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Short communication

Direct evidence for an angiotensin AT_1 receptor type in rat vas deferens

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Abstract

Physiological experiments suggest that the angiotensin AT_1 receptor type predominates in rat vas deferens. Membrane binding experiments, using ^{125}I-[Sar^1,Ile^8]angiotensin II, confirm the presence of angiotensin AT_1 receptors and the absence of angiotensin AT_2 receptors in this tissue. Angiotensin II and the angiotensin AT_1 receptor-specific antagonist, losartan, bind to rat vas deferens membranes with comparable affinity, with K_D equal to 22.7 and 34.1 nM, respectively. The affinities of angiotensin AT_2 receptor-specific ligands are 3 orders of magnitude lower. According to the numbers of binding sites and Western blotting of membrane proteins, the concentration of angiotensin AT_1 receptors in the rat vas deferens is rather low. The fact that similar numbers of binding sites were obtained from binding data for angiotensin II and losartan further supports the hypothesis of exclusive existence of angiotensin AT_1 receptor type in rat vas deferens.

Keywords: Vas deferens; Receptor binding; Angiotensin II; Angiotensin AT_1 receptor; Antibody against angiotensin AT_1 receptor

1. Introduction

Angiotensin II causes contractions of smooth muscle cells in vas deferens and serves as a modulator of sympathetic nerves in vas deferens via adrenergic and purinergic pathways (Magnan and Regoli, 1978; Ellis and Burnstock, 1989). In many tissues, two main binding sites for angiotensin—angiotensin AT_1 and AT_2 receptors—were identified using receptor-specific ligands (Timmermans et al., 1993; Speth and Kim, 1990; Whitebread et al., 1989).

The presence of only the angiotensin AT_1 receptor type was deduced from the physiological data for vas deferens. Losartan, a well-known antagonist specific for the angiotensin AT_1 receptor, was proved to antagonize modulatory effects of angiotensin on purinergic neurotransmission in the field-stimulated rabbit vas deferens. The angiotensin AT_2 receptor agonist, *p*-[Phe(*p*-NH_2)^6]Ang, as well as the antagonist, (*S*)-1-[[4-(dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1-*H*-imidazo[4,5-c]pyridine-6-carboxylic acid (PD 123319), did not affect purinergic neurotransmission in rabbit vas deferens (Hedge and Clarke, 1993).

Angiotensin II enhances the production of prostacyclin in vas deferens. The release of prostacyclin in the epididymal part of rabbit vas deferens is mediated via the angiotensin AT_1 receptor only. However, in the prostatic part of vas deferens, an angiotensin AT_2 receptor agonist as well as an antagonist affected prostacyclin excretion, but to a lesser extent than did losartan (Catalioto et al., 1994).

The purinergic electrically stimulated twitch responses in rat vas deferens were enhanced with angiotensin II and reduced with both losartan and PD 123319 (Cox et al., 1995). However, the authors admitted that, at high concentrations, PD 123319 did not act selectively and blocked angiotensin AT_1 receptors also.

When the epididymal and prostatic parts of vas deferens were studied separately, it was found that direct smooth muscle cell contraction as well as noradrenaline release were predominantly mediated via angiotensin AT_1 receptors in the epididymal part of the vas deferens (Sum and Cheung, 1995). In the prostatic part of vas deferens, angiotensin II did not cause a direct contractile response, but potentiated purinergic neurotransmission via angiotensin AT_1 receptors (Sum et al., 1996).

The present study aims to define the angiotensin receptor type population in rat vas deferens by means of direct binding experiments with ligands specific for both main

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angiotensin receptor types as well as with an antibody specific for angiotensin AT_1 receptor protein.

2. Materials and methods

2.1. Materials

Wistar rats (250–350 g) were obtained from the Institute of Pharmacy and Biochemistry (Konárovice, Czech Republic). ^{125}I-[Sar^1,Ile^8] angiotensin II ([^{125}I]angiotensin II analogue) was purchased from DuPont NEN (Bad Homburg, Germany). Angiotensin II, CGP 42112A ([Nicotinyl-Tyr-](N$^{\alpha}$-benzyloxycarbonyl-Arg)Lys-His-Pro-Ile-OH) and [Phe(*p*-NH_2)^6]angiotensin II, as well as a prestained molecular weight standard mixture were obtained from Sigma (St. Louis, MO, USA). Losartan was a generous gift from DuPont-Merck (Wilmington, DE, USA). Rabbit anti-angiotensin AT_1 receptor antibody was a product of Boehringer (Ingelheim, Germany). The antibody was raised against the sequence 225–237 of rat angiotensin AT_1 receptor (Phillips et al., 1993); it does not recognize any protein from PC12 cells that predominantly express the angiotensin AT_2 receptor (data given by the producer). Bacitracin was purchased from Fluka (Buchs, Switzerland), and the ECL kit was from Amersham. All other reagents were from Serva (Heidelberg, Germany).

2.2. Preparation of liver membranes

Liver membranes were prepared from female Wistar rats according to Gunther (1984). Liver was homogenized in 10 volumes of ice-cold 50 mM Tris–HCl buffer, pH 7.4, containing 2 mM EGTA, 100 μM bacitracin and 100 μM phenylmethylsulfonyl fluoride, for 20 s using an Ultra-Turrax polytron (Janke and Kunkel KG, Ika Werk, Stanfen i. Breisgau, Germany) at setting 7, followed by homogenization in a teflon-glass homogenizer (Glass-Col, Terre Haute, IN, USA), six times at setting 30. The suspension was then centrifuged for 10 min at $1000 \times g$, the sediment was discarded and the supernatant was centrifuged at $16\,000 \times g$ for 15 min at 4°C. The resulting pellets were resuspended in 50 mM Tris–HCl buffer, pH 7.4 and stored as aliquots at −70°C.

2.3. Preparation of membranes from vas deferens

Membranes were prepared from vas deferens of male Wistar rats according to the method used by Chen et al. (1996) for uterine membranes. Tissue from 20 rats was minced thoroughly at 4°C and then homogenized in buffer as described for liver membranes. The homogenates were filtered through one layer of gauze and divided into 2 fractions: particles sedimenting at $1000 \times g$ and at $50\,000 \times g$. The homogenate was centrifuged at $1000 \times g$ for 10 min at 4°C and the $1000 \times g$ pellet was resuspended in 50 mM Tris–HCl buffer, pH 7.4 and stored as aliquots at −70°C. The supernatant was centrifuged at $50\,000 \times g$ for 60 min at 4°C. The $50\,000 \times g$ pellet (named vas deferens membranes) was resuspended in 50 mM Tris–HCl buffer, pH 7.4, and stored as aliquots at −70°C.

2.4. Binding assay

Binding of ^{125}I-[Sar^1,Ile^8]angiotensin II to both types of membranes was performed in modified Tyrode buffer containing 114 mM NaCl, 30 mM $NaHCO_3$, 10 mM NaH_2PO_4, 6.2 mM KCl, 0.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 100 μM phenylmethylsulfonyl fluoride, 100 μM bacitracin and 1 mg/ml bovine serum albumin.

Liver membranes and vas deferens membranes (approximately 50 μg and 400 μg of protein per sample, respectively) were incubated in the presence of 0.05 nM ^{125}I-labelled angiotensin II analogue and various concentrations of tested substances (range 10^{-10}–10^{-4} M) in a total volume of 0.25 ml for 45 min at 25°C or 30 min at 30°C, respectively. In both cases the reaction was terminated by the addition of ice-cold 120 mM NaCl followed by quick filtration on a Brandel cell harvester (Biochemical Research and Development Laboratories, Gaithersburg, MD, USA). The radioactivity associated with the membranes was measured in a gamma counter Minigamma 1275 (LKB-Wallac, Sweden) with an efficiency for ^{125}I of 75%. The total binding (i.e., binding in the absence of unlabelled angiotensin II) was about 10% of the total radioactivity in the sample. Non-specific binding (i.e., binding in the presence of 1 μM angiotensin II) amounted to less than 15% of the total binding.

Using vas deferens membranes, the time-dependence (0–60 min) of the binding and membrane concentration dependence (50–1000 μg of protein per tube) was also determined.

2.5. Western blot of angiotensin AT_1 receptor in membrane preparation of vas deferens

The membrane preparation (for vas deferens see Section 2.3, for liver see Section 2.2) was resuspended in the reducing sample buffer to a final concentration of 4 μg/μl and kept 2 min at 95°C. The protein samples (20 μg and 40 μg) were subjected to 4%/10% discontinuous polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) according to Laemmli (1970). Separated proteins were blotted from the gels to nitrocellulose membranes in 0.025 M Tris–HCl (pH 8.3), 0.15 M glycine, 0.01% SDS at 25 V at 4°C overnight. The membranes with transferred proteins were washed with washing buffer containing 0.01 M Tris–HCl (pH 7.6), 0.15 M NaCl, 0.02% Tween-20 and then blocked with either 3% bovine serum albumin or 3% non-fat dried milk in washing buffer for 1 h at room temperature. After rinsing with washing buffer, the membranes were incubated with

rabbit anti-rat angiotensin AT_1 receptor antibody, diluted 1:1000 in washing buffer containing either 0.3% bovine serum albumin or 0.3% non-fat dried milk for 1 h at room temperature. The excess of antibody was washed out with washing buffer and the membranes were treated with swine anti-rabbit immunoglobulin (IgG) conjugated to horseradish peroxidase, diluted in the same way as the first antibody, for 1 h at room temperature. After rinsing with washing buffer, some membranes were exposed to 10^{-4}% H_2O_2 substrate solution in 0.2 M Tris–HCl (pH 7.4) with 5×10^{-2}% 4-chloro-1-naphtol and 10^{-3}% $CoCl_2$. The reaction was stopped after 5 min by rinsing with water. Other membranes were developed using the ECL kit. X-ray film was exposed to the membrane for 1 min.

2.6. Analysis of binding data

The dissociation constants (K_d) and concentration of receptor sites (B_{max}) were determined from the competition curves transforming the data to Scatchard plots and using a linear least squares curve fitting program to fit the data to a one-site model based on the equation $B = B_{max} \times F/F + K_d$ (Hulme and Birdsall, 1992) (B = ligand bound, F = free ligand concentration). Calculations were based on a single ligand model in which the affinities for labelled angiotensin II analogue and unlabelled angiotensin II were considered to be equal (Gunther, 1984; Samanen et al., 1988; Campanille et al., 1982).

All data are expressed as means ± S.E.M.

3. Results

3.1. Binding studies

The binding affinity of the ^{125}I-angiotensin II analogue to the $1000 \times g$ and $50\,000 \times g$ pellet was determined. Binding to the $1000 \times g$ pellet was equal to the non-specific binding up to 1000 μg/mg protein per tube, and these particles were excluded from further experiments. The specific binding to the $50\,000 \times g$ fraction was dependent on protein concentration in the range of 50 to 400 μg of protein per tube.

The time course of binding (i.e., dependence of ^{125}I-angiotensin II analogue binding on time of incubation) was also examined. The binding reached a steady state in 20 min at 30°C and remained constant for an additional 30 min. Thus, further binding experiments were carried out for 30 min.

Fig. 1A shows the displacement binding curves for angiotensin II, losartan (angiotensin AT_1 receptor antagonist), CGP 42112A (angiotensin AT_2 receptor antagonist) and *p*-[Phe(*p*-NH_2)^6]angiotensin II (angiotensin AT_2 receptor agonist) to vas deferens membranes. Both angiotensin II and losartan had high affinity to the binding sites in vas deferens tissue compared to CGP 42112A or

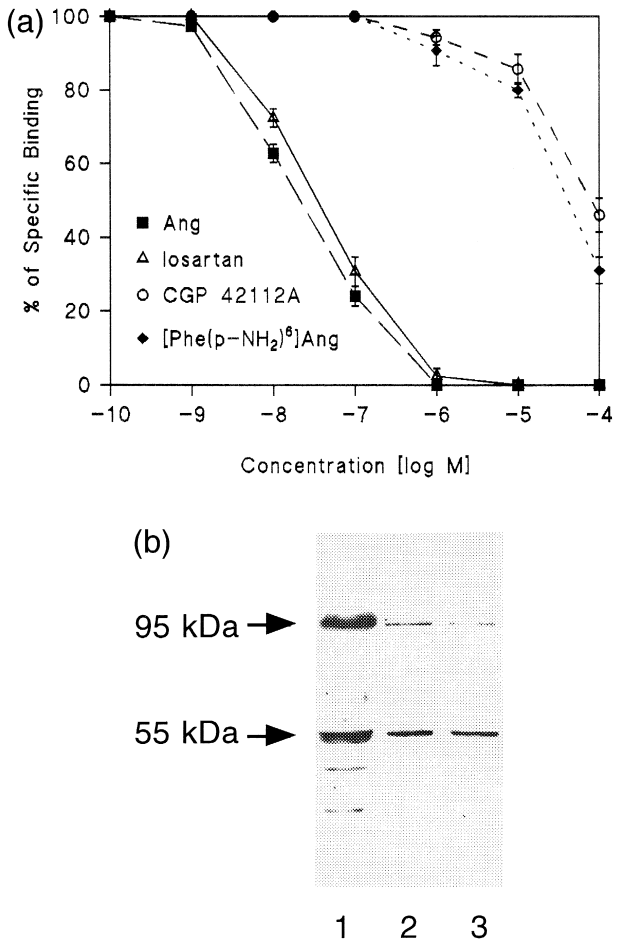

Fig. 1. (A) Displacement of ^{125}I-[Sar^1,Ile^8]angiotensin II binding to vas deferens membranes by angiotensin II and its analogues. The values are expressed as percentage of total binding. (The results shown are means ± S.E.M. of at least three independent experiments carried out in duplicates). (B) Western blot of membrane proteins detected with antibody against angiotensin AT_1 receptor. Liver (lane 1, 40 μg of membrane protein) and vas deferens (lane 2, 40 μg of membrane protein and lane 3, 20 μg of membrane protein).

p-[Phe(*p*-NH_2)^6]angiotensin II, which had very low affinity. When the data were transformed according to Scatchard, a linear plot was obtained which fitted optimally a one-site model. Table 1 summarizes K_d values for the analogues tested and the concentration of receptor sites

Table 1
Affinity of angiotensin II and angiotensin II analogues to vas deferens membranes

Compound	K_d (M)	Concentration of receptor sites (fmol/mg protein)
Angiotensin II	$(2.27 \pm 0.59) \times 10^{-8}$	386 ± 146
Losartan	$(3.41 \pm 1.31) \times 10^{-8}$	503 ± 133
CGP 42112A	$(9.00 \pm 1.73) \times 10^{-5}$	
[Phe(*p*-NH_2)^6] angiotensin II	$(4.00 \pm 2.83) \times 10^{-5}$	

Means ± S.E.M. of at least three separate experiments.

based on calculations from competitive displacement curves for angiotensin II and losartan.

Binding to liver membranes was used as a standard in our study. Liver membranes contain both angiotensin AT_1 and AT_2 receptors (Gunther, 1984). In our test, K_d equal to $(4.60 \pm 0.57) \times 10^{-9}$ M was obtained for angiotensin II, which is in agreement with the literature (Campanille et al., 1982; Gunther, 1984). The concentration of binding sites for vas deferens membranes was approximately 10 times lower than for liver membranes.

Similarly, compared to that in liver membranes, the affinity of angiotensin II to vas deferens membranes was about five times lower.

3.2. Western blot of vas deferens membranes and identification of angiotensin AT_1 receptor by specific antibody

The antibody against the sequence 225–237 of the angiotensin AT_1 receptor recognized the 55 kDa band only in vas deferens and liver, when the angiotensin AT_1 receptor was detected with the 4-chloro-1-naphtol colour reaction (not shown). If the ECL kit was used, both the 55 kDa major band and the 95 kDa minor band were seen in vas deferens membrane samples. In liver, the 95 kDa band had an intensity similar to that of the 55 kDa band. Based on equal amounts of membrane protein, the 55 kDa band in liver was more intense than that in vas deferens. (Fig. 1B).

4. Discussion

Although the presence of angiotensin receptors in vas deferens had been demonstrated, neither direct binding studies nor determination of the angiotensin AT_1 receptor in vas deferens with antibody had been done. It was found that binding of angiotensin II to angiotensin AT_1 receptors caused direct contractions of smooth muscle cells and influenced neurotransmission (Hedge and Clarke, 1993; Cox et al., 1995; Sum and Cheung, 1995; Sum et al., 1996). Our study therefore concerned the direct detection of angiotensin II binding sites in vas deferens tissue in binding experiments and through angiotensin AT_1 receptor determination using an antibody specific to the angiotensin AT_1 receptor.

To classify the angiotensin receptor population in rat vas deferens, receptor type specific ligands were used: losartan (AT_1), CGP 42112A and [Phe(*p*-NH_2)^6] angiotensin II (AT_2). All of them, even at high concentrations, are considered to be receptor type specific. Table 1 shows K_d values calculated from a competitive binding study. It is evident that while K_d values for angiotensin II and losartan are comparable, both angiotensin AT_2 receptor-specific analogues have a very low affinity to vas deferens membranes, about 3 orders of magnitude lower than that of angiotensin II.

It was found that there was a lower concentration of AT_1 receptor in vas deferens membranes than in liver ones. On the other hand, similar numbers of binding sites were found according to calculations from angiotensin II and losartan binding curves. This supports the presence of angiotensin AT_1 receptor type only. The presence of angiotensin AT_1 receptor in vas deferens was further confirmed by specific antibody detection on the Western blots of the membrane proteins. The major band corresponding to the glycosylated form of the angiotensin AT_1 receptor was detected with staining methods. The very sensitive ECL detection revealed the minor band of molecular mass 95 kDa in vas deferens. A similar band (95 kDa) had been observed in liver, kidney and adrenals, using an antibody against the 14–23 sequence of the angiotensin AT_1 receptor (Železná et al., 1992).

The K_d value for angiotensin II binding to vas deferens membranes determined in this study, $(2.27 \pm 0.59) \times 10^{-8}$ M, was consistent with EC_{50} values for angiotensin II found in published physiological studies. This includes EC_{50} values for the field-stimulated contractile response of rat vas deferens, i.e., $(6.02 \pm 0.78) \times 10^{-8}$ M (Magnan and Regoli, 1978), the epididymal part of rat vas deferens, i.e., $(3.45 \pm 0.45) \times 10^{-8}$ M (Sum and Cheung, 1995) or rabbit vas deferens, i.e., 1×10^{-8} M (Hedge and Clarke, 1993), as well as angiotensin-induced release of prostacyclin from rabbit vas deferens ($EC_{50} = 2 \times 10^{-8}$ M).

The K_d value for angiotensin II binding to vas deferens membranes found by us was about five times higher than that in most other tissues containing the angiotensin AT_1 receptor (aorta, liver, adrenocortical membranes), but was still lower than the K_d determined for angiotensin II in breast tissue membranes, 60 nM (Inwang et al., 1997). The values of K_d for losartan and for angiotensin II in vas deferens membranes determined by us were of the same order of magnitude (10^{-8}). This could be explained by the prevalent existence of the angiotensin AT_{1A} receptor subtype which has a higher affinity to losartan than the angiotensin AT_{1B} receptor subtype (Zhou et al., 1993). For the vascular angiotensin AT_1 receptor, where mostly the AT_{1A} subtype is present, K_d determined for losartan was only four times higher than that for angiotensin II (Murphy et al., 1991). The fact that vas deferens is morphologically similar to vascular tissue supports the subtype hypothesis.

In conclusion, the presence of angiotensin AT_1 receptors and the absence of angiotensin AT_2 receptors was confirmed in this study, both in a binding assay and by Western blot with angiotensin AT_1 receptor specific antibody. Our data are in agreement with conclusions drawn from earlier physiological assays.

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