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Evidence for a cholera-toxin-sensitive G-protein involved in the regulation of phosphatidylinositol 4-phosphate kinase of rat liver membranes

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Studies on the phosphorylation of inositol phospholipids of rat liver membranes have shown that [γS]pppG stimulates ^{32}P incorporation from [γ-^{32}P]ATP into PI and PIP. This effect appeared specific for stable GTP analogues and could not be reproduced by other compounds. ADP-ribosylation of the membranes with cholera toxin resulted in a large decrease of PIP_2 without changes in the level of PIP. Since an activation of phospholipase C can be ruled out, the lowering of PIP_2 is explained on the basis of an inhibition of PIP kinase (EC 2.7.1.68). From these results it appears that a novel cholera-toxin-sensitive G-protein is involved in the regulation of PIP kinase.

Introduction

Various hormones, transmitters and growth factors exert their action, after binding to specific receptors on the plasma membrane through an activation of PLC. This leads to an increase in the PI turnover whereby PI is phosphorylated by specific kinases to PIP and consequently to PIP_2, the latter undergoing hydrolysis by PLC to yield the two intracellular messengers IP_3 and DAG (for review see Ref. 1). At present it appears that three different classes of G-protein, distinguished by their sensitivity to bacterial toxins, are involved in receptor-induced regulation of PLC: one that is inactivated by pertussis toxin (G_i-like protein), one that is inactivated by cholera toxin [2], and one that is not influenced by either toxin (review, see Ref. 3).

In contrast to PLC, little is known concerning the regulation of the phosphatidylinositol kinases, though they are responsible for supplying the substrate for PLC. A pull mechanism has been proposed, implying product control of PI and PIP kinases which is released on activation of PLC [4]. Cockroft and Gomperts [5] have raised the point that the substrate supply for PLC (and hence the production of two important messengers) is limited and that the inositol lipid kinases may be under separate control. We have recently reported that in human placenta membranes [γS]pppG stimulates the phosphorylation of PIP, suggesting that

Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP, inositol monophosphate; IP_2, inositol 1,4-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PA, phosphatidic acid; [γS]pppG, guanosine 5′-*O*-(thiotriphosphate); PLC, phospholipase C (phosphoinositide phosphodiesterase) (EC 3.1.4.3); p[NH]ppG, guanylylimidodiphosphate; p[NH]ppA, adenylylimidodiphosphate; DTT, dithiothreitol; BSA, bovine serum albumin; PE, phosphatidylethanolamine; PS, phosphatidylserine; PMSF, phenylmethylsulfonyl fluoride; EGF, epidermal growth factor; DTE, dithioerythritol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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(a) G-protein(s) is (are) involved in the activation of PIP kinase [6]. As shown in the present work, stable GTP analogues increased the phosphorylation of PIP – and to a lesser extent of PI – also in rat liver membranes. Moreover, PIP phosphorylation was greatly reduced after treatment of the membranes with cholera toxin, pointing to the participation of a novel G-protein susceptible to inhibition and ADP-ribosylation by cholera toxin. This G-protein appears, however, to be different from the G-proteins which are involved in receptor–PLC coupling.

Materials and Methods

PI, PIP, PIP_2, PA, Triton X-100 and [Arg^8] vasopressin were from Sigma (St. Louis). [γS]-pppG, p[NH]ppG and ATP were from Boehringer, Mannheim. [γ-^{32}P]ATP was from New England Nuclear, Dreieich, and all other chemicals were from Sigma or Merck, Darmstadt.

Preparation of liver and fat cell plasma membranes

Plasma membranes from rat livers were prepared according to Ref. 7. In brief, for a standard preparation, six livers from 250 g male Sprague Dawley rats killed by decapitation were perfused in situ with 25 ml each of ice-cold 0.9% NaCl and placed in 100 ml of an ice-cold solution consisting of 250 mM saccharose/5 mM Hepes/1 mM EGTA (pH 7.4)/1 mM mercaptoethanol/50 μM PMSF. After mincing with scissors, the livers were homogenized with the above buffer with 20 strokes using a loose-fitting Dounce homogenizer (Braun Melsungen) and diluted to give a 6% homogenate. The homogenate was centrifuged 10 min at 1464 $\times g$ and the resulting pellet was resuspended in the same medium to give a 6% suspension. The suspension was mixed with Percoll (Pharmacia) in a ratio 7.43 : 1 and was centrifuged at 34450 $\times g$ for 30 min. The membrane layer close to the top of the tubes was harvested, washed with 5 vol. of 250 mM saccharose in 50 mM Tris-HCl (pH 8.0), resuspended in the same medium yielding a protein concentration of about 12 mg/ml, frozen and kept at −30 °C up to several months.

Fat cell membranes were prepared from isolated rat fat cells (Rodbell) as in Ref. 8 by homogenization in 0.25 M saccharose/10 mM Tris-HCl/2 mM EGTA (pH 7.4)/1 mM mercaptoethanol/50 μM PMSF. After centrifugation at 30000 $\times g$ for 30 min the pellet was resuspended in a self-forming Percoll gradient with density of 1.05 and centrifugated at 10000 $\times g$ for 15 min. The plasma membrane fraction was then washed with 5 vol. of 0.15 M NaCl/10 mM Tris-HCl/1 mM EGTA (pH 7.4) and sedimented by centrifugation at 10000 $\times g$ for 20 min.

Determination of PI kinase and PIP kinase activities

Unless otherwise stated 50 μl rat liver plasma membranes corresponding to 800 μg protein were incubated for 2 min at 25 °C in 140 μl of a mixture containing 25 mM Tris-HCl (pH 7.5)/0.5 mM EGTA/10 mM $MgCl_2$/0.5 mM [γ-^{32}P]ATP (100000 cpm/nmol). The concentration of free calcium, [Ca^{2+}], was adjusted by buffering EGTA and $CaCl_2$ and calculated as in Ref. 9. The incubations were stopped as described in Ref. 6 by addition of 150 μl ice-cold 2.5 mM HCl and 400 μl chloroform/methanol (2 : 1, v/v). After centrifugation, the water phase was re-extracted with 150 μl chloroform and the combined organic extracts were washed three times with 500 μl of a 1 : 1 (v/v) mixture of methanol and 1 mM HCl. Phospholipids were further separated on TLC plastic sheets (silica gel 60 F_{254}, Merck) using a mixture of chloroform/methanol/3.3 M ammonia (43 : 38 : 12, v/v) according to Ref. 17. PIP, PIP_2 and PA were spotted by autoradiography on Kodak X-Omat films, excised and counted on a 1219 Rackbeta liquid scintillation counter (LKB, Wallac). PIP, PIP_2 and PA standards were run on the same plates and stained with iodine.

Determination of PLC activity

PLC activity was assayed for 30 min at 37 °C as described [10]. Liver membranes corresponding to 30 μg protein were incubated in 200 μl of a mixture containing 50 mM Hepes (pH 7.0), 100 mM NaCl, 0.1 mg BSA, 2.5 mM $MgCl_2$, and 0.2 mM [^{3}H]PIP_2 (750 cpm/nmol) added as liposomes consisting of PIP_2 : PS : PE = 1 : 2 : 2 (molar ratio). Free Ca^{2+} concentrations were maintained with Ca^{2+}/EGTA buffers with 2 mM EGTA according to Ref. 9. Assays were initiated by the addition of the membranes and terminated by

addition of 200 μl 10% (v/v) trichloroacetic acid followed by 100 μl 1% (v/v) BSA. After 10 min on ice, the reaction mixtures were centrifuged, and 400-μl portions of the supernatants were counted for radioactivity. Blank assays were run in which trichloroacetic acid was added immediately after the membranes. In some experiments, the individual labelled inositol phosphates were separated by anion-exchange chromatography according to Ref. 11.

ADP-ribosylation by cholera toxin

ADP-ribosylation of the liver membranes was done according to [12]. 1 mg cholera toxin was dissolved in 200 μl 10 mM Tris-HCl (pH 7.5) diluted with 200 μl 100 mM DTT, and activated for 60 min at 25 °C. The toxin was then diluted with 400 μl of 0.1% BSA in the same buffer and the solution was kept frozen at −70 °C until use. For ADP-ribosylation, 50 μl liver membranes corresponding to 800 μg protein were incubated 30 min at 30 °C in 120 μl of 300 mM potassium phosphate buffer (pH 7.0) containing 10 mM thymidine, 1 mM ATP, 0.1 mM GTP, 1 mM EDTA, 0.5 mM EGTA, 10 mM $MgCl_2$, 1.66 mM DTT, 0.1 mM NAD or 0.01 mM [^{32}P]NAD ($6 \cdot 10^7$ cpm/nmol) and 100 μg/ml cholera toxin. PI kinase and PIP kinase activities of the ADP-ribosylated membranes was then measured as described before. Controls were run where membranes were incubated under the same conditions as for ADP-ribosylation, but without cholera toxin.

Results and Discussion

Previously reported work from this laboratory suggested the participation of a guanine nucleotide binding protein (G-protein) in the regulation of PIP kinase activity of placenta membranes [6]. This possibility was further investigated in the present work and phosphoinositide phosphorylation in rat liver plasma membranes was studied.

Fig. 1 shows the rates of ^{32}P incorporation into PI (left side) and PIP (right side) as a function of increasing [γS]pppG concentrations, and with a constant amount of vasopressin (hatched bars). [Ca^{2+}] was kept at 3 μM throughout. Thus, at low [γS]pppG concentrations (0.1–1 μM) there is a decline of labelling of PI and PIP which is markedly enhanced by vasopressin. These changes may be explained to be due to an activation of PLC which, in agreement with other observations [10,13], is demonstrable under these conditions, i.e., at 0.1 μM [γS]pppG alone or in combination with vasopressin (Table I, Expt. 1). In contrast, high (100 μM) doses of [γS]pppG led to a marked

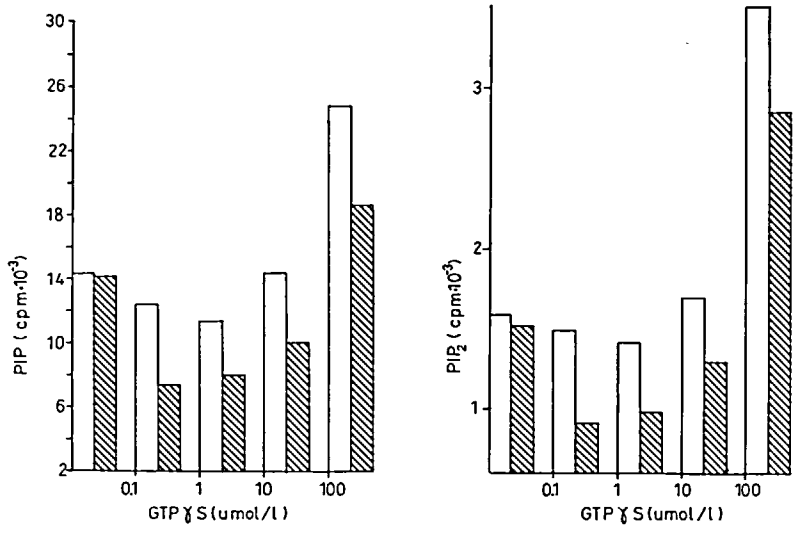

Fig. 1. Effect of [γS]pppG and vasopressin on phosphorylation of PI and PIP. Liver plasma membranes were phosphorylated for 2 min as described in Materials and Methods in the presence of 3 μM Ca^{2+}. The washed phospholipids were separated on silica-gel plates, excised and counted as described. Open bars, no vasopressin; hatched bars, 0.5 μM vasopressin.

TABLE I

STIMULATION OF PLC ACTIVITY IN LIVER MEMBRANES BY [γS]pppG AND VASOPRESSIN

Liver membranes were incubated at 37°C for 30 min with [^{3}H]PIP_2-containing liposomes as indicated in the Materials and Methods section, and the water-soluble radioactivity was counted. The free Ca^{2+} concentration was 0.2 μM. The radioactivity of the blanks (645 dpm) was subtracted. Data represent the average dpm of duplicate incubations from one membrane preparation in Expt. 1 and another in Expt. 2.

Additions	Water-soluble radioactivity (dpm)
Expt. 1	
Control	1026±323
[γS]pppG 0.1 μmol/l	2494±295
[γS]pppG 0.1 μmol/l + vasopressin 0.5 μmol/l	4604±442
vasopressin 0.5 μmol/l	1227±335
Expt. 2	
control	624±183
[γS]pppG 100 μmol/l	6481±418

increase of the phosphorylation of phosphoinositides. We propose that this effect of [γS]pppG is due to an activation of lipid kinases by a G-protein, which differs from the G-proteins involved in PLC activation by having lower affinity for [γS]pppG. The possibility that the higher PIP_2 and PIP formation rates might be attributable to an inhibition of PLC at the high concentration of [γS]pppG is ruled out by Expt. 2 of Table I, which shows a clear increase rather than a decrease of PLC activity at 100 μM [γS]pppG. An inhibition of phosphomonoesterase(s) at the high [γS]pppG concentrations could also explain the observed accumulation of PIP and PIP_2. To check this possibility we have measured the membrane catalyzed release of [^{32}P]P_i from [^{32}P]phosphoinositides and found it to be unchanged in the presence or absence of 100 μM [γS]pppG (data not shown).

Although a pull mechanism has been proposed for the formation of PIP and PIP_2 [4], a push mechanism is more likely. In our experiments (Fig. 1) PLC activation was always accompanied by a decrease rather than an increase in ^{32}P incorporation into PI and PIP. The enhancement of ^{32}P incorporation into the phosphoinositides in response to the high doses of [γS]pppG therefore suggests activation of the kinases by a push rather than a pull mechanism. This is compatible with the experiments in Fig. 2, where the effects of [γS]pppG (100 μM) on PIP kinase activity in the absence (panel A) or presence of Ca^{2+} (panel B) are compared. In the incubations where PLC activity is low by lack of Ca^{2+}, the stimulation of PIP kinase activity by [γS]pppG is much greater (panel A) than with Ca^{2+} (panel B) when PLC is active. Similar results were obtained when the phosphorylation of PI was followed (data not shown). Direct assay of PLC activity without Ca^{2+} following the cleavage of [^{3}H]PIP_2 did not show any measurable enzyme activity for up to 30 min, either in the absence or in the presence of 100 μM [γS]pppG (data not shown).

The specificity of the effect of stable GTP analogues on the phosphorylation of PI and PIP is demonstrated in Table II. Thus the stable ATP analogue, p[NH]ppA, pyrophosphate, GDP and CTP were all ineffectual. The data in Table II also show that labelling of PI is much faster than that of PIP, suggesting that in liver membranes, as in membranes from other tissues such as placenta, adipocytes, erythrocytes and thrombocytes (Ref. 6

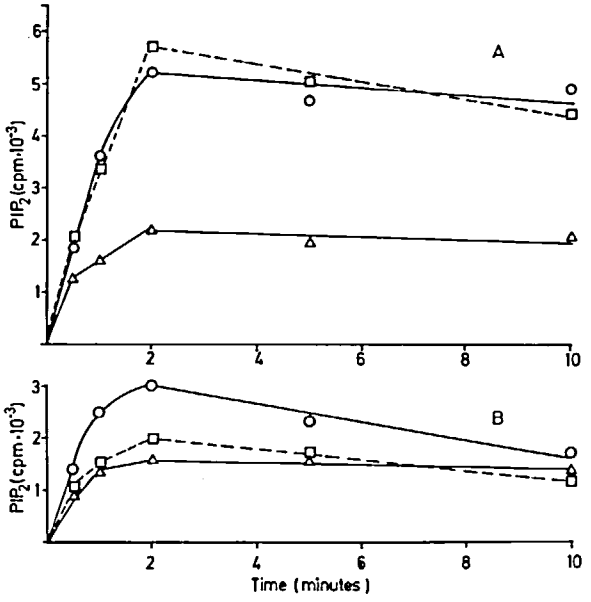

Fig. 2. Time-course of PIP phosphorylation in rat liver membranes. (A) No added Ca^{2+}, EGTA 0.5 mM; (B) with Ca^{2+}-EGTA to give free [Ca^{2+}] = 3 μM. Symbols in (A) and (B) denote no effectors (△), [γS]pppG, 100 μM (○), [γS]pppG, 100 μM, plus vasopressin, 0.5 μM (□).

TABLE II

SPECIFICITY OF THE EFFECT OF GTP ANALOGUES ON PHOSPHOINOSITIDE PHOSPHORYLATION

Liver plasma membranes were phosphorylated with [γ-^{32}P]ATP as indicated in Materials and Methods in the presence of the compounds listed in the table. The extracted phospholipids were separated on silica-gel plates and the radioactivity of PIP and PIP_2 was counted. Data represent the average pmol/min per mg protein of duplicate incubations of two different membrane preparations.

Compound (100 μM)	PIP				PIP_2			
	Expt. 1 (pmol·min^{-1}·mg^{-1})	change (%)	Expt. 2 (pmol·min^{-1}·mg^{-1})	change (%)	Expt. 1 (pmol·min^{-1}·mg^{-1})	change %	Expt. 2 (pmol·min^{-1}·mg^{-1}	change (%)
Control	292±28	–	124± 5	–	49±4	–	22±2.1	–
[γS]pppG	440± 2,2	+50	180± 3	+45	135±2	+175	52±1	+136
p[NH]ppG	403±12.9	+38	164± 7.8	+32	104±3.1	+112	42±2	+91
GTP	329±13.8	+13	131± 6	+6	77±5	+57	30±1	+36
GDP	312±15.9	+6	123± 2	−1	54±7	+10	24±2	+9
p[NH]ppA	307±11	+5	124± 5	–	56±8	+14	23±2	+4
CTP	294±17	+1	122±11	−2	51±2.1	+5	22±1	–
Sodium pyrophosphate	273± 2.2	−7	114± 1.1	−8	46±3	−6	19±1	−14

and unpublished observations), PIP kinase is the rate-limiting enzyme.

The activation by [γS]pppG points to the existence of an unknown G-protein involved in the activity control of the lipid kinases that catalyze the phosphorylation of PI and PIP. To characterize this putative G-protein further we have studied the influence of cholera toxin and pertussis toxin on the phosphorylation of the phosphoinositides. As illustrated in Fig. 3, pretreat-

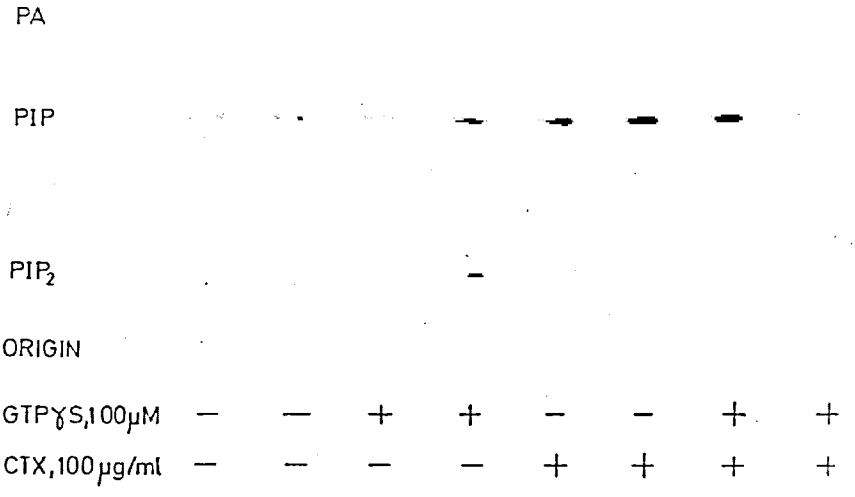

Fig. 3. Effect of cholera toxin treatment on ^{32}P-labeling of PI and PIP with [γ-^{32}P]ATP. Liver membranes were phosphorylated with [γ-^{32}P]ATP for 2 min at 25 °C in presence or absence of 100 μM [γS]pppG following preincubation of the membranes with (right side) or without (left side) 100 μg/ml cholera toxin as described in Materials and Methods. Phospholipids were extracted and separated on silica-gel plates as described. The labeled fractions were excised and counted. The average of duplicate determinations of ^{32}P incorporated in PIP_2 was 458±44 cpm in the absence, and 957±59 cpm in the presence of [γS]pppG. The corresponding values after cholera toxin treatment were 167±5 cpm and 198±0 cpm, respectively. The corresponding values for radioactivity in PIP were 957±48, 892±6, 886±4, 906±50 cpm.

ment of the membranes with cholera toxin virtually abolished ^{32}P incorporation into PIP, irrespective of the presence or absence of [γS]pppG. On the other hand, the PIP levels remained essentially unchanged by the toxin treatment. It may also be noted that, under these experimental conditions, i.e., after 30 min preincubation of the membranes, PIP synthesis – in contrast to PIP_2 formation – was no longer stimulated by [γS]pppG. To exclude the possibility that the inhibition of PIP labelling in the presence of cholera toxin is mediated by a cAMP-dependent protein kinase A, we have tested the influence of different concentrations of db-cAMP, forskolin and catalytic subunit of protein kinase A on the rate of phosphatidylinositol phophorylation. None of these agents produced inhibition of phosphorylation, with or without [γS]pppG (data not shown). It has been shown that an ADP-ribosylation of $G_{s\alpha}$ by cholera toxin decreases its affinity to $G_{\beta\gamma}$ [18] and that $G_{\beta\gamma}$ can attenuate the action of the GTP-binding subunit [19]. Although this could offer another explanation for the inhibition of PIP kinase, it remains to be established that an increase of free βγ-subunits by cholera toxin does occur in the intact membrane.

The autoradiographs shown in Fig. 4 confirm that cholera toxin did stimulate ADP-ribosylation of several membrane proteins, two of them in the 45–50 kDa region where one should expect the α-subunits of G proteins. The nature of a faster-migrating fraction which also appeared in the absence of cholera toxin is not known. It appears to be a substrate for an endogenous NAD-glucohydrolase activity. Treatment of liver membranes with pertussis toxin as described [12] affected neither the activation of PIP kinase by [γS]pppG nor the basal non-activated rate of PIP phosphorylation.

That the reduced labeling of PIP after treatment of the membranes with cholera toxin is due to an enhanced degradation of radioactive PIP_2 by PLC rather than an inhibition of PIP kinase seems unlikely. Since diacylglycerol formed by the cleavage of PIP_2 is rapidly converted to phosphatidate, the latter would be expected to rise when PLC activity increases [6]. This was, however, not the case, as shown in Fig. 3. This agrees with other studies, which failed to observe activation of PLC

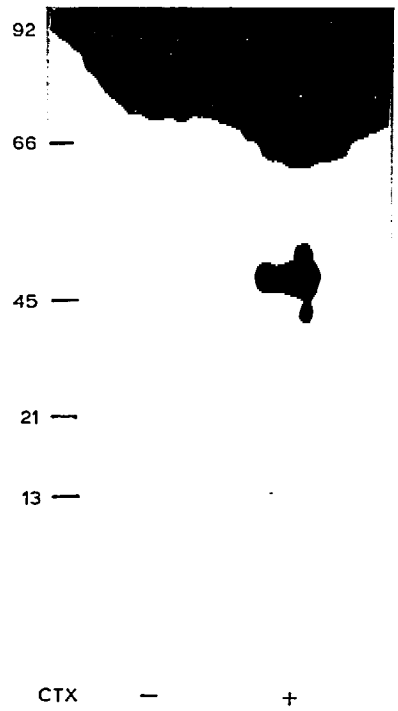

Fig. 4. ADP-ribosylation pattern of liver membranes after cholera toxin treatment. Liver membranes were incubated in the presence or in the absence of 100 μg/ml cholera toxin (CTX) as indicated in methods except that 10 μmol/l [^{32}P]NAD ($6 \cdot 10^7$ cpm/nmol) instead of unlabelled NAD was used, and the incubation mixture was fortified with a cocktail of proteinase inhibitors containing (final concn.) 0.25 mM PMSF, 1 mM benzamidine, 10 μg/ml trypsin inhibitor, 0.2 U/ml aprotinin, 25 μg/ml leupeptin, 10 μg/ml bacitracin. After incubation, 20 μl of a solution of 2 M DTE containing 10% SDS were added, and after keeping 1 h at 56°C the samples received 10 μl of glycerol. 50 μl thereof were loaded on a 10% polyacrylamide gel and the electrophoresis was run for 16 h at 40 V. Molecular mass is indicated on the left (kDa) and standards were: lysozyme, 14.4; soybean trypsin inhibitor, 21.5; carbonic anhydrase, 31; ovalbumin, 45; bovine serum albumin, 66.2; phosphorylase *b*, 97,5.

after treatment of liver membranes with cholera toxin [10]. To the contrary, recent reports in the literature suggest that PLC is inhibited by cholera toxin, suggesting a cholera-toxin-sensitive G-protein distinct from G_s linked to adenylate cyclase [2,3].

A reduction of polyphosphoinositide labelling in rat liver plasma membranes upon preincubation with cholera toxin has also been described by Biffen and Martin [14]. It was suggested that this results from ADP-ribosylation of a G-protein and

consequent inhibition of PI kinase. In our experiments (Fig. 3), cholera toxin treatment affected only the synthesis of PIP_2, suggesting that PIP kinase rather than PI kinase is the target of inhibition. Stimulation of the formation of PIP by EGF has been described in A 431 cells [15] and in membranes derived from A 431 cells treated with EGF [16]. Treatment of whole cells (but not of membranes) with cholera toxin abolished the stimulatory effect of EGF on PI phosphorylation, suggesting involvement of a guanine-nucleotide-binding protein in PI phosphorylation [16].

In conclusion, this study shows that in liver membranes the enzymes of the polyphosphoinositide pathway: PI kinase and PIP-kinase are subject to activation by stable GTP analogues. Furthermore, cholera toxin suppressed the activation of PIP kinase which appears to be the rate-limiting step in the conversion of PI to PIP_2. This suggests the involvement of a novel G-protein with an affinity for [γS]pppG that is lower than that of the G-proteins which activate PLC.

Acknowledgements

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