Changes in inositol lipids and phosphates after stimulation of the MAS-transfected NG115-401L-C3 cell line by mitogenic and non-mitogenic stimuli

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A neuronal cell line (NG115-401L-C3) was stimulated by mitogenic (angiotensin) and non-mitogenic (bradykinin) peptides and examined for the time course of changes in the levels of radiolabelled inositol phosphates and phospholipids. Both peptides stimulated the time-dependent production of $\text{Ins}(1,4,5)\text{P}_3$ and related metabolites. Bradykinin caused a much larger increase in $\text{Ins}(1,4,5)\text{P}_3$ than did angiotensin. However, both peptides stimulated similar rises in the levels of $\text{Ins}(1,3,4)\text{P}_3$ and InsP_4 . Bradykinin, but not angiotensin, caused a rapid (within 2 s) fall in the levels of $\text{PtdIns}(4,5)\text{P}_2$ and $\text{PtdIns}(4)\text{P}$. Serum pretreatment of the cells caused a 2–3-fold potentiation of both the responses to bradykinin and angiotensin. Although significant levels of $\text{PtdIns}(3)\text{P}$ were detected in resting cells, neither mitogenic (angiotensin, insulin-like growth factor I, transforming growth factor β) nor non-mitogenic (bradykinin, nerve growth factor, interleukin-1) receptor activation changed its levels, arguing against regulation of either PtdIns 3-kinase or $\text{PtdIns}(3)\text{P}$ phosphatase. We conclude that, as judged by the levels of its product, $\text{PtdIns}(3)\text{P}$, the enzyme PtdIns 3-kinase is not activated. This questions the significance of this activity in the receptor-mediated initiation of DNA synthesis.

INTRODUCTION

Although the importance of inositol phosphates and phospholipids in regulating cell activity has been known for some years (Michell, 1975; Berridge & Irvine, 1989), there are numerous unanswered questions as to the roles of individual inositol-containing compounds. One of the most controversial areas concerns the role of the inositol phospholipid signalling system in activating cell division. Although many growth factors can interact with this pathway (Berridge & Irvine, 1984), the nature of the mitogenic mechanism remains elusive. Recently attention has moved away from water-soluble inositol phosphates to the new class of inositol phospholipids which are phosphorylated on the 3-position of the inositol ring (Stephens *et al.*, 1989; Whitman *et al.*, 1988). The simplest member of this class of compounds, $\text{PtdIns}(3)\text{P}$, has been identified in transformed and non-transformed fibroblasts (Lips *et al.*, 1989), astrocytoma cells (Stephens *et al.*, 1989) and smooth-muscle cells (Auger *et al.*, 1989). Additionally $\text{PtdIns}(3,4)\text{P}_2$ and $\text{PtdIns}(3,4,5)\text{P}_3$ have been reported to occur in non-mitogenic contexts on stimulation of smooth-muscle cells (Auger *et al.*, 1989) and neutrophils (Traynor-Kaplan *et al.*, 1988). The enzyme responsible for introducing a phosphate on the 3-position of inositol phospholipids has been termed type 1 PtdIns kinase or PtdIns 3-kinase. Independent lines of evidence suggest that this enzyme is activated by growth-promoting receptors and oncogenes (Bishop, 1985; Coughlin *et al.*, 1989; Varticovski *et al.*, 1989; Ulug *et al.*, 1990). The receptor system which has been studied in most detail is stimulation by platelet-derived growth factor (PDGF) of DNA synthesis in fibroblasts and smooth-muscle cells. The PDGF receptor is a multi-domain transmembrane protein with intrinsic tyrosine kinase activity, but the tyrosine kinase region is split by an 'insert domain' of about 50 amino acids (Williams, 1989). If the insert domain is removed, the mutant receptor loses the ability to initiate mitogenesis, and also can no longer interact

with a variety of proteins, including PtdIns 3-kinase and the GTPase-activating protein (GAP) associated with *ras* (Coughlin *et al.*, 1989). In quiescent smooth-muscle cells, stimulation with PDGF causes the appearance of two new minor inositol lipids, postulated to be $\text{PtdIns}(3,4)\text{P}_2$ and $\text{PtdIns}(3,4,5)\text{P}_3$, accompanied by a transient fall in the levels of $\text{PtdIns}(3)\text{P}$ (Auger *et al.*, 1989). Similar results have been reported for the actions of another tyrosine kinase receptor, that for CSF-1, on fibroblasts (Varticovski *et al.*, 1989).

Taken together, these results suggest that activation of PtdIns 3-kinase may play an essential role in signalling pathways of specific receptors, especially mitogenic tyrosine kinase receptors. We have examined the kinetic control of inositol lipid and inositol phosphate metabolism by a structurally distinct mitogenic receptor, the MAS/angiotensin receptor. This receptor belongs to the seven transmembrane domain superfamily of G-protein receptors and activates phospholipase C (PLC)-mediated cleavage of $\text{PtdIns}(4,5)\text{P}_2$ (Jackson *et al.*, 1988; Jackson & Hanley, 1989). The stable MAS-transfectant neuroblastoma cell line, NG115-401L-C3, has an endogenous bradykinin receptor which is also coupled to PLC cleavage of $\text{PtdIns}(4,5)\text{P}_2$ (Jackson *et al.*, 1987), but, unlike activation of the MAS/angiotensin receptor, cannot initiate DNA synthesis. Thus a comparison of the fate of inositol phospholipids and phosphates after stimulation with angiotensin or bradykinin allowed assessment of the proposal that activation of PtdIns 3-kinase is an obligatory step in stimulation of DNA synthesis. Additionally, a comparison of the production of water-soluble inositol phosphates would be instructive to see if these differed between the two stimuli, since there are differences in angiotensin- as compared to bradykinin-stimulated cytosolic Ca^{2+} responses (Jackson *et al.*, 1988; Hanley *et al.*, 1990). Finally, the experiments described below represent the most complete comparison of inositol metabolites between two stimuli which operate via PLC-mediated cleavage of $\text{PtdIns}(4,5)\text{P}_2$.

Abbreviations used: GroPIns, GroPInsP and GroPIns P_2 , glycerophosphoinositol and its mono- and bis-phosphates; PLC, phospholipase C; PDGF, platelet-derived growth factor; CSF, colony-stimulating factor; NGF, nerve growth factor; IL-1, interleukin 1; IGF, insulin-like growth factor; TGF, transforming growth factor; G-protein, GTP-binding protein; DMEM, Dulbecco's modified Eagle's Medium; FCS, fetal-calf serum.

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METHODS

Cell culture

NG115-401L-C3 cells were grown in antibiotic-free Dulbecco's Modified Eagle's Medium (DMEM)/10% (v/v) fetal-calf serum (FCS) (Gibco) as described by Jackson *et al.* (1987). The cells were plated at approx. 2×10^5 cells/3.5 cm Petri dish, and grown for 72 h. The medium was then replaced with inositol-free DMEM, containing 20 μ Ci of [3 H]inositol per plate (Amersham; 20 Ci/mmol) and 10 μ M-inositol. In certain experiments this was supplemented with 10% FCS (dialysed against Hanks solution). The cells were left for a further 24 h. The [3 H]inositol-containing medium was then removed, and the cells were washed briefly with 2×2 ml of DMEM before challenge with fresh medium (37 °C) containing agonist at the required final concentration. Incubations were terminated by adding trichloroacetic acid (20%, v/v) and extracting on ice (15 min).

Extraction and measurement of water-soluble inositol phosphates

Cells were scraped into 2 ml of 20% trichloroacetic acid, centrifuged briefly, and the supernatant was removed for analysis. The pellet was washed once with 1 ml of 5% trichloroacetic acid/2 mM-EDTA, and this extract was combined with the previous supernatant. The combined extracts were neutralized with 9 ml of 1,1,2-trichlorotrifluoroethane (Freon)/tri-n-octylamine (1:1, v/v), and the upper aqueous phase was stored at -20 °C. H.p.l.c. analysis of trichloroacetic acid extracts used a Partisil SAX 10 μ m column (25 cm; Jones Chromatography) with a flow rate of 1.25 ml/min and gradient elution with water (A) and 3.5 M-ammonium formate/ H_3PO_4 , pH 3.7 (B), as follows: 5 min of 100% A, 10 min of 0–20% B, 45 min of 20–50% B, 20 min of 50–100% B, followed by a further 12 min wash in 100% B. In later experiments this gradient was shortened to: 5 min of 100% A, 10 min of 0–10% B, 25 min of 10–38% B, 20 min of 38–100% B and 6 min wash in 100% B. Both gradients gave a full separation of the relevant inositol phosphate isomers present in the mixture. Samples were collected every 20 s. Samples were diluted with 1 ml of methanol/water (1:1, v/v) and mixed with 4 ml of scintillant (Quicksint, Zinser). Peak identification was by internal ^{14}C (GroPIns, $InsP_1$) and ^{32}P ($InsP_4$, $InsP_5$ and $InsP_6$) standards, in calibration experiments (Jackson *et al.*, 1987).

Extraction and measurement of inositol phospholipids

The methods were described in detail by Stephens *et al.* (1989). Briefly, the washed cell membrane pellet was extracted with acidic chloroform/methanol. The lipid-containing phase was deacylated by methylamine treatment, and resulting glycerophosphoinositides were extracted in water and stored at -20 °C until h.p.l.c. analysis on a Partisphere SAX 10 μ m column (12.5 cm, Whatman) (flow, 1 ml/min) by using gradients formed from water (A) and 1.25 M-ammonium phosphate/ H_3PO_4 , pH 3.8 (B). The first gradient was used in the initial studies and consisted of 5 min of A, 40 min of 0–12% B, 7 min of 12–20% B, 12 min of 20–100% B, followed by a 12 min wash in 100% B. The alternative, shorter, gradient used in subsequent studies was 4 min of 0–1% B, 21 min of 1–8% B, 10 min of 8–100% B, followed by a 10 min wash in 100% B. The sample collection rate was 3 fractions min, and each sample was mixed with methanol/water and scintillant as described for the water-soluble inositol compounds.

Measurement of [3 H]thymidine incorporation into DNA

The rate of DNA synthesis in confluent cultures of 401L-C3 cells in response to a variety of cytokines and growth factors was

determined as uptake of [3 H]thymidine into trichloroacetic acid-precipitable material essentially as described previously (Benton *et al.*, 1989). Cells were grown to confluence in 24-well plates in DMEM/5% FCS, placed in lower serum concn. (1%) for 24 h and cultured in the presence of growth factors or cytokines (10 ng/ml) and 0.25 μ Ci of [3 H]thymidine (Amersham; 20 Ci/mmol)/ml for 16–24 h. Medium was removed and cold trichloroacetic acid added to each well and incubated on ice for 20 min. Cell layers were washed twice with ethanol and taken up into 0.5 ml of 0.1 M-NaOH/2% (w/v) $NaHCO_3$. The radioactivity in each fraction was determined by liquid-scintillation counting. IGF-1 and TGF β were from British Biotechnology, IL-1 α and -1 β were from Immunex, and NGF was purified by standard methods from mouse submaxillary glands.

Functional receptors were identified for IL-1 by stimulation of prostaglandin E_2 synthesis as described previously (Benton *et al.*, 1989) and for NGF by stimulation of neurite outgrowth. Neither NGF nor IL-1 caused any stimulation of DNA synthesis.

RESULTS

Bradykinin-stimulated changes in inositol phosphates and phospholipids

Bradykinin treatment stimulated production of $Ins(1,4,5)P_3$ to a maximal level (~500% over basal) at 8–15 s after addition.

Fig. 1. Time course of production of water-soluble inositol phosphates in serum-starved cells in response to 10 μ M-bradykinin

(a) ●, GroPIns; ○, $InsP_1$; ▲, $InsP_2$; ■, $Ins(1,4,5)P_3$. (b) □, $Ins(1,3,4)P_3$; △, $InsP_4$; ▽, $InsP_5$; ▼, $InsP_6$. Each point represents the mean $\pm$ S.E.M. for 3 determinations. Control (100%) values were: GroPIns 20100 c.p.m.; $InsP_1$ 41100 c.p.m.; $InsP_2$ 3200 c.p.m.; $Ins(1,3,4)P_3$ 160 c.p.m.; $Ins(1,4,5)P_3$ 1600 c.p.m.; $InsP_4$ 790 c.p.m.; $InsP_5$ 9900 c.p.m.; $InsP_6$ 2300 c.p.m.

Fig. 2. Representative h.p.l.c. elution profile of water-soluble inositol phosphates found in unstimulated NG115-401L-C3 cells

Preparation of sample and separation were as described in the Methods section, the longer of the two gradients being used. Peaks were identified as follows: 1, free inositol; 2, *GroPI*ns; 3, *InsP*₁; 4, *Ins*(1,4)*P*₂; 5, *Ins*(1,3,4)*P*₃; 6, *Ins*(1,4,5)*P*₃; 7, *InsP*₄; 8, *InsP*₅; 9, *InsP*₆. Broken lines indicate levels of *Ins*(1,3,4)*P*₃ and *Ins*(1,4,5)*P*₃ after 60 s stimulation with 10 μM-bradykinin.

Fig. 3. Time course of the production of water-soluble inositol phosphates in serum-starved cells in response to 10 μM-angiotensin III

(a) ●, *GroPI*ns; ○, *InsP*₁; ▲, *InsP*₂; ■, *Ins*(1,4,5)*P*₃. (b) □, *Ins*(1,3,4)*P*₃; △, *InsP*₄; ▽, *InsP*₅; ▾, *InsP*₆. Each point is the mean ± S.E.M. for 4 determinations. Points indicated with an asterisk are significantly different from control values ($P < 0.05$, Student's t test). Control values were the same as for bradykinin stimulation (Fig. 1).

Fig. 4. Time course of the changes in inositol phospholipids

Cells were challenged with either 10 μM-bradykinin (a) or 10 μM-angiotensin III (b) and lipids were extracted, deacylated and separated by h.p.l.c. as described in the Methods section: ●, *GroPI*ns(3)*P*; ▲, *GroPI*ns(4)*P*; ■, *GroPI*ns(4,5)*P*₂. Each point is the mean ± S.E.M. for 4 determinations. Control (100%) values were: *GroPI*ns(3)*P* 2300 c.p.m.; *GroPI*ns(4)*P* 41 600 c.p.m.; *GroPI*ns(4,5)*P*₂ 41 000 c.p.m.

(Figs. 1 and 2). This response was closely followed by a peak of *InsP*₂ which lagged slightly behind the *Ins*(1,4,5)*P*₃ peak, but was of a similar magnitude, and was presumably derived from it by 5-phosphatase activity. By 5 min the *Ins*(1,4,5)*P*₃ had declined to levels not significantly different from control. Bradykinin also stimulated a slower and less pronounced *InsP*₄ increase, which reached a peak around 30 s. The largest proportional change was seen with levels of *Ins*(1,3,4)*P*₃, which were elevated to 800% of control by 30 s, continuing up to 60 s. By 5 min this had declined to 250% of control, still significantly elevated. Levels of *Ins*(1,3,4)*P*₃ did not change until after 10 s from peptide addition. There were no significant changes in the measured levels of any of the other water-soluble inositol phosphate fractions: *GroPI*ns, *InsP*₁, *InsP*₅ and *InsP*₆.

Bradykinin stimulated marked decreases in *PtdIns*(4)*P* and *PtdIns*(4,5)*P*₂ (Fig. 4a), as judged by their fractionated deacylation products. The most rapid decrease was seen with *PtdIns*(4,5)*P*₂, which was decreased to 60% of control levels within 10 s. *PtdIns*(4)*P* was decreased to a similar value by 15 s. The levels of these lipids remained decreased for up to 60 s, with subsequent recovery to basal levels completed by 5 min (results not shown). The levels of *PtdIns*(3)*P* showed no significant changes from 2 s up to 15 min after stimulation.

Fig. 5. Representative h.p.l.c. elution profile of glycerophosphoinositides

Inositol phospholipids were extracted, deacylated and separated as described in the Methods section by using the longer of the two gradients. (a) Unstimulated cells. Peaks were identified as follows: 1, GroPIIns; 2, GroPIIns(3)P; 3, GroPIIns(4)P; 4, GroPIIns(4,5)P₂. (b) Partial h.p.l.c. elution profile of phospholipids after stimulation of cells with angiotensin III for 15 s: 1, GroPIIns(3)P; 2, GroPIIns(4)P; 3, GroPIIns(4,5)P₂.

Angiotensin-stimulated changes in inositol phosphates and phospholipids

The time course of changes in inositol phosphates generated by angiotensin was distinct from that seen with bradykinin (Fig. 3). There was only a limited (40% over basal at maximum) increase in Ins(1,4,5)P₃ at 30–60 s after addition of peptide. This had returned to control values by 5 min. InsP₄ appeared to increase slightly, although there was no statistically significant elevation over control levels. By contrast, there was a large increase in Ins(1,3,4)P₃, which approached that seen in response to bradykinin. The response was slow, and levels continued to rise at 60 s, but by 5 min had declined to 160% of control. As with bradykinin, there was no significant changes in the levels of GroPIIns, InsP₁, InsP₅ and InsP₆.

There were no significant changes in the levels of the inositol

Fig. 6. Time course of the changes in water-insoluble inositol phosphates in serum-pretreated cells in response to 10 μ M-bradykinin

(a) ●, GroPIIns; ○, InsP₁; ▲, InsP₂; ■, Ins(1,4,5)P₃. (b) □, Ins(1,3,4)P₃; △, InsP₄; ▽, InsP₅; ▼, InsP₆. Each point is the mean $\pm$ S.E.M. for 3 determinations. Control values were: GroPIIns 24800 c.p.m.; InsP₁ 55400 c.p.m.; InsP₂ 2900 c.p.m.; Ins(1,3,4)P₃ 130 c.p.m.; Ins(1,4,5)P₃ 1900 c.p.m.; InsP₄ 600 c.p.m.; InsP₅ 7100 c.p.m.; InsP₆ 1900 c.p.m.

phospholipids PtdIns(3)P, PtdIns(4)P and PtdIns(4,5)P₂ at any time up to 15 min after stimulation (Fig. 4b). Nor was there any sign of receptor-stimulated production of other minor inositol phospholipids such as PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ in response to angiotensin (Fig. 5). If the production of these lipids was stimulated, we estimate that they could have risen to no more than 5–10% of the levels of PtdIns(3)P.

Effects of FCS

In the course of preliminary experiments it was noted that, if serum remained in the medium until peptide incubations were initiated, then the inositol phosphate response to agonists was potentiated relative to serum-starved cells. This effect was examined for both bradykinin and angiotensin. As shown in Fig. 6, the serum-pretreated cell production of Ins(1,4,5)P₃ in response to bradykinin was 3 times greater at its maximum, which reached a peak earlier. The production of Ins(1,3,4)P₃ was actually decreased under these conditions, compared with the serum-starved condition. The increased production of Ins(1,4,5)P₃ was paralleled by larger decreases in the inositol lipids, with PtdIns(4)P and PtdIns(4,5)P₂ decreasing to 20% of control levels. There was no change in the levels of PtdIns(3)P (Fig. 8a). The actions of angiotensin were also potentiated by FCS pretreatment (Fig. 7). There was a doubling of the production of Ins(1,4,5)P₃ with serum pretreatment, without any

Fig. 7. Time course of the changes in water-soluble inositol phosphates in serum-pretreated cells in response to 10 μ M-angiotensin III (a) $\bullet$, GroPIns; $\circ$, InsP₁; $\blacktriangle$, InsP₂; $\blacksquare$, Ins(1,4,5)P₃. (b) $\square$, Ins(1,3,4)P₃; $\triangle$, InsP₄; $\blacktriangledown$, InsP₅; ∇ , InsP₆. Each point is the mean $\pm$ S.E.M. for 4 determinations. Points indicated with an asterisk are significantly different from control values ($P < 0.05$, Student's t -test). Control values were as for bradykinin treatment (Fig. 6).

apparent change in production of Ins(1,3,4)P₃, contrasting with the results obtained with bradykinin. Despite the enhanced production of Ins(1,4,5)P₃, there was no detectable decrease in PtdIns(4,5)P₂ and neither did the amount of PtdIns(3)P change (Fig. 8b). There was no evidence for the production of PtdIns(3,4)P₂ or PtdIns(3,4,5)P₃.

Actions of cytokines and growth factors

In view of the reported activation of PtdIns 3-kinase by PDGF, it was important to evaluate the effects of other classes of stimulants acting on distinct functional receptors. We have

Fig. 8. Time course of the changes in inositol phospholipids in serum-pretreated cells Cells were challenged with either 10 μ M-bradykinin (a) or 10 μ M-angiotensin III (b). Lipids were extracted, deacylated and separated by h.p.l.c. as described in the Methods section: $\bullet$, GroPIns(3)P; $\blacktriangle$, GroPIns(4,5)P₂; $\blacksquare$, GroPIns(4,5)P₂. Each point is the mean $\pm$ S.E.M. for 3 determinations. Control values were: GroPIns(3)P 2600 c.p.m.; GroPIns(4)P 47000 c.p.m.; GroPIns(4,5)P₂ 41200 c.p.m.

identified several cytokine or growth-factor receptors, including two, TGF β and IGF-I, which have a mitogenic action in these cells, and two for non-mitogenic factors, IL-1 and NGF. These stimulants were examined for actions on inositol lipids. The results are shown in Table 1. None of these agents altered the amounts of PtdIns(3)P relative to control cells at 1 min or 5 min, times at which PDGF has been reported to be producing large effects. Additionally, no production of PtdIns(3,4)P₂ or

Table 1. Effects of cytokines and growth factors on inositol phospholipid levels

Values are expressed as percentages of control. For DNA-synthesis measurements, each value is the mean $\pm$ S.E.M. ($n = 3$) with control values in the range of 10000–20000 c.p.m. Under these conditions maximal stimulation of DNA synthesis (2–5% FCS) was no more than 220%. Neither NGF nor IL-1 caused a significant change from basal levels of [3 H]thymidine incorporation. PtdIns isomer measurements are means $\pm$ S.D. ($n = 2$). PtdIns measurements, controls are: PtdIns(3)P, 750 c.p.m.; PtdIns(4)P, 27700 c.p.m.; PtdIns(4,5)P₂, 14700 c.p.m.

Addition	DNA synthesis	1 min			5 min		
		PtdIns(3)P	PtdIns(4)P	PtdInsP ₂	PtdIns(3)P	PtdIns(4)P	PtdInsP ₂
NGF	–	104 $\pm$ 13	96 $\pm$ 1	107 $\pm$ 9	116 $\pm$ 14	109 $\pm$ 13	98 $\pm$ 10
IL-1 α/β	–	98 $\pm$ 8	97 $\pm$ 2	89 $\pm$ 8	117 $\pm$ 2	103 $\pm$ 9	104 $\pm$ 2
IGF-I	165 $\pm$ 7	107 $\pm$ 5	105 $\pm$ 1	99 $\pm$ 1	99 $\pm$ 1	108 $\pm$ 3	96 $\pm$ 3
TGF β	158 $\pm$ 11	92 $\pm$ 8	88 $\pm$ 5	97 $\pm$ 10	101 $\pm$ 3	118 $\pm$ 3	116 $\pm$ 14

PtdIns(3,4,5) P_3 could be detected. Therefore the inability to detect receptor modulation of PtdIns(3) P is not specific to one type of growth-promoting stimulus; rather, three distinct factors with mitogenic effects, including one, IGF-I, whose receptors are of the tyrosine kinase class, do not appear to regulate PtdIns(3) P as part of their mechanism.

DISCUSSION

Two closely related peptide activators of inositol lipid events, angiotensin and bradykinin, produce distinct temporal patterns of changes in inositol phosphates and phospholipids in the NG115-401L-C3 cell line. Neither peptide alters the levels of PtdIns(3) P or, under the conditions described here, stimulates the production of detectable amounts of novel inositol lipids, thought to be markers for the activation of PtdIns 3-kinase. Thus, contrary to PDGF activation of fibroblasts and smooth-muscle cells, angiotensin activation of DNA synthesis in transfectants is not correlated with the regulation of PtdIns 3-kinase. This conclusion is reinforced by the fact that two agents which are growth-promoting in these cells and which act through distinct receptors, TGF β and IGF-I, are also without effects on PtdIns(3) P levels.

The behaviour of PtdIns(3) P seen when PDGF is added to smooth-muscle cells (Auger *et al.*, 1989) and CSF-1 to fibroblasts (Varticovski *et al.*, 1989) is quite similar to that reported here, in that there were no net increases in its levels, as would be expected from activation of PtdIns3-kinase. On the contrary, the major effects reported were the appearance of new inositol lipids, thought to be PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3 , accompanied by no change or a decline in PtdIns(3) P . If only these new lipids and not PtdIns(3) P are being produced by 'PtdIns 3-kinase', then its behaviour is significantly different from that observed *in vitro*, where it is readily able to produce PtdIns(3) P from PtdIns. We suggest that the production of the novel lipids may reflect a different enzyme activity. Moreover, stimulation of astrocytoma cells with carbachol causes a different type of PtdIns(3) P response, namely a dramatic, relatively slow, decrease in the levels of PtdIns(3) P (Stephens *et al.*, 1989), which is unlike responses seen with PDGF-receptor activation. This decline in PtdIns(3) P does not appear to proceed via hydrolysis by PLC, as four distinct purified PLC isoforms showed no ability to hydrolyse PtdIns(3) P *in vitro* (Lips *et al.*, 1989; Serunian *et al.*, 1989). Recently, PtdIns(3) P was found to be a substrate for a novel phosphomonoesterase (Lips & Majerus, 1989), suggesting that the latter enzyme might be responsible for observed decreases in PtdIns(3) P levels, as seen with astrocytoma cells. Our results demonstrate that the stimuli used here do not activate either a PLC or a phosphomonoesterase which hydrolyses PtdIns(3) P in intact 401L-C3 cells. Moreover, at the level of labelling used in these experiments, we have found no evidence for the appearance of novel inositol lipids under any resting or stimulated condition of this cell line. We cannot rule out the possibility that these lipids are being produced, but in significantly smaller quantities than observed in the work of Auger *et al.* (1989) and Varticovski *et al.* (1989). However, it may be that the occurrence and possible significance of PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3 is cell-specific. Indeed, PtdIns3-kinase and the related lipid products may have roles outside those which have been so far proposed for it, perhaps in the regulation of the cytoskeleton and shape changes. For example, the production of PtdIns(3,4,5) P_3 in fMet-Leu-Phe-stimulated neutrophils is not connected with DNA synthesis (Traynor-Kaplan *et al.*, 1988). Indeed Coughlin *et al.* (1989) specifically conclude that activation of PtdIns 3-kinase cannot be sufficient to initiate DNA synthesis in PDGF action.

A striking difference between angiotensin and bradykinin time courses is in the smaller magnitude of the Ins(1,4,5) P_3 production achieved by the former (less than 10% of the bradykinin response). Intriguingly, this appears to make no difference to the resulting maximal Ca^{2+} signal (Jackson *et al.*, 1988), but could be the basis of slower kinetics of the angiotensin Ca^{2+} response. It would seem that there must be an effective 10-fold receptor reserve for Ins(1,4,5) P_3 to explain these results. In a similar vein, angiotensin stimulation appeared to produce a striking elevation of Ins(1,3,4) P_3 , whereas the level of its presumptive precursor, Ins P_4 , was not elevated to a comparable extent. This might point to a tight coupling between the initial production of Ins(1,4,5) P_3 and subsequent conversion steps, which is more evident for a lower-efficiency agonist, angiotensin, than for a high-efficiency agonist, bradykinin. The production of Ins(1,4,5) P_3 in response to angiotensin did not seem to be longer lasting than that seen with bradykinin.

Surprisingly, there was no detectable decrease in any of the inositol phospholipids with angiotensin stimulation, despite the appearance of PLC-mediated breakdown products such as Ins(1,4,5) P_3 . Once again, this might imply that there is very efficient homeostatic control of inositol lipid resynthesis during angiotensin activation, whereas the intensity of the bradykinin response might exceed the capacity of the lipid recovery events.

Pretreatment of the cells with serum before stimulation with agonist caused a 2–3-fold potentiation of responses to both angiotensin and bradykinin. With bradykinin, it was an initial, very rapid, phase of Ins(1,4,5) P_3 production that was augmented, suggesting an underlying biphasic mechanism to bradykinin activation of PLC, whereas the angiotensin-generated Ins(1,4,5) P_3 response was potentiated throughout its time course. The mechanism behind this potentiation is unclear, but, given the recent observations that growth-factor receptors can phosphorylate PLC isoforms *in vivo* (Meisenhelder *et al.*, 1989; Margolis *et al.*, 1989), it is tempting to conclude that serum-derived factors may act on one or more components of the transduction cascade to alter the efficiency of the messenger production.

If, as has been suggested above, activation of PtdIns 3-kinase by angiotensin is not a step in its initiation of DNA synthesis, then the nature of the difference in activity between angiotensin and bradykinin remains elusive. It may well be due to a precise pattern of receptor-triggered events acting as a code. The unusual pattern of angiotensin-induced inositol phosphate production may be suggestive of this possibility. It would clearly be of interest to compare the actions of angiotensin and bradykinin on protein kinase C. The Na^+/H^+ antiport system is a marker of activation of this kinase, and its activity is enhanced for a period of several minutes by angiotensin (Jackson & Hanley, 1989).

In conclusion, changes in levels of PtdIns(3) P cannot be demonstrated with activation by either mitogenic or non-mitogenic stimuli. Although PtdIns(3) P is present in a neuronal population, demonstrating the presence of its biosynthetic enzyme PtdIns 3-kinase, PtdIns(3) P levels appear unregulated by the stimuli used here. The related lipids PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3 were undetected. Thus enthusiasm for an obligate role for PtdIns(3) P and its synthetic enzyme as signal-transduction intermediates in growth-factor receptor mechanisms must be tempered with an awareness of its lack of universality. Moreover, other functions for PtdIns(3) P should be considered (Hanley, 1989).

D. R. P. is supported by a fellowship from CellTech, P. T. H. is a Lister Research Fellow, and M. R. H. is the recipient of a Research Award from the ILSI Research Foundation.

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Received 26 March 1990/4 June 1990 ; accepted 26 June 1990