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Cloning and expression of canine glucagon receptor and its use to evaluate glucagon receptor antagonists *in vitro* and *in vivo*

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Abstract

Glucose homeostasis is maintained by the combined actions of insulin and glucagon. Hyperglucagonemia and/or elevation of glucagon/insulin ratio have been reported in diabetic patients and in animal models of diabetes. Therefore, antagonizing glucagon receptor function has long been considered a useful approach to lower hyperglycemia. Dogs serve as an excellent model for studying glycemic control and various aspects of glucagon biology *in vivo*; however, the amino acid sequence of the dog glucagon receptor has not been reported. To better understand the pharmacology of the dog glucagon receptor and to characterize glucagon receptor antagonists, we cloned a cDNA corresponding to the glucagon receptor from dog liver RNA. The dog glucagon receptor shares a significant (>75%) homology at both nucleotide and amino acid levels with the glucagon receptor from human, monkey, mouse, and rat. The protein is highly conserved among all species in areas corresponding to the 7 transmembrane domains. However, it shows significant divergence at the carboxy terminus such that the receptor from dog has the longest cytoplasmic tail among all species examined. When expressed in chinese hamster ovary cells, the dog glucagon receptor bound [^{125}I]Glucagon with a K_d of 477±106 pM. Glucagon stimulated the rise of intracellular cAMP levels in these cells with an EC_{50} of 9.6±1.7 nM and such effects could be blocked by known peptidyl and non-peptidyl small molecule antagonists. In addition we show that a small molecule glucagon receptor antagonist with significant activity in cell based assays also blocked the ability of glucagon to induce elevation in blood glucose in beagle dogs. These data demonstrate that the cloned cDNA encodes a functional dog glucagon receptor. The availability of the dog cDNA will facilitate the understanding of glucagon pharmacology and aid in the characterization of novel glucagon antagonists that may serve as anti-hyperglycemic treatment for type 2 diabetes mellitus.

Index words: Glucagon; Dog Glucagon Receptor; Receptor Antagonist

1. Introduction

Glucagon is a 29 amino acid polypeptide produced in the pancreatic α-cells and secreted in response to falling glucose levels during the fasting period (Burcelin et al., 1996). Glucagon stimulates glucose production by promoting glycogenolysis and gluconeogenesis in the liver and attenuates the ability of insulin to inhibit these processes (Consoli, 1992). Both increased glucagon secretion during the fasting state and/or lack of suppression of glucagon production in the post-parandial state result in increased glucagon levels that are linked to hyperglycemia observed in diabetics (Muller et al., 1970; Reaven et al., 1987; Shah et al., 2000; Woerle et al., 2006). Thus, suppression of glucagon action has long been considered as a way to control hyperglycemia in the diabetic state. Indeed, studies using potent peptide antagonists demonstrated significant blood glucose lowering in diabetic models (Johnson et al., 1982; Unson et al., 1989). Furthermore, it has been demonstrated that immunoneutralization of glucagon in animal models of type 2 diabetes mellitus effectively diminished glucagon-stimulated hyperglycemia. Such data provide proof of concept for mitigation of hyperglycemia by antagonism of the glucagon receptor. Not surprisingly, a number of small molecules

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glucagon receptor antagonists have been reported in recent years by several groups [reviewed in (Kurukulasuriya and Link, 2005; Sloop et al., 2005)].

Development of novel therapeutics requires extensive testing in animal models prior to evaluation in humans. Dogs have long been used as one of the mainstay preclinical species for such evaluations (Parkinson and Grasso, 1993). In addition, dogs have been found to develop a diabetes phenotype that resembles human type 2 diabetes mellitus and they respond well to many antidiabetic therapies used to treat humans (Catchpole et al., 2005; Davison et al., 2003). Therefore, dogs have served as a useful model for studying the effects of numerous antidiabetic agents (Cherrington et al., 1998; Chu et al., 2000; Edgerton et al., 2005; Nelson et al., 2004; Uchino et al., 2005). In this regard, dogs have been extensively utilized to study the biological effects of glucagon and the interaction of glucagon and insulin to maintain glucose homeostasis (Cherrington et al., 1998, 1978; Dobbins et al., 1991; Edgerton et al., 2004; Jacobson et al., 2005; Wasserman et al., 1989).

Despite such wide use of dogs to study glucagon action, the sequence of the dog glucagon receptor has not been determined. Here, we report the cloning of a functional dog glucagon receptor and the characterization of novel small molecule glucagon antagonists using the cloned receptor. In addition, we demonstrate the inhibition of glucagon-induced glucose excursion by a glucagon receptor antagonist in dog.

2. Material and methods

2.1. Reagents

Chinese hamster ovary (CHO) cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal calf serum, 1 mM L-Glutamine, and antibiotic (GIBCO/Invitrogen Life Sciences, Carlsbad, CA). Glucagon and des-His$^{[1]}$-Glu$^{[9]}$-glucagon-Amide were obtained from Bachem (Bachem California Inc, Torrance, CA) and dissolved in 0.01 N Acetic Acid. Compounds evaluated as antagonists were synthesized as described previously {Compound 1 (Cpd **1)** is *N*-[3-cyano-6-(1, 1-dimethylpropyl)-4, 5, 6, 7-tetrahydro-1-benzothien-2-yl]-2-ethylbutanamide (Duffy et al., 2005; Qureshi et al., 2004), Compound 2 (Cpd **2)** is 4-({9-*tert*-butyl-2-oxo-3-[4-(trifluoromethoxy)phenyl]-1,3-diazaspiro[5.5]undec-1-yl}methyl)-*N*-1*H*-tetrazol-5-ylbenzamide (Shen et al., 2005), and Compound 3 (Cpd **3)** is 4-({(trans-4-*tert*-butylcyclohexyl)[1-methyl-5-(trifluoromethyl)-1*H*-benzimidazol-2-yl]amino}methyl)-*N*-1*H*-tetrazol-5-ylbenzamide (Kim et al., 2005)}, dissolved in 100% dimethyl sulphoxide to obtain a stock solution, and subsequently diluted in buffers to yield appropriate concentrations of compounds prior to their addition into the assays described below. All other materials were from commercial sources.

2.2. Cloning of cDNA for dog glucagon receptor

To facilitate cloning of the dog glucagon receptor we searched a Merck proprietary database of sequences expressed in dog liver. This resulted in identification of a sequence that shared a high degree of homology with the human glucagon receptor. To obtain a full-length cDNA that could be used to functionally characterize the dog glucagon receptor *in vitro* we synthesized primers based on this sequence that would bind upstream of the putative start codon, upstream and downstream of a unique Bam HI site present within the center of the cDNA sequence, and just upstream of the terminal sequence (~150 bases downstream of the last in frame stop codon). The sequence of the primers used to amplify each segment is

Segment I: 5′ of cDNA to Bam HI Site
Forward: 5′-ACCCTTTGCCCCAGCTGTGT-3′
Reverse: 5′-GATGAGGAGGACGCGGATGA-3′
Segment II: Bam HI to 3′ end of cDNA
Forward: 5′-CTGGACCAGCAACGACAA-3′
Reverse: 5′-CCGCTCCGCAGACTCTTT-3′.

With the aid of these primers we amplified sequences from total RNA prepared from a beagle dog liver using a reverse transcription (RT) and polymerase chain reaction (PCR) kit according to the instructions of the manufacturer (Access-RT-PCR, Stratagene®, La Jolla, CA). This resulted in amplification of full-length glucagon receptor sequence in two segments. The amplified PCR products were cloned into pCRII-TOPO (Invitrogen, Carlsbad, CA) following the manufacturer's protocol and clones containing full-length inserts were identified by digestion with restriction enzymes. The fidelity of these clones was further confirmed by sequencing them using a PCR-based sequencing protocol (ACGT Inc. Wheeling, IL) and comparing the sequences obtained to the dog sequence found in the database described above as well as to known glucagon receptor sequences from various species. A correct full-length cDNA was then subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) to generate pcDNA3.dGCGR.

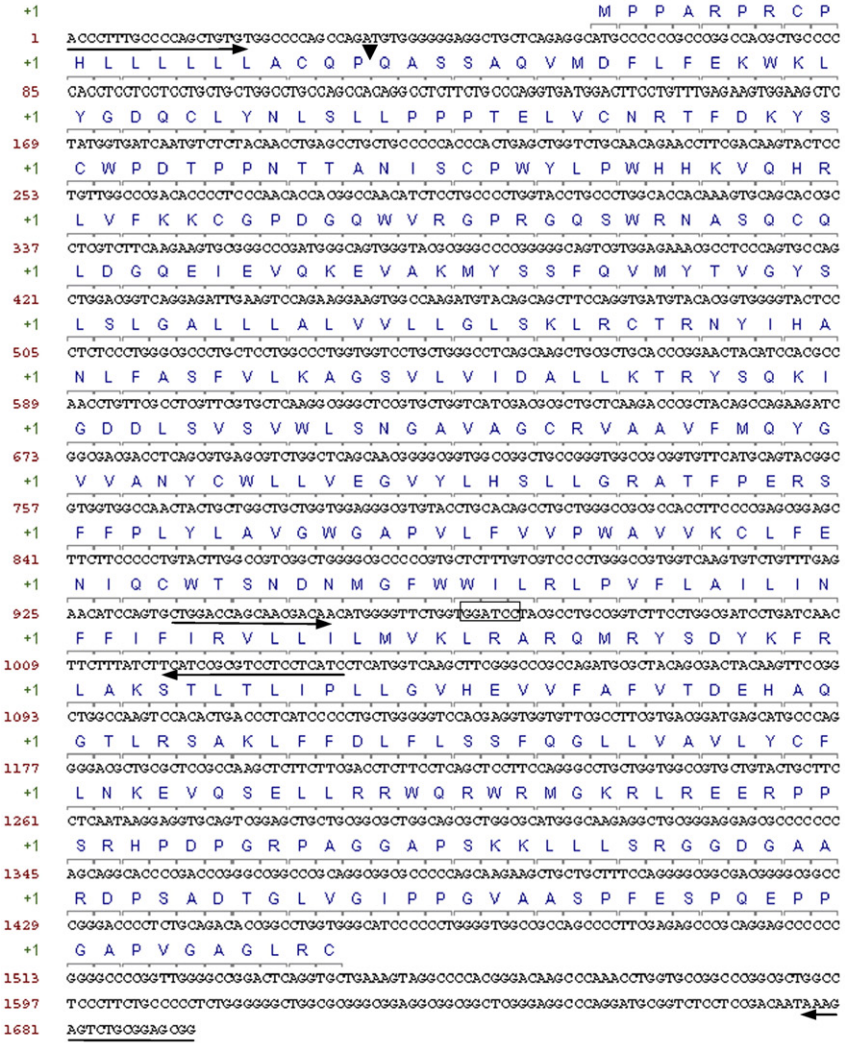
2.3. Expression of cloned dog glucagon receptor in CHO cells

20 μg of pcDNA3.dGCGR DNA was transfected into CHO cells using $CaPO_4$ precipitation technique (Sambrook et al., 1989). Stable clones expressing the dog glucagon receptor were first selected in 400 μg/ml Geneticin® (G418 sulphate, Invitrogen, Carlsbad, CA) for 14 days and then by picking isolated colonies. Each colony was expanded and then further accessed for response to human glucagon in an adenylyl cyclase activation assay described below and subcloned one time to obtain pure clonal lines. 2–3 lines were obtained and evaluated for cAMP formation in response to glucagon.

2.4. Adenylyl cyclase activation assay

Exponentially growing CHO cells expressing dog glucagon receptor (CHO.dGCGR) and untransfected CHO cells were harvested with the aid of enzyme-free dissociation media (Mediatech, Herndon, VA) and used to establish a glucagon dose response as described (Qureshi et al., 2004). The activities of known glucagon antagonists were examined as described previously (Qureshi et al., 2004).

A



B

	Dog	Human	Monkey	Mouse	Rat
Dog	100	81	82	76	76
Human		100	92	81	82
Monkey			100	78	78
Mouse				100	95
Rat					100

C

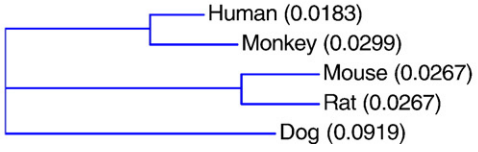
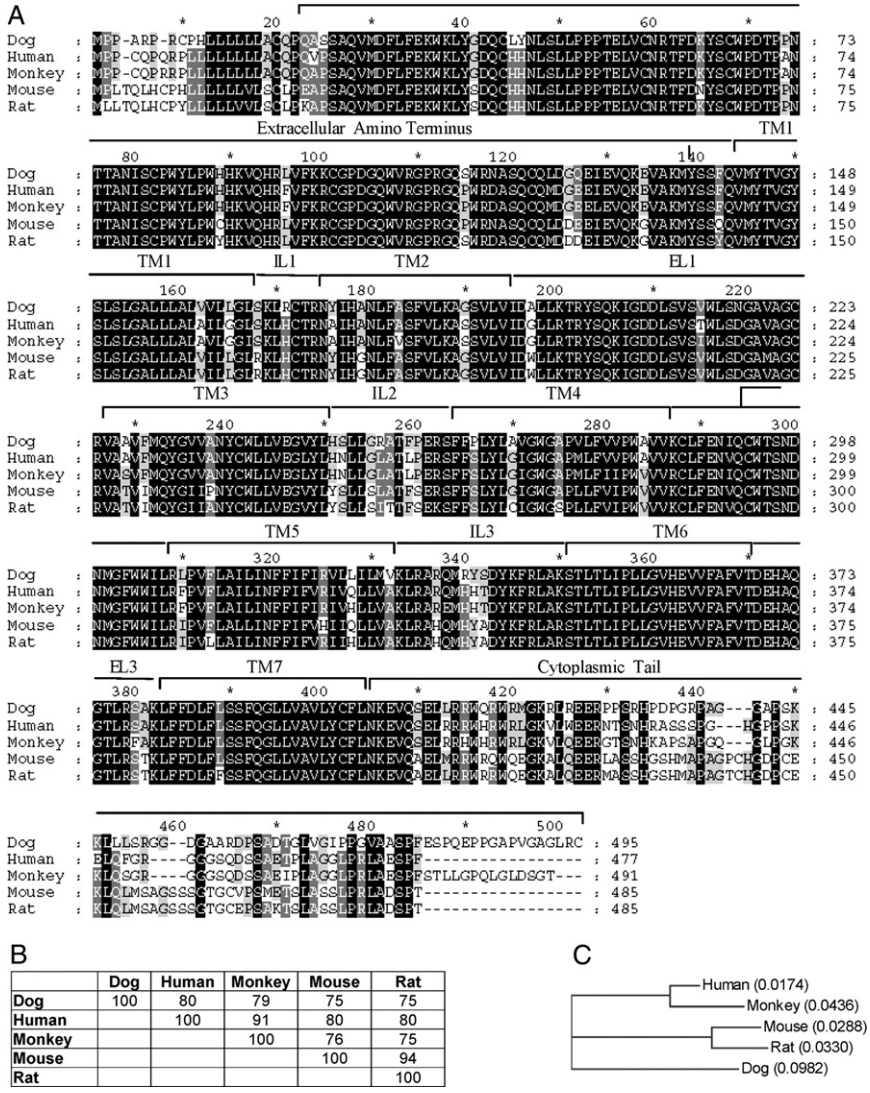


Fig. 1. Sequence of the dog glucagon receptor and comparison with the same from other species. (A) Sequence of the full-length dog glucagon receptor with *in silico* translation. Arrows indicate the position and direction of the primers for amplification of this sequence by RT-PCR. A Bam HI site in the middle of the sequence used to ligate the amplified fragments is boxed. ▼ marks the position of predicted cleavage site occurring after the putative signal sequence. (B) Homology of glucagon receptor from different species. Table shows % homology between the coding region of glucagon receptor gene between each species as determined by using AlignX function of software Vector NTI Suite 9.0.0 (Informix Inc., CA). (C) Phylogenetic relationship between the glucagon receptors of different species. The genetic dendrogram was created using AlignX function of vector NTI Suite 9.0.0 (Informix Inc., CA) using the coding region of glucagon receptor from each species as described above following the algorithm proposed by Saitou and Nei (Saitou and Nei, 1987).



	Dog	Human	Monkey	Mouse	Rat
Dog	100	80	79	75	75
Human		100	91	80	80
Monkey			100	76	75
Mouse				100	94
Rat					100

Fig. 2. Homology between the dog glucagon receptor and the same from other species at the amino acid level. (A) Alignment of the primary amino acid sequence of dog glucagon receptor with the same from human, monkey, mouse, and rat. Amino acid sequences were compared using AlignX function of software Vector NTI Suite. Positions of different regions of the receptor are indicated above the sequence as has been proposed for human glucagon receptor (Macneil et al., 1994) and labeled as follow; transmembrane region as TM1-7, extracellular loops regions as EL1-3, intracellular loop regions as IL 1-3, amino and carboxyl termini as such. (B) Homologies between glucagon receptors from dog, human, monkey, mouse and rat. (C) Phylogenetic relationship at the amino acid level levels. The dendrogram was created following the algorithm of Saitou and Nei (Saitou and Nei, 1987) with the aid of software package Vector NTI Suite 9.0.

2.5. Whole cell binding assay

For equilibrium binding studies, CHO.dGCGR cells were plated at 90% confluence. The next day, cells were washed twice with serum free medium and incubated at 4 °C for 1 h in DMEM plus 10% fetal calf serum with or without 2 μM unlabelled glucagon to establish non specific binding. After that [^{125}I]Glucagon (PerkinElmer, Boston, MA) was added to individual wells to achieve a final concentration of [^{125}I] Glucagon in the range of 0.03–3.0 nM. After overnight incubation at 4 °C an aliquot of supernatant was removed to establish unbound glucagon in each well and each well was washed three times with ice-cold serum free medium. Cells in each well were lysed by addition of 500 μl of 0.1% SDS and 450 μl of the lysate was counted in a gamma counter (Packard Instruments, Downers Grove, IL) to establish the amount of [^{125}I]Glucagon bound in each well. The data obtained from these studies was processed in Graphpad PRISM software using single site binding curve fit and regression analysis to establish K_d and B_{max}. For competition curve using cold glucagon shown in Fig. 4, cells plated as above were washed twice with serum free IMDM and incubated with increasing concentrations of cold glucagon in Gavin's buffer (100 mM HEPES, 120 mM sodium chloride, 1.2 mM magnesium sulphate, 15 mM sodium acetate, 0.25% bovine serum albumin) at 4 °C for 1 h. Subsequently, [^{125}I]Glucagon (20,000 cpm/well) was added and incubation was continued for another 18 h at 4 °C. After that cells were washed three times with serum free medium and lysed by addition of 500 μl of 0.1% SDS. 450 μl of the lysate was counted as described above.

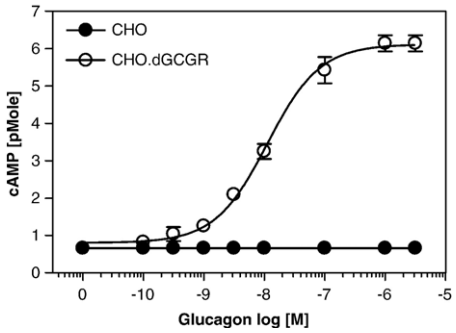


Fig. 3. Adenylyl cyclase activation in CHO cells expressing the dog glucagon receptor cDNA. CHO.dGCGR and untransfected CHO cells were stimulated with glucagon at the doses indicated for 30 min and cAMP levels were measured with the aid of a competitive RIA as described in the "Material and methods" section. Data is the Mean ± S.D. of 2–3 replicates. Similar data were obtained in three independent experiments.

2.6. Evaluation of glucagon antagonism by a small molecule antagonist in beagle dogs

Male beagle dogs (Marshall Farms, North Rose, NY) weighing between 8–15 kg were fasted for 15 h prior to the procedure. Fasted animals were given an oral dose (10 mg/kg) of Cpd **3** resuspended in 0.5% methylcellulose (vehicle) or vehicle only in a volume of 2 ml/kg. One hour after oral dosing, animals were submitted to glucagon challenge by a single intramuscular injection (5 μg/kg) of glucagon (Glucagon, rDNA origin, Eli Lilly, Indianapolis, IN). Glucose levels at each time point were established with the aid of a hand held glucose meter (OneTouch® Ultra, LifeScan Inc., Milpitas, CA). All animal care and procedures were conducted in accordance with institutional guidelines.

2.7. Data analysis

All data analysis was performed with the aid of software package GraphPad Prism (GraphPad Software Inc., San Diego, CA) and Vector NTI Suite 9 (InforMax/Invitrogen, Carlsbad, CA). The following sequences were used to compare the dog glucagon receptor sequence with that from other species; Human [(Lok et al., 1994), GenBank accession no. L20316], Monkey (Mcnally et al., 2004), Mouse [(Burcelin et al., 1995), GenBank accession no. L38613], and Rat [(Jelinek et al., 1993), GenBank accession no. M96674].

3. Results

3.1. Cloning of cDNA corresponding to dog glucagon receptor

cDNA sequences corresponding to glucagon receptor were amplified from beagle dog liver RNA with the aid of RT-PCR using primers based on a sequence in our internal data base that bore a high degree of homology to human glucagon receptor (Fig. 1A). This cloned dog sequence is 81%, 82%, 76%, and 76% homologous to glucagon receptor sequences from the human, monkey, mouse, and rat, respectively (Fig. 1B). Comparison of the dog glucagon receptor sequence with that of other species indicates that the dog sequence has diverged significantly from those of human, monkey, rat and mouse. Further comparison demonstrates that glucagon receptor sequences from human and monkey, and mouse and rat are more homologous to each other than to dog. Nevertheless, each of these two sequence pairs shows significant divergence from each other as well as from the dog sequence (Fig. 1C).

In silico translation of the dog cDNA suggests that this sequence can produce a protein of 495 amino acids that shows notable homology to glucagon receptors from other species (Fig. 2A). Specifically, at the amino acid level, the putative dog protein shares an 80%, 79%, 75%, and 75% homology to the glucagon receptor from human, monkey, mouse, or rat respectively (Fig. 2B). Comparison of the dog protein sequence with that of the other species indicates the same trend as was seen at the nucleotide levels: the dog sequence clusters away from the other sequences whereas human and rhesus, and mouse and rat sequences cluster together (Fig. 2C). While the dog glucagon receptor sequence bears a significant similarity to other species in the transmembrane (TM) and extracellular loop regions, it displays significant divergence from all species past the seventh TM region. In addition, the dog glucagon receptor sequence has additional 17 amino acids at the carboxyl terminus. Further comparison of the glucagon receptor sequences shows a high degree of conservation among all species in the seven transmembrane domains and in the extracellular loop region, but less conservation in the intracellular loop regions and at the carboxyl terminus (Fig. 1C).

3.2. Expression of the cloned sequences in CHO cells

The binding of glucagon to glucagon receptors from various species exogenously expressed in CHO cells has previously been shown to increase intracellular cAMP levels (Cascieri et al., 1999; Mcnally et al., 2004; Parker et al., 2000). To demonstrate that the cloned dog cDNA as described above could produce a biologically active protein capable of conferring a glucagon response to an otherwise non-responsive cell line, we transfected it into CHO cells under the control of

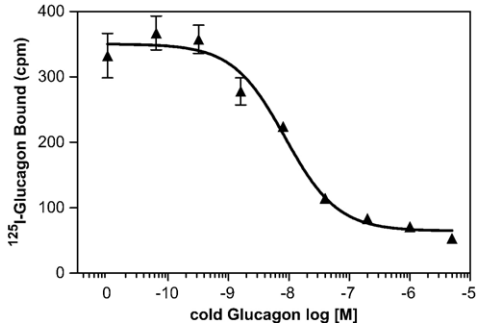
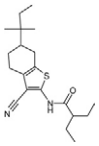
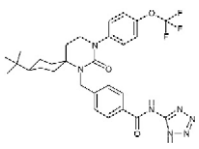
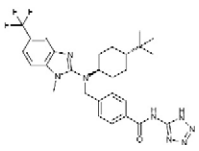


Fig. 4. Binding of [^{125}I]Glucagon and its displacement by unlabelled glucagon. CHO.dGCGR cells were incubated with [^{125}I]Glucagon at 4 °C for 18–20 h in presence or absence of cold glucagon as described in the "Material and methods" section. Data shows amount of ^{125}I-Glucagon bound as dpm per well and is a Mean ± S.E.M. of three replicates. Invisibility of the error bars is due to a smaller error bar size than the symbol size. Similar data were obtained in three independent experiments.

cytomegalovirus promoter as described in the "Material and methods" section. As shown in Fig. 3, glucagon induced dose-responsive increases in the cAMP levels with an EC_{50} of 9.6±1.7 nM in cells expressing the dog cDNA (CHO.dGCGR) whereas no such effect was observed in untransfected CHO cells. Additional CHO.dGCGR cell lines also showed a similar dose response (not shown). In order to determine the affinity of glucagon towards the dGCGR, we performed equilibrium binding studies using CHO.dGCGR cells and [^{125}I]Glucagon (as described in 'Material and methods'). The analysis of these data suggested that [^{125}I]Glucagon binds to these cells with a K_d of 476±110 pM and this clone expresses (B_{max}) 42,267±3869 receptors per cell (Date is Mean±S.E.M. of three independent determinations performed in quadruplicate). Furthermore, cold glucagon inhibited the binding of [^{125}I]Glucagon to these cells with an IC_{50} of 9.6±2.0 nM (Fig. 4). Taken together, these data demonstrate that expression of our cloned dog cDNA in CHO cells confers binding of glucagon that results in glucagon-dependent induction of intracellular cAMP levels in a manner similar to what has been observed with glucagon receptors from other species. Thus, this novel sequence represents a *bona fide* dog glucagon receptor.

Table 1
Inhibition of dog and human glucagon receptor activation by glucagon receptor antagonists[1]

Antagonist	Structure	IC_{50} (nM) dog	IC_{50} (nM) human
des-His-Glucagon-amide	Peptide	21.4±3.5	19.4±1.2
Cpd **1**		525.5±47.1	129.0±33.0[a]
Cpd **2**		359.8±50.6	92.0±29.0[b]
Cpd **3**		472.5±133.6	58.0±15.4[c]

[1]CHO cells expressing either dog or human glucagon receptors were incubated with compounds at various doses for 30 min and then treated with glucagon (2 nM or 0.25 nM for cells expressing dog or human receptors respectively) to induce cAMP formation as described in "Material and methods". Data shows the IC_{50} for each antagonist on respective cell line and is a Mean±S.E.M. of 3–4 independent determinations. Data for the activities of the compounds on human glucagon receptor has been described previously [[a] in (Duffy et al., 2005) as compound **2**, [b] in (Shen et al., 2005) as compound **15** and [c] in (Kim et al., 2005) as compound **2**].

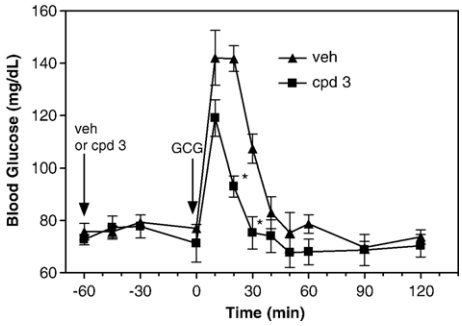


Fig. 5. Blockade of glucagon response by a glucagon receptor antagonist in beagle dog. Glucose levels in overnight fasted beagle dogs that were dosed with 0.5% methylcellulose (veh, ▲) or cpd **3** at 10 mg/kg in the same vehicle (cpd 3, ■) at time −60 min and challenged with a single intramuscular dose (5 μg/kg) of glucagon (GCG) at time zero. The data is Mean±S.E.M. (n=3 in each group). $*p<0.5$ *vs* veh at each time point; student t test.

3.3. Activity of glucagon receptor antagonists

A number of peptidyle and non-peptidyle glucagon receptor antagonists have been reported in recent years (De Laszlo et al., 1999; Duffy et al., 2005; Kurukulasuriya et al., 2004; Madsen et al., 2002; Parker et al., 2000; Petersen and Sullivan, 2001; Qureshi et al., 2004; Shen et al., 2005; Unson et al., 1989). des-His$^{[1]-}$Glu$^{[9]-}$glucagon-amide is a potent peptidyle antagonist that has been shown to inhibit the activity of glucagon on human, rat and rabbit receptors (Unson et al., 1989, 1987). Compounds (Cpd) **1**–**3** are small molecule antagonists that have been shown to block the activity of human glucagon receptor *in vitro* and *in vivo* (Duffy et al., 2005; Kim et al., 2005; Qureshi et al., 2004; Shen et al., 2005). To determine if these antagonists also display affinity for the dog glucagon receptor, we evaluated them as inhibitors of glucagon-stimulated cAMP formation in a CHO.dGCGR cell line. We found that des-His-glucagon blocked the ability of human glucagon to stimulate cAMP production with an IC_{50} of 20.4±3.5 nM, while Cpd **1**–**3** blocked this response with IC_{50} of 525.5±47.1 nM, 359.8±47.1 nM, and 472.5±133.6 nM, respectively (Table 1). The activity of des-His-glucagon in this cell line was similar to that which has been observed in cells expressing the human glucagon receptor (Duffy et al., 2005). In contrast, the small molecule antagonists were less potent in blocking glucagon-induced cAMP production in CHO.dGCGR cells than in cells expressing the human glucagon receptor as illustrated by higher IC_{50} in the former cells (Table 1). Nevertheless, these data demonstrate that this new generation of glucagon antagonists can abrogate glucagon activation of the dog glucagon receptor by glucagon and, therefore, might prove useful in studying the impact of glucagon antagonism in dogs.

3.4. Effect of small molecule glucagon antagonist in beagle dog

The ability of Cpd **3** to suppress the effects of glucagon *in vivo* was examined in overnight fasted beagle dogs. Treatment of the dogs with exogenous glucagon resulted in increases in blood glucose levels as has previously been observed in humans and rodent (Petersen and Sullivan, 2001;

Qureshi et al., 2004). However, this hyperglycemic effect was significantly blunted in animals treated orally with Cpd **3** (10 mg/kg) 1 h prior to the glucagon challenge (Fig. 5). These data demonstrate that a glucagon antagonist can inhibit glucagon activation of the endogenous glucagon receptor in beagle dogs.

4. Discussion

Whole body glucose homeostasis is maintained by the competing actions of insulin and glucagon. Whereas insulin stimulates clearance of excess glucose in the post-prandial state by promoting glucose uptake by the skeletal muscle and adipose tissue while inhibiting glucose output by the liver, glucagon defends against falling glucose levels in the post-absorptive state by inducing hepatic glucose production (Burcelin et al., 1996; Consoli, 1992). Insufficient insulin secretion, reduced efficacy of insulin and elevated glucagon levels all have been implicated in the development of type 2 diabetes mellitus (Unger, 1978; Unger and Orci, 1975). Thus, agents that trigger insulin production, such as sulfonylureas (Dailey, 2005), increase insulin sensitivity, such as PPARγ agonists (Berger and Moller, 2002), or reduce hepatic glucose production, such as metformin (Davidson and Peters, 1997), have proven efficacious in the treatment of type 2 diabetes mellitus patients. While glucagon has long been recognized as a major player in the regulation of endogenous glucose production, finding small molecules that suppress the activity of glucagon *in vivo* has been extremely challenging. Although a number of small molecule glucagon receptor antagonists have been reported in recent years, only one has been evaluated in normal subjects (Petersen and Sullivan, 2001) and none in diabetic patients to date.

Development of a new chemical entity to treat a human condition requires extensive characterization in a variety of *in vitro* assays and animal models prior to evaluation of its efficacy in humans. Dogs are routinely used for the safety assessment of new potential therapeutics. In addition, dogs have also been used to study the effects of insulin and glucagon *in vivo*, and have served as a very attractive model for understanding of human diabetes. In light of this, we undertook the cloning of the dog glucagon receptor as a first step towards the evaluation of novel glucagon antagonists.

The glucagon receptor belongs to family B of guanine binding protein coupled receptors (GPCR), which is characterized by the presence of a relatively long amino terminus that is displayed extracellularly on the target cells. This feature is also conserved in the cloned dog glucagon receptor. Glucagon is a 29 amino peptide that binds to the extracellular surfaces of its receptor. Since the glucagon sequence is completely conserved in human, monkey, mouse and rat, it is not surprising that the glucagon receptors from these species as well as dog show a high sequence homology in the regions that compose the glucagon binding site: the amino terminus and extracellular loops. Similarly, since the transmembrane regions of the receptor are believed to form a highly ordered tertiary structure based on the crystal structure of rhodopsin (Palczewski et al., 2000), the observed high sequence homology of these domains is not unanticipated. While the glucagon receptor from dog as well as from other species show a high degree of homology up to the seventh transmembrane region, the rest of the sequence bears little similarity amongst the species examined. This is not unexpected as this part of the glucagon receptor is believed to be involved in interactions with downstream signaling molecules (Bockaert et al., 2003, 2004), and has been found to be divergent in other glucagon receptors (Lenzen et al., 1998). The heterogeneity in this sequence may represent an adaptation to compensate for changes in those components that may occur independently during the course of evolution. Aside from generalized divergence in the primary amino acid sequence, the dog glucagon receptor also contains an extension at the carboxyl terminus that is not present in sequences from human, mouse or rat. However, such an extension is not unique to dog and has been reported in the glucagon receptor from various monkey species (Mcnally et al., 2004). While understanding the precise role of this extension in glucagon signal transduction within the dog will require further investigation, it may be necessary for the hormone to attain its maximal biological effects in this species.

Since several known small molecule antagonists exhibit different potencies towards human and rodent glucagon receptor (Cascieri et al., 1999; Djuric et al., 2002; Jiang and Zhang, 2003), we examined the activities of several recently described glucagon antagonists on the dog glucagon receptor. des-His^{1}-[Glu^{9}]-glucagon is a modified glucagon peptide in which histidine at position 1 has been removed, aspartic acid at position 9 has been with substituted with glutamic acid, and an amide has been added at the carboxyl terminus while the rest of the molecule is identical to the native glucagon (Unson et al., 1987). des-His^{1}-[Glu^{9}]-glucagon competes with the binding of glucagon to its receptor binding and thereby inhibits activation of human, rat and rabbit glucagon receptor by the hormone receptors (Unson et al., 1989, 1987), thus, this synthetic peptide appears to be able to tolerate differences that may exist in sequences required for its interaction with the glucagon receptors of different species. While the precise coordinates for its binding are not known, it is reasonable to hypothesize that it may bind to the glucagon receptor in more or less the same way as native glucagon. Indeed, when tested as an antagonist against the dog glucagon receptor this peptide exhibited a potency that is similar to what was observed with the human receptor. In contrast, all 3 small molecule antagonists tested here exhibited reduced potencies on the dog glucagon receptor relative to the human receptor, consistent with the previous observation that small molecule antagonists are sensitive to sequence changes in the receptor's transmembrane domains (Chicchi et al., 1997). While the precise interaction site of these small molecules needs to be established, it is possible that these may not complex solely to the glucagon receptor intracellular domain as has been observed in the past (Chicchi et al., 1997).

Activation of the glucagon receptor *in vivo* results in the rapid elevation of circulating glucose levels. This paradigm has previously been used to demonstrate the ability of glucagon antagonists to block glucagon-induced hyperglycemia in non-

diabetic rodents and humans (Kurukulasuriya et al., 2004; Petersen and Sullivan, 2001; Qureshi et al., 2004; Shen et al., 2005). An important benefit of having the cloned dog glucagon receptor in hand is that it allows us to establish the precise intrinsic potency of novel glucagon receptor antagonists *in vitro* before evaluating their efficacy *in vivo* in this species. Indeed, when we tested Cpd **3** that had shown significant antagonism on the dog glucagon receptor we found that it also inhibited the glucagon-stimulated hyperglycemia in a normal beagle. These results not only demonstrate the utility of the dog in characterizing the next generation of glucagon receptor antagonists, but also indicate that we have identified a novel antagonist that should prove useful to researchers studying the pharmacology and biology of glucagon in this model.

In summary, we have reported the sequence of a glucagon receptor cloned from dog, which produces biological responses ascribed to the action of glucagon when expressed in CHO cells. We have used this dog receptor to demonstrate the *in vitro* activity of several novel glucagon receptor antagonists, one of which also blocked glucagon induction of circulating glucose levels *in vivo*. The availability of this new clone will aid in furthering our understanding of dog glucagon receptor pharmacology. Furthermore, it may also assist in identification and characterization of novel glucagon antagonists to treat diabetes in humans and dogs alike.

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