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ORIGINAL ARTICLE

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Differential effects of eprosartan and losartan at prejunctional angiotensin II receptors

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Abstract A comparison was made of the influence of losartan and eprosartan on angiotensin II effects at pre- and postjunctional receptors of the canine pulmonary artery and at prejunctional receptors of the rat left ventricle. To study postjunctional contractile responses to angiotensin II, non-cumulative concentration-response curves were determined; to study prejunctional effects of angiotensin II, the tissues were preincubated with [^{3}H]noradrenaline and then superfused and electrically stimulated (1 Hz, 2 ms, 50 mA, 5 min).

Postjunctionally, both losartan and eprosartan caused a parallel shift of the concentration-response curve of angiotensin II to the right (pK_d of 8.15 and 8.28, respectively). At the prejunctional level, while eprosartan, in concentrations similar to those which were effective postjunctionally (30–100 nM), antagonized the facilitatory effect on noradrenaline release in both the dog pulmonary artery and the rat ventricle, losartan was ineffective in concentrations up to 1 μM. It is concluded that prejunctional receptors for angiotensin II in the canine pulmonary artery and in the rat left ventricle are different from postjunctional receptors of the canine pulmonary artery. It is proposed that the prejunctional receptors of these tissues are atypical AT_1 or "AT_{1B}-like" receptors.

Keywords Eprosartan · Losartan · PD123319 · Angiotensin II receptors · Rat ventricle · Canine pulmonary artery

Introduction

Angiotensin II is able to cause vasoconstriction both by a direct action on smooth muscle cells (Helmer 1964) and indirectly through the facilitation of noradrenaline release from postganglionic sympathetic axons (Zimmerman and Whitemore 1967; Peach 1977; Starke 1977). While it is generally accepted that the receptors mediating the direct postjunctional effect of angiotensin II at the effector organ are AT_1 (Dudley et al. 1990; Rhaleb et al. 1991; Cox et al. 1995; Guimarães et al. 1998), the situation is not so clear for the prejunctional effects for angiotensin II. On the basis of results obtained with selective AT_1 and AT_2 antagonists, the prejunctional effect of angiotensin II is considered as being mediated by AT_1 receptors in the majority of the tissues: canine kidney (Wong et al. 1991; Suzuki et al. 1992), guinea-pig atria (Brasch et al. 1993), rabbit iris ciliary body (Ohia and Jumblatt 1993), rat atria (Gironacci et al. 1994), human kidney (Rump et al. 1995), rat trachea (Boicos et al. 1998) and mouse atria (Cox et al. 1999). However, on the same basis, there are also several tissues where the prejunctional receptors of angiotensin II cannot be classified as AT_1: in the rat tail artery the effect of angiotensin II was inhibited to the same extent by selective AT_1 (losartan) and AT_2 (PD123319) antagonists (Cox et al. 1995), and in the rat left ventricle (Moura et al. 1997) as well as the canine mesenteric and pulmonary arteries (Guimarães et al. 1998) the prejunctional effect of angiotensin II was not changed by selective concentrations of either antagonist. Similarly, in the rabbit *vas deferens* losartan was ineffective against the prejunctional effect of angiotensin II (Trachte et al. 1990).

Also in pithed rats, results were obtained which do not support that prejunctional angiotensin II receptors are typical AT_1. Ohlstein et al. (1997) reported that sub-pressor doses of angiotensin II which shifted to the left the frequency-response curves for increases in blood pressure due to spinal cord stimulation, most probably due to an enhancement of sympathetic transmitter release, were not changed by losartan but were inhibited by the nonpeptide angiotensin II receptor antagonist eprosartan. Having in mind this finding we compared the influence of eprosartan and losartan on angiotensin II effects at both the pre- and postjunctional receptors of the canine pulmonary artery and at the prejunctional receptors of rat left ventricle.

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Part of the present results were presented at the second Joint Meeting of the British and Portuguese Pharmacological Societies (Moura et al. 1999).

Materials and methods

The experiments were carried out on canine pulmonary arteries and rat left ventricles. In the municipal dog pound, mongrel dogs, 9–16 kg in weight, of either sex, were sacrificed by an overdose of pentobarbitone sodium (100 mg/kg). Immediately after removal, pulmonary arteries were placed in bottles containing aerated (95% O_2 and 5% CO_2) and cold modified Krebs-Henseleit solution (Guimarães and Osswald 1969) of the following composition (mM): NaCl, 119; KCl, 4.70; $CaCl_2$, 2.52; KH_2PO_4, 1.18; $MgSO_4$, 1.23; $NaHCO_3$, 25.0; glucose, 10.0. The arteries were then transported to the laboratory where they were cut into small strips (of about 1.5×20 mm) to study prejunctional effects or into small rings (of about 5 mm length) to study postjunctional effects.

Male normotensive Wistar rats weighing 200–250 g (Biotério do Instituto Gulbenkian de Ciências, Oeiras, Portugal) were kept on a 12-h light/dark cycle and given a standard laboratory chow and water *ad libitum*. After overnight fasting the animals were anaesthetized with pentobarbitone sodium (50 mg/kg, i.p.) and the hearts were removed and immediately placed in warmed, aerated modified Krebs-Henseleit solution (see above). Six slices (across the entire wall) of about 50 mg were taken from the left ventricle.

Prejunctional effects. The strips of canine pulmonary artery or the rat ventricle slices were preincubated for 30 min in 3 ml medium containing 1 mM pargyline (to inhibit monoamine oxidase), 40 μM hydrocortisone and 40 μM U-0521 (3,4-dihydroxy-2-methylpropiophenone) to inhibit extraneuronal removal (Guimarães et al. 1978). After preincubation, the strips were exposed for 60 min to [^{3}H]noradrenaline (0.2 μM). All drugs present during preincubation and incubation with [^{3}H]noradrenaline (except pargyline) plus 10 μM cocaine (which was added after the exposure to the tritiated amine) were kept in the medium for the remainder of the experiment. Thereafter the strips were mounted in a perifusion chamber and perifused with amine-free medium (aerated and at 37°C) during 110 min at a flow rate of 0.8 ml/min. From t=110 min (t=0 min being the onset of the perifusion) the perifusion fluid was collected continuously in samples of 5 min. Three periods of transmural electrical stimulation (1 Hz, 2 ms, 50 mA, 5 min; Stimulator II X; Hugo Sachs Elektronik, March-Hugstetten, Germany) were applied at min 120 (S_1), 160 (S_2), and 200 (S_3). The first period of electrical stimulation was disregarded, the second was taken as control and the third one was used to study the influence exerted by angiotensin II (in the absence or in the presence of antagonists). Angiotensin II was added to the perifusion fluid 20 min before S_3. Angiotensin II antagonists were added to the perifusion fluid 30 min before S_1.

After the experiment, the tissues were kept overnight in 2 ml of 0.2 M perchloric acid. Radioactivity was measured by liquid scintillation counting (liquid scintillation counter 1209 Rackbeta; LKB Wallac, Turku, Finland) in 2-ml aliquots of perifusate (or 0.5 ml of the acid extract of the tissue + 1.5 ml of Krebs-Henseleit solution), after addition of 8 ml of scintillation mixture (OptiPhase "HiSafe" 3; LKB, Loughborough, UK). The outflow of tritium was calculated as a fraction of the amount of tritium in the tissue at the start of the respective collection period (fractional rate of loss per min).

For the calculation of the overflow induced by electrical stimulation those 5-min samples were taken into account in which the overflow of tritium exceeded that in the last pre-stimulation control sample (usually this applied to the three or four samples collected during and after stimulation). The spontaneous outflow measured in the last pre-stimulation sample was assumed to represent the spontaneous outflow in subsequent samples; it was subtracted from the overflow determined in stimulation and post-stimulation samples. The "total overflow of transmitter" was the sum of all increases (induced by a period of stimulation) above the spontaneous level of outflow of tritium. The fractional release per shock (FR) was calculated by dividing evoked tritium overflow by tritium present in the tissue at the beginning of the stimulation period and by the number of shocks. Drug effects are expressed as the percentage increase of tritium overflow evoked by S_3 compared to that evoked by S_2. Each result was corrected for time-dependent changes as determined in parallel drug-free control experiments.

The prejunctional effect of angiotensin II was determined as the increase of the overflow of tritium evoked by electrical stimulation. $EC_{30\%}$ values represent the molar concentration of angiotensin II that increased the evoked overflow by 30% and $pEC_{30\%}$ the negative logarithm of $EC_{30\%}$. $EC_{30\%}$ represents the means of $EC_{30\%}$ values obtained in different tissues. Each $EC_{30\%}$ value was determined by interpolation from the concentration-response curve to angiotensin II obtained on six segments from the same tissue perifused in parallel using different concentrations of angiotensin II.

Postjunctional effects. Rings of about 5 mm length of the canine pulmonary artery were mounted in a 10-ml bath containing aerated modified Krebs-Henseleit solution at 37°C. Two stainless steel wires (diameter 0.05 mm) were introduced into the lumen and then moved to stretch the vessel wall to a resting tension of 9.8–14.7 mN. One of the wires was fixed to the bottom of the bath and the other to the isometric transducer. The mechanical responses were recorded on a Harvard Universal Oscillograph. The rings were allowed to stabilize for 2 h.

Two concentration-response curves (separated by an interval of 60 min) were obtained on each ring by non-cumulative additions of agonists with half-log increments. After the response to a given concentration had reached the maximum, the tissue was repeatedly washed out. Desensitization has been reported when repeated additions of angiotensin II were made; indeed, in the canine pulmonary artery some desensitization was observed whenever the interval between two successive additions was less than 10 min (Guimarães et al. 1998). To avoid this phenomenon, the additions of angiotensin II to the medium were made with intervals of at least 45 min.

pD_2 values, i.e. negative logarithms of the molar concentration of angiotensin that caused 50% of the maximal contraction, were calculated from the concentration-response curves by interpolation. pK_d values were calculated according to the method of van Rossum (1963) from the equation: $pK_d = pA_x + \log(x-1)$ in which x represents the shift of the concentration-response curve to the right and pA_x the negative logarithm of the molar concentration of the antagonist which caused this shift. The antagonists were added to the bath 30 min before starting the determination of the second concentration-response curve.

Statistics. The results are expressed as arithmetic means ± SEM. One-way analysis of variance was used to test differences between unpaired results. A probability level of 0.05 or less was considered statistically significant.

Drugs. Angiotensin II acetate (Sigma, St. Louis, Mo., USA); cocaine hydrochloride (Uquipa, Lisbon, Portugal); eprosartan hydrochloride (SmithKline-Beecham, King of Prussia, Pa., USA); hydrocortisone 21-hemisuccinate sodium (Sigma); losartan (Merck Portuguesa, Lisbon, Portugal); [^{3}H]7-(-)-noradrenaline (18.2–21.1 Ci/mmol; New England Nuclear, Dreieich, Germany); pargyline hydrochloride (Sigma); PD123319 ditrifluoroacetate (*S*(+)-1-((4-(dimethylamino)-3-methylphenyl)methyl)-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazol(4,5-c)pyridine-6-carboxylic acid ditrifluoroacetate; Research Biochemicals, Natick, Mass., USA); saralasin ((Sar^1,Val^5,Ala^8)-angiotensin II acetate; Sigma); U-0521 (3,4-dihydroxy-2-methylpropiophenone; Upjohn, Kalamazoo, Mich., USA).

Results

There is some overlap with results published by Guimarães et al. (1998). The results now shown for the canine

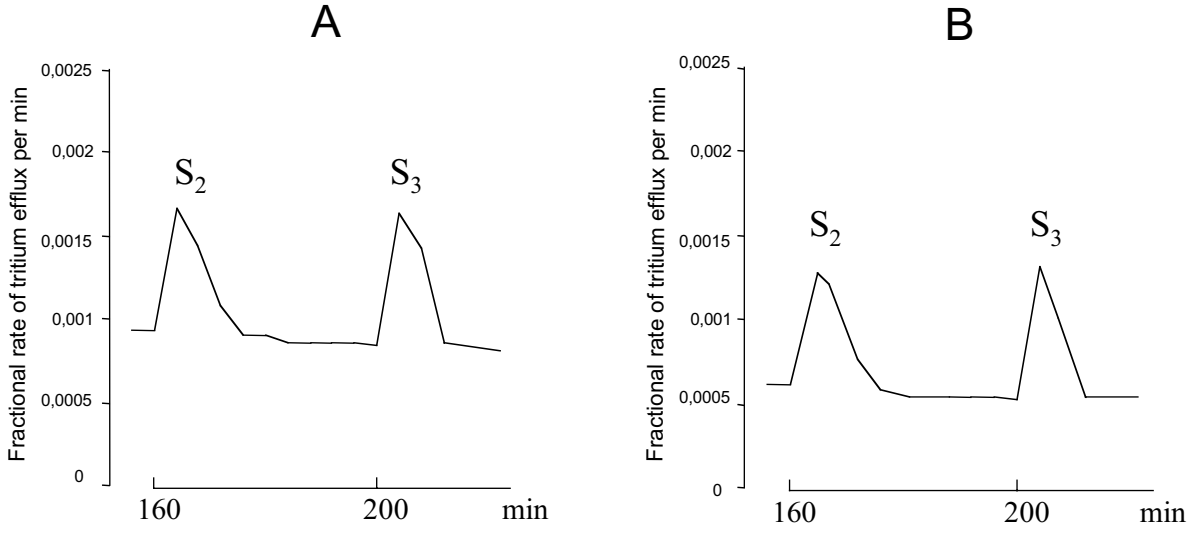

Fig. 1 Outflow of tritium from **A** rat left ventricle and **B** canine pulmonary artery. Effect of electrical stimulation (S_2 and S_3 consisted of 300 pulses, 1 Hz). *Abcissae:* superfusion in min

pulmonary artery, in which losartan and PD123319 were used (both pre- and postjunctionally), represent the means of results already published plus new results obtained in new experiments carried out under the same conditions.

Prejunctional effects of angiotensin II

In the absence of drugs, the basal efflux of tritium decreased slowly with time. However, the fractional rate of loss remained constant in the course of the experiment: $7.88\pm0.98\times10^{-4}$ per min (n=10) for the rat left ventricle and $5.78\pm0.42\times10^{-4}$ per min (n=15) for the canine pulmonary artery (Fig. 1). Furthermore, the overflow of tritium evoked by electrical stimulation remained also constant throughout the experiment, as shown by the ratio S_3/S_2 which was close to unity (0.99±0.10, n=10, for the ventricle and 0.97±0.08, n=15, for the pulmonary artery).

Neither angiotensin nor the antagonists caused any change of the basal tritium efflux (data not shown).

Angiotensin II caused a concentration-dependent enhancement of tritium overflow evoked by electrical stimulation in both tissues, the maximum effect representing an increase by 115.3±14.0% (n=6) in the rat ventricle and by 65.0±6.3% (n=9) in the pulmonary artery (Fig. 2). The $pEC_{30\%}$ values for angiotensin II were 8.60±0.27 (n=5) in the rat ventricle and 8.79±0.15 (n=17) in the pulmonary artery (P>0.05).

At 100 nM, a concentration usually reported as selective for AT_1 receptors (3–100 nM; Ernsberger et al. 1992), losartan did not change the enhancement by angiotensin II of tritium overflow evoked by electrical stimulation in either tissue (Fig. 2). Even at 1 μM (the highest concentration which was tested), losartan did not change the prejunctional angiotensin II effect (Fig. 2). However, another selective AT_1 receptor antagonist, eprosartan (Edwards et al. 1992), in nearly identical concentrations (30, 50 and 100 nM), concentration-dependently antagonized the effect of angiotensin II, causing a progressive reduction of the maximum effect in both tissues (Fig. 3).

At 100 nM, a concentration which blocks AT_2 angiotensin II receptors (Dudley et al. 1990), PD123319 did

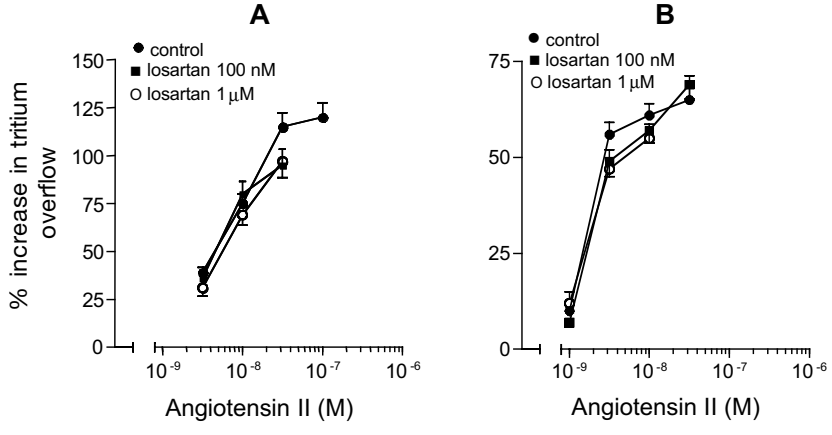

Fig. 2 A Rat left ventricle and **B** canine pulmonary artery: effects of losartan on the enhancement by angiotensin II of tritium overflow evoked by electrical stimulation (1 Hz, 2 ms, 50 mA, during 5 min). Shown are results obtained in the absence (*control*) and in the presence of losartan. The results are means ± SEM of 5–6 experiments

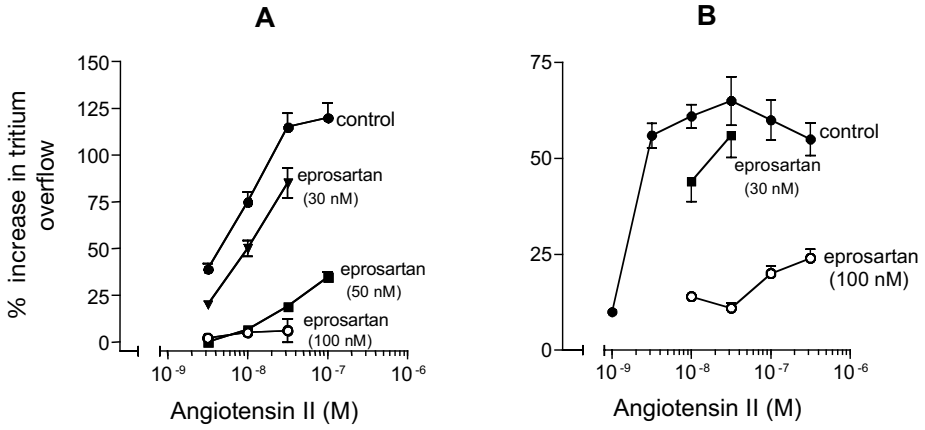

Fig. 3 A Rat left ventricle and **B** canine pulmonary artery: effects of eprosartan on the enhancement by angiotensin II of tritium overflow evoked by electrical stimulation (1 Hz, 2 ms, 50 mA, during 5 min). Shown are results obtained in the absence (*control*) and in the presence of eprosartan. The results are means ± SEM of 5–6 experiments

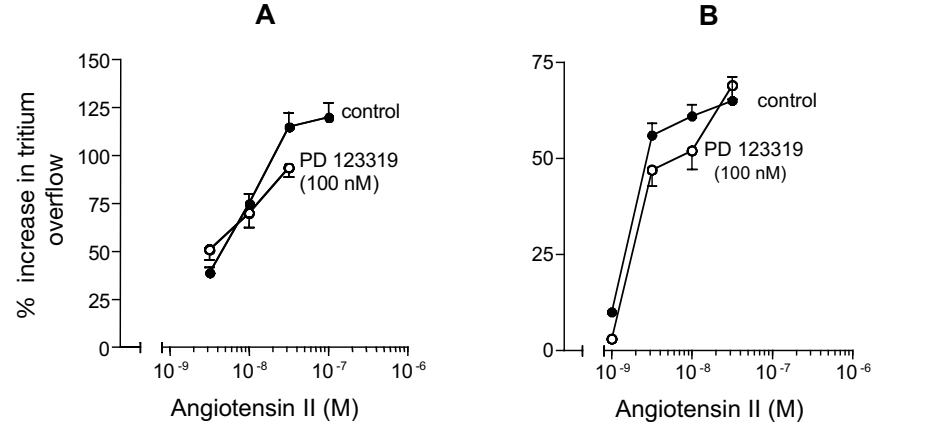

Fig. 4 A Rat left ventricle and **B** canine pulmonary artery: effects of PD123319 on the enhancement by angiotensin II of tritium overflow evoked by electrical stimulation (1 Hz, 2 ms, 50 mA, during 5 min). Shown are results obtained in the absence (*control*) and in the presence of PD123319. The results are means ± SEM of 5–6 experiments

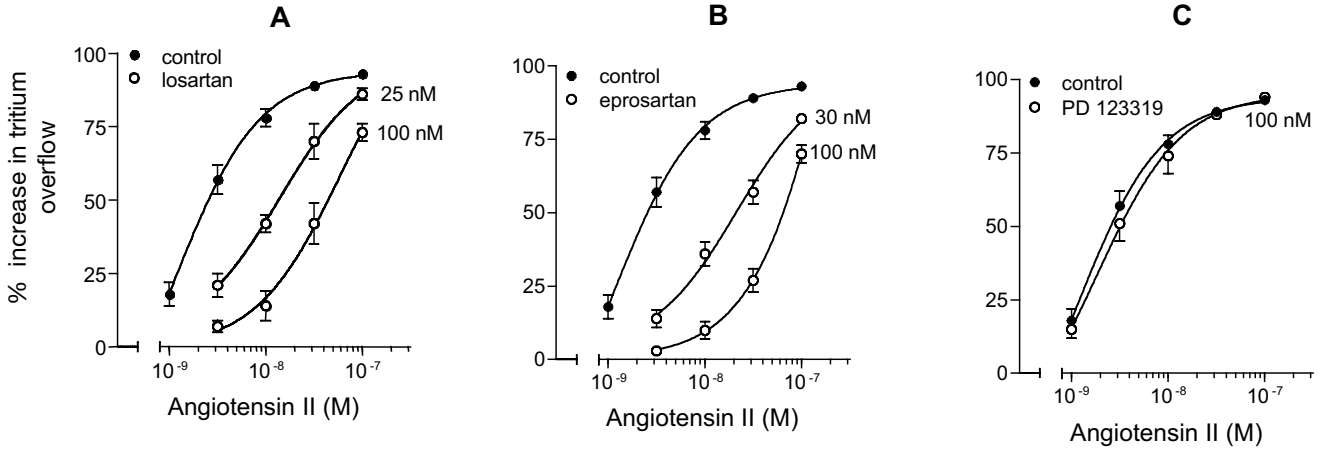

Fig. 5 Canine pulmonary artery: concentration-contraction curves to angiotensin II obtained in the absence and in the presence of **A** losartan, **B** eprosartan or **C** PD123319. The results are means ± SEM of 5–6 experiments

not change the facilitatory effect of angiotensin II on the electrically evoked tritium overflow in either tissue (Fig. 4).

Postjunctional effects of angiotensin II

Angiotensin II (1–100 nM) caused concentration-dependent contractions of canine pulmonary artery rings, the maximum effect reaching 1.28±0.12 mN/mg of tissue (n=15). The pD_2 for angiotensin II was 8.48±0.10 (n=15). As shown in Fig. 5, both losartan (25 nM and 100 nM) and eprosartan (30 nM and 100 nM) caused marked displacement of the concentration-response curve for angiotensin II to the right (pK_d=8.15±0.14, n=12, for losartan and 8.28±0.21, n=10, for eprosartan).

In contrast to losartan and eprosartan, PD123319 (up to 1 μM) had no effect on the response of the pulmonary artery to angiotensin II.

Discussion

AT_1 receptors mediate nearly all of the known postjunctional effects of angiotensin II (Wong et al. 1990, 1991; Chiu et al. 1991; Barbella et al. 1993). AT_1 receptors are selectively blocked by losartan (Dudley et al. 1990; Bumpus et al. 1991; Duncia et al. 1992; Guimarães et al. 1998) and also by the more recently synthesized compounds eprosartan (Edwards et al. 1992), irbesartan (Cazaubon et al. 1993) and valsartan (Criscione et al. 1993).

The results reported in the present publication show that both losartan and eprosartan antagonize in a competitive manner the postjunctional response of the canine pulmonary artery to angiotensin II, as previously shown for losartan by Guimarães et al. (1998). At the same concentration, losartan and eprosartan caused similar antagonism. These results confirm that postjunctional angiotensin II receptors are AT_1. However, at the prejunctional level, the antagonist potencies of losartan and eprosartan were totally different: while losartan in concentrations up to 1 μM was ineffective, as previously shown by Guimarães et al. (1998), eprosartan was very active, showing that in the canine pulmonary artery, the receptors mediating the prejunctional responses to angiotensin II differ from those mediating postjunctional ones.

Cox et al. (1999) recently reported that in mouse atria and spleen, angiotensin II facilitated the release of noradrenaline evoked by electrical stimulation via a prejunctional AT_1 receptor, since angiotensin II effects were antagonized by losartan (0.1–1 μM). In the canine pulmonary artery, while the concentrations of eprosartan required to inhibit pre- and postjunctional effects of angiotensin II were in the same range, the concentrations of losartan required to act at prejunctional level, although not quantified, were about 400 times higher than those acting at a postjunctional one. These results very clearly show a difference between pre- and postjunctional receptors for angiotensin II in the canine pulmonary artery.

In experiments carried out in normal humans, Worck et al. (1997) showed that losartan in doses that completely blocked vascular AT_1 receptors did not affect the sympathoadrenal response in terms of adrenaline release evoked by insulin-induced hypoglycemia. Accordingly, these authors concluded that the facilitation by angiotensin II of adrenaline release in humans is not mediated through AT_1 receptors. In the pithed rat, Ohlstein et al. (1997) showed that while losartan, valsartan and irbesartan failed to antagonize pressor responses induced by spinal cord stimulation, eprosartan, at a dose identical to that used with other antagonists, inhibited the pressor response evoked by spinal cord stimulation in a manner similar to that observed with saralasin. However, these authors did not advance any explanation for this differential effect observed between eprosartan and the other nonpeptide angiotensin II receptor antagonists.

The present results show that while one "typical" AT_1 receptor antagonist (eprosartan), at the same concentration, blocks pre- and postjunctional effects of angiotensin II, another "typical" AT_1 antagonist blocks postjunctional angiotensin II effects but has no influence on prejunctional ones, unless its concentration is increased by a factor of about 400, as shown by Guimarães et al. (1998). Most recently, Shetty and Delgrande (2000) reported that in rat atria the rank order of antagonistic potency against the prejunctional facilitatory effect of angiotensin II was: valsartan > irbesartan > eprosartan > losartan. However, these authors did not compare the antagonistic potency of the same antagonists on postjunctional effects of angiotensin II. Thus it is impossible to conclude on the relative potency of each of those compounds at typical AT_1 receptors and prejunctional angiotensin II receptors.

The classification of α-adrenoceptors into α_1- and α_2-subtypes was established on the basis of a smaller difference of pA_2 values for yohimbine at pre- and postjunctional level (Starke et al. 1975). A similar differential effect was observed in the canine mesenteric artery, where losartan was about 800 times more potent at post- than at prejunctional angiotensin II receptors (Guimarães et al. 1998).

Eprosartan is chemically different from all other nonpeptide angiotensin II receptor antagonists; it is a nonbiphenyl, non-tetrazole compound, while losartan is a biphenyl-tetrazole derivative. This may well explain why losartan distinguishes between pre- and postjunctional angiotensin II receptors and eprosartan does not.

On the basis of data obtained in both cloning and receptor binding studies, a subdivision of AT_1 receptors into AT_{1A} and AT_{1B} subtypes was proposed (Murphy et al. 1991; Ernsberger et al. 1992; Iwai and Inagami 1992; Zhou et al. 1993). According to Ernsberger et al. (1992), AT_{1A} are sensitive to nanomolar concentrations of losartan, AT_{1B} are sensitive to micromolar concentrations of losartan and AT_2 are sensitive to millimolar concentrations of losartan (Ernsberger et al. 1992).

We suggest that postjunctional angiotensin II receptors are AT_{1A} because they are sensitive to nM concentrations of losartan, while prejunctional angiotensin II receptors are atypical AT_1 or "AT_{1B}-like" receptors since they are sensitive to μM concentrations of losartan, as shown by Guimarães et al. (1998).

According to Ernsberger et al. (1992), AT_{1B} receptors should be sensitive to inhibition by PD123319. This did not happen in the present study. However, the comparison of binding affinities at AT_{1B} receptor with antagonist potencies in the adenylyl cyclase assay shows that PD123319 is approximately 1,000-fold less potent than expected from membrane binding assays (Zhou et al. 1993). This was explained in terms of a partial agonist action of PD123319 at AT_{1B} receptors. Our suggestion is based on functional studies, and this may explain the lack of influence of PD123319 observed in the present experiments.

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