Vol. 99, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 16, 1981 Pages 275-283

A SENSITIVE ASSAY OF PHOSPHOLIPASE USING THE FLUORESCENT PROBE 2-PARINAROYLLECITHIN

CLAUDE WOLF, LUCIEN SAGAERT, GILBERT BEREZIAT

Laboratoire de Biochimie
C.H.U. Saint-Antoine, 27 rue Chaligny, 75012-Paris

Received January 5, 1981

Summary

A_2-phospholipase from Crotalus Adamanteus venom has been assayed using the fluorescent probe 2-all trans-parinoyllecithin. The hydrolysis of the lecithin in an "albumin-rich" incubation medium is paralleled with a fluorescence hyperpolarization. The mechanism of the hyperpolarization is discussed. The application of the method to assay phospholipases, allows detection of an activity under 1 nanomole/min and continuous monitoring of the hydrolysis.

Introduction

The assay of phospholipase has been described by various procedures with a variable sensitivity. Titrimetry (1, 2) allows determination in the range of 100-1000 nmoles/min. The assay with radiolabeled phospholipids(3) has been requested for assay of activities of crude intracellular phospholipases preparations in the range of 0.5-10 nmoles/min/mg of protein. The disadvantages of radiochemical methods (time-consuming and fixed-time assay) are overcome by the continuous spectrophotometric assay described by Aarsman and coll (4) using acyl-thioester analogs of phospholipids. Disturbances of the method by the Ellman reagent, the different hydrolysis of acylthioester bonds and of their corresponding oxyesters, and the disturbance by monospecific thioesterases have been already discussed (4). A successful assay of lipoprotein-lipase using a fluorescent dansylphosphatidylethanolamine-labeled substrate has been recently published by J.D.Johnson (5). The availability of the fluorescent phospholipid analog 2-parinoyl-

0006-291X/81/050275-09$01.00/0

phosphatidylcholine described by Sklar et al (6) and the sensitiveness of parinaric acid with respect to the environnement, as it is measured by fluorescence polarization, have been combined in the present work to perform a simple, continuous and sensitive assay of A_2-phospholipase.

Material and methods

Synthesis of 2-all trans-parinoylphosphatidylcholine.

All-trans parinaric acid (9, 11, 13, 15-octadecatetraeneoic acid in the all-trans configuration) is purchased from Molecular Probes [Texas, USA] or prepared from the seed of Parinari glaberrimum [kindly provided by Dr. M.Lambert, Westem Samoa]. Lyso-1-acyl-phosphatidylcholine was prepared from egg yolk lecithin by the action of Crotalus Adamanteus venom [Sigma] and purified by repeated washing with anhydrous ether (7). The acylation conditions differ in 2 aspects from the conditions described by Sklar (6) :
1) Pure parinaric anhydride was made by the Lapidot procedure (8)
2) The conditions described by Robles (9) were substituted by the efficient procedure of Gupta (10) using the N, N-dimethyl-4-aminopyridine catalyst [Merck]. The ratio lysocompound/catalyst is 1.5. The yield is 40 % determinated by phosphate analysis after 24 h. The absorption spectra were consistent with those described by Sklar (6) and were repeated with minor changes during storage of the fluorescent phospholipid in chloroform at -18°C.

Incubation conditions for phospholipase assay.

A_2-phospholipase [from Crotalus Adamanteus (Sigma)] is assayed at 37°C in an albumin-rich (10 mg/ml) buffer (4 mM $CaCl_2$, 250 mM NaCl, 10 mM Tris HCl (pH 8.5)). The high ionic strenght achieved by NaCl prevents the segregation of released fatty acids by Ca^{++} and hence favours the binding onto albumin. [Sigma ; "essentialy fatty acids free" grade]. The substrate is added as phosphatidylcholine vesicles prepared by sonication until optical clarity of the following mixture : ovolecithin (1 mg), 2-all trans-parinoyllecithin (1 µg) and butylhydroxytoluene as antioxydant (10 nmoles) for 2 ml of carefully deoxygenated buffer.

Fluorescence measurement

Fluorescence polarization is continuously monitored in the spectrofluorometer JY3 [Jobin Yvon] fitted with 2 linear polarizers [Polaroïd HPN'B]. The temperature is measured by a thermistance immersed in the cuvette. The incubation medium is continuously stirred by a micro-magnet.
The fluorescence polarization (P) is calculated according to the formula

$$P = \frac{(I_{\parallel} - I_{\parallel}\ \text{Albumin}) - (I_{\perp} - I_{\perp}\ \text{Albumin}) \times G}{(I_{\parallel} - I_{\parallel}\ \text{Albumin}) + (I_{\perp} - I_{\perp}\ \text{Albumin}) \times G} \quad \text{where ;}$$

$I_{\parallel}$ and $I_{\perp}$ are the intensities of fluorescence at 432 nm polarized parallel and perpendicular to the polarized exitation light at 320 nm, $I_{\parallel}$ Albumin and $I_{\perp}$ Albumin are the fluorescence intensities in both directions from the incubation medium without the fluorescent phospholipid vesicles and G is the correction factor for instrumental anisotropy (11).

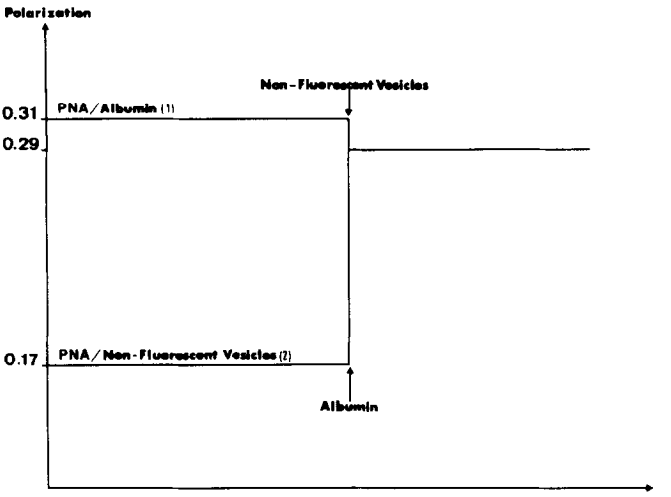

Fig.1
Experimental diagram of fluorescence polarization variations for free parinaric acid bound onto albumin or intercalated in phospholipid vesicles at 38°C.

3 nmoles of parinaric acid (PNA) in 1 µl tetrahydrofuran are added under continuous stirring in 2.5 ml aqueous buffer containing either 400 nmoles of fatty acid-free albumin (1) or 40 nmoles of non-fluorescent lecithin vesicles (2). Fluorescence polarization (λ excitation = 320 nm ; λ emission = 432 nm) is recorded and then,either non-fluorescent lecithin vesicles (1) or fatty acid-free albumin (2) is added and the fluorescence polarization recorded again.

Results

The principles of the assay by fluorescence polarization is summarized in fig.1 where free parinaric acid has been alternatively bound to lecithin vesicles or to albumin. When parinaric acid is bound to albumin its rotational diffusion is strongly hindered and the fluorescence polarization reaches 0.31. On the contrary, when parinaric acid is intercalated in phospholipid vesicles, the fluidity of this medium at 38°C allows a high fluorescence depolarization by fast rotational diffusion.

During the hydrolysis of 2-parinoyllecithin by A_2-phospholipase an increasing amount of released parinaric acid will move off the fluid phospholipid environnement to be uptaken in the tight hydrophobic pockets of albumin. Hence the hydrolysis of parinoyllecithin is paralleled with a fluorescence hyperpolarization. It is noticeable in figure 1 that most of the parinaric acid is bound onto albumin even in the presence of vesicles

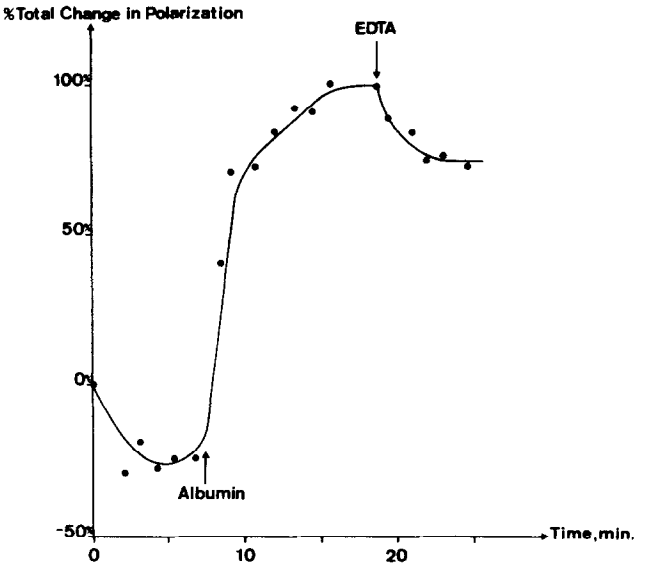

Fig. 2
Albumin-dependence of fluorescence hyperpolarization recorded during the hydrolysis of 2-parinoyllecithin by A_2-phospholipase.

The fluorescence of 2-parinoyllecithin is excitated by a polarized beam at 320 nm and analysed continuously at 432 nm through parallel and perpendicular polarizers. Experiment is started at time 0 in a albumin-free buffer by addition of the enzyme. At time 7.5 min, the albumin powder is added. The hydrolysis is stopped by addition of 100 mM EDTA.

and then the polarization is close to its maximum value even in the presence of vesicles.

The dependence on albumin of the fluorescence hyperpolarization is seen in figure 2. The hydrolysis of 2-parinoyllecithin by the phospholipase is not paralleled by an increased polarization in the absence of albumin. On the contrary a low depolarization is observed. But as early as albumin is added, a burst of hyperpolarization accounts for the released parinaric acid accumulated in the vesicles. The experiment shown in figure 2 has been ended by addition of EDTA since Crotalus Adamanteus A_2-phospholipase is Ca^{++} dependent (2) and the slight depolarization due to the release of the Ca^{++}-segregated PNA.

In figure 3 the experimental recordings of the fluorescence through the paralled ($I_{\parallel}$) and the perpendicular ($I_{\perp}$) polarizer are presented. The dependence on pH of the snake phospholipase is seen (optimum pH

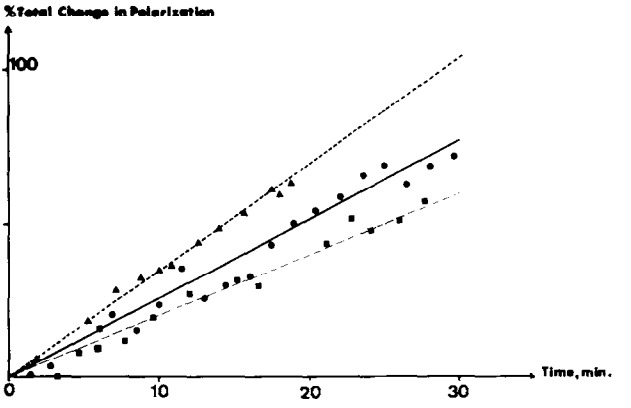

Fig. 3
Phospholipase activity in function of the pH of incubation medium.
Crotalus Adamenteus venom is added at time 0 in an albumin-rich medium buffered with 10 mM Tris-HCl at pH 7, 8 or 9. The fluorescence of parinaric acid is continuously monitored through the parallel and perpendicular polarizers (see Method).

around 9) in agreement with the revious results of M. A. Wells (2). Since I remains stable and I increases, the fluorescence total intensity I_{tot}(I_{tot}=I + 2 I) is assumed to increase simultaneously with the polarization. This variation of I_{tot} correlated with the variation of polarization (p) throughout the release of fatty acids by the venom is displayed in figure 4. In this experiment aliquots of the incubation medium throughout the hydrolysis process have been extracted by the Dole-method as reported by S. A. Ibrahim (12) and the fluorescence of the remaining phospholipid has been recorded. First, it is seen that the hyperpolarization is correlated with the disappearance of the fluorescent phospholipid, i.e. with its hydrolysis by the venom. Secondarily the binding of parinaric acid onto albumin is correlated with an increase in polarization as well as with an increase in the fluorescence total intensity (I_{tot}). This increase of I_{tot} is easily explained if an increased quantic yield is assumed for parinaric acid bound onto albumin. Further evidences from fluorescence lifetimes measurements will be given to support this assumption (not published). In figure 5 the linear change in polarization with the incubation time is displayed for various

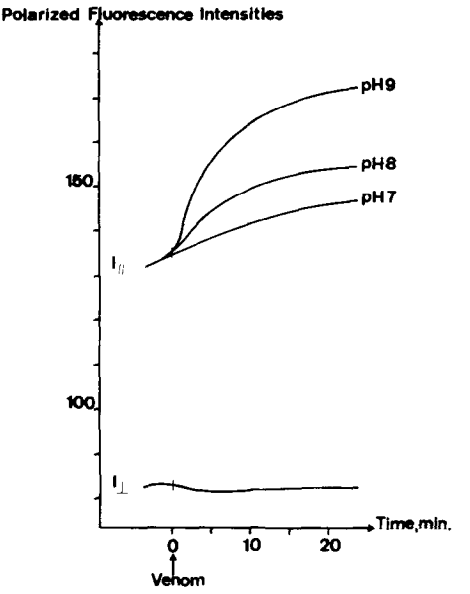

Fig. 4
Relationships between hyperpolarization (p), total fluorescence intensity ($I_{tot} = I_{\parallel}+2x\,I_{\perp}$) and release of fatty acids from the fluorescent 2-parinoyl-lecithin by A_2-phospholipase.

Incubation is carried out under the standard conditions (final volume = 4ml) in the cuvette and $I_{\parallel}$ and $I_{\perp}$ are continuously monitored. At fixed-times 0.4 ml aliquots are sampled and extracted according to the Dole procedure. Release of FA was calculated from the fluorescence of the remaining substrate.

quantities of enzyme in the presence of a non-limiting concentration of substrate. From the lowest activity (6 µl of venom) added, the sensitivity of the assay has been calculated and reaches 0.3 nanomoles of substrate hydrolysed/min. It is to say that the release of 1.5 nanomoles of fatty acids after a 5 min incubation will be easily detected.

Discussion

Thanks to the availability of the fluorescent probe 2-parinoyllecithin, previously described by Sklar (6), we have been able to monitor the hydrolysis of lecithin by the A_2-phospholipase from Crotalus Adamenteus venom. The analysis of the polarization of fluorescence emitted by the probe incorporated in the substrate at a very low ratio (10^{-3}) or bound onto albumin after hydrolysis, is efficient to assay an activity of 0.3 nanomoles/min. This sensitivity is very close

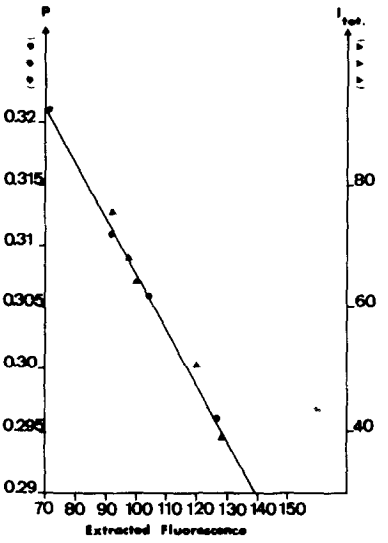

Fig. 5
Rate of hyperpolarization in function of Crotaleus Adamanteus A_2-phospholipase concentration.

Various concentrations of crude venom ((■--■) 6 µl, (●—●) 12 µl, (▲--▲) 25 µl) are added to 10 µg of lecithin vesicles (ovolecithin/ 2-parinoyllecithin : 1 000/1) suspended in the albumin-rich buffer (300 mM NaCl, 4mM $CaCl_2$, 10 mM Tris-HCl, 1 % albumin ["free fatty-acid" grade], (pH 8.5)). Incubation is carried out in the temperature-controled cuvette (37°C) under continuous stirring and fluorescence polarization is recorded in function of time.

to the sensitivity of the radiochemical methods and the fluorescence method has the advantages to be less time-consuming (no separation of the reaction products) and to allow continuous assays. With regard to its structural similarity with the natural lecithin, the 2-parinoyllecithin is probably closer to the ideal formula than the dansylphosphatidylethanolamine (5) and than the thioester analogs (4) used previously to monitor by spectrometry the enzyme lipolysis.

The 2 main disadvantages of the parinoyl-substrate, i.e. sensibility to oxygen and to photobleaching,have been overcome by the following tricks :

1) a careful deoxygenation of the buffer used to sonicate lecithin and addition of butylhydroxytoluene as antioxydant

2) fluorescence is excitated under a low illumination by the use of a

polaroid polarizer preferably to the optically clear Nichols polarizer (at λ excitation = 320 nm).

The use of albumin as "hindered" parinaric acid binding site offers the particularity of a high affinity as well as a low specificity for the reaction products (it binds parinaric acid as well as lysolecithin). Then the procedure is able to assay A_1-and A_2-phospholipase. The natural triglyceride extracted from Parinari glaberrimum seed could as well be used for monitoring a lipase activity. Finally, the fluorescence energy transfert from the tryptophyl residues of albumin to parinaric acid is assumed to be another interresting fluorescence parameter usable to monitor in the "albumin" system the hydrolysis of parinoyl-substrate. Nevertheless it is noticeable that this energy transfert has been completely masked by the "inner filter" effect in the standard "albumin-rich" buffer used throughout the present work.

Acknowlegment

Grants from DRET (79137) and DGRST (7970791) have generously supported this work.

References

1. de Haas, G.H., Bonsen, P.P.M., Pieterson, W.A. and Van Deenen, L.L.M. (1971) Biochim. Biophys. Acta, 239, 252-262.

2. Wells, M.A. (1972) Biochem., 11, 1030-1036.

3. Zieve, F.J. (1973) Anal. Biochem. 51, 436-441.

4. Aarsman, A.J., Van Deenen, L.L.M. and Van den Bosch,H. (1976) Bioorgan. Chem. 5, 241-253.

5. Johnson, J.D., Taskinen, M-R., Matsuoka, N. and Jackson, R.L. (1980) J. Biol. Chem. 255, 3461-3465.

6. Sklar, L.A., Hudson, B.S. and Simoni, R.D. (1977) Biochem. 16, 819-828.

7. Hanahan, D.J. (1952) J. Biol. Chem. 195, 199-210.

8. Salinger, Z. and Lapidot, Y. (1966) J. Lipid Res. 7, 174-175.

9. Robles, E.C. and Van Den Berg, D. (1969) Biochim. Biophys. Acta, 187, 520-526.

10. Gupta, C.M., Radhakrishman, R. and Khorana, H.G. (1977) Proc. Natl. Acad. Sci. USA 74, 4315-4319.

11. Azumi, T. and Mc Glynn, S.P. (1962) J. Chem. Phys. 37, 2413-2416.

12. Ibrahim, S.A. (1967) Biochim. Biophys. Acta, 137, 413-419.