Activation of phosphatidylinositol 4,5-bisphosphate supply by agonists and non-hydrolysable GTP analogues

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PtdIns(4,5) P_2 serves as a precursor of a diverse family of signalling molecules, including diacylglycerol (and hence phosphatidic acid), Ins(1,4,5) P_3 [and hence Ins(1,3,4,5) P_4] and PtdIns(3,4,5) P_3 . The production of these messengers can be activated by agonists, and therefore the rate of utilization of PtdIns(4,5) P_2 can vary dramatically. Although cells can only meet these large changes in demand for PtdIns(4,5) P_2 by increasing its synthesis and/or by continuously cycling it at a rate that exceeds its potential consumption (avoiding the need for a co-ordinated activation mechanism), no satisfactory explanation

for how this is achieved in agonist-stimulated cells has yet been provided. We show here that, in streptolysin-O-permeabilized neutrophils, *N*-formylmethionyl-leucyl-phenylalanine (FMLP), platelet-activating factor (PAF) and non-hydrolysable GTP analogues can cause large activations of PtdIns4P 5-kinase, suggesting that cells can accommodate agonist-activated rates of consumption of PtdIns(4,5) P_2 without having to sustain continuous, comparably rapid and energetically expensive 'futile cycling' reactions.

INTRODUCTION

PtdIns4P and PtdIns(4,5) P_2 are normal constituents of mammalian cells (e.g. [1]). They can be synthesized by PtdIns 4-kinase and PtdIns4P 5-kinase [1–6] and can be degraded by PtdIns(4,5) P_2 5-phosphomonoesterase and PtdIns4P 4-phosphomonoesterase [1,7]. Both these lipid kinases and phosphomonoesterases are thought to be active in unstimulated cells and thereby form two potential, linked, 'substrate cycles' [8] (see Figure 1).

On cell stimulation by a variety of agonists, PtdIns(4,5) P_2 serves as a substrate for two apparently independent signalling mechanisms; one involving activation of a PtdIns(4,5) P_2 -directed phospholipase C (PIC) [9,10] and one involving activation of a PtdIns(4,5) P_2 -directed phosphoinositide 3-kinase (PI3K) [11,12] (Figure 1). Thus, in certain circumstances, the demand for PtdIns(4,5) P_2 can rise dramatically. In a number of examples of PIC activation, the entire cellular complement of PtdIns(4,5) P_2 is consumed by this enzyme approximately twice per minute for ≥ 30 min [8]. Obviously this demand on PtdIns(4,5) P_2 must be met by an increase in its net synthesis (i.e. synthesis in excess of degradation).

In principle, increased net synthesis of PtdIns(4,5) P_2 during agonist stimulation could be supplied by an increase in the rate of a PtdIns4P 5-kinase and/or a decrease in the rate of a PtdIns(4,5) P_2 -degrading reaction that was active before stimulation. In the context of the metabolism to which PtdIns(4,5) P_2 is known to be subject (Figure 1), the latter possibility would only be supplied by a decrease in the rate of the PtdIns(4,5) P_2 5-phosphatase. For a decrease in PtdIns(4,5) P_2 5-phosphatase activity to be solely responsible for the rise in net synthesis, it would have to be more rapid in unstimulated cells than the maximum rates at which PtdIns(4,5) P_2 is degraded in stimulated cells [13]. This would require that a highly active 'futile' or substrate cycle is continuously maintained. Estimates of the

Figure 1 Schematic diagram of the basal routes of metabolism of the inositol phospholipids for which good evidence exists in at least some mammalian cells (see the text)

Upon appropriate hormonal stimulation, either or both of two basally inactive enzyme activities, PIC and/or PI3K, can be stimulated, which cause the further metabolism of PtdIns(4,5) P_2 to form Ins(1,4,5) P_3 or PtdIns(3,4,5) P_3 respectively (see the text). Abbreviation: DG, diacylglycerol.

degree of, or indeed even whether, this form of substrate cycling exists in unstimulated cells have been notoriously hard to derive [8].

One of the major problems in resolving these issues is the difficulty in measuring the rate of the relevant reactions (e.g. a PtdIns4P 5-kinase) in intact cells. The most obvious way of tackling this problem is to use [32 P] P_i -tracer analysis, measuring the rate of incorporation of 32 P into defined phosphoinositide phosphate groups [e.g. the 5-phosphate of PtdIns(4,5) P_2]. However, the relatively slow entry of [32 P] P_i into most mammalian cells severely compresses the time window during which 32 P incorporation into the appropriate phospholipid phosphate groups is largely dictated by the rates of the relevant enzymes (rather than the rates of labelling of the 32 P-lipid precursors, i.e. intracellular [32 P] P_i and hence [γ - 32 P]ATP). This has meant that

Abbreviations used: PtdIns, phosphatidylinositol [monoester phosphates located by numbering relative to the D-1 phosphodiester phosphate of PtdIns as defined in Biochem. J. (1989) 258, 1–2], e.g. PtdIns(4,5) P_2 , phosphatidyl-D-inositol 4,5-bisphosphate; PtdOH, phosphatidic acid; PIC, PtdIns(4,5) P_2 -directed phospholipase C; PI3K, PtdIns(4,5) P_2 -directed phosphoinositide 3-kinase; FMLP, *N*-formylmethionyl-leucyl-phenylalanine; PAF, platelet-activating factor; GTP[S], guanosine 5'-[γ -thio]triphosphate; p[NH]ppG, guanosine 5'-[β -imidazo]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; GTP[β S]- R_p or - S_p , R_p or S_p diastereoisomers of guanosine 5'-[β -thio]triphosphate; PMA, phorbol 12-myristate 13-acetate ('TPA').

substrate cycling (as opposed to basal PIC-mediated turnover) is only known to occur in erythrocytes [3,14]. Studies of agonist-sensitive cells with these techniques are limited [15–18] and, although there are some indications that the rate of PtdIns4P and PtdIns(4,5) P_2 synthesis may be increased in cells activated by PIC-coupled receptors [15–17], these are ambiguous, and furthermore the potential role that decreases in PtdIns(4,5) P_2 phosphatase activity might make to increased net synthesis of PtdIns(4,5) P_2 is not clear.

There are a number of reports of agonist-activated cells in which the levels of PtdIns(4,5) P_2 appear to rise immediately (e.g. [19]), remain unchanged (e.g. [20]), or fall and then rebound towards controls (e.g. [9,18,21]) despite maintained activation of PIC. These data all suggest that the agonists had activated PtdIns(4,5) P_2 production. However, the doubt over whether substrate cycling occurs in cells means that these observations could be supplied by an increase in PtdIns4P 5-kinase and/or an inhibition of a PtdIns(4,5) P_2 5-phosphatase. We have aimed to determine the roles played by phosphoinositide kinases and phosphatases, and of cycles that they might form, in the mechanism by which agonist-activated cells can increase net synthesis of PtdIns(4,5) P_2 . To achieve this, we have attempted to circumvent the accessibility problems associated with tracer analysis of intact cells by measuring the rate of synthesis of PtdIns(4,5) P_2 in streptolysin-O-permeabilized neutrophils. This experimental system allows the introduction of [γ - 32 P]ATP directly into the interior of the cells and yet maintains large agonist {*N*-formylmethionyl-leucyl-phenylalanine (FMLP), platelet-activating factor (PAF) and/or guanosine 5'-[γ -thio]triphosphate (GTP[S])}-sensitive activations of PIC and PI3K which are similar to those seen in intact cells [22,23].

MATERIALS AND METHODS

Materials

Materials were from sources defined previously [4,6,22]. Radioisotopes were obtained from Amersham International. GTP[S], guanosine 5'-[β - γ -imido]triphosphate (p[NH]ppG), guanosine 5'-[β -thio]diphosphate (GDP[S]) and ATP were obtained from Boehringer-Mannheim. The R_p and S_p isomers of guanosine 5'-[β -thio]triphosphate (GTP[β S]- R_p and S_p respectively) were generously given by F. Eckstein (Max-Planck Institut für Experimentelle Medizin, Germany). PAF, FMLP and phorbol 12-myristate 13-acetate (PMA) were from Sigma. Streptolysin-O was from Wellcome Diagnostics, Beckenham, Kent, U.K. Staurosporine was from Calbiochem. RO31-7549 and RO31-8220 were generously given by T. Hallam, Roche Pharmaceuticals, Welwyn, Herts., U.K.

Intact-cell experiments

Freshly isolated human neutrophils were prepared and suspended in a HEPES-buffered balanced salts solution at room temperature, as described previously [6,22].

Neutrophils were labelled with [3 H]inositol (1.25 mCi/ml for 2.5 h), washed and challenged with agonists at 37 °C as described previously [6,22]. Incubations were terminated with 4% (v/v) HClO₄. Phospholipid and aqueous extracts were prepared, deacylated or neutralized respectively, and then resolved by h.p.l.c. [24].

Neutrophils were labelled with [32 P]P_i, and 32 P-labelled polyphosphoinositides were quantified by deacylation and anion-exchange h.p.l.c. as described previously [6,22].

Neutrophils were treated with pertussis toxin (1.5 μ g/ml for 2.5 h; heat-denatured toxin was used as the control) as described

previously [22]; the extent to which G_i-proteins had been ADP-ribosylated was assessed by comparing the extent to which 40–45 kDa proteins in particulate fractions derived from either toxin-treated or control cells could be ADP-ribosylated *in vitro*.

Permeabilized-cell experiments

Neutrophils were permeabilized with streptolysin-O as described in detail in [22]. Briefly, the PIC-linked ATP-sensitive receptor on neutrophils was routinely inactivated before permeabilization by incubation with ATP and dithiothreitol at 30 °C [22]. The cells were then resuspended in ice-cold isotonic 'intracellular' buffer [22] (8×10^7 cells/ml), and samples (40 μ l) were permeabilized with streptolysin-O (120 μ l, containing 0.9 unit of streptolysin-O/ml in intracellular buffer supplemented with 160 μ M MgATP) at 37 °C. After 20 s, [γ - 32 P]ATP [20 μ l; 100 μ M MgATP (30 μ Ci) in intracellular buffer; i.e. finally 3.55×10^3 d.p.m./pmol of ATP] with or without agonists and/or GTP analogues was added. In some experiments the streptolysin-O, GTP[S], FMLP and [γ - 32 P]ATP (finally 3.5×10^4 d.p.m./pmol) were added together (see the Results section, Figure 3). Reactions were quenched with chloroform/methanol/water [750 μ l; 32.6:65.3:2.1, by vol.; in most cases containing $(5-8) \times 10^3$ d.p.m. of [3 H]PtdIns(4,5) P_2], extracted, and 3 H- and 32 P-labelled lipids were quantified by deacylation and anion-exchange h.p.l.c. as described previously [22]. These methods were designed specifically to quantify deacylated 32 P-labelled phosphoinositides, but a measure of glycerol[32 P]phosphate (derived from [32 P]PtdOH) could also be obtained, which was a useful indicator of the degree of activation of PIC (see the Results section). However, the low polarity of glycerol[32 P]phosphate meant it was often eluted with other contaminating 32 P-labelled compounds (of unknown identity, but probably originating from contaminants in the source of [γ - 32 P]ATP), and thus a 'zero-time blank' was routinely subtracted from each of the values presented.

[32 P]ATP levels in these assays were routinely monitored [22]; in none of the experiments reported in this paper was greater than 9% hydrolysed.

An analysis of the [32 P]PtdIns(4,5) P_2 derived from permeabilized neutrophils incubated with [γ - 32 P]ATP in the presence of GTP[S] and FMLP (1 min) showed that 85–95% of its 32 P was in the 5-position and that its structure was entirely consistent with expectation (see [4,6,22]).

Other assays

PtdIns(4,5) P_2 and PtdIns4P concentrations in samples of intact or permeabilized neutrophils were determined as described [22,25].

Assays of PtdIns4P 5-kinase activities in neutrophil homogenates used exogenously added lipid substrates [in the form of unilamellar phospholipid vesicles containing, in mol %, 26.9% PtdEtn, 26.9% PtdIns, 12.26% PtdCho, 25.5% PtdSer, 2.8% sphingomyelin, 2.8% PtdIns4P, 2.8% PtdIns(4,5) P_2 ; to give a final lipid concentration of 2 mg/ml and 60 μ M PtdIns(4,5) P_2] and [γ - 32 P]ATP, as described [22].

RESULTS

FMLP stimulates net synthesis of PtdIns(4,5) P_2 in intact neutrophils

FMLP binding to human neutrophils stimulates both PIC and PI3K [23,26]. It has been estimated previously that the activation of PIC accounts for approx. 85% of the initial consumption of

Table 1 FMLP-induced changes in [3 H]PtdIns(4,5) P_2 and [3 H]InsPs in human neutrophils

[3 H]Ins-labelled human neutrophils were incubated with 10 mM LiCl, then challenged with FMLP (1 μ M final concn.) or vehicle for 8 or 30 s, and the quantities of [3 H]inositol phosphates and [3 H]PtdIns(4,5) P_2 were determined as described in the Materials and methods section (the concentration of PtdIns(4,5) P_2 was also determined and used to estimate the specific radioactivity of [3 H]PtdIns(4,5) P_2 and hence the concentration of inositol phosphates). Data shown (d.p.m.) are means $\pm$ S.E.M. ($n = 3$) from one experiment. A further experiment taking measurements at 10 and 30 s gave similar results.

3 H-labelled metabolite	Time	3 H (d.p.m.)		FMLP-induced changes	
		Control	FMLP	3 H (d.p.m.)	(pmol/mg)
PtdIns(4,5) P_2	8 s	15145 $\pm$ 485	11845 $\pm$ 265	-3300 $\pm$ 750	-58
InsPs	8 s	1205 $\pm$ 91	5010 $\pm$ 113	+3805 $\pm$ 204	+67
PtdIns(4,5) P_2	30 s	15201 $\pm$ 495	12183 $\pm$ 306	-3018 $\pm$ 801	-53
InsPs	30 s	1391 $\pm$ 84	13456 $\pm$ 806	+12845 $\pm$ 890	+225

Table 2 Total or PIC-mediated turnover of PtdIns(4,5) P_2

Estimates of the rate of turnover of PtdIns(4,5) P_2 [% of control levels of PtdIns(4,5) P_2 /min] in control or maximally stimulated intact (data derived from this and other reports) or permeabilized (data from this report) cells are shown. (A) Data from Table 1: the initial rate of depletion of PtdIns(4,5) P_2 is probably a good estimate of its PIC-driven turnover; the negative value is a consequence of PtdIns(4,5) P_2 levels returning towards control during this period. (B) Data from Table 1: these are minimum estimates of PIC-catalysed (and are therefore minimum estimates of the total) turnover of PtdIns(4,5) P_2 , based on accumulation of inositol phosphates in the presence of 10 mM LiCl. (C) Estimates based on changes in PtdIns(4,5) P_2 concentration [like those in (A)] derived from a number of other studies using different ligands and 32 P- or 3 H-labelled cells [6,9,18,21,52]. (D) The data are derived from other studies [like those in (B)] using [3 H]Ins-labelled cells [18,21]. (E) An estimate of the total turnover of PtdIns(4,5) P_2 [8] based on its predicted rate of synthesis. (F) Data from Figure 2 similar in principle to those in (A) and (C). (G) Data from Figure 2, an estimate of the total turnover of PtdIns(4,5) P_2 at later times that is largely unaffected by possible activation of PtdIns(4,5) P_2 synthesis. (H) Data from Figure 3 pooled with data from 5 other experiments (means $\pm$ S.E.M.) to estimate directly the total turnover of PtdIns(4,5) P_2 in an analogous manner, but with more accurately defined data, to (E). (I) Estimates of PtdIns4P synthesis; data from Figure 3.

Cell preparation	Source of data	Assays	Total or PIC-mediated turnover of PtdIns4P or PtdIns(4,5) P_2 (% of control/min)		
			Basal	Maximum activation	
				Initial	Late
Intact cells	A	Loss of [3 H]PtdIns(4,5) P_2		163 $\pm$ 27 (0-8 s)	-5 $\pm$ 14 (8-30 s)
	B	Accumulation of [3 H]-InsPs	3.3 $\pm$ 2.2	188 $\pm$ 7 (0-8 s)	149 $\pm$ 15 (8-30 s)
	C	Loss of PtdIns(4,5) P_2		150 $\rightarrow$ 250 (0-15 s)	0 $\rightarrow$ 15 (15-30 s)
	D	Accumulation of InsPs	3 $\rightarrow$ 15	175 $\rightarrow$ 250 (0-15 s)	125 $\rightarrow$ 250 (15-30 s)
	E	PtdIns(4,5) P_2 synthesis	5 $\rightarrow$ 35		
Permeabilized cells	F	Loss of [3 H]PtdIns(4,5) P_2		130 $\pm$ 21 (0-15 s)	16 $\pm$ 19 (30-60 s)
	G	Loss of [32 P]PtdIns(4,5) P_2			132 (30-40 s)
	H	PtdIns(4,5) P_2 synthesis	9 $\pm$ 1	19 $\pm$ 5 (0-15 s)	155 $\pm$ 25 (45-60 s)
	I	PtdIns4P synthesis	55 $\pm$ 18	65 $\pm$ 15 (9-30 s)	54 $\pm$ 12 (30-60s)

PtdIns(4,5) P_2 by these two enzymes [6]. Thus, over the first 8 s of stimulation, the rapid fall in the levels of PtdIns(4,5) P_2 is matched by an approximately stoichiometric increase in the accumulation of inositol phosphates (Table 1). However, after 8-10 s, PtdIns(4,5) P_2 begins to return towards control levels, but inositol phosphate production continues unabated for at least 30 s (Table 1). Taken within the context of (a) the known metabolism of PtdIns(4,5) P_2 (Figure 1), (b) the observations that the levels of PtdIns and PtdIns4P do not fall significantly in this time frame (results not shown), (c) only 18 % of the total inositol phosphates which accumulate in response to FMLP are inositol mono-phosphates (results not shown), and (d) that, although LiCl was included in these experiments as an inhibitor of inositol phosphate

degradation (the approximately linear accumulation of inositol phosphates in stimulated cells suggests that this had been effective), the fact that this blockade will not be complete means that this estimate of the extent of activation of PtdIns(4,5) P_2 production will be a minimum value; these data imply that FMLP must stimulate a substantial increase in net synthesis of PtdIns(4,5) P_2 . Furthermore, this increased net synthesis, although delayed relative to activation of PIC, is nevertheless rapidly established after receptor stimulation.

The rates of PtdIns(4,5) P_2 depletion and inositol phosphate accumulation during the initial (0-8 s) and late (8-30 s) phases of FMLP stimulation are shown in Table 2 (rows A and B). These values are remarkably similar to estimates of equivalent values

Figure 2 Guanine-nucleotide- and agonist-regulated PtdIns4P 5-kinase in permeabilized neutrophils

Two forms of data are shown in the Figure. [^3H]Ins-labelled human neutrophils were permeabilized with streptolysin-O, then challenged with GTP[S] and FMLP (●; final concns. 10 and 1 μM respectively) or vehicle (○), and the quantity of [^3H]PtdIns(4,5) P_2 was determined and expressed as a percentage of the [^3H]PtdIns(4,5) P_2 in unstimulated neutrophils which had been permeabilized with streptolysin-O for 20 s (see the Materials and methods section). The concentrations of PtdIns(4,5) P_2 and PtdIns4P in these samples were 203 and 160 pmol/mg of protein respectively (right-hand ordinate). Data are means $\pm$ S.E.M. ($n = 3-4$) from two independent experiments. The concentrations of PtdIns4P in control and GTP[S] + FMLP-stimulated permeabilized neutrophils fell to $85 \pm 3\%$ and $82 \pm 4\%$ respectively of their initial value by 60 s. Data are also shown from experiments in which streptolysin-O-permeabilized neutrophils were incubated with [γ - ^{32}P]ATP and either GTP[S] and FMLP (■; final concns. 10 and 1 μM respectively) or their vehicle (□) during permeabilization (i.e. streptolysin-O, agonists and [γ - ^{32}P]ATP were added simultaneously): after 30 s, 6 mM MgATP was added (arrow) and the quantity of [^{32}P]PtdIns(4,5) P_2 was determined after various times (see the Materials and methods section). At the point of addition of the unlabelled ATP, there were 10 495 and 1740 d.p.m. in the GTP[S] + FMLP-inactivated and control samples respectively. Data shown (d.p.m.) are means ($n = 2$) from 2 independent experiments.

derived from a number of other studies of receptor-activated PIC in various cells (where PIC undergoes prolonged activation by G-protein-linked receptors); see Table 2 (rows C and D). This suggests that agonist-stimulated net synthesis of PtdIns(4,5) P_2 is a general phenomenon.

FMLP activates a PtdIns4P 5-kinase activity in permeabilized neutrophils

Streptolysin-O-permeabilized neutrophils maintained intact-cell levels of PtdIns4P and PtdIns(4,5) P_2 (results not shown; see [22]) and, at least over the first 60 s of stimulation, similar rates of FMLP-stimulated PtdIns(4,5) P_2 depletion to those seen in intact cells (Figure 2, and Table 2, rows F and G).

The rates of synthesis of PtdOH, PtdIns4P and PtdIns(4,5) P_2 in control, GTP[S]- and FMLP/GTP[S]-stimulated permeabilized neutrophils were estimated from the initial rates of ^{32}P incorporation into these compounds in permeabilized cells incubated with [γ - ^{32}P]ATP (Figure 3 and Table 2, rows H and I). Previous work suggests that the large and sustained increase in ^{32}P incorporation into PtdOH stimulated by GTP[S] and GTP[S]/FMLP is partly due to the combined actions of a stimulated PIC and a diacylglycerol kinase (e.g. [27]); this is likely to be an overestimate of PIC activation, however, since some diacylglycerol may be liberated from other sources on stimulation [28]. GTP[S] and FMLP/GTP[S] also stimulated a small increase in ^{32}P incorporation into PtdIns4P and a very substantial increase in ^{32}P incorporation into PtdIns(4,5) P_2 .

Figure 3 GTP[S] + FMLP-activated synthesis of PtdIns4P, PtdIns(4,5) P_2 and PtdOH in streptolysin-O-permeabilized neutrophils

Human neutrophils were permeabilized with streptolysin-O and then incubated with [γ - ^{32}P]ATP (30 μCi per assay) and either GTP[S] + FMLP (■, ●, ▲; final concns. 10 and 1 μM respectively) or their vehicle (□, ○, △). Reactions were quenched and PtdOH (□, ■), PtdIns4P (○, ●) and PtdIns(4,5) P_2 (△, ▲) were quantified as described in the Materials and methods section. Data shown (d.p.m.) are means $\pm$ S.E.M. ($n = 4$) from two independent experiments. Samples of these preparations of neutrophils contained 205 pmol of PtdIns(4,5) P_2 /mg of protein and 280 pmol of PtdIns4P/mg of protein. The quantities of PtdIns(4,5) P_2 and PtdIns4P synthesized during the assays were calculated from the specific radioactivity of the ATP, and are shown in the Figure.

The [^{32}P]PtdIns(4,5) P_2 produced after 1 min stimulation with FMLP/GTP[S] was taken for further study. Its structure was confirmed to be PtdIns(4,5) P_2 , and 85–90% of its label was found in the 5-position (results not shown). Therefore, since FMLP/GTP[S] stimulated the initial rate of ^{32}P incorporation into this compound (Figure 3), while the levels of PtdIns4P were falling (see the legend to Figure 2), a PtdIns4P 5-kinase activity must have been substantially stimulated by FMLP/GTP[S] under these conditions. Further, a consideration of the time courses for FMLP/GTP[S]-stimulated incorporation of ^{32}P into PtdIns(4,5) P_2 and PtdIns4P suggests that the stimulation of PtdIns4P 5-kinase occurs with a lag of 10–20 s (Figure 3).

Characteristics of G-protein-stimulated PtdIns4P 5-kinase activity

The ability of GTP[S] to stimulate a PtdIns4P 5-kinase activity in permeabilized neutrophils strongly suggested that this enzyme might be under the control of a receptor-operated G-protein. This idea is confirmed by observations that FMLP and GTP[S] act synergistically to stimulate PtdIns4P 5-kinase, that pertussis toxin can block both PAF- and FMLP-induced activations of this enzyme (but not the response to GTP[S]), and that both FMLP- and GTP[S]-induced activations of this enzyme are blocked by GDP[S] (Table 3).

The characteristics of G-protein-stimulated activation of PIC

Table 3 Regulation of PtdIns4P 5-kinase in permeabilized neutrophils

Freshly isolated human neutrophils were incubated with pertussis toxin (either heat-denatured or active), resuspended in intracellular buffer and permeabilized in the absence or presence of 150 μ M GDP[S] (assays with GDP[S] finally contained 400 μ M ATP: controls which included 400 μ M ATP showed that this did not differentially affect the responses to GTP[S] and FMLP; consequently the data from GDP[S]-containing assays were calibrated via their controls to the rest of the data shown). After 30 s permeabilization, GTP[S] (10 μ M) and/or FMLP (1 μ M) or PAF (0.1 μ g/ml) were added with [γ - 32 P]ATP (25 μ Ci/assay, except for assays containing GDP[S], which contained 100 μ Ci assay $^{-1}$). Assays were quenched after 30 s, and [32 P]PtdIns(4,5) P_2 was quantified as described in the Materials and methods section. Data shown (d.p.m.) are means $\pm$ S.E.M. or ranges ($n = 2-4$) from 5 separate experiments.

Pretreatment	Stimulus	[32 P]PtdIns(4,5) P_2
Control	Control	3361 $\pm$ 151
	GTP[S]	6096 $\pm$ 392
	PAF	6151 $\pm$ 163
	FMLP	3918 $\pm$ 157
	FMLP + GTP[S]	15262 $\pm$ 304
Pertussis toxin	Control	1865 $\pm$ 105
	GTP[S]	5987 $\pm$ 60
	PAF	2093 $\pm$ 109
	FMLP	1920 $\pm$ 84
	FMLP + GTP[S]	8153 $\pm$ 817
GDP[S]	Control	2453 $\pm$ 94
	GTP[S]	2706 $\pm$ 111
	FMLP	2043 $\pm$ 111

Figure 4 Guanine-nucleotide-activated PtdIns(4,5) P_2 synthesis in streptolysin-O-permeabilized neutrophils

Streptolysin-O-permeabilized human neutrophils were incubated with [γ - 32 P]ATP in the presence of various concentrations of p[NH]ppG (■), GTP[S] (▲), GTP[β S]-Rp (●) and GTP[β S]-Sp (◆). Reactions were quenched after 30 s, and [32 P]PtdIns(4,5) P_2 was quantified as described in the Materials and methods section and expressed as mean percentages of the response to 35 μ M GTP[S], $\pm$ S.E.M. ($n = 3-6$ from 4 independent experiments).

Figure 5 Ca^{2+} -regulated PtdIns(4,5) P_2 and PtdOH accumulation in permeabilized neutrophils

Human neutrophils were permeabilized with streptolysin-O and then challenged with GTP[S] + FMLP (○, △; final concns. 10 and 1 μ M, respectively) or vehicle alone (●, ▲) in the presence of various concentrations of free Ca^{2+} . Reactions were quenched after 30 s, and PtdOH (●, ○) and PtdIns(4,5) P_2 (▲, △) were quantified as described in the Materials and methods section. Data shown (d.p.m.) are means $\pm$ S.E.M. ($n = 3-4$) from two experiments.

and PtdIns4P 5-kinase were investigated by studying the ability of a range of different guanine nucleotides to elicit these responses (Figure 4) and their sensitivity to the concentration of free Ca^{2+} (Figure 5). GTP[β S] [29], particularly the S_p diastereoisomer, was able to elicit relatively larger accumulations of [32 P]PtdIns(4,5) P_2 than of [32 P]PtdOH, though the relative effects of GTP[S] and p[NH]ppG were very similar. Ca^{2+} , over the range 0–1 μ M, caused a very large potentiation of GTP[S]-stimulated PtdOH formation, in agreement with previous work, which has indicated that Ca^{2+} in this range potentiates a G-protein-activated PIC in neutrophils [23]. However, equivalent concentrations of Ca^{2+} caused a much smaller potentiation of GTP[S]-stimulated [32 P]PtdIns(4,5) P_2 formation, such that at essentially zero Ca^{2+} concentration GTP[S] was able to elicit approx. 75% of its maximal stimulation of [32 P]PtdIns(4,5) P_2 formation (100% at 100 μ M Ca^{2+} is an approx. 4-fold stimulation), whereas its stimulation of [32 P]PtdOH formation was virtually undetectable (100% at 750 μ M Ca^{2+} is an approx. 12-fold stimulation).

Protein kinase C activators have been suggested to stimulate synthesis of PtdIns(4,5) P_2 and/or PtdIns4P [30]. PMA increased accumulation of PtdIns4P in both basal and FMLP-activated neutrophils (Table 4). In contrast, it had no effects on the accumulation of PtdIns(4,5) P_2 under these circumstances, and indeed decreased inositol phosphate production in response to FMLP (Table 4). Protein kinase C inhibitors (RO31-8220, RO31-7549) failed to reveal evidence of protein kinase C-mediated activation of PtdIns(4,5) P_2 production in either basal or FMLP-activated cells, and this was confirmed by findings with the potent but less selective protein kinase inhibitor staurosporine in both intact (see Table 4: these treatments abolished FMLP-activated production of reactive oxygen intermediates in neutrophils and PMA-stimulated PKC-type activity in permeabilized neutrophils [22,31,32]) and permeabilized cells (Table 5). The manner in which some of these reagents selectively elevated or decreased PtdIns4P accumulation, without effect on PtdIns(4,5) P_2 levels, indicates not only that PtdIns 4-kinase is subject to complex control, but also that PtdIns(4,5) P_2 synthesis is not limited by its lipid substrate. Staurosporine is also a potent inhibitor of many protein tyrosine kinases [33], and has been demonstrated to inhibit the ability of FMLP and granulocyte/

Table 4 Effects of PMA and protein kinase inhibitors

[32 P] P_i - or [3 H]Ins-labelled human neutrophils were incubated with PMA (treatments 1, 2, 3 and 9; final concn. 100 ng/ml, for 1.5, 3.0, 4 and 4 min respectively), staurosporine (ST; treatments 4, 5, 10 and 11; 1 or 5 μ M for 3 min), RO31-7549, RO31-8220 (treatments 6, 7, 12 and 13; all 10 μ M for 3 min) and/or FMLP (treatments 8–13; final concn. 1 μ M, for the last 8 s of the relevant treatment protocols). Reactions were quenched and the indicated 32 P-labelled lipids or a total [3 H]inositol phosphate fraction were quantified as described in the Materials and methods section. Data are means $\pm$ S.E.M. ($n = 3-8$) from 10 independent experiments (8 with [32 P] P_i -labelled cells) which were normalized via the amount of [3 H]- or [32 P]-PtdIns(4,5) P_2 in samples of unstimulated cells, from each of the experiments.

				Radioactivity (d.p.m.) in:		
				[32 P]PtdIns4P	[32 P]PtdIns(4,5) P_2	[3 H]-Ins P_2 -Ins P_4
Control				10618 $\pm$ 493	28089 $\pm$ 2165	1055 $\pm$ 42
PMA	1.5 min	1		17021 $\pm$ 1321	31194 $\pm$ 522	1098 $\pm$ 22
	3.0 min	2		18993 $\pm$ 702	28134 $\pm$ 2111	1103 $\pm$ 81
	4.0 min	3		20491 $\pm$ 1673	27166 $\pm$ 2311	991 $\pm$ 46
ST	1 μ M	4		10639 $\pm$ 783	25599 $\pm$ 1308	
	5 μ M	5		15432 $\pm$ 836	27066 $\pm$ 1343	
RO31-7549		6		10788 $\pm$ 23	27489 $\pm$ 1359	
RO31-8220		7		11205 $\pm$ 536	27691 $\pm$ 2100	
FMLP		8		10952 $\pm$ 555	24172 $\pm$ 2031	8945 $\pm$ 464
FMLP + PMA		9		19432 $\pm$ 1943	26351 $\pm$ 1473	5841 $\pm$ 371
FMLP + ST	1 μ M	10		10639 $\pm$ 386	25599 $\pm$ 1323	
	5 μ M	11		15432 $\pm$ 664	27066 $\pm$ 873	
FMLP + RO31-7549		12		12188 $\pm$ 1101	24033 $\pm$ 2639	
FMLP + RO31-8220		13		12161 $\pm$ 936	23011 $\pm$ 1666	

Table 5 Effects of staurosporine on PtdIns(4,5) P_2 and PtdIns4P synthesis in permeabilized neutrophils

Human neutrophils were permeabilized with streptolysin-O in the absence or presence of staurosporine (ST; final concn. 5 μ M), then incubated with [γ - 32 P]ATP with p[NH]ppG (final concn. 300 μ M), FMLP (final concn. 1 μ M) or their vehicle for 40 s. The assays were quenched, and 32 P (d.p.m.) accumulated in PtdIns4P and PtdIns(4,5) P_2 was quantified as described in the Materials and methods section. Data shown are means $\pm$ S.E.M. ($n = 3-5$, from 2 independent experiments).

Treatment	32 P accumulated (d.p.m.)	
	PtdIns4P	PtdIns(4,5) P_2
Control	31794 $\pm$ 140	6063 $\pm$ 113
Control + ST	30251 $\pm$ 898	6339 $\pm$ 47
p[NH]ppG	32041 $\pm$ 124	25642 $\pm$ 581
p[NH]ppG + ST	26338 $\pm$ 1598	22053 $\pm$ 241
FMLP	34149 $\pm$ 3132	6799 $\pm$ 257
FMLP + ST	34473 $\pm$ 976	6550 $\pm$ 50

macrophage colony-stimulating factor to activate *src*-type protein-tyrosine-kinase-driven processes in neutrophils [31,32]. Hence the resistance of the p[NH]ppG-activated accumulation of PtdIns(4,5) P_2 to staurosporine (see Table 5) also suggests these effects are unlikely to be coupled via *src*-type protein tyrosine kinases.

DISCUSSION

A comparison of estimates for the basal rates of synthesis of PtdIns4P and PtdIns(4,5) P_2 in permeabilized neutrophils, with estimates for the basal rates of synthesis of inositol phosphates in intact cells (Table 2, rows B and D versus H and I), suggests that substantial levels of substrate cycling of PtdIns4P, but only low

levels of substrate cycling of PtdIns(4,5) P_2 , occur in basal cells. The prediction that a low level of substrate cycling of PtdIns(4,5) P_2 occurs in cells is consistent with less accurately defined data estimating the basal rate of PtdIns(4,5) P_2 synthesis in [32 P] P_i -labelled intact cells [18] (Table 2, row E). This suggests that substrate-cycling reactions could supply a large proportion of the synthesis of PtdIns4P, but only some 5–8% of the synthesis of PtdIns(4,5) P_2 , that is required on cell stimulation (Table 2).

It is clear from the data obtained with permeabilized neutrophils that agonists can stimulate a PtdIns4P 5-kinase activity via a pertussis-toxin-sensitive G-protein. The increased rate of synthesis of PtdIns(4,5) P_2 produced by this mechanism is of similar magnitude to that predicted to be required to maintain the activity of PIC in intact neutrophils (Table 2). Thus, at least in neutrophils, it appears that agonists such as FMLP and PAF solve their problems of stimulating increased net synthesis of PtdIns(4,5) P_2 by substantially activating a PtdIns4P 5-kinase. This conclusion is similar to that drawn from more ambiguous studies of either increased 32 P-labelling [15,16], or increased levels [19,30,34–36], of PtdIns(4,5) P_2 in other agonist-stimulated intact cells. It is also apparently consistent with previous reports of agonist or GTP[S] stimulation of PtdIns4P 5-kinase activities in other cell-free assays [37–39,49], though the relatively small effects of stimulation (1.5–2.0-fold increases in activity) and possible ambiguities of interpretation [40] in these studies suggest that we may be studying a different phenomenon.

The picture regarding the rates of synthesis of PtdIns4P in basal and agonist-stimulated neutrophils is less clear (see also [16]). The much larger basal rates of synthesis of PtdIns4P, and the relatively small activations of PtdIns 4-kinase by agonists and/or GTP[S] in permeabilized neutrophils, suggest that it is possible that agonists could increase net synthesis of PtdIns4P by substantial inhibition of a PtdIns4P 4-phosphatase activity. However, even though our estimates of the relevant activities are relatively crude, they do suggest that the activity of PtdIns 4-

kinase in permeabilized neutrophils is insufficient to maintain the observed rates of PIC activation in intact cells (Table 2), and thus it remains plausible that, for some reason, an activation of PtdIns 4-kinase activity is underestimated in these experiments.

The substantially lagged activation of PtdIns4P 5-kinase that occurs in both intact and permeabilized cells, and the almost complete loss of responsiveness to GTP[S] and/or agonists that occurs on homogenization of neutrophils (results not shown), indicate that the pathway may involve more than one step. In principle, the receptor activation of PtdIns4P 5-kinase could be mediated by a signal that is independent of, or alternatively represents feedback from, a PtdIns(4,5) P_2 -directed effector system. The latter class of mechanism includes a huge array of candidates, including: product inhibition of PtdIns4P 5-kinase, diacylglycerol, PtdOH, PtdIns(3,4,5) P_3 and Ca^{2+} , Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 [37,38,41,42]. However, some evidence suggests that these mechanisms are unlikely to be responsible for coupling receptor activation to PtdIns4P 5-kinase. Hence, the small changes in PtdIns(4,5) P_2 levels that occur on agonist stimulation limit the potential scope of product-derived feedback, unless PtdIns4P 5-kinase is speculated to experience local concentrations of its product that are substantially lower than those present in the whole cell. The pharmacological sensitivity of the response suggests that diacylglycerol and PKC are not implicated in coupling (see Table 4 and 5). Similarly, the fact that GTP[β S]-Sp can stimulate production of PtdIns(4,5) P_2 , but not PtdOH (Table 3) or PtdIns(3,4,5) P_3 (results not shown; [22]), would appear to indicate that neither of these phospholipids serves as messages in this context. The observations that the response is insensitive to Ca^{2+} and is still fully coupled in permeabilized cells indicates that Ca^{2+} , Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 are also poor candidates. Moreover, although a post-effector-derived modulatory input can generate an input-dependent feedback signal, it would be inflexibly associated with activation of its effector, and would not readily accommodate the receptor specificity which we have seen exemplified in the potent activation of PtdIns4P 5-kinase by PAF compared with FMLP (Table 5), despite their similar effects on inositol phosphate accumulation (results not shown). Pathways for PtdIns4P 5-kinase activation that do not utilize PtdIns(4,5) P_2 -directed effectors require additional mechanisms to tailor supply to consumption; however, a combination of product inhibition and a limited level of substrate cycling could achieve this and retain the greater regulatory flexibility of this type of system.

An issue related to this question is whether identical G-proteins are responsible for the activation of PtdIns(4,5) P_2 synthesis and PtdIns(4,5) P_2 consumption. The agonist-stimulated activations of PtdIns4P 5-kinase, PIC and PI3K are all sensitive to inhibition by pertussis toxin, and hence are all likely to be mediated by G_i -family proteins [22,23,26] (G_{i2} or G_{i3} in neutrophils). It is not clear, however, if the same G_i proteins, or their subunits (PIC β_2 is likely to be activated by a G_i -protein's $\beta\gamma$ subunits in neutrophils [43]), are involved in coupling to these three responses. The relative abilities of GTP[β S] diastereomers to activate PtdOH synthesis, PtdIns(4,5) P_2 synthesis and PtdIns(3,4,5) P_3 synthesis provide some indirect evidence that separate G-proteins may be involved. The possibility that the differential effects of these GTP[β S] isomers are a result of specific effects on diacylglycerol kinase cannot be discounted; however, neither of these GTP[β S] compounds inhibited PtdIns(4,5) P_2 3-kinase activity assayed with exogenous lipid substrates [22]. It is unlikely that this issue will be fully resolved until the appropriate responses can be reconstituted *in vitro* with defined G-protein subunits.

The speed of the agonist-induced increase in PtdIns4P 5-kinase activity, and the fact that it occurs in permeabilized cells, suggest that it did not result from an increase in the mass of PtdIns4P 5-kinase in the cell, nor, because 85 % of the activity in unstimulated neutrophils is particulate ([44]; results not shown), was it likely to be due to its translocation to the plasma membrane. It is plausible, however, that agonists might stimulate an association of PtdIns4P 5-kinase with the cytoskeleton [45–47], thereby 'activating' the enzyme by causing a local reorientation of the lipid kinase and/or PtdIns4P (i.e. increasing effective 'substrate availability'), rather than a classical allosterically- or covalent-modification-driven activation of its catalytic domain. A requirement for stimulated association with the cytoskeleton would also provide simple explanations for the lag built into the process of activation and the loss of this response on homogenization.

The results described above begin to answer the question of how G-protein-coupled agonists such as FMLP and PAF co-ordinate the activation of PtdIns(4,5) P_2 consumption and supply in neutrophils. The principles on which this regulation is based are likely to be the same in a wide variety of different cellular contexts (see the Introduction), though their operation in detail is likely to depend on numerous cell-specific factors, such as the nature and intensities of agonist stimulation, the relative sizes of the reservoirs of PtdIns4P and PtdIns(4,5) P_2 and the precise degree of substrate cycling between them. In the case of agonist stimulation via protein tyrosine kinase-mediated mechanisms, e.g. via receptors for growth factors such as epidermal growth factor [48] or the T-cell antigen receptor complex [34], precedent suggests that any enzymes directly regulated will carry SH2 domains and/or tyrosine phosphate adaptations required for control by these mechanisms [12] and are thus likely to be different to equivalent enzymes directly regularly by G-proteins [10]. Perhaps the recent identification of multiple isoforms of PtdIns4P 5-kinase [50,51] and observations of agonist-sensitive translocations of PtdIns4P 5-kinase activity to growth-factor-receptor complexes [45] are indeed evidence of more general regulation of this enzyme activity.

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