The dependence on Ca^{2+} of the guanine-nucleotide-activated polyphosphoinositide phosphodiesterase in neutrophil plasma membranes

Shamshad COCKCROFT

Department of Experimental Pathology, School of Medicine, University College London, London WC1E 6JJ, U.K.

The requirement for Ca^{2+} for the activation of polyphosphoinositide phosphodiesterase was studied with the guanine nucleotide analogue guanosine 5'-[γ -thio]triphosphate (GTP γ S). Levels of Ca^{2+} that pertain in unstimulated neutrophils (100 nM) are obligatory for the full expression of enzyme activity stimulated with GTP γ S. Reduction of Ca^{2+} to 1 nM leads to inhibition. Increasing the level of Ca^{2+} from 100 nM to 1000 nM does not alter enzyme activity. Guanosine 5'-[β -thio]diphosphate (GDP β S) does not stimulate the phosphodiesterase but is an effective inhibitor of activation by GTP γ S. Ca^{2+} in the millimolar range can also activate the phosphodiesterase alone and this is not inhibited by GDP β S. It is also shown that Sr^{2+} in the millimolar range can stimulate enzyme activity similarly to Ca^{2+} .

INTRODUCTION

Stimulation of a wide variety of cells with their appropriate ligands leads to the generation of two signalling molecules, diacylglycerol and IP_3 , both derived from PIP_2 hydrolysis (Berridge, 1984). IP_3 is responsible for mobilizing Ca^{2+} from endoplasmic reticulum and diacylglycerol is the activator of protein kinase C (Berridge & Irvine, 1984; Nishizuka, 1984). The enzyme responsible for causing the hydrolysis of the inositol lipids, polyphosphoinositide phosphodiesterase (PPI-pde), is present at the plasma membrane and is coupled to the receptor via a guanine nucleotide binding protein (N_p) (Cockcroft *et al.*, 1984; Cockcroft & Gomperts, 1985; Lucas *et al.*, 1985; Uhing *et al.*, 1985, 1986).

Activation of neutrophils with fMet-Leu-Phe leads to stimulation of various functional responses including chemotaxis, oxidative burst and secretion of two populations of granules (Kreutzer *et al.*, 1978; Bennett *et al.*, 1980; Becker *et al.*, 1985). Coupled to the activation of the functional responses is the stimulation of inositol lipid metabolism (Cockcroft *et al.*, 1981, 1985; Volpi *et al.*, 1983; Bradford & Rubin, 1985; Di Virgilio *et al.*, 1985) and a rise in cytosol Ca^{2+} (Pozzan *et al.*, 1983; Lew *et al.*, 1984). Studies of inositide metabolism in intact neutrophils have suggested that Ca^{2+} is required for the full expression of stimulated inositol lipid metabolism using fMet-Leu-Phe as the agonist (Cockcroft *et al.*, 1980a,b, 1981; Cockcroft, 1984; Volpi *et al.*, 1983; Di Virgilio *et al.*, 1985).

The PPI-pde of neutrophil plasma membranes can be activated by Ca^{2+} in the millimolar range or by guanine nucleotides in the presence of 100 nM- Ca^{2+} (Cockcroft *et al.*, 1984; Cockcroft & Gomperts, 1985). In this paper the calcium requirement for the guanine-nucleotide-activatable PPI-pde is examined in an isolated plasma membrane preparation from human neutrophils. It is concluded that resting levels of Ca^{2+} that pertain in unstimulated cells (100 nM) are obligatory to support

PPI-pde activation in the presence of GTP γ S and a reduction in Ca^{2+} levels to 1 nM leads to a loss of activity. On the other hand, increasing the level of Ca^{2+} from 100 nM to 1000 nM (the level attained in stimulated cells) does not influence the activity of the PPI-pde. It is also demonstrated that GDP β S does not support activity of PPI-pde but is an effective inhibitor of GTP γ S-activated, but not Ca^{2+} -activated, PPI-pde.

METHODS

Human peripheral neutrophils were purified from buffy coat residues as described previously (Cockcroft, 1984). Neutrophils (10^8 cells/ml) were preincubated in a balanced salt solution comprising 137 mM-NaCl, 2.7 mM-KCl, 1 mM- CaCl_2 , 1 mM- MgCl_2 , 20 mM-Hepes, 5.6 mM-glucose, 1 mg of bovine serum albumin/ml and supplemented with [^{32}P]phosphate (100 $\mu\text{Ci}/\text{ml}$) for 90 min at 37 °C to prelabel the phospholipids including PIP and PIP_2 . A light membrane fraction enriched in plasma membranes was prepared as described previously (Bennett *et al.*, 1982). The plasma membrane fraction was suspended in 20 mM-Pipes buffer containing 150 mM-KCl and 100 μM -EGTA, pH 6.8, at a protein concentration of 1–2 mg/ml. Samples (100 μl) were transferred to tubes containing guanine nucleotides and various concentrations of Ca^{2+} buffered with EGTA (3 mM-EGTA final) or EGTA alone in an equal volume to give final concentrations as indicated. After 10 min at 37 °C, the reaction was quenched with acidified chloroform/methanol (1:2, v/v). Lipid extraction, separation and quantification of polyphosphoinositides was as described previously (Cockcroft & Gomperts, 1985). For the determination of the water-soluble inositol phosphates, the upper (aqueous) phase was neutralized, made up to 10 ml with water and analysed on Dowex-1 (formate form) as described previously (Downes & Michell, 1981). The formulations for the Ca^{2+} -EGTA buffers were calculated as previously described (Barrowman *et al.*, 1986a,b).

Abbreviations used: PPI-pde, polyphosphoinositide phosphodiesterase; GTP γ S, guanosine 5'-[γ -thio]triphosphate; GDP β S, guanosine 5'-[β -thio]diphosphate; PIP, PIP_2 , phosphatidylinositol 4-phosphate and 4,5-bisphosphate; IP_2 , IP_3 , inositol 1,4-bisphosphate and 1,4,5-trisphosphate.

Fig. 1. Ca^{2+} dependence of $\text{GTP}\gamma\text{S}$ -induced polyphosphoinositide hydrolysis

^{32}P -labelled neutrophil membranes were incubated for 10 min at 37°C in the presence of Ca -EGTA buffers (EGTA 3 mM) to give the indicated free Ca^{2+} concentrations in the presence (closed symbols) or absence (open symbols) of $\text{GTP}\gamma\text{S}$ ($100\ \mu\text{M}$). The radioactivity in the presence of 3 mM-EGTA was designated as 100% and the remainder of the samples are expressed as a percentage of that. The data points presented are pooled from five individual experiments done either in triplicate or quadruplicate. The samples were quenched with acidified chloroform/methanol and the lipids extracted and analysed as described previously (Cockcroft *et al.*, 1985).

RESULTS

Calcium requirement of the $\text{GTP}\gamma\text{S}$ -stimulated PPI-pde

The resting level of cytosol Ca^{2+} in neutrophils, as in other cells, is 120 nM (Pozzan *et al.*, 1983). In previous studies it has been demonstrated that preincubation of neutrophils in the absence of external Ca^{2+} leads to the inhibition of fMet-Leu-Phe-stimulated inositol lipid metabolism (Cockcroft *et al.*, 1980a,b, 1981; Cockcroft, 1984; Volpi *et al.*, 1983; Di Virgilio *et al.*, 1985). Here the Ca^{2+} requirement for the activation of the PPI-pde by $\text{GTP}\gamma\text{S}$ in neutrophil membranes is demonstrated. Membranes were made from ^{32}P -labelled neutrophils and incubated with $\text{GTP}\gamma\text{S}$ ($100\ \mu\text{M}$) in the presence of different concentrations of Ca^{2+} ranging from 1 to 100 nM (buffered with 3 mM-EGTA). The hydrolysis of both PIP and PIP_2 was diminished as the level of Ca^{2+} was reduced from 100 nM to 1 nM (Fig. 1). Total inhibition of PIP_2 hydrolysis was never observed in any experiment. In the presence of 3 mM-EGTA alone, the

percentage hydrolysis of PIP_2 (but not PIP) was always greater than that at 1 nM- Ca^{2+} . The decline in PIP_2 and PIP with $\text{GTP}\gamma\text{S}$ was matched by a corresponding increase in IP_2 and IP_3 , showing that the loss was via a phosphodiesterase C-type cleavage as demonstrated previously (Fig. 2) (Cockcroft & Gomperts, 1985). The Ca^{2+} -dependency of IP_2 and IP_3 generation in the presence of $\text{GTP}\gamma\text{S}$ is identical with the pattern observed for PIP and PIP_2 hydrolysis (Figs. 1 and 2).

We have recently shown that $\text{GTP}\gamma\text{S}$ is able to cause secretion from permeabilized neutrophils in the presence of 3–30 mM-EGTA (Barrowman *et al.*, 1986a). We argued that secretion due to $\text{GTP}\gamma\text{S}$ was not due to activation of PPI-pde but due to activation of a guanine nucleotide regulatory protein (N_e , distinct from N_p). Here it is shown that increasing the concentration of EGTA from 3 mM to 30 mM leads to a progressive loss of the $\text{GTP}\gamma\text{S}$ activation of PIP_2 , but not PIP, hydrolysis

Fig. 2. Ca^{2+} dependence of $\text{GTP}\gamma\text{S}$ -induced IP_2 (a) and IP_3 (b) formation

^{32}P -labelled neutrophil membranes were incubated for 10 min at 37°C in the presence of Ca -EGTA buffers (EGTA 3 mM) to give the indicated free Ca^{2+} concentrations in the presence or absence of $\text{GTP}\gamma\text{S}$ ($10\ \mu\text{M}$ or $100\ \mu\text{M}$). The radioactivity in the IP_2 and IP_3 fractions in the presence of 3 mM-EGTA alone was designated as 100% and the remainder of the samples are expressed as a percentage of that. The results are an average of triplicate determinations $\pm$ S.D. from a single experiment. $\circ$, $\square$, Controls; $\bullet$, $\blacksquare$, $\text{GTP}\gamma\text{S}$ ($100\ \mu\text{M}$); $\blacktriangle$, $\triangle$, $\text{GTP}\gamma\text{S}$ ($10\ \mu\text{M}$).

Table 1. Effect of different concentrations of EGTA on the activation of PPI-pde by GTP γ S

³²P-labelled neutrophil membranes were incubated in the presence of various concentrations of EGTA in the presence or absence of GTP γ S (100 μ M) for 10 min. The experiment presented is typical of three others and the results are expressed as c.p.m. in PIP and PIP₂ $\pm$ S.E.M. ($n = 4$).

EGTA (mM)	PIP			PIP ₂		
	Control (c.p.m.)	+ GTP γ S (c.p.m.)	(% of control)	Control (c.p.m.)	+ GTP γ S (c.p.m.)	(% of control)
3	3609 $\pm$ 195	3590 $\pm$ 94	99	3228 $\pm$ 283	2642 $\pm$ 176	82
10	3506 $\pm$ 90	3672 $\pm$ 131	105	3308 $\pm$ 218	2943 $\pm$ 99	88
30	3660 $\pm$ 271	3796 $\pm$ 84	104	3797 $\pm$ 187	3650 $\pm$ 224	96

Table 2. Effect of Ca²⁺ on GTP γ S-activated PPI-pde

³²P-labelled neutrophil membranes were incubated with Ca²⁺ buffers in the presence and absence of GTP γ S (10 μ M). After 10 min of incubation the samples were quenched and processed as described in Fig. 1 legend. The experiment present is one of three and the results are presented as c.p.m. in PIP and PIP₂ $\pm$ S.E.M. ($n = 4$), with percentages of control values in parentheses.

Ca ²⁺ (M)	PIP (c.p.m.)		PIP ₂ (c.p.m.)	
	Control	GTP γ S (10 μ M)	Control	GTP γ S (10 μ M)
10 ⁻⁷	10124 $\pm$ 220 (100)	8435 $\pm$ 390 (83)	6529 $\pm$ 59 (100)	5428 $\pm$ 120 (83)
10 ⁻⁶	10595 $\pm$ 213 (100)	8463 $\pm$ 174 (80)	6411 $\pm$ 370 (100)	5396 $\pm$ 183 (84)
10 ⁻⁵	10475 $\pm$ 296 (100)	8407 $\pm$ 204 (80)	6685 $\pm$ 418 (100)	5430 $\pm$ 59 (81)

Table 3. Comparison between the effect of Ca²⁺ and Sr²⁺ on PPI-pde

³²P-labelled neutrophil membranes were incubated in the presence of Ca²⁺ or Sr²⁺ (1 mM) for 10 min and the samples processed as in Fig. 1 legend. The results are expressed as c.p.m. in PIP and PIP₂ $\pm$ S.E.M. ($n = 3$), with percentages of control values in parentheses.

	³² P (c.p.m.) in:	
	PIP	PIP ₂
Control	5727 $\pm$ 347 (100)	6612 $\pm$ 550 (100)
Ca ²⁺ (1 mM)	2689 $\pm$ 68 (47)	3039 $\pm$ 88 (46)
Sr ²⁺ (1 mM)	4647 $\pm$ 90 (81)	5103 $\pm$ 67 (77)

(Table 1). The level of PIP₂ recovered in the presence of 30 mM-EGTA was always greater compared with 3 mM and this may reflect the ability of EGTA to chelate cations and so improve the extraction of PIP₂.

In stimulated neutrophils the level of Ca²⁺ rises from 120 nM to 800 nM (Pozzan *et al.*, 1983). We therefore tested the effect of Ca²⁺ in the range 10⁻⁷–10⁻⁵ M in the presence and absence of 10 μ M-GTP γ S (Table 2). A submaximal dose of GTP γ S was chosen in order to study any potentiating effect of Ca²⁺ (Cockcroft & Gomperts, 1985). Ca²⁺ up to 10 μ M is ineffective in stimulating PPI-pde as reported previously (Cockcroft *et al.*, 1984) although there is a difference between the two sets of experiments. In the previous experiments the requirement for Ca²⁺ was studied under low ionic strength compared with the experiments presented here. It has been reported

that ionic strength can modulate the requirement for Ca²⁺ for the activation of the PPI-pde in erythrocyte membranes (Downes & Michell, 1982). Increasing the level of Ca²⁺ in the presence of a submaximal concentration of GTP γ S does not potentiate the activation of the PPI-pde (Table 2 and Fig. 2).

Ca²⁺ in the millimolar range alone is able to stimulate hydrolysis of polyphosphoinositides and this is accompanied by release of inositol phosphates and diacylglycerol (Cockcroft *et al.*, 1984). Ca²⁺ in many instances

Table 4. Effect of GDP β S on GTP γ S- and Ca²⁺-induced polyphosphoinositide hydrolysis

³²P-labelled human neutrophil membranes were suspended in buffer containing 100 nM-Ca²⁺ and transferred to tubes containing the appropriate additions as indicated. The results are presented as c.p.m. in PIP and PIP₂ $\pm$ S.E.M. ($n = 4$) and are representative of two other experiments. Percentages of control values are given in parentheses.

	³² P (c.p.m.) in:	
	PIP	PIP ₂
Control	1931 $\pm$ 126 (100)	1148 $\pm$ 82 (100)
GTP γ S (100 μ M)	1390 $\pm$ 63 (72)	752 $\pm$ 15 (65)
GTP γ S (100 μ M) + GDP β S (1 mM)	1730 $\pm$ 32 (90)	1063 $\pm$ 30 (93)
Ca ²⁺ (0.5 mM)	1123 $\pm$ 70 (58)	669 $\pm$ 34 (58)
Ca ²⁺ (0.5 mM) + GDP β S (1 mM)	1252 $\pm$ 51 (65)	732 $\pm$ 44 (64)
GDP β S (1 mM)	1983 $\pm$ 65 (102)	1178 $\pm$ 14 (102)

can be replaced partially by Sr^{2+} (Douglas & Rubin, 1964; Foreman & Mongar, 1972; Allan & Thomas, 1981) and here it is shown that the Ca^{2+} activation of the PPI-pde can be mimicked by Sr^{2+} (Table 3).

Inhibition of $\text{GTP}\gamma\text{S}$ activation of PPI-pde by $\text{GDP}\beta\text{S}$

Activation of the PPI-pde by $\text{GTP}\gamma\text{S}$ was inhibited by $\text{GDP}\beta\text{S}$ (Table 4). The inhibition was specific for $\text{GTP}\gamma\text{S}$ in that Ca^{2+} (1 mM)-dependent activation of the PPI-pde was not inhibited by $\text{GDP}\beta\text{S}$ at the same concentration. $\text{GDP}\beta\text{S}$ alone had no effect on the PPI-pde (Table 4).

DISCUSSION

In this study it is demonstrated that optimal activation of PPI-pde in neutrophil membranes occurs with the stable guanine nucleotide analogue, $\text{GTP}\gamma\text{S}$, when the level of Ca^{2+} is fixed at 100 nM or above (see Fig. 1 and Table 2). This is the level of intracellular Ca^{2+} maintained in many cell types under physiological conditions (Tsien *et al.*, 1982; Hallam *et al.*, 1984; Thomas *et al.*, 1984; Capponi *et al.*, 1985). Reducing the Ca^{2+} level from 100 nM to 1 nM leads to the loss of PPI-pde activity in the membrane preparation. This result helps to reconcile the studies in the intact cell where Ca^{2+} deprivation led to a loss of the inositol lipid response (Cockcroft *et al.*, 1980a,b, 1981; Cockcroft, 1984; Volpi *et al.*, 1983; Di Virgilio *et al.*, 1985). The Ca^{2+} -dependency of the 'PI response' in the neutrophil did not quite fit with the idea that inositol lipid metabolism was responsible for mobilizing Ca^{2+} (see Cockcroft, 1981; Michell *et al.*, 1981, for discussion on this point). However, the earlier studies can now be reinterpreted. Incubation of cells, including neutrophils, in Ca^{2+} -free media leads to a reduction in the resting cytosolic levels of free Ca^{2+} (Pozzan *et al.*, 1983; Capponi *et al.*, 1985; Tatham *et al.*, 1986). It is possible that extensive depletion of intracellular Ca^{2+} had occurred during the prolonged incubations in Ca^{2+} -free media in the experiments presented on neutrophils (Cockcroft *et al.*, 1981). Thus it is more than likely that inhibition of fMet-Leu-Phe-stimulated secretion and inositol lipid metabolism in Ca^{2+} -depleted cells was a consequence of the Ca^{2+} -dependency of the PPI-pde.

Thus it is clear that the PPI-pde is a Ca^{2+} -dependent enzyme but that its requirements are fulfilled by the presence of resting levels of cytosol Ca^{2+} . Furthermore, the enzyme activity is not enhanced when cytosol Ca^{2+} is raised to the micromolar range, levels which occur in stimulated cells.

A guanine nucleotide-regulated PPI-pde has been identified in liver plasma membranes and was found to be dependent on Ca^{2+} in the nanomolar range (Uhing *et al.*, 1985, 1986). On the other hand, the guanine nucleotide-regulated PPI-pde in GH3 cell membranes does not show an absolute dependence on Ca^{2+} but was enhanced when Ca^{2+} was greater than 10^{-9} M (Lucas *et al.*, 1985). Thus Ca^{2+} dependency of the PPI-pde may be general but may differ only in the level of sensitivity to the presence of Ca^{2+} .

A somewhat unexpected result was the increase in PIP_2 loss when no Ca^{2+} was added to the preparation but 3 mM-EGTA was present. One possible explanation could be due to chelation of some heavy metal cation that releases its inhibitory effect on the PPI-pde. However, increasing the EGTA concentration from 3 to 30 mM led

to a loss of PIP_2 -pde activity. This result should be contrasted with the ability of $\text{GTP}\gamma\text{S}$ to cause secretion from permeabilized neutrophils in the presence of 30 mM-EGTA (Barrowman *et al.*, 1986a). Both these observations taken together strongly support the proposal that another guanine-nucleotide regulatory protein (termed N_e) is involved in the exocytotic mechanism (Barrowman *et al.*, 1986a; Gomperts *et al.*, 1986).

$\text{GDP}\beta\text{S}$ has been shown to be an inhibitor of guanine nucleotide regulatory proteins, e.g. N_s , the activator of adenylate cyclase (Eckstein *et al.*, 1979). Here it is shown that only activation by $\text{GTP}\gamma\text{S}$ is inhibited by $\text{GDP}\beta\text{S}$ and not that induced by millimolar concentrations of Ca^{2+} . This implies that activation by high concentrations of Ca^{2+} does not occur via a guanine nucleotide regulatory protein but by a direct effect either on the enzyme or on the substrate, which will bind Ca^{2+} (Buckley & Hawthorne, 1972). It has been recently demonstrated that $\text{GDP}\beta\text{S}$ also inhibits the liver plasma membrane PPI-pde activated with $\text{GTP}\gamma\text{S}$ (Uhing *et al.*, 1986).

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