Predominant Expression of β_1-Adrenergic Receptor in the Thick Ascending Limb of Rat Kidney

Absolute mRNA Quantitation by Reverse Transcription and Polymerase Chain Reaction

Jean-Marc Elalouf, Jean-Marie Buhler,* Christel Tessiot, Anne-Christine Bellanger, Isabelle Dublineau, and Christian de Rouffignac

*Département de Biologie Cellulaire et Moléculaire, Service de Biologie Cellulaire, and *Service de Biochimie et de Génétique Moléculaire, Centre d'études de Saclay, 91191 Gif-sur-Yvette Cedex, France*

Abstract

β_1- and β_2-adrenergic receptor (β-ARs) expression in the thick ascending limb of rat kidney was studied at the level of mRNA and receptor coupling to adenylyl cyclase. Absolute quantitation of β_1- and β_2-AR mRNAs in microdissected nephron segments was performed with an assay based on reverse transcription and polymerase chain reaction, using in vitro transcribed mutant RNAs as internal standards. In the cortical thick ascending limb (CTAL), the number of mRNA molecules / mm of tubular length was 2,806±328 (n = 12) for β_1-AR and 159±26 for β_2-AR ($P < 0.01$). Lower levels were obtained in the medullary thick ascending, β_1-AR mRNA still being predominant. The pharmacological properties of β-ARS was also studied in the CTAL. Cyclic AMP accumulation was stimulated by β-agonist with a rank order of potency of isoproterenol >norepinephrine >epinephrine. This observation, and the higher efficiency of a β_1 than of a β_2 antagonist to inhibit isoproterenol-induced cAMP accumulation, establish the typical β_1-AR sensitivity of the CTAL. No detectable contribution of atypical or β_3-ARs to adenylyl cyclase stimulation could be found. In conclusion, this study, which shows markedly different levels of β_1- and β_2-AR mRNAS in the CTAL, provides a molecular basis for the predominant expression of the β_1 receptor subtype in this nephron segment. (*J. Clin. Invest.* 1993. 91:264–272.) Key words: quantitative reverse-transcription polymerase chain reaction • site-directed mutagenesis • nephron microdissection • cyclic adenosine monophosphate • catecholamines

Introduction

Catecholamine action through adenylyl cyclase–coupled β-adrenergic receptors (β-ARs)[1] has been shown to influence a variety of renal transport processes, including Cl (1), Na (1, 2), Ca and Mg (2) reabsorption as well as K (3, 4) and bicarbonate (5) secretion. In both rat and mouse kidney, catecholamine-sensitive adenylyl cyclase activity is mainly present in the thick ascending limb of Henle's loop and the collecting duct (6). In the thick ascending limb, the β-agonist isoproterenol increases Na, Cl, Ca and Mg reabsorption (2), all effects that are also elicited by several peptide hormones, including vasopressin, glucagon, calcitonin, and parathyroid hormone (7). To date, the physiological significance of such multiple hormonal control of the thick ascending limb functions has not been elucidated. However, it was proposed that the pluri-hormonal control of NaCl reabsorption could preserve the corticomedullary gradient in water diuresis (i.e., in the absence of vasopressin) and thus might play a role in the urinary concentrating process (7). On the other hand, the hormone-dependent increase of Mg reabsorption in the thick ascending limb is generally responsible for a reduction of the Mg excretion rate (7). This observation opens the question of the hormonal control of Mg balance.

β-ARs are membrane receptors that stimulate adenylyl cyclase. Three subtypes of β-ARs, termed β_1, β_2, and β_3, have been characterized (8–10). Nothing is known, however, about the contribution of the various β-AR subtypes to the action of catecholamines in the thick ascending limb as well as in other nephron segments. This question gains importance in view of the different affinities of these receptors for epinephrine and norepinephrine. Norepinephrine is the preferential ligand of both β_1- and β_3-ARs (8, 10, 11) whereas epinephrine acts preferentially on the β_2-AR (8). In addition recent studies revealed that glucocorticoids (12) or obesity (13) alters differentially the various β-ARs at both the mRNA and protein levels. Thus, establishing the physiological significance of catecholamine action first requires a detailed characterization of the β-ARs expressed in the target cells.

Considering the structural and functional heterogeneity of the tubular segments, it is essential to determine the distribution of the various receptor subtypes in well-delineated nephron portions. Probably because of this heterogeneity, previous Northern hybridization analysis performed on whole kidney RNAs failed to detect either β_1-, β_2-, or β_3-AR mRNAs in the rat or mouse kidney (8, 11). More sensitive methods must be used to obtain this information. The amplification through reverse transcription and polymerase chain reaction (RT-PCR) offers the opportunity to detect specific mRNAs in single or small numbers of cells (14). Accordingly, we developed a quantitative RT-PCR assay to measure β_1- and β_2-AR mRNAs in single pieces of nephron segments. This method was used to measure the steady state levels of β_1- and β_2-AR mRNAs in microdissected rat cortical thick ascending limbs (CTAL) and revealed that the amounts of β_1- and β_2-AR mRNAs greatly differed in this nephron portion. From a physi-

Address correspondence to Jean-Marc Elalouf, Departement de Biologie Cellulaire et Moléculaire, Service de Biologie Cellulaire, Centre d'études de Saclay, 91191 Gif-sur-Yvette, Cedex, France.

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1. *Abbreviations used in this paper:* β-AR, β-adrenergic receptor; CTAL, cortical thick ascending limb; MTAL, medullary thick ascending limb; RT-PCR, reverse transcription-polymerase chain reaction; TBE, Tris-borate EDTA; TE, Tris-EDTA.

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ological perspective it was also important to assess whether the expression of the corresponding membrane receptors correlated with these differential mRNA levels. For this purpose, intracellular cAMP accumulation induced by β-agonists and its inhibition by specific antagonists were measured in microdissected nephron segments. These experiments allowed us to establish which β-AR subtypes are functionally coupled to the adenylyl cyclase system.

Methods

The quantitative RT-PCR assay we have elaborated requires the availability of a standard as close as possible to the mRNA of interest. This assay, as the one described by Becker-André and Hahlbrock (15), was performed using mutant RNAs that display a single or double base substitution to create a new endonuclease restriction site in the corresponding cDNA. To generate such mutated RNAs the entire coding region of the rat β_1- and β_2-AR genes were amplified by PCR from genomic DNA and cloned into a plasmid vector carrying promoters of T3 and T7 RNA polymerases. Site-directed mutagenesis was performed on the cloned DNA fragments and known amounts of β_1 and β_2 mutant RNAs were produced by in vitro transcription. Precise amounts of the mutant RNAs were used as internal standards to determine the amounts of coamplified RNAs extracted from isolated tubules. After conversion to cDNA and PCR, the DNA fragments were treated with the appropriate restriction enzyme. Wild-type and mutant DNA fragments were discriminated by gel electrophoresis, allowing a quantitative determination of natural mRNA amounts introduced in the assay.

All the methods used for recombinant DNA procedures and RNA manipulation were from Ausubel et al. (16). The solutions used for experiments on RNAs were prepared in diethyl pyrocarbonate–treated water and were sterilized by filtration or autoclaving as appropriate. Glassware and surgery instruments were heated at 180°C for 8–12 h and were subsequently always handled with gloves. To prevent contamination, PCR and RT-PCR experiments were performed using specially designed pipettes. Restriction enzymes were from New England Biolabs (Beverly, MA).

Primer design and synthesis. For PCR and RT-PCR experiments the primers were designed with the help of Oligo software (MedProbe, Oslo, Norway). Specific β_1 or β_2 primers with Tm >60°C were selected. Each pair of upstream and downstream primers had closely similar Tm (Table *I*). They were also checked for minimal self-priming and upper/lower dimer formation. The primers were synthetized on a DNA synthesizer (Model 380B; Applied Biosystems, Inc., Foster City, CA) by β-cyanoethyl-phosphoramidite chemistry, and then ammonia deprotected overnight and ethanol precipitated. Working solutions were stored at −20°C in 10 mM Tris-HCl, pH 8; 1 mM EDTA (TE) at a concentration of 5 pmol/μl.

PCR cloning of rat β_1 and β_2-AR templates. A 1,586-bp DNA fragment encompassing the entire coding region of the rat β_1-AR gene (17) was amplified from 1 μg of Sprague-Dawley rat genomic DNA (Stratagene, Inc., San Diego, CA) by PCR using primers listed in Table I. The 5′ end of the upper primer located 14 nucleotides upstream to the ATG codon, and the 5′ end of the lower primer located 271 nucleotides downstream to the stop codon. Amplification was carried out using a Hybaid Thermal reactor with 0.2 μM of each primer, 100 μM dNTP, 4 mM $MgSO_4$, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM NH_4SO_4, 0.1% Triton X-100 and 100 μg/ml of Dnase–Rnase-free acetylated BSA in a final volume of 75 μl. The reagents were mixed in a polypropylene tube kept on ice and then placed in the thermocycler at a holding temperature of 80°C. To avoid cooling of the sample, the enzyme (1 U of Vent Polymerase [New England Biolabs] in a volume of 25 μl) was slowly added along the tube wall. The solution was mixed by repeated filling of the pipette tip. It was then overlaid with three droplets of mineral oil and processed for 35 cycles of three temperature steps: 98°C (15 s, denaturation), 68°C (30 s, annealing), and 72°C (2.5 min, elongation). One additional cycle was performed using an elongation time of 10 min.

With regards to the β_2-AR gene, a 1,842-bp DNA fragment encompassing the entire coding region of the rat β_2-AR (18, 19) was also produced by PCR from 1 μg of Sprague-Dawley rat genomic DNA. The upstream primer was a 23-mer (5′ end located 55 nucleotides upstream to the initiation codon) and the downstream primer was a 25-mer (5′ end located 532 nucleotides downstream the stop codon) (see Table I). Amplification was carried out with 1.25 U of Thermus aquaticus DNA polymerase (Taq polymerase; Beckman Instruments, Inc., Fullerton, CA), 0.25 μM of each primer, 75 μM dNTP, 3 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM DTT, and 100 μg/ml gelatin in a final volume of 100 μl. The tubes were processed as described above and submitted to 35 cycles as follows: 97°C for 15 s, 61°C for 30 s, and 72°C for 1.5 min. One additional cycle with an elongation time of 5 min was carried out.

The PCR products were analyzed by agarose gel electrophoresis. To further characterize the DNA to be cloned, the 1,586-bp (β_1) and the 1,842-bp (β_2) fragments were recovered from the gel with Gene Clean (BIO 101, Inc., Vista, CA). Digestion of these fragments with a set of restriction enzymes always gave products of the expected size (data not shown).

Site-directed mutagenesis of β_1 and β_2-AR templates. The β-AR DNA fragments were cloned into P Bluescript (BSSK+) vector linearized at SmaI (β_1) or EcoRV (β_2) sites. The clones selected displayed positive and reverse orientation for β_1 and β_2, respectively. Mutants were generated according to the method of Kunkel et al. (20) using primers containing a single (β_1) or a double (β_2) mismatch (see Table I) to produce a new (XhoI) restriction site.

In vitro transcription. Mutated sense RNAs were transcribed from 1 μg of EcoRI-cut DNA templates. Transcription was performed in a 25-μl reaction volume containing 20 mM Tris-HCl (pH 8), 4 mM

Table I. Oligonucleotide Primers Used in this Study

	β_1			β_2		
Application	Sequence	Position	Tm	Sequence	Position	Tm
			°C			°C
PCR-cloning	5′-CTCCGAAGCTCGGCATGGGCGC-3′	−14	72.0	5′-TGCACCCGCTGAGAGAGTCTGGG-3′	−55	66.4
	5′-TGGGCTTCGCATTCACCTGCTTTCGG-3′	1,571	72.3	5′-TCCCTCAAATCCCTGCCTTCAACAC-3′	1,786	64.9
RT-PCR	5′-CGCTCACCAACCTCTTCATCATGTCC-3′	272	63.6	5′-TCTTCGAAAACCTATGGGAACGGC-3′	1,036	63.0
	5′-CAGCACTTGGGGTCGTTGTAGCAGC-3′	647	64.6	5′-GGATGTGCCCCTTCTGCAAAATCT-3′	1,378	63.1
Mutagenesis	5′-ACAGGGTCTCGAGGCTGGCCGTCAC-3′	448	63.0	5′-GCATATCAGCTCGAGCAGGAGAA-3′	1,130	50.7

Positions of the 5′ ends of the primers are numbered from the ATG initiation codon of the β_1- and β_2-AR genes. Tm values were calculated with the program OLIGO. Base substitutions (underlined) are as follows: G replaces T in β_1; C and A replace G residues in β_2.

$MgCl_2$, 1 mM spermidine, 250 mM NaCl, 0.4 mM of each nucleotide, 10 μCi of α(^{32}P)UTP (400 Ci/mmol), and 3 U of T3 or T7 RNA polymerase (Stratagene, Inc.) as appropriate. Incubation was carried out at 37°C for 30 min. DNA templates were then digested at 37°C with 5 U of Rnase-free Dnase (Stratagene, Inc.) during an additional 1-h incubation period. The mixture was then treated with Proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany), extracted with phenol-chloroform-isoamylalcohol (PCI), and precipitated with ethanol. The pellet was resuspended in RNA dilution buffer (TE pH 7.6; DTT, 2 mM; actylated BSA, 100 μg/ml; Rnasin (Promega Corp., Madison, WI), 40 U/ml). The amount of RNA synthesized was measured by liquid-scintillation counting of the TCA-precipitated material. 4–5 pmol of RNA were obtained per reaction. Size and homogeneity of the product were checked by agarose gel electrophoresis and autoradiography.

RT-PCR of β_1 and β_2-AR mRNAS. The β_1 primers corresponded to bases 272–298 and 623–647 of the coding region, giving a DNA fragment of 376 bp. The β_2 primers corresponded to bases 1,036–1,059 (upstream primer, located in the coding region) and 1,355–1,378 (downstream primer, located in the 3′ untranslated region), giving a DNA fragment of 343 bp. To minimize sample handling and contamination, RT and PCR steps were performed sequentially in the same reaction tube. To a final volume of 25 μl, the following compounds were added and maintained on ice: 2.5 μl of 10× PCR buffer (200 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 1 mg/ml gelatin), RNAs obtained from isolated segments, known amounts of β_1 or β_2 mutant RNAs (used as internal standards), and the corresponding downstream primer (6.25 pmol). The tubes were heated 2 min at 80°C in the thermocycler to break up secondary structures and then equilibrated at 42°C. Each sample was supplemented with 25 μl of RT mix containing 2.5 μl of PCR buffer, 200 μM of each dNTP, 1 μCi/nmol of α[^{32}P]dCTP, 4 mM $MgCl_2$, 6.4 mM DTT with or without 200 U of Moloney Murine Leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD). The RT reaction lasted 45 min and was carried out at 42°C to prevent excessive mispriming and possible RNA refolding. After completion of RT, the temperature was raised to 96°C for 30 s to inactivate the enzyme and denaturate the RNA–DNA hybrid and then was equilibrated at 80°C. The amplification reaction was initiated by adding 50 μl of a mix containing 5 μl of PCR buffer, 6.25 pmol of the upper primer, and 1.25 U of Taq polymerase. The samples were overlaid with mineral oil and submitted to the following sequential steps: 96°C, 30 s; 64°C, 30 s; and 74°C, 1 min. 28 PCR cycles were performed for experiments performed with α[^{32}P]-dCTP; 35 cycles were used when DNA was analyzed by ethidium-bromide staining. In all cases, the final elongation step lasted 10 min.

Quantitative analysis of RT-PCR products. To discriminate DNAs formed from wild-type and mutant RNAs, the PCR samples were XhoI digested (90 min at 37°C) by adding 10 μl of the adequate digestion buffer, 10 U of enzyme, and 100 μg/ml of acetylated BSA. It was checked that these conditions ensure complete digestion of mutant DNA fragments. 15 μl of the reaction was electrophoresed through a 3% (1% agarose, 2% low melting point agarose) agarose slab gel in TBE. The gel was fixed in 10% acetic acid, dried on 3MM paper (Whatman Inc., Clifton, NJ) at 70°C, and submitted to autoradiography. Several exposures were always performed to obtain nonsaturated images of all samples. Band intensity was recorded by densitometry. In each experiment, serial dilutions of RNAs obtained from nephron segments were always amplified simultaneously to fixed amounts of internal standards. The number of mRNA molecules in isolated tubules was calculated from samples in which the ratio of the signals obtained from the wild-type DNA to the mutant DNA was close to unity (see Figs. 4 and 5). Results are expressed as number of mRNA molecules per millimeter of tubular length.

Isolation of nephron segments. The experiments were performed on male Sprague-Dawley rats that had free access to water and standard laboratory chow until anesthesia. Nephron segments were obtained by microdissection, as previously described (21). After anesthesia (Nembutal, 5 mg/100 g), the left kidney was perfused with collagenase (2 mg/ml), dissolved in the microdissection solution with the following composition (mM): 137 NaCl, 5 KCl, 0.8 $MgSO_4$, 0.34 NaH_2PO_4, 0.44 KH_2PO_4, 1 $MgCl_2$, 1 $CaCl_2$, 4 $NaHCO_3$, 10 $CH3CO_2Na$, 5 D-Glucose, 20 Hepes, and protease-free BSA (1 mg/ml, Fraction V; Miles Inc., West Haven, CT) (pH 7.5). For RNA extraction experiments this medium was made from Hanks' sterile solution (Eurobio, Les Ulis, France) and for cAMP accumulation studies it also contained 0.5 mM ascorbic acid, as antioxydant, and 5 μM indomethacin (21). Thin pyramids cut along the corticomedullary axis were incubated for 20–25 min at 30°C in the microdissection solution containing 1 mg/ml collagenase, continuously bubbled with air. The pyramids were then rinsed thoroughly in ice-cold microdissection solution and kept on ice in a Petri dish containing the same medium. The segments were microdissected at 4°C under stereomicroscopic observation using thin needles. CTAL were obtained from the medullary rays, and medullary thick ascending limbs (MTAL) were obtained from the inner stripe of the outer medulla. Tubular length was measured with an ocular micrometer either directly in the microdissection Petri dish or, for cAMP accumulation studies, after transfer of the tubules onto glass slides (21).

RNA extraction from isolated tubules. Total RNAs were extracted using a microadaptation of the method described by Chomczynski and Sacchi (22). After microdissection, the tubules were transferred by pipetting to a second Petri dish containing fresh microdissection solution. They were dragged from one side to the other of the dish with microdissection needles to get structures free of contaminating cells or debris. Either single segments or pools of tubules (1–40 mm) were transferred with 5–10 μl of microdissection solution into 400 μl of denaturing solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl, and 0.1 M β-mercaptoethanol. 10 μg of yeast RNA (Pharmacia Inc., Piscataway, NJ), used as carrier for microextraction, was added just before transferring the tubular segments. The suspension was immediately mixed by vortexing. Sequentially, 40 μl of 2 M CH_3CO_2Na (pH 4), 400 μl of water-saturated phenol, and 80 μl of chloroform were added, with 20 s of mixing by vortexing after addition of each reagent. The final suspension was cooled on ice for 15 min and then centrifuged at 10,000 *g* for 20 min at 4°C. The supernatant was transferred to a fresh tube, mixed with 450 μl of isopropanol, and cooled 15 min at 4°C. The tubes were centrifuged at 17,000 *g* for 30 min at 4°C and the RNA pellet was dissolved in 100 μl of denaturing solution. After adding 100 μl of ice-cold isopropanol, centrifugation (17,000 *g*) was again performed for 20 min at 4°C. The RNA pellet was washed two times in 75% ethanol, sedimented, and vacuum dried. The final pellet was dissolved in 10–20 μl of RNA dilution buffer and stored at −80°C until RT-PCR. All these steps were performed in 1.5-ml polypropylene tubes.

The yield of the entire procedure was evaluated by extracting in the same way a radiolabeled RNA synthetized from the cloned β_1-AR gene. Less than 10% (8±2%, $n = 5$) of TCA-precipitated radioactivity was lost in the organic and ethanol phases, indicating that RNA recovery was essentially complete. Another approach consisted of adding into the denaturing solution both tubular segments and known amounts of β_1 or β_2 mutant RNAs (see Fig. 5). In this case, the number of mRNA molecules per nephron segment was calculated, after RT-PCR and XhoI digestion, from the amount of mutant molecules introduced initially; the possible loss during extraction being considered equivalent for all RNA species. This protocol gave results identical to those obtained when the mutant RNAs were added only for the RT-PCR reaction (see Figs. 4 and 6 for comparison).

cAMP accumulation studies. The nephron segments, stored on sealed glass slides in 2 μl of microdissection solution supplemented with bacitracin (1 mg/ml), were preincubated 10 min at 30°C. Incubation was initiated by adding 2 μl of microdissection solution containing 3-isobutyl-1-methylxanthine (1 mM final concentration) and the tested agent(s). For studies on the inhibition of cAMP accumulation, antagonists and isoproterenol were added simultaneously. The incubation (4 min at 35°C) was stopped by transferring the segments by pipette onto a 10-μl droplet of formic acid in absolute ethanol (5%, vol/vol) in a polypropylene tube. After overnight evaporation to dry-

ness, 50 μl of 50 mM potassium phosphate buffer (pH 6.2) was added and the samples were kept frozen at −20°C until determination of cAMP content. cAMP was measured on acetylated samples using an enzyme immunoassay enabling amounts of cAMP of between 5 and 130 fmol per tube to be measured accurately (21). Each experiment consisted of several replicate samples (mean±SD, 7±2) for each experimental condition.

Results

Specificity of the oliginucleotide primers. The possibility of amplifying specific β-AR genes was first investigated on genomic DNA. As shown in Fig. 1, amplification of 0.1 μg of DNA with the β_2 primers gave a single band of the expected size (343 bp). Under our conditions, a DNA fragment was detected by ethidium-bromide staining when amplification was performed from as low as 0.1 ng (~30 targets) of genomic DNA (data not shown). Fig. 1 also shows that a set of restriction enzymes chosen from the published sequence (18, 19) always gave bands of the expected size. This restriction profile definitively identifies the amplification product as a β_2 fragment. The same kind of experiment also validated the primers designed for β_1 amplification (data not shown).

Detection of β_1 and β_2-AR mRNAs in isolated thick ascending limbs by RT-PCR. The following experiments were performed to assess β-AR mRNA expression in the CTAL and MTAL (Fig. 2). In all cases, single bands of the expected size (β_1, 376 bp; β_2, 343 bp) were obtained. In both the CTAL and MTAL, amplification with the β_2 primers gave very faint bands (Fig. 2). With the β_1 primers, the band intensities were definitely higher. The β_1 signal obtained from the CTAL RNA sample appears more intense than the one obtained with the MTAL RNAs. The amplification was fully RNA dependent, as shown by the uniformly negative results obtained without reverse transcriptase on the same RNA samples.

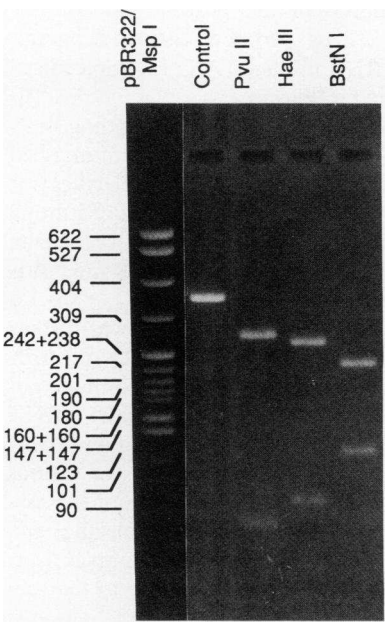

Figure 1. Amplification of rat genomic DNA with specific β_2 primers. Amplification was performed from 0.1 μg of rat genomic DNA using 35 PCR cycles. DNA was then extracted with phenol-chloroform-isoanyl alcohol, precipitated with ethanol, and left uncut (*Control*) or digested with the indicated enzyme. The fragments were fractionated on a 3% agarose gel and stained with ethidium bromide. The expected band sizes were (bp): control, 343; PvuII, 265 + 78; HaeIII, 247 + 96; BstnI, 220 + 123. The left lane shows the molecular weight marker (1 μg of pBR322 digested with MspI).

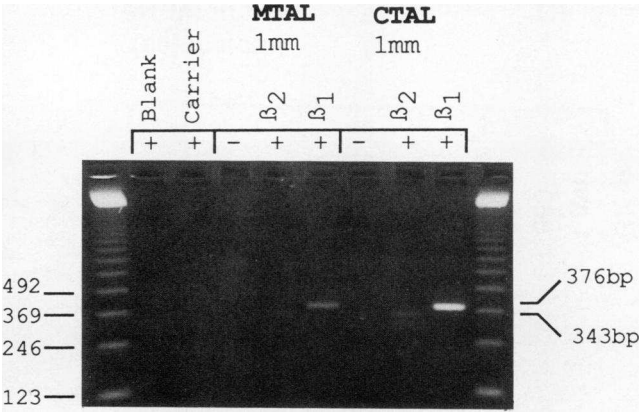

Figure 2. Detection of β_1- and β_2-AR mRNAs in isolated thick ascending limbs by RT-PCR. RNAs were extracted from pools of microdissected CTAL or MTAL segments (total length, 15 mm). Aliquots corresponding to RNAs originating from 1 mm of tubule were submitted to RT-PCR (35 PCR cycles). 20 μl of the RT-PCR reaction were loaded on a 2% agarose gel and the DNA fragments were stained with ethidium bromide. The assay was carried out in the absence (−) or presence (+) of reverse transcriptase. *Blank,* reaction performed without RNA; *Carrier,* reaction performed on a sample treated similarly to the tubular segments and containing 10 μg of yeast RNA but no isolated tubules. These reactions were carried out with the β_2 primers and were also negative when using the β_1 primers. Left and right lanes shows the molecular weight marker (123-bp DNA ladder; Bethesda Research Laboratories).

Attempts to quantify even roughly the absolute mRNA levels in this experiment were impeded by the lack of external or internal standards in the RT-PCR assay and the low sensitivity of ethidium-bromide staining. This detection method requires a high number of PCR cycles to generate detectable amounts of DNA molecules. Under these conditions, however, exponential accumulation is not maintained throughout the PCR reaction, resulting in a lack of proportionality between the amount of RNA in the sample and the DNA product (23). To overcome these limitations, a quantitative assay was set up. To reduce the number of cycles and lower the detection threshold of the method, a radiolabeled deoxynucleotide was included in the assay.

Quantitative analysis of β_1 and β_2-AR mRNA. The requirements for a quantitative RT-PCR assay, especially the number of molecules to be introduced for a given number of cycles, were first studied by amplifying separately wild-type and mutant mRNAs. Fig. 3 illustrates such an experiment. As expected, single bands of identical sizes were obtained from CTAL and mutant RNAs. More importantly, Fig. 3 shows that, under our conditions, the amount of DNA molecules formed clearly increased with the number of β_1-AR mRNA molecules introduced in the assay. Scanning analysis of the autoradiograms revealed a linear increase of up to 2,500 starting molecules. Between 2,500 and 12,500 the output signal increased by a factor of 4.3, suggesting that linearity was not fully maintained. In a similar experiment performed using 12,500 mutant RNA molecules as starting material, we calculated from the radioactivity incorporated that the end product concentration was 0.5×10^{-8} M. On the basis of the observation that the concentration of Taq polymerase in the assay becomes limiting when DNA concentration reaches 10^{-8} M

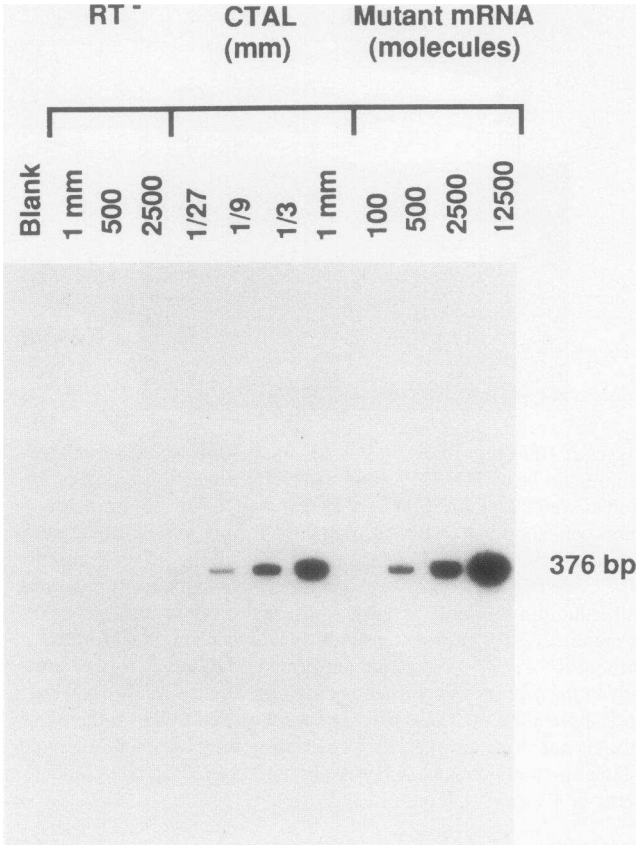

Figure 3. RT-PCR of β_1-AR mRNA from isolated CTAL and mutant β_1-AR mRNA. The figure shows an autoradiogram of DNA fragments formed by RT-PCR from CTAL RNAs or known amounts of mutated β_1 RNA. CTAL samples (serial dilutions corresponding to 0.04–1 mm) and mutated β_1 RNAs were amplified using 28 PCR cycles. Note that a single band of the expected size (376 bp) was obtained from both targets. The assay was performed in the absence (−) or presence (+) of RT enzyme. Blank, reaction carried out in the absence of RNA but in the presence of RT and Taq polymerase.

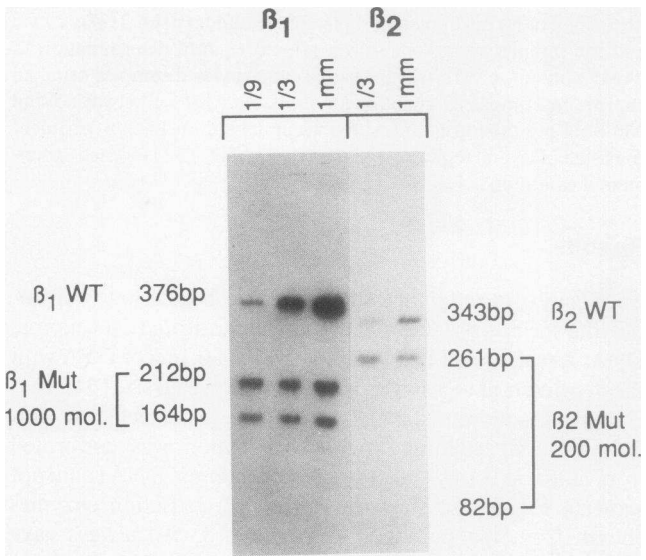

Figure 4. Quantitative analysis of β_1- and β_2-AR mRNAs in the CTAL: coamplification of wild-type and mutant RNAs. CTAL samples and mutated β-AR RNAs were amplified simultaneously using variable amounts of CTAL RNAs and constant amounts (β_1, 1,000 molecules; β_2, 200 molecules) of the internal standard. The DNA fragments formed from wild-type and mutant RNAs were discriminated by digestion with XhoI. Wild-type DNA fragments (*bp*): β_1, 376; β_2, 343. Mutant DNA fragments (*bp*): β_1, 212 + 164; β_2, 262 + 81. These fragments were fractionated on a 3% agarose slab gel and detected by autoradiography.

(23), we defined conditions for obtaining 10 times lower DNA concentrations. Accordingly, to allow exponential accumulation throughout the PCR reaction and thus to avoid competition between wild-type and mutant DNA targets, we decided to introduce a maximum of 2,500 molecules in the assay.

The number of β-AR mRNA molecules in tubular segments was measured through coamplification with mutant RNA (Fig. 4). The corresponding DNA products were clearly identified through XhoI digestion. The number of β_1-AR mRNA molecules per millimeter calculated from serial dilutions of CTAL RNAs was 2,803, 3,564, and 2,972 for nine-, three-, and onefold dilutions, respectively. The three independent determinations therefore gave very similar results. In addition, Fig. 4 shows that the number of β_2-AR mRNA molecules, measured on the same CTAL sample, was markedly different: it was only 185 molecules per mm, i.e., nearly 20-fold lower than the number of β_1-AR mRNA molecules.

Since catecholamines might act in both the CTAL and MTAL (2), β-AR mRNA levels were also measured in this latter nephron segment. Fig. 5 shows that the β_1-AR mRNA level was clearly lower than in the CTAL, being only 529 molecules/mm. This is consistent with reports indicating a lower stimulation of adenylyl cyclase by isoproterenol in the MTAL than in the CTAL (2, 6) (In both the mouse and rat kidney, isoproterenol-sensitive adenylyl cyclase was first reported to be present in CTAL but not in MTAL [6]. More recently, it was demonstrated that adenylyl cyclase is activated by isoproterenol in both segments in the mouse [2]. In preliminary studies we compared isoproterenol-dependent cAMP accumulation in the rat MTAL and CTAL. We found a small but significant increase of cAMP accumulation in the MTAL [~30% of that observed in the CTAL].). This experiment also confirms the very low level of β_2-AR mRNA in the CTAL (137 molecules/mm for the sample analyzed in Fig. 5).

β_1- and β_2-AR mRNA levels in the thick ascending limb were measured in several experiments, which are summarized in Table II. In the CTAL, the number of mRNA molecules was always markedly higher for β_1- than for β_2-AR, demonstrating the preponderance of the β_1-AR mRNA. In the MTAL, despite the low number of observations, it is clear that β_1-AR mRNA is also predominant.

The accuracy of the previous determinations was further tested by adding known amounts of mutant β_1 mRNA during the extraction procedure. This approach should eliminate potential losses as contributing to underestimation of the mRNA content of biological samples. Fig. 6 gives results of an experiment in which RNAs were isolated from CTALs of various length (range 0.4–2.15 mm). A significant correlation was observed between the number of β_1-AR mRNA molecules and the tubular length. β_1-AR mRNA levels in these samples averaged 2,500 molecules per mm, a value very close to that ob-

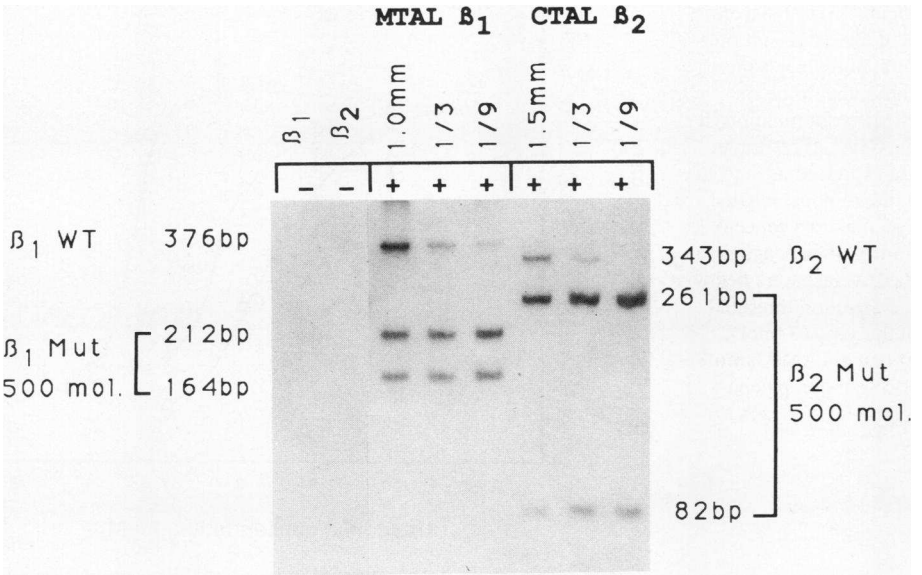

Figure 5. Quantitative analysis of β-AR mRNAS in the MTAL and the CTAL. Serial dilutions of MTAL or CTAL samples were amplified with constant amounts (500 molecules) of mutated β_1- and β_2-synthetic RNA, respectively (28 PCR cycles). The DNA fragments were then digested with XhoI, fractionated on a 3% agarose gel, and analyzed by autoradiography. Wild-type DNA fragments (*bp*): β_1, 376; β_2, 343. Mutant DNA fragments (*bp*): β_1, 212 + 164; β_2, 262 + 81. The reaction was carried out in the absence (−) or presence (+) of reverse transcriptase.

tained when the internal standard was added only for the RT-PCR reaction step. This experiment demonstrates that our microprocedure of extraction allows complete RNA recovery.

Pharmacological properties of β-ARs in the CTAL. The markedly different levels of β_1- and β_2-AR mRNAs in the CTAL prompted us to study its functional consequences on agonist-induced cAMP accumulation. As shown in Fig. 7, the rank order of potency for stimulating cAMP accumulation was isoproterenol >norepinephrine >epinephrine, a profile entirely typical of a β_1-AR (8, 17). Moreover, the β_1 antagonist CGP 20712A was 15 times more potent than the β_2 antagonist ICI 118551 in inhibiting isoproterenol-induced cAMP accumulation (Fig. 8). CGP 12177A, a nonselective β-AR antagonist (10), inhibited isoproterenol action with a K_i even lower than that of CGP 20712A (Fig. 8). This is in agreement with the different affinities of CGP 12177A and CGP 20712A for β_1-AR (24). On the other hand, CGP 12177A has partial agonistic properties on β_3-AR (11, 24). Nevertheless, as shown on Fig. 8, it completely abolished isoproterenol-dependent cAMP accumulation and, when tested alone, did not measurably increase cAMP formation (data not shown). These data indicate that β_3-AR should not contribute significantly to cAMP synthesis in this nephron segment, and consequently, that β_1-AR is the predominant receptor subtype responsible for adenylyl cyclase activation.

Table II. β_1- and β_2-AR mRNA Levels in the CTAL and MTAL

	mRNA content			
	CTAL		MTAL	
Experiment	β_1	β_2	β_1	β_2
	molecules/mm			
1	3,078	244	529	117
2	5,453	138	—	—
3	2,392	90	—	—
4	3,385	185	—	—
5	1,895	137	—	—
6	—	—	565	119
Mean	3,241	159*	547	118
SEM	611	26		

RNAs extracted from isolated CTALs or MTALs were analyzed by quantitative RT-PCR, as described in Figs. 4 and 5. The same RNA preparation was always tested for both β_1 and β_2 mRNA levels.
* Significant difference between the number of β_1 and β_2 mRNA molecules in the CTAL ($P < 0.01$, paired t test).

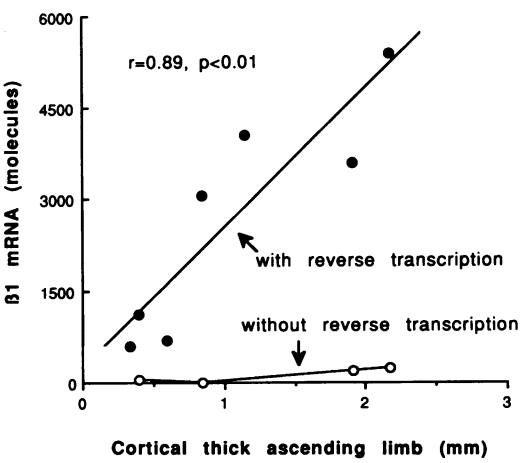

Figure 6. Correlation between the amount of β_1-AR mRNAs and the tubular length. RNAs were extracted from microdissected CTALs (0.4–2.15 mm). Known amounts of mutated β_1 RNAs were added just before extraction. RT-PCR (28 PCR cycles) was then performed as described in Fig. 4 except that the mutated β_1 RNA was not further added to the reaction. Some samples were halved to perform the assay in the presence or absence of RT enzyme, as indicated. Linear regression equation: $y = 2{,}257x + 267$.

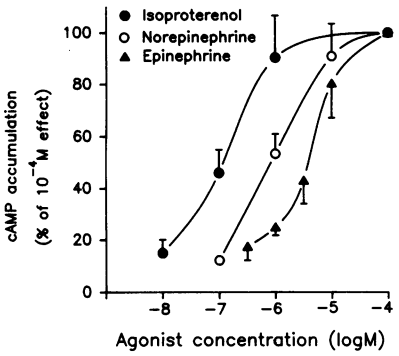

Figure 7. Dose-dependent effects of isoproterenol, norepinephrine, and epinephrine on cAMP accumulation in the CTAL. The results are expressed as percent of the response elicited by a maximal concentration of each agonist. Basal values were below the detection threshold of the assay (3 fmol). Maximal cAMP (fmol/mm · 4 min) accumulations were isoproterenol, 58±8; norepinephrine, 53±12; epinephrine, 58±15 (n = 3–5 experiments for each agonist).

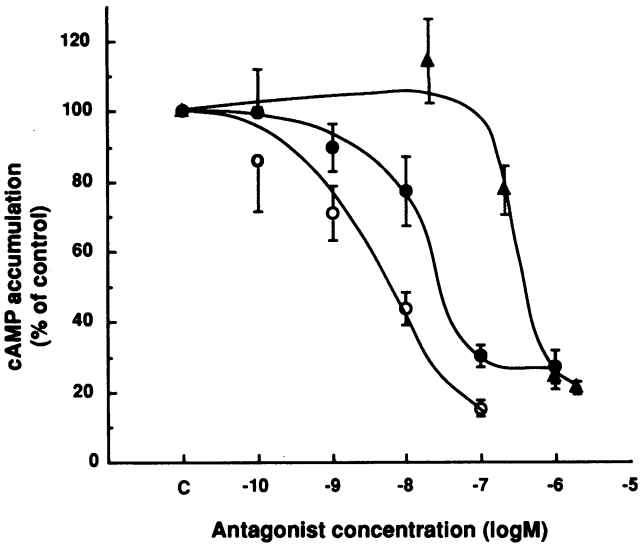

Figure 8. Inhibition of isoproterenol-induced cAMP accumulation by nonselective (CGP 12177A), β_1-(CGP 20712A), and β_2- (ICI 118551) adrenergic antagonists. Each point is the mean value of 7 to 17 replicate samples obtained from two experiments. The results are expressed as percent of the effect of isoproterenol (0.2 μM) – induced cAMP accumulation, which averaged 28±3 fmol/mm · 4 min (n = 6 experiments). cAMP accumulation in the presence of 10^{-7}–10^{-4} M concentrations of CGP 12177A was below the detection threshold of the assay (<3 fmol/mm), indicating complete inhibition. ○, CGP 12177A; ●, CGP 20712A; ▲, ICI 118551.

Discussion

This study demonstrates the differential expression of β_1 and β_2-AR mRNA in the thick ascending limb of Henle's loop. The high levels of β_1-AR mRNAS were associated with a profile of agonist-induced cAMP accumulation typical of β_1-ARs. These data were obtained using a method that allows absolute mRNA quantitation through a RT-PCR assay that is sensitive enough to detect 100 target copies. The method can be applied to measure mRNAs obtained from as few as 30 cells.

Several methods using RT-PCR have already been proposed for relative or absolute quantitation of RNAs. Relative quantitation has been used to detect aldose reductase, Na/H antiporter, and ANF receptor mRNAs in single nephron segments (25–27). This method, although useful to roughly evaluate gene expression, is limited in its applications. Robinson and Simon (28) attempted to quantitate mRNA levels using an external standard. In this case, known amounts of synthetic RNAs and experimental samples were amplified in separate RT-PCR reactions. However, this method does not correct the yield of product for reaction-to-reaction variation and its application for accurate quantitation is uncertain (29). This problem is circumvented and reliability is greatly improved through coamplification with a single pair of primers of the natural mRNA target and of a synthetic RNA, used as an internal standard. The internal standard is either of different size than the wild-type species (29, 30) or only differs by a single base substitution to introduce a new restriction site in the cDNA (15, 29). In each case, the products obtained from synthetic and wild-type RNAs can be separated by gel electrophoresis.

Our method is close to the one described by Becker-André and Hahlbrock (15), i.e., the PCR-aided transcript titration assay (PATTY) method, with notable modifications that make it easier. Our assay is performed in the presence of one labeled deoxynucleotide. This allows direct detection of the product by autoradiography without additional steps for transfer and hybridization. The amounts of natural and synthetic RNAs introduced in the assay are 1,000-fold lower than in other methods (15, 29) to ensure an exponential accumulation all along the PCR reaction. If such was not the case, the DNA strands not used as templates could partly reassociate and form heterodimeric DNA species resistant to XhoI digestion.

To demonstrate the absence of heteroduplex molecules in our assay, the CTAL samples amplified with the β_1 primers and described in Table II (line *1*) were 10 or 100 times diluted, fully denaturated, and submitted to an additional PCR cycle with fresh enzyme before XhoI digestion and electrophoresis (15). The ratio of the wild-type and mutant DNA molecules was unaltered. Consequently, the calculated mRNA level was unchanged, being 3,078, 3,133, and 2,703 for native, 10 times, and 100 times diluted samples, respectively. Therefore, our experimental conditions rule out the presence of heterodimeric molecules. With the advantage of labeling the product, it was possible to quantitate 100 molecules after only 28 PCR cycles.

Physiological significance of the differential expression of β_1 and β_2-AR mRNAS. β_1-AR mRNA levels in the CTAL averaged 3,000 molecules/mm. Considering that the number of cells approximates 300/mm in this nephron segment (30a), then the amount of β_1-AR mRNA is of about 10 copies/cells. Conversely, the β_2-AR mRNA level should be < 1 copy/cell. This very faint level may correspond to background expression of the β_2-AR gene. The predominance of β_1-AR mRNA is in close agreement with the typical β_1-AR sensitivity of the CTAL and demonstrates coordinated differences of β-AR subtypes at the protein and mRNA levels. The contribution of a third or "atypical" β-AR to catecholamine action in the CTAL is unlikely since CGP 12177A, which elicits 30–40% of the effect of isoproterenol in cells bearing β_3-ARs (24), was unable to stimulate cAMP accumulation and completely inhibited isoproterenol action. In addition, when RT-PCR was performed using β_3-AR selective primers, no detectable product (assessed by ethidium-bromide staining) was obtained from the CTAL whereas, as expected (11, 13), β_3-AR transcripts were evi-

denced in the brown adipose tissue (unpublished observations from our laboratory). All these data, therefore, reveal a molecular basis for the predominant expression of the β_1-AR in the CTAL and are consistent with previous autoradiographic studies showing that β-adrenoceptors of the thick ascending limb are almost exclusively of the β_1 subtype (31–33).

Norepinephrine, the neurotransmitter of sympathetic nerves, is the preferential ligand of β_1-ARs. Consequently, the thick ascending limb should be more sensitive to variations of the local concentrations of norepinephrine than to circulating epinephrine. The thick ascending limb has monoaminergic innervation (34), and low frequency renal nerve stimulation has been shown to increase Na and Cl reabsorption in the loop of Henle (35). We therefore suggest that norepinephrine, through β_1-ARs, is responsible for many of the physiological effects assigned to β-agonists in this nephron segment, including the stimulation of NaCl, Ca and Mg reabsorption (2).

The lack of significant expression of the β_2-AR gene in the CTAL indicates that this nephron segment will be a useful model to study the regulation of the β_1-AR and to assess the factors contributing to the emergence of β_2-AR. Recently, a human neurotumoral cell line that expresses β_1- but not β_2-ARs has been used to study the mechanism of β_1-AR desensitization (36). The cAMP-dependent protein kinase A, but not the β-adrenergic receptor kinase, was responsible for desensitization of the human β_1-AR to isoproterenol. The mechanisms of desensitization of the rat and human β_1-AR may nevertheless be different since the rat β_1-AR has more potential phosphorylation sites for β-adrenergic receptor kinase (8, 17). Desensitization to isoproterenol has also been reported in the CTAL (21), and investigating the kinase(s) involved in this process is an attractive area for future studies. Furthermore, our RT-PCR assay will make possible to study the regulatory mechanisms of the β_1-AR at the mRNA level. Regulation of the β_2-AR has been shown to involve alterations of both the receptor protein and its mRNA (37, 38). However, nothing is known on the possible effects of adrenergic agents on the steady state level of β_1-AR mRNA.

In conclusion, this study demonstrates the possibility of measuring minute amounts of β_1 and β_2-AR mRNAs by RT-PCR. This assay is sensitive enough to detect mRNA in 30 cells and will be useful to characterize β-AR subtypes and establish their regulation. In the thick ascending limb, β_1-AR mRNA levels were 20-fold higher than those of β_2-AR. The corresponding adrenergic sensitivity supports the notion that norepinephrine, rather than epinephrine, is responsible for the stimulation of ion transport in this portion of the nephron.

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