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Effect of haemoglobin on liposome permeability to Rb^+ and other solutes

PIETRO CALISSANO, STEFANO ALEMÀ and GRAZIELLA RUSCA
Laboratory of Cell Biology, C.N.R., Via Romagnosi 18A, Rome (Italy)
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SUMMARY

The action of beef haemoglobin on the diffusion at pH 7.5 of Rb^+, choline, 4-aminobutyric acid and glucose across artificial lipid membranes has been investigated. The results indicate that the interaction of the protein with liposomes is followed by a differential stimulation, resulting in a 90–100-fold increase in the Rb^+ leak and a lower (choline), negligible (4-aminobutyric acid) or undetectable (glucose) effect on the other solutes.

The use of liquid crystals[1] formed by two or more concentric lipid layers (liposomes) as a model of biological membranes, has opened new paths to the study concerning membrane permeability and the compounds (antibiotics, steroids, anaesthetics, *etc.*) interfering with such a process.

Among these substances, the proteins represent a particularly important class for investigation, due to their postulated role as structural components and possible carriers in biological membranes. During a study on the effect of two brain specific proteins[2] on cation diffusion in liposomes, beef haemoglobin, used as a control protein, revealed a marked effect on $^{86}Rb^+$ diffusion. The importance of this protein, as well as its unique localization at a very high concentration, prompted a deeper investigation of this effect as compared to a possible analogous action on other solutes.

The method of liposome preparation was described in detail elsewhere[3]. 1 ml of 5 mM Tris–HCl buffer, pH 7.5, containing 60 mM KCl, 1 mM Rb^+ *plus* 10–20 μCi of $^{86}Rb^+$, was added to a mixture of phospholipids previously evaporated to dryness. Other liposomes, containing 5 mM glucose, 4-aminobutyric acid of choline, were prepared by adding 20 μCi of the corresponding labelled compound to the same buffer.

The liposomes were sonicated to clearness under nitrogen. The untrapped radioactive isotope was removed by gel filtration through a Sephadex G-25 fine column

equilibrated with the same solution used to prepare liposomes. Proteins, dissolved in the same buffer, were added in 0.5-ml portions to liposomes previously placed in 8/32 inch Visking dialysis bags. The mixture in the dialysis bags was then allowed to incubate in tubes containing 10 ml of the same buffer. At time intervals the bags were then transferred to another set of tubes and the incubation continued. At the end of the experiment the contents of the dialysis bags were emptied out and the remaining labelled material counted. Radioactive counting was performed in a Beckman LS-250 liquid scintillation system. Preparation of globin and haemin was performed according to the method of Rossi Fanelli *et al.*[4] from the same batch of beef haemoglobin used for the other experiments. Phosphatidylserine as sodium salt and phosphatidylcholine grade I were supplied by Lipid Products. Beef haemoglobin twice crystallized, as a mixture of approximately 75% methaemoglobin and 25% oxyhaemoglobin, was from Sigma and it was used as such in all the experiments reported. Horse heart cytochrome *c* type III, bovine serum albumin, pepsin twice crystallized and ribonuclease type IIIA from bovine pancreas were purchased from Sigma; Pronase B grade was from Calbiochem; polyglutamate, mol. wt 175 000 from Mann Research Lab.

Fig. 1 reports the effect of haemoglobin on the diffusion of $^{86}Rb^+$, [^{3}H]choline, 4-[^{3}H]aminobutyric acid and [^{14}C]glucose across the liposome membrane prepared from a mixture of 75% phosphatidylserine–25% phosphatidylcholine. Since there are slight variations from batch to batch in the permeability of liposomes to K^+ (ref. 5), the $^{86}Rb^+$ leak has been used as an internal control for following the diffusion of liposomes loaded with the other solutes.

As it can be seen, the haemoglobin has its maximum effect on the $^{86}Rb^+$ leak which increases from 0.41% per hour in control experiments to 37–39% per hour in presence of protein, corresponding to a 90–100-fold increase. In the same liposome preparation the leak of choline is also stimulated by the protein although to a lower extent. Thus, the approximate 1% per hour leak of choline in control experiments is increased to 26–36% per hour. Further experiments are necessary in order to elucidate whether this apparent differential stimulation by haemoglobin of Rb^+ and choline leak is also detectable for Rb^+ and Na^+ diffusion. A different situation occurs when the effect of the haemoglobin is measured on the diffusion of 4-aminobutyric acid or glucose. In fact the diffusion of the amino acid is only slightly increased (10–15%) and that of glucose unaffected by the protein, while the strong effect on the Rb^+ leak is maintained. Ca^{2+}, at a concentration of 1 mM in the incubation medium, did not alter the effect of haemoglobin on the Rb^+ leak.

In a further experiment the effect of haemoglobin has been followed on liposomes with phosphatidylserine–phosphatidylcholine–cholesterol (1:1:1) sequestring the usual buffer, $^{86}Rb^+$ *plus* 5 mM glutamate. In these conditions the glutamic acid leak was increased by 1-fold as compared to 22-fold for Rb^+ (P. Amaldi, G. Levi and P. Calissano, unpublished data).

When liposomes were prepared by substituing an equimolar concentration of phosphatidylethanolamine to phosphatidylserine, the $^{86}Rb^+$ diffusion induced by the intact beef haemoglobin was reduced to 15–17-fold.

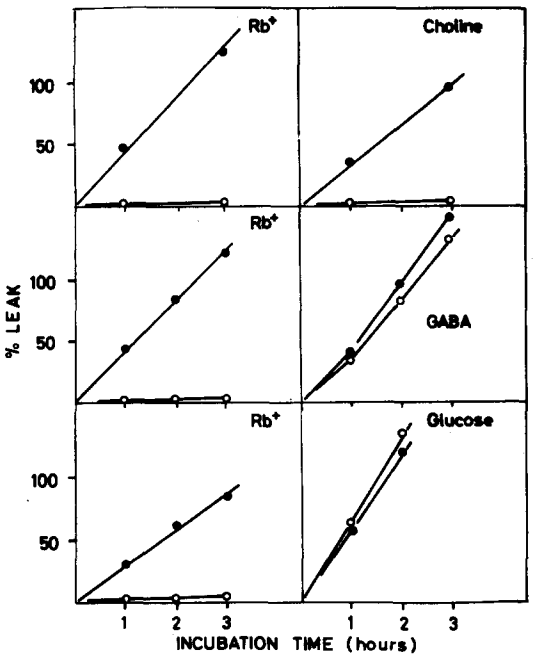

Fig. 1. Effect of haemoglobin on $^{86}Rb^+$, [^{3}H] choline, 4-[^{3}H] aminobutyric acid (GABA) and [^{14}C]-glucose leak. Liposomes prepared with 75% phosphatidylserine–25% phosphatidylcholine were incubated in an air atmosphere at 37 °C at a final concentration of 4 μmoles of phospholipid/ml. The leak is calculated by adding the counts lost by the liposomes during each incubation period and expressing this loss as per cent of the counts present in the liposomes at the beginning of each period of incubation. ○—○, controls; ●—●, haemoglobin, 0.5 mg/ml.

In order to see whether the induced leak by the protein was due to the intact structure or to one of the two constituents, globin and haemin were separated by the method of Rossi Fanelli *et al.*[4] and tested separately on the $^{86}Rb^+$ leak in liposomes prepared as in previous experiments. This experiment showed that when equimolar concentrations of haemin, globin and haemoglobin were used, haemin had no effect, and globin had half the effect of haemoglobin, which induced the usual 90–100-fold increase in Rb^+ diffusion.

None of the many other proteins tested in the same experimental conditions (Table I) had an effect similar to that elicited by haemoglobin on $^{86}Rb^+$ diffusion. Recently[6,7] it has been shown that lysozyme, cytochrome *c* and spectrin are capable of inducing a marked increase in Na^+ permeability. However, these effects cannot be compared to those reported in the present report, for the experimental conditions were different (effect of spectrin only at pH 3.5–4.5) or much higher protein concentrations were used (100–500 μM lysozyme or cytochrome *c*).

Experiments have also been performed in order to measure the amount of haemoglobin bound to liposomes in the same experimental conditions used to measure the diffusion of Rb^+. Different concentrations of haemoglobin, ranging from 0.7 to 7.3

nmoles/ml were incubated for 30 min at 37 °C in the presence of liposomes prepared from 75% phosphatidylserine–25% phosphatidylcholine at a concentration of 4 μmoles phosphate/ml. After incubation, the mixture was passed through a Sephadex G-75 column. The amount of haemoglobin eluted with the peak of liposomes was taken as a measure of the protein bound to the lipid membranes. Previous experiments had indicated that liposomes are eluted from this column with the void volume and are well separated from proteins with molecular weights ranging between 68 000 and 12 000. From these experiments it could be calculated that approximately 0.3–0.4 nmole/ml haemoglobin are bound per μmole of phospholipid in the experimental conditions described in Fig. 1 and Table I.

TABLE I

EFFECT OF SOME PROTEINS ON $^{86}Rb^+$ LEAK IN LIPOSOMES

The assay conditions were those as described in the legend of Fig. 1. The protein concentration was 0.5 mg/ml. 0.1 mM EDTA was present both in the protein samples and in the incubation medium.

Protein	*% $^{86}Rb^+$ leak*	
	1 h	*2 h*
None	0.41	0.78
Polyglutamic acid	0.46	0.85
Pepsin	0.67	1.24
Bovine serum albumin	0.48	0.90
Pronase	0.43	0.84
Ribonuclease	0.53	0.93
Cytochrome *c*	1.90	3.80
Haemoglobin	38.7	79.0

Recently it has been shown that haemoglobin binds several phosphate-containing compounds like adenine nucleotides and glycolytic intermediates[8,9].

The fact that the induced diffusion is different for different solutes but linear within the incubation time for a given substance, indicates that the interaction of haemoglobin with the liposomes is quite specific and does not result in a breakdown of the lipid barrier. Any conclusion on the intimate mechanism of action of this protein on liposomes resulting in an increased leak, as well as its possible biological significance, is premature. It is tempting to speculate that the higher leak flux of monovalent cations in a red cell as compared to a liposome[6,10] is due, besides to the different lipid composition, to some of its proteins, among which haemoglobin, due to its high concentration and differential stimulation, could play a significant role. Further experiments under controlled conditions of oxygen saturation could give additional information on the possible relationship between forms of haemoglobin (*i.e.* oxy-, met-, cyanohaemoglobin, *etc.*) and effect on liposome permeability.

In addition, since these artificial lipid membranes can be prepared with a definite

composition and charge, and because the full structure of the haemoglobin is known, its differential stimulation on the diffusion of several solutes might represent a good model to investigate the interplay between proteins and lipid membranes.

REFERENCES

1 A.D. Bangham, M.M. Standish and J.C. Watkins, *J. Mol. Biol.*, 13 (1965) 238.
2 P. Calissano and A.D. Bangham, *Biochem. Biophys. Res. Commun.*, 43 (1971) 504.
3 S.M. Johnson and A.D. Bangham, *Biochim. Biophys. Acta*, 193 (1969) 82.
4 A. Rossi Fanelli, E. Antonini and A. Caputo, *Biochim. Biophys. Acta*, 30 (1958) 608.
5 A.D. Bangham, in L. Bolis *et al.*, *Permeability and Function of Biological Membranes*, North Holland Publ. Co., 1970.
6 H.K. Kimelberg and D. Papahadjopoulos, *J. Biol. Chem.*, 246 (1971) 1142.
7 R.L. Juliano, H.K. Kimelberg and D. Papahadjopoulos, *Biochim. Biophys. Acta*, 241 (1971) 894.
8 D.P. Zahn, R.G. Klinger, G.A. Cumme and H.E. Frunder, *Abstr. Commun. 7th Meet. Eur. Biochem. Soc.*, (1971) 91.
9 R. Benesch, R. Benesch and Chi Ing Yu, *Proc. Natl. Acad. Sci. U.S.*, 59 (1968) 526.
10 R. Whittam, *Transport and Diffusion in Red Blood Cells*, Edward Arnold, London, 1964.