Ca²⁺-induced polyphosphoinositide breakdown due to phosphomonoesterase activity in chicken erythrocytes

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Treatment of chicken erythrocytes with ionophore A23187 and Ca²⁺ caused the breakdown of a large proportion of the cellular polyphosphoinositides. Since no diacylglycerol or phosphatidate was generated, but there was a small increase in the level of phosphatidylinositol, it was concluded that breakdown occurred as a result of phosphomonoesterase activation. Experiments with subcellular fractions showed that the phosphomonoesterase activity was present in the cytosolic fraction of the cells.

INTRODUCTION

Erythrocytes normally maintain very low levels of intracellular Ca²⁺ and respond markedly to an increase in their internal Ca²⁺ concentration. These responses, which show clear species variations, include K⁺ efflux, degradation of polyphosphoinositides, proteolysis of membrane polypeptides, morphological changes and loss of microvesicles (Allan & Michell, 1977). A particularly bizarre morphological response to a rise in intracellular Ca²⁺ induced by A23187 is shown by chicken erythrocytes, which appear to undergo cytoskeletal alterations resulting in fusion of nuclear membrane with plasma membrane and release of microvesicles from the resulting hybrid membrane (Allan *et al.*, 1982; Thomas *et al.*, 1983).

Nuclear membranes from chicken erythrocytes have a very different phospholipid composition from plasma membranes, being relatively rich in phosphatidylcholine and PI whereas plasma membranes are rich in phosphatidylserine and sphingomyelin. Little is known about polyphosphoinositides in chicken erythrocytes, but it was initially reasoned that, considering the close resemblance between the overall phospholipid composition of chicken and human plasma membranes (Allan *et al.*, 1982; Nelson, 1972), there was likely to be a relative localization of PIP and PIP₂, together with the associated inositide kinases, in the plasma membrane of chicken cells, as there is in their mammalian counterparts (see Allan, 1982; Irvine, 1982). We decided to examine the polyphosphoinositide levels in A23187/Ca²⁺-treated chicken erythrocytes in order to see if, following the putative fusion of nuclear and plasma membranes, there was evidence for phosphorylation of PI contributed by nuclear membrane by kinase present in plasma membrane. This might be seen as an increase in the chemical amount and radioactivity of polyphosphoinositide in the hybrid membrane resulting from the fusion event, and particularly in the microvesicles released from this hybrid membrane.

In the event, the results showed no evidence for an enhanced phosphorylation of PI in chicken cells treated with A23187/Ca²⁺. However, there was an increased synthesis of PIP₂, which could have been due to phosphorylation of PIP derived from nuclear membrane, but was more likely to be a consequence of enhanced

turnover of PIP₂ resulting from the activation of a phosphomonoesterase present in the cytosolic fraction of the cells.

MATERIALS AND METHODS

Materials

[³²P]P_i (carrier-free, 10 mCi/ml) was obtained from Amersham International. All other reagents were from either Sigma or BDH.

Preparation and incubation of cells

Blood was obtained from the wing vein of Leghorn hens; EDTA was used as anticoagulant. Fresh blood samples were centrifuged at 1000 *g* for 5 min and the pelleted red cells were washed three times with 150 mM-NaCl with removal of buffy coat. The cells were given a final wash with 20 mM-Mops/NaOH buffer, pH 7.1, containing 150 mM-NaCl (Mops/saline). Washed erythrocytes were resuspended at approx. 25% haematocrit in Mops/saline containing 10 mM-glucose, 1 mM-Mg²⁺ and 100 μCi of [³²P]P_i/ml of packed cells and incubated at 37 °C for 90 min. After 90 min the labelled cells were washed with Mops/saline and were resuspended in the same volume of the buffer. The cells were incubated at 37 °C either alone or in the presence of 5 μM-ionophore A23187 together with (a) 1 mM-EGTA, (b) 1 mM-MgCl₂ or (c) 1 mM-CaCl₂ and at various times 0.5 ml aliquots were added to 8 ml of ice-cold lysis buffer containing 10 mM-Hepes/NaOH, pH 7.5, plus 0.1 mM-EGTA. After centrifugation at 39000 *g* for 5 min in a Sorvall RC5B centrifuge, the supernatant solution was discarded, the pellet resuspended in 0.5 ml of lysis buffer and this suspension was added to 1.9 ml of methanol/chloroform (2:1, v/v) at 20 °C. Microvesicles were isolated from the remaining cells treated with A23187/Ca²⁺ for 20 min as described by Allan *et al.* (1982) and were extracted with methanol/chloroform as above.

Preparation of cell fractions

Cells were fractionated to give plasma membranes and nuclei essentially as described previously (Beam *et al.*, 1979) with the following modifications. In addition to

Abbreviations used: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate.

Table 1. Inositide content of chicken erythrocytes and derived subfractions

Cells were incubated at 37 °C for 20 min and the amount of each lipid was determined as a percentage in molar terms of total phospholipid (4500 ± 300 nmol/ml of packed cells) as described under 'Materials and methods'. The percentage of total phospholipid recovered in the plasma membrane fraction was 47 ± 19 (s.d.) and in the nuclear fraction 53 ± 19 (s.d.). Values are presented as the means ($\pm$ s.d.) of results from four different experiments, in each of which triplicate or quadruplicate determinations were made. RSA = specific ^{32}P radioactivity relative to control cells (100). Radioactivity was expressed as c.p.m./nmol of lipid (calculated as 33% of PIP_2 phosphorus, 50% of PIP phosphorus and 100% of PI phosphorus).

Fraction	PIP_2		PIP		PI
	(mol%)	(RSA)	(mol%)	(RSA)	
Control cells	1.2 ± 0.2	100	1.1 ± 0.2	100	6.3 ± 0.3
Cells + A23187/EGTA	1.2 ± 0.1		1.1 ± 0.3		6.6 ± 0.4
Cells + A23187/ Ca^{2+}	0.3 ± 0.1	298 ± 53	0.7 ± 0.1	110 ± 11	7.5 ± 0.4
Cells + A23187/ Mg^{2+}	1.3 ± 0.2		1.2 ± 0.3		6.4 ± 0.2
Microvesicles	0.6 ± 0.1	155 ± 19	0.9 ± 0.3	120 ± 15	6.8 ± 0.5
Plasma membranes	2.2 ± 0.2		0.8 ± 0.1		3.2 ± 1.2
Nuclei	0.4 ± 0.1		0.5 ± 0.2		8.4 ± 1.5

10 mM- MgCl_2 , the lysis buffer (10 mM-Tris/HCl, pH 8.0) also contained 0.1 mM-EGTA and 0.1 mM-phenylmethanesulphonyl fluoride. The ghosts produced by this procedure were sedimented by centrifugation at 12000 *g* for 10 min. The pellet of washed ghosts was resuspended in 1 ml of lysis buffer and then sonicated for 20 s at an amplitude of 20 μm (peak-to-peak) on a MSC sonicator. Aliquots (0.2 ml) of either plasma membranes or nuclei in Mops/saline were treated with 1 mM- Ca^{2+} , 1 mM- Mg^{2+} , 1 mM- Ca^{2+} + 1 mM- Mg^{2+} or cytosolic fraction from 50 μl of packed cells for 15 min at 37 °C in a total volume made up to 0.4 ml with 0.15 M-NaCl. After incubation, 1.5 ml of methanol/chloroform (2:1, v/v) was added to the samples and lipids were extracted as described below.

For preparation of the soluble fraction a volume of cells was added to an equal volume of lysis buffer. The samples were kept at 4 °C for 15–20 min, after which time they were centrifuged at 30000 *g* for 5 min. The supernatant was removed and designated the 'cytosolic fraction'.

Lipid analysis

The suspension of cells or cell fractions in methanol/chloroform was centrifuged at 1000 *g* for 5 min, the supernatant solution (neutral extract) was removed and the pellet was re-extracted with methanol/chloroform/conc. HCl/water (2:1:0.05:0.8, by vol.). Both extracts were partitioned by the method of Bligh & Dyer (1959) by adding 0.6 ml of chloroform and either 0.6 ml of 3 M-NaCl (for the neutral extract) or 0.6 ml of 1 M-HCl (for the acid extract) and the chloroform phase from each was dried under vacuum. The phospholipids from the neutral extract were separated on 5 mM-EDTA-impregnated t.l.c. plates by the method of Allan & Cockcroft (1983) and those from the acid extract were separated on 1% oxalate-impregnated silica gel H plates with a solvent consisting of chloroform/methanol/acetone/acetic acid/water (40:13:15:12:7, by vol.). Haem pigments were only present in acid extracts from lysed cells but these pigments did not interfere with chromatography of lipids.

Iodine-staining spots were identified by comparison with standard phospholipids and after digestion with 70% HClO_4 at 180 °C for 1 h were analysed for

phosphorus by the method of Bartlett (1959). After dilution of these samples in 10 ml of water, Čerenkov radiation from radioactive lipids was counted in a Searle 6880 liquid-scintillation counter.

A portion of the lipid from cells treated with A23187/ Ca^{2+} was analysed for diacylglycerol as described previously (Allan *et al.*, 1978).

Measurement of ATP specific radioactivity

This was carried out by the h.p.l.c. procedure of Anderson & Murphy (1976) using samples of the aqueous layer derived from the Bligh & Dyer (1959) partition. The ATP was quantified chemically using a Waters Lambda Max spectrophotometer set at 260 nm, in conjunction with a Shimadzu C-R1B integrator. ATP radioactivity was counted by its Čerenkov radiation in 10 ml of water.

RESULTS

The inositide content of chicken erythrocytes and various subfractions derived from them is shown in Table 1. It is clear that PIP_2 is particularly concentrated in the plasma membrane fraction with relatively little in the nuclei, but PIP seems to be less specifically concentrated in the plasma membrane. Most of the PI is in the nuclear fraction, although generally the amounts measured in the present work are somewhat larger than were seen previously (Allan *et al.*, 1982), possibly because 3 M-NaCl was used in the Bligh & Dyer (1959) partition (see the Materials and methods section) instead of the 2 M-KCl used formerly.

Treatment of [^{32}P]P_i-labelled chicken red cells with A23187/ Ca^{2+} under the conditions previously described (Allan *et al.*, 1982) led to a pronounced drop in the cell content of PIP_2 which decreased to 25–50% of its original level after 20 min (Table 1 and Fig. 1a). PIP also decreased, but less dramatically. A small but reproducible increase (approx. 15–20%) in the cell content of PI was seen after 20 min (Table 1). Cells treated under identical conditions with A23187 and 1 mM- MgCl_2 or 1 mM-EGTA showed no changes in the levels of PIP_2 , PIP or PI (Table 1). During the incubation ATP levels in the cells fell by 20–30% but the increase in PIP_2 did not seem to be a

Fig. 1. Time course of polyphosphoinositide and ATP breakdown in chicken erythrocytes treated with A23187/Ca²⁺

[³²P]P_i-labelled cells were treated with A23187/Ca²⁺ for various times at 37 °C and samples were analysed for PIP₂, PIP and ATP (mass and radioactivity) as described under 'Materials and methods'. Microvesicles were isolated after 20 min incubation and analysed similarly; (a) PIP₂ (▲) and PIP (●) mass changes; (b) PIP₂ (▲) and PIP (●) radioactivity; (c) ATP mass (○) and radioactivity (●) changes. Open circles and triangles in (a) and (b) refer to control incubations without addition of A23187. Inositol specific radioactivity is expressed as c.p.m./nmol of inositol calculated from phosphate values as 33% of PIP₂ phosphorus and 50% of PIP phosphorus. ATP specific radioactivity is expressed as c.p.m./nmol of adenine. This Figure demonstrates the results of a representative experiment (one of four) and each point shown is the mean of triplicate determinations. The arrows mark the values for microvesicle PIP₂ content and specific radioactivity.

consequence of ATP depletion, since in other experiments where cells were treated with fluoride (5 mM) and deoxyglucose (1 mM), which reduced their ATP levels to < 10% of normal within 60 min, little change was seen in concentrations of inositides. The specific radioactivity of ATP and PIP did not change significantly during the incubation with A23187/Ca²⁺ (Figs. 1b and 1c) but there was a pronounced rise in the specific radioactivity of PIP₂. Assuming two radioactive phosphates (β and γ) in ATP, the specific radioactivity of the γ-phosphate was about

3000 c.p.m./nmol (Fig. 1c). If the increment in radioactivity in PIP₂ was entirely due to phosphorylation at the 5 position on the inositol ring, then the specific radioactivity of the 5-phosphate after 20 min incubation was about 3 × 1000, i.e. the 5-phosphate was in isotopic equilibrium with the γ-phosphate of ATP.

In some experiments, small increases in ³²P specific radioactivities of PI and phosphatidic acid were observed following treatment with A23187, but this labelling was not seen when careful precautions were taken to remove all leukocytes. It appears therefore that increased labelling of lipids other than the polyphosphoinositides was due to white cell contamination (see Allan & Michell, 1978).

The above results indicated that Ca²⁺ entry into the cells caused degradation of polyphosphoinositides (especially PIP₂) to PI, presumably by activation of a phosphomonoesterase. The possibility that some Ca²⁺-dependent diesterase activity also occurred was rendered unlikely by the failure to see any significant production of diacylglycerol or phosphatidate in cells treated with A23187/Ca²⁺, even though the cells possess an active diacylglycerol kinase (see Allan & Michell, 1977). Diacylglycerol content of control cells was 4 ± 2 nmol/ml of packed cells, and that of A23187/Ca²⁺-treated cells was 6 ± 2 nmol/ml (mean ± s.d. for three separated preparations). This compares with a loss of polyphosphoinositides equivalent to 1.3% of total phospholipid or about 60 nmol/ml of packed cells (see Table 1).

In an attempt to localize the activity responsible for degrading polyphosphoinositides in intact cells, studies were carried out on isolated plasma membranes and nuclei. Plasma membranes treated with Ca²⁺ alone showed little change in their content of polyphosphoinositides (Table 2). Mg²⁺ by itself or in combination with Ca²⁺ likewise had negligible effect. However, incubation of plasma membranes with cytosol prepared in MgCl₂/EGTA medium and especially with cytosol + Ca²⁺ caused large and consistent declines in PIP₂ and to a lesser extent PIP. Similar results were seen with the nuclear fraction, although in this case there was some slight indication that Mg²⁺ alone could promote polyphosphoinositide degradation.

DISCUSSION

In commencing this work it had been hoped to demonstrate an enhancement of PI phosphorylation resulting from the fusion of the PI-rich nuclear membrane with the PI-poor plasma membrane, assuming that the plasma membranes might possess the necessary kinase. The experimental results, however, show no evidence for an enhancement of PI phosphorylation in cells treated with A23187/Ca²⁺. There is however a clear increase in PIP phosphorylation as judged by the increased specific radioactivity of PIP₂ (Fig. 1a), and this could be due to phosphorylation of PIP contributed by nuclear membranes. Alternatively, this change could be related to an increase in PIP₂ turnover induced by the prior stimulation of PIP₂ phosphomonoesterase, which reduces the mass of PIP₂ to less than 50% of its original value. PIP did not accumulate as a result of PIP₂ breakdown; a small amount was reconverted to PIP₂ as judged by the increased labelling in this lipid, but most of it was further degraded to PI which increased as PIP₂ fell.

Curiously, microvesicles released from cells during a

Table 2. Content of PIP₂ and PIP in plasma membranes and nuclei under various conditions

Plasma membranes, nuclei and cytosolic fraction were prepared and incubated under various conditions for 15 min at 37 °C as described under 'Materials and methods'. The amounts of PIP₂ and PIP after various treatments are shown as percentages of zero-time control values and represent the means $\pm$ S.D. derived from four separate experiments in each of which determinations were carried out in triplicate.

Conditions	Plasma membranes		Nuclei	
	PIP ₂	PIP	PIP ₂	PIP
Control (zero time)	100	100	100	100
No addition	97 $\pm$ 5	98 $\pm$ 5	102 $\pm$ 8	89 $\pm$ 10
1 mM-CaCl ₂	96 $\pm$ 4	86 $\pm$ 9	98 $\pm$ 10	91 $\pm$ 10
1 mM-MgCl ₂	100 $\pm$ 6	102 $\pm$ 10	73 $\pm$ 4	75 $\pm$ 5
1 mM-MgCl ₂ + 1 mM-CaCl ₂	102 $\pm$ 12	93 $\pm$ 12	76 $\pm$ 11	65 $\pm$ 12
Cytosolic fraction	33 $\pm$ 10	76 $\pm$ 1	43 $\pm$ 4	45 $\pm$ 2
Cytosolic fraction + 1 mM-CaCl ₂	16 $\pm$ 5	47 $\pm$ 5	35 $\pm$ 6	34 $\pm$ 7

20 min incubation period with A23187/Ca²⁺ did not show the same inositide pattern as cells at 20 min. The inositide composition and specific radioactivity of the microvesicles instead resembled that of cells treated for only 2–3 min (Table 1 and Fig. 1). Since it is at this time that most microvesicles are released (Allan *et al.*, 1982) the data suggest that, subsequent to their release, microvesicles undergo little change in their inositide content and metabolism, in marked contrast to the situation in the remaining cells. A possible explanation for this observation arises from the results of cell subfractionation, which suggest that the Ca²⁺-sensitive enzyme(s) which degrades PIP₂ is located in the cytosol (Table 2). Microvesicles have a very much reduced cytosol:membrane ratio due to their small size and therefore may be relatively deficient in phosphomonoesterase activity. Similar conditions may make the maintenance of ATP levels and ionic gradients more difficult in small vesicles, so that pH changes could cause a decrease in phosphomonoesterase activity in the microvesicles. Such changes would equally well contribute to the failure to observe inositide kinase activity in the microvesicles.

In cases where erythrocyte PIP₂ phosphomonoesterase activity has been measured previously, it has been found mainly in the soluble fraction (Irvine, 1982; Roach & Palmer, 1981; but see Koutouzov & Marche, 1982) and the activity in chicken erythrocytes appears also to be soluble. It is less clear whether the enzyme really has a requirement for Ca²⁺ (Table 2), although there is an apparent precedent in the Ca²⁺-activated enzyme described by Nijjar & Hawthorne (1977). Cytosol prepared in EGTA-containing medium seems to be able to degrade the polyphosphoinositides but Ca²⁺ does augment this breakdown. The effect of Ca²⁺ in causing breakdown of polyphosphoinositides in intact cells could be due to an indirect action of Ca²⁺ on presentation of substrate to the enzymes rather than to a direct effect on the phosphomonoesterase. A similar effect has been described for chicken erythrocyte sphingomyelinase (Allan *et al.*, 1982).

Since PI seems to accumulate at the expense of PIP₂ and PIP in Ca²⁺-treated chicken erythrocytes there must also be some PIP phosphomonoesterase activity present. Mack & Palmer (1984) have identified a similar enzyme in human erythrocyte membranes. The total decrease in

polyphosphoinositides (1.3 mol%) appears to be similar in magnitude to the rise in PI (1.2 mol%) (Table 1). A similar rise in PI at the expense of polyphosphoinositides has been described in metabolically-depleted human erythrocytes and this change has been associated with morphological alterations in these cells (Ferrell & Huestis, 1984). We cannot say at present whether these lipid changes make an essential contribution to the morphological responses of chicken erythrocytes.

There seems to be no evidence for the presence in chicken erythrocytes of the Ca²⁺-activated polyphosphoinositide phosphodiesterase which is observed in some mammalian cells (Allan & Michell, 1977, 1978; Moore & Appel, 1984; Cockcroft *et al.*, 1984). In this respect chicken erythrocytes seem to resemble those of sheep, pigs and cattle, although in these cases it is not known if the cells show any Ca²⁺-dependent degradation of inositides by other metabolic routes.

Regarding the subcellular distribution of polyphosphoinositides in the chicken erythrocyte, it is apparent from Table 1 that, whereas PIP₂ is largely concentrated in the plasma membrane fraction, PIP seems to be distributed more evenly between plasma membrane and nuclei. Assuming that there are approximately equal amounts of total phospholipid in the two fractions (Blanchet, 1974), it appears that there is reasonable agreement between the total amounts of PI and PIP₂ in the separate fractions and in the intact cells. Thus the mean of the values for PIP₂ in plasma membrane and nuclei is 1.3% compared with a value of 1.2% for intact cells. The figures for PIP suggest that some breakdown of this lipid occurs during the preparation of the cellular subfractions, since only about 60% of the PIP of the original cells was recovered in the isolated nuclei and plasma membranes.

The observed distribution of polyphosphoinositides between the two subcellular fractions is consistent with the work of Smith & Wells (1983), who showed that nuclear envelope from liver possessed PI kinase activity, whereas PIP kinase appears to be localized in the plasma membrane (Michell *et al.*, 1967; Seyfred *et al.*, 1984; Cockcroft *et al.*, 1985). Thus the relatively small amount of PIP₂ in chicken nuclear membrane seems likely to be due to an absence of PIP kinase from this membrane.

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REFERENCES

- Allan, D. (1982) *Cell Calcium* **3**, 451–465
- Allan, D. & Cockcroft, S. (1982) *J. Lipid. Res.* **23**, 1373–1374
- Allan, D. & Michell, R. H. (1977) *Biochem. J.* **166**, 495–499
- Allan, D. & Michell, R. H. (1978) *Biochim. Biophys. Acta* **508**, 277–286
- Allan, D., Thomas, P. & Michell, R. H. (1978) *Nature (London)* **276**, 289–290
- Allan, D., Thomas, P. & Limbrick, A. R. (1982) *Biochim. Biophys. Acta* **693**, 53–67
- Anderson, F. E. & Murphy, R. C. (1976) *J. Chromatogr.* **121**, 251–262
- Bartlett, G. (1959) *J. Biol. Chem.* **234**, 466–468
- Beam, K. G., Alper, S. L., Palade, G. E. & Greengard, P. (1979) *J. Cell Biol.* **83**, 1–15
- Blanchet, J. P. (1974) *Exp. Cell Res.* **84**, 159–166
- Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- Cockcroft, S., Baldwin, J. M. & Allan, D. (1984) *Biochem. J.* **221**, 477–482
- Cockcroft, S., Taylor, J. A. & Judah, J. D. (1985) *Biochim. Biophys. Acta* **845**, 163–170
- Ferrell, J. E. & Huestis, W. H. (1984) *J. Cell Biol.* **98**, 1992–1998
- Irvine, R. F. (1982) *Cell Calcium* **3**, 295–309
- Koutouzov, S. & Marche, P. (1982) *FEBS Lett.* **144**, 16–20
- Mack, S. E. & Palmer, F. B. St. C. (1984) *J. Lipid Res.* **25**, 75–85
- Michell, R. H., Harwood, T. L., Coleman, R. & Hawthorne, J. N. (1967) *Biochim. Biophys. Acta* **144**, 649–658
- Moore, R. B. & Appel, S. H. (1984) *Can. J. Biochem. Cell Biol.* **62**, 363–368
- Nelson, G. J. (1972) in *Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism* (Nelson, G. J., ed.), pp. 317–388, Interscience, New York
- Nijjar, M. W. & Hawthorne, J. N. (1977) *Biochim. Biophys. Acta* **480**, 390–402
- Roach, P. D. & Palmer, F. B. St. C. (1981) *Biochim. Biophys. Acta* **661**, 323–333
- Seyfred, M. A., Smith, C. D., Farrell, L. E. & Wells, W. W. (1984) in *The Chilton Conference on Inositol and Phosphoinositides* (Bleasdale, J. E. *et al.*, eds.), Humana Press, Clifton, NJ, in the press
- Smith, C. D. & Wells, W. W. (1983) *J. Biol. Chem.* **258**, 9368–9373
- Thomas, P., Limbrick, A. R. & Allan, D. (1983) *Biochim. Biophys. Acta* **730**, 351–358

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