Calcium Ion-Dependent Diacylglycerol Accumulation in Erythrocytes is Associated with Microvesiculation but not with Efflux of Potassium Ions

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Erythrocytes from several different species were exposed to Ca^{2+} and the bivalent-cation ionophore A23187. The lipid composition, morphology and K^+ permeability of the treated cells were investigated. Erythrocytes from human, rat, guinea pig and rabbit (a) showed an increased concentration of 1,2-diacyl-*sn*-glycerol and enhanced labelling of phosphatidate with ^{32}P , (b) underwent echinocytosis and outward vesiculation, and (c) rapidly released much of their intracellular K^+ . Pig cells showed only the K^+ loss, and ox and sheep (high- K^+) cells showed none of these Ca^{2+} -evoked effects. All of the cells underwent stomatocytosis and inward vesiculation when treated externally with *Clostridium perfringens* phospholipase C. These results support the idea that there is a correlation between the asymmetric insertion of diacylglycerol (or ceramide) into the membrane and the shape-changes leading to microvesiculation, but they indicate that Ca^{2+} -triggered K^+ efflux and diacylglycerol production are unrelated events. Erythrocytes of chicken and turkey showed no Ca^{2+} -stimulated K^+ efflux. They showed slight ionophore A23187-stimulated vesiculation, but this appeared to be associated with the appearance in the membrane of ceramide rather than of diacylglycerol. Phospholipase C treatment caused very similar changes in morphology and phosphatidate labelling to those seen in mammalian erythrocytes.

An increase in cytosolic Ca^{2+} concentration in human erythrocytes leads to accumulation of 1,2-diacyl-*sn*-glycerol and phosphatidate, and the accumulation of diacylglycerol is associated with echinocytosis and outward vesiculation of the plasma membrane (Allan & Michell, 1975, 1976; Allan *et al.*, 1976a,b). There is also a pronounced efflux of K^+ from the cells when the intracellular Ca^{2+} concentration is raised (Reed, 1976; Lew & Ferreira, 1976; Romero, 1976), and we wondered, as did Lew & Ferreira (1976), whether accumulation of diacylglycerol or phosphatidate might influence the permeability of the cells to K^+ . This possibility seemed attractive, since it has been suggested that in a variety of physiologically stimulated cells the production of diacylglycerol and/or phosphatidate from phosphatidylinositol might be involved in the control of cell-surface Ca^{2+} channels (Michell, 1975; Michell *et al.*, 1976). In addition, Tyson *et al.* (1976) have observed that phosphatidate has cationophoric properties in model systems. To clarify these relationships we have examined erythrocytes from various species of animals after exposure to a Ca^{2+} ionophore: we have compared cell morphology, K^+ efflux and changes in lipid composition. Our findings indicate that microvesiculation only occurs in response to a rise in intracellular Ca^{2+} when diacyl-

glycerol (and perhaps ceramide) are produced in the membrane. In contrast, Ca^{2+} -dependent K^+ efflux is not always associated either with microvesiculation or with changes in lipid composition.

Methods

Ca^{2+} -ionophore A23187 was a gift from Eli Lilly, Indianapolis, IN, U.S.A. A freeze-dried culture filtrate from *Clostridium perfringens*, supplied by Burroughs Wellcome, Beckenham, Kent, U.K., provided the source of phospholipase C (EC 3.1.4.3) activity.

Sheep, pig, ox, chicken and turkey blood samples were obtained from slaughterhouses; blood samples from laboratory rats, rabbits and guinea pigs were collected by heart puncture. All samples were rapidly mixed with 5 mM-EDTA (final concn.) to prevent clotting. Outdated human blood (in acid citrate/dextrose anticoagulant) was obtained from The Regional Blood Transfusion Service. Erythrocytes were sedimented at 100g for 5 min and washed three times with 0.9% NaCl containing 20 mM-Hepes†/NaOH buffer, pH 7.4 (Allan *et al.*, 1976b).

Diacylglycerol production and microvesiculation

Packed cells were resuspended in an equal volume of the same medium, but with the addition of 1 mM-

†Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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CaCl₂, and 5 ml samples were incubated at 37°C for 2 h with or without 2 µg of ionophore A23187/ml. Samples (1 ml) of control and ionophore-treated cell suspensions were extracted with methanol/chloroform (2:1, v/v) (Allan *et al.*, 1976b) and neutral lipids analysed as described by Freeman & West (1966). 1,2-Diacyl-*sn*-glycerol was determined by densitometry on lipid spots separated by t.l.c. (Allan *et al.*, 1976b). The remaining cells were sedimented at 500g for 5 min and the supernatant was centrifuged at 15000g for 20 min at 2°C. Any red pellet of microvesicular material that was observed at this stage was resuspended in 1 ml of the same medium and samples were taken for lipid analysis and for electron microscopy (Allan *et al.*, 1976a).

Phosphatidate labelling

For studies of ³²P incorporation into phosphatidate, cells were resuspended in 4 vol. of Hepes/Ringer medium (Allan *et al.*, 1976b) containing 1 mM-glucose and 10 µCi of ³²P_i/ml and incubated for 1 h at 37°C. Ionophore A23187 (2 µg/ml) or *Clostridium perfringens* phospholipase C (5 µg/ml) were added to 1 ml samples, and incubation was continued for a further 15 min before extraction and analysis of the radioactive lipids (Allan *et al.*, 1975, 1976b).

K⁺ efflux

Ca²⁺-stimulated K⁺ efflux from cells treated with 1 mM-Ca²⁺ and 2 µg of ionophore A23187/ml was measured at 20°C by using a K⁺-sensitive electrode as described by Reed (1976). The electrode was obtained from Orion Electronics, Cambridge, MA, U.S.A.

Results

Echinocytosis and microvesiculation

The erythrocytes of rat, rabbit and guinea pig become echinocytic and produced well-defined microvesicles very like those derived from human cells (Allan *et al.*, 1976a) when treated with Ca²⁺ and ionophore A23187. Supernatants from cells treated in this way were cloudy and on centrifugation gave red pellets that were shown by electron microscopy to consist of microvesicles similar to those from human cells (see Allan *et al.*, 1976a). In addition to the circular profiles seen previously, there were also indications of tubular forms. In each type of cell the released vesicles contained about 10–20% of the total lipid of the original cells (Table 1). On the other hand, when cells from ox, pig or sheep were treated in an identical fashion they produced no detectable microvesicles.

Diacylglycerol production and phosphatidate labelling

A marked increase in the cell content of 1,2-diacyl-*sn*-glycerol was seen with human, rat, rabbit and

guinea-pig cells when they were treated with Ca²⁺ and ionophore A23187, but this was not observed in ox, sheep or pig cells (Table 1). In these cells in which diacylglycerol was produced, it appeared to be relatively enriched in the microvesicles compared with the residual cells, as was previously shown for human erythrocytes (Allan *et al.*, 1976a).

In previous experiments (Michell *et al.*, 1973) it was found that when rat erythrocyte 'ghosts' were treated with phospholipase C the liberated diacylglycerol did not accumulate, but was rapidly degraded to monoacylglycerol and unesterified fatty acids by an endogenous lipase in the membrane. In the present experiments on intact cells we have observed no contribution of this lipase activity; diacylglycerol accumulated in the Ca²⁺-treated cells, and diacylglycerol was available to diacylglycerol kinase in both the Ca²⁺- and phospholipase C-treated cells.

Rat cells differed from those of the other species tested in that ionophore A23187 treatment made the cells more susceptible to rupture. This appeared to be due to crystallization of haemoglobin.

K⁺ efflux

Human, rabbit, rat, guinea-pig and pig cells all showed a pronounced Ca²⁺-triggered efflux of K⁺, whereas sheep (high-K⁺) and ox cells showed no such efflux (Table 1 and Fig. 1). The species differences are the same as those reported by Jenkins & Lew (1973), except that they did not include pig cells in their survey. In the cells that showed a K⁺ efflux, this was inhibited more than 90% by 0.1 mM-quinine (e.g. Fig. 1), as described for human cells by Armando-Hardy *et al.* (1975). Generally the efflux could be elicited by addition of ionophore even in the absence of added extracellular Ca²⁺, but extracellular Ca²⁺ had to be added for the full effect to be observed with pig cells; this suggests that the mechanism responsible for triggering the efflux may have a lower affinity for Ca²⁺ in pig cells than in the other species.

Shape change and phosphatidate labelling in erythrocytes treated with phospholipase C

When human erythrocytes are treated with concentrations of *Clostridium perfringens* phospholipase that are insufficient to cause cell lysis, the only phospholipids attacked are phosphatidylcholine and sphingomyelin, the major phospholipids of the outer leaflet of the membrane. There are corresponding rises in the concentrations of 1,2-diacyl-*sn*-glycerol plus phosphatidate and of ceramide (D. Allan, unpublished work; Allan *et al.*, 1975). There is no increase in lysophosphatidylcholine, indicating the absence of appreciable phospholipase A activity in the bacterial enzyme preparation.

Table 1. Effects of Ca²⁺-ionophore A23187 and phospholipase C treatment on the erythrocytes of various species

Each numerical value given represents a separate experiment. Averages $\pm$ s.d. are given where justified. Numbers in parentheses show the numbers of experiments performed. Diacylglycerol production is expressed as nmol/ml of packed cells. Untreated cells contained <5 nmol of diacylglycerol/ml. Phosphatidate labelling is expressed as the ratio d.p.m. in phosphatidate in treated cells/d.p.m. in phosphatidate in control cells. Values for microvesiculation are expressed as percentage of total lipid P found in microvesicular fraction (see Allan *et al.*, 1976a); abbreviation: ND, none detected. K⁺ efflux is expressed as percentage of total cell K⁺ lost in the first 5 min after addition of ionophore. In the right-hand column, + indicates change in shape from biconcave disc to stomatocyte within 30 min of phospholipase C addition; cells of most species showed endocytosis by this time.

	Ca ²⁺ -stimulated diacyl-glycerol production	Ca ²⁺ -stimulated phosphatidate labelling	Ca ²⁺ -stimulated microvesiculation	Ca ²⁺ -stimulated K ⁺ efflux	Phospholipase C-stimulated phosphatidate labelling	Phospholipase C-stimulated stomatocytosis/endocytosis
Human	30 $\pm$ 5 (4)	22 $\pm$ 4 (7)	18 $\pm$ 4 (12)	28 $\pm$ 4 (5)	155 $\pm$ 31 (8)	+
Rat	30, 30 (2)	17, 25 (2)	10, 12 (2)	25, 28 (2)	80, 40 (2)	+
Guinea pig	40, 50 (2)	14, 16 (2)	13, 19 (2)	31, 26 (2)	84, 100 (2)	+
Rabbit	50 $\pm$ 10 (3)	18 $\pm$ 3 (3)	18 $\pm$ 3 (3)	30 $\pm$ 3 (3)	54 $\pm$ 13 (3)	+
Pig	<5 (4)	1.3 $\pm$ 0.5 (3)	ND (4)	31 $\pm$ 4 (4)	6 $\pm$ 3 (4)	+
Ox	<5 (2)	1.7, 1.3 (2)	ND (2)	0.5 (2)	1.4, 0.9 (2)	(slow)
Sheep (high K ⁺)	<5 (2)	1.1 $\pm$ 0.4 (3)	ND (4)	0.5 (3)	1.2 $\pm$ 0.3 (4)	+
Chicken	<5 (2)	2.0, 1.2 (2)	0.9 $\pm$ 0.5 (3)	0.2 (3)	186, 114 (2)	+
Turkey	(but ceramide produced)	1.3, 1.2 (2)	1.2, 2.1 (2)	0.1 (2)	150, 100 (2)	+
	(but ceramide produced)

Fig. 1. Effect of Ca²⁺-ionophore A23187 on K⁺ efflux from pig and sheep erythrocytes

Cell suspension (10 ml; 2×10^9 /ml) in 0.15 M-NaCl containing 20 mM-Hepes/NaOH buffer, pH 7.4, was incubated at 20°C while being agitated with a magnetic stirrer. Ca²⁺ (1 mM) and ionophore A23187 (2 μ g/ml) were added at the times shown. The concentration of K⁺ in the medium was measured with a K⁺-sensitive electrode. O, Pig cells; ●, pig cells in the presence of 0.1 mM-quinine; ▲, sheep cells in the presence or absence of quinine. The addition of Ca²⁺ and/or quinine in the absence of ionophore had no effect on K⁺ efflux.

Under the same conditions, cells become cup-shaped (stomatocytic) and bud-off membrane vesicles inwards, and they also show a marked labelling of phosphatidate with ³²P (Allan *et al.*, 1975). The latter effect is due to the phosphorylation of the diacylglycerol liberated by the action of the phospholipase on the phosphatidylcholine of the outer leaflet of the membrane. The same effects were observed in rat, guinea-pig and rabbit cells (Table 1). In ox and sheep there is very little phosphatidylcholine in the erythrocyte membrane. In these species, phospholipase C treatment liberates ceramide from the sphingomyelin that constitutes almost all of the external lipid leaflet of the membrane. Thus there is no phospholipase C-dependent production of 1,2-diacyl-*sn*-glycerol or labelling of phosphatide. However, inward vesiculation of the cells did occur, probably owing to the production of ceramide from sphingomyelin (Table 1, and Allan *et al.*, 1975). Pig cells contain a substantial amount of phosphatidylcholine, but they are not very effectively attacked by the phospholipase C at the enzyme concentration that was used here; thus they show a small enhancement of phosphatidate labelling after phospholipase treatment and a slow and incomplete morphological change.

Effects of ionophore A23187 and phospholipase C on nucleated avian erythrocytes

Vos *et al.* (1976), after the reports of membrane vesiculation in ionophore-A23187-treated erythrocytes by Allan *et al.* (1976a), looked for formation of membrane vesicles in ionophore-A23187-treated chicken erythrocytes; evidence for vesiculation was obtained by freeze-fracture electron microscopy. However, we have found no evidence that elevation of the intracellular Ca^{2+} concentration with an ionophore causes any production of diacylglycerol in either turkey or chicken cells (Table 1). Neither was there any increase in labelling of phosphatidate in the ionophore-A23187-treated cells. There was, after prolonged incubation (4–5 h), a loss of discoid shape in about 10% of the cells, together with slight microvesiculation; the yield of microvesicles was only sufficient to account for about 1–2% of the membrane lipid of the cells, compared with 10–20% for the mammalian species, in which ionophore treatment readily induced microvesiculation. One non-polar lipid, not diacylglycerol, was enriched in the microvesicles compared with the original cells, and this was tentatively identified as ceramide because it co-chromatographed with authentic ceramide (derived from sphingomyelin by phospholipase C treatment) during t.l.c. in three different solvent systems [benzene/diethyl ether/ethanol/acetic acid (250:200:10:1 or 250:250:40:1, by vol.) and chloroform/methanol/acetic acid/water (85:15:10:4, by vol.)]. It was concentrated about four- to five-fold in the released vesicles compared with the residual cells. Thus it seems that a rise in the intracellular Ca^{2+} concentration in the avian erythrocyte can stimulate a lipase that attacks an unidentified sphingolipid which is presumably present in the inner leaflet of the membrane's lipid bilayer.

These cells, like mammalian cells, clearly have an active diacylglycerol kinase, since they form substantial quantities of labelled phosphatidate if they are exposed to attack by extracellular phospholipase C (Table 1). They also show morphological changes very similar to those seen in mammalian cells after attack by phospholipase C.

Neither the chicken nor the turkey cells showed any efflux of K^+ when treated with Ca^{2+} and ionophore A23187 (Table 1). Campbell & Siddle (1976) have found that this response is also absent from pigeon erythrocytes.

Discussion

In six of the seven types of mammalian erythrocytes that we studied there was a correlation between the occurrence of a Ca^{2+} -stimulated K^+ efflux and the occurrence of diacylglycerol production, echinocytosis and outward membrane vesiculation. How-

ever, in a single species, the pig, this correlation broke down; pig cells showed a rapid Ca^{2+} -stimulated K^+ efflux, but did not show either diacylglycerol production or the associated morphological changes. Thus these results give substantial support to our previous suggestion (Allan & Michell, 1975; Allan *et al.*, 1976a) that there is a causal relationship between Ca^{2+} -stimulated diacylglycerol production and the Ca^{2+} -induced morphological effects, but they indicate that the Ca^{2+} -stimulated efflux of K^+ is a process that is mechanistically unrelated to the effects of Ca^{2+} on lipid composition and on morphology.

The comparison between the mammalian and avian cells has proved interesting, especially in view of the possibility that ionophore-A23187 treatment activates a sphingolipid-specific lipase in the avian cells. Possibly this enzyme is the sphingomyelinase described by Hirschfield & Loyter (1975). Ceramide has marked similarities to diacylglycerol, and it seems likely, as in phospholipase C-treated ox and sheep cells, that the morphological effects on the avian cells of ionophore-A23187 treatment were caused by the asymmetric accumulation of ceramide in the lipid bilayer (see also Allan *et al.*, 1975).

Among the mammalian erythrocytes studied, it is clear that those cells which produce diacylglycerol in response to increased intracellular Ca^{2+} also possess an active diacylglycerol kinase that can phosphorylate diacylglycerol, whether endogenously generated or produced by exogenous phospholipase C treatment (Table 1). We have suggested previously that this kinase activity may counteract morphological changes induced by diacylglycerol (Allan & Michell, 1977). However, we have no idea what could be the function of diacylglycerol kinase in avian cells, which appear to lack an endogenous mechanism for generating diacylglycerol (Table 1). Our information does not allow us to decide whether ox and sheep cells possess this enzyme.

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