Biochimica et Biophysica Acta 1850 (2015) 343–351

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Pancreatic polypeptide regulates glucagon release through PPYR1 receptors expressed in mouse and human alpha-cells☆

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ARTICLE INFO

Article history:
Received 2 July 2014
Received in revised form 1 November 2014
Accepted 4 November 2014
Available online 8 November 2014

Keywords:
Pancreatic polypeptide
Islets
Glucagon secretion
Glucose
Y4
PPYR1

ABSTRACT

Background: Plasma levels of pancreatic polypeptide (PP) rise upon food intake. Although other pancreatic islet hormones, such as insulin and glucagon, have been extensively investigated, PP secretion and actions are still poorly understood.
Methods: The release of PP upon glucose stimulation and the effects of PP on glucagon and insulin secretion were analyzed in isolated pancreatic islets. Expression of PP receptor (PPYR1) was investigated by immunoblotting, quantitative RT-PCR on sorted pancreatic islet cells, and immunohistochemistry.
Results: In isolated mouse pancreatic islets, glucose stimulation increased PP release, while insulin secretion was up and glucagon release was down. Direct exposure of islets to PP inhibited glucagon release. In mouse islets, PPYR1 protein was observed by immunoblotting and quantitative RT-PCR revealed PPYR1 expression in the FACS-enriched glucagon alpha-cell fraction. Immunohistochemistry on pancreatic sections showed the presence of PPYR1 in alpha-cells of both mouse and human islets, while the receptor was absent in other islet cell types and exocrine pancreas.
Conclusions: Glucose stimulates PP secretion and PP inhibits glucagon release in mouse pancreatic islets. PP receptors are present in alpha-cells of mouse and human pancreatic islets.
General significance: These data demonstrate glucose-regulated secretion of PP and its effects on glucagon release through PPYR1 receptors expressed by alpha-cells.

1. Introduction

Pancreatic polypeptide (PP) was initially obtained as a by-product of insulin purification from chicken pancreatic extracts and later characterized in mammals [1,2]. It is a 36 amino acid protein, mainly produced in PP-cells (also named F-cells) of the endocrine pancreas [3]; as well as by a small fraction of the exocrine pancreas, gastrointestinal tract and rat adrenal medulla [4]. The presence of PP was also reported in the central nervous system (CNS) [5,6], although this point is still controversial [7]. Protein- and fat-rich meals elicit a strong stimulatory effect on PP release [8], resulting in elevated plasma levels up to 6 h after food ingestion in humans [9]. The food intake-induced rise in PP is abolished by total pancreatectomy, indicating that the pancreas is the main source of the polypeptide [10]. Some observations suggest that PP release is mainly controlled by peripheral neural elements, the main mediator of PP release being the vagal cholinergic innervation [10,11]. Adrenergic stimulation may also promote PP release after insulin-induced hypoglycemia and exercise [12–15]. In addition, cholecystokinin secreted by intestinal L-cells increases PP levels [8,10].

PP belongs to the neuropeptide Y (NPY) family of proteins, which includes NPY, peptide YY (PYY) and PP. These proteins induce cellular responses that are mediated through the Y family of inhibitory G-protein-coupled receptors (Y1-Y5, y6) [16]. NPY proteins exhibit a wide range of affinities for the different Y receptors, PP exhibiting higher affinity for Y4 (also named PPYR1) and lower for Y5 [17]. PPYR1 has been detected in the CNS in both rodents [18–21] and humans [22,23] by *Northern blot*, receptor autoradiography, in situ hybridization, histoimmunochemistry, and RT-PCR. *Northern blot* expression studies suggest that a wide range of peripheral tissues may also contain PPYR1 [20–24]. Presence of PPYR1 mRNA has been reported in human total pancreas preparations, containing both exocrine and endocrine fractions [22,24], although not in rodent pancreas.

PP has recently gained interest due to the major role this peptide plays in the control of appetite. Indeed, peripheral PP acts as a long term satiety signal both in mice and humans [25,26] and the islets of obese *ob/ob* mice contain relatively few PP-producing cells [27]. Studies in knockout mice lacking PPYR1 revealed that these receptors are

Abbreviations: CNS, central nervous system; FACS, Fluorescence Activated Cell Sorting; PP, pancreatic polypeptide; PPYR1, pancreatic polypeptide receptor

☆ Disclosure statement: The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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http://dx.doi.org/10.1016/j.bbagen.2014.11.005
0304-4165/

necessary for the food-intake inhibitory properties of PP [28]. However, the mechanisms involved in the inhibitory effects of PP on food intake have not been clarified. Such anorexic response might involve central mechanisms through specific brain nuclei and/or peripheral circuits associated with vagal afferent pathways. Other peripheral biological actions of PP have been proposed; such as the regulation of hepatic glucose production, delay of gastric emptying, decreased intestinal motility, reduction in bile acid output and pancreatic exocrine secretion [29]; although the detailed mechanisms remain unknown.

Crosstalk between different cell types composing the endocrine pancreas participates to the fine tuning of endocrine function in the control of metabolic homeostasis. Intra-islet paracrine functions have been described for the pancreatic hormones insulin [30,31] and somatostatin [30,32,33]. In this context, we hypothesized that the effects of PP might be mediated by intra-islet paracrine regulations. The present study investigated the peripheral functions of PP in pancreatic islets. We observed expression of the PP receptor PPYR1 in the mouse and human endocrine pancreas, as well as glucose-regulated stimulation of PP secretion and its modulatory effects on glucagon release from mouse pancreatic islets.

2. Material and methods

2.1. Insulin, glucagon and pancreatic polypeptide secretion experiments

Pancreatic islets from male C57BL/6 mouse were isolated by collagenase digestion, in accordance with the Institutional Animal Ethics Committee's policies, and cultured overnight free-floating in RPMI-1640 medium before use. For static incubations, islets were washed and preincubated for 30 min in 2.8 mM glucose Krebs–Ringer bicarbonate HEPES buffer [KRBH, containing in mM: 135 NaCl, 3.6 KCl, 10 HEPES (pH 7.4), 5 NaHCO3, 0.5 NaH2PO4, 0.5 MgCl2, 1.5 CaCl2, 0.1% bovine serum albumin]. Then, batches of 3–4 islets were hand-picked and incubated for 60 min at 37 °C at basal (2.8 mM) or stimulatory (15 mM) glucose concentrations. For the measurements of PP release, batches of 7 islets were hand-picked and incubated for 60 min at 37 °C at basal (2.8 mM) or stimulatory (15 mM) glucose concentrations. Insulin, glucagon and PP concentrations were measured by Mouse Endocrine Multiplex Immunoassay from Linco (Millipore, Billerica, MA, USA).

2.2. Source and species of pancreatic polypeptide

Mouse pancreatic polypeptide (GenBank: EDL34086.1, 100 aa), used for stimulation of isolated mouse pancreatic islets, was synthesized by Proteomics Core Facility in Pompeu Fabra University-Centre for Genomic Regulation (Barcelona, Spain).

2.3. Perifusion of mouse pancreatic islets

Islet perifusion was carried out using 200 hand-picked islets per chamber, thermostated at 37 °C (Brandel, Gaithersburg, MD, USA). The flux was set at 250 μL/min and fractions were collected every minute, after a 30 min preincubation period at basal glucose (2.8 mM), in tubes containing 500 KUI/mL aprotinine (Applichem). At minute 18, PP (10 nM) was added to the perifusion buffer. At the end of the perifusion period, islets were collected from the chamber and acid-EtOH extracts were prepared to measure the remaining islet glucagon contents. Glucagon levels were determined by RIA kit according manufacturer's instructions (Millipore GL-32K). Glucagon secretion was expressed as pg/mL.

2.4. Western blotting

Tissues were obtained from C57/BL6J male mice (Charles River) and stored at −80 °C. Frozen samples were dounce-homogenized in 30 volumes of lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 g/mL aprotinin, 1 μg/mL leupeptine, 1 μg/mL pepstatin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 100 mM sodium fluoride, 5 mM sodium pyrophosphate, and 40 mM beta-glycerolphosphate) plus 1% Triton X-100. After 10 min at 4 °C, samples were centrifuged at 16,000 *g* for 30 min to remove debris and protein concentrations in the supernatants were determined by DC-micro plate assay (Bio-Rad, Madrid, Spain). Equal amounts of lysates were mixed with denaturing 5x Laemmli loading buffer and boiled for 5 min. Samples with equal amounts of total protein (20 μg per lane) were separated in 10% sodium dodecyl sulfate-polyacrylamide gel before electrophoretic transfer onto nitrocellulose membrane (Bio-Rad, Spain). Membranes were blocked for 1 h at 21 °C in Tris-buffered saline (TBS) (100 mM NaCl, 10 mM Tris, pH 7.4) with 0.1% Tween-20 (TBS-T) and 5% non-fat milk. Afterwards, membranes were incubated overnight with the primary antibodies, rabbit polyclonal antibody against rat PPYR1 (SA-644, 1:200; Enzo Life Sciences, Farmingdale, NY, USA) and mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bound primary antibodies were detected with horseradish peroxidase-conjugated antibodies to rabbit or mouse antibodies (Pierce, diluted 1:5000) and visualized by enhanced chemiluminescence detection (West-Femto-SuperSignal, Pierce). Representative cropped immunoblots for display were processed with Adobe Photoshop 7.0.

2.5. FACS sorting of pancreatic islet cells

Pancreatic islets from male C57BL/6 mouse fed *ad libitum* were isolated by collagenase digestion, in accordance with the Institutional Animal Ethics Committee's policies, and cultured free-floating overnight in RPMI-1640 medium before use. Isolated islets were dispersed into individual cells by treatment with trypsin, and the freshly dissociated cells were subjected to Fluorescence Activated Cell Sorting (FACS) using a FACS-IV instrument. An argon laser illuminated the cells at 488 nm, and emission was monitored at 510–550 nm. This technique yields two populations of cells, one enriched in alpha cells and one enriched in beta cells.

2.6. Quantitative RT-PCR

Total RNA was extracted from FACS-sorted islet cells by use of the acid guanidinium phenol preparation TRIZOL (#15596-026, Invitrogen, CA, USA) following the manufacturer's instructions. Total RNA obtained from sorted islet cells was reverse transcribed by Superscript III reverse transcriptase (#18080, Invitrogen). The oligonucleotides (Sigma Aldrich, MO, USA) used to perform subsequent cDNA amplifications by quantitative RT-PCR were: 61-mPPYR1.for: 5′-GCT TCC TAG CCA GGA CTT GGT-3′, 185-mPPYR1.rev: 5′-AAA GGG CCT ACT TCA GAG ATG C-3′, 46-mGLUC.for: 5′-GGA CTC CCT CTG TCT ACA CCT GTT-3′, 146-mGLUC.rev: 5′-GCA CCA GCA TTA TAA GCA ATC CA-3′, 104-mINS.for: 5′-CTG GTG GGC ATC CAG TAA CC-3′, 210-mINS.rev: 5′-GGG TAG GAA GTG CAC CAA CAG-3′. Hypoxanthine-guanine phosphoribosyl transferase (HPRT) mRNA was analyzed as an internal control by using oligonucleotides 711-mHPRT.for: 5-GTT GGA TAT GCC CTT GAC TAT AAT GAG TA-3′ and 790-mHPRT.rev: 5′-TTG GCT TTT CCA GTT TCA CTA ATG-3′. Quantitative determination of PPYR1, insulin, glucagon and HPRT mRNA levels was performed simultaneously in triplicate by using Power SYBR® Green PCR Master Mix, (#4367659, Applied biosystems, CA, USA). qRT-PCR and data collection were performed on the ABI Prism 7900HT system. All quantifications were normalized to the endogenous control (HPRT).

2.7. Preparation and cryostat sectioning of mouse and human pancreatic tissue

C57/BL6J male mice were anesthetized by *i.p.* administration of sodium pentothal 100 mg/kg body weight and immediately perfused with