Production of 1,2-Diacylglycerol and Phosphatidate in Human Erythrocytes Treated with Calcium Ions and Ionophore A23187

By DAVID ALLAN,* RODNEY WATTS† and ROBERT H. MICHELL*

* Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, and † Department of Experimental Pathology, The Medical School, Birmingham B15 2TJ, U.K.

(Received 10 October 1975)

1. When the ionophore A23187 and Ca^{2+} were added to normal human erythrocytes, the incorporation of ^{32}P into phosphatidate was enhanced within 1 min, but there was only slight labelling of other phospholipids. 2. Labelling of phosphatidate in these cells did not continue to increase after about 20 min at 37°C ; by this time, radioactivity in phosphatidate was about ten times higher in ionophore A23187-treated cells than in controls. A net synthesis of phosphatidate was measured in response to the increase in intracellular Ca^{2+} concentration; the content of this phospholipid in the cell was increased by $\sim 50\%$. 3. In the presence of 2.5 mM-Ca^{2+} a maximum effect was seen with about $0.5\text{ }\mu\text{g}$ of ionophore/ml. 4. The concentration of Ca^{2+} giving half-maximal labelling of phosphatidate in the presence of $10\text{ }\mu\text{g}$ of ionophore A23187/ml was about $10\text{ }\mu\text{M}$. 5. A rapid decrease of ATP content in the cell occurred in ionophore-treated cells. 6. Labelling of phosphatidate appeared to be secondary to the production of 1,2-diacylglycerol in the cells; accumulation of 1,2-diacylglycerol was only seen after about 15 min. After 60 min, the 1,2-diacylglycerol content of the cells was five to seven times that of untreated control cells. 7. The change in the shape of erythrocytes treated with Ca^{2+} and ionophore appeared to be related to accumulation of 1,2-diacylglycerol. 8. The source of 1,2-diacylglycerol has not been definitely identified, but its fatty acid composition was similar to that of phosphatidylcholine. However, it had an unusually high content of hexadecenoic acid, a fatty acid not common in the major erythrocyte phospholipids. 9. Accumulation of 1,2-diacylglycerol also occurred in energy-starved cells, even in the absence of calcium; in this case it appeared to be produced by phosphatidate breakdown.

Erythrocytes or erythrocyte 'ghosts' with an increased internal Ca^{2+} concentration change in shape from biconcave discs to spiculed spheres (echinocytes) (Weed & Chailly, 1972; White, 1974; Palek *et al.*, 1974), and it was suggested that this change might be controlled by a Ca^{2+} -sensitive contractile protein (spectrin?) present on the internal surface of the membrane (Palek *et al.*, 1974). We found that an increased intracellular Ca^{2+} concentration leads to enhanced production of 1,2-diacylglycerol, and suggested that this Ca^{2+} -dependent change in membrane-lipid composition, rather than any direct effect on protein conformation, might be responsible for the change in shape (Allan & Michell, 1975*a,b*).

The present paper describes the changes in diacylglycerol and phosphatidate concentrations which occur in human erythrocytes when their intracellular Ca^{2+} concentration is increased after treatment with the calcium ionophore A23187. The results suggest that 1,2-diacylglycerol, even in relatively small quantities, may influence membrane morphology and fusibility in erythrocytes. In addition, they emphasize the role of diacylglycerol kinase as a regulator of

diacylglycerol concentration in membranes and thus as an activity involved in the maintenance of normal membrane morphology. These observations also raise the possibility that in physiological situations where both a rise in intracellular Ca^{2+} concentration and changes in the morphology, fluidity and permeability of the plasma membrane occur, the morphological changes may be brought about by similar Ca^{2+} -dependent effects on membrane lipid composition.

Materials and Methods

The bivalent cationophore A23187 (Reed & Lardy, 1972*a,b*) was obtained as a gift from Eli Lilly and Co., Indianapolis, IN, U.S.A. It was dissolved at 1 mg/ml in ethanol and stored at -20°C . [^{32}P]Phosphate (PBS-1, carrier-free) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. 1,2-Diacylglycerol was prepared from egg phosphatidylcholine by treatment with *Clostridium perfringens* phospholipase C (Mangiapane *et al.*, 1973); the product was 1,2-diacylglycerol with a trace of con-

taminating 1,3-diacylglycerol. ATP (disodium salt) was from Boehringer Corp. (London) Ltd., Lewes, East Sussex BN7 1LG, U.K. Human blood (group O, Rh-positive) was obtained from the Birmingham Regional Blood Transfusion Centre. Erythrocytes were centrifuged down and washed three times with 0.9% (w/v) saline at 4°C. The buffy coat was aspirated after each centrifugation, and in the final suspension leucocytes accounted for less than one cell in 10^4 . An erythrocyte membrane preparation was used in some experiments; this was made by lysis of packed erythrocytes in 80 vol. of 1 mM-EGTA,* 10 mM-Hepes/NaOH buffer, pH 7.4. The membranes were recovered by centrifugation at 15000g for 15 min (10 × 100 ml angle rotor in a Beckman model L centrifuge) and were resuspended in lysing buffer to the same volume as the original cell suspension.

Erythrocytes were usually incubated at a concentration of 2×10^9 cells/ml in a modified Krebs-Ringer solution (Allan & Michell, 1975c) (hereinafter referred to as 'Hepes/Ringer') buffered with 18 mM-Hepes/NaOH, pH 7.4, and with the addition of 11 mM-glucose. Before treatment with ionophore, the intracellular phosphate pools of the cells were labelled by incubation for 1 h with $10 \mu\text{Ci}$ of $[^{32}\text{P}]\text{P}_i$ /ml. Equilibration of the label with the γ -phosphate of ATP was largely complete after 1 h (see Fig. 3). Ionophore A23187 was dried from ethanol solution in test tubes and portions (1 ml) of the ^{32}P -labelled erythrocyte suspension were then added. Incubation was terminated at various times, and lipids were extracted by addition of 2.5 ml of methanol followed by 1.25 ml of chloroform. After 30 min, a further 1.25 ml of chloroform and 1.25 ml of 2M-KCl were added and mixed vigorously. The phases were separated by centrifugation and the lower phase was removed and evaporated to dryness *in vacuo*. For isolation of phosphatidate, the lipid samples were dissolved in $100 \mu\text{l}$ of chloroform and run on formaldehyde-treated papers (Lapetina & Michell, 1972) in the upper phase from a mixture of butanol/formic acid/water (4:1:5, by vol.). This solvent gave a separation of phosphatidyl-inositol from the major phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin) similar to that obtained with the acetic acid solvent originally used (Lapetina & Michell, 1972), but in addition separated phosphatidate from all other phospholipids. Phosphatidate ran near the solvent front together with neutral lipids (Allan *et al.*, 1975). In most experiments, the area of the Nile Blue-stained chromatogram corresponding to phosphatidate was digested with HClO_4 , analysed for phosphorus (Bartlett, 1959) and its radioactivity determined by Čerenkov counting after dilution to

10 ml in water. In other experiments the spot near the solvent front was eluted with methanol, evaporated to dryness and then run on a silica-gel G t.l.c. plate in a solvent system in which neutral lipids migrate but phosphatidate remains at the origin (Freeman & West, 1966). 1,2-Diacylglycerol moved with an R_F of about 0.8 and was well separated from all other lipids. Samples of phosphatidate and 1,2-diacylglycerol isolated by this method were used for determination of fatty acid composition (see below).

In some experiments, erythrocyte phosphatidate was labelled to isotopic equilibrium with P_i and ATP by incubation of cells for 20 h at 37°C with $[^{32}\text{P}]\text{P}_i$ ($10 \mu\text{Ci}/\text{ml}$) in the presence of antibiotics (penicillin, 200 units/ml; streptomycin, $100 \mu\text{g}/\text{l}$). The cells were washed with 20 vol. of non-radioactive medium and added to tubes containing ionophore A23187. After incubation for various periods of time, the lipids were extracted and analysed as described above. Radioactivity in P_i was also measured.

The effects of metabolic poisons on unlabelled erythrocytes incubated in Hepes-Ringer were investigated either in the presence of 2 mM-EGTA (no calcium) or 2 mM- CaCl_2 . Diacylglycerol and phosphatidate contents were measured as described below.

Phospholipids were determined as phosphate (Bartlett, 1959) after digestion with HClO_4 . 1,2-Diacylglycerol was determined either by a photographic procedure or by g.l.c. The first method involved photography of an iodine-stained t.l.c. plate used for separation of the neutral lipids. The absorbances of the 1,2-diacylglycerol spots were measured on the photographic negative by using a Joyce-Loebl microdensitometer and the value was compared with the absorbances of known amounts of authentic diacylglycerol (derived from egg phosphatidylcholine) run as standards on the same plate. Although this method is probably only semi-quantitative, it gave results in good agreement with those obtained by g.l.c. ($\pm 10\%$). The g.l.c. was performed on a glass column ($45 \text{ cm} \times 0.65 \text{ cm}$) containing 3% SE-30 (methyl polysiloxysilicone gum) on 100–120-mesh universal support (Jones Chromatography Ltd., Llanbradach, Glamorgan, Wales, U.K.). It was found unnecessary to silylate the diacylglycerol to obtain quantitative column recoveries. The quantity of diacylglycerol was measured relative to the cholesterol of the erythrocyte lipid extract as an internal standard. The fatty acid compositions of erythrocyte 1,2-diacylglycerol and phospholipids were determined by g.l.c. after methylation of the separated lipids. Each class of lipid was methylated by a modified procedure of Hartman & Lago (1973) and the methyl esters were analysed on a column ($165 \text{ cm} \times 0.65 \text{ cm}$) of 5% (w/w) polyethylene glycol succinate.

ATP present in aqueous supernatants after partition of lipid extracts (without addition of KCl) was isolated on paper chromatograms (Bock *et al.*,

* Abbreviations: EGTA, ethanedioxybis(ethylamine)-tetra-acetic acid; Hepes, 2-(N-2-hydroxymethylpiperazin-N'-yl)ethanesulphonic acid; ATPase, adenosine triphosphatase.

1956) and assayed spectrophotometrically by its E_{260} ($E_{260}^{1\%1\text{cm}} = 15000$ at pH 7.4).

Diacylglycerol kinase activity of erythrocyte membranes was measured by using a modification of the method of Hokin & Hokin (1963). 1,2-Diacylglycerol (derived from egg phosphatidylcholine) was sonicated in water by using an M.S.E. ultrasonic generator (5 min at 0°C) and various amounts were added to portions (1 ml) of the membrane preparation (equivalent to 2×10^9 cells). 1 mM-MgATP²⁻ was added, and the tubes were incubated at 37°C for 1 h in the presence of either 1 mM-EGTA or 1 mM-CaCl₂ in 10 mM-Hepes/NaOH buffer, pH 7.4. The incubations were terminated by addition of 3.75 ml of methanol/chloroform (2:1, v/v), and phosphatide in the samples was separated and determined as described above.

Results

³²P incorporation into erythrocyte phosphatide was markedly increased as early as 1 min after addition of ³²P-labelled cells to ionophore A23187 in the presence of 2.5 mM-Ca²⁺, and labelling continued for 15–20 min before reaching a plateau (Fig. 1). After 20 min, labelling of phosphatide in ionophore A23187-treated cells was about ten times greater than in controls; this change in radioactivity was accompanied by an increase of about 50% in the chemically determined cell phosphatide content (Table 1). When cells were incubated with ³²P_i for 20 h labelling of phosphatide reached a plateau, with the specific radioactivity of phosphatide becoming approximately equal to that of the P_i of the cells (~1.3 μCi/μmol; see the legend to Fig. 2). After treatment of these cells with ionophore A23187 for 20 min, an increase of about 70% was observed in phosphatide radioactivity, consistent with the observed rise in phosphatide content (Fig. 2 and Table 1). Of the other phospholipids, only polyphosphoinositides were labelled with ³²P; their radioactivity was about

10% of that in phosphatide in untreated cells and was slightly decreased in the presence of ionophore and Ca²⁺.

In the presence of ionophore A23187 there was a dramatic fall in cellular ATP concentration to about 10% of normal within 30–60 min (Fig. 3). In addition, a pronounced increase in the cellular content of 1,2-diacylglycerol was observed after 15–20 min, leading after 1 h to a five- to seven-fold rise in ionophore A23187-treated cells as compared with controls

Fig. 1. Time-course of 1,2-diacylglycerol production and labelling of phosphatide in erythrocytes treated with ionophore A23187 and Ca²⁺

Untreated controls: ○, labelling of phosphatide; △, diacylglycerol content; cells treated with ionophore A23187: ●, labelling of phosphatide; ▲, diacylglycerol content. The Figure shows the results of a single typical experiment (one of four giving similar results). Diacylglycerol was determined by the photographic method (see the Materials and Methods section).

Table 1. Content of 1,2-diacylglycerol and phosphatide in human erythrocytes treated with ionophore A23187 or metabolic poisons

Values (±s.d.) represent the average of duplicate (diacylglycerol) or triplicate (phosphatide) determinations from a single typical experiment (one of three giving similar results). Diacylglycerol was determined by g.l.c., and phosphatide as described in the Materials and Methods section. The basic incubation system was Hepes–Ringer (see the Materials and Methods section), without addition of Ca²⁺. The metabolic poisons were a mixture of 2 mM-arsenate, 2 mM-iodoacetate and 2 mM-deoxyglucose.

	Incubation period (h)	Additions to the basic incubation system				Content (nmol/10 ¹⁰ cells)	
		Glucose	Ca ²⁺	EGTA	Metabolic poisons	Diacylglycerol	Phosphatide
Control	1	+	+	—	—	4 ± 1	45 ± 2
Ionophore A23187 (1 μg/ml)	1	+	+	—	—	21 ± 2	68 ± 4
Control	20	+	—	+	—	3 ± 1	47 ± 4
Energy-starved	20	—	—	+	+	11 ± 1	34 ± 2

Fig. 2. Time-course of labelling of phosphatidate in cells labelled to isotopic equilibrium with $[^{32}\text{P}]\text{P}_i$, resuspended in non-radioactive medium and then exposed to ionophore A23187

○, Untreated cells; ●, cells treated with ionophore A23187 ($1\mu\text{g/ml}$). Phosphatidate specific radioactivity was unchanged over the final 2 h of the 20 h preincubation; it was $1.2\mu\text{Ci}/\mu\text{mol}$ and the corresponding specific radioactivity of P_i was measured as $1.3\mu\text{Ci}/\mu\text{mol}$. The decrease in specific radioactivity of P_i compared with that originally present in the medium presumably reflected equilibration with the erythrocyte pool of P_i and organic phosphates; this accounted for about $6\mu\text{mol}$ of phosphorus/ 2×10^9 cells in addition to the $1\mu\text{mol}$ of phosphate/ml present in the incubation medium. Similar results were obtained in a further experiment.

(Fig. 1, Table 1). The rise in diacylglycerol content was accompanied by a change in cell shape from the original biconcave disc to a spiculed spherical (echinocyte) form (Allan & Michell, 1975b). Diacylglycerol accumulation and the shape-change were more rapid in cells incubated without glucose.

Although increased Ca^{2+} concentration within erythrocytes led to enhanced labelling of phosphatidate, this did not appear to be due to stimulation of diacylglycerol kinase activity by Ca^{2+} . The activity of this enzyme in erythrocyte membranes was the same in the presence of EGTA or Ca^{2+} (Fig. 4).

The most likely explanation of these results appeared to be that an increase in intracellular Ca^{2+} concentration caused the activation of an enzyme that liberated diacylglycerol, and that this was then converted into phosphatidate so long as ATP was available. Diacylglycerol production and its phosphorylation are difficult to dissociate in the intact erythrocyte (see the Discussion section) and during the first

Fig. 3. ATP concentrations in erythrocytes incubated with ionophore A23187

Radioactivity in ATP: ○, untreated controls; ●, incubated with ionophore ($1\mu\text{g/ml}$); ATP content of cell (nmol/ 2×10^9 cells): △, untreated controls; ▲, incubated with ionophore ($1\mu\text{g/ml}$). The results in this Figure are from the same experiment as Fig. 1. After 60–90 min, labelling of ATP (largely in the γ -phosphate, since ADP was only weakly labelled) had reached equilibrium. This labelling corresponded to a sp. radioactivity of about $4.5\mu\text{Ci}/\text{ml}$ as compared with a value of about $5\mu\text{Ci}/\mu\text{mol}$ for P_i . Very similar results were obtained in two other experiments using other batches of cells.

15 min of incubation with maximally effective concentrations of Ca^{2+} and ionophore A23187 the liberated diacylglycerol is converted almost quantitatively into phosphatidate (Fig. 1). We therefore chose labelling of phosphatidate in a 15 min incubation as an indirect but moderately accurate measure with which to undertake an initial study of the reaction giving rise to the diacylglycerol.

In the presence of 2.5mM-Ca^{2+} , the optimum concentration of ionophore A23187 for stimulating labelling of phosphatidate was about $0.5\text{--}1\mu\text{g/ml}$, with no further change up to $10\mu\text{g/ml}$ (Fig. 5). Phosphatidate labelling depended on the presence of Ca^{2+} in the medium, being decreased to control values in the presence of EGTA. By using various proportions of Ca^{2+} and EGTA to define the free concentration of ionized calcium in the external medium, it was found that in the presence of a high concentration of ionophore A23187 ($10\mu\text{g/ml}$) labelling of phosphatidate was half-maximal at a Ca^{2+} concentration of about $10\mu\text{M}$ (Fig. 6). *o*-Phenanthroline, a chelator of transition metals, did not decrease the ionophore-stimulated labelling of phosphatidate.

Fig. 4. Lack of effect of Ca^{2+} on 1,2-diacylglycerol kinase activity in human erythrocyte membranes

1,2-Diacylglycerol kinase activity was measured as described in the Materials and Methods section. $\circ$, Incubations in the presence of 2mM-EGTA; $\bullet$, incubations in the presence of 2mM- Ca^{2+} . One other similar experiment gave identical results.

Fig. 5. Effect of ionophore concentration on phosphatidate labelling in human erythrocytes

Portions of cells (2×10^9 /ml) labelled by 1 h preincubation with $10 \mu\text{Ci}$ of ^{32}P /ml in Hepes-Ringer containing 2.5mM- Ca^{2+} were treated for 15 min with various amounts of ionophore A23187. The radioactivity of phosphatidate was measured as described in the text. These results were closely similar to those obtained with two other batches of erythrocytes under identical conditions.

Fig. 6. Dependence of phosphatidate labelling in ionophore A23187-treated erythrocytes on the concentration of Ca^{2+} in the medium

Portions of cells (2×10^9 /ml) labelled by preincubation with $10 \mu\text{Ci}$ of ^{32}P /ml in Ca^{2+} -free Hepes-Ringer were incubated for 15 min with $10 \mu\text{g}$ of ionophore A23187/ml in the presence of various concentrations of Ca^{2+} . Phosphatidate was isolated and its radioactivity determined as described in the text. $\bullet$, Values obtained at various free Ca^{2+} concentrations obtained by using 2mM- Ca^{2+} /EGTA buffers (Raaflaub, 1960). $\circ$, Values obtained at various concentrations of total Ca^{2+} added to each incubation in the absence of EGTA. $\blacktriangle$, Radioactivity in phosphatidate immediately before incubation with ionophore A23187. This experiment was repeated on three other occasions with different batches of erythrocytes; the results were almost identical.

The fatty acid compositions of phosphatidate and 1,2-diacylglycerol isolated from erythrocytes are shown in Table 2 and are compared with the fatty acid composition of total cell phospholipids. The compositions of the two minor lipids were very similar both in control and in ionophore-treated cells and were quite similar to that of erythrocyte phosphatidylcholine (White, 1973). However, both phosphatidate and 1,2-diacylglycerol contained moderate amounts of hexadecenoic acid ($\text{C}_{16:1}$), which is only a very minor constituent in the other erythrocyte glycerolipids (Table 2; White, 1973).

Cells treated with metabolic inhibitors (arsenate, iodoacetamide and deoxyglucose) over periods of

Table 2. *Fatty acid compositions of phospholipids and diacylglycerol from human erythrocytes with and without treatment with Ca²⁺ and ionophore A23187*

Values are quoted as a percentage ($\pm$ s.d.) of total fatty acid recovered. The numbers in parentheses indicate the numbers of separate experiments in each case. (C), Untreated controls; (A), A23187-treated cells. Lipids were isolated and their fatty acid compositions analysed as described in the Materials and Methods section. Tr., trace.

		Fatty acids						
		C _{16:0}	C _{16:1}	C _{18:0}	C _{18:1}	C _{18:2}	C _{20:3}	C _{20:4}
Total phospholipids (1)	(C)	23.5	1.4	19.7	19.2	13.1	2.5	20.6
	(A)	22.7	2.1	17.8	18.2	12.0	2.3	24.9
Phosphatidate (2)	(C)	34.8 $\pm$ 1.0	7.7 $\pm$ 2.1	17.1 $\pm$ 0.7	25.0 $\pm$ 1.4	6.0 $\pm$ 1.4	Tr.	7.4 $\pm$ 1.7
	(A)	32.1 $\pm$ 1.1	7.5 $\pm$ 1.8	16.0 $\pm$ 1.5	23.8 $\pm$ 1.3	9.0 $\pm$ 1.0	Tr.	8.5 $\pm$ 1.8
Diacylglycerol (4)	(C)	39.9 $\pm$ 0.8	9.4 $\pm$ 0.7	18.2 $\pm$ 0.7	22.5 $\pm$ 1.2	5.3 $\pm$ 1.3	Tr.	5.4 $\pm$ 0.6
	(A)	38.5 $\pm$ 1.2	7.6 $\pm$ 1.2	19.4 $\pm$ 1.3	23.1 $\pm$ 1.4	7.0 $\pm$ 1.7	Tr.	5.4 $\pm$ 0.4

20–24 h at 37°C also accumulated diacylglycerol and showed a shape-change (Allan & Michell, 1975b). This occurred even in the absence of calcium and in the presence of EGTA (Table 1) and coincided with an approximately equivalent decrease in the phosphatidate content of the cells. Complete lysis of cells was observed after several hours when cells were incubated with the above inhibitors and 2 mM-Ca²⁺; results for these cells are therefore not included in Table 1.

Discussion

The concentration of ionized calcium in physiologically healthy erythrocytes is very low ($\leq 1 \mu\text{M}$) and the cells have efficient mechanisms for maintaining this ion at a low concentration (Schatzmann, 1975). When the intracellular concentration of Ca²⁺ is artificially increased by using the ionophore A23187, a complex and interrelated series of changes occur; these include a change in shape (Allan & Michell, 1975b), an efflux of K⁺ (Reed & Lardy, 1972b), a rapid decrease in cell [ATP] and modifications in the metabolism and composition of membrane lipids, particularly of phosphatidate and 1,2-diacylglycerol. In the present paper we describe the latter three changes and attempt to define the relationship between them.

The earliest event which we observed when ³²P-labelled erythrocytes were treated with ionophore A23187 and Ca²⁺ was an increase in the specific radioactivity of phosphatidate (Figs. 1 and 2). This occurred within minutes of addition of cells to the ionophore, and appeared to reflect a net synthesis of phosphatidate, whose concentration was increased by about 50% within about 20 min. The newly synthesized phosphatidate had a specific radioactivity of 3–4 $\mu\text{Ci}/\mu\text{mol}$, which is similar to that of the γ -phosphate of ATP (see the legend to Fig. 3). This

suggested that the increase in radioactivity in phosphatidate could be largely accounted for in terms of increased synthesis of this phospholipid, although the possibility of some turnover was not excluded.

Phosphatidate synthesis could account for only a small proportion of the decrease in ATP concentration which follows ionophore treatment (Fig. 3). Only about 20 nmol of phosphatidate was synthesized per 1×10^{10} cells, whereas the decrease in ATP concentration was at least 250 nmol in 1 h (Fig. 3); this value does not take into account any synthesis of ATP by the cells during this period, which may have been of the order of 400 nmol ATP/h per 10^{10} cells (Duhm, 1975). The most likely cause of the decrease in ATP concentration is that the elevated intracellular Ca²⁺ concentration activated the Ca²⁺-sensitive ATPase associated with calcium-pump activity in the membrane of the erythrocyte (Schatzmann, 1975). This enzyme has the capacity to hydrolyse 600–1000 nmol of ATP/h per mg of protein (Bramley & Coleman, 1972) and, since 1 mg of membrane protein is approximately equivalent to 10^9 cells, this activity is more than sufficient to account for the Ca²⁺-induced decrease in cell ATP concentration.

The decrease in ATP concentration probably explains why phosphatidate synthesis ceased 15–20 min after exposure of cells to ionophore A23187. Diacylglycerol kinase activity is the only significant route for synthesis of phosphatidate in the human erythrocyte membrane (Hokin & Hokin, 1963) and if this enzyme has a relatively low affinity for ATP, then it will not effectively phosphorylate diacylglycerol when the ATP concentration is decreased. There was certainly no deficiency of diacylglycerol to serve as its substrate, since the content of this lipid in the cell increased in the period 15–60 min after addition of cells to ionophore A23187 (Fig. 1).

The simplest way of explaining our observations is that phosphatidate synthesis is only a secondary

consequence of the production of diacylglycerol by a Ca^{2+} -stimulated process, even though it is the earliest event that we have observed. The evidence in support of this suggestion includes the following: (a) diacylglycerol accumulated when the cell no longer had the ability to phosphorylate it; (b) a net increase in phosphatidate content of the cells was observed (Table 1; Fig. 2), and therefore the diacylglycerol that was phosphorylated was derived from some glycerolipid other than phosphatidate; (c) phosphatidate had a very similar fatty acid composition to diacylglycerol (Table 2); and (d) diacylglycerol kinase activity was independent of Ca^{2+} (Fig. 4) and therefore seemed unlikely to be the step controlling the Ca^{2+} -dependent labelling of phosphatidate. The conclusion that diacylglycerol production was the primary event is further strengthened by the observation (D. Allen & R. H. Michell, unpublished work) that diacylglycerol was produced when Ca^{2+} was added to erythrocyte membranes isolated in the presence of EDTA. This also provides evidence that the enzyme activity giving rise to diacylglycerol is membrane-bound.

It is interesting that no significant accumulation of diacylglycerol was seen during the early stages of treatment of erythrocytes with ionophore A23187; during this time it was entirely converted into phosphatidate. When erythrocytes are treated with phospholipase C, a similar phenomenon is seen in that phosphatidate rather than diacylglycerol accumulates (Allan *et al.*, 1975). The high activity of erythrocyte diacylglycerol kinase may be connected with the fact that diacylglycerol has marked morphological effects on erythrocyte and other membranes. For example, this lipid was shown to be associated with membrane-fusion events in a variety of situations (Ahkong *et al.*, 1973; Allan *et al.*, 1975; Bansbach *et al.*, 1974). Membrane fusion also occurs in ionophore A23187-treated erythrocytes, in that during the accumulation of diacylglycerol, which occurs 20–60 min after addition of ionophore A23187, the erythrocytes undergo a change in shape which leads to the echinocyte form and also to the formation and release of microvesicles (Allan & Michell, 1975a,b). Under conditions of energy depletion there is a more rapid accumulation of diacylglycerol and a faster change to the echinocyte form. Diacylglycerol kinase activity can therefore be seen as an influence that tends to prevent accumulation of diacylglycerol and thus prevents morphological changes caused by it. Ca^{2+} -dependent production of 1,2-diacylglycerol, followed by diacylglycerol kinase activity, may explain the results of Redman (1972), who showed that labelling of phosphatidate in erythrocyte 'ghosts' incubated with [γ - ^{32}P]ATP was increased in the presence of Ca^{2+} .

The source of the diacylglycerol produced when erythrocytes are treated with Ca^{2+} and ionophore

A23187 is still not known. The fatty acid compositions of diacylglycerol and of phosphatidate were similar both in untreated cells and in those exposed to ionophore A23187 (Table 2), and in general they resembled that of phosphatidylcholine rather than those of phosphatidylethanolamine or phosphatidylserine. They did, though, differ from phosphatidylcholine in their unusually high content of hexadecenoic acid. This suggests that diacylglycerol and phosphatidate might be derived from a limited portion of the erythrocyte phosphatidylcholine, presumably through the action of a Ca^{2+} -dependent phospholipase C. However, the production of diacylglycerol and phosphatidate which we have observed would be accounted for by breakdown of only about 1% of the total cell glycerolipids or 3% of the phosphatidylcholine. Unfortunately it has not yet proved practicable to make reliable measurements of such changes.

In the present studies, increased labelling of phosphatidate with ^{32}P was very valuable as an indirect but sensitive detector of 1,2-diacylglycerol production in response to elevated intracellular Ca^{2+} concentrations. It should, however, be remembered that this reaction provides no information on the source or mechanism of production of the diacylglycerol. In the present study it seems possible that the diacylglycerol was derived from Ca^{2+} -stimulated breakdown of phosphatidylcholine, but in previous investigations, labelling of phosphatidate (and sometimes of phosphatidylinositol) has been used as an indication of the production of diacylglycerol from either phosphatidylinositol (Durell *et al.*, 1969; Michell, 1975) or phosphatidate (Schacht & Agranoff, 1974) in response to extracellular stimuli, or from phosphatidylcholine in phospholipase C-treated cells (Allan *et al.*, 1975). In addition, phosphatidate (and other anionic lipids derived therefrom) may become labelled with ^{32}P by biosynthesis *de novo*, as in studies of the re-direction of glycerolipid biosynthesis by amphiphilic amines (Eichberg & Hauser, 1974; Allan & Michell, 1975c; Brindley *et al.*, 1975); diacylglycerol plays no part in this pathway. Steps should therefore always be taken to ensure that, when interpreting such labelling studies, one can distinguish between the numerous routes by which [^{32}P]phosphatidate may arise in ^{32}P -labelled cells. In the case of human erythrocytes there are few opportunities for such ambiguities to occur, since they contain very little phosphatidylinositol (White, 1973) and also have no capacity for synthesis of lipids *de novo* (Hokin & Hokin, 1963). This was confirmed in our experiments in which the erythrocytes showed no detectable incorporation of ^{32}P into lipids other than phosphatidate and polyphosphoinositides.

It is well known that when erythrocytes are starved of energy, they tend eventually to become spherical and probably adopt the echinocyte form (Brecher & Bessis, 1972); this was confirmed in the present

experiments. The shape-change occurred more readily in the presence of Ca^{2+} in the starved cells, as would be expected if the cells were less able to pump out this ion, but somewhat similar changes were still seen when EGTA was substituted for Ca^{2+} (Allan & Michell, 1975b). However, an accumulation of diacylglycerol was observed in cells incubated for 20 h with EGTA; it was, though, less marked than that seen after 1 h incubation with ionophore A23187 and Ca^{2+} (Table 1). A decrease in the cell phosphatidate content was also seen, and this was approximately equivalent to the increase in diacylglycerol content. Thus it appears possible that, under conditions of energy starvation, diacylglycerol accumulates as a result of phosphatidate phosphohydrolase activity that cannot be balanced by diacylglycerol kinase activity. This activity is probably not significant during the much shorter incubations used for ionophore A23187 treatment (see Fig. 2).

We are grateful to the Medical Research Council for financial support and to Eli Lilly and Co. for gifts of ionophore A23187.

References

- Ahkong, Q. F., Fisher, D., Tampion, W. & Lucy, J. A. (1973) *Biochem. J.* **136**, 147–155
- Allan, D. & Michell, R. H. (1975a) *Biochem. Soc. Trans.* **3**, 751–752
- Allan, D. & Michell, R. H. (1975b) *Nature (London)* **258**, 348–349
- Allan, D. & Michell, R. H. (1975c) *Biochem. J.* **148**, 471–478
- Allan, D., Low, M. G., Finean, J. B. & Michell, R. H. (1975) *Biochim. Biophys. Acta* **413**, 309–316
- Banschbach, M. W., Geison, R. L. & Hokin-Neaverson, M. R. (1974) *Biochem. Biophys. Res. Commun.* **58**, 714–718
- Bartlett, G. (1959) *J. Biol. Chem.* **234**, 466–468
- Bock, R. M., Ling, N. S., Morell, S. A. & Lipton, S. H. (1956) *Arch. Biochem. Biophys.* **62**, 253–264
- Bramley, T. A. & Coleman, R. (1972) *Biochim. Biophys. Acta* **290**, 219–228
- Brecher, G. & Bessis, M. (1972) *Blood* **40**, 333–344
- Brindley, D. N., Allan, D. & Michell, R. H. (1975) *J. Pharm. Pharmacol.* **27**, 462–464
- Duhm, J. (1975) *Biochim. Biophys. Acta* **385**, 68–80
- Durell, J., Garland, J. T. & Friedel, R. O. (1969) *Science* **165**, 862–866
- Eichberg, J. & Hauser, G. (1974) *Biochem. Biophys. Res. Commun.* **60**, 1460–1467
- Freeman, C. P. & West, D. (1966) *J. Lipid Res.* **7**, 324–327
- Hartman, L. & Lago, R. C. A. (1973) *Lab. Pract.* **22**, 475–476
- Hokin, L. E. & Hokin, M. R. (1963) *Biochim. Biophys. Acta* **67**, 470–484
- Lapetina, E. G. & Michell, R. H. (1972) *Biochem. J.* **126**, 1141–1147
- Mangiapane, E. H., Lloyd-Davies, K. A. & Brindley, D. N. (1973) *Biochem. J.* **134**, 103–112
- Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
- Palek, J., Stewart, G. & Lionetti, F. J. (1974) *Blood* **44**, 584–597
- Raaflaub, J. (1960) *Methods Biochem. Anal.* **3**, 301–325
- Redman, C. M. (1972) *Biochim. Biophys. Acta* **282**, 122–134
- Reed, P. W. & Lardy, H. A. (1972a) *J. Biol. Chem.* **247**, 6970–6973
- Reed, P. W. & Lardy, H. A. (1972b) in *The Role of Membranes in Metabolic Regulation* (Mehlman, M. A. & Hanson, R. W., eds.), pp. 111–131, Academic Press, New York
- Schacht, J. & Agranoff, B. W. (1974) *J. Biol. Chem.* **249**, 1551–1557
- Schatzmann, H. J. (1975) in *Current Topics in Membranes and Transport* (Bronner, F. & Kleinzeller, A., eds.), pp. 125–168, Academic Press, New York
- Weed, R. I. & Chailly, B. (1972) *Nouv. Rev. Fr. Hemat.* **12**, 715–746
- White, D. A. (1973) in *Form and Function of Phospholipids* (Ansell, G. B., Dawson, R. M. C. & Hawthorne, J. N., eds.), pp. 441–482, Elsevier, Amsterdam
- White, J. G. (1974) *Am. J. Pathol.* **77**, 507–517