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Subcellular localization of inositol lipids in blood platelets as deduced from the use of labelled precursors

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1. By rapid fractionation of blood platelet lysates on Percoll density gradients at alkaline pH (9.6), a very pure plasma-membrane fraction was obtained, as well as discrimination between endoplasmic reticulum and lysosomes. 2. Labelling of intact platelets with [^{32}P]P_i followed by subcellular fractionation showed an exclusive localization of all inositol lipids in the plasma membrane. 3. Preincubation of whole platelets with *myo*-[^{3}H]inositol in a buffer containing 1 mM-$MnCl_2$ allowed incorporation of the label into PtdIns (phosphatidylinositol) of both plasma and endoplasmic-reticulum membrane, whereas [^{3}H]PtdIns4*P* (phosphatidylinositol 4-phosphate) and [^{3}H]PtdIns(4,5)P_2 (phosphatidylinositol 4,5-bisphosphate) were exclusively found on the plasma membrane. 4. It is concluded that PtdIns4*P* and PtdIns(4,5)P_2 are exclusively localized in the plasma membrane, whereas PtdIns is present in both plasma and endoplasmic-reticulum membranes. This could provide an explanation for previously reported data on hormone-sensitive and -insensitive inositol lipid pools.

INTRODUCTION

It has been known for more than three decades that inositol-lipid metabolism is modified during cell activation (Hokin & Hokin, 1953; reviewed by Michell, 1975; Berridge, 1984; Hokin, 1985). These modifications are induced by receptor–agonist interaction at the plasma membrane, followed by a phosphodiesteratic cleavage of PtdIns(4,5)P_2 (possibly also of the other inositol lipids, PtdIns4*P* and PtdIns) leading to the accumulation of DAG and InsP_3. This event is common to a wide variety of cells, including blood platelets (Lloyd *et al.*, 1973; Mauco *et al.*, 1978, 1983, 1984*b*; Lapetina & Cuatrecasas, 1979; Rittenhouse, 1983; McIntyre & Pollock, 1983; Agranoff *et al.*, 1983; Vickers *et al.*, 1984). However, although this hydrolysis is well documented, the subcellular localization of inositol lipids and of their metabolism is yet unclear. Phosphorylation and dephosphorylation are fast events, which rapidly equilibrate the 4- and 5-phosphate groups of PtdIns4*P* and PtdIns(4,5)P_2 with the γ-phosphate of ATP (Hokin & Hokin, 1964; Dangelmaier *et al.*, 1986). This is assumed to occur at the plasma membrane, since agonist-induced hydrolysis of inositol lipids appears to be a plasma-membrane event (Rana *et al.*, 1986; Tooke *et al.*, 1984; Seyfred & Wells, 1984*a*,*b*). However, PtdIns phosphorylation was also shown in nuclear membranes, endoplasmic reticulum (Smith & Wells, 1983), Golgi membranes (Lundberg *et al.*, 1985) and endocytic vesicles (Campbell *et al.*, 1985). On the other hand, PtdIns synthesis from PtdA via CMP-PtdA is believed to occur in the microsomal fraction from liver (Carter & Kennedy, 1966; Thompson & McDonald, 1977; Takenawa & Egawa, 1977) and also from platelets (Call & Williams, 1970; Call & Ruppert, 1973). Furthermore, some intriguing data show that two pools of hormone-sensitive and hormone-insensitive PtdIns and PtdIns(4,5)P_2 can be found in WRK-1 cells (Monaco, 1982; Koreh & Monaco, 1985) or in pancreatic slices (Schoepp, 1985).

However, no data are available on the subcellular localization of inositol lipids in platelets, and we decided to investigate these aspects of inositol lipid metabolism.

In this paper we describe the localization of ^{32}P- and [^{3}H]inositol-labelled lipids. We took advantage of our rapid method of platelet subcellular fractionation (Perret *et al.*, 1979; Mauco *et al.*, 1984*a*; Fauvel *et al.*, 1986) and show that PtdIns is distributed between plasma and endoplasmic-reticulum membranes, whereas PtdIns4*P* and PtdIns(4,5)P_2 are exclusively located in the plasma membrane.

EXPERIMENTAL

Labelling of blood platelets

Washed platelets were obtained from platelet concentrates and processed less than 26 h after blood collection (Centre de Transfusion Sanguine, Toulouse, France). The procedure was identical with that described by Ardlie *et al.* (1970), with the following modifications in order to incorporate the different radioactive labels.

In preliminary experiments, platelet suspensions were incubated with [^{3}H]concanavalin A (100 nCi/ml) in Ca^{2+}-free Tyrode's buffer A [145 mM-NaCl, 3.5 mM-KCl, 1 mM-$MgCl_2$, 25 mM-$NaHCO_3$, 2 mM-NaH_2PO_4, 5 mM-glucose, 2% (w/v) bovine serum albumin, 1 mM-Na_2EGTA, pH 6.5] for 15 min to label the plasma membrane.

myo-[^{3}H]Inositol (1 mCi/platelet concentrate) was incorporated by a platelet suspension obtained in a modified Ca^{2+}-free Tyrode's buffer A (0.1 mM-glucose and 1 mM-$MnCl_2$) for 2 h at room temperature. Further labelling was obtained on addition of 5 mM-glucose and incubation for 30 min more at room temperature.

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns4*P*, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; PtdA, phosphatidic acid, InsP_3, inositol 1,4,5-trisphosphate.

^{32}P-labelled platelets were obtained by incubation with $Na_3{}^{32}PO_4$ (0.1 mCi/ml) in a Ca^{2+}-free Tyrode's buffer without added phosphate, at room temperature for 90 min.

In all three labelling procedures, further washings were carried out as described by Ardlie *et al.* (1970).

Cell lysis

Platelets were finally suspended in 25 mM-Tris/HCl (pH 7.4)/100 mM-KCl/3 mM-ATP/3 mM-$MgCl_2$ and transferred to a Kontes Mini Bomb (Vineland, NJ, U.S.A.). After equilibration for 20 min at 70 atm N_2 and 4 °C, lysis occurred on decompression to atmospheric pressure. The product is referred to as 'homogenate'.

Percoll-density-gradient ultracentrifugation

The supernatant after centrifugation at 1500 *g* for 10 min at 4 °C was then obtained; 6 ml of this was mixed with 10.4 ml of Percoll, 12 ml of 50 mM-Tris/200 mM-KCl/6 mM-ATP/6 mM-$MgCl_2$ and 1.6 ml of water. pH was carefully adjusted to 9.6 with 1 M-NaOH as previously described (Mauco *et al.*, 1984*a*; Fauvel *et al.*, 1986). The mixture was then spun for 9.8×10^{10} rad^2/s in a Beckman 60 Ti rotor at 28500 rev./min at 4 °C, in polycarbonate centrifuge tubes (Kontron, Basle, Switzerland); 2 ml fractions were collected from the top of the tube and immediately assayed for their radioactive phospholipid content and subcellular markers. The total activity measured on the homogenate obtained after N_2 decompression was taken as 100%.

Subcellular markers

Lysosomes were detected by measuring *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30) (Day *et al.*, 1969; Perret *et al.*, 1979) and/or β-D-glucuronidase (EC 3.2.1.31) by using a commercial kit (Sigma, St. Louis, MO, U.S.A.). NADH dehydrogenase (EC 1.6.99.3), also called NADH diaphorase, was used to localize the endoplasmic reticulum (Wallach & Kamat, 1966; Record *et al.*, 1982).

In preliminary experiments plasma membranes were detected by measuring bound [^{3}H]concanavalin A.

Lipid analysis

All extraction procedures were carried out at 4 °C, by a modified Bligh & Dyer (1959) procedure. Addition of 20 mM-EDTA to 1.8 ml of Percoll-density-gradient fractions was followed by 6 ml of cold methanol and 2 ml of chloroform. After mixing and standing on ice for about 10 min, 0.150 ml of 12 M-HCl was then added, and the tubes were centrifuged (3000 *g* for 10 min at 4 °C). Lipids were extracted into the organic layer by adding 4 ml of chloroform and 3 ml of water to the supernatant.

T.l.c. was performed on oxalate-impregnated silica-gel plates (Merck, Darmstadt, Germany), with chloroform/methanol/4 M-NH_3 (9:7:2, by vol.) (Lloyd *et al.*, 1973) as eluent.

Radioactive spots containing [^{3}H]inositol were detected and quantified by using a LC 283 monitor (Berthold, Munich, Germany). Those containing ^{32}P were detected by autoradiography (Kodirex films; Kodak, Rochester, NY, U.S.A.), scraped off and counted for radioactivity in a liquid-scintillation counter with automatic quenching correction (Intertechnique, Paris, France).

Materials

[^{32}P]P_i (carrier free; 1 mCi/ml), *N*-[^{3}H]acetylated concanavalin A and *myo*-[2-^{3}H]inositol (10–20 Ci/mmol) were all from Amersham International (Amersham, Bucks., U.K.).

RESULTS AND DISCUSSION

Subcellular fractionation of blood platelets

We have previously described a two-step Percoll procedure for the fractionation of human platelets, which allowed the purification of plasma membranes and dense tubular system (i.e. endoplasmic reticulum) from other organelles (Mauco *et al.*, 1984*a*; Fauvel *et al.*, 1986). To decrease the time and therefore to decrease the risk of any modifications during the preparation, we designed a simplified one-step procedure, taking advantage of the effect of pH on the apparent density of membranes. By using a highly alkaline pH (9.6) we obtained a very good purification of plasma membranes from intracellular components (Fig. 1). Even if lysosomes were not well separated from endoplasmic reticulum, the reproducibility of Percoll-gradient centrifugation allowed us to distinguish reasonably between the two organelles, as lysosomes display a lower density than the dense tubular system. Granules and mitochondria were described to localize at the very bottom of the tube (Perret *et al.*, 1979; Mauco *et al.*, 1984*a*). The high resulting pH had no significant effect on the enzymic markers that we used, since 105±12% of the 1500 *g* supernatant NADH diaphorase and 80.6±8.1% of *N*-acetyl-β-D-glucosaminidase were recovered in gradient

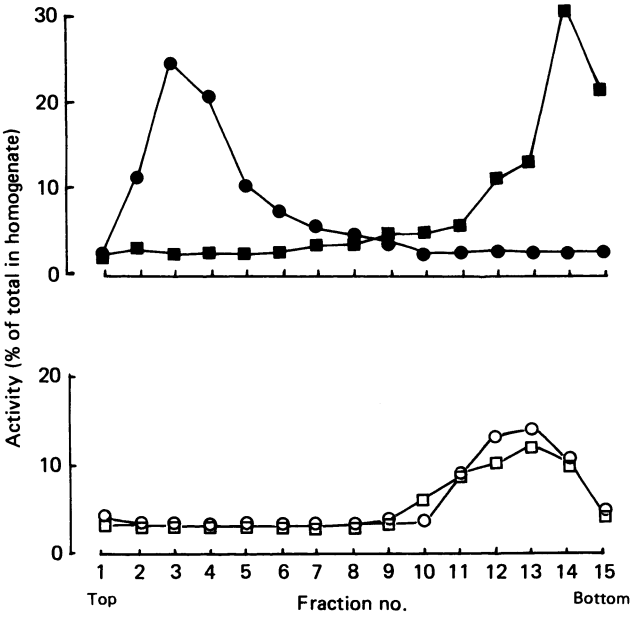

Fig. 1. Distribution of subcellular markers on the Percoll gradient

[^{3}H]Concanavalin A-prelabelled platelets were processed as indicated in the Experimental section. Results are expressed as percentages of the total activity of the homogenate (representative of four experiments). ●, [^{3}H]Concanavalin A; ■, NADH diaphorase; ○, *N*-acetyl-β-D-glucosaminidase; □, β-D-glucuronidase.

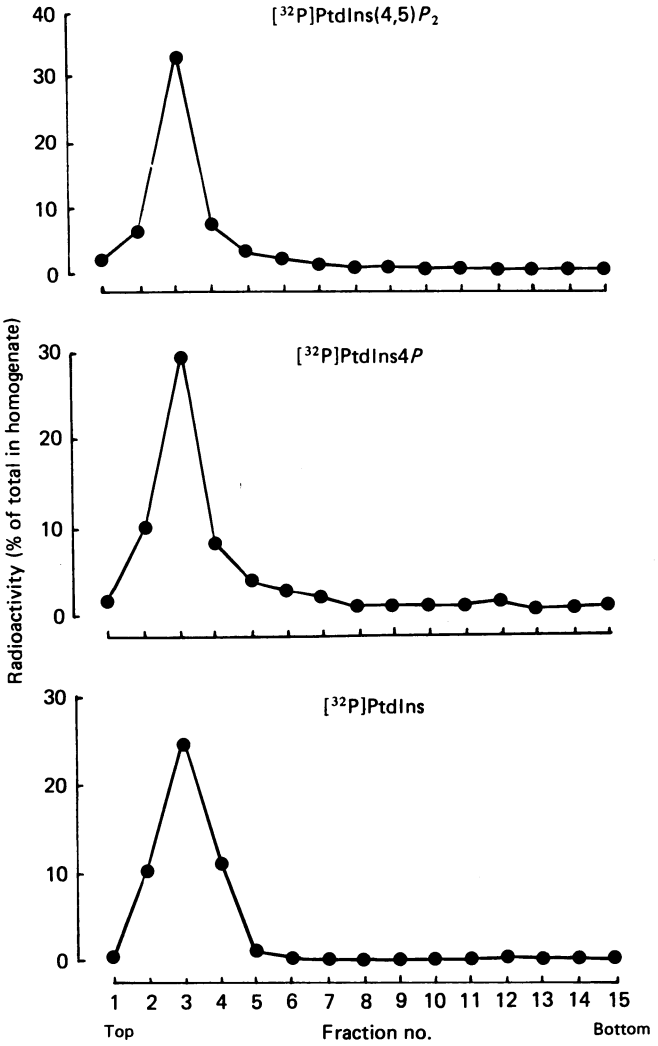

Fig. 2. [^{32}P]Inositol-lipid distribution on Percoll density gradient

Results are expressed as percentages of the total radioactivity measured in the homogenate for each phospholipid, after preincubation of the cells with [^{32}P]P_i (representative of three quite similar independent experiments).

fractions, as well as 90±8.1% of [^{3}H]concanavalin; these recoveries confirm previously published data on Percoll gradients at alkaline pH (Fauvel *et al.*, 1986; Record *et al.*, 1982) as well as at neutral pH (Perret *et al.*, 1979; Mauco *et al.*, 1984*a*). This rapid procedure (less than 1 h after cell lysis) therefore appeared suitable for obtaining well-purified plasma membranes with a high yield and can be very useful for avoiding post-lytic modifications of cell composition.

Subcellular fractionation of ^{32}P-labelled platelets

Platelets incorporate P_i at a high rate, and the 4- and 5-positions of inositol phospholipids reach rapid isotopic equilibrium with the γ-phosphate of ATP (Lloyd *et al.*, 1973; Dangelmaier *et al.*, 1986). This is also observed for other cells (Hokin & Hokin, 1964), and therefore allows one to consider radioactive measurements to be a good indication of masses. Fig. 2 shows the results obtained after subcellular fractionation. [^{32}P]PtdIns(4,5)P_2, [^{32}P]PtdIns4*P* and [^{32}P]PtdIns+PdtA are exclusively located in the plasma membrane. The relative labelling of each individual phospholipid was similar in plasma membranes and in whole platelets (Table 1), suggesting that no significant modification occurred during the whole procedure. The results agree very well with the proposal of Rawlyer *et al.* (1984), who suggested the use of polyphosphoinositide phosphorylation as a specific marker for plasma membrane in Friend erythroleukaemic cells. Assuming that the radioactivity of the phosphodiester could be neglected, it can be calculated from Table 1 that the specific radioactivity of each of the monophosphate groups of PtdIns(4,5)P_2 is 1.8 times that of PtdIns4*P*. This could be due to an overestimation of PtdIns4*P* in our mass study (Mauco *et al.*, 1984*b*), since it has been shown that an unknown lipid co-migrates with PtdIns4*P* on t.l.c. (Tysnes *et al.*, 1985). However, we can assume that all the phosphorylated derivatives of PtdIns were located in the plasma membrane. Such a conclusion cannot be made for PtdIns, since the labelling of this lipid was extremely low under the conditions used. One should then notice that ^{32}P-labelling of PtdIns could have been obtained either by synthesis *de novo* or by a minimal activation of the inositol-lipid cycle. It is not known, however, if such a turnover of the phosphodiester should be attributed to a slight activation of platelets during the preparation or to a basal activity of the cycle in resting platelets.

Subcellular localization of [^{3}H]inositol-labelled lipids

Inositol was not incorporated in significant amounts into whole platelets in platelet-rich plasma or in balanced saline substitutes such as Tyrode's buffers (results not shown). However, lowering the glucose concentration to 0.1 mM and adding 1 mM-$MnCl_2$ to a Ca^{2+}-free Tyrode's buffer allowed us to incorporate large amounts of inositol into whole platelets. Table 1 shows that inositol labelling reached isotopic equilibrium between the three classes of inositol lipids, since the relative amounts of radioactivity paralleled those previously described for masses in platelets (Mauco *et al.*, 1984*b*). As shown in Fig. 3, PtdIns4*P* and PtdIns(4,5)P_2 were again exclusively located on plasma membranes, whereas PtdIns was distributed between plasma (35.9±4.6%) and endoplasmic-reticulum membranes (49.7±4.0%) (means±S.E.M., $n = 3$). This confirms previously reported data obtained by mass measurement of PtdIns in subcellular fractions of platelets (Lagarde *et al.*, 1982; Fauvel *et al.*, 1986). Furthermore, these results are consistent with the current hypothesis of an intracellular synthesis of PtdIns followed by a redistribution to the other membranes (Call & Williams, 1970; Call & Ruppert, 1973; reviewed by Esko & Raetz, 1983). This is assumed to be catalysed by phospholipid-transfer proteins, which are present in various cells, including blood platelets (Laffont *et al.*, 1981; George & Helmkamp, 1985). A dual localization of inositol lipid metabolism is also suggested by other published data on hormone-sensitive and -insensitive inositol lipid pools. Schoepp (1985) showed that Mn^{2+} stimulated incorporation of [^{3}H]inositol into a PtdIns fraction which was poorly hydrolysed on stimulation of brain slices with noradrenaline and carbachol. Monaco (1982) also reported the existence of a hormone-insensitive PtdIns pool in WRK-1 cells. Koreh & Monaco (1985) showed that hormone-sensitive PtdIns(4,5)P_2 was derived exclusively from the hormone-sensitive PtdIns pool, at least

Table 1. Inositol-lipid composition of total platelets, plasma membranes and endoplasmic reticulum

Results are means ± S.E.M. of three independent experiments, except for those indicated by *, which are taken from Mauco *et al.* (1984*b*). For each cellular compartment the sum of all three inositol lipids was taken as 100, since lysophospholipids were barely detectable. Abbreviation: ND, not detected.

	PtdIns	PtdIns4*P*	PtdIns(4,5)P_2
Total platelets mass*	81.7	13.5	4.8
[^{3}H]Inositol-labelled	75.8±6.8	12.3±4.2	11.9±2.6
^{32}P-labelled	8.0±4.0	40.0±2.4	51.6±6.5
Plasma membranes			
[^{3}H]Inositol-labelled	66.1±3.7	19.4±2.6	14.5±0.1
^{32}P-labelled	3.7±0.4	42.6±1.9	53.7±2.2
Endoplasmic reticulum			
[^{3}H]Inositol-labelled	99.0±0.8	0.5±0.1	0.5±0.1
^{32}P-labelled	ND	ND	ND

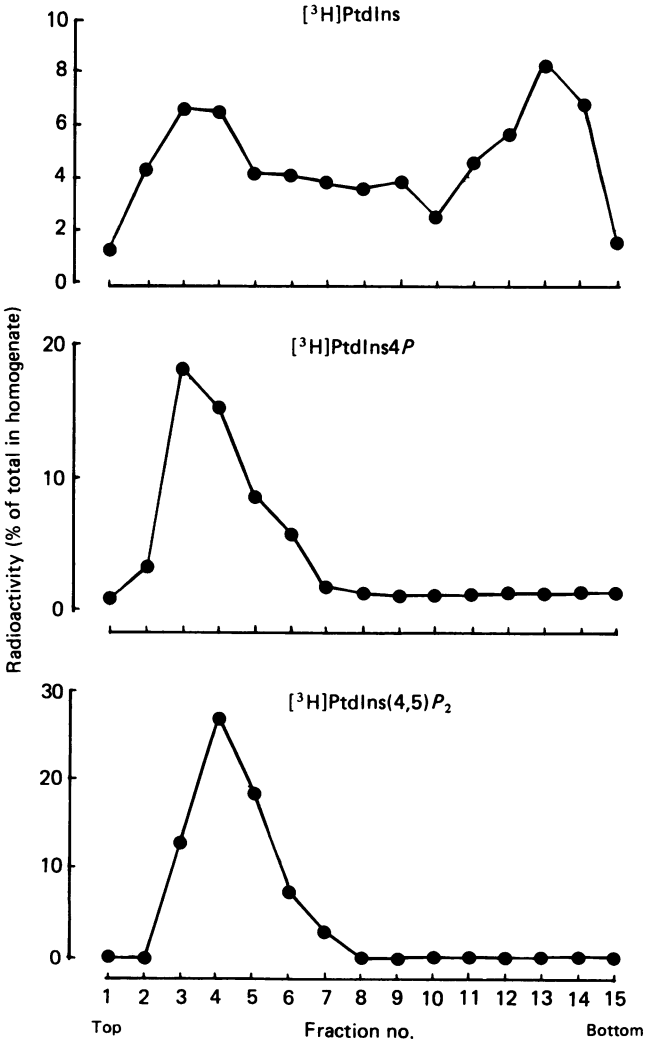

Fig. 3. [^{3}H]Inositol-lipid distribution on Percoll density gradient

Results are expressed as percentages of the total radioactivity measured in the homogenate for each phospholipid, after preincubation of the cells with [^{3}H]inositol (representative of three quite similar independent experiments).

during long incubation times in the presence of vasopressin. Subcellular localization of stimulable PtdIns(4,5)P_2 hydrolysis was not studied in platelets, since this response is maximal 10 s after thrombin addition (Billah & Lapetina, 1982; Agranoff *et al.*, 1983; Mauco *et al.*, 1984*b*), followed by rapid PtdInd(4,5)P_2 resynthesis, so that practical problems were expected. However, it should be emphasized that hormone-sensitive pools are likely to exist in platelets, since only 30–35% of the total PtdIns(4,5)P_2 can be hydrolysed during stimulation by ADP (Vickers *et al.*, 1984), thrombin (Billah & Lapetina, 1982; Mauco *et al.*, 1984*b*) or platelet-activating factor (PAF-acether) (Mauco *et al.*, 1983). No doubt further investigations are needed to understand the organization of membrane inositol-lipid micro-domains.

In conclusion, our study brings clear evidence for a highly specific (if not exclusive) localization of polyphosphoinositides in the platelet plasma membrane, whereas PtdIns, whose synthesis probably occurs in the endoplasmic reticulum, seems to equilibrate between different membranes. Since PtdIns has been shown to be confined in the internal leaflet of the plasma membrane, owing to membrane asymmetry (Chap *et al.*, 1977; Perret *et al.*, 1979), it is tempting to propose a similar localization for polyphosphoinositides, which display metabolic equilibrium with cytosolic ATP. Such an arrangement provides further support to the well-accepted role of PtdIns(4,5)P_2 in trans-membrane signalling, including coupling of PtdIns(4,5)P_2 hydrolysis to agonist/receptor complex by G protein (Haslam & Davidson, 1984*a*,*b*; Baldassare & Fisher, 1986*a*,*b*), generation of DAG in a membrane monolayer rich in phosphatidylserine facing cytosolic protein kinase C (Nishizuka, 1984) and diffusion of InsP_3 from the plasma membrane to its target organelles, i.e. dense tubular system (O'Rourke *et al.*, 1985; Adunyah & Dena, 1985; Authi & Crawford, 1985; Brass & Joseph, 1985).

REFERENCES

Adunyah, S. E. & Dena, W. L. (1985) Biochem. Biophys. Res. Commun. **128**, 1274–1280

Agranoff, B. W., Murthy, P. & Seguin, E. B. (1983) J. Biol. Chem. **258**, 76–78

Ardlie, N. G., Packham, M. A. & Mustard, J. F. (1970) Br. J. Haematol. **19**, 7–17

Authi, K. S. & Crawford, N. (1985) Biochem. J. **230**, 247–253
Baldassare, J. J. & Fisher, G. J. (1986*a*) Biochem. Biophys. Res. Commun. **137**, 801–805
Baldassare, J. J. & Fisher, G. J. (1986*b*) J. Biol. Chem. **261**, 11942–11944
Berridge, M. J. (1984) Biochem. J. **220**, 345–360
Billah, M. M. & Lapetina, E. G. (1982) J. Biol. Chem. **257**, 12705–12708
Bligh, E. G. & Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–918
Brass, L. F. & Joseph, S. K. (1985) J. Biol. Chem. **260**, 15172–15179
Call, F. L., II & Ruppert, W. J. (1973) J. Lipid Res. **14**, 466–474
Call, F. L., II & Williams, W. J. (1970) J. Clin. Invest. **49**, 1949–1955
Campbell, C. R., Fishman, J. B. & Fine, R. E. (1985) J. Biol. Chem. **260**, 10948–10951
Carter, J. R. & Kennedy, E. P. (1966) J. Lipid Res. **7**, 678–683
Chap, H., Zwaal, R. F. A. & Van Deenen, L. L. M. (1977) Biochim. Biophys. Acta **467**, 146–164
Dangelmaier, L. A., Daniel, J. L. & Smith, J. P. (1986) Anal. Biochem. **154**, 414–419
Day, H. J., Holmsen, H. & Hovig, T. (1969) Scand. J. Haematol. Suppl. **7**, 3–35
Esko, J. D. & Raetz, C. R. H. (1983) Enzymes 3rd Ed. **16**, 207–253
Fauvel, J., Chap, H., Roques, V., Levy-Toledano, S. & Douste-Blazy, L. (1986) Biochim. Biophys. Acta **856**, 155–164
George, P. Y. & Helmkamp, G. M. (1985) Biochim. Biophys. Acta **836**, 176–184
Haslam, R. J. & Davidson, M. M. L. (1984*a*) FEBS Lett. **174**, 90–95
Haslam, R. J. & Davidson, M. M. L. (1984*b*) Biochem. J. **222**, 351–361
Hokin, L. E. (1985) Annu. Rev. Biochem. **54**, 205–235
Hokin, L. E. & Hokin, M. R. (1964) Biochim. Biophys. Acta **84**, 563–575
Hokin, M. R. & Hokin, L. E. (1953) J. Biol. Chem. **203**, 967–977
Koreh, K. & Monaco, M. E. (1985) J. Biol. Chem. **261**, 88–91
Laffont, F., Chap, H., Soula, G. & Douste-Blazy, L. (1981) Biochem. Biophys. Res. Commun. **102**, 1366–1371
Lagarde, M., Guichardant, M., Menashi, S. & Crawford, N. (1982) J. Biol. Chem. **257**, 3100–3104
Lapetina, E. G. & Cuatrecasas, P. (1979) Biochim. Biophys. Acta **573**, 394–402
Lloyd, J. V., Nishizawa, E. E., Joist, H. T. & Mustard, J. F. (1973) Br. J. Haematol. **24**, 589–604
Lundberg, G. A., Jergil, B. & Sundler, R. (1985) Biochim. Biophys. Acta **846**, 379–387
Mauco, G., Chap, H., Simon, M. F. & Douste-Blazy, L. (1978) Biochimie **60**, 653–661
Mauco, G., Chap, H. & Douste-Blazy, L. (1983) FEBS Lett. **153**, 361–365
Mauco, G., Fauvel, J., Chap, H. & Douste-Blazy, L. (1984*a*) Biochim. Biophys. Acta **796**, 169–177
Mauco, G., Dangelmaier, C. A. & Smith, J. B. (1984*b*) Biochem. J. **224**, 933–940
McIntyre, D. E. & Pollock, W. K. (1983) Biochem. J. **212**, 433–437
Michell, R. H. (1975) Biochim. Biophys. Acta **415**, 81–147
Monaco, M. E. (1982) J. Biol. Chem. **257**, 2137–2139
Nishizuka, Y. (1984) Nature (London) **308**, 693–698
O'Rourke, F. A., Halenda, S. P., Zavoico, G. B. & Feinstein, M. B. (1985) J. Biol. Chem. **260**, 956–962
Perret, B., Chap, H. & Douste-Blazy, L. (1979) Biochim. Biophys. Acta **556**, 434–446
Rana, R. S., Kowluru, A. & McDonald, M. J. (1986) Arch. Biochem. Biophys. **245**, 411–416
Rawlyer, A. J., Roelofsen, B., Wirtz, K. W. A. & Op Den Kamp, J. A. F. (1982) FEBS Lett. **148**, 140–144
Record, M., Bes, J. C., Chap, H. & Douste-Blazy, L. (1982) Biochim. Biophys. Acta **688**, 47–54
Rittenhouse, S. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 5417–5420
Schoepp, D. D. (1985) J. Neurochem. **45**, 1481–1486
Seyfred, M. A. & Wells, W. W. (1984*a*) J. Biol. Chem. **259**, 7659–7665
Seyfred, M. A. & Wells, W. W. (1984*b*) J. Biol. Chem. **259**, 7666–7672
Smith, C. D. & Wells, W. W. (1983) J. Biol. Chem. **258**, 9360–9368
Takenawa, T. & Egawa, K. (1977) J. Biol. Chem. **252**, 5419–5423
Thompson, W. & McDonald, G. (1977) Can. J. Biochem. **55**, 1153–1158
Tooke, N. E., Hales, N. & Hutton, C. (1984) Biochem. J. **219**, 471–480
Tysnes, O.-B., Aarbakke, G. M., Verhoeven, A. J. M. & Holmsen, H. (1985) Thromb. Res. **40**, 329–338
Vickers, J. D., Kinlough-Rathbone, R. L. & Mustard, J. F. (1984) Biochem. J. **224**, 399–405
Wallach, D. F. H. & Kamat, V. F. (1966) Methods Enzymol. **8**, 164–192

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