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Long acting analogue of the calcitonin gene-related peptide induces positive metabolic effects and secretion of the glucagon-like peptide-1 ☆

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ABSTRACT

The pharmacological potential of Calcitonin gene-related peptide (CGRP) beyond vasodilation is not completely understood and studies are limited by the potent vasodilatory effect and the short half-life of CGRP. In particular, the effects of CGRP on metabolic diseases are not clarified. A peptide analogue of the α form of CGRP (αAnalogue) with prolonged half-life (10.2 ± 0.9 h) in rodents was synthesised and used to determine specific metabolic effects in 3 rodent models; normal rats, diet-induced obese rats and the Leptin deficient mouse model (ob/ob mice). The αAnalogue (100 nmol/kg) induced elevated energy expenditure and reduced food intake after single dosing in normal rats. In addition, the αAnalogue increased levels of circulating Glucagon-Like Peptide-1 (GLP-1) by >60% and a specific concentration dependent CGRP-induced GLP-1 secretion was verified in a murine L-cell line. Two weeks treatment of the type 2 diabetic ob/ob mice with the αAnalogue caused reduction in fasting insulin levels (199 ± 36 pM vs 332 ± 68 pM) and a tendency to reduce fasting blood glucose (11.2 ± 1.1 mM vs 9.5 ± 0.5 mM) and % glycosylated haemoglobin (HbA1c) (5.88 ± 0.17 vs 5.12 ± 0.24), demonstrating a potential anti-diabetic effect. Furthermore, two weeks treatment of diet-induced obese rats with the αAnalogue caused reduction in food intake and a significant decline in body weight (3.6 ± 1.9 g vs. −36 ± 1.1 g). We have demonstrated that long-acting CGRP analogues may have a therapeutic potential for the treatment of type 2 diabetes through positive metabolic effects and effect on GLP-1 secretion.

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1. Introduction

Calcitonin gene-related peptide (CGRP) is a 37 amino acid neuropeptide, which belongs to the calcitonin peptide family (Morris et al., 1984; Russell et al., 2014). Two distinct homologues of CGRP have been identified, αCGRP and βCGRP. αCGRP is generated by tissue specific alternative splicing of the calcitonin gene and βCGRP is encoded by a closely related gene (Rosenfeld et al., 1983; Amara et al., 1985). αCGRP is widely distributed in the central, peripheral and enteric nervous system (Skofitsch and Jacobowitz, 1985; Takami et al., 1985; Su et al., 1987; Kruger et al., 1988). It has also been proposed to act as a neurotransmitter being released in the neuromuscular junction in response to physiological stimuli (Takami et al., 1985; Uchida et al., 1990). The role of CGRP in enteric nerves is currently unknown. CGRP is one of the most potent vasodilators known (Brain et al.,1985) and CGRP mediated neurogenic arterial vasodilation has been suggested to be important in the pathology of migraine (Benemei et al., 2007; Crowley et al., 2015; Karsan and Goadsby, 2015). Selective CGRP antagonists for treatment of migraine are under development and have shown efficacy in phase III studies (Edvinsson and Ho, 2010).

CGRP exerts its effect through CGRP receptors containing a 7TM receptor moiety (CLR) and a Receptor activity modifying protein 1 (RAMP1) molecule (McLatchie et al., 1998). The receptor family has been reviewed (Poyner, 1995; Barwell et al., 2013). In isolated muscle and muscle cell lines, CGRP has been shown to inhibit glycogen formation (Pittner et al., 1996).

The effect of CGRP on metabolic processes has been given less attention, although a number of studies demonstrate both pro- and anti-diabetic roles of CGRP (Russell et al., 2014). It has been shown that CGRP can reduce food intake in rodents after

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peripheral as well as central administration (Krahn et al., 1984; Lutz et al., 1997) and Danaher et al. (2008) have shown that αCGRP plays a role in controlling systemic lipid availability and utilisation.

In isolated skeletal muscle, αCGRP increased muscle free-fatty acids (FFA) content, reduced triglycerides (TG) levels and increased fatty acid oxidation. In vivo, αCGRP infusion led to an increase in FFA and reduction of TG in soleus muscle, liver and plasma. These studies indicate that by reducing muscle and liver lipids, CGRP might be able to increase insulin sensitivity (McGarry, 2002).

In contrast, it has been implicated that CGRP impairs insulin action in vitro in liver and skeletal muscle (Leighton and Cooper, 1988; Rossetti et al., 1993). The ability of CGRP to acutely induce insulin resistance has been confirmed in vivo in insulin clamp studies, showing that CGRP inhibits the ability of insulin to stimulate peripheral glucose uptake as well as inhibit hepatic glucose output (Molina et al., 1990; Rossetti et al., 1993). Furthermore, it was recently published that mice lacking αCGRP displayed increased energy expenditure and reduction in respiratory quotient as compared to wild type mice (Walker et al., 2010). When challenged with a high fat diet, mice were protected against high fat diet induced weight gain. This was also reflected in an improved glucose homoeostasis. These data indicate that CGRP display negative metabolic effects.

To explore the role of CGRP on metabolic effects, we have investigated the effect of our αCGRP analogue that has a protracted pharmacokinetic profile using a technology as described previously (Jonassen et al., 2012; Lau et al., 2015). This analogue was used for 2 weeks once daily treatment of ob/ob mice and Diet Induced Obese (DIO) rats and for acute single dose studies in normal rats and demonstrated a net positive effects on body weight and glucose homeostasis.

2. Material and methods

2.1. CGRP analogue synthesis and purification

An analogue of the α-form of the CGRP (Serinyl-α-CGRP (2–37)-amide) with an albumin binding fatty acid moiety in the N-terminus (αAnalogue, Fig. 1) was synthesised on a ChemMatrix PAL resin using a ABI433A peptide synthesiser. 9H-fluoren-9-ylmethoxycarbonyl protected amino acid based peptide chemistry was used with standard peptide chemistry coupling protocols. Pseudoprolins (Novabiochem) in position 5–6, 8–9 and 16–17 was employed. The albumin binding moiety was attached to a Ser residue at the N-terminus in a stepwise manner as a continuation of the peptide synthesis using 2-[2-[2-(9H-fluoren-9-ylmethoxycarbonylamino)ethoxy]ethoxy]acetic acid (4S)-5-tert-butoxy-4-(9H-fluoren-9-ylmethoxycarbonylamino)-5-oxo-pentanoic acid and the mono-t-butyl ester of octadecane dioic acid as the coupling agents. Deprotection (trifluoroacetic acid (TFA), water, triisopropyl silane, 95/2.5/2.5) and purification (preparative high-pressure liquid chromatography on a reverse-phase C18 column using acetonitrile, water, TFA) was standard.

The prolonged αCGRP analogue has a half-life of $10.2 \pm 0{,}9$ h and will be nominated αAnalogue.

Native human αCGRP was purchased from Bachem.

2.2. In vitro biology

2.2.1. CGRP-induced cAMP accumulation in CGRP receptor expressing cells

Chinese hamster ovary (CHO) cells were stably transfected with calcitonin receptor-like receptor (CRLR), receptor activity modifying protein-1 (RAMP1) and CRE luciferase (cAMP responsive element for control of luciferase gene expression). Luciferase is a reporter-gene reporting accumulation of intracellular cAMP.

Cells were cultured in F12 media supplemented with GlutaMAX™ (Gibco), Foetal calf serum (FCS) (10%, Gibco), Penicillin–streptomycin (1%, Lonza), G418 (400 μg/ml, Gibco), Blasticidine (10 μg/ml, Corning), Hygromycin (400 μg/ml, Calbiochem).

Cells were seeded in 96 well plates, (20,000 cells/well) on the day before the experiment. On the day of the experiment, media was replaced by 50 μl/well assay medium (Dulbecco's media w/o phenol red (Gibco) supplemented with 10% Foetal calf serum (FCS) (10%, Gibco), Hepes (10 mM, Gibco), GlutaMAX™ (2 mM).

Peptides were diluted in assay media before added to cells for stimulation, 50 μl/well.

Cells were incubated 5% CO_2, 37 °C for 4 h.

Assay medium and peptides were replaced by phosphate buffered saline (PBS), 100 μl/well and LucLite™ (PerkinElmer), 100 μl/well were added. The plate was sealed before incubation for 30 min at room temperature (21 °C). Luciferase activity (luminescens, 7 s/well) was measured (TopCount®, NXT, Packard).

Dose–response curves were fitted by use of GraphPad Prism software and pEC_{50} values were calculated.

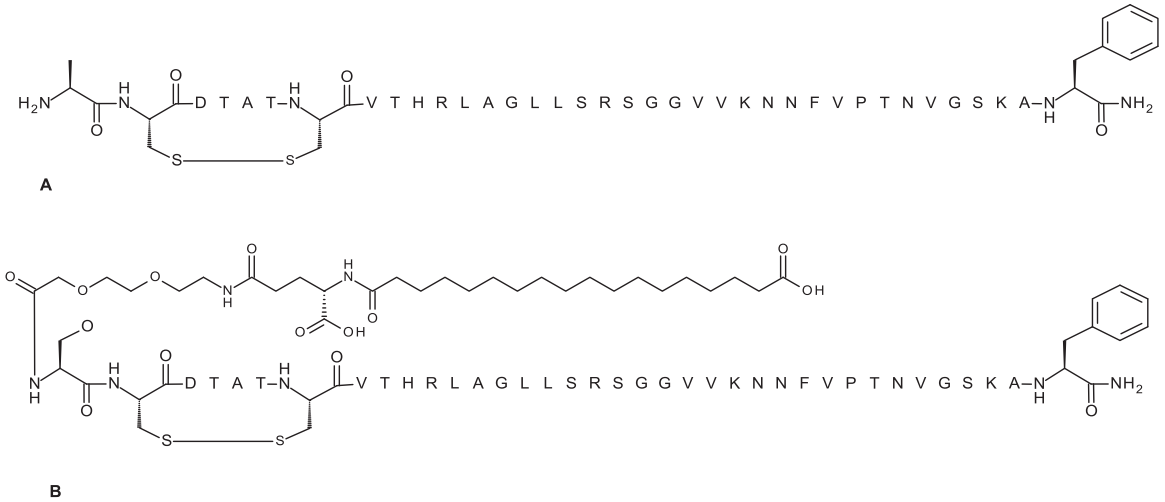

Fig. 1. Structure of αCGRP (A) and αAnalogue (B).

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