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Protection of insulin from enzymatic degradation by its association to liposomes

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Summary

The degradative action of three digestive enzymes (pepsin, α-chymotrypsin and trypsin) was investigated against insulin associated or entrapped in positively charged liposomes. Under the chosen experimental conditions, both insulin preparations were protected against enzymatic degradation. Furthermore, the nature of the interaction between external phospholipidic bilayer and insulin was found to result from an ionic association. Finally, the protective role of liposomes appeared highly dependent upon the molar proportion phospholipid/hormone.

Introduction

Many active polypeptidic drugs, such as insulin, cannot be orally administered, especially because they are denatured by digestive enzymes. Liposomes have been proposed as a carrier to allow the oral administration of insulin.

Although successful attempts were made by several authors (Dapergolas and Gregoriadis, 1976; Gregoriadis et al., 1976; Patel and Ryman, 1976), some conflicting results were reported concerning the efficiency of the absorption of liposomal insulin from the gastrointestinal tract. Dapergolas and Gregoriadis (1976) claimed

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that oral administration of liposomally-entrapped insulin to normal rats produced a significant fall in blood glucose. Patel and Ryman (1977) as well as Hashimoto and Kawada (1979) showed, however, the lack of hypoglycaemic effects in normal rats, whereas a significant reduction of the blood glucose level was observed after oral administration of liposomally-associated insulin to diabetic animals. Moreover, the poor reproducibility of the hypoglycaemic effect was often reported in the literature (Patel and Ryman, 1977; Hashimoto and Kawada, 1979; Shenfield and Hill, 1982; Arrieta-Molero et al., 1982).

We previously described (Weingarten et al., 1981) the significant hypoglycaemic effect obtained after intrabuccal administration of insulin-associated liposomes, although that preparation was ineffective after intragastric administration to normal rats. Surprisingly, the hypoglycaemic effect was also recorded after intrabuccal instillation of a mixture containing empty liposomes with free insulin. This preparation was, however, ineffective after intragastric administration. Despite the fact that a few authors (Hashimoto and Kawada, 1979; Patel and Ryman, 1977) have studied the in vitro protective action of liposomes against proteolytic degradation of insulin, no work was systematically carried out in order to investigate not only the carriage of free insulin associated with empty liposomes, but also the exact role of each enzyme concerned in the hormone's degradation. Therefore, the aim of the present paper is to describe the degradative action of three digestive enzymes (pepsin, α-chymotrypsin and trypsin) on liposome-associated insulin.

Materials and Methods

Chemicals

Dipalmitoylphosphatidylcholine (DPPC), stearylamine and enzymes (pepsin, titer 440 IU/mg, trypsin from porcine pancreas, titer 15000 BAEE IU/mg, and chymotrypsin from bovine pancreas, titer 52 IU/mg) were purchased from Sigma, Cholesterol came from Rhône-Poulenc, soja phosphatidylcholine from Lecithos-France and bovine insulin (titer 25 IU/mg) from Organon. All chemicals were used as purchased.

Preparations

Free insulin solution was prepared after dissolving insulin powder (800 IU) in 10 ml HCl 2×10^{-2} N. After adding 5 ml of distilled water, the pH of the insulin solution was then rapidly raised up until solubilization, by use of NaOH 4×10^{-2} N. This insulin stock solution was then diluted in a buffer solution pH 7 (KH_2PO_2–$Na_2HPO_4 \cdot 2H_2O$ 7 mM, NaCl and phenol 25 mM) to obtain solutions of different concentrations (2.5–10 IU/ml).

Preparation of insulin-entrapped liposomes was made according to the method previously described by Gregoriadis et al. (1971). Briefly, DPPC or soja phosphatidylcholine/cholesterol/stearylamine (7 : 2 : 1 molar ratio) were dissolved in a chloroform medium, which was evaporated at 37°C in a rotary dryer under reduced pressure. The residual film was dispersed at 50°C, in the buffered insulin solution, in

order to adjust lipidic concentration to 27 μmol/ml. The liposomal suspension was left for 2 h at room temperature and sonicated (Branson, Danbury, CT, U.S.A.) for 2 min in a nitrogen atmosphere at 40°C. Non-entrapped insulin was separated by gel filtration (Ultrogel A_6, IBF, Gennevilliers, France) on a column 26 × 150 mm and by using PBS solution (phosphate buffer 6.7 mM + Na_3N 24 mM and NaCl 0.15 M) as eluant. With a rate of 0.5 ml/min, insulin-entrapped liposomes were found in the fractions 7–13, and free insulin in fractions 20–30. The final liposomal vesicles were oligolamellar, with a size of about 200 nm. This preparation is called "insulin-entrapped liposomes". Determination of insulin was performed by HPLC (high-performance liquid chromatography), according to the method described by Biemond et al. (1979). Dosage of phospholipids was performed by an enzymatic method, according to Takayama et al. (1979).

The liposomal suspension devoid of insulin was prepared according to the method previously described, but the lipidic film was dispersed in a buffer solution 7 mM at pH 7.0, without insulin. Preformed empty liposomes were then mixed with free insulin solution, in order to reach a lipidic concentration of 27 μmol/ml. The mixture was kept for 15 min at room temperature and non-associated insulin was separated by gel filtration, in the same conditions as previously. This preparation is called "insulin-associated liposomes".

Enzymatic degradation study

Three enzymatic solutions were used: a pepsin solution (5 IU/ml) in a glycin buffer adjusted to pH 1.34, a trypsin solution (7350 IU/ml) and a chymotrypsin solution 23.5 IU/ml in a phosphate buffer at pH 7.84. Each enzymatic solution (100 μl) was incubated for 30 min at 37°C with free or liposomal insulin entrapped or associated preparations (100 μl).

The insulin concentration in the incubation medium was 2 IU/ml for entrapped insulin preparation and 0.5 IU/ml for associated one. Free insulin solutions were used as control in the same conditions of concentration.

Likewise incubations of free insulin solutions were performed with empty liposomes in the same experimental conditions.

Results and Discussion

A satisfying encapsulation rate "for insulin" was obtained after preparing liposomes according to the method described by Gregoriadis et al. (1971) (Table 1). Indeed, we found it possible to trap about 10% w/w of insulin inside phospholipidic vesicles. Furthermore, under the chosen experimental conditions, the amount of trapped insulin doesn't seem dependent upon the nature of the phospholipid used (Table 1). Insulin can also be associated to liposomes by simply mixing it with empty phospholipidic vesicles (Table 1). However, binding capacity was importantly reduced (2% w/w) probably because of possible association of insulin to the external phospholipidic bilayer. Besides, the interaction of the hormone at the outer surface of the carrier has been previously suggested by Wiessner and Hwang (1982).

TABLE 1

EVIDENCE OF TRAPPED INSULIN IN DIFFERENTS TYPES OF PREPARATIONS

Method of preparation	Nature of phospholipid	Concentration of insulin in the liposomal peaks [a]	
		(μg/μg/ of phospholipid)	(μmol/μmol of phsopholipid [b])
Bangham	DPPC	$91\ (\pm 4)\ \times 10^{-3}$	$11.24(\pm 0.55)\times 10^{-3}$
	soja phosphatidylcholine	$96.8(\pm 5)\ \times 10^{-3}$	$11.9\ (\pm 0.65)\times 10^{-3}$
Mixture and filtration	DPPC	$19.7(\pm 2)\ \times 10^{-3}$	$2.4\ (\pm 0.23)\times 10^{-3}$
	soja phosphatidylcholine	$11.1(\pm 1.3)\times 10^{-3}$	$1.4\ (\pm 0.16)\times 10^{-3}$

[a] Each value was the mean ± S.E.M. of 3 batches of preparations.
[b] 1 mol of DPPC = 740 g; 1 mol of soja phosphatidylcholine = 780 g.

Insulin-entrapped or -associated liposomes were incubated in presence of pepsin, trypsin or chymotrypsin. The experimental results showed that insulin was protected against enzymatic degradation after entrapping it inside liposomes, whereas free insulin solutions, used as control, were found to be completely degraded after incubation under the same conditions (Fig. 1A). Likewise, insulin associated to the external phospholipidic bilayer was found to be protected, whatever the nature of the phospholipid used (Fig. 1B). That observation is in contradiction with the results obtained by Adrian and Huang (1979) who claimed that proteins associated to the outer surface of the liposomes were not resistant to the trypsinic digestion. The exact mechanism of the protective action of liposome-adsorbed insulin is not clear. As presumed previously, the hormone could become protected against the digestive enzymes because of its tight association to the external lipidic bilayer by which is

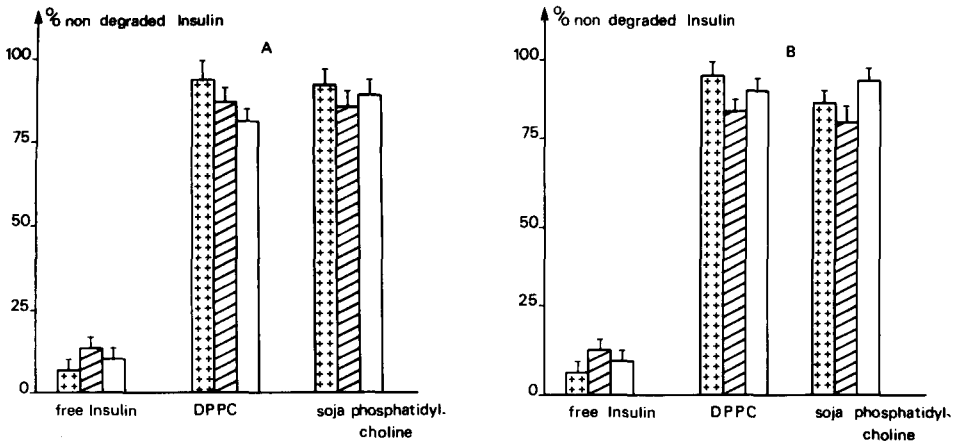

Fig. 1. Protection of liposomally entrapped (A) or associated (B) insulin towards enzymatic degradation (⊞, pepsin; ▨, trypsin; and □, α-chymotrypsin).

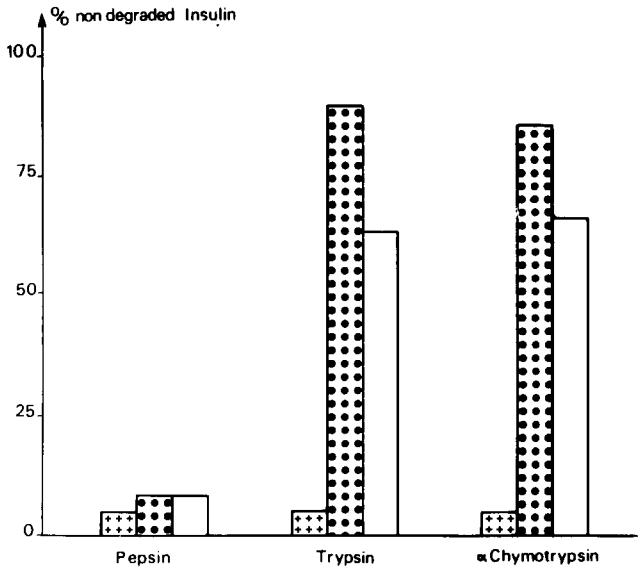

Fig. 2. Enzymatic activity of pepsin, trypsin or α-chymotrypsin towards free insulin (⊡) or insulin incubated with empty liposomes of DPPC (⊡) or soja phosphatidylcholine (□).

probably implied that the segments are sensitive to the enzymatic degradation. Moreover, enzymes themselves could be associated to the phospholipidic bilayers making them inactive against insulin. In order to decide between these two hypotheses, we studied the enzymatic activity after mixing extemporaneously empty liposomes, free insulin and proteolytic enzymes. The pH of these mixtures was 1.84 for the pepsinic incubation medium and 7.4 for the trypsinic and α-chymotrypsinic ones. As shown in Fig. 2, only the presence of empty liposomes in the incubation medium provided valuable protection of insulin against trypsin and α-chymotrypsin activity. In contrast, complete insulin degradation was observed after pepsinic incubation. These unexpected results could be explained by the pH used in the enzymatic incubation medium. Indeed, at pH 1.84 (for the pepsinic activity), insulin becomes positively charged whereas at pH 7.4 (for the trypsinic and α-chymotrypsinic activity) it appears negatively charged. Because of the positive charge of the used liposomes, it can be supposed that the association between external phospholipidic bilayer and insulin occurs only for a pH above its isoelectric point (pH = 5.5). From these results, it can be concluded that when the mixing was performed at pH 1.84, the hormone couldn't bind to liposomes and was destroyed by pepsin. On the contrary, when insulin was prebound to liposomes, by mixing of empty liposomes and free insulin solution at pH 7.0, the hormone appeared resistant to the pepsinic activity even if, for the enzymatic study in pepsinic buffer at pH 1.34, the net negative charge of insulin was reversed.

In order to state precisely the ratio of insulin to phospholipids necessary to maintain insulin undestroyed from enzymatic degradation, the same experiment was done but after modifying either insulin (Fig. 3A) or phospholipidic concentration

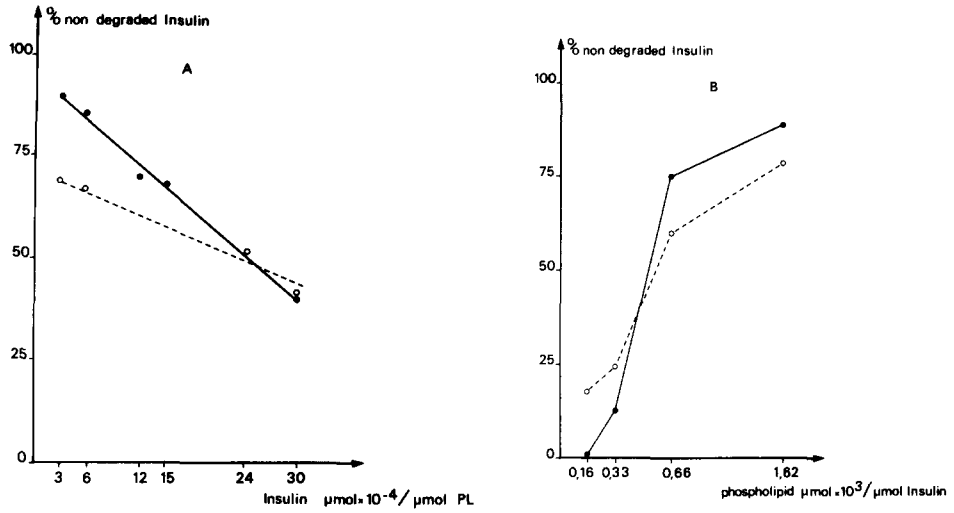

Fig. 3. Influence of insulin (A) or phospholipid (B) concentration on the protection of the insulin towards α-chymotrypsinic degradation. DPPC, ———; soja phosphatidylcholine, - - - - - -.

(Fig. 3B). The results obtained showed that protection of hormone towards chymotrypsinic degradation was highly dependent upon molar proportion of insulin to phospholipid. Indeed, when the ratio was higher than 12×10^{-4}, protection of insulin became weaker with both DPPC and soja phosphatidylcholine.

The presented data constitute an original approach for the interaction of insulin with liposomes after their mixing. Indeed, from our study it can be concluded that binding of the hormone to phospholipids results more from an ionic interaction than from a hydrophobic association. Furthermore, this adsorption to the external phospholipidic bilayer seems to be able to protect insulin from proteolytic enzymes. This could suggest that the failure of intragastric administration which we previously observed (Weingarten et al., 1981) was probably due to the degradation of the liposomal membrane by bile salts in the intestinal lumen rather than by the destruction of the hormone by gastric enzymes.

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