0026-895X/93/010070-06$03.00/0

MOLECULAR PHARMACOLOGY, 44:70–75

Human α_2-Adrenergic Receptor Subtype Distribution: Widespread and Subtype-Selective Expression of α_2C10, α_2C4, and α_2C2 mRNA in Multiple Tissues

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Received January 8, 1993; Accepted April 7, 1993

SUMMARY

At present, molecular cloning and pharmacological studies have delineated three human α_2-adrenergic receptor (α_2AR) subtypes, α_2C10, α_2C4, and α_2C2. Assignment of the α_2AR subtypes to specific functions has been limited by an unclear definition of tissue α_2AR expression outside of the central nervous system. It has been suggested that α_2C4 expression is confined to the brain, that α_2C2 expression is only in the liver and kidney, and that there is nearly ubiquitous expression of α_2C10. However, this is based on studies of a limited number of rat tissues or on studies using non-species-specific approaches. Therefore, to define α_2C10, α_2C4, and α_2C2 tissue expression, we used reverse transcription of total RNA isolated from 20 human tissues, followed by amplification of α_2AR cDNA using the polymerase chain reaction. This technique provided two advantages: high sensitivity and, with the use of subtype-specific oligonucleotide primers and probes, differentiation between the α_2AR subtypes. The tissues studied were aorta, vena cava, heart (epicardium and endocardium), lung, skeletal muscle, liver, pancreas (head and tail), fat (perinephric and subcutaneous), kidney (cortex and medulla), prostate, stomach, ileum, jejunum, colon, adrenal gland, and spleen. We found that the majority of these tissues expressed α_2C10, with the exceptions being the head of the pancreas, subcutaneous fat, colon, and spleen. In marked distinction to other studies, however, we found a prolific expression of the α_2C4 and α_2C2 subtypes. Expression of α_2C4 was found in all tissues with the exception of liver, fat, stomach, and colon, and a virtually ubiquitous expression of α_2C2 was found, with the exception of epicardium. Of all tissues studied, only colon and subcutaneous fat expressed a single α_2AR subtype, which was α_2C2. Thus, the α_2AR subtypes do not have a confined expression but appear to be widely distributed in humans and display subtype-specific expression in some tissues.

Heterogeneity of α_2ARs has been well documented by several approaches, including physiological studies, radioligand binding, and molecular cloning (reviewed in Refs. 1–4). Three human α_2AR subtypes have been cloned to date (5–8) and are referred to here as α_2C10, α_2C4, and α_2C2, according to their chromosomal localization to human chromosomes 10, 4, and 2, respectively. Recently, the basis of such subtype differentiation has begun to be ascertained. For example, we and others have recently described differences in G protein coupling of α_2AR subtypes (9, 10). We have also shown that the three human subtypes undergo different patterns of agonist-dependent desensitization during short and long term continuous exposure to agonists (11). Thus, the subtypes of the α_2AR may have evolved to meet differing physiological needs either for coupling to G proteins or for adaptive regulation.

Paramount to a full understanding of how these findings integrate with radioligand binding and physiological data is a knowledge of the tissue distribution of the three subtypes. Lorenz *et al.* (12) used the three human α_2AR cDNAs as probes and performed Northern blots on selected rat tissues. They reported virtually ubiquitous expression of α_2C10, confinement of expression of α_2C4 to the central nervous system, and expression of α_2C2 in only liver and kidney. Other studies using probes for the rat homologues of these receptors have delineated the distribution in a limited number of rat peripheral tissues (13–16) and, more extensively, in rat brain (17, 18). Because of differences between subtype homologues among various species (see Discussion), it has become apparent that tissues are best studied with species-specific approaches. In the current study, we have used a sensitive and highly specific method of identifying human α_2AR subtype mRNAs and applied it to an expanded array of 20 human tissues to establish the tissue distribution of α_2C10, α_2C4, and α_2C2.

Materials and Methods

RNA preparation. Freshly excised human tissues (1 g/sample) were obtained from patients during surgery, frozen in liquid nitrogen,

ABBREVIATIONS: α_2AR, α_2-adrenergic receptor; G protein, guanine nucleotide-binding protein; PCR, polymerase chain reaction; RT, reverse transcription; bp, base pair(s); SSC, standard saline citrate.

and stored at −70°. Total RNA was isolated from these tissues using the guanidinium thiocyanate method described by Chomczynski and Sacchi (19). To eliminate any contaminating DNA, RNA from each tissue was incubated with RNase-free DNase I (Stratagene) at a concentration of 1 unit/μg of total RNA, in the presence of 3 units/μg placental RNase inhibitor (Promega), in 50 mM Tris·HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM dithiothreitol, for 1 hr at 37°. Reactions were stopped by the addition of sodium dodecyl sulfate and EDTA to final concentrations of 0.2% and 10 mM, respectively. Samples were then extracted once with phenol/chloroform (1:1) and once with chloroform, followed by salt precipitation of RNA.

Constructs and oligonucleotides. The constructs consisting of the cDNAs for human α_2C10, α_2C4, and α_2C2 inserted into the vector pBC12BI (pBC10, pBC4, and pBC2, respectively) have been described previously (11). For PCR, sense and antisense primer pairs (20-mers) for each α_2AR subtype were chosen such that there was little homology between the nucleic acid sequences of the primers between subtypes. Primers for α_2C10, 5′-AAACCTCTTCCTGGTGTCTC-3′ (sense) and 5′-AGACGAGCTCTCCTCCAGGT-3′ (antisense), provided for amplification of a 691-bp sequence, from nucleic acids 204 to 894. For α_2C4, the primers 5′-GTGGTGATCGCCGTGCTGAC-3′ (sense) and 5′-CGTTTTCGGTAGTCGGGGAC-3′ (antisense) amplified a 574-bp sequence, from nucleic acids 214 to 787. For α_2C2, the primers 5′-CCTGGCCTCCAGCATCGGAT-3′ (sense) and 5′-CAGAGCACAAAAACGCCAT-3′ (antisense) were chosen and provided amplification of a 630-bp sequence, from nucleic acids 519 to 1148. As shown below (see Results), these primers indeed provided for specificity between the α_2AR subtypes, as tested using the cloned α_2C10, α_2C4, and α_2C2 genes. In addition, the efficiency of amplification provided by each primer pair was the same for each receptor subtype cDNA (data not shown). Primers used for amplification of β-actin sequence were 5′-ATCATGAAGTGTGACGTGGAC-3′ (sense) and 5′-AACCGACTGCTGTCACCTTCA-3′ (antisense). These primers allowed for amplification of a region that included an intron of 110 bp (20). Thus, cDNA produced from mRNA during RT would provide a product 110 bp smaller than product amplified from genomic DNA (444 versus 554 bp). This provided an internal control for the detection of contamination of RNA with genomic DNA. For each α_2AR subtype, oligonucleotides (50-mers) corresponding to a sequence within the region amplified by the respective primer pairs, but not inclusive of the sequence for either primer oligonucleotide, were chosen as probes for use with Southern hybridization analysis. Sequences of the oligonucleotide probes were as follows: α_2C10, 5′-GACGCGCTGGACCTGGAGGAGAGCTCGTCTTCCGACCACGCCGAGCGGCC-3′; α_2C4, 5′-AACGACGAGACCTGGTACATCCTGTCCTCCTGCATCGGCTCCTTCTTCGC-3′; α_2C2, 5′-GGGTGGTTTGTGGGCATCTCCAGAGGATGAAGCTGAAGAGGAGGAAGAGG-3′. Additional validation of the PCR products was undertaken with restriction digests. Products obtained from PCR amplification of pBC10, pBC4, and pBC2 were purified using Centricon 100 columns and were digested with restriction enzymes for which there was a unique restriction site within the sequence of the expected product. For α_2C10, the expected amplification product contained one *Bsp*HI site, which after digestion would result in two products, 429 and 262 bp in size. For α_2C4 and α_2C2, the expected products each contained one *Bst*XI site. After digestion, the resultant products would be 225 and 349 bp and 300 and 330 bp, respectively. For each digestion the purified PCR products were incubated alone (for control reactions) or with the appropriate enzyme for 1 hr at 37°.

RT-PCR. Identification of α_2AR subtype mRNA was carried out using a RT-PCR method similar to that of Fraser and colleagues (21). Studies with each tissue were performed at least twice and used the same amount of total RNA from each tissue. RT of mRNA was carried out using random hexamers as primers. For each RT reaction, total RNA (5 μg) was added to 100 pmol of random hexamers and incubated at 70° for 10 min. RT buffer, providing for a final concentration of 50 mM Tris·HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, and 200 μM each of dATP, dGTP, dTTP, and dCTP, was then added and reaction mixtures were preincubated at 42° for 2 min before the addition of 400 units of Superscript (GIBCO/BRL) RNase H^- reverse transcriptase (a recombinant form of Moloney murine leukemia virus reverse transcriptase from which the RNase H sequence has been deleted). Samples were incubated for 1 hr at 42° and then reactions were terminated by incubation for 10 min at 95°.

For amplification of cDNA, each RT reaction was diluted to 100 μl in a mixture containing 20 mM Tris·HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 4 mM $MgSO_4$, 40 μM each of appropriate sense and antisense primers, 200 μM concentrations of all four deoxynucleoside triphosphates, and 40 units of Vent(exo−) DNA polymerase (New England Biolabs). Additionally, 10% dimethylsulfoxide was added to reactions in which α_2AR DNA was amplified. PCR was performed in a Perkin-Elmer GeneAmp 9600 thermocycler with the following protocol: 3 min at 95°, 35 cycles of 1 min at 95° followed by 1 min at 60°, and then a final extension at 60° for 7 min. PCR products (40 μl) were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. Gels were transferred via capillary action to 0.22-μm nitrocellulose filters (Micron Separations, Inc.), in 20× SSC (1× SSC is 15 mM sodium citrate, pH 7.0, 150 mM NaCl).

Southern blots. Oligonucleotide probes discussed above were end-labeled with [γ-^{32}P]ATP, using T4 polynucleotide kinase, to a specific activity of ~2 × 10^8 dpm/μg. Filters were prehybridized for 2 hr at 68° in a buffer consisting of 5× SSC, 5× Denhardt solution, and 100 μg/ml salmon testes DNA. Radiolabeled probes were added at a concentration of 2 × 10^6 dpm/ml and hybridization was carried out for 12–16 hr at 68°. Filters were rinsed twice in 2× SSC, washed twice under high stringency conditions (30 min in 0.1× SSC at 65°), dried, and exposed to Amersham Hyperfilm.

Northern blots. To confirm some of the findings that were obtained with RT-PCR, Northern blots were performed using poly(A)$^+$ RNA from selected tissues when sufficient total RNA was available. Probes for these consisted of PCR-amplified portions of α_2C10 and α_2C4 cDNA from nucleotides ~600 to ~1150, representing the third intracellular loops of the encoded receptors. These were labeled with [^{32}P]dCTP to a specific activity of ~2 × 10^9 dpm/μg, using the random primer technique. Hybridizations were carried out for 18 hr at 65° using a probe concentration of 3 × 10^6 dpm/ml. Filters were washed under high stringency conditions (0.1× SSC at 65°) and exposed to film, with intensifying screens, for 10 days at −70°.

Results and Discussion

RT-PCR was chosen for these studies because of the small amounts of human tissue that we had available and the high degree of specificity that can be obtained using specific primers. Shown in Fig. 1, *upper*, is the specificity of the primer pairs used in the PCR portion of the RT-PCR method. Each primer pair was used in a PCR with each of the cDNAs for the three α_2AR subtypes as template. As can be seen, a single specific product of the expected molecular size was obtained only when primers were matched with cDNA of the respective subtype. The lack of signals in unmatched lanes even after 35 cycles of PCR suggests highly specific primers. To further validate the PCR product, Southern blots using nested oligonucleotide probes (Fig. 1, *lower*) and restriction digests (Fig. 2) were performed. As shown in Fig. 1, *lower*, the PCR products hybridized strongly, under high stringency conditions, to oligonucleotide probes that were internal to the PCR primers. Each PCR product was also extracted and digested with restriction enzymes that were predicted to provide specifically sized fragments. As shown in Fig. 2, *Bsp*HI and *Bst*XI digestion gave products of the expected molecular sizes.

We next approached the potential problem of amplification

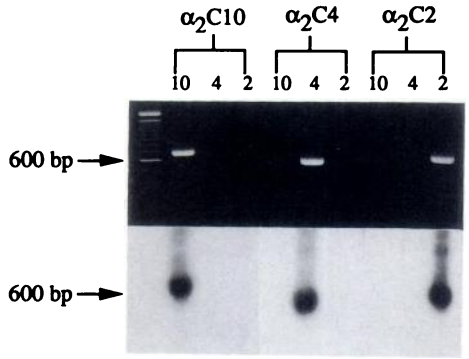

Fig. 1. Specificity of oligonucleotide primers between α_2AR subtypes. The subtype specificity of oligonucleotide primer pairs (denoted *above the brackets*) for the α_2AR subtypes was evaluated by performing PCR using each pair with pBC10, pBC4, and pBC2 (*10*, *4*, and *2*, respectively). *Far left lane*, DNA size markers, in 100-bp increments, indicate the size of the PCR product obtained for each subtype. In each case, the primers provided a product only when PCR was performed with the appropriate cDNA template (*upper*). For α_2C10, α_2C4, and α_2C2, the products migrated according to the expected molecular weights (691, 574, and 630 bp, respectively). In Southern blots the PCR products hybridized strongly to nested probes under high stringency conditions (*lower*).

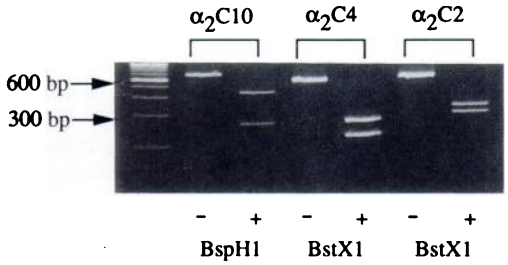

Fig. 2. Restriction digests of α_2AR PCR amplification products. PCR products were digested with restriction enzymes for which a unique site was present in the expected product. For α_2C10, digestion with *Bsp*HI resulted in the expected 429- and 262-bp products. Similarly, for α_2C4 and α_2C2 digestion with *Bst*XI resulted in the expected 225- and 349-bp products and 300- and 330-bp products, respectively.

by the PCR of contaminating genomic DNA, rather than the DNA generated by RT of mRNA. We used three methods to eliminate or identify such amplification. First, all total RNA preparations were treated with DNase. Second, after inactivation of the DNase, primers for human β-actin were used in a mock RT-PCR where no reverse transcriptase was included. Thus, any product resulting from this reaction would represent amplified genomic DNA. Finally, we chose β-actin primers whose product spans an intron in genomic DNA (20) and thus provide a product 110 bp larger than would occur if DNA were transcribed from RNA. The validity of this approach is shown in Fig. 3A. In the absence of pretreatment with DNase, liver RNA gave two products for β-actin, one at 444 bp from reverse transcribed RNA and one at 554 bp from genomic DNA. However, with DNase treatment, only one product (Fig. 3A, *middle lane*) was obtained, at the molecular size indicative of reverse transcribed RNA. Finally, when reactions were run without reverse transcriptase no product was obtained (Fig. 3A, *right lane*). In all tissues studied, reactions were first carried out using these β-actin primers under control (i.e., no reverse transcriptase added) and standard RT-PCR conditions. In every case, no signal was observed from reactions that lacked reverse transcriptase, and in those that had reverse transcriptase the molecular size of the β-actin signal was 444 bp. Both

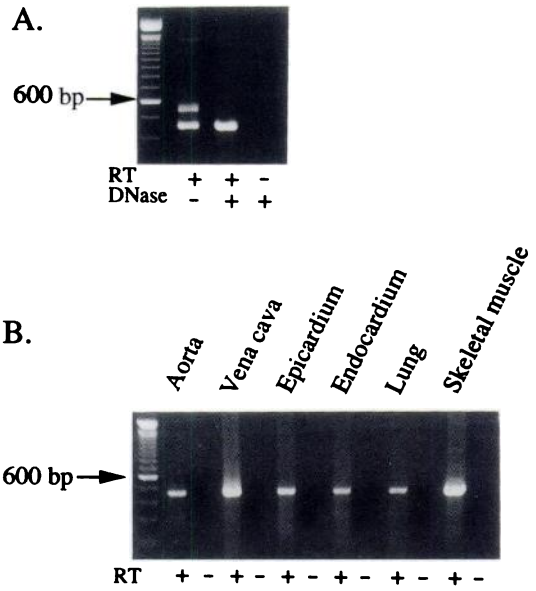

Fig. 3. Methods of detection of contaminating genomic DNA in RNA tissue samples. Primers for β-actin were chosen such that the amplified sequence spanned an intron in genomic DNA, yielding a 554-bp product versus a 444-bp product obtained from cDNA produced by RT of RNA. A, Results from RT-PCR performed with untreated and DNase-treated RNA, as described in Materials and Methods. Whereas reactions containing untreated RNA resulted in both 554- and 444-bp products, DNase treatment eliminated the 554-bp product, leaving the 444-bp product intact. Mock RT-PCR performed without the addition of reverse transcriptase yielded no product. B, Results from RT-PCR (+) and mock RT-PCR (−) using DNase-treated RNA from six representative tissues.

of these results thus confirmed that the product resulted from amplification of reverse transcribed mRNA. Fig. 3B shows these results from six representative tissues.

Results of RT-PCR performed with the RNA from the 20 tissues using the aforementioned α_2AR subtype-specific primers are shown in Fig. 4 and Table 1. We have previously validated and used a similar technique for quantitative analysis of changes in mRNA (22). However, for the current study, which uses so many different tissue sources of mRNA, we suggest that these results represent only semiquantitative estimates. RT-PCR using α_2C10 primers in these tissues provided for a single band at the expected molecular weight (Fig. 4, *upper*). For α_2C4 and α_2C2 the appropriately sized product was obtained along with another product of lower molecular weight (Fig. 4). This smaller product was not evident when the filters were washed under very stringent conditions, and its origin is not clear. α_2C10 mRNA was present in most tissues, with the following exceptions: head of the pancreas, subcutaneous fat, colon, and spleen (Fig. 4; Table 1). α_2C2 mRNA was present in every tissue except the left ventricular epicardium. α_2C4 mRNA had a more restricted distribution than did α_2C2, being absent in liver, subcutaneous and perinephric fat, stomach, and colon. It should be noted that, in all cases where a negative signal was obtained for one subtype, in that same tissue a positive signal was obtained for β-actin and at least one of the other α_2AR subtypes.

In assessing the presence of α_2AR subtype mRNA in individual tissues, several interesting observations can be made. First, no tissue studied was without expression of at least one of the three α_2AR subtypes. A number of tissues appear to possess all three of the subtypes, namely, aorta, vena cava, endocardium,

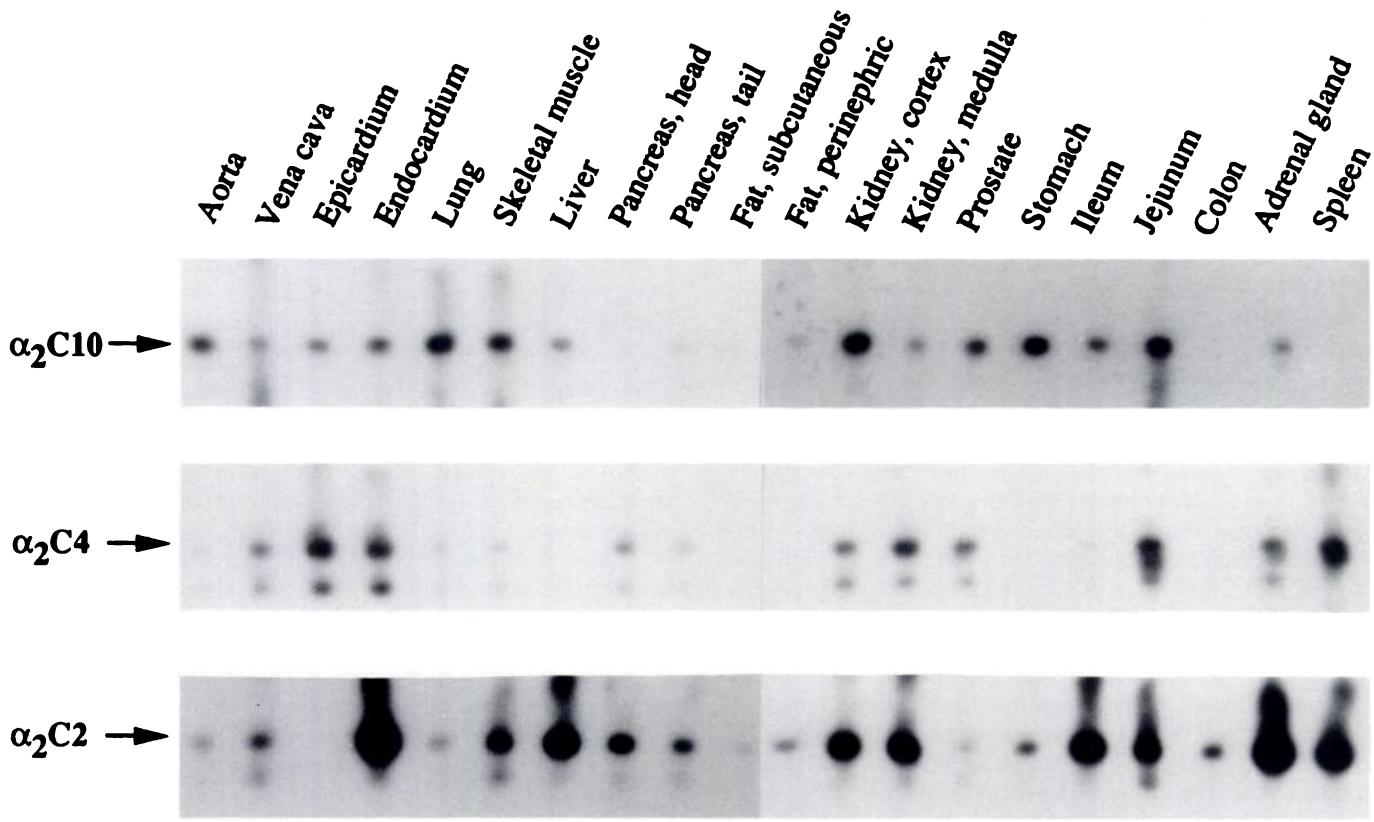

Fig. 4. Expression of α_2C10, α_2C4, and α_2C2 in human tissues. Total RNA was reverse transcribed and the cDNA product was subjected to PCR amplification using primers specific for the separate α_2AR subtypes, as described in Materials and Methods. PCR products were electrophoresed through 2% agarose gels, transferred to nitrocellulose filters, and hybridized to specific nested oligonucleotide probes.

TABLE 1
α_2AR subtype mRNA distribution

	Expression		
	α_2C10	α_2C4	α_2C2
Aorta	+	+	+
Vena cava	+	+	+
Epicardium	+	+	−
Endocardium	+	+	+
Lung	+	+	+
Skeletal muscle	+	+	+
Liver	+	−	+
Pancreas, head	−	+	+
Pancreas, tail	+	+	+
Fat, subcutaneous	−	−	+
Fat, perinephric	+	−	+
Kidney, cortex	+	+	+
Kidney, medulla	+	+	+
Prostate	+	+	+
Stomach	+	−	+
Ileum	+	+	+
Jejunum	+	+	+
Colon	−	−	+
Adrenal gland	+	+	+
Spleen	−	+	+

lung, skeletal muscle, tail of the pancreas, kidney, prostate, small intestine, and adrenal gland. Several key metabolic tissues appear to express only one or two α_2AR subtypes. Notably, the α_2ARs in liver, which may be important in glycogen metabolism, appear to be α_2C10 and α_2C2. In the head of the pancreas, where α_2ARs inhibit insulin secretion from β cells, the subtypes present appear to be α_2C4 and α_2C2. Although it is not clear which part of the pancreas was studied, it should be pointed out that, in rat tissue probed with the rat homologue of α_2C4, Felder and colleagues (13) reported an absence of α_2C4 expression in pancreas. Fat cells express an α_2AR that inhibits lipolysis (23). Certain characteristics of this response appear to be dependent on the type of fat (i.e., deep or subcutaneous). In the current study, we find that the subcutaneous fat α_2AR subtype appears to be α_2C2. In conflict with these results, however, are those from a recent radioligand binding study of human subcutaneous fat, which suggests that the predominant α_2AR subtype is α_{2A}(C10) (24). It is intriguing to note that the antilipolytic function of the α_2AR in hamster subcutaneous fat appears to undergo little short term agonist-promoted desensitization (23). Although it is difficult to compare desensitization patterns between different cell types, this is consistent with our current findings of this subtype being α_2C2 and our recent studies that show minimal α_2C2 functional desensitization in response to short term agonist exposure (11). Perinephric fat, in contrast, expressed α_2C10 and α_2C2 but not α_2C4. Both the vasculature of the coronary circulation and cardiac myocytes are known to express α_2AR and, interestingly, our study shows a distinct absence of α_2C2 from the epicardium, with strong α_2C10 and α_2C4 signals. In contrast, the endocardium expressed all three subtypes. In the stomach, where α_2AR may participate in adrenergic modulation of motility, the α_2AR subtypes appear to be α_2C10 and α_2C2. We found no evidence of α_2C10 or α_2C4 in the colon; this tissue and subcutaneous fat were the only tissues that expressed a single α_2AR subtype.

To further confirm our results, we used Northern blots with

poly(A)$^+$ RNA from selected tissues where an ample supply of total RNA was available. These blots were performed specifically to confirm selected positive and negative results from RT-PCR. As shown in Fig. 5, α_2C10 was expressed in heart, liver, and kidney, whereas α_2C4 was expressed in heart and kidney but was not expressed in liver. These findings are in accord with results obtained using RT-PCR from these tissues.

A systematic delineation of α_2AR subtype distribution in multiple non-central nervous system tissues by using either mRNA detection or radioligand binding has not been performed previously. The latter is difficult, because there are no subtype-specific ligands available that unequivocally distinguish between the subtypes. There are certain criteria, such as the affinities for prazosin and oxymetazoline, that are, nevertheless, helpful in identifying a particular α_2AR subtype. Even with these criteria, however, there have been discrepancies between radioligand binding studies and mRNA analysis. For example, it has been suggested that rat brain expresses primarily the α_{2A} and α_{2B} subtypes, as determined by radioligand binding (25). However, mRNA analysis has shown a predominance of RG20 and RG10 subtypes, with virtually no expression of the RNG subtype (12, 17, 18). Thus, in tissues with more than one subtype, present subtype classification is problematic using radioligand binding, especially with pharmacologically similar subtypes. Furthermore, few recent radioligand binding studies have been performed in human tissue, now that it is clear that there are at least three α_2AR subtypes. RNA studies with nonhuman tissues may not be indicative of the distribution in humans, due to the apparent interspecies differences between subtype homologues. For example, the rat α_2AR subtype, designated RG20, has ~89% overall amino acid sequence identity (and 98% identity in the transmembrane domains) with the human α_2C10 receptor (15). Thus, these appear to be molecular homologues. However, the affinities for yohimbine, rauwolscine, BAM 1303, and SKF104078 are clearly different between the two receptors (15), suggesting that they are not pharmacological homologues, which has led to the classification of the rat receptor as α_{2D}. The mouse α_2AR, denoted Mα_2-10H, which appears to be the molecular homologue of α_2C10, has different binding affinities for yohimbine, WB-4101, and rauwolscine (26). In the case of yohimbine, this appears to be due to a single amino acid difference between the two receptors (26). Finally, there appear to be two structurally distinct nonglycosylated α_{2B}-like subtypes in rat, designated RNGα_2 (15) and RBα_{2B} (14). It is not clear whether probes intended for identification of RBα_{2B} mRNA also identify RNGα_2 transcripts or an α_2C4-like receptor with which RBα_{2B} shares greater sequence homology (14).

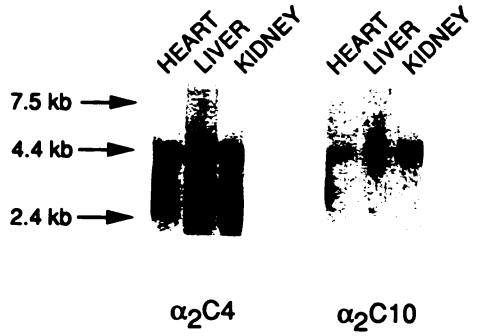

Fig. 5. Northern blot analysis of α_2AR subtypes. Two micrograms of poly(A)$^+$ RNA from human heart (endocardium), liver, and kidney (cortex) were loaded in each lane and, after transfer, hybridized to the probes for α_2C10 and α_2C4, as described in Materials and Methods. The α_2C4 probe identified a species of the expected molecular size (2.8 kilobases) in heart and kidney. (This probe also cross-hybridized with α_2C10 mRNA at 3.8 kilobases.) The α_2C10 probe hybridized to a single mRNA species at 3.8 kilobases that was present in all three tissues.

The current study is the first to delineate the distribution of all three cloned α_2AR subtypes in a large array of tissues using a technique that has high specificity and that uses human tissues and human probes. The study by Lorenz *et al.* (12), as discussed above, used Northern blots (with human probes) and rat tissues and gave results that were at variance with several molecular biology and radioligand binding studies. From a molecular standpoint, the α_2C4 was cloned from a human kidney cDNA library (7), yet the Lorenz study failed to detect mRNA in rat kidney. Similarly, those investigators did not show expression of α_2C2 in kidney or neonatal rat lung. However, neonatal rat lung is a rich source of the α_{2B} subtype, as delineated by radioligand binding, and the subsequently cloned rat α_2C2 homologue has been shown to be abundantly expressed in kidney (16). These results and those from other studies (13–18) have suggested a confined expression of α_2C4 and α_2C2 or their species homologues. Our study, in contrast, shows a greatly expanded distribution of the α_2C4 and α_2C2 subtypes.

The significance of the various α_2AR subtypes continues to be somewhat obscure. However, we have recently shown that the three cloned human α_2AR subtypes appear to differ in the degree to which they couple to G_s but not to G_i (9). In addition, we have shown that these three subtypes differ in their patterns of agonist-promoted desensitization, sequestration, and down-regulation (11). Thus, the α_2AR may have evolved based on a tissue-specific need for either G protein coupling or agonist-promoted regulation. We have shown in the current study that, indeed, the α_2AR subtypes are expressed in a wide variety of tissues, as opposed to the confined distribution of α_2C4 and α_2C2, and that in some instances there is subtype-specific localization. With the development of more subtype-specific agonists and antagonists these distributions can be explored *in vivo*, and their physiological significance ascertained.

Acknowledgments

The authors thank Naomi Sims for typing the manuscript.

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