The Ca^{2+} -activated polyphosphoinositide phosphodiesterase of human and rabbit neutrophil membranes

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Addition of Ca^{2+} to a plasma-membrane fraction derived from human or rabbit neutrophils led to the specific breakdown of polyphosphoinositides. The degradation products were identified as diacylglycerol and inositol bis- and tris-phosphate, thus demonstrating the presence of a Ca^{2+} -activated phospholipase C. The newly generated diacylglycerol resembled the polyphosphoinositides in its fatty acid composition, and in the presence of MgATP^{2-} it was converted into phosphatidate. These results therefore demonstrate the presence in neutrophil plasma membranes not only of polyphosphoinositide phosphodiesterase but also of diacylglycerol kinase.

The polyphosphoinositides are quantitatively minor components of most tissues, but they usually show rapid metabolic turnover, especially of the monoester phosphate groups attached to the inositol ring, which are subject to degradation by an ubiquitous polyphosphoinositide phosphomonoesterase (Thompson & Dawson, 1964a; Hawthorne & White, 1975; Michell, 1975). Hydrolysis of polyphosphoinositides can also occur through the action of a phosphodiesterase yielding diacylglycerol and InsP_2 and InsP_3 (Thompson & Dawson, 1964b; Allan & Michell, 1978; Downes & Michell, 1981), and it has recently been suggested that the activity of this enzyme may be a function of receptor occupancy in a variety of cell types, e.g. hepatocytes (Creba *et al.*, 1983), parotid gland (Weiss *et al.*, 1982), platelets (Billah & Lapetina, 1982, 1983), blowfly salivary gland and brain (Berridge *et al.*, 1983). These cellular situations where cleavage of polyphosphoinositide occurs in response to an extracellular agonist are also those where a rise in intracellular Ca^{2+} seems to be involved in the final physiological responses, and it

has been hypothesized that receptor-controlled breakdown of polyphosphoinositide may actually be an essential part of the mechanism by which Ca^{2+} is mobilized (Michell *et al.*, 1981). According to this view, polyphosphoinositide breakdown occurs before changes in Ca^{2+} and thus should not be controlled by Ca^{2+} . Some data, however, are difficult to reconcile with this suggestion. For example, some cells, e.g. iris smooth muscle (Akhtar & Abdel Latif, 1978a, 1980), fusing myoblasts (Wakelam & Pette, 1982), islets of Langerhans (Laychock, 1983) and hepatocytes (Rhodes *et al.*, 1983), do not show agonist-stimulated breakdown of polyphosphoinositides unless Ca^{2+} is present in the external medium. In other cases polyphosphoinositide phosphodiesterase can be activated by external Ca^{2+} in the presence of the Ca^{2+} ionophore A23187, e.g. synaptosomes (Griffin & Hawthorne, 1978; Fisher & Agranoff, 1981), erythrocytes (Allan & Thomas, 1981), islets of Langerhans (Laychock, 1983), and iris smooth muscle* (Akhtar & Abdel Latif, 1980), and a Ca^{2+} -dependent polyphosphoinositide phosphodiesterase can be identified in a plasma-membrane fraction from some of these cell types (Akhtar & Abdel Latif, 1978b; Allan & Michell, 1978; Downes & Michell, 1981; Van Rooijen *et al.*,

Abbreviations used: InsP_2 and InsP_3 , inositol bis- and tris-phosphates; PtdIns , phosphatidylinositol; PtdIns4P , phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2 , phosphatidylinositol 4,5-bisphosphate; polyphosphoinositides = $\text{PtdIns4P} + \text{PtdIns(4,5)P}_2$; Ptd (when used alone), phosphatidate; PtdCho , phosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; PtdEtn , phosphatidylethanolamine; PtdSer , phosphatidylserine; fMet-Leu-Phe, formylmethionyl-leucylphenylalanine.

* The effect of ionophore in this case may be indirect, owing to the release of noradrenaline in the tissue (A. A. Abdel Latif, personal communication). Nevertheless, noradrenaline-induced polyphosphoinositide breakdown still requires external Ca^{2+} (Akhtar & Abdel Latif, 1980).

1983). These observations seem more consistent with an alternative view, namely that polyphosphoinositide breakdown is one of the consequences of a rise in intracellular Ca^{2+} and is not directly controlled by agonist-receptor interaction. However, as has been pointed out by Downes & Michell (1981, 1982), the phosphodiesterase does not seem to be sensitive to Ca^{2+} at the levels that prevail inside intact cells, so that the possibility must be considered that control over this enzyme activity could be exerted by a mechanism that does not directly involve Ca^{2+} .

In the work presented here we show that the plasma membranes of neutrophils possess a Ca^{2+} -dependent phosphodiesterase that specifically degrades polyphosphoinositides and also a kinase that phosphorylates diacylglycerol generated by the phosphodiesterase.

Methods

Human peripheral neutrophils were prepared as described by Segal *et al.* (1980) from buffy-coat residues obtained from St. George's Hospital, Tooting, London S.W.17, U.K. Neutrophils were purified first by dextran sedimentation of red cells and then on a Ficoll-Hypaque gradient. Any contaminating red cells were lysed by hypo-osmotic shock with distilled water for 20s. Rabbit peritoneal neutrophils were obtained as described pre-

viously (Bennett *et al.*, 1980). A light membrane fraction enriched in plasma membranes was prepared as described previously for rabbit neutrophils (Bennett *et al.*, 1982).

The plasma-membrane fraction was resuspended in 20mM-Hepes and 0.1mM-EGTA at pH7.4. Ca^{2+} (0.5mM) and ionophore A23187 (5 μM) was added to the membranes at 37°C. The reaction was terminated at the appropriate times and an excess of EGTA was added to chelate the Ca^{2+} . An equal volume of 2M- HClO_4 was added and the resulting precipitate was extracted with acidified chloroform/methanol as described previously (Allan & Michell, 1978). The aqueous supernatant was neutralized with KOH and the KClO_4 precipitate was removed by centrifugation. The clear supernatant was analysed for InsP_2 and InsP_3 on Dowex 1 (formate form) as previously described (Downes & Michell, 1981). Phospholipids and diacylglycerols were separated and assayed as previously described (Allan & Cockcroft, 1983). [γ - ^{32}P]ATP was prepared by the method of Glynn & Chappell (1964) and the specific radioactivity of ATP was measured by h.p.l.c. as previously described (Anderson & Murphy, 1976).

Results

Breakdown of polyphosphoinositides by phosphodiesterase action in human neutrophil membranes

When the light membrane fraction (plasma-membrane-enriched fraction) that had been prepared from human neutrophils prelabelled with [^{32}P]P_i was incubated with Ca^{2+} and ionophore A23187, there was a specific loss of radioactivity from the polyphosphoinositides (Tables 1 and 2). This loss was accompanied by an equivalent decrease in mass, so that there was no change in the specific radioactivity of these lipids (Table 1). Approx. 40–60% (about 1.3 nmol/mg of protein) of the polyphosphoinositides disappeared on Ca^{2+} addition. A similar loss of label from rabbit neutrophil membranes was observed under identical incubation conditions (Table 1).

In ^{32}P -labelled human neutrophil membranes, 80% of the radioactivity in the lipids is accounted for by the polyphosphoinositides. The remainder is distributed between Ptd, PtdIns and PtdCho (Table 2). Addition of Ca^{2+} leads specifically to the breakdown of the polyphosphoinositides only. Mass changes in sphingomyelin, PtdCho, Ptd, PtdEtn, PtdSer and PtdIns were not observed (results not shown).

The breakdown of the polyphosphoinositides could proceed via either phosphodiesterase or phosphomonoesterase action. To determine the precise route of hydrolysis, the water-soluble products were analysed. On addition of Ca^{2+} to mem-

Table 1. Ca^{2+} -dependent breakdown of polyphosphoinositides from human and rabbit neutrophil light membrane fraction

Light membrane fractions were prepared from ^{32}P -labelled human and rabbit neutrophils. Duplicate samples were incubated in the presence or absence of Ca^{2+} (0.5mM) for 10min. A 1ml sample (3mg of protein/ml for human and 0.35mg of protein/ml for rabbit) was used per incubation. Results are presented as the means ($\pm$ S.D. where appropriate) of duplicate (human) or triplicate (rabbit) determinations in single experiments. Duplicates did not vary by more than 10%. One further identical experiment with human membranes and two with rabbit membranes gave results closely similar to those presented here.

Membranes	Radioactivity (c.p.m.) in:		
	PtdIns(4,5)P ₂	PtdIns4P	Ptd
Human			
– Ca^{2+}	14974	15230	2639
	(2.15 nmol)	(4.0 nmol)	(2.1 nmol)
+ Ca^{2+}	5981	5500	2794
	(0.7 nmol)	(1.4 nmol)	(2.3 nmol)
Rabbit			
– Ca^{2+}	3884 $\pm$ 24	4008 $\pm$ 40	998 $\pm$ 112
+ Ca^{2+}	2894 $\pm$ 39	2406 $\pm$ 253	992 $\pm$ 26

Table 2. *Loss of label from polyphosphoinositide and an increase in label in inositol phosphates when neutrophil membranes are treated with Ca²⁺*

Duplicate samples of light membrane fraction from [³²P]_i-labelled human neutrophils were incubated for 10 min in the presence and absence of Ca²⁺ (0.5 mM). The decrease in the polyphosphoinositides was compared with the increase in InsP₂ and InsP₃. Of the increase in the water-soluble components, about 40% was accounted for by InsP₂ and InsP₃ and the remainder of the activity was in P_i. The results (c.p.m.) are expressed as an average of duplicate determinations, which did not differ from each other by more than 10%. Similar results were obtained in two other experiments.

(a) Lipids

	Radioactivity (c.p.m.) in:				
	PtdIns(4,5)P ₂	PtdIns4P	Ptd	PtdCho	PtdIns
– Ca ²⁺	9586	11803	1325	1750	2650
+ Ca ²⁺	6307	6605	1326	1710	2815
Ca ²⁺ -dependent decrease in labelling	3279	5198			

(b) Water-soluble constituents

	Radioactivity (c.p.m.) in:		
	InsP ₂	InsP ₃	P _i
– Ca ²⁺	3555	3756	72666
+ Ca ²⁺	7495	10064	88363
Ca ²⁺ -dependent increase in labelling	3904	6308	15697

branes there is an increase in the water-soluble fraction, approx. 60% of which was accounted for by P_i and the remainder by the inositol phosphates InsP₂ and InsP₃ (Table 2). The loss of radioactivity from the polyphosphoinositides (8477 c.p.m. in this experiment) could be all accounted for by the increase in inositol phosphates (10212 c.p.m.) (Table 2). The slightly higher recovery in the water-soluble components probably reflects the losses of polyphosphoinositides during extraction. The increase in P_i most likely results from phosphoprotein degradation.

The Ca²⁺-stimulated production of inositol phosphates suggests that the polyphosphoinositides were hydrolysed via phospholipase C action; therefore diacylglycerol should be the other reaction product. This was confirmed by isolation of the diacylglycerol and assay by fatty acid analysis (Allan & Cockcroft, 1983). Diacylglycerol accounted for about 50% of the breakdown of polyphosphoinositide at 10 min, and the fatty acid composition of the 'new' diacylglycerol closely resembled that of polyphosphoinositides (Table 3).

A substantial proportion of the diacylglycerol generated in this way could be phosphorylated to form Ptd when MgATP²⁻ was added, thus confirming the presence of diacylglycerol kinase in neutrophil membranes. Other workers (Call & Rubert, 1973) have failed to demonstrate this enzyme activity in neutrophils. When unlabelled membranes were treated with Ca²⁺ and then incubated with [³²P]ATP of known specific radio-

Table 3. *Fatty acid composition of polyphosphoinositides (PPI) from human neutrophils and of diacylglycerol (DG) from human neutrophil plasma membranes*

Diacylglycerol was isolated from neutrophil membranes that had been incubated in the absence or presence of Ca²⁺ (0.5 mM) for 10 min. Polyphosphoinositides were extracted from either untreated membranes or cells. The fatty acid composition of the individual polyphosphoinositides [PtdIns4P and PtdIns(4,5)P₂] were determined and did not differ significantly from each other. Results are therefore expressed as pooled data from five independent experiments. For the diacylglycerols the results are presented as an average (±S.D.) of determinations in three separate experiments. Lipids were subjected to methanolysis in methanolic 15% (w/v) BF₃, and esterified fatty acids were determined as described previously (Allan & Cockcroft, 1983).

Fatty acid	Proportions of fatty acids (mol %) in DG (a, b, c) and in PPI (d)			
	(a)	(b)	(c)	(d)
	Control	+ Ca ²⁺	'New DG'	PPI
C _{16:0}	11 ± 4	5 ± 1	3	7 ± 2
C _{18:0}	31 ± 6	37 ± 4	37	46 ± 5
C _{18:1}	22 ± 2	17 ± 2	12	12 ± 3
C _{18:2}	18 ± 4	12 ± 3	7	4 ± 3
C _{20:4}	20 ± 1	30 ± 1	37	31 ± 4

activity, the mass of Ptd synthesized could be calculated. The measured activity of diacylglycerol kinase in neutrophil membranes was similar to

Table 4. *Demonstration of diacylglycerol kinase activity in membranes by measuring Ptd formation from endogenously generated diacylglycerol*

Light membrane fractions from human and rabbit neutrophils or red-cell ghosts were incubated in triplicate with or without Ca^{2+} (0.5 mM) and ionophore A23187 (5 μM) in the presence of 3 mM- $\text{Mg}[\text{}^{32}\text{P}]\text{ATP}^{2-}$ (specific radioactivity 1000–3000 c.p.m./nmol) for 10 min. The level of radioactivity in Ptd was determined and the quantity of Ptd formed was calculated from the specific radioactivity of ATP. Results are expressed either as an average for three experiments ($\pm$ S.D.) for human cells, or as individual values from two separate experiments for rabbit cells. For the human red cells a time course of Ptd formation was monitored and was linear for up to 10 min. Abbreviation used: N.D., not detectable.

	Ptd formed (pmol/min per mg of protein)		
	Human neutrophils	Rabbit neutrophils	Human red cells
– Ca^{2+}	6 $\pm$ 2	7, 4	N.D.
+ Ca^{2+}	44 $\pm$ 3	35, 28	46

Table 5. *Requirements for Ca^{2+} in the breakdown of polyphosphoinositides*

Light membrane fraction prepared from ^{32}P -labelled human neutrophils were suspended in 20 mM-Hepes and 0.1 mM-EGTA. Sufficient bivalent cation or ionophore was added to give the net values indicated, and the samples incubated for 10 min. The radioactivity in the individual polyphosphoinositides was measured as described in the Methods section. Results are expressed as mean ($\pm$ S.D.) percentage breakdown in three individual experiments. In the case of the sample using 5 μM - Ca^{2+} (final concn.) the membranes were suspended in 20 mM-Hepes only and Ca^{2+} /HEDTA buffer was added to give the required free Ca^{2+} concentration as described previously (Allan & Thomas, 1981). The results are expressed as mean ($\pm$ S.D.) percentage breakdown from five independent experiments.

	Percentage breakdown of:	
	PtdIns4P	PtdIns(4,5) P_2
Control	(100)	(100)
Ca^{2+} (400 μM)	39 $\pm$ 2	38 $\pm$ 4
A23187 (5 μM)	98 $\pm$ 7	100 $\pm$ 2
A23187 (5 μM) + Ca^{2+} (5 μM)	98 $\pm$ 3	102 $\pm$ 4
A23187 (5 μM) + Ca^{2+} (50 μM)	49 $\pm$ 1	44 $\pm$ 5
A23187 (5 μM) + Ca^{2+} (400 μM)	39 $\pm$ 2	34 $\pm$ 3
A23187 (5 μM) + Mg^{2+} (400 μM)	117 $\pm$ 4	86 $\pm$ 3
fMet-Leu-Phe (0.1 μM)	98 $\pm$ 2	100 $\pm$ 4

that found in red cell ghosts when diacylglycerol was generated from endogenous polyphosphoinositides (Table 4).

Fig. 1. *Time course of polyphosphoinositide hydrolysis and of generation of the water-soluble products in human neutrophil membranes treated with Ca^{2+}*

Light membrane fractions prepared from ^{32}P -labelled human neutrophils were incubated without or with Ca^{2+} (0.5 mM) and ionophore A23187 (5 μM) for the indicated times. The radioactivity in the individual polyphosphoinositides (a) and the water-soluble components (b) was measured as described in the Methods section. Very similar results were obtained in two other experiments. Symbols: triangles, PtdIns4P; squares, PtdIns(4,5) P_2 ; circles, water-soluble products; open symbols, without Ca^{2+} ; closed symbols, with Ca^{2+} and ionophore A23187.

Time course of polyphosphoinositide hydrolysis

The time course of polyphosphoinositide hydrolysis is illustrated in Fig. 1(a). By 2 min, 90% of the hydrolysable substrate had disappeared. Fig. 1(b) shows the time course of the corresponding increase in the total water-soluble radioactivity.

Absolute requirement for Ca^{2+}

Initially, ionophore A23187 was routinely used in all experiments to allow Ca^{2+} accessibility to both sides of the membrane. However, it was later found that ionophore A23187 addition was not obligatory (Table 5). The phosphodiesterase acti-

vity was only observed in the presence of Ca^{2+} . With Mg^{2+} there was a small conversion of $\text{PtdIns}(4,5)\text{P}_2$ to $\text{PtdIns}4\text{P}$, suggesting that this cation could activate a phosphomonoesterase. Such an activity has been observed for erythrocytes (Koutouzov & Marche, 1982). Maximum polyphosphoinositide phosphodiesterase activity was seen at $300\text{--}500\text{ }\mu\text{M}\text{-Ca}^{2+}$ and activity was decreased to about 80% in the presence of $50\text{ }\mu\text{M}\text{-Ca}^{2+}$ and to zero at $5\text{ }\mu\text{M}\text{-Ca}^{2+}$. These findings make it unlikely that the enzyme could be activated at those levels of Ca^{2+} found even in stimulated neutrophils (Pozzan *et al.*, 1983). The agonist fMet-Leu-Phe was without effect on polyphosphoinositide degradation when added to membranes (Table 5).

Discussion

In this paper we demonstrate the presence of a Ca^{2+} -activated phosphodiesterase of the phospholipase C type which specifically degrades endogenous $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ in a plasma-membrane-enriched fraction of human or rabbit neutrophils. The initial reaction products are InsP_2 and InsP_3 and diacylglycerol, but, in the presence of ATP, some of the diacylglycerol is converted into Ptd through the action of diacylglycerol kinase. Call & Rubert (1973) failed to demonstrate any diacylglycerol kinase activity in human neutrophils, possibly because insufficient precautions were taken to inactivate endogenous proteinase. The initial rate of the polyphosphoinositide phosphodiesterase was $1.8\text{ nmol/min per mg of protein}$. At 10 min, 1.3 nmol of polyphosphoinositide disappeared, but only 0.65 nmol of diacylglycerol was recovered per mg of protein. In the presence of ATP, 0.37 nmol of Ptd was formed per mg of protein in 10 min. It is possible that the remainder of the diacylglycerol was removed by alternative routes, e.g. by the action of diacylglycerol lipase.

In neutrophils there is clear evidence that the fMet-Leu-Phe-dependent changes in metabolism of PtdIns and Ptd are consequences of Ca^{2+} mobilization (Cockcroft *et al.*, 1981), and it would be consistent with this view if the rise in internal Ca^{2+} induced by fMet-Leu-Phe (Pozzan *et al.*, 1983) were to activate the plasma-membrane polyphosphoinositide phosphodiesterase in intact cells. Indeed, Volpi *et al.* (1983) did observe a transient loss of label from polyphosphoinositides in rabbit neutrophils treated with fMet-Leu-Phe and concluded that this effect was most likely to be a consequence of a rise in intracellular Ca^{2+} . However, we have repeatedly failed to observe any change in levels of polyphosphoinositides in human neutrophils stimulated with fMet-Leu-Phe, so that we

cannot at present ascribe a physiological role to the phosphodiesterase that attacks polyphosphoinositides in plasma membranes. *In vitro* this enzyme activity seems to require levels of Ca^{2+} far higher than could be achieved even in stimulated cells, so that the possibility must be considered (as suggested by Downes & Michell, 1981) that an alternative mode of activation, which does not depend on Ca^{2+} , may allow such an enzyme to have a role in the complex changes in lipid metabolism which follow receptor activation in neutrophils.

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