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MICROVESICULATION AND SPHINGOMYELINASE ACTIVATION IN CHICKEN ERYTHROCYTES TREATED WITH IONOPHORE A23187 AND Ca^{2+}

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Treatment of chicken erythrocytes with ionophore A23187 and Ca^{2+} leads to the disappearance of the marginal band of microtubules and to a release of the constraints which normally maintain the nucleus in a central position in the cells. The consequent close apposition of the nucleus to the plasma membrane may allow nuclear-plasma membrane fusion to occur and subsequently results in the release of microvesicles from the hybrid surface membrane. The remnant cells are spherical, and have nuclei which appear to be partly exocytosed. Concomitant with these morphological changes, there is a breakdown of 20–30% of the total cell sphingomyelin by an endogenous sphingomyelinase which does not require Ca^{2+} and which releases phosphorylcholine only into the cell interior. It is suggested that the pool of sphingomyelin which is broken down as a consequence of Ca^{2+} entry into the cells is present in the nuclear membrane and that it becomes available to the plasma membrane sphingomyelinase as a result of the close apposition of nucleus and plasma membrane induced by Ca^{2+}.

Introduction

A wide variety of biochemical and morphological alterations occur in the plasma membranes of erythrocytes when the normally low concentration of Ca^{2+} in these cells is artificially elevated [1,2]. Among the most dramatic Ca^{2+}-mediated changes are those in which membrane fusion events appear to occur, either in the course of cell-cell fusion [3–6] or during the release of microvesicles [1,2,4,7–10]. Microvesiculation of human cells may depend upon a direct interaction of Ca^{2+} with membrane phosphatidylserine, but in addition has a requirement for cell shrinkage and possibly also for a Ca^{2+}-dependent breakdown of membrane polyphosphoinositides to 1,2-diacylglycerol [2,7,10–13]. In contrast, Ca^{2+}-dependent release of microvesicles from chicken and turkey erythrocytes [1,4] appears to occur without gross cell shrinkage or production of 1,2-diacylglycerol [1], and this would argue that neither cell shrinkage nor breakdown of polyphosphoinositides are essential for microvesiculation to occur. However, treatment of these avian erythrocytes with ionophore A23187 and Ca^{2+} does lead to the accumulation of ceramide [1], a lipid which is structurally similar to diacylglycerol and which in principle could have analogous effects on membrane structure. Ceramide could be generated by the action of a sphingomyelinase which is present in chicken erythrocyte plasma membrane but this enzyme is not activated by Ca^{2+} and does not appear to function in intact cells [14,15].

The present work was undertaken to characterize more fully the release of microvesicles from chicken erythrocytes and to investigate the relation of sphingomyelinase activity to microvesiculation.

Materials

Ionophore A23187 was a gift from Dr. R. Hamill, Eli Lilly Co. Indianapolis, IN, U.S.A. A freeze-dried culture filtrate of *Clostridium welchii* was used as a source of phospholipase C activity and was a gift from Burroughs Wellcome Research, Beckenham, Kent, U.K.

Distol grade solvents for GLC were supplied by Fisons Scientific Apparatus, Loughborough, Leics., U.K. and other reagents and chemicals for GLC were obtained from Chromatography Services, Hoylake, Merseyside, U.K.

Deoxyribonuclease I, phosphorylcholine, phosphorylethanolamine, phosphorylserine and phosphorylinositol, standard phospholipids and neutral lipids were from Sigma Chemical Co., Poole, Dorset, U.K. All other reagents were Analar grade and were supplied by BDH, Poole, Dorset, U.K.

Methods

Preparation and incubation of cells

Blood was obtained from the wing vein of either Light Sussex or Rhode Island Red hens, using EDTA as anticoagulant. The red cells were sedimented at $500 \times g$ for 5 min in a MSE bench centrifuge and plasma and buffy coat were removed by aspiration. The cells were washed three times with 0.15 M NaCl with further removal of buffy coat and were given a final wash with 0.13 M NaCl, 20 mM morpholinopropanesulphonic acid (Mops)/NaOH (pH 7.1).

Except when otherwise stated, incubations were carried out with 0.1 ml cells in 0.9 ml of 0.13 M NaCl, 20 mM Mops/NaOH (pH 7.1) with the addition of 1 mM $CaCl_2$ and 5 μM A23187, at 37°C. Incubations were terminated by addition of 50 μl of 100 mM EGTA and rapid cooling in ice. Cells were sedimented at $500 \times g$ for 5 min and the supernatant was removed and recentrifuged at $16\,000 \times g_{av}$ for 20 min in a Sorvall RC5B machine in order to sediment any microvesicles. Cell lysis was determined by measurement of the proportion of the total cell haemoglobin which was found in the high-speed supernatant [16]. Microvesicles were resuspended in 1 ml of 0.15 M NaCl and were assayed by measurement of their lipid content as described below and occasionally also by measurement of the haemoglobin contained within them [16].

In some experiments where a range of buffered Ca^{2+} concentrations was required, 10 mM *N'*-(2-hydroxyethyl)ethylenediamine-*N*,*N*,*N'*-triacetic acid (HEDTA) or 10 mM citric acid together with various amounts (1–10 mM) of $CaCl_2$ were added to the basic Mops/NaOH buffer. Free Ca^{2+} concentrations corresponding to different ratios of Ca^{2+} to chelator were calculated from the data of Raaflaub [17] and were checked with a Ca^{2+}-sensitive electrode (Pye-Unicam, Cambridge, U.K.) [10].

In other experiments cells were energy-depleted by incubation for 1 h in the standard medium with the addition of 1 mM iodoacetamide and 2 mM deoxyglucose. This procedure reduced ATP levels to less than 10% of normal.

The action of exogenous phospholipase C on the cells was measured by incubating 100 μl of packed cells in 100 μl of 0.13 M NaCl, 20 mM Mops/NaOH, 1 mM $CaCl_2$ (pH 7.1) to which was added 5 μg of *C. welchii* phospholipase C. Incubation was at 37°C for up to 10 min and was terminated by the addition of 2 μl of 10 mM *o*-phenanthroline hydrochloride. Lipids were extracted as described below.

Cell lysis and the accompanying activation of endogenous sphingomyelinase was measured in 50 μl aliquots of cells subjected to incubation at 37°C for 30 min in 950 μl of 5 mM Mops/NaOH buffer (pH 7.1) containing 1 mM EGTA and various hypo-osmotic concentrations of NaCl. 200 μl of suspension was used for estimation of lysis and lipids were extracted from the remaining 800 μl and analysed as described below.

Preparation of erythrocyte membranes and nuclei

Plasma membranes were prepared from chicken erythrocytes according to the procedure of Record et al. [15]. Total cell membranes were prepared by osmotic lysis of cells in 20 vol. of ice-cold 20 mM Hepes/NaOH buffer + 1 mM EGTA (pH 7.4), followed by centrifugation at $16\,000 \times g_{av}$ for 20 min at 4°C. The resulting pellet was washed a further three times in the same medium and then was incubated at 37°C for 10 min with 50 μg/ml DNAase I, 5 mM $MgCl_2$, 1 mM EGTA. Nuclei were prepared according to Beam et al. [18].

Polypeptide analysis

Analysis of polypeptides present in various cell fractions was carried out by polyacrylamide gel electrophoresis on 7.5% gels according to the procedure of Laemmli [19]. Protein was determined as described by Lowry et al. [20].

Lipid analysis

Lipids were extracted from cells, microvesicles and membranes as previously described [2]. Phospholipids were separated on silica gel HR (Merck) plates according to the procedure of Skipski et al. [21] except that bicarbonate was not incorporated into the slurry. In some experiments phospholipids were also separated on formaldehyde-treated papers using *n*-butanol/water/acetic acid (4 : 5 : 1, v/v) as solvent which resolved phosphatidylserine and phosphatidylinositol. Phospholipid was determined as phosphate after digestion of the spots with 70% perchloric acid [22].

Neutral lipids were separated on silica gel HR plates according to the method of Freeman and West [23] and were identified by comparison with standard samples of ceramide, 1,2-diolein, palmitic acid and cholesterol. Cholesterol was determined as described by Brown et al. [24]. Ceramide separated on a tlc plate was converted to sphingosine by the procedure of Morrison [25] and the free base was determined as its *N,O*-bistrimethylsilylacetamide derivative by GLC on a 1.5 m column of 1% Dexsil on 80–100 mesh Supelcoport 100G at 200°C, using a Pye 204 gas chromatograph.

Plasma membrane preparations possessed sphingomyelinase activity which led to somewhat variable results for recovery of sphingomyelin. In this case, total sphingomyelin was determined as (sphingomyelin plus ceramide).

Analysis of phosphorylcholine

Aqueous fractions containing phosphorylcholine were desalted on a mixed bed resin (Dowex 1 and Dowex 50W) and dried on a rotary evaporator at 50°C. Samples were redissolved in 20 μl of distilled water and 10-μl aliquots were chromatographed on silica gel HR (Merck) TLC plates with a solvent consisting of methanol/98% formic acid/water (80 : 13 : 7, v/v). Phosphorus-containing spots were located by spraying the dried plate with 0.1% $FeCl_3$/7% sulphosalicylic acid [26] and were analysed for phosphate as described above. Standard phosphorylcholine (Sigma) was run in separate lanes as a marker; in this TLC system it ran slowly (R_F 0.33) whereas all other intracellular phosphates ran near the front. Standard inositol and serine phosphates ran with the solvent front and ethanolamine phosphate ran with R_F0.67.

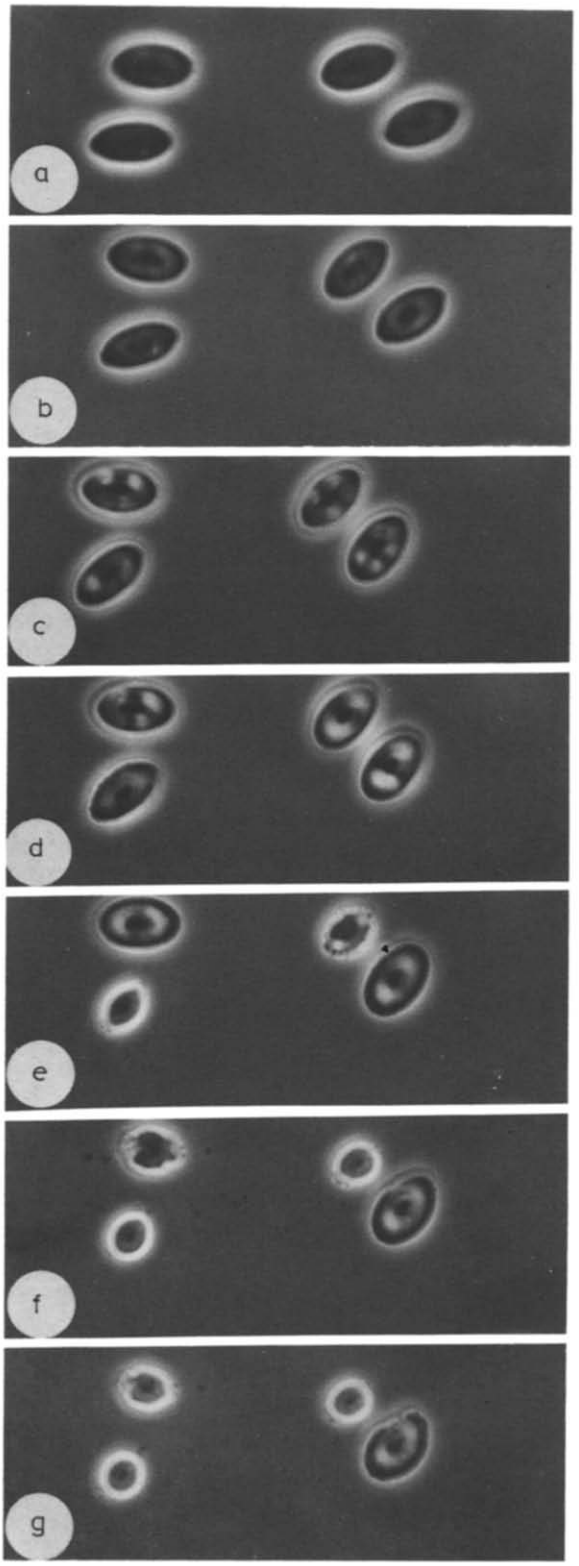
Light and electron microscopy

Samples incubated at 37°C were fixed in suspension by addition of glutaraldehyde to a final concentration of 1.5% in 0.13 M NaCl, 20 mM Mops/NaOH buffer (pH 7.1). They were examined initially under phase contrast in a Leitz (Wetzlar) SM-Lux microscope at either ×400 or ×1000 magnification. Differential cell counts were made in triplicate on each sample, counting not less than 100 cells in separate fields. Cells were assessed as normal, 'spoked' or refractile after treatment with A23187 and Ca^{2+} (see Results). Cells were also stained with 1% Toluidine blue to identify nuclei. For light photomicrography cells were incubated at 20°C and unfixed samples were examined on glass slides in a Zeiss photomicroscope (Mk 2). Phase contrast optics were employed and the intensity of illumination was adjusted to give short exposure times. Ilford Pan F film was used. Electron microscopy was as described previously [8].

Results

Effects of A23187 and Ca^{2+} on erythrocyte morphology

Untreated chicken erythrocytes were uniformly lenticular in shape and their nuclei were difficult to resolve in unstained specimens (Fig. 1a). Addition of A23187 and Ca^{2+} caused drastic alterations in cell morphology. The earliest change (1–2 min) observable in the light microscope was an increase in phase contrast of the cell nucleus (Fig. 1b) and this was rapidly followed (2–5 min) by the development of radial (spoke-like) structures apparently joining the nucleus to the periphery of the cell (Fig. 1c). These 'spokes' rapidly coalesced to form a dark bar across the middle of the cells (Fig. 1d). Subsequently the cells lost their original ellipsoidal shape and became more irregular, with



the nucleus displaced to the periphery of the cell. They appeared brighter (more refractile) under phase contrast (Figs. 1e and 1f). At this stage (5–15 min) it was evident that vesicular material of various sizes from 0.1 μm to 1 μm diameter was being shed from the cells. Eventually (by 30 min) the cells were mostly small bright and spherical and the nucleus was only discernable after staining (Fig. 1g). Individual cells varied considerably in the rate at which they progressed through these changes but in general, the transition was rapid from the ellipsoidal shape to the refractile stage when the cells were shedding microvesicles. During the whole process, cell lysis was less than 2% and no cell-cell fusion was observed. All of the above morphological changes were halted by the addition of chelators of Ca^{2+} at any time during the incubation.

Electron microscopy (Fig. 2) generally confirmed the observations made with the light microscope. However in control cells and cells treated for relatively short periods with ionophore and Ca^{2+}, an electron-transparent area surrounding the nucleus was apparent (Fig. 2a) although this area seemed to be less noticeable in cells treated for longer periods (Figs. 2d, 2e). At various points this space was bridged by sections of amorphous material (arrowed in Fig. 2a) which in many cases had a bar of denser-staining substance running across (arrowed in Fig. 2b). It was evident that not only did the treated cells release vesicular material as they became progressively more spherical but in marked contrast to untreated cells where the nucleus was always located centrally (Fig. 2a), the nuclei of treated cells were often found closely abutting the plasma membrane (Figs. 2d, 2e). In many cases there was no space apparent between plasma membrane and nucleus (Figs. 2d, 2e) and there were indications of partial exocytosis of nuclei (Fig. 2f) particularly in cells which had become spherical, although no evidence was obtained for complete nuclear exocytosis. It was dif-

Fig. 1. Light microscopy of chicken erythrocytes treated with A23187 and Ca^{2+} at 20°C. Samples were examined and photographed as described under Methods. Incubation times (min) are given in the text. Microvesicles are visible as small dots, in e–g.

ficult to determine the precise disposition of membranes in the erythrocytes due to the large background staining of haemoglobin, but in regions where nuclei abutted the plasma membrane, small membraneous blebs were often visible (arrowed in Fig. 2d). Similar but less electron-dense blebs were also often seen in regions of the cell membrane distant from the site of its interaction with the nucleus (arrowed in Fig. 2d); these may have represented sites where microvesicles had detached from the cells. The nuclei of obviously deformed cells appeared in general to be more spherical than those of normally-shaped erythrocytes. Cells which could be directly related to the 'spoked' erythrocytes seen under phase contrast were not apparent in the electron microscope. The marginal band of microtubules described previously [27] was readily observed in control cells (Figs. 2a and 2c) and in treated cells which still retained an ellipsoidal shape. However, these microtubules were not visible in cells with an irregular or spherical shape (Figs. 2d, 2e, 2f).

Thin sections of the microvesicles released from chicken cells under the above conditions revealed that they consisted predominantly of dense membrane-bounded spheres (Fig. 2g). Apart from their greater size range (0.1–1 μm), they appeared to be analogous to microvesicles released from human erythrocytes treated with A23187 and Ca^{2+} [7,8]. Appendages similar to those present on microvesicles from human cells were seen on some of the microvesicles (arrowed in Fig. 2g).

Determinants of microvesicle release

The time-course of microvesicle release from chicken erythrocytes treated with ionophore and Ca^{2+} is shown in Fig. 3b. At 37°C release was linear for the first five minutes of treatment and had largely ceased by 15–20 min, by which time up to 25% of the total cell phospholipid and 4–5% of the haemoglobin had been lost as microvesicles. A correlation was generally observed between microvesicle release and the production of bright (refractile) cells, (Fig. 3a) and chains of microvesicles could often be seen apparently being shed from these cells. Removal of Ca^{2+} from the medium by addition of EDTA caused an abrupt cessation of microvesicle release (Fig. 3b); release was maximal at about 1 mM Ca^{2+} and half maximal at about 0.1 mM Ca^{2+}. As with human erythrocytes, higher concentrations of Ca^{2+} (> 2 mM) were associated with a progressive decrease in microvesicle release (Fig. 4). Unlike human cells [2] microvesiculation was not accompanied by gross cell shrinkage (unpublished results and Ref. 28) and was not affected by replacement of KCl for NaCl in the external medium nor by addition of quinine or elevation of ionic strength (Fig. 1b). The optimum temperature for microvesicle release was 35–40°C with inhibition occurring at higher temperatures.

The composition of cells, plasma membranes and microvesicles

Electrophoresis on polyacrylamide gels revealed profound differences in the polypeptide composition of lysed cells, plasma membranes and microvesicles (Fig. 5). The pattern for plasma membranes resembled that obtained with human erythrocytes and the bands in Fig. 5 which correspond directly with those in human membranes were numbered according to current convention [29]. The lysed cells, as expected, contained some polypeptides characteristic of the plasma membrane (M1, M2, etc.), some which were identified with the soluble cytoplasm (S1, S2, etc.) and others (N1, N2, etc.), which by comparison with the findings of other workers were probably proteins derived from the cell nucleus [30,31]. It is possible that the band designated N1 (M_r 55000) could have been tubulin. Only one prominent polypeptide (X), of molecular weight about 70000, was not easily attributable to any of these categories and in accordance with earlier data [30,31] was provisionally identified as a polypeptide derived from the nuclear membrane.

The polypeptide pattern of the microvesicles was very different from those of either lysed cells or plasma membranes; most obviously the microvesicles did not contain either nuclear proteins or those bands which can be identified with the plasma membrane skeleton, i.e. M1, M2 (spectrin) and M5 (actin), or with tubulin. In addition to bands which are characteristic of the soluble cytoplasm (e.g. S3 and haemoglobin) the microvesicles contained the major plasma membrane polypeptide M3 and possibly some of the components referred to above which could be derived from

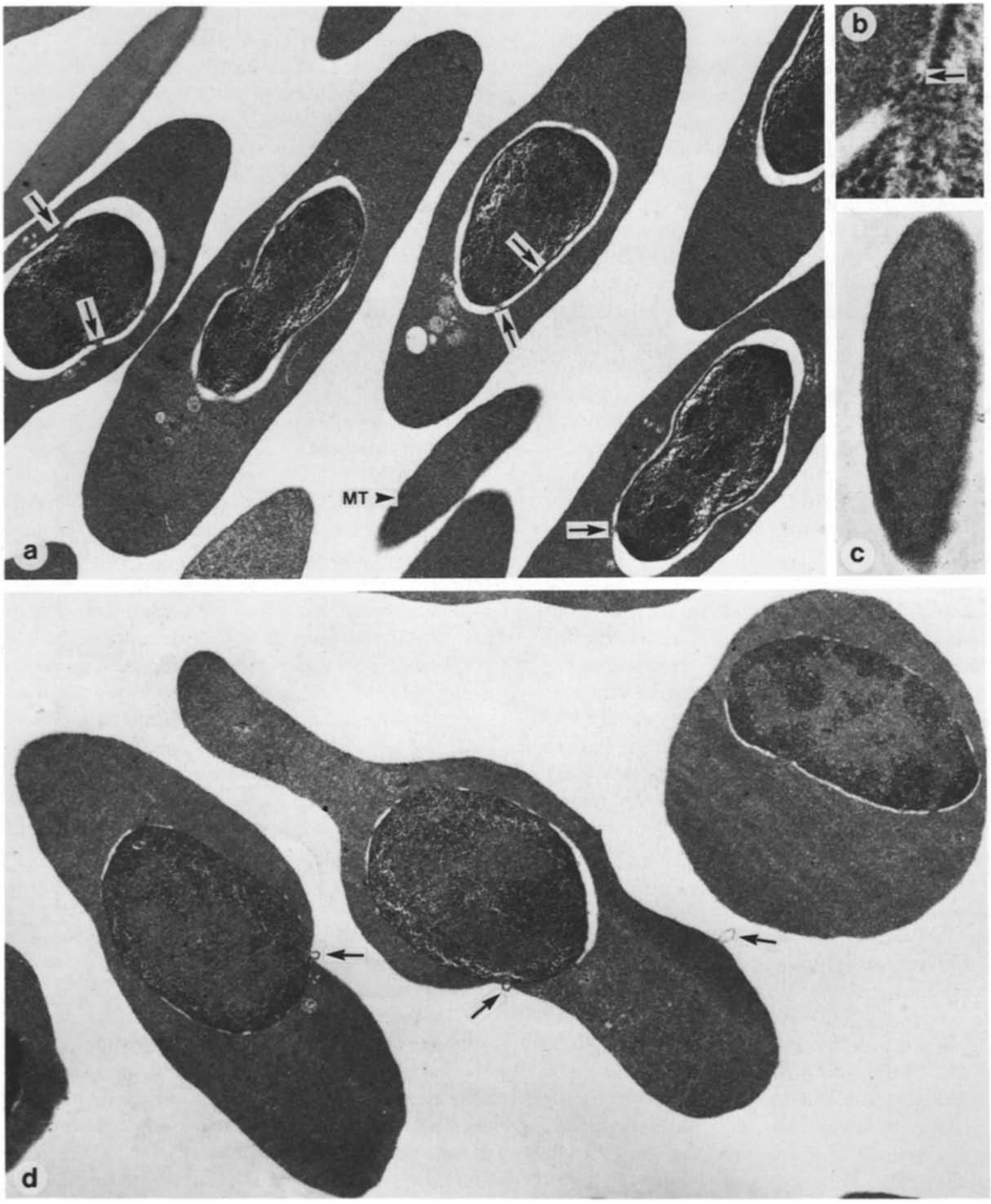
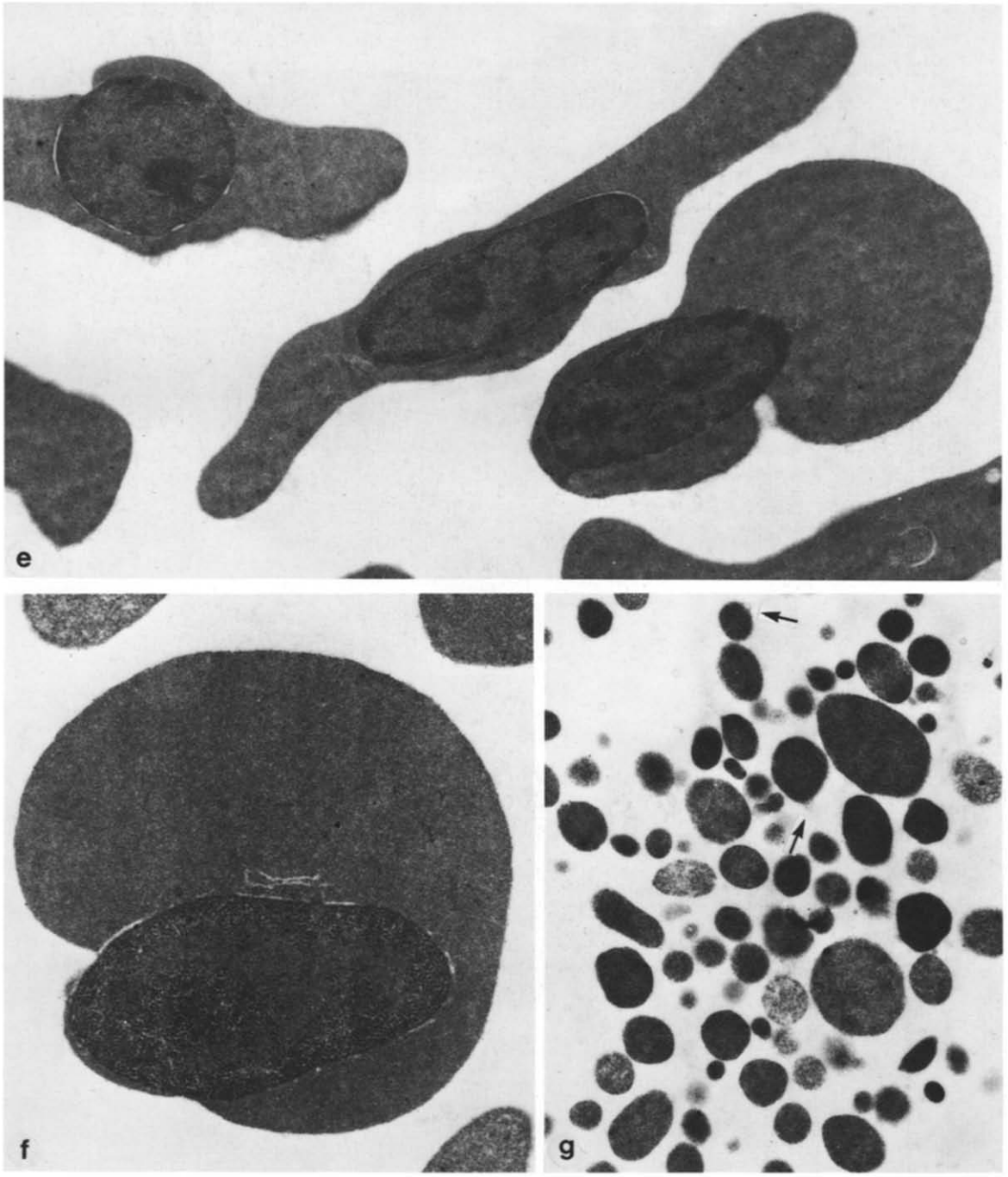


Fig. 2. Electron micrographs of chicken erythrocytes before and after treatment with A23187 and Ca^{2+} at 37°C. (a) Untreated cells (×10000). Arrows indicate bridges between nucleus and cytoplasm. MT indicates where marginal microtubules are visible. (b) Detail of bridge (×60000). (c) Detail of microtubules in a tangential section from a control cell (×15000). (d), (e) Cells treated with A23187 and Ca^{2+} at 37°C for 5 min and 20 min, respectively ((d)×12500, (e)×10000). Arrows in (d) indicate membrane blebs. (f) Detail of cell after 20 min incubation showing apparent partial exocytosis of nucleus (×17500). (g) Microvesicles (×12500). Cells were



incubated as described under Methods and were fixed in suspension with 1.5% glutaraldehyde, washed several times with isotonic NaCl buffer (pH 7.4) and post-fixed with 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4). The preparation was dehydrated in ethanol and embedded in Spurr resin. Sections were cut with an LKB ultramicrotome, stained in methanolic uranyl acetate followed by Sato's lead stain and examined using a JEOL 100CX electron microscope.

TABLE I

LIPID COMPOSITION OF CELLS, PLASMA MEMBRANE, NUCLEI AND MICROVESICLES.

Values for phospholipid composition are expressed at % of total phospholipid. All results are given as mean ± S.D. with number of experiments in parentheses. Abbreviations: SM, sphingomeylin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PL, total phospholipid; Chol, cholesterol; Hb, haemoglobin. Combined values for PI + PS are given were indicated.

	SM	PC	PI	PS	PE	PL/Hb (nmol/mg)	Chol/Hb (nmol/mg)	Chol/PL (mol/mol)
Control cells	17.0 ± 1.3 (10)	41.2 ± 1.4 (10)	3.9 ± 0.3 (5)	8.9 ± 1.2 (5)	29.8 ± 1.9 (10)	36.1 ± 1.9 (6)	18.7 ± 2.1 (6)	0.48 ± 0.06 (14)
Treated cells (20 min)	14.0 ± 1.6 (10)	42.9 ± 1.6 (10)	3.8 ± 0.6 (5)	9.6 ± 1.6 (5)	30.7 ± 2.4 (10)	32.3 ± 2.8 (4)	17.4 ± 1.8 (4)	0.50 ± 0.07 (14)
Plasma membranes	24.0 ± 3.2 [a] (7)	26.5 ± 3.1 (7)	1.5 ± 1.5 (7)	16.6 ± 4.1 (7)	31.4 ± 1.9 (7)	–	–	0.69 ± 0.11 (9)
Nuclei	6.0 ± 0.4 [a] (4)	52.4 ± 0.5 (4)	7.5 ± 0.5 (4)	4.2 ± 0.3 (4)	29.9 ± 0.4 (4)	–	–	0.21 ± 0.08 (6)
Microvesicles 2 min	18.0 ± 0.5 (3)	38.9 ± 1.0 (3)	13.7 ± 1.3 (3)		29.4 ± 1.5 (3)	– –	– –	–
Microvesicles 5 min	17.7 ± 0.4 (4)	39.6 ± 1.2 (4)	13.3 ± 0.6 (4)		29.4 ± 1.0 (4)	–	–	–
Microvesicles 10 min	16.6 ± 0.9 (5)	38.8 ± 0.9 (5)	13.9 ± 0.6 (5)		30.7 ± 1.0 (5)	–	–	–
Microvesicles 15 min	16.3 ± 1.0 (5)	39.2 ± 1.3 (5)	14.2 ± 1.7 (5)		30.3 ± 1.7 (5)	–	–	–
Microvesicles 20 min	14.8 ± 2.2 (6)	40.8 ± 2.1 (6)	4.9 ± 1.2 (4)	11.5 ± 2.6 (4)	29.1 ± 3.5 (6)	174.8 ± 4.0 (4)	87.5 ± 2.0 (4)	0.55 ± 0.07 11)
Microvesicles 30 min	14.4 ± 1.0 (5)	39.6 ± 0.3 (5)	15.0 ± 0.9 (5)		31.0 ± 0.9 (5)	–	–	–

[a] Including ceramide (see Methods).

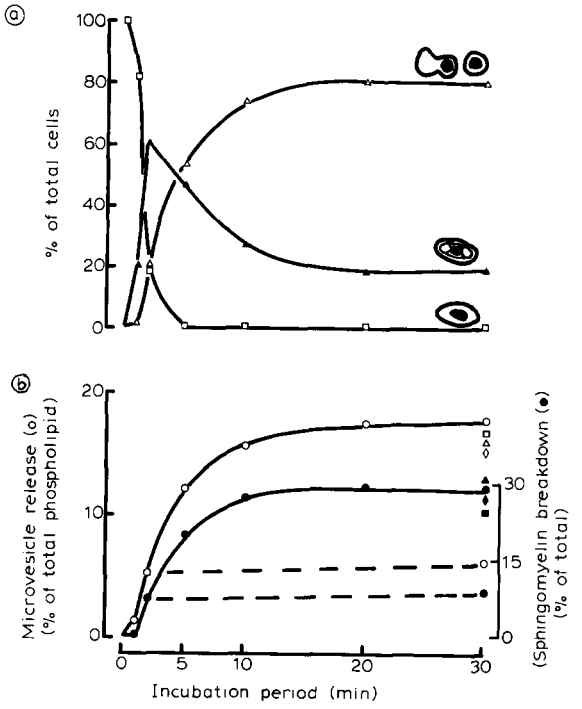


Fig. 3. Time-course of (a) changes in cell morphology (b) microvesicle release and sphingomyelin breakdown in chicken erythrocytes treated with A23187 and Ca^{2+} at 37°C. Results are shown for a single representative experiment (one of four similar experiments). In (a) cells were counted differentially after fixation with glutaraldehyde as described under Methods. Points are means of triplicate determinations. □, normal cells; ▲, 'spoked' cells; △, refractile cells. In (b) microvesiculation (open symbols) and sphingomyelinase activity (closed symbols) were measured as described under Methods. Points are means of duplicate determinations. Dashed lines refer to samples where EGTA was added at 2 min and incubation was continued for a further 28 min. Squares, triangles and diamonds refer to incubations with hypertonic NaCl, quinine (0.4 mM) or isotonic KCl, respectively.

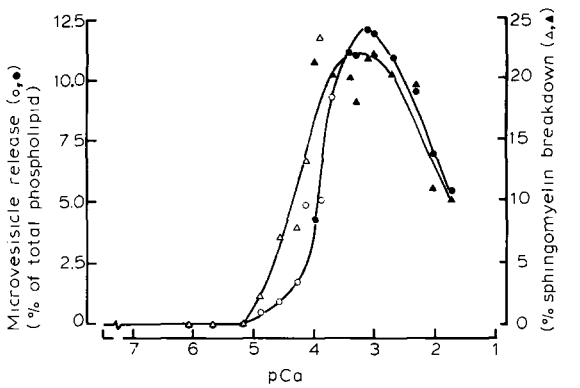


Fig. 4. Dependence of microvesiculation and sphingomyelinase activity on Ca^{2+} in chicken erythrocytes treated with A23187. 5 min incubations at 37°C were carried out as described under Methods. Results are shown for a representative experiment (one of five similar experiments). Microvesiculation (△, ▲) is expressed as % of total cell phospholipid released. Sphingomyelin breakdown (○, ●) is expressed as % of total sphingomyelin present in the cells. Open symbols (△, ○) refer to incubations in HEDTA-Ca buffers. Closed symbols (▲, ●) refer either to incubations in citrate-Ca buffers (up to p*Ca* 3) or to incubations with unbuffered $CaCl_2$ (above p*Ca* 3). Half maximum microvesiculation was seen at p*Ca* 3.9±0.1 and 2.0±0.2; half maximum sphingomyelin breakdown was at p*Ca* 4.2±0.2 and 2.0±0.2 (means of five experiments).

nuclear membrane. No significant alterations in polypeptide pattern were seen with microvesicles isolated after various times of incubation (Fig. 5).

The lipid composition of whole chicken erythrocytes, plasma membranes, nuclei and microvesicles is shown in Table I. The values for whole erythrocytes are similar to those found by previous investigators with the exceptions that we have measured substantial quantities of phosphatidylserine, which others have not detected [32] and also that cholesterol content was somewhat lower than found previously [32,33]. This latter finding did not appear to be due to a methodological error, since human erythrocytes assayed under the same conditions gave cholesterol values close to those in the literature [34].

The overall lipid composition of the plasma membranes agreed broadly with previous reports [32,33] except that cholesterol content was lower (but was nevertheless markedly enriched relative to whole cells) and phosphatidylserine was present in comparatively large amounts. From these figures, the plasma membrane appeared to account for most of the phosphatidylserine of the cells, assuming that plasma membrane represented about half of the total membranes of these cells [32]. In contrast the nuclear fraction contained most of the phosphatidylinositol of the cells and was relatively poor in sphingomyelin and cholesterol.

Microvesicles had a lipid composition which was distinctly different from that of plasma membranes or nuclei and resembled much more closely the composition of whole cells. The microvesicles had a substantially lower sphingomyelin content than untreated cells but in this respect resembled treated cells which lost about 20% of their original

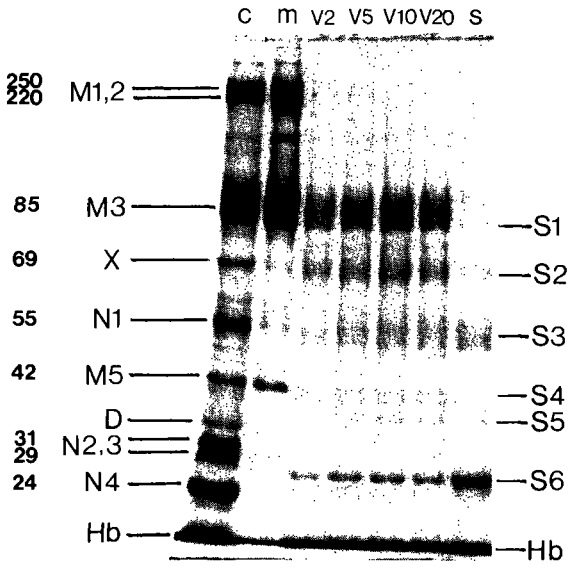


Fig. 5. Polyacrylamide gel electrophoresis of cells and fractions. The procedure used for electrophoresis is described under Methods. Loading was about 20 nmol lipid phosphorus for each sample except for the sample of cell supernate where about 400 μg of protein was loaded. Molecular weights ($\times 10^{-3}$) of the various components are shown on the left of the figure. Abbreviations: c, particulate fraction from lysed cells; m, plasma membranes; v2, v5, v10, v20, microvesicles isolated after 2, 5, 10, 20 min incubation with A23187 + Ca^{2+}; s, supernate from lysed cells. Bands labelled M are believed to be derived from the plasma membrane; bands labelled N are believed to be derived from the nucleus or nuclear membrane; bands labelled S are believed to be from the soluble fraction of the cells. The origin of band X is not certain but it may be a nuclear membrane protein. D is deoxyribonuclease which was added to sample c. Hb, haemoglobin.

sphingomyelin during the course of the incubation with A23187 and Ca^{2+} (Table I).

Sphingomyelinase activity in chicken erythrocytes

The loss of sphingomyelin from cells treated with A23187 and Ca^{2+} appeared to be due to the action of an endogenous phospholipase C (sphingomyelinase) since the products of breakdown were identified as ceramide (*N*-acylsphingosine) and phosphorylcholine (see Methods). A maximum of 20–30% of the total sphingomyelin in the cells could be broken down by treatment with A23187 and Ca^{2+} and very significantly, phosphorycholine was not released into the medium: it was retained inside the cells (Table II), suggesting that the pool of sphingomyelin which was attacked by the endogenous enzyme was in the cell interior. Consistent with this interpretation was the observation that non-lytic attack of *C. welchii* phospholipase C on intact chicken erythrocytes degraded a maximum of 65–70% of the total sphingomyelin (Fig. 6). When however, untreated cells were lysed osmotically and incubated, even in the presence of EGTA, almost complete degradation of the sphingomyelin occurred. (Fig. 7). It was therefore clear that in lysed cells, the endogenous enzyme not only had access to the total pool of sphingomyelin but also did not require Ca^{2+} for its activity.

These findings regarding the activity and Ca^{2+} independence of the endogenous sphingomyelinase were consistent with previous reports [14,15] but

TABLE II

PHOSPHORYLCHOLINE IS RELEASED INSIDE CHICKEN ERYTHROCYTES TREATED WITH A23187 AND Ca^{2+}

Cells were incubated for 20 min with or without A23187 and Ca^{2+} (see Methods) and then were sedimented. The supernate was retained for analysis of extracellular phosphorylcholine. The cells were extracted with methanol/chloroform (see Methods) and the upper (aqueous) layer from the Bligh and Dyer separation [2] was analysed for phosphorylcholine (assumed to be derived from the cell interior). Determinations of sphingomyelin and phosphorylcholine were as described under Methods. Values are expressed as nanomoles ± S.D. per 0.2 ml of packed cell (number of determinations in parentheses). The results are derived from two experiments on separate samples but almost identical results were obtained in one further experiment. Recoveries were not corrected for losses occurring during isolation of phosphorylcholine.

	Control	A23187 + Ca^{2+}	Phosphoryl-choline	% Recovery
Sphingomyelin	263 ± 15 (6)	205 ± 6 (6)	58	-
Phosphorylcholine				
Extracellular	6.1 ± 0.6 (6)	6.5 ± 0.8 (6)	0.5	1
Intracellular	32 ± 5 (6)	72 ± 9 (6)	40	69

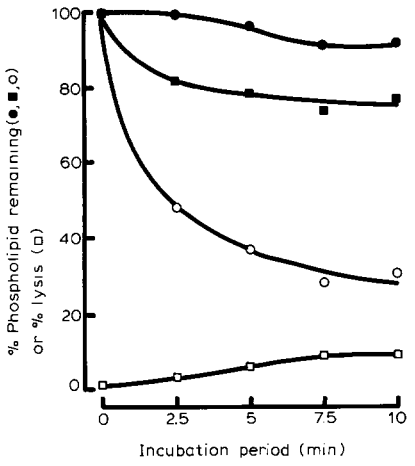


Fig. 6. The action of *C. welchii* phospholipase C on chicken erythrocytes. Incubations and lipid analyses were carried out as described under Methods. Points represent means of duplicate determinations which differed by less than 5% and are derived from a single experiment. In three such experiments, the mean maximum % breakdown of sphingomyelin was 63% and of phosphatidylcholine was 21%. ●, phosphatidylethanolamine + phosphatidylserine; ■, phosphatidylcholine; ○, sphingomyelin; □, cell lysis.

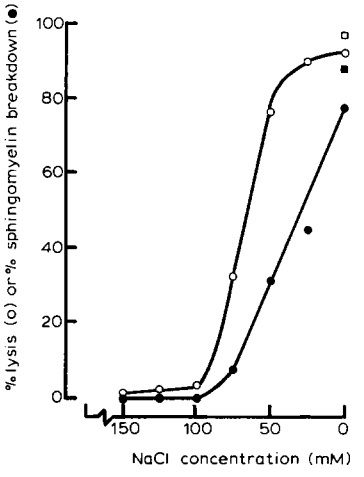


Fig. 7. Sphingomyelin breakdown and cell lysis in chicken erythrocytes exposed to hypotonic media in the presence of EGTA. Incubation conditions and measurement of lysis (○, □) and sphingomyelin breakdown (●, ■) are described under methods. Squares refer to incubations to which were added 1% Triton X-100. Points represent means of duplicate determinations in a single representative experiment (one of three similar experiments in which the mean maximum breakdown of sphingomyelin was 82%).

appeared to conflict with the data on intact cells treated with A23187 and Ca^{2+} (Fig. 3b) where breakdown of the internal pool of sphingomyelin occurred only in the presence of Ca^{2+}.

In general, Ca^{2+}-dependent breakdown of sphingomyelin occurred in parallel with release of microvesicles, having a similar time course (Fig. 3b), Ca^{2+} dependency (Fig. 4) and temperature dependency (not shown). Although it has been reported that the endogenous sphingomyelinase in plasma membranes is temperature-sensitive [14], we were unable to permanently inactivate the enzyme by exposure of whole cells to 50°C for 10 min (results not shown).

The possibility was considered that microvesiculation and/or sphingomyelinase activation in chicken cells could result from a depletion of intracellular ATP caused by A23187 and Ca^{2+}. Human erythrocyte ATP levels are decreased dramatically by a similar treatment [11,35] and there is evidence that ATP depletion per se may cause microvesiculation and also may increase the susceptibility of membrane lipids to attack by exogenous phospholipases [38–41]. However, treatment of chicken erythrocytes with iodoacetamide and deoxyglucose, which decreased ATP levels to less than 10% of normal did not induce either microvesiculation or breakdown of sphingomyelin (results not shown).

Discussion

The morphology of chicken cells trated with A23187 and Ca^{2+}

The normal chicken erythrocyte is lenticular in shape with a nucleus which is invariably found near the centre of the cell. Treatment with A23187 and Ca^{2+} leads to a complex variety of morphological alterations, some of which have been described previously [4,6]. The initial change, which is observable by phase-contrast microscopy, is characterised by an increase in contrast of the nucleus which develops 'spoke-like' structures radiating from it (Fig. 1). Corresponding structures are not apparent in the electron microscope either because they are not fixed under the conditions we have used or because they represent an optical effect possibly due to a redistribution of

water within the cells *. Other workers [27] have described a small (10%) shrinkage of chicken cells induced by A23187 + Ca^{2+} but we have not observed a consistent change in cell volume during this treatment. A 4-5% decrease in volume would be in accord with the observed loss of haemoglobin as microvesicle contents.

Subsequent changes in the morphology of treated cells lead to the formation of cells which are refractile in the phase-contrast microscope and which become progressively more spherical, probably due to the concomitant loss of surface membrane as microvesicles (Figs. 1, 2). The nucleus of these cells is no longer located centrally but is often found immediately adjacent to the plasma membrane, suggesting that the constraints which normally confine the nucleus to the centre of the cell are not operative in the presence of high levels of intracellular Ca^{2+}. It has been suggested [42,43] that a fibrillar network exists in avian erythroctyes which may have the function of holding the nucleus in place and although we have no direct evidence for its existence, the dissolution of such a structure could explain the altered appearence of the nucleus under phase-contrast optics immediately following treatment with A23187 and Ca^{2+}. The apparent disappearance of marginal microtubules in treated cells seems not to parallel these early changes but to be related to the morphological transition from ellipsoidal cells to irregular cells which possess an eccentric nucleus and which release microvesicles.

Although Vos et al. [4] and Volsky and Loyter [6] induced extensive cell-fusion by treatment of chicken erythrocytes with A23187 and Ca^{2+}, we were unable to find evidence for such an effect under our experimental conditions. This disparity probably arises because the above authors used cells which were neuraminidase-treated and incubated at 47°C [4] or cells which were ATP-depleted [6], whereas we worked with fresh cells at 20 or 37°C. However we did obtain good evidence for the occurrence of two different membrane fusion events, one which involved the release of microvesicular material from the cells and the other which appeared to represent a completely novel type of membrane fusion viz. the fusion of nuclear membrane with plasma membrane.

Although the possibility of fusion between nuclear membrane and plasma membrane in intact cells does not seem to have been considered previously, there is no intrinsic reason why such a process should be any less likely than fusion between a secretory granule membrane and plasma membrane. Conclusive proof of nuclear-plasma membrane fusion might be provided by the observation of exocytosis of nuclei lacking a nuclear membrane, but in our experiments, such 'bald' nuclei or ennucleated cells were not observed following treatment with A23187 + Ca^{2+}. However there are several lines of evidence that support the idea that fusion of nuclear and plasma membranes does occur under our experimental conditions. Firstly, the direct evidence of electron microscopy shows that a very close approach between nuclear and plasma membranes occurs in treated cells, and that at these membrane junctions, blebs appear to form (Fig. 2d), which are qualitatively similar to those observed by Lawson et al. [45] in mast cells undergoing exocytosis. Secondly, the lipid composition of the microvesicles released from the cell surface is not characteristic of the composition of the plasma membrane (as it is in human erythrocytes treated in the same fashion [7]) but instead resembles the lipid composition of whole cells (Table I). This implication that the microvesicle lipids are derived from both plasma and nuclear membranes, is consistent with the hypothesis that fusion of these two membranes occurs prior to microvesicle release. It is difficult to imagine how nuclear membrane lipid could appear outside the cells, free of the skeletal proteins of the plasma membrane (spectrin, actin, etc.) and in the absence of significant lysis, unless fusion had occurred between nuclear and plasma membranes. A further factor which supports this idea is that the amount of lipid which is released as microvesicles (up to 25% of the total lipid) is too large to be

* The electron-transparent region surrounding the nucleus seen in sections from control cells and from cells treated for short periods with A23187 + Ca^{2+} has been observed previously [42] and whilst it may be an artifact of the preparative procedure for electron microscopy, it could represent a real morphological feature of these cells. It is not clear why cells treated for longer periods do not show this feature and the significance of the amorphous material bridging the gap between nucleus and cytoplasm (Fig. 2 and also reported in Ref. 42) is not understood. One possibility is that the amorphous material represents the sites of nuclear pores [44].

accounted for solely in terms of plasma membrane loss. It also seems possible that loss of lipid from the cell nucleus may explain the observed tendency of the nuclei of treated cells to become more spherical.

There was some indication that one putative membrane protein (X in Fig. 5) was present in the microvesicles. The failure to find other such nuclear membrane proteins in the microvesicles may simply indicate that nuclear membranes do not possess many intrinsic proteins. This interpretation is supported by the work of Dwyer and Blobel [30] who found that most of the nuclear membrane proteins were associated with nuclear pores and their connecting lamina which would not be expected to partition into microvesicles.

Our failure to observe exocytosis of 'bald' nuclei could be due to restraints on the nucleus exercised by the bridges of amorphous material (Figs. 2a, b) which may represent the sites of nuclear pores.

The relation of nuclear-plasma membrane fusion to microvesiculation and to Ca^{2+}

Assuming the absence of membrane microheterogeneity, it seems likely that the composition of the microvesicles released at any given moment would represent the instantaneous composition of the lipid bilayer portion of the surface membrane. Now even at the earliest incubation times, the microvesicles released had the composition of a mixed nuclear-plasma membrane (Table I) and this suggested that fusion of the nuclear membrane with the plasma membrane occurred prior to release of microvesicles. Indeed, it is possible that the expansion of the bilayer portion of the plasma membrane by nuclear membrane material and in the absence of a similar expansion of the membrane skeleton (spectrin, actin, etc.) could be the motive force which led to loss of bilayer material in the form of microvesicles. However, this factor cannot by itself be sufficient to cause microvesiculation since removal of Ca^{2+} from the incubation at any time during incubation immediately halted microvesiculation (Fig. 3b). Unless microvesicle release occurred very rapidly and completely after nuclear-plasma membrane fusion in each individual cell, then these findings would suggest a requirement similar to that in human erythrocytes for the continuous presence of Ca^{2+} in order for microvesiculation to occur [2].

In human cells, the crucial role of Ca^{2+} in microvesiculation might depend on its interaction with phosphatidylserine [2]. It has been proposed that this anionic lipid plays a vital role in the maintenance of lipid bilayer structure [46,47] and also that interactions between phosphatidylserine and spectrin stabilize the association between the bilayer and the membrane skeleton [48]. Titration of phosphatidylserine with Ca^{2+} is thus supposed to cause destabilisation. In a previous report no phosphatidylserine was found in chicken erythrocytes [33] but we have found substantial quantities of this lipid, which appears to be primarily localised in the plasma membrane (Table I). Despite the close similarities in composition between chicken and human plasma membranes it does not appear from our results that Ca^{2+} induces microvesiculation of the original plasma membrane in chicken cells as it does in human erythrocytes. Microvesiculation in chicken cells only seems to occur when nuclear membrane material has been inserted into the surface membrane but it is possible that an interaction of Ca^{2+} with plasma membrane phosphatidylserine is a crucial factor in enhancing the likelihood of fusion between the two initially separate membranes.

Sphingomyelinase activation in chicken erythrocytes

Sphingomyelinase activity in chicken erythrocyte plasma membranes has been described before [14,15] but until now, it had not been demonstrated to occur in intact cells. Our results show that the introduction of Ca^{2+} into intact chicken erythrocytes leads to the rapid breakdown of up to one third of the total sphingomyelin. This occurs despite the apparent Ca^{2+} independence of enzyme activity (Ref. 14) and significantly, the sphingomyelin which is broken down seems to be present on the inside of the cells since the phosphorylcholine which is produced appears in the cell interior, not in the medium (Table II).

The precise localisation of the internal pool of sphingomyelin is unknown, but the data for the lipid composition of isolated nuclei and plasma membranes (Table I) suggests that about 20–25% of the total cell sphingomyelin is in the nuclear membrane. It therefore seems likely that a considerable proportion of the sphingomyelin that is

degraded in cells treated with A23187 and Ca^{2+} could be nuclear membrane sphingomyelin. In normal cells, the sphingomyelinase which is present in the plasma membrane would not have access to sphingomyelin in the nuclear membrane, since cytoskeletal elements in the cytoplasm appear to prevent a close approach of plasma membrane to nucleus. This factor could at least in part explain the apparent latency of sphingomyelinase in untreated cells.

The effect of introducing Ca^{2+} into chicken erythrocytes appears to be the dissolution of cytosolic cytoskeletal structures, thus allowing a very close approach of the nucleus to the plasma membrane (Fig. 2). This may allow access of the plasma membrane sphingomyelinase to its substrate in the nuclear membrane with a consequent breakdown of the 20–30% of the total cell sphingomyelin.

At present we cannot exclude the interesting possibility that the breakdown of nuclear membrane sphingomyelin may then be important in mediating the fusion between nucleus and plasma membrane which appears to occur and which leads subsequently to the release of microvesicles from the cells.

Further work will be required in order to establish the precise relationships between the Ca^{2+}-induced dissolution of cytoskeletal structures, the apposition of nucleus and plasma membrane, sphingomyelin breakdown, nuclear-plasma membrane fusion and microvesiculation in this complex system.

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