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Genomic cloning and species-specific properties of the recombinant canine β_3-adrenoceptor

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Abstract

A molecular clone encoding a β_3-adrenoceptor was isolated from a canine genomic library. The cloned receptor exhibited a pharmacological profile similar to that of other species: in particular, high efficiency of the two selective β_3-adrenoceptor agonists, CL 316,243 (disodium(*R*,*R*)-5[2[[2-(chlorophenyl)-2hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate) and ICI 201651 ((*R*)-4-(2-hydroxy-3-phenoxypropylaminoethoxy)-*N*-(2-methoxyethyl)phenoxy acetic acid) and a low affinity for the radioligand (−)-[3-^{125}I]-iodocyanopindolol. Interestingly, CGP 12177A ((±)-4-(3-*t*-butylamino-2-hydroxypropoxy)benzimidazol-2-one), which is described as a partial agonist for the human receptor, was a full agonist for the canine receptor. After expression and stimulation of the canine β_3-adrenoceptor in stably transfected Chinese hamster ovary cells there was a very low accumulation of cAMP, suggesting weak coupling to Gs-protein and adenylyl cyclase. However, the response was much better in human embryonal kidney cells transfected with the canine β_3-adrenoceptor gene. The cloning of the canine β_3-adrenoceptor and the insights gained from its pharmacological characterization may allow the development of selective compounds for use in the treatment of obese dogs.

Keywords: β_3-Adrenoreceptor; Molecular cloning; Pharmacology; (Canine)

1. Introduction

Three β-adrenoceptor subtypes respond to the endogenous catecholamines, epinephrine and norepinephrine, by modulating the activation of adenylyl cyclase. The β_3-adrenoceptor, first recognized functionally in rodents, has now been cloned and completely sequenced for six species (reviewed by Strosberg, 1997), including human, rhesus monkey (Walston et al., 1997), cow, mouse and rat, and has been partially sequenced in guinea pig (Atgié et al., 1996), hamster (Strosberg, 1997) and pig (GenBank accession number U55858).

The β_3-adrenoceptor is preferentially localized in adipose tissue. In white adipose tissue, stimulation of β-adrenoceptors by catecholamines induces the activation of adenylyl cyclase via the Gs-protein, increases intracellular cAMP levels and leads to activation of the hormone-sensitive lipase involved in triglyceride hydrolysis. The β_3-adrenoceptor plays a significant role in the control of thermogenesis in the brown adipose tissue of rodents and humans (Arch and Kaumann, 1993; Enocksson et al., 1995). Brown adipose tissue has been demonstrated to play an important role in compensatory thermogenesis in small rodents (Himms, 1990). Active brown adipose tissue is also present in neonates and young animals of others species including cats (Holloway et al., 1991), cattle (Casteilla et al., 1989), dogs (Ashwell et al., 1987) and humans (Cannon and Needergard, 1994). The amount of brown adipose tissue decreases in adulthood. Interestingly, stimulation of the β_3-adrenoceptor with a selective agonist (ICI D7114) has been shown to reactivate dormant brown adipose tissue in adult dogs (Champigny et al., 1991) and in adipocytes of adult humans (Champigny and Ricquier, 1996).

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Finally, the Trp-to-Arg polymorphism at amino acid residue position 64 ($Trp^{64} \rightarrow Arg$) in the human β_3-adrenoceptor has been shown to be associated with morbid obesity in a large number of studies; carriers suffer from increased weight gain, early-onset diabetes, insulin resistance and an increased waist-to-hip ratio (Clement et al., 1995). Because of the possible role of the β_3-adrenoceptor in fat metabolism, this discovery increased the importance of investigations of the function and species-specific pharmacological characterization of this receptor. Several reports describing the effects of β-adrenoceptor compounds have revealed the functional involvement of the three β-adrenoceptors in canine fat cells (Langin et al., 1991; Galitzky et al., 1993b). The potency of physiological catecholamines in causing the activation of lipolysis was proposed to be different: lipolysis could be initiated at low concentrations of norepinephrine by activation of β_1-adrenoceptors whereas at higher concentrations of catecholamines activation of β_3-adrenoceptors could occur (Galitzky et al., 1993b). More precisely, it was shown that canine adipocytes express functional β_3-adrenoceptors which, when stimulated with selective β-adrenoceptor agonists, induce lipid mobilization (Galitzky et al., 1993a; Bousquet-Melou et al., 1994). The canine β_3-adrenoceptor apparently mediates additional effects in different tissues and organ systems such as decreased blood pressure and increased cutaneous blood flow (Berlan et al., 1994), relaxation of bronchial smooth muscle (Tamaoki et al., 1993), and modulation of colonic motility (De Ponti et al., 1995).

The present study describes the molecular analysis and the pharmacological characterization of the canine β_3-adrenoceptor. This receptor appeared to be highly similar to the bovine and human β_3-adrenoceptors and the expressed protein had a pharmacological profile similar, but not identical to, that of β_3-adrenoceptors from other species. This was examined by stably expressing the cloned canine β_3-adrenoceptor in Chinese hamster ovary cells (CHO-K1) and human embryonal kidney cells (HEK293). In CHO-K1 cells, when coupling to G-protein and adenylyl cyclase of the canine β_3-adrenoceptor was investigated, we observed a very modest accumulation of cAMP after stimulation with agonists. In contrast, coupling was much better when the canine β_3-adrenoceptor was expressed in human HEK293 cells. This observation suggested that the canine β_3-adrenoceptor does not efficiently interact with the hamster G-protein present in the CHO-K1 cells. Detailed knowledge of the structure and function of the canine β_3-adrenoceptor might lead to the development of selective agonists for use in dogs.

2. Materials and methods

Standard molecular biological methods were used (Sambrook et al., 1989). All chemical reagents were molecular biology or analytical grade. Restriction endonucleases and DNA-modifying enzymes were from New England Biolabs. Sequenase version 2.0 sequencing kits and nylon hybridization membranes were obtained from Amersham. Further suppliers were Life Technologies, Promega and Perkin Elmer.

2.1. Pharmacological compounds

Use in the text, in the table or in the figures of the name of β-adrenoceptor ligands without qualification implies that the (±)-isomer was used. (−)-Norepinephrine, (−)-epinephrine, (−)-isoproterenol, IBMX (3-isobutyl-1-methyl xanthine), bovine serum albumin, and polyethylenimine were from Sigma (St. Louis, MO, USA). (−)-[3-^{125}I]-Iodocyanopindolol (specific activity 2000 Ci/mmol) was from Dr. J. Sulon, Faculté de Médecine Vétérinaire, B41 B4000-Liège, Belgium. cAMP levels were measured with the Amersham [^{3}H]cAMP assay kit. CGP 12177A (±)-4-(3-*t*-butylamino-2-hydroxypropoxy)benzimidazol-2-one and CGP 20712A [(±)-(2-(3-carbamoyl-4-hydroxyphenoxy)ethylamino)-3-(4-(1-methyl-4-trifluoromethyl-2-midazolyl)-phenoxy)-2-propanol methane sulfonate] were generous gifts from Ciba-Geigy (Basel, Switzerland). ICI 201651 (*R*)-4-(2-hydroxy-3-phenoxypropylaminoethoxy)-*N*-(2-methoxyethyl) phenoxy acetic acid, ICI D7114 (*S*)-4-[2-hydroxy-3-phenoxypropylaminoethoxy]-*N*-(2-methoxyethyl) phenoxyacetamide and ICI 118551 [(±)-D-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol were from Tocris Cookson. CL 316,243 (disodium(*R*,*R*)-5[2[[2-(chlorophenyl)-2hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate was a generous gift from American Cyanamid and (−)-bupranolol [1-(2-chloro-5-methylphenoxy)-3-((1,1-dimethylethyl)amino)-2-propanol] was a generous gift from Schwarz Pharma (Monheim, Germany).

2.2. Probes

Polymerase chain reaction (PCR) experiments were performed with canine genomic DNA using a primer set from the human β_3-adrenoceptor gene (GenBank M29932). Sense primer 1269 (5′-ATG GCT CCG TGG CCT CAC-3′ positions 38 to 55) and antisense primer 1263 (5′-GGT AGA AGG AGA CGG AGG-3′ positions 663 to 680) were able to amplify a 643-bp fragment spanning the initiation codon ATG to the coding region of transmembrane domain 5 under the following conditions: 700 ng of canine genomic DNA was amplified by 29 temperature cycles (94°C, 15 s; 56°C, 30 s; 72°C, 30 s) followed by 3 min of extension at 72°C in a Gene Amp PCR system 9600 (Perkin Elmer) in 50 μl PCR buffer containing both primers at 0.25 μM, 10% (v/v) dimethylsulfoxide, 2.5 U of Taq polymerase (Promega) and 0.25 mM dNTP. The supplied reaction buffer was supplemented with 1.5 mM $MgCl_2$.

The 643-bp PCR product was subcloned into M13 cloning vectors tg130 and tg131 (Amersham) and sequenced. Sequencing data showed strong homology with known β_3-adrenoceptor sequences, indicating that the cloned fragment was part of the canine β_3-adrenoceptor gene.

Secondly we amplified a 1041-bp fragment of canine genomic DNA using the following human β_3-adrenoceptor primer set: sense primer 1269 as described above and antisense primer TR2 (5′-GCA GTA GAT GAG CGG GTT GAA GGC A-3′ positions 1054 to 1078). The amplified fragment spans the gene from the initiation codon ATG to the end of the coding region of transmembrane domain 7. PCR conditions were (94°C, 15 s; 63.6°C, 30 s; 72°C, 30 s.) × 30, followed by 3 min of extension at 72°C in 50 μl PCR buffer as described above.

Both fragments were used as probes in Southern hybridization assays and are referred to as *probe 1*: 643 bp = ATG-transmembrane domain 5 and *probe 2*: 1041 bp = ATG-transmembrane domain 7.

2.3. Southern blot analysis

Canine genomic DNA was digested by the following restriction enzymes: *Xba*I, *Bam*HI, *Hin*dIII, *Eco*RI and by combinations of *Xba*I/*Hin*dIII, *Xba*I/*Bam*HI, *Bam*HI/*Hin*dIII, *Eco*RI/*Xba*I, *Eco*RI/*Hin*dIII or *Eco*RI/*Bam*HI. DNA fragments were run on a 0.7% agarose gel and transferred to a nylon membrane (Hybond N + ; Amersham). Hybridization was carried out using ^{32}P random priming-labelled probe 1 corresponding to the 5′ region of the canine β_3-adrenoceptor open reading frame (ATG-transmembrane domain 5). Prehybridization and hybridization were carried out at 42°C in the following solution: 600 mM NaCl; 60 mM Na-citrate, 8 mM Tris–HCl pH 7.5, 50 mM Na-phosphate, 1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin, 40% formamide, 0.2% sodium dodecyl sulfate (SDS) and 100 μg/ml salmon sperm DNA. Radiolabelled probe (10^6 cpm/ml) was added and incubated overnight. Final washes were at 50°C with 3 mM NaCl, 0.3 mM Na-citrate and 0.05% SDS for 30 min.

2.4. Library construction and screening

Canine genomic DNA (200 μg) was digested with *Eco*RI, separated on a 0.7% agarose gel and split into three fractions in the range 4.8–8 kb. Small portions of these fractions were separated on an agarose gel, blotted onto nylon membrane and probed with probe 1. Hybridization showed that fraction 2 (fragment sizes 6 to 7.2 kb) contained the canine β_3-adrenoceptor gene. A library was constructed by ligating fraction 2 into the lambda phage GEM-2 (Promega) and then 370,000 λ phages were screened with canine β_3-adrenoceptor probe 2. Hybridization and washing conditions were as previously described. Three positive clones were identified by repeated rounds of screening, and each of them showed the same *Eco*RI insert size. The 6-kb *Eco*RI insert of one clone, designated λ 20.1, was subcloned into M13 tg131 and sequenced on both strands by dideoxynucleotide chain-termination sequencing and automated sequencer ABI 373A, using dye terminator chemistry (Perkin Elmer).

2.5. Stable expression of the canine β_3-adrenoceptor in CHO-K1 and HEK293 cells

A *Bsp*EI/*Eco*RI fragment, containing the entire open reading frame of the canine β_3-adrenoceptor, was excised from a larger clone containing the β_3-adrenoceptor genomic fragment as follows. The restriction site *Bsp*EI was cleaved and blunt ended by using Klenow DNA polymerase. Subsequent cleavage with *Eco*RI generated a 2.5-kb fragment. This fragment was ligated into the mammalian expression vector pcDNA3 (Invitrogen), which itself had been restricted at the polylinker with *Bam*HI, blunt ended with Klenow and then cleaved with *Eco*RI. The integrity of the resulting plasmid was confirmed by restriction endonuclease analysis and end sequencing. This vector contains a SV 40-driven neomycin resistance gene for the establishment of stable clones. The (pcDNA3)-canine β_3-adrenoceptor construct was introduced into CHO-K1 cells by lipofectine (Gibco), and stable transfectants were selected in the presence of 400 μg/ml G418 (Gibco BRL). Isolated colonies were expanded and screened for [^{125}I]iodocyanopindolol binding in the presence (non-specific binding) or absence (total binding) of the β_3-adrenoceptor antagonist bupranolol as described below. The (pcDNA3)-canine and (pRC/CMV)-human β_3-adrenoceptors (Pietri-Rouxel et al., 1997) were introduced in HEK293 cells by DOTAP (Boehringer Mannheim), and stable transfectants were selected in the presence of 500 μg/ml G418 (Gibco BRL). Isolated colonies were expanded and screened for [^{125}I]iodocyanopindolol binding in the presence (non-specific binding) or absence (total binding) of the β_3-adrenoceptor antagonist bupranolol as described below.

2.6. Radioligand binding analysis

Radioligand binding analysis was carried out with HEK293 (or CHO-K1) membranes. Membranes were prepared from detached pre-confluent cells by initial lysis in hypotonic lysis buffer (25 mM Tris–HCl (pH 7.5), 1 mM EDTA, containing protease inhibitors) followed by three cycles of homogenization and low-speed centrifugation. Homogenates were pooled and centrifuged for 30 min at 50,000 × *g* at 4°C. Pelleted membranes were resuspended and stored in storage buffer (25 mM Tris–HCl (pH 7.5), 1 mM EDTA, 10% glycerol, containing protease inhibitors) at −80°C until required. Saturation experiments were performed with [^{125}I]iodocyanopindolol in concentrations

ranging from about 10 to 20,000 pM. Non-specific binding was determined in the presence of 10 μM bupranolol (non-selective β-adrenoceptor antagonist). Briefly, 20–25 μg membrane proteins were incubated with compounds for 30 min at 37°C. The reaction was stopped by dilution with ice-cold phosphate-buffered saline (PBS) and rapid filtration over Whatmann GF/C glass fiber filters soaked in 0.3% polyethylenimine, using a Brandel cell harvester, followed by extensive washing with ice-cold PBS buffer. Radioactivity was assessed by γ-counting. Computer-aided Scatchard analyses were carried out to estimate the K_d (equilibrium dissociation constant) and the B_{max} (maximum number of binding sites). Competition analysis was carried out using a sub-saturating concentration of 5 nM [^{125}I]iodocyanopindolol, and increasing concentrations of the selective β_3-adrenoceptor agonists.

2.7. cAMP Accumulation assay

HEK293 cells were seeded 24 h before experiments in 6-well plates at 0.3×10^6 cells/well. After being washed with 1 ml Ham's F12 medium, buffered with 20 mM HEPES (pH 7.4) and supplemented with 1 mM isobutylmethyl xanthine, cell monolayers were incubated for 20 min at 37°C in 1 ml Ham's/HEPES buffer, with or without 100 μM (−)-isoproterenol, or 100 μM forskolin, or 1 pM to 100 μM of ligand. At the end of incubation, cells were harvested and collected in microcentrifuge tubes to be centrifuged at $700 \times g$ for 10 min at 4°C. The reaction was stopped by addition of 400 μl 1 N NaOH to the pellet. After 20 min at 4°C, the dissolved cells were buffered with 1 N acetic acid and centrifuged at $3000 \times g$ for 10 min at 4°C. The total amount of cAMP contained in an aliquot of supernatant was determined by using the Amersham kit according to the manufacturer's instructions. Adenylyl cyclase stimulation experiments with CHO-K1 cells expressing β_3-adrenoceptor were performed as described by Pietri-Rouxel et al. (1995).

2.8. Statistical analyses

The data are expressed as the mean ± standard error of the mean (S.E.M.) of two to five independent experiments performed in duplicate or triplicate. Saturation experiments were analyzed using the EBDA program (Biosoft; Elsevier, Cambridge, UK). IC_{50} (50% inhibitory concentration) values and K_{act} (activation constant) parameters, for the binding experiments and the activation of adenylyl cyclase, were determined by computerized iterative non-linear regression curve fitting using the INPLOT-4 program (GraphPad software; © 1987 by H.J. Motulsky). K_i (inhibition constant) values were calculated from the Cheng and Prussof equation (Cheng and Prusoff, 1973).

3. Results

3.1. Southern blot analysis of canine genomic DNA

Southern blot analysis was performed on canine genomic DNA to establish a restriction map before construction of a genomic library (Fig. 1). Restriction enzymes were used in single or combined digestions. Probe 1 detected only one fragment in each lane. The 3.7-kb

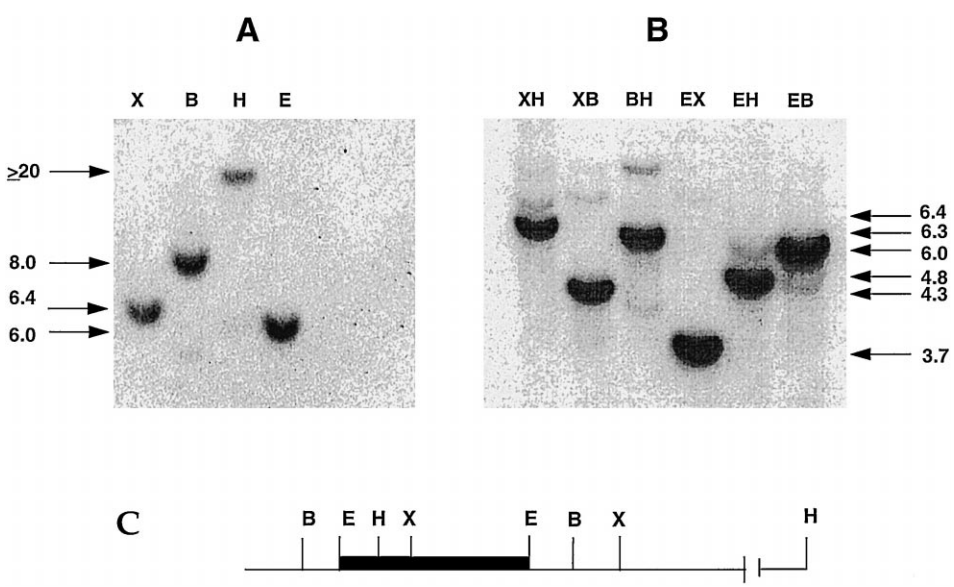

Fig. 1. Southern blot analysis of canine genomic DNA. Restriction enzymes used were *Xba*I: X, *Bam*HI: B, *Hin*dIII: H, *Eco*RI: E, (A); and *Xba*I/*Hin*dIII: XH, *Xba*I/*Bam*HI: XB; *Bam*HI/*Hin*dIII: BH, *Eco*RI/*Xba*I: EX, *Eco*RI/*Hin*dIII: EH, *Eco*RI/*Bam*HI: EB, (B); the sizes of the hybridizing fragments are indicated in kb on the left (A) and on the right (B). Restriction map of the canine genomic fragment containing the β_3-adrenoceptor gene (C). The cloned 6-kb *Eco*RI fragment is indicated as a black box. Original autoradiographies were scanned by using Studio Scan IIsi (Agfa) and Adobe Photoshop 3.0.

*Xba*I/*Eco*RI fragment was the smallest hybridizing fragment.

3.2. Cloning and sequence analysis of the canine β_3-adrenoceptor

Screening of the canine genomic library with a canine 1041-bp β_3-adrenoceptor PCR fragment (probe 2) led to the isolation of three clones; one clone was analyzed by dideoxynucleotide chain-termination sequencing on both strands. Data for the 2649-bp fragment revealed a single open reading frame interrupted by an intron (Fig. 2), thus displaying a structure similar to the human β_3-adrenoceptor gene (Van Spronsen et al., 1993). A splice donor signal (GAC GG/**gt**) found in position 1309 and a splice acceptor site (ttttc**ag**/G) in position 2011 were located 703 bp

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   1                                CCCGGGAAGCGCTCCCACGCCCCGCTGGCCCCTTCCCTGAGCTGG
  46 GGGAAGGGACCCGTCCGGAAGGGAGACCCCTCCTCCCTTCCCCTCCCGCCCCACTCGCGCCGCGGGG
     Met Ala Pro Trp Pro His Gly Asn Gly Ser Val Ala Ser Trp Pro Ala Ala     17
 113 ATG GCT CCG TGG CCT CAC GGG AAC GGC TCT GTG GCC TCG TGG CCG GCT GCC
     Pro Thr Pro Thr Pro Asp Ala Ala Asn Thr Ser Gly Leu Pro Gly Ala Pro     34
 164 CCC ACC CCG ACG CCC GAT GCC GCC AAC ACG AGT GGG CTG CCA GGG GCG CCC
     Trp Ala Val Ala Leu Ala Gly Ala Leu Leu Ala Leu Glu Val Leu Ala Thr     51
 215 TGG GCG GTG GCC TTG GCG GGG GCG CTG TTG GCG CTG GAG GTG CTG GCC ACC
     Val Gly Gly Asn Leu Leu Val Ile Val Ala Ile Ala Arg Thr Pro Arg Leu     68
 266 GTG GGA GGC AAC CTG CTG GTC ATC GTG GCC ATC GCT CGG ACG CCA AGA CTG
     Gln Thr Met Thr Asn Val Phe Val Thr Ser Leu Ala Thr Ala Asp Leu Val     85
 317 CAG ACC ATG ACC AAC GTG TTC GTG ACG TCG CTG GCC ACC GCG GAC CTG GTG
     Val Gly Leu Leu Val Val Pro Pro Gly Ala Thr Leu Ala Leu Thr Gly Arg    102
 368 GTG GGG CTC CTG GTA GTG CCG CCG GGG GCC ACC TTG GCG CTG ACG GGC CGC
     Trp Pro Leu Gly Ala Thr Gly Cys Glu Leu Trp Thr Ser Val Asp Val Leu    119
 419 TGG CCT CTG GGC GCC ACC GGT TGC GAG CTG TGG ACC TCA GTG GAC GTG CTG
     Cys Val Thr Ala Ser Ile Glu Thr Leu Cys Ala Leu Ala Val Asp Arg Tyr    136
 470 TGT GTG ACA GCC AGC ATC GAA ACC CTG TGC GCC CTG GCG GTG GAC CGC TAC
     Leu Ala Val Thr Asn Pro Leu Arg Tyr Gly Ala Leu Val Thr Lys Arg Arg    153
 521 CTG GCC GTG ACC AAC CCG CTG CGC TAC GGC GCC CTG GTC ACC AAA CGG CGC
     Ala Arg Ala Ala Val Val Leu Val Trp Val Val Ser Ala Ala Val Ser Phe    170
 572 GCC CGG GCG GCA GTG GTC CTG GTG TGG GTC GTG TCC GCC GCG GTG TCG TTC
     Ala Pro Ile Met Ser Lys Trp Trp Arg Val Gly Ala Asp Ala Glu Ala Gln    187
 623 GCG CCC ATC ATG AGC AAG TGG TGG CGC GTG GGA GCC GAC GCC GAG GCG CAG
     Arg Cys His Ser Asn Pro His Cys Cys Ala Phe Ala Ser Asn Ile Pro Tyr    204
 674 CGC TGC CAC TCC AAC CCG CAC TGC TGC GCC TTC GCC TCC AAC ATA CCC TAC
     Ala Leu Leu Ser Ser Ser Val Ser Phe Tyr Leu Pro Leu Leu Val Met Leu    221
 725 GCG CTG CTC TCC TCC TCC GTC TCC TTC TAC CTT CCG CTT CTG GTG ATG CTC
     Phe Val Tyr Ala Arg Val Phe Leu Val Ala Thr Arg Gln Leu Arg Leu Leu    238
 776 TTC GTC TAC GCG CGC GTT TTC CTC GTG GCT ACG CGC CAA CTG CGC CTG CTG
     Arg Arg Glu Leu Gly Arg Phe Pro Pro Ala Glu Ser Pro Pro Ala Ala Ser    255
 827 CGC CGG GAG CTG GGC CGC TTC CCG CCC GCG GAG TCT CCG CCG GCC GCG TCT
     Arg Ser Arg Ser Pro Gly Pro Ala Arg Arg Cys Ala Ser Pro Ala Ala Val    272
 878 CGC TCC CGG TCC CCC GGC CCG GCC CGG CGG TGC GCT TCG CCC GCC GCG GTG
     Pro Ser Asp Arg Leu Arg Pro Ala Arg Leu Leu Pro Leu Arg Glu His Arg    289
 929 CCC TCC GAC CGC CTG CGG CCC GCG CGC CTC CTG CCT CTG CGG GAG CAC CGG
     Ala Leu Arg Thr Leu Gly Leu Ile Val Gly Thr Phe Thr Leu Cys Trp Leu    306
 980 GCC CTG CGC ACC CTG GGC CTC ATC GTG GGC ACC TTC ACT CTC TGC TGG TTG
     Pro Phe Phe Val Ala Asn Val Met Arg Ala Leu Gly Gly Pro Ser Leu Val    323
1031 CCC TTC TTC GTG GCC AAC GTG ATG CGC GCT CTC GGG GGG CCC TCT CTG GTT
     Pro Ser Pro Ala Leu Leu Ala Leu Asn Trp Leu Gly Tyr Ala Asn Ser Ala    340
1082 CCC AGC CCG GCC CTC CTG GCC CTT AAC TGG CTG GGC TAC GCC AAC TCT GCC
     Phe Asn Pro Leu Ile Tyr Cys Arg Ser Pro Asp Phe Arg Ser Ala Phe Arg    357
1133 TTC AAC CCG CTC ATC TAC TGC CGC AGC CCC GAC TTC CGC AGC GCT TTC CGC
     Arg Leu Leu Cys Arg Cys Arg Arg Glu Glu His Arg Ala Ala Ala Ser Pro    374
1184 CGC CTA CTG TGC CGC TGC CGG CGG GAG GAG CAC CGC GCC GCC GCC TCC CCG
     Pro Gly Asp Pro Ser Ala Ala Pro Ala Ala Leu Thr Ser Pro Ala Glu Ser    391
1235 CCG GGC GAC CCC TCG GCC GCC CCT GCG GCC CTG ACC AGC CCC GCG GAG TCC
     Ser Arg Cys Gln Ala Leu Asp Gly                                       399
1286 AGC CGG TGC CAA GCG CTC GAC GG/ GTGGGTAACTGAGGCGAGGAGGCCGGCGGTTCAGG
1344 GTCAGAAGGCATTCGGAGTCTCTTTGGGCCATTTCTCAGAGTTTGGGGTTCGGTAGGATAAGGTGGG
1411 GTTGGAGACGTCTCTGCGGCGAAAGAAGGGGGGACCTGGAGTAGGGAACCAACATGGAAGCCCGGAC
1478 CCTTCCGTCTCCCGCGGCCGAGCACCTGCCCCAGGACGGAAGCAAAGAGGGCAGCAGATTGTTGTTC
1545 ACCCCAGGACCTAGTGCGGTCCGGGGAATGCGGCTGTATCCTGAGCCGGCTCGGTCAGCTCCGCATT
1612 TTCTAGCTGAGTTCTTTGGCCTCCCAGATCTCTTGCCACCCCTTGGGCCGGCTTTGACTTGCAGGGA
1679 AGACGAGAGGCCTTCTCAGACTCAAGGCCTGAGCTCTGGTTTCTTTGAAAGGTGTGATAGCTACGGA
1746 GTGATGGTGAGAATCCACTCGAGGTCTGAAGGATAAGCGGGAGTTGGGGAGGGGGTGAGGACTGTGA
1813 GTGTCAATTCTTCTCCTGGGTAGGAGCAGGCCCCGTTGGAGGTGGGGGGTGGGTATTTTGTGGCTGG
1880 GTGGAGCCCGGATGCTTCTGCGAGATTGTGGACAAATGCTTCCCAGCGTCCCTGACCTTTGCTCCTT
1947 CCCTCTACTGGCCCTGTCTCCACCCTGTGCCCCTCACCCC
                                Ala Ser Trp Gly Ile Ser Stop                405
1987 AAGATATGTTATCTCCATTTTTCAG/G GCT TCC TGG GGA ATC TCT TAG GTCCTGAACGA
2045 CAAGAAACAACTCTGTCAATCCAGAACTTTTGGAAAGCCTCTCCTGGCCTCTGTTTAGAATGGGCCC
2112 TGTGGAACTTCCCAGCTGGAAATCTCTGACCTCCAGAAACTGATGACTTGGCCTTGGGGTGGGGAGG
2179 CGGAGGTTGGGAGGGGCAACCCTTACCAAGTGAGTTTTCACCATCCTCTTGTCTCTCTCTTCCTGAG
2246 AAGAGTTTTCTAAACCCCACCCCTGAATTTTACCACTACCTCAGCAGCTGAAGTACCCAGCAGCCTG
2313 CTCTCAGCTGCCCTCGGAGTCCCCATTAGCTTTGGTGGCCCACCTGTCACCTTGCTCACTTCTGTGC
2380 TGCGTGCTTAGGGCAAAGAGGTCTCTCCTCCTTCTATTCTTTCTGCTGCCTGTGGACCTGATGGACC
2447 ACTGAGTGTCCTTCAGGCTCGGTGGGCAAGGCTGGGAGCAGAAAGCTATAAAAGGTCCGGGTTTGGG
2514 GTTCTGTCCCTGACTCCATCACTACAGATTCCTAAGCACCAGCCTTCCCCCCTTTGGATACAGGACA
2581 GCTCTGATCTACCTCACAGCAGTGTCAGGAGGACTTCTCCAGGGTTTGAGGAGGGTGGAGGGTGAATTC
                                                                    EcoRI
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Fig. 2. Nucleotide sequence and deduced protein sequence of the canine β_3-adrenoceptor gene. ATG start codon at position 113 is shown in boldface. The open reading frame is shown translated. mRNA splice sites at positions 1309 and 2011 are indicated in boldface and underlined. The *Eco*RI cloning site is underlined.

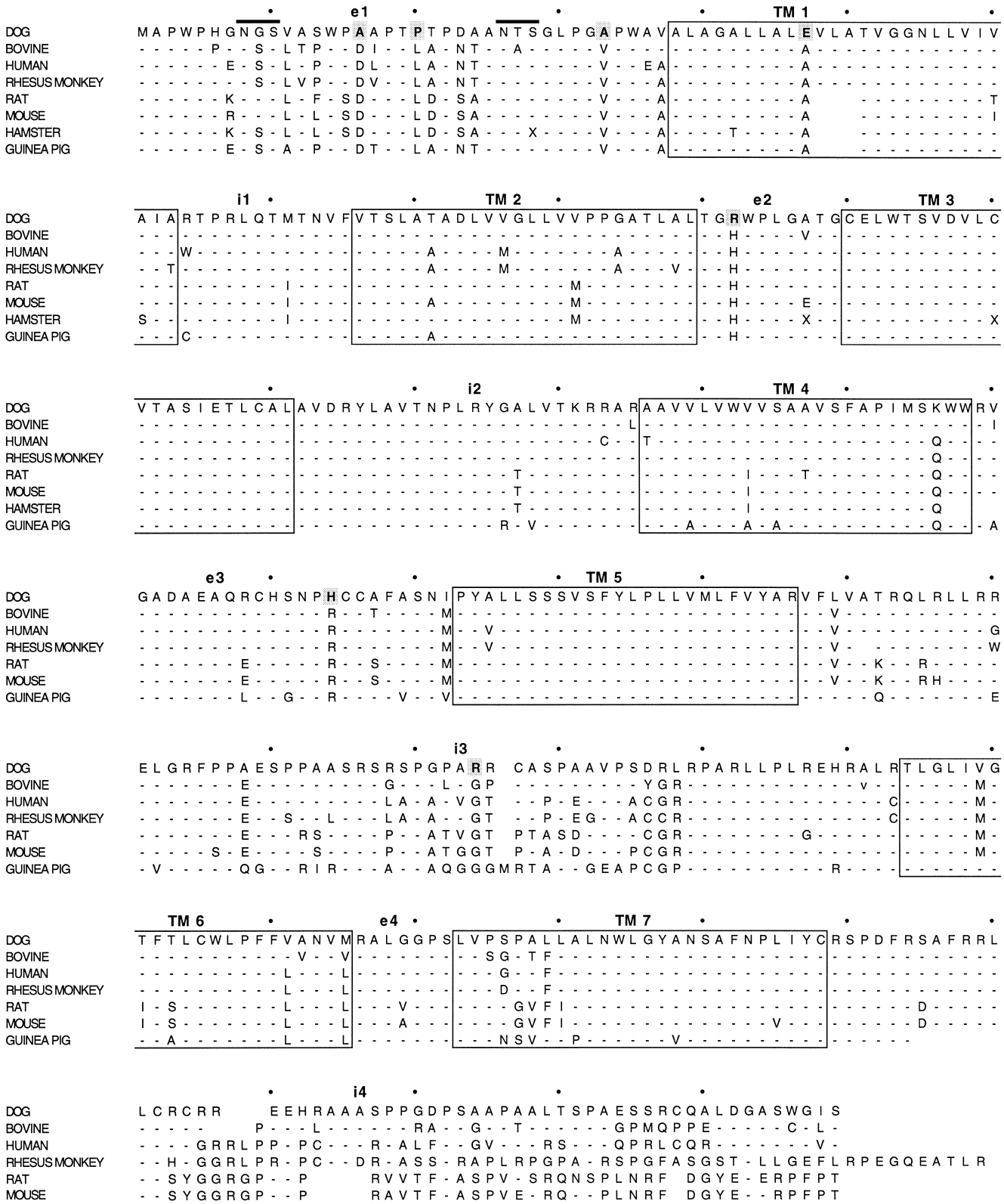

Fig. 3. Comparison of β_3-adrenoceptor amino acid sequences. The canine β_3-adrenoceptor protein sequence is compared to the β_3-adrenoceptor sequence of seven different species. Similarities between dog and other species are indicated (−). To maximize homologies, gaps represented by empty spaces have been introduced at different positions. Residues which are only found in canine β_3-adrenoceptor are shaded. The seven presumed α-helical membrane spanning domains (TM1 to TM7) are boxed and separated by extracellular (e1 to e4) and intracellular (i1 to i4) loops. In the NH_2-terminal region consensus sequences for asparagine-linked glycosylation sites (N–X–S) are highlighted by bars. Every tenth amino acid is indicated (•).

from each other, suggesting that there is an intronic sequence which interrupts the coding block (Padgett et al., 1986). The coding sequence started in exon 1 with the ATG initiation codon in position 113. Exon 2 contained a 22-bp sequence coding for the six carboxy-terminal residues and the TAG stop codon. The following 616 nucleotides were part of the 3′-untranslated region (UTR); there was no polyadenylation signal. After splicing of the intron, the ATG started an open reading frame of 1218 bp. The corresponding 405 amino acid residues formed a protein of 43,057 Da prior to post-translational modifications. Comparison of the derived sequences of previously reported β_3-adrenoceptors with the canine β_3-adrenoceptor protein sequence showed that it was most similar to the bovine β_3-adrenoceptor (86%) and the human β_3-adrenoceptor (82%). It was less similar to the rodent β_3-adrenoceptor sequences (76%).

Further sequence analysis of our clone confirmed that the corresponding protein belonged to the seven hydrophobic domain, G-protein-coupled family of receptors. For small ligand receptors, the seven potential transmembrane regions are postulated to form the hydrophobic ligand-binding site of the receptor. The protein sequence also contained two long intracellular domains, i3 and i4, which are likely to interact with G-protein during signal transduction (Guan et al., 1995).

The amino-terminal extracellular domain contained two consensus signals for N-linked glycosylation (N–G–S; N–T–S), which are highly conserved in β-adrenoceptor proteins (Fig. 3). The canine β_3-adrenoceptor contains several conserved residues that have been shown to be important for catecholamine binding and β-adrenoceptor activation process, namely Asp^{83}, Asp^{117} (Strader et al., 1987b; Gros et al., 1998) Ser^{209}, Ser^{212} (Strader et al., 1987a), and several conserved cysteine residues (Fraser, 1989).

The canine β_3-adrenoceptor displayed several structural features that are also found in bovine, monkey and human, but not in rodent β_3-adrenoceptors. These include a three-residue insertion in helix 1 at positions 48 to 50 (Val–Leu–Ala), and a three-residue insertion in the C-terminal domain at positions 370 to 372 (Ala–Ala–Ala). Within the transmembrane domains, the canine β_3-adrenoceptor had several residues which were different from the those of the bovine, human and monkey β_3-adrenoceptors. These included Glu^{47} in transmembrane domain 1 (usually Ala), Val^{298} in transmembrane domain 6 (usually Met), and Leu^{328} in transmembrane domain 7 (usually Phe).

The human β_3-adrenoceptor is three residues longer than the canine β_3-adrenoceptor, but shares a similar carboxy-terminal, which suggests that the human and canine receptors have a common intron/exon structure. The human β_3-adrenoceptor is encoded by two exons, the first of which codes for a polypeptide of 402 amino acid residues and the second exon for a further six amino acids (Granneman et al., 1993; Van Spronsen et al., 1993). Exon 1 of the canine β_3-adrenoceptor sequence was very similar to exon 1 of the human β_3-adrenoceptor and there was a consistent splice donor site at position 1309 (GG/gtgggt) (Padgett et al., 1986), as there is in the human sequence (GG/gtaggt) (Van Spronsen et al., 1993).

3.3. Stable expression of the canine β_3-adrenoceptor in CHO-K1 and HEK293 cells

Transfection of the canine β_3-adrenoceptor in CHO-K1 cells resulted in the generation of 20 clones that were selected for the ability to bind the [^{125}I]iodocyanopindolol ligand. Binding experiments revealed that transfected CHO-K1 cells expressed approximately 138 and 82 fmol/10^6 cells of human or canine β_3-adrenoceptors, respectively. Adenylyl cyclase activity was investigated in the human and canine clones (Fig. 4). Very little cAMP was accumulated when the canine β_3-adrenoceptor was stimulated by agonists. The effects of (−)-isoproterenol and CGP 12177A were normalized to the maximal effect obtained with 100 μM forskolin in each cell line. cAMP accumulation in CHO-K1 expressing the canine β_3-adrenoceptor corresponded to 32% (for (−)-isoproterenol) and 23% (for CGP 12177A) of the effects measured in cells expressing human β_3-adrenoceptor (Fig. 4). Similar results were found in another independent CHO-K1 clone that expressed the canine β_3-adrenoceptor at the same extent. The human HEK293 cell line was selected for a new transfection of the canine β_3-adrenoceptor. Of the 10 positive clones, one was used for the binding and adenylyl cyclase experiments.

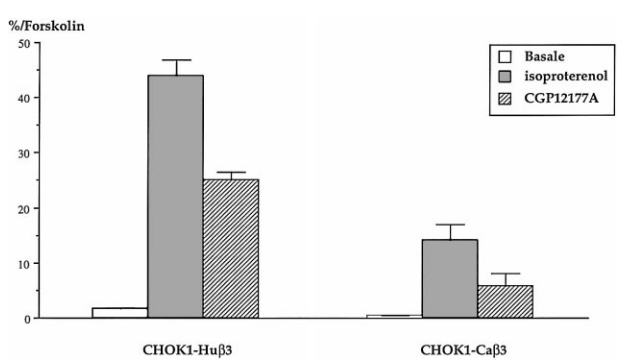

Fig. 4. Specificity of adenylyl cyclase activation properties of CHO-K1 canine- and human-β_3-adrenoceptor cells. cAMP accumulation following exposure of canine β_3-adrenoceptor (CHOK1-Caβ3) and human (CHOK1-Huβ3) cells to 100 μM (−)-isoproterenol, CGP 12177A or buffer (Basal) was measured. Data expressed in pmoles of cAMP/10^6 cells were respectively for CHOK1-Huβ3 and CHOK1-Caβ3 basal, 42 ± 3 and 5 ± 0, respectively; forskolin, 2558 ± 307 and 935 ± 73, respectively; isoproterenol, 1130 ± 71 and 132 ± 27, respectively; and CGP 12177A, 643 ± 37 and 54 ± 23, respectively. Results shown were normalized as the mean percentage of the response induced by forskolin (100 μM) in each cell line. Results are from a typical experiment performed three times in duplicate.

Table 1
Comparison of pharmacological properties of the canine and human β_3-AR expressed in HEK293 cells

	Canine β_3-AR			Human β_3-AR		
	K_i (μM)	K_{act} (nM)	IA	K_i (μM)	K_{act} (nM)	IA
β_1-β_2-β_3-Adrenoceptor agonists						
(−)-Isoproterenol	7.92 ± 1.08	60.4 ± 14.8	0.96 ± 0.06	2.37 ± 0.59	268 ± 22	0.91 ± 0.03
(−)-Norepinephrine	11.35 ± 0.52	701 ± 211	0.92 ± 0.05	5.68 ± 1.18	514 ± 216	0.71 ± 0.39
β_3-Adrenoceptor agonists / β_1-β_2-adrenoceptor antagonists						
CGP 12177A	0.40 ± 0.00	24 ± 4	1.23 ± 0.00	0.30 ± 0.02	3199 ± 1983	0.61 ± 0.09
ICI 201651	3.20 ± 1.76	528 ± 399	1.04 ± 0.27	0.60 ± 0.21	7424 ± 473	0.37
CL 316,243	0.22 ± 0.05	0.45 ± 0.27	0.74 ± 0.00	0.63 ± 0.064	3636 ± 298	0.54 ± 0.25
β_1-β_2-β_3-Adrenoceptor antagonists						
(−)-Bupranolol	0.76 ± 0.02	antagonist		6.79 ± 1.22	antagonist	
ICI 118 551	11.23 ± 1.04	antagonist		3.44 ± 0.70	antagonist	
CGP 20712A	16.03 ± 4.47	antagonist		18.11 ± 7.43	antagonist	

Binding competition experiments were carried out with membrane preparations in the presence of [^{125}I]ICYP as described in Section 2. Accumulation of cAMP experiments were performed with intact cells incubated or not with increasing concentration of ligands. Competition/response curves were fitted, using least-square regression analysis, and the determined constants, K_i and K_{act}, are expressed in micromolar or nanomolar concentrations, respectively. Intrinsic activity (IA) values were calculated relative to (−)-isoproterenol-induced maximal cAMP accumulation.

3.4. Pharmacological characterization of the canine β_3-adrenoceptor expressed in HEK293 cells

Binding experiments were performed with HEK293 membranes. A series of saturation analyses allowed the determination of the K_d and B_{max} values for [^{125}I]iodocyanopindolol. The K_d determined for the canine β_3-adrenoceptor was 10.00 ± 1.95 nM with a B_{max} of 2.8 ± 0.6 pmol/mg of protein. This K_d value was equivalent to the value determined for HEK293 expressing human β_3-adrenoceptors (Pietri-Rouxel et al., 1997) and in the same order of magnitude as values previously described for human (Emorine et al., 1989), bovine (Pietri-Rouxel et al., 1995), mouse (Nahmias et al., 1991) and rat (Granneman et al., 1991) β_3-adrenoceptors. Binding competition experiments were carried out with membrane preparations, and adenylyl cyclase stimulation and cAMP measurements were performed with whole HEK293 cells (Table 1).

Pharmacological parameters determined in [^{125}I]iodocyanopindolol binding competition experiments and adenylyl cyclase dose–response curves revealed that the canine β_3-adrenoceptor was similar to its human, bovine and rodent counterparts but with some significant characteristics (Table 1). The potency order for the canine β_3-adrenoceptor was: CL 316,243 > CGP 12177A > isoproterenol > ICI 201651 > norepinephrine; for the human β_3-adrenoceptor the potency order is: isoproterenol > norepinephrine > CGP 12177A > CL 316,243 > ICI 201651. Isoproterenol and norepinephrine displayed nearly the same affinity (K_i values) and the same potency for adenylyl cyclase stimulation (K_{act} values) for the canine β_3-adrenoceptor as for the human β_3-adrenoceptor. The β_3-selective compound ICI 201651 is the in-vivo metabolite of ICI D7114. This full agonist, which is able to stimulate brown adipose tissue in the dog (Champigny et al., 1992), was 14 times more efficient for the canine β_3-adrenoceptor than for the human receptor. CL 316,243 exhibited the highest affinity and was the most potent agonist for the canine β_3-adrenoceptor but possessed partial agonist activity (IA = 0.74 ± 0.00). This specific agonist was 8080 times more potent for canine than for human β_3-adrenoceptors. CGP 12177A, which is a partial agonist for the human receptor, was a full agonist for the canine receptor (IA = 1.23 ± 0.00). ICI 118,551 (a β_2-adrenoceptor selective antagonist) and CGP 20712A (a β_1-adrenoceptor selective antagonist) exhibited low affinity for human and canine β_3-adrenoceptors (Strosberg and Pietri-Rouxel, 1996). Bupranolol, an antagonist for mouse and human β_3-adrenoceptors (Strosberg and Pietri-Rouxel, 1996) and a partial agonist on the bovine receptor, displayed antagonist activity for the canine β_3-adrenoceptor.

4. Discussion

Since the cloning of the human β_3-adrenoceptor in 1989 (Emorine et al., 1989), and thereafter the rodent and bovine homologues (Granneman et al., 1991; Nahmias et al., 1991; Pietri-Rouxel et al., 1995), considerable effort has been expended in the identification of their exact physiological function and potential pathophysiological role. Convincing evidence has emerged to show that the β_3-adrenoceptor has the ability to modulate both lipolysis and heat generation in brown adipose tissue. This has led to speculation as to whether the use of a potent specific β_3-adrenoceptor agonist, or upregulation of the β_3-adrenoceptor in adipose tissues, could have an anti-obesity effect. We report here the cloning of the canine adrenoceptor gene. The canine β_3-adrenoceptor gene encodes a

polypeptide with an approximate molecular weight of 43 kDa and is most similar to its bovine counterpart, showing 86% sequence identity (Pietri-Rouxel et al., 1995). The coding block of the canine gene is interrupted by a 703-bp intronic sequence at positions 1309 and 2011. As in the human β_3-adrenoceptor, exon 2 contains a sequence coding for six carboxy-terminal amino acids and the stop codon. The carboxy-terminal intracellular sequence of β_3-adrenoceptors is highly conserved up to Cys^{361}, but there is less homology in the rest of the protein sequence. There was strong homology in the C-terminal sequence after Leu^{396} between dog, bovine and human β_3-adrenoceptor protein sequences (Fig. 3). This suggests that these genes share a common one intron/two exon organization (Van Spronsen et al., 1993), and not the two intron/three exon structure found in rodents (Granneman et al., 1992, 1993; Lelias et al., 1993; Van Spronsen et al., 1993). Reverse transcriptase-polymerase chain reaction (RT-PCR) studies revealed that the β_3-adrenoceptor is expressed in canine: brown adipose tissue, white adipose tissue, colon and bladder (data not shown), but not or only slightly in most other tissues. Further RT-PCR analysis was performed on vascular tissues (arteries and veins) to investigate the presence of β_3-adrenoceptor transcripts (Berlan et al., 1994). Despite high amplification, very little β_3-adrenoceptor expression was revealed by hybridization assays on PCR fragments. In contrast, β_2-adrenoceptor mRNA was readily detectable in most of these vessels.

When the coupling to G-protein and adenylyl cyclase of the canine β_3-adrenoceptor expressed in CHO-K1 cells was investigated, we observed very low cAMP accumulation after stimulation with agonists. Because the expression levels of the human and canine β_3-adrenoceptors are highly similar, the differences observed in terms of receptor coupling are probably due to species-specific differences. Coupling was much better when the canine β_3-adrenoceptor was expressed in human HEK293 cells. Taken together, these observations suggested that the canine β_3-adrenoceptor could not efficiently interact with the hamster G-protein present in the CHO-K1 cells. This could be due to several substitutions of crucial amino residues within the third intracellular loop and the carboxy-terminal tail of the canine β_3-adrenoceptor. These two regions, which are involved in the coupling to G-proteins (Guan et al., 1995), show poor sequence homology when β_3-adrenoceptors from all the species cloned so far are compared (Fig. 3). Species differences in the G-protein selectivity have also been observed for human and bovine A_1-adenosine receptors (Jockers et al., 1994). The differences found between the canine and the human β_3-adrenoceptors illustrate the importance of characterizing the structural and the functional properties of homologues of other species in the same cellular system.

Studies with HEK293 cells stably transfected with human or canine β_3-adrenoceptors showed that these had similar pharmacological profiles. Nevertheless, differences were apparent with the β_3-adrenoceptor agonists CGP 12177A, CL 316,243 and ICI 201651, which were more selective and more potent towards the canine than the human β_3-adrenoceptor. These three ligands, described as potent agonists for the human β_3-adrenoceptor expressed in CHO-K1 cells (Strosberg and Pietri-Rouxel, 1996), displayed in HEK293 cells partial agonistic properties with a K_{act} higher than 1 μM (Table 1). CL 316,243 is the most selective β_3 agonist developed so far, but has been shown to be 97 times less active on human receptors than on mouse or rat receptors expressed in CHO cells (see for review Strosberg, 1997). This ligand was the most potent agonist tested here and was 8080 times more active on canine than on human β_3-adrenoceptors (Table 1). The effect of ICI 201651 (or its metabolic precursor ICI D7114) has been well studied in the dog: the compound is a potent stimulator of the expression of uncoupling protein in brown adipose tissue (Champigny et al., 1992) and increases whole-body oxygen consumption (Holloway et al., 1991). Its high potency for canine β_3-adrenoceptors could facilitate its use in the treatment of obesity in dogs.

Animal models are now becoming available for the study of β_3-adrenoceptors in vivo. In transgenic mice expressing a toxic gene driven by a brown adipose tissue-specific promoter there is a temporary ablation of brown adipose tissue, and a severely obese phenotype related to the loss of β_3-adrenoceptor activity (Lowell et al., 1993). The targeted knockout of the β_3-adrenoceptor has been reported (Revelli et al., 1997). This deletion leads to a complete lack of effect of β_3-adrenoceptor agonist on the metabolic rate of homozygous mice, the body fat accumulation was favored with a slight increase in food intake. This shows that β_3-adrenoceptor disruption creates conditions which predispose to the development of obesity. The physiological relevance of the presence of β_3-adrenoceptors in canine adipose tissue has been demonstrated both in vivo (Champigny et al., 1991) and in vitro (Galitzky et al., 1993b). In this species, in contrast to rodents, there is little organized brown adipose tissue in adult animals but, as in humans, dormant brown adipose tissue can be reactivated by β_3-adrenoceptor agonists (Champigny and Ricquier, 1996; Champigny et al., 1991).

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sequence databases and is available under the accession number U92468.

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