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Glycerophosphoinositol 4-Phosphate, a Putative Endogenous Inhibitor of Adenylylcyclase*

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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 phosphoinositide-specific phospholipase A_2, 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 adenylylcyclase. 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 fluoroaluminate. 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 thymidine 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 adenylylcyclase and phospholipase A_2 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 transducing enzymes have been investigated extensively. There is good evidence for the involvement of the product of the RAS gene in the regulation of adenylylcyclase in yeast (1). By contrast, in mammalian cells no direct link between *ras*-p21 and adenylylcyclase has been found, whereas there is increasing evidence for an involvement of *ras*-p21 in the regulation of phospholipid metabolism (3, 4).

Recently, we have shown that the expression of oncogenic *ras*-p21 correlates with an increased phospholipase A_2 basal activity (as measured by arachidonic acid release) in KiKi cells, a continuous line derived from FRTL5 cells (a differentiated normal rat thyroid cell line) by transformation with the k-*ras* oncogene (5). In these cells, the levels of a metabolite deriving from the sequential activity of phospholipase A_2 and phospholipase A_1 on membrane phosphoinositides, glycerophosphoinositol (GroPIns),[1] are 5–10-fold higher than in normal FRTL5 cells (5). Similar results are observed in a temperature-sensitive clone of rat thyroid cells under permissive conditions (33 °C) for the expression of oncogenic *ras*-p21 (5). A similar increase in GroPIns has been reported in NIH3T3 fibroblasts transformed by *ras* and other cytosolic oncogenes (6, 7). A correlation between *ras* transformation and increased phospholipase A_2 activity has been hypothesized in several studies (5–8). Bar-Sagi and Feramisco (8) demonstrated an increase in lysophosphatidylcholine and lysophosphatidylethanolamine 30 min after microinjection of *ras*-p21, suggesting that *ras*-p21 might stimulate phospholipase A_2. High levels of glycerophosphocholine in *ras*-transfected mouse fibroblasts also led to the hypothesis of an increased activity of this enzyme (9).

In this study we have investigated the biological activities of glycerophosphoinositols. We found two forms of glycerophosphoinositols in KiKi cells, the GroPIns and the glycerophosphoinositol 4-phosphate (GroPIns-4-P). GroPIns-4-P, at concentrations similar to those found in KiKi cells, inhibits the stimulation of adenylylcyclase both in membrane preparations and in whole FRTL5 cells. As most of the thyroid-differentiated functions in FRTL5 cells are modulated by cAMP (10–14), these data suggest that glycerophosphoinositols levels might be functionally important in these cells. In addition, GroPIns-4-P inhibited the stimulation of adenylylcyclase activity in other cell lines, suggesting that this compound might act as an endogenous modulator of adenylylcy-

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[1] The abbreviations used are: GroPIns, glycerophosphoinositol; Ins, inositol; InsP, inositol monophosphate; $InsP_2$, inositol bisphosphate; $InsP_3$, inositol trisphosphate; GroPIns-4-P, glycerophosphoinositol 4-phosphate; GroPIns-4,5-P, glycerophosphoinositol 4,5-bisphosphate; CT, cholera toxin; BSA, bovine serum albumin; Ins-1-P, inositol 1-monophosphate; Ins-4-P, inositol 4-monophosphate; Ins-1,4-P_2, inositol 1,4-bisphosphate; Ins-1,4,5-P_3, inositol 1,4,5-trisphosphate; HBSS, Hanks' balanced salt solution; HPLC, high pressure liquid chromatography.

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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 glycerophosphoinositol were from Sigma. Tissue culture materials were from Gibco. Glycerophosphoinositol 4-phosphate and glycerophosphoinositol 4,5-bisphosphate were from Boehringer Mannheim. Cholera toxin was from Calbiochem, and sodium fluoride and aluminium chloride were from Fluka Chem. Corp. cAMP radioimmunoassays, [α-^{32}P]ATP, [^{3}H]cAMP, [^{3}H]thymidine, and [^{3}H]*myo*-inositol were purchased from Du Pont-New England Nuclear. [^{3}H]$InsP_3$ 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, cortisol, somatostatin, and glycyl-L-histidyl-L-lysine acetate). They were grown at 37 °C in a humified 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 *ras*-p21, lose the thyroid-differentiated functions, and grow in a hormone-independent manner. They are routinely cultured in the same medium of the FRTL5 cells and deprived of the hormone mixture (17–19). Smooth muscle cells were kindly provided by A. De Blasi and cultured as previously described (20).

Thymidine Uptake—DNA synthesis was evaluated by measuring [^{3}H]thymidine uptake as described with minor modifications (21). Briefly, FRTL5 were seeded in 96-well plates at a density of 8–10 × 10^3 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, stimulants were added for an additional 48 h, and a pulse of [^{3}H]thymidine (1 μCi/well) was given 16 h before stopping the reaction by washing twice with Hanks' balanced salt solution (HBSS). The [^{3}H]thymidine incorporation into trichloroacetic acid-insoluble material was evaluated as described previously (13).

cAMP Assay—The intracellular cAMP content of FRTL5 cells was measured by a method previously reported (22). Briefly, FRTL5 cells were grown in 96-well plates to 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 experiments shown refer to cells preincubated with different glycerophosphoinositols for 1 h; thyrotropin and other substances were added for an additional 30 min. Different times of preincubation (0–120 min) were also evaluated. For preincubation ≥10 min, data were not different from those reported. The intracellular cAMP content was measured using a commercial radioimmunoassay. Results are expressed as picomoles of cAMP/ml. Experiments were performed in triplicate.

Membrane Preparation—FRTL5 cell membranes were prepared as previously described (23). Briefly, cells were washed once with HBSS, followed by addition of 5 mM Tris chloride (pH 8) containing 0.5 mM $MgCl_2$ and 0.1 mM EGTA, collected and homogenized in a Teflon glass homogenizer. Cell homogenate was centrifuged for 10 min at 600 × *g* to pellet unbroken cells and nuclei from the crude membrane preparation; the supernatant was then centrifuged for 40 min at 25,000 × *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 μg of membrane protein/sample were used in a final volume of 50 μl of 25 mM Tris chloride (pH 7.3); the reaction was carried on for 30 min at 37 °C. AMF is a mixture of 20 mM NaF, 50 μM $AlCl_3$, and 5 mM $MgCl_2$. Adenylylcyclase activity was calculated from duplicate samples as the mean of [α-^{32}P]cAMP/min/mg of protein.

Steady-state Iodide Content—Iodide content in FRTL5 cells was evaluated as previously described (11, 22). Cells were grown in 24-well plates 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 μCi of carrier-free Na^{125}I and 10 μM NaI for 40 min (11, 22).

ADP-ribosylation Assay—ADP-ribosyltransferase activity was measured by following the incorporation of [^{32}P]ADP-ribose into membrane components as described (26). Cholera toxin (1 mg/ml) was dialyzed 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 M urea gels (27). Autoradiography was performed using Kodak X-R5 films. The density of the radiolabeled bands was measured by an LKB Ultroscan-XL densitometer equipped with an internal integrator.

Extraction and HPLC Analysis of [^{3}H]Inositol Derivatives—Cells were labeled in Medium 199 containing [^{3}H]*myo*-inositol (2.5 μC/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 × *g* for 15 min. The aqueous phase was lyophilized and stored at −80 °C.

Separation of [^{3}H]inositol-labeled compounds by HPLC analysis was done by a modification of the method described by Morgan and Catt (29) with a Partisil 10 SAX (4.6 mm × 25 cm) analytical column (Whatman) using a 0–1 M ammonium phosphate gradient (pH 3.35). The elution was as follows: H_2O at 1 ml/min flow for the first 5 min, followed by a linear gradient of 0–30 mM ammonium phosphate (5–55 min) to resolve GroPIns and InsPs separation, then a gradient of 30 mM–1 M (55–115 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 eluate 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 ^{3}H-labeled standards (Du Pont-New England Nuclear) included inositol 1-monophosphate (Ins-1-P), inositol 4-monophosphate (Ins-4-P), inositol 1,4-bisphosphate (Ins-1,4-P_2), and inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3). Alkaline hydrolysis in monomethylamine (30) of inositol-labeled phosphoinositide standards was used to produce the corresponding glycerophosphoinositols (GroPIns, GroPIns-4-P, and GroPIns-4,5-P). Methylamine reagent (1 ml) (40% aqueous methylamine/H_2O/*n*-butyl alcohol/methanol, 36:8:9:47, v/v) was added to the phosphoinositide standards and incubated at 50 °C for 45 min. The mixture was dried and added with 1 ml of a mixture of *n*-butyl alcohol/petroleum ether/ethyl formate (20:4:1, v/v). Glycerophosphoinositides were extracted twice with 1 ml of H_2O.

Some batches of the commercial GroPIns-4-P (Boehringer Mannheim) required further purification. This was done using a Partisil 10 SAX (4.6 mm × 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 purified 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 × 10^6 cells/sample. The cell suspension was incubated at 37 °C in HBSS containing 10 mM LiCl and 10 mM HEPES pH 7.3 for 45 min. Samples were then added with 1 ml of ice-cold HBSS, pelleted, and used in the radioimmunoassay.

GroPIns-4-P Incorporation—FRTL5 cells were grown to confluency (12-well plates) followed by addition of 500 μl of HBSS containing 0.4% BSA, 1 mM HEPES pH 7.3, 50 μM GroPIns-4-P, and [^{3}H]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 μl of ethanol for 10 min. The samples were collected, dried under a stream of nitrogen, reconstituted in 500 μl of HBSS, and the associated radioactivity was detected by liquid scintillation counting. The amount of [^{3}H]GroPIns-4-P in the ^{3}H-lipids recovered was evaluated by analyzing the cell extract by HPLC as described above. After 90 min of incubation (the time used in most experiments reported), [^{3}H]GroPIns-4-P represented ~10% of the total ^{3}H-lipids. The intracellular concentration of GroPIns-4-P was then determined considering that 1 × 10^6 cells correspond to a volume of 1.9 μl, as measured by Coulter Channelyzer 256; this volume is in good agreement with data from equilibrium uptake of 3-*O*-methyl-D-[^{3}H]glucose (31). The intracellular concentration of GroPIns-4-P was ~80 μM

after 90 min of incubation with extracellular GroPIns-4-P (50 μM).

Statistical Analysis—All experiments are presented as the average of duplicate or triplicate determinations repeated at least three times. Statistical analysis was carried out either by Student's *t*, Dunnett ANOVA, or Mann-Whitney test.

RESULTS AND DISCUSSION

Glycerophosphoinositols in ras-transformed (KiKi) and Normal (FRTL5) Thyroid Cells—The expression of oncogenic *ras*-p21 in rat thyroid cells (KiKi) correlates with an increase in phospholipase A_2 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. 1*A*); in normal FRTL5 cells, the levels of GroPIns were markedly lower, and the phosphorylated forms were undetectable (Fig. 1*B*) (5). A summary of several HPLC elution patterns is reported in Table I, which shows that the phospholipase C metabolites, inositol monophosphate (InsP), and bisphosphate ($InsP_2$) were also elevated in transformed cells (albeit much less than the GroPIns), whereas the inositol trisphosphate ($InsP_3$) 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 aluminium, magnesium, and fluoride (AMF, see "Materials and Methods"). AMF is an activator of adenylylcyclase that acts via the stimulatory protein G_s (32); it mimicks the γ-phosphate of the guanine nucleotide thereby promoting the dissociation of G-protein $\beta\gamma$ subunits and allowing the stimulatory interaction of the α subunit with the enzyme (32). The AMF stimulation of adenylylcyclase was inhibited by ~48% in the presence of 50 μM GroPIns-4-P. A dose-response of the effects of GroPIns, GroPIns-4-P, and GroPIns-4,5-P is shown in Fig. 2*A*. The IC_{50} of GroPIns-4-P was ~30 μM. GroPIns-4,5-P was ineffec-

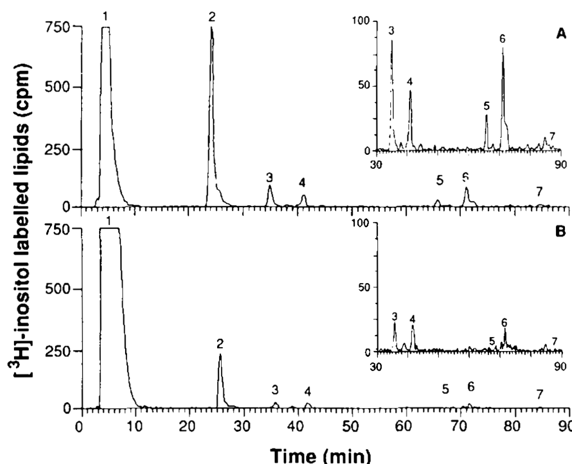

FIG. 1. **HPLC elution patterns of water-soluble [³H]inositol-labeled metabolites of *ras*-transformed KiKi (*panel A*) and normal FRTL5 (*panel B*) cell extracts.** Labeling and extraction procedures are detailed under "Materials and Methods." *Numbers* indicate the elution positions of the different metabolites as identified by coelution with commercially available standards (see "Materials and Methods"). *1*, Ins; *2*, GroPIns; *3*, Ins-1-P; *4*, Ins-4-P; *5*, GroPIns-4-P; *6*, Ins-1,4-P; *7*, Ins-1,4,5-P. The elution was analyzed by an on-line flow detector (see "Materials and Methods"). The areas of the peaks (expressed in counts/min) in this elution were as follows: *1*, 23830 (KiKi) and 303448 (FRTL5); *2*, 2785 (KiKi) and 972 (FRTL5); *3*, 367 (KiKi) and 95 (FRTL5); *4*, 159 (KiKi) and 94 (FRTL5); *5*, 91 (KiKi) and ND (FRTL5); *6*, 177 (KiKi) and 101 (FRTL5); *7*, 45 (KiKi) and 49 (FRTL5). The *inset* shows a magnified area of the elution pattern between 30 and 90 min.

TABLE I

Levels of [³H]inositol-labeled lipids in normal (FRTL5) and ras-transformed thyroid cells (KiKi)

	³H-Labeled[a]	
	FRTL5 cells	KiKi cells
	cpm/well	
GroPIns	1006 ± 144	2953 ± 206[b]
Ins-1-P	150 ± 27	356 ± 53[b]
Ins-4-P	155 ± 38	213 ± 32
GroPIns-4-P	ND[c]	111 ± 22[b]
$Ins-1,4-P_2$	83 ± 44	316 ± 35[b]
$Ins-1,4,5-P_3$	38 ± 10	38 ± 5

[a] HPLC analysis was performed as described under "Materials and Methods." The data represent counts/min after background subtraction and are the mean ± S.E. of 14 (FRTL5) and 18 (KiKi) independent elution patterns. Each sample was counted for 10 min to minimize the counting error. Each experiment was performed in duplicate. Alternatively, elutions were evaluated by an on-line flow detector (see "Materials and Methods"). The levels of GroPIns-4,5-P are not included in the table since this compound was detected only in eight cases in KiKi cells but never in FRTL5 cells.

[b] $p < 0.05$ by the Mann-Whitney test.

[c] Not detectable.

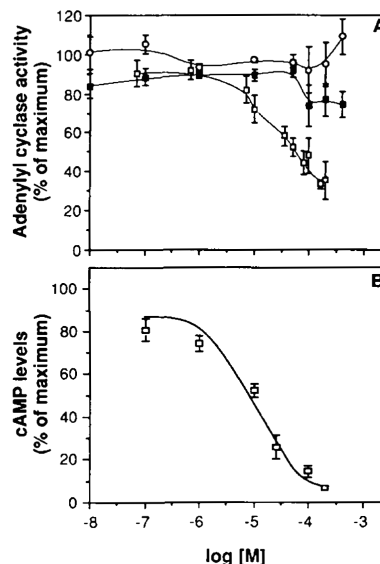

FIG. 2. *Panel A*, dose-response of the effect of GroPIns-4-P (*open square*), GroPIns (*filled square*), and GroPIns-4,5-P (*circle*) on AMF-stimulated adenylylcyclase activity. Data in *panel A* are expressed as percent of the lipid effect on the AMF-stimulated adenylylcyclase activity and are the mean ± S.E. of 3–14 experiments performed in duplicate. 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 μM $AlCl_3$, and 5 mM $MgCl_2$). *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 CT-induced increase in cAMP levels and are the mean ± S.E. of four experiments performed in triplicate. Basal cAMP levels were 1.9 ± 0.2 pmol/ml and increased ~30-fold in the presence of 10 nM CT. The GroPIns-4-P inhibition is statistically significant at concentrations ≥10 μM in cells ($p < 0.01$) and ≥50 μM in cell membranes ($p < 0.02$). The figures present the data best fitted curve from which IC_{50} of 30 μM (*panel A*) and 14 μM (*panel B*) were obtained (GraphPAD Institute for Scientific Information, 1987).

tive at up to 400 μM, whereas GroPIns induced an apparent inhibition (20%) at concentrations from 100 to 400 μM (Fig. 2*A*). Similar effects of GroPIns-4-P were observed in KiKi cell membranes (data not shown). As GroPIns-4-P can be taken up by intact cells (see below), the effects of the three glycerophosphoinositols were evaluated on the thyrotropin- and cholera 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_s-protein coupled to the enzyme (23, 33), increased 30-fold the basal cAMP levels. GroPIns-4-P (50 μM) reduced this stimulation by ~67% (Fig. 3*A*). At the same concentration, GroPIns and GroPIns-4,5-P were inactive (Fig. 3*A*). The GroPIns-4-P IC_{50} in this series of experiments was ~14 μM (Fig. 2*B*). 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 (~20%) in the presence of 50 μM GroPIns-4-P. Both GroPIns and GroPIns-4,5-P (at up to 100 μM) were ineffective in thyrotropin-stimulated cells (Fig. 3*B*). 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_s, these results are compatible with the possibility that the G_s-protein might be the site of action of GroPIns-4-P. In line with this possibility 100 μM 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 GroPIns-4-P (data not shown).

If the action of GroPIns-4-P is exerted on G_s, the ADP-ribosylation of this protein by CT might be affected by GroPIns-4-P. Indeed, at concentrations ranging from 10 to 200 μM GroPIns-4-P significantly inhibited the ADP-ribosylation of the G_s-protein by CT in FRTL5, KiKi, and AtT20 (a pituitary line, see below) cell membrane preparations (Fig. 4, *A* and *B*).

As mentioned above, GroPIns-4-P is active not only on membranes but also on intact cells. Further, we examined the possibility that GroPIns-4-P could penetrate living cells, and this was achieved by using [^{3}H]GroPIns-4-P, as described under "Materials and Methods." GroPIns-4-P, added to the extracellular medium at a 50 μM concentration, reached an intracellular concentration of ~80 μM in different experi-

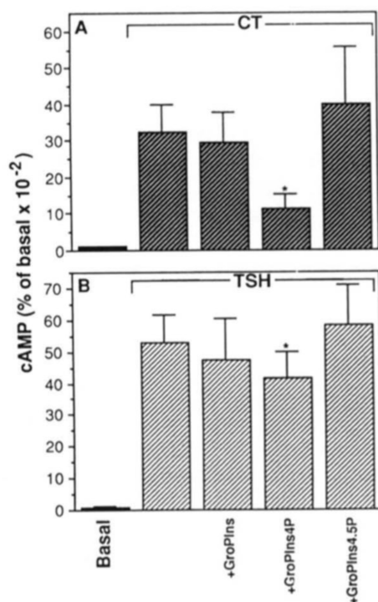

FIG. 3. **Effect of GroPIns, GroPIns-4-P, and GroPIns-4,5-P at a 50 μM concentration on CT- (*panel A*) and thyrotropin- (*panel B*) induced increase in cAMP levels.** FRTL5 cells were preincubated for 60 min with the different compounds followed by thyrotropin or CT, both at a 10 nM concentration for 30 min. Basal cAMP levels were 1.1 ± 0.4 pmol/ml and are the mean ± S.E. of 17 triplicate determinations. Data are expressed as percent of stimulation over the basal and are the mean ± 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, $p < 0.01$.

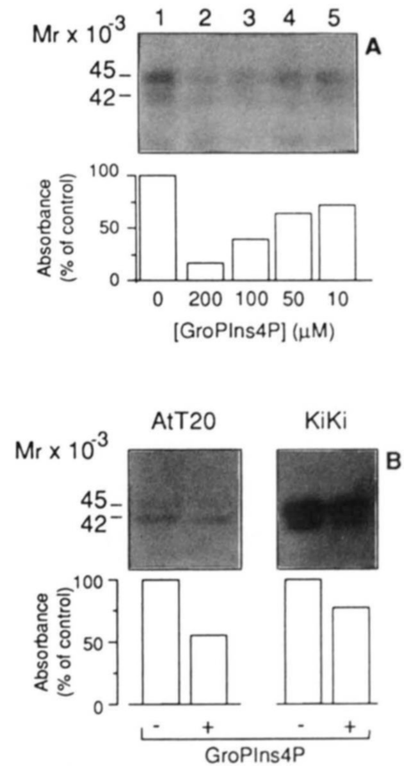

FIG. 4. *Panel A*, dose-response of the effect of GroPIns-4-P on the CT-dependent [^{32}P]ADP-ribosylation of membrane proteins from FRTL5 cells. The experiment shown is representative of four performed in duplicate. *Panel B*, effect of 50 μM GroPIns-4-P on the CT-dependent [^{32}P]ADP-ribosylation on membrane proteins from AtT20 and KiKi cells. The experiment shown is representative of 3–5 performed in duplicate. Samples were analyzed by SDS-polyacrylamide 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*).

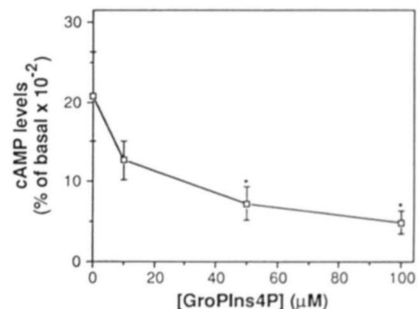

FIG. 5. **Dose-response of the GroPIns-4-P inhibition on the CT-induced cAMP levels in smooth muscle cells.** Data are expressed as percent of stimulation over basal and are the mean ± S.E. of six experiments performed in triplicate. Basal cAMP levels were 17.5 ± 6.8 pmol/ml and are the mean ± S.E. of six triplicate determinations. See Fig. 3 for further details. *, Significantly different from control (*i.e.* CT-induced stimulation), $p < 0.02$.

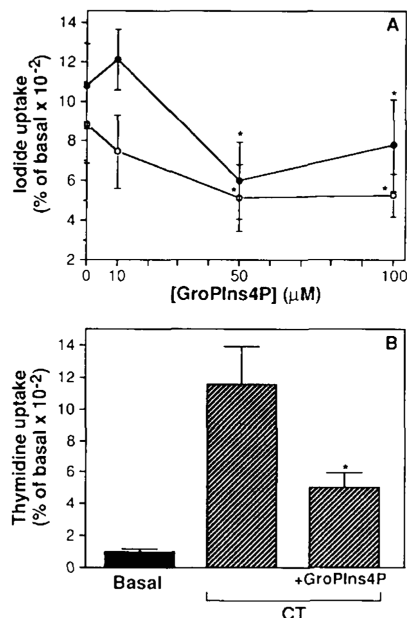

FIG. 6. **Effect of GroPIns-4-P on iodide uptake and thymidine incorporation in FRTL5 cells.** *Panel A*, dose-response of the effect of GroPIns-4-P on thyrotropin- (*open circles*) and CT- (*filled circles*) induced steady-state iodide content. Thyrotropin and CT were both at 10 nM. Basal iodide content was 189.6 ± 10.4 cpm/well (mean ± S.E.). Data are expressed as percent of stimulation over basal and are 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 μM, 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, $p < 0.01$.

ments after 90 min of incubation (the incubation time used in the cAMP assay, see above), a concentration similar to that which inhibited the adenylylcyclase in the membrane assay (see above).

Estimation of the GroPIns-4-P Intracellular Levels—These data demonstrate that GroPIns-4-P is generated in mammalian cells and that it has a partial but clear inhibitory effect on G_s-mediated stimulation of the adenylylcyclase. It was therefore of interest to determine whether the endogenous levels of this compound are similar to those found effective in our adenylylcyclase assays. Since no direct method to evaluate the mass of glycerophosphoinositols is presently available, we made the reasonable assumption that the inositol pool was labeled to equilibrium (5) and that the specific activities of all inositol derivatives are similar; then the concentrations of GroPIns and GroPIns-4-P can be deduced from the intracellular concentrations of Ins-1,4,5-P_3, which can be measured by a radioimmunoassay. Thus, the Ins-1,4,5-P_3 levels were 1.0 ± 0.4 pmol/10^6 in FRTL5 cells and 3.5 ± 1.5 pmol/10^6 in KiKi cells. Since the volume of FRTL5 cells was 1.9 μl/10^6 cells and that of KiKi cells was 5.7 μl/10^6 cells (see "Materials and Methods"), the concentration of Ins-1,4,5-P_3 is 0.5 ± 0.3 μM in FRTL5 cells and 0.7 ± 0.2 μM in KiKi cells; these concentrations are in good agreement with the values reported in other cell systems (34). As GroPIns and GroPIns-4-P levels were 80- and 3-fold those of $InsP_3$ (Table I), in KiKi cells the two glycerophosphoinositols can be estimated to be present intracellularly at ~56 and ~2.1 μM, respectively. This concentration of GroPIns-4-P, which induces a small inhibition on the adenylylcyclase activity (~20%) (Fig. 2), should be considered the lower limit of the actual intracellular levels due to some approximations used in the calculations. The measured volume of the cells includes nucleus, organelles, and endoplasmic reticulum, which is very developed in the thyroid; moreover, GroPIns-4-P has been considered homogeneously distributed in the whole cells whereas it could be present at higher concentrations in those compartments where it should exert its action. Thus, while more direct methods being developed in our laboratory will improve the precision of these measurements, the above data already indicate that the concentrations of GroPIns-4-P found in KiKi cells may reach those active in inhibiting adenylylcyclase. It could be speculated that GroPIns-4-P could play a role in modulating adenylylcyclase in KiKi cells. Indeed, we have previously reported that the adenylylcyclase of KiKi cells is less sensitive to CT stimulation (35). The generality of this phenomenon is presently being evaluated in our laboratory using cell lines of different origin transformed by the *ras* oncogenes.

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 μM, and the maximal inhibition was nearly 80%. A partial inhibition (~20%) was also observed when smooth muscle cells were stimulated by 10 μM isoproterenol (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 μM GroPIns-4-P by ~45% (a dose-response of the inhibitory effect is shown in Fig. 6*A*). GroPIns-4-P also affected the iodide uptake stimulated by CT; the inhibition in this case was ~38% (Fig. 6*A*). In addition, GroPIns-4-P inhibited the CT-induced thymidine uptake (~60%, Fig. 6*B*) 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 A_2 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 A_2, 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 A_2.

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