Glycerophosphoinositol 4-Phosphate, a Putative Endogenous Inhibitor of Adenylyl cyclase*

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In a continuous line of rat thyroid cells transformed by the k-ras oncogene (KiKi), the expression of ras-p21 correlates with an increased activity of a phosphoinoside-specific phospholipase A2, which leads to elevated levels of glycerophosphoinositols. In this study we have characterized the biological activities of these compounds. Growth and differentiation in thyroid cells are mainly regulated by the activation of adenylyl cyclase. Therefore, we have studied the effects of glycerophosphoinositols on the activity of this enzyme using a normal thyroid cell line (FRTL5). Micromolar concentrations of glycerophosphoinositol 4-phosphate (GroPIns-4-P) caused a ~50% inhibition of the adenylylcyclase activity in FRTL5 membranes stimulated by the GTP-binding protein activator forskolin. Similar concentrations of GroPIns-4-P were detected in KiKi cells but not in the normal FRTL5 line.

Micromolar GroPIns-4-P was found to be taken up by intact FRTL5 cells and to induce nearly 50% inhibition of the thyrotropin- and cholera toxin-induced increase in cAMP levels. Similar results were also observed in other cell lines (smooth muscle, pituitary cells, and pneumocytes). GroPIns-4-P inhibited cAMP-dependent cellular functions such as iodide uptake and thyroglobulin incorporation in FRTL5 cells when stimulated by thyrotropin and cholera toxin but not when induced by forskolin. These results are consistent with GroPIns-4-P exerting an inhibitory effect on the GTP-binding protein that stimulates adenylylcyclase.

We propose that GroPIns-4-P might mediate a mechanism of cross-talk between adenylyl cyclase and phospholipase A2 in thyroid as well as in other cell systems.

The protein encoded by the ras protooncogene (ras-p21) is a monomeric GTP-binding protein of 21 kDa that has been associated with the regulation of growth and differentiation in several cell systems (1-3). The effects of ras-p21 on transforming growth factor receptors and in whole FRTL5 cells.

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 clase under conditions of phospholipase $A_2$ activation in several cell types.

MATERIALS AND METHODS

Hormones used in the tissue culture media, Coon's modified Ham's F-12 medium, forskolin, and glycrophosphoinositols were from Sigma. Tissue culture materials were from Gibco. Glycrophosphoinositols 4-phosphate and glycrophosphoinositols 4,5-bisphosphate were from Boehringer Mannheim. Cholera toxin was from Calbiochem, and sodium fluoride and aluminum chloride were from Fluka Chem. Corp. cAMP radioimmunoassays, [3H]-cAMP, [3H]-thymidine, and [3H]-myo-inositol were purchased from Du Pont-New England Nuclear. [3H]InsP$_3$ radioimmunoassay kit was purchased from Amersham Corp. All chemicals were obtained from commercial sources as the highest purity material available.

Cell Culture—FRTL5 are differentiated cells derived from Fisher rat thyroids. Their growth conditions have already been described (10, 15, 16). Briefly, the cells were maintained in Coon's modified F-12 medium supplemented with 5% calf serum, 20 mM glutamine, and a mixture of six hormones (thyrotropin, insulin, transferrin, cortisone, somatostatin, and glycyl-L-histidyl-L-lysine acetate). They were grown at 37°C in a humidified atmosphere of 5% CO$_2$, 95% air; the culture medium was changed every 4 days. KiKi cells are derived from FRTL5 cells infected by kiMSV-kiMuLV. They express high levels of rat $A_2$ adenylyl cyclase under conditions described previously (21). Briefly, FRTL5 were seeded in 96-well plates at a density of 5-10 $\times$ 10$^4$ cells/well in growth medium. After 3 days the cells were refed with culture medium deprived of serum and hormones and containing 0.3% bovine serum albumin (BSA). After a 45-h starvation, stimuliants were added for an additional 45 h, and a pulse of [3H]-thymidine (1 $\times$ 10$^6$ cpm/well) was added. The reaction was stopped by washing twice with Hank's balanced salt solution (HBSS). The [3H]-thymidine incorporation into trichloroacetic acid-insoluble material was evaluated as described previously (13).

Thymidine Uptake—DNA synthesis was evaluated by measuring [3H]-thymidine uptake as described with minor modifications (21). Briefly, FRTL5 were seeded in 96-well plates at a density of 5-10 $\times$ 10$^4$ cells/well in growth medium. After 3 days the cells were refed with culture medium deprived of serum and hormones and containing 0.3% bovine serum albumin (BSA). After a 48-h starvation, stimuliants were added for an additional 48 h, and a pulse of [3H]-thymidine (1 $\times$ 10$^6$ cpm/well) was added. The reaction was stopped by washing twice with Hank's balanced salt solution (HBSS). The [3H]-thymidine incorporation into trichloroacetic acid-insoluble material was evaluated as described previously (15).

cAMP Assay—The intracellular cAMP content of FRTL5 cells was measured by a method previously reported (22). Briefly, FRTL5 were grown in Petri dishes to 70% confluency, then they were shifted for 48 h to Coon's modified Ham's F-12 medium containing 0.3% BSA and 20 mM glutamine. Cells were washed twice with HBSS, and incubations were continued at 37°C in HBSS containing 0.4% BSA, 10 mM HEPES, and 0.5 mM 3-isobutyl-1-methylxanthine. The extracellular medium contained 10 mM MgCl$_2$ and 20 mM EGTA, collected and homogenized in a glass homogenizer. Cell homogenate was centrifuged for 15 min at 600 $\times$ g to pellet unbroken cells and nuclei from the crude membrane preparation; the supernatant was then centrifuged for 40 min at 25,000 $\times$ g. The pellet was resuspended in HBSS and buffered with 20 mM HEPES pH 7.6. Protein concentration was determined by a modification of the Lowry procedure (24).

Adenylylcyclase Assay—Adenylylcyclase activity in cell membranes was determined according to Salomon (25). 40 $\mu$g of membrane protein/sample were used in a final volume of 50 $\mu$l of 25 mM Tris chloride (pH 7.5); the reaction was carried out for 30 min at 37°C. AMP is a mixture of 20 mM NaF, 50 $\mu$M AIC$_3$, and 5 mM MgCl$_2$. Adenylylcyclase activity was calculated from duplicate samples as the mean of [3H]-cAMP/min/mg of protein.

Steady-state Iodide Content—Iodide content in FRTL5 cells was evaluated as described previously (11, 22). Cells were grown in Petri dishes to 70% confluency, then starved from thyrotropin for 4 days. The different compounds were then added and incubation carried on for 72 h. The steady-state iodide content was evaluated by incubating FRTL5 cells with 0.5 ml of HBSS buffered with 10 mM HEPES pH 7.36 containing 0.1 M NaI and 10 $\mu$M NaI for 40 min (11, 22).

ADP-ribosylation Assay—ADP-ribosyltransferase activity was measured by following the incorporation of [3H]ADP-ribose into membrane components (26). Cells were incubated in phosphate buffer (pH 7.5) and activated by dilution with an equal volume of 40 mM dithiothreitol for 10 min at 30°C. The samples were analyzed on 8% SDS-polyacrylamide, 4 $\mu$m gels (27). Autoradiography was performed using Kodak X-R5 films. The density of the radiolabeled bands was measured by an LKB Ultrascan XL densitometer equipped with an internal integrator.

Extraction and HPLC Analysis of [PH]Inositol Derivatives—Cells were labeled in Medium 199 containing [3H]-myo-inositol (2.5 $\mu$Ci/ml); the cell extract was obtained as previously described (28). Briefly, cells were washed three times with HBSS and preincubated for 15 min in 1 ml of HBSS containing 10 mM HEPES and 10 mM LiCl (pH 7.4) at 37°C. An extraction of the membrane lipid was performed at this point using methanol/chloroform/water (28). After 1 h at room temperature with occasional stirring the aqueous and organic phases were separated by centrifugation at 600 $\times$ g for 15 min. The aqueous phase was lyophilized and stored at -80°C.

Separation of [3H]inositol-labeled compounds by HPLC was done by a modification of the method described by Morgan and Catt (29) with a Partisil 10 SAX (4.6 mm $\times$ 25 cm) analytical column (Whatman) using a 0.1 M ammonium phosphate gradient (pH 3.35). The elution was as follows: H$_2$O at 1 ml/min flow for the first 5 min, followed by a linear gradient of 30-55% ammonium phosphate (55-55 min) to resolve GroPIns and InsPs separation, then a gradient of 30 min from 55-155 min (55-155 min) for the separation of other inositol derivatives. Fractions were collected at 30-60 s intervals and evaluated by liquid scintillation counting; alternatively, radioactivity in the eluates was monitored with an on-line radioactivity flow detector (Packard FLO ONE A-525). The data obtained by the two methods were virtually identical. Commercial [3H]-labeled standards (Du Pont-New England Nuclear) included inositol 1-monophosphate (Ins-1-P), inositol 4-monophosphate (Ins-4-P), inositol 1,4,5-trisphosphate (Ins-1,4,5-P$_3$), and inositol 1,4,5,6-tetrakisphosphate (Ins-1,4,5,6-P$_4$). Glycerophosphoinositol-4-phosphate was used to produce the corresponding glycerophosphoinositols (GroPIns, GroPIns-4-P, and GroPIns-4,5-P). Methylamine reagent (1 ml) (40% aqueous methylamine/H$_2$O/n-butyl alcohol/methanol, 36:8:9:47, v/v) was added to the phosphoinositide standards and incubated at 37°C for 45 min. The mixture was dried and added with 1 ml of a mixture of n-butyl alcohol/petroleum ether/ethyl formate (42:1:4, v/v). Glycerophosphoinositides were extracted twice with 1 ml of H$_2$O.

Some batches of the commercial GroPIns-4-P (Boehringer Mannheim) required further purification. This was done using a Partisil 10 SAX (4.6 mm $\times$ 25 cm) analytical column. The fractions eluted from the column were dried under speed-vacuum, resuspended in water, and pH-adjusted to 7.6. The recovery of GroPIns-4-P from the column, calculated from the radiolabeled standard elution, was ~70%. The eluted compound was used only in the adenylylcyclase assay.

Ins-1,4,5-P$_3$ levels were also determined by a commercial radioimmunoassay. Briefly, cells grown in Petri dishes to 70% confluency were detached adding 0.3 M EGTA, washed once with HBSS, and aliquoted as 2.5 $\times$ 10$^5$ cells/sample. The cell suspension was incubated at 37°C in HBSS containing 10 mM LiCl and 10 mM HEPES pH 7.3 for 40 min. The cell suspension was then added with 1 ml of ice-cold HBSS, pelleted, and used in the radioimmunoassay. GroPIns-4-P Incorporation—FRTL5 cells were grown to confluence (12-well plates) followed by addition of 500 $\mu$l of HBSS containing 0.4% BSA, 10 mM HEPES pH 7.3, 50 $\mu$M GroPIns-4-P, and [3H]GroPIns-4-P (33,000 cpm/sample). Incubation was stopped at the indicated time by washing the cells once with cold HBSS and adding 500 $\mu$l of ethanol for 10 min. The samples were collected, dried under a stream of nitrogen, reconstituted in 500 $\mu$l of HBSS, and the associated radioactivity was detected by liquid scintillation counting.

The concentration of GroPins-4-P was evaluated by analyzing the cell extract by HPLC as described above. After 90 min of incubation (the time used in most experiments reported), [3H]GroPIns-4-P represented ~10% of the total [3H]-lipids. The intracellular concentration of GroPIns-4-P was then determined by subjecting the cell samples to a volume of 4 ml that, as measured by Coulter Counter, corresponded to a volume of 4 ml. For 20 samples, the final concentration was 400 $\mu$l, and a good agreement with data from equilibrium uptake of 3-O-methyl-d-[3H]glucose (31). The intracellular concentration of GroPIns-4-P was 80 $\mu$M.
RESULTS AND DISCUSSION

Glycerophosphoinositols in ras-transformed (KiKi) and Normal (FRTL5) Thyroid Cells—The expression of oncopgenic ras-p21 in rat thyroid cells (KiKi) correlates with an increase in phospholipase A2 activity as measured by arachidonic acid and GroPIns release (5). Fig. 1 describes the presence and relative abundance of glycerophosphoinositols in normal and ras-transformed thyroid cells. Detectable amounts of GroPIns and GroPIns-4-P were found in ras-transformed cells (Fig. 1A); in normal FRTL5 cells, the levels of GroPIns were markedly lower, and the phosphorylated forms were undetectable (Fig. 1B). A summary of several HPLC elution patterns is reported in Table I, which shows that the phospholipase C metabolites, inositol monophosphate (InsP), and bisphosphate (InsP2) were also elevated in transformed cells (albeit much less than the GroPIns), whereas the inositol trisphosphate (InsP3) levels as previously reported (5) were not significantly changed.

Effect of Glycerophosphoinositols on cAMP Generation—FRTL5 cells are more suitable than KiKi cells for these studies because they have undetectable endogenous levels of GroPIns-4-P. In FRTL5 cells the regulation of cellular growth and differentiation is under the control of adenylylcyclase (10-14). The effects of GroPIns, GroPIns-4-P, and glycerophosphoinositol 4,5-bisphosphate (GroPIns-4,5-P) were first evaluated on the adenylylcyclase activity in isolated membranes treated with a mixture of aluminum, magnesium, and fluoride (AMF; see “Materials and Methods”). AMF is an activator of adenylylcyclase that acts via the stimulatory subunit with the enzyme (32). The AMF stimulation of adenylylcyclase was inhibited by ~48% in the presence of AMF-stimulated adenylylcyclase activity. Data in panel A are expressed as percent of the GroPIns-4-P inhibition is statistically significant (p < 0.02). The figures present the data best fitted curve of GroPIns-4-P on the CT-induced increase in cAMP levels in FRTL5 cells. Data in panel B are expressed as percent of the GroPIns-4-P effect on the CTA-induced increase in cAMP levels and are the mean ± S.E. of four experiments performed in triplicate. Basal adenylylcyclase activity was 1.9 ± 0.2 pmol/min/mg of protein and increased ~6-fold in the presence of AMF (20 mM NaF, 50 mM AlCl₃, and 5 mM MgCl₂). Panel B, dose-response of the effect of GroPIns-4-P on the CT-induced increase in cAMP levels in FRTL5 cells. Data in panel B are expressed as percent of the GroPIns-4-P effect on the CTA-induced increase in cAMP levels and are the mean ± S.E. of four experiments performed in triplicate. Basal adenylylcyclase activity was 1.9 ± 0.2 pmol/min/mg of protein and increased ~6-fold in the presence of AMF (20 mM NaF, 50 mM AlCl₃, and 5 mM MgCl₂). Panel B, dose-response of the effect of GroPIns-4-P on the CT-induced increase in cAMP levels in FRTL5 cells. Data in panel B are expressed as percent of the GroPIns-4-P effect on the CTA-induced increase in cAMP levels and are the mean ± S.E. of four experiments performed in triplicate. Basal adenylylcyclase activity was 1.9 ± 0.2 pmol/min/mg of protein and increased ~6-fold in the presence of AMF (20 mM NaF, 50 mM AlCl₃, and 5 mM MgCl₂).
tive at up to 400 \( \mu M \), whereas GroPlns induced an apparent inhibition (20%) at concentrations from 100 to 400 \( \mu M \) (Fig. 2A). Similar effects of GroPlns-4-P were observed in KiKi cell membranes (data not shown). As GroPlns-4-P can be taken up by intact cells (see below), the effects of the three glycerophosphoinositols were evaluated on the thyrotropin and cholinergic toxin (CT)-induced increase in cAMP levels in intact FRTL5 monolayers (Fig. 3). CT, which is known to stimulate the adenylylcyclase by ADP-ribosylating the G\(_{\beta}\) protein coupled to the enzyme (23, 33), increased 30-fold the basal cAMP levels. GroPlns-4-P (50 \( \mu M \)) reduced this stimulation by \( \sim 67\% \) (Fig. 3A). At the same concentration, GroPlns and GroPlns-4,5-P were ineffective (Fig. 3A). The GroPlns-4-P IC\(_{50}\) in this series of experiments was \( \sim 14 \) \( \mu M \) (Fig. 2B). Thyrotropin, the main hormonal activator of adenylylcyclase in thyroid cells, also increased the cAMP levels by 50-fold, and this increase was inhibited, albeit to a lesser extent than that induced by CT (\( \sim 20\% \)) in the presence of 50 \( \mu M \) GroPlns-4-P. Both GroPlns and GroPlns-4,5-P (at up to 100 \( \mu M \)) were ineffective in thyrotropin-stimulated cells (Fig. 3B). Interestingly the basal level of intracellular cAMP was not affected by any of the three glycerophosphoinositols (data not shown). As both thyrotropin and CT act through G\(_{\beta}\), these results are compatible with the possibility that the G\(_{\beta}\)-protein might be the site of action of GroPlns-4-P. In line with this possibility, 100 \( \mu M \) forskolin, a compound that directly acts on the enzyme catalytic subunit and increases the cAMP levels in FRTL5 cells by up to 15-fold, was not inhibited by GroPlns-4-P (data not shown).

If the action of GroPlns-4-P is exerted on G\(_{\beta}\), the ADP-ribosylation of this protein by CT might be affected by GroPlns-4-P. Indeed, at concentrations ranging from 10 to 200 \( \mu M \) GroPlns-4-P significantly inhibited the ADP-ribosylation of the G\(_{\beta}\)-protein by CT in FRTL5, KiKi, and AtT20 (a pituitary line, see below) cell membrane preparations (Fig. 4, A and B).

As mentioned above, GroPlns-4-P is active not only on membranes but also on intact cells. Further, we examined the possibility that GroPlns-4-P could penetrate living cells, and this was achieved by using \([H]GroPlns-4-P\), as described under “Materials and Methods.” GroPlns-4-P, added to the extracellular medium at a 50 \( \mu M \) concentration, reached an intracellular concentration of \( \sim 80 \) \( \mu M \) in different experi-

**FIG. 3.** Effect of GroPlns, GroPlns-4-P, and GroPlns-4,5-P at a 50 \( \mu M \) concentration on CT- (panel A) and thyrotropin-(panel B) induced increase in cAMP levels. FRTL5 cells were preincubated for 60 min with the different compounds followed by thyrotropin or CT, both at a 10 nM concentration for 30 min. Basal cAMP levels were 1.1 \( \pm 0.4 \) pmol/ml and are the mean \( \pm S.E. \) of 17 triplicate determinations. Data are expressed as percent of stimulation over the basal and are the mean \( \pm S.E. \) of 7-17 experiments performed in triplicate. See “Materials and Methods” for further details. *, Significantly different from CT- (panel A) and thyrotropin-induced (panel B) stimulation, \( p < 0.01 \).

**FIG. 4.** Panel A, dose-response of the effect of GroPlns-4-P on the CT-dependent \([32P]ADF-ribosylation\) of membrane proteins from FRTL5 cells. The experiment shown is representative of four performed in duplicate. Panel B, effect of 50 \( \mu M \) GroPlns-4-P on the CT-dependent \([32P]ADF-ribosylation\) on membrane proteins from AtT20 and KiKi cells. The experiment shown is representative of 3-5 performed in duplicate. Samples were analyzed by SDS-polycrylamide gel electrophoresis and autoradiography. Only the region of 45 kDa is shown. The apparent molecular masses are indicated. The densitometric analysis of the experiments presented is also reported. The absorbance of the different bands is presented as percent of control (lane 1 in panel A).

**FIG. 5.** Dose-response of the GroPlns-4-P inhibition on the CT-induced cAMP levels in smooth muscle cells. Data are expressed as percent of stimulation over basal and are the mean \( \pm S.E. \) of six experiments performed in triplicate. Basal cAMP levels were 17.5 \( \pm 6.8 \) pmol/ml and are the mean \( \pm S.E. \) of six triplicate determinations. See Fig. 3 for further details. *, Significantly different from control (i.e. CT-induced stimulation), \( p < 0.02 \).
the intracellular concentrations of \( \text{Ins-1,4,5-P}_3 \), which can be activities of all inositol derivatives are similar; then the concentration over basal and are the mean ± S.E. of three experiments performed in triplicate. *, Significantly different from control, \( p < 0.05 \). Panel B, effect of GroPIns-4-P on CT-stimulated thymidine uptake. CT and GroPIns-4-P were 10 nM and 50 \( \mu M \), respectively. Basal thymidine uptake was 1140 ± 169 cpm/well. Data are expressed as percent of stimulation over basal and are the mean ± S.E. of 17 experiments performed in triplicate. See "Materials and Methods" for further details. *, Significantly different from control, \( p < 0.01 \).

**Effect of GroPIns-4-P on Adenylylcyclase in Other Cell Types**—The effect of GroPIns-4-P on adenylylcyclase activity was examined also in unrelated cell types to verify whether it might be general or thyroid-specific. GroPIns-4-P inhibited adenylylcyclase in smooth muscle cells stimulated by 10 nM CT (Fig. 5). The \( IC_{50} \) was 10 \( \mu M \), and the maximal inhibition was nearly 80%. A partial inhibition (−20%) was also observed when smooth muscle cells were stimulated by 10 \( \mu M \) isoprenaline (data not shown). Similar effects were also observed in pituitary cells (AtT20) and in pneumocytes (A549) (data not shown). These data indicate that the effect of GroPIns-4-P is not restricted to the thyroid system and that this compound could be a general regulator of adenylylcyclase.

**Effect of GroPIns-4-P on cAMP-dependent Functions in FRTL5 Cells**—We also investigated whether the cAMP-dependent functions of thyroid cells might be affected by GroPIns-4-P. Iodide transport in the thyroid is modulated by cAMP as well as by other second messengers (10-14, 22, 36-40). Indeed, the thyrotropin-induced increase in iodide uptake (15-fold at 10 nM) was inhibited by 50 \( \mu M \) GroPIns-4-P by ~45% (a dose-response of the inhibitory effect is shown in Fig. 6A). GroPIns-4-P also affected the iodide uptake stimulated by CT; the inhibition in this case was ~38% (Fig. 6A). In addition, GroPIns-4-P inhibited the CT-induced thymidine uptake (−60%, Fig. 6B) and by ~20% the thyrotropin-stimulated thymidine uptake, an effect that is largely dependent on cAMP in FRTL5 cells (10, 12, 13) (data not shown). GroPins and GroPIns-4,5-P were inactive in all of the above assays (data not shown). It is not clear why the CT- and thyrotropin-induced iodide uptake are inhibited to the same extent by GroPIns-4-P, while the adenylylcyclase stimulation by CT is reduced by GroPIns-4-P more effectively than that caused by thyrotropin. The reason for this apparent discrepancy might be due to the complexity of the signal cascades initiated by thyrotropin (10-14, 37, 40).

**Concluding Remarks**—Taken together, these data suggest that the activation of phospholipase A2 can produce a phosphoinositide metabolite, GroPIns-4-P, that is able to modulate the activity of adenylylcyclase. In thyroid cells this cross-talk mechanism could play a role in the pathways modulating differentiation and/or growth (see above).

Examples of cross-talk have been previously reported in FRTL5 cells. Thyrotropin via the cAMP cascade regulates the expression of adrenergic receptors, which in turn activate phospholipase C and phospholipase A2, enzymes important in the regulation of thyroid hormone formation (41, 42). Other examples of cross-talk between second messenger cascades have been reported (for reviews see Refs. 43 and 44). GroPIns-
4-P might therefore represent a novel regulatory mechanism of CAMP-dependent processes, which might be initiated by receptor activation of phospholipase A2.

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