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Molecular cloning of the canine angiotensin II receptor

An AT1-like receptor with reduced affinity for DuP753

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Abstract

Canine aortic strip studies revealed insensitivity of angiotensin II (AII)-induced aortic contraction to inhibition by the non-peptide antagonist DuP753 ($pK_B = 6.7 \pm 0.1$). In order to determine the origin of this phenomenon we cloned the canine homologue of the AT_1 AII receptor. Expression of this cDNA in COS-7 cells indicated a low affinity of DuP753 for the cloned receptor ($K_D = 92$ nM). The predicted amino acid sequence is highly homologous to other mammalian AT_1 receptors; sequence comparisons suggest the pharmacological difference may be the result of a threonine residue in position 163 in the IVth transmembrane domain.

Key words: Angiotensin receptor; Non-peptide antagonists; cDNA cloning; Dog aorta; Vascular contractility; COS cell

1. Introduction

The renin angiotensin system has a major role in the regulation of salt and water metabolism and hence blood pressure, and there is abundant evidence of its involvement in human cardiovascular pathology. Investigation of its role has been facilitated in recent years by the development of non-peptide angiotensin receptor antagonists [1]. The use of these drugs has revealed the presence of two distinct subtypes of angiotensin receptors termed AT_1 and AT_2 which show selectivity for the non-peptide antagonist DuP753 (Losartan) and PD123177, respectively [2].

The canine aortic strip model has been widely used in pharmacological studies of angiotensin and other vasoactive substances. In the course of investigations into the pharmacology of various non-peptide AT_1 angiotensin receptor antagonists we found that the canine aortic angiotensin receptor was significantly less sensitive to blockade by DuP753 than the rabbit. As virtually all the physiological functions of angiotensin including aortic contraction are mediated through the AT_1 receptor in other mammals [3], it seemed most probable that the canine vasoconstrictor receptor was a molecular variant of the AT_1 receptor of other species. An understanding of the molecular basis of the interaction of non-peptide antagonists with the receptor could be a valuable advantage in the design of more effective agents of this type, and thus we set out to clone this receptor and to compare its pharmacology with that observed in functional studies in dog isolated aortic strips.

2. Materials and methods

2.1. Functional studies in aortic strips

Isometric tension measurements were made from endothelium denuded helical strips of rabbit or dog aorta, suspended at an initial tension of 0.5 g in glass baths containing physiological salt solution (Na^+, 143.4 mM; K^+, 5.9 mM; Mg^{2+}, 0.6 mM; Ca^{2+}, 1.3 mM; Cl^-, 124.5 mM; $H_2PO_4^-$, 1.2 mM; SO_4^{2-}, 0.6 mM; HCO_3^-, 25 mM; glucose, 11.1 mM) at 37°C. This also contained indomethacin (30 μM) and ascorbic acid (100 μM), and was continually gassed with 95% O_2/5% CO_2. The following protocol was then followed in four matched preparations of rabbit or dog aorta. KCl (80 mM) was added to all tissues to determine the contractile viability of the preparations. Once the contraction had reached plateau, the tissues were washed. After a further 15 min a cumulative priming 10-fold concentration response curve to AII (0.1–100 nM) was constructed. After washing the preparations were allowed to equilibrate for a further 30 min at which point a 3-fold concentration–response curve to AII was constructed. After washing, three preparations were exposed to different concentrations of DuP753 or PD123177 whilst the fourth preparation acted as a time-matched vehicle control. Two hours later, in the continued presence of antagonist or vehicle, a final 3-fold concentration–response curve was constructed. Where AII log concentration–response curves in the presence of antagonists were parallel with those observed in control preparations, AII concentration ratios were calculated at the EC_{50} levels of the control curve. Antagonist pA_2 estimates were then made [4]. Where the slope of the Schild regression did not differ significantly from unity, the slope was constrained to unity and apparent pK_B values calculated.

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2.2. Northern blot analysis

Poly-(A)$^+$ mRNA was prepared from various dog tissues and was loaded on a 1% agarose gel containing formaldehyde (6.3%) in 20 mM MOPS buffer (pH 7.0). After electrophoresis, RNA was transferred to supported nitrocellulose (Amersham), baked, pre-hybridized, and hybridized for 16 h at 42°C in the presence of 50% formamide. The probes used were either the full-length human or canine AT_1 receptor cDNA clone, or a rat β-actin cDNA probe. Probes were labelled to high specific activity (>10^9 cpm/μg) using [^{32}P]dCTP (6000 Ci/mmol, Amersham) and the random hexanucleotide priming technique [5]. Blots were washed in 0.1 × SSC at 55 or 60°C (final wash) and subjected to autoradiography for 18 h to 5 days (AII receptor) or 1.5 h (β-actin) with Kodak XAR-2 film.

2.3. Library screening

An oligo-dT primed canine liver cDNA library cloned in λgt10 was obtained from Clontech and approximately 250,000 plaques were plated and screened with a canine-derived AT_1 receptor probe [6]. This was synthesised by PCR using canine genomic DNA as template and oligonucleotide primers hAT_1-211 (5′-GCACTGGCTGACTTATGC-3′) and hAT_1-858 (5′-GATAGGCATGGCCGTGTC-3′) based on the human AT1 receptor cDNA sequence [7,8]. PCR products were separated on a gel, and the 647 bp band excised and quantitated prior to being labelled as above. Three successive rounds of library screening were performed as described [6] and resulted in two strongly positive clones. The largest cDNA insert from these was excised with *Eco*RI and subcloned into the plasmid pGEM7 (Promega).

2.4. DNA sequencing

Double-stranded dideoxynucleotide chain termination sequencing with Sequenase (USBC) was performed using successive oligonucleotide primers. Sequence data was analyzed with PCGENE software (Intelligenetics).

2.5. Expression vector construction and transfection

The full-length *Eco*RI fragment from the pGEM clone was excised and inserted into the *Eco*RI site of the plasmid expression vector pcDNA1 (Invitrogen). The orientation of the insert was checked in transformed colonies, and the correctly orientated plasmid was used in transfection studies. These were performed by cationic lipid mediated transfection with DOTAP (Boehringer) [9] into COS-7 cells (CRL No.1651, ATCC). After transfection, cells were plated in 6-well plates for binding studies, which were performed 48 h after addition of the DNA.

2.6. AII binding studies

Displacement binding studies were performed on whole cells in multiwell plates using ^{125}I-labelled [Sar_1,Ile_8]-AII (2000 Ci/mmol, Amersham). Displacement was with either AII, DuP753 or PD123177 (synthesized by Glaxo Group Research), and incubations were for 60 min at 37°C. After washing on ice, cells were lysed in 0.1 M sodium hydroxide, scraped and counted in a multichannel gamma counter. Data were analyzed with LIGAND software [10].

3. Results

AII (0.1–100 nM) evoked concentration-related contractions of rabbit and dog aortic strips with mean EC_{50} values (95% confidence interval) of 1.8 nM (0.7–4.4) and 3.3 nM (1.9–5.7)(n = 4–7), respectively (Fig. 1). DuP753 acted as a competitive antagonist of the effects of AII in aortic strips from both species causing parallel concentration-related rightward displacement of the AII concentration–response curve (Fig. 1). DuP753 also tended to increase the maximum response to AII. Fig. 1 illustrates that DuP753 is a significantly more potent antagonist of AII in the rabbit (mean apparent $pK_B = 8.4 \pm 0.02$, $n = 4$) compared to dog aortic strips (mean apparent $pK_B = 6.7 \pm 0.1$, $n = 7$). In contrast to DuP753, the AT_2 antagonist PD123177 (1 μM) caused no inhibition of the effects of AII in rabbit or dog aortic strips.

Northern blot analysis using the human angiotensin receptor as a probe with mRNA from various dog tissues suggested that a single AT_1-like mRNA species of 2.9 kb was present in most tissues, but was most prevalent in the canine liver and adrenal. For this reason, a canine liver cDNA library was screened. In order to undertake high stringency hybridization the canine gene was amplified by PCR using primers based on human AT_1 receptor transmembrane domains II and VII.

The cDNA library was screened as described and resulted in the isolation of two strongly positive plaques. One of these (λ9a) contained a 1.8 kb cDNA which was subcloned into pGEM7. This clone was sequenced and revealed a cDNA encoding a polypeptide of 359 residues with marked homology to other cloned AT1 receptors (Fig. 2). Interspecies comparison indicates that this clone diverges insignificantly from other mammalian AT_1 angiotensin receptors, being most similar to the porcine receptor (95.5% identical over the whole length).

Use of this clone as a probe in northern blots of mRNA from various canine tissues indicates high prevalence of this mRNA in the adrenal and liver, with smaller quantities in aorta, kidney, lung, testis and heart (Fig. 3).

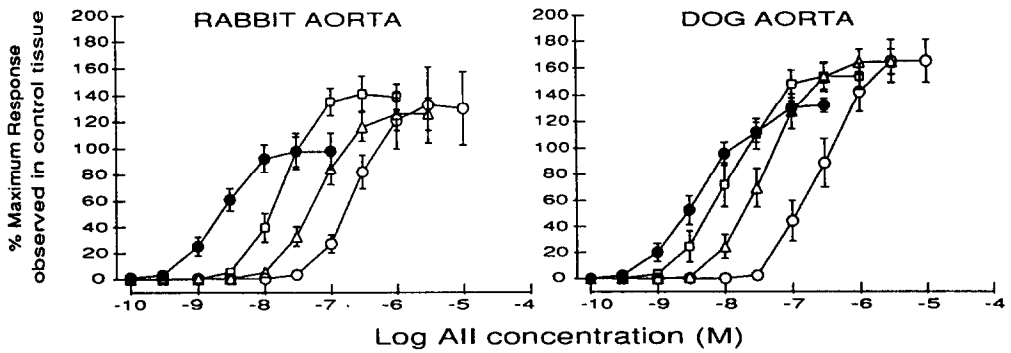

Fig. 1. Antagonism of the contractile effects of AII in rabbit and dog aortic strips by DuP753. Rabbit aorta (AII in the absence, ●; and presence of DuP753 at 0.03 μM, □; 0.1 μM, △; and 0.3 μM, ○; n = 4). Dog aorta (AII in the absence ●; and presence of DuP753 at 0.1 μM, □; 1.0 μM, △; and 10 μM, ○; n = 7). Values are mean ± S.E.M.

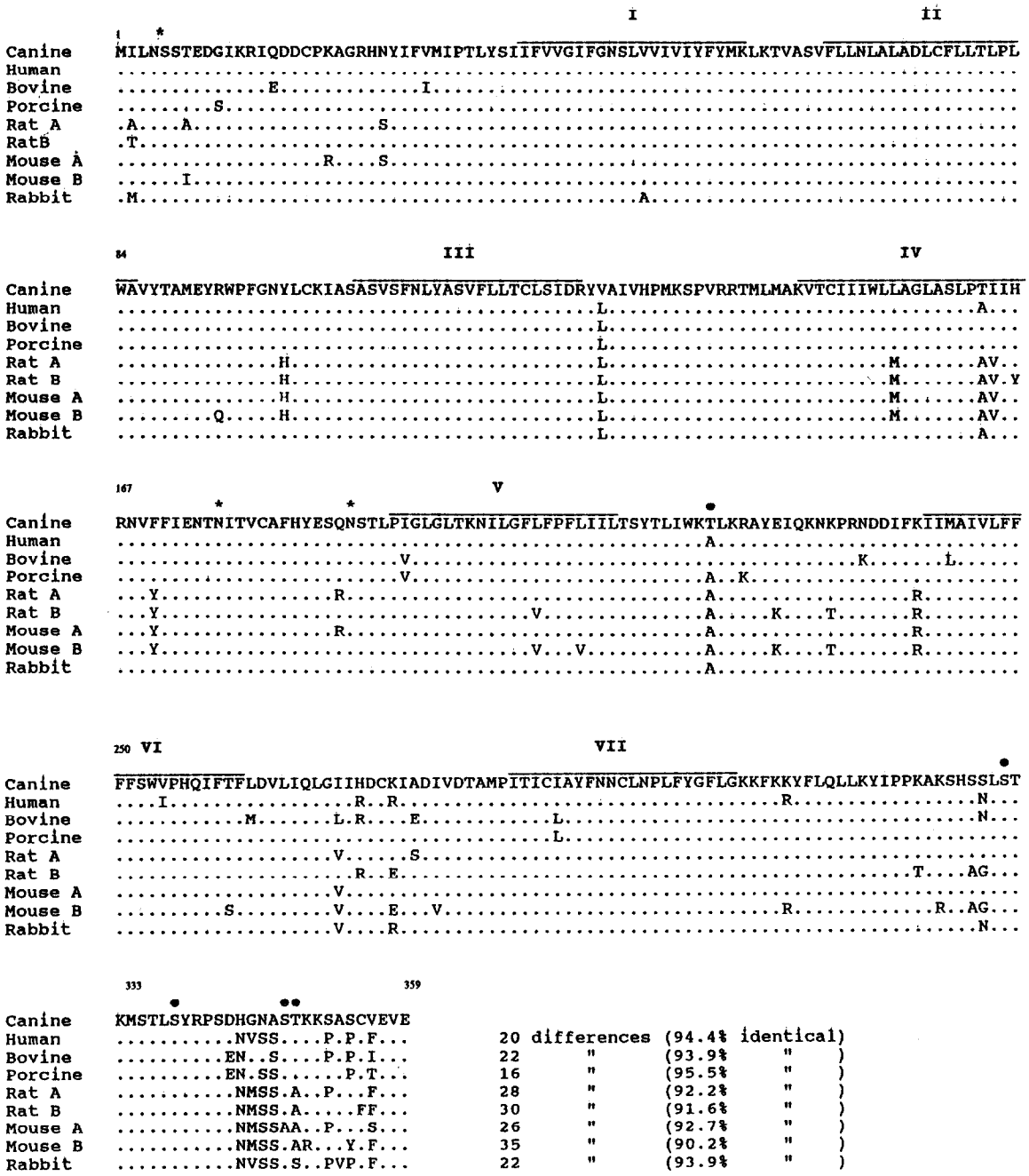

Fig. 2. Comparison of the amino acid sequence of the canine AT1 receptor with other mammalian AT_1 receptors. Identical amino acids are represented by (–). Potential N-linked glycosylation sites are represented by (*), and potential protein kinase C phosphorylation sites in the canine receptor by (●). The single letter amino acid code is used throughout.

Confirmation that this clone is an angiotensin receptor and characterization of its pharmacology requires its expression in a eukaryotic system. Therefore the full-length cDNA was inserted downstream of the CMV promoter in the plasmid pcDNA1, and this was used in transient expression transfection experiments in COS-7 cells, which we have previously found to be almost devoid of endogenous AT_1 receptor [11]. Expression was assessed by binding of ^{125}I-labelled [Sar_1,Ile_8]AII 48 h after transfection. Fig. 4 illustrates the displacement curves obtained from a mean of three binding experiments in which either AII or Dup 753 was used to displace the labelled ligand. The K_D's calculated by Scatchard analysis were 5.29×10^{-9} M for AII and 92.2×10^{-9} M for DuP753. The AT_2 specific non-peptide antagonist, PD123177 at a concentration of 10^{-6} M had no effect on binding.

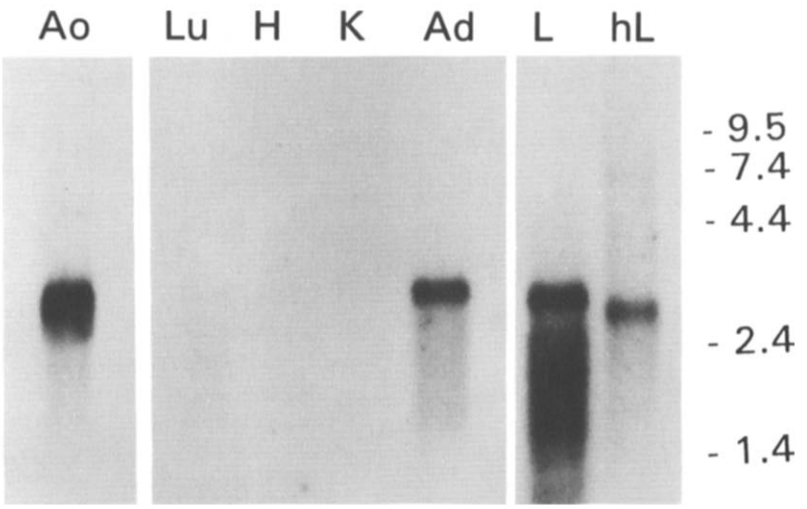

Fig. 3. Northern blot analysis of various canine tissues probed with the canine AT_1-like receptor cDNA probe. RNA markers are shown on the right. Ao = aorta, Lu = lung, H = heart, K = kidney, Ad = adrenal, L = liver, hL = human liver. 2.5 μg of poly $(A)^+$ mRNA were loaded in each lane except for aorta which was 20 μg of poly $(A)^+$ mRNA.

4. Discussion

Development of the non-peptide angiotensin receptor antagonists has resulted in a rapid expansion of our understanding of the role of angiotensin in physiology, and they have great potential value as therapeutic agents in the treatment of hypertension and cardiac failure. Since the original cloning of the rat and bovine AT_1 receptors [12,13], there has been the opportunity to understand the molecular nature of the interaction between the AT_1-selective non-peptide antagonists and target receptors. This might in turn ultimately lead to the development of new antagonists. Clues as to the basis of this interaction could come from mutagenesis studies of the receptor, or from functional studies of these antagonist–receptor interactions in other species, essentially exploiting naturally occurring 'mutagenesis' experiments. The discovery that DuP753 has low affinity for AT_1 receptors in functional studies in dog aortic strips provided the basis for this study.

Our cloning and sequencing studies reveal that the canine receptor has a sequence that is remarkably similar to that of the AT_1 receptor of other mammalian species, being 95.5% identical at the amino acid level to the porcine sequence over its entire length. The sequence predicts the existence of a number of potential protein kinase C phosphorylation sites in the third intracellular loop (one site) and in the C-terminal tail (four sites), and three potential N-linked glycosylation sites in identical positions to those of the other mammalian AT_1 receptors (see Fig. 2).

When expressed in COS-7 cells the canine receptor exhibits very similar pharmacology to the bovine [12,14] and porcine receptor [16,17] in terms of its high affinity for AII, insensitivity to PD123177, and reduced affinity for DuP753 when compared with the rat, results which are consistent with the canine aortic strip studies. Thus the aortic strip studies yielded an estimated K_B of 199.5 nM for DuP753 which is similar to the calculated K_D of 92.2 nM. However comparison of the canine, bovine and porcine receptor sequences reveals little that is common between them yet different from the other DuP753 sensitive receptors. The only obvious common feature in a location that might be involved in DuP753 binding is the threonine at position 163 in the IVth transmembrane domain which is an alanine in other receptors (see Fig. 2). Domain swapping and mutagenesis experiments could be used to determine the role of the molecular differences between the canine and other receptors in their interaction with non-peptide antagonists.

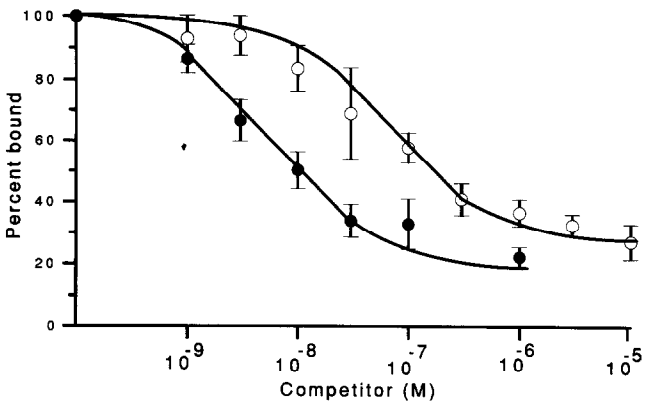

Fig. 4. Displacement of ^{125}I-labelled $[Sar_1,Ile_8]$-AII with angiotensin II (●) and DuP753 (○). These results represent the mean displacement from three binding experiments, and error bars represent the S.E.M.

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