Tyrosine-phosphorylation-dependent and Rho-protein-mediated control of cellular phosphatidylinositol 4,5-bisphosphate levels

Ulrich RÜMENAPP^{*1}, Martina SCHMIDT^{*}, Simone OLESCH^{*}, Sabine OTT^{*}, Christoph von EICHEL-STREIBER[†] and Karl H. JAKOBS^{*}

^{*}Institut für Pharmakologie, Universitätsklinikum Essen, Hufelandstrasse 55, D-45122 Essen, Germany, and [†]Institut für Medizinische Mikrobiologie und Hygiene, Universität Mainz, Obere Zahlbacher Strasse 63, D-55101 Mainz, Germany

The polyphosphoinositide PtdIns(4,5) P_2 , best known as a substrate for phospholipase C isozymes, has recently been recognized to be involved in a variety of other cellular processes. The aim of this study was to examine whether the cellular levels of this versatile phospholipid are controlled by tyrosine phosphorylation. The studies were performed in human embryonic kidney (HEK)-293 cells stably expressing the M_3 muscarinic acetylcholine receptor. Inhibition of tyrosine phosphatases by pervanadate induced an up-to-approx.-2.5-fold increase in the total cellular level of PtdIns(4,5) P_2 , which was both time- and concentration-dependent. In contrast, the tyrosine kinase inhibitors, genistein and tyrphostin 23, caused a rapid and specific fall in the cellular PtdIns(4,5) P_2 level and prevented the stimulatory effect of pervanadate on PtdIns(4,5) P_2 formation. Inactivation of Rho proteins by *Clostridium difficile* toxin B caused a similar fall in

the HEK-293 cell PtdIns(4,5) P_2 level, which was not altered by additional genistein treatment. Furthermore, toxin B treatment abolished the pervanadate-induced increase in PtdIns(4,5) P_2 levels. As PtdIns(4,5) P_2 is an essential stimulatory cofactor for phospholipase D (PLD) enzymes, we finally examined the effects of the agents regulating PtdIns(4,5) P_2 levels on PLD activity in HEK-293 cells. Inhibition of tyrosine phosphatases by pervanadate caused an increase in PLD activity, which was susceptible to genistein and tyrphostin 23, and which was abolished by prior treatment with toxin B. In conclusion, the data presented indicate that the cellular level of the multifunctional phospholipid, PtdIns(4,5) P_2 , in HEK-293 cells is controlled by a tyrosine-kinase-dependent mechanism and that this process apparently involves Rho proteins, as found similarly for tyrosine-phosphorylation-induced PLD activation.

INTRODUCTION

Altered metabolism of phosphoinositides, specifically of PtdIns(4,5) P_2 , plays an essential role in various cellular processes. This polyphosphoinositide serves as a substrate for phosphoinositide-specific phospholipase C enzymes, generating the second messengers Ins(1,4,5) P_3 and diacylglycerol [1,2]. In addition, PtdIns(4,5) P_2 can be converted by PtdIns-3-kinases to PtdIns(3,4,5) P_3 , which is apparently involved in cellular signalling of various growth factor receptors [3]. Furthermore, PtdIns(4,5) P_2 has been shown to both bind to and regulate the activity of numerous proteins, including various actin-binding proteins, many pleckstrin-homology-domain-containing proteins and even ion channels [4–9]. The major pathway of PtdIns(4,5) P_2 synthesis is apparently mediated by type I PtdIns4P 5-kinases, three isoforms of which have recently been cloned [10–12]. The numerous cellular functions attributed to PtdIns(4,5) P_2 suggest strongly that the level of this phospholipid is tightly controlled.

Phospholipase D (PLD) enzymes, which hydrolyse phosphatidylcholine into phosphatidic acid and choline, have recently been recognized to belong with the many enzymes whose activity is regulated by PtdIns(4,5) P_2 . While PtdIns(4,5) P_2 is, thus far, the only known regulator of PLD2 enzymes, PLD1 enzymes are regulated additionally by ADP-ribosylation factor (ARF) and Rho proteins, two low-molecular-mass GTPases [13–16]. Both ARF and Rho proteins not only stimulate PLD, but can apparently also increase PtdIns(4,5) P_2 synthesis by PtdInsP 5-kinases, although a direct stimulation of these kinases has not

been reported so far [17–21]. Rather, the PLD product, phosphatidic acid, is the best known activator of type I PtdInsP 5-kinases [10–12].

Receptor regulation of PLD activity in human embryonic kidney (HEK)-293 cells, stably expressing the human M_3 muscarinic acetylcholine receptor (mAChR) subtype, involves both ARF and Rho proteins [22,23]. Studies on the mechanisms of PLD inhibition by *Clostridium difficile* toxin B and *C. botulinum* C3 exoenzyme, which inactivate Rho family GTPases [24], indicated that these toxins decrease the cellular level of PtdIns(4,5) P_2 [25,26]. As receptor stimulation of PLD activity in HEK-293 cells additionally involves a tyrosine-kinase-dependent mechanism [27], as reported similarly for other cell types [28,29], we have examined in this study whether the cellular level of PtdIns(4,5) P_2 is under the control of tyrosine phosphorylation. We report here that a tyrosine-kinase-dependent mechanism, apparently involving Rho proteins, regulates the synthesis of PtdIns(4,5) P_2 in HEK-293 cells.

MATERIALS AND METHODS

Materials

myo-[³H]inositol (25 Ci/mmol) was obtained from Biotrend (Köln, Germany) and [³H]oleic acid (10 Ci/mmol) from New England Nuclear (Köln, Germany). Genistein and tyrphostin 23 were obtained from Biomol (Hamburg, Germany). *C. difficile* toxin B was purified as described previously [30]. Pervanadate

Abbreviations used: ARF, ADP-ribosylation factor; HBSS, Hank's balanced salt solution; HEK, human embryonic kidney; mAChR, muscarinic acetylcholine receptor; PLD, phospholipase D; PtdEtOH, phosphatidylethanol.

¹To whom correspondence should be addressed (e-mail ulrich.ruemenapp@uni-essen.de).

(10 mM stock solution) was produced from a combination of 20 mM H_2O_2 and 20 mM orthovanadate, and allowed to stand at room temperature for at least 15 min. All other materials were from sources described previously [22,26,27].

Cell culture

Culture conditions of HEK-293 cells, stably expressing the M_3 mAChR, were set up as described previously [27]. For these experiments, cells subcultured in Dulbecco's modified Eagle's medium/F-12 medium were grown to near-confluency (in 175- cm^2 culture flasks or 35- and 145-mm culture dishes).

Phosphoinositide analysis

HEK-293 cell phosphoinositide levels were determined as described previously [25,26]. Briefly, phosphoinositides were labelled by incubating nearly confluent monolayers of cells for 20–24 h with [^3H]inositol (0.5–1 $\mu\text{Ci}/\text{ml}$) in inositol-free growth medium. In studies on the effects of toxin B on phosphoinositide levels, the labelling medium was then replaced by normal growth medium, followed by treatment of the cells for 24 h with or without toxin B at the indicated concentrations. Thereafter, cells were detached from the culture flasks, washed once in PBS containing 137 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl_2 , 0.5 mM MgCl_2 , 6.5 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 , pH 7.2, and resuspended at a cellular concentration of about 2×10^7 cells/ml in Hank's balanced salt solution (HBSS), containing 118 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 and 5 mM D-glucose, buffered at pH 7.4 with 15 mM Hepes. After incubation for 60 min (or the indicated periods of time) at 37 °C in the absence or presence of various test agents, the phosphoinositides of approximately 6×10^7 cells were extracted, and an aliquot containing about 50000 c.p.m. total labelled phosphoinositides was analysed for the levels of [^3H]PtdIns, [^3H]PtdIns4P and [^3H]PtdIns(4,5) P_2 by TLC and liquid-scintillation counting, as described previously [25,26]. The difference between total [^3H]phosphoinositides applied on to the TLC plates (50000 c.p.m.) and the total of [^3H]PtdIns, [^3H]PtdIns4P and [^3H]PtdIns(4,5) P_2 (about 30000 c.p.m.) recovered from the plates was due to quenching by the TLC material; a difference in total [^3H]inositol incorporation between control and treated cells was not observed for any of the agents studied.

Assay of PLD activity

PLD activity was determined as described previously [27]. Briefly, cellular phospholipids were labelled by incubating nearly confluent monolayers of cells for 20–24 h with [^3H]oleic acid (2–2.5 $\mu\text{Ci}/\text{ml}$) in growth medium. Thereafter, the cells were detached from the culture flasks, washed once in PBS and finally resuspended in HBSS. Accumulation of the specific PLD product, [^3H]phosphatidylethanol ([^3H]PtdEtOH), was then measured in the presence of ethanol (1.75%, v/v) for the indicated periods of time at 37 °C in a total vol. of 200 μl , containing 100 μl of the cell suspension (1×10^6 cells). Termination of the reactions and isolation of radiolabelled phospholipids were performed as described previously [27]. Formation of [^3H]PtdEtOH is expressed as the percentage of total counts in the phospholipid fraction.

Assay of ARF translocation

Translocation of ARF proteins was monitored as described previously [22]. Briefly, HEK-293 cells were detached from culture flasks, washed once with PBS and resuspended in PBS at

a density of about 2×10^6 cells/ml. After 10 min incubation at 37 °C in the absence or presence of 1 mM carbachol, cells were pelleted and resuspended in 500 μl of buffer A [135 mM KCl/5 mM NaHCO_3 /5 mM EGTA/5.6 mM D-glucose/2 mM ATP/4 mM MgCl_2 /1.5 mM CaCl_2 /20 mM Hepes (pH 7.2)] without or with 8 μM digitonin, and incubated for 15 min at 37 °C. Thereafter, cells were pelleted by high-speed centrifugation, and proteins in the supernatant fraction were analysed for ARF protein content with a polyclonal anti-(recombinant ARF1) antibody.

Assay of tyrosine phosphorylation

HEK-293 cells were detached from culture flasks, washed once with PBS and resuspended in HBSS. Approx. 1×10^6 cells were preincubated for 15 min at 37 °C in the absence or presence of 100 μM genistein or tyrphostin 23, followed by incubation for 2 min in the absence or presence of 1 mM pervanadate (total vol. of 60 μl). The reaction was stopped by adding 30 μl of lysis buffer, containing 6% (w/v) SDS, 18% (w/v) 2-mercaptoethanol, 30% (v/v) glycerol, 1 mM Na_3VO_4 and a trace amount of Bromophenol Blue dye in 200 mM Tris/HCl, pH 7.5, followed by heating for 5 min at 95 °C. SDS/PAGE, transfer of proteins to nitrocellulose filters and detection of phosphotyrosine-containing proteins using anti-phosphotyrosine antibodies and the enhanced chemiluminescence Western blotting detection system (Amersham) have been described previously [27].

RESULTS

Regulation of HEK-293 cell PtdIns(4,5) P_2 levels by tyrosine phosphorylation

To study a tyrosine-phosphorylation-dependent regulation of phosphoinositide levels in HEK-293 cells, we examined the effects of the protein tyrosine phosphatase inhibitor, pervanadate [31], and the tyrosine kinase inhibitors, genistein and tyrphostin 23, on the total cellular levels of PtdIns, PtdIns4P and PtdIns(4,5) P_2 . To meet this aim, HEK-293 cells prelabelled with [^3H]inositol were treated with the above-mentioned agents, followed by extraction and quantification of the labelled phosphoinositide species. As shown in Table 1, treatment of HEK-293 cells for 60 min with 1 mM pervanadate did not alter the level of [^3H]PtdIns. In contrast, the level of [^3H]PtdIns4P, and particularly that of [^3H]PtdIns(4,5) P_2 , were increased markedly by pervanadate, amounting to about 150% and 250% respectively of those in untreated control cells. On the other hand,

Table 1 Effect of pervanadate on the level of phosphoinositides in HEK-293 cells

The level of [^3H]phosphoinositides was quantified in HEK-293 cells prelabelled with [^3H]inositol and subsequently treated for 60 min with either H_2O_2 , orthovanadate or pervanadate (1 mM each). Data are given as percentages of control (untreated) cells. The radioactivity associated with [^3H]PtdIns, [^3H]PtdIns4P and [^3H]PtdIns(4,5) P_2 in control cells amounted to $27.5 \pm 0.15 \times 10^3$ c.p.m., $0.73 \pm 0.01 \times 10^3$ c.p.m. and $0.67 \pm 0.04 \times 10^3$ c.p.m. respectively. Data represent means $\pm$ S.D. ($n = 3$); *, significantly different from the value in control, untreated cells ($P < 0.005$).

Addition	Phosphoinositide (% of control)		
	PtdIns	PtdIns4P	PtdIns(4,5) P_2
H_2O_2	103 $\pm$ 22	111 $\pm$ 1	110 $\pm$ 29
Orthovanadate	92 $\pm$ 5	111 $\pm$ 6	96 $\pm$ 8
Pervanadate	91 $\pm$ 8	151 $\pm$ 7*	280 $\pm$ 38*

Figure 1 Concentration-dependence and time course of pervanadate-induced PtdIns(4,5) P_2 formation in HEK-293 cells

The level of [3 H]PtdIns(4,5) P_2 was determined in [3 H]inositol-prelabelled HEK-293 cells after treatment for either 60 min with the indicated concentrations of pervanadate (**A**) or the indicated periods of time with 1 mM pervanadate (**B**). [3 H]PtdIns(4,5) P_2 in control (untreated) cells amounted to $1.20 \pm 0.01 \times 10^3$ c.p.m. and $0.34 \pm 0.01 \times 10^3$ c.p.m. in (**A**) and (**B**) respectively. Data represent means $\pm$ S.D. ($n = 3$).

neither orthovanadate nor H_2O_2 (at 1 mM each), from which the pervanadate was generated, altered the level of any of the phosphoinositides. The stimulatory effect of pervanadate on the [3 H]PtdIns(4,5) P_2 level was half-maximal at about 100 μ M, and a maximal increase was observed at 1 mM pervanadate (Figure 1A). The time course of pervanadate-induced [3 H]PtdIns(4,5) P_2 accumulation (at 1 mM) was almost linear for the first 30 min, with no major changes occurring at later time points (Figure 1B).

The stimulatory effect of pervanadate on the level of PtdIns(4,5) P_2 was apparently due to inhibition of tyrosine phosphatases, as determined with the tyrosine kinase inhibitors, genistein and tyrphostin 23. Pretreatment of HEK-293 cells for 15 min with either genistein or tyrphostin 23 (100 μ M each) almost completely prevented the increase in PtdIns(4,5) P_2 level

Table 2 Inhibition of pervanadate-induced PtdIns(4,5) P_2 increase by tyrosine kinase inhibitors

HEK-293 cells prelabelled with [3 H]inositol were pretreated without or with 100 μ M genistein or tyrphostin 23 for 15 min. Thereafter, the cells were incubated for 15 min with pervanadate (100 μ M or 1 mM), and the level of [3 H]PtdIns(4,5) P_2 was quantified. Data are given as percentages of [3 H]PtdIns(4,5) P_2 in control (untreated cells), amounting to $1.24 \pm 0.17 \times 10^3$ c.p.m. Data represent means $\pm$ S.D. ($n = 3$); *, significantly different from the value in control, untreated cells ($P < 0.01$).

Pretreatment	PtdIns(4,5) P_2 (% of control)	
	100 mM Pervanadate	1 mM Pervanadate
None	151 $\pm$ 8*	222 $\pm$ 10*
Genistein	103 $\pm$ 7	127 $\pm$ 8
Tyrphostin 23	91 $\pm$ 22	103 $\pm$ 11

induced by pervanadate, both at its half-maximally and maximally effective concentrations (Table 2).

The tyrosine kinase inhibitors did not only prevent the stimulatory effect of pervanadate, but also caused, by themselves, a significant reduction in the level of PtdIns(4,5) P_2 . As shown in Figure 2(A), treatment of HEK-293 cells for 60 min with either genistein or tyrphostin 23 (100 μ M each) had no effect on the levels of [3 H]PtdIns and [3 H]PtdIns4 P , compared with untreated control cells. However, the cellular level of [3 H]PtdIns(4,5) P_2 was significantly decreased by either of the tyrosine kinase inhibitors, and amounted to only 50–60 % of that in untreated controls. Time course studies performed with genistein demonstrated that the inhibitory effect of the tyrosine kinase inhibitor on the HEK-293 cellular [3 H]PtdIns(4,5) P_2 level was rather rapid. An almost maximal decrease in the level of [3 H]PtdIns(4,5) P_2 was observed in cells treated for only 5 min with genistein (100 μ M), with only minor further changes occurring at later time points (Figure 2B).

Pervanadate and the tyrosine kinase inhibitors exhibited the expected effects on protein tyrosine phosphorylation. As shown in Figure 3, treatment of HEK-293 cells with pervanadate (1 mM) strongly increased the tyrosine phosphorylation of a large number of proteins. The stimulatory effect of pervanadate was inhibited strongly by prior treatment with tyrphostin 23 (100 μ M), which also lowered the tyrosine phosphorylation of several proteins in cells not treated with pervanadate. Similar effects were observed with genistein (results not shown).

Role of Rho proteins in tyrosine-phosphorylation-dependent PtdIns(4,5) P_2 synthesis

Since PtdIns(4,5) P_2 synthesis in HEK-293 cells is apparently controlled by both tyrosine phosphorylation and Rho proteins [25,26], the relationship between these two regulatory mechanisms was examined. Therefore we studied the effects of Rho protein inactivation by *C. difficile* toxin B on both the genistein-induced fall and the pervanadate-induced rise in phosphoinositide levels. Hence, HEK-293 cells prelabelled with [3 H]inositol were first treated for 24 h with or without toxin B, followed by a second incubation for 60 min with or without genistein or pervanadate. As shown in Figure 4, both toxin B (50 pg/ml) and genistein (100 μ M) alone reduced the cellular [3 H]PtdIns(4,5) P_2 level by about 50 %. Most importantly, subsequent treatment of toxin-B-treated cells with genistein did not result in a further decrease in [3 H]PtdIns(4,5) P_2 level. As found in HEK-293 cells not treated with toxin B, genistein did not affect the levels of [3 H]PtdIns and [3 H]PtdIns4 P , and thus genistein did

Figure 2 Effects of genistein and tyrphostin 23 on the level of phosphoinositides in HEK-293 cells

(A) The level of [³H]phosphoinositides was quantified in HEK-293 cells prelabelled with [³H]inositol and subsequently treated for 60 min with 100 μM genistein (left panel) or 100 μM tyrphostin 23 (right panel). Data are given as percentages of control (untreated) cells. The radioactivity associated with [³H]PtdIns, [³H]PtdIns4P and [³H]PtdIns(4,5)P₂ in control cells was $26.5 \pm 1.6 \times 10^3$ c.p.m., $0.76 \pm 0.09 \times 10^3$ c.p.m. and $0.80 \pm 0.06 \times 10^3$ c.p.m. respectively (left panel), and $28.6 \pm 0.8 \times 10^3$ c.p.m., $0.77 \pm 0.02 \times 10^3$ c.p.m. and $0.87 \pm 0.06 \times 10^3$ c.p.m. respectively (right panel). (B) The level of [³H]PtdIns(4,5)P₂ was determined in [³H]inositol-prelabelled HEK-293 cells after treatment with 100 μM genistein for the indicated periods of time. The radioactivity associated with [³H]PtdIns(4,5)P₂ in control cells at zero time point was $1.22 \pm 0.07 \times 10^3$ c.p.m. Data represent means $\pm$ S.D. ($n = 3$).

Figure 3 Pervanadate-induced tyrosine phosphorylation and its inhibition by tyrphostin 23 in HEK-293 cells

HEK-293 cells pretreated for 15 min without (lanes 2 and 3) or with 100 μM tyrphostin 23 (lanes 1 and 4) were incubated for 2 min in the absence (lanes 1 and 2) or presence of 1 mM pervanadate (lanes 3 and 4). Proteins were analysed for phosphotyrosine content as described in the Materials and methods section. An autoluminogram of a Western blot with molecular masses of marker proteins (kDa) is shown.

not synergize with toxin B in reducing the cellular PtdIns(4,5)P₂ level. These data therefore suggest that the genistein-sensitive tyrosine kinase and Rho proteins regulate a singular pool of cellular PtdIns(4,5)P₂ in concert.

The pervanadate-induced rises in the cellular levels of [³H]-PtdIns4P and [³H]PtdIns(4,5)P₂ were differentially affected by Rho protein inactivation. Pretreatment of HEK-293 cells with toxin B (2 ng/ml, for 24 h) did not alter the pervanadate-induced increase in [³H]PtdIns4P level (at 1 mM pervanadate), which was

Figure 4 Effects of toxin B and genistein on the level of phosphoinositides in HEK-293 cells

HEK-293 cells prelabelled with [³H]inositol were treated for 24 h with or without 50 pg/ml toxin B. Thereafter, the level of [³H]phosphoinositides was quantified in cells treated additionally for 60 min without or with 100 μM genistein. Data are given as percentages of control cells not treated with toxin B or genistein. The radioactivity associated with [³H]PtdIns, [³H]PtdIns4P and [³H]PtdIns(4,5)P₂ in control cells was $24.0 \pm 2.1 \times 10^3$ c.p.m., $0.47 \pm 0.07 \times 10^3$ c.p.m. and $0.75 \pm 0.02 \times 10^3$ c.p.m. respectively. Data represent means $\pm$ S.D. ($n = 3$).

increased in both control and toxin-B-treated cells similarly, by about 50% (Table 3). In contrast, the rise in [³H]PtdIns(4,5)P₂ level (> 2-fold) caused by pervanadate in control cells was completely absent in cells pretreated with toxin B, the data for which suggest that the tyrosine-phosphorylation-dependent increase in PtdIns(4,5)P₂ levels is mediated by Rho proteins.

As found in other cell types, ARF proteins have been implicated in the control of PtdIns(4,5)P₂ synthesis [17,18]. We therefore examined whether the activity state of these proteins in HEK-293 cells is controlled by tyrosine phosphorylation. Activation of either ARF or Rho proteins is associated with a translocation of the proteins from the cytosol to the membranes.

Table 3 Effect of toxin B on the pervanadate-induced increases in the level of phosphoinositides

HEK-293 cells were labelled with [3 H]inositol and subsequently treated in the presence or absence of toxin B (2 ng/ml, 24 h). Thereafter, the level of [3 H]phosphoinositides was quantified after 60 min incubation with or without 1 mM pervanadate. Changes in the cellular levels of [3 H]PtdIns4P and [3 H]PtdIns(4,5) P_2 are given as percentages of control cells treated with neither toxin B nor pervanadate. The radioactivity associated with [3 H]PtdIns4P and [3 H]PtdIns(4,5) P_2 in control cells was $0.27 \pm 0.06 \times 10^3$ c.p.m. and $1.19 \pm 0.07 \times 10^3$ c.p.m. respectively. Data represent means $\pm$ S.D. ($n = 3$); *, significantly different from the value in untreated cells ($P < 0.001$).

	Phosphoinositide (% of control)	
	PtdIns4P	PtdIns(4,5) P_2
Untreated cells	153 ± 10	217 ± 10
Toxin-B-treated cells	149 ± 20	$100 \pm 3^*$

Figure 5 Lack of effect of genistein on M_3 -mAChR-stimulated ARF translocation

HEK-293 cells pretreated without or with genistein (100 μ M) for 30 min were incubated for 10 min without or with carbachol (1 mM), followed by permeabilization with digitonin and analysis of proteins released into the supernatant for ARF immunoreactivity. Lane 1, recombinant ARF1 (1 μ g); lane 2, supernatant of non-permeabilized cells; lanes 3–6, supernatants of permeabilized cells pretreated without (lane 3) or with carbachol (lane 4), genistein (lane 5) or genistein followed by carbachol (lane 6). An autoluminogram of a representative immunoblot is shown. Other immunoreactive bands were not detected. Migration of ARF proteins (19 kDa) is indicated by the arrow.

We have recently reported that M_3 mAChR activation induces translocation of ARF and RhoA proteins from the cytosol to the membranes in HEK-293 cells [22,32]. Furthermore, the mAChR-induced RhoA translocation was prevented by genistein and tyrphostin 23, and mimicked by pervanadate [32]. Similar experiments performed on the subcellular distribution of ARF proteins showed that treatment of HEK-293 cells with pervanadate (1 mM, 30 min) did not affect the cytosol–membrane distribution of ARF proteins (results not shown). Moreover, as shown in Figure 5, pretreatment of HEK-293 cells for 30 min with genistein (100 μ M) did not decrease basal or carbachol-induced reductions in cytosolic ARF content, measured as immunoreactive ARF proteins (19 kDa) released from digitonin-permeabilized cells. Thus in contrast with RhoA activation, ARF activation in HEK-293 cells is apparently not controlled by a tyrosine-phosphorylation-dependent process.

Regulation of PLD activity by tyrosine phosphorylation

In common with other PLD enzymes, HEK-293 cell PLD activity is stimulated by PtdIns(4,5) P_2 [26]. Therefore we examined the effects of the agents regulating PtdIns(4,5) P_2 levels under very similar conditions on PLD activity in HEK-293 cells. First, as reported previously [27] with HEK-293 cell monolayers, the tyrosine phosphatase inhibitor, pervanadate, increased PLD activity in HEK-293 cell suspensions (Figure 6A). The maximal

Figure 6 Stimulation of HEK-293 cell PLD activity by pervanadate

(A) [3 H]Oleic-acid-labelled HEK-293 cells were incubated with pervanadate at the indicated concentrations for 60 min, and [3 H]PtdEtOH formed was analysed. (B) [3 H]Oleic-acid-labelled HEK-293 cells were incubated without (Basal) and with 1 mM pervanadate for the indicated periods of time to measure [3 H]PtdEtOH formation. Data represent means $\pm$ S.D. ($n = 3$).

increase in [3 H]PtdEtOH formation was observed at 1 mM pervanadate, being half-maximally effective at about 30 μ M. As shown in Figure 6B, maximal levels of [3 H]PtdEtOH were attained after 15–30 min stimulation with pervanadate, and further [3 H]PtdEtOH formation was negligible at later time points. In contrast with pervanadate, neither orthovanadate nor H_2O_2 (1 mM each) had any effect on PLD activity in HEK-293 cells (results not shown). Secondly, the stimulatory effect of pervanadate (100 μ M or 1 mM) on HEK-293 cell PLD activity was prevented by prior treatment of the cells with either genistein or tyrphostin 23 (100 μ M each) (Table 4), indicating the involvement of tyrosine phosphorylation in pervanadate's action on PLD activity. Thirdly, the effect of inactivation of Rho protein by toxin B on PLD stimulation by pervanadate was examined. As shown in Table 4, pretreatment of HEK-293 cells with toxin B (2 ng/ml, 24 h) completely prevented the stimulatory effect of pervanadate (1 mM) on PLD activity.

Table 4 Inhibition of pervanadate-stimulated PLD activity by tyrosine kinase inhibitors and toxin B

HEK-293 cells prelabelled with [3 H]oleic acid were pretreated without or with genistein or tyrphostin 23 (100 μ M each) for 15 min or toxin B (2 ng/ml) for 24 h. Thereafter, basal and pervanadate (100 μ M or 1 mM)-stimulated [3 H]PtdEtOH formation were measured for 30 min. Data represent means $\pm$ S.D. ($n = 3$). *, significantly different from the basal value ($P < 0.001$); †, significantly different from the value in untreated cells ($P < 0.001$). N.D., not determined.

Pretreatment	PtdEtOH formation (% of phospholipids)		
	Basal	100 μ M Pervanadate	1 mM Pervanadate
None	0.14 $\pm$ 0.03	0.51 $\pm$ 0.03*	0.67 $\pm$ 0.03*
Genistein	0.13 $\pm$ 0.00	0.24 $\pm$ 0.01*†	0.23 $\pm$ 0.01*†
Tyrphostin 23	0.14 $\pm$ 0.02	0.18 $\pm$ 0.04†	0.32 $\pm$ 0.01*†
Toxin B	0.14 $\pm$ 0.02	N.D.	0.08 $\pm$ 0.04†

DISCUSSION

Regulation of PLD activity involves various signalling molecules, including the two low-molecular-mass GTPases, ARF and Rho, and the phosphoinositide, PtdIns(4,5) P_2 [16]. In addition, as demonstrated here for HEK-293 cells stably expressing the M $_3$ mAChR, an as-yet-unidentified tyrosine kinase is apparently involved in PLD stimulation. First, treatment of HEK-293 cells with the phosphotyrosine phosphatase inhibitor, pervanadate, which induced accumulation of several tyrosine-phosphorylated proteins, increased markedly PLD activity. Secondly, PLD stimulation by pervanadate was prevented by the tyrosine kinase inhibitors, genistein and tyrphostin 23. Thirdly, these inhibitors also prevented PLD stimulation caused by mAChR activation or by direct activation of heterotrimeric G-proteins [27], indicating that the putative tyrosine kinase plays a essential role in receptor signalling to PLD.

In studies on the mechanisms of PLD inhibition by *C. difficile* toxin B and *C. botulinum* C3 exoenzyme in HEK-293 cells, we observed that these Rho-protein-inactivating toxins decrease the cellular level of PtdIns(4,5) P_2 [25,26]. Therefore we examined in this study whether the level of PtdIns(4,5) P_2 in HEK-293 cells is regulated by a tyrosine-phosphorylation-dependent mechanism. In addition, we studied whether Rho and/or ARF proteins are involved in this process, since both GTPases have been reported to stimulate PtdIns(4,5) P_2 synthesis [17–21]. We demonstrate here that the level of PtdIns(4,5) P_2 in HEK-293 cells is controlled by a tyrosine-kinase-dependent mechanism, and that this process apparently involves Rho proteins. First, treatment of HEK-293 cells with the two structurally distinct tyrosine kinase inhibitors, genistein and tyrphostin 23, caused both a specific and rapid reduction, by about 50 %, in the cellular level of PtdIns(4,5) P_2 , without altering the levels of PtdIns and PtdIns4P, as reported similarly before for toxin B and C3 exoenzyme [25,26]. Comparable with our observations in HEK-293 cells, genistein has been reported to decrease the level of PtdIns(4,5) P_2 in human platelets [33] and rat pancreatic acini [34], although slightly increasing that of PtdIns4P. Secondly, genistein did not synergize with toxin B in reducing the level of PtdIns(4,5) P_2 in HEK-293 cells, suggesting that the genistein-sensitive tyrosine kinase and Rho proteins regulate the same pool of PtdIns(4,5) P_2 in concert and do not act independently of each other. Thirdly, treatment of HEK-293 cells with the phosphotyrosine phosphatase inhibitor, pervanadate, but not with orthovanadate or H $_2$ O $_2$, markedly increased the level of PtdIns(4,5) P_2 by about 2.5-fold, whereas that of PtdIns4P was increased by only about 50 %, with

no change in the level of PtdIns. The observation that even relatively large changes in the level of PtdIns(4,5) P_2 , i.e. a 2.5-fold increase in response to pervanadate, did not lead to significant changes in the levels of PtdIns and PtdIns4P is best explained by the fact that the level of PtdIns is 20–30 times higher than that of PtdIns(4,5) P_2 , and that in intact cells the depleted PtdIns4P is rapidly replenished by PtdIns kinases. Fourthly, the pervanadate-induced increase in PtdIns(4,5) P_2 levels was prevented by genistein and tyrphostin 23, indicating that the effect of pervanadate is indeed due to inhibition of tyrosine phosphatases. Finally, inactivation of Rho proteins by toxin B treatment of HEK-293 cells, which did not affect the rather small increase in PtdIns4P, eliminated completely the pervanadate-induced elevation in PtdIns(4,5) P_2 level.

Direct inhibition (by genistein and tyrphostin 23) and activation (by pervanadate) of PtdIns4P 5-kinases are unlikely to cause the observed changes in PtdIns(4,5) P_2 level for the following reasons. First, it would be highly fortuitous if the same agents inhibiting or increasing tyrosine phosphorylation had the same effects on PtdIns4P 5-kinase activities. Secondly, genistein decreased the PtdIns(4,5) P_2 level to a similar extent as toxin B, but in a non-additive manner; thus if genistein does directly inhibit PtdIns4P 5-kinase, it would be expected that this direct inhibition would be additive to the (indirect) inhibitory effect of toxin B. Finally, the stimulatory effect of pervanadate on PtdIns(4,5) P_2 level was not only inhibited by genistein and tyrphostin 23, but also by toxin B, which specifically inactivates Rho proteins [23].

All of these data suggest that synthesis of PtdIns(4,5) P_2 in HEK-293 cells is stimulated by a tyrosine-phosphorylation-dependent mechanism, and that Rho proteins act downstream of the putative tyrosine kinase. A similar concept of a signalling cascade involving a tyrosine kinase upstream of Rho proteins has also been proposed for the regulation of actin polymerization and stress fibre formation in Swiss 3T3 fibroblasts by lysophosphatidic acid [35,36]. On the other hand, we have found no evidence that ARF proteins, which have been reported to stimulate PtdIns(4,5) P_2 synthesis in other cell types [17,18], are involved in tyrosine-kinase-mediated PtdIns(4,5) P_2 synthesis in HEK-293 cells. First, whereas pervanadate and mAChR activation induced activation of RhoA proteins [32], activation of ARF proteins was only detected upon mAChR activation, and not with pervanadate. Secondly, genistein and tyrphostin 23 inhibited the mAChR-induced RhoA activation, whereas the receptor-induced ARF activation was not affected by these tyrosine kinase inhibitors. In agreement with these findings, we recently observed that overexpression of wild-type RhoA, but not of wild-type ARF1, constitutively active ARF1 or ARNO (a guanine-nucleotide-exchange factor for ARF proteins), increased PtdIns(4,5) P_2 levels in HEK-293 cells (results not shown).

There was a close correlation between the observed changes in PtdIns(4,5) P_2 level and PLD activity. First, formation of PtdIns(4,5) P_2 was stimulated solely by pervanadate (produced from a combination of orthovanadate and H $_2$ O $_2$), but not by either of these two agents alone, as found similarly in the increase in PLD activity mediated exclusively by pervanadate. Secondly, both effects of pervanadate were blocked by genistein and tyrphostin 23. Thirdly, both the time courses and the concentration-response curves for the pervanadate-induced synthesis of PtdIns(4,5) P_2 and its stimulatory effect on PLD activity were rather similar. Finally, and most importantly, treatment of HEK-293 cells with the Rho-inactivating toxin B not only prevented the pervanadate-induced increase in PtdIns(4,5) P_2 synthesis, but also abolished the pervanadate-induced PLD stimulation, suggesting that Rho proteins mediate the stimulatory

effect of tyrosine phosphorylation on PLD activity as well. In contrast with the PtdIns(4,5) P_2 -regulated PLD, the PtdIns(4,5) P_2 -hydrolysing phospholipase C was stimulated in HEK-293 cells by both orthovanadate and pervanadate (by about 2-fold), and this effect was relatively insensitive to the presence of the tyrosine kinase inhibitors (results not shown).

Despite the close correlation observed between PtdIns(4,5) P_2 synthesis and PLD activity, it remains to be determined whether the increased synthesis of the stimulatory PLD cofactor, PtdIns(4,5) P_2 , is solely responsible for the increase in PLD activity induced by pervanadate in intact HEK-293 cells. The interpretation is complicated, particularly in view of the fact that, on one hand, PtdIns(4,5) P_2 can stimulate PLD enzymes [13–16], and on the other hand, phosphatidic acid generated by PLD enzymes can stimulate type I PtdIns4P 5-kinases that are responsible for PtdIns(4,5) P_2 synthesis [10–12]. Furthermore, Bourgoin and coworkers [37] reported recently that hPLD1 in HL-60 cells is tyrosine-phosphorylated by pervanadate. Although no functional data were provided, it is possible that this tyrosine phosphorylation increases PLD activity in itself, or alters its sensitivity to stimulatory cofactors, such as Rho and PtdIns(4,5) P_2 . Thus although, on the basis of the toxin B data, a direct PLD stimulation is unlikely, it remains a possibility that pervanadate not only activates (Rho) and increases the level [PtdIns(4,5) P_2] of PLD cofactors, but in addition, it may sensitize PLD for these cofactors by inducing tyrosine phosphorylation of the PLD itself.

In conclusion, we have provided evidence that the synthesis of the multifunctional phospholipid, PtdIns(4,5) P_2 , best known as the substrate of phospholipase C isozymes but apparently also involved intimately in a variety of other cellular processes (including PLD activation), is regulated by a tyrosine-kinase-dependent mechanism in HEK-293 cells, and that this process apparently involves Rho proteins, as does tyrosine-phosphorylation-induced PLD activation. Whether or not synthesis of PtdIns(4,5) P_2 (mediated by tyrosine phosphorylation and Rho proteins) is under active receptor control, and the identification of the tyrosine kinase acting upstream of Rho, are areas for further investigation.

We thank Dr. E. G. Peralta for providing the M₃-mAChR-expressing HEK-293 cells, and Dr. J. B. Helms for the gift of the ARF1 antibody. This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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