Original Article

The Vasodepressor Function of the Kidney: Further Characterization of Medullipin and a Second Hormone Designated Angiolysin

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The objective of this study was to further characterize the antihypertensive properties of medullipin and a second hormone designated angiolysin physiologically. Angiolysin and medullipin were tested in coronary and aortic rings from cows, sheep, pigs, mice and rats. *In vivo* animal experiments were performed using spontaneously hypertensive rats. Medullipin was successfully separated from another antihypertensive agent at the polar end of the polarity continuum. It is an extremely potent vasodilator. With the methods available today, it is not possible to make a galenical preparation of medullipin for *in vivo* analysis. The newly discovered antihypertensive agent is another extremely potent vasodilator, even stronger than medullipin, and was therefore named angiolysin. The vasodilatory activity of both medullipin and angiolysin persisted for hours, on rat and mouse aortae, and on the coronary arteries of pigs, cows and sheep. Both substances exerted their effects even in animal rings that were precontracted with 100 mmol/l K⁺. Angiolysin reduced the resting tension in blood vessels from mice and rats even without precontraction. A single injection of angiolysin resulted in a dose-dependent reduction of blood pressure, independent of the initial blood pressure, even to zero if the dosage was sufficient. The effect persisted for several hours. In conclusion, both hormones are extremely potent vasodilators, and are expected to lead to paradigmatic changes in the treatment of hypertension. With regard to potency, only sodium nitroprusside is comparable, but the effects of medullipin and angiolysin persist for hours after a single injection. (*Hypertens Res* 2006; 29: 533–544)

Key Words: hypertension, angiolysin, medullipin, blood pressure, renal function

Introduction

Although the kidneys contribute to hypertension through the renin–angiotensin–aldosterone system, they also exhibit an antihypertensive activity, which appears to originate in the renal medulla (1). The search for renal substances that mediate this antihypertensive action has led to the discovery of three substance entities: the prostaglandins (2–4), the antihypertensive polar renomedullary lipid (APRL) (5), and the antihypertensive neutral renomedullary lipid (ANRL) (6),

whose structure has yet to be elucidated. APRL is a semisynthetic product that does not occur naturally (7). ANRL, later called medullipin, could not be separated from the other renal medullary lipids with the means available at the time (8). Thus, the initial goal of this study was to achieve the further chromatographic separation of a renal medullary extract. To our great surprise, medullipin as a neutral lipid was separated from a much stronger agent. As a result, the goals of our study were the following: 1) characterization of the fraction containing ANRL and its activity; 2) galenical preparation of the fraction containing ANRL; 3) characterization of the fraction

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containing the newly discovered hormone and of the hormone itself, and clarification of its mechanism of action.

The newly-discovered antihypertensive agent was named angiolysin due to its extremely potent vasodilatory properties.

Methods

Materials

The prostaglandins A₂, E₂ and F_{2α}, norepinephrine and acetylcholine chloride were obtained from Sigma (St. Louis, USA). Prostacyclin (synonymous with prostaglandin I₂) was procured from ICN Biomedical Inc. (Eschwege, Germany). Pig kidneys were purchased from Westfleisch (Coesfeld, Germany). Potassium chloride, sodium chloride, calcium chloride, potassium dihydrogen phosphate, magnesium sulfate, sodium hydrogen carbonate, edetate calcium disodium, acetylcholine chloride, serotonin, norepinephrine chloride, and sodium nitroprusside dihydrate were obtained from Sigma Chemicals Inc. (Deisenhofen, Germany). All organic solvents used were of analytical grade and were obtained from Riedel De Haen (Seelze, Germany). *Aqua bidestillata* was used in the preparation of aqueous solutions. Duran® glass was used for all organ chambers, dissection baths, and laboratory equipment.

Dissection of the Renal Medulla

The renal medulla was obtained from pig kidneys. Immediately following the slaughter of the animals and excision of their kidneys, the latter were placed in crushed ice and after approx. 45 min were flash frozen in liquid nitrogen and stored at -40°C until dissection. The frozen kidneys were then cut lengthwise into 1–2 mm thick slices. The renal medulla was then carefully removed and immediately replaced in the liquid nitrogen. After this, the frozen specimens were pulverized in the liquid nitrogen-cooled steel beaker of a Thermomix 3300 electric mixer (Vorwerk, Wuppertal, Germany) and subsequently freeze-dried (Suwelack, Billerbeck, Germany).

Extraction

To extract the medulla, the freeze-dried material was sprinkled with chloroform and subsequently mixed with a mixing rod (Ultra-Turrax®; Janke & Kunkel, Staufen, Germany) for 10 min. After filtration, the material was extracted exhaustively until the filtrate became colorless. The solution was then dried at room temperature *in vacuo* until a thick, dark yellow oil remained. This residue was subsequently spread, along with some chloroform, on ICN silica gel (irregular particles of size 32–64 µm; pore size 60 Å), blended and then desiccated.

Chromatography

Column chromatography was carried out in a glass column filled with the ICN silica gel mentioned above. Elution was performed through a gradient, beginning with hexane and continuing through hexane–diethylether, hexane–diethylether–ethanol, diethylether–ethanol, diethylether–ethanol–methanol, ethanol–methanol to pure methanol.

Preparation of Specimens for the Animal Experiments

To prepare the specimens for the animal experiments, the lipid residue was first sprinkled with ethanol (Merck, Darmstadt, Germany) and then mixed with water (Braun, Melsungen, Germany) in a 1:19 (v/v) ratio. This mixture was then homogenized in a Branson Sonifier 250 microsuspension device (Branson Inc., Danbury, USA), in pulse mode using a 1/8 s microtip (1–3 pulses at 5 to 30 W for 15 to 30 s).

Calculation of the Doses for Tests on Animals and on Isolated Vessels

General-dose findings led to the injection of an arbitrary 10 mg fraction per kg of body weight in an aqueous suspension. To calculate dose dependency in the current study, doses of 5, 10, and 20 mg per kg of body weight were used. These doses were then carried over to the tests of the isolated vessels, *i.e.*, they were converted to the organ bath volume, and concentrations of 100 ng, 1 µg, 10 µg, and 100 µg per ml were tested in the organ bath.

Calculation of Size Distribution of the Injected Suspensions

The turbidimetric determination of particle size distribution in the aqueous solution produced through microsuspension was carried out with the aid of photon correlation spectroscopy (PCS). An autosizer outfitted with a multi-8 correlator was used for this purpose (Malvern autosizer 2c series 7032; Malvern Instruments, Herrsching, Germany).

Gas Chromatography Coupled with Mass Spectrometry Trace Analysis of the Prostaglandins A₂, E₂, F_{2α} and I₂

Capillary gas chromatography coupled with mass spectrometry (GC-MS) was used to detect traces of the prostanoid substances prostaglandin A₂, E₂ and F_{2α} and I₂ (prostacyclin). After derivatization in a typical manner, analysis was carried out with a GC-Q instrument (Finnigan MAT, Bremen, Germany), outfitted with a 30 m DB-5 capillary GC column. The detection limit for prostaglandins in the fraction was 2.2 ppm.

Fig. 1. Vascular tone of a mouse aorta, plotted in g on the y-axis, against the time in h on the x-axis. Application of 100 mmol/l K^+ (arrow) and subsequent application of the fraction VC 17–27, medullipin (arrow), resulted in almost complete abolition of the contraction.

Dissection of Coronary Artery and Aortic Rings

Hearts from cows, sheep and pigs were used for the dissection of the coronary arteries. These were rinsed and filled with a 4°C Krebs solution and cooled down to 1–4°C. Dissection of the coronary arteries was carried out in a 4°C Krebs-Ringer bicarbonate solution (composition in mmol/l: $CaCl_2$ 2.5; KCl 4.7; KH_2PO_4 1.2; $MgSO_4$ 1.2; NaCl 118.6; $NaHCO_3$ 25.1; calcium disodium EDTA 0.026, and glucose 11.1). The vessels were transected into rings 2–3 mm wide. Aortas were excised from laboratory mice (Crl:CFW®(SW) BR) after pentobarbital anesthesia (50 mg/kg body weight of pentobarbital intraperitoneally) with the aid of a magnifier. The vessels were filled with a 4°C Krebs-Ringer bicarbonate solution as soon as feasible, isolated, removed and placed into a 4°C Krebs-Ringer bath (pH 7.3). The arteries were dissected under a microscope. Rings of 2–3 mm width were used in the organ chamber experiments. The same procedure was employed for dissecting the rat aortas. Study design and protocols followed the guidelines set by the institutional animal care committee and the American Heart Association for research animal use.

Vascular Function

Aortic and coronary artery rings, respectively, were suspended from tungsten stirrups in organ chambers (IOA-5300; Foehr Medical Instruments, Seeheim, Germany) containing 10 ml Krebs bicarbonate solution (37°C, pH 7.4, 95% O_2 and 5% CO_2). They were allowed an equilibration period of 60 min. Next, the resting tension was gradually increased in 7 to 10 increments, in order to avoid overexpansion. Rings were repeatedly exposed to 100 mmol/l KCl until the optimal tension for generation of force during isometric contraction was reached. There was no difference in precontraction among the different groups of aortas and coronary arteries and also no significant difference among the groups in the contractions after exposure to KCl (data not shown). After equilibration for 30 min, the rings were again precontracted with 100 mmol/l KCl until a stable plateau was reached. The control

solution consisted of 6 parts of ethyl alcohol mixed with 143 parts of water (v/v). Alternatively, the experiments were carried out without precontraction or after prior precontraction with norepinephrine. These substances were generally applied cumulatively. Constriction experiments with norepinephrine and serotonin, respectively, and relaxation experiments with acetylcholine (1×10^{-10} to 3×10^{-5} mol/l) and sodium nitroprusside (1×10^{-10} to 3×10^{-5} mol/l) were subsequently performed in order to demonstrate the vitality and functionality of the rings. Norepinephrine contractions were completely abolished by acetylcholine, proving that the endothelium was functional after the experiments. The diameters of the arterial rings were calculated. The dimensions of the arterial rings used in this study did not differ significantly (data not shown).

Animal Experiments

For the animal experiments, adult spontaneously hypertensive Wistar rats (SHR/N Crl BR) with an average weight of about 400 g were used (20–30 weeks; Charles River, Sulzbach, Germany). Experiments on 10–20-week-old Lewis rats (Charles River) were carried out as well. Under ketamine-diazepam anesthesia (50 mg/kg body weight and 3 mg/kg body weight, respectively), each of the extracts in a volume of 1.5 ml was injected over a period of approximately 10 s into the superior vena cava. Blood pressure was measured intraarterially in the common iliac artery or the distal aorta, and was recorded with a sampling frequency of 17 Hz. All animal experiments were assessed by the Animal Protection Commission of the Westfälische Wilhelms University of Münster, and approved by the District Government Office in Münster (G42/99 RP MS). The NIH guidelines for care and use of laboratory animals were followed strictly.

Data Analysis, Statistics, and Protocol Design

All data are given as the means $\pm$ SEM. Relaxations are expressed as absolute values in g, and as percentages of precontractions. Endothelium-dependent relaxation was calcu-

Fig. 2. Blood pressure in the femoral artery of a spontaneously hypertensive rat, plotted on the y-axis against the time on the x-axis. There was an almost instantaneous drop in blood pressure following the application of 20 mg/kg body weight of fraction VC 47–73, angiolysin (arrow). Several attempts were made to counteract the drop in blood pressure at approximately 110 mmHg with plasma expanders (1 ml each time) (arrows; v, volume). Nevertheless, 3 h after application of the fraction, angiolysin continued to lower the blood pressure.

lated as the maximum vasodilatory response of the vessel to acetylcholine, referring to the maximum constrictor response to norepinephrine and serotonin, respectively. Relaxations were normalized to the maximal contractile response to 100 mmol/l KCl. Due to the very limited quantity of material, the determination of EC_{50} could only be approximated. Where not explicitly described, contraction and endothelium-dependent relaxation capacity were demonstrated by administering norepinephrine and acetylcholine, respectively, at the conclusion of the experiments. The subsequent contractions and relaxations were statistically significant in all groups studied and thus are not always indicated separately due to space limitations. The maximum blood pressure readings or tension readings at the peak point were compared with the respective blood pressure readings or tension readings immediately before injection of the mixtures. The Mann-Whitney *U* test was used where appropriate. In most cases, the Wilcoxon matched-pairs test was used. One-way ANOVA and post tests for linear trends were carried out with the aid of the program Graph Pad Prism (Graph Pad, San Diego, USA) according to

current guidelines (9). A *p* value <0.05 was considered to indicate statistical significance.

Results

Active Fractions

The two active fractions designated VC 17–27 and VC 47–73 each appeared as a highly viscous yellowish amorphous residue. Fraction VC 17–27 was an eluate, which was eluted with a mixture of hexane and diethyl ether in a ratio of 80:20 v/v. To extract the total fraction using the polarity gradient, the percentage of diethyl ether was raised to 40%. Upon obtaining a hexane to diethyl ether ratio of 60:40 v/v, a new fraction was initiated, whose inefficacy could be proven (data not shown). Fraction VC 17–27, eluted with solvent mixtures of slight to medium polarity, contained the ANRL described by Muirhead (6), *i.e.*, medullipin. Fraction VC 47–73 was at the polar end of the polarity gradient chromatography. It was eluted from the column with large volumes of diethyl ether, ethanol, and finally with pure methanol, and contained the newly discovered hormone, which was named angiolysin.

A preliminary physicochemical characterization placed angiolysin in the rather polar range of lipids covered by chloroform extraction. Angiolysin behaved neutrally, its molecular weight was less than 2,000 amu, and its overall chromatographic behavior indicated what today is considered a “small” molecule. It was stable at neutral to moderately acidic pH and at up to 50°C. The average dosage was estimated for a single constituent based on high performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) profiling to be in the few $\mu\text{g/kg}$ body weight range or below, which corresponds to typical physiological concentrations of hormonal substances.

GC-MS Trace Detection of Prostaglandins and Turbidimetry of the Fractions

Capillary GC-MS revealed no traces of the vasodilatory prostaglandins A_2 , E_2 , $F_{2\alpha}$, and I_2 mentioned above. On the basis of the detection limits it can be stated with certainty that if these substances were present in the samples injected into the animals at all, their concentrations would have been less than 10^{-11} mol per animal. Such low concentrations could not be responsible for the effects detected.

Particle Size

The turbidimetrically determined particle size found in the specimen of fraction VC 47–73 was 184 ± 9.8 nm ($n=6$), which is well below the upper limit that ensures capillary passage through the lung. Furthermore, the particle size distribution was found to be of Gaussian lineshape. The particle size in the specimen of fraction VC 17–27, however, could not be reduced to suitable values. Despite the use of a variety of

Fig. 3. Systolic (triangles) and diastolic (closed squares) drop in blood pressure 2 min (a) and 60 min (b) after completion of the application of fraction VC 47–73 (angiolysin) in four dosages: 5 ($n=6$), 10 ($n=7$), 20 ($n=5$), and 50 mg/g ($n=2$) body-weight. The drop in blood pressure was plotted in mmHg on the y-axis against the concentration of the fraction in mg/g body weight on the x-axis. The percentage drop in blood pressure (y-axis), relative to the initial blood pressure before application of fraction VC 47–73 (angiolysin, x-axis) is shown 2 min after completing the injection (c) and 60 min after completing the injection (d).

state-of-the-art techniques, including emulsification, for the galenic optimization of the fraction, flocculation occurred consistently and prevented the determination of a meaningful average particle size. The fraction could thus not be prepared in a suitable galenic form and, like fraction VC 1–16, was only used in organ chamber experiments.

Responses to Control Solution, Other Fractions, and Kidney Cortex

Baseline blood pressure prior to intravenous administration of 1.5 ml of the control solution was constant. Two min after injection, blood pressure remained almost constant ($-0.7 \pm 2.9/0.1 \pm 3.2$ mmHg, $n=6$, $p>0.05$), as well as at 1 h following application ($0.14 \pm 3.9/0.4 \pm 3.8$ mmHg), indicating that the control solution had no effect on blood pressure ($p>0.05$). The other fractions showed no effect on blood pressure. Upon injection of an extract prepared in analogous fashion from the kidney cortex, virtually no change of blood

pressure was observed (increase of 0.1 ± 1.3 mmHg, $n=7$, $p>0.05$, 1 h after injection).

Vascular Response to Medullipin

Applying fraction VC 17–27 to the mouse aorta after precontraction with 100 mmol/l K^+ brought about a reduction of tension from 0.86 ± 0.12 g to 0.13 ± 0.06 g ($n=17$; $p<0.0001$, Wilcoxon test). The potassium contraction was thus nearly abolished. The average reduction was $-88.7 \pm 17.1\%$ of the initial contraction, whereas a non-significant increase of $2.0 \pm 6.2\%$ ($n=8$, $p=0.4796$, Mann-Whitney U test) was caused by applying the control solution. The difference from the control group, at $p<0.0001$, was also statistically significant. The cumulative administration of noradrenaline up to a dose of 10^{-6} mol/l caused a contraction of 0.83 ± 0.19 g ($n=8$; $p=0.0078$, Wilcoxon test) compared to the baseline value. The subsequent cumulative administration of acetylcholine up to a dose of 10^{-6} mol/l caused a reduction of 0.80 ± 0.47 g

Fig. 4. *a*: Vascular tone of a mouse aorta, plotted on the y-axis in g against the time in h on the x-axis. Fraction VC 47–73 (angiolysin) was applied after precontraction with 40 mmol/l K⁺. *b*: Relaxation as a percentage of the initial K⁺ contraction is plotted on the y-axis, against the inversely plotted K⁺ concentration on the x-axis.

($n=8$; $p=0.0039$, Wilcoxon test). The rings were thus shown to be vital. Figure 1 provides an example of relaxation after the administration of fraction VC 17–27.

Blood Pressure Response to Fraction VC 47–73 (Angiolysin)

Figure 2 shows the drop in blood pressure after administration of 10 mg/kg body weight of fraction VC 47–73. The onset of antihypertensive activity was detected almost immediately following application. This response was initially obscured, partly due to the presence of a hypertensive substance, which briefly triggered a rise in blood pressure. This blood pressure–increasing substance, however, can be separated from the blood pressure–lowering substance as previously described (10).

Concentration Dependency of the Blood Pressure Response to Fraction VC 47–73 (Angiolysin)

The concentration dependency of the action of angiolysin was established using four concentrations, *i.e.*, 5, 10, 20, and 50 mg/kg body weight. Baseline blood pressure of the rats prior to administration of the fractions was constant. The blood pressure decrease was statistically significant, as determined by comparing the baseline blood pressure in each group with the mean blood pressure 2 min or 1 h after administration of the specimens, respectively (Wilcoxon test, $p<0.05$ for each

comparison).

Figure 3a shows the drop in blood pressure in spontaneously hypertensive rats 2 min after completing the injection of 5, 10, 20, and 50 mg/kg body weight of fraction VC 47–73. There was an asymptotic approach in the drop in blood pressure to a limit, which was around 30 mmHg diastolic and 40 mmHg systolic. Accordingly, the ANOVA, at $p=0.2330$ for the systolic and $p=0.2602$ for the diastolic blood pressure, did not exhibit statistical significance. In contrast, as shown in Fig. 3b, blood pressure dropped 1 h after completion of the application relative to the dose; at higher doses, the reduction was more than 100 mmHg ($p<0.0001$, ANOVA and post test for linear trend, systolic and diastolic values). A plot of the percentage drop in blood pressure relative to the initial blood pressure against the concentration demonstrated that the observed blood pressure reduction was clearly dose-dependent (Fig. 3d).

Heart Rate during Administration of Fraction VC 47–73 (Angiolysin)

The heart rate was determined from the continuous analogue blood pressure measurements. Prior to the administration of the samples, both the heart rate and the blood pressure remained constant. Immediately after injection, a sharp heart rate decline occurred due to the volume load, but there was no difference between the control group and the angiolysin group. A few seconds after injection, the heart rate returned to

Table 1. Murine Aortic Rings: Contractions upon Administration of 20, 40, and 100 mmol/l of K^+ , as Well as upon Administration of 10^{-6} mol/l Norepinephrine (g); Tension after Relaxation Observed upon Subsequent Application of Fraction VC 47–73; Vascular Responses upon Administration of Fraction 47–73 in % Referring to the Initial Contractions; Number of Experiments; and p Value of the Wilcoxon Test in Each Group

	Contraction (g)	10 μ g/ml angiolsin (tension, g)	Relaxation (% of K^+ contraction)	n	p (Wilcoxon test)
Potassium 100 mmol/l	0.62 ± 0.05	0.43 ± 0.05	35.5	20	<0.0001
Potassium 40 mmol/l	0.41 ± 0.06	0.22 ± 0.05	50.8	9	0.0020
Potassium 20 mmol/l	0.23 ± 0.04	0.06 ± 0.07	73.9	4	not tested
Norepinephrine 10^{-6} mmol/l	0.21 ± 0.04	-0.03 ± 0.03	-100	9	0.0020

Fig. 5. a: Vascular tone of a rat aorta, plotted on the y-axis in g against the time in h on the x-axis. Fraction VC 47–73 (angiolsin) was applied following precontraction with 40 mmol/l K^+ . b: Relaxation as a percentage of the initial K^+ contraction is plotted on the y-axis against the inversely plotted K^+ concentration on the x-axis. c shows the relaxation after the cumulative precontraction with 10^{-6} mmol/l norepinephrine (NE) and application of fraction VC 47–73 (angiolsin).

initial levels in both groups, with $101.3 \pm 5.4\%$ of the initial value in the control group, and $98.2 \pm 6.1\%$ of the initial value in the angiolsin group 2 min after injection ($p > 0.05$). Subse-

quently, the heart rate remained stable throughout the whole experiment (1 h after injection), showing no significant difference compared to the initial heart rate at any time, and no

Table 2. Rat Aortic Rings and Sheep, Cow and Pig Coronary Arteries: Contractions upon Administration of 20 (Rat Aortic Rings), 40 (Rat Aortic Rings), and 100 mmol/l of K⁺ (Rat Aortic Rings and Sheep, Cow and Pig Coronary Arteries); Tension after Relaxation Observed upon Subsequent Application of Fraction VC 47–73; Vascular Responses upon Administration of Fraction VC 47–73 in % Referring to the Initial Contractions; Number of Experiments; and *p* Value of the Wilcoxon Test in Each Group

	Contraction (g)	10 µg/ml angiotensin (tension, g)	Relaxation (% of K ⁺ contraction)	<i>n</i>	<i>p</i> (Wilcoxon test)
Rat aortic ring					
Potassium 100 mmol/l	6.43±0.4	4.32±0.34	32.8	11	0.0005
Potassium 40 mmol/l	6.60±0.62	3.87±1.36	41.4	8	0.0357
Potassium 20 mmol/l	2.74±0.36	1.16±0.27	57.7	5	0.0313
Sheep coronary ring					
Potassium 100 mmol/l	10.1±2.8	7.5±2.8	25.7	4	not tested
Cow coronary ring					
Potassium 100 mmol/l	9.4±1	6.6±1.3	29.8	4	not tested
Pig coronary ring					
Potassium 100 mmol/l	12.0±1.8	9.7±1.9	19.2	3	not tested

statistically significant trend.

Vascular Response to Fraction VC 47–73 (Angiotensin) after Precontraction

Figure 4a shows the following sequence of events and responses in a mouse aortic ring: precontraction with 40 mmol/l K⁺; the immediate onset of relaxation after subsequent application of 10 µg/ml of fraction VC 47–73; deceleration of relaxation after a few minutes; and nearly constant relaxation until the recording was discontinued about 60 min later. Figure 4b represents the relaxation as a percentage of the initial K⁺ contraction. The lower the concentration of K⁺ for precontraction of the vessels, the stronger the relative relaxation after application of the fraction ($p=0.0002$, ANOVA and post test for linear trend).

Table 1 shows the extent of contraction in the aorta of mice upon administration of 20, 40, and 100 mmol/l of K⁺, as well as of 10⁻⁶ mol/l norepinephrine, and the relaxation observed upon subsequent application of fraction VC 47–73. The contraction upon administration of 20 mmol/l K⁺ was almost completely abolished; the contraction upon administration of 10⁻⁶ mmol/l norepinephrine was reduced to even less than the initial tension level. The aortic rings were shown to be fully vital at the end of the experimental protocol by terminal acetylcholine relaxation experiments (cumulative: 10⁻⁶ mol; relaxation of 0.24±0.05 g; $p<0.0001$). The EC₅₀ value was calculated by analyzing a series of concentrations of the fraction at 1, 10, and 50 µg/ml. The concentration could not be further increased due to the lack of sample material. The relaxation was dose-dependent ($p=0.0004$, ANOVA; $p<0.0001$, post test for linear trend; $n=9$ for each concentration). The EC₅₀ of fraction VC 47–73 on the mouse aorta was thus determined to be 62 µg/ml.

Analogous results were obtained for the aortic rings from

the rat. Figure 5a shows the reaction of a rat aortic ring to the application of 10 µg/ml of fraction VC 47–73 after precontraction with 40 mmol/l of K⁺. The application of the fraction caused an immediate onset of relaxation, which decelerated after a few minutes and then remained constant until the end of the recording about 120 min later. Figure 5b shows the relaxation as a percentage of the initial K⁺ contraction. The lower the concentration of K⁺ for precontraction of the vessels, the stronger the relative relaxation after application of the fraction ($p=0.0197$, ANOVA; $p=0.0057$, post test for linear trend). Figure 5c shows the relaxation after the cumulative precontraction with 10⁻⁶ mmol/l norepinephrine and application of fraction VC 47–73.

Table 2 shows the extent of contraction in the aorta of rats upon administration of 20, 40, and 100 mmol/l of K⁺, and the relaxations upon subsequent application of fraction VC 47–73. All differences were statistically significant ($p<0.05$ each). The aortic rings were shown to be fully vital at the end of the experimental protocol by terminal acetylcholine relaxation experiments. Figure 6a shows an example of the complete abolition of a potassium-induced contraction by application of fraction VC 47–73.

Reduction of the Resting Tension of Mouse and Rat Aortas

Figure 6b and c show the reduction of resting tension after the application of fraction VC 47–73 to the mouse and rat aortas. This effect persisted for several hours. The precontraction for the mouse was 1.88±0.04 g for these trials. This value was reduced after application of fraction VC 47–73 to 1.72±0.07 g (Fig. 7, $n=11$, $p=0.0005$; Wilcoxon test). For the rat, the precontraction was 9.33±1.67 g. After application of fraction VC 47–73, this value was reduced to 8.34±1.79 g (Fig. 7, $n=13$, $p=0.0001$; Wilcoxon test).

Fig. 6. a: Vascular tone of a rat aorta plotted on the y-axis in g, against the time in h on the x-axis. Twenty mmol/l K⁺ (arrow) was applied, followed by fraction VC 47–73 (angiolysin, arrow). This treatment completely abolished the contraction and lowered the tone in resting tension. b shows the vascular tone of a mouse aorta, and c that of a rat aorta, after application of fraction VC 47–73 (angiolysin) without precontraction with potassium. The resting tension was reduced in both cases.

Fig. 7. Reduction of resting tension after application of fraction VC 47–73 (angiolysin) in mice and rats (n=11 and 13, respectively). Vascular tone is given in g. PC, precontraction; A, angiolsin.

Coronary Arteries of Cows, Pigs, and Sheep

Tests on coronary arteries of all three species resulted in vasodilation analogous to the results shown above. The data are shown in Table 2.

Response to the Fraction VC 47–73 in Normotensive Rats

Two normotensive Lewis rats were also administered 10 mg/kg body weight of fraction VC 47–73. The changes in blood pressure in these animals following application of the fraction are not illustrated due to the limited space, but there was no appreciable difference between these changes and the observations in spontaneously hypertensive rats.

Discussion

In summary, the results of this study show that there are two extremely potent vasodilatory components in the renal medulla. The first of these components corresponds to the

long-postulated medullipin. For the first time, medullipin was shown to be a potent vasodilator. Purified medullipin is thus far not available in a galenic quality adequate for *in vivo* tests. *In vitro*, it almost completely abolished a potassium-induced vasoconstriction of the mouse aorta, and the effect persisted for several hours. The bioavailability for the arterial wall was sufficient for a further bioassay-guided fractionation in the selected galenic preparation. The second vasodilatory component was at the polar end of the polarity continuum of the extract. It lowered blood pressure to any level desired, dependent on the dose, and independent of the initial blood pressure; at sufficiently high doses, it could even lower the blood pressure to zero. Because of its extreme potency as vasodilator, it was named angiolysin. After one injection of the fraction VC 47–73 (angiolysin), the antihypertensive or vasodilatory effect persisted for hours. It can be speculated that a central effect is involved, since the heart rate remains unaffected despite the extremely potent vasodilatory activity. Moreover, whether the baseline values are hypertensive or normotensive was irrelevant to the antihypertensive effect of angiolysin. The fraction VC 47–73 (angiolysin) reduced the resting tension of blood vessels even without precontraction. The results on the rat could be reproduced in the mouse and were the same in the coronary arteries of the cow, pig, and sheep, demonstrating the universal nature of the effect. The vasodepressor effect was shown to be specific for the kidney medulla, and could not be obtained using the kidney cortex. This finding was consistent with prior results, demonstrating the combined kidney medulla organ and tissue specificity (1, 11, 12). Galenic preparation as used here is straightforward, and appears to be suitable for a bioassay-guided fractionation.

In 1967, prostaglandin E₂ was identified as the most significant antihypertensive component in the renal medulla (2), but the fraction described in the present study did not contain this substance. The existence of renal antihypertensive properties has been known ever since Grollman *et al.* (13) and Page *et al.* (14) reported that renal extracts could reverse changes in the ocular fundus in patients with arterial hypertension (14) as well as the hypertension itself (13). Later it was clearly demonstrated that this antihypertensive activity occurs in the renal medulla and that kidney explantation-induced renoprival hypertension can be forestalled through replantation of homogenates from whole kidneys and the renal medulla, but not through replantation of homogenates from the renal cortex (1). This report marked the beginning of intensive efforts to identify and describe the substances responsible for the antihypertensive mechanism(s) underlying this effect, regarding which the following three hypotheses have thus far been proposed.

Hypothesis 1: In 1967 the prostaglandins A₂, E₂ and F_{2α} were isolated from the renal medulla (2, 3). Prostaglandin E₂ was described as the main vasodepressor mechanism in the renal medulla (2).

Hypothesis 2: Prewitt *et al.* described a lipid which they designated an antihypertensive polar renomedullary lipid

(APRL) based on its physicochemical characteristics (7). The natural provenance of this substance is highly questionable, however, as it can only be obtained semi-synthetically. The authors themselves have stated that APRL is “semisynthetic” and “may not be an endogenous agent” (7). Be that as it may, the phenomenon observed by Page cannot be attributed to the antihypertensive properties of APRL, since the vasodepressor effect of APRL is of short duration (6).

Hypothesis 3: Another vasodepressor lipid described by Muirhead was initially termed an “antihypertensive neutral renomedullary lipid” (ANRL) due to its physicochemical characteristics (6), and was later renamed medullipin (15). The fraction containing medullipin in this study was free of prostaglandin, as shown by GC-MS analysis (limit of detection: 2.2 ppm). In addition, this study shows that medullipin can be further purified using modern chromatographic methods. It was proved for the first time that medullipin is a potent vasodilator. Despite the fact that it is impossible at the present time to make a qualitatively adequate galenic preparation of the fraction, it can be used for a bioassay-guided fractionation, which will ultimately allow structure analysis.

The antihypertensive activity described in fraction VC 47–73 containing the designated angiolysin differs markedly, however, from that of the groups of compounds alluded to above for the following three reasons: First, the activity cannot be accounted for by the presence of prostanoid substances. The antihypertensive substances under consideration here were not present in fraction VC 47–73 (angiolysin). Secondly, the activity cannot be accounted for by the presence of the vasodilator APRL (7). Whereas APRL is a semisynthetic substance, the activity we investigated here was isolated from porcine renal medulla. Thirdly, the mechanism cannot be accounted for by the presence of medullipin, as medullipin has been classified as a neutral lipid (16), which in our study has been separated from the activity of fraction VC 47–73 (angiolysin). Medullipin eluted from silica gel with a mixture of hexane and diethyl ether into fraction VC 17–27 and, thus, was found to be of medium polarity. In contrast, the angiolysin activity of fraction VC 47–73 occurs on the polar to very polar end of the polarity continuum.

Recently, substantial progress has been made regarding the role of vasoactive hormonal peptides such as adrenomedullin (17), vasopressin (18), or angiotensinogen (19), whereas comparatively little progress has been made in the characterization of small non-peptidic vasoactive substances, such as the endogenous digitalis-like factor (20) or the medulla-derived vasoactive factors discussed herein. In the latter case, the difficulty of characterization can be attributed to the following. 1) Because prostaglandin E₂ was described early on as the main vasodepressor mechanism in the renal medulla (2), this topic was not revisited until now. As it turns out, however, the medulla extract and fractions used in the present study were free of vasoactive prostaglandins. 2) The fact that prostaglandins were reported to be present in previously described kidney medulla extracts has been challenging the

hypothetical existence of medullipin. The present work shows that a neutral and strongly vasodilatory activity exists in a prostaglandin-free extract (evidence for "medullipin"), and that it can be prepared for *in vitro* testing, but presently not for *in vivo* testing. In addition, this activity can be distinguished from a more polar, similar biological activity, *i.e.*, that of angiolysin. Overall, this confirms the existence of Muirhead's "medullipin." 3) While previous attempts to further purify medullipin have failed for different reasons (16, 21), the vasodilatory *in vitro* properties of the medullipin activity observed here enable bioassay-guided fractionation without the need for *in vivo* bioassay. The fact that the polarity and analytical and chromatographical behavior of medullipin overlaps that of fatty substances is an inherent problem. However, the present work shows that the difficulties in galenic preparation of a suitable sample for bioassay can be overcome by homogenization and particle size characterization. The same applies to angiolysin, except that it is more polar and, therefore, less problematic to prepare as an aqueous injectable. 4) Because it had to be considered that the presence of vasoactive, blood pressure-lowering prostaglandins had been obscuring and/or superimposing the effects of medullipin, Muirhead *et al.* tried to discriminate between medullipin and prostaglandins based on particular features of the blood pressure responses to the different substances (22). However, although they were able to make a successful distinction, these differences cannot practically be utilized for a bioassay to guide fractionation. Based on the knowledge of the preparation of a vasodepressor prostaglandin-free extract and the separation of medullipin from angiolysin activities in the present study, a rather routine bioassay capable of measuring vasodilatation can now be considered for interference-free bioassay-guided fractionation. The widespread availability, demonstrated suitability and relatively simplicity of a stable biological method will allow for further characterization of active substances through targeted bioassay-guided fractionation (23–25).

The effect of angiolysin directly depends on the administered dose. Angiolysin activity corresponds to that of kidney extracts originally described by Page (14), especially with regard to the long duration. Both vasodepressor substances, the neutral medullipin and the polar angiolysin, appear to be chromatographically stable and, thus, suitable for isolation. Angiolysin has the unique ability to lower blood pressure to any desired level in a dose-dependent fashion. The only known substance with a comparable effect is sodium nitroprusside. Unlike nitroprusside, however, the effect of angiolysin persists for hours rather than seconds. Due to these inimitable characteristics of angiolysin, it is reasonable to envision that this substance has enormous potential to influence cardiovascular physiology and lead to paradigmatic changes in the treatment of hypertension.

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