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A NOVEL INOSITOL GLYCOPHOSPHOLIPID (IGPL) AND THE SERUM DEPENDENCE OF ITS METABOLISM IN BOVINE ADRENOCORTICAL CELLS

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Adrenocortical cells in primary culture actively incorporated [^{3}H]-inositol into phosphatidylinositol (PI) and its mono (PIP) and bisphosphate (PIP_2) derivatives. In addition to these well known phosphoinositides, a novel inositol-containing component was detected in the cell lipid extract when analyzed by proper chromatographic systems. This component was also labeled when the cells were provided with ^{32}P or radioactive fatty acids and a distinctive character was its ability to incorporate [^{3}H]-glucosamine. This novel phospholipid was thus characterized as an inositol glycophospholipid (IGPL). Study of IGPL metabolism in adrenocortical cells disclosed that the presence of serum in the culture medium strikingly increased glucosamine as well as inositol incorporation by a factor of about 10 and 5, respectively, within 36 hours. These observations suggest that IGPL turnover rate, especially at the level of its inositol-glycan moiety may be regulated by extracellular signals. A possible role of IGPL in membrane signalling systems and cell regulation remains to be clarified.

Although quantitatively minor cellular components, inositol lipids are currently concentrating considerable interest, due to their fundamental role in a wide-spread cellular transmembrane signalling system (1, 2). The polyphosphoinositide degradation is considered to provide the source of two major intracellular messengers i.e. diacylglycerol and inositol phosphates (especially the trisphosphate IP_3), respectively proposed as activators of protein kinase C and intracellular calcium mobilization (1-3). Activation of phosphoinositide turnover is triggered by a number of extracellular signals, including hormones and several growth factors acting through specific receptors in their target cells (1, 2). Bovine adrenocortical cells represent an example in which the differentiated functions (i.e. synthesis and secretion of active corticosteroid hormones) are acutely activated by two different peptide hormones i.e. adrenocorticotropin (ACTH) and angiotensin II (A II). Whereas ACTH is believed to act through a cyclic AMP dependent pathway (4), A II stimulates adrenocortical phosphoinositide turnover and has been proposed to act through a protein kinase C and calcium activation pathway (5-7).

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During studies of adrenocortical phosphoinositide metabolism, cell labeling with [^{3}H]-inositol and use of proper analytical separation methods pointed to the fact that, in addition to phosphatidylinositol (PI) and its mono (PIP) and diphosphate (PIP_2) derivatives, a lipidic component could incorporate [^{3}H]-inositol. This communication reports the characterization of this inositol lipid moiety as a novel inositol glycophospholipid (IGPL). Study of IGPL in adrenocortical cells under various culture conditions revealed that its content in labeled precursors (especially glucosamine and inositol) was strikingly enhanced in the presence of serum.

These observations suggest that IGPL metabolism is amenable to study in intact cells, with the aim of understanding its possible role in cellular regulation, especially in relation with the mechanism of action of specific effectors of adrenocortical cell functions.

MATERIALS AND METHODS

. **Radioisotopes and chemicals.** [1,6-^{3}H]-glucosamine (42.5 Ci/mmol), myo-[2-^{3}H]-inositol (17.1 Ci/mmol), [9,10-^{3}H]-myristate (22.4 Ci/mmol) and [1-^{14}C]-arachidonate were purchased from New England Nuclear. [^{32}P]-phosphate (10 mCi/150 µl), DME / F-12 culture medium (powder), bovine serum albumin (BSA : fatty acid free) were obtained from Sigma. Horse serum was from Boehringer-Mannheim and fetal calf serum was produced by Flow Laboratories (France). Trypsin, penicillin and streptomycin were from Bio-Mérieux (Lyon, France). Unless otherwise indicated other chemicals including standard phospholipids were reagent grade and purchased from Sigma.

. **Cell culture and labeling-experiments.** Dispersed bovine adrenocortical (BAC) cells were prepared from adrenal cortex tissue by successive tryptic digestion of fasciculata zone slices, as previously described (8). Cells were seeded into 16 mm or 35 mm diameter culture dishes in DME / F-12 medium supplemented with 12.5 % horse serum and 2.5 % fetal calf serum, HEPES (15 mM, pH 7.4), gentamycin sulfate (0.01 %), penicillin (25 U/ml) and streptomycin (12.5 µg/ml) in an air/CO_2 (95:5) atmosphere at 37°C.
Labeling experiments were performed with confluent cells (days 4-5 of culture). Confluent BAC cells were maintained in fresh serum-containing medium in the presence of the radioisotope, as indicated, for 36 hours. Under these conditions, no significant change in the cell number was detected during this time lapse.
For the experiments testing the effect of serum, the culture medium was replaced by a serum-free DME / F-12 mixture containing 0.01 % gentamycin sulfate, 50 µg/ml BSA. After 2 hours, the medium was removed and replaced by a fresh medium with or without serum (12.5 % horse serum and 2.5 % fetal calf serum) containing the indicated radiolabel. The cells were cultured for a 36 hour period under these conditions, before analysis.

. **Extraction and analysis of cellular labeled lipids.** Lipid extraction from the cell layer was carried out by the method of Bligh and Dyer (9) using an acidified $CHCl_3/CH_3OH$ mixture (10) with slight modification. Briefly, labeled cells ($4x10^6$ cells) were washed with phosphate buffer saline (PBS) (Ca^{2+} and Mg^{2+} free) and scrapped off in 1 ml of 0.1 N HCl solution. After transfering to extraction tubes, 3.75 ml of a $CHCl_3/CH_3OH$ (1:2) mixture was added followed by vigorous shaking. For phase separation, 1.25 ml $CHCl_3$, 1.25 ml 0.1 NHCl and 50 µl acetone were added, and the mixture was shaken and centrifuged. The organic phase was evaporated to dryness, dissolved in 50 µl

of $CHCl_3/CH_3OH/0.1$ N HCl (75/25/2, v/v), and analyzed by thin-layer chromatography on a silica gel G plate (Merk), developed in a solvent system consisting of $CHCl_3/CH_3OH$/acetic acid/H_2O (50/30/8/4, v/v) (11), or on potassium oxalate-impregnated silica G plates developed in a solvent consisting of $CHCl_3$ /CH_3OH/4 M NH_4OH containing 0.1% 1,2-cyclohexanediamine-tetraacetic acid (CDTA) (45/35/10, v/v) (12).

. **Other analytical methods.** Cell number in culture dish was determined by counting dye-stained nuclei after citrate treatment (13). Organic phosphorus was determined by the method of Ames and Dubin (14), modified by using ascorbic acid as a reducing reagent. Radioactive determination was performed using aquasol-2 (New England Nuclear) or a toluene-Triton X-100 scintillation mixture.

RESULTS

A novel lipid component in adrenocortical cell incorporates inositol, glucosamine and phosphorus.

When confluent adrenocortical cells were maintained in the presence of either [^{3}H]-inositol or [^{3}H]-glucosamine, independent experiments showed that the recovery of the radiolabels in the lipidic cell extract steadily increased to reach a plateau after about 36 hours of culture. At this time, labeling of the cell lipids could be examined following extraction and thin layer chromatography analysis. As illustrated in figure 1B, [^{3}H]-inositol was found associated with the major expected phosphoinositides. PI was the prominantly labeled component, while PIP and PIP_2 (not resolved from the origin in the chromatographic system shown in figure 1), incorporated much less of the label. However, an additional inositol-containing moiety was detected, with a Rf of about 0.30 in the system used, and a mobility that was similar to that of sphingomyelin introduced as a marker (figure 1). It may be noticed that this additional inositol lipid was clearly separated from lyso-PI in this chromatographic system. When adrenocortical cells were labeled with [^{3}H]-glucosamine, a major glucosamine-containing component of the lipid extract was detected which comigrated with the aforementioned new inositol lipid (figure 1A). This identical chromatographic mobility was confirmed in two different chromatographic systems (not shown).

The same type of phospholipid analysis was applied to adrenocortical cell extract following culture in the presence of ^{32}P-phosphate. As shown in table I, ^{32}P, as expected, was actively incorporated into PI while PIP and PIP_2 were also ^{32}P labeled. In addition, ^{32}P was also associated with the new inositol glycolipid of Rf 0.3 in the chromatographic system of figure 1. One might thus conclude that this lipid has the structural properties of an inositol glycophospholipid (IGPL). The lipidic nature of IGPL was further confirmed by the fact that it was labeled following cell culture in the presence of ^{3}H- or ^{14}C-labeled fatty acids (table I).

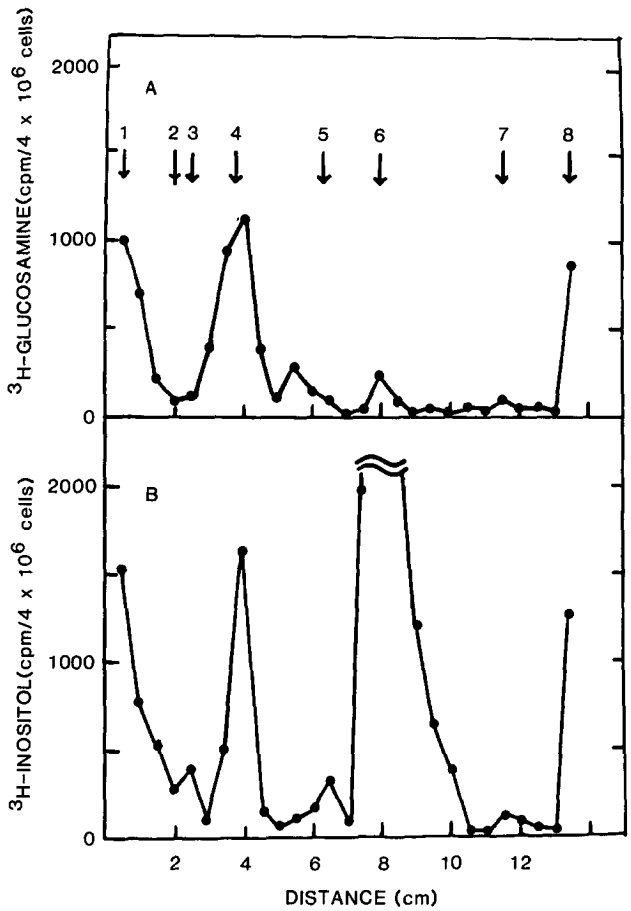

Figure 1 . Identification of a novel inositol glycophospholipid (IGPL) in bovine adrenocortical cells.
After reaching confluency (day 4 of culture), BAC cells were maintained in fresh DME / F-12 medium supplemented with 12.5 % horse serum and 2.5 % fetal calf serum in the presence of 2 µCi/ml of [1,6-^{3}H]-glucosamine (A), or 2 µCi/ml of myo-[2-^{3}H]-inositol (B) for 36 hours. Lipids were extracted and separated by thin layer chromatography with silica G plates developed in a solvent system consisting of $CHCl_3/CH_3OH$/acetic acid/H_2O (50/30/8/4, v/v). Five millimeter-wide bands were scrapped off the gel and counted for radioactivity. Positions of the authentic phospholipids separately developed on the same plate and used as markers are indicated by numbers : 1 : PIP ; 2 : lysophosphatidylcholine ; 3 : Lyso PI ; 4 : sphyngomyelin ; 5 : phosphatidylcholine ; 6 : PI ; 7 : phosphatidylethanolamine ; 8 : neutral lipids.

Serum-dependence of IGPL labeling activities in BAC cells.

In a search for optimal IGPL labeling conditions, confluent adrenocortical cells were maintained in culture either in a serum free medium or in a medium supplemented with horse serum and fetal calf serum. As illustrated in figure 2, glucosamine incorporation into IGPL was strikingly increased by almost 10 fold within 36 hours when serum was present in the culture medium. It may be noticed that, whereas IGPL was the prominent labeled moiety, a small labeling contribution appeared in the PI fraction. The nature of this labeled moiety associated with PI is not clear at the present time. This

TABLE I

Incorporation of various radioactive precursors into inositol-phospholipids of BAC cells in culture in the absence or presence of serum

Radioisotope	Serum	IGPL	PI	PIP	PIP_2
		cpm/4x10^6 cells/36 h			
^{3}H-glucosamine	-	150	100	30	20
	+	1 440 * (960)	320 (320)	23 (77)	15 (75)
^{3}H-inositol	-	300	23 510	110	50
	+	1 560 (520)	64 310 (273)	460 (418)	90 (180)
^{3}H-myristate	-	660	54 310	280	170
	+	3 740 (566)	79 420 (146)	450 (161)	310 (182)
^{14}C-arachidonate	-	980	5 660	250	320
	+	1 190 (121)	5 710 (101)	350 (140)	450 (141)
^{32}P	+	3 690	281 000	4 370	830

* % of control (-serum)

Confluent BAC cells were kept in culture in a fresh medium in the absence (-) or in the preence (+) of 12.5 % horse serum + 2.5 % fetal calf serum. The medium contained either ^{3}H-glucosamine (2 µCi/ml), ^{3}H-myo inositol (2 µCi/ml), ^{3}H-myristate (1 µCi/ml), ^{14}C-arachidonate (0.1 µCi/ml) or ^{32}P-sodium phosphate (4 µCi/ml). After 36 hours, cell phospholipids were extracted and analysed by thin layer chromatography in a solvent system made of $CHCl_3/CH_3OH/4$ M NH_4OH (45:35:10, v/v) containing 0.1 % CDTA. Under these conditions, the four inositol lipids (PI, PIP, PIP and IGPL) were completely separated. The gel area corresponding to each lipid was scrapped off and counted for radioativity.
The values listed were representative ones from two independent experiments with similar results.

labeling was increased as a function of [^{3}H]-glucose contamination in the labeled glucosamine preparation used. This might suggest that this label could represent PI-incorporated inositol derived from glucose metabolism or PI-incorporated fatty acyl moieties coming from a glucose contribution to acyl-CoA metabolism.

On the other hand, inositol incorporation into IGPL was increased by 5 to 6 fold (table I). The level of IGPL labeling by ^{14}C- or ^{3}H- fatty acids was dependent upon the nature of the fatty acid provided (table I) : myristic acid was preferentially incorporated into IGPL in a serum-dependent manner, as compared to arachidonate whose incorporation remained at a low level even when serum was present. From these data, it appeared that, as compared to that of other inositol lipids examined (table I), the presence of serum in

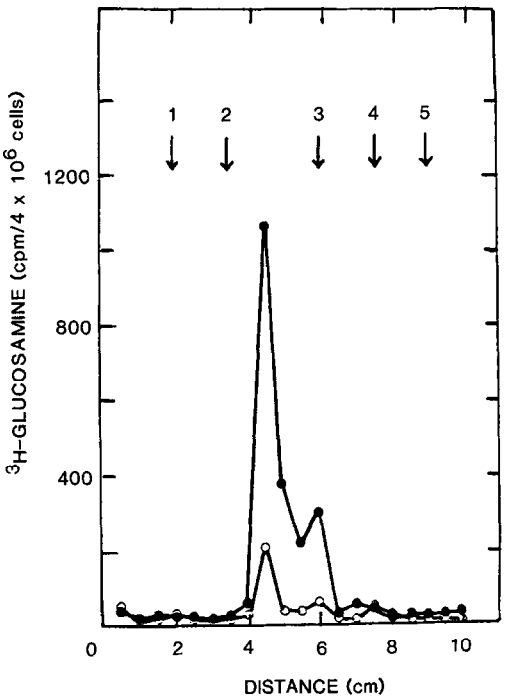

Figure 2. Effect of serum on ^{3}H-glucosamine incorporation into BAC cells IGPL
Confluent BAC cells were maintained in fresh DME / F-12 medium in the absence or in the presence of serum (12.5 % horse serum + 2.5 % fetal calf serum) containing 2 µCi/ml of [1,6-^{3}H]-glucosamine. After 36 hours, the cell lipid fraction was extracted and analyzed by TLC on potassium-oxalate-impregnated silica gel G plate which was developed in a solvent consisting of $CHCl_3/CH_3OH$/4 M NH_4OH containing 0.1 % CDTA (45/35/10, v/v). Five millimeter-wide bands were scrapped off and the gel counted for radioactivity.
The numbers indicate the positions of authentic phospholipids : 1 : PIP_2 ; 2 : PIP ; 3 : PI ; 4 : phosphatidylcholine ; 5 : phosphatidylethanolamine.

the culture medium induced a selective activation of inositol as well as myristate incorporation into IGPL.

DISCUSSION

The present data demonstrate that, together with the well characterized glycero phosphoinositides (PI, PIP, PIP_2), bovine adrenocortical cells contain an additional inositol lipid which incorporates fatty acids, phosphate and glucosamine. It can be therefore characterized at the present time as an inositol glycophospholipid (IGPL). Thorough identification of the IGPL structure remains to be established and is currently under study. Labeling experiments using corresponding precursors disclosed that the presence of serum in the culture medium induced a striking activation of IGPL labeling, especially with glucosamine. It remains to be examined whether this increased labeling corresponds to *de novo* synthesis or to an activation of the turnover of IGPL. The latter possibility may be more likeky in view of

the fact that serum-induced incorporation was quantitatively different with different individual precursors (e.g. glucosamine vs inositol vs fatty acids). The highest incorporation rate was observed with glucosamine and may reflect a selectively increased turnover of the osamine moiety in IGPL under these conditions. A detailed study of the metabolic pathways involved in IGPL synthesis and degradation is required to fully understand the present observations. It will be of special interest to examine whether IGPL may derive from a phosphatidylinositol lipid and whether its occurrence may be related to the activity of the phosphoinositide cyclic pathway.

The properties of the IGPL characterized in this work are reminiscent of those of the recently described membrane-associated glycan-phosphatidyl-inositol lipids serving as an anchor for various membrane-associated proteins, including trypanosome variant surface protein (15), Thy-1 antigen (16), acetylcholine esterase (17) and alkaline phosphatase (18). It remains to be determined whether IGPL could be part of such a protein glyco-phospholipid complex in adrenocortical cells, and whether in this case it may be released from such membrane-anchored complexes by specific phospholipase C treatment (19).

IGPL also appears similar to the phosphatidylinositol glycan moiety recently described in hepatoma cells (20) and rat liver membranes (21) and proposed as the precursor of an inositol phosphate-glycan messenger which could regulate cAMP phosphodiesterase or phospholipid methyl transferase activities (20-23). This pathway was proposed as a possible mechanism of action of insulin which following binding to its receptor, may activate a phosphatidylinositol glycan specific phospholipase C (20-23). At the same time, insulin was shown to release specific diacylglcyerol moieties which were suggested to result from the PI-glycan hydrolysis (23). The adrenocortical IGPL moiety characterized in the present work may thus be representative of a novel type of inositol glycolipid possibly involved in transmembrane-signalling processes. Current work is in progress to examine whether this may be the case in the regulation of adrenocortical cell functions by well defined hormonal effectors and specific growth factors.

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