

## RESEARCH COMMUNICATION

**Thrombin receptors modulate insulin-stimulated phosphatidylinositol 3,4,5-trisphosphate accumulation in 1321N1 astrocytoma cells**

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Thrombin and insulin receptor signalling via phosphoinositide (PI)-specific phospholipase C (PLC) and PI 3-kinase was studied in [<sup>3</sup>H]inositol-labelled 1321N1 cells. Thrombin stimulated a dramatic, transient activation of PLC which is probably mediated via receptors of the 'tethered-ligand' type, since it was both reproduced by, and abolished following, pretreatment of cells with a synthetic peptide (SFLLRN) corresponding to the ligand domain of the human thrombin receptor. However, neither thrombin nor SFLLRN stimulated PI 3-kinase. By contrast, insulin did not influence [<sup>3</sup>H]InsP<sub>3</sub> concentrations but stimulated accumulation of [<sup>3</sup>H]PtdIns(3,4,5)P<sub>3</sub> and [<sup>3</sup>H]PtdIns(3,4)P<sub>2</sub>, the

relative steady-state concentrations of which may indicate degradation of [<sup>3</sup>H]PtdIns(3,4,5)P<sub>3</sub> by 5- and 3-phosphatases. The independent coupling of thrombin and insulin receptors to PLC and PI 3-kinase respectively in 1321N1 cells allowed interactions between these systems to be examined. Thus insulin-stimulated [<sup>3</sup>H]PtdIns(3,4,5)P<sub>3</sub> accumulation was attenuated on co-stimulation of the thrombin receptor, whereas concentrations of [<sup>3</sup>H]PtdIns(3,4)P<sub>2</sub> were transiently enhanced but then reduced. These results indicate that thrombin receptors in 1321N1 cells do not activate PI 3-kinase, but can modulate signalling by this enzyme.

## INTRODUCTION

It is apparent that inositol phospholipids are central components of two systems for the transmission of signals across the plasma membrane. The first involves the activation of phospholipases C (PLC) by cell-surface receptors, including many coupled to heterotrimeric GTP-binding proteins (G-proteins) and others possessing intrinsic tyrosine kinase activity, resulting in the hydrolysis of PtdIns(4,5)P<sub>2</sub> and release of the second messengers Ins(1,4,5)P<sub>3</sub> and diacylglycerol [1]. The second system involves phosphoinositide (PI) 3-kinase [2–4], and although most attention to date has focused on the activation of this enzyme by receptor tyrosine kinases, more recent evidence has demonstrated similar activities regulated by the G<sub>βγ</sub>-subunits of heterotrimeric G-proteins [5,6] and by small G-proteins [7–9]. Many details of this system are still uncertain, but it is likely that the primary substrate for receptor-regulated PI 3-kinases is also PtdIns(4,5)P<sub>2</sub> [10,11], and it is widely accepted that the PtdIns(3,4,5)P<sub>3</sub> produced may function as a second messenger, perhaps by regulating the activity of certain isoforms of protein kinase C [12] or of protein kinase B [13,14]. As both PLC and PI 3-kinase share PtdIns(4,5)P<sub>2</sub> as their common substrate, it is probable that signalling via these systems will be closely integrated, particularly as some receptors (e.g. thrombin, platelet-derived growth factor or formylmethionyl-leucylphenylalanine; see the above-cited references) possess the capacity to stimulate both pathways.

The potential for interaction between these systems was examined here in 1321N1 astrocytoma cells. These cells are an appropriate model for such studies as they express numerous receptors coupled to PLC [15], including that for thrombin [16] and an insulin [or insulin-like growth factor-1 (IGF-1)] receptor which mediates a marked activation of PI 3-kinase (see below), both of which responses may be assayed directly and quantitatively after the labelling of phospholipid pools to steady state

with [<sup>3</sup>H]inositol. The initial results presented suggest that thrombin activates PLC in these cells through a 'tethered-ligand' receptor similar to that thought to mediate many of the actions of thrombin in diverse cell types ([17]; for a review, see [18]), including the stimulation of both PLC and PI 3-kinase in platelets [19]. Significantly, however, activation of the thrombin receptor in 1321N1 cells evoked a profound stimulation of PLC, but failed to stimulate PI 3-kinase. Conversely, insulin stimulated a marked accumulation of PtdIns(3,4,5)P<sub>3</sub>, but did not affect concentrations of InsP<sub>3</sub>. As many receptors activate both PLC and PI 3-kinase, interactions between these signalling systems are difficult to resolve and none have yet been reported. The observation that thrombin and insulin (or IGF-1) receptors respectively couple separately to PLC and PI 3-kinase in 1321N1 cells allowed each response to be characterized independently and the potential for cross-regulation to be examined. Thus analysis of the PI 3-kinase response to insulin, and of the effects on this resulting from co-stimulation of the thrombin receptor, revealed complex control of PtdIns(3,4,5)P<sub>3</sub> concentrations in 1321N1 cells, indicating that these may be modulated by two mechanisms after activation of PLC.

## MATERIALS AND METHODS

## Materials

1321N1 cells were obtained from the European Tissue Culture Collection, and materials for cell culture were from Gibco. Thrombin-receptor-activating peptide (SFLLRN) was synthesized by the MRC Protein Phosphorylation Unit at the University of Dundee. Human thrombin, bovine insulin, carbachol and Folch fraction 1 were from Sigma. AGIX8 resin (200–400 mesh, formate form) was from Bio-Rad, HPLC columns were from LaserChrom, and *myo*-[2-<sup>3</sup>H]inositol (~

Abbreviations used: PI, phosphoinositide; PLC, phospholipase C; IGF, insulin-like growth factor; SFLLRN, thrombin-receptor-activating peptide; GroPIns, glycerophosphoinositol.

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20 Ci/mmol) was from New England Nuclear. Other reagents were of analytical grade and purchased from BDH or Sigma.

### Cell culture and radiolabelling

1321N1 cells were grown to confluence over 6 days in 6- or 24-multiwell plates under inositol-depleting conditions as described previously [15], then labelled with [ $^3\text{H}$ ]inositol ( $\sim 2\text{--}10 \mu\text{Ci/ml}$ ) for 2–3 days. These conditions initially reduce cellular free inositol, but not phosphoinositide concentrations, allowing subsequent steady-state [ $^3\text{H}$ ]inositol labelling of inositol phospholipids to relatively high specific radioactivity [15]. For experiments designed to measure activation of PI 3-kinase, cells were washed several times with serum and inositol-free Dulbecco's modified Eagle's medium after [ $^3\text{H}$ ]inositol labelling and maintained overnight in the same medium supplemented with either 0.05% (v/v) dialysed foetal-calf serum or 0.5% (w/v) BSA before incubation as described below.

### Measurement of PLC and PI 3-kinase activity

Culture medium was aspirated, and [ $^3\text{H}$ ]inositol-labelled cells washed and incubated as indicated and then fixed by the addition of ice-cold 0.5 M trichloroacetic acid as described previously [15,20]. The analysis of  $^3\text{H}$ -labelled metabolites arising from assays designed to measure PLC was also as described previously [15,20], but a modification of this procedure was used for the measurement of the [ $^3\text{H}$ ]PtdIns(3,4,5) $P_3$  and [ $^3\text{H}$ ]PtdIns(3,4) $P_2$  arising from stimulation of PI 3-kinase. Briefly, trichloroacetic acid-precipitated cell debris was collected by centrifugation and the supernatant processed for analysis of [ $^3\text{H}$ ]inositol phosphates as indicated above. Cell pellets were washed twice with 2 ml of 5% (w/v) trichloroacetic acid containing 1 mM EDTA and  $^3\text{H}$ -labelled lipids then extracted for  $\sim 15$  min into 0.75 ml of methanol/chloroform/12 M HCl (80:40:1, by vol.) and 0.5 mg of Folch fraction 1 added as carrier. Chloroform (0.25 ml) and 0.1 M HCl (0.45 ml) were then added, the samples mixed and centrifuged, and the lower organic phase containing the [ $^3\text{H}$ ]phospholipid collected and stored on ice. The upper phases were washed twice with synthetic lower phase and the pooled organic phases then neutralized with 0.5 M  $\text{NH}_4\text{OH}$  in methanol and dried under vacuum. The dried  $^3\text{H}$ -labelled lipids were then deacylated exactly as described by Clarke and Dawson [21], but using only 0.1–0.2 ml of methylamine reagent. The resulting water-soluble [ $^3\text{H}$ ]glycerophosphoinositol (phosphates) were separated by anion-exchange HPLC as described previously for inositol phosphates [15] and quantified by liquid-scintillation counting. Sample [ $^3\text{H}$ ]GroPIns(3,4,5) $P_3$  and [ $^3\text{H}$ ]GroPIns(3,4) $P_2$  were identified by comparison with retention times for authentic  $^{32}\text{P}$ -labelled standards prepared essentially as described previously [22] (GroPIns is glycerophosphoinositol).

## RESULTS AND DISCUSSION

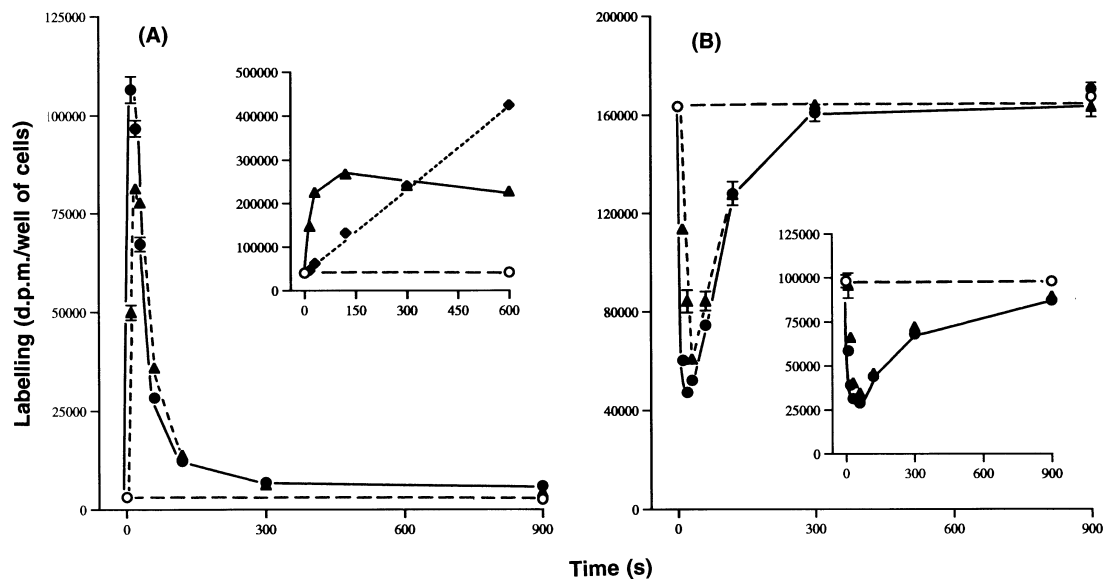
### 1321N1 cells express a 'tethered-ligand' receptor for thrombin which couples to PLC but not PI 3-kinase

Activation of the G-protein-coupled receptor through which thrombin exerts many of its actions [18] occurs by an unusual mechanism [17] involving proteolytic cleavage of the receptor within the extracellular domain, leaving a new N-terminal sequence (SFLLRN) [23] which functions as a 'tethered ligand' recognized by regions of the receptor at the cell surface [24]. In platelets this receptor couples to the activation of both PLC and PI 3-kinase [19]. As only thrombin which is proteolytically active is able to stimulate PLC in 1321N1 cells [16], we examined

the possibility that these cells express a similar receptor capable of regulating both PI-signalling systems.

Figure 1 shows that thrombin and the thrombin receptor-activating peptide (SFLLRN) both evoke a marked and rapid, but transient, stimulation of PLC in [ $^3\text{H}$ ]inositol-labelled 1321N1 cells. This was reflected by a dramatic increase in the concentration of [ $^3\text{H}$ ]Ins $P_3$  (typically  $\geq 10\text{--}20$ -fold basal), which peaked after 20–30 s and 10–20 s in response to thrombin and SFLLRN respectively, and then returned close to control values as illustrated in Figure 1(A). The modestly faster response to SFLLRN is consistent with its presumed action as a direct receptor ligand, circumventing the proteolytic step catalysed by thrombin. Figure 1(B) shows that thrombin and SFLLRN each also evoked a contemporary decrease in the concentration of [ $^3\text{H}$ ]PtdIns $P_2$ , which fell by 60–70% at 20–30 s before returning to control values over the subsequent 2–5 min. The inset to Figure 1(B) shows similar changes in the concentration of [ $^3\text{H}$ ]PtdInsP ([ $^3\text{H}$ ]PtdIns concentrations were essentially unaffected by stimulation; results not shown). These responses suggest a similar activation by thrombin and SFLLRN of a PtdIns $P_2$ -selective PLC with access to a large fraction of the total cellular poly-PI pool. Moreover, their transient nature emphasizes not only the stimulated rates at which Ins $P_3$  and PtdIns $P_2$  are metabolized, but also implies a rapid desensitization of thrombin- and SFLLRN-stimulated PLC, which is characteristic of similar responses mediated by the thrombin receptor in other cell types [25–27]. Desensitization of thrombin-stimulated PLC in 1321N1 cells has been suggested previously [16] and is confirmed by the data presented in the inset to Figure 1(A). These show that, in the presence of  $\text{Li}^+$  ions to trap the [ $^3\text{H}$ ]inositol phosphates released by PLC, thrombin-stimulated accumulation of [ $^3\text{H}$ ]products was limited to the initial 1–2 min, whereas that evoked by stimulation of the muscarinic receptor, which mediates persistent activation of PLC in these cells [15], was continuous. These data also emphasize the magnitude of the initial PLC response to thrombin, particularly as that to maximal muscarinic receptor activation is approximately equivalent to turnover of the total cellular PI pool once per hour.

The ability of SFLLRN to stimulate PLC activity in 1321N1 cells, and the similarity in the pattern of this response to that elicited by thrombin, are consistent with the expression of a tethered-ligand-type receptor. Detailed, comparative dose relationships for stimulation of PLC were not defined, but preliminary data indicated maximal and half-maximal responses to thrombin or peptide respectively at 100 nM and 20 nM or 100  $\mu\text{M}$  and 10  $\mu\text{M}$ . These values indicate a lower potency for thrombin than reported previously in 1321N1 cells [16], though this probably reflects methodological differences. The requirement for much higher concentrations of peptide is consistent with both the different mechanisms by which thrombin and SFLLRN activate the receptor and results reported previously [19,28,29]. To confirm that both stimuli act through a common receptor, 1321N1 cells were pretreated for 30 min either as control or with thrombin (50 nM) or SFLLRN (100  $\mu\text{M}$ ) and the stimulation of PLC (increase in Ins $P_3$ ) evoked by a subsequent 30 s exposure to the same or opposing stimulus was then measured in the continued presence of the primary activator. Pretreatment with SFLLRN or thrombin reduced the response on secondary challenge with thrombin and SFLLRN by  $> 95\%$  and  $> 95\%$  or by  $> 95\%$  and  $\sim 70\%$  respectively, but did not influence a secondary stimulation of PLC by the muscarinic receptor agonist carbachol (1 mM) (values are the means for three experiments). These results clearly imply a common receptor target for SFLLRN and thrombin and are compatible with similar data obtained in other cell types [27]. The residual signal



**Figure 1** Thrombin and SFLLRN activate PLC with comparable kinetics

Cells cultured in 24-multiwell plates and labelled with [ $^3\text{H}$ ]inositol ( $\sim 2 \mu\text{Ci}/\text{ml}$ ) for 2–3 days were washed with HEPES-buffered modified Krebs–Henseleit buffer (see [15]), then incubated in the same buffer in the absence ( $\circ$ ) or presence of thrombin (50 nM,  $\blacktriangle$ ) or SFLLRN (100  $\mu\text{M}$ ,  $\bullet$ ) as indicated before extraction and analysis of [ $^3\text{H}$ ]metabolites. (A) and (B) show changes in the concentrations of [ $^3\text{H}$ ]Ins $P_3$  and [ $^3\text{H}$ ]PtdIns $P_2$  respectively and the inset to (B) shows data for [ $^3\text{H}$ ]PtdIns4 $P$ . Labelling of [ $^3\text{H}$ ]PtdIns under control and stimulated conditions was  $\sim 1.7 \times 10^6$  d.p.m./well of cells. Data are the means  $\pm$  S.E.M. for triplicate incubations in one experiment representative of three. The inset to (A) shows the desensitization of thrombin-, but not muscarinic-receptor-mediated PLC activity. Cells were labelled and incubated as above, but in buffer supplemented with 10 mM LiCl and in the absence ( $\circ$ ) or presence of thrombin (50 nM,  $\blacktriangle$ ) or carbachol (1 mM,  $\blacklozenge$ ) before extraction and measurement of a total [ $^3\text{H}$ ]Ins $P_{1-4}$  fraction. Data are the means of duplicate determinations from a single experiment. Similar, less detailed data, were obtained on two further occasions.

**Table 1** Effects of thrombin, SFLLRN and insulin on the accumulation of [ $^3\text{H}$ ]PtdIns(3,4,5) $P_3$  and [ $^3\text{H}$ ]Ins $P_3$

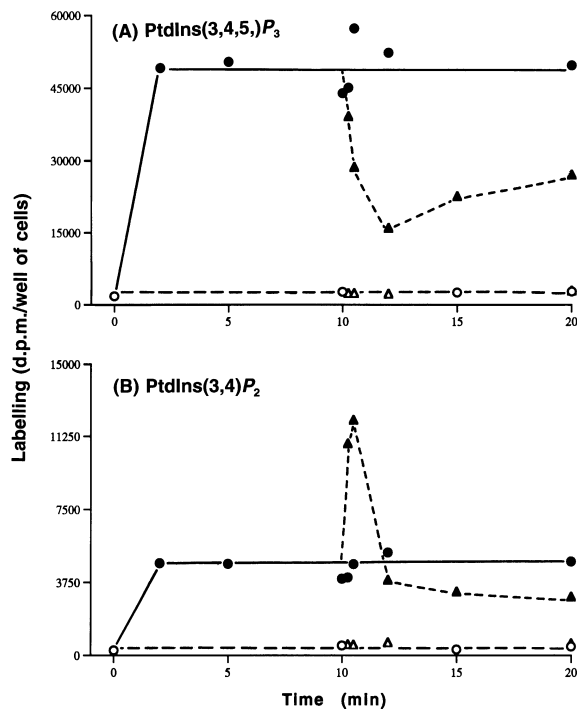
1321N1 cells cultured in six-well plates and labelled with [ $^3\text{H}$ ]inositol (10  $\mu\text{Ci}/\text{ml}$ ) for 2 days were incubated overnight under serum-free conditions, then washed with buffer as described for Figure 1 and incubated for 30 s or 10 min in the presence of thrombin (50 nM), SFLLRN (100  $\mu\text{M}$ ), insulin (10  $\mu\text{g}/\text{ml}$ ) or buffer vehicle before extraction and analysis of  $^3\text{H}$ -labelled metabolites. Results are from single incubations in one experiment, but similar, directly comparable data across all conditions were obtained on one further occasion and independently for each condition in at least one to five other, separate, experiments. The apparent ability of thrombin or SFLLRN to reduce basal PtdIns $P_3$  was not significant. From previous measurements [15] the specific radioactivity of [ $^3\text{H}$ ]metabolites under these conditions is  $\sim 3000$ –6000 d.p.m./pmol.

Incubation time	Stimulus	$^3\text{H}$ -labelled metabolite ...	Labelling (d.p.m./well of cells)		
			[ $^3\text{H}$ ]PtdIns(4,5) $P_2$	[ $^3\text{H}$ ]Ins $P_3$	[ $^3\text{H}$ ]PtdIns(3,4,5) $P_3$
30 s	Control		2703168	124832	3977
	Thrombin		876543	1158391	2032
	SFLLRN		576522	994337	1989
	Insulin		2651339	92634	37782
10 min	Control		2897364	118717	2867
	Thrombin		2837687	153028	2622
	SFLLRN		2183406	81631	1451
	Insulin		2946814	107421	49356

to SFLLRN in thrombin-pretreated 1321N1 cells may reflect the processing and recycling of initially internalized receptors to the cell surface, as reported in human erythroleukaemia and Children's Hospital Research Foundation cell line no. 288 cells [30].

These results provide strong, though preliminary evidence, that the thrombin receptor expressed in 1321N1 cells is very similar to the tethered ligand-type described in other systems. However, the data in Table 1 show that, in contrast with responses in platelets and human embryo retinoblast 10 cells [19,31], stimulation of the thrombin receptor in 1321N1 cells did

not activate PI 3-kinase, whereas PLC responses to thrombin receptor stimulation within the same samples were consistent with those reported above. Table 1 also shows that, under the same conditions, insulin did not elevate [ $^3\text{H}$ ]Ins $P_3$  concentrations nor diminish those of [ $^3\text{H}$ ]PtdIns(4,5) $P_2$ , but did evoke a rapid and sustained increase in concentrations of [ $^3\text{H}$ ]PtdIns(3,4,5) $P_3$  consistent with an  $\sim 10$ –20-fold stimulation of PI 3-kinase activity. The latter observation, which presumably reflects an insulin- or IGF-1-receptor-mediated recruitment and activation of the heterodimeric (p85p110) isoform of PI 3-kinase by insulin receptor substrate 1 or 2 (see e.g. [32]), clearly demonstrates the



**Figure 2** Thrombin-receptor activation modifies insulin-stimulated accumulation of  $[^3\text{H}]\text{PtdIns}(3,4,5)\text{P}_3$  and  $[^3\text{H}]\text{PtdIns}(3,4)\text{P}_2$

Cells labelled with  $[^3\text{H}]\text{inositol}$  as described for Table 1 were incubated either as control (open symbols) or with insulin ( $10\ \mu\text{g}/\text{ml}$ , closed symbols) for up to 10 min. SFLLRN ( $100\ \mu\text{M}$ , triangles) or vehicle (circles) was then added and incubations continued as shown before extraction and analysis of (A)  $[^3\text{H}]\text{PtdIns}(3,4,5)\text{P}_3$  or (B)  $[^3\text{H}]\text{PtdIns}(3,4)\text{P}_2$ . Results are from single incubations in one experiment representative of four similar ones. Comparison across experiments by paired Student's *t* test showed a significant fall in  $[^3\text{H}]\text{PtdIns}(3,4,5)\text{P}_3$  after 10 min with insulin + SFLLRN compared with 20 min with insulin alone ( $P = 0.01$ ), a significant rise in  $[^3\text{H}]\text{PtdIns}(3,4)\text{P}_2$  after 30 s with insulin + SFLLRN compared with 10 min with insulin alone ( $P = 0.02$ ) and a significant, secondary fall in  $[^3\text{H}]\text{PtdIns}(3,4)\text{P}_2$  after 10 min with insulin + SFLLRN compared with 20 min with insulin alone ( $P = 0.04$ ).

capacity of 1321N1 cells to generate a  $\text{PtdIns}(3,4,5)\text{P}_3$  signal in response to an appropriate stimulus. Thus the failure of thrombin and SFLLRN to evoke  $\text{PtdIns}(3,4,5)\text{P}_3$  accumulation in these cells implies either (i) greater heterogeneity of thrombin receptors than is yet apparent, (ii) differences in their effector coupling or (iii) differences in the expression of relevant effectors in distinct cell types. These alternatives are currently under investigation in Rat-1 fibroblasts in which PLC responses to thrombin and SFLLRN are similar in all respects to those currently reported in 1321N1 cells (P. A. Thomason and C. P. Downes, unpublished work).

#### Thrombin-receptor activation modifies insulin-stimulated accumulation of $\text{PtdIns}(3,4,5)\text{P}_3$ and $\text{PtdIns}(3,4)\text{P}_2$

The presence of thrombin and insulin (or IGF-1) receptors, coupled apparently mutually exclusively to the stimulation of PLC and to PI 3-kinase respectively, provided the opportunity to study potential interactions between these two  $\text{PtdIns}(4,5)\text{P}_2$ -dependent signalling pathways. Consequently, the insulin-stimulated accumulation of 3-phosphorylated phosphoinositides and the influence of thrombin receptor stimulation on this was examined as illustrated in Figure 2. Figure 2(A) shows that insulin alone evoked a marked and persistent increased accumulation of  $[^3\text{H}]\text{PtdIns}(3,4,5)\text{P}_3$ , concentrations of which

achieved a new steady state,  $\sim 15\text{--}20$ -fold control values, within 2–5 min. Figure 2(B) illustrates similar effects of insulin on the concentrations of  $[^3\text{H}]\text{PtdIns}(3,4)\text{P}_2$ , which were also increased maximally by 2–5 min and then maintained. At apparent steady state, the insulin-stimulated increase in concentrations of  $[^3\text{H}]\text{PtdIns}(3,4,5)\text{P}_3$  exceeded that of  $[^3\text{H}]\text{PtdIns}(3,4)\text{P}_2$  by  $\sim 10\text{--}20$ -fold. Since it is generally accepted that receptor-regulated PI 3-kinases phosphorylate  $\text{PtdIns}(4,5)\text{P}_2$  to  $\text{PtdIns}(3,4,5)\text{P}_3$ , and that metabolism of this lipid probably accounts for the stimulated accumulation of  $\text{PtdIns}(3,4)\text{P}_2$  [4,10], this is unexpected, and implies either that  $[^3\text{H}]\text{PtdIns}(3,4)\text{P}_2$  is metabolized much more rapidly than  $[^3\text{H}]\text{PtdIns}(3,4,5)\text{P}_3$  or that metabolism of the latter proceeds predominantly by routes other than removal of the 5-phosphate (i.e. by a 3-phosphatase) in 1321N1 cells.

Figures 2(A) and 2(B) respectively also show the effects of thrombin receptor activation on the steady-state accumulation of  $[^3\text{H}]\text{PtdIns}(3,4,5)\text{P}_3$  and  $[^3\text{H}]\text{PtdIns}(3,4)\text{P}_2$  stimulated by insulin. Figure 2(A) shows that the addition of SFLLRN markedly reduced the insulin-stimulated accumulation of  $[^3\text{H}]\text{PtdIns}(3,4,5)\text{P}_3$  which declined rapidly, achieving a new apparent steady state which by 10 min was  $55 \pm 1\%$  (mean  $\pm$  S.E.M.,  $n = 4$ ) of that in response to insulin alone. By contrast, insulin-stimulated concentrations of  $[^3\text{H}]\text{PtdIns}(3,4)\text{P}_2$  were initially further enhanced ( $250 \pm 24\%$ , mean  $\pm$  S.E.M.,  $n = 4$ ), but then diminished rapidly and by 10 min after SFLLRN addition were  $71 \pm 6\%$  of concentrations in the presence of insulin alone. Resting concentrations of neither lipid were increased in response to SFLLRN, while peptide-stimulated changes in the concentrations of  $[^3\text{H}]\text{PtdIns}(4,5)\text{P}_2$  and  $[^3\text{H}]\text{InsP}_3$  were consistent with those shown in Figure 1, and from two experiments were similar both with or without insulin. These results imply a rapid turnover of  $[^3\text{H}]\text{PtdIns}(3,4,5)\text{P}_3$  and  $[^3\text{H}]\text{PtdIns}(3,4)\text{P}_2$  in insulin-stimulated 1321N1 cells, but also indicate complex effects of thrombin-receptor activation on metabolism of the former lipid. The ability of SFLLRN to attenuate stimulated concentrations of  $[^3\text{H}]\text{PtdIns}(3,4,5)\text{P}_3$  suggests that the thrombin receptor mediates either an inhibition of insulin-stimulated PI 3-kinase and/or an acceleration of  $[^3\text{H}]\text{PtdIns}(3,4,5)\text{P}_3$  removal. The initial, very rapid, increase in insulin-stimulated concentrations of  $[^3\text{H}]\text{PtdIns}(3,4)\text{P}_2$  in response to SFLLRN is clearly consistent with an increased degradation of  $[^3\text{H}]\text{PtdIns}(3,4,5)\text{P}_3$  by a 5-phosphatase. However, as the increased accumulation of  $[^3\text{H}]\text{PtdIns}(3,4)\text{P}_2$  on SFLLRN addition is transient, but the reduction in  $[^3\text{H}]\text{PtdIns}(3,4,5)\text{P}_3$  is persistent, thrombin-receptor activation must exert a secondary influence on  $[^3\text{H}]\text{PtdIns}(3,4,5)\text{P}_3$  concentrations either by accelerating catabolism by an additional route (e.g. 3-phosphatase) or by inhibition of PI 3-kinase.

Further studies will be required to establish the mechanisms underlying these effects, though several points are apparent. As thrombin-receptor activation evokes a profound decrease in  $\text{PtdIns}(4,5)\text{P}_2$  concentrations, this may account for both the initial fall in insulin-stimulated  $\text{PtdIns}(3,4,5)\text{P}_3$  concentrations and the transient rise in those of  $\text{PtdIns}(3,4)\text{P}_2$  if insulin-stimulated PI 3-kinase operates under first-order conditions with respect to its lipid substrate and if a single 5-phosphatase degrades both  $\text{PtdIns}(4,5)\text{P}_2$  and  $\text{PtdIns}(3,4,5)\text{P}_3$  [33,34]. However, as preliminary data indicate that muscarinic-receptor activation in 1321N1 cells exerts effects similar to those of SFLLRN, but which are not accompanied by dramatic reductions in  $\text{PtdIns}(4,5)\text{P}_2$  concentrations, this seems unlikely. Furthermore, substrate depletion cannot account for the later reduction in insulin-stimulated  $[^3\text{H}]\text{PtdIns}(3,4,5)\text{P}_3$  concentrations observed in response to SFLLRN, since this persists even when  $\text{PtdIns}(4,5)\text{P}_2$  concentrations have recovered fully. Thus it

appears that the thrombin receptor either inhibits PI 3-kinase and/or enhances degradation of PtdIns(3,4,5) $P_3$  by (an)other mechanism(s). It is noteworthy that the transient increase in PtdIns(3,4) $P_2$  following thrombin-receptor stimulation is contemporary with elevated Ins $P_3$  concentrations and thus may be mediated by the consequent rise in intracellular  $Ca^{2+}$  concentrations reported previously [16]. Similarly, although the thrombin receptor appears to be rapidly desensitized and its activation of PLC (and phospholipase D [35]) is largely transient, it is clear that downstream signals may be more prolonged, for example the  $\epsilon$ -isoform of protein kinase C remains persistently membrane-associated following thrombin stimulation in 1321N1 cells [35]. Thus it is possible that such persistent signals may account for the ability of the thrombin receptor to modify PtdIns $P_3$  metabolism. This is currently under investigation.

The present study has thus demonstrated that the thrombin receptors expressed in 1321N1 cells are similar to the tethered-ligand type. These receptors activate PLC but not PI 3-kinase, presumably reflecting either their specific G-protein coupling or the absence of appropriate isoforms of PI 3-kinase in 1321N1 cells. Importantly, however, the same receptors are able to modulate signalling via PI 3-kinase mediated through the insulin (or IGF-1) receptor. Since it may indicate feedback control of PtdIns(3,4,5) $P_3$  concentrations through a mechanism downstream of PLC, this may reflect a primary means by which PI signalling pathways are integrated. Clearly, such mechanisms are to be anticipated and are likely to exert a pivotal influence on second-messenger concentrations, which may be of particular significance for receptors which couple to both PLC and PI 3-kinase.

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