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Characterization of an Angiotensin Type-1 Receptor Partial cDNA from Rat Kidney: Evidence for a Novel AT_{1B} Receptor Subtype

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SUMMARY: We sought to determine if multiple forms of mRNA for the angiotensin type-1 (AT_1) receptor could be detected in rat kidney using the polymerase chain reaction (PCR) procedure. Amplification of rat kidney cDNA with oligonucleotide primers derived from the second and sixth transmembrane domains of the rat AT_1 receptor yielded a single cDNA fragment 528bp in size. Sequence analysis indicated, however, that the cDNA fragment was a mixture of two highly similar gene products: the first cDNA was identical to the previously cloned AT_1 receptor (termed here AT_{1A}) whereas the second cDNA (termed here AT_{1B}) was 92% identical at the nucleotide level and 96% identical at the amino acid level. Nucleotide substitutions were dispersed throughout the cDNA and 80% (33 of 41) were conservative. Significant levels of AT_{1A} and AT_{1B} mRNA were detected by PCR amplification of kidney poly(A)$^+$ RNA and restriction enzyme analysis. These results indicate that at least two distinct AT_1 receptor genes are expressed in rat kidney.

Angiotensin II (Ang II) decreases renal blood flow and increases proximal tubule sodium reabsorption (1). Ang II receptors in the kidney have been shown to be concentrated on renal vascular smooth muscle, mesangial cells and proximal tubule brush border and basolateral membranes (1) and linked to stimulation of polyphosphoinositol (PI) hydrolysis and inhibition of adenylyl cyclase (AC) (2). Ang II receptors have been characterized pharmacologically as type 1 and type 2 (AT_1 and AT_2) (3,4). The AT_1 receptor is a G-protein coupled receptor that stimulates PI hydrolysis and mobilizes intracellular calcium (3), whereas AT_2 receptors are not coupled to G proteins and their effector system are still unknown (5). The majority of the effects of Ang II on the kidney appear to be mediated by AT_1 receptors (4).

Recently, several highly similar cDNAs encoding an AT_1 receptor have been cloned from bovine adrenal, rat vascular smooth muscle, rat kidney, and human liver (6-9); greater than 90% homology at the amino acid level. The gene for the human AT_1 receptor has also been cloned (10). Functional studies indicate that the cloned AT_1 receptor stimulates

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PI hydrolysis and mobilizes intracellular calcium (6-8). It is not known, however, whether the same AT_1 receptor couples to multiple effector systems (i.e. PLC and AC) or whether there are multiple AT_1 receptor subtypes. Northern blot analysis of rat kidney RNA revealed labeling of two bands, a heavily labeled 2.3 kb band and a weakly labeled 3.5 kb band (7). It was not known whether the 3.5 kb band represented a highly similar gene product, alternatively spliced mRNA, or nuclear AT_1 receptor RNA (7). In addition to a heterogenous distribution within the kidney (1), previous pharmacological and physiological studies have suggested that multiple Ang II receptors may be expressed in the kidney (2). Here we sought to determine if multiple forms of mRNA for the AT_1 receptor could be detected in rat kidney by the polymerase chain reaction (PCR) procedure.

MATERIALS AND METHODS

Male Sprague-Dawley rats (150-175 g, Charles River Breeding Laboratories, Wilmington, MA) were decapitated and the kidney cortex dissected and frozen in liquid nitrogen. Total RNA was extracted from rat kidney by the method of Chomczynski and Sacchi (11). Poly(A)$^+$ RNA was isolated by passage over an oligo(dT) cellulose column. First strand cDNA synthesis was conducted with poly(A)$^+$ RNA as template in the presence of reverse transcriptase and an oligo(dT) primer (Superscript RNA reverse transcriptase, BRL). PCR primers were constructed based on the sequence of the rat AT_1 receptor (7,8). The sense oligonucleotide primer was from the carboxyl region of the second transmembrane domain (nucleotides 250-270, 5'-TGGGCAGTCTATACCGCTATG-3') terminating in the codon for methionine and the antisense codon was from the sixth transmembrane domain (nucleotides 757-777, 5'-AATATTTGGTGGGGGACCCA-3') terminating in the codon for tryptophan. Each primer also included a 5' linker sequence (5'-CAACTCGAG-3') that encoded an *XhoI* restriction site. Kidney cDNA (20 ng) was submitted to 25 cycles of PCR amplification with PCR primers in the presence of *Taq* DNA polymerase in the following sequence: first cycle --- 5 min at 94°C, 2 min at 55°C, 3 min at 72°C; subsequent cycles --- 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C; last cycle --- 1 min at 94°C, 2 min at 55°C, and 10 min at 72°C. The PCR products were then separated on a 1% agarose gel and the bands examined under UV illumination. PCR controls included a) amplification with template but without primers, b) amplification with mRNA without the reverse transcriptase step, and c) amplification without template.

The major PCR-amplified product was subcloned into the TA cloning vector (Invitrogen, San Diego, CA) and used for transformation of INVαF' competent *E. coli*. Positive transformants containing plasmids with inserts were selected by growing bacteria on LB plates containing kanamycin, IPTG and Bluo-Gal. Plasmids were isolated from minicultures and digested with *XhoI*. Plasmids containing the PCR product were then sequenced directly by the dideoxynucleotide chain-termination method with sequence-specific oligonucleotide primers (Sequenase, US Biochemical Corp.).

Relative levels of AT_1 receptor mRNA in rat kidney were determined by amplification of rat kidney cDNA by PCR followed by restriction enzyme (RE) digestion of the PCR product. cDNA was incubated with appropriate restriction enzymes under optimal conditions and then electrophoresed on a 1% agarose gel and viewed under UV illumination.

RESULTS AND DISCUSSION

PCR primers were constructed based on the second and sixth transmembrane regions of the rat AT_1 receptor cDNA (7,8). Amplification of rat kidney cortex cDNA yielded a

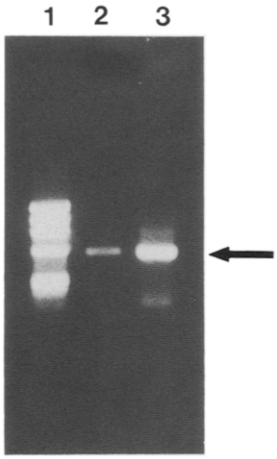

Figure 1. Agarose gel electrophoresis of PCR products from amplification of rat kidney cortex cDNA with AT_1 receptor oligonucleotide primers. Lane 1: DNA markers (PhiX 174 digested with *HaeIII*), lane 2: 25 cycles, lane 3: second round of 25 cycles.

single product of the predicted size (Figure 1, arrow). The PCR-amplified cDNA product was subcloned into the TA cloning vector and four individual clones sequenced. Two of the clones were identical to the previously published rat kidney AT_1 cDNA (8), and differed from the rat vascular AT_1 cDNA by several nucleotides (7). The remaining two clones were identical to each other and highly similar, but not identical, to the rat AT_1 receptor cDNA. Sequence analysis (Figure 2) indicated that the novel cDNA was 92%

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                        G     C              T                    G        G
TGGGCAGTCTATACCGCTATGGAATACCGATGGCCCTTCGGCAACCACCTATGTAAGATCGCTTCTGCCAGCGTC  324
TrpAlaValTyrThrAlaMetGluTryArgTrpProPheGlyAsnHisLeuCysLysIleAlaSerAlaSerVal  108

 CG     C     C           C  T                      C              C
AGTTTCAATCTCTATGCCAGTGTGTTTCTGCTCACGTGTCTCAGCATCGATCGCTACCTGGCCATTGTCCACCCA  399
SerPheAsnLeuTyrAlaSerValPheLeuLeuThrCysLeuSerIleAspArgTyrLeuAlaIleValHisPro  133
Thr
              T                    G                          G
ATGAAGTCTCGCCTCCGCCGCACGATGCTGGTAGCCAAAGTCACCTGCATCATCATCTGGCTAATGGCTGGCTTG  474
MetLysSerArgLeuArgArgThrMetLeuValAlaLysValThrCysIleIleIleTrpLeuMetAlaGlyLeu  158

              T      C       T     C                                G     G
GCCAGTTTGCCAGCCGTCATCTACCGAAACGTATATTTCATCGAGAACACCAATATCACAGTTTGCGCTTTTCAT  549
AlaSerLeuProAlaValIleTyrArgAsnValTyrPheIleGluAsnThrAsnIleThrValCysAlaPheHis  183
                       His
    G    G   T  G  G        A  G     C  T  C     T             T
TATGAATCTCAGAACTCAACACTCCCCATTGGACTGGGTCTAACAAAGAACATTCTGGGCTTCGTGTTCCCTTTC  624
TyrGluSerGlnAsnSerThrLeuProIleGlyLeuGlyLeuThrLysAsnIleLeuGlyPheValPheProPhe  208
        Arg                                                      Leu
                        C              T                G       A       AA
CTTATCATTCTCACCAGCTATACTCTTATTTGGAAAGCCCTAAAGAAGGCTTATAAAATTCAGAAGAACACGCCA  699
LeuIleIleLeuThrSerTyrThrLeuIleTrpLysAlaLeuLysLysAlaTyrLysIleGlnLysAsnThrPro  233
                                                        Glu             Lys
    C
AGAAATGATGACATCTTTAGGATAATTATGGCGATTGTGCTTTTCTTCTTCTTTTCCTGGGTCCCCCACCAAATATTC  777
ArgAsnAspAspIlePheArgIleIleMetAlaIleValLeuPhePhePhePheSerTrpValProHisGlnIlePhe  259
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Figure 2. Nucleotide and deduced amino acid sequence of the partial AT_{1B} cDNA from rat kidney. Nucleotides that were different between the AT_{1B} and rat kidney AT_1 cDNA are shown above and amino acids that were different are shown below. The nucleotide sequence of the AT_{1B} cDNA in the region used for the PCR primers (underlined) is unknown. Numbering corresponds to the scheme used by Murphy et al. (7).

homologous with the rat AT_1 receptor cDNA at the nucleotide level and 96% homologous at the amino acid level. This pattern of substitution strongly suggests that there are two distinct AT_1 genes expressed in rat kidney: the previously cloned AT_1 receptor (referred to here as AT_{1A}) and a novel gene that will be referred to as AT_{1B}.

As noted above, the nucleotide sequence of the AT_{1A} and AT_{1B} cDNAs were highly similar, with the majority of the nucleotide substitutions (33 of 41) being conservative substitutions in the third base position of the codon. The greatest divergence between the AT_{1A} and AT_{1B} cDNAs was seen in the region extending from the putative second extracellular loop to the amino portion of the fifth transmembrane domain, with 11 of 46 nucleotides being different. There were only two amino acid substitutions predicted for the AT_{1B} receptor in the transmembrane regions II-VI (i.e. serine for Thr-109 and valine for Leu-205) suggesting that the AT_{1A} and AT_{1B} receptors may bind angiotensin peptides with a similar profile. Other areas of the cDNA thought to encode functionally significant amino acid residues were conserved, including two putative phosphorylation sites, Ser-136 and Thr-141 in the second intracellular loop, two asparagine-linked glycosylation sites, Asn-176 and Asn-188 in the second extracellular loop, and two cysteine residues, Cys-101 and Cys-180, that are belieeved to stabilize the receptor by formation of a disulfide bond between the first and second extracellular loops. Particular attention was paid to the portion of the cDNA encoding the putative third intracellular loop (CIII), between the fifth and sixth transmembrane domains. This region is highly variable among G protein linked receptors (12). In receptors that couple to G_s, G_o or G_q the CIII loop is small, whereas in receptors that couple to G_i the CIII loop is quite large. If the kidney expressed two closely related forms of the AT_1 receptor, i.e. one that inhibits AC activity by coupling to G_i and another that stimulates PLC by coupling to G_o or G_q, then two distinct PCR products would have been predicted. However, PCR amplification of rat kidney cDNA yielded a single band of the predicted size for the AT_1 receptor. Comparison of the nucleotide sequence between the AT_{1B} and AT_{1A} cDNAs in the region of the CIII loop indicated a high degree of conservation at both the nucleotide and amino acid levels. Interestingly, two amino acid substitutions --- lysine for Glu-227 and threonine for Lys-232 --- are predicted based on the sequence of the AT_{1B} cDNA. It is not clear whether these substitutions would result in a difference in the coupling properties of the AT_{1B} receptor to G proteins, but the substitution of Thr-232 for Lys-232 introduces a potential protein kinase C (PKC) consensus phosphorylation site (S/T-X-R/K) into the AT_{1B} sequence. The AT_{1A} receptor subtype does not have a PKC phosphorylation site in the CIII region. This difference may have functional significance, since Ang II receptor mediated breakdown of PI leads to formation of diacylglycerol, the physiological activator of PKC. Furthermore, PKC-mediated phosphorylation of receptors has been shown to be involved in receptor desensitization mechanisms (12). Thus, it is possible that the AT_{1A} and AT_{1B} receptors may be regulated differently with regards to homologous and heterologous desensitization.

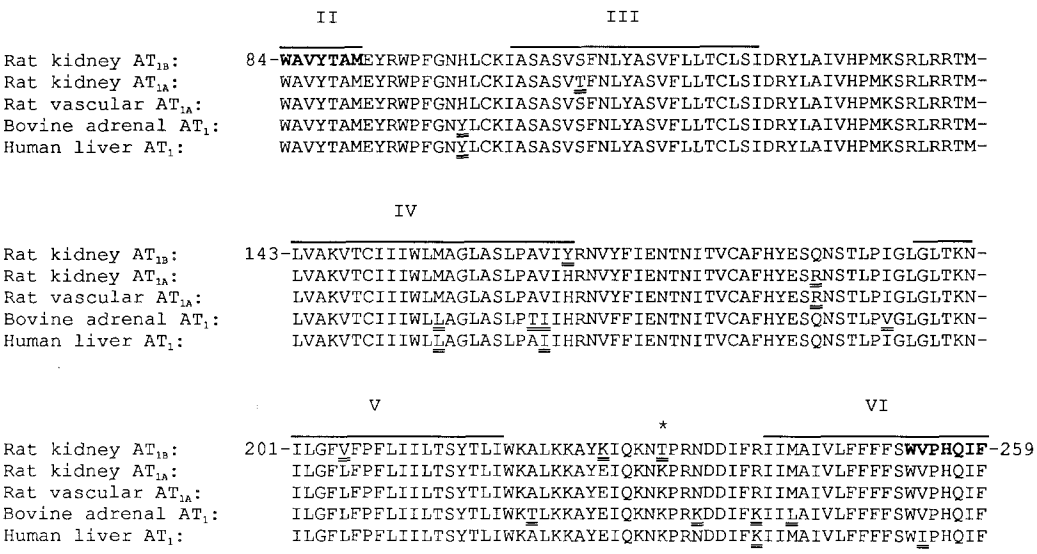

Figure 3. Comparison of the amino acid sequences of the rat AT_{1B} cDNA with AT_1 receptors from rat kidney (8), rat vascular (7), bovine adrenal (6), human liver (9). Amino acids that are not found in the majority of receptors are double-underlined. The potential PKC phosphorylation site in the AT_{1B} receptor (T-232) is marked with an asterisk.

The nucleotide and deduced amino acid sequences of the AT_{1B} receptor cDNA were also compared against the sequences for bovine adrenal and human liver AT_1 receptors. The AT_{1B} cDNA sequence was 85% and 86% identical to the bovine and human AT_1 receptors at the nucleotide level and 92% and 94% identical at the amino acid level (Figure 3), respectively. Since there appears to be multiple forms of the AT_1 receptor expressed in rat, it is probable that additional AT_1 receptors will be identified from bovine and human. Moreover, since only one form of the AT_1 receptor has been cloned from bovine and human, it is not clear which form of the rat AT_1 receptor the bovine and human AT_1 receptor cDNAs represent. Within the area of comparison prescribed by the partial AT_{1B} cDNA from rat, the amino acid sequence of the human liver AT_1 receptor was 96% and 94% identical with the rat kidney AT_{1A} and AT_{1B} receptors, respectively. Therefore, it is uncertain whether the AT_1 receptors cloned from bovine and human are more closely related to the rat AT_{1A} or AT_{1B} receptor.

The high degree of similarity between the AT_{1A} and AT_{1B} cDNAs would seemingly preclude the use of hybridization methods to measure tissue mRNA levels. Indeed, Northern blot hybridization of kidney RNA with either the AT_{1A} or AT_{1B} receptor cDNAs resulted in labeling of a ~2.3 kb band (data not shown). We utilized an alternative approach to determine the relative levels of each mRNA in the kidney. Sequence analysis indicated that there were unique restriction enzyme (RE) sites within the AT_{1A} and AT_{1B} cDNA sequences. Rat kidney cortex cDNA was amplified with AT_1 primers and the PCR product then digested with REs. *EcoRI* or *PvuII* treatment, specific REs for the AT_{1A}, yielded the predicted smaller bands (Figure 4) as well as intact material that was RE-

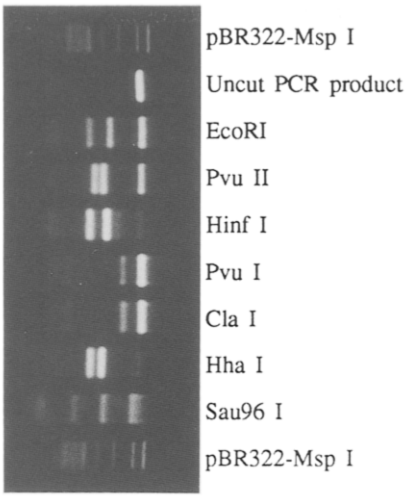

Figure 4. Restriction enzyme (RE)-PCR analysis of AT_1 receptor expression in rat kidney cortex. Oligonucleotide primers for the AT_1 receptor were used to amplify rat kidney cDNA by PCR and the PCR product digested with various REs and separated on a 1.2% agarose gel, stained with ethidium bromide and viewed under UV illumination. DNA markers were pBR322 digested with MspI: 622 bp, 527, 404, 309, 242, 238, 217, 201, 190, 180 etc. The uncut PCR product was ~540 bp (cDNA plus linkers) in size. Digestion of the AT_{1A} and AT_{1B} 540bp cDNAs with REs were predicted to yield the following products (in base pairs):

RE	AT_{1A}	AT_{1B}
EcoRI	321,219	-
PvuII	293,247	-
HinfI	313,227	313,227
PvuI	-	405,135
ClaI	-	409,131
HhaI	301,239	239,170,131
Sau96I	304,174,41,21	408,41,21

Digestion with EcoRI and PvuII, enzymes which should cut AT_{1A} but not AT_{1B}, yielded bands of the predicted size for AT_{1A} digestion, but also a major uncut band at 540 bp, which may represent the AT_{1B} cDNA. HinfI sites were identical for AT_{1A} and AT_{1B} and almost completely digested the PCR product. Digestion with PvuI and ClaI enzymes which should cut AT_{1B} but not AT_{1A}, yielded bands of the predicted size for AT_{1B} digestion, but also a significant uncut band at 540 bp, which probably represents the AT_{1A} cDNA. There is one HhaI site in AT_{1A} and two sites in AT_{1B} and digestion gave rise to the predicted products. Finally, there are three Sau96I sites in AT_{1A} and two sites in AT_{1B}. Note that following Sau96I digestion there is a prominent 408 bp band, corresponding to AT_{1B}.

resistant, presumably corresponding to AT_{1B} cDNA. Likewise, treatment with *PvuI* and *ClaI*, specific REs for the AT_{1B} cDNA, gave rise to a smaller band of the predicted size, as well as a RE-resistant band that presumably corresponds to AT_{1A} cDNA. Treatment with REs with sites in both cDNAs resulted in virtually complete digestion of the PCR product. These results indicate that there are lower levels of the AT_{1B} receptor mRNA than there AT_{1A} receptor mRNA in the kidney, perhaps accounting for the latter form being the first to be cloned. Nevertheless, these results clearly indicate the kidney cortex contains two species of mRNA for the AT_1 receptor.

In conclusion, these results indicate that there are at least two AT_1 receptor genes expressed in the kidney. Cloning and expression of the full length AT_{1B} cDNA will be necessary to determine whether the AT_{1A} and AT_{1B} receptors can be distinguished on the basis of their functional properties or cellular localizations. In addition, it will be important to determine whether the AT_{1B} receptor is expressed in tissues other than the kidney, since the high degree of similarity between the AT_{1A} and AT_{1B} cDNAs suggests that previous hybridization studies would have failed to distinguish between the two forms of mRNA. Therefore, the tissue distribution of the AT_1 receptor based on hybridization studies needs to be re-examined using sequence-specific probes.

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REFERENCES

1. Ichikawa, I., and Harris, R. C. (1991) Kidney Int. 40, 583-596.
2. Douglas, J.D. (1987) Am. J. Physiol. 253, F1-F7.
3. Chiu, A.T., Herblin, W. F., McCall, D. E., Ardecky, R. J., Carini, D. J., Duncia, J. V., Pease, L. J., Wong, P. C., Wexler, R. R., Johnson, A. L., and Timmermans, P. B. M. W. M. (1989) Biochem. Biophys. Res. Comm. 165, 196-203.
4. Edwards, R. M., Stack, E. J., Weidley, E. F., Aiyar, N., Keenan, R. M., Hill, D. T., and Weinstock, J. (1992) J. Pharmacol. Exp. Ther. 260, 933-938.
5. Dudley, D. T., Hubbell, S. E., and Summerfelt, R. M. (1991) Mol. Pharmacol. 40, 360-367.
6. Sasake, K., Yamano, T., Bardhan, S., Iwai, N., Murray, J. J., Hasegawa, M., Matsuda, Y., and Inagami, T. (1991) Nature 351, 230-233.
7. Murphy, T.J., Alexander, R. W., Griendling, K. K., Runge, M. S., and Bernstein, K. E. (1991) Nature 351, 233-236.
8. Iwai, N., Yamano, Y., Chaki, S., Konishi, F., Bardhan, S., Tibbetts, C., Sasaki, K., Hasegawa, M., Matsuda, Y., and Inagami, T. (1991) Biochem. Biophys. Res. Comm. 177, 299-304.
9. Takayanagi, R., Ohnaka, K., Saka, Y., Nakao, R., Yanase, T., Haji, M., Inagami, T., Furuta, H., Gou, D. F., Nakamuta, M., and Nawata, H. (1992) Biochem. Biophys. Res. Comm. 183, 910-916.
10. Furuta, H., Guo, D. F., and Inagami, T. (1992) Biochem. Biophys. Res. Comm. 183, 8-13.
11. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
12. Raymond, J. R., Hnatowich, M., Lefkowitz, R. J., and Caron, M. C. (1990) Hypertension 15, 119-131.