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PRODUCTION OF 1,2-DIACYLGLYCEROL IN HUMAN ERYTHROCYTE MEMBRANES EXPOSED TO LOW CONCENTRATIONS OF CALCIUM IONS

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SUMMARY

A specific increase in the membrane content of 1,2-diacylglycerol occurred when erythrocytes were lysed at 20 °C in media which did not include a chelator of Ca^{2+} and also when Ca^{2+} was added to haemoglobin-free erythrocyte ghosts which had been prepared in the presence of ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA). The maximum increase was about 20-fold. The production of 1,2-diacylglycerol appeared to be caused by an endogenous membrane-bound phospholipase C which was half-maximally activated at less than 1 μM Ca^{2+} and which had access to only about 0.6–0.8 % of the cells' glycerolipids. This activity was optimal at pH 7.0–7.2 in the presence of 0.1 mM Ca^{2+}; under these conditions diacylglycerol production was complete within 5–10 min. Enzyme activity was markedly decreased at low temperatures, and was abolished by heating at 100 °C for 1 min.

INTRODUCTION

We recently showed that an increase in the intracellular Ca^{2+} concentration in human erythrocytes causes a rise in the cell content of 1,2-diacylglycerol and it seems probable that this effect is caused by the activation of a Ca^{2+}-dependent phospholipase C [1–4]. Although these observations suggested that the enzyme responsible for this reaction might be membrane-bound, this was not certain. We have now shown that this enzyme is a membrane component and that, unless a chelator is present to remove the traces of Ca^{2+} present in the lysing buffers, diacylglycerol is produced when cells are lysed at temperatures $\geqslant 10$ °C. In addition, we have examined some of the characteristics of the enzyme in haemoglobin-free ghosts.

METHODS

Group O human red cells were obtained and washed as described before [3]. The buffy coat was retained and washed separately under the same conditions.

Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid.

Haemoglobin-free ghosts which had not been exposed to Ca^{2+} were obtained from erythrocytes by lysis and washing three times in a medium containing 20 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (Hepes)/NaOH buffer, pH 7.0, 1 mM $MgCl_2$ and 1 mM EGTA. Approximately 1 ml of packed ghosts was derived from 1 ml of packed cells. These ghosts were then incubated under various conditions either with no further additions or with the addition of Ca^{2+}. Appropriate concentrations of ionised Ca^{2+} were established using Ca^{2+}/EGTA buffers calculated from the data of Raaflaub [5]. Lipids were extracted and analysed for diacylglycerol and phosphatidate as described previously [3]; the diacylglycerol assays were by a method involving densitometry of photographs of thin-layer chromatograms [3]. Duplicate determinations by this procedure differed by less than 10 % except at very low values. Fatty acid analyses were carried out by Dr. R. Watts as previously described [3]. When membranes containing [^{32}P]phosphatidate were needed they were obtained from cells which had been incubated for 60 min in a Hepes-Ringer medium [3] containing ^{32}P and 11 mM glucose.

In some experiments 1 ml aliquots of the packed cells were lysed simultaneously in different media containing 1 mM $MgCl_2$, all of which were buffered with 20 mM Hepes/NaOH, pH 7.0, and which varied in their contents of other materials (Ca^{2+}, chelators, etc). After 30 min at 20 °C the haemolysates were centrifuged at 15,000 × *g* for 15 min. Lipids were extracted from the pellets and analysed in the same way as in the experiments with haemoglobin-free ghosts.

RESULTS

When washed human erythrocytes were lysed at room temperature (approx. 20 °C) in dilute Hepes/NaOH buffer alone, the diacylglycerol content of their membranes was increased (Table 1). This increase did not occur if lysis was carried out in the presence of EGTA and therefore it seemed likely that it was caused by the small

TABLE I

THE INFLUENCE OF Ca^{2+} ON THE GENERATION OF 1,2-DIACYLGLYCEROL IN ERYTHROCYTE HAEMOLYSATES

0.5 ml aliquots of packed washed cells were lysed in 40 vol. of 20 mM Hepes/NaOH buffer pH 7.0 containing 1 mM $MgCl_2$ and other additions as shown. Details of incubation procedures and lipid extractions are given in the text. Results are averages of duplicate determinations on a single batch of cells, but very similar values were obtained in three other identical experiments using different batches.

	Additions to lysing buffer	1,2-Diacylglycerol content (nmol)
Intact washed packed cells (0.5 ml)	—	0.4
Haemolysate	—	2.5
	EGTA (1 mM)	0.5
	$CaCl_2$ (0.1 mM)	10.5
	$CaCl_2$ (0.1 mM) + *o*-phenanthroline (1 mM)	11.1

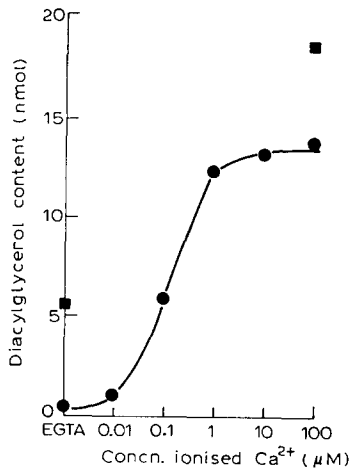

Fig. 1. The dependence of 1,2-diacylglycerol production in red cell membranes on the concentration of ionised Ca^{2+} in the medium. 1.0 ml aliquots of packed membranes in 20 mM Hepes/NaOH buffer pH 7.0, containing 1 mM $MgCl_2$, and 1 mM EGTA were preincubated for 5 min at 37 °C. Sufficient $CaCl_2$ was added to each sample to give defined concentrations of ionised Ca^{2+} as calculated from the data of Raaflaub [5] and incubation was continued for a further 5 min. The reaction was stopped by addition of 3.75 ml of methanol/chloroform 2 : 1 and lipids were analysed as described previously [3]. This figure shows the results of a typical experiment (one of four giving similar results). The membrane preparations were prepared either from erythrocyte suspensions [$\approx$2 leukocytes per 10^5 erythrocytes, (●)]; or from a buffy-coat suspension [$\approx$ 500 leukocytes/10^5 erythrocytes, (■)].

TABLE II

THE EFFECT OF TEMPERATURE ON Ca^{2+}-DEPENDENT DIACYLGLYCEROL PRODUCTION IN HUMAN ERYTHROCYTE GHOSTS

Diacylglycerol was measured in 1.0 ml aliquots of packed, washed ghosts which were treated as indicated in the table. The results are expressed as means of duplicate determinations in each of two separate experiments.

Treatment	1,2-Diacylglycerol content (nmol per ml of packed ghosts)
1 mM EGTA, not incubated	0.8
1 mM EGTA, 5 min at 37 °C	0.7
0.1 mM Ca^{2+}, 5 min at 0 °C	0.8
0.1 mM Ca^{2+}, 5 min at 10 °C	1.8
0.1 mM Ca^{2+}, 5 min at 20 °C	4.2
0.1 mM Ca^{2+}, 5 min at 37 °C	15.3
1 mM EGTA, 1 min at 100 °C, followed by 0.1 mM Ca^{2+}, 5 min at 37 °C	0.6

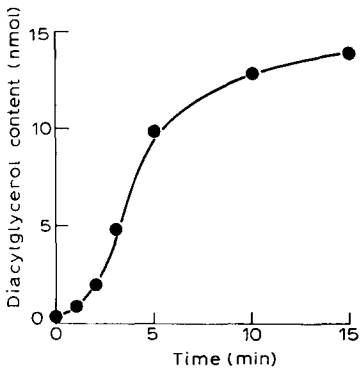

Fig. 2. Time course of 1,2-diacylglycerol accumulation in red cell membranes exposed to 0.1 mM Ca^{2+}. Aliquots of membrane were preincubated as in the legend to Fig. 1 and then 110 μl of of 10 mM $CaCl_2$ was added to each sample, giving a final ionised Ca^{2+} concentration of 0.1 mM. Incubation was continued for various periods of time before extracting lipids as described previously [3]. The figure shows the results of a typical experiment (one of three similar experiments).

TABLE III

FATTY ACID COMPOSITIONS OF 1,2-DIACYLGLYCEROL FROM HUMAN RED CELL MEMBRANES INCUBATED WITH OR WITHOUT ADDITION OF Ca^{2+}

These results represent averages of determinations on two different preparations of membrane and are expressed as mol % of total fatty acid recovered.

	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2	Other (mainly 20 : 4)
No Ca^{2+}	38	6	21	26	5	4
+0.1 mM Ca^{2+}	34	6	22	28	7	5
Phosphatidylcholine*	33	2	12	19	18	6

* The values for phosphatidylcholine are averages of several sets of values collected in Ref. 6.

quantities of Ca^{2+} present in the cells and the nominally calcium-free buffers. Diacylglycerol production was not influenced by Mg^{2+} or by *o*-phenanthroline, a chelator of transition metals, and was maximally stimulated by addition of approx. 0.1 mM Ca^{2+}. The diacylglycerol content of ghosts produced in the presence of 0.1 mM Ca^{2+} was about 20-fold greater than in ghosts isolated in EGTA.

A rise in diacylglycerol content of about the same magnitude was seen when Ca^{2+} was added to incubations at 37 °C containing haemoglobin-free ghosts which had been prepared in the presence of EGTA (Fig. 1). Optimal activity was observed at pH 7.0–7.2. This demonstrated that the activity responsible for diacylglycerol production is membrane-bound and also provided a much more controlled system in which to study the characteristics of this activity.

The washed red cell suspensions used in these experiments contained about 2–5 leukocytes/10^5 erythrocytes. In order to exclude the possibility that diacylglycerol production was due to these contaminating white cells, experiments were

TABLE IV

LACK OF EFFECT OF Ca^{2+} ON ERYTHROCYTE MEMBRANE PHOSPHATIDATE CONTENT AND LABELLING WITH ^{32}P

^{32}P-labelled membranes prepared in 20 mM Hepes/NaOH buffer, 1 mM $MgCl_2$, 1 mM EGTA as described in Methods were incubated at 37 °C with or without addition of Ca^{2+}. The results of a single typical experiment are shown with values given as average ±S.D. The figures in brackets refer to the number of determinations in each case.

	Phosphatidate content (nmol/ml packed ghosts)	^{32}P-labelling of phosphatidate (dpm)
Unincubated control: 1 mM EGTA (2)	48±2	2260±210
15 min at 37 °C: 1 mM EGTA (4)	50±2	2390±130
15 min at 37 °C: 1 mM EGTA and 2 mM Ca^{2+} (4)	50±3	2300±110

carried out using washed buffy-coat cell suspensions which contained about 500 leukocytes/10^5 erythrocytes. The results of this comparison (Fig. 1) demonstrated that although leukocytes did contribute some diacylglycerol, the quantity was only about one-hundredth of that needed to account for the calcium-dependent diacylglycerol production in purified red cell suspensions.

Heating of ghosts at 100 °C for 1 min abolished Ca^{2+}-stimulated diacylglycerol production, suggesting that an enzyme was responsible for diacylglycerol liberation (Table II). This enzyme reaction was sensitive to Ca^{2+}, giving half-maximum diacylglycerol production between 0.1 μM and 1 μM Ca^{2+} (Fig. 1). Substitution of strontium (0.1 mM) for Ca^{2+} gave very much lower activities and lanthanum (1 mM) completely abolished diacylglycerol production in the presence of 0.1 mM Ca^{2+}. Diacylglycerol production in the ghost preparation was essentially complete within 5–10 min of the addition of Ca^{2+} and 0.6–0.8 % of the membrane glycerolipids were hydrolysed in this time (Fig. 2). No changes were apparent in the concentrations of monoacylglycerol or of unesterified fatty acids. Diacylglycerol production was temperature-dependent and was greatly reduced below 10–15 °C (Table II). As a result, erythrocyte ghosts prepared at low temperatures (0–5 °C) did not show raised diacylglycerol contents even when prepared in the presence of 10–100 μM Ca^{2+} (Allan, D. and Billah, M. M., unpublished data).

The fatty acid composition of the liberated diacylglycerol (Table III) was similar to that reported previously for whole cells [3]. As noted before [3], the pattern showed much more resemblance to that of erythrocyte phosphatidylcholine (Table III) than to other erythrocyte glycerolipids [6] but it was enriched in hexadecenoic acid and depleted in linoleic acid even in comparison with phosphatidylcholine. The possibility that diacylglycerol was liberated from phosphatidate by the action of phosphatidate phosphohydrolase was also considered, but was rejected when it was found that incubation of membranes containing [^{32}P] phosphatidate with Ca^{2+} caused neither a loss of radioactivity from phosphatidate nor a decrease in their phosphatidate content (Table II). Triacylglycerol is another source from which diacylglycerol can be liberated in response to raised intracellular Ca^{2+}, at least in lymphocytes [20], but our erythrocyte membrane preparations, like those of

previous workers [6], contained far too little triacylglycerol for this to be the origin of the diacylglycerol in the experiments being reported here.

DISCUSSION

Our previous studies have shown that an enzyme which liberates diacylglycerol is activated when human erythrocytes are treated with Ca^{2+} and a divalent cation ionophore (compound A23187) to raise their intracellular Ca^{2+} concentration: it seems quite likely that this enzyme is a Ca^{2+}-dependent phospholipase C which has access to a small fraction of the phosphatidylcholine of the cell [1–4]. When ATP is available in the treated erythrocytes the liberated diacylglycerol is rapidly converted to phosphatidate by diacylglycerol kinase [3]. The results reported here show that Ca^{2+}-dependent diacylglycerol production also occurs in haemoglobin-free erythrocyte ghosts and therefore that the enzyme responsible for this reaction is a component of the plasma membrane, presumably being located at its cytoplasmic surface. This appears to be the first indication of a phospholipase C other than phosphatidate phosphohydrolase as a constituent of the plasma membrane of human red cells, although there is already substantial evidence that diacylglycerol kinase may be an important constituent of human red cell membranes [7, 8].

Despite the fact that it brings about a 20–30 fold increase in the membrane content of diacylglycerol, the enzyme which causes Ca^{2+}-dependent diacylglycerol production only causes the breakdown of about 1 % of the glycerolipids of the cell. Although the lipid which is the substrate for this reaction has not been identified with certainty it seems most likely that it is a fraction (relatively rich in hexadecenoic acid and depleted in linoleic acid) of the phosphatidylcholine of the inner leaflet of the plasma membrane [3, 4]. About one-third of human erythrocyte glycerolipid is phosphatidylcholine, and one-third of this is in the inner leaflet [9], so it appears that only 10 % of the phosphatidylcholine of the inner leaflet is available for conversion to diacylglycerol. The reason for this is not known.

It appears possible from these studies that some of the erythrocyte membrane preparations which are used routinely for studies of membrane structure and function might contain a small amount of diacylglycerol which is not a component of the membrane in the healthy parent erythrocytes. Although the quantity of diacylglycerol involved is small, recent studies have suggested that it can lead to major changes in membrane morphology and to a vesiculation process which involves both curvature and fusion of membranes [1–4, 8]. Previously we have only studied this process in intact cells but it is clear that ghosts exposed to Ca^{2+} undergo morphological changes similar to those exhibited by cells whose intracellular calcium concentration has been raised using an ionophore [2, 10, 11]. These morphological changes in ghosts have previously been attributed by Palek et al. [10] to effects of Ca^{2+} on the actin-spectrin meshwork at the inner surface of the membrane, but one should now consider whether echinocytosis of ghosts, like echinocytosis of intact cells, might occur as a result of the increased 1,2-diacylglycerol content of the inner lipid leaflet of membranes whose cytoplasmic surfaces have been exposed to Ca^{2+} [4].

Fortunately, it appears that production of diacylglycerol is insignificant at low temperatures, so that membranes isolated and maintained at 0–5 °C would not accumulate diacylglycerol even in the absence of Ca^{2+} chelators. However, it seems

possible that diacylglycerol accumulation might be significant during procedures which require higher temperatures and which only occur in the absence of chelators (e.g. resealing of ghosts) [12]. If ATP is also present during these procedures, then the released diacylglycerol may be phosphorylated by diacylglycerol kinase. This would account for the observation that Ca^{2+} causes an increase in labelling of phosphatidate in erythrocyte ghosts incubated with ^{32}P-labelled ATP [13].

It seems probable that the normal intracellular concentration of ionised calcium in the human red cell is below 0.1 μM [12], and it is therefore clear that the enzyme which liberates diacylglycerol will be appreciably activated whenever the ability of cells to maintain their low intracellular Ca^{2+} concentration is diminished. Such activation may lead to morphological changes resulting in membrane vesiculation [4]. These effects might be significant in at least two physiological situations in which erythrocytes tend to lose membrane material into their environment, namely during ageing [14, 15] when intracellular ATP levels become depressed, and in hereditary spherocytosis [16], when the activity of the Ca^{2+}-pumping ATPase system of the membranes may be decreased [17]. Recently, we have isolated membrane vesicles from the plasma of aged blood: these vesicles are enriched in diacylglycerol [21]. In hereditary spherocytosis there is an increase in the labelling of phosphatidate [18, 19], which would be expected as a consequence of a Ca^{2+}-induced increase in membrane diacylglycerol content [3].

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