Metabolism of inositol 1,3,4,5-tetrakisphosphate by human erythrocyte membranes

A new mechanism for the formation of inositol 1,4,5-trisphosphate

Claire DOUGHNEY, Margaret A. McPHERSON and Robert L. DORMER

Department of Medical Biochemistry, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, U.K.

Human erythrocyte membranes metabolize inositol 1,3,4,5-tetrakisphosphate [$\text{Ins}(1,3,4,5)\text{P}_4$] to inositol 1,3,4-trisphosphate [$\text{Ins}(1,3,4)\text{P}_3$] in the presence of Mg^{2+} . In the absence of Mg^{2+} a less rapid conversion of $\text{Ins}(1,3,4,5)\text{P}_4$ into $\text{Ins}(1,4,5)\text{P}_3$ was revealed. Such an enzyme activity, if present in hormonally sensitive cells, could provide a mechanism for maintaining constant concentrations of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$, important for stimulation of Ca^{2+} entry after Ca^{2+} mobilization.

INTRODUCTION

The rapid formation of $\text{Ins}(1,3,4,5)\text{P}_4$ was first described in cholinergically stimulated brain cortical slices (Batty *et al.*, 1985), and has since been demonstrated in stimulated rat parotid slices (Hawkins *et al.*, 1986), submandibular and pancreatic acini (Doughney *et al.*, 1987*a,b*), insulin-secreting tumour cells (Biden & Wolheim, 1986) and rat hepatocytes (Hansen *et al.*, 1986). A parent phospholipid (phosphatidylinositol 3,4,5-trisphosphate), which would be broken down by phospholipase C action to produce $\text{Ins}(1,3,4,5)\text{P}_4$, was not detectable in rat parotid (Hawkins *et al.*, 1986), and evidence suggests that $\text{Ins}(1,3,4,5)\text{P}_4$ is produced from $\text{Ins}(1,4,5)\text{P}_3$ by the action of a 3'-kinase (Irvine *et al.*, 1986), which is dependent on Ca^{2+} in some cells (Biden & Wolheim, 1986; Tennes *et al.*, 1987; Yamaguchi *et al.*, 1987; Zilberman *et al.*, 1987). A 5'-phosphomonoesterase in human erythrocyte membranes, originally shown to hydrolyse $\text{Ins}(1,4,5)\text{P}_3$ (Downes *et al.*, 1982), also degrades $\text{Ins}(1,3,4,5)\text{P}_4$ to $\text{Ins}(1,3,4)\text{P}_3$ (Batty *et al.*, 1985). $\text{Ins}(1,3,4)\text{P}_3$ is formed in a variety of cells after stimulation, with a slower time of onset than for $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ (Irvine *et al.*, 1985; Biden & Wolheim, 1986; Hansen *et al.*, 1986; Doughney *et al.*, 1987*a,b*; Zilberman *et al.*, 1987), and has not yet been ascribed any biological function.

The present study originated from our initial studies of inositol phosphate formation in cultured sweat duct cells (Doughney *et al.*, 1987*c*). Since the pathways have not previously been studied in detail in this cell type, we wished to demonstrate that an inositol polyphosphate formed, after cholinergic stimulation, which was eluted in the same position on anion-exchange columns as $\text{Ins}(1,3,4,5)\text{P}_4$, was degraded in the expected manner by the 5'-phosphomonoesterase of the erythrocyte membrane. Preliminary control experiments using authentic $\text{Ins}(1,3,4,5)\text{P}_4$ revealed a previously undescribed 3'-phosphomonoesterase activity in human erythrocyte membranes incubated in the absence of Mg^{2+} , such that the major product was $\text{Ins}(1,4,5)\text{P}_3$.

MATERIALS AND METHODS

Preparation and incubation of human erythrocyte membranes

The methods used were based on those described by Downes *et al.* (1982). Freshly drawn blood in heparin, from healthy volunteers, was centrifuged, and the erythrocytes were washed three times in 154 mM-NaCl/1.5 mM-Hepes/NaOH, pH 7.2, and lysed by mixing with 8 vol. of 2.5 mM-Hepes/NaOH, pH 7.0, containing 1 mM-EGTA at 4 °C. After centrifugation for 15 min at 23000 g, the membranes were washed six times with lysis buffer and then twice with 154 mM-NaCl/1.5 mM-Hepes/NaOH, pH 7.2. The resulting membranes were incubated in 30 mM-Hepes/NaOH, pH 7.0, at a concentration of 1 mg of membrane protein/ml for various times at 37 °C in the presence of 5 nCi of [^3H] $\text{Ins}(1,3,4,5)\text{P}_4$ /ml [0.1 Ci/mmol, from NEN, Dupont (U.K.), Stevenage, Herts., U.K.]. When applicable, EDTA was added from a stock solution of 10 mM-NaEDTA, pH 7.0. Reactions were terminated by the addition of an equal volume of 20% (w/v) trichloroacetic acid, and the precipitated proteins were removed by centrifugation. Trichloroacetic acid was removed from the supernatant by extraction with 4 × 4 vol. of water-saturated diethyl ether, and the extracts were neutralized with 1 M- K_2HPO_4 . Mannitol was added to a concentration of 5 mM, and the extracts were freeze-dried.

Separation of ^3H -labelled inositol phosphate products

The extracts were dissolved in 1 ml of water for injection on to a Partisil SAX 10 high-performance anion-exchange column (Technicol, Stockport, U.K.) equilibrated with water. The inositol phosphates were eluted with a modification of an ammonium formate gradient previously described for extracts of exocrine acini (Doughney *et al.*, 1987*a,b*). The eluent was increased linearly to 0.71 M-ammonium formate (adjusted to pH 3.7 with H_3PO_4) over 10 min to elute [^3H] InsP_2 and held at this concentration for a further 2 min. [^3H] $\text{Ins}(1,3,4)\text{P}_3$

Abbreviations used: InsP_2 , InsP_3 , InsP_4 , inositol bis-, tris- and tetrakis-phosphate (numbers in parentheses denote positions of phosphate groups in defined isomers).

Table 1. Metabolism of Ins(1,3,4,5) P_4 by human erythrocyte membranes

Erythrocyte membranes were incubated for 1 h at 37 °C with [^3H]Ins(1,3,4,5) P_4 , and ^3H -labelled inositol phosphates formed were separated by high-performance anion-exchange chromatography, as described in the Materials and methods section. Results for single incubations are means $\pm$ S.E.M. for four incubations using membranes derived from two individuals. For the sequential 1 h incubations (bottom two lines) results from two incubations using membranes from two individuals are shown.

Incubation conditions	^3H content (% of total recovered)			
	Ins(1,3,4,5) P_4	Ins(1,3,4) P_3	Ins(1,4,5) P_3	Ins P_2
No membranes + 2 mM-Mg $^{2+}$	89.8 $\pm$ 1.0	5.8 $\pm$ 2.9	2.2 $\pm$ 0.5	0.7 $\pm$ 0.4
+ membranes + 2 mM-Mg $^{2+}$	8.1 $\pm$ 1.0	82.9 $\pm$ 2.4	0.8 $\pm$ 0.03	11.0 $\pm$ 2.6
+ membranes, no added Mg $^{2+}$	32.2 $\pm$ 1.1	52.5 $\pm$ 3.4	9.3 $\pm$ 1.5	8.0 $\pm$ 1.1
+ membranes + 1 mM-EDTA	41.3 $\pm$ 6.8	14.8 $\pm$ 1.9	40.9 $\pm$ 4.6	3.2 $\pm$ 0.8
+ membranes + 2 mM-Mg $^{2+}$ (1 h), then + 10 mM-EDTA (1 h)	3.5, 3.8	81.0, 79.7	6.6, 0.6	8.4, 16.0
+ membranes + 1 mM-EDTA (1 h), then + 3 mM-Mg $^{2+}$ (1 h)	2.1, 1.5	24.6, 23.8	1.3, 0.4	72.0, 74.2

and [^3H]Ins(1,4,5) P_3 were then eluted by a linear gradient up to 1.1 M-ammonium formate/ H_3PO_4 over 11 min. This concentration was held for a further 2 min and then increased over 10 min to 1.7 M-ammonium formate/ H_3PO_4 to elute [^3H]Ins(1,3,4,5) P_4 . In all cases, recovery of radioactivity applied was at least 85 %, and results are expressed as a percentage of the total recovery in all peaks.

RESULTS AND DISCUSSION

As shown in Table 1, erythrocyte membranes, incubated for 1 h at 37 °C in the presence of 2 mM-Mg $^{2+}$, hydrolysed approx. 90 % of the [^3H]Ins(1,3,4,5) P_4 added, of which 92 % was recovered as Ins(1,3,4) P_3 . Ins P_2 was also formed, of which 10–15 % was presumably due to breakdown of Ins(1,4,5) P_3 (Downes *et al.*, 1982), some of which is a contaminant of the Ins(1,3,4,5) P_4 preparation. The source of the additional Ins P_2 produced cannot be deduced from the present data without separation of Ins P_2 isomers.

When the Mg $^{2+}$ concentration was lowered by omitting it from the incubation medium or depleted to less than nanomolar concentration by addition of 1 mM-EDTA, the amount of Ins(1,3,4,5) P_4 hydrolysed was progressively decreased, although 54 % was still hydrolysed in 1 h in the presence of EDTA. The time courses (Fig. 1) show that hydrolysis is markedly slower in the presence of EDTA, being linear up to 1 h. However, the unexpected finding was that, as the Mg $^{2+}$ concentration was lowered, a different product was formed such that, in the presence of EDTA, only 15 % was Ins(1,3,4) P_3 and 80 % was Ins(1,4,5) P_3 after 1 h (Table 1). The amount of Ins P_2 produced was also decreased, presumably because of the Mg $^{2+}$ -dependence of Ins(1,4,5) P_3 5'-phosphomonoesterase activity (Downes *et al.*, 1982).

To test whether the effects of EDTA were due specifically to removal of Mg $^{2+}$, the effect of incubation of [^3H]Ins(1,3,4,5) P_4 for 1 h in the presence of 5 mM-EGTA + 2 mM-MgCl $_2$ was compared with incubation with 2 mM-MgCl $_2$ alone, with the same membrane preparation. Under both conditions the free Mg $^{2+}$ concentration was the same, but in the presence of EGTA the concentration of any residual free Ca $^{2+}$ would

be decreased to < 1 nM, and that of any contaminating transition metals by at least 5 orders of magnitude. The percentages of total ^3H radioactivity recovered in each inositol phosphate after incubation with 5 mM-EGTA/2 mM-MgCl $_2$ were 10.5 % Ins(1,3,4,5) P_4 , 85.6 % Ins(1,3,4) P_3 , < 0.1 % Ins(1,4,5) P_3 , 3.9 % Ins P_2 , compared with 6.5 % Ins(1,3,4,5) P_4 , 86.6 % Ins(1,3,4) P_3 , 0.5 % Ins(1,4,5) P_3 and 6.3 % Ins P_2 in the presence of 2 mM-MgCl $_2$ alone (compare also with Table 1). Thus removal of Ca $^{2+}$ or of transition-metal ions alone had no marked effect on Ins(1,3,4,5) P_4 breakdown, suggesting that the effects of EDTA were due to Mg $^{2+}$ removal.

In order to confirm that the compound formed in the presence of EDTA was Ins(1,4,5) P_3 , in two experiments (Table 1) a 1 h incubation in the presence of EDTA was followed by a further 1 h after addition of 3 mM-MgCl $_2$. As shown, after the second hour, there was virtually no Ins(1,4,5) P_3 present, and 72 % of the radioactivity was Ins P_2 . This is consistent with the Ins(1,4,5) P_3 formed in the first hour being broken down in the second hour by a Mg $^{2+}$ -dependent 5'-phosphomonoesterase.

The sequential incubations for 1 h in the presence of Mg $^{2+}$ followed by 1 h with excess EDTA added produced similar amounts of Ins(1,3,4) P_3 and Ins P_2 to those formed in 1 h in the presence of Mg $^{2+}$ (Table 1). Under these conditions, very low amounts of Ins(1,4,5) P_3 were formed (Table 1), as would be expected, since after the first hour the Ins(1,3,4,5) P_4 would have been present at not more than 10 % of its starting concentration. Similarly, in the sequential incubations in EDTA followed by Mg $^{2+}$, less Ins(1,3,4) P_3 was formed than would have been predicted, when the Ins(1,3,4,5) P_4 concentration would have been decreased by 50–60 % after the first hour.

The results therefore confirm previous reports (Batty *et al.*, 1985; Hawkins *et al.*, 1986; Hansen *et al.*, 1986) that Ins(1,3,4,5) P_4 can be hydrolysed by a Mg $^{2+}$ -dependent 5'-phosphomonoesterase. However, the finding of a 3'-phosphomonoesterase activity at very low Mg $^{2+}$ concentrations was unexpected, although it is noteworthy that a 3'-phosphomonoesterase activity against Ins(1,3) P_2 has been described in calf brain which was active in the absence of Mg $^{2+}$ (Bansal *et al.*, 1987). The data do not allow any conclusion to be drawn as to

Fig. 1. Time course of Ins(1,3,4,5)P₄ metabolism by human erythrocyte membranes

Erythrocyte membranes were incubated for the times shown at 37 °C with [³H]Ins(1,3,4,5)P₄ as described in the Materials and methods section. (a) Incubation in the presence of 2 mM-Mg²⁺; (b) incubation in the presence of 1 mM-EDTA. ³H-labelled inositol phosphates formed were separated by high-performance anion-exchange chromatography as described in the Materials and methods section: ▲, Ins(1,3,4,5)P₄; ○, Ins(1,3,4)P₃; ●, Ins(1,4,5)P₃; ■, InsP₂. Results are from a single experiment which is representative of three experiments, each using a membrane preparation from a different individual.

whether the 3'-esterase activity is the same enzyme as the 5'-esterase with altered specificity at low Mg²⁺ concentration, or a distinct enzyme whose activity is masked by the 5'-esterase at millimolar Mg²⁺, but is more active in the absence of Mg²⁺. In the latter case, the existence of a distinct 3'-phosphomonoesterase in a normally regulated cell, compartmented from 5'-phosphomonoesterases, might act with Ins(1,4,5)P₃ 3'-kinase to maintain constant concentrations of Ins(1,3,4,5)P₄ and Ins(1,4,5)P₃ by a futile cycle, more favourable energetically than by the cycling of Ins(1,4,5)P₃ via inositol and phosphatidylinositol 4,5-bisphosphate (Downes & Wusterman, 1983; Berridge & Irvine, 1984). This may be

important for the concerted action of Ins(1,3,4,5)P₄ and Ins(1,4,5)P₃ in regulating increased Ca²⁺ entry in stimulated cells, as suggested by previous studies (Irvine & Moor, 1986, 1987; Morris *et al.*, 1987) and consistent with maintenance of Ins(1,4,5)P₃ concentrations, which may be required to prevent re-filling of the intracellular Ca²⁺ store during continuous stimulation (Putney, 1986; Brown *et al.*, 1987; Dormer *et al.*, 1987).

In summary, we have shown that in erythrocyte membranes a mechanism exists by which Ins(1,3,4,5)P₄ is hydrolysed to Ins(1,4,5)P₃. Like other enzymes of inositol phosphate metabolism previously described (Downes *et al.*, 1982; Batty *et al.*, 1985), although this may not be important in the physiology of the mature erythrocyte, it might have a counterpart in hormonally regulated cells and reveal a new complexity in our understanding of the interaction of these important intracellular messengers.

We are very grateful to the Cystic Fibrosis Research Trust, U.K., for financial support.

REFERENCES

- Bansal, V. S., Inhorn, R. C. & Majerus, P. W. (1987) *J. Biol. Chem.* **262**, 9444–9447
- Batty, I. R., Nahorski, S. R. & Irvine, R. F. (1985) *Biochem. J.* **232**, 211–215
- Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
- Biden, T. J. & Wolheim, C. B. (1986) *J. Biol. Chem.* **261**, 11931–11934
- Brown, G. R., Richardson, A. E. & Dormer, R. L. (1987) *Biochim. Biophys. Acta* **902**, 87–92
- Dormer, R. L., Brown, G. R., Doughney, C. & McPherson, M. A. (1987) *Biosci. Rep.* **7**, 333–344
- Doughney, C., Dormer, R. L. & McPherson, M. A. (1987a) *Biochem. J.* **241**, 705–709
- Doughney, C., Brown, G. R., McPherson, M. A. & Dormer, R. L. (1987b) *Biochim. Biophys. Acta* **928**, 341–348
- Doughney, C., Pedersen, P. S., McPherson, M. A. & Dormer, R. L. (1987c) *Pediatr. Pulmonol. Suppl.* **1**, 119 (abstr. 45)
- Downes, C. P. & Wusterman, M. M. (1983) *Biochem. J.* **216**, 633–640
- Downes, C. P., Mussat, M. C. & Michell, R. H. (1982) *Biochem. J.* **203**, 169–177
- Hansen, C. A., Mah, S. & Williamson, J. R. (1986) *J. Biol. Chem.* **261**, 8100–8103
- Hawkins, P. T., Stephens, L. & Downes, C. P. (1986) *Biochem. J.* **238**, 507–516
- Irvine, R. F. & Moor, R. M. (1986) *Biochem. J.* **240**, 917–920
- Irvine, R. F. & Moor, R. M. (1987) *Biochem. Biophys. Res. Commun.* **146**, 284–290
- Irvine, R. F., Anggard, E. E., Letcher, A. J. & Downes, C. P. (1985) *Biochem. J.* **229**, 505–511
- Irvine, R. F., Letcher, A. J., Heslop, J. P. & Berridge, M. J. (1986) *Nature (London)* **320**, 631–634
- Morris, A. P., Gallacher, D. V., Irvine, R. F. & Petersen, O. H. (1987) *Nature (London)* **330**, 653–655
- Putney, J. W. (1986) *Cell Calcium* **7**, 1–12
- Tennes, K. A., McKinney, J. S. & Putney, J. W. (1987) *Biochem. J.* **242**, 797–802
- Yamaguchi, K., Hirata, M. & Kuriyama, H. (1987) *Biochem. J.* **244**, 787–791
- Zilberman, Y., Howe, L. R., Moore, J. P., Hesketh, T. R. & Metcalfe, J. C. (1987) *EMBO J.* **6**, 957–962