Selective exocytosis of zymogen granules induces non-parallel secretion in short-term cholecystokinin-stimulated rats

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Abstract

Parallel studies on pancreatic enzyme secretion and zymogen granule enzyme composition have been carried out in rats subjected to infusion of cholecystokinin (CCK) (1.25 µg/kg per h) over 30 min. Flow cytometric analysis showed a significant decrease in the mean value of granule size after CCK stimulation. The amount of trypsinogen stored in each individual zymogen granule was significantly lower at 30 min of CCK infusion, but no variation in intragranular amylase content was observed. As a result, the amylase/trypsinogen ratio was significantly increased in the zymogen granules that remained in the pancreas of

rats stimulated with CCK for 30 min. A significantly greater proportion of trypsin than amylase was secreted after 30 min CCK infusion. Our results support the existence of different types of granules loaded with different proportions of enzymes. We conclude that short-term CCK stimulation induces the selective release of large granules containing a high proportion of trypsinogen, which leads to a non-parallelism of enzyme secretion.

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Introduction

Cholecystokinin (CCK) is a gastrointestinal hormone that has a key integrative role in nutrient digestion and assimilation (Rehfeld 1989). Among other effects, CCK regulates the exocrine pancreatic function, and its major action on acinar cells is to stimulate digestive enzyme secretion (Williams & Yule 1993). Under some conditions (Dagorn 1978, Grendell *et al.* 1984, de Dios *et al.* 1997b), the proportion of different enzymes is 'non-parallel'. A selective exocytosis of zymogen granules with different enzyme content has been suggested (Adelson & Miller 1989), however, the mechanism for this phenomenon is still not completely understood. To prove the existence of different types of granules, it is necessary to measure the enzyme content in individual zymogen granules. Flow cytometry has proved to be a rapid, objective, sensitive and quantitative method for the analysis of a large number of individual granules of rat pancreas under different physiological, pharmacological and pathological conditions (de Dios *et al.* 1995, 1997b, Garcia-Montero *et al.* 1995, Manso *et al.* 1997, 1998, Rodriguez *et al.* 1997). The storage of enzymes in the pancreas represents the intermediate stage between synthesis and secretion, and consequently the understanding of this process is the key to assessing the different pancreatic responses to specific stimuli; nevertheless, little is known about it. In the

present study, we have analysed the distribution patterns of amylase and trypsinogen in individual zymogen granules, together with the enzyme composition of pancreatic juice, in rats subjected to a CCK infusion over 30 min, with the aim of finding out whether short-term CCK stimulation induces a selective release of different types of granules.

Materials and Methods

Chemicals

Sucrose, MgSO₄, starch, potassium sodium tartrate and 3–5-dinitrosalicylic acid were from Merck (Darmstadt, Germany); maltose, bovine trypsin type III, *N*-α-benzoyl L-arginine-*p*-nitroaniline (BAPNA), ethylene glycol-bis-(*b*-aminoethyl ether) *N,N,N',N'*-tetra-acetic acid (EGTA), 3-(*N*-morpholino) propanesulphonic acid (MOPS), phenylmethylsulphonyl fluoride (PMSF), 2-(*N*-morpholino) ethanesulphonic acid (MES), Percoll, bovine serum albumin (BSA), CCK-8 (98% purity) and rabbit anti-amylase antiserum were purchased from Sigma Chemical Company (St Louis, MO, USA); rabbit anti-trypsinogen antiserum was obtained in our laboratory as previously described (de Dios *et al.* 1997b) and goat anti-rabbit immunoglobulin G antiserum was from Caltag (Burlingame, CA, USA).

Animals and treatments

Male Wistar rats weighing about 250 g were used. A standard laboratory diet (Panlab, rat chow) and water were available *ad libitum*. The rats were maintained at 24 °C on a 12 h light : 12 h darkness cycle. Three groups of animals were randomly established:

Group 1 (controls): Rats that received an intravenous infusion of saline solution containing 0.2% BSA over 15 min.

Group 2: Rats that received an intravenous infusion of saline solution containing 0.2% BSA over 15 min and thereafter CCK-8 (1.25 µg/kg per h) resuspended in saline plus 0.2% BSA for 5 min.

Group 3: Rats that received an intravenous infusion of saline plus 0.2% BSA over 15 min and thereafter CCK-8 (1.25 µg/kg per h) resuspended in saline plus 0.2% BSA for 30 min.

Analysis of zymogen granules

Isolation of zymogen granules Zymogen granules were isolated from the pancreas of control rats (group 1), considered to represent basal conditions, and of CCK-infused rats (groups 2 and 3) after pancreatic juice had been collected. For each experiment, pancreata of two rats were pooled and put on ice-cold homogenization medium pH 6.5 containing 250 mM sucrose, 5 mM MOPS, 0.1 mM MgSO₄ and 0.1 mM PMSF at 40 ml/g wet weight pancreas. They were finely minced with scissors and homogenized for 30 s in an electric homogenizer (Omni 2000 International) at low speed. Heavy components were removed by centrifugation at low speed. Zymogen granules were isolated according to the method of De Lisle *et al.* (1984) by centrifuging at 100 000 *g* for 20 min in a 40% Percoll gradient composed of a homogenization buffer, pH 5.5 (250 mM sucrose, 50 mM MES, 0.1 mM MgSO₄, 0.1 mM PMSF and 2 mM EGTA) mixed with Percoll. Finally, the granules were washed twice (1300 *g*, 15 min) with buffer pH 6.5 (0.3 M sucrose, 0.1 mM MgSO₄, 2 mM MOPS, 1 mM EGTA).

Measurements of amylase and trypsinogen in zymogen granules Specific intragranular staining was carried out according to methods previously reported (de Dios *et al.* 1995, 1997b). Briefly, zymogen granules were incubated for 15 min with rabbit anti-amylase antiserum (0.7 µg/µl) or with rabbit anti-trypsinogen antiserum (1 µg/µl). The unbound antisera were removed by centrifuging twice in buffer (2 × 1300 *g* for 15 min). The pellet was resuspended and incubated in the dark for 15 min with goat anti-rabbit immunoglobulin antiserum labelled with phycoerythrin (1 × 10⁻² µg/µl). To remove the unbound antiserum, centrifugation medium was added twice (2 × 1300 *g* for 15 min). Neither selective nor significant loss of the enzymes was observed in the

Table 1 Enzyme activity in the supernatants before and after incubating zymogen granules with specific antisera. Values are means ± S.E.M.; *n*=number of experiments

	Before primary antiserum	After secondary antiserum
Amylase (U/ml)	0.052 ± 0.02 (n=5)	0.041 ± 0.014 (n=5)
Trypsin (U/ml)	0.016 ± 0.006 (n=5)	0.012 ± 0.04 (n=5)

supernatants of the centrifugation steps during preparation for flow cytometry (Table 1). Flow cytometric measurement of intragranular enzyme content had previously been validated with biochemical assays of enzyme activity (de Dios *et al.* 1997b).

Flow cytometric analysis of zymogen granules All flow cytometric measurements of zymogen granules were performed for at least 10 000 events per test in each experiment. A FACScan flow cytometer (Becton/Dickinson, San José, CA, USA) equipped with an argon ion laser set at 488 nm and 15 mW was used, as we have described previously (de Dios *et al.* 1995, 1997a,b, Garcia-Montero *et al.* 1995, Manso *et al.* 1997, 1998). Sample (medium washing)/sheath (FACS flow, Becton/Dickinson) differential pressure was set at low speed in order to obtain a minimum diameter of the sample part of the flow at the intersection of the laser beam. During the entire measurement process, the zymogen granules remained suspended in the washing buffer, which was surrounded by the sheath fluid, and no admixture occurred between the two fluids, in accordance with the principles of FACScan flow cytometer analysis (Kachel *et al.* 1990). Calibration of the instrument was performed on a daily basis using Calibrite beads (Becton/Dickinson). Fine adjustments were performed using unstained zymogen granules and fluorescence compensation was established by measuring anti-amylase- or anti-trypsinogen-stained zymogen granules. Each day, zymogen granules from CCK-infused and control rats were measured in parallel. The Lysis II software program was used for data acquisition and analysis; in each experiment, the mean value per individual zymogen granule was calculated using this software. Results were expressed as the mean value of at least five experiments.

Study of pancreatic secretion

Collection of pancreatic juice Rats were anaesthetized with sodium pentobarbitone (3 mg/100 g body weight, i.p.). After tracheotomy, a median laparotomy was performed, the bile duct was cannulated at its exit from the liver to divert bile flow to the exterior, and the pancreatic bile duct was cannulated at its exit into the duodenum, to collect pure pancreatic juice. The jugular vein was

catheterized for infusion of 0.15 M NaCl containing 0.2% BSA over 15 min, considered as the basal period, and for infusion of 1.25 µg/kg per h CCK-8 resuspended in 0.15 M NaCl containing 0.2% BSA over 5 min (group 2) or 30 min (group 3) thereafter. Infusion was carried out with a peristaltic pump (LKB, model 2132) at a flow rate of 0.5 ml/h.

The animals were unconscious during the entire period of juice collection, and body temperature was kept at $37 \pm 1^\circ\text{C}$ by placing the rats on a heating pad and monitoring rectal temperature with a thermometer. Pancreatic juice was collected in pre-weighed Eppendorf tubes on ice, to preserve the enzyme content.

Finally, the pancreas was quickly removed and freed from fat and lymph nodes for the isolation of zymogen granules.

Biochemical assays Pancreatic juice volume was estimated by direct weighing on an electronic balance, assuming the density of the juice to be 1.0 g/ml. According to the method of Noelting & Bernfeld (1948), amylase activity in pancreatic juice was determined immediately as follows. A solution of 2% of starch (w/v) in 0.02 M saline phosphate buffer was used as a substrate, and 100 µl pancreatic juice containing 100 µg/µl protein was incubated with 5 ml of this starch solution at 38°C for 5 min. The maltose produced as a result of enzymic action was quantified by the addition of a solution containing 0.02 M 3–5-dinitrosalicylic acid, 0.1 M NaOH and 0.4 M potassium sodium tartrate, in a ratio of 1:5 (v/v), incubation at 100°C for 10 min, dilution with water in a ratio of 1:10 (v/v), and measurement at 520 nm. Maltose spontaneously released was measured from a tube containing 2% starch, but without pancreatic juice, and subjected to the same reaction conditions; the absorbance value was subtracted from the values obtained in the tubes with pancreatic juice. The amount of amylase in pancreatic juice was calculated by comparison with a standard curve, using known concentrations of maltose.

The measurement of trypsin activity was carried out by the method of Caldwell & Sparrow (1970) modified by Manso *et al.* (1992). Pancreatic juice diluted to a protein concentration of 100 µg/ml was incubated during 24 h at 4°C with a solution of bovine trypsin type III (25 µg/ml) in 0.01 M Tris-HCl buffer, pH 7.6 containing 0.05 M CaCl_2 , in order to activate the trypsinogen. BAPNA (0.2 M) in Tris-HCl buffer was used as a substrate, and 100 µl activated pancreatic juice was incubated with 2.4 ml of this BAPNA at 37°C for 20 min. The reaction was halted by the addition of 500 µl 20% acetic acid, and *p*-nitroaniline was measured at 410 nm, as a product of the enzyme reaction. The absorbance obtained from a tube containing 25 µg/ml bovine trypsin was subtracted. Trypsin activity in pancreatic juice was calculated by extrapolation with a standard curve, using known concentrations of bovine trypsin.

Figure 1 Forward scatter (FSC) distribution of zymogen granules from control rats (black) and rats CCK-stimulated for 5 and 30 min (grey).

Statistical analysis

Results are expressed as means $\pm$ S.E.M. Statistical analysis of results from the different experimental groups was by analysis of variance (ANOVA) followed by the Scheffé test. Values of $P < 0.05$ were considered to be statistically significant in all cases.

Results

Figure 1 shows a representative example of the zymogen granule distribution according to forward light scatter (FSC). FSC is a parameter directly related to the

Figure 2 Forward scatter (FSC) of zymogen granules of control rats (basal conditions, $n=12$ animals) and under CCK stimulation for 5 min ($n=12$) and 30 min ($n=12$). Values are mean channels $\pm$ S.E.M. (arbitrary units scaled from 0 to 10 000). ANOVA revealed significant differences: $F(2,23)=13.28$, $P<0.001$. Significant differences compared with basal: ♦♦ $P<0.01$, ♦♦♦ $P<0.001$ (Scheffé test).

size – mainly reflecting the cross-sectional area – of the granule (Salzman *et al.* 1990). As can be seen in the figure, the amount of granules of larger size diminished after CCK infusion, so a shift in the size–frequency distribution towards a lower range was observed under these conditions. As a result of this, the mean value of granule size was significantly decreased in CCK-stimulated rats, from the first 5 min of CCK infusion (Fig. 2).

Figure 3 shows, on a logarithmic scale, a set of representative flow cytometric bivariate distributions of the zymogen granules from control rats and rats stimulated with CCK for 30 min, according to the FSC and amylase or trypsinogen content. Two histograms at the top show the negative fluorescence of the samples. As can be seen, the zymogen granule population displayed a high degree of heterogeneity, indicated by the wide range of staining with specific antisera.

Analysis of the mean values of enzyme content in individual zymogen granules (Fig. 4) revealed no variation in amylase and a significant ($P<0.001$) decrease in trypsinogen in CCK-stimulated rats over 30 min, at which time the amylase/trypsinogen ratio was, consequently, significantly ($P<0.01$) increased.

The amylase/FSC ratio increased significantly ($P<0.001$) 5 and 30 min after CCK infusion. In addition, CCK stimulation induced an increase in the trypsinogen/FSC ratio, but statistical significance ($P<0.001$) of the change with respect to basal values was achieved only at 5 min after CCK infusion (Fig. 5).

Table 2 shows the amylase/trypsin ratio in pancreatic juice under basal conditions and with CCK stimulation. A decrease in the enzyme ratio was observed in response to CCK infusion and reached statistical significance ($P<0.05$) after 30 min.

Discussion

Long-term CCK treatment and protein-enriched diet feeding induce a greater secretion of proteases than of other pancreatic enzymes. This is associated with a modulatory effect of CCK on biosynthesis, acting preferentially on serine proteases (Renaud *et al.* 1986, Pradel *et al.* 1993, Lloste *et al.* 1994, de Dios *et al.* 1997b). In agreement with previous reports (Dagorn 1978, Grendell *et al.* 1984), our results also show a non-parallel secretion of enzymes in response to short-term CCK stimulation. Under these conditions, differential release of a previously synthesized product may be taking place. As CCK-stimulated secretion tends to occur via the 'regulated' pathway (Palade 1973), which mainly depends on the exocytosis of zymogen granules, a non-parallel exocytosis should also be considered. Analysis of zymogen granules by flow cytometry shows that they display a large heterogeneity regarding physical properties, enzyme content and glycoprotein membrane composition (Garcia-Montero *et al.* 1995, de Dios *et al.* 1997a, Rodriguez *et al.* 1997), which suggests the possibility of achieving a great diversity of pancreatic responses to specific stimuli.

CCK induces the discharge of larger granules, as deduced by the shift in the size–frequency distribution of granules in rats subjected to CCK infusion. Morphological studies had also reported the same effect after feeding (Ermak & Rothman 1981, Aughtsteen & Cope 1987) and pharmacological stimulation (Beaudoin *et al.* 1984). Ermak & Rothman (1981) suggested that these changes reflected the loss of protein across the membrane, as proposed in the equilibrium hypothesis (Rothman 1975). Previous studies have shown that some cytosolic proteins were secreted in the extracellular medium via a non-classical pathway (Rubartelli *et al.* 1990, Christmas *et al.* 1991, Lindstedt *et al.* 1993). However, these proteins are characterized by a lack of signal sequence in their primary structure. The proteins known to be devoid of signal sequence do not include any of the pancreatic digestive enzymes (Muesch *et al.* 1990). Therefore, according to the classical scheme, granule content is released by exocytosis (Palade 1973) and the reduction in mean size of the granules may result from a preferential release of the largest zymogen granules in response to CCK.

Zymogen granules remaining in the pancreas 5 min after CCK infusion showed contents of amylase and trypsinogen similar to those under basal conditions. However, the enzyme/FSC ratio indicated that the two enzymes were more concentrated in each zymogen granule, because the granules were smaller in CCK-stimulated rats. In agreement with the findings of Goncz *et al.* (1995), our results suggest that the concentrations of the intragranular enzyme do not depend on physical limitations, but can be physiologically regulated. The intragranular amylase/trypsinogen ratio revealed that the granules released at the beginning of CCK infusion carry a

Figure 3 Flow cytometric characterization of zymogen granules. Histograms show the distribution of FSC- and fluorescence 2 (FI2)-negative controls. Dot plots are log/log representation of the distribution pattern of zymogen granules from control rats (basal conditions) and from rats stimulated with CCK for 30 min, according to FSC and enzyme (phycoerythrin, PE) fluorescence (FI2).

proportional admixture of both enzymes. Nevertheless, as the infusion continued, CCK preferentially induced the exocytosis of granules with a greater content of trypsinogen than of amylase. In fact, a significant decrease in the quantity of trypsinogen was observed in individual granules 30 min after CCK infusion. As a consequence, the

mean value of the amylase/trypsinogen ratio in single granules was significantly increased. This finding reflects the presence of different types of granules and suggests an ordered partitioning of digestive enzymes within them.

Variation in granule content has been demonstrated by immunocytochemical (Gingras & Bendayan 1992) and

Figure 4 Amylase and trypsinogen content of individual zymogen granules of control rats (basal conditions, $n=10$ animals) and in rats stimulated with CCK for 5 min ($n=6$) and 30 min ($n=8$). Values are expressed as mean channels of phycoerythrin fluorescence $\pm$ S.E.M. (arbitrary units scaled from 0 to 10 000). The histogram below represents the mean values of amylase/trypsinogen ratio in each zymogen granule. ANOVA revealed significant differences in trypsinogen content ($F(2,22)=22.51$, $P<0.001$) and in amylase/trypsinogen ratio ($F(2,22)=13.00$, $P<0.001$). The Scheffé test revealed significant differences compared with basal values ($\blacklozenge$ $P<0.01$, $\blacklozenge$ $P<0.001$) and compared with 5 min CCK stimulation ($\circ$ $P<0.001$).

microfluorimetric (Mroz & Lechene 1986) methods. However, in both studies, a small population of granules was examined. It would be possible to demonstrate multivariate distribution of enzymes, were a large enough sampling of the population to be undertaken. In this respect, flow cytometry provides the possibility of analysing 10 000 granules in each test, which offers a high statistical reliability. The use of specific antisera against

Figure 5 Amylase/FSC ratio and trypsinogen/FSC ratio in zymogen granules of control rats (basal conditions, $n=10$ animals) and in rats stimulated with CCK for 5 min ($n=6$) and 30 min ($n=8$). Values are expressed as means $\pm$ S.E.M. For both ratios, ANOVA revealed significant differences ($F(2,22)=19.60$, $P<0.001$ and $F(2,22)=15.82$, $P<0.001$, respectively). The Scheffé test revealed significant differences compared with basal conditions ($\blacklozenge$ $P<0.001$) and compared with 5 min CCK stimulation ($\circ$ $P<0.05$).

intracellular enzymes also provides reliable measurements compared with traditional biochemical methods (de Dios *et al.* 1997b), with the added advantage that individual granules can be analysed and a high degree of heterogeneity in the zymogen granule population can be observed in terms of their amylase and trypsinogen content (coefficient of variation $>99\%$). The selective release of the different types of granules would explain the non-parallel secretory response of the pancreas to 30 min of

Table 2 Amylase/trypsin ratio in pancreatic secretion. Values are means $\pm$ S.E.M.; n =number of animals

	Basal	CCK-8 (1.25 μ g/kg per h)	
		5 min	30 min
Amylase/trypsin ratio	34.76 $\pm$ 1.66 ($n=9$)	30.12 $\pm$ 2.83 ($n=6$)	26.17 $\pm$ 2.34* ($n=7$)

Significant differences: between the three ratios ($F(2,19)=4.63$, $P<0.05$; ANOVA); between ratio at CCK 30 min and basal secretion (* $P<0.05$, Scheffé test).

CCK infusion. The significant change in the proportion of amylase and trypsin in pancreatic juice could account for the non-parallel exocytosis of granules, with quantitative rather than qualitative differences regarding their enzyme composition.

Non-parallel exocytosis would also require some mechanism to distinguish among different granule types in order that they be selectively discharged from the cell. Glycoproteins in the zymogen granule membrane seem to be involved in the recognition process between zymogen granules and the inner surface cell membrane (Scheele *et al.* 1994), and different types of zymogen granule membranes have been reported in the exocrine pancreas of the rat as regards morphology and glycoprotein composition (Beaudoin & Grondin 1991). Lectin-binding studies carried out recently (de Dios *et al.* 1997a) demonstrated that glycoconjugates of granule membrane involved in CCK-regulated exocytosis contain large amounts of *N*-acetyl D-glucosamine. Further studies should be undertaken to find out whether *N*-acetyl D-glucosamine-enriched glycoproteins in the zymogen granule membrane are more abundant in those granules with greater proportions of trypsinogen and if it is for this reason that they are preferentially released in response to short-term CCK stimulation.

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