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Cloning and Expression of the α_{2C}-Adrenergic Receptor from the OK Cell Line

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SUMMARY

The α_2-adrenergic receptors have been divided into four pharmacological subtypes, α_{2A}, α_{2B}, α_{2C}, and α_{2D}. The OK cell line, a cell line derived from an opossum kidney, expresses the α_{2C}-adrenergic receptor and is the prototypical cell line for the α_{2C} receptor subtype. The cloned human α_{2C}-C4 and rat RG10 receptors have been shown to express α_{2C} pharmacology. Here we report the cloning and expression of the OK α_{2C}-adrenergic receptor, OKc2. The receptor has 64% deduced amino acid identity and 21% similarity to the α_2-C4 receptor, giving an overall similarity of 85%. The clone, expressed in Chinese hamster ovary cells, has a pharmacology that correlates very well (r = 0.97) with that of the native OK cell α_{2C}-adrenergic receptor, and it is negatively coupled to adenylyl cyclase.

Four pharmacological subtypes of α_2-adrenergic receptors have been defined and are classified as α_{2A}, α_{2B}, α_{2C}, and α_{2D}. Human platelets and the HT29 human adenocarcinoma cell line are the prototypical tissues for α_{2A} receptors, whereas neonatal rat lung and the NG108 cell line are prototypical tissues for the α_{2B}-adrenergic receptor (1-3). The characteristics of the α_{2A} subtype include high affinity for oxymetazoline and low affinity for prazosin, spiroxatrine, and ARC-239. In contrast, the α_{2B} subtype has low affinity for oxymetazoline and high affinity for prazosin, spiroxatrine, and ARC-239. The α_{2B} subtype is unique in that it is not glycosylated (4).

The α_{2C} receptor was initially characterized in OK cells, a cell line derived from the kidney of the North American opossum, and subsequently in opossum kidney (5, 6). The α_{2C}-adrenergic receptor has a high affinity for rauwolscine, prazosin, spiroxatrine, and ARC-239. In addition to its higher affinity for rauwolscine, the α_{2C} receptor is distinguished from the α_{2B} subtype by its higher affinity for BAM1303 and WB4101.

The α_{2D}-adrenergic receptor was initially characterized in the bovine pineal gland (7). It has low affinity for rauwolscine, SKF104078, and BAM1303. The pharmacology of the α_{2D}-adrenergic receptor in the bovine pineal gland correlated well with the pharmacology previously described for the rat submaxillary gland (8). The gene coding for the human platelet α_{2A}-adrenergic receptor has been cloned and expressed. It is localized to chromosome 10 and thus has been termed α_2-C10 (9). Two other genes coding for human α_2-adrenergic receptors have been cloned and similarly designated by their chromosomal location, i.e., α_2-C2 (10, 11) and α_2-C4 (12). The α_2-C2 receptor expresses the pharmacology of the α_{2B} subtype, whereas the α_2-C4 receptor expresses the pharmacology of the α_{2C} subtype (13).

Orthologous genes for the human α_2-C10 (α_{2A}) receptor have been isolated from pigs (14), rats (15, 16), and mice (17). The deduced amino acid sequences have identities of 93%, 89%, and 92%, respectively, to α_2-C10. The porcine clone expresses the α_{2A} subtype pharmacology. However, the rat and mouse clones express α_{2D} subtype pharmacology. Because Southern blot analyses of human (9) and rat (18) genomic DNA indicate only three α_2-adrenergic receptor subtypes, it may be more useful to refer to these rat and mouse clones and the adrenergic receptor of the bovine pineal gland as $\alpha_{2A/D}$. Recent immunological data also support the close genetic relationship between the pharmacologically defined α_{2A}- and α_{2D}-adrenergic subtypes (19).

The orthologous genes for the human α_2-C2 (α_{2B} subtype) receptor have been cloned from rats (20) and mice (21, 22). These both show amino acid sequence identity of 82% to the human gene. In addition to the human α_2-C4 receptor, the α_{2C} subtype has been cloned from rats (16, 23, 24) and mice (17), with 90% and 89% sequence identities, respectively. We report here the molecular cloning, sequencing, and functional expression of the OK cell α_{2C}-adrenergic receptor.

Materials and Methods

Cloning and DNA sequencing. Total RNA was isolated from OK cells and opossum kidney by a modified guanidinium thiocyanate/

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ABBREVIATIONS: PCR, polymerase chain reaction; RT, reverse transcription; RACE, rapid amplification of cDNA ends; CHO, Chinese hamster ovary; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase(s); 5-HT, 5-hydroxytryptamine.

phenol chloroform method (25). For Northern blot analysis poly(A)$^+$ RNA was isolated using the Fast Track mRNA isolation kit (Invitrogen, La Jolla, CA). Two micrograms of total OK RNA were reverse transcribed using random primers (Promega, Madison, WI) and Superscript reverse transcriptase (Life Technologies, Gaithersburg, MD), to obtain cDNA. This cDNA was amplified by PCR (26) using degenerate oligonucleotides that were designed to the second and seventh transmembrane regions of α_2-C10 and α_2-C4, 5′-GGGAATTCGCCCCCCA-GAACCTGTTCCTGGTG-3′ and 5′-GGACTAGTGTTGCAGTAGC-CGA(T/A)CCAGAAGAA-3′. The oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). The cycling conditions were 25 cycles of 95° for 1 min, 55° for 1 min, and 72° for 3 min. The 1032-base pair product from this RT-PCR was cloned into the *Eco*RI/*Spe*I sites of pBluescript KS(+) for sequencing. The 5′ and 3′ ends of the full length clone were obtained by the RACE technique (27), using 3′ and 5′ RACE systems (Life Technologies). In brief, the 3′ clone was obtained by using the oligo(dT) primer and the oligonucleotide to the second transmembrane region described above and the 5′ clone was obtained by using the anchor primer and a gene-specific primer, 5′-GGGAATTCAGGAGG-TGCAGAAGAGGACGTCTA-3′.

Sequencing was performed both manually and by automated techniques. Single-stranded DNA was sequenced manually by the dideoxy chain termination method (28) using Sequenase 2.0 (United States Biochemical Co., Cleveland, OH). Automatic sequencing was performed on an ABI 373A automatic sequencer (Applied Biosystems, Inc.) with the *Thermus aquaticus* polymerase PCR method, using fluorescently tagged primers or fluorescently labeled dideoxy terminators and Sequenase reactions with fluorescent primers.

To obtain the complete coding region of the opossum α_{2C}-adrenergic receptor, sequence data from 5′ and 3′ RACE clones were used to design oligonucleotides to enable RT-PCR to be performed on OK cDNA, i.e., 5′-GGAAGCTTTGTCAGTGGAAAGAGGAGACG3-′ and 5′-GGACTAGTCCAC(C/G)GGGGTCACTGCAAGGA-3′, respectively. The resulting 1555-base pair product was cloned into the *Hind*III/*Xba*I sites of the eukaryotic expression vector pRc/CMV (Invitrogen, San Diego, CA). The resulting plasmid was sequenced as described above.

RNA analysis. Eight micrograms of poly(A)$^+$ OK RNA were fractionated on a 1% agarose-formaldehyde gel (28) with a 0.24–9.5-kb RNA ladder (Life Technologies) and were transferred to a Magnagraph filter (Micron Separations, Inc., Westborough, MA). The 1032-base pair cDNA was labeled with [^{32}P]dCTP (ICN, Irvine, CA) using a random primer kit (Boehringer-Mannheim, Indianapolis, IN), hybridized to the filter overnight at 65° in 6× SSC, 0.5% SDS, 5× Denhardt's solution, 100 μg/ml sheared herring sperm DNA, washed at 65° in 0.1× SSC, 0.1% SDS, and exposed for 8 hr to Kodak XAR film with an intensifier.

Southern analysis. A 390-base pair PCR probe, which included transmembrane regions 1–4, was generated from α_2-C10 (American Type Culture Collection, Rockville, MD). The RT-PCR product was electrophoresed on a 1% Seakem GTG (FMC, Rockland, ME) agarose gel. The DNA was transferred to a Magnagraph filter (MSI), probed with the ^{32}P-labeled 390-base pair PCR probe, washed at high stringency (0.1× SSC, 0.1% SDS, at 65°), and exposed overnight to Kodak XAR film.

RNase protection assay. A 320-base pair *Sac*I fragment from the 1032-base pair RT-PCR product was subcloned into pBluescript KS(+). This subclone includes the fifth transmembrane region and 250 base pairs of the third cytoplasmic loop. Antisense ^{32}P-labeled RNA was prepared from this clone by utilizing the T7 DNA-dependent RNA polymerase binding site of pBluescript KS(+), using the Maxiscript kit (Ambion, Austin, TX), and was gel purified on an 8 M urea-6% acrylamide gel. OK and opossum kidney RNA was hybridized overnight at 45° to this probe (1×10^5 cpm), digested with RNase A/RNase T1 using a RPA II kit (Ambion), and run on an 8 M urea-6% acrylamide gel with a 0.16–1.77-kb RNA ladder (Life Technologies). The gel was then exposed to Kodak XAR film overnight.

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ATGGATCTCCAGCTGACAACCAACAGTACCGACTCTGGCGACCGAGGGGGATCTTCCAAC    60
METAspLeuGlnLeuThrThrAsnSerThrAspSerGlyAspArgGlySerSerAsn      20

GAGTCCCTGCAGAGGCAGCCCCCGAGCCAATACTCGCCTGCAGAGGTGGCAGGCTTGGCC  120
GluSerLeuGlnArgGlnProProSerGlnTyrSerProAlaGluValAlaGlyLeuAla   40

GCGGTGGTGAGCTTTCTCATCGTCTTCACCATCGTGGGTAACGTGTTGGTGGTGATTCCC  180
AlaValValSerPheLeuIleValPheThrIleValGlyAsnValLeuValValIlePro   60

GTGTTGACCAGCCGAGCTCTGAAAGCCCCCCAGAACCTGTTCCTGGTGTCCCTGGCCAGC  240
ValLeuThrSerArgAlaLeuLysAlaProGlnAsnLeuPheLeuValSerLeuAlaSer   80

GCTGACATTCTGGTGGCTACTCTGGTCATGCCTTTCTCACTAGCCAATGAGCTCATGAAT  300
AlaAspIleLeuValAlaThrLeuValMetProPheSerLeuAlaAsnGluLeuMetAsn  100

TACTGGTACTTCGGGAAAGTGTGGTGCGACATTTACCTGGCGCTAGACGTCCTCTTCTGC  360
TyrTrpTyrPheGlyLysValTrpCysAspIleTyrLeuAlaLeuAspValLeuPheCys  120

ACCTCCTCCATAGTGCACCTGTGCGCCATCAGCCTGGACCGCTACTGGTCCGTGACGCAG  420
ThrSerSerIleValHisLeuCysAlaIleSerLeuAspArgTyrTrpSerValThrGln  140

GCGGTGGGGTACAACCTGAAACGCACCCCGCGCCGAATCAAGGGCATTATCGTAACGGTG  480
AlaValGlyTyrAsnLeuLysArgThrProArgArgIleLysGlyIleIleValThrVal  160

TGGCTCATCTCCGCGGTCATCTCCTTTCCTCCGCTCATCTCCCTCTACCGGGACCCGGAG  540
TrpLeuIleSerAlaValIleSerPheProProLeuIleSerLeuTyrArgAspProGlu  180

GACGACCTGTACCCCCAGTGCGAGCTCAACGACGAGACGTGGTACATCCTTTCGTCCTGC  600
AspAspLeuTyrProGlnCysGluLeuAsnAspGluThrTrpTyrIleLeuSerSerCys  200

ATTGGTTCCTTCTTCGCGCCCTGCATCATCATGGTGCTGGTCTACGTTCGCATCTACCGA  660
IleGlySerPhePheAlaProCysIleIleMetValLeuValTyrValArgIleTyrArg  220

GTGGCCAAGCTGCGGACACGGACGCTGTCGGAGAAGCGCACGGTGCCCGAGGGCTCGTCC  720
ValAlaLysLeuArgThrArgThrLeuSerGluLysArgThrValProGluGlySerSer  240

CAGACCGAGAACGGGCTGAGCCGACCGCCGGTGGGGGCAGGGCCCTCGACCGCCGCAGCT  780
GlnThrGluAsnGlyLeuSerArgProProValGlyAlaGlyProSerThrAlaAlaAla  260

GCCGCCGCCTCCCTACGTCTCCAGGCTGGCGAAAATGGGCACTATCACCTCCACCACCAC  840
AlaAlaAlaSerLeuArgLeuGlnAlaGlyGluAsnGlyHisTyrHisLeuHisHisHis  280

CACCACCACCTTCATCATCACCATCACCATCACCATCACCAGCTGCGCAAGAGTGCGGAG  900
HisHisHisLeuHisHisHisHisHisHisHisHisHisGlnLeuArgLysSerAlaGlu  300

CTCGAGGACATCGAACTGGAGGAGAGTAGCACCTCCGAGAACCGGCGCAGGAGGCGCAGC  960
LeuGluAspIleGluLeuGluGluSerSerThrSerGluAsnArgArgArgArgArgSer  320

CGAGAGGAGGCGGCCCGCCCCAAGGGCAGGCCGCGCTTCTCCTTTCTTTTCTCCTCACC 1020
ArgGluGluAlaAlaArgProLysGlyArgProArgPheSerPheSerPheSerSerThr  340

AAAGGAGGCCAGTCTGCTGGTGCTGGGAGCCGCCTGTCCCGGGCCAGCAACCGCTCCCTG 1080
LysGlyGlyGlnSerAlaGlyAlaGlySerArgLeuSerArgAlaSerAsnArgSerLeu  360

GAGTTCTTCTCGTCCCACCGGCGCCGCAAGCGCAGCCAGCCTGTGCCGCCGAAAGGTGACC 1140
GluPhePheSerSerHisArgArgArgLysArgSerSerLeuCysArgArgLysValThr  380

CAGGCCAGAGAGAAGCGCTTCACATTCGTGCTCGCTGTGGTCATGGGCGTATTCGTCGTG 1200
GlnAlaArgGluLysArgPheThrPheValLeuAlaValValMetGlyValPheValVal  400

TGCTGGTTCCCTTTCTTTTTCACCTACAGCCTGTACGGCATCTGCAGGGAGGCCTGCCAG 1260
CysTrpPheProPhePhePheThrTyrSerLeuTyrGlyIleCysArgGluAlaCysGln  420

GTGCCCGAGACTCTCTTCAAGTTCTTCTTCTGGTTCGGCTATTGCAATAGCTCCCTCAAC 1320
ValProGluThrLeuPheLysPhePhePheTrpPheGlyTyrCysAsnSerSerLeuAsn  420

CCGGTTATCTACACCATCTTCAACCAAGACTTCCGGAGGTCCTTCAAGCACATCCTGTTC 1380
ProValIleTyrThrIlePheAsnGlnAspPheArgArgSerPheLysHisIleLeuPhe  460

AAAAAGAAGAAGAAGACCTCCTTGCAGTGA 1410
LysLysLysLysLysThrSerLeuGln---  469
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Fig. 1. Nucleotide and deduced amino acid sequences of the OKc2 clone. *Arrowheads*, putative *N*-glycosylation sites. •, Consensus site for phosphorylation by cAMP-dependent protein kinase. The seven stretches of hydrophobic amino acids are *overlined*. The nucleotide sequence shown is a composite of sequence obtained from the 1032-base pair RT-PCR product and two *Sac*I subclones, the 1555-base pair RT-PCR product and two *Apa*I subclones, and the 3′ RACE and 5′ RACE products. The 1032-base pair RT-PCR product, the two *Sac*I subclones, the two *Apa*I subclones of the 1555-base pair RT-PCR product, and the 3′ RACE and 5′ RACE clones were sequenced in pBluescript using M13 and M13 reverse sequencing primers. The 1407-base pair RT-PCR product was sequenced in pRc/CMV using T7 and Sp6. The expression clone has a four-histidine deletion comprising nucleotides 870–881.

Drugs and chemicals. Sources for the drugs used in this study have been reported previously (3, 6). Drugs were prepared as 10 mM stock solutions in 5 mM HCl or in ethanol (yohimbine) and were subsequently diluted in 5 mM HCl. Sources for all other chemicals used in this study have been reported previously (3, 5).

Cell culture. CHO-K1 cells (American Type Culture Collection) were maintained in Ham's F-12 medium (GIBCO) with 10% fetal bovine serum (Hyclone, Logan, UT). OK cells were grown as described previously (5), in Dulbecco's modified Eagle's medium supplemented with high glucose and 5% fetal bovine serum. All cells were grown in 95% air/5% CO_2 at 37° in a humid atmosphere.

Transfections. The vector pRc/CMV was used to establish stable transfectants in CHO-K1 cells using the calcium phosphate precipita-

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                 I                                  II
OKC2   MD-------LQLTT----NSTDSGDRG---GSSNESLQRQPPS-QYSPAE    35
C4     MASPALAAALAVAAAAGPNASGAGERGSGGVANASGASWGPPRGQYSAGA    50
RC4    MASPALAAALAAAAAEGPNGSDAGEWGSGGGANASGTDWGPPPGQYSAGA    50
       *.         *. ..    *....*..*     .....  . **. ***...

OKC2   VAGLAAVVSFLIVFTIVGNVLVVIPVLTSRALKAPQNLFLVSLASADILV    85
C4     VAGLAAVVGFLIVFTVVGNVLVVIAVLTSRALRAPQNLFLVSLASADILV   100
RC4    VAGLAAVVGFLIVFTVVGNVLVVIAVLTSRALRAPQNLFLVSLASADILV   100
       ********.******.*********.*******.*****************

                                    III
OKC2   ATLVMPFSLANELMNYWYFGKVWCDIYLALDVLFCTSSIVHLCAISLDRY   135
C4     ATLVMPFSLANELMAYWYFGQVWCGVYLALDVLFCTSSIVHLCAISLDRY   150
RC4    ATLVMPFSLANELMAYWYFGQVWCGVYLALDVLFCTSSIVHLCAISLDRY   150
       **************.*****.***..************************

                                 IV
OKC2   WSVTQAVGYNLKRTPRRIKGIIVTVWLISAVISFPPLISLYRDPEDDLYP   185
C4     WSVTQAVEYNLKRTPRRVKATIVAVWLISAVISFPPLVSLYRQPDGAAYP   200
RC4    WSVTEAVEYNLKRTPRRVKATIVAVWLISAVISFPPLVSFYRRPDGAAYP   200
       ****.**.*********.*..**.*************.*.** *...  **

                   V
OKC2   QCELNDETWYILSSCIGSFFAPCIIMVLVYVRIYRVAKLRTRTLSEKRTV   235
C4     QCGLNDETWYILSSCIGSFFAPCLIMGLVYARIYRVAKRRTRTLSEKRAP   250
RC4    QCGLNDETWYILSSCIGSFFAPCLIMGLVYARIYRVAKLRTRTLSEKRGP   250
       **.*******************.** ***.******* *********.

OKC2   --PEGSSQT-ENGLSRPPVGAGPSTAAAAAASLRLQAGENGHYHLHHHHH   282
C4     VGPDGASPTTENGLGAAAGEARTGTARPRPPT-------------WSRTR   287
RC4    AGPDGASPTTENGLGKAAGENGHCAPPRTEVE-------------PDESS   287
       *.*.*.* ****.  .. ..    ..      .

OKC2   HLHHHHHHHHHQLRKSAELEDIELEESSTSENRRRRRSREEAARPKGRPR   332
C4     AAQRPRGGAPGPLRRGGRRRAGAEGGAGGADGQGAGPGAAQSG-------   330
RC4    AAERRR--RRGALRRGGRRREGAEGDTGSADGPGPGLAAEQ-G-------   327
        ....   . .**...   . . .........    . .. .

OKC2   FSFSFSSTKGGQSAGAGSRLSRASNRSLEFFSSHRRRKRSSLCRRKVTQA   382
C4     ------ALTASRSPGPGGRLSRASSRSVEFFLSRRRRARSSVCRRKVAQA   374
RC4    ------ARTASRSPGPGGRLSRASSRSVEFFLSRRRRARSSVCRRKVAQA   371
             . ....*.*.*.******.**.*** *.*** ***.*****.**

                      VI
OKC2   REKRFTFVLAVVMGVFVVCWFPFFFTYSLYGICREACQVPETLFKFFFWF   432
C4     REKRFTFVLAVVMGVFVLCWFPFFFIYSLYGICREACQVPGPLFKFFFWI   424
RC4    REKRFTFVLAVVMGVFVLCWFPFFFSYSLYGICREACQLPEPLFKFFFWI   421
       *****************.******* ************.*..*******.

          VII
OKC2   GYCNSSLNPVIYTIFNQDFRRSFKHILFKKKKKTSLQ     469
C4     GYCNSSLNPVIYTVFNQDFRPSFKHILFRRRRRGFRQ     461
RC4    GYCNSSLNPVIYTVFNQDFRRSFKHILFRRRRRGFRQ     458
       **************.******.*******...... .*
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Fig. 2. Alignment of the deduced amino acid sequence of OKc2 with the rat (23) and human (12) α_{2C}-adrenergic receptor sequences. *, Identical amino acids; •, well conserved amino acids. Sequences were aligned using CLUSTAL (37).

A

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          -120      -110      -100      -90       -80       -70
OKc2   TGTCAGTGGAAAGAGGAGACGGCGATGAAGGCACTGTGTCCGAGGAGGGAGCACC
C4
RC4    GGGCTTCGTGGCCCCCAGCGGCGCCGAGGAGCAGCGCGGCGATGCAGACGCGACT

               -60       -50       -40       -30       -20
OKc2   AGAGCGGACTCTGCTCCTGTGCACGGGGACACAAGGGGACCCCTTTGCTGCTGCT
C4                                             CCGGCTCCAGGAGGGGCGGCGTAG
RC4    CTGGCTGTGGGCGCCCAGCTGCGGAGCTGCCCCCGGCTCCTGGCAGGACGCGTAG

          -10       1
OKc2   CTTGTAGTGGGGTCATG
C4     CCCGCGGGAGGACCATG
RC4    CTCTCGGGAGGACCATG
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B

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          1410      1420      1430          1440
OKc2   TGACCCCGGTGGGCTCGGGGTGGGGCTGGGAGGAGGAGGGAT
C4     TGACTCGCACCCGTCTGGGAATCCTGGACAGCTCCGCGCTCG
RC4    TGACCCTCTGCTGCCTGGGACTTGGCCCCACTGACCTCCTGG
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Fig. 3. Nucleotide sequences for 5′ untranslated OKc2, rat (*RC4*) (23), and human (*C4*) (12) α_{2C}-adrenergic receptors (A) and 3′ untranslated sequences (B). The numbering for the 3′ untranslated sequences is based on that for OKc2.

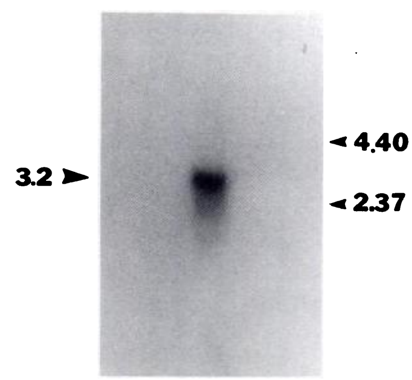

Fig. 4. A Northern blot of OK cell mRNA (8 μg) was probed with the ^{32}P-labeled 1032-base pair RT-PCR product from OK cell RNA. A single band of approximately 3.2 kb is present after an 8-hr exposure with an intensifier. Size markers, 0.24–9.5-kb RNA ladder (Life Technologies).

tion technique (28). Stable transfectants were selected with 500 μg/ml G-418 (Geneticin; Life Technologies) and screened by intact cell binding (29) using [^{3}H]rauwolscine. Briefly, replica 24-well plates were washed twice with 37° HEPES-buffered Ham's F-12 medium, and 300 μl of 3 nM [^{3}H]rauwolscine were added. Mock-transfected CHO-K1 cells were used as negative controls. The plates were incubated at room temperature for 1 hr and then washed three times with HEPES-buffered Ham's F-12 medium. The monolayers were then dissolved in 0.5 ml of 0.2 N NaOH, neutralized with 100 μl of 1 N HCl, and transferred to scintillation vials, and 5 ml of Budget-solve (Research Products Inc., Mount Prospect, IL) were added. CHO cells transfected with the α_{2A}-adrenergic receptor receptor were used as positive controls. The amount of radioactivity present was determined by liquid scintillation counting.

Membrane preparation. Membranes were prepared for radioligand binding assays by scraping cells from confluent monolayers in ice-cold phosphate-buffered saline and pelleting cells by centrifugation at 2000 × *g* for 10 min at 4°. The cell pellet was resuspended in ice-cold 50 mM Tris buffer, pH 8.0, and homogenized using a Tissuemizer (Tekmar, Cincinnati, OH) at a setting of 90 for 20 sec. A crude membrane pellet was obtained by centrifugation of the homogenate at 49,000 × *g* for 10 min at 4°. This wash step was repeated once again and the final pellet was frozen at −80° until used in the radioligand binding assay.

Radioligand binding assays. Competition and saturation binding experiments were performed as described previously (3, 5, 30). Briefly, for competition experiments membrane pellets were thawed and resuspended in 25 mM glycylglycine buffer, pH 7.6. To duplicate tubes were added 10 μl of unlabeled competing drug, 970 μl of membrane suspension, and 20 μl of [^{3}H]rauwolscine at a single concentration near its K_d for that tissue. After a 30-min incubation at 23°, the suspension was filtered rapidly through GF/B glass fiber filter strips (Whatman, Clifton, NJ) using a 48-sample manifold (Brandel cell harvester; Biomedical Research and Development, Gaithersburg, MD). Tubes and filters were washed twice with 50 mM Tris·HCl, pH 8.0, at 4° and radioactivity retained on the filters was determined by liquid scintillation counting. Inhibition experiments, routinely consisting of 11 concentrations of the unlabeled ligand, were analyzed using nonlinear, least-squares, parametric, curve-fitting programs (GraphPAD Software, San Diego, CA), to obtain IC_{50} values. IC_{50} values were converted to K_i values by the method of Cheng and Prusoff (31) and are presented as geometric means. Saturation experiments used six concentrations of [^{3}H]rauwolscine (final concentration ranged approximately from 20 pM to 2.5 nM). Nonspecific binding was determined using 100 μM (−)-norepinephrine. K_d and B_{max} values were calculated by nonlinear regression of the data

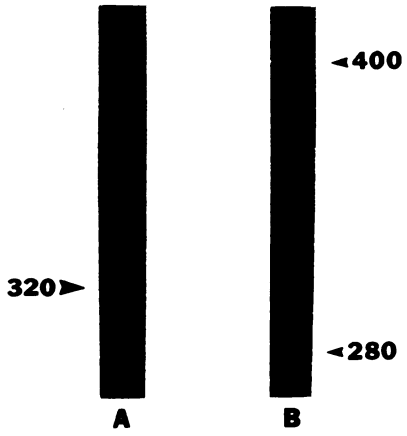

Fig. 5. RNase protection assay using 100 μg of total RNA from OK cells (A) and opossum kidney (B). A single 320-base pair protected band is seen in each lane. Size markers, 0.16–1.77-kb single-stranded RNA ladder (Life Technologies).

and are given as geometric and arithmetic means, respectively. Protein concentrations were determined by the method of Lowry *et al.* (32), using bovine serum albumin as the standard.

cAMP production assay. The [^{3}H]adenine prelabeling technique was used to assay cAMP production in intact transfected CHO cells. The cells were preincubated for 1 hr at 37° with 2 μCi/well [^{3}H]adenine (in six-well dishes), washed three times with 2.5 ml of HEPES-buffered Ham's F-12 medium, and then incubated for 2 min at 37° with 1 ml of HEPES-buffered Ham's F-12 medium containing 30 μM forskolin (to stimulate cAMP production) and various concentrations of UK14,304 (to inhibit adenylyl cyclase). The assays were terminated by aspiration of the medium and rapid addition of 1 ml of trichloroacetic acid (5%, w/v). [^{3}H]ATP and [^{3}H]cAMP fractions were isolated by ion exchange column chromatography (33) and the radioactivity was measured by liquid scintillation counting (Beckman Instruments, Irvine, CA). The data were calculated as percent conversion [cAMP/(ATP + cAMP)] and are presented as percentages of control values.

Results

A single band of 1032 base pairs was obtained from the RT-PCR performed on OK RNA using the oligonucleotides directed to the second and seventh transmembrane regions of α_2-C10 and α_2-C4. This band hybridized strongly to the 390-base pair probe generated from α_2-C10, indicating that the RT-PCR product coded for an α_2-adrenergic receptor. The deduced amino acid sequence showed an overall sequence identity of 67%, with a similarity of 19%, to the human α_2-C4. The 5′ and 3′ RACE clones hybridized strongly to the 1032-base pair piece and showed sequence data identical to this 1032-base pair RT-PCR product in the respective regions that overlapped. In addition, they contained the amino and carboxy termini, respectively, of the receptor.

The RT-PCR product obtained from OK cDNA using primers designed from 5′ and 3′ RACE sequences yielded a product, OKc2, that contained an open reading frame of 1407 base pairs. Fig. 1 shows the OKc2 receptor sequence. Hydropathy analysis of the coding sequence according to the method of Kyte and Doolittle (34) revealed seven hydrophobic regions, which may serve as membrane-spanning domains (data not shown). The deduced amino acid sequence (Fig. 2) shows an overall sequence identity of 64% to human α_2-C4, with 21% similarity, giving an overall similarity of 85%. Both receptors have two potential *N*-linked glycosylation sites in the amino terminus (Asn-X-Ser/Thr). OKc2 shares with its human orthologue a single consensus site in the third cytoplasmic loop (Arg/Lys-Arg/Lys-X-Ser/Thr), Thr^{388}, for phosphorylation by cAMP-dependent protein kinase. Fig. 3 compares the nucleotide sequences for the 5′ and 3′ untranslated regions of the OKc2, rat, and human α_{2C}-adrenergic receptors. There is no apparent sequence similarity among the three species.

Northern analysis (Fig. 4) showed a single band with an estimated size of 3.2 kb. The RNase protection assay (Fig. 5) showed a single protected band from both OK RNA and opossum kidney RNA.

Saturation studies with [^{3}H]rauwolscine were performed on membranes from two CHO cell clones that had been stably transfected with the expression vector pRc/CMV-OKc2. [^{3}H]Rauwolscine indicated the presence of a single class of high affinity binding sites for both clones. The K_d value (mean ± standard error) from three experiments was 0.24 ± 0.03 nM and the B_{max} was 227 ± 33 fmol/mg of protein for the F12 clone; the K_d value was 0.11 ± 0.01 nM and the B_{max} was 307 ± 98 fmol/mg of protein for the B9 clone. Consistent with our previous results (5), membranes from the OK cell line had a similar K_d value (0.12 ± 0.02 nM, five experiments). Membranes from mock-transfected CHO cells demonstrated no specific

TABLE 1

Drug affinities for the cloned OK α_{2C}-adrenergic receptor

Values are mean ± standard error for inhibition of [^{3}H]rauwolscine binding.

Drug	CHO-OKc2 cells				OK cells				COS-C4 cells, K_i[a]
	K_i	pK_i	n[b]	Slope	K_i	pK_i	n	Slope	
	nM				nM				nM
Rauwolscine	0.24 ± 0.03[c]	9.60	3		0.17[d]				0.15
Yohimbine	0.11 ± 0.01	9.97	2	0.90	0.09 ± 0.01	10.04	3	1.01	0.37
BAM1303	0.10 ± 0.04	10.00	4	1.03	0.17 ± 0.06	9.77	3	1.04	0.47
ARC-239	144 ± 6	6.84	4	0.95	33 ± 3	7.48	3	1.11	4.65
Spiroxatrine	0.51 ± 0.21	9.30	3	0.95	0.23 ± 0.01	9.64	2	1.10	0.15
Oxymetazoline	29 ± 4	7.54	2	0.91	31[d]				35
7-Hydroxychlorpromazine	312 ± 9	6.51	2	1.04	223[d]				
WB4101	1.38 ± 0.21	8.86	2	1.01	0.63[d]				0.48
Norepinephrine	40 ± 6	7.40	4	0.73	96 ± 10	7.02	2	0.57	

[a] K_i values from Bylund *et al.* (13).
[b] n, number of experiments.
[c] K_d value.
[d] K_i values from Blaxall *et al.* (6).

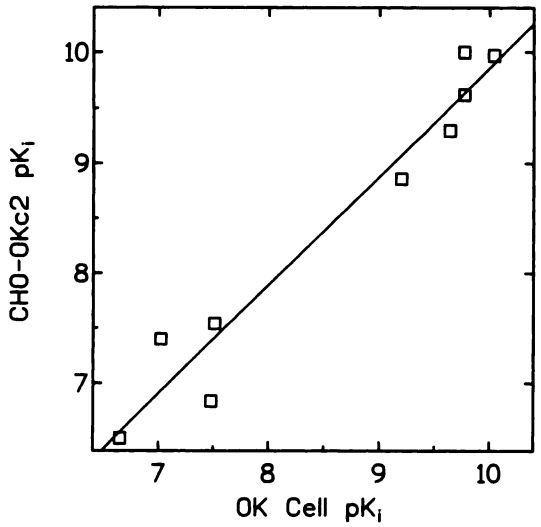

Fig. 6. Correlations between pK_i values of various adrenergic drugs for OKc2 and pK_i values for the OK cell α_{2C}-adrenergic receptor. Values are from Table 1. The correlation coefficient is 0.97.

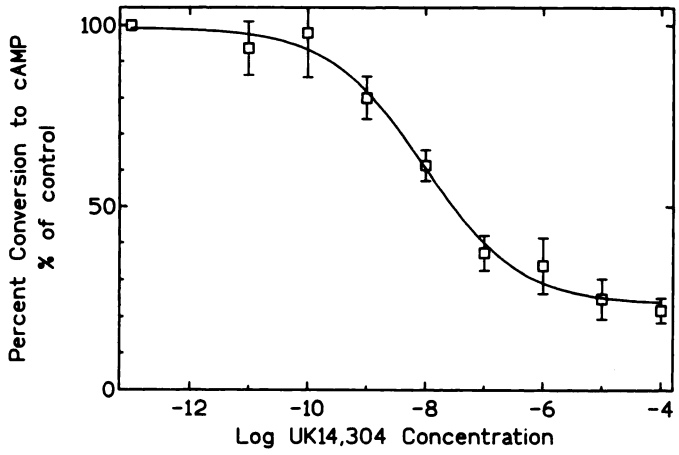

Fig. 7. Inhibition by UK14,304 of forskolin-stimulated cAMP production. CHO cells transfected with the OKc2 clone were prelabeled with [^{3}H] adenine and then incubated with 30 μM forskolin, without or with the indicated concentrations of UK14,304. The percent conversion of ATP to cAMP was calculated for each of three experiments. The mean basal value (no forskolin) was 0.032 ± 0.004% conversion, and the mean control value (30 μM forskolin, no UK14,304) was 0.63 ± 0.07% conversion. The means of the normalized data are presented as percentages of control. The EC_{50} from these data is 9.2 nM. The mean ± standard error of the EC_{50} value from the three individual experiments is given in the text.

binding. Several competing ligands were used in inhibition binding studies to characterize pharmacologically the expressed receptor in the F12 clone (Table 1). A correlation plot (Fig. 6) of the pK_i values of the cloned OK α_{2C}-adrenergic receptor for the drugs listed in Table 1 plotted against the pK_i values for the same drugs in the OK cell line gives a correlation coefficient (r) of 0.97, with a slope of 0.98. This very high correlation between the values of the CHO cells expressing the OKc2 clone and those of the native OK cell α_{2C}-adrenergic receptor indicates that OKc2 is indeed the cloned OK α_{2C}-adrenergic receptor.

To demonstrate that the expressed receptor was functional, a cAMP production assay was used. Transfected cells (B9 clone) were incubated with 30 μM forskolin without or with various concentrations of the α_2-adrenergic receptor agonist UK14,304 (Fig. 7). UK14,304 maximally inhibited forskolin-stimulated cAMP production by 77%. The EC_{50} for inhibition was 11 ± 2 nM (three experiments), which agrees with the EC_{50} of 12 ± 3 nM in OK cells (5).

Discussion

The OK cell α_{2C}-adrenergic receptor shows 64% identity to the human α_2-C4 receptor and has a similarity of 21%, giving an overall similarity of 85%. The human and rat orthologues have 90% identity. Within the transmembrane regions, the protein encoded by OKc2 has 89% identity to human α_2-C4; however, if conservative substitutions are included the overall similarity becomes 99%. The amino terminus and third cytoplasmic loop are the least conserved regions. The lower sequence identity of the OK cell α_{2C}-adrenergic receptor to the human, rat, and mouse orthologues may be a reflection of the greater evolutionary separation between metatherian (marsupial) and eutherian (placental) mammals. The opossum parathyroid hormone receptor (35) has 78% identity to the rat parathyroid receptor (36) and the OK serotonin 5-HT_{1B} receptor has 82% identity to the human 5-HT_{1B} receptor (38).

Kurose *et al.* (19) reported that antibodies directed to epitopes within the third cytoplasmic loop of α_2-C4 failed to immunoprecipitate the OK cell α_{2C}-adrenergic receptor and similar antibodies derived from RG10 precipitated only relatively small amounts of the OK α_{2C}-adrenergic receptor. These data are in agreement with the sequence data presented here, which show very low sequence homology within the third cytoplasmic loop.

The α_{2C}-adrenergic receptor is encoded by a 3.2-kb message in OK cells. The message for the rat α_{2C}-adrenergic receptor was reported by Voigt *et al.* (23) to be of two sizes, 2.9 and 2.4 kb. The opossum kidney RNA and OK cell RNA both show the same size protected band in the RNase protection assay using a probe that includes part of the third cytoplasmic loop, indicating that opossum kidney expresses the same α_{2C}-adrenergic receptor as do OK cells.

In conclusion, we have cloned, sequenced, and expressed a functional OK cell α_{2C}-adrenergic receptor. This clone, OKc2, expresses the pharmacology of the α_{2C} subtype and is negatively coupled to adenylyl cyclase. The 64% overall deduced amino acid sequence identity to α_2-C4 shows that pharmacological subtype prediction cannot be based solely on sequence similarities.

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