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COLD-INDUCED LEAKAGE OF AMYLASE FROM THE ZYMOGEN GRANULE AND SEALING OF ITS MEMBRANE BY SPECIFIC LIPIDS

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SUMMARY

Zymogen granules isolated from rat parotid gland are cold-labile after washing at 25°. Transfer to 0° causes rapid and extensive release into the medium of amylase stored inside the granules. Addition of defatted serum albumin further increases the leakage of amylase in the cold and also causes the release of this enzyme at 30°. Enzyme leakage from the granules in the cold is completely prevented by the prior addition of the following lipids and related compounds: lauric acid (10^{-4} M), lauryl alcohol ($2 \cdot 10^{-5}$ M) and glyceryl monoolein ($2 \cdot 10^{-5}$ M). Phospholipids are ineffective. The ionic strength of the medium in which the granules are suspended is not a critical factor since buffered 0.3 M sucrose or 0.15 M KCl serve equally well for the demonstration of enzyme leakage and its prevention. It is concluded that the limiting membrane of the zymogen granule becomes leaky due to modification of its lipid structure at low temperatures. Certain lipids, when added at low concentration, are taken up by the membrane and thus prevent leakiness of the granule in the cold.

INTRODUCTION

A number of investigators have reported that certain microorganisms release intracellular constituents and lose viability when the cells are rapidly but briefly chilled[1–3]. It was recently argued that the reduced temperature may cause a physical modification of the lipid structures in cellular membranes so as to increase their permeability[4,5].

The present communication demonstrates that the zymogen granules of rat parotid gland which store and secrete digestive enzymes (*cf.* ref. 6) are cold-labile. The limiting membrane of the isolated washed granules becomes leaky in the cold and amylase stored inside the granule is released into the medium. It is further shown that when certain lipids are added they prevent enzyme leakage in the cold, which indicates that the permeability of the membrane can be controlled by its lipid structure. A note on some of the earlier findings has been published[4].

EXPERIMENTAL

Isolation of zymogen granule fractions

Rat parotid glands were removed from the animal after overnight starvation[7] and immediately transferred to Krebs–Ringer bicarbonate medium at 37° which was continuously gassed with a mixture of 95% O_2:5% CO_2. Homogenization of the glands and subsequent isolation of the zymogen granule fractions by differential centrifugation were carried out at 4° or at 25°. As will be shown, the temperature of isolation markedly affected the properties of the granules. The glands were homogenized[7] in a medium containing 0.3 M sucrose, 10 mM Tris buffer (pH 7.6) or phosphate buffer (pH 7.0), 0.5 mM EDTA and 1 μg/ml of diphenyl-*p*-phenylenediamine. The latter reagent, which prevents lipid peroxidation[8], was added to the sucrose medium from a fresh solution in ethanol containing 5 mg/ml. Eight glands were homogenized in 30 ml of the above medium. A 0–250 $\times$ *g* fraction was obtained by centrifugation of the homogenate for 5 minutes. The supernatant was centrifuged at 1000 $\times$ *g* or 1300 $\times$ *g* for 10 minutes. Each fraction from 30 ml of homogenate was washed once in 10 ml of the above sucrose medium. The sediment was finally suspended in the homogenizing medium to contain 3000–8000 amylase units per ml. About 90% of the zymogen granules, containing roughly 50% of the total amylase, were sedimented by centrifugation of the homogenate for 10 minutes at 1000 $\times$ *g*. The highest specific activity of amylase was obtained in the 250–1000 $\times$ *g* fraction which contained about 25% of the total amylase of the homogenate[9].

Test system for amylase release from the granules as a function of temperature

Amylase was assayed according to BERNFELD[10], the unit of activity being defined as previously described[7]. Zymogen granules were prepared fresh before each experiment. Unless otherwise stated, the granule fraction sedimenting from the homogenate between 250 and 1000 $\times$ *g* was used. The sucrose medium for testing amylase release was the same as that employed for the isolation of the granules. The buffer, Tris or phosphate, is given under each experiment. When the effect of added lipids was tested, these were thoroughly dispersed in the sucrose medium prior to addition of the granules.

A 0.2-ml aliquot of the washed granule suspension was added to 0.8 ml of sucrose medium at 30°. After preincubation for 2 minutes at 30°, the mixture was transferred to an ice bath at 0° for 10 minutes. Duplicate tubes were kept at 30° for the same time period. Aliquots of 0.1 ml were removed for assay of total amylase and the mixtures were all centrifuged at 25° for 10 minutes at 1300 $\times$ *g*. The supernatant was decanted and assayed for amylase released from the granules. The total amylase activity of the system was defined as 100%. The amount of amylase released from the granules into the supernatant was expressed as % of total.

Control experiments showed that the lipids and other additions in these experiments had no effect on subsequent amylase determinations which served as a measure of enzyme release from the granules.

Assay of radioactivity

[^{14}C]Laurate was assayed in a Packard scintillation counter. The vials contained 0.2 ml of the aqueous sample and 10 ml BRAY's solution[11]. Counting efficiency was

60%. For the counting of lipids separated on thin-layer chromatograms, the silica gel was scraped off the glass plate and introduced into the scintillator solution. Counting efficiency was not significantly affected by the silica gel powder.

Extraction and separation of lipids

Suspensions of zymogen granules were extracted with chloroform–methanol (2:1) by shaking at room temperature for a few minutes. The clear solvent phase was readily separated after centrifugation and was concentrated to a small volume under a stream of nitrogen. Neutral lipids and fatty acids were separated by thin-layer chromatography on silica gel G according to FREEMAN AND WEST[12]. For the assay of radioactivity, chromatograms were exposed to iodine vapor and the various spots and blank areas were scraped off the glass plates and counted. Non-radioactive oleic acid was added to the sample on the chromatoplate to ensure accurate location, by iodine, of the free fatty acid spot. For sensitive qualitative detection of the lipid components present in the zymogen granule, some chromatograms were sprayed with 50% sulfuric acid and heated[13].

Major groups of phospholipids present in the solvent extract of zymogen granules were identified by thin-layer chromatography on silica gel G with the solvent mixture, chloroform–methanol–water–acetic acid (65:25:2:2, by vol.)[13].

Materials

Asolectin, a soy-bean phospholipid concentrate was purchased from Associated Concentrates Inc., Woodside, Long Island; purified lecithin and a sphingomyelin-rich fraction were products of Sylvana Co., Orange, New Jersey; [^{14}C]lauric acid was obtained from the Radiochemical Centre, Amersham, England. Diolein phosphatidic acid was synthesized in this department by Dr. LAPIDOT. Other lipids were purchased at the highest grade available and their purity was checked by thin-layer chromatography.

Crystalline bovine and human serum albumin were products of Calbiochem and Mann Research Co., respectively. These two products were defatted according to GOODMAN[14]. Bacterial phospholipase C was a product of Worthington Co., New Jersey. Purified gas gangrene antiserum which specifically inhibits the bacterial phospholipase C (*cf.* ref. 15) was obtained from the Pasteur Institute, France.

RESULTS

The membranes obtained from purified fractions of zymogen granules of rat parotid gland contain about 10% of the total protein of the granules and the ratio of phospholipid to protein in the membrane is about 1:5 (ref. 9). Thin-layer chromatography revealed the presence of the major classes of phospholipids, cholesterol, cholesterol ester, some triglyceride and trace amounts of material corresponding in mobility to fatty acid and to monoglyceride. While such trace components might be of special significance there is no doubt that in its gross composition the zymogen granule membrane resembles other known lipoprotein membranes.

The importance of the intact lipid structure of the membrane in preventing the escape of amylase stored inside the zymogen granule could be readily demonstrated. Incubation with phospholipase C caused rapid release of amylase from the granules

TABLE I

AMYLASE RELEASE FROM ZYMOGEN GRANULES BY PHOSPHOLIPASE C

Zymogen granules were isolated at 4° in sucrose–Tris medium. Systems contained, zymogen granules (1000 amylase units), Tris–sucrose medium and other additions in 1 ml. The phospholipase C antiserum was preincubated with phospholipase for 1 min at 30° prior to the final addition of 0.1 ml of zymogen granules. Incubation, 15 min at 30°. The final dilution of antiserum (v/v) is shown in the table.

Expt.	*Phospholipase C (μg)*	*Additions*	*Amylase released from granules % of total*
I	0	Ca^{2+} 5 mM	9
	20	Ca^{2+} 5 mM	90
	20	Mg^{2+} 5 mM	9
II	0	Ca^{2+} 5 mM	8
	100	Ca^{2+} 5 mM	100
	100	Ca^{2+} 5 mM, antiserum 1:1000	11
	100	Ca^{2+} 5 mM, antiserum 1:10 000	100
	10	Ca^{2+} 5 mM	50
	10	Ca^{2+} 5 mM, antiserum 1:10 000	8

(Table I). The reaction required calcium ions and was inhibited by an antiserum specific for the bacterial phospholipase C. It should be noted that 5–15% of the amylase was usually released from the granules as a result of damage caused during resuspension after centrifugation.

Leakage of amylase from the granules in the cold

Zymogen granules isolated and washed at 4° showed a variable degree of amylase leakage in the cold. It was noted that transfer of the granules to 30° prevented leakage. Granules were therefore prepared and washed at 25°. When such a suspension of granules was cooled in ice-water, rapid and extensive release of amylase took place. A marked loss in the turbidity of the granule suspension was also often observed. The extent of amylase release varied somewhat with each preparation for unknown reasons. The presence of nuclei which are sedimented mainly in the 0–250 × *g*

TABLE II

COLD LABILITY OF ZYMOGEN GRANULE FRACTIONS ISOLATED AT ROOM TEMPERATURE

Fractions were isolated from the homogenate at 25° in sucrose–Tris medium as outlined under EXPERIMENTAL. The fraction sedimented at 1300 × *g* was washed once at 1300 × *g*. All other fractions were washed once at 1000 × *g*. Granules (800 amylase units) were incubated in the above medium for 10 minutes at 30° and 0°.

Granule fraction (× g)	*Amylase released from granules % of total*	
	At 0°	*At 30°*
0–250	61	15
0–1000	62	11
250–1000	54	14
0–1300	50	15

TABLE III

SENSITIZATION TO COLD SHOCK OF GRANULES ISOLATED IN THE COLD

Zymogen granules were isolated at 4° in sucrose–phosphate medium. Aliquots of the granule suspension in the above medium, containing 800 amylase units, were tested. For the pretreatment duplicate tubes were set up. For the final incubation of 10 minutes one tube was incubated at 30° while its duplicate was kept at 0°.

Pretreatment	*Amylase released % of total*	
	At 0°	*At 30°*
Granules in 1 ml, 40 minutes at 30°	17	9
Granules in 1 ml, 40 minutes at 0°	14	13
Granules in 1 ml, sedimented and resuspended in 1 ml, all at 30°	18	13
Granules diluted to 10 ml at 30°	15	13
Granules diluted to 10 ml at 0°	15	15
Granules diluted to 10 ml, sedimented and resuspended in 1 ml, all at 30°	42	17

fraction and mitochondria which heavily contaminate the 1300 × *g* fraction seemed to have little effect on amylase release (Table II).

Granules isolated in the cold and demonstrating a sluggish and variable release of amylase on further incubation at 0° became cold-labile only after washing with a relatively large volume of warm medium (Table III). These findings indicated that a component which is essential for keeping the membrane impermeable in the cold is removed by washing the granules at 25°. In further support of this conclusion it was found that serum albumin, which efficiently binds certain lipids, markedly increased amylase release from the granules in the cold. Furthermore, addition of serum albumin induced release of amylase even at 30° (Table IV).

TABLE IV

EFFECT OF DEFATTED SERUM ALBUMIN ON AMYLASE RELEASE FROM ZYMOGEN GRANULES

Expt. I. The granule fraction was isolated at 25° in sucrose–phosphate medium. Incubation mixtures contained granules (600 amylase units) and bovine serum albumin in the above medium. Expt. II. The granule fraction was isolated in sucrose–phosphate medium at 4°. In order to induce cold lability portions of the zymogen granule suspension containing 800 amylase units were washed at 25° with 10 ml of the above medium as shown in Table III. The final incubation time for both experiments was 10 minutes at 0° and 30° in a volume of 1 ml.

Expt.	*Granule fraction*	*Additions*	*Amylase released % of total*	
			At 0°	*At 30°*
I	Prepared at 25°	None	46	11
		Bovine serum albumin, 0.25%	78	—
		0.50%	91	43
		1.00%	88	46
II	Prepared in the cold, washed at 25°	None	42	17
		Human serum albumin 1.2%	—	49

The removal of extremely small amounts of a lipid component of the membrane appeared to be involved so that isolation and identification of the active compound was not yet feasible.

As an alternative approach, a search was conducted for lipids or related compounds which would protect the granule against leakage of amylase. The survey revealed a most interesting pattern of specificity. Low concentrations of glyceryl monoolein, lauryl alcohol and long-chain fatty acids, especially lauric acid, completely protected the granules against leakage of amylase in the cold (Table V). Sodium lauryl sulfate showed only partial protection within a narrow range of concentration above which it increased amylase leakage.

TABLE V

PROTECTION AGAINST AMYLASE LEAKAGE BY LIPIDS AND RELATED COMPOUNDS

Expt. I. The zymogen granule fraction was isolated in sucrose–Tris medium at 25°. Each test mixture contained zymogen granules (700 amylase units) in the above medium and additions as shown in the table. Expt. II. Same as described for Expt. II of Table IV. Incubation in both experiments was 10 minutes at 0° and 30°.

Expt.	*Additions (mM)*		*Amylase released % of total*	
			At 0°	*At 30°*
I	None		37	6
	Monoolein,	0.1	11	8
	Monoolein,	0.02	11	—
	Monoolein,	0.005	39	—
	Lauryl alcohol,	0.1	9	—
	Lauryl alcohol,	0.02	9	—
II	None		75	7
	Lauric acid,	0.1	9	8
	Lauryl sulfate,	0.01	65	—
	Lauryl sulfate,	0.02	46	—
	Lauryl sulfate,	0.04	100	—

The following compounds showed no significant protection against leakage of amylase: laurylamine, caprylic acid, cholesterol, cholesterol ester, lecithin, diolein phosphatidic acid, sphingomyelin and sonicated asolectin[16].

The amount of the added lipid compound actually taken up by the granule, presumably by its membrane, was determined in the case of lauric acid (Table VI). It was found that even trace amounts of lauric acid taken up by the granule will partially protect against leakage of amylase in the cold. It was further demonstrated that the [^{14}C]lauric acid taken up by the granules remained unesterified. At the termination of the experiment shown in Table VI the pellets of granules were pooled and the lipids were extracted and separated by thin-layer chromatography[12]. Of the total radioactivity placed on the chromatoplate (1700 counts/min) more than 99% was located in the free fatty acid spot.

Leakage of amylase and its prevention in KCl medium

Isolation of zymogen granules at 4° in a medium in which 0.15 M KCl replaced 0.3 M sucrose resulted in a low yield of amylase in the washed granule fraction. Such

TABLE VI

UPTAKE OF [^{14}C]LAURIC ACID BY ZYMOGEN GRANULES AND ITS EFFECT ON AMYLASE RELEASE IN THE COLD

The granule fraction was prepared in sucrose–phosphate medium at 25°. The systems contained zymogen granules, 1.5 mg protein (1400 amylase units) and [^{14}C]laurate in the above medium in a final volume of 2 ml. The incubation time was 5 minutes at 30° and 0°. The specific radioactivity of [^{14}C]laurate was $2.5 \cdot 10^8$ counts/min per μmole. The amount of [^{14}C]laurate bound to the granules was determined by difference, from the radioactivity of the system before and after removal of the granules by centrifugation. As explained in the text the precipitate of granules was saved for analysis by thin-layer chromatography.

Incubation temperature	*[^{14}C]Laurate added (mM)*	*[^{14}C]Laurate bound to the granules (μmoles per mg protein)*	*Amylase released % of total*
30°	—	—	15
0°	—	—	66
30°	0.20	0.04	18
0°	0.20	0.02	10
30°	0.07	0.01	12
0°	0.07	$<$ 0.01	30

losses of amylase from the granules did not occur when the isolation in KCl medium was performed at 25°. The granules prepared in KCl are thus already sensitive to cold during the isolation of the crude fraction and the first wash. Granules isolated in sucrose medium at 25° but subsequently tested in KCl medium were also cold-sensitive and could be efficiently protected by lauric acid against leakage of amylase (Table VII). It is thus evident that these phenomena are not specific for sucrose, which is usually used as an isoosmotic medium, and that the ionic strength of the solution is not a critical factor.

DISCUSSION

The zymogen granule appears to have no substructure apart from its limiting lipoprotein membrane[7,17,18]. Inside the granule, digestive enzymes accumulate and

TABLE VII

AMYLASE RELEASE AND ITS PREVENTION TESTED IN KCl MEDIUM

The granule fraction was isolated in sucrose–phosphate medium at 25°. Aliquots of 0.2 ml of the granule suspension (1500 amylase units) were added to 1.8 ml of medium in which 0.15 M KCl replaced 0.3 M sucrose. The buffer was phosphate. The incubation time was 5 minutes at 30° and 0°.

Additions (mM)	*Amylase released % of total*	
	At 0°	*At 30°*
None	59	16
Lauric acid 0.25	17	20
Lauric acid 0.12	23	16
Lauric acid 0.06	25	—

reach an extremely high concentration prior to secretion by the gland cell[18]. Because of these factors the leakage of enzymes from the isolated granule appears to occur readily and may serve as a sensitive indicator of minor changes in the structure of the granule membrane. It should be noted that leakage of enzyme protein from the granule is probably a secondary event. In the primary reaction a change in the structure of the membrane apparently permits the entry from the outside medium of small solute molecules such as sucrose or KCl which are intended to stabilize the granules as an isoosmotic medium. The entry of solute and water causes swelling which finally permits the release of protein through the membrane.

The fact that the zymogen granule became leaky under certain conditions in the cold should probably be ascribed to a change in the state of aggregation of the lipids in the granule membrane. ADAM[19] showed that monomolecular films of lipid will contract on cooling within a certain temperature range which is specific for each lipid. It is also established that salts of long-chain fatty acids in solution will leave the micellar state and form microcrystals on cooling to a certain characteristic temperature which is known as the Krafft point and is almost independent of the concentration of the substance[20]. The specific temperature at which these phenomena occur is mainly dependent on the chemical structure and composition of the lipids tested. It is therefore of interest to note that enzymatic modification of a lipid cell membrane which increased its permeability in the cold has already been reported. Incubation of sheep erythrocytes with minimal amounts of phospholipase C caused lysis on subsequent cooling, while at 37° the cells remained quite stable[21]. There are also a number of other reports which link the effect of temperature on biological membranes with their lipid structure[22].

Taking into account the properties of lipids outlined above, the release of amylase from the zymogen granule in the cold is best interpreted in the following way. A lipid component on the granule membrane is removed by washing at 25° or by binding to serum albumin. This permits the remaining lipid structure to undergo rearrangement on cooling which renders the membrane permeable. The identity and origin of the lipid removed from the membrane is as yet unknown. Since there is some evidence that the zymogen granules are cold-labile within the cell[4] it is even possible that the lipid which is removed is not a true component of the membrane but is adsorbed on it during homogenization.

The findings on added lipids which reseal the zymogen granule membrane and thus prevent amylase leakage are of special interest. They serve to demonstrate that specific lipid compounds are taken up by the membrane and dramatically modify its permeability properties. Of the series of compounds having a C_{12} chain, lauryl alcohol, lauric acid and lauryl sulfate, the alcohol was most efficient in preventing leakage from the granules. The carboxylic acid was almost as good while the sulfate showed only partial protection and total lysis at slightly higher concentration. Monoolein, however, which has two hydroxyl groups, was about as efficient as lauryl alcohol. Taking into account also the series of other lipids and phospholipids tested, the following rather tentative description of a compound which will be taken up within the lipid membrane structure and reseal it, seems meaningful. The molecule should not be too bulky (like cholesterol or phospholipids) and should form a stable aqueous solution or dispersion at a concentration of about 1 mM at 25°. The hydro-

philic group should preferably be a hydroxyl while the hydrophobic group should be an open hydrocarbon chain.

It is important to point out that addition of such a lipid to a suspension of granules creates a state of competition between the suspended lipid and the lipids in the membrane. Lipids of the granule membrane may dissolve in the added lipid micelles which would lead to lysis. On the other hand, the lipid in the medium may dissolve in the membrane to a limited extent and prevent lysis. The result depends on the nature of lipid added and on its concentration. Thus there is a relatively large concentration range in which lauric acid protects the granules against cold leakage, while with lauryl sulfate the concentrations which protect and those which cause lysis almost overlap (see als ref. 4).

Rather small amounts of added fatty acids and monoglycerides such as monoolein, were shown to affect the permeability of the zymogen granule membrane. Such compounds may well occur under certain conditions in the living cell. The present findings therefore raise the interesting possibility that intermediates of lipid metabolism may play a role in regulating the permeability properties of cellular membranes.

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