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LIMITED BREAKDOWN OF CYTOSKELETAL PROTEINS BY AN ENDOGENOUS PROTEASE CONTROLS Ca^{2+}-INDUCED MEMBRANE FUSION EVENTS IN CHICKEN ERYTHROCYTES

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The profound morphological changes which follow the treatment of chicken erythrocytes with the ionophore A23187 and Ca^{2+} are associated with a concomitant breakdown of certain membrane-associated proteins including α-spectrin, goblin and microtubule-associated proteins (MAPS) which undergo a limited proteolysis to give large, well-defined fragments. The Ca^{2+}-sensitive protease responsible for these changes appears to be present in the soluble fraction of the cells. Treatment with TLCK or iodoacetamide inhibits both the major morphological changes and the proteolytic events but these agents do not prevent the dissociation of microtubules or the activation of endogenous sphingomyelinase which occur in cells with raised levels of intracellular Ca^{2+}. It is suggested that the sphingomyelinase is activated as a consequence of a Ca^{2+}-induced loss of phospholipid asymmetry in the plasma membrane.

Introduction

Of the variety of different biochemical events which result from the introduction of abnormally high levels of Ca^{2+} into erythrocytes [1], perhaps the most striking are the morphological alterations. These include echinocytosis, loss of microvesicles [1–3] and in some circumstances, cell fusion [4]. The mechanism of these changes is still obscure even in such simple cells as human erythrocytes although there are indications that Ca^{2+}-mediated degradation of proteins and lipids may be involved [1,5]. Chicken erythrocytes are considerably more complicated since they possess a nucleus with its associated nuclear membranes and a well-developed cytoskeleton which includes a marginal band of microtubules [6] and intermediate filaments [7–9].

Recent work [10] from this laboratory has shown that chicken erythrocytes treated with A23187 and Ca^{2+} undergo complex changes in morphology and composition. Under the influence of Ca^{2+}, the nuclei of these cells appear to undergo fusion with plasma membrane to form a hybrid surface membrane from which spectrin-free microvesicles are released into the extracellular medium. In addition there is concomitant activation of a normally latent plasma membrane sphingomyelinase which breaks down internal sphingomyelin and also a loss of the marginal band of microtubules. We now present evidence that well-defined proteolytic events occur

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Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; Mops, 3-(*N*-morpholino)propanesulphonic acid; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone.

in parallel with the morphological changes and suggest that breakdown of certain proteins associated with the membrane skeleton is a vital part of the mechanism of nucleus-plasma membrane fusion and microvesicle release.

Materials

Ionophore A23187 was obtained from Calbiochem-Behring Corp. (CP Laboratories Ltd., Bishop Stortford, Herts, U.K.). Molecular weight standards were supplied by Boehringer, Mannheim, F.R.G. and all other reagents were from either Sigma Chemical Co. (Poole, Dorset, U.K.) or BDH (Poole, Dorset, U.K.).

Methods

Preparation and incubation of cells. Blood was obtained as described previously [10] from Rhode Island Red hens, using EGTA as anticoagulant. The erythrocytes 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 150 mM NaCl with removal of any remaining buffy coat and were then given a final wash with 130 mM NaCl, 20 mM Mops/NaOH (pH 7.1).

Except where otherwise stated, cells were incubated at a haematocrit of 10% in 130 mM NaCl, 20 mM Mops/NaOH (pH 7.1), with 1 mM $CaCl_2$ and 5 μM A23187 for 5 min at 37°C. Incubations were stopped by addition of 100 mM EGTA to a final concentration of 5 mM. Cells and microvesicles were isolated as described previously [10], and the microvesicles were quantified by measurement of their phospholipid content [10].

Preparation of cytosolic fraction. The soluble fraction of the cells was obtained by lysis of one volume of cells in one volume of 10 mM Hepes/NaOH, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.4. The nucleated ghosts, free nuclei and plasma membranes were sedimented by centrifugation at $30\,000 \times g$ for 30 min in a Sorvall RC-5B superspeed centrifuge and the supernatant was removed and designated the 'cytosolic fraction'.

Preparation and incubation of plasma membranes. Plasma membranes for SDS-polyacrylamide gel electrophoresis were prepared from chicken erythrocytes using the method of Beam et al. [11], with addition of 1 mM EGTA to the lysing medium. Plasma membranes used for incubation were prepared similarly but the Tris/HCl buffer was replaced by 10 mM Hepes/NaOH at pH 7.4.

In experiments designed to localise the Ca^{2+}-sensitive protease activity 50-μl aliquots of plasma membrane were incubated for 5 min at 37°C with: (a) 50 μl of 10 mM Hepes/NaOH buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM EGTA with or without addition of 2 mM $CaCl_2$; (b) 50 μl cytosolic fraction with or without the addition of 2 mM $CaCl_2$; (c) 50 μl of boiled cytosolic fraction with or without $CaCl_2$ and (d) 10 mM cysteine with or without $CaCl_2$. Samples were run on polyacrylamide gels as described below.

Preparation of Triton shells. 0.1 ml of packed washed cells were incubated for 10 min at 37°C in 0.9 ml of a medium containing 130 mM KCl, 20 mM Mops/KOH (pH 7.1), 5 μM A23187 with the addition of: (a) 1 mM EGTA; (b) 1 mM $CaCl_2$; (c) 1 mM $CaCl_2$ + 2 mM TLCK. Incubation was terminated by addition of EGTA to a final concentration of 4 mM and then the cell suspension was diluted in 10 ml of 100 mM Mops/KOH, 5 mM $MgCl_2$, 1 mM EGTA (pH 6.8) containing 0.5% Triton X-100. After centrifugation at $750 \times g$ for 1 min, the supernate was discarded and the pellet was resuspended in the above buffer without Triton. Samples were fixed with glutaraldehyde (2% final concentration) for 30 min, washed four times with water and then examined in the electron microscope using negative staining.

Polypeptide analysis and quantification. Analysis of the polypeptide composition was carried out by SDS-polyacrylamide gel electrophoresis on 3–13% gradient gels using the buffer system of Laemmli [12] with omission of the stacking gel and the addition of sucrose as the gradient former.

Polypeptides were quantified, after staining of the gels with Coomassie Brilliant Blue, by scanning with a Joyce-Loebl Chromoscan 201 linked to a Shimadzu C-R1B integrator.

All other procedures were as described previously [10].

Results

Fig. 1 (b,c) shows the dramatic changes which occur in the polypeptide pattern of plasma mem-

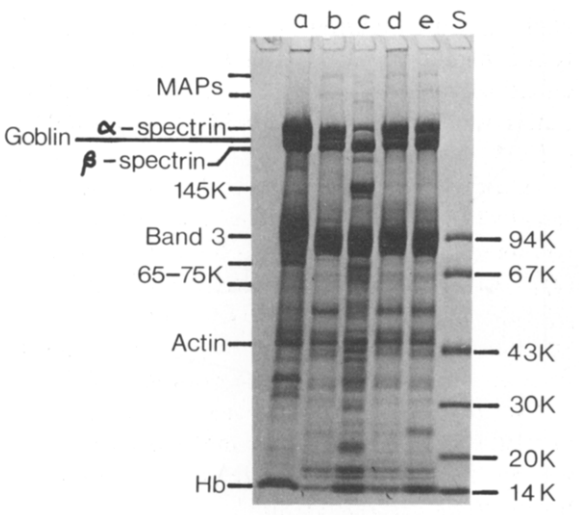

Fig. 1. Polyacrylamide gel patterns of erythrocyte membranes from cells treated with ionophore A23187 and Ca^{2+} and with TLCK or iodoacetamide. Chicken cells were incubated with A23187 and Ca^{2+} with or without TLCK or iodoacetamide and plasma membranes prepared as described under Methods. Samples containing about 20 μg of protein were run on gradient (3–13%) polyacrylamide gels. (a) Human erythrocyte membranes; (b) control chicken erythrocyte plasma membranes from cells incubated without A23187 for 2 min at 37°C; (c) chicken plasma membranes from cells incubated for 2 min with A23187 and Ca^{2+} at 37°C; (d) and (e) as (c) but with the addition of 1 mM TLCK (d) or 10 mM iodoacetamide (e). (S) standard molecular weight markers. Similar results were seen in five other experiments.

branes when cells are treated with A23187 and Ca^{2+}. The major alterations appear to affect the high molecular weight proteins, in the range 240 000 to 340 000. The heaviest components, of molecular weight 340 000 and 320 000 may correspond to the microtubule-associated proteins which have been reported previously [13], and these appear to be converted into somewhat lower molecular weight polypeptides (330 000, 300 000) in treated cells. α-Spectrin (M_r 270 000) and a protein migrating between α- and β-spectrin which may correspond to the protein 'goblin' reported in turkey erythrocytes [11], underwent the most marked changes when cells were exposed to A23187 and Ca^{2+}. The amounts of these polypeptides were reduced by 60–90% and there was a reciprocal increase in the amounts of lower molecular weight polypeptides (mainly 145, 70 and 20 kDa but with smaller and more variable quantities of components at 200 and 130 kDa). It was not easy to decide which of these assumed breakdown products were derived from α-spectrin and which from goblin but it seemed possible that polypeptides of similar molecular weight were produced from both high molecular weight components, since the 145 kDa product was often resolved as a doublet whilst two separate products were always seen in the 65–75 kDa region.

A loss of components of molecular weight 50–55 kDa possibly corresponding to tubulin and vimentin [7,14,15] was also seen in membranes from treated cells but since no identification of breakdown products was made it was not clear whether these components were actually degraded or whether they could have been lost physically into the soluble fraction of the treated cells.

The above changes in polypeptide pattern correlated well with the morphological changes previously described [10] which led to the release of microvesicles from the cells (Figs. 2,3). At 5°C, loss of microvesicles occurred with a concomitant breakdown of α-spectrin but with little indication of any attack on β-spectrin or band 3 polypeptide (Fig. 2a). However at 37°C, loss of up to a third of the band 3 protein and β-spectrin was also observed (Fig. 2b).

The changes in polypeptide pattern and the microvesicle release induced by A23187 and Ca^{2+} were almost completely prevented by preincubation of the cells with 1 mM TLCK or 10 mM iodoacetamide (Fig. 1 and Table I) but not by preincubation with other well known protease inhibitors such as TPCK, phenylmethylsuphonyl fluoride or diisopropylfluorophosphate (each 2 mM). TLCK or iodoacetamide arrested the Ca^{2+}/A23187-treated cells in the intermediate morphological state previously described as 'spoked cells' [10] (Fig. 4b). These cells were obviously deformed, and possessed a more rounded nucleus with a smaller perinuclear space than either control cells [10] or cells treated only with TLCK (Fig. 4a). The nuclei of spoked cells were never observed to come into contact with plasma membrane, in marked contrast to cells which were treated with Ca^{2+}/A23187 in the absence of TLCK or iodoacetamide where nuclear-plasma membrane interaction has been shown to precede release of microvesicles [10].

Examination of 'Triton shells' derived by ex-

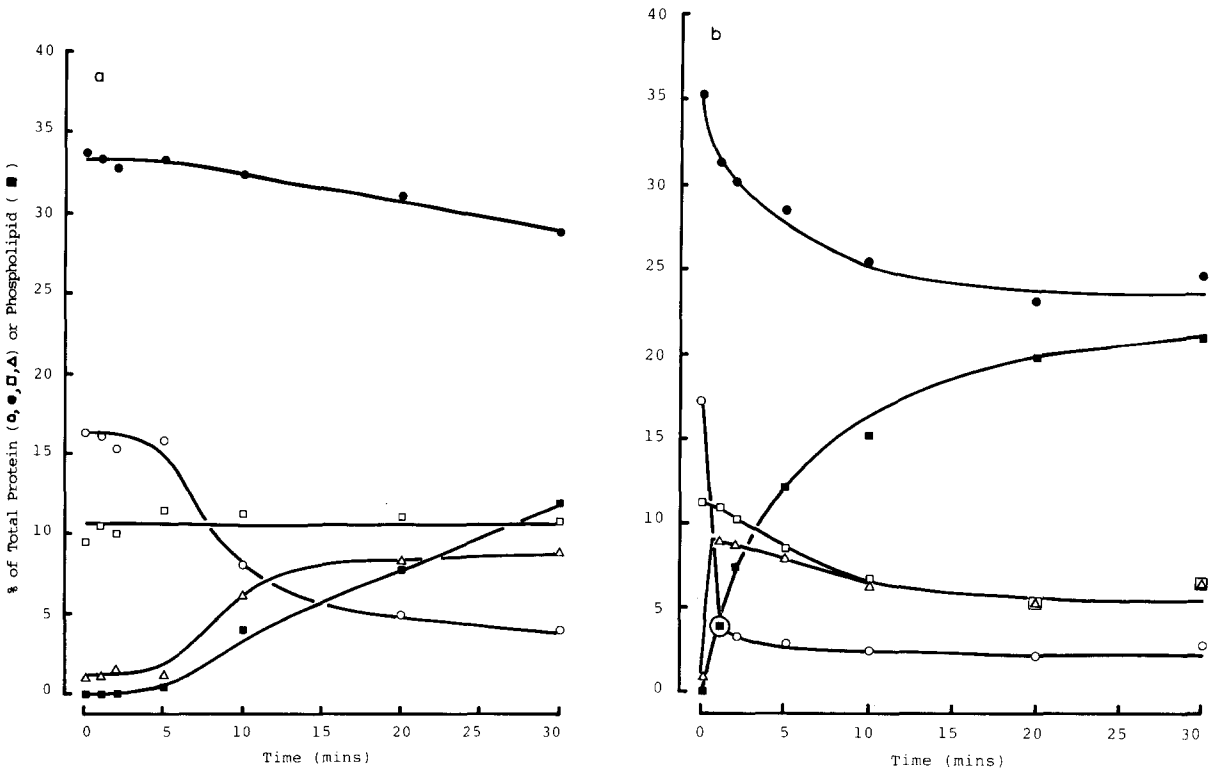

Fig. 2. Time course of changes in polypeptides and of microvesicle release in chicken erythrocytes treated with ionophore A23187 and Ca^{2+}. Cells were incubated with A23187 and Ca^{2+} as described under Methods at (a) 5°C or (b) 37°C. Membranes were prepared from cells treated for various times up to 30 min and polypeptides were analysed by polyacrylamide gel electrophoresis. Results are expressed as the percentage of total polypeptide present in each individual band as measured by densitometric scanning. The total amount of staining material was unchanged in the treated samples compared with controls. ●, Band 3; ○, α-spectrin; □, β-spectrin; △, 145 kDa polypeptide; ■, % of total phospholipid released as microvesicles. Similar results were obtained in three further experiments.

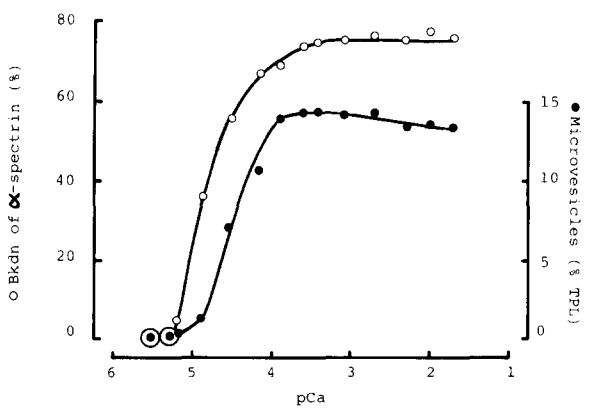

Fig. 3. The influence of Ca^{2+} concentration on breakdown of α-spectrin and on release of microvesicles from cells treated with ionophore A23187. Cells were incubated for 5 min at 37°C as described under Methods in the presence of A23187 (5 μM) and different concentrations of Ca^{2+}. Below 1 mM Ca^{2+}, calcium-citrate buffers were employed. Microvesicle release (●) was measured as % of total cell phospholipid and α-spectrin breakdown (○) was determined after isolation of plasma membranes and analysis of polypeptides by polyacrylamide gel electrophoresis.

traction of normal and treated cells with Triton X-100 confirmed previous suggestions [10] that the marginal band of microtubules and other cytoskeletal structures observed in normal cells (presumably including the plasma membrane skeleton and intermediate filaments) were disaggregated when cells were exposed to A23187 and Ca^{2+} (Fig. 5). In fact only 'bald' nuclei remained (Fig. 5d). It was of particular interest that when spoked cells were extracted with Triton, the marginal band of microtubules was seen to have disappeared whereas the other cytoskeletal elements seemed to be intact (Fig. 5c). This indicated that elevation of

TABLE I

THE EFFECT OF TLCK AND IODOACETAMIDE ON BIOCHEMICAL CHANGES INDUCED BY A23187/Ca^{2+} IN CHICKEN ERYTHROCYTES

Cells were incubated for 5 min at 37°C with A23187 (5 μM) and Ca^{2+} (1 mM) as described under Methods. Some samples were incubated additionally with TLCK (1 mM) or iodoacetamide (10 mM). Values presented are means ±S.D. from six separate experiments.

	Microvesicle loss (% phospholipid)	Sphingomyelin (% breakdown)	α-Spectrin (% breakdown)
A23187 + Ca^{2+}	21.3 ± 6.6	24.9 ± 3.8	82.1 ± 4.9
A23187 + Ca^{2+} + iodoacetamide	0	26.9 ± 14.1	2.3 ± 2.3
A23187 + Ca^{2+} + TLCK	0	22.7 ± 6.3	14.9 ± 9.5

Ca^{2+} in TLCK or iodoacetamide-treated cells was sufficient to disaggregate the microtubules, but not to cause marked changes in other cytoskeletal structures. The ruffled aspect of the remaining cytoskeletal elements in these cells might be related to the spoke-like structures seen in the light microscope (Figs. 4b and 5c).

Significantly, addition of TLCK or iodoacetamide seemed to inhibit the loss of 50–55 kDa protein from plasma membrane (see above and Figs. 1 and 6) but had no effect on the breakdown of sphingomyelin which is induced by treatment with A23187 and Ca^{2+} (Ref. 10 and Table I).

Isolated plasma membranes did not undergo proteolytic degradation on addition of Ca^{2+} (Fig. 6f) but a limited proteolysis leading to accumulation of the 145 kDa polypeptide was observed when cytosolic fraction was added to plasma

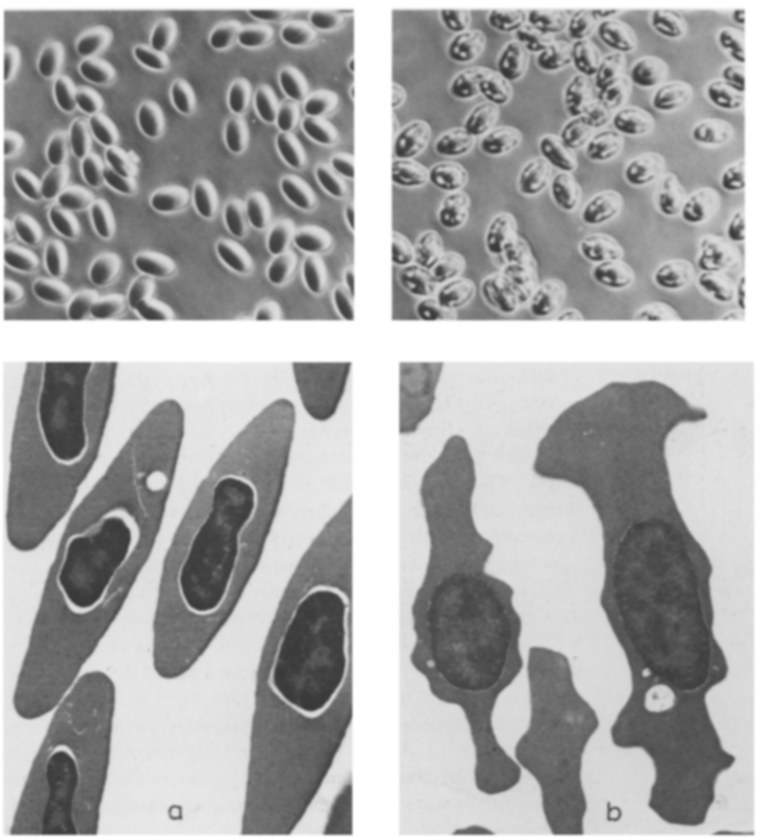

Fig. 4. The morphology of cells treated with Ca^{2+}/A23187 and TLCK. Cells were incubated for 5 min at 37°C with 1 mM TLCK and 5 μM A23187 either in the presence of 1 mM EGTA (a) or with 1 mM Ca^{2+} (b). Samples were fixed with glutaraldehyde and examined under phase contrast optics or by transmission electron microscopy as described previously [10]. Magnification is ×320 in light micrographs and ×5200 in electron micrographs.

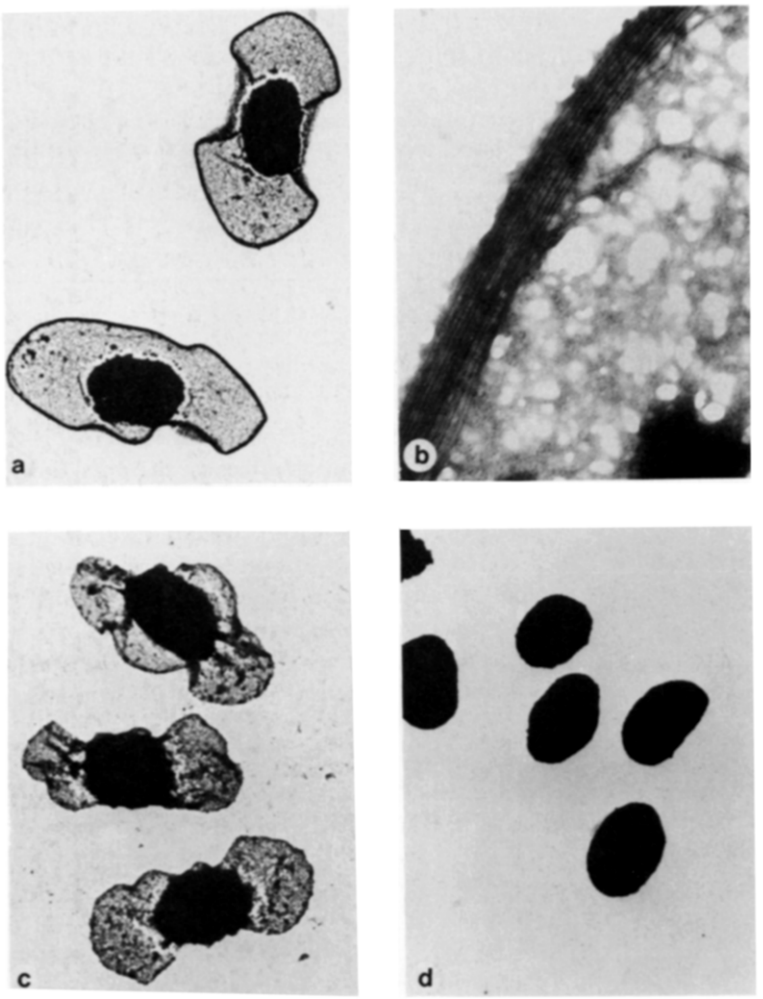

Fig. 5. The morphology of Triton shells of cells treated with Ca^{2+}/A23187 and TLCK. Cells were incubated for 10 min at 37°C with 5 μM A23187 in the presence of (a) EGTA, (c) 1 mM $CaCl_2$ + 2 mM TLCK, (d) 1 mM $CaCl_2$ and then were extracted with Triton X-100 as described under Methods. After fixation with glutaraldehyde, Triton shells were examined in the electron microscope using negative staining with phosphotungstate [23]. Magnification ×2800. (b) A detail of the marginal band of microtubules in (a). Magnification ×36800.

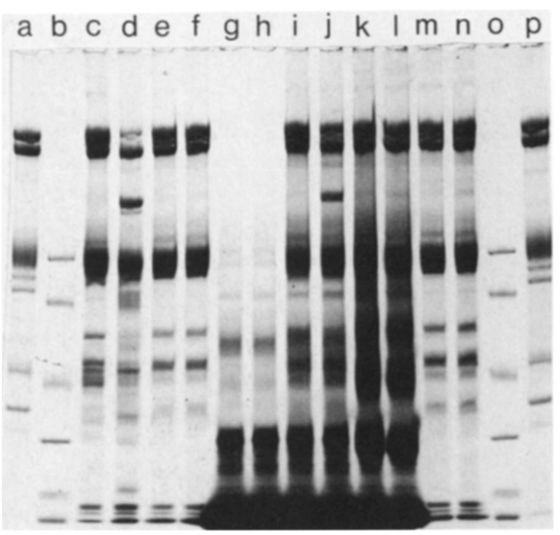

Fig. 6. Localisation of Ca^{2+}-dependent protease activity in the soluble fractions of the cells. Experimental details of polyacrylamide gel electrophoresis are described under Methods. (a) and (p) are samples of human erythrocyte plasma membranes. (b) and (o) are samples containing molecular weight standards (94K, 67K, 43K, 30K, 20K and 14K). (c,d) are plasma membranes isolated from chicken erythrocytes which had been preincubated with A23187 and EGTA (c) or Ca^{2+} (d). (e,f) are plasma membranes isolated from control chicken erythrocytes. The membranes were subsequently incubated with EGTA (e) or Ca^{2+} (f). (g,h) are samples of cytosolic fractions which were incubated with EGTA (g) or Ca^{2+} (h). (i,j) are plasma membranes isolated as for (e,f) to which were added samples of the cytosolic fraction together with EGTA (i) or Ca^{2+} (j). (k,l) are plasma membranes incubated with boiled cytosolic fraction in the presence of EGTA (k) or Ca^{2+} (l). (m,n) are plasma membranes incubated with 10 mM cysteine in the presence of EGTA (m) or Ca^{2+} (n). Membrane samples contained about 20 μg protein and cytosolic samples about 500 μg protein.

membranes in the presence of Ca^{2+} (Fig. 6j). Boiled cytosolic fraction did not induce proteolysis (Fig. 6l). These results suggested that the enzyme responsible for Ca^{2+}-sensitive degradation of plasma membrane polypeptides resided in the cytosolic fraction and not in the membrane. Despite this conclusion, there was no evidence that the putative cytosolic protease could attack soluble proteins (Fig. 6g,h).

Discussion

It is clear from our present results that the limited proteolysis of certain plasma membrane-associated polypeptides, especially microtubule-associated proteins, α-spectrin and goblin is a vital part of the mechanism by which Ca^{2+} promotes the microvesiculation of chicken erythrocytes. This conclusion arises from the observation that breakdown of microtubule-associated proteins and α-spectrin parallels the release of microvesicles induced by A23187 and Ca^{2+} (Figs. 2,3 and Table I) and that agents which block the proteolytic events (TLCK or iodoacetamide) completely inhibit the release of microvesicles (Fig. 1, Table I).

It appears also from electron microscopy of normal and treated cells (Figs. 4,5) that dissolution of internal cytoskeletal structures (intermediate filaments?) is also required in order for nuclei to come into contact with plasma membrane and thus initiate the events which lead to microvesicle release, but we are uncertain whether or not proteolytic degradation of intermediate filaments is necessary for the observed loss of cytoskeletal elements.

Other cellular alterations induced by A23187 + Ca^{2+}, e.g. the activation of sphingomyelinase (Table I), the disappearance of the marginal band of microtubules (Fig. 5), and the conversion of the cells to the 'spoked' form observed by phase contrast microscopy (Fig. 4 and Ref. 10) are not affected by TLCK or iodoacetamide and thus do not seem to depend on protease activity. It is worth emphasising that elevation of intracellular Ca^{2+} alone is sufficient to dissociate the microtubules and breakdown of microtubule-associated proteins is not necessary for this to happen (Fig. 1d and Fig. 5c).

In the electron microscope the spoked form seems to be characterised by a more irregular shape and by the possession of a more rounded nucleus which lacks the wide perinuclear space seen in control cells (Fig. 4). It seems possible that these morphological alterations are related to the breakdown of sphingomyelin and to the disappearance of the marginal band of microtubules which occur concomitantly. However, it appears that neither sphingomyelin breakdown nor microtubule dissociation are by themselves sufficient to allow the close approach of nuclear and plasma membranes which seems to be the necessary prelude to the membrane fusion events leading to microvesiculation [10]. In cells treated with A23187, Ca^{2+} and TLCK it does not appear that the nucleus and plasma membrane come into contact, (Fig. 4) so that our previous hypothesis [10] that sphingomyelin breakdown in the nucleus is brought about by a sphingomyelinase present in the plasma membrane, seems untenable. Possibly there is sphingomyelinase activity in either the nucleus or in the cytoplasm of the cells in addition to the well-established activity in the plasma membrane [16]. However a more attractive explanation is that perhaps Ca^{2+} can cause a disturbance of the normal lipid orientation in the plasma membrane so that some of the originally outward-facing sphingomyelin moves into the inner leaflet of the bilayer where it is then accessible to the plasma membrane sphingomyelinase. This interpretation is consistent with recent suggestions that Ca^{2+} may disrupt the spectrin-phosphatidylserine interactions [17] which are thought to be vital to the maintenance of normal phospholipid assymetry in the bilayer [18,19]. Such localised structural alterations, which free the bilayer from its membrane skeleton may increase the likelihood of membrane fusion events [4,5,10].

There are sound reasons why dissolution of microtubules and cytoskeletal elements might be essential in order for nuclei to approach and fuse with the plasma membrane. The marginal band of microtubules and the spectrin-actin framework on the inner surface of the plasma membrane would be expected to impede close apposition of the nuclear membrane to the lipid bilayer portion of the plasma membrane. Recent evidence [7–9] suggests that a network of 'intermediate filaments' apparently composed of vimentin (52 kDa) and synemin (230 kDa) maintains the nucleus in its

central position in the cell and it seems quite possible that dissolution of this network would be necessary in order for direct interactions to occur between nucleus and plasma membrane. The fact that Ca^{2+} can apparently cause the breakdown of all these protein barriers to fusion in chicken erythrocytes could have important implications for membrane fusion events in other cell types. However, it is not clear in the chicken erythrocyte whether the removal of these protein barriers is sufficient for fusion or whether other events such as breakdown of sphingomyelin are also necessary components of the process which leads to quasi-exocytosis of the nucleus and to release of microvesicles.

An interesting feature of the Ca^{2+}-dependent breakdown of proteins in chicken erythrocytes is the limited nature of the process. The very high molecular weight proteins presumed to correspond to microtubule-associated proteins are reduced in molecular weight only by about 10% whilst α-spectrin is selectively degraded to components of molecular weights about 145 000, 70 000 and 20 000–30 000 under conditions where β-spectrin and band 3 protein are largely unaltered. The protein which may correspond to goblin also undergoes proteolytic attack although the breakdown products have not been definitely identified. It is not clear whether polypeptides in the 50 000 molecular weight region (probably including tubulin and vimentin) are degraded or whether they are solubilised from cell membranes prepared from treated cells. Solubilisation of these components could follow degradation of membrane skeletal proteins to which they may be attached.

Preliminary evidence (Fig. 6) suggests that the breakdown of proteins promoted by Ca^{2+} entry is due to a soluble protease. This would indicate that the apparent specificity of the breakdown is not due to a particular localisation of the enzyme, but to an intrinsic specificity of its active site. Based on the inactivation of the breakdown by TLCK and iodoacetamide, the enzyme appears to have a trypsin-like activity, and depends on an active sulphydryl group.

Recently, Murachi et al. [20] have noted the presence in a wide range of tissues of a cytosolic, SH-dependent neutral protease which is activated by Ca^{2+} and which attacks endogenous high molecular weight polypeptides. The presence of such proteases (termed 'calpains') has been demonstrated most clearly in rat liver [20], human erythrocytes [20], human platelets [21] and rat synaptosomes [22]. The results presented here appear to link the degradation of cytoskeletal proteins by such a calpain with membrane fusion events in chicken erythrocytes and may suggest a more general role for calpains in the control of events such as secretion which involve the fusion of an intracellular granule with the plasma membrane.

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