Diverse Tissue Expression of Rat \(\alpha_2\)-Adrenergic Receptor Genes

Diane E. Handy, Christos S. Flordellis, Natalia N. Bogdanova, Margaret R. Bresnahan, and Haralambos Gavras

Previously, we have reported two major \(\alpha_2\)-adrenergic receptor transcripts in rat brain of 3.8 and 3.0 kb and the cloning and characterization of the rat brain complementary DNA (cDNA) (RB\(\alpha_2\)) specific for the 3.0-kb messenger RNA. In this report, we used rat brain cDNAs specific for the 3.0 and 3.8 kb transcripts, which encode the \(\alpha_2\)- and \(\alpha_2\)-adrenergic receptors, respectively, and the RNG\(\alpha_2\) cDNA, which encodes for the nonglycosylated \(\alpha_2\)-adrenergic receptor in rat, to study tissue-specific expression of the three \(\alpha_2\)-adrenergic receptor genes in rat. To eliminate cross-hybridization of probes with transcripts from other \(\alpha_2\) genes, we subcloned fragments that encode for the highly divergent third cytoplasmic loop of each rat \(\alpha_2\)-adrenergic receptor cDNA and used RNase protection analysis to detect specific transcripts. We show that the three rat \(\alpha_2\)-adrenergic receptor genes have diverse patterns of tissue expression, and although transcripts specific for each \(\alpha_2\)-adrenergic receptor gene are found in brain and kidney, the levels of expression of each subtype differ in these tissues. We speculate on the significance of tissue-specific expression of the \(\alpha_2\)-adrenergic receptor genes. (Hypertension 1993;1:861-865)

KEY WORDS • receptors, adrenergic, alpha • molecular biology • RNA, messenger

The \(\alpha_2\)-adrenergic receptors are a heterogeneous group of receptors that bind to the naturally occurring ligands, epinephrine and norepinephrine, and mediate their effects through the G class of G proteins. Ligand binding studies have provided evidence for the existence of four different pharmacological subtypes of \(\alpha_2\)-adrenergic receptors, \(\alpha_2A\), \(\alpha_2B\), \(\alpha_2C\), and \(\alpha_2D\), through an analysis of \(\alpha_2\)-adrenergic receptors from different species. Recent cloning, sequencing, and expression of recombinant \(\alpha_2\)-adrenergic receptors have shown that in both human and rat there are three distinct \(\alpha_2\)-adrenergic receptor subtypes that are unique in sequence and ligand binding profiles. We have been interested in defining the heterogeneity and distribution of \(\alpha_2\)-adrenergic receptors in rat tissues. In previous work, the human \(\alpha_2\)-adrenergic receptor gene was used to define the distribution of various \(\alpha_2\)-specific transcripts, suggesting the expression of more than one \(\alpha_2\)-adrenergic receptor gene in rat. To further characterize these transcripts, we have screened a rat brain complementary DNA (cDNA) library and isolated two classes of \(\alpha_2\)-adrenergic receptor clones, RB\(\alpha_2\) and RB\(\alpha_2\). (In Reference 16, the rat brain \(\alpha_2\)-adrenergic receptor cDNA was called RB\(\alpha_2\) and the receptor subtype referred to as an \(\alpha_2\)-subtype. In this report, we have renamed this receptor an \(\alpha_2\)-subtype [RB\(\alpha_2\) cDNA] and follow the subtype designations suggested in Reference 5.) As we have previously reported, the RB\(\alpha_2\) cDNA specifically hybridizes to a 3.0-kb transcript in rat brain, whereas the RB\(\alpha_2\) cDNA specifically hybridizes with 3.8- and 4.6-kb transcripts in rat brain. A third rat \(\alpha_2\)-subtype receptor clone, RNG\(\alpha_2\), was isolated from a rat kidney cDNA library and encodes for the \(\alpha_2\)-nonglycosylated adrenergic receptor found in neonatal rat lung. In this article, we report the expression of \(\alpha_2\)-adrenergic receptor genes in rat tissues by using probes that distinguish among subtype transcripts in RNase protection assays.

Methods

Isolation and Sequencing of Genomic and Complementary DNA Clones

A 1.6-kb Neo I/HindIII fragment of the human platelet \(\alpha_2\)-adrenergic receptor gene (\(\alpha_2\)-) was labeled by the random-priming DNA method with \([\text{32P}]\) deoxyctydine triphosphate (3,000 Ci/mmol, Du Pont/NEN) and used to screen 10\(^6\) phage plaques from a Sprague-Dawley rat whole-brain, oligo(dT)-primed cDNA library in \(\lambda\)-ZAP II as previously described. Inserts from purified clones were subcloned into pBluescript II SK. DNA sequences were determined by the dideoxy chain termination method using double-stranded templates, Sequenase, and \([\text{35S}]\) deoxyadenosine triphosphate (1,350 Ci/mmol, Du Pont/NEN).

RNA Extraction and Northern Blotting

Total cellular RNA was extracted from rat tissues by a modification of the guanidinium thiocyanate procedure. Poly (A)\(^+\) RNA was selected by passage over an oligo(dT)-cellulose column twice. Poly (A)\(^+\) RNA was separated on a 1% agarose/3% formaldehyde gel prepared in 20 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (pH 7.0)/5 mM sodium acetate/1 mM

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Two sets of clones were isolated, one clone (RBa2c) had high homology with the human α2-c4 cDNA16-12; the derived from the human α^-adrenergic receptor gene.

Subtype-Specific Probes and Tissue Distribution

By taking advantage of the divergence of the third cytoplasmic loop, we were able to subclone regions of each of the three rat α₂-adrenergic receptor cDNAs into SP6 promoter–containing vectors, psP65 or psP64 (Promega), to allow the synthesis of antisense RNA probes that are specific for transcripts from each α₁C-adrenergic receptor gene. To detect the α₁α messenger RNA (mRNA), we subcloned a 333-bp Kpn I/XmnI 1 fragment that encompasses most of the third cytoplasmic loop region of RBa2A into puc19 at the Kpn I/HincII sites and then directionally cloned it into psP64 as an EcoRI/HindIII fragment. A 269-bp Pst I fragment of RNGα2 was cloned into the Pst I site of psP65 to create a probe specific for the transcripts that encode the α₁A-adrenergic receptor. (RNGα2 cDNA15 was a kind gift of Dr. Kevin R. Lynch, University of Virginia School of Medicine, Charlottesville.) Similarly, a 466-bp Nae I/Stu I fragment that encompasses most of the third cytoplasmic loop region and some of the VI transmembrane region of RBa2C was subcloned into the Sma I site of psP65 to produce a probe specific for α₁C transcripts.

To study the expression of the α₁-adrenergic receptor subtypes, we isolated total RNA from tissues of six Wistar rats as described above. (Neonatal rat lung RNA was kindly provided by Dr. Martin Joyce-Brady, Boston University School of Medicine.) RNase protection was performed by methods previously described.24 Briefly, antisense psP65 and psP64 clones were linearized at the HindIII site or EcoRI site, respectively, and antisense RNA was synthesized from the SP6 promoter in the presence of SP6/T7 quality [32P]cytidine triphosphate (Pipes, pH 6.7. Single-stranded RNA was digested by a mixture of RNase A and RNase T1 (Boehringer Mannheim Corp., Indianapolis, Ind.) at 34°C for 1 hour. Protected fragments were visualized on a denaturing 6 M urca/6% polyacrylamide gel. RNA probes of known size and end-labeled pBR322 HindII fragments were used as size markers. In our experiments, we used RNA from transfected CHO cell lines that permanently express the α₁-adrenergic receptors encoded by RBa2A or RBa2C cDNAs (Handy et al, unpublished) as hybridization controls.

Results

A rat brain cDNA library was screened with a probe derived from the human α₂-adrenergic receptor gene.11 Two sets of clones were isolated, one clone (RBa2C) contained sequences that encoded for a receptor that had high homology with the human α₂-c4 cDNA16,12; the other set of clones (RBa2A) had high homology with the human α₂-C10 gene,11 which encodes for an α₂A subtype of adrenergic receptor. Using the entire RBa2A cDNA as a probe on a Northern blot (Figure 1), a 3.8-kb transcript can be detected in brain, spleen, and kidney. Also detectable are a 2.3-kb transcript in spleen and a 4.6-kb transcript in kidney, spleen, and brain. From these results, it was not clear whether the multiple bands represented transcripts from other α₂-adrenergic receptor genes or alternative transcripts from the rat α₂A-adrenergic receptor gene locus. To eliminate cross-hybridizing bands and to characterize the tissue distribution of each of the three α₂ subtypes, we subcloned fragments overlapping the highly divergent third cytoplasmic loop of the RBa2C, RBa2A, and RNGα2 cDNAs to create probes specific for the α₂C, α₂A, and α₂B-adrenergic receptor gene transcripts, respectively (Figure 2). RNase protection was used as a method of detection to eliminate cross-hybridization among related receptor sequences and to maximize the sensitivity of detection.

Tissue Distribution of α₂ Subtype Transcripts

Protection assays using the α₂A-specific probe (Figure 3A) show high expression in brain, spleen, and submandibular and sublingual glands, followed by detection of α₂A transcripts in kidney, adult lung, and thymus. No α₂C transcript was detected in neonatal lung (not shown). RNA from the permanently transfected CHO cell line expressing the recombinant α₂A-adrenergic receptor (CHO A in Figure 3A) shows a protected band, whereas RNA from the CHO transfectant expressing the recom-
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FIGURE 2. Diagram shows common structure of α2-adrenergic receptors and relative positions of rat α2-adrenergic receptor subtype probes. Boxed line shows common structural organization of α2-adrenergic receptors: N, extracellular N-terminus; T1–T7, transmembrane regions 1 through 7; C1–C3, intracellular (cytoplasmic) loops 1 through 3; E1–E3, extracellular loops 1 through 3; C, intracellular C-terminus. Lines represent location of probes used in RNase protection assays relative to structural organization of the receptors. The α2A probe is derived from a 333-bp Kpn I/Xmn I fragment that encompasses only C3 (third cytoplasmic loop) regions of RBα2A; α2C probe is derived from a 466-bp Nae I/Stu I fragment17 that contains mostly C3 regions plus T6 sequences of RBα2C; α2B probe is derived from a 269-bp Pst I fragment17 that encompasses only C3 regions of RNGα2. The third cytoplasmic loops of the three rat α2-adrenergic receptor subtypes have no significant homology (<30%).

binant α2c-adrenergic receptor (CHO-C) shows no specific band.

The α2B probe showed strong hybridization in kidney and neonatal rat lung and weak hybridization in brain, heart, adult lung, and liver (Figure 3B). No signal was detected in salivary gland and thymus or in the CHO cells expressing either the α2A or α2c-adrenergic receptors.

The α2C probe showed the strongest hybridization in brain, very low expression in whole-kidney RNA, and no detectable levels of expression in other tissues, including neonatal rat lung, adult rat lung, heart, and liver (Figure 3C). A comparison of the CHO-A and CHO-C lanes shows the specificity of the α2C probe, which only detects transcripts in RNA from the CHO-C transfectants. These results are summarized in Table 1 and indicate that all three subtypes were expressed in rat brain and kidney, although at different levels.

TABLE 1. Relative Tissue Distribution of α2-Adrenergic Receptor Transcripts

<table>
<thead>
<tr>
<th>Tissue</th>
<th>α2A</th>
<th>α2B</th>
<th>α2C</th>
</tr>
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<tbody>
<tr>
<td>Kidney</td>
<td>++</td>
<td>+</td>
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</tr>
<tr>
<td>Brain</td>
<td>+</td>
<td>++</td>
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<td>Spleen</td>
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<td>Liver</td>
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<td>Thymus</td>
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<tr>
<td>Adult lung</td>
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<td>+</td>
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<tr>
<td>Neonatal lung</td>
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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>+</td>
<td>+</td>
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Approximate relative amounts of messenger RNA detected by subtype-specific probes are indicated: -, no expression; +, barely detectable expression. Number of + indicates increasing levels of expression. Data were obtained from analysis of four to six tissue samples except for neonatal rat lung, which was pooled from several rats.

Discussion

We have isolated two rat α2-adrenergic receptor cDNAs, RBα2C and RBα2A, that represent the major forms of α2-adrenergic receptor transcripts in brain. Northern blots have shown that the RBα2C cDNA hybridizes primarily to a 3.0-kb transcript, whereas the RBα2A cDNA hybridizes to a 3.8- and 4.6-kb transcript in brain. To eliminate the detection of cross-hybridizing bands, we have used the RNase protection assay and probes specific for each rat α2 cDNA in studies of tissue distribution of α2-adrenergic receptor gene transcripts.

RNase protection analysis has allowed for the detection of the α2A transcripts in a number of tissues, including kidney, spleen, brain, and salivary glands. We suggest that the rat α2A-receptor encoded by RBα2A represents the adrenergic receptor subtype previously characterized in bovine pineal glands and rat salivary glands that has a lower affinity for rauwolscine than the other α2-adrenergic receptors.5,9,10 This is consistent with studies in our laboratory (Bresnahan et al, unpublished data, 1993) and by others17 which show that receptors encoded by RBα2A cDNA or the nearly identical RG20 cDNA17 have a much lower affinity for rauwolscine than other characterized α2-receptors. This has led to the use of the designation "α2D" to reflect the unique pharmacology of the rat α2D-receptor.17

Using RNase protection, we studied the expression of the α2B-adrenergic receptor-specific transcripts. Lorenz et al,25 using a probe derived from the human α2B gene (α2-c2), did not detect transcripts in neonatal rat lung, although they did detect transcripts in kidney and liver. Furthermore, in their study, bands detected in brain and adult lung after hybridization with the α2-c2 probe were dismissed as background from previous hybridizations of the Northern blot filter.25 In our studies, we show α2B transcripts in neonatal and adult lung and brain as well as in kidney, liver, and heart. We attribute some of these differences in detection to the methods used: by relying on Northern blots and cross-species probes, it is difficult to distinguish between cross-hybridizing bands and the possible existence of alternative mRNA transcripts.

Our analysis of tissue-specific distribution of α2C transcripts shows that the α2C transcripts are most highly expressed in rat brain and weakly expressed in rat kidney. The identification of the α2C-adrenergic receptor transcripts in kidney is consistent with the previous isolation of the human cDNA encoding the α2C-receptor from a human kidney cDNA library.12 Unlike our sensitive RNase protection analysis with species- and subtype-specific probes, Lorenz et al,25 using a probe from the human α2-c4 cDNA, failed to detect any α2-c4-specific transcripts from rat kidney on Northern blot analysis. In addition, it has been suggested that the RBα2C-receptor may be the rat counterpart of the α2-adrenergic receptor in opossum kidney cells.5,7 Our detection of this transcript in kidney is also consistent with the expression of this subtype in the OK cells6 derived from kidney.

While this article was in preparation, Zeng and Lynch26 reported the localization of all three α2-adrenergic receptor subtypes in rat brain using Northern blot analysis and probes specific for each of the rat α2-adrenergic receptor genes. Their results also show that the α2A and α2C transcripts are predominant throughout
FIGURE 3. Gels show RNase protection of RNA from rat tissues with \( \alpha_2 \)-adrenergic receptor subtype-specific probes. Twenty micrograms of RNA from various tissues was hybridized to subtype-specific probes. After digestion by RNases, fragments were separated on a denaturing 5% polyacrylamide gel. CHO-A and CHO-C samples were taken from CHO lines permanently expressing the protein-coding regions of RBoA and RBoC complementary DNAs, respectively. Because of the high expression in transfectants, less than 1 \( \mu \)g CHO-A or CHO-C RNA was used in the protection assays. Markers lane indicates pBr322 HindIII fragments as described below. Large arrowhead indicates specific protected band, small arrowhead the full-length probe. Panel A: Protection assays with \( \alpha_{2A} \) probe; markers are 396, 344 (doublet), and 298 nucleotides; panel B: protection assays with \( \alpha_{2B} \) probe; markers are 517/506, 396, 344 (doublet), 298, and 222/221 nucleotides; panel C: protection assays with \( \alpha_{2C} \) probe; markers are 517/506, 396, 344 (doublet), 298, and 222/221 nucleotides.

the brain, whereas the \( \alpha_{2B} \) transcripts are present at a lower level. In their study, multiple transcripts were detected with the \( \alpha_{2A} \) probe. Our results with RNase protection analysis show expression of all three subtypes of \( \alpha_2 \)-adrenergic receptor genes in rat brain and eliminate any question of probe specificity. Another report used the technique of in situ hybridization to study the expression of two \( \alpha_2 \)-adrenergic receptor
genes, referred to as $\alpha_{2R}$ for kidney form ($\alpha_{2K}$), and $\alpha_{2B}$ for brain form ($\alpha_{2C}$). The in situ hybridization detected both $\alpha_{2R}$ and $\alpha_{2B}$ transcripts in kidney but did not detect $\alpha_{2K}$ ($\alpha_{2C}$) in brain. This may be due to a lack of sensitivity in the detection method; by using end-labeled oligonucleotide probes in in situ hybridizations, it may be impossible to detect low-abundance transcripts. This in situ hybridization study also claims that the $\alpha_{2R}$ ($\alpha_{2B}$) is only detected in kidney, whereas the $\alpha_{2C}$ ($\alpha_{2K}$) is found in liver, heart, and lung as well as in brain. This is not supported by our RNase protection experiments, which show the broad tissue distribution of the $\alpha_{2B}$ transcripts and a restricted distribution of the $\alpha_{2C}$ to brain and kidney. Our results are supported by data reported elsewhere.\(^2,2^6\) Clearly, in our protection experiments, our probes do not cross-hybridize with transcripts specific for other subtypes, as each probe shows unique tissue distribution, and in addition, RNA isolated from the CHO transfectants only hybridizes with the appropriate probe. Repeating in situ hybridization studies either with the same probes as reported in Reference 27 or with other probes will probably clarify the in situ results.

With the advent of molecular techniques, clear-cut evidence for the existence of three separate $\alpha_2$-adrenergic receptors in both human and rat has been shown. We have isolated two cDNAs representing the major expressed $\alpha_2$-adrenergic receptor transcripts in rat brain. By using the sensitive method of RNase protection and subtype-specific probes, we have shown that the three $\alpha_2$-adrenergic receptor genes have unique tissue-specific distribution. All three rat $\alpha_2$-adrenergic receptor genes are expressed in rat brain and in kidney, although it appears that these receptors have a unique distribution within these tissues. We suggest that the tissue-specific and subregion-specific expression of these related receptors may prove important in defining their biological function. Segregation of these similar receptors to different cells may allow for subtle differences in responses to catecholamines, perhaps by allowing the activation of different intracellular signaling pathways according to the $\alpha_2$-adrenergic receptor subtype that is expressed. Or perhaps cell-specific expression may result in a differential ability of a cell to upregulate or downregulate the number of $\alpha_2$-adrenergic receptors depending on the $\alpha_2$ subtype gene expressed. A closer scrutiny of tissue-specific expression and tissue-specific function of subtypes of $\alpha_2$-adrenergic receptors will clarify their role in regulating complex biological functions.

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