Cloning and expression of a fish α_2 -adrenoceptor

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1 Pigment granule aggregation in specialized cells (melanophores) from the skin of teleost fishes has been shown to be mediated by receptors with an α_2 -adrenoceptor pharmacology. We now report the cloning of the α_2 -F, a fish skin α_2 -receptor from the cuckoo wrasse (*Labrus ossifagus*).

2 Degenerate oligonucleotides corresponding to conserved regions of the human α_2 -adrenoceptor subtypes were used in a polymerase chain reaction (PCR) with cDNA prepared from mRNA isolated from the skin of the cuckoo wrasse. An 876 base pair (bp) product was obtained that was homologous with that of the human α_2 -adrenoceptor and was used to screen a genomic library from the cuckoo wrasse.

3 A clone (pTB17BS) consisting of ~5 kb of genomic DNA was obtained which contained the nucleotide sequence of the initial PCR product. In addition, it contained an open reading frame that encoded a protein of 432 amino acids and ~2 kb of 5'-untranslated sequence. The deduced amino acid sequence of this protein showed 47–57% identity with the human α_2 -adrenoceptors and thus appeared to encode a fish α_2 -adrenoceptor.

4 In the 5'-untranslated region of the gene, nucleotide sequences were present suggesting that transcription of the α_2 -F might be regulated by cyclic AMP, calcium and/or steroids.

5 The α_2 -F was expressed in COS-7 cells and radioligand binding studies were performed with [³H]-rauwolscine. The binding was of high affinity and it was saturable with a K_D of 0.8 ± 0.1 nM and a B_{max} of 5.7 ± 1.0 pmol mg⁻¹ of protein.

6 Competition curves for the displacement of specific [³H]-rauwolscine binding showed the following order of potency: for agonists, medetomidine > clonidine > *p*-aminoclonidine > B-HT 920 > (–)-nora-drenaline; for antagonists, rauwolscine > atipamezole > yohimbine > phentolamine > prazosin.

7 These results show that α_2 -F has characteristics of both the human α_2 -C10 and α_2 -C4 and that it might represent an ancestral α_2 -adrenoceptor subtype.

Keywords: Adrenoceptor; G-protein coupled receptor; melanophore; yohimbine; *Labrus ossifagus*

Introduction

Mammalian α_2 -adrenoceptors belong to the growing family of G-protein-coupled receptors (Regan & Cotecchia, 1992). The receptor proteins within this family consist of a single polypeptide chain that is postulated to span the cell membrane seven times (Dohlman *et al.*, 1991). To date, three α_2 -adrenoceptor subtypes have been identified which mediate a variety of tissue-specific responses including; presynaptic inhibition of neurotransmitter release, smooth muscle contraction, inhibition of lipolysis, inhibition of insulin release and platelet aggregation (Nichols & Ruffolo, 1991). In the skin of many lower vertebrates, such as frogs and fish, receptors with an α_2 -adrenoceptor pharmacology, have been shown to mediate pigment granule aggregation in specialized cells called melanophores (Berthelsen & Pettinger, 1977; Andersson *et al.*, 1984). The aggregation, or dispersion, of these granules is responsible for colour changes in these animals. The fact that these changes can be easily evaluated with a photometer or light microscope have made the melanophores from fish skin an ideal model system for functional studies of α_2 -adrenoceptors (Karlsson *et al.*, 1989; Svensson *et al.*, 1991a). Although similar in some respects to its mammalian counterparts, the fish melanophore α_2 -adrenoceptor has a unique pharmacology. For example, the imidazoline, UK 14304, which is an agonist at mammalian α_2 -adrenoceptors, is an antagonist at the α_2 -adrenoceptors present in fish melanophores (Karlsson *et al.*, 1989).

We were, therefore, interested in cloning and expressing DNA encoding the fish melanophore α_2 -adrenoceptor in order to correlate functional properties of the receptor with

its protein structure. Using mRNA isolated from the skin of the cuckoo wrasse, a PCR product was obtained that appeared to encode an α_2 -adrenoceptor. This was used to screen a genomic library and a gene was isolated which yielded a complete sequence for the α_2 -F. This is the first nonmammalian α_2 -adrenoceptor to be cloned and it shows some interesting structural and pharmacological characteristics with respect to the human α_2 -adrenoceptor subtypes.

Methods

Reverse transcriptase/polymerase chain reaction

mRNA was isolated from the skin of the cuckoo wrasse (*Labrus ossifagus*) with the Fast Track Kit (Invitrogen). cDNA was synthesized from 100 ng of mRNA in a 20 μ l reaction containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatin, 40 u RNasin inhibitor (Invitrogen), 0.5 mM dNTP's (Pharmacia), 2 μ g random primers (Invitrogen) and 15 u of AMV reverse transcriptase (United States Biochemical). The reaction mix was incubated for 10 min at room temperature, then 60 min at 42°C. It was stopped by heating at 95°C for 5 min.

Oligonucleotide primers were prepared corresponding to the 3rd (primer A) and 7th (primer B) transmembrane domains of the human α_2 -adrenoceptors and included a BamHI site (primer A) and a SacII site (primer B) to facilitate cloning. The sequences of the primers are shown below: primers A and B were 16 fold and 4 fold degenerate, respectively.

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primer A
 5'CGTCC ATCGG GATCC TGTGC GCCAT C^T/_A^C/CC
 TGGAC^C/_AG^G/_CTA3'
 primer B
 5'GTAGA TCCGC GGGTT GAGCG AGCTG TTGCA
 GTAGC CGA^T/_C^A/_G CCA3'

To the reverse transcriptase reaction, a PCR-mixture containing 100 pmol of primer A, 50 pmol of primer B, 5% dimethylsulphoxide (DMSO) and 1.25 u of AmpliTaq DNA polymerase (Perkin Elmer/Cetus) was added. Sixty cycles of PCR were performed using the following temperature profile: 95°C, 1 min; 50°C, 2 min; 72°C, 3 min. An 876 bp product was obtained and was named CW1. CW1 was cloned into pBluescript SK⁺ (Stratagene) and the nucleotide sequence of both strands was determined by primer extension and dideoxynucleotide sequencing with Sequenase (United States Biochemical; Sanger *et al.*, 1977).

Southern blot analysis

Genomic DNA was extracted from cuckoo wrasse brains according to the method of Ausubel *et al.* (1988); 10 μ g was digested with either EcoRI, HindIII or PstI. The DNA fragments were separated on 1% agarose gels and were blotted on to nitrocellulose filters (Sambrook *et al.*, 1989). The PCR product, CW1, was labelled with ³²P by the method of nick translation (Gibco/BRL) and used as a probe. Hybridization was performed overnight at 40°C in 50% formamide, 25 mM KPO₄, 5 $\times$ SSC and 0.25% nonfat powdered milk. Final washing conditions were for 30 min at 55°C in 2XSSC/0.1% SDS. Autoradiographs were obtained using Kodak XAR film and overnight exposures at -70°C.

Construction and screening of a size-selected fish genomic library

EcoRI-digested genomic DNA was fractionated by centrifugation at 20°C for 24 h at 25,000 r.p.m. (SW28 rotor) over a 10–40% sucrose-gradient and DNA fragments ranging in size from 14–18 kb were pooled. These were used to prepare a library in the vector Lambda Dash II (Stratagene), according to the manufacturer's instructions. Approximately 1.4×10^6 plaque-forming-units were obtained of which 80% had inserts; without amplification, 400,000 were used to infect SRB-P2 cells at a titer of 20,000 plaques/plate. Nitrocellulose lifts were taken and were screened using the nick-translated ³²P-CW1 as a probe (see above). Pre-hybridization was done at 37°C for 2 h in 70 ml of 50% formamide, 1% SDS, 1 M NaCl, and 100 μ g ml⁻¹ herring sperm DNA. The probe was added ($\sim 5 \times 10^6$ d.p.m. ml⁻¹) and hybridized overnight at 37°C. The filters were washed at 53°C for 90 min in 2 $\times$ SSC/0.1% SDS and overnight exposures were made at -70°C using Kodak XAR film and cassettes containing intensifying screens. A positive signal was found and the responsible clone, TB17, was isolated following 2 additional rounds of plating and screening. TB17 was amplified in plate lysates and DNA was prepared using LambdaSorb (Promega). A 16.5 kb insert was present which hybridized with ³²P-CW1 in Southern blot analysis.

Construction of a full-length clone encoding α_2 -F

Using Southern blot analysis, a 3 kb BamHI fragment from TB17 was identified which hybridized with ³²P-CW1. It was subcloned into pBluescript SK⁺ to yield pTB17B and was sequenced. The sequence of CW1 was found within an open reading frame of 1293 bases, but the BamHI fragment began with this open reading frame, presenting the possibility that the coding sequence was short. A BamHI/SacII fragment of pTB17B, which contained 227 bases of the 5'-end of the open reading frame, was used as a probe in a Southern blot of SacII digested TB17. A 2 kb SacII fragment was identified and cloned into pBluescript. The 2 kb SacII fragment was

then cloned into SacII-digested pTB17B to yield pTB17BS. [SacII cleaves pTB17B twice: once in the multiple cloning site, just upstream of the BamHI site, and again 227 bases downstream of the BamHI site.] In pTB17BS the open reading frame was extended an additional 57 bases beyond the BamHI site; however, the first methionine in this 1350 base open reading frame was only 3 bases upstream of the BamHI site. The length of open reading frame, therefore, starting with the first ATG (methionine) is 1296 nucleotides.

Construction of a eukaryotic expression vector encoding α_2 -F

pTB17BS was used as a template in a PCR reaction with a sense primer containing adjacent SacII and DraI restriction sites (5'CCGCGGTTTAA . . .), and corresponding to nucleotides -136 to -119 of α_2 -F (Figure 2), and an antisense primer corresponding to nucleotides 401–423. A 571 bp product was obtained which was cleaved with SacII yielding a 374 bp fragment that was isolated and cloned into SacII digested pTB17BS. This created pA2F which could be cleaved with DraI to yield a 1.5 kb blunt-ended fragment containing the full coding sequence of α_2 -F along with 136 bases of 5'-untranslated sequence and 68 bases of 3'-untranslated sequence. The eukaryotic expression vector, pBC12BI (Cullen, 1987), was digested with HindIII and BamHI, blunt-ended with Klenow, and ligated with the 1.5 kb DraI fragment to give pBCA2F.

Expression and radioligand binding studies

pBCA2F was expressed transiently in COS-7 cells and the binding [³H]-rauwolscine (77.9 Ci mmol⁻¹) to membranes was examined as previously described (Regan *et al.*, 1988). For saturation curve analysis, nonspecific binding was defined with 100 μ M phentolamine. For competition curves analysis, a final concentration of 1 nM [³H]-rauwolscine was used. Data were analysed by computer using the logistic function described by Parker & Waud (1971). K_i values were calculated by the Cheng-Prusoff conversion. Protein concentrations were determined by Bradford assay (Biorad).

Drugs

Drugs were obtained as follows: atipamezole and medetomidine were gifts from the Orion Corp./Farmos (Turku, Finland); yohimbine, prazosin, clonidine, (-)-noradrenaline, methoxamine and *p*-aminoclonidine were from Sigma Chemicals (St. Louis, MO, U.S.A.); SKF 104078 (6-chloro-9-[3-methyl-2-butenyl]oxy]-3-methyl-1H2,3,4,5-tetrahydro-3-benzopine) was a gift from Smith Kline & French (Hearts, UK); phentolamine and guanfacine were gifts from CIBA-Geigy (Basel, Switzerland); UK 14304 (5-bromo-6-N-(2,4,5-dihydroimidazolyl) quinoxaline) was a gift from Pfizer Central Research (Sandwich, UK); idazoxan was a gift from Reckitt & Colman (Kingston-upon-Hull, UK); B-HT 920 (2-amino-6 allyl-5,6,7,8-tetrahydro-4H-thiazolo-[4,5-d]-azepine dihydrochloride) was a gift from Dr Thomae (Biberach, Germany); oxymetazoline was a gift from Astra Draco (Lund, Sweden); rauwolscine was from Carl Roth (Karlsruhe, Germany); [³H]-rauwolscine was from New England Nuclear/Dupont (Boston, MA, U.S.A.).

Results

Cloning and sequence of α_2 -F

mRNA was isolated from the skin of the cuckoo wrasse and was used as a template in a polymerase chain reaction (PCR) with degenerate primers from conserved regions of the human α_2 -adrenoceptors. An 876 base pair (bp) product was obtained from a set of primers that corresponded to the third and seventh membrane domains of the human α_2 -adreno-

Figure 1 Southern blot of cuckoo wrasse (*Labrus ossifagus*) genomic DNA, digested with the indicated restriction enzymes and probed with 32 P-CW1, a PCR-product generated from fish skin mRNA and degenerate primers designed from the mammalian α_2 -adrenoceptors. Restriction enzyme digests and blotting conditions were as described in Methods. The positions of molecular size standards are indicated on the left.

ceptors. When sequenced, this PCR product was found to have similarity with the human α_2 -adrenoceptors and it could be aligned with them.

Difficulties with the preparation of skin cDNA libraries led to the screening of a genomic library in order to obtain the full coding sequence of this putative fish α_2 -AR. Prior to this, genomic DNA from the cuckoo wrasse was used in a Southern blot with the 32 P-labelled PCR product as a probe (Figure 1). Under conditions of moderate stringency (55°C, $2 \times$ SSC) only a single band was present in DNA that had been cleaved with either EcoRI, HindIII or PstI. EcoRI-digested genomic DNA was fractionated by sucrose density gradient centrifugation and DNA in the size range of 14–18 kb was used to make a library in lambda phage. The library was screened with the 32 P-labelled PCR product and a phage was cloned with a 16.5 kb genomic insert that hybridized with the probe.

Restriction enzyme mapping, Southern blotting and subcloning resulted in the construction of a plasmid (pTB17BS) that contained a 1350 base open reading frame (ORF) and approximately 2 kb of additional sequence both 5' and 3' to the ORF. Figure 2 shows the nucleotide sequence for 2898 bases of this clone and the deduced amino acid sequence for 1296 bases of the ORF. The deduced amino acid sequence codes for a protein of 432 amino acids the beginning of which, was defined as the first ATG (methionine) in the ORF and by the presence of an optimal consensus sequence for the initiation of translation (Kozak, 1980). Within this open reading frame was the sequence of the PCR product that had been obtained from the skin mRNA (nt. 363–1218). The identity of the complete deduced amino acid sequence with the sequences of the human α_2 -adrenoceptor subtypes was as

follows: α_2 -C4, 57%; α_2 -C10, 50%; and α_2 -C2, 47%. We named this intronless gene from fish as the α_2 -F.

Hydropathy analysis (Kyte & Doolittle, 1982) of the deduced amino acid sequence of the α_2 -F indicated seven domains of hydrophobic amino acids, each separated by loops of hydrophilic residues. These domains are thought to span the cell membrane and give the receptor a topological structure similar to rhodopsin (Dohlman *et al.*, 1991). This model as applied to the α_2 -F is shown in Figure 3. The amino acid sequence identity of the α_2 -F compared with other G-protein coupled receptors was highest in these putative transmembrane domains. Amino acid identities in the transmembrane domains between α_2 -F and other adrenoceptor receptors are as follows: α_2 -C4, 82%, α_2 -C10, 77%; α_2 -C2, 78%, α_1 B, 43%, α_1 C, 41%; β_1 , 41%; β_2 , 38%; β_3 , 41%, D_2 , 49%. In the third intracellular loop, which represents the most divergent part of the α_2 -F as compared with its mammalian counterparts, serines and threonines were found that may represent sites for regulatory phosphorylation by protein kinases such as protein kinase A and β -adrenoceptor kinase (Benovic *et al.*, 1989). As in two of the mammalian α_2 -adrenoceptor subtypes, potential sites for N-linked glycosylation (Asn-X-Ser or Asn-X-Thr) were found in the amino terminus.

A variety of nucleotide consensus sequences have been identified that are involved in the transcriptional regulation of eukaryotic genes (Maniatis *et al.*, 1987). In the 5'-untranslated sequence of the α_2 -F, two TATA-like sequences are present at nt. -21 and -157 which could represent part of the promoter for RNA polymerase. Consensus sequences for other transcription factors that could potentially regulate the expression of the α_2 -F were also found in the 5' untranslated region. As listed in Table 1, these included activation protein-2 (AP-2, Mitchell *et al.*, 1987), adenosine 3':5'-cyclic monophosphate (cyclic AMP) response element (CRE, Montminy *et al.*, 1990), steroid hormone receptor binding site (SRE, Beato, 1986) and a CAAT-box.

Expression

The ligand recognition properties of the α_2 -F were determined in membranes prepared from COS-7 cells that had been transiently transfected with pBCA2F, a plasmid derived from the eukaryotic expression plasmid, pBC12BI and the coding region of the α_2 -F (see Methods). In membranes prepared from the transfected COS-7 cells, [3 H]-rauwolscine bound in a specific and saturable manner (Figure 4). The binding of [3 H]-rauwolscine was linear with protein concentration and reached equilibrium after 20 min (data not shown). The affinity of [3 H]-rauwolscine for the α_2 -F, as determined by Scatchard analysis of saturation binding data, was 0.8 ± 0.1 nM (K_D) with a B_{max} of 5.7 ± 1.0 pmol mg^{-1} protein. Competition binding studies were used to characterize the pharmacology of the α_2 -F (Table 2). The potency series for putative agonists was as follows: medetomidine > clonidine > B-HT 920 > guanfacine > UK14304 > (-)-noradrenaline >> isoprenaline and methoxamine. For putative antagonists, the potency series was: rauwolscine > atipamezole > yohimbine > phentolamine > prazosin. The α_2 -F, indeed, has the pharmacological characteristics of an α_2 -adrenoceptor.

Table 1 Potential upstream promoter elements in the gene encoding the α_2 -F^a

Promoter	Sequence ^b	Location ^c	Activator
AP-2	CCCCACAC	- 758/- 749	Cyclic AMP/ Ca^{2+}
CRE	AAAGGTCA	- 1040/- 1033	Cyclic AMP
SRE	GGTCT---TGAGC	- 871/- 859	Steroids
CAAT-box	CAAT	- 219/- 216	

^aAbbreviations: AP-2, activation protein 2; CRE, cyclic AMP response element; SRE, steroid hormone response element.

^bNucleotide sequence in the α_2 -F, nonhomologous bases are shown in small caps.

^cAccording to the nucleotide sequence shown in Figure 2.

[illegible]

Figure 2 Nucleotide and deduced amino acid sequence of the fish pigment cell α_2 -adrenoceptor (α_2 -F). Consensus sequences for potential regulators of transcription (Table 1) are indicated by the single underline. Probable transmembrane domains are indicated by the double underlines. Three restriction sites that were used during the cloning procedures are indicated in bold-face type (BamHI, nt. 3–8; SacII, nt. 227–232; DraI, nt. 1361–1366).

Discussion

The α_2 -F, an adrenoceptor from the skin of the cuckoo wrasse, has been cloned and heterologous expression in a mammalian cell line has shown a unique pharmacology but one that is characteristic of the α_2 -adrenoceptor family. The α_2 -F appears to be that receptor in fish skin which controls

the aggregation of pigment granules in melanophores. The melanophores of frog skin were one of the first cell types where functional postsynaptic α_2 -adrenoceptors were identified (Berthelsen & Pettinger, 1977). Recent work has also identified the presence of postsynaptic α_2 -adrenoceptors on the melanophores from several species of teleost fishes (Andersson *et al.*, 1984; Svensson *et al.*, 1989). In functional

Table 2 Binding affinities of adrenoceptor ligands at the α_2 -F^a

Ligand	K _i (nM)
Rauwolscine	0.3 ± 0.05
Atipamezole	0.6 ± 0.1
Yohimbine	1.1 ± 0.2
SKF 104078	24 ± 4
Idazoxan	45 ± 4
Phentolamine	104 ± 12
Prazosin	469 ± 79
UK 14304	1612 ± 218
Medetomidine	46 ± 2
Oxymetazoline	49 ± 12
Clonidine	83 ± 11
<i>p</i> -Aminoclonidine	92 ± 17
B-HT 920	209 ± 13
Guanfacine ^b	1404 ± 149
Noradrenaline	2849 ± 286
Amiloride	23000 ± 3400
Isoprenaline	67468 ± 10417
Methoxamine	87440 ± 23096

^aAs determined in radioligand binding studies using [³H]-rauwolscine (1 nM) and membranes prepared from COS-7 cells transfected with the α_2 -F. Data are the means ± s.e.mean for 3 experiments.

^b*n* = 2.

Table 3 K_i ratios for prazosin/oxymetazoline and prazosin/yohimbine^a

Subtypes	Prazosin/ Oxymetazoline	Prazosin/ Yohimbine
α_2 A	164	1125
α_2 B	0.19	31
α_2 C	0.66	44
α_2 -F	10	426

^aData for the α_2 -F are derived from Table 2. Data for the human α_2 A (α_2 -C10), α_2 B (α_2 -C2) and α_2 C (α_2 -C4) are from Harrison *et al.* (1991).

Figure 4 Saturation curve (a) and Scatchard plot (b) for the binding of [³H]-rauwolscine to membranes prepared from COS-7 cells transiently transfected with the α_2 -F. A representative experiment is shown, conducted as described in Methods. Total binding (□), nonspecific (●) and specific (■) binding. **Figure 3** Seven transmembrane domain model for the deduced amino acid sequence of the α_2 -F. The circles represent individual amino acids (single letter code). Amino acids that are conserved between the α_2 -F and the human α_2 -C4 are shown in black. Sites for potential N-linked glycosylation are indicated with arrows.

studies, the melanophore α_2 -adrenoceptor is pharmacologically similar to its mammalian counterparts; however, UK 14304, an imidazoline which is a full agonist at mammalian α_2 -adrenoceptors, is an antagonist at the α_2 -adrenoceptors on melanophores (Karlsson *et al.*, 1989). It was of interest, therefore, to determine the primary sequence of this receptor because differences between it and the mammalian α_2 -adrenoceptors might be revealing with respect to what it takes to activate an α_2 -adrenoceptor.

Molecular cloning has identified 3 mammalian genes encoding α_2 -adrenoceptor subtypes in both man (α_2 -C2, α_2 -C4, α_2 -C10) and rats (Kobilka *et al.*, 1987; Regan *et al.*, 1988; Lomasney *et al.*, 1991; Weinshank *et al.*, 1990; Zeng *et al.*, 1990; Chalberg *et al.*, 1990; Lanier *et al.*, 1991). Based largely on radioligand binding studies, Bylund (1988) has also defined 3 α_2 -adrenoceptor subtypes (α_2 A, α_2 B, α_2 C). Biochemical, molecular biological and pharmacological studies have correlated the α_2 -C10 with the α_2 -A, the α_2 -C2 with the α_2 B and the α_2 -C4 with the α_2 C (Lanier *et al.*, 1988; Lorenz *et al.*, 1990; Harrison *et al.*, 1991; Regan & Cotecchia, 1992; Bylund *et al.*, 1992).

The deduced amino acid sequence of the α_2 F shows similarity with its mammalian counterparts. Overall sequence identity suggests that α_2 -F is most closely related to the α_2 -C4 (57% for the α_2 -C4 versus 50% for the α_2 -C10 and 47% for the α_2 -C2). This level of identity is significantly less than has been observed between α_2 -adrenoceptor subtypes from more closely related species. For example, there is 88% overall identity between the human α_2 -C4 and its equivalent in the rat. With respect to various regions of the mammalian α_2 -adrenoceptors, the greatest identity with the α_2 -F is in the membrane spanning domains where ligand binding is thought to occur (Dohlman *et al.*, 1991).

Several amino acids that are highly conserved in the adrenoceptor family, and which are thought to be part of the ligand binding site, are also found in the α_2 -F. For example, present in the α_2 -F are 2 serines in the fifth transmembrane domain and an aspartic acid in the third transmembrane domain which may interact with noradrenaline by way of its catechol hydroxyl groups and amino nitrogen, respectively (Dixon *et al.*, 1988). An amino acid that is not conserved in the α_2 -F, as compared with the mammalian α_2 -adrenoceptors, is a phenylalanine in the seventh membrane spanning domain. This residue which corresponds to F⁴¹² in the human α_2 -C10 appears to be deleted in the α_2 -F (if present, it would be between F³⁹⁵ and W³⁹⁶). Interestingly, F⁴¹² has been shown to be critical in dictating antagonist binding specificity (Suryanarayana *et al.*, 1991). Mutation of F⁴¹² from phenylalanine to asparagine results in a receptor with low affinity for the α_2 -selective antagonist, yohimbine, and high affinity for the β -selective antagonist, alprenolol. In the human β_2 -adrenoceptor, an asparagine is present at the equivalent position (N³¹²). Whether the absence of this residue in the α_2 -F can explain any of the unusual aspects of its pharmacology remains to be tested.

The transiently expressed α_2 -F displayed high affinity and saturable binding with [³H]-rauwolscine as a radioligand. To characterize further the pharmacological profile of the α_2 -F, the potency of a variety of adrenoceptor ligands was examined. The α_2 -F behaved like an α_2 -adrenoceptor. Using regression analyses, the data for the compounds listed in Table 2 showed the best correlation with the human α_2 -C4 subtype, perhaps reflecting the greater structural identity of the α_2 -F with the α_2 -C4. Using the analysis as suggested by Harrison *et al.* (1991), however, somewhat different results were obtained. In this analysis, the ratios of prazosin to oxymetazoline and prazosin to yohimbine are compared: Table 3 lists these ratios. Using the prazosin to oxymetazoline ratio, it is hard to find any good correlation, but using the prazosin to yohimbine ratio, α_2 -F appears more like the α_2 -C10. This pharmacology, with characteristics of both the α_2 -C4 and α_2 -C10, suggests that α_2 -F is not a simple homologue of the

human α_2 -C4 and that it might represent a common ancestor. Obviously, more work will be needed to answer this question.

Another question concerns whether or not the α_2 -F represents the α_2 -receptor controlling the pigment granule aggregation in melanophores. Although the PCR product used to clone the α_2 -F was obtained from fish skin, and its sequence was present in the clone, we cannot at this time unequivocally prove that it is the melanophore α_2 -adrenoceptor. This is a consequence of the possibility of more than one α_2 -receptor being present in the skin. Several lines of evidence suggest, however, that it is the fish melanophore α_2 -adrenoceptor. The first is that when the α_2 -F was used as a radiolabelled probe, only one hybridizing band was observed in a Southern blot of fish genomic DNA (Figure 1). With similar hybridization and wash conditions, experiments done with a human α_2 -C10 probe invariably identified the other α_2 -adrenoceptor subtypes in Southern blots of human genomic DNA (Kobilka *et al.*, 1987; Regan & Cotecchia, 1992). A second line of evidence is that the pharmacology of the α_2 -F and the melanophore α_2 -adrenoceptor are very similar. For example, both receptors are rather insensitive to prazosin (Table 2; Svensson *et al.*, 1993). Additionally, in melanophores Schild plot analysis gave a pA₂ of 6 for UK 14304 which is in good agreement with the K_i obtained for UK 14304 in binding studies with the expressed α_2 -F (1.6 μ M).

In studies of the functional activity of fish melanophore α_2 -adrenoceptor, scales containing the pigment cells are removed from the skin and are maintained in tissue culture. Because the scales are innervated, the process of removing them results in degeneration of the intrinsic sympathetic nerve endings going to the melanophores. With time, there develops a supersensitivity of the α_2 -adrenoceptors for noradrenaline which is probably related to the loss of nerve endings (Karlsson *et al.*, 1988; Svensson *et al.*, 1991b).

The molecular mechanism of this denervation supersensitivity is unknown but it could involve increased expression of the α_2 -adrenoceptor. It is interesting that there is a consensus sequence for a cyclic AMP response element (CRE) in the 5'-untranslated region of the α_2 -F (Table 1). Stimulation of the melanophore α_2 -adrenoceptor by noradrenaline decreases intracellular cyclic AMP and causes pigment granule aggregation (Andersson *et al.*, 1984). It is possible, therefore, that loss of a tonic inhibition could raise intracellular cyclic AMP levels and increase receptor expression by increasing transcription. There is precedence for this, the genes for the β_2 -adrenoceptor (Collins *et al.*, 1989) and the α_2 -C10 (Sakaue & Hoffman, 1991) both contain CRE's and seem to be regulated by their second messenger, i.e. cyclic AMP.

Other promoters may also operate to regulate the expression of the α_2 -F. Thus, consensus sequences were found in the 5'-untranslated region of the α_2 -F that could lead to regulation of expression by calcium and/or steroids (Table 1). Understanding the possible role of these promoters may do more than just helping us to understand the fish melanophore α_2 -adrenoceptor: a consensus site for AP-2 and a CRE have also been found in the 5'-untranslated region of the α_2 -C10 (Fraser *et al.*, 1989). It is hoped that future studies on the α_2 -F and its gene will provide us with a better understanding of the function, pharmacology and expression of all the members of the α_2 -adrenoceptor family.

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References

- ANDERSSON, R.G.G., KARLSSON, J.O. & GRUNDSTRÖM, N. (1984). Adrenergic nerves and the α_2 -adrenoceptor system regulating melanosome aggregation within fish melanophores. *Acta Physiol. Scand.*, **121**, 173–179.
- AUSUBEL, F.M., BRENT, R., KINGSTON, R.E., MOORE, D.D., SEIDMAN, J.G., SMITH, J.A. & STRUHL, K. (1988). In *Short Protocols in Molecular Biology*. Harvard Medical School.
- BEATO, M. (1986). Gene regulation by steroid hormones. *Cell*, **56**, 335–344.
- BENOVIC, J.L., DEBLASI, A., STONE, W.C., CARON, M.G. & LEFKOWITZ, R.J. (1989). β -Adrenergic receptor kinase: primary structure delineates a multigene family. *Science*, **246**, 235–240.
- BERTHELSEN, S. & PETTINGER, W.A. (1977). A functional basis for classification of alpha-adrenergic receptors. *Life Sci.*, **21**, 595–606.
- BYLUND, D. (1988). Subtypes of α_2 -adrenoceptors: pharmacological and molecular biological evidence converge. *Trends Pharmacol. Sci.*, **9**, 356–361.
- BYLUND, D.B., BLAXALL, H.S., IVERSEN, L.J., CARON, M.G., LEFKOWITZ, R.J. & LOMASNEY, J.W. (1992). Pharmacological characteristics of α_2 -adrenergic receptors: comparison of pharmacologically defined subtypes with subtypes identified by molecular cloning. *Mol. Pharmacol.*, **42**, 1–5.
- CHALBERG, S.C., DUDA, T., RHINE, J.A. & SHARMA, R.K. (1990). Molecular cloning, sequencing and expression of an alpha-2 adrenergic receptor complementary DNA from rat brain. *Mol. Cell. Biochem.*, **97**, 161–172.
- COLLINS, S., BOUVIER, M., BOLANOWSKI, M.A., CARON, M.G. & LEFKOWITZ, R.J. (1989). cAMP stimulates transcription of the β_2 -adrenergic receptor gene in response to short-term agonist exposure. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 4853–4857.
- CULLEN, B.R. (1987). Use of eukaryotic expression technology in the functional analysis of cloned genes. *Methods Enzymol.*, **152**, 684–704.
- DIXON, R.A.F., SIGAL, I.S. & STRADER, C.D. (1988). Structure-function analysis of the β -adrenergic receptor. *Cold Spring Harbor Symp. Quant. Biol.*, **53**, 487–497.
- DOHLMAN, H.G., THORNER, J., CARON, M.G. & LEFKOWITZ, R.J. (1991). Model system for the study of seven-transmembrane-segment receptors. *Annu. Rev. Biochem.*, **60**, 653–688.
- FRASER, C.M., ARAKAWA, S., MCCOMBIE, W.R. & VENTER, J.C. (1989). Cloning sequence analysis, and permanent expression of a human α_2 -adrenergic receptor. *J. Biol. Chem.*, **264**, 11754–11761.
- HARRISON, J.K., D'ANGELO, D.D., ZENG, D. & LYNCH, K.R. (1991). Pharmacological characterization of rat α_2 -adrenergic receptors. *Mol. Pharmacol.*, **40**, 407–412.
- KARLSSON, J.O.G., ANDERSSON, R.G.G., GRUNDSTRÖM, N. & ANDERSSON, R.G.G. (1988). Pronounced supersensitivity of post-junctional alpha adrenoceptors after denervation of fish melanophores. *J. Pharmacol. Exp. Ther.*, **245**, 345–351.
- KARLSSON, J.O.G., ANDERSSON, R.G.G. & GRUNDSTRÖM, N. (1989). Characterization of pigment aggregating α_2 -adrenoceptors of fish melanophores using different agonists after partial irreversible receptor inactivation. *Br. J. Pharmacol.*, **97**, 222–228.
- KOBILKA, B.K., MATSUI, H., KOBILKA, T.S., YANG-FENG, T.L., FRANCKE, U., CARON, M.G., LEFKOWITZ, R.J. & REGAN, J.W. (1987). Cloning, sequencing and expression of the gene coding for the human platelet α_2 -adrenergic receptor. *Science*, **238**, 650–656.
- KOZAK (1983). Comparison of initiation of protein synthesis in prokaryotes, eukaryotes, and organelles. *Microbiol. Rev.*, **47**, 1–45.
- KYTE, J. & DOOLITTLE, R.F. (1982). A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.*, **157**, 105–132.
- LANIER, S.M., HOMCY, C.J., PATENAUE, C. & GRAHAM, R.M. (1988). Identification of structurally distinct α_2 -adrenergic receptors. *J. Biol. Chem.*, **263**, 14491–14496.
- LANIER, S.M., DOWNING, S., DUZIC, E. & HOMCY, C.J. (1991). Isolation of rat genomic clones encoding subtypes of the α_2 -adrenergic receptor: identification of a unique receptor subtype. *J. Biol. Chem.*, **266**, 10470–10478.
- LOMASNEY, J.W., LORENZ, W., ALLEN, L.F., KING, K., REGAN, J.W., YANG-FENG, T.L., CARON, M.G. & LEFKOWITZ, R.J. (1990). Expansion of the α_2 -adrenergic receptor family: Cloning and characterization of a human α_2 -adrenergic receptor subtype, the gene for which is located on chromosome 2. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 5094–5098.
- LORENZ, W., LOMASNEY, J.W., COLLINS, S., REGAN, J.W., CARON, M.G. & LEFKOWITZ, R.J. (1990). Expression of three α_2 -adrenergic receptor subtypes in rat tissues: Implications for α_2 -receptors classification. *Mol. Pharmacol.*, **38**, 599–603.
- MANIATIS, T., GOODBOURN, S. & FISCHER, J.A. (1987). Regulation of inducible and tissue-specific gene expression. *Science*, **236**, 1237–1245.
- MITCHELL, P.J., WANG, C. & TJIAN, R. (1987). Positive and negative regulation of transcription in vitro: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. *Cell*, **50**, 847–861.
- MONTMINY, M.R., GONZALES, G.A. & YAMAMOTO, K.K. (1990). Regulation of cAMP-inducible genes by CREB. *Trends Neurosci.*, **13**, 184–188.
- NICHOLS, A.J. & RUFFOLO, R.R. (1991). Functions mediated by α -adrenoceptors. In *α -Adrenoceptors: Molecular Biology, Biochemistry and Pharmacology*, ed. Ruffolo, R.R. London: Karger.
- PARKER, R.B. & WAUD, D.R. (1971). Pharmacological estimation of drug receptor dissociation constant. Statistical evaluation. I. Agonists. *J. Pharmacol. Exp. Ther.*, **177**, 1–12.
- REGAN, J.W. & COTECCHIA, S. (1992). The α -adrenergic receptors: New subtypes, pharmacology and coupling mechanism. In *Molecular Biology of G-Protein-Coupled Receptors*, ed. Brann, M.R. pp. 76–112. Boston, MA: Birkhauser Inc.
- REGAN, J.W., KOBILKA, T.S., YANG-FENG, T.L., CARON, M.G., LEFKOWITZ, R.J. & KOBILKA, B.K. (1988). Cloning and expression of a human cDNA for an α_2 -adrenergic receptor subtype. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 6301–6305.
- SAKAUE, M. & HOFFMAN, B.B. (1991). cAMP regulates transcription of the α_2A adrenergic receptor gene in HT-29 cells. *J. Biol. Chem.*, **266**, 5743–5749.
- SAMBROOK, J., FRITSCH, E.F. & MANIATIS, T. (1989). In *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- SANGER, G., NICKLEN, S. & COULSON, A.R. (1977). DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 5463–5467.
- SURYANARAYANA, S., DAUNT, D.A., VON ZASTROW, M. & KOBILKA, B.K. (1991). A point mutation in the seventh hydrophobic domain of the α_2 -adrenergic receptor increases its affinity for a family of β receptor antagonists. *J. Biol. Chem.*, **266**, 15488–15492.
- SVENSSON, S.P., KARLSSON, J.O.G. & GRUNDSTRÖM, N. (1989). Melanophores in isolated scales of *Labrus bergylta* (Ascanius): innervation and alpha-2-adrenoceptor-mediated pigment aggregation. *Comp. Biochem. Physiol.*, **93C**, 247–251.
- SVENSSON, S.P., MÄRTENSSON, L.G.E., GRUNDSTRÖM, N., ANDERSSON, R.G.G., CRAGOE, E.J. & KARLSSON, J.O.G. (1991a). Antagonistic effect of amiloride on alpha-2 adrenoceptor-mediated pigment aggregation: pharmacological heterogeneity between B-HT 920 and noradrenaline. *J. Pharmacol. Exp. Ther.*, **258**, 447–451.
- SVENSSON, S.P., ANDERSSON, R.G.G. & KARLSSON, J.O.G. (1991b). Reciprocal changes in sensitivity to MCH and noradrenaline after denervation of teleost melanophores. *Pigment Cell Res.*, **4**, 252–254.
- WEINSHANK, R.L., ZGOMBICK, J.M., MACCHI, M., ADHAM, N., LICHTBLAU, H., BRANCHEK, T.A. & HARTIG, P.R. (1990). Cloning, expression, and pharmacological characterization of a human α_2B -adrenergic receptor. *Mol. Pharmacol.*, **38**, 681–688.
- ZENG, D.J., HARRISON, J.K., D'ANGELO, D.D., BARBER, C.M., TUCKER, A.L., LU, Z. & LYNCH, K.R. (1990). Molecular characterization of a rat α_2B -adrenergic receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 3102–3106.

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