR-2828 was similar to that obtained with CNP, while the effect of levosimendan was found to be smaller than that produced by the known peptidergic vasomotor regulator.

OR-2828 produced less potency and efficacy values in dilating porcine coronary artery than that presented previously with levosimendan in the same type of coronary arteries [56]. The maximum hyperpolarizing effect of OR-2828, however, appears to be larger than that measured with levosimendan. The effect of both synthetic inodilators was compared to CNP because this natriuretic peptide has recently been shown to act as EDHF in rat mesenteric artery [21]. It was also demonstrated that CNP is one of the potential endogenous hyperpolarizing mediators in epicardial coronary artery of the pig [23, 38]. In this artery the maximum response to CNP was found to be already –3.3 mV at 1.4 μM of the peptide [57].

Therefore, we used only this concentration of CNP as reference for comparing the magnitude of hyperpolarization induced by OR-2828 or levosimendan.

While hyperpolarization is the main vasodilating mechanism in resistance arteries, conduit arteries, like epicardial large coronary vessels, do not belong to the category of 'EDHF-type' arteries [58]. This is an explanation why the magnitude of changes in membrane potential was relatively small in the present study and the finding is in agreement with those measured by other authors. Our values of CNP-induced hyperpolarization (-3.6 mV on average) are comparable to those obtained with the peptide (~ -4-5 mV) or with an ATP-sensitive potassium channel opener, levcromakalim (~ -4 mV), in other experiments [38,59].

We presented the first evidence that at the maximal vasorelaxing concentrations of levosimendan (~ 1-3 μM) [56], the inodilator hyperpolarizes the coronary artery. The hyperpolarizing sensitivity of levosimendan is similar to that obtained in a resistance type of arteries (levosimendan EC_{50}=2.9 μM) [60]. It is important to note that the magnitude of hyperpolarization is much less in our epicardial coronary artery preparations than that produced maximally in a resistance type artery, such as the rat mesenteric artery [21, 60].

However, some mV changes in the membrane potential might considerably influence vascular tone. In our present observations correlation between the hyperpolarizing and relaxing effects of OR-2828 showed that 1 mV increase of membrane potential is coupled with 4.2 % relaxation. This value is almost the same as found in rat mesenteric artery (4.3%/1 mV) [61].

In porcine coronary artery, voltage-dependent potassium channels regulate the tone both at resting and stimulated conditions [62,63]. Concerning the mechanism of action, OR-2828 appears to activate, at least in part, the 4-aminopyridine-sensitive voltage-dependent potassium channels. In this respect, the drug resembles levosimendan which relaxes porcine coronary arteries partly through voltage-dependent as well as calcium-dependent potassium channels [64]. 4-aminopyridine-sensitive potassium channels were proposed to play a role in the coronary artery vasomotor tone under pathological conditions, as demonstrated in human coronary arteries [65]. Beneficial haemodynamic effects of both OR-2828 (our unpublished observation) and levosimendan [66] have been presented in experiments performed in pathological cardiac models in the dog, and with levosimendan in human severe heart failure [67]. In addition, more recent observations have revealed direct inhibition by high-fat diet and

cholesterol of 4-aminopyridine-sensitive potassium channels, thereby disturbing adenosine-mediated coronary autoregulation [68,69]. High glucose level also directly impairs 4-aminopyridine-sensitive voltage-dependent potassium channels [70].

5.2. Resistance arteries

Our findings provide, for the first time, evidence suggesting that CNP possesses the characteristics of an EDHF contributing to vasodilation of human penile small arteries (see Figure 16, 17). Thus, direct electrophysiological measurements revealed that CNP hyperpolarizes penile arterial smooth muscle. CNP caused potent relaxations which were also present after inhibition of NO synthase and cyclooxygenase. These EDHF type relaxations were inhibited in the presence of blockers of calcium-activated K^+ channels, apamin plus charybdotoxin.

As mentioned in the Introduction, several candidates may contribute to EDHF-type relaxation in arteries. CNP is a likely candidate for an EDHF in human penile arteries. CNP receptors are present in the corpus cavernosum of different species [71-73] and as we demonstrated in the present study, CNP evoked relaxations in the presence of inhibitors of NO synthase and cyclooxygenase and in arteries without endothelium. The biological activity of CNP can be mediated by activation of NPR-B, that is present in the corpus cavernosum of different species including man [71-73], and is linked to particulate guanylyl cyclase and the formation of cyclic guanosine monophosphate (GMP) [74]. Activation of the cyclic GMP pathway has been found to lead to activation of both Na^+/K^+-ATPase and BK_{Ca} in horse penile arteries [75,76], and of Na/K-ATPase in human corpus cavernosum [77]. Moreover, CNP evoked relaxation in systemic arteries was described to be inhibited by iberiotoxin, a blocker of BK_{Ca} channels, [78-81]. In the present study iberiotoxin markedly reduced CNP relaxation suggesting BK_{Ca} channels are involved in CNP induced relaxation in human penile small areries.

6. SUMMARY AND CONCLUSIONS

The results presented in this thesis can be summerized as follows:

1. CNP was proved to have significant relaxant effect on rat isolated systemic arteries. The order of maximal relaxation was found to be as follows: carotid arteries > mesenteric arteries > femoral arteries.

2. The relaxant effect of CNP was found to be endothelium-independent in porcine isolated coronary arteries. The vasorelaxant effect of low concentration of CNP was augmented by the endothelin inhibitor, PD142893, in the presence but not in the absence of endothelium.

3. The NEP-inhibitors (phosphramidon, thiorphan) potentiated the relaxant effect of CNP both in endothelium intact and endothelium deprived porcine isolated coronary artery preparations.

4. CNP hyperpolarized the porcine isolated coronary arteries. The magnitude of OR-2828 exerted hyperpolarization was similar, the maximal effect of levosimendan was found to be smaller compared to the endogenous peptide, CNP.

5. Correlation was found between hyperpolarization and relaxation induced by OR-2828. K_v channels partly mediated hyperpolarization as it was proved by the decrease of OR-2828 induced relaxation by the K_v channel blocker, 4-AP.

6. The relaxant and hyperpolarizing effect of CNP was endothelium independent in resistance type penile arteries isolated from humans. Hyperpolarization was preserved in the endothelium deprived preparations, facing to the effect of the endothelium dependent relaxant, Ach, that was abolished.

7. CNP revealed to be a non-adrenergic/non prostaglandin type relaxing agent in human penile resistance arteries. BK_{Ca} channels were involved in the relaxing effect of the peptide as it was proved by using a selective inhibitor of the channels, iberiotoxin.

The vascular effect, the functional consequence of NEP inhibition on the blood vessel tone, largely depends on the kind of the peptide substrates for the enzyme available at the targeted area of circulation [37,82-84]. While the source of certain substrates of the enzyme, such as bradykinin, substance P and endothelin is the vascular endothelium, CNP has also been found in the medial and adventitial layers of the coronary arteries [39]. Thus, local

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delivery of CNP and the inhibition of its degradation may have a crucial role in the improvement of vasorelaxing capacity of large coronary arteries with damaged endothelium, e.g. atherosclerotic lesions [85]. NEP 24.11. is constitutively expressed in endothelial and smooth muscle cells [44,86]. The functional role of NEP in CNP degradation is proved in NEP/ACE inhibitor, omapatrilat, pretreated rat myocardial arterioles and capillaries by identification of the amplified antihypertrophic effect of the peptide compared with the effect of an ACE inhibitor, fosinopril [87].

The present observations raise the possibility that enhancement of hyperpolarizing and vasodilator effect of C-type natriuretic peptide by the inhibition of the NEP activity could moderate the vasospastic tendency of diseased coronary segments with endothelial dysfunction or with complete endothelial defect. The results suggest that NEP inhibitors could endue this endogenous peptide with therapeutic potency against vasospasm and strengthen the effect of CNP as an endogenous defensive factor against intimal hyperplasia in severe coronary artery diseases.

The results of the comparative studies raise the possibility that coronary artery relaxants with hyperpolarizing effect, such as OR-2828 and levosimendan, might also be advantageous in some metabolic alterations leading to coronary artery diseases.

In point of the results on human penile arteries an apparent limitation of the examined samples could be that they were obtained from patients undergoing transsexual operations, and we cannot entirely exclude that hormonal treatments influence the endothelial responses. However, the advantage, on the other hand, is that none of the patients had cardiovascular disease, which is known to alter endothelium-dependent vasodilation in both systemic [88] and penile arteries [89–91]. Thus, in erectile tissue and penile arteries from patients with diabetes, endothelium dependent relaxation is blunted [92,93]. The same applies to the penile arteries and erectile tissue isolated from hypertensive and diabetic animals [94,95]. In penile arteries mainly, the EDHF-type relaxation is impaired in hypertensive animals [94]. Our data suggest that CNP possesses the characteristics of an EDHF in human penile resistance arteries, because it causes hyperpolarization of the penile smooth muscle. CNP causes potent relaxations sensitive to the blockers of K^+ channels present both in the vascular smooth muscle cells and the endothelium. Modulation of this pathway opens for new treatment modalities of erectile dysfunction.

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