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Renal mesangium is a target for calcitonin gene-related peptide

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Renal mesangium is a target for calcitonin gene-related peptide. Rat calcitonin gene-related peptide (CGRPα; EC_{50}, 1 nM) was shown to stimulate cAMP formation in cultured rat renal mesangial cells. CGRP concentration dependently (EC_{50}, 1 nM) also inhibited contraction of mesangial cells by angiotensin II (10 nM). Angiotensin II (10 nM) caused a transient increase of the intracellular calcium concentration from 140 nM to 480 nM in the mesangial cells, but these calcium transients were not altered by CGRP. CGRP (10 nM) decreased vascular resistance in the isolated rat kidney perfused at constant pressure (100 mm Hg; $P < 0.01$). The decreased vascular resistance was accompanied by a rise of the glomerular filtration fraction. CGRP, moreover, attenuated the effects of angiotensin II on renal vascular resistance and glomerular filtration ($P < 0.01$). In conclusion, CGRP causes relaxation of renal mesangial cells and decreases renal vascular resistance. As a result CGRP raises glomerular filtration and the filtration fraction. The effect may be linked to cyclic AMP formation. Thus, regulation of renal vascular and glomerular function may represent a novel action of CGRP apart from its cardiovascular effects.

Calcitonin gene-related peptide (CGRP) [1] is a 37 amino acid polypeptide synthetized as a result of tissue specific processing of the primary RNA-transcript of the calcitonin gene [1–4]. CGRP has been identified in the central and peripheral nervous system [2, 3], and it is considered to act as neurotransmitter or neuromodulator [5, 6], but CGRP is also present in thyroid C-cells [7]. Important actions of CGRP are on the cardiovascular system where CGRP causes vasodilation, and has positive chronotropic and inotropic effects on the heart [8–12]. Recently, CGRP was shown to directly stimulate renin secretion in renal juxtaglomerular cells [13]. To this end, CGRP immunoreactive nerve fibers were recognized in the proximity of renal glomeruli [13]. This localization indicates that CGRP may affect glomerular function through relaxation of mesangial cells. Since mesangial cells are modified vascular smooth muscle cells, and CGRP causes vasodilation, it seemed reasonable to assess the effect of CGRP on the function of mesangial cells. We have therefore examined the effect of rat CGRPα on cAMP formation and contractility of cultured rat mesangial cells. In addition, the effect of rat CGRPα on renal vascular resistance and glomerular filtration in the isolated perfused rat kidney was investigated. The results suggest that CGRP causes a cAMP mediated relaxation of renal mesangial cells, and as a result increases the glomerular filtration rate.

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Methods

Cultures of rat mesangial cells

Rat mesangial cells were cultured as described previously [14]. Briefly, renal glomeruli from male Sprague-Dawley rats were isolated by a sieving procedure. Isolated glomeruli were seeded in 75 cm^2 flasks and 7 cm^2 petri dishes in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 mg/ml insulin and 100 μg streptomycin and 100 IU penicillin per ml, and were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Twenty-one days after seeding, the cells were passaged for the first time using trypsin-EDTA. For the experiments mesangial cells from the primary culture and the first passage were used.

Cyclic 3', 5'-AMP measurements

For determination of cAMP accumulation the culture medium was withdrawn and replaced by Leibovitz-Medium (L-15) supplemented with 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX) and graded amounts of rat CGRPα. The culture dishes were placed on a heating block at 37°C. After incubation for 10 minutes the L-15 medium was withdrawn, and the dishes were placed on an ice block. Ice-cold buffer (0.4 ml) containing 5 mM potassium phosphate, 2 mM EDTA, 0.5 mM IBMX and 150 mM KCl, pH 6.8 were then added, and the cells were scraped with a teflon policeman. The obtained suspension was sonicated, boiled for five minutes and centrifuged. The supernatants were assayed for cAMP using a specific binding assay [15]. The pellets were lysed with 1 N NaOH and used for protein determination [16]. The L-15 medium withdrawn at the end of the incubations was centrifuged, and the supernatants were also assayed for cAMP.

Contraction studies

Mesangial cell cultures, grown in 2 cm^2 culture trays (24-well; Nunc, Wiesbaden, FRG), were washed twice, and incubated for two minutes at 37°C in Hanks' balanced salt solution (without bicarbonate, pH 7.2) supplemented with 1 mM calcium, 0.1% bovine serum albumin and graded amounts of

CGRPα. Angiotensin II was then added at a final concentration of 10 nM. Shape changes of the cells were followed by phase contrast microscopy. Mesangial cell shape changes were studied four minutes after the addition of angiotensin II, because recent studies indicate that angiotensin induced contraction may be followed by autogeneous relaxation after 10 to 30 minutes [17].

Photomicrographs were enlarged to a final magnification of 3000 and the contors of the cells were copied onto transparent paper. Areas and perimeters of the cells were determined by tracing these contors on a graphic tablet Hewlett-Packard (HP) 9111A by a morphometrical and graphic software written in HP-BASIC. The algorithms to determine areas and perimeters were based on routines provided by R.R. Mize [18].

Calcium measurements

Trypsin-EDTA dispersed rat mesangial cells were loaded for 20 minutes at 37°C with 1 μM fura-2/AM in a medium containing: 137 mM NaCl, 5.5 mM KCl, 0.8 mM Na_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.01 mM diethyltriaminopentaacetic acid, 20 mM Hepes, pH 7.45, and supplemented with 0.1% glucose and 0.1% BSA essentially as described [19]. Washed cells were incubated for an additional 10 minutes at 37°C in medium without fura-2/AM. Loaded cells were kept at room temperature until fluorescence measurements were performed. Fluorescence was measured at 37°C by spectrofluorimetry (Perkin Elmer 650-10 S, Norwalk, Connecticut, USA) with respective excitation and emission wavelengths of 340 nm and 500 nm, and slit widths of 4 nm and 10 nm. The fura-2 signal was calibrated by the addition of 50 μM digitonin for F_{max} and 20 mM EGTA, pH 8.5 for F_{min}. $(Ca^{2+})_i$ was calculated from the equation: $(Ca^{2+})_i = K_d(F - F_{min})/(F_{max} - F)$, assuming a K_d of 224 nM.

Isolated perfused rat kidney

Male Wistar rats (260 to 290 g) were fed a commercial chow (Altromin standard pellets). Isolated rat kidneys were perfused as described [20]. Effective perfusion pressure was held constant at 100 mm Hg by feedback regulation of the perfusion pump. The pressure signal was taken through the inner part of a double barreled cannula within the aorta directly at the origin of the renal artery. The basic perfusion medium was Krebs-Henseleit physiologic saline with 8.7 mM glucose and 5% bovine serum albumin. A mixture of substrates and amino acids, and a 5 to 6% hematocrit fraction of freshly drawn and five-times-washed human erythrocytes were added. The perfusate (200 ml at the beginning, 150 ml during recirculation) was dialyzed continuously against 5000 ml of a protein free solution which was gassed with 95% O_2 and 5% CO_2. The basic medium (perfusate and dialysate) contained 0.3 mM pyruvate, 2.0 mM L-lactate, 1.0 mM α-ketoglutarate, 1.0 mM maleate, 6.0 mM urea, and 21 physiological amino acids in concentrations between 0.2 mM and 2 mM. Neomycin-sulfate (10 mg/liter), polyfructosan (1 g/liter) and antidiuretic hormone (10 mU/liter) were also added. An in-line filter (12 μm pore size) was used during the preperfusion period, before erythrocytes had been added. Angiotensin II and rat CGRPα, dissolved in freshly drawn dialysate, were infused in front of the kidney into a micro-magnetic stirrer chamber. The flow signal of the perfusion pump was used to achieve a flow proportional infusion rate of 1% of the flow rate. For parallel two electronically-regulated pumps were used (workshop adaption of an injectomat, Fresenius, Homburg, FRG).

Sodium and potassium were analyzed by flame photometry (IL 543 digital flame photometer) with an internal lithium standard. Osmolality was measured by a Wescor vapor pressure osmometer. Glucose was measured by hexokinase/glucose-6-phosphate dehydrogenase method [21], and polyfructosan after acid hydrolysis by a phosphohexose isomerase reaction. Glomerular filtration rate was determined from the polyfructosan clearance rate.

Materials

Cell culture media, penicillin-streptomycin, pyruvate and the enzymes used for polyfructosan and glucose analysis were obtained from Boehringer (Mannheim, FRG). Fetal bovine serum was from Gibco (Karlsruhe, FRG). Insulin, trypsin, angiotensin II, and bovine serum albumin were purchased from Sigma International, and fura-2AM from Calbiochem. Glutamine, α-ketoglutarate and malate was provided by Merck (Darmstadt, FRG), L-lactate as sodium salt by Serva (Heidelberg, FRG) and neomycin sulfate by Byk-Gulden (Konstanz, FRG). L-amino acids were purchased from Braun/Melsungen, (FRG, aminoplasmal paed. free of carbon hydrates). Polyfructosan was supplied by Laevosan (Salzburg, Austria and ADH as Pitressin (aqueous solution) from Parke-Davis (Munich, FRG). Rat CGRP-α was obtained from Peninsula Laboratories (Belmont, California, USA).

Results

Incubation of cultured rat mesangial cells with rat CGRPα enhanced cellular and extracellular cAMP accumulation (Fig. 1). The EC_{50} of cellular cAMP formation was 1 nM. At a concentration of 1 nM we also examined the effects of salmon and rat calcitonin on cAMP formation in comparison with that of CGRPα. Basal cAMP formation was 4.3 ± 0.8 pmol cAMP per 10 minutes and mg of protein (mean ± SEM of five experiments), and 19.8 ± 2.0, 5.9 ± 0.5, and 4.4 ± 0.9 in presence of rat CGRPα, salmon calcitonin and rat calcitonin, respectively.

There is evidence that cAMP is able to attenuate the contractility of mesangial cells [22–24]. We therefore examined the effect of CGRP on the contraction of cultured rat mesangial cells brought about by angiotensin II (A II). In Figure 2 cells responding typically to AII (10 nM) are shown. Contraction was quantitated by measuring planar cellular surface and planar cellular circumference. Four minutes after addition of AII planar surface and circumference of the cells were significantly reduced by 38% and 36%, respectively, in the absence of CGRP. Two minutes of preincubation with CGRP concentration dependently (EC_{50}, 1 nM) attenuated the reduction of planar surface and circumference in response to AII (Fig. 3). At 10 nM CGRP the contraction evoked by 10 nM AII was blunted.

Raised cytosolic calcium in mesangial cells after addition of AII is considered to be an essential step through which AII causes contraction [25]. Even though CGRP reduced the contraction brought about by AII, the amplitude and the duration of cytosolic calcium responses remained unchanged (Fig. 4, Table 1). It was confirmed in control experiments that CGRP increases cAMP formation also in trypsinized cells used for the calcium measurements.

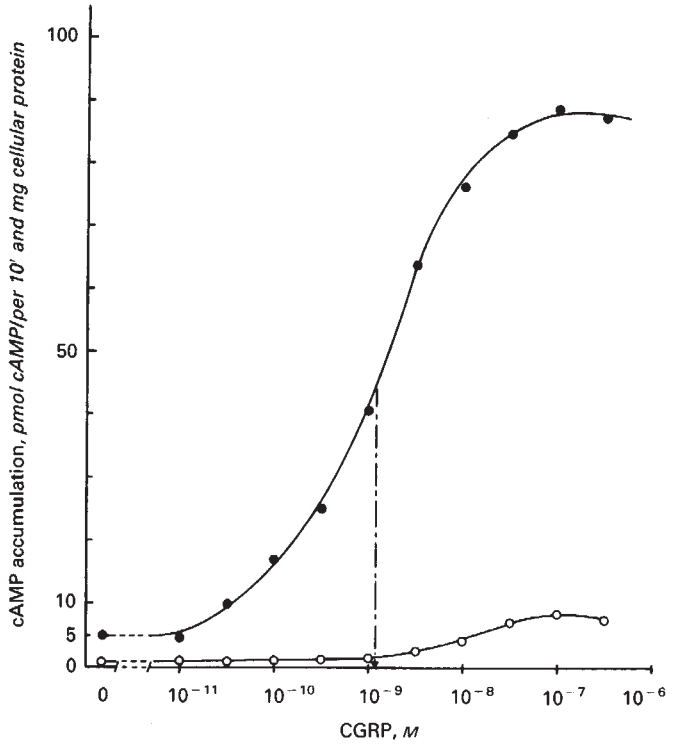

Fig. 1. *Dose response curve for rat CGRPα on accumulation of intracellular (●) and extracellular (○) cAMP in cultured rat renal mesangial cells.* Data are means of quadruplicates. Maximal stimulation of cAMP formation by CGRP as determined in four different culture preparations ranged between 9.5- to 21-fold over basal.

Table 1. Effect of rat CGRPα on rise of the $(Ca^{2+})_i$ in response to AII (10 nM) in rat renal mesangial cells

Concentration of CGRPα M	$(Ca^{2+})_i$ nM	
	Basal	Peak
0	141 ± 9	475 ± 62[b]
10^{-8}	138 ± 14	427 ± 86[a,b]
10^{-7}	156 ± 3	548 ± 96[a,b]
10^{-6}	135 ± 12	469 ± 74[a,b]

Results are means ± SEM of four determinations.
[a] $P > 0.1$ vs. control group.
[b] $P < 0.001$ vs. basal values (paired t-test).

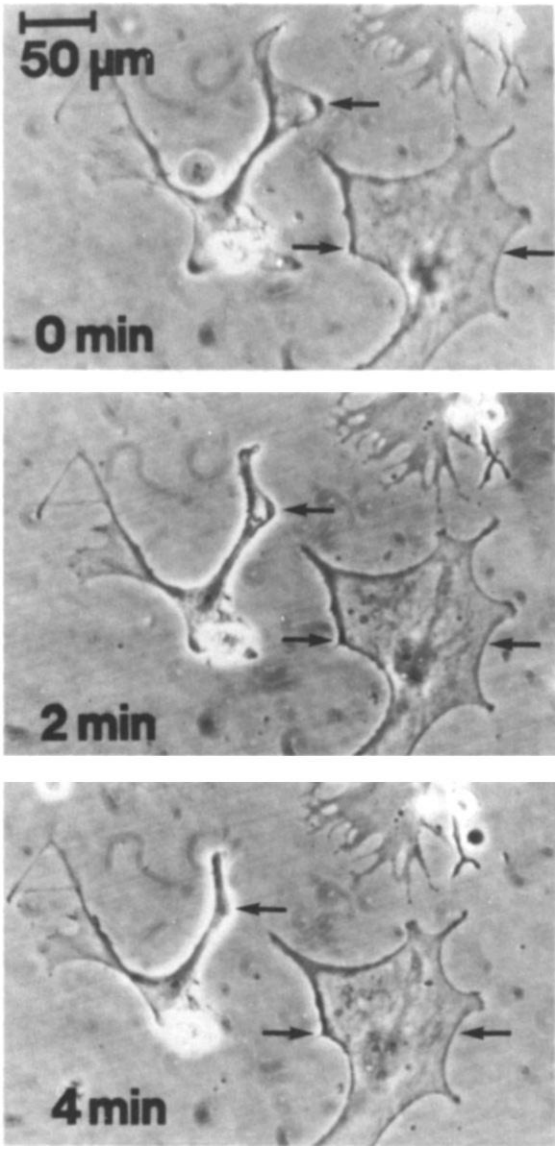

Fig. 2. *Shape of cultured rat renal mesangial cells before, 2 and 4 minutes after the addition of 10 nM AII.*

Effects of CGRP in the absence and presence of AII were, moreover, analyzed in the isolated perfused rat kidney. Figure 5 demonstrates the effect upon perfusion flow rate at a constant effective perfusion pressure (100 mm Hg). After a control phase AII was first examined. AII (24 pM) caused a significant decrease of flow rate, glomerular filtration and filtration fraction (Fig. 5, Table 2). With CGRP (10 nM) alone, both the flow rate and GFR increased (Fig. 5, Table 2). The filtration fraction rose from 4.15% ± 0.47% to 5.16% ± 0.43%. When CGRP (10 nM) and AII (24 pM) were infused together, the effects of AII were blunted. It should be noted that the effects of AII were not subject to homologous desensitization (insert in Fig. 5). Even when CGRP was withdrawn and infusions with AII were continued, the flow rate and GFR did not return immediately to control values, which indicates prolonged action of CGRP. AII and CGRP alone had opposite effects on urinary flow rates. They were decreased during infusions of AII, and were doubled in response to CGRP. Though CGRP raised GFR from 1.04 ± 0.08 to 1.34 ± 0.07 ml/min per g kidney, fractional sodium reabsorption decreased only from 90 to 85.3% when the filtered sodium load was increased from 134 to 163 µmol/min per g. Free water reabsorption in this phase was increased out of proportion during CGRP infusion, and was blunted after subsequent addition of AII.

In addition we measured the renin activity in the venous effluate during the experimental periods. As documented in Table 2 CGRP enhanced renin secretion in the isolated perfused rat kidney.

Discussion

Here we have examined effects of CGRPα on rat renal mesangial cells. Much like in blood vessels, the heart and the

Table 2. Functional parameters of the isolated perfused rat kidney at constant pressure (effective aortic pressure 100 mm Hg)

Midpoint of time int. *min*	Control 54	AII 73	Control 87	CGRP 101	CGRP/AII 114	AII 125
RVR *mm Hg · min · g per ml*	4.21 ± 0.23	5.81[a] ± 0.44	4.07 ± 0.28	3.61[a] ± 0.22	4.08 ± 0.23	4.22 ± 0.27
GFR *μl/min · g*	1124 ± 43	791[a] ± 57	1041 ± 78	1342[a] ± 72	1162 ± 75	1054 ± 62
FF %	5.08 ± 0.28	4.62[a] ± 0.26	4.15 ± 0.47	5.16[a] ± 0.43	4.70 ± 0.35	4.37 ± 0.22
UF *μl/min · g*	69.0 ± 20.4	46.2 ± 9.9[a]	119 ± 23.3	213 ± 38.5[a]	194 ± 25	188 ± 19.7
U_{Na} *μmol/min · g*	6.73 ± 0.28	4.32 ± 0.31[a]	13.4 ± 1.0	24.0 ± 1.3[a]	22.1 ± 1.7	20.5 ± 1.2
RA *ng AI/h · ml*	6.43 ± 1.28	6.40 ± 1.4	6.85 ± 0.43	21.9 ± 2.18[a]	20.1 ± 4.20	20.8 ± 3.40

Concentrations of AII and CGRP were 24 pM and 10 nM, respectively. Data are means ± SEM of 7 experiments. Abbreviations are: RVR, renal vascular resistance; GFR, glomerular filtration rate; FF, filtration fraction; UF, urinary flow; U_{Na}, urinary sodium excretion; RA, renin activity in venous effluate. Midpoints of time intervals are indicated in Figure 5.

[a] $P < 0.05$ vs. respective control.

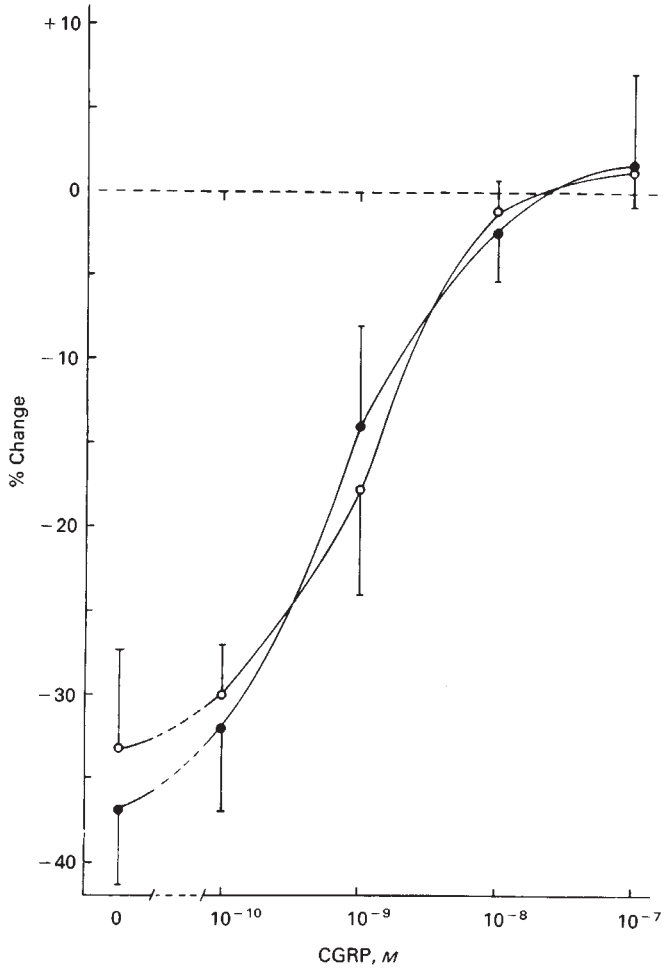

Fig. 3. *Percentage of change of planar surface area (●) and circumference (○) of cultured rat renal mesangial cells five minutes after addition of 10 nM angiotensin II.* Cells were preincubated for two minutes with different concentrations of rat CGRPα. Data are mean ± SEM of 13 to 20 experiments. Area and circumference of cells before the addition of angiotensin II were 4026 ± 1083 μm^2 and 584 ± 105 μm (mean ± SEM; $N = 13$), respectively. Both parameters were not significantly altered by preincubation with rat CGRPα ($P > 0.1$). Percentage of cells contracting in response to AII was 69%, 72%, 45%, 20% and 10% after preincubation with 0, 0.1 nM, 1 nM, 10 nM and 100 nM CGRP, respectively.

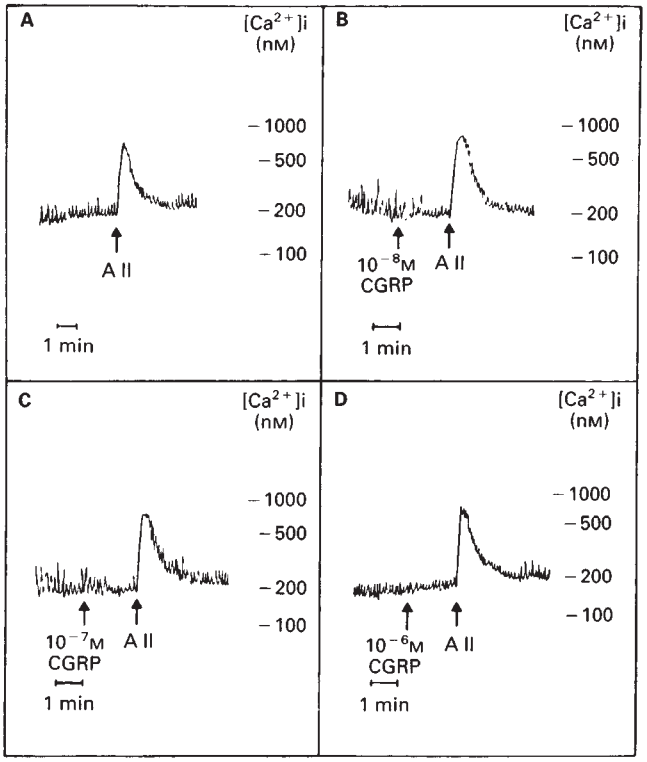

Fig. 4. *Recordings of cytosolic calcium concentrations in suspensions of rat renal mesangial cells after subsequent addition of rat CGRPα and angiotensin II (10 nM).*

spleen [26–28] CGRP stimulated cAMP formation in mesangial cells. In view of the structural homology between CGRP and calcitonin and the cross tachyphylaxis observed between the two peptides, it was essential to also investigate possible effects of rat and salmon calcitonin on cAMP formation in the same cells. On a molar basis CGRP was more active in stimulating cAMP production than salmon calcitonin and rat calcitonin was inactive. We infer from these results that activation of adenylate cyclase in mesangial cells is mediated by specific receptors for CGRP rather than for calcitonin. This conclusion is supported by the observation that calcitonin receptors could not be demonstrated in renal glomeruli [29].

There is evidence that elevations of cAMP in mesangial cells are capable to attenuate contraction [22–24]. In control experiments we observed that a 30 minute preincubation of mesangial with 500 μM dibutyryl cAMP reduces the percentage of cells contracting upon angiotensin II (10 nM) from 52% to 9%. Our observation that among different cell preparations between 50%

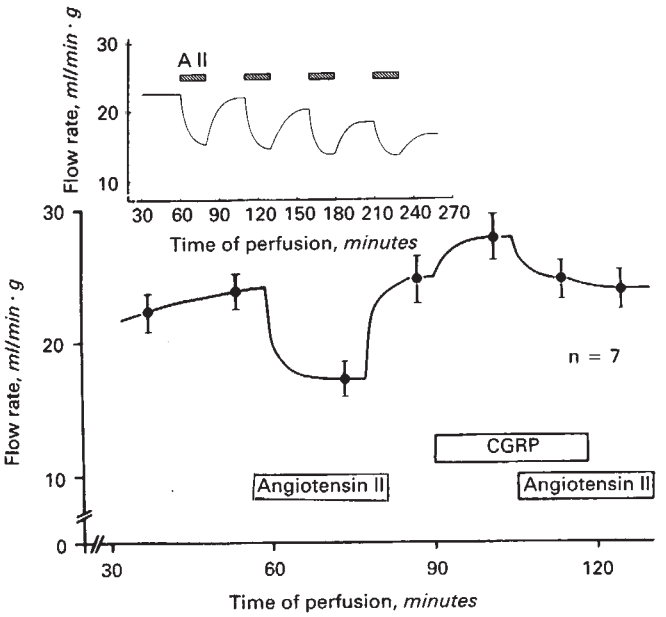

Fig. 5. *Flow rate through isolated rat kidneys perfused at 100 mm Hg after application of angiotensin II (24 pM) and rat CGRPα (10 nM).* Data are mean (± SEM of seven independent experiments. Insert: Typical experiment showing flow rate through isolated perfused rat kidney after repetitive application of AII (24 pM).

to 70% of the cells contract in response to AII is in accordance with previous reports [17, 25]. We also examined CGRP caused relaxation of mesangial cells contracted with angiotensin II. The numerical similarity of EC_{50} values of cAMP production and inhibition of contraction by AII are consistent with a close linkage between activation of adenylate cyclase by CGRP and relaxation of mesangial cells.

The contraction of vascular smooth muscle cells and mesangial cells by AII is initiated by a transient rise of the cytosolic-free calcium concentration [25]. Nevertheless, CGRP did not alter the calcium transients, although it enhanced cAMP formation. This suggests that CGRP did not affect $(Ca)_i$ and therefore the generation of the essential second messenger of AII. CGRP therefore may inhibit contraction of mesangial cells through mechanisms distal of increase of cytosolic calcium, such as the myosin light chain kinase, which is inactivated by cAMP dependent phosphorylation [30, 31].

The contraction of renal mesangial cells is considered as an important determinant of glomerular filtration [32–34]. Contraction of mesangial cells reduces the filtering surface resulting in a decreased glomerular filtration rate. To analyze a possible effect of CGRP on the glomerular filtration rate in an intact kidney model, we have used the isolated perfused rat kidney. This model was chosen to avoid possible indirect effects of CGRP on renal function caused by stimulation of the central nervous system [9], fall of blood pressure [10, 13] or stimulation of the renin-angiotensin-aldosterone system [13]. Effects of AII and CGRP on flow rate and glomerular filtration rate have been examined at constant perfusion pressure. The present results demonstrate that CGRP lowers renal vascular resistance, enhances glomerular filtration rate, and also filtration fraction. The rise of filtration fraction could be brought about by in an increase of intraglomerular perfusion pressure, or more likely by an increase of glomerular hydraulic permeability due to an increase of the filtering surface. This finding would thus be compatible with a direct effect of CGRP on glomerular filtration through interaction with mesangial cells. Our results also demonstrate that CGRP is a potent vasodilator that antagonizes in part the renal vasoconstrictory action of AII. This finding agrees with the observation that systemic application of CGRP increases renal blood flow in rats [35]. The reduced but still present action of angiotensin II during CGRP infusion (Fig. 5) may be due to submaximal amounts of CGRP or independent effects of AII.

We have recently shown that CGRP enhances renin release in humans as well as from isolated rat renal juxtaglomerular cells [13]. Here we extended these findings and demonstrate in perfused rat kidneys that CGRP also stimulates the release of renin (Table 2).

CGRP is believed to act on its target cells by a local release from nerve fibers [5, 12]. We and others have demonstrated the presence of CGRP immunoreactive nerve fibers in the vicinity of renal blood vessels and renal glomeruli [13, 36, 37]. This may also indicate that CGRP physiologically regulates renal function such as glomerular filtration, renal perfusion and the secretion of renin.

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