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Biochimica et Biophysica Acta 1326 (1997) 131–137

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Cholecystokinin regulates glycoprotein membrane composition of rat pancreatic zymogen granules

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Received 4 December 1996; accepted 17 January 1997

Abstract

Lectin-binding studies were performed on rat pancreatic zymogen granules to investigate the alterations in the carbohydrate membrane composition under both chronic CCK stimulation and long-term CCK blockade for 3, 7 and 15 days. By flow cytometry using FITC-WGA – which specifically binds to N-acetylglucosamine and sialic acid – we measured the amount of WGA molecules bound to each individual granule. Parallel studies on pancreatic secretion were also carried out. CCK treatment displayed a differential effect on two zymogen granule subpopulations (Z_1 and Z_2) identified by flow cytometry on the basis of their light scatter properties: no effects on Z_2 zymogen granules were observed in CCK-treated rats, while Z_1 granules showed a significant increase in WGA binding at day $+7$ which coincides with an increase in protein secretion in response to the hormone. On the contrary, a significant decrease in the amount of WGA receptors was observed in zymogen granule membrane of both the Z_1 and Z_2 subsets of rats subjected to a long-term CCK blockade. Again, these changes parallel to the reduction observed in protein secretion. Our results suggest that glycoconjugates of zymogen granule membrane involved in CCK-regulated exocytosis contain N-acetylglucosamine and sialic acid residues whose quantities are regulated by CCK.

Keywords: Cholecystokinin; Exocrine pancreas; Glycoconjugate; Zymogen granule membrane; (Rat)

1. Introduction

Zymogen granules are important organella in the secretory system of the exocrine pancreas, which are involved in the enzyme packaging and secretion of acinar cells. It has been reported that the protein components of the zymogen granule membrane play an important role in granule formation [1]. Moreover, in response to appropriate stimuli, the exocrine pancreas secretes enzymes by exocytosis, a process that involves the fusion of the zymogen granule membrane with the apical plasma membrane of the acinar cells. This phenomenon implies the recognition of the two membranes and proteins are known to be involved in the process of membrane fusion [2–4]. Intracellular mechanisms leading to secretion and the structure and enzymatic activities of the digestive

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PII S0005-2736(97)00015-1

secretory enzymes are well-established [5]. However, comparatively little is known about the protein components of the granule membrane despite their relevant role in the secretion process.

Lectins are carbohydrate-binding compounds which have been widely used to identify cell surface glycoproteins and glycolipids [6–8]. Fracture-label studies revealed lectin binding sites in the zymogen granule membrane and a heterogenous population of granules has been defined according to the lectin-labeling density [9]. Flow cytometry becomes a rapid, objective, sensitive and quantitative method for the analysis and quantification of large numbers of single subcellular components [10,11], including the lectin receptors of zymogen granules in control rats [12].

This work is focused to investigate how the glycoconjugates of the zymogen granule membrane of rat pancreas are affected by two contrasting experimental situations which have a clear influence on the exocytosis phenomenon: long-term CCK and L-364,718 (a CCK-receptor antagonist) treatments. The aim of this study is to know whether a relationship between secretion of zymogen granules and their membrane composition could be established.

2. Materials and methods

2.1. Products and companies

L-364,718, a CCK receptor antagonist, was kindly donated by Merck Sharp and Dohme (Madrid, Spain), Sucrose (Merck), CCK-8 (fragment 26-33 amide, 99% purity), dimethylsulfoxide (DMSO), Ethyleneglycol-bis-(b-aminoethylether), N, N, N′, N′ tetraacetic acid (EGTA), 3-(N-morpholino) propanesulfonic acid (MOPS), phenyl-methylsulfonil fluoride (PMSF), 2-(N-morpholino) ethanesulfonic acid (MES), Percoll, FITC conjugated *Triticum vulgaris* lectin (Wheat germ agglutinin) (FITC-WGA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals and treatments

Male Wistar rats weighing about 250 g body weight were used. Rats were fed ad libitum with standard laboratory diet (Panlab rat chow) and had free access to water. They were randomly separated into different groups:

- Controls
- Rats treated with subcutaneous injections of CCK-8 (5 μg/kg/12 h) for 3, 7 and 15 days
- Rats treated with subcutaneous injections of L-364,718 (0.1 mg/kg/day) in 10% DMSO as depot carrier for 3, 7 and 15 days. A solution of 10% DMSO was administered to rats in previous studies [13,14] and no significant effect was observed in pancreatic function.

Different animals were used to analyze zymogen granules and pancreatic secretion.

Twelve hours after the last injection and while fasting, the animals used to study zymogen granules were killed by decapitation and their pancreases were quickly removed and freed from fat and lymph nodes. After 18 h fasting the animals used to study pancreatic secretion were surgically prepared for the collection of pancreatic juice as detailed below.

2.3. Isolation of zymogen granules

The pancreases from three animals were pooled and put on ice-cold homogenization medium containing 250 mM sucrose, 5 mM MOPS (pH 7.0), 0.1 mM $MgSO_4$ 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. [15] by centrifuging at $100\,000 \times g$ 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 $MgSO_4$, 0.1 mM PMSF and 2 mM EGTA) mixed with Percoll. Lastly, the granules were washed twice ($1300 \times g$, 15 min) with a solution containing 0.3 M sucrose, 0.1 mM Mg SO_4, 2 mM MOPS, 1 mM EGTA (pH 6.5).

2.4. Staining of zymogen granules with FITC-WGA

Zymogen granules were incubated with lectin from *Triticum vulgaris* (Wheat germ agglutinin) (WGA) conjugated with fluorescein isothiocyanate (FITC) at saturating concentration (1 μg/μl) [12] at 4°C in the dark for 45 min. The unbound FITC-WGA was

removed by centrifugation twice in washing buffer ($2 \times 1300 \times g$, 15 min, 4°C). The specificity of the binding of FITC-WGA to the zymogen granules was assessed by using as a control the same concentration of lectin but in the presence of N-acetyl glucosamine as has been previously reported [12].

2.5. Flow cytometric analysis of the zymogen granules

All flow cytometry measurements of zymogen granules were performed for at least 10 000 events/test of a pooled pancreas of five animals per experiment. A FACScan flow cytometer (Becton/Dickinson, San Jose, Ca, USA) equipped with an argon ion laser tuned at 488 nm and 15 mWatts was used, as previously described [12,16]. 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 whole measurement process the zymogen granules remained resuspended in the washing buffer which is surrounded by the sheath fluid, and no admixture between the two fluids occurs in accordance with FACScan flow cytometer procedure [17]. Calibration of the instrument was performed on a daily basis using CALIBRITE beads (Becton/Dickinson). Fine adjustments were performed using unstained zymogen granules. Each day zymogen granules from control and CCK or L-364,718-treated rats were measured in parallel. The Lysis II software program was used for data acquisition and analysis. Results were expressed as the mean value of at least six experiments, and in each experiment the mean value per individual zymogen granule was calculated using Lysis II software.

The amount of FITC-WGA bound to individual zymogen granules was measured using molecule equivalent soluble fluorescein (MESF) units. A mixture of five different beads with well-established amounts of FITC (QuickCal beads, Flow Cytometry Standards Corporation) was used to obtain a regression equation for each measurement that allowed the calculation of the mean MESF content of zymogen granules based on its mean fluorescence channels (scaled from 0 to 1023 channels arbitrary units). The amount of lectin bound to each zymogen granule was expressed as the number of molecules of lectin calculated from the ratio of the number of MESF for FITC obtained per granule and the number of MESF for FITC per one molecule of lectin.

2.6. Collection of pancreatic juice

Rats were anaesthetized with sodium pentobarbital (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 animals were unconscious during the entire juice collection period and body temperature was kept at 37 ± 16°C by placing the rats on a heating pad and monitoring rectal temperature with a thermometer. Pancreatic juice was collected in preweighed Eppendorf tubes on ice to preserve the enzyme content. Total protein concentration in pancreatic juice was determined by the Bradford method [18].

2.7. Statistical analysis

Results are expressed as means ± S.E.M. Comparative analysis between control and CCK-treated rats and between control and L-364,718-treated rats were made using the analysis of Variance (ANOVA) test followed by the Scheffé test. An unpaired Student's *t*-test was applied to the results obtained in the two subpopulations of zymogen granules in each experimental group. In all cases *P*-values lower than 0.05 were considered to be statistically significant.

3. Results

Fig. 1 shows a set of representative flow cytometric bivariate patterns of the isolated zymogen granules distributed according to their forward light scatter (FSC) and their side light scatter (SSC) properties from control rats (Fig. 1A) and according to the FSC and WGA-FITC labelling (Fig. 1C). Fig. 1B shows and isotype-matched negative control. Each dot plot is representative of one experiment.

On the basis of both parameters FSC and SSC, a similar distribution of zymogen granules was ob-

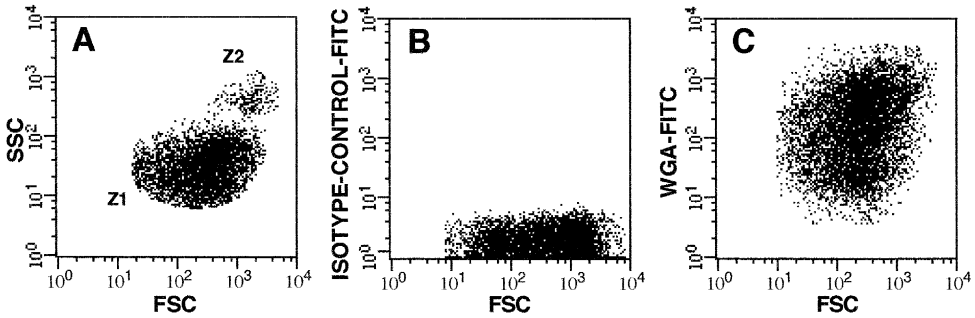

Fig. 1. Flow cytometric characterization of pancreatic Z_1 and Z_2 zymogen granules of the control rat pancreas (log./log. representation). Forward scatter (FSC)/Side scatter (SSC) distribution pattern (A); FSC/fluorescence obtained for an isotype matched negative control (B); FSC/WGA-FITC associated fluorescence (C). Each dot plot is a representative example of one experiment.

served in controls, CCK- and L-364,718-treated rats. Accordingly, two well-differentiated populations of zymogen granules have been defined in all experimental groups: a major population (Z_1) and a minor subset (Z_2) with larger and more complex granules than in Z_1.

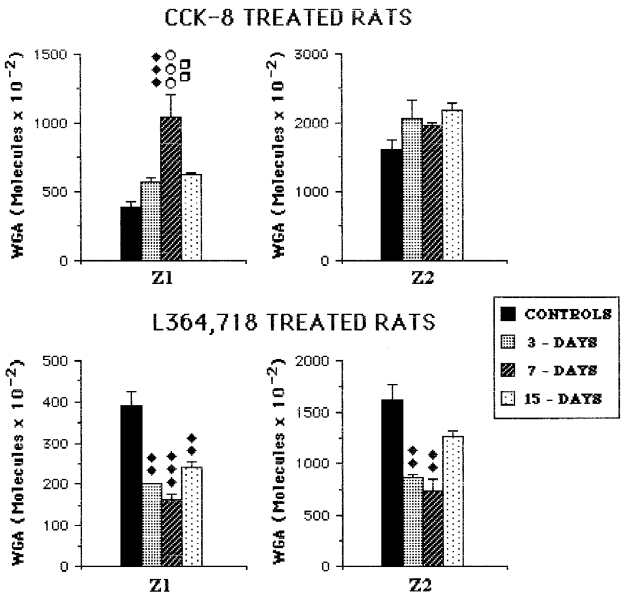

Fig. 2. Number of WGA molecules bound to each individual zymogen granule of control rats ($n=12$), CCK-treated rats (5 μg/kg/12 h) for 3 days ($n=6$), 7 days ($n=5$) and 15 days ($n=6$) and L-364,718 treated rats (0.1 mg/kg/day) for 3 days ($n=5$), 7 days ($n=6$) and 15 days ($n=6$). n, number of experiments. Values are means ± S.E.M. Application of ANOVA test followed by Scheffé test revealed significant differences with respect to controls (◆◆ $P<0.01$, ◆◆◆ $P<0.001$), 3 days CCK-treated rats (○○ $P<0.01$) and 15 days treated rats (□□ $P<0.01$).

The measurement of the mean number of WGA molecules bound to each individual zymogen granule belonging to both subpopulations (Fig. 2) revealed a significant increase ($P<0.001$) in Z_1 zymogen granules from pancreas of rats treated with CCK for 7 days. However, CCK treatment did not display any variation in the binding of FITC-WGA to Z_2 zymogen granules. On the contrary, a significant decrease in the number of WGA molecules bound to zymogen granules from L-364,718-treated rats was observed in both the Z_1 and Z_2 subsets at days +3 and +7 of the treatment. After fifteen days of L-364,718 treatment a significant reduction of WGA receptors was observed in Z_1 zymogen granules but not in those belonging to the Z_2 subset. In all animal groups a

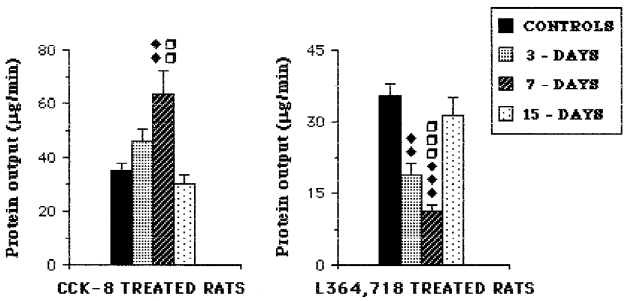

Fig. 3. Protein output in control rats ($n=11$), CCK-treated rats (5 μg/kg/12 h) for 3 days ($n=6$), 7 days ($n=6$) and 15 days ($n=6$) and L-364,718-treated rats (0.1 mg/kg/day) for 3 days ($n=6$), 7 days ($n=7$) and 15 days ($n=8$). n, number of experiments. Values are means ± S.E.M. Application of ANOVA test followed by Scheffé test showed significant differences when compared with controls (◆◆ $P<0.01$, ◆◆◆ $P<0.001$) and with 15 days CCK-treated rats (□□ $P<0.01$, □□□ $P<0.001$).

Table 1
Comparative analysis of the number of WGA molecules ($\times 10^{-2}$) bound to Z_1 and Z_2 zymogen granules

	Controls	CCK-8 (5 μg/kg/day)			L-364,718 (0.1 mg/kg/day)		
	($n = 12$)	3 days ($n = 6$)	7 days ($n = 6$)	15 days ($n = 6$)	3 days ($n = 6$)	7 days ($n = 6$)	15 days ($n = 6$)
Z_1	389.7 ± 35.6	570.5 ± 31.0	1038.6 ± 167.4	623.4 ± 15.4	200.6 ± 2.1	164.6 ± 11.4	240.1 ± 13.5
Z_2	1604.3 ± 155.7 *	2065.7 ± 258.9 *	1963.0 ± 36.6 *	$2192{,}1 \pm 91.1$ *	867.1 ± 26.3 *	739.2 ± 113.4 *	1261.1 ± 62.2 *

Values are expressed as means ± S.E.M. n = number of experiments.
(* $P < 0.001$).

significant higher number of WGA molecules bounds to Z_2 zymogen granules than to those from Z_1 subset (Table 1).

Fig. 3 shows the results observed in pancreatic secretion. A progressively higher increase in protein output was observed after 3 and 7 days of CCK treatment but the secretion returned to control values after 15 days. L-364,718 treatment displayed the opposite effect since protein secretion decreased after 3 and 7 days, while at day +15 values close to those of controls were reached.

4. Discussion

Previous studies have demonstrated that the protein composition of pancreatic zymogen granules of different species is relatively simple, containing 8–10 major proteins [19–21]. Ronzio et al. [22] reported that N-acetylglucosamine and sialic acid are important carbohydrates in the composition of granule membrane glycoproteins. The amount of these glucidic radicals can be easily detected by labeling with WGA, a lectin that specifically binds to them. Since the content within zymogen granules from rat pancreas fails to bind with WGA [7], this lectin is useful for analyzing the changes in the quantity of N-acetylglucosamine and sialic acid in the membrane that occur in response to stimuli such as CCK, which is known to have effect on both the formation of new granules [23] and on exocytosis [24], two processes involving membrane glycoproteins [1–4].

Despite the high content of N-acetylglucosamine and sialic acid reported in pancreatic zymogen granules [22], no binding of WGA to the granule surface has been observed in previous studies [25]. Therefore, the majority of N-acetylglucosamine and sialic acid residues must be orientated towards the inside of the zymogen granule. Intact zymogen granules allow the passage of protein across their membrane [26] and previous studies on intact and permeabilized granules have demonstrated that antisera which come up against intragranular enzymes are able to cross the membrane of non-permeabilized granules, showing a higher fluorescence intensity in intact granules than in permeabilized ones when incubated with FITC-lectins [12]. Therefore FITC lectins can specifically bind to carbohydrates of membrane glycoproteins localized outside or inside zymogen granules. Ronzio's work [22] reported three major glycoproteins as firmly bound constituents of zymogen granule membrane: GP-1, GP-2 and GP-3. Of these, GP-2 was shown to be the most abundant, accounting for 40% of total zymogen granule membrane proteins. Recently, a new glycoprotein identified as GP-4, with similar molecular mass to that of GP-3 has been purified from the zymogen granule membrane of pig pancreas [27] and from human pancreatic juice [28]. GP-1 is the only one exposed on the external face of zymogen granules [22]. Although these glycoproteins have been characterized on the molecular level [27–30] their functions remain unclear. It has been assumed that GP-2 has a role in granule formation [31]. GP-3 is released into the pancreatic juice and its secretion is strongly regulated by cholecystokinin [32]; however, it has been suggested that this glycoprotein is not part of the general exocytotic machinery [33]. GP-4 has demonstrated ATP-diphosphohydrolase activity [27] and it is released into pancreatic juice [28]. Cytochemical studies have observed ATP-diphosphohydrolase activity on the plasma membrane of the acinar cells of rat pancreas [34]. Therefore it is likely that GP-4 is involved in the fusion of mature zymogen granules with the acinar cell membrane during exocytosis.

In a similar fashion to that observed in control [12]

and adrenalectomized rats [16], two different subsets of zymogen granules are clearly identified in rats subjected to long-term CCK and L-364,718 treatments on the basis of FSC and SSC characteristics. FSC is a parameter directly related to the cross-sectioned area [35], and therefore reflects the zymogen granule size while SSC mainly reflects their internal complexity [36]. Accordingly, Z_1 zymogen granules are smaller and less complex which could be related to the newer granules as has been suggested previously [37], while the Z_2 zymogen granules could represent more mature granules formed as a result of the fusion of granules that come into close contact with each other. Moreover, significant differences in the amount of amylase and trypsinogen stored in zymogen granules have been found between Z_1 and Z_2 zymogen granules in rats treated with CCK [38]. Regarding the glycosylation of their membrane differences between zymogen granules of both subsets have also been observed in this study, the Z_2 granules displaying a significantly higher FITC-WGA labelling than those of Z_1. This finding could have a physiological significance taking into account other distinctive characteristics between both zymogen granule subsets, in fact Z_2 granules are bigger, more complex and have higher enzyme content than Z_1 ones; thereby these seem to be the best prepared to release the enzyme secretion by exocytosis. The high degree of glycosylation that characterizes Z_2 zymogen granules could be required in the step of recognition between the granule and acinar apical membrane in the exocytosis process.

FITC-WGA labelling demonstrates a clear effect of CCK on the composition of zymogen granule membrane glycoconjugates. It is noteworthy that the amount of sialic acid and N-acetylglucosamine significantly decreased when CCK was blocked by L-364,718 (the most specific CCK-receptor antagonist [39]), and only increased when CCK exerted its highest secretagogue effect, at day $+7$ of continuous treatment. The return of WGA receptors to control values observed at day $+15$ of CCK administration coincides with a failure in CCK to stimulate pancreatic secretion. Both findings could be related to a desensitization of the pancreas to long-term CCK treatment as has previously been reported [38,40]. Since CCK stimulates the formation of new granules and exocytosis, it would seem logical to suppose that glycoconjugates involved in these functions would increase in the zymogen granule membrane of rats treated with CCK for 7 days. As such, our results suggest that N-acetylglucosamine and sialic acid increases could be related to these CCK regulated functions. Moreover, long-term CCK administration displayed a differential effect on Z_1 and Z_2 zymogen granules. Taking into account that only an increase in WGA receptors was observed in granules belonging to the Z_1 subset – the newest granules –, but not in the Z_2 zymogen granule subset, the finding could support the notion that N-acetylglucosamine and sialic acid could be part of the composition of glycoproteins involved in zymogen granule formation.

On the other hand, the fact that the amount of WGA molecules bound to the zymogen granule membrane decreases in rats whose secretion is also significantly reduced by the CCK-receptor antagonist effect suggests that N-acetylglucosamine and sialic acid could become a ‘label’ in the recognition step between zymogen granule and the apical plasma membranes in the exocytosis process. This hypothesis would also be supported by the results observed in CCK-treated rats in which the pattern of protein secretion is similar to the changes in the amount of WGA receptors in the membrane.

We cannot state with certainty which glycoconjugate is the most influenced by CCK levels but our results suggest that some glycoprotein involved in the secretion process would be the most likely candidates. GP-3 is strongly regulated by cholecystokinin, although its labelling with WGA-coupled horseradish peroxidase on a Western blot system has been reported to be weak [32]. On the other hand, GP-4 is a highly glycosylated protein with diphosphohydrolase activity [27] that has recently been associated with secretory process. Since the hydrolysis of ATP could provide energy required by the exocytosis, it could be one of the glycoproteins influenced by the treatment. Nor should the possibility of an alteration in the glycosylation of glycoproteins in response to CCK levels be discarded: i.e., the binding site may change while the number of glycoproteins stays stable. In any case, it is noteworthy that CCK exerts a direct effect on the glucidic composition of the zymogen granule membrane in rat pancreas and a correlation between the amount of N-acetylglucosamine and sialic acid and the pancreatic protein secretion can be estab-

lished. Later studies should be carried out in order to find out whether any specific glycoprotein or the general glycosylation of zymogen granule membrane is affected by CCK or its antagonist.

Acknowledgements

This study was supported by a grant from DGICYT (PB 93-0638), Spain. Thanks are due to Mark Andersson for revision of the manuscript.

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