Vol. 128, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1985 Pages 68-74

INSULIN-MEDIATED FUSION OF NEGATIVELY CHARGED PHOSPHOLIPID VESICLES AT LOW pH

Ricardo N.Farías, Alberto López Viñals and Roberto D.Morero

Departamento de Bioquímica de la Nutrición
Instituto Superior de Investigaciones Biológicas
(INSIBIO), CONICET-UNT.
Chacabuco 461, 4,000, San Miguel de Tucumán, Tucumán (Argentina)

Received January 19, 1985

SUMMARY: Fusion of negatively charged phospholipid vesicles by bovine insulin was studied. The fusion induced by the hormone was demonstrated by resonance energy transfer, sepharose chromatography, light scattering and electron microscopy. The insulin effect was more effective when the pH was in the range of 3.6 - 3.9. The action of insulin also depends on the phosphatidylcholine: phosphatidic acid molar ratio, and buffer and vesicles concentration. At optimal conditions, half-maximal effect was obtained at 2 x 10^{-8}M. The insulin-mediated fusion is non especific. The potencial importance of these studies is discussed.

Membrane fusion is a widely observed phenomenon, still not well understood, in spite of intensive research. Fusion phenomena are a prerequisite in exocitosis, endocytosis, and formation of secondary lysosomas (1), events that could be important in the insulin action. It is proposed that a fraction of the receptor-bound insulin is internalized by endocytosis, part of the internalized hormone is descomposed in lysosomes or lysosome-like vesicles, and a fraction of the internalized insulin is discharged back into the extracellular medium by the mechanisms of exocytosis (2) as intact hormone. The insulin undergoes rapid dissociation from its receptors when the pH drops below 5.0 (3). An intermediate endosome compartment (which lacks lysosomal enzymes) may serve as an acid wash that promotes the ligand-receptor dissociation (4). Because of its cationic properties at low pH, insulin could be expected to interact with negatively charged phospholipid in biological membranes. Some effects of insulin on membrane fluidity have been described (5-8).

In this report, we show that insulin, at concentrations ten times higher than physiological ones, induces fusion of

0006-291X/85 $1.50

unillamelar vesicles containing acidic phospholipids, in a pH range from 3.0 to 4.0.

MATERIALS AND METHODS

Vesicles preparations: Phospholipid vesicles were prepared by the ethanol-injection method of Batzri and Korn (9). Phospholipids were dissolved in ethanol, and aliquots of 100 ul were injected into a stirred 10 ml solution of buffer 10 mM Tris ClH pH 7.4, at room temperature. For some experiments, the vesicles were also prepared in a similar buffer by ultrasonication in a sonicator bath under N_2. Various lipid compositions were used as indicated in the text. Phosphatidylcholine (PC) was purified from egg yolk (10). Phosphatidic acid (PA) was prepared by cabbage phospholipase D action (11). NBD-PE was synthesized from dipalmitoylethanolamine and NBD-chloride as described by Monti et al. (12). All phospholipids were checked for purity on thin-layer chromatography. The concentration of both phospholipids and liposomes are expressed as μM Pi.

Resonance energy transfer assay: The RET has been described in detail by Hoekstra (13). The fluorescence donor CA9C (cholesterol antracene 9 carboxylate), and aceptor NBD-PE (N-7-nitro-2 1,3 benzoxadiazol 4-yl phosphatidylethanolamine) were incorporated at different vesicles populations. The probes constituted 1.0 mole % or 3.0 mole % of the total lipid respectively. Equal volumens of CA9C-labeled vesicles and NBD-PE vesicles were mixed and fusion was initiated by the addition of 5-40 μl aliquots of hormone solution. The mixtures (2 ml) were incubated for 1 min at 22°C. Fusion of phospholipid vesicles results in the intermixture of lipids, which brings the CA9C and NBD-PE on close proximity. The efficiency of resonance energy transfer (RET) is defined by the equation (14).

$$RET = 1 - F/Fo$$

Where Fo and F are the fluorescence emission peak (470 nm) of CA9C in the absence and in the presence of the hormone respectively. Steady state fluorescence emission spectra of samples containing both CA9C and NBD-PE were obtained by exciting at 380 nm, in an Aminco Bowman spectrophotofluorometer. At low pHs (2.4 to 4.0) a little but consistent reduction of the fluorescence intensity of CA9C-vesicles in the presence of NBD-PE vesicles as a function of time was found, even in the absence of insulin (about 5-15 %, depending on experimental conditions). For this reason, control experiments to measure CA9C fluorescence without insulin addition were carried-out, and the results substracted from those obtained with the hormone. This disminution was not observed in the absence of NBD-PE vesicles. Light scattering changes were recorded with the excitation and emission monochromators set at 380 nm.

Electron microscopy: an aliquot of each sample (phospholipid vesicles in absence and presence of insulin) was pipetted onto formvar-coated grids. After approximately 1 min, the grids were blotted but not allowed to dry. A drop of 2 % phosphotungstic acid, pH 6.5 was applied. After 1 min, the grids were blotted, allowed to dry, and examined using a Zeiss, EM 109, 50 Kv.

Analysis of vesicle fusion by gel filtration: a vesicles mixture containing donor (CA9C) and aceptor (NBD-PE) probes was subjected to insulin-induced fusion, and applied to a sepharose 4B column (1.0 cm x 20 cm) equilibrated, and eluted with 20 mM

Tris-ClH pH 7.2. Fractions of 1.2 ml were collected, and the fluorescence of NBD-PE was recorded exciting at 470 nm. Bovine insulin: from Sigma Chemical, Co (St.Louis) was dissolved in 3 mM ClH, and adequated dilutions were obtained with destilled water. Porcine insulins from Sigma and Eli-Lilly Co (Indianapolis) (Lot LDG 04-94204) all of 24 units per mg, also were assayed.

RESULTS AND DISCUSSION

The vesicle fusion could be quantitated by a fluorescence resonance energy transfer assay, according to Uster and Deamer (15). As can be seen in Fig. 1 the donor fluorescence emission peak in the vesicles mixture, decreases in the presence of insulin. A simultaneous increase in the light scattering, which was consistent with the results of RET, was found in all the analyzed cases (inset of Fig. 1). Insulin did not affect the spectra and quantum yield of both CA9C labeled vesicles and NBD-PE labeled vesicles separately, but the light scattering was changed as expected considering the experiments performed when both types of vesicles were simultaneously present. The results described in Fig. 1 were unchanged in the presence of 2 mM EDTA. This fact ruled out the participation of some divalent cations in the observed phenomena. Identical results were obtained with vesicles prepared by sonication, or ethanol

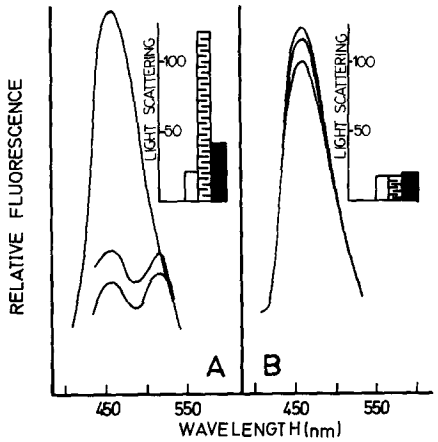

Fig. 1.- Effect of insulin on the emission spectra of mixed CA9C, and NBD-PE-labeled vesicles. Fusion mixture (2 ml final volume) 10 mM phosphate-citrate buffer pH 3.9 contained 60 nmoles phospholipids (PC:PA 8:2), 0.6 nmole of CA9C, and 1.8 nmole of NBD-PE. A; the emission spectra of fusion mixtures represent in descending order: cero time, 1 min after addition of 1 x 10^{-6}M insulin, and when the pH reaction mixture was raised up to 6.6 by the addition of 0.12 ml of NaOH 0.2 N. B; control experiment. Inset shows light scattering changes from same experiments; cero time (□), after 1 min (⊟) and after NaOH addition (■).

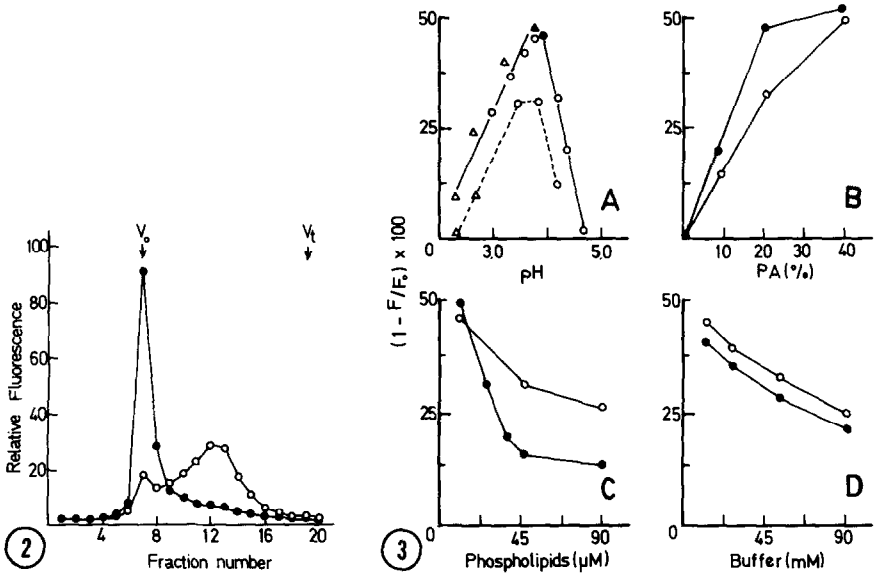

Fig. 2.- Analysis of phospholipid vesicles mixture by gel filtration prior (○) and after insulin-induced fusion (●). Experiment was performed as indicated in Fig. 1. After raising the pH to 6.6, the samples were applied to a Sepharose 4B column.

Fig. 3.- A; effect of pH; 10 μM of labeled phospholipid vesicles (PC:PA; 8:2) were incubated in the presence of 5 x 10^{-7}M (—), and 1 x 10^{-7}M (--) of insulin. The buffer concentration was of 10 mM of each phosphate-citrate (○), acetate-NaOH (●) glycine-ClH (△) and glycilglycine-ClH (▲). B; effect of phosphatidic acid concentration in vesicles; 10 μM of labeled phospholipid vesicles with increasing PA content were incubated in the presence of 5 x 10^{-7} M (○) and 1 x 10^{-6} (●) of insulin in 10 mM phosphate-citrate buffer pH 3.9. C; the effect of liposomes concentration; phospholipid vesicles composed of PC:PA at 8:2 (○) or 6:4 (●) molar ratio, were incubated in the presence of 5 x 10^{-7}M of insulin in 10 mM phosphate-citrate buffer pH 3.9. The vesicles (PC:PA; 6:4) were obtained by sonication. D; the effect of buffer concentration; 20 μM of labeled phospholipid vesicles (PC:PA; 8:2) were incubated in the presence of 5 x 10^{-7}M (○) or 1 x 10^{-6}M (●) of insulin in increasing buffer phosphate-citrate concentration, pH 3.9.

injection. The elution pattern of Sepharose 4B, showed in Fig.2, confirmed the conversion of small vesicles into fused forms. The electrom micrographs of negatively stained preparations of both types of vesicles (fused and non fused) indicated that the mean diameter of unillamelar vesicles increased from 200-300 Å to 1000 Å in the presence of insulin (data no shown). The fusion of vesicles induced by insulin was pH dependent (Fig. 3A), the optimal pH was in the range of 3.6 to 3.9. Decrease of PC:PA molar ratio increased the insulin fusion effect. Vesicles of PC lacking PA did not fuse (Fig. 3B). Increasing the concentrations of vesicles (Fig. 3C) or buffer (Fig. 3D) decreased the fusogenic action of the insulin. At optimal conditions of pH, PC:PA molar

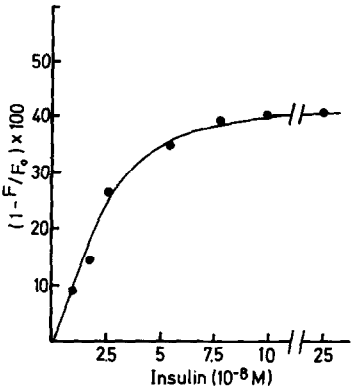

Fig. 4.- Resonance energy transfer as a function of insulin concentration; 10 μM of phospholipid vesicles (PC;PA; 8:2) were incubated in the presence of increasing insulin concentration in 20 mM phosphate-citrate buffer at pH 3.6.

ratio, and vesicles and buffer concentrations, insulin was capable to induce fusion at concentrations of 10^{-8}M or even less (Fig. 4). We also found that porcine insulin has e similar dose response effect that bovine insulin (data no shown). It can be calculated that there are about three to six hormone molecules per vesicle at e concentration of 2 x 10^{-8}M of insulin (the half-maximal effect on vesicles fusion). Insulin, in this range of concentrations, and under acidic pH behaves as a monomeric molecule (16). When the dose-response curve for insulin was performed in the presence of 50 μM of phospholipid vesicles (instead of 10 μM, see Fig. 4), the half maximal values were about 4-5 fold higher, indicating that the vesicles/insulin concentration ratio remained constant. According to this result , it is possible to asume that at physiological concentrations (10^{-9}M), insulin could induce fusion of 1 μM of phospholipid vesicles. Experiments with concentrations of phospholipid vesicles lower than 10 μM can not be performed since the fluorofors concentrations to be used are not detected spectrofluorometrically.

A previous report showed that the direct titration of PA micelles gave pK_1 values in the range from 2.8 to 3.8 and pK_2 values in the range from 8.0 to 8.6 (17). It has also been observed that a mixture of PA with PC seems to lower the pKs of PA. The anionic sites on PA, when diluted out with PC, are fully ionized at pH 5.5 and two net negative charges are present (18). The net positive charges of insulin were three and ten at pH 4.0 and 2.6 respectively (19). The electrostatic attraction between phospholipid PC:PA vesicles (whose pK_1 values

would probably be below 3.0) and insulin (isolectric point about 5.3 (19))could favor the aggregation of the vesicles in the pHs ranged from 3.0 to 5.3, but not at pHs lower than 3.0 or higher than 5.0. The results shown in Fig. 3A support this possibility. Thus, charge-charge interactions between phospholipids and hormone, promote both aggregation and fusion. In the fusion event, it could be considered that a non-polar interaction had taken place after the initial ionic association occurred at low pH. The X-ray crystallographic data showed that the nonpolar groups A_{19} Tyr, B_{12} Val, B_{16} Tyr, B_{24} Phe, and B_{26} Tyr, form a "hydrophobic core" on the surface of each molecule of insulin (20-21). This "hydrophobic core" could be involved in the fusion effect showed in this paper.

The relevance of insulin-induced fusion of negatively charged vesicles is debatable, since serum albumin (22), glucagon and human growth hormone (data no shown) were able to induce vesicle fusion under low pH conditions. However, the lack of specificity in the phospholipid vesicles system, could be not present in the case of interaction of insulin with biological membrane. In the latter type of interaction there is a specificity of action of the hormone, which is mediated by a receptor. Thus, the participation of insulin in the membrane fusion events that ocurred in the process of internalization of the hormone-receptor complex has to be considered since the insulin mechanism remains unclear, and that insulin interacts with citoplasm and intracellular membranes (23). In addition, the vesicle fusion approach has the advantage of a better defined system, and it could already provide some insight of the mechanism of phospholipids-insulin interaction.

ACKNOWLEDGMENTS

This work was supported by a grant form Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Consejo de Ciencia y Técnica de la Universidad Nacional de Tucumán (UNT). R.N.F. and R.D.M. are career investigator and A. L.V. is a fellow of the CONICET. We wishto thank Miss Susana Bustos for her secretarial aid, Mrs.María Orce for his assitance in the preparation of the English manuscript and Dr. R.Santolaya for having made the electron microscopy observations.

REFERENCES

1. Poste G. and Allison A.C. (1973) Biochim.Biophys.Acta 300, 421-465.
2. Suzuki K. and Kono T. (1979) J.Biol.Chem. 254, 9786-9794.

3. Posner B.I., Bergeson J.J.M., Josefsberg Z., Khan M.N., Khan R.J., Paterl B.A., Sikstrom A., Verma A.K. (1981) Recent. Prog.Horm.Res. 37, 539-579.
4. Farquhar M.G. (1983) Fed.Proc. 42, 2407-2431.
5. Massa E.M., Morero R.D., Bloj B. and Farías R.N. (1975) Biochem. Biophys.Res.Commun. 66, 115-122.
6. Moreno H. and Farías R.N. (1976) Biochem.Biophys.Res.Commun. 72, 74-80.
7. Luly P. and Shinitzky M. (1979) Biochemistry 18, 445-452.
8. Farías R.N. (1981) Adv.Lipid.Res. 17, 251-282.
9. Batzri S. and Korn E.D. (1973) Biochim.Biophys.Acta 298, 1015-1020.
10. Bloj B. and Zilversmit D.B. (1978) Biochemistry 15, 1277-1283.
11. Yank S.F. (1969) Methods Enzymol. 14, 208-211.
12. Monti J.A., Christian S.T. and Shaw W.A. (1978) J.Lipids Res. 19, 222-228.
13. Hoeckstra D. (1982) Biochemistry 21, 2833-2840.
14. Fung B.K.K. and Stryer L. (1978) Biochemistry 17, 5241-5248.
15. Uster P.A. and Deamer P.W. (1981) Arch.Biochem.Biophys. 209, 385-395.
16. Packer Y. and Biswas S.B. (1981) Biochemistry 20, 2354-2361.
17. Abramson M.B., Katzman R., Wilson C.E. and Gregor H. (1964) J.Biol.Chem. 239, 4066-4072.
18. Hauser H. and Dawson R.M.C. (1967) Eur.J.Biochem. 1, 61-69.
19. Tanford C. (1961) Physical Chemistry of Macromolecules (Ed. John Waley & Sons In.) 242 and 520.
20. Puller R.A., Lindsay D.G., Wood S.P., Tickle I.J., Blundell T.L. Wollner A., Krail G., Bandenburg D., Zahn H., Glieman J. and Gammeltoft S. (1976) Nature 259, 371-373.
21. De Meyts P. Van Obberghen E., Roth J., Wollmer A. and Brandenburg D. (1978) Nature 273, 504-509.
22. Schenkman S., Aranjo P.S., Dijkman R., Quina F.H. and Chaimovich H. (1981) Biochim.Biophys.Acta 649, 633-641.
23. Goldfine I.D. (1981) Biochem.Biophys.Acta 650, 53-67.