Biochimica et Biophysica Acta, 778 (1984) 449–456
Elsevier

BBA 72348

EVIDENCE FOR A HIGHLY ASYMMETRIC ARRANGEMENT OF ETHER- AND DIACYL-PHOSPHOLIPID SUBCLASSES IN THE PLASMA MEMBRANE OF KREBS II ASCITES CELLS

MICHEL RECORD, AHMED EL TAMER, HUGUES CHAP and LOUIS DOUSTE-BLAZY *

INSERM Unité 101, Biochimie des Lipides, Hôpital Purpan, 31059 Toulouse (France)

(Received July 23rd, 1984)

Key words: Ether phospholipid; Plasma membrane; Lipid asymmetry; Phospholipase; (Krebs ascites cells)

(1) Krebs II ascites cells were taken as a model of the neoplastic cells to investigate the transverse distribution of phospholipids in the plasma membrane. The experimental procedure was based on non-lytic degradation of phospholipids in the intact cell by *Naja naja* phospholipase A_2 and *Staphylococcus aureus* sphingomyelinase C and on phospholipid analysis of purified plasma membranes. It was shown that the three major phospholipids, i.e., phosphatidylcholine, phosphatidylethanolamine and sphingomyelin, are randomly distributed between the two halves of the membranes, whereas phosphatidylserine remains located in the inner leaflet. (2) The membrane localization of phosphatidylcholine and phosphatidylethanolamine subclasses (diacyl, alkylacyl and alkenylacyl) was also examined, using a new procedure of ether-phospholipid determination. The method involves a selective removal of diacyl species by guinea pig pancreas phospholipase A_1 and of alkenylacyl species by acidolysis. This analysis revealed a 50% increase of ether phospholipids in the plasma membrane as compared to the whole cell (36.5 and 23.1% of total phospholipid, respectively). Furthermore, a strong membrane asymmetry was demonstrated for the three phosphatidylcholine subclasses, since 1-alkyl-2-acyl-*sn*-glycerol-3-phosphocholine (alkylacyl-GPC) was entirely found in the inner leaflet, whereas both diacyl- and alkenylacyl-GPC displayed an external localization. The same pattern was observed for phosphatidylethanolamine subclasses, except for 1-alkenyl-2-acyl-*sn*-glycero-3-phosphoethanolamine, which was found randomly distributed. These results are discussed in relation to the process of cell malignant transformation and to the biosynthesis of platelet-activating factor (PAF-acether or 1-alkyl-2-acetyl-GPC).

Introduction

The transverse distribution of membrane phospholipids has been studied in numerous cells, including viruses, bacteria and normal eucaryotic cells [1,2]. In the latter case, as shown for instance for erythrocytes and platelets, choline-containing phospholipids were located in the external leaflet of the membrane, whereas phosphatidylethanolamine and anionic phospholipids were found in the inner monolayer [3–8]. However, no data are yet available on such an asymmetry in neoplastic cells. It was of interest to start investigations in this field, in so far as procoagulant activity has been described for some tumour cells [9–12]. Indeed, membrane phospholipid asymmetry might regulate coagulation by maintaining procoagulant phosphatidylserine at the inside of red cells and resting platelets, whereas platelet activation leads to an increased exposure of this phospholipid on the outside of the cell [13–15].

In the present study, phospholipid distribution

* To whom correspondence should be addressed.

Abbreviations: GPC, *sn*-glycero-3-phosphocholine; GPE, *sn*-glycero-3-phosphoethanolamine.

was investigated by submitting intact cells to non-lytic degradation by phospholipase A_2 and sphingomyelinase C. This method was previously applied to various cell types, like erythrocytes [4] and platelets [7,8], and its reliability was established by comparison with other methods, using chemical labelling of phospholipid-exchange proteins [1,2,5,]. Its main advantage is the possibility to study at once all the phospholipid classes.

In a previous work, we devised a new method of ether phospholipid determination, based on the removal of diacyl phospholipids by guinea pig phospholipase A_1 and of plasmalogens by acidolysis [16,17]. This analysis revealed a high occurrence of ether phospholipids in Krebs ascites cell, and thus offered an interesting model to study the membrane sidedness of ether phospholipids. Indeed, this sidedness remains presently unknown, the only exception being recent works devoted to *Clostridium butyricum* [18] and myelin [19].

By coupling our method of ether phospholipid determination to the non-lytic degradation of phospholipids by phospholipases, it became possible to study the distribution of diacyl, alkylacyl and alkenylacyl phospholipids between the membrane outer leaflet and the cell interior. Further analysis on membranes purified according to our previously published procedure [20] allowed us to describe this phospholipid distribution at the membrane level.

Material and Methods

Enzymes. Purified sphingomyelinase C from *Staphylococcus aureus* and phospholipase A_2 from *Naja naja* were kindly supplied by Professor R.F.A. Zwaal [4]. Phospholipase A_2 from guinea pig pancreas was prepared in the laboratory by Fauvel et al. [21]. In this report, we used a fraction partially purified by DEAE-Sepharose chromatography, with a specific activity of 175 I.U. $\cdot$ mg^{-1}.

Krebs cell preparation and isolation of purified plasma membranes. Krebs ascites strain was maintained in Swiss mice as already described [20]. Cell pellet was washed twice with 50 vol. of a calcium-free Tyrode's buffer (pH 6.5) and finally suspended in Tyrode's buffer (pH 7.4) lacking albumin but supplemented in calcium (10 mM) and glucose (20 mM), as previously reported [7,8]. The suspension was adjusted to $5 \cdot 10^6$ cells/ml after numeration with a Nageotte counting cell.

Plasma membranes were purified from non-treated cells after preliminary labelling with [^{3}H]concanavalin A, as previously described [20].

Treatment of intact cells and of plasma membranes with lipolytic enzymes. Cells previously lysed by nitrogen cavitation were incubated with phospholipases under conditions identical to those used with intact cells. For the experiments depicted in Table I, incubations were performed in the same way, using purified plasma membranes. In both cases, lipids were extracted according to Bligh and Dyer [22], after addition of 200 000 dpm [^{3}H]cholesterol (Amersham Internation, Amersham, U.K.) as an internal standard.

Treatment of intact cells with lipolytic enzymes. Aliquots of the cell suspension in silicone-coated test tubes (2.5 ml) were incubated at 37°C under gently stirring with 10 I.U. phospholipase A_2 and 2 I.U. sphingomyelinase C for up to 60 min. Cells were then spun down at $600 \times g$ for 2 min and the supernatant was assayed for lactate dehydrogenase activity [23]. The pellets received 15 mM EDTA and lipids were extracted according to Reed et al. [24].

Diacyl- and ether-phospholipid determination. This was achieved according to our previously reported method, with some minor modifications [16,17]. Briefly, lipid extracts corresponding to 400 nmol phospholipids were dried under vacuum and dispersed by sonication in 1 ml of 200 mM Tris-HCl buffer (pH 8.0)/2.4 mM sodium deoxycholate. After incubation for 3 h at 42°C in the presence of 1 I.U. phospholipase A_1, one half of the mixture was extracted according to Bligh and Dyer [22], the other half being extracted with acidified chloroform/methanol [25], in order to hydrolyse plasmalogens.

Three kinds of lipid extracts were then submitted to bidimensional thin-layer chromatography [26]: (1) non-treated sample; (2) phospholipase A_1-treated sample; (3) phospholipase A_1 and acid-treated sample. The same procedure was applied to extracts obtained from intacts cells, from phospholipase-treated cells or from purified plasma membranes. The various spots detected after exposure to iodine vapor were determined for phosphorus according to Böttcher et al. [27].

During non-lytic degradation of phospholipids by phospholipases, the sum lysophosphatidylcholine + phosphatidylcholine was taken as an internal standard, as previously described [4,7,8]. In chromatography dealing with phospholipase A_1 and phospholipase A_1 + acid-treated samples, the amount of sphingomyelin present in the control extract was taken as an internal standard, as previously described [16,17].

Results

Phospholipid degradation in lysed cells and isolated plasma membranes

Upon incubation of cells lysed by nitrogen cavitation, *S. aureus* sphingomyelinase C degraded 95% of sphingomeylin, whereas 92–100% of all glycerophospholipids became susceptible to phospholipase A_2. Since this incomplete hydrolyses might be due to the presence of sealed structures possibly coming from intracellular organelles, the same incubation was repeated with isolated plasma membranes. As shown in Table I, this allowed for a complete hydrolysis of glycerophospholipids and of sphingomyelin by phospholipase A_2 and sphingomyelinase C, respectively, when used alone or in combination. In these experiments, the yield of lysophosphatidylcholine and lysophosphatidylethanolamine was below 100%, which is probably due to an incomplete extraction by the Bligh and Dyer procedure [22]. Under these conditions, lysophosphatidylserine and lysophosphatidylinositol were never detected on the plates and were certainly eliminated in the upper aqueous phase.

Phospholipid degradation in intact cells

In agreement with previous observations made on platelets [7,8], phospholipase A_2 from *N. naja* hardly degraded phospholipids in intact cells, probably because of too high a surface pressure in Krebs cell surface membrane [4,28]. However, this degradation could be achieved by combining phospholipase A_2 with *S. aureus* sphingomyelinase C. Fig. 1 shows that no significant cell lysis occurred under these conditions, as detected by the low amounts of lactate dehydrogenase released into the supernatants.

Phospholipid hydrolysis started rapidly and levelled off between 30 and 60 min, at which time 23% of total phospholipids were hydrolysed, comprising 50% of sphingomyelin, 17% of phosphatidylcholine and of phosphatidylserine, 28% of phosphatidylethanolamine and 10% of phosphatidylinositol. No measurable hydrolysis of cardiolipin could be detected at that time.

These conditions of incubation with intact cells were thus taken as those allowing a maximal degradation of phospholipids under non-lytic conditions and were currently used in further experiments where ether-phospholipid analysis was in-

TABLE I

DEGRADATION OF PHOSPHOLIPIDS BY PHOSPHOLIPASE A_2 AND SPHINGOMYELINASE C IN PLASMA MEMBRANES ISOLATED FROM KREBS ASCITES CELLS

Purified plasma membranes (5.4 mg protein, 750 nmol phospholipids) were incubated for 60 min at 37°C in the presence of *S. aureus* sphingomyelinase C (2 IU) and (or) *Naja naja* phospholipase A_2 (10 IU) in 2.5 ml Tyrode's buffer (pH 7.4) containing 10 mM $CaCl_2$. Results (means ± S.D., three experiments) represent the nmoles of each phospholipid recovered after different incubations. The minor compound cardiolipin was not considered in these experiments.

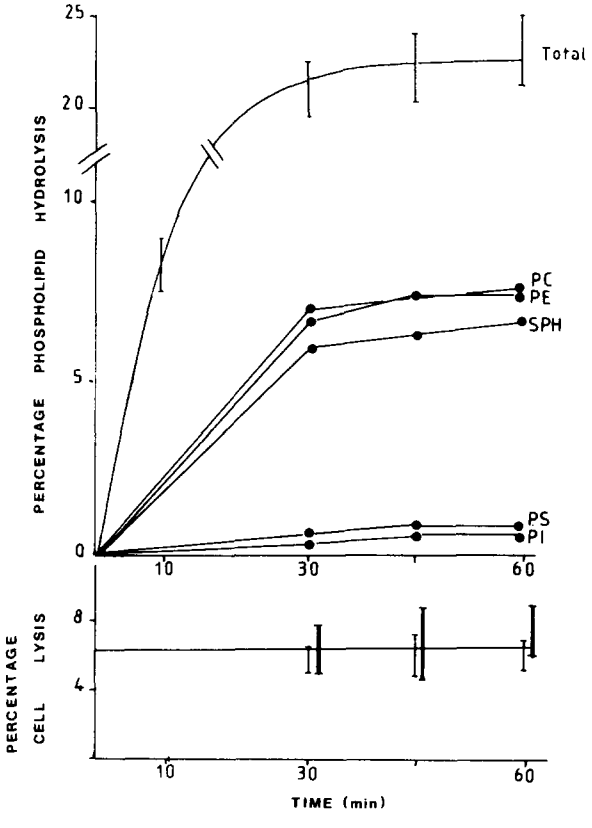
Phospholipids	Incubation conditions			
	no phospholipase	phospholipase A_2	sphingomyelinase C	phospholipase A_2 + sphingomyelinase C
Sphingomyelin	68 ± 16	77 ± 4	0	0
Phosphatidylcholine	375 ± 39	0	420 ± 25	0
Phosphatidylethanolamine	196 ± 19	0	213 ± 37	0
Phosphatidylserine	35 ± 4	0	26 ± 1.7	0
Phosphatidylinositol	6 ± 1	0	5 ± 0.4	0
Lysophosphatidylcholine	0	310 ± 4	0	285 ± 48
Lysophosphatidylethanolamine	0	135 ± 7	0	113 ± 25

TABLE II

NON-LYTIC DEGRADATION OF PHOSPHOLIPIDS BY PHOSPHOLIPASE A_2 AND SPHINGOMYELINASE C IN KREBS ASCITES CELLS

Incubation conditions are reported in Material and Methods. Results are means ± S.D. of nine determinations.

Phospholipid	Phospholipid composition (% of total phospholipid)	Phospholipid degradation	
		% of total phospholipid	% of each phospholipid
Sphingomyelin	13.0 ± 0.8	6.4 ± 0.5	49.2
Phosphatidylcholine			
Total class	47.3 ± 1.0	8.2 ± 0.6	17.3
Diacyl-GPC	35.2 ± 0.9	6.5 ± 0.6	18.5
Alkylacyl-GPC	8.5 ± 1.5	0	0
Alkenylacyl-GPC	3.6 ± 0.8	1.7 ± 0.7	47.2
Phosphatidylethanolamine			
Total class	23.6 ± 1.1	6.7 ± 1.0	28.4
Diacyl-GPE	11.5 ± 0.8	3.4 ± 0.6	29.6
Alkylacyl-GPE	7.5 ± 0.3	1.2 ± 0.7	16.0
Alkenylacyl-GPE	4.6 ± 0.5	2.1 ± 0.9	45.7
Phosphatidylserine	6.0 ± 0.4	1.0 ± 0.6	16.7
Phosphatidylinositol	7.1 ± 0.4	0.7 ± 0.3	9.9
Cardiolipin	2.5 ± 0.4	0	0
Total phospholipid	99.5	23.0 ± 3	



cluded. Data obtained with intact cells incubated for 60 min with phospholipase A_2 and sphingomyelinase C are reported in Table II. They are identical to those depicted in Fig. 1, when considering the various phospholipid classes. However, separation of diacyl- and ether-subclasses of phosphatidylcholine and phosphatidylethanolamine revealed different susceptibility of the various phospholipid species to phospholipase A_2. For instance, 47% of 1-alkenyl-2-acyl-*sn*-glycero-3-phosphocholine (alkenylacyl-GPC) were hydrolysed under non-lytic conditions, against 18% of diacyl-GPC and 0% of alkylacyl-GPC. Although to a lower extent, similar differences were observed within phosphatidylethanolamine.

Fig. 1. Non-lytic degradation of Krebs cell phospholipids during prolonged incubation with *N. naja* phospholipase A_2 and *S. aureus* sphingomyelinase C. Cells were incubated as described under Material and Methods. Degradation is expressed in percentage of total phospholipids. Cell lysis in control (———) and sample (_____) correspond to percentages of total lactate dehydrogenase activity in the cell. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylsérine; PI, phosphatidylinositol; SPH, sphingomyelin. Results are the means ± S.D. from nine determinations.

Phospholipid distribution in the plasma membrane

Results from the previous paragraph correspond to percentages of degradation of phospholipids as expressed relatively to total cell phospholipids. To have access to the transverse distribution of phospholipids in the membrane itself, one must express phospholipid hydrolysis as percentages of total phospholipids from the plasma membrane. This requires the determination of the proportion of total cell phospholipids located in the plasma membrane, which was achieved by using a specific labelling of the surface membrane with [^{3}H]concanavalin A. The ratio of specific radioactivity (expressed in dpm/μmol phospholipid) in the total cell and in the purified plasma membrane gives the proportion of cell phospholipid present in the plasma membrane. We found a value of 53.6%, in agreement with our previously reported value [20].

As previously depicted for platelets [7,8], we could thus calculate that 23.3% of total cell phospholipid hydrolysis corresponds to 23/0.536 = 43.1% of plasma membrane phospholipid degradation. The same calculation was performed with each phospholipid, and the results are shown in the second column of Table III.

The first column of Table III gives the phospholipid composition of purified plasma membranes. This greatly differed from that found for whole cells (Table II), since for instance sphingomyelin increased by 100% in the membrane at the expense of phosphatidylcholine. Great differences also appeared for ether phospholipids, which increased from 23% of total phospholipid in the cells to 36.5% in the purified membrane.

By comparing data compiled in columns 1 and 2 of Table III, it became possible to calculate for each phospholipid the proportion of it hydrolysed by phospholipases in the plasma under non-lytic conditions. As shown in column 3 of Table II, roughly 50% of sphingomyelin, phosphatidylcholine and phosphatidylethanolamine were degraded. This value decreased to 30% for phosphatidylinositol, 20% for phosphatidylserine and 0% for cardiolipin.

However, results obtained with total classes did not reflect concerning the various subclasses. For instance, 92% of diacyl-GPC and 86% of alken-

TABLE III

NON-LYTIC DEGRADATION OF PHOSPHOLIPIDS BY PHOSPHOLIPASE A_2 AND SPHINGOMYELINASE C IN THE PLASMA MEMBRANE OF KREBS ASCITES CELLS

Phospholipid composition is the mean ± S.D. of nine determinations.

Phospholipid	Phospholipid composition (% of total phospholipid)	Phospholipid degradation	
		% of total phospholipid [a]	% of each phospholipid
Sphingomyelin	25.8 ± 1.3	12.0	46.5
Phosphatidylcholine			
Total class	30.3 ± 0.9	15.3	51.5
Diacyl-GPC	13.1 ± 1.2	92.3	
Alkylacyl-GPC	13.4 ± 0.9	0	0
Alkenylacyl-GPC	3.7 ± 0.5	3.2	86.5
Phosphatidylethanolamine			
Total class	28.1 ± 0.9	12.6	44.8
Diacyl-GPE	8.7 ± 1.3	6.4	73.6
Alkylacyl-GPE	12.2 ± 1.4	2.2	18.0
Alkenylacyl-GPE	7.2 ± 0.5	4.0	55.6
Phosphatidylserine	9.8 ± 1.1	1.9	19.4
Phosphatidylinositol	3.5 ± 0.3	1.3	31.2
Cardiolipin	2.5 ± 0.4	0	0
Total phospholipid	100.0	43.1	

[a] Calculated, as described in the text, from data of Table I (column 2).

ylacyl-GPC were hydrolysed against 0% of alkylacyl-GPC. A similar pattern was obtained with phosphatidylethanolamine, except that alkenylacyl-GPE was less susceptible to phospholipase A_2 (56% hydrolysis) compared to the choline analogue.

Discussion

Determination of phospholipid asymmetry in a cell membrane with purified phospholipases is based on the assumption that phospholipid degradation performed in an intact cell remains limited to those phospholipids directly exposed at the outside of the cell, i.e., forming the outer monolayer of the plasma membrane [1,2,5]. On the other hand, all the phospholipids must be hydrolysed upon cell lysis, in which case both leaflets of the membrane become accessible to the enzyme attack. This has been successfully achieved on human erythrocytes [4] and platelets [7,8], and results were shown to be in very good agreement with other investigations based on different methodologies [1,2,5].

The same procedure was thus applied to Krebs ascites cells. Our data show that all phospholipids including ether phospholipids, were totally hydrolysed by phospholipase A_2 and sphingomyelinase C upon incubation of lysed cells or isolated plasma membranes, whereas phospolipid degradation remained limited in the case of non-lytic incubations with intact cells. Moreover, phospholipid hydrolysis reached a plateau under these conditions, suggesting that transmembrane movement of phospholipids (flip flop) was not induced by phospholipase treatment. This is indeed a very critical point of the procedure, since certain intracellular membranes (endoplasmic reticulum) can undergo a rapid exchange of phospholipids between the two faces of the membrane, owing probably to the existence of non-bilayer structures [1,2,5]. In the latter case, contracdictory or inconclusive result about phospholipid sidedness were reported in the literature [1,2,5].

In the present study, we thought it reasonable to conclude that non-lytic incubation of Krebs ascites cells with the two enzymes leads to a complete hydrolysis of phospholipids forming the outer monolayer of the surface membrane. On the contrary, non-degraded phospholipids should correspond to those displaying an intracellular localization, since they become accessible to phospholipases upon cell lysis or in isolated membranes.

However, with Krebs ascites cells, as with platelets, the problem is more complex than with erythrocytes, in so far as internal phospolipids not only represent those forming the inner leaflet of the plasma membrane but also those located in other intracellular membranes (endoplasmic reticulum, lysosomes, mitochondria, etc.). As previously discussed [7,8], data obtained on intact cells can be extrapolated to the plasma membrane itself, using the calculation procedure described under Results. This requires the estimation of the proportion of total cell phospholipids located in the plasma membrane and determination of the phospholipid composition of this purified membrane. Such a calculation benefited from our previously published method of plasma membrane isolation using Percoll gradients [20]. As seen in Table II, it revealed that 43% of phospholipids of the plasma membrane are located in the exterior half. This value is similar to those previously obtained with red cells [4] and platelets [7,8] and fits rather well with the model of the bilayer [29,30].

However, at variance with cells previously investigated, we found a rather symmetric distribution for all phospholipid classes, including sphingomyelin, except for phosphatidylserine and cardiolipin, which display an internal localization. This again raises the problem of the factors regulating membrane phospholipid sidedness, which is partially understood for phosphatidylserine only. Indeed, for erythrocyte some evidence exists indicating that a specific interaction of this phospholipid with spectrin might determine a preferential localization in the inner leaflet [31]. Thus, a similar interaction with proteins of the cytoskeleton might be involved in other cells like platelets or Krebs ascites cells. As for the other phospholipids, our data lead to the question as to whether a random distribution does represent a character accompanying malignant transformation. Further studies on other tumour cells are still required to elucidate this problem. Anyway, the phospholipid composition of the outer leaflet reveals that phosphatidylserine accounts for only 3% of total phospholipids, which is certainly insufficient to pro-

mote a procoagulant activity [13–15]. Other tumour cells presenting such an activity might thus be interesting to investigate in regard to their phospholipid distribution.

In the present study, a new approach to investigate the membrane sidedness of ether phospholipids was used. Analysis was performed with a newly devised procedure using guinea pig pancreas phospholipase A_1 [16,17]. Surprisingly, it revealed a highly asymmetric partition of the various subclasses of phosphatidylcholine and phosphatidylethanolamine, which contrasts with the apparent random distribution observed within the total classes. To our knowledge, this represents the first observation of a topological separation of various phospholipid species in a plasma membrane. Of course, this result must be viewed with a certain caution, as it corresponds to only one cell type. The same arguments as those raised 10 year ago about this very special cell, that is erythrocyte, could also be used again. Thus, studies of other cells rich in ether phospholipids are still necessary before it can be concluded that such an asymmetry represents a general rule.

One of the most striking features of the phospholipid asymmetry described here is certainly the exclusive localization of alkylacyl-GPC in the internal half of the membrane. Increasing evidence is not available indicating that this phospholipid could be the precursor of platelet-activating factor (PAF-acether or 1-akyl-2-acetyl-GPC) [32–35], the synthesis of which would require the sequential action of a phospholipase A_2 and of a specific aceyltransferase [35–42] It is tempting to compare the present result to our previous observation that arachidonic acid, another precursor of potent lipid mediators, is mostly located in the inner leaflet of the platelet plasma membrane [8]. It is currently suggested that the biosynthesis of PAF-acether and of arachidonate metabolites could be triggered by the same phospholipafse A_2 acting on 1-alkyl-2-arachidonoyl-GPC [43,44]. If extended to other cells producing these various mediators in large amounts, our finding might give further support to this hypothesis.

Acknowledgements

This study was supported by a grant from the Fédération National des Centres de Lutte contre le Cancer. One of us (H.C.) wants to express this gratitude to Prof. L.L.M. Van Deenen and Prof. R.F.A. Zwaal, who helped him to continue studies on membrane phospholipid sidedness after his stay in their laboratory by providing the samples of purified phospholipases. Thanks are also due to Dr. J. Fauvel and Mrs. V. Roques for prepararing the phospholipase A_1, to Mrs. Y. Jonquière for correcting the English manuscript and to Miss M.C. Vialette for typing the text.

References

1 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71
2 Etemadi, A.H. (1980) Biochim. Biophys. Acta 604, 423–475
3 Bretscher, M.S. (1972) J. Mol. Biol. 71, 523–528
4 Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and Van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83–96
5 Van Deenen, L.L.M. (1981) FEBS Lett. 123, 3–15
6 Schick, P.K., Kurica, K.B. and Chacko, G.K. (1976) J. Clin. Invest. 57, 1221–1226
7 Chap, H., Zwaal, R.F.A. and Van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 467, 146–164
8 Perret, B., Chap, H. and Douste-Blazy, L. (1979) Biochim. Biophys. Acta 556, 434–446
9 Colucci, M., Giavazzi, R., Alessandri, G., Semeraro, N., Mantovani, A. and Donati, M.B. (1981) Blood 57, 733–735
10 Bastida, E., Ordinas, A., Giardina, S.L., Jamieson, G.A. (1982) Cancer Res. 42, 4348–4352
11 Gasic, G.J. and Gasic, T.B. (1982) in Interaction of Platelets and Tumor Cells (Jamieson, G.A. and Scipio, A.R., eds.), pp. 429–444, Alan R. Liss, New York
12 Pearlstein, E., Ambroglio, C., Gasic, G. and Karpatkin, S. (1982) in Interaction of Platelets and Tumor Cells (Jamieson, G. and Scipio, A.R., eds.), pp. 479–502, Alan R. Riss, New York
13 Zwaal, R.F.A., Comfurius, P. and Van Deenen, L.L.M. (1977) Nature 268, 358–360
14 Zwaal, R.F.A. (1978) Biochim. Biophys. Acta 515, 163–205
15 Bevers, E.M., Comfurius, P., Van Rijn, J.L.M.L., Hemker, H.C. and Zwaal, R.F.A. (1982) Eur. J. Biochem. 122, 429–436
16 El Tamer, A., Record, M., Fauvel, J., Chap, H. and Douste-Blazy, L. (1983) Biochim. Biophys. Acta, 793, 213–220
17 Diagne, A., Fauvel, J., Record, M., Chap, H. and Douste-Blazy, L. (1983) Biochim. Biophys. Acta, 793, 221–231
18 Goldfine, M., Johnston, N.C. and Bishop, D.G. (1982) Biochem. Biophys. Res. Commun. 108, 1502–1507
19 Kirschner, D.A. and Ganser, A.L. (1982) J. Mol. Biol. 157, 635–658
20 Record, M., Bes, J.C., Chap, H. and Douste-Blazy, L. (1982) Biochim. Biophys. Acta 688, 57–65
21 Fauvel, J., Bonnefis, M.J., Sarda, L., Chap, H., Thouvenot, J.P. and Douste-Blazy, L. (1981) Biochim. Biophys. Acta 663, 446–456

22 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 1277–1283
23 Wroblewski, F. and La Dyne, J.S., (1955) Proc. Soc. Exp. Biol. 90, 210–215
24 Reed, C.F., Swisher, S.N., Marinetti, G.V. and Ede, G.V. (1960) J. Lab. Clin. Med. 56, 281–289
25 Mauco, G., Chap, H., Simon, M.F. and Douste-Blazy, L. (1978) Biochimie 60, 653–661
26 Esko, J.D. Raetz, C.R.H. (1980) J. Biol. Chem. 255, 4474–4480
27 Böttcher, C.J.e., Van Gent, C.M. and Pries, C. (1961) Anal. Chim. Acta 24, 203–204
28 Demel, R.A., Geurts van Kessel, W.S.M., Zwaal, R.F.A., Roelofsen, B. and Van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 97–107
29 Gorter, E. and Grendel, F. (1925) J. Exp. Med. 41, 439–446
30 Singer, S.J. and Nicolson, G.L. (1972) Science 175, 720–731
31 Haest, C.W.M. (1982) Biochim. Biophys. Acta 694, 331–352
32 Demopoulos, C., Pinckard, R.N. and Hanahan, D.J. (1979) J. Biol. Chem. 254, 9355–9358
33 Benveniste, J., Tencé, M., Varenne, P., Bidault, J., Boullet, C. and Polonsky, J. (1979) C.R. Acad. Sci. Ser. D 289, 1037–1040
34 Blank, M.L., Snyder, F., Byers, L.W., Brooks, B. and Muirhead, E.E. (1979) Biochem. Biophys. Res. Commun. 90, 1194–1200
35 Hanahan, D.J., Demopoulos, C.A., Liehr, J and Pinckard, R.N. (1980) J. Biol. Chem. 255,l 5514–5516
36 Chap, H., Mauco, G., Simon, M.F., Benveniste, J. and Douste-Blazy, L. (1981) Nature 289, 312–314
37 Wykle, R.L., Malone, B. and Snyder, F. (1980) J. Biol. Chem. 255, 10256–10260
38 Ninio, E., Mencia-Huerta, J.M., Heymans, F. and Benveniste, J. (1982) Biochim. Biophys Acta 710, 23–31
39 Mencia-Huerta, J.M., Roubin, R., Morgat, J.L. and Benveniste, J. (1982) J. Immunol. 129, 804–808
40 Alonso, F., Gil, M.G., Sanchez-Crespo, M. and Mato, J.M. (1982) J. Biol. Chem. 257, 3376–3378
41 Lee, T.C., Malone, B., Wasserman, S.I., Fitzgerald, V. and Snyder, F. (1982) Biochem. Biophys. Res. Commun. 105, 1303–1308
42 Albert, D.H. and Snyder, F. (1983) J. Biol. Chem. 258, 97–102
43 Wykle, R.L., Chilton, F.H., Mueller, H.W., Swendsen, C.L. and O'Flaherty, J.T. (1983) J. Pharmacol. 14, Suppl. 1, 32
44 Touqui, L., Dumarey, C., Jacquemin, C. and Vargaftig, B. (1983) J. Pharmacol. 14, Suppl. 1, 34