Receptor regulation of phosphoinositide 3-hydroxykinase in the NG115-401L-C3 neuronal cell line: stimulation by insulin-like growth factor-I

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The activation of phosphoinositide 3-hydroxykinase (PI3K) is currently believed to represent the critical regulatory event which leads to the production of a novel intracellular signal. We have examined the control of this pathway by a number of cell-surface receptors in NG115-401L-C3 neuronal cells. Insulin-like growth factor-I stimulated the accumulation of 3-phosphorylated inositol lipids in intact cells and the appearance of PI3K in anti-phosphotyrosine-antibody-directed immunoprecipitates pre-

pared from lysed cells, suggesting that PI3K had been activated by a mechanism involving a protein tyrosine kinase. In contrast, PI3K in these cells was not regulated by a variety of G-protein-coupled receptors, nerve growth factor acting via a low affinity receptor, or receptors for transforming growth factor- β and interleukin-1. The receptor-specificity of PI3K activation in these cells places significant constraints on the possible physiological function(s) of this pathway.

INTRODUCTION

An inositol lipid 3-hydroxykinase, often referred to as phosphoinositide 3-hydroxykinase (PI3K) introduces a phosphate group on the 3-position of inositol head groups in phosphoinositides, giving rise *in vitro* to PtdIns3P, PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ [1,2]. There is evidence that *in vivo*, however, the preferred substrate of PI3K is PtdIns(4,5)P₂ [3,4], which explains the rapid accumulation of PtdIns(3,4,5)P₃ that is seen in cells stimulated by agonists which are thought to activate PI3K [2,5–7]. Many of these agonists act through growth-promoting receptors at the cell surface and, because a number of species of intracellular oncogenic tyrosine kinases also bind and stimulate PI3K [1,8–10], there have been numerous suggestions that the activation of PI3K might be an obligate step in the stimulation of mitogenesis (e.g. [11]). Subsequent work [12,13], and the observation that agonists can stimulate PI3K in non-dividing cells such as platelets or neutrophils [5,6,14], has indicated that it is neither obligatory for, nor restricted to, mitogenic signalling, and so PtdIns(3,4,5)P₃ must have other roles.

Many different agonists have been reported to cause activation of PI3K. Some of these agonists, such as platelet-derived growth factor (PDGF) [9], epidermal growth factor (EGF) [15], colony-stimulating factor-1 (CSF-1) [16] and insulin [7], act through receptors which have intrinsic tyrosine kinase activity. Others, such as interleukin 2 [17] and granulocyte-macrophage CSF (S. Corey, A. Eguinoa, T. R. Jackson, P. T. Hawkins and L. R. Stephens, unpublished work) are thought to act through the activation of non-receptor tyrosine kinases (e.g. those of the *src* family). A purified PI3K from bovine brain has been shown to consist of a heterodimer of a catalytic 110 kDa subunit and an 85 kDa subunit [18–20]. The gene encoding the 85 kDa subunit has been cloned and found to contain paired SH2 domains, supporting a role for intermolecular interactions with phosphotyrosine residues in the regulation of the enzyme [21–23]. It is

also likely that there are paths for receptor activation of PI3K which do not involve tyrosine phosphorylation, e.g. in neutrophils, *N*-formylmethionyl-leucyl-phenylalanine appears to activate the formation of PtdInsP₃ via a G-protein-dependent mechanism (L. R. Stephens, T. R. Jackson and P. T. Hawkins, unpublished work).

The physiological role of receptor-stimulated 3-phosphorylated inositol-lipid metabolism is unknown. Evaluation of the ability of different receptors to regulate the production of these novel inositol lipids may identify a correlation between their synthesis and the regulation of particular cell functions. We now report the results of such a survey carried out in the NG115-401L-C3 neuronal-like cell clone. Insulin-like growth factor-I (IGF-I), presumably acting via its cognate tyrosine kinase-containing receptor, was the only mediator examined which activated this pathway in these cells.

METHODS

Materials

[³²P]P_i (carrier- and HCl-free), [³H]inositol (80 Ci/mmol), [¹²⁵I]-labelled nerve growth factor (NGF) (2000 Ci/mmol) and interleukin 1 were purchased from Amersham International. IGF-I was purchased from Calbiochem. Other mediators and reagents were obtained from Sigma. Anti-phosphotyrosine antibody (mouse hybridoma, PY20) was purchased from ICN Biochemicals. [³²P]Glycerophosphoinositol 3,4,5-trisphosphate {[³²P]GroPIns(3,4,5)P₃} was given by Dr. E. Ulug (it was prepared by using PI3K associated with a middle-T-antigen-directed immunoprecipitate, as described in [24]).

Cell culture

NG-115-401L-C3 cells were grown as described previously [25]. Before an experiment they were plated in 9 cm-diam. Petri dishes

Abbreviations used: PI3K, phosphoinositide 3-hydroxykinase; IGF-I, insulin-like growth factor-I; DMEM, Dulbecco's modified Eagle medium; GroPIns3P, GroPIns(3,4)P₂ and GroPIns(3,4,5)P₃, glycerophosphoinositol 3-phosphate, 3,4-bisphosphate and 3,4,5-trisphosphate; NGF, nerve growth factor; TGF- β , transforming growth factor- β ; Gp, pertussis-toxin-insensitive G-protein coupled to phospholipase C; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; CSF-1, colony-stimulating factor-1.

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(antibody-precipitation studies) or glass Wheaton 'Shorty' vials ($[^{32}\text{P}]\text{P}_i$ labelling). Cells were allowed to grow to approx. 70% confluence in 10% fetal-calf serum/Dulbecco's modified Eagle medium (DMEM). At 24 h before an experiment this supernatant was removed, 1 ml of the supernatant was diluted 10-fold with serum-free DMEM, and then this mixture (1% conditioned fetal-calf serum/DMEM) was replaced on the cells. In some experiments cells were labelled with 100 μCi of $[^3\text{H}]\text{inositol}$ /well, in inositol-free DMEM as described previously [26].

Analysis of $[^3\text{H}]\text{inositol}$ -labelled phospholipids

Cells were challenged with 10 ng of IGF-I/ml and lipids were extracted and deacylated as described previously [26]. Glycerophosphoinositols were analysed on a 12.5 cm Partisphere SAX (10 μm particle size) column, flow rate 1 ml/min, with a gradient formed from water (A) and 1.25 M-ammonium phosphate/ H_3PO_4 , pH 3.8 (B), as follows: 4 min, 0–1% B; 21 min, 1–8% B; 25 min, 8–100% B; followed by a 10 min wash in 100% B. The sample collection rate was 3 min^{-1} , and each sample was mixed with 1 ml of methanol/water (1:1, v/v) and 4 ml of Quicksint scintillant (Zinsser). Peaks were recovered from scintillant and desalted as described elsewhere [27,28]. These were re-chromatographed with internal standards of $[^{32}\text{P}]\text{GroPIns}(3,4,5)\text{P}_3$ and $[^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$ on a Partisphere SAX column as above, but with a gradient of 4 min 0–10% B, 85 min 10–100% B.

Anti-phosphotyrosine-antibody-directed immunoprecipitation of PI3K

Cells were stimulated by agonists and the appearance of PI3K in anti-phosphotyrosine-antibody-directed immunoprecipitates was measured, as described in [7,29].

Analysis of $[^{32}\text{P}]\text{P}_i$ -labelled phospholipids

Cells were grown in Wheaton glass 'Shorty' vials (capacity approx. 4 ml), to permit subsequent manipulations involving ^{32}P or organic solvents to be carried out in a single glass container. Labelling and subsequent steps were carried out as described in detail elsewhere [7,26,29]. Briefly, cells were incubated (70 min) in phosphate-free Hanks' solution containing $[^{32}\text{P}]\text{P}_i$ (100 μCi per bottle). This was discarded, cells were challenged with mediators, and incubations were stopped and polyphosphoinositides extracted with acidic chloroform/methanol [29]. Lipids were deacylated with methylamine and analysed by h.p.l.c. with a 25 cm Partisphere SAX (15 μm particle size) column, flow 1 ml/min, with a gradient composed of water (A) and 2.5 M NaH_2PO_4 (B), as follows: 1 min, 1% B; 29 min, 4% B; 1 min, 9% B; 29 min, 16% B; 1 min, 20% B; 20 min, 30% B; 1 min, 100% B; followed by a 3 min wash in 100% B. Fraction collection rate was 2 min^{-1} ; the first 132 were mixed with 0.5 ml of methanol/water (1:1, v/v), and the remainder were mixed with 0.5 ml of water. A 3.8 ml portion of either Zinsser Quicksint or Packard 299 scintillation fluid was added before determining radioactivity.

^{125}I -NGF binding

Crude membranes were prepared from NG115-401L-C3 cells by scraping cells into 1 mM EDTA/20 mM Tris, pH 7.5 (7.5 ml/15 cm plate), homogenizing with 12 up-and-down strokes of a pestle, and centrifuging (35000 g, 20 min). The pellet was resuspended in 1 mM MgCl_2 /20 mM Tris/HCl (pH 7.5)/0.3% BSA, at 0.5 mg of membrane protein/ml.

Samples (0.5 ml) of membrane suspension were transferred into micro-centrifuge tubes, and incubated for 60 min with 27 pM ^{125}I -NGF and various concentrations of unlabelled NGF to produce a displacement curve. Incubations were terminated by centrifugation (15000 g, 5 min), the supernatant was discarded, the pellets were washed twice with water, and membrane-bound ^{125}I -NGF determined by γ -radiation counting.

RESULTS

Measurement of PI3K activity in anti-phosphotyrosine-antibody-directed immunoprecipitates

A number of agonists have been shown to stimulate the appearance of PI3K in anti-phosphotyrosine-antibody-directed immunoprecipitates prepared from lysed cells (e.g. [10,16,17,30]), providing a sensitive assay for a tyrosine kinase-activated form of the enzyme. In NG115-401L-C3 cells, IGF-I (30 ng/ml) was the only agonist tested which stimulated the appearance of PI3K in precipitates of this type (Table 1). The time course of this response is shown in Figure 1(a).

Production of $\text{PtdIns}(3,4,5)\text{P}_3$ and $\text{PtdIns}(3,4)\text{P}_2$ in $[^{32}\text{P}]\text{P}_i$ -labelled cells

Of all the mediators tested in Table 1, only IGF-I (and NGF when the product of the *trk* proto-oncogene is expressed; see below) is thought to activate signal-transduction pathways by direct tyrosine phosphorylation. Since it now appears that PI3K can be activated by tyrosine-phosphorylation-independent pathways, in particular '7-transmembrane segment' receptors working through G-proteins (L. R. Stephens, T. R. Jackson and P. T. Hawkins, unpublished work), it was necessary to test the ability of agonists to activate PI3K in NG115-401L-C3 cells by a means independent of assuming the mechanism by which it takes place. This is most obviously achieved by measuring the products of PI3K activation in the intact cell, i.e. the stimulated formation of $\text{PtdIns}(3,4)\text{P}_2$ and $\text{PtdIns}(3,4,5)\text{P}_3$. This was achieved by labelling cells with $[^{32}\text{P}]\text{P}_i$ and analysing the ^{32}P -labelled lipids as previously described [3,29]. The methods used for the quantification of $[^{32}\text{P}]\text{PtdIns}(3,4)\text{P}_2$ and $[^{32}\text{P}]\text{PtdIns}(3,4,5)\text{P}_3$ in experiments of this type have been rigorously characterized [3]. Furthermore, the identity of the ^{32}P -labelled lipid head groups in the final chromatographic separation were confirmed by dual-labelling cells with $[^{32}\text{P}]\text{P}_i$ and $[^3\text{H}]\text{inositol}$ and, in the case of $[^3\text{H}]\text{GroPIns}(3,4,5)\text{P}_3$ in IGF-I-stimulated extracts, by co-chromatography with an internal standard of authentic $[^{32}\text{P}]\text{GroPIns}(3,4,5)\text{P}_3$ (Figure 2).

Table 1 Appearance of PI3K in anti-phosphotyrosine-antibody precipitates

Cells were stimulated for 1 min and then antibody precipitates were assayed for $\text{PtdIns}3\text{P}$ synthesis *in vitro*, as described in the Methods section. Values represent means $\pm$ S.E.M. of *n* determinations and are expressed as percentages of control (no addition; 2550 c.p.m.). Concentrations of mediators are supra-maximal for other biological effects.

Addition	<i>n</i>	$\text{PtdIns}3\text{P}$ production (%)
30 ng/ml IGF-I	11	439 $\pm$ 60
10 ng/ml Interleukin 1- α/β	4	116 $\pm$ 2
5 ng/ml TGF- β	4	108 $\pm$ 9
100 ng/ml NGF	3	112 $\pm$ 8
10 μM Bradykinin	3	139 $\pm$ 28
10 μM Angiotensin II	3	142 $\pm$ 23
10 μM Met-enkephalin	2	138 $\pm$ 24

Figure 1 Time course of appearance of PI3K in an anti-phosphotyrosine-antibody precipitate and production of 3-phosphorylated inositol lipids by IGF-I

(a) Production of [32 P]PtdIns3P by an anti-phosphotyrosine-antibody precipitate made from cells challenged for various times with 30 ng/ml IGF-I. Control (100%) values represent 2550 ± 550 c.p.m. Values are means $\pm$ S.E.M. of 2–11 determinations. (b) Production of 32 P-labelled inositol lipids in intact [32 P] P_i prelabelled cells challenged with 30 ng/ml IGF-I. Lipids were deacylated and separated by h.p.l.c. as described in the Methods section: ∇ , PtdIns(3,4,5) P_3 ; Δ , PtdIns(3,4) P_2 ; $\circ$, PtdIns3P; $\square$, PtdIns(4,5) P_2 . Each point is the mean $\pm$ S.E.M. for 3 determinations, except for the 1 min point, where $n = 7$. Control (100%) values represent: PtdIns(4,5) P_2 , $297\,200 \pm 5300$ c.p.m.; PtdIns(3,4,5) P_3 , 665 ± 250 c.p.m.; PtdIns(3,4) P_2 , 4165 ± 1203 c.p.m.; PtdIns3P, 8290 ± 1630 c.p.m.

The following mediators were examined for their ability to stimulate the formation of [32 P]PtdIns(3,4) P_2 and [32 P]PtdIns(3,4,5) P_3 in [32 P] P_i -labelled cells: 30 ng/ml IGF-I,

Figure 2 Production of 3 H-labelled PtdIns(3,4,5) P_3 by IGF-I

Preparation of samples and separations were as described in the Methods section. (a) Cells were incubated for 1 min with either 10 ng/ml IGF (●) or buffer (○) before inositol phospholipids were extracted, deacylated and separated by h.p.l.c. The region of the chromatogram shown is the area of GroPIns(4,5) P_2 elution. This experiment has been repeated once with similar results. (b) The putative GroPIns(3,4,5) P_3 (●) from (a) (i.e. peak centred on fraction 120 in Figure 2a) was rechromatographed with 32 P-labelled GroPIns(3,4,5) P_3 and Ins(1,4,5) P_3 standards (○) (marked 1 and 2 respectively).

Table 2 Levels of 32 P-labelled inositol lipids in [32 P] P_i -prelabelled NG115-401L-C3 cells stimulated with various agonists

Values are expressed as percentages of controls (no addition), which were: PtdIns3P, 8290 c.p.m.; PtdIns4P, 176900 c.p.m.; PtdIns(3,4) P_2 , 4165 c.p.m.; PtdIns(4,5) P_2 , 297200 c.p.m.; PtdIns(3,4,5) P_3 , 665 c.p.m. Each value is the mean $\pm$ S.E.M. of three determinations, except for IGF-I, which is from seven determinations. Cells were stimulated for 1 min, with the same concentrations of additions as in Table 1.

Addition	PtdIns3P	PtdIns4P	PtdIns(3,4) P_2	PtdIns(4,5) P_2	PtdIns(3,4,5) P_3
IGF-I	116 $\pm$ 16	107 $\pm$ 7	362 $\pm$ 107	111 $\pm$ 7	2010 $\pm$ 438
Interleukin 1- α/β	106 $\pm$ 6	109 $\pm$ 26	107 $\pm$ 2	97 $\pm$ 1	127 $\pm$ 39
TGF- β	80 $\pm$ 2	94 $\pm$ 2	91 $\pm$ 9	95 $\pm$ 7	138 $\pm$ 33
NGF	82 $\pm$ 5	98 $\pm$ 3	108 $\pm$ 8	102 $\pm$ 12	137 $\pm$ 56
Bradykinin	85 $\pm$ 3	32 $\pm$ 3	107 $\pm$ 11	61 $\pm$ 5	122 $\pm$ 54
Angiotensin II	81 $\pm$ 6	85 $\pm$ 7	103 $\pm$ 3	89 $\pm$ 4	96 $\pm$ 27
Enkephalin	111 $\pm$ 21	98 $\pm$ 17	104 $\pm$ 11	96 $\pm$ 3	122 $\pm$ 39

Figure 3 Displacement of ^{125}I -NGF bound to NG115-401L-C3 membranes by unlabelled NGF

Membranes prepared from NG115-401L-C3 cells were incubated with 27 pM ^{125}I -NGF and the indicated concentrations of unlabelled NGF, as described in the Methods section. The curve represents the least-squares best fit to a logistic (Hill) equation, where $\text{IC}_{50} = 1.2 \pm 0.2$ nM, and the Hill coefficient = 1.00 ± 0.14 . The data are pooled from three separate experiments, with each point representing the mean of a duplicate determination. Maximum c.p.m. bound/incubation was typically 20000 (60% specific binding).

100 ng/ml NGF, 5 ng/ml transforming growth factor- β (TGF- β), 10 ng/ml interleukin 1, 10 μM bradykinin, 10 μM angiotensin II, 10 μM [Met]enkephalin; the results are shown in Table 2. Only IGF-I stimulated a measurable accumulation of [^{32}P]PtdIns(3,4) P_2 and [^{32}P]PtdIns(3,4,5) P_3 . The time course of IGF-I-stimulated production of [^{32}P]PtdIns(3,4) P_2 and [^{32}P]PtdIns(3,4,5) P_3 is shown in Figure 1(b). Bradykinin caused a significant decrease in [^{32}P]PtdIns4P and [^{32}P]PtdIns(4,5) P_2 after 1 min of stimulation, consistent with it being a powerful stimulus of phospholipase C in these cells [26].

Binding characterization of the NGF receptor in NG115-401L-C3 cells

The inability of NGF to stimulate PI3K was unlike results in other cell types [31]. Accordingly, we examined the NGF receptor by radioligand binding. As shown in Figure 3, ^{125}I -NGF binds to a single population of sites (Hill coefficient 1.00 ± 0.14) with a dissociation constant of 1.2 ± 0.2 nM. This suggests that undifferentiated NG115-401L-C3 cells express predominantly a low-affinity NGF receptor.

DISCUSSION

In NG115-401L-C3 cells, IGF-I rapidly stimulated the production of ^{32}P -labelled PtdIns(3,4,5) P_3 in [^{32}P]P $_i$ -labelled intact cells, and the appearance of a PI3K in anti-phosphotyrosine-antibody precipitates prepared from lysed cells. Thus the IGF-I receptor activates PI3K in these cells, probably via a mechanism involving tyrosine phosphorylation, either directly or indirectly. A number of other tyrosine kinase growth-factor receptors, including those for PDGF, EGF, CSF-1 and insulin [7,11,15,16], also stimulate PI3K, possibly via interactions between phosphotyrosine-containing domains on the receptor and SH2 domains

in the enzyme's 85 kDa subunit [32]. These results are consistent with previous data which demonstrated that IGF-I caused association of PI3K with anti-phosphotyrosine-antibody precipitates from CHO cells and 3T3 cells [29,33].

The pattern of 3-phosphorylated phosphoinositides generated by IGF-I is of interest. The most rapid rise was seen with PtdIns(3,4,5) P_3 production. There was a distinct lag before levels of PtdIns(3,4) P_2 increased, and PtdIns3P levels did not change significantly during the period of stimulation examined. This pattern is typical of agonist-stimulated formation of 3-phosphorylated inositol lipids in a number of different cell types. In both *N*-formylmethionyl-leucyl-phenylalanine-stimulated neutrophils [3] and PDGF-stimulated 3T3 cells [4], this pattern is thought to arise via a primary stimulation of PtdIns(4,5) P_2 3-phosphorylation (by PI3K) to form PtdIns(3,4,5) P_3 , and subsequent 5-dephosphorylation of PtdIns(3,4,5) P_3 [by a specific PtdIns(3,4,5) P_3 5-phosphatase] to form PtdIns(3,4) P_2 . This interpretation places emphasis on PtdIns(3,4,5) P_3 as the likely output signal from this pathway. IGF-I did not alter PtdIns(4,5) P_2 or PtdIns4P levels, consistent with it not activating an inositol-lipid-specific phospholipase C. This is unlike other tyrosine kinase-type receptors such as the PDGF receptor, where production of Ins(1,4,5) P_3 and Ca^{2+} mobilization can be observed [11]. Little is known of the transduction process employed by IGF-I, beyond that tyrosine phosphorylation is involved, and activation of PI3K is one of the few targets identified.

At short time periods, there is no evidence that other signalling mechanisms can stimulate PI3K in NG115-401L-C3 cells. TGF- β works through a receptor with an intrinsic serine/threonine kinase activity [34], and interleukin 1 acts by completely unknown signalling pathways. Angiotensin and bradykinin work via pertussis-toxin-insensitive G-protein (Gp)-coupled receptors, which activate phospholipase C [26]. This leads to the production of diacylglycerol and Ins(1,4,5) P_3 , and hence protein kinase C stimulation, and increases in intracellular Ca^{2+} . Although it has been suggested that, in platelets, thrombin activates PI3K via protein kinase C [35], there is no evidence for this happening in the NG115-401L-C3 cells at short time periods. Met-enkephalin interacts with a receptor coupled to a form of G_i [36]. Together, these results indicate that PI3K is not likely to be under direct or indirect control of a G-protein, at least of the G_p or G_i families, in this cell line.

The inability of NGF to activate PI3K in NG115-401L-C3 cells is unlike its reported action in PC12 cells, where it can promote PtdIns(3,4,5) P_3 formation [31]. A number of NGF receptors exist. The low-affinity form (INGF-R: $K_d \geq 1$ nM) has no recognized signalling mechanism [37]; however, the *trk* proto-oncogene, either alone or in association with the INGF-R, binds NGF with high affinity ($K_d \leq 1$ nM) and shows intrinsic tyrosine kinase activity. It is the latter form which is found in PC12 cells [38]. The binding data described in this study suggest that NG115-401L-C3 cells express the low-affinity form of the receptor. This form does not have tyrosine kinase activity, and it does not appear to activate PI3K in these cells.

The function of IGF-I-receptor-regulated PI3K in NG115-401L-C3 cells is unknown. It is clearly not a universal signal for mitogenesis, since some of the agents tested which failed to stimulate PI3K are mitogens for these cells (e.g. angiotensin [26]). Further, although IGF-I is itself mitogenic, the fact that the enzyme is activated in non-mitogenic contexts, e.g. in platelets and neutrophils, suggests that its product, PtdIns(3,4,5) P_3 , may be directly involved in some plasma-membrane event such as shape change, endocytosis or secretion. Little is known of the action of IGF on neuronal cells, although, in addition to mitogenesis, it has been reported to act as a trophic factor [39]

supporting the secretion of neurotransmitters [40]. A lower-molecular-mass subtype of the IGF-I receptor has been found exclusively in neurons, which may induce transcriptional activity and exert neurotrophic effects [41]. A thorough exploration of IGF-I actions on NG-115-401L-C3 cells may allow identification of roles for PI3K and its product PtdIns(3,4,5) P_3 in cellular signalling.

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