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The rat α_2-C4 adrenergic receptor gene encodes a novel pharmacological subtype

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A rat gene and brain cDNA (pA2d) encoding the homologue of the human α-C4 adrenergic receptor subtype were isolated and characterized. RNA blots indicate that this gene is expressed in brain, heart and kidney but not in lung, liver or pancreas. Yohimbine, WB-4101 and prasozin all exhibited high affinity for this receptor in binding studies. Clonidine was more potent and efficacious than norepinephrine in inhibiting forskolin-stimulated cAMP production in CHO cells expressing pA2d. Together, these data suggest that the α_2-C4 gene product defines a previously undescribed pharmacological subtype of α_2-adrenergic receptor.

α_2-Adrenoreceptor; Gene expression; Gene coupling; cAMP inhibition

1. INTRODUCTION

Traditionally, α_2-adrenergic receptor activation in brain has been linked to inhibition of adenylate cyclase activity [1]. Recent studies have suggested that α_2-adrenergic receptor activation can lead to perturbations of multiple cellular processes in a cAMP-independent manner through coupling with guanine nucleotide binding proteins (G-proteins). Such events include activation of K^+ channels [2], alterations in Na^+/H^+ exchange that lead to intracellular pH changes [3], and inhibition of voltage-dependent Ca^{2+} channels [4]. In keeping with these multiple functions of α_2-adrenergic receptors, pharmacological evidence derived from studies utilizing non-neuronal cell cultures suggests the existence of multiple α_2 subtypes. A classification scheme defining these subtypes as α_2A, α_2B and α_2C has been suggested based upon the rank order of potencies for a large number of antagonists [5]. More conclusive evidence for the existence of multiple α_2 subtypes has come from recent molecular cloning experiments which have demonstrated the existence of at least three genes encoding α_2-adrenergic receptors in the human designated α_2-C2, -C4 and -C10 [6-8] and of a subtype in the rat (RNGα_2) homologous to the α_2-C2 [9]. Preliminary evidence suggests that the α_2-C10 gene encodes an adrenergic receptor of the α_2A subtype and the α_2-C2 gene encodes a receptor of the α_2B subtype. The classification of the α_2-C4 gene product has yet to be determined.

In order to study the characteristics and functions of multiple α_2-adrenergic receptors in the brain, we have set about to clone the genes and cDNAs encoding members of this receptor family expressed in rat brain. In this report the pharmacology and distribution of expression of a rat gene and a cognate cDNA encoding an α_2-adrenergic receptor protein homologous to the human α_2-C4 adrenergic receptor is described. This receptor exhibited binding and functional properties that were dissimilar to those for the pharmacologically characterized A, B and C subtypes. The findings presented here suggest that the α_2-C4 gene product defines a pharmacologically novel adrenergic receptor subtype.

2. EXPERIMENTAL

2.1 *Isolation of genomic and cDNA clones*

Approximately 1.2×10^6 recombinants of a λCharon-4A rat genomic library (Clontech Laboratories, Palo Alto, CA) were screened by filter hybridization [11] in 6×SSC, 10 mM EDTA, 0.1% sodium pyrophosphate, 0.2% SDS, 100 μg/ml denatured herring sperm DNA at 60°C with two kinased oligonucleotides (Genetic Designs, Houston, TX) derived from sequences present in the third (amino acids 106–122) and fourth (amino acids 161–175) transmembrane domains of the human platelet α_2 receptor [6]. Filters were washed in 3×SSC at 65°C and exposed to X-ray film at −70°C. Eight positive clones were identified and one, λgA2d, was chosen for further study. A rat brain cDNA library in λgt11 (a gift of Dr Hemin Chin, NINDS/NIH) screened with a genomic fragment containing the putative receptor coding region from λgA2d yielded six clones. The largest of the six was 2.8 kb, and contained 900 bp of 5′-untranslated

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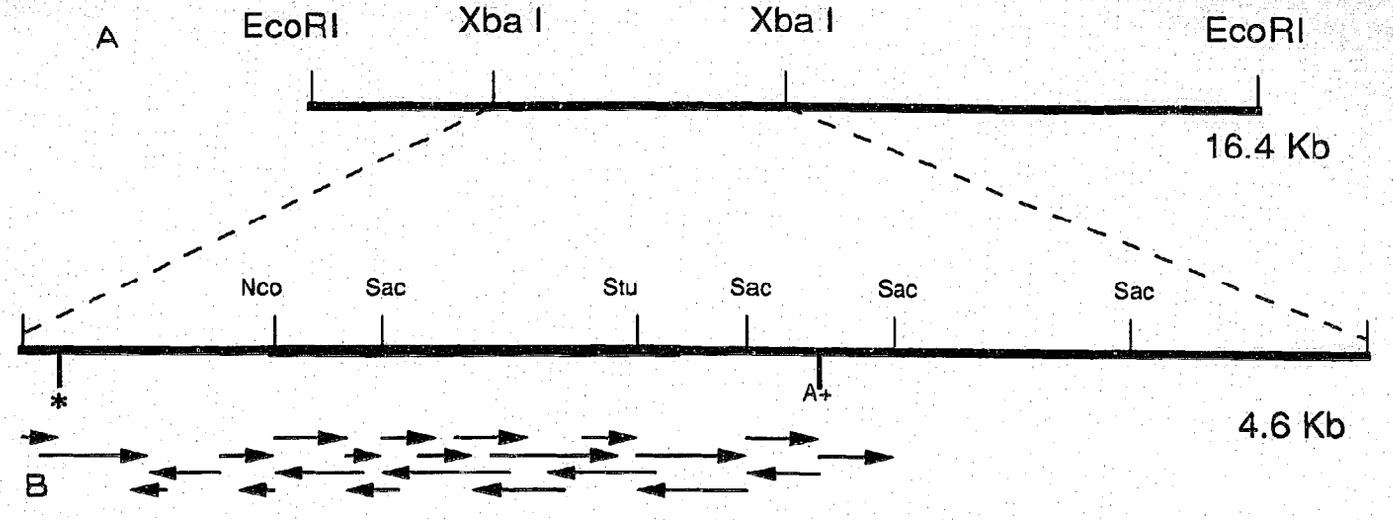

C

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          1
     aggaccATGGCGTCCCCAGCGCTGGCCGCGGCGCTGGCGGCGGCGGCGGCTGAGGGCCCC   54
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     AsnGlySerAspAlaGlyGluTrpGlySerGlyGlyGlyAlaAsnAlaSerGlyThrAsp
     TGGGGACCACCGCCGGGCCAGTACTCCGCAGGTGCTGTGGCAGGGCTGGCAGCCGTGGTG  174
     TrpGlyProProProGlyGlnTyrSerAlaGlyAlaValAlaGlyLeuAlaAlaValVal
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     LeuValAlaThrLeuValMetProPheSerLeuAlaAsnGluLeuMetAlaTyrTrpTyr
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     PheGlyGlnValTrpCysGlyValTyrLeuAlaLeuAspValLeuPheCysThrSerSer
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     IleValHisLeuCysAlaIleSerLeuAspArgTyrTrpSerValThrGluAlaValGlu
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     TyrAsnLeuLysArgThrProArgArgValLysAlaThrIleValAlaValTrpLeuIle
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     SerAlaValIleSerPheProProLeuValSerPheTyrArgArgProAspGlyAlaAla
     TACCCGCAGTGCGGCCTCAACGATGAGACCTGGTACATCTTGTCCTCCTGCATAGGCTCC  654
     TyrProGlnCysGlyLeuAsnAspGluThrTrpTyrIleLeuSerSerCysIleGlySer
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     PhePheAlaProCysLeuIleMetGlyLeuValTyrAlaArgIleTyrArgValAlaLys
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     LeuArgThrArgThrLeuSerGluLysArgGlyProAlaGlyProAspGlyAlaSerPro
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     ThrThrGluAsnGlyLeuGlyLysAlaAlaGlyGluAsnGlyHisCysAlaProProArg
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     ThrGluValGluProAspGluSerSerAlaAlaGluArgArgArgArgArgGlyAlaLeu
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     ArgArgGlyGlyArgArgArgGluGlyAlaGluGlyAspThrGlySerAlaAspGlyPro
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     GlyProGlyLeuAlaAlaGluGlnGlyAlaArgThrAlaSerArgSerProGlyProGly
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     GlyArgLeuSerArgAlaSerSerArgSerValGluPhePheLeuSerArgArgArgArg
     GCGCGCAGCAGTGTGTGCCGCCGCAAGGTGGCCCAGGCCGCGAGAAACGCTTCACCTTC 1134
     AlaArgSerSerValCysArgArgLysValAlaGlnAlaArgGluLysArgPheThrPhe
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     ValLeuAlaValValMetGlyValPheValLeuCysTrpPheProPhePhePheSerTyr
     AGCCTGTATGGCATCTGCCGTGAGGCCTGCCAGCTGCCAGAACCGCTCTTTAAGTTTTTC 1254
     SerLeuTyrGlyIleCysArgGluAlaCysGlnLeuProGluProLeuPheLysPhePhe
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     cctttgtaattaagtgttaggtgcattggtcagtcctaagccagggtgtgagagaactc
     tccagatcttgccctgagagccctgcacaggcacacactagagtatagagtgctctgaaa
     actggagttctaactgccttttttcatgaggaagagaggggctctcctttaaacggttaa
     cgggtttgggtatatgcaaagtatatactccaaaattcttttcctttcagg          2065
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Fig. 1. Restriction map, sequencing strategy and nucleotide and deduced amino acid sequence for clone λgA2d. (A) Partial restriction map for the genomic clone. (B) Map of the *Xba*I restriction fragment that holds the exon containing the entire coding region of the receptor. The coding region is identified by a thickened bar, and the sequencing strategy illustrated by arrows, representing individually sequenced clones, under the fragment. Nucleotide numbering beings from the first ATG of the coding region of the gene. The star indicates the position of the 5′-terminus of the longest cDNA clone obtained, and the poly(A) addition signal is represented by the A^+. (C) Nucleotide (above) and deduced amino acid (below) sequences of the portion of the *Xba*I fragment containing the coding region of the protein. The untranslated regions of the mRNA are in lowercase letters and the assumed translated portion of the gene is in uppercase letters.

sequence was extended 3′ to the poly(A) tail and was designated λcA2d. Sequences of all clones were obtained using a chain termination protocol with T7 DNA polymerase (Sequenase, US Biochemicals, USA) and M13 vectors as suggested by the manufacturer.

2.2. *Mammalian cell transfection and radioligand binding studies*

Chinese hamster ovary (CHO) cells and COS-7 cells were grown as previously described [11]. The cDNA insert from λcA2d was subcloned into pCDNA-1 (Invitrogen, San Diego, CA) for transient expression studies in COS-7 cells, or into pRc/CMV (Invitrogen) for production of stably-transfected cells. COS-7 and CHO cells were transfected by calcium phosphate precipitation as previously described [12], with stably-transfected CHO cells selected in medium containing G418 (500 μg/ml). Clonal cell lines expressing the receptor were verified by radioligand binding assays. The COS-7 cell membranes were prepared 48–72 h after transfection. Membrane preparation and radioligand binding using [^{3}H]rauwolscine (Amersham, Chicago, IL) was performed as previously described [13]. Clonidine, corynanthine, chlorpromazine, epinephrine, norepinephrine and oxymetazoline were from Sigma Chemical Co. (St. Louis, MO), prazosin, serotonin, yohimbine and WB-4101 were from Research Biochemicals (Natick, MA). Measurement of the inhibition of forskolin-stimulated cAMP accumulation in whole cells was performed as previously described [11].

2.3. *Northern blot analysis*

Total RNA from various tissues was obtained using the guanidine thiocyanate-cesium chloride gradient method [10]. Poly(A) containing RNA was then prepared, size-fractionated in formaldehyde-containing 1% agarose gels and transferred to Nytran filters (Schleicher and Schuell, Keene, NH) [10]. Blots, probes with uniformally-labeled cRNA synthesized using T7 RNA polymerase (Promega, Madison, WI) were hybridized in 6×SSC, 5×Denhardt's, 100 μg/ml denatured salmon sperm DNA, 2 mM EDTA, 0.2% SDS, 0.1% $NaPP_i$ and 50% formamide at 71°C, washed to a final stringency level of 0.5×SSC/0.5% SDS at 80°C and X-ray film was then exposed to the filters at −70°C.

3. RESULTS AND DISCUSSION

A restriction map of the 16.4 kb insert of the genomic clone λgA2d is shown in Fig. 1A. The putative coding region was localized to the internal 4.6 kb *Xba*I fragment (Fig. 1B), which was then sequenced (Fig. 1C).

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Rat      1   MASPALAAALAAAAAEGPNGSDAGEWGSGGGANASGTDWGPPPGQYSAGA   50
Human        MASPALAAALAVAAAAGPNASGAGERGSGGVANASGASWGPPRGQYSAGA   50

                    1                                  2
     51  VAGLAAVVGFLIVFTVVGNVLVVIAVLTSRALRAPQNLFLVSLASADILV   100
         VAGLAAVVGFLIVFTVVGNVLVVIAVLTSRALRAPQNLFLVSLASADILV   100

                                        3
    101  ATLVMPFSLANELMAYWYFGQVWCGVYLALDVLFCTSSIVHLCAISLDRY   150
         ATLVMPFSLANELMAYWYFGQVWCGVYLALDVLFCTSSIVHLCAISLDRY   150

                                   4
    151  WSVTEAVEYNLKRTPRRVKATIVAVWLISAVISFPPLVSFYRRPDGAAYP   200
         WSVTQAVEYNLKRTPRRVKATIVAVWLISAVISFPPLVSLYRQPDGAAYP   200

                         5
    201  QCGLNDETWYILSSCIGSFFAPCLIMGLVYARIYRVAKLRTRTLSEKRGP   250
         QCGLNDETWYILSSCIGSFFAPCLIMGLVYARIYRVAKRRTRTLSEKRAP   250

    251  AGPDGASPTTENGLGKAAGENGHCAPPRTEVEPDESSAAERRR..RRGAL   298
         VGPDGASPTTENGLGAAAGEARTGTARPRPPTWSRTRAAQRPRGGAPGPL   300

    299  RRGGRRREGAEGDTGSADGPGPGLAAEQ.GARTASRSPGPGGRLSRASSR   347
         RRGGRRRAGAEGGAGGADGQGAGPGAAQSGALTASRSPGPGGRLSRASSR   350

                                                  6
    348  SVEFFLSRRRRARSSVCRRKVAQAREKRFTFVLAVVMGVFVLCWFPFFFS   397
         SVEFFLSRRRRARSSVCRRKVAQAREKRFTFVLAVVMGVFVLCWFPFFFI   400

                                 7
    398  YSLYGICREACQLPEPLFKFFFWIGYCNSSLNPVIYTVFNQDFRRSFKHI   447
         YSLYGICREACQVPGPLFKFFFWIGYCNSSLNPVIYTVFNQDFRPSFKHI   450

    448  LFRRRRRGFRQ   458
         LFRRRRRGFRQ   461
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Fig. 2. Comparison of predicted amino acid sequences for the rat A2D clone and human α_2-C4 cDNA. Straight lines indicate conserved amino acid changes, while dots indicate non-conserved residues. Amino acid omissions are shown by a period. The putative transmembrane regions are delineated by a heavy line and are numbered from 1 to 7.

An open reading frame of 1374 bp was present which encoded a 458 amino acid protein with a predicted molecular mass of 48 900 Da. The sequence surrounding the initiating methionine codon fits the Kozak consensus sequence [14]. During the sequencing of this clone a human kidney cDNA encoding an α_2-adrenergic receptor, the α_2-C4, was reported [7]. There is a high degree of identity, 88% at the predicted amino acid level, between clone λgA2d and the human kidney α_2-adrenergic receptor (Fig. 2). The few non-conservative amino acid substitutions are present in regions of the protein thought not to be involved with either ligand binding or effector coupling, such as the amino-terminus region and the middle of the third intracellular loop [15]. This suggests that λgA2d encodes the rat homologue of the human kidney α_2-C4 adrenergic receptor. The sequence of the longest brain cDNA clone obtained, λcA2d, was co-linear with that of the gene, suggesting that this portion of the gene is intronless. However, the presence of an intron in the extreme 5′-untranslated region cannot be excluded. The human α_2-C10 adrenergic receptor gene has also been reported to the intronless [6].

Tissue-specific expression of the rat α_2-C4 adrenergic receptor gene is shown in Fig. 3. Two mRNAs, 2.9 and 2.4 kb, encoding this receptor were found to be most abundant in the brain, with lower levels in kidney and heart and no detectable signal present in liver, lung or pancreas. The weak 4.5 kb band seen in lung was most likely due to non-specific hybridization to residual 28 S rRNA present in the sample. The low signal in heart could be due to gene expression in the coronary artery endothelium, which has been shown to exhibit α_2 receptor-mediated responses [16]. As this gene does not

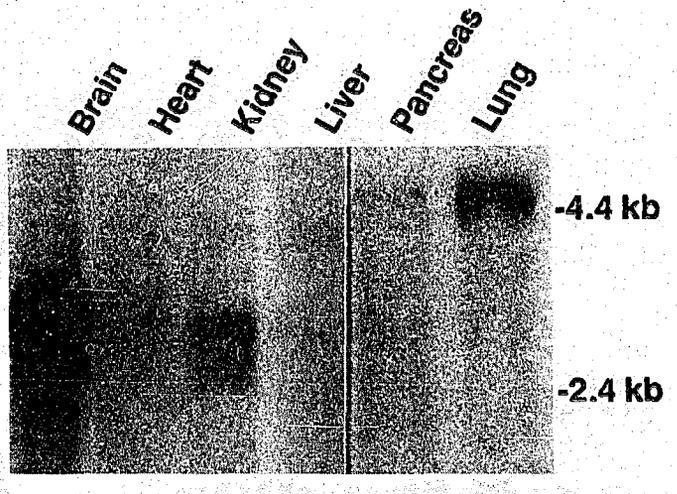

Fig. 3. RNA blot analysis of rat α_2-C4 adrenergic receptor expression in various rat tissues. Northern blots were prepared and probed as described in section 2. Each lane contained 5 μg of poly(A)$^+$ RNA from the identified tissue. Positions of co-electrophoresed RNA size markers (BRL) are shown.

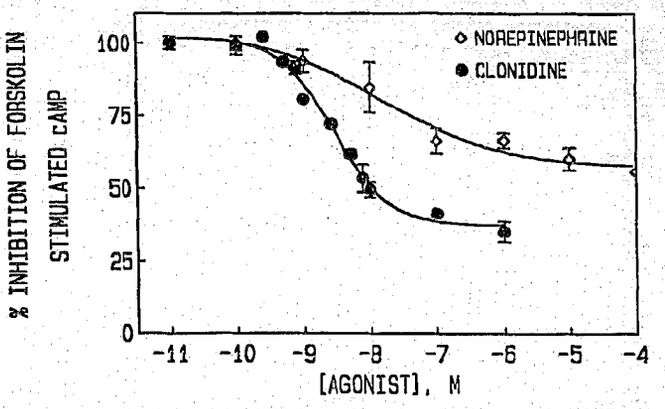

Fig. 4. Agonist-induced inhibition of forskolin-stimulated cAMP accumulation in CHO cells. Clonidine and norepinephrine inhibited forskolin (500 nM)-stimulated cAMP accumulation in CHO cells stably expressing the pA2D clone. IC_{50} for clonidine, 2.8 ± 0.15 nM, for norepinephrine, 12.5 ± 0.9 nM, $n = 3$. Data are the mean ± SE of triplicate determinations and are representative of at least 3 experiments performed in triplicate.

appear to be expressed in such highly vascular organs as the lung and liver, it suggests that a different α_2-adrenergic receptor subtype gene is expressed in these tissues. In order to see the signal present in heart and kidney it was necessary to expose the blot to X-ray film for a length of time that resulted in overexposure of the brain mRNA lane. The 2.9 kb signal appears to correspond to the cDNA clone isolated. Both the 2.9 and 2.4 kb species were detected after very stringent washing conditions (0.5 × SSC at 90°C). These findings suggest that at least two size classes of transcripts are produced from this gene. Based on 3′-genomic sequence, this does not appear to be due to alternative polyadenylation. It is not known whether the two species are products of alternative splicing at the 5′-end of the transcript or of alternative promotor usage.

In effector-coupling studies of the expressed rat α_2-C4 receptor cDNA (Fig. 4), the agonists norepinephrine and clonidine inhibited forskolin-stimulated cAMP accumulation, with clonidine being both more potent and more efficacious than norepinephrine. Both agonists could also inhibit prostaglandin E_2-stimulated cAMP accumulation (data not shown). These results are in direct contrast to those found for the α_2B adrenergic receptors on NG-108 cells (personal observation and [17]) and those reported for the α_2C adrenergic receptor on OK-1 cells [18]: in both cases clonidine was found to have little or no efficacy or potency at these receptors. Clonidine has, however, been reported to have agonist activity at the human α_2A (α_2-C10) receptor. Radioligand binding studies on the rat α_2-C4 cDNA (Fig. 5A,B) demonstrated conclusively that this receptor does not belong in the α_2A adrenergic receptor subtype category, as prazosin has a higher affinity than oxymetazoline. The calculated K_i values of various antagonists for the rat receptor clone are shown in Table

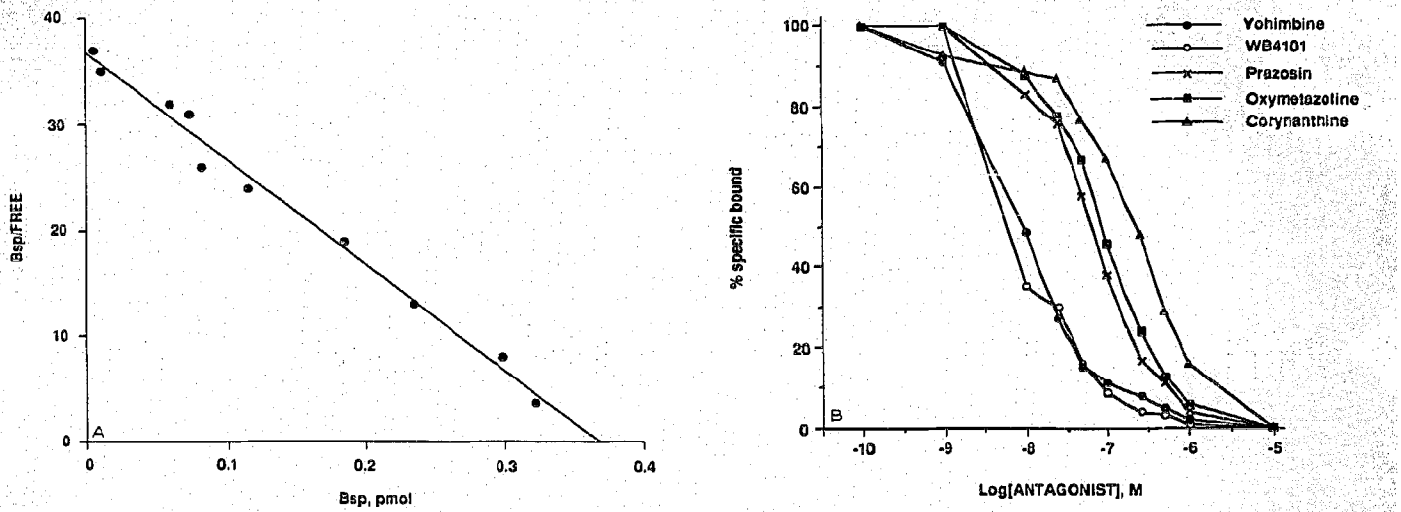

Fig. 5. [^{3}H]Rauwolscine binding to membrane homogenates from COS-7 cells expressing the pA2d clone. (A) Saturation analysis was carried out using [^{3}H]rauwolscine as described in section 2. The concentrations of labeled ligand used spanned the range from 0.05 nM to 10 nM, with non-specific binding defined using 100 nM yohimbine. The data from a typical experiment were converted into a Rosenthal plot and are depicted here. Data are representative of 3 separate experiments, with each point in triplicate. (B) Competition curves of various ligands for the receptor from a typical experiment are depicted here. Binding was carried out as described in section 2, using 0.5 nM [^{3}H]rauwolscine per assay tube. This experiment is representative of at least 3 experiments per drug, with each point being performed in triplicate.

I. To help in comparing α_2-adrenergic receptor subtype pharmacology, values from the literature [13,18] for the α_2A, α_2B, and α_2C, obtained using the same binding buffer as this study, are also shown. The rat receptor clone exhibits a pharmacological profile similar to that of the α_2B and α_2C adrenergic subtypes, due to the high affinity of prazosin and low affinity of oxymetazoline. Taken together, the combination of effector-coupling and radioligand binding data suggests strongly that the receptor encoded by the cloned rat α_2-C4 homologue defines a new pharmacological subtype of adrenergic receptor, one of which prazosin has high affinity and clonidine strong efficacy.

The findings presented in this report underscore the inherent difficulties of performing radioligand binding in a complex tissue such as brain. Thus, while past studies have used prazosin to unmask the presence of 'α_2B' receptors in brain, the results from this paper suggest that at least a portion of these 'α_2B' receptors are in fact receptor-encoded by the α_2-C4 gene. Further studies into the cell-type expression and coupling mechanisms for this receptor class are needed in order to shed more light on its functional significance in neural transmission.

Table I

Comparison of K_i values, in nM, of various ligands for the four putative α_2 receptor subtypes

	α_2A	α_2B	α_2C	rat α_2-C4
yohimbine	1.0	0.7	0.2	1.5
prazosin	270	3.7-5.4	7.5	20
WB-4101	0.8	6.4	0.3	1.6
chlorpromazine	396	20	26	28
corynanthine	91-144	70	28	81
oxymetazoline	0.8	40	10	34

Values for the α_2A, α_2B and α_2C were obtained from [13,18], and those of the rat α_2-C4 were calculated from the curves shown in Fig. 4B using the Cheng-Prusoff equation [19]. The results for the rat α_2-C4 represent the means of at least 3 separate experiments.

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