The fatty acid composition of 1,2-diacylglycerol and polyphosphoinositides from human erythrocyte membranes

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The fatty acid compositions of 1,2-diacylglycerol and polyphosphoinositides have been determined in human erythrocyte membranes that have been incubated in the presence or in the absence of Ca^{2+} . The results show that the diacylglycerol that is formed in Ca^{2+} -treated membranes has a fatty acid composition closely similar to that of the inositides, consistent with previous indications that Ca^{2+} stimulates the activity of a polyphosphoinositide phosphodiesterase in the membranes. In contrast with some previous results, it appears that these plasma-membrane inositides and their derived diacylglycerols are rich in stearic acid and arachidonic acid.

Previous work has shown that human erythrocyte membranes contain an enzyme that in the presence of Ca^{2+} specifically degrades endogenous polyphosphoinositides (phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate) to 1,2-diacylglycerol and inositol bis- and tris-phosphates (Allan & Michell, 1976, 1977, 1978; Downes & Michell, 1981). Although the role of this polyphosphoinositide phosphodiesterase is unclear the enzyme is of interest for several reasons. First, it may be involved in the morphological changes undergone by human red cells whose internal Ca^{2+} levels have increased (Allan *et al.*, 1976; Allan & Thomas, 1982). Secondly, it is the only known example of a phospholipase C that has an undoubted plasma-membrane localization. Thirdly, a knowledge of this enzyme may be relevant to the study of related enzymes believed to be important in the enhanced metabolism of plasma-membrane inositides, which seems to accompany stimulation of many cell types by extracellular agonists whose final effect is mediated by Ca^{2+} (Michell, 1975). Recent work has emphasized the possibility that the polyphosphoinositides, which, unlike phosphatidylinositol, seem to be localized in plasma membranes, may be of particular importance in the mediation of events at the cell surface (Michell *et al.*, 1981).

When the polyphosphoinositide phosphodiesterase of human erythrocytes was first studied, it appeared from the fatty acid composition of the 1,2-diacylglycerol produced in plasma membranes exposed to Ca^{2+} that the precursor of the diacylglycerol must have a composition rather like that of

phosphatidylcholine, with relatively high levels of palmitic acid and oleic acid (Allan & Michell, 1976). This contrasted markedly with the composition generally observed for inositides, which in many tissues are rich in stearic acid and arachidonic acid (Holub & Kuksis, 1978). The results suggested the interesting possibility that the fatty acid composition of plasma-membrane inositides might be significantly different from that of inositides present in intracellular membranes (see Colbeau *et al.*, 1971). Re-examination of this work now suggests that some of the original findings were erroneous and that the fatty acid composition of diacylglycerol generated in Ca^{2+} -treated erythrocyte membranes is closely similar to that of the inositol lipids, which, as in other tissues, are rich in stearic acid and arachidonic acid.

Methods

Fresh human erythrocytes were generously donated by Members of the Department of Human Tumour Immunology, University College London. Cells were washed, labelled with [^{32}P]P_i and used for the preparation of membranes as described previously (Allan & Michell, 1978).

Membranes were incubated at a concentration of 1 μmol of lipid phosphorus/ml in a medium containing 10 mM-Mops/NaOH buffer with the addition of either 0.1 mM-EGTA or 1 mM- CaCl_2 . After 15 min at 37°C, incubation was ended by the addition of 50 μl of 100 mM-EDTA/ml of medium followed by 3.75 vol. of methanol/chloroform/conc. HCl

Fig. 1. *Autoradiogram of phospholipid separation by t.l.c.* Lipids were separated by t.l.c. and autoradiographed as described in the Methods section. Identity of spots: O, origin; 1, phosphatidylinositol 4,5-bisphosphate; 2, phosphatidylinositol 4-phosphate; 3, phosphatidate; 4, sphingomyelin; 5, phosphatidylcholine; 6, phosphatidylserine and phosphatidylinositol; 7, phosphatidylethanolamine. Spots 3–7 were located by staining with I_2 vapour.

(200:100:1, by vol.). Phase separation was achieved by the procedure of Bligh & Dyer (1959) and the lower (chloroform) phase was evaporated under N_2 at 25°C. For isolation of diacylglycerol a sample of the lipid from each incubation was chromatographed on a silica gel H t.l.c. plate in a solvent consisting of benzene/diethyl ether/conc. NH_3 (1000:800:1, by vol.). Standard lipids including palmitic acid (R_F 0.08), cholesterol (R_F 0.43), 1,2-dioleoylglycerol, (R_F 0.57), 1,3-dioleoylglycerol (R_F 0.64), trioleoylglycerol (R_F 0.70) and cholesteryl palmitate (R_F 0.81) were run in separate lanes on the same plate. After spraying with 0.1% Rhodamine 6G in acetone the area of the sample lane corresponding to the standard dioleoylglycerol was removed for fatty acid analysis.

For isolation of polyphosphoinositides the concentrated chloroform solution of membrane lipids was diluted with 4 vol. of ethanol and was left overnight at -30°C under N_2 . The resulting precipitate of acidic lipids was centrifuged and redissolved in the minimum necessary volume of chloroform. A sample of this material was separated by two-dimensional t.l.c. on oxalate-impregnated plates [first dimension, chloroform/methanol/28% NH_3 (13:7:1, by vol.); second dimension, chloroform/methanol/acetone/acetic acid/water (40:13:15:12:8, by vol.)] and the plate was subsequently subjected to autoradiography.

Three radioactive spots corresponding to phosphatidate, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate were observed (Fig. 1). The individual inositides were removed from the plate for fatty acid analysis. To minimize lipid peroxidation, all chromatographic procedures were carried out in an N_2 atmosphere and 0.5 mg of butylated hydroxytoluene/ml was added to solvents.

Fatty acids were analysed as their methyl esters after BF_3 /methanol treatment of diacylglycerol and polyphosphoinositide samples as described by Morrison & Smith (1964). Methyl esters were quantified by g.l.c. using a 1.5 m glass column packed with 10% EGSS-Y on 80–100 mesh Supelcoport. Isothermal analysis at 200°C in a Pye 204 apparatus gave separations closely comparable with those of Dodge & Phillips (1967). Peaks were quantified using a Shimadzu RC1-B computing integrator.

Results and discussion

The fatty acid compositions of 1,2-diacylglycerols and polyphosphoinositides from human red-cell membranes are shown in Table 1. It is clear from this Table that the fatty acid composition of 1,2-diacylglycerol from membranes incubated in the absence of Ca^{2+} contrasts markedly with that of diacylglycerol in Ca^{2+} -treated membranes. In particular, the content of palmitic acid and oleic acid is much lower and the content of stearic acid and arachidonic acid much higher in membranes exposed to Ca^{2+} . The calculated composition of the new Ca^{2+} -generated diacylglycerol (column *e*) is almost identical with that of the polyphosphoinositides (columns *a* and *b*) and unlike that of any other erythrocyte lipid (Dodge & Phillips, 1967) with the exception of monophosphoinositide (Breckenridge & Palmer, 1982). This supports previous indications that Ca^{2+} promotes the breakdown of polyphosphoinositides to 1,2-diacylglycerol in red-cell membranes (Allan & Michell, 1978; Downes & Michell, 1981). A previous suggestion that the fatty acid composition of the diacylglycerol generated in Ca^{2+} -treated red-cell membranes resembled that of phosphatidylcholine (Allan & Michell, 1976) appears now to be erroneous, although the reason for this is not clear. One possibility is that in the early experiments diacylglycerol may have been contaminated with non-esterified fatty acid that separated poorly in the original solvent system employed. The modified system used in the present work allowed a clear separation of 1,2-diacylglycerol from possible contaminants. In addition, it is likely that the precautions taken to guard against fatty acid oxidation have allowed much better recoveries of arachidonic acid than in previous work.

Table 1. *Fatty acid composition of polyphosphoinositides and diacylglycerols from human erythrocyte membrane*
Results are means $\pm$ s.d. and are derived from duplicate determinations in three separate experiments using membranes from different donors. The level of diacylglycerol in control membranes was 4.2 ± 1.4 compared with $16.0 \pm 0.9 \mu\text{g/ml}$ of packed ghosts in membranes treated with Ca^{2+} .

	Content (mol %)				
	Phosphatidylinositol 4-phosphate	Phosphatidylinositol 4,5-bisphosphate	Diacylglycerol from:		'New' diacylglycerol (calculated from c and d)
	(a)	(b)	Control membrane (c)	Membrane + Ca^{2+} (d)	(e)
$\text{C}_{16:0}$	8 ± 1	8 ± 1	32 ± 6	12 ± 2	7 ± 1
$\text{C}_{18:0}$	42 ± 6	43 ± 6	20 ± 4	36 ± 1	43 ± 5
$\text{C}_{18:1}$	9 ± 1	9 ± 1	26 ± 9	14 ± 1	8 ± 3
$\text{C}_{18:2}$	5 ± 1	5 ± 2	9 ± 3	7 ± 1	7 ± 1
$\text{C}_{20:3}$	3 ± 1	2 ± 1	<1	2 ± 1	3 ± 1
$\text{C}_{20:4}$	32 ± 1	32 ± 2	6 ± 1	26 ± 4	33 ± 2
Other ($\text{C}_{22:5}$, $\text{C}_{22:6}$)	<1	<1	<1	<1	<1

The overall fatty acid composition of the inositides and inositide-derived diacylglycerol from red-cell membranes resembles that of inositides from other cells (Holub & Kuksis, 1978). The only respect in which red-cell plasma-membrane inositides may differ somewhat lies in their content of palmitate, which appears to be somewhat higher than in the inositides of other tissues (see also Breckenridge & Palmer, 1982).

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