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ACCELERATED COMMUNICATION

Expression of Three α_2-Adrenergic Receptor Subtypes in Rat Tissues: Implications for α_2 Receptor Classification

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SUMMARY

Based on biochemical and ligand binding studies in various tissues and species, evidence for several α_2-adrenergic receptor subtypes has accumulated. The current α_2-adrenergic receptor classification (α_{2A}, α_{2B}, α_{2C}) is based exclusively on pharmacological criteria. The molecular cloning of three distinct genes for human α_2-adrenergic receptors has confirmed the existence of multiple α_2-adrenergic receptor subtypes. According to their localization on different human chromosomes, the receptor genes were termed α_2-C10, α_2-C4, and α_2-C2. The relationship, however, between the pharmacologically characterized α_2-adrenergic receptors and the isolated genes has yet to be clarified. Using Northern blot hybridization, we analyzed the expression of the three cloned α_2-adrenergic receptor genes in 13 rat tissues, as well as in cell lines previously described as model systems for the pharmacologically defined α_2-adrenergic receptor subtypes. The α_2-C10 receptor corresponds to the α_{2A} subtype and is expressed in rat brainstem, cerebral cortex, hippocampus, pituitary gland, cerebellum, kidney, aorta, skeletal muscle, spleen, and lung. Messenger RNA coding for the α_2-C4 receptor was detected only in brain regions, not in peripheral tissues, whereas the α_2-C2 message was found only in liver and kidney. Hybridization experiments with RNA derived from tissues and cells from which the pharmacological α_2-receptor classification has been developed lead to the conclusion that the α_{2B} subtype represents two distinct receptor molecules, the α_2-C4 and a subtype previously undetected by classical ligand binding approaches. Furthermore, our results suggest that the α_{2C} subtype characterized in opossum kidney cells is an interspecies variation of α_2-C4 rather than a separate subtype. Finally, the cloned α_2-C2 receptor was found to be "α_{2B}-like" and not covered by the current pharmacological classification.

The ARs are members of a family of integral membrane proteins mediating the actions of hormones and neurotransmitters on target cells via guanine nucleotide-binding protein-linked signal transduction pathways (1). The current classification of AR subtypes is largely based on their pharmacological properties. The molecular cloning of the genes or cDNAs of several ARs confirmed, and even expanded, the previously postulated receptor heterogeneity for the β- (2–4), α_1- (5, 6), and α_2-AR subtypes (7–10).

Based on ligand binding studies, three subtypes of α_2-ARs have been postulated by Bylund and co-workers. The human (11) and the rat (12) brain were shown to contain two α_2-AR subtypes, which differ in their affinity for prazosin. The α_{2A} subtype has a low affinity for prazosin, is typically represented by the platelet α_2-AR, and is expressed in the human colonic adenocarcinoma cell line HT29 (12, 13). The α_{2B} receptor has a 30- to 40-fold higher affinity for prazosin and is expressed in the neonatal rat lung and in the neuroblastoma × glioma hybrid cell line NG 108-15 (13–15). Evidence for a third α_2-AR subtype (16), termed α_{2C} (14), was revealed by pharmacological characterization of α_2-ARs in OK cells.

In addition to pharmacological evidence for three α_2-ARA subtypes, three different genes or cDNAs coding for α_2 receptors have been cloned and localized to three different human chromosomes. The first α_2-AR gene to be cloned was the gene for the human platelet α_2-AR (7). According to its localization on chromosome 10, the corresponding receptor was termed α_2-C10. Its pharmacological characterization clearly assigned it to the α_{2A} subtype (7, 14). The second α_2-AR gene, cloned from a human kidney cDNA library, resides on chromosome 4 (α_2-C4). Its pharmacological properties differ from those of α_2-C10 (8). To date it cannot be assigned unequivocally to one of the

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ABBREVIATIONS: AR, adrenergic receptor; bp, base pairs; kb, 1000 base pairs; DMEM, Dulbecco's modified Eagle's medium; OK, opossum kidney cell line; SSC, standard saline citrate; SDS, sodium dodecyl sulfate.

pharmacologically defined subtypes, although it closely resembles the α_{2B}-AR. Utilizing the polymerase chain reaction we have recently cloned, from human genomic DNA, the gene for a third α_2-AR that localizes to chromosome 2 (9). This novel α_2-AR (α_2-C2) has a unique pharmacology and cannot be identified clearly as one of the present pharmacologically defined α_2-AR subtypes. From a rat kidney cDNA library an α_2-AR has been cloned and assigned to the α_{2B} subtype (10).

In order to investigate the relationship between the pharmacologically defined α_2-ARs and those isolated by molecular cloning, we have used DNA probes for α_2-C2, α_2-C4, and α_2-C10 to map their mRNA distribution in various rat tissues and cell lines that are known to express the three pharmacologically defined α_2-ARs.

Materials and Methods

Cell culture. HT29 human colonic adenocarcinoma cells were obtained from the American Type Culture Collection. Monolayer cultures were grown in high glucose (4.5 g/liter) DMEM (GIBCO) supplemented with 5% (v/v) fetal bovine serum and 5% (v/v) newborn calf serum. OK cells, a generous gift of J. Raymond (Department of Nephrology, Duke University Medical Center, Durham, NC), were grown in high glucose DMEM supplemented with 10% fetal calf serum. NG 108-15 neuroblastoma × glioma hybrid cells were obtained from K. Harden (Department of Pharmacology, University of North Carolina, Chapel Hill, NC). Cells were cultured as monolayers in DMEM supplemented with 10% newborn calf serum, 0.1 mM hypoxanthine, 1.0 μM aminopterin, and 16 μM thymidine. All cells were grown at 37° in a humidified atmosphere of 5% CO_2/95% air.

Poly(A)$^+$ RNA preparation and Northern analysis. Total cellular RNA was isolated from fresh rat tissues (Sprague-Dawley of either sex) and from freshly harvested cells by the guanidinium isothiocyanate/cesium chloride gradient method (17). Neonatal rat lungs were excised from 1-day-old Sprague-Dawley rats. Poly(A)$^+$ RNA was selected using two cycles of oligo(dT)-cellulose chromatography (18). Following denaturation by glyoxylation, the RNA was fractionated by electrophoresis on 1.2% agarose gels (19), transferred to Biotrans membranes (ICN Biochemicals) by capillary blotting, and hybridized (20, 21) with specific ^{32}P-labeled probes. After hybridization, the filters were washed successively in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% SDS, at room temperature, and 0.1× SSC, 0.1% SDS, at 55°, and were exposed to Kodak X-OMAT AR film at −70° with two intensifying screens. Sizes of mRNAs were estimated by comparison with an RNA ladder (Bethesda Research Laboratories) stained with methylene blue after blotting (22).

DNA probes. To obtain the α_2-C10 probe, the 0.95-kb *Pst*I fragment derived from the coding block of the gene for the human platelet α_2-AR was subcloned (7). This fragment contains the coding sequence corresponding to the putative first transmembrane domain through most of the third intracellular loop. The α_2-C4 probe consisted of the *Eco*RI fragment containing the entire coding block of the human kidney α_2-AR cDNA (8). A 900-bp polymerase chain reaction product amplified from human genomic DNA and subcloned was used for the α_2-C2 probe (9). This subclone contains the coding sequence from the putative third transmembrane domain through the carboxy end of the putative third cytoplasmatic loop. After self-ligation of the isolated fragments, the probes were labeled to a specific activity of 4×10^8 to 1×10^9 dpm/μg of DNA by nick translation (23) with [α-^{32}P]dCTP (DuPont-New England Nuclear). For hybridizations, 2×10^6 dpm/ml were used.

Binding studies. Radioligand binding studies were performed with [^{3}H]yohimbine (DuPont-New England Nuclear), using phentolamine (Ciba-Geigy) to determine nonspecific binding, as previously described (8). Membranes were prepared as described (8). Protein concentrations were determined by the method of Bradford (24).

Results

Poly(A)$^+$ RNA prepared from a series of rat tissues was analyzed by Northern blotting and hybridization with DNA probes specific for each of the three cloned human α_2-AR subtypes, α_2-C2, α_2-C4, and α_2-C10 (Fig. 1). The 3.8-kb mRNA of α_2-C10, the equivalent of the α_{2A} subtype (7), is most abundant in brainstem, followed by cerebral cortex, hippocampus, pituitary gland, cerebellum, kidney, aorta, skeletal muscle, spleen, and lung (Fig. 1A). Weak hybridization to a band of 4.5 kb could be detected in RNA from heart and aorta. The identity of this mRNA is presently unknown. The additional mRNA species of 2.9 kb hybridizing with the α_2-C10 probe in cerebral cortex, cerebellum, brainstem, and hippocampus was shown to result from cross-hybridization to the α_2-C4 mRNA (Fig. 1B). Although the α_2-C4 cDNA clone was isolated from a human kidney cDNA library (8), the expression of this receptor subtype in rat appears to be restricted to brain regions. Whereas high levels of α_2-C4 mRNA are present in cerebral cortex, cerebellum, brainstem, and hippocampus, no hybridization could be detected in rat kidney. The third probe used in our experiments was a probe derived from the recently cloned α_2-C2 receptor (9). A 4.1-kb message encoding this subtype was found only in two peripheral tissues, liver and kidney (Fig. 1C). The weak bands of 3.8 and 2.9 kb seen in RNA from adult lung and the brain regions are due to the previous hybridization of this membrane with the α_2-C10 probe, which could not be completely washed off.

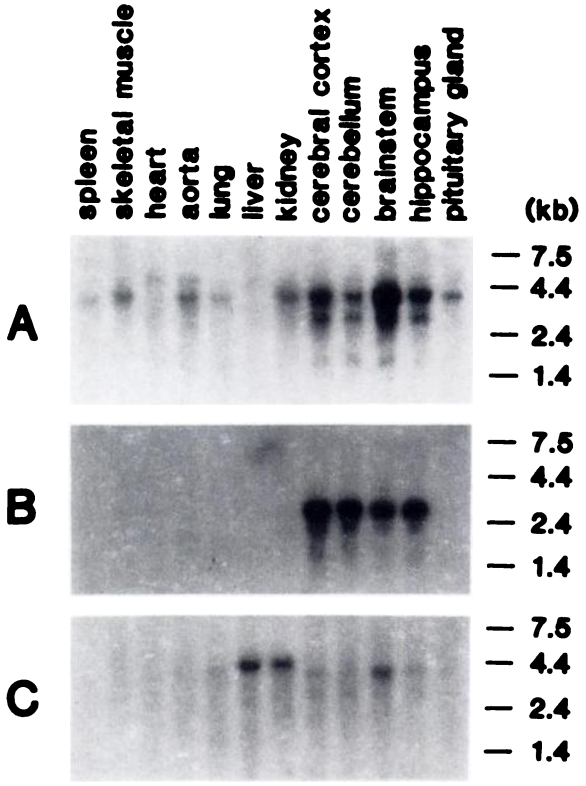

Fig. 1. Northern blot analysis of poly(A)$^+$ RNA prepared from rat tissues. Ten micrograms of RNA were applied to each lane and electrophoresed, blotted, and hybridized as described in Materials and Methods. The final washing conditions were 0.1× SSC, 0.1% SDS, at 55°. The respective rat tissues are indicated above A. The sizes of the molecular weight markers are indicated in kb. A–C, Hybridizations with the probes α_2-C10, α_2-C4, and α_2-C2, respectively.

In order to compare the three cloned α_2-AR subtypes with the pharmacologically defined α_2-AR subtypes, we also examined the expression of α_2-ARs at the mRNA level in cell lines and tissues that had been shown by binding studies to contain a single α_2-AR subtype. At least one model system has been described for each of the pharmacological α_2-AR subtypes, α_{2A}, α_{2B}, and α_{2C}. The human colonic adenoma cell line HT29 has been shown to express only the α_{2A} receptor (12, 13). Neonatal rat lung and the neuroblastoma × glioma cell line NG 108-15 have been described as model systems for the α_{2B} subtype (13–15). The α_2-AR expressed in OK cells has a slightly different pharmacology from that of the α_{2B} subtype (16) and has been termed α_{2C} (14). Ligand binding experiments were performed with [^{3}H]yohimbine to confirm the expression of the three α_2-ARs in these cell lines and tissue. In membrane preparations from HT29, NG 108-15, and OK cells and neonatal rat lung, B_{max} values for α_2-AR binding were 260, 200, 410, and 370 fmol/mg of protein, respectively.

Hybridization of poly(A)$^+$ RNA from HT29, NG 108-15, and OK cells and from neonatal rat lung with probes from the three cloned α_2-ARs are shown in Fig. 2, A-C. Blots containing these RNAs were also hybridized with an actin probe (Fig. 2D). The α_2-C10 probe detects a message of 3.8 kb in HT29 RNA and cross-hybridizes weakly with a 3.3-kb mRNA in OK cells (Fig. 2A). The α_2-C4 probe hybridizes with a 2.9-kb mRNA in NG 108-15 and with a 3.3-kb mRNA in OK cells (Fig. 2B). The relatively low extent of hybridization of the α_2-C4 probe to NG 108-15 RNA could result from expression of the corresponding mouse gene in this hybridoma cell line and weaker cross-hybridization of the probe with the mouse message, as compared with the rat homologue. In HT29 RNA, the α_2-C4 probe cross-hybridizes with the 3.8-kb message of α_2-C10 and with a 1.4-kb mRNA of unknown identity (Fig. 2B), which also hybridizes weakly with the α_2-C2 probe (Fig. 2C). Besides this cross-hybridization, the α_2-C2 probe did not detect a message in any of the four analyzed model systems for α_2-AR subtypes (Fig. 2C). Furthermore, none of the probes used in our experiments recognized a specific mRNA in the neonatal rat lung preparation. The quality of this RNA preparation was verified by hybridization with the actin probe (Fig. 2D). In addition, a hamster α_{1B} probe strongly hybridized to a 2.4-kb mRNA species in this tissue (data not shown).

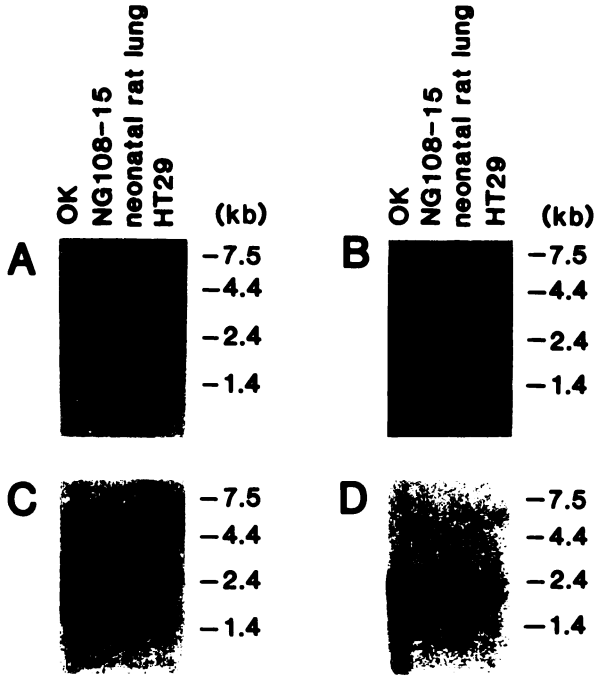

Fig. 2. Northern blot analysis of poly(A)$^+$ RNA prepared from a tissue and cell lines expressing only one α_2-AR subtype. Ten micrograms of RNA were applied to each lane and electrophoresed, blotted, and hybridized as described in Materials and Methods. The final washing conditions were 0.1× SSC, 0.1% SDS, at 55°. The respective tissues are indicated above A and B. The sizes of the molecular weight markers are indicated in kb. A–D, Hybridizations with the probes α_2-C10, α_2-C4, α_2-C2, and actin, respectively.

Discussion

The results of this study clearly show that at least three different α_2-AR genes are expressed in the rat and these α_2-AR subtypes have a unique tissue distribution. The α_2-C10 receptor is expressed at high levels in various brain regions and at lower levels in most of the peripheral tissues analyzed. The α_2-C4 subtype was detected only in rat brain, whereas α_2-C2-specific mRNA was found only in rat liver and kidney.

It has been unclear how the cloned α_2-AR subtypes correspond to the pharmacologically defined α_2-AR subtypes. The pharmacological definition of the subtypes α_{2A}, α_{2B}, and α_{2C} is based on the differential potencies of the ligands prazosin, yohimbine, oxymetazoline, ARC-239, and chlorpromazine. The compound oxymetazoline is selective for the α_{2A} subtype (K_i = 0.7–1.1 nM), which has a low affinity for prazosin (K_i = 240–340 nM) and a K_i ratio of prazosin to yohimbine of 240–570 (11, 12, 16, 25). The ligands ARC-239 (K_i = 1.6–1.7 nM) and chlorpromazine (K_i = 20 nM) are selective for the α_{2B} subtype, which in addition has a 30- to 40-fold higher affinity for prazosin (K_i = 3.7–5.4 nM) and a K_i ratio of prazosin to yohimbine of 5.4 (11, 12, 16, 25). The α_{2C} subtype has a high affinity for prazosin (K_i = 7.6 nM) and a K_i ratio of prazosin to yohimbine of 40 (16).

Ligand binding experiments with COS-7 cells expressing the α_2-C10 receptor have shown a high affinity of this receptor for oxymetazoline (K_i = 11 nM), a low affinity for prazosin (K_i = 1800 nM), and a K_i ratio of prazosin to yohimbine of 1125 (8). Based on these results, α_2-C10 was assigned to the α_{2A} subtype. The hybridization of a message in RNA from the cell line HT29, a model for the α_{2A} receptor (12, 13), confirms these results. From binding studies on α_2-ARs in rat brain, the cerebral cortex, hippocampus, and corpus striatum were found to contain the α_{2A} and α_{2B} subtypes in roughly equal proportions (12). The occurrence of an α_2-C10 message in various regions of the rat brain is consistent with these findings.

Expression of the α_2-C4 receptor in COS-7 cells has revealed pharmacological properties similar to those of the α_{2B} subtype (8, 9). Its affinity for prazosin is 40-fold higher (K_i = 41 nM) than that of α_2-C10, whereas the affinity of α_2-C4 for oxymetazoline is lower by a factor of 6 (K_i = 62 nM). However, the ratio of the K_i value for prazosin to that for yohimbine is 44 for α_2-C4 and, thereby, more similar to that for the α_{2C} subtype than that for the α_{2B} subtype. When viewed together with our finding of an α_2-C4 message in several regions of the rat brain, these data suggest that α_2-C4 is the equivalent of the α_{2B} subtype. Neonatal rat lung and the cell line NG 108-15 have been described as model systems that contain α_2-ARs exclusively of the α_{2B} subtype (13). In our experiments, the α_2-C4

probe detected a specific mRNA only in the NG 108-15 cells, but not in RNA from neonatal rat lung. These results suggest that, whereas the α_2-ARs expressed in NG 108-15 and neonatal rat lung have similar pharmacological properties, they may be encoded by separate genes and, hence, be closely related α_2-AR subtypes. Additional biochemical data indicate that the α_{2B} subtypes of rat cerebral cortex and neonatal lung might be two distinct receptors. In contrast to the α_2-ARs in cerebral cortex and human platelets, the α_2-AR in neonatal rat lung has been shown to lack *N*-glycosylation (26).

The α_2-C4 probe also hybridized to a mRNA species in OK cells, a model for the α_{2C} subtype. However, the message size in rat (2.9 kb) was different from that in opossum (3.3 kb). This difference in message size may be due to the distant evolutionary relationship between the two species. The differences between the pharmacological profile of the α_2-AR in OK cells and that of the α_2-AR expressed in NG 108-15 cells are slight. The affinity of the receptor in OK cells for yohimbine (K_d = 0.23 nM) is closer to that of the α_{2A} subtype (K_d = 0.59 nM in HT29 cells) than to that of the receptor in NG 108-15 cells (K_d = 2.2 nM) and its ratio of the K_i value for prazosin to that for yohimbine is intermediate between those of α_{2A} and α_{2B} receptors. On the other hand, the affinities of prazosin, ARC-239, and oxymetazoline (K_i = 7.6, 8.1, and 10 nM, respectively) and the rank order of potencies of the antagonists, yohimbine > prazosin = phentolamine > chlorpromazine = corynanthine, are similar to those of the receptor expressed in NG 108-15 cells. Therefore, the different pharmacological properties of the α_2-AR in OK cells may represent a species variation rather than a genuine third α_2-AR subtype. We propose that α_2-C4 represents both the pharmacologically defined α_{2B} subtype expressed in rat brain and the α_{2C} subtype expressed in OK cells.

Recently, the gene for a third human α_2-AR has been cloned [α_2-C2 (9)]. Examination of its ligand-binding characteristics after expression in COS-7 cells showed that α_2-C2 is an α_2-AR with unique pharmacological properties, as compared with α_2-C4 and α_2-C10. A relatively high affinity for prazosin (K_i = 293 nM) and low affinity for oxymetazoline (K_i = 1506 nM) suggest that α_2-C2 is an "α_{2B}-like" receptor. However, in our experiments the α_2-C2 probe did not detect a specific mRNA in regions of the rat brain, neonatal lung, NG 108-15 cells, or OK cells. These data suggest that α_2-C2 is not equivalent to the pharmacologically defined α_{2B} subtype and that this receptor is not represented in the model systems that form the basis of the current pharmacological α_2-AR classification. This receptor would, therefore, represent a third distinct α_2-AR subtype.

None of the probes used in our experiments hybridized to mRNA from neonatal rat lung. This suggests that the neonatal lung receptor, although pharmacologically very similar to the α_{2B} subtype of rat brain and NG 108-15 cells, is encoded by a gene distinct from α_2-C4, which encodes the α_{2B}-AR expressed in rat brain.

Recently, the cloning of an α_2-AR from a rat kidney cDNA library has been described (10). Pharmacological studies after expression of the cloned receptor in COS-1 cells have shown it to be an α_{2B}-AR. It has a high affinity for prazosin (K_i = 27 nM), a relatively low affinity for oxymetazoline (K_i = 613 nM), and a K_i ratio of prazosin to yohimbine of 5.2. The amino acid sequence of this rat α_2-AR is very similar to that of α_2-C2 (84% amino acid identity). This suggests that this cDNA is derived from the rat homologue of the human α_2-C2 gene. The reported mRNA size of 4 kb in rat kidney is consistent with our data. We cannot explain the fact that the rat cDNA probe hybridizes with a specific mRNA in neonatal rat lung, whereas in our hands the human probe does not detect a message in the same tissue.

The compound SKF104078 has been found to discriminate between pre- and postsynaptic α_2-ARs (27). It has a low affinity for the prejunctional site (K_b = 3–30 μM) and a moderately high affinity for the postjunctional site (K_b = 70–150 nM). All three cloned α_2-AR subtypes have a moderately high affinity for SKF104078 (K_b = 33–105 nM). Although the differences in affinities of α_2-ARs located at the two sites could be due to other factors, e.g., different receptor numbers, these data suggest that none of the cloned receptors represents the presynaptic α_2-AR, if presynaptic α_2-ARs do indeed represent distinct entities. This suggests that there may be as many as five distinct α_2-AR molecules. A brief report has appeared describing the cloning of an additional human α_2-AR (28). The information available does not allow a comparison of this receptor with the other α_2-ARs cloned.

Our study highlights the fact that the tools of pharmacology alone are not sufficient to describe receptor heterogeneity. Over the past few years a number of new guanine nucleotide-binding protein-coupled receptors have been cloned that were not predicted to exist by pharmacological definition. A definitive and reliable classification of α_2-ARs should be available when all of the genes for this subclass of ARs have been cloned and their expressed products characterized pharmacologically.

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