Co-secretion of carboxypeptidase H and insulin from isolated rat islets of Langerhans

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The release of carboxypeptidase H activity from isolated rat islets was determined and compared to the secretion of immunoreactive insulin. Analysis of pancreatic islet cells sorted into β and non- β types indicated that approx. 80 % of islet carboxypeptidase H activity is present in the β cell. The release of both insulin and carboxypeptidase H was stimulated markedly by increasing the glucose concentration in the medium from 2.8 to 28 mM. The fractional release was in accordance with the observed cellular distribution of both proteins. The secretory response was biphasic with time, with an initial rapid transient phase of release within 5 min, followed by a more sustained response. The concentration-dependencies of glucose stimulation of release of insulin and carboxypeptidase H were similar, with a threshold for stimulation around 5.6 mM-glucose and maximal stimulatory response at 16.7–28 mM-glucose. The release of both proteins was inhibited by 20 mM-mannoheptulose, removal of Ca^{2+} from the medium and addition of 1 μM -noradrenaline. The combination of 10 mM-4-methyl-2-oxopentanoate and 10 mM-glutamine stimulated the release of carboxypeptidase H and insulin, as did 3-isobutyl-1-methylxanthine and 350 μM -tolbutamide in the presence of glucose. It is evident that carboxypeptidase H is released from the pancreatic β -cell by an exocytotic process from the same intracellular compartment as insulin. The release of carboxypeptidase H by a constitutive process was at best equivalent to 0.4 %/h, or less than 2 % of the maximal rate of release via the regulated pathway. It is concluded that carboxypeptidase H can be used as a sensitive index of β -cell secretion and an alternative marker to the insulin-related peptides.

INTRODUCTION

Carboxypeptidase H (EC 3.4.17.10) is a cobalt-stimulated carboxypeptidase B-like enzyme which removes C-terminal basic amino acids from the peptide substrates produced by endoproteolytic cleavage of precursor proteins. It is broadly distributed in tissues secreting polypeptide hormones and neurotransmitters, where it is concentrated in secretory granules along with the major secreted products (Fricker & Snyder, 1982, 1983; Docherty & Hutton, 1983). In the pancreatic β -cell it constitutes approx. 2–5 % of the total granule protein (Hutton, 1984), and is present mostly as a soluble granule matrix constituent (Davidson & Hutton, 1987). Subcellular-fractionation studies indicate that the enzyme may serve as a useful marker for the insulin secretory granule compartment (Docherty & Hutton, 1983; Davidson *et al.*, 1987), and it is thus potentially an alternative to measurement of insulin and C-peptide as a means of determining the secretory response of the cell.

The principal objective of the present studies was to document the secretion of carboxypeptidase H from isolated rat islets in comparison with that of insulin, and to evaluate the data within the context of the exocytotic model of insulin secretion as proposed originally by Palade (1975). A number of additional factors, however, need to be considered. The β -cell is not the only glucose-sensitive cell of the islet likely to contain carboxypeptidase H activity. Also, the enzyme is not necessarily concentrated to the same extent as insulin within the

secretory-granule compartment, especially as it may play a role in the processing of other precursor proteins, which are either secreted constitutively or segregated to other cellular locations. Finally, the biosynthesis of the enzyme is not responsive to insulinotropic stimuli (Guest *et al.*, 1989), and its intragranular concentration could thus vary relative to that of insulin, depending on the physiological status of the cell. This could also be manifest as differences in the relative composition of insulin and carboxypeptidase H between separate pools of granules within the same cell or between cells which exhibit different thresholds for the activation of insulin secretion or biosynthesis (Pipeleers, 1987).

EXPERIMENTAL

Materials

Analytical-grade biochemicals were obtained from Sigma Chemical Co. or BDH Chemicals (both of Poole, Dorset, U.K.), unless specified otherwise. Guanidino-mercaptosuccinic acid was obtained from Cambridge Biosciences (Cambridge, U.K.), [2,5- ^3H (n)]benzoyl-Phe-Ala-Arg (30 Ci/mmol) from New England Nuclear (Stevenage, Herts., U.K.) and benzoyl-Phe-Ala-Arg from Neosystems Laboratories (Strasbourg, France).

Preparation of islets

Pancreatic islets of Langerhans were isolated from 7–12-week-old New England Deaconess Hospital rats by

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collagenase digestion, as described previously (Guest *et al.*, 1989), and collected into an incubation medium consisting of modified Krebs bicarbonate buffer (120 mM-NaCl, 5 mM-KCl, 1 mM-MgSO₄, 2.5 mM-CaCl₂, 24 mM-NaHCO₃), containing 20 mM-Hepes (pH 7.4), 0.1% bovine serum albumin and 2.8 mM-glucose.

Static incubations

Batches of 10 islets were incubated in 500 μ l of incubation media, with the indicated modifications, for 90 min at 37 °C under O₂/CO₂ (19:1) in 1.8 ml-capacity sealed cryotubes (Nunc Cryotubes, Gibco, Paisley, Scotland, U.K.). Islets were then recovered by centrifugation for 10 s at 800 *g* (MSE bench-top centrifuge) and the media retained. The islets were sonicated for 10 s at 25 W (MSE Sonifer) in 500 μ l of a lysis buffer consisting of 0.025 M-Tris/HCl (pH 8.0), 2% (w/v) albumin and 1% Triton X-100. The lysates were centrifuged for 3 min at 13000 *g* (MSE Microcentaur micro-centrifuge) and the supernatants retained.

Perfusion

The perfusion chamber was a 1 ml syringe (Monoject, Crawley, W. Sussex, U.K.) containing a 20 μ l bed of Bio-Gel P-2 resin (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.), overlying a layer of packed glass wool (2–5 mm) in the tip. Groups of 200 islets in incubation media were transferred on to the gel, and the chamber was made to a volume of 30 μ l by insertion of the rubber tip of the syringe plunger. The chamber was connected to the medium reservoirs through a syringe needle (Monoject; 0.5 mm $\times$ 16 mm), attached to 0.41 mm-internal-diam. tubing (Technicon, New York, NY, U.S.A.), inserted through the plunger tip. The islets were perfused at a flow rate of 2 ml/min (PI peristaltic pump; Pharmacia, Stockholm, Sweden), with incubation media containing either 2.8 mM or 28.0 mM-glucose at 37 °C under O₂/CO₂ (19:1). The perfusate was collected (Frac-100 fraction collector; Pharmacia) in 1 ml fractions into 1.5 ml-capacity micro-centrifuge tubes (Alpha, Eastleigh, Hants., U.K.). The islets were finally recovered from the chamber and lysed as described above.

Preparation of soluble and membrane fractions

Islets were first sonicated for 15 s at 25 W in 500 μ l of 10 mM-Tris/HCl (pH 8.0), containing 1 M-NaCl. The lysates were centrifuged for 5 min at 13000 *g* (MSE Microcentaur) and the supernatants (soluble activity) were retained. The pellets were sonicated as described above in the same buffer containing 1% Triton X-100, centrifuged, and the supernatants (membrane-bound activity) retained.

Preparation of islet cells

Single β and non- β islet cells were prepared as described previously (Van De Winkel *et al.*, 1982; Pipeleers *et al.*, 1985) by autofluorescence-activated cell sorting, which distinguishes β -cells from the smaller non- β -cells by their greater light-scatter activity and FAD autofluorescence. β -cell and non- β -cell purity was assessed by immunostaining for the hormones insulin, glucagon, somatostatin and pancreatic polypeptide (Pipeleers *et al.*, 1985), and cross-contamination was typically less than 5%. The DNA content of islet cells was measured by the method of Downs & Wilfinger (1983).

Assay procedures

Carboxypeptidase H activity was determined by a modification of the radiometric method of Rossier *et al.* (1989). Briefly, islet lysates and media were made to 25 μ l with lysis buffer in 1.5 ml-capacity micro-centrifuge tubes (Alpha) and preincubated at 37 °C for 10 min with 25 μ l of 0.1 M-sodium acetate (pH 5.6) containing 2% albumin, 4 mM-CoCl₂, and with or without 50 μ M-guanidinomercaptosuccinic acid. The reactions were initiated with 50 μ l of 0.1 M-sodium acetate (pH 5.6) containing 0.2 mM unlabelled substrate benzoyl-Phe-Ala-Arg and 16 nM ³H-labelled substrate (approx. 30000 c.p.m.). Reactions were terminated with 680 μ l of 0.25 M-HCl/CH₃CN (10:7, v/v). The tubes were transferred into 20 ml screw-capped scintillation vials, and 4 ml of toluene containing 0.5% 2,5-diphenyloxazole and 0.02% 1,4-bis-(5-phenyloxazol-2-yl)benzene was added. The vials were shaken by hand for 10 s, left overnight at 4 °C and subjected to liquid-scintillation counting. Under these conditions the product, [³H]-benzoyl-Phe-Ala, is partitioned quantitatively into the scintillant and its radioactivity is thus registered, whereas the uncleaved substrate remains in the aqueous phase. The difference between the radioactivity registered in the presence and absence of guanidinomercaptosuccinic acid was taken to represent carboxypeptidase H activity.

Insulin immunoreactivity was assayed by a two-site immunoradiometric technique as described by Sobey *et al.* (1989), by using rat insulin as standard and an immunoadsorbent prepared from guinea-pig polyclonal antisera raised against bovine insulin. Lactate dehydrogenase was determined by the method of Nielsens (1955), modified as described by Lowry & Passonneau (1972) for the indirect fluorimetric measurement of the reaction product NAD⁺.

Presentation of results

Results are expressed as means $\pm$ S.E.M. with the numbers of separate observations indicated in parentheses or in the Figure legends. The statistical significance of differences for unpaired values was estimated by Student's *t* test.

RESULTS

Enzymic properties

Carboxypeptidase H activity in freshly isolated rat islets, determined in the presence of 1 mM-CoCl₂ was 238.6 ± 21.8 pmol/min per islet (6). Other activities capable of hydrolysing the same substrate, benzoyl-Phe-Ala-Arg, in the presence of the active-site-directed inhibitor guanidinomercaptosuccinic acid accounted for less than 1.5% of the carboxypeptidase H activity. The extent of cobalt activation (4–5-fold) and the pH optimum (5.5) of the islet activity were indistinguishable from those of the enzyme purified from transplantable rat insulinoma (Davidson & Hutton, 1987), and the *K_m* for the substrate was 81 μ M.

Carboxypeptidase H activity was present in both β -cells and non- β -cells from fractionated rat islets (Table 1). The specific activity of the enzyme expressed in terms of either cell number or cellular DNA content was approx. 2-fold higher in the β -cell population. Since β -cells constitute approx. 70% of the islet cell number (Baetens *et al.*, 1979) it is evident that around 84% of the

Table 1. Content of immunoreactive insulin and carboxypeptidase H in pancreatic islet cells

Isolated β - and non- β -cells were analysed for insulin immunoreactivity and carboxypeptidase H activity as described in the text. The tabulated values are means $\pm$ S.E.M. of six independent observations.

Cell preparation	DNA content (fg/cell)	Insulin (fmol/cell)	Carboxypeptidase H (fmol/min per cell)
Purified β -cells	5.31 ± 0.54	6.69 ± 0.80	69.61 ± 1.65
Non- β -cells	5.75 ± 0.24	0.12 ± 0.04	31.20 ± 1.50

Fig. 1. Glucose dependency of insulin and carboxypeptidase H release

Islets were incubated for 90 min at the indicated glucose concentrations, and the release of insulin (○) and carboxypeptidase H (●) was determined by radio-immunoassay and enzymic assay respectively. All results are means $\pm$ S.E.M. of six independent observations, and are expressed as percentage of the islet content at the end of the experiment (insulin 13.8 ± 0.4 pmol/islet, carboxypeptidase H 263.2 ± 50.5 pmol/min per islet).

total islet activity is in the β -cell. In addition, $91.2 \pm 1.5\%$ ($n = 6$) of the islet carboxypeptidase H activity is present in a soluble form. Thus, if the soluble enzyme were localized exclusively with insulin in the β -cell, around 75% of the total islet activity would be available for release.

Effect of glucose on release of insulin and carboxypeptidase H from islets

The content of carboxypeptidase H activity in the medium after a 90 min static incubation of islets in the presence of 2.8–5.6 mM-glucose was $1.03 \pm 0.01\%$ (24) of the islet content. The corresponding value for immunoreactive insulin was $0.40 \pm 0.05\%$ (6) ($P < 0.001$). The higher percentage release of carboxypeptidase H compared with insulin could not be related to contamination of the islet preparation with acinar tissue, since the specific activity of carboxypeptidase-H-like enzymes in whole pancreas was less than 5% of that present in islets, and acinar tissue constitutes less than 5% of the cells in isolated islets (Sener *et al.*, 1978). Under basal conditions the content of both proteins in media varied by approx.

3-fold between experiments, thus suggesting that a component of their release might be related to non-specific damage to islets during the isolation procedure. The release of lactate dehydrogenase, a cytosolic marker, in a separate series of experiments ranged from 0.3 to 1% of the cellular content, further supporting this idea.

The release of carboxypeptidase H activity was markedly stimulated by incubation of islets for 90 min in the presence of 16.7–28 mM-glucose. This was significantly lower than the corresponding insulin release when expressed as a percentage of the total islet content [$6.95 \pm 0.71\%$ (24) versus $9.41 \pm 0.88\%$ (24) respectively ($P < 0.05$)], but did not differ when carboxypeptidase H release was expressed in terms of total β -cell content [$8.48 \pm 0.87\%$ (24)].

Both insulin and carboxypeptidase H were released from islets with a sigmoidal dependence on the concentration of glucose in the incubation medium (Fig. 1). The release of both proteins showed a threshold for stimulation above 5.6 mM-glucose, with half-maximal response at approx. 10 mM-glucose and maximal stimulation at 16.7–28.0 mM-glucose. Linear-regression analysis showed a close correlation ($r^2 = 0.987$) between insulin and carboxypeptidase H release at stimulatory glucose concentrations. The slope of the regression (0.62) approaches the estimated ratio of soluble carboxypeptidase H in the β -cell to the total islet content (0.75; see above). Over the range of glucose concentrations which induced release, there was no indication of a divergence from a linear relationship. This suggested that both the secretory granules and cells from which release was occurring had the same relative contents of insulin and carboxypeptidase H.

Modifiers of release

The release of carboxypeptidase H activity and immunoreactive insulin induced by 16.7 mM-glucose was markedly decreased in Ca^{2+} -depleted media containing 1 mM-EGTA (Table 2). Mannoheptulose (20 mM), which has been shown to inhibit glucose metabolism in islets (Ashcroft & Randle, 1970), and noradrenaline (1 μM) also reversed significantly the stimulatory effect of glucose on the release of both proteins. Tolbutamide (350 μM) in the presence of 5.6 mM-glucose and the combination of 10 mM-4-methyl-2-oxopentanoate and 10 mM-glutamine were potent stimuli for the release of both proteins (Table 3). There was a close correlation between release of carboxypeptidase H and insulin under all these conditions. Linear-regression analysis of the data in Tables 1 and 2, excluding the incubations performed in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1 μM), indicated that the release of

Table 2. Effect of inhibitors on release of insulin and carboxypeptidase H

Islets were incubated for 90 min as indicated, and the release of insulin and carboxypeptidase H was determined by radioimmunoassay and enzymic assay respectively (see the text for further details). All results are means $\pm$ S.E.M. of at least six independent observations, and are expressed as percentage released of the islet content at the end of the experiment (insulin 13.1 ± 1.2 pmol/islet, carboxypeptidase H 241.9 ± 7.9 pmol/min per islet). Levels of significance for difference between value and control value (16.7 mM-glucose): * $P < 0.001$.

Glucose (mM)	Modification	Release (%)	
		Insulin	Carboxypeptidase H
2.8	—	0.36 ± 0.10	0.42 ± 0.11
16.7	—	9.60 ± 1.60	6.71 ± 0.73
16.7	(+) 1 mM-EGTA (no extracellular Ca^{2+})	$1.13 \pm 0.13^*$	$2.55 \pm 0.23^*$
16.7	(+) 20 mM-Mannoheptulose	$0.14 \pm 0.03^*$	$0.55 \pm 0.06^*$
16.7	(+) 1 μM -Noradrenaline	$1.04 \pm 0.39^*$	$1.43 \pm 0.30^*$

Table 3. Effect of secretagogues on release of insulin and carboxypeptidase H

Islets were incubated for 90 min as indicated, and the release of insulin and carboxypeptidase H was determined by radioimmunoassay and enzymic assay respectively (see the text for further details). All results are means $\pm$ S.E.M. of at least six independent observations, and are expressed as percentage released of the islet content at the end of the experiment (insulin 10.0 ± 0.9 pmol/islet, carboxypeptidase H 179.4 ± 25.5 pmol/min per islet). Abbreviations: IBMX, 3-isobutyl-1-methylxanthine; KIC, 4-methyl-2-oxopentanoate ('ketoisocaproate').

Glucose (mM)	Modification	Release (%)	
		Insulin	Carboxypeptidase H
5.6	—	0.19 ± 0.06	1.05 ± 0.10
5.6	(+) 350 μM -Tolbutamide	5.05 ± 0.77	2.86 ± 0.20
0	(+) 10 mM-Glutamine + 10 mM-KIC	5.45 ± 1.52	4.93 ± 1.40
16.7	—	7.93 ± 2.41	6.78 ± 2.65
16.7	(+) 1 μM -IBMX	19.08 ± 1.57	18.20 ± 1.57

carboxypeptidase H relative to that of insulin was 0.66 ($r^2 = 0.913$), a value similar to that observed above at different glucose concentrations.

Proportionately more carboxypeptidase H release occurred in the presence of 3-isobutyl-1-methylxanthine (Table 3). The possibility that non- β islet cells contributed to its release in this case warrants investigation.

Dynamics of release

The release of carboxypeptidase H from perfused islets was stimulated markedly by increasing the glucose concentration in the medium from 2.8 to 28 mM (Fig. 2). This increase in release followed a pattern similar to the biphasic pattern characteristic of insulin release (Curry *et al.*, 1968). In both cases stimulation occurred after a delay of 90 s, reached an initial peak at 3 min, followed by a nadir at 5 min and a more sustained response over the subsequent 10 min. Linear-regression analysis again showed a close correlation between the release of both proteins ($r^2 = 0.899$) and a slope (0.63) which approximated to the estimated content of soluble carboxypeptidase H in the β -cell relative to the total islet content. It was apparent that the release of carboxypeptidase H was not limited by its diffusion from the interstitial space within the islet, even though the protein has a molecular size almost 10-fold that of insulin. Since

carboxypeptidase H was released in the same proportion to insulin during the first phase of secretion as in longer-term static incubations, it is evident that the secretory-granule pools and cells involved in the first and second phases of release have the same average relative compositions of the two proteins.

DISCUSSION

Carboxypeptidase H is widely distributed in the secretory granules of endocrine and neural tissues, where it is involved in the proteolytic processing of polypeptide hormones and neuropeptides (Fricker & Snyder, 1982, 1983). High levels of the enzyme are present in the pituitary and pancreatic islets, with substantially lower levels in the adrenal medulla and various brain regions. There is little evidence for an extracellular function of the enzyme, since it is optimally active at the acidic pH of the secretory granule and retains very little activity at the neutral pH of plasma (Fricker & Snyder, 1982; Docherty & Hutton, 1983).

Carboxypeptidase H has been shown previously to be co-released with pro-corticotropin/endorphin-derived 16 kDa peptide from AtT-20 cells (Mains & Eipper, 1984) and with [Met]enkephalin from chromaffin cells (Hook & Eiden, 1985). The relationship between the

Fig. 2. Dynamics of glucose-stimulated release of insulin and carboxypeptidase H

Groups of 200 islets were perfused at a flow rate of 2 ml/min for 30 min (the first measurements were made after 25 min) with a medium containing 2.8 mM-glucose and then for 15 min with 28.0 mM-glucose. The vertical bar indicates the time at which the medium was changed. The release of insulin (●) and carboxypeptidase H (■) was determined by radioimmunoassay and enzymic assay respectively. All results are means $\pm$ S.E.M. of three independent observations, and are expressed as percentage released of the islet content at the end of the experiment (insulin 13.11 ± 2.13 pmol/islet, carboxypeptidase H 202.9 ± 25.3 pmol/min per islet).

release of carboxypeptidase H and the hormones in question from these tissues has often not been a precise one, with considerable variation in the relative compositions of secreted and cellular material. Non-parallel release of secretory-granule constituents has also been reported in other tissues (see Burgess & Kelly, 1987), and in some cases was construed as evidence against the exocytotic model of secretion (Rothman, 1975). The present studies with isolated rat islets, however, show that the releases of carboxypeptidase H and insulin were almost exactly parallel under a wide range of conditions. It was evident that the β -cell is a major cellular source of carboxypeptidase H in islets. Along with previous morphological data (Kakiuchi & Sobue, 1983) and studies on the co-secretion of insulin and C-peptide (Rubenstein *et al.*, 1969; Melani *et al.*, 1970), these data provide strong experimental support for the concept that secretion from the pancreatic β -cell occurs

by exocytosis of secretory granules, which results in the quantal release of the soluble contents of the granule.

Whether carboxypeptidase H release from the β -cell occurs exclusively by a regulated process remains an open question, since the basal release of the enzyme consistently exceeded that of insulin both under non-stimulatory conditions and when release by the regulated pathway was inhibited. One possible explanation for this difference is that it constitutes basal or stimulated release from non- β islet cells, the majority of which contain the enzyme (P. C. Guest, L. Orci & J. C. Hutton, unpublished work). It is also conceivable that release of carboxypeptidase H occurred by a constitutive process from the β -cell. However, it was not experimentally feasible to test this possibility, since the basal rates of release were of the same order as the extent of cellular lysis, as measured by the release of a cytosolic marker protein. As a consequence, only an upper estimate of the rate of constitutive release could be derived. This was equivalent to 0.4% of the cellular contents/h, or around 2% of the maximal rate of secretion via the regulated pathway (as represented by release in the presence of 16.7 mM-glucose plus 3-isobutyl-1-methylxanthine).

These studies demonstrate that carboxypeptidase H secretion from isolated rat islets occurs in response to the same intracellular signals effecting the secretion of insulin. We conclude that its measurement provides an alternative and independent method of determining the rates of exocytosis from the pancreatic β -cell *in vitro*. Assays for this enzyme should find general application in experimental situations in which immunoassay of insulin is not feasible, for example where the secreted product is structurally altered or in studies where the effects of immunologically cross-reactive peptides on insulin secretion are of interest.

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