Stimulation of phosphatidylinositol 4-phosphate phosphorylation in human placenta membranes by GTPγS

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In human placenta membranes the rate limiting enzyme for PIP_2 formation from PI is PIP kinase. GTPγS is shown to activate PIP kinase by increasing V_{max} of the enzyme. It is suggested that a guanine nucleotide regulatory protein is involved in the activation of PIP kinase although coupling with a specific receptor is not yet known. Since PIP_2 is the preferred substrate of phospholipase C, the possibility exists that an increase of PIP_2 due to activation of PIP kinase leads to an enhancement of phospholipase C activity and hence to an increased production of IP_3 and DAG.

Placenta membrane *Guanine nucleotide regulatory protein* *Phosphatidylinositol-4-phosphate kinase* *Phospholipase C*

1. INTRODUCTION

Little is known about the regulation of the lipid kinases of the phosphatidylinositol cycle: PI kinase, PIP kinase and DAG kinase. Studies from this laboratory [1] recently confirmed by others [2] had shown that PI kinase activity remains closely associated with the tyrosine protein kinase activity during extensive purification of the insulin receptor from human placenta membranes. Moreover, these studies also provided evidence for a stimulatory effect of insulin on PI phosphorylation although this was not a consistent finding. The next enzyme, PIP kinase which catalyses the phosphorylation of PIP to form PIP_2 is also present in placenta membranes. This enzyme is important in providing the substrate proper for phospholipase C that cleaves PIP_2 into IP_3 and DAG. These products possess specific messenger functions the former for mobilisation of cytosolic calcium, and the latter for the activation of protein kinase C (review [3]). There is now accumulating evidence suggesting that a guanine nucleotide regulatory (G) protein is important in regulating the activity of phospholipase C [4]. On the other hand, since phospholipase C uses preferably PIP_2 as substrate [5–7] an increase in the concentration of the latter could also play a regulatory role by enhancing the activity of the enzyme. On investigating this latter possibility we have observed that ^{32}P incorporation into endogenous and exogenously added PIP by placenta membranes is enhanced upon addition of GTPγS. These results suggest that GTPγS leads to an activation of PIP kinase through the participation of a guanine nucleotide regulatory protein.

Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinotisol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP, inositol monophosphate; IP_2, inositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PA, phosphatidic acid; GTPγS, guanosine 5′-*O*-(thiotriphosphate); NaDoCh, Na-deoxycholate; TX-100, Triton X-100

2. MATERIALS AND METHODS

PI, PIP, PIP_2, bovine insulin, NaDoCh, TX-100, EGF, vasopressin and phenylephrine were

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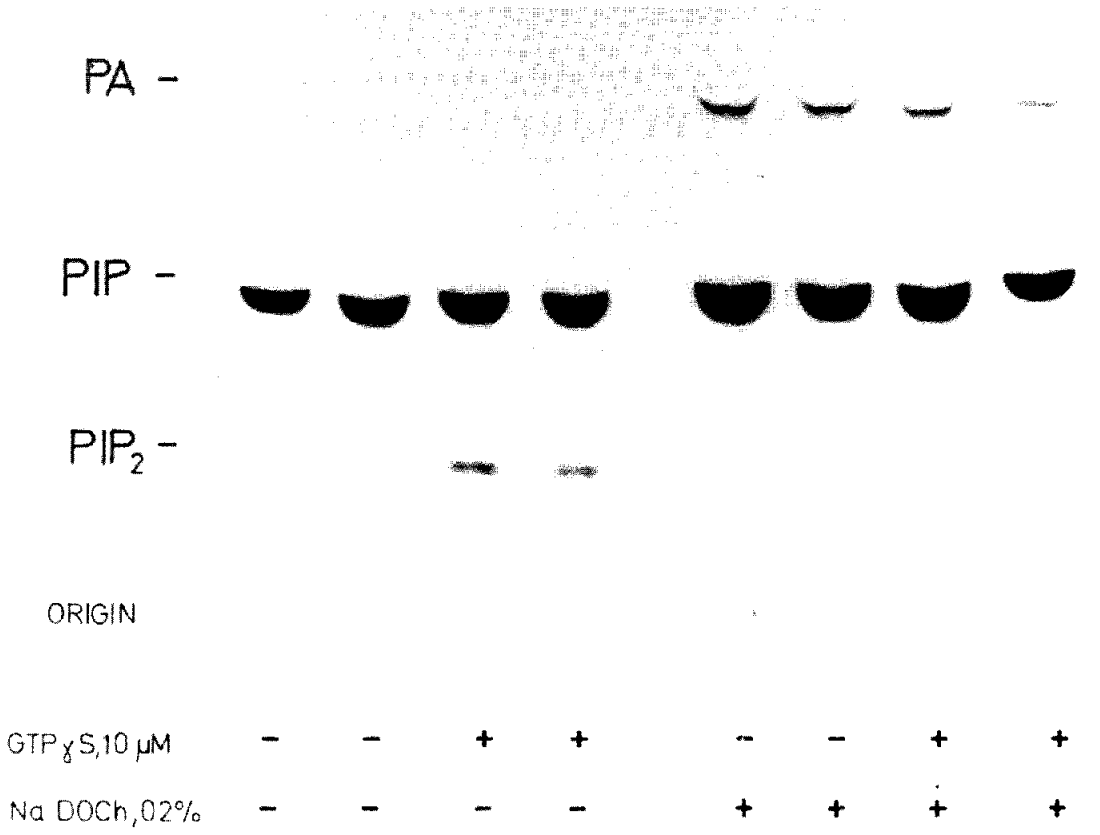

Fig.1. Effect of GTPγS on PIP_2 formation of placenta membranes in the absence and presence of NaDoCh. Placental membranes were phosphorylated with [γ-^{32}P]ATP for 2 min at 25°C after 15 min preincubation with or without GTPγS, 10 μmol/l, in the absence (left panel) or presence (right panel) of 0.2% NaDoCh. Phospholipids were extracted and separated on silica gel plates as described in section 2.1 The labelled fractions were scraped off and counted. ^{32}P incorporated in PIP_2 in the absence and presence of GTPγS was 740 ± 36 and 1332 ± 52 cpm (left panel), and 186 ± 7 and 192 ± 31 cpm (right panel), respectively. The corresponding values for PIP were 9865 ± 320 and 10 675 ± 411 cpm (left panel) and 11 318 ± 109 and 10 641 ± 211 cpm (right panel).

from Sigma (St. Louis, USA), and GTPγS and ATP were from Boehringer, Mannheim. Phosphatidyl[2-^{3}H]inositol was from Amersham, Braunschweig, and [γ-^{32}P]ATP from NEN, Dreieich. All other chemicals were from Sigma or Merck, Darmstadt. Membranes from human placenta were prepared as described in [8].

2.1. *Determination of PI kinase and PIP kinase*

10 μl membranes corresponding to 250 μg protein were incubated at 25°C in 140 μl of a mixture containing 50 mmol/l Tris-Maleate, pH 7.4, 10 mmol/l $MgCl_2$, 1 mmol/l EDTA, 1 mmol/l EGTA, 0.2 mmol/l dithiothreitol, 0.1 mmol/l vanadate, 0.1 mmol/l phenylmethylsulfonyl, fluoride 0.1 μmol/l leupeptin, 2 IU/l aprotinin, 0.14% TX-100. PI and PIP when added as exogenous substrates were given in amounts of 100 and 50 μg, respectively. After 15 min preincubation with or without GTPγS the reaction was initiated by addition of [γ-^{32}P]ATP (100 000 cpm/nmol), final concentration 2 mmol/l, and incubation continued for the times indicated in section 3. Phospholipids were extracted according to [9] by addition of 150 μl 2.5 mmol/l HCl and 400 μl of 2:1 (v/v) chloroform/methanol. The aqueous phase was reextracted with 150 μl chloroform and the combined organic extracts

were washed 3 times with 500 μl of a 1:1 (v/v) mixture of methanol and 1 mol/l HCl. Phospholipids were further separated on TLC plastic sheets (silica gel 60 F_{254} Merck) using a mixture of chloroform/methanol/3.3 mol/l ammonia (43:38:12, v/v) according to [9]. PIP, PIP_2 and PA were spotted by autoradiography on Kodak X-Omat films, excised and counted on 1219 Rackbeta liquid scintillation counter from LKB Wallac. PIP, PIP_2 and PA standards were run on the same plates and stained with iodine vapour.

2.2. *Determination of phospholipase C*

Phospholipase C in placenta membranes was determined essentially according to [10] using phosphatidyl[2-^{3}H]inositol as a tracer. In general, 20 μl membrane suspension (corresponding to 500 μg protein) were incubated in a total volume of 140 μl containing 50 mmol/l Tris-Maleate, pH 7.5, 1 mmol/l phosphatidyl[2-^{3}H]inositol (60000 dpm, 2 mmol/l $CaCl_2$, 0.2% NaDoCh, and where added ATP was 2 mmol/l. After incubation water soluble radioactivity was separated according to [10] and counted.

3. RESULTS AND DISCUSSION

The possibility that a change in substrate (PIP, PIP_2) supply might bear on the regulation of phospholipase C focussed our interest on the regulatory properties of the responsible enzyme's PI kinase and PIP kinase. On studying a variety of hormones and other effectors we observed that $GTP\gamma S$ stimulated the ^{32}P incorporation from [γ-^{32}P]ATP into PIP_2. The labelling of PIP remained unchanged (fig.1). In these experiments the phosphorylation of endogenous membrane inositol phospholipid was studied. However, as indicated in table 1 the phosphorylation of exogenously added PIP was likewise stimulated by $GTP\gamma S$. Table 1 further shows that $GTP\gamma S$ produced a significant effect at 1 μmol/l, and a maximal effect at 10 μmol/l. As shown in table 2 the $GTP\gamma S$ effect depends on the presence of TX-100 displaying a maximal response at 0.36% TX-100.

It is important to note in fig.1 (right panel) that the effect of $GTP\gamma S$ on PIP_2 formation is no longer demonstrable in incubations containing NaDoCh. Under these conditions the phosphorylated fraction corresponding to PIP_2 has vanished and a new phosphorylated band corresponding to PA appears. This is explained by the fact that phospholipase C of placenta membranes becomes activated by NaDoCh while it is essentially inactive in its absence (not shown). As a consequence, PIP_2 is rapidly cleaved, and one of the products, DAG appears in form of PA after phosphorylation by DAG kinase.

We believe that the effects of $GTP\gamma S$ on PIP phosphorylation is attributable to an activation of PIP kinase rather than an inhibition of phospholipase C. The latter possibility seems rather unlikely as under our assay conditions (1 mmol/l EGTA and no NaDoCh) phospholipase C showed no measurable activity even up to 30 min of incubation (not shown).

Kinetic data on endogenous PIP phosphorylation by placenta membranes (fig.2) show linear reaction rates at least up to 5 min both in the absence and presence of $GTP\gamma S$. From these data one can calculate a specific PIP kinase activity of

Table 1

Effect of $GTP\gamma S$ on PIP_2 formation by human placenta membranes

	PIP_2 formed (cpm)					
	No PIP added			50 μg PIP added		
$GTP\gamma S$ (μmol/l)	Expt 1	Expt 2	Expt 3	Expt 1	Expt 2	Expt 3
–	7101	5099	3091	63376	24451	30137
1	10275	7339	4267	80635	32387	40672
10	12724	8711	5927	99412	39401	58371
100	12692	9427	5140	103602	41865	54108

Table 2

Dependence of stimulated and unstimulated PIP kinase on TX-100

TX-100 (%)	PIP kinase $-GTP\gamma S$ (cpm)	PIP kinase $+GTP\gamma S$ (cpm)	$\Delta GTP\gamma S$ (%)
0	3809	4147	9
0.07	5921	8346	41
0.14	6168	9809	59
0.36	2162	4343	101
0.72	44	47	0

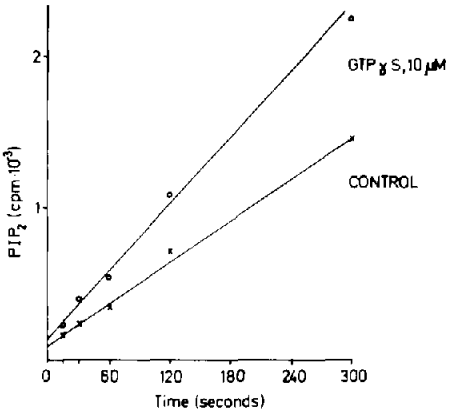

Fig.2. Time dependence of PIP_2 formation. After 15 min preincubation of placenta membranes with (○—○) or without (×—×) $GTP\gamma S$, 10 μmol/l, phosphorylation was started by addition of [^{32}P]ATP, and 100 μl aliquots were removed from each batch at the times indicated on the abscissa for lipid extraction and separation on TLC as indicated in section 2.1

0.4 mU/mg protein and about double that value in the presence of $GTP\gamma S$. Fig.3 illustrates the dependence of PIP kinase activity on ATP concentrations. From the Lineweaver-Burk plot it appears that $GTP\gamma S$ acts on PIP kinase by increasing V_{max} while $K_{m\ app}$ for ATP (0.26 mmol/l) remains unchanged. Guanosine 5′-(β,γ-imido)triphosphate between 10 and 100 μmol/l showed a slightly weaker effect than $GTP\gamma S$ whereas GTP itself had no effect at concentrations between 1 μmol/l and 1 mmol/l.

That the activation of PIP kinase by $GTP\gamma S$ and the ensuing increase in PIP_2 formation demonstrated in this study does in fact pertain to phospholipase C activity is shown in fig.4. In these experiments exogenously added PIP was first coincubated with placenta membranes and labelled ATP either in the presence of absence of $GPT\gamma S$. Phospholipids were then extracted, and after sonification used as substrates for phospholipase C of another batch of placenta membranes. It is clear that phospholipase C was about twice as active in the presence of the liposomes obtained after $GTP\gamma S$ pretreatment of the membranes as compared to the controls.

In conclusion our findings suggest that PIP

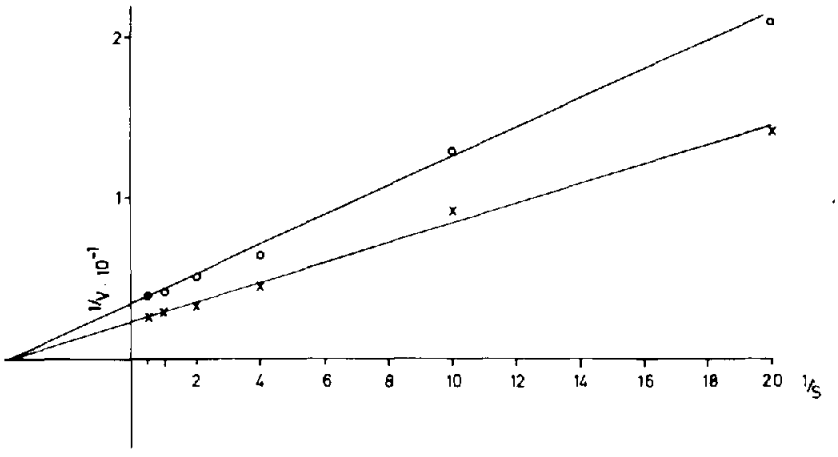

Fig.3. PIP_2 formation as a function of ATP concentration. Placental membranes were incubated at different ATP concentrations and with a constant tracer amount of [γ-^{32}P]ATP corresponding to 11.2 × 10^6 cpm/sample after preincubation with (○—○) or without (×— ×) $GTP\gamma S$, 10 μmol/l, and PIP_2 formation measured as described in section 2.1.

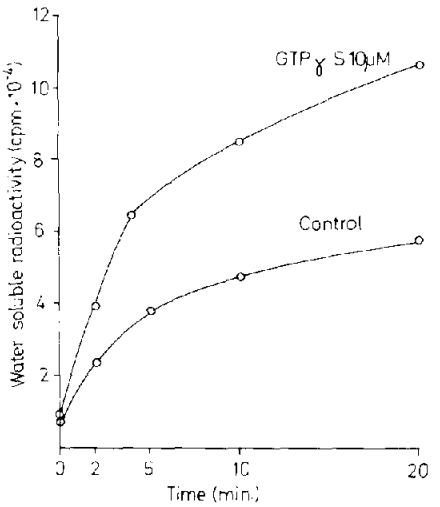

Fig.4. Time course of phospholipase C activity in response to different PIP_2 concentrations generated by GTPγS. 100 μl placental membranes corresponding to 2.5 mg protein and 1 mg PIP, were incubated 15 min with or without GTγS, 10 μmol/l. Phosphorylation was then started by addition of 0.5 mmol/l [γ-^{32}P]ATP (100000 cpm/nmol) and continued for 10 min. Thereafter phospholipids were extracted as in [9] and after sonification [10] added as liposomes to another batch of placental membranes. Phospholipase C activity is represented by ^{32}P radioactivity of the aqueous phase during incubation in the medium described in section 2.2 except that no tritium labelled PI and no Ca^{2+} but 1 mmol/l EGTA was added. ^{32}P radioactivity of the liposomes obtained from GTPγS treated membranes was 29 101 and 3032 cpm in PIP_2 and PIP, respectively. The corresponding values of the controls were 17 235 and 2720 cpm.

kinase of human placenta membranes may be a regulatable enzyme, and that a nucleotide binding protein is involved in its regulation. In this respect it is worth comparing the rate of PI kinase of 10 mU/mg protein with that of PIP kinase of 0.4 mU/mg protein (both calculated from the present study). This would mean that PIP kinase represents the rate limiting step during the formation of PIP_2, and that an activation of this step, through an increase of PIP_2, might exert control on phospholipase C. That the substrate supply for phospholipase C is limited and that the inositol lipid kinases may therefore be under separate control have also been discussed elsewhere [11].

Previous findings from this laboratory that insulin activates phospholipase C in fat cells [10] seem to have been confirmed recently [12,13]. So far, in our hands placenta membranes did not respond to insulin with activation of phospholipase C nor of PIP kinase. Likewise, other hormones such as vasopressin, angiotensin II, phenylephrine, carbachol and oxytocin had no effect on PIP kinase either alone or together with GTPγS. Hence the receptor(s) and agonist(s) to which the postulated G-protein may be coupled remain to be identified. In this context it seems noteworthy that a novel G-protein, G_p, of unknown function, was recently purified from human placenta membranes [14].

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