Ca²⁺-induced biochemical changes in human erythrocytes and their relation to microvesiculation

David ALLAN and Paul THOMAS

Department of Experimental Pathology, School of Medicine, University College London, University Street
London WC1E 6JJ, U.K.

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1. Human erythrocytes were treated with Ca²⁺ and ionophore A23187 and measurements were made of K⁺ efflux, polyphosphoinositide breakdown, 1,2-diacylglycerol accumulation, phosphatidate synthesis, changes in membrane polypeptide pattern and release of microvesicles. 2. It was shown that neither transamidase-mediated protein cross-linking, proteolysis of polypeptides 2.1 (ankyrin) or 4.1, nor accumulation of diacylglycerol or phosphatidate appeared to be necessary for microvesiculation to occur. 3. Microvesicles were released only under conditions where KCl efflux leading to cell shrinkage occurred and where polyphosphoinositides were broken down. These circumstances were sufficient to cause microvesiculation only in the presence of increased intracellular concentrations of Ca²⁺.

The release of small haemoglobin-containing vesicles (microvesicles and nanovesicles) from human erythrocytes treated with Ca²⁺ and ionophore A23187 appears to involve a membrane-fusion event (Allan *et al.*, 1976a, 1980) and provides a convenient model system that may be relevant to more general aspects of biological membrane fusion. Besides the release of microvesicles, treatment of erythrocytes with Ca²⁺-ionophore induces a bewildering variety of concomitant biochemical changes including K⁺ efflux (Lew & Ferreira, 1978), breakdown of polyphosphoinositides (phosphatidylinositol 4-phosphate + phosphatidylinositol 4,5-bisphosphate) with a consequent rise in 1,2-diacylglycerol and phosphatidate concentrations (Allan *et al.*, 1976a,b; Allan & Michell, 1978; Ponnappa *et al.*, 1980) and several alterations in membrane polypeptide pattern, notably the aggregation of protein components catalysed by transamidase (Anderson *et al.*, 1977; Lorand *et al.*, 1978), the conversion of polypeptide 2.1 (mol.wt. about 200 000) into lower-molecular-weight components, especially polypeptide 2.3 (mol.wt. about 175 000) (Anderson *et al.*, 1977; Allen & Cadman, 1979) and the apparent proteolysis of polypeptide 4.1 (mol.wt. about 78 000).

In the present work we have attempted to analyse the inter-relationships between the biochemical changes occurring in cells treated with Ca²⁺ and

ionophore and to determine which (if any) are essential for the release of microvesicles.

Materials and methods

Ionophore A23187 was obtained from Eli Lilly Co., and valinomycin, nigericin and gramicidin from Sigma Chemical Co. BDH Chemicals supplied quinine sulphate.

Preparation and incubation of cells

Fresh human erythrocytes were obtained from a number of different normal donors and were washed in heparinized 0.15 M-NaCl as described previously (Allan *et al.*, 1980). Portions (0.5 ml) of packed cells were incubated at 37°C in 4.5 ml of a medium containing 0.13 M-NaCl, 20 mM-4-morpholinepropanesulphonic acid/NaOH buffer, pH 7.1, and 1 mM-CaCl₂, together with ionophore A23187 (usually 5 µM) and appropriate concentrations of various inhibitors etc., as described in the text. Incubations were terminated (usually after 3 min) by the addition of 200 µl of 100 mM-EDTA and the cells were immediately sedimented at 500g for 5 min. The supernatant solution was carefully removed and was centrifuged at 16 000g_{av} for 20 min to sediment microvesicles as described by Allan *et al.* (1980). The resulting supernatant solution was retained for measurement of K⁺ content with a K⁺-sensitive electrode (Pye–Unicam, Cambridge, U.K.) (Reed, 1976) and for determination of cell lysis from measurements of A₅₅₀. Microvesicles were quantified

Abbreviation used: Tos-Lys-CH₂Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK').

by measurement of their phospholipid content, after extraction with methanol/chloroform (2:1, v/v) (Allan *et al.*, 1980). Packed-cell volumes were determined by centrifugation in microhaematocrit tubes using a Hawksley centrifuge. Samples were reduced in volume from the original 5 ml by centrifugation before the haematocrit measurement.

In some experiments cells were energy-depleted by pre-incubation for 1 h with 5 mM-iodoacetamide and 5 mM-deoxyglucose before incubation as above. These cells had ATP concentrations less than 10% those of normal cells.

Preparation of membranes and analysis of membrane polypeptides

Haemoglobin-free membranes were prepared from cells by lysis at 2°C in 20 vol. of 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer, pH 7.4, containing 0.2 mM-EGTA and 1 mM-MgCl₂, followed by four washes in the same medium. Analysis of membrane polypeptides was carried out on sodium dodecyl sulphate/polyacrylamide slab gels by the procedures of Laemmli (1970) and Fairbanks *et al.* (1971), with acrylamide concentrations of 7.5% and 5.6% respectively. Molecular-weight calibration was achieved by using a mixture of standard proteins (Combithek; Boehringer, Mannheim, Germany). Gels were scanned using a Joyce-Loebl Chromoscan 201 densitometer, and peaks were quantified by excision and weighing.

Lipid analysis

For investigations of polyphosphoinositide and phosphatidate concentrations, cells were pre-labelled for 90 min with [³²P]P_i (The Radiochemical Centre, Amersham, Bucks., U.K.) as described by Allan & Michell (1978). Lipids were extracted from membranes prepared as above, separated on formaldehyde-treated papers and their phosphate and radioactivity contents were measured (Allan & Michell, 1978). Total polyphosphoinositide was taken as the sum of half the phosphate measured in phosphatidylinositol 4-phosphate plus one-third of the phosphate measured in phosphatidylinositol 4,5-bisphosphate. Synthesis of phosphatidate was determined from its radioactivity by comparison with ATP specific radioactivity as measured below. The pre-incubation period was sufficient to allow equilibration of added ³²P into the γ -phosphate of ATP. In some experiments, neutral lipids were extracted with methanol from the solvent front of the paper chromatograms and were separated by t.l.c. (Freeman & West, 1966). Plates were sprayed with 20% (NH₄)₂SO₄ and lipid spots were charred by heating for 15 min at 180°C, after which the amount of 1,2-diacylglycerol was quantified by scanning in a Joyce-Loebl Chromoscan 201 densitometer. 1,2-

Dioleoylglycerol (Sigma Chemical Co.), chromatographed on the same t.l.c. plates, was used as a standard.

ATP in haemolysates was measured using a kit (366-UV) supplied by Sigma Chemical Co. and ATP radioactivity was determined after paper chromatography of the protein-free haemolysate (Bock *et al.*, 1956).

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms of membranes from cells treated with Ca²⁺ and ionophore A23187

Erythrocyte membrane polypeptides were separated (a) on 7.5% gels by the procedure of Laemmli (1970) or (b) on 5.6% gels by the method of Fairbanks *et al.* (1971). For (b), only the top portion of the gel is illustrated. The numbers below (b) refer to the time period (in minutes) of incubation with ionophore A23187 before quenching with EDTA (see Materials and methods section). C, T and Q refer to incubations that included respectively cystamine (5 mM), Tos-Lys-CH₂Cl (1 mM) or quinine (0.4 mM) in addition to ionophore A23187 (5 μ M). -A refers to the absence of ionophore A23187. V represents the polypeptide pattern of microvesicles generated by incubation of cells for 30 min with ionophore A23187. Membranes were prepared from pig erythrocytes that had been incubated for 3 min with (P+) or without (P-) 5 μ M-ionophore A23187, and were analysed on gels as above. Loading for membrane and microvesicle samples was equivalent to about 20 nmol of phospholipid. The nomenclature of the polypeptide bands (shown on the left of the gel photographs) was based on that of Steck (1974). It is noteworthy that our preparation of membranes (see the Materials and methods section) did not contain a band corresponding to Steck's band 6 (mol.wt. 36 000). Abbreviation used: Hb, haemoglobin.

Fig. 2. The time courses of Ca²⁺-induced changes in erythrocytes

Conditions were as described in the Materials and methods section. Results are all derived from a

Results and discussion

Time-dependent changes in polypeptide pattern

Fig. 1 shows the changes in polypeptide pattern that occur when intact fresh erythrocytes were treated with ionophore A23187 and Ca²⁺. The only notable changes were (a) an increase in the intensity of polypeptide 2.3 (mol.wt. 175 000), (b) a decrease in the intensity of polypeptide 4.1 (mol.wt. 78 000) as observed by Anderson *et al.* (1977) and Allen & Cadman (1979) and (c) an increased amount of stain at the top of the gels, presumed to be due to cross-linking of proteins by a Ca²⁺-sensitive transamidase activity (Anderson *et al.*, 1977; Lorand *et al.*, 1978). Changes (b) and (c) were relatively slow (Figs. 1 and 2a) and were arrested immediately after addition of excess EDTA, but the production of polypeptide 2.3 appeared to be much faster and was not stopped by addition of EDTA as early as 30s from the start of incubation with ionophore A23187. When resolved in another electrophoretic system (Fairbanks *et al.*, 1971) (Fig. 1) it was confirmed that the increase in polypeptide 2.3 was matched by a decrease in the staining of polypeptide 2.1 (ankyrin), the molecule that apparently links spectrin to polypeptide 3 in the intact erythrocyte membrane (Luna *et al.*, 1979; Bennett & Stenbuck, 1979; Tyler

single preparation. (a) Polypeptides. The ratio of polypeptides 2.3 (O) or 4.1 (●) to polypeptide 1 was measured by scanning polyacrylamide gels of membranes isolated at various times after addition of ionophore A23187 (see the Materials and methods section and Fig. 1a). Δ Represents the percentage of total cellular polypeptide 3 that was recovered in microvesicles. (b) Microvesiculation. Microvesiculation was measured in cells that had normal ATP concentrations (●) or that were metabolically depleted (O) (see the Materials and methods section). (c) K⁺ efflux and cell lysis. K⁺ efflux and cell lysis were measured as described in the Materials and methods section. O and ● refer to K⁺ efflux from ATP-depleted and normal cells respectively. Cell lysis with depleted cells was somewhat less than that measured with normal cells, which is shown as Δ. (d) Lipid changes. Measurements of polyphosphoinositide (■), phosphatidate (●, O) and 1,2-diacylglycerol (▲, Δ) contents in membranes from erythrocytes treated with ionophore A23187 and Ca²⁺ were made as described in the Materials and methods section. Filled symbols refer to normal cells and open symbols to cells that had been metabolically depleted. Polyphosphoinositide loss from depleted cells is not shown for the sake of clarity, but was little different from the loss from normal cells. In (a)–(d), □ refers to a value obtained at 30min incubation after addition of EDTA at 2min.

et al., 1980). The inability of EDTA to quench the polypeptide 2.1→2.3 change [in contrast with the effect of EDTA on all the other biochemical changes induced by Ca²⁺ (Fig. 2)], suggested either that the Ca²⁺-dependent proteinase responsible for this change binds Ca²⁺ very strongly or that the release of the enzyme in an active form is achieved at low Ca²⁺ concentrations but the enzyme itself does not depend on Ca²⁺ for activity.

Microvesiculation

As reported previously, up to 20% of the total membrane lipid of the treated erythrocytes was lost in the form of microvesicles (Allan *et al.*, 1976a, 1980; Shukla *et al.*, 1978). Microvesiculation was not affected by depletion of intracellular ATP concentration (Fig. 2b).

The microvesicles were largely free of spectrin (Fig. 1) but retained polypeptide 3 in amounts approximately proportional to their share of the total cell lipid (Figs. 1 and 2a). This suggested that polypeptide 3, the major penetrating protein of erythrocytes, partitions freely with the membrane lipids between microvesicles and remnant cells. Polypeptide 4.1 was decreased in the microvesicles compared with untreated cells (Fig. 1) so that the loss of this component from cells treated with Ca²⁺ and ionophore cannot be ascribed to its partition into microvesicles. The major component of the loss of polypeptide 4.1 is presumably due to its proteolytic degradation (Allen & Cadman, 1979) and/or to its incorporation into aggregates with spectrin (Anderson *et al.*, 1977).

Effects of inhibitors on Ca²⁺-dependent biochemical changes

Fig. 1 shows the effect of various putative inhibitors on the changes in polypeptide pattern induced by Ca²⁺ and ionophore A23187. The clearest finding was that cystamine consistently blocked transamidase-mediated protein aggregation (Fig. 1) as shown by Lorand *et al.* (1978) but had little effect on any of the other biochemical changes including microvesiculation (Fig. 1, Table 1). This appears to exclude protein cross-linking as a possible cause of microvesiculation or as a major reason for the decrease in the amount of polypeptide 4.1. In the experiment illustrated in Fig. 1 there did appear to be an inhibition by cystamine of band 4.1 loss, but this was not seen in any other experiment.

A variety of compounds reported to inhibit proteinases were used in an attempt to block the Ca²⁺-dependent breakdown of polypeptides 2.1 and 4.1 (Fig. 1, Table 1). These included Tos-Lys-CH₂Cl, 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one ('TPCK'), bisulphite, diisopropyl fluorophosphate and phenylmethane-sulphonyl fluoride. Of these compounds, only

Table 1. The effect of various inhibitors on Ca²⁺- and ionophore-dependent changes in erythrocytes

Cells were incubated in the standard medium (see the Materials and methods section) with the various additions indicated. Results are means ± s.d. from several separate experiments (number of experiments shown in parentheses). For data from only two experiments, the separate values are given.

Conditions	Microvesiculation (% of total cell phospholipid)	Polypeptide 2.3 (% of polypeptide 1)	Polypeptide 4.1 (% of polypeptide 1)	Cell content of polyphosphoinositides (nmol/10 ¹⁰ cells)	Net synthesis of phosphatidate (nmol/10 ¹⁰ cells)	K ⁺ efflux (% of total in cells)
EGTA (1 mM)	0 min 30 min	<0.1 (3) <0.1 (3)	30 ± 3 (4) 28 ± 2 (4)	34 ± 5 (4) 32 ± 2 (4)	2 (4) 1.5 (4)	<1 (4) 2 (4)
EGTA (1 mM)	3 min	4.3 ± 0.9 (5)	28 ± 4 (4)	24 ± 4 (4)	8 ± 2 (4)	33 ± 4 (4)
Ca ²⁺ (1 mM)	30 min	14.0 ± 2.0 (5)	12 ± 4 (4)	9 ± 2 (4)	18 ± 2 (4)	78 ± 7 (5)
Ca ²⁺ (1 mM)	3 min	0.1 ± 0.05 (5)	15 ± 2 (4)	23 ± 3 (3)	6 ± 2 (3)	2 (5)
Ca ²⁺ and quinine (0.4 mM)	30 min	0.2 ± 0.1 (5)	16 ± 1 (4)	11 ± 4 (3)	15 ± 4 (3)	4 (5)
	3 min	3.8 ± 0.4 (5)	27 ± 4 (4)	21, 26	7 ± 1 (3)	33 ± 2 (3)
Ca ²⁺ and Tos-Lys-CH ₂ Cl (1 mM)	30 min	12.7 ± 1.5 (5)	26 ± 2 (4)	8, 13	15 ± 2 (3)	81 ± 9 (3)
	3 min	3.8 ± 0.7 (3)	27 ± 5 (3)	27, 21	7 ± 2 (3)	30 ± 2 (3)
Ca ²⁺ and cystamine (5 mM)	30 min	15.2 ± 1.5 (3)	16 ± 4 (3)	6, 11	16 ± 4 (3)	75 ± 7 (3)

Tos-Lys-CH₂Cl produced significant changes in the polypeptide pattern (Fig. 1); the remainder caused little perceptible alteration in either polypeptide pattern or any of the other biochemical changes induced by Ca²⁺ and ionophore A23187, including microvesiculation. Tos-Lys-CH₂Cl reproducibly blocked the breakdown of polypeptide 4.1 and diminished the increase in polypeptide 2.3 (Figs. 1 and 2a). It was confirmed that breakdown of polypeptide 2.1 (ankyrin) was inhibited by Tos-Lys-CH₂Cl (Fig. 1). Tos-Lys-CH₂Cl did not significantly diminish microvesiculation or any of the other biochemical changes induced by Ca²⁺ and ionophore and it was therefore concluded that none of these changes, including microvesiculation, depended on breakdown of polypeptides 4.1 or 2.1. Polypeptide 2.1 (ankyrin) is thought to connect the spectrin-actin framework with the membrane bilayer through its dual interactions with spectrin and polypeptide 3 (Bennett & Stenbuck, 1980b) and in some ways it would be an attractive idea if breakdown of this bridging molecule were critical to the release of part of polypeptide 3 in the form of spectrin-free microvesicles. Since, however, there is only sufficient ankyrin to bind to about 10% of the total polypeptide 3 (Bennett & Stenbuck, 1980b), it may be that ankyrin does not markedly restrict the tendency of polypeptide 3 to partition into microvesicles.

Lipid changes

The alterations in lipid content of cells treated with ionophore A23187 and Ca²⁺ were consistent with those described previously (Allan *et al.*, 1976b, 1978), whereby Ca²⁺ induces a breakdown of polyphosphoinositides to 1,2-diacylglycerol, which can then be phosphorylated to give phosphatidate if ATP is available (Fig. 2d). No effective inhibitor of polyphosphoinositide breakdown was found, and we could not exclude the possibility that this reaction, which is promoted by a Ca²⁺-dependent membrane-bound phospholipase C (Allan & Michell, 1978) was also an essential component of the microvesiculation mechanism. However, our results do enable us to conclude that neither 1,2-diacylglycerol nor phosphatidate accumulation arising as a consequence of polyphosphoinositide breakdown are essential for microvesiculation to occur. Using fresh cells in these experiments, and in contrast with our previous results using stored cells (Allan *et al.*, 1976a,b), we found little accumulation of 1,2-diacylglycerol under conditions where microvesiculation was rapid and extensive; this neutral lipid was smoothly and efficiently converted into phosphatidate (Fig. 2d). On the other hand, under conditions of energy depletion where synthesis of phosphatidate was prevented and 1,2-diacylglycerol accumulated in proportion to the breakdown of

polyphosphoinositide there was again no effect on the microvesiculation process (Fig. 2d). Thus neither of the products of polyphosphoinositide breakdown appear to be essential for microvesiculation and we are left with the suggestion that breakdown itself may be the vital factor (Allan *et al.*, 1978). One piece of circumstantial evidence that supports this view is that pig erythrocytes treated with Ca²⁺ and ionophore A23187 show no accumulation of phosphatidate or diacylglycerol that would have resulted from phosphoinositide breakdown and no sign of microvesiculation (Allan & Michell, 1977), although in other respects they respond like human cells, e.g. they show Ca²⁺-dependent K⁺ efflux (Allan & Michell, 1977) and a well-defined 2.1→2.3 polypeptide change (Fig. 1). This observation again suggests that polyphosphoinositide breakdown may be more significant than the polypeptide change in the control of microvesiculation.

Cell shrinkage and microvesiculation

Experiments with quinine revealed a clear role of cell shrinkage in the mechanism of microvesiculation. Quinine has been shown previously (Armando-Hardy *et al.*, 1975; Reichstein & Rothstein, 1981) to block the Ca²⁺-dependent increase in permeability to K⁺ (the Gardos effect), which is exhibited by the erythrocytes of several animal species including man (Jenkins & Lew, 1973). With cells suspended in an NaCl medium, addition of Ca²⁺ and ionophore A23187 results in net efflux of KCl and water with a consequent shrinkage of the cells (Lew & Ferreira, 1978). In our experiments, 0.4 mM-quinine suppressed not only K⁺ efflux and cell shrinkage but also completely eliminated microvesiculation (Fig. 3, Tables 1 and 2). Inhibition of K⁺ efflux, cell shrinkage and microvesiculation showed the same dependence on quinine concentration (Fig. 3), suggesting that K⁺ efflux and/or cell shrinkage were vital factors in the control of microvesiculation. Cell shrinkage and consequent crenation would increase the likelihood of the intramembrane interactions, which must be involved in the budding process that leads to microvesiculation.

The link between K⁺ efflux leading to cell shrinkage and microvesiculation was confirmed by the experiment illustrated in Fig. 4, where it was shown that with iso-osmotic KCl replacing NaCl, cell shrinkage and microvesiculation were both suppressed. Furthermore, the quinine inhibition of microvesiculation could be completely reversed by addition of valinomycin (Table 2), which induces KCl efflux and cell shrinkage in a Ca²⁺-independent manner (Hunter, 1977).

Valinomycin by itself was quite unable to produce microvesiculation (Table 2), so that it seemed clear that other factors besides K⁺ efflux and cell

Fig. 3. The effect of quinine on K^+ efflux and microvesiculation in human erythrocytes treated with Ca^{2+} and ionophore A23187

Cells were incubated for 3 min in the presence of ionophore A23187 and various amounts of quinine. K^+ efflux (●) and microvesicle release (O) and haematocrits (□) were measured as described in the Materials and methods section. Cell lysis was less than 1%. Values are means $\pm$ s.d. (represented by the bars) from three experiments.

Fig. 4. The effect of extracellular K^+ on microvesiculation of erythrocytes treated with Ca^{2+} and ionophore A23187

Microvesicle release (●) and haematocrit (O) were measured from erythrocytes that were incubated for 3 min in the presence of Ca^{2+} , ionophore A23187 and various amounts of KCl and NaCl. Cell lysis was less than 1%. Results are means $\pm$ s.d. (represented by the bars) from three experiments.

Table 2. Microvesiculation, K^+ efflux and cell shrinkage in erythrocytes exposed to ionophore A23187, quinine and various K^+ ionophores

Ionophore A23187, valinomycin and nigericin were added at $5 \mu M$ final concentration; gramicidin was $0.3 \mu M$ and quinine was $400 \mu M$. Each separate incubation was for 5 min at $37^\circ C$, in the standard incubation medium (see the Materials and methods section) with the addition indicated. The above results were obtained with a single sample of cells; similar experiments with three different samples gave almost identical results.

Incubation conditions	K^+ efflux (%)	Packed-cell		Microvesiculation	
		volume (%)	Shrinkage (%)	(% of maximum value)	
1. No addition	0.8	41	0	<1	
2. Quinine	0.7	41	0	<1	
3. Ionophore A23187	41	24	41	100	
4. Ionophore A23187 + quinine	1.5	38	7	<1	
5. Valinomycin	47	24	41	<1	
6. Nigericin	75	36	12	<1	
7. Gramicidin	89	40	2	<1	
8. Ionophore A23187 + quinine + valinomycin	48	24	41	98	
9. Ionophore A23187 + quinine + nigericin	89	36	12	<1	
10. Ionophore A23187 + quinine + gramicidin	89	40	2	<1	
11. As 5, then +ionophore A23187	74	24	41	92	
12. As 6, then +ionophore A23187	92	39	5	<1	
13. As 7, then +ionophore A23187	92	41	0	<1	
14. As 4, then added EDTA (4 mM), washed and re-incubated with Ca^{2+} -free buffer.	0.5	37	9	<1	
15. As 14, +valinomycin	50	24	41	3	
16. As 14, + Ca^{2+} (1 mM) + ionophore A23187	25	24	41	90	

shrinkage must also be involved, presumably including some of the Ca²⁺-dependent biochemical changes referred to above.

It was apparent from experiments with other ionophores that K⁺ efflux in itself did not promote microvesiculation. Thus neither nigericin nor gramicidin, which support K⁺ efflux in exchange for H⁺ or Na⁺ respectively and which cause little cell shrinkage, were able to relieve the quinine inhibition of Ca²⁺-ionophore-induced microvesiculation. Pre-incubation with nigericin or gramicidin, which dissipated the K⁺ gradient and hence made it impossible for ionophore A23187 and Ca²⁺ alone to cause cell shrinkage, completely blocked microvesiculation (Table 2). It seemed therefore that only agents that caused net efflux of KCl and hence cell shrinkage would permit microvesiculation. If so, then it should be possible to relieve the quinine inhibition of microvesiculation by other procedures that caused cell shrinkage e.g. by treatment with hyper-osmotic medium. However, Fig. 5 demon-

strates that microvesiculation was still completely blocked by quinine even in the presence of NaCl concentrations that were sufficient to cause similar degrees of cell shrinkage to those obtained with valinomycin or with ionophore A23187 in the absence of quinine. This unexpected result could be partly accounted for by the parallel observation that microvesiculation in the absence of quinine was markedly decreased by increased NaCl concentrations (Fig. 5). It is known that hyper-osmotic media greatly decrease the deformability of erythrocytes (Mohandas *et al.*, 1980), probably by increasing the normally very high intracellular haemoglobin concentration to a limiting value, and it therefore appears plausible that the inhibition of microvesiculation by high NaCl concentrations could be due to the same phenomenon since it seems likely that microvesiculation could only occur in a flexible cell. The drawback to this argument is that cells treated with ionophore A23187 also become inflexible (Kirkpatrick *et al.*, 1975) and yet exhibit a copious release of microvesicles. We have considered the possibility that microvesicle release may occur in ionophore A23187-treated cells before KCl release is maximal (Fig. 2) and hence before the cells become rigid, but this argument seems to fall down on the evidence that pretreatment of cells with valinomycin, which causes acute cell shrinkage, produces no significant inhibition of microvesiculation when the cells are subsequently exposed to ionophore A23187 (Table 2).

It should be emphasized, however, that cell shrinkage induced by hyper-osmotic media is by no means equivalent to the shrinkage that follows exposure to valinomycin or ionophore A23187; in the former case, the concentration of internal KCl will rise in proportion to the external osmolarity, whereas in the latter situations, where net KCl efflux occurs, there should be little change in intracellular tonicity. This difference in the intracellular ionic conditions may be the crucial factor in determining the susceptibility of the cells to microvesiculation.

Quinine and protein aggregation

It was noticeable that cells treated with Ca²⁺, ionophore and quinine showed little evidence of protein aggregation after polyacrylamide-gel electrophoresis of their membranes (Fig. 1). This might have been a consequence of the inhibition by quinine of cell shrinkage and microvesiculation, but that possibility was rendered unlikely by the observation that a similar inhibition induced by high KCl medium did not prevent protein aggregation (P. Thomas & D. Allan, unpublished work; Lorand *et al.*, 1978). It therefore appears that quinine at rather low concentrations (0.4 mM and below) exerts a direct inhibitory effect on the transamidation reactions that give rise to protein aggregation. Quinine

Fig. 5. The influence of NaCl concentration on microvesiculation and cell volume of erythrocytes treated with Ca²⁺ and ionophore A23187 in the presence or absence of quinine

Microvesiculation (O, ●) and packed-cell volume (Δ, ▲) were measured as described in the Materials and methods section. Cells were incubated for 5 min with various amounts of NaCl added to the standard medium, which contained Ca²⁺ and ionophore A23187. Incubations were carried out in the absence (open symbols) or presence (filled symbols) of 0.4 mM-quinine. Microvesiculation data are means $\pm$ s.d. from four separate experiments.

seems to be an exception to the rule that inhibitors of this aggregation possess a primary amino function (Lorand *et al.*, 1978).

The importance of raised intracellular Ca^{2+} concentrations

Finally we can ask whether the various Ca^{2+} -induced changes considered here are in themselves sufficient to cause microvesiculation or whether they are only effective in the presence of elevated intracellular Ca^{2+} concentrations. Experimentally this question could be answered quite simply since it was possible to produce all the lipid and polypeptide changes by incubation of the cells with Ca^{2+} , ionophore A23187 and quinine and then, after washing away all these agents, to produce KCl efflux and cell shrinkage by treatment with valinomycin (Table 2). It was quite clear that no significant microvesiculation occurred under these conditions, although microvesicles were released profusely on further addition of ionophore A23187, thus indicating the importance of a raised intracellular Ca^{2+} concentration in the microvesiculation process. The influence of Ca^{2+} is considered more fully in the following paper (Allan & Thomas, 1981).

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References

- Allan, D. & Michell, R. H. (1977) *Biochem. J.* (1977) **166**, 495–499
- Allan, D. & Michell, R. H. (1978) *Biochim. Biophys. Acta* **508**, 277–286
- Allan, D. & Thomas, P. (1981) *Biochem. J.* **198**, 441–445
- Allan, D., Billah, M. M., Finean, J. B. & Michell, R. H. (1976a) *Nature (London)* **261**, 58–60
- Allan, D., Watts, R. & Michell, R. H. (1976b) *Biochem. J.* **156**, 225–232
- Allan, D., Thomas, P. & Michell, R. H. (1978) *Nature (London)* **276**, 289–291
- Allan, D., Thomas, P. & Limbrick, A. R. (1980) *Biochem. J.* **188**, 881–887
- Allen, D. W. & Cadman, S. (1979) *Biochim. Biophys. Acta* **551**, 1–9
- Anderson, D. R., Davis, J. L. & Carraway, K. L. (1977) *J. Biol. Chem.* **252**, 6617–6623
- Armando-Hardy, M., Ellory, J. C., Ferreira, H. G., Fleminger, S. & Lew, V. L. (1975) *J. Physiol. (London)* **250**, 32p–33p
- Bennett, V. & Stenbuck, P. J. (1979) *J. Biol. Chem.* **254**, 2533–2541
- Bennett, V. & Stenbuck, P. J. (1980a) *J. Biol. Chem.* **255**, 2540–2548
- Bennett, V. & Stenbuck, P. J. (1980b) *J. Biol. Chem.* **255**, 6424–6432
- Bock, R. M., Ling, N. S., Morell, S. A. & Lipton, S. H. (1956) *Arch. Biochem. Biophys.* **62**, 253–264
- Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617
- Freeman, G. P. & West, D. (1966) *J. Lipid Res.* **7**, 324–327
- Hunter, M. J. (1977) *J. Physiol. (London)* **268**, 38–49
- Jenkins, D. M. & Lew, V. L. (1973) *J. Physiol. (London)* **234**, 41p–42p
- Kirkpatrick, F. H., Hillman, D. G. & LaCelle, P. L. (1975) *Experientia* **31**, 653–654
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lew, V. L. & Ferreira, H. G. (1978) *Curr. Top. Membr. Transp.* **10**, 217–277
- Lorand, L., Siefring, G. E., Jr. & Lowe-Krentz, L. (1978) *J. Supramol. Struct.* **9**, 427–440
- Luna, E. J., Kidd, G. H. & Branton, D. (1979) *J. Biol. Chem.* **254**, 2526–2532
- Mohandas, N., Clark, M. R., Jacobs, M. S. & Shohet, S. B. (1980) *J. Clin. Invest.* **66**, 563–573
- Ponnappa, B. C., Greenquist, A. C. & Shohet, S. B. (1980) *Biochim. Biophys. Acta* **598**, 494–501
- Reed, P. (1976) *J. Biol. Chem.* **251**, 3489–3494
- Reichstein, E. & Rothstein, A. (1981) *J. Membr. Biol.* **59**, 57–63
- Shukla, S. D., Berriman, J., Coleman, R., Finean, J. B. & Michell, R. H. (1978) *FEBS Lett.* **90**, 289–291
- Steck, T. L. (1974) *J. Cell Biol.* **62**, 1–19
- Tyler, J. M., Reinhardt, B. N. & Branton, D. (1980) *J. Biol. Chem.* **255**, 7034–7039