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Partial characterization of a thyrotropin releasing hormone-sensitive glycosyl-phosphatidylinositol in pituitary lactotrophes

Lourdes Benítez[a], Luisa F. Fanjul[a], C.M. Ruiz de Galarreta[a], J. Quintana Aguiar[a], J. González Reyes[a], Inmaculada Hernández[a], Pino Santana Delgado[a], J. Cabrera Oliva[a], R. Alonso Solís[b], F. Estévez Rosas[a,*]

[a]*Departamento de Endocrinología Celular y Molecular, Universidad de Las Palmas de Gran Canaria, Apdo. 550, Las Palmas 35080, Spain*
[b]*Departamento de Fisiología, Facultad de Medicina, Universidad de La Laguna, Tenerife 38320, Spain*

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Abstract

Metabolic labelling experiments performed with cultured pituitary lactotrophes revealed the presence of a glycosyl-phosphatidylinositol (GPtdIns) structurally related to GPtdIns lipids isolated from other cell types as demonstrated by: (i) metabolic incorporation of [^{3}H]galactose, [^{3}H]glucosamine and [^{3}H]inositol into the polar inositolphosphoglycan moiety (InsPG) and [^{3}H]myristate and [^{3}H]-palmitate into the diacylglycerol (DAG) backbone of GPtdIns; (ii) sensitivity of the [^{3}H]labelled GPtdIns to nitrous acid deamination and; (iii) sensitivity of GPtdIns to phosphatidylinositol (PtdIns)-specific phospholipase C (PLC) hydrolysis. In cultured pituitary cells labelled to isotopic steady state with 10 μCi/ml of [^{3}H]glucosamine, treatment with hypothalamic TRH (10^{-6} M) induced a rapid and transient hydrolysis (ca. 50%) of the labelled GPtdIns. Moreover, as demonstrated in [^{3}H]inositol labelled cells, treatment with thyrotropin releasing hormone (TRH) elicited the cleavage of [^{3}H]GPtdIns in a similar manner, and this effect was followed by the phosphoinositide (PtdIns, PtdInsP and PtdInsP$_2$) hydrolysis 30 s later. These results suggest that the phosphodiesterase cleavage of GPtdIns could be an early event implicated in TRH action in pituitary lactotrophes.

Keywords: Thyrotropin releasing hormone; Glycosyl-phosphatidylinositol; Phosphatidylinositols; Inositol phosphoglycan

It is generally accepted that a result of thyrotropin releasing hormone (TRH) receptor occupancy in pituitary lactotrophes involves the signal transduction pathway which entails phospholipase-C (PtdIns-PLC) activated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdInsP$_2$) followed by inositol-1,4,5-trisphosphate (InsP$_3$) stimulated burst in intracellular Ca^{2+} and second messenger DAG mediated protein kinase C (PKC) activation (for a review, see Ref. [1]). Recent evidence demonstrates that ligand-activated PtdInsP$_2$ hydrolysis is not the only source of second messenger DAG species in mammalian cells [2,3]. Moreover activation of membrane receptors for hormones or growth factors can activate additional phospholipases that promote the phosphodiesterase cleavage not only from phosphatidylcholine (PtdCho) or phosphatidylethanolamine (PtdEt), the major structural building blocks of the lipid bilayer [2,3] but also from minor membrane constituents such as glycosyl-phosphatidylinositol (GPtdIns) lipids (reviewed in Ref. [4]).

The initial identification of GPtdIns molecules as membrane anchors for covalently attached proteins [5] was followed by the demonstration that, upon interaction with its receptor, insulin stimulated the hydrolysis of GPtdIns and the generation of DAG, and a polar head group of the lipid, an inositol-phosphoglycan (InsPG) containing inositol, glucosamine, galactose, galactosamine and several phosphates [6–9].

Moreover, these initial reports suggesting a role for GPtdIns lipids as mediators of insulin action were later supported by the demonstration that the polar InsPG was endowed with widespread intracellular effects that include activation of protein kinases and phosphatases [10,11] and insulin mimetic effects on glucose transport and intermediary metabolism [12–14]. Thereafter, new observations demonstrating agonist-stimulated cleavage of GPtdIns lipids by other growth factors that activate

* Corresponding author, Tel.: +34 28 45 14 40; Fax: +34 28 33 22 48.

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receptors with tyrosine kinase activity [15,16] or hormones like ACTH, TSH and LH/hCG previously shown to activate cellular responses as a consequence of a guanine nucleotide (G) protein mediated stimulation of adenylyl cyclase [17–19], have stimulated new insight into the field of signal transduction, and support the notion that more than a single transduction mechanism is involved in receptor-activated cellular responses [2–4]. Although the recent cloning of the TRH receptor revealed that it is composed of a single polypeptide chain with seven transmembrane segments characteristic of the superfamily of G protein-coupled receptors [20,21], recent evidence demonstrates that TRH can also activate alternative signalling pathways in pituitary cells that include tyrosine phosphorylation of MAP kinase, and activation of phospholipase A_2 [22–24]. The relative roles of these different signalling mechanisms in TRH receptor activation are difficult to asses [1–3] and are a strong rationale for the studies presented here.

We investigated whether the phosphodiesterase cleavage of these purported second messenger precursor GPtdIns lipid could be implicated in TRH action on pituitary lactotrophs. Enriched cultures of pituitary lactotrophes, obtained from female Sprague–Dawley rats weighing 180–200 g (Lettica, Barcelona), were used in all experiments. Upon arrival, animals were bilaterally ovariectomized, implanted with diethylestilbestrol-filled sylastic canulae, a treatment known to dramatically increase the number of pituitary lactotrophs [25,26], and kept in individual cages in an environment of controlled temperature, humidity and light-dark cycle. Animals were killed by decapitation 20 days later, and the adenohypophysis carefully separated from the neurointermediate lobe, cut into small pieces (1 × 1 mm) and enzymatically dispersed into single cells in 25 mM HEPES buffer (pH 7.4), containing 0.4% (w/v) collagenase (Worthington Biochemical Co., 162 U/mg), 10 μg/ml DNase (Gibco, 2100 U/mg) and 1% bovine serum albumin [22,27,28]. Cells were collected by centrifugation (250 × g for 5 min), washed five times with 25 mM HEPES buffer supplemented with 0.1% BSA, allowed to sediment at unit gravity, and inoculated (~10^6 viable cells/dish) into Falcon (35 × 10 mm) tissue culture dishes containing 1 ml of Ham's F-10 medium supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin sulphate), 15% horse serum and 2.5% foetal calf serum. After 2 days in culture, the cells were washed and cultured for an additional 2 days in the same medium under serum-free conditions [27–29] and used thereafter to investigate the presence of GPtdIns molecules in pituitary lactotrophes (Fig. 1).

Results show that pituitary lactotrophes incorporated [^{3}H]palmitate or [^{3}H]myristate into the DAG backbone and [^{3}H]glucosamine, [^{3}H]galactose and [^{3}H]inositol into the polar head group of a GPtdIns lipid in a time-dependent manner. Isotopic steady state was observed after 24 h labelling with [^{3}H]fatty acids or 48 h in the presence of [^{3}H]saccharides (Fig. 1). In contrast, no significant incorporation of [^{3}H]mannose into the glycosyl-phosphatidylinositol fraction could be observed. The isotopic integrity of the metabolic label was also investigated in acid hydrolysates of [^{3}H]PtdInsG labelled with different [^{3}H]saccharides. Samples of [^{3}H]saccharide-labelled GPtdIns, were incubated (110°C for 24 h) with 1 ml of 4 N HCl in vacuum-sealed test tubes [6,7,18]. The reaction products were redissolved in 50 μl of water, supplemented with the appropriate acid-treated saccharide standards and separated by TLC using a mobile phase of pyridine/ethylacetate/glacial acetic acid/water (5:5:1:3, by vol.). The radioactivity was recovered (92–98%) in fractions that co-migrated with the same retardation factor as the precursor molecules [^{3}H]inositol (R_F 0.26), [^{3}H]-glucosamine, (R_F 0.50) and [^{3}H]galactose (R_F 0.88), treated in a similar manner (results not shown).

Although the detailed structure of GPtdIns is not known, chemical and enzymatic modifications have suggested that hormone-sensitive GPtdIns lipids consist of a core structure of phosphatidyl-inositol glycosidically linked to a non-acetylated glucosamine, which is itself cou-

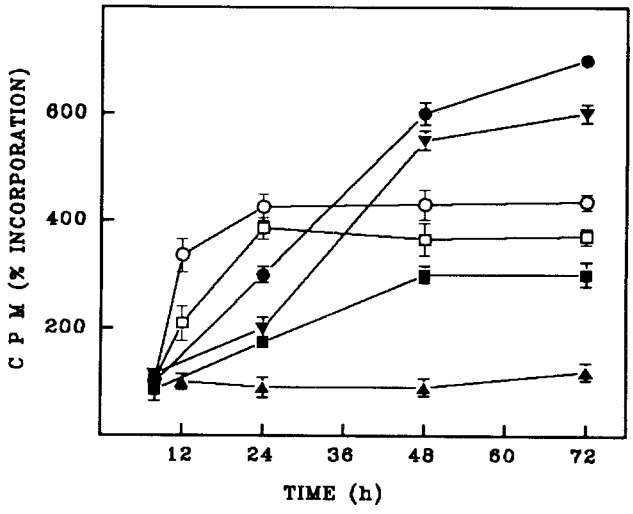

Fig. 1. Metabolic labelling of the glycosyl-phosphatidylinositol. Triplicate or quadruplicate cultures of cells (~10^6 viable cells/dish) were metabolically labelled for different time periods (5–72 h) with 5 μCi/ml fatty acids: [^{3}H]palmitate (○), [^{3}H]myristate (□) or 10 μCi/ml of the labelled monosaccharides [^{3}H]glucosamine (▼), [^{3}H]galactose (●), [^{3}H]mannose (▲) and [^{3}H]inositol (■). After the time periods indicated, media were aspirated and phospholipids extracted, and separated by sequential thin layer chromatography (TLC) on activated silica gel G plates (Merck, Darmstadt, Germany) in the system chloroform/ acetone/methanol/glacial acetic acid/water (79:35.5:15.5:15.5:8 by vol.). In this acid mobile phase, $PtInsP_2$ remains in the origin and other phospholipids migrated with retardation factors (R_F) of 0.12, 0.25 and 0.31 for PtdInsP, GPtdIns and PtdIns, respectively. The GPtdIns fraction was eluted from the plates with 2 ml methanol at 37°C, and rechromatographed in a basic mobile phase of chloroform/methanol/ammonia/ water (65.2:65.2:5:2 by vol.) to separate PtdInsP (R_F 0.15) from GPtdIns and PtdIns that migrated with retardation factors of 0.32 and 0.65, respectively. Values represent the mean ± SE of quadruplicate cultures. Similar results were obtained in three different experiments.

Table 1
Modification of glycosyl-phosphatidylinositol isolated from pituitary lactotrophes

	Radioactivity recovered (%)	Labelled precursor	Product recovered
Nitrous acid	97%	[^{3}H]Palmitate	[^{3}H]PtdIns
PtdIns-PLC	95%	[^{3}H]Myristate	[^{3}H]DAG

Samples (3000–5000 cpm) of [^{3}H]fatty acid-labelled GPtdIns, were incubated for 12 h at 37°C in 0.2 ml of 0.33 M sodium nitrite in 25 mM sodium acetate (pH 3.5) or the same volume of 20 mM sodium borate buffer (pH 7.5) supplemented with 1 unit of phosphatidylinositol-specific phospholipase C (PLC) purified from *Bacillus thuringiensis* (a generous gift of Dr. S. Udenfriend, Roche Institute of Molecular Biology, Nutley, NJ). The lipids were extracted and separated by the sequential TLC procedure described. Results are presented on a percent basis and were corrected for non-specific conversion from control incubations conducted at pH 3.5 in the absence of sodium nitrite, or at pH 7.4 in the absence of PtdIns-PLC from *Bacillus cereus*. Similar results were obtained in three other experiments.

pled to additional monosaccharides [6–8]. The GPtdIns isolated from pituitary cells meets the basic structural features of GPtdIns lipids isolated from other cell types (Table 1), as demonstrated by (i) the glucosamine C-1 is linked to the lipid moiety through a glycosidic bond as demonstrated by [^{3}H]PtdIns generation after nitrous acid deamination of [^{3}H]fatty acid-labelled GPtdIns [6,18]; and (ii) treatment with PtdIns-specific phospholipase C (PLC) of [^{3}H]palmitate-labelled GPtdIns generates [^{3}H]-diacylglycerol [5,6].

The possible involvement of GPtdIns lipids in TRH mediated cellular responses was also investigated (Fig. 2). Results show that TRH (10^{-6} M) stimulated the rapid cleavage of GPtdIns in cells labelled with [^{3}H]galactose or [^{3}H]inositol. Moreover the hydrolysis of [^{3}H]inositol labelled GPtdIns was followed 30 s later by the subsequent hydrolysis of [^{3}H]phosphoinositides (PtdIns, PtdInsP and $PtdInsP_2$). These findings suggest that the early and rapid cleavage of GPtdIns and the subsequent generation of an InsPG and DAG could be involved in TRH action, and support recent evidence showing that the classical $PtdInsP_2$ hydrolysing phospholipase C ($PtdInsP_2$-PLC) pathway is not the only mechanisms that operates after TRH stimulation of pituitary lactotrophes [22–24]. The consequence of TRH-mediated hydrolysis of GPtdIns is difficult to assess, and whether the activation and/or crosstalk of these two different signalling systems are physiologically relevant for TRH action in pituitary lactotrophes remains unknown. Nevertheless, since DAG species generated in response to GPtdIns hydrolysis can activate a subset of PKC family members that have little or no requirement for calcium ions [30] and the bioeffector InsPG moiety is endowed with regulatory properties on protein kinases and phosphatases [10,12,15], it is tempting to speculate that TRH-stimulated hydrolysis of GPtdIns could be an early event implicated in the activation of PtdIns-PLC and the subsequent activation of other calcium dependent PKC family members. This possibility seems reasonable, but definitive evidence for a role of GPtdIns hydrolysis in TRH action awaits the demonstration that second messenger DAG species or InsPG moiety generated in response to TRH receptor activation could mimic some effects of TRH on pituitary lactotrophes.

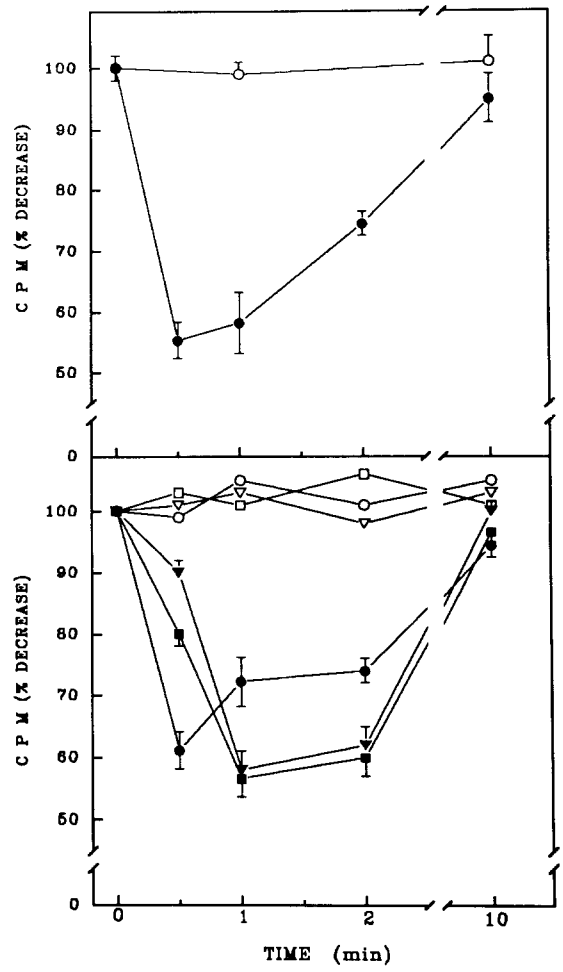

Fig. 2. Effects of TRH on glycosyl-phosphatidylinositol and phosphoinositide turnover. Cultured cells (~10^6 viable cells/dish) were labelled to isotopic steady state with 25 μCi/ml of [^{3}H]glucosamine (top) or [^{3}H]inositol (bottom). Before experiments, media were aspirated and cells allowed to equilibrate for 30 min at 37°C in fresh medium. Treatment with 10^{-6} M TRH (●) or 50 μl of vehicle (○) was added at decreasing times to triplicate or quadruplicate cultures and experiments terminated by adding 1 ml of ice-cold methanol to each culture. The lipids were extracted and GPtdIns separated by sequential TLC and quantitated by liquid scintillation counting. In cells labelled with [^{3}H]inositol (bottom) the radioactivity associated with GPtdIns (●) was separated from PtdIns (■) and PtdInsP + $PtdInsP_2$ (▼) as described in Fig. 1. In vehicle-treated cells, GPtdIns (○), PtdIns (□) or PtdInsP + $PtdInsP_2$ (▽) remained unchanged. Similar results were obtained in three different experiments.

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