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Pancreastatin Activates Pertussis Toxin-Sensitive Guanylate Cyclase and Pertussis Toxin-Insensitive Phospholipase C in Rat Liver Membranes

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Abstract We have recently found the calcium dependent glycogenolytic effect of pancreastatin on rat hepatocytes and the mobilization of intracellular calcium. To further investigate the mechanism of action of pancreastatin on liver we have studied its effect on guanylate cyclase, adenylate cyclase, and phospholipase C, and we have explored the possible involvement of GTP binding proteins by measuring GTPase activity as well as the effect of pertussis toxin treatment of plasma liver membranes on the pancreastatin stimulated GTPase activity and the production of cyclic GMP and myo-inositol 1,4,5-triphosphate.

Pancreastatin stimulated GTPase activity of rat liver membranes about 25% over basal. The concentration dependency curve showed that maximal stimulation was achieved at 10^{-7} M pancreastatin (EC_{50} = 3 nM). This stimulation was partially inhibited by treatment of the membranes with pertussis toxin. The effect of pancreastatin on guanylate cyclase and phospholipase C were examined by measuring the production of cyclic GMP and myo-inositol 1,4,5-triphosphate respectively. Pancreastatin increased the basal activity of guanylate cyclase to a maximum of 2.5-fold the unstimulated activity at 30°C, in a time- and dose-dependent manner, reaching the maximal stimulation above control with 10^{-7} M pancreastatin at 10 min (EC_{50} = 0.6 nM). This effect was completely abolished when rat liver membranes had been ADP-ribosylated with pertussis toxin. On the other hand, adenylate cyclase activity was not affected by pancreastatin. Phospholipase C activity of rat liver membranes was rapidly stimulated (within 2–5 min) at 30°C by 10^{-7} M pancreastatin, reaching a maximum at 15 min. The dose response curve showed that with 10^{-7} M pancreastatin, maximal stimulation was obtained (EC_{50} = 3 nM). GTP (10^{-5} M) stimulated the membrane-bound phospholipase C as expected. However, the incubation of rat liver membranes with GTP partially inhibited the stimulation of phospholipase C activity produced by pancreastatin, whereas GTP enhanced the activation of phospholipase C by vasopressin. This inhibition by GTP was dose dependent and 10^{-5} M GTP obtained the maximal inhibition (about 40%). The inhibitory effect of GTP on the stimulatory effect of pancreastatin on phospholipase C activity was completely abolished when rat liver membranes had previously been ADP-ribosylated with pertussis toxin. The presence of 8-Br-cGMP mimics the effect of GTP, whereas GMP-PNP increased both basal and pancreastatin-stimulated phospholipase C, suggesting a role of the cyclic GMP as a feed-back regulator of the synthesis of myo-inositol 1,4,5-triphosphate. However, the pretreatment of membranes with pertussis toxin did not modify the production of myo-Inositol 1,4,5-triphosphate stimulated by pancreastatin.

In conclusion, pancreastatin activates guanylate cyclase activity and phospholipase C involving different pathways, pertussis toxin-sensitive, and -insensitive, respectively.

Key words: phosphatydilinositol 1,4,5-triphosphate, cGMP, peptide, hepatocyte, calcium

INTRODUCTION

Pancreastatin, a 49 amino acid residues comprising peptide with a C-terminal glycinamide, was isolated from porcine pancreas by Tatemoto et al. [1986] and a role as a regulatory gastroenteropancreatic hormone has been established in the light of a variety of biological effects which could be assigned to the C-terminal part of the molecule [see Schmidt and Creutzfeldt, 1991 for review]. From several lines of evidence, it is now

Abbreviations: AMP-PNP, adenosyl-5′-yl-imidophosphate; cGMP, cyclic GMP; 8-Br-cGMP, 8-Bromo-cyclic GMP; GMP-PNP, guanosyl-5′-imidophosphate; $InsP_3$, myo-inositol 1,4,5-triphosphate.

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established that pancreastatin arises from proteolytic cleavage of its precursor chromogranin A, a glycoprotein present in endocrine and neuronal cells [Iancangelo et al., 1986, 1988a,b]. Rat chromogranin A cDNA revealed the existence of a pancreastatin-like sequence, homologous to porcine pancreastatin [Hutton et al., 1988: Abood and Eberwine, 1990; Iancangelo et al., 1988a; Parmer et al., 1989]. Synthetic rat pancreastatin has also been shown to have biological activity in different tissues [Funakoshi et al., 1989; Miyasata et al., 1990; Peiró et al., 1991]. With regard to the mechanism of action of pancreastatin, it has been shown the reversion of the inhibition of insulin secretion by pertussis toxin pretreatment in RINm5F cells [Lorinet et al., 1989] as well as the increase in the intracellular free calcium concentration in the same cell line [Sánchez-Margalet et al., 1992a].

We have previously described that pancreastatin produces hepatic glycogenolysis both in vivo and in vitro [Sánchez et al., 1990; Sánchez-Margalet et al., 1992a; Sánchez et al., 1992], an effect dependent on calcium. Recently, we have also demonstrated the effect of rat pancreastatin on intracellular calcium concentration in rat hepatocytes [Sánchez-Margalet et al., 1993]. This effect of pancreastatin increasing intracellular calcium turned out to be mediated by both pertussis toxin-sensitive and -insensitive mechanisms. We have also identified and characterized rat pancreastatin receptors coupled to a GTP-binding protein in rat liver membranes [unpublished data]. These findings suggested the role of pancreastatin as a calcium-mobilizing hormone with a receptor-mediated effect in the liver.

In many systems, hormones whose transduction system involves a GTP-binding protein increase specific GTPase activity. Thus, hormone-stimulated GTPase activity have been used as evidence for the involvement of a GTP-binding protein in hormone responses [Fain et al., 1985; Fitzgerald et al., 1986].

There is experimental evidence indicating a role for $InsP_3$ as a second messenger for receptor-mediated intracellular calcium mobilization in liver [see Exton, 1988 for review] and other tissues [Berridge, 1987; Guillon et al., 1992], involving several GTP-binding protein [see Harden, 1992 for review]. Guanine-nucleotide and hormone regulation of membrane-bound polyphosphoinositide phospholipase C activity in rat liver plasma membranes have been demonstrated [Uhing et al., 1986; Taylor and Exton, 1987]. Pertussis toxin is known to inhibit the function of the inhibitory G protein of the adenylate cyclase system by ADP-ribosylation of its alpha subunit [Gilman, 1984], and also inhibits agonist- and guanine nucleotide-stimulated phosphoinositide breakdown in some cell types [see Cockcroft, 1987 for review] but not in liver [Uhing et al., 1986].

Cyclic GMP is considered as an important second messenger across the Animal Kingdom in almost every type of cell [see Chinkers, 1991 for review], although its cellular function is not completely known. Membrane-bound guanylyl cyclases represent a recently discovered family of proteins whose activity is stimulated by different peptide hormones such as atrial natriuretic and related peptides [see Koesling et al., 1991 for review]. Changes in the intracellular concentration of cyclic GMP may also affect the inositol phosphate metabolism by inhibiting hormone-stimulated phospholipase C and GTPase activity, implying that cyclic GMP may be involved in a feedback inhibition of phosphatidylinositol hydrolysis [Hirata et al., 1990; Takai et al., 1981; Nakashima et al., 1986], as well as the removal of calcium from the cytoplasm by activating Ca^{2+}-ATPase [Nakashima et al., 1986; Cornwell and Lincoln, 1989].

The purpose of the present study was to examine the molecular mechanism underlying the glycogenolytic effect of pancreastatin on rat liver and the mechanism of its calcium mobilization by investigating the possible role of three transduction systems, i.e., the membrane-bound phospholipase C, adenylate-cyclase, and guanylate-cyclase, on the receptor-mediated pancreastatin action in the liver, as well as the possible role of GTP-binding proteins by measuring the pancreastatin-stimulated GTPase activity and studying the influence of pertussis toxin on the pancreastatin-stimulated production of these second messengers.

MATERIALS AND METHODS

Membranes Preparation

Animal used were male Wistar rats (150–200 g) fed ad libitum. Rat liver membranes were prepared according to Neville [1968] up to step 11. The protein concentrations were determined using a protein assay kit (Bio-Rad) based on the method of Bradford [1976], with bovine serum albumin as standard.

Pertussis toxin treatment of liver membranes. The treatment of membranes with thiol-preactivated pertussis toxin was carried out as described by Ribeiro-Neto et al. [1985]. The control membranes were treated in an identical manner except there was no toxin present in the incubation. The membrane suspension was then centrifuged and the pellet washed twice and finally resuspended in Hepes buffer (20 mM, pH 7.5), containing bacitracin (0.1 mg/ml) and leupeptin (0.1 mg/ml). ADP ribosylation was assessed by the reversion of the inhibitory effect of angiotensin II on glucagon-stimulated cAMP production as previously described [Pobiner et al., 1985].

GTPase assay. The GTPase activity of the membranes (20 μg) was measured in the presence of Hepes buffer (30 mM, pH 7.5) containing EDTA (0.3 mM), bovine serum albumin (2 mg/ml), bacitracin (0.2 mg/ml), benzamidin (0.5 mM), leupeptin (0.1 mg/ml), NaCl (100 mM), $MgCl_2$ (3 mM), ATP (1 mM), AMP-PNP (1 mM), GTP (0.01 μM), [γ-^{32}P]GTP (0.1 μM, 225 Ci/mmol), and an ATP regenerating system consisting in creatine kinase (0.1 mg/ml) and creatine phosphate (2 mM). The assay was preincubated for 3 min at 25°C and the reaction was initiated by the addition of the radiolabeled nucleotide and further incubated for 15 min at 25°C in a final volume of 100 μl. The assay was stopped by the addition of 900 μl of a suspension of activated charcoal in 20 mM KH_2PO_4, pH 2. The amount of radioactivity in 700 μl of the supernatant was quantitated in a scintillation counter (Wallac 1409). The non-specific amount of [γ-^{32}P]GTP hydrolyzed in the presence of an excess of GTP (30 μM) was discounted in every experiment.

Adenylate cyclase assay. In a final volume of 0.2 ml, the incubation medium contained 50 mM tris-HCl buffer, pH 7.5, bovine serum albumin (2 mg/ml), bacitracin (0.2 mg/ml), leupeptin (0.1 mg/ml), 5 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine, 0.1 mM ATP, and the ATP regenerating system described above. The incubation medium was equilibrated for 3 min at 30°C, and the reaction was initiated by adding membranes (60 μg), conducted for 15 min at 30°C, and stopped by adding 300 μl ice-cold ethanol. The proteins were pelleted and the cyclic AMP was determined in the dried supernatant by radioimmunoassay (Amersham International plc, U.K.)

Guanylate cyclase assay. In a final volume of 0.1 ml, the incubation medium contained 40 mM tris-HCl buffer, pH 7.5, bovine serum albumin (2 mg/ml), bacitracin (0.2 mg/ml), leupeptin (0.1 mg/ml), 6 mM $MgCl_2$, 2 mM 3-isobutyl-1-methylxanthine, 1 mM GTP, and the GTP regenerating system (creatine kinase/creatine phosphate). The incubation medium was equilibrated for 3 min at 30°C, and the reaction initiated by the addition of membranes (200 μg), further incubated for 15 min at 30°C, and stopped by the addition of 300 μl ice-cold ethanol. Cyclic GMP was determined, after ethanol precipitation of proteins, in the dried supernatant by a radioimmunoassay (Amersham International plc, U.K.).

Phospholipase C assay. Phospholipase activity was assayed at 30°C, in a final volume of 0.5 ml, containing 50 mM Hepes, pH 7.4, bovine serum albumin (2 mg/ml), bacitracin (0.2 mg/ml), leupeptin (0.1 mg/ml), and $MgCl_2$ (5 mM). Free Ca^{2+} concentrations were maintained at 0.5 μM with Ca^{2+}/EGTA buffers with 0.5 mM EGTA and 0.5 mM Ca^{2+}. When GTP was present, creatine phosphate (2 mM) and creatine kinase (0.1 mg/ml) was also added. The incubation medium was equilibrated for 3 min at 30°C. The assay was initiated by the addition of membranes (500 μg) and terminated with 100 μl ice-cold 20% (v/v) perchloric acid and kept on ice for 20 min. Proteins are sedimented by centrifugation and supernatants are tritiated to pH 7.5 with 10 M KOH and kept ice cold. Precipitated $KClO_4$ is sedimented by centrifugation and myo-Inositol 1,4,5-triphosphate was determined in the supernatant by a radioimmunoassay from Amersham (U.K.) using D-myo-Inositol 1,4,5-triphosphate[^{3}H] as a tracer. Further purification on 1 ml anion exchange columns (trimethylaminopropyl SAX from Amersham plc, U.K.) did not improve direct $InsP_3$ assay of samples.

Materials

Rat pancreastatin was provided by Peninsula Laboratories Europe (Merseyside, U.K.). Arg-vasopressin, 3-isobutyl-1-methylxanthine, creatine kinase, and creatine phosphate were from Boehringer Mannheim (Mannheim, Federal Republic of Germany). Porcine glucagon was obtained from Novo Biolabs (Bagsvaerd, Denmark) Pertussis toxin, Bacitracin, leupeptine, dithiothreitol, bovine serum albumin (fraction V), GTP, AMP, AMP-PNP, and EGTA were from Sigma (St. Louis, MO). [γ-^{32}P]GTP was

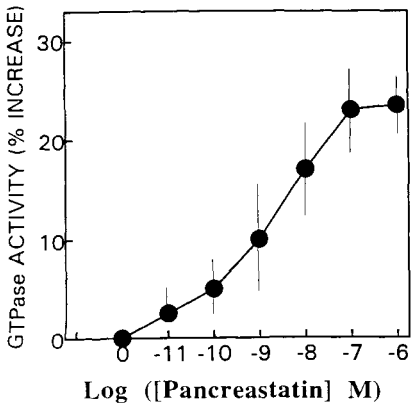

Fig. 1. Dose-response of the pancreastatin-stimulated GTPase activity. Membranes (20 μg) were incubated at 30°C for 15 min in the presence of different pancreastatin concentrations to determine GTPase activity as described under Materials and Methods. Basal GTPase activity was about 1 pmol/min/mg protein. The values of each experiment were based on cuadriplicates tubes. Data are means ± SEM (n = 4) of the percentage of increase above basal GTPase activity.

from New England Nuclear (Du Pont de Nemours, Germany).

RESULTS

Pancreastatin Stimulation of GTPase Activity

Specific high-affinity basal GTPase activity ranged from 0.6 to 1,6 pmol/min/mg of protein with a mean of 1 pmol/min/mg of protein. In the presence of pancreastatin (10^{-7} M), the GTPase activity was increased 20–30% over the basal activity. The dose-response curve for activation of specific GTPase by pancreastatin is shown in Figure 1. Maximal stimulation was observed at 10^{-7} M pancreastatin. Treatment of membranes with pertussis toxin partially inhibited this effect, and pancreastatin only increased 10–20% over the basal GTPase activity, which was also decreased (8–10%) compared to untreated membranes. However, the effect of pertussis toxin on the Pancreastatin stimulated GTPase activity was significant (14.1 ± 1% vs. 24 ± 5%, $P < 0.05$).

Pancreastatin Effect on Guanylate Cyclase Activity

The time-course of cGMP formation from liver membranes at 30°C in the presence of phosphodiesterase inhibitor is shown in Figure 2. Pancreastatin (10^{-7} M) markedly increased the activity of plasma membrane-bound guanylate cyclase to a maximum of 2.5-fold the unstimulated activity at 10 min. The dose response of pancreastatin effect on cyclic GMP production is represented in Figure 3. Maximal elevation upon control values was achieved at 10^{-7} M, with half maximal effect at 0.6×10^{-9} M. This effect was completely inhibited when liver membranes had been pretreated with pertussis toxin (Table I).

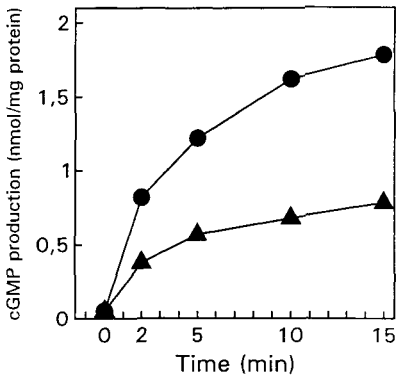

Fig. 2. Time course of membrane-bound guanylate cyclase activity. Effect of pancreastatin. Membranes (200 μg) were assayed at 30°C as described under Materials and Methods in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, with (closed circles) or without 10^{-7} M pancreastatin (closed triangles). Data are the values of one experiment representative of three other.

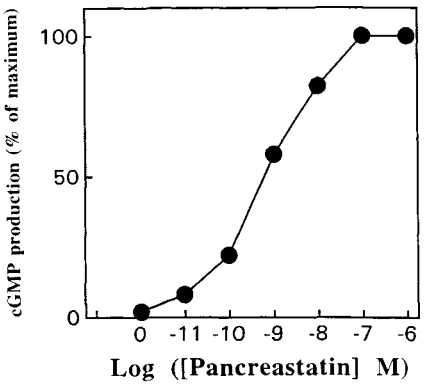

Fig. 3. Concentration dependency of the pancreastatin-stimulated guanylate cyclase activity. Membrane-bound guanylate cyclase was assayed as a function of the pancreastatin concentration in the presence of 1 mM GTP, the GTP regenerating system and the phosphodiesterase inhibitor 3-isobutylmethylxanthine as described under Materials and Methods. Values are means ± SEM (n = 4) of the percentage of maximal cGMP production.

Lack of Effect of Pancreastatin on Adenylate Cyclase Activity

Pancreastatin did not affect basal activity of membrane-bound adenylate cyclase in the presence of phosphodiesterase inhibitor, as expected

TABLE I. Effect of Pertussis Toxin Treatment of Rat Liver Membranes on Guanylate Cyclase Stimulation by Pancreastatin*

Condition	Cyclic GMP production (nmol/mg protein)	
	−PT	+PT
Control	0.8 ± 0.07	0.7 ± 0.09
Pancreastatin (10^{-7} M)	1.8 ± 0.2[a]	0.8 ± 0.08

*Plasma liver membranes (1 mg/ml) were pretreated with thiol-preactivated pertussis toxin (10 μg/ml) in the presence of 10 mM NAD^+, 1 mM ATP, 0.1 mM GTP, 5 mM $MgCl_2$, 1 mM dithiotreitol, bacitracin (0.2 mg/ml), and leupeptin (0.1 mg/ml) in hepes 20 mM, pH (7.4), for 30 min at 30°C. Control membranes of pertussis toxin treatment (PT Control) were incubated in the same conditions but the pertussis toxin was omitted. Membranes were diluted with ice-cold hepes 20 mM, collected by centrifugation and resuspended in Tris-HCl 40 mM, bacitracin (0.2 mg/ml) and leupeptin (0.1 mg/ml). Reaction was conducted in 40 mM tris-HCl buffer, pH 7.5, bovine serum albumin (2 mg/ml), bacitracin (0.2 mg/ml), leupeptin (0.1 mg/ml), 6 mM $MgCl_2$, 2 mM 3-isobutyl-1-methylxanthine, 1 mM GTP, and the GTP regenerating system. The reaction mixture was preincubated for 3 min at 30°C before the addition of the agonists, and further incubated for 15 min. Data are means ± SEM of four separate experiments.
[a]$P < 0.001$ vs. control.

for the results of cAMP production in isolated hepatocytes [Sánchez et al., 1992]. Positive controls of increased production of cAMP were carried out by stimulating adenylate cyclase directly with forskolin, by stimulation of G_S with GTP or NaF, and by receptor-mediated mechanism with glucagon (Table II). Pancreastatin did not modify the cAMP production stimulated by forskolin, GTP, NaF, or glucagon.

Pancreastatin Effect on Membrane-Bound Phospholipase C Activity

Pancreastatin (10^{-7} M) caused a 7-fold stimulation of basal phospholipase C activity in the absence of GTP. The time-course of the pancreastatin-stimulated $InsP_3$ production is shown in Figure 4. In the presence of 10^{-5} M GTP, pancreastatin produced only 60% of the $InsP_3$ produced in its absence. However, GTP (10^{-5} M) increased 2-fold basal phospholipase C activity of liver membranes (Fig. 4). On the other hand, the effect of vasopressin on phospholipase C activity was strongly stimulated by GTP (10^{-5} M) (Table III), as should be expected [Uhing et al., 1986; Taylor and Exton, 1987]. Moreover, GMP-NHP (10^{-5} M) increased 5-fold basal $InsP_3$ production and enhanced the phospholipase C activity stimulated by pancreastatin or vasopres-

TABLE II. Effect of Pancreastatin on Adenylate Cyclase Activity*

Condition	Cyclic AMP production (pmol/min/mg protein)
Control	21 ± 5
Pancreastatin (10^{-7} M)	27 ± 6
GTP (10^{-5} M)	42 ± 7[a]
Glucagon (10^{-7} M)	100 ± 10[a]
Forskolin (10^{-4} M)	145 ± 12[a]
NaF (10^{-2} M)	140 ± 11[a]
Pancreastatin (10^{-7} M) + GTP (10^{-5} M)	48 ± 9[a]
Pancreastatin (10^{-7} M) + Glucagon (10^{-7} M)	104 ± 14[a]
Pancreastatin (10^{-7} M) + Forskolin (10^{-4} M)	143 ± 11[a]
Pancreastatin (10^{-7} M) + NaF (10^{-2} M)	142 ± 12[a]

*Membranes (60 μg) were incubated for 15 min at 30°C in 50 mM tris-HCl buffer, pH 7.5, bovine serum albumin (2 mg/ml), bacitracin (0.2 mg/ml), leupeptin (0.1 mg/ml), 5 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine, 0.1 mM ATP, and the ATP regenerating system. The reaction mixture was preincubated for 3 min at 30°C before the addition of the agonists. The values of each experiment were based on the means of duplicate tubes. Data are means ± SEM of five separated experiments, using two different membranes preparation.
[a]$P < 0.001$ vs. control.

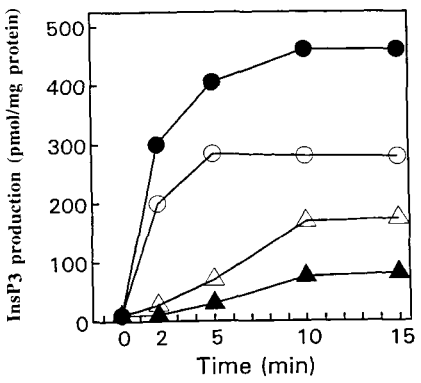

Fig. 4. Time-course of membrane-bound phospholipase C activity. Effect of pancreastatin and GTP. Membranes (500 μg) were incubated at 30°C as described under Materials and Methods in the presence of 0.5 μM Ca^{2+} to determine phospholipase C activity. The reaction was started by the addition of the membranes to the tubes under the following conditions: buffer (closed triangles), 10^{-5} M GTP (open triangles), 10^{-7} M pancreastatin (closed circles), or 10^{-7} M pancreastatin + 10^{-5} M GTP (open circles). Results are the values of one experiment representative of three other.

TABLE III. Effect of Pertussis Toxin Pretreatment of Liver Membranes on Pancreastatin-Stimulated and/or GTP-Stimulated Phospholipase C Activity*

Condition	$InsP_3$ production (pmol/mg protein) −PT	+PT
Control	70 ± 8	66 ± 7
GTP (10^{-5} M)	170 ± 14[a]	162 ± 12[a]
Pancreastatin (10^{-7} M)	460 ± 18[a]	446 ± 17[a]
Pancreastatin (10^{-7} M) + GTP	180 ± 12[a,c]	434 ± 21[a,b]
Pancreastatin + GMP-PNP	540 ± 23[a]	535 ± 22[a]
Vasopressin	370 ± 15[a]	364 ± 16[a]
Vasopressin + GTP	482 ± 15[a,c]	480 ± 17[a,c]
Vasopressin + GMP-PNP	513 ± 20[a,c]	510 ± 22[a,c]

*Membranes were pretreated with or without pertussis toxin as described in the legend to Table I. Liver plasma membranes (500 μg) were incubated for 10 min at 30°C in Hepes 50 mM containing 0.5 μM free Ca^{2+} concentration with 0.5 mM EGTA and 0.5 mM Ca^{2+}. When GTP was present, creatine phosphate (2 mM) and creatine kinase (0.1 mg/ml) was also added. The incubation medium was equilibrated for 3 min at 30°C before the addition of the agonists (10^{-7} M) and further incubated for 10 min. Data are means ± SEM (n = 5).

[a]$P < 0.001$ vs. control.

[b]$P < 0.001$ vs. the same condition (−PT).

[c]$P < 0.001$ vs. the same condition (−GTP).

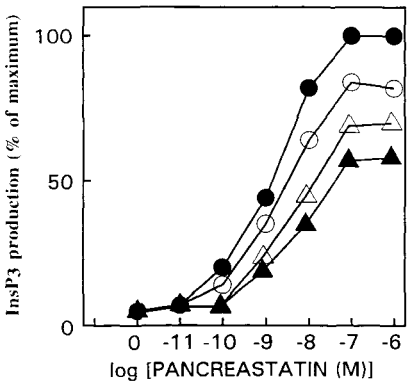

Fig. 5. Concentration dependency of the pancreastatin-stimulated phospholipase C activity and the GTP inhibition of this effect. Membrane-bound phospholipase activity was measured as a function of pancreastatin concentration in the absence (closed circles) or presence of different GTP concentrations: No GTP (closed circles), 10^{-7} M GTP (open circles), 10^{-6} M (open triangles), and 10^{-5} M (closed triangles). Data are the means of three separate experiments.

sin. The stimulatory effect of pancreastatin was dose-dependent as well as the partial inhibition of this effect by GTP (Fig. 5).

Maximal $InsP_3$ production was obtained with 10^{-7} M pancreastatin and maximal inhibition of this effect was achieved at 10^{-5} M GTP. GTP did not modify the ED_{50} of pancreastatin (3 nM) but impaired its maximal effect in a dose-dependent manner. The treatment of liver membranes with pertussis toxin did not affect the ability of pancreastatin to stimulate $InsP_3$ production (Table III). However, such a treatment completely abolished the inhibitory effect of GTP on pancreastatin-stimulated $InsP_3$ production, but not the stimulatory effect of vasopressin or GMP-PNP as expected [Berridge, 1987]. Besides, the enhancement in both basal and pancreastatin-stimulated phospholipase C activity by GMP-PNP was not affected by pretreatment of plasma liver membranes with pertussis toxin.

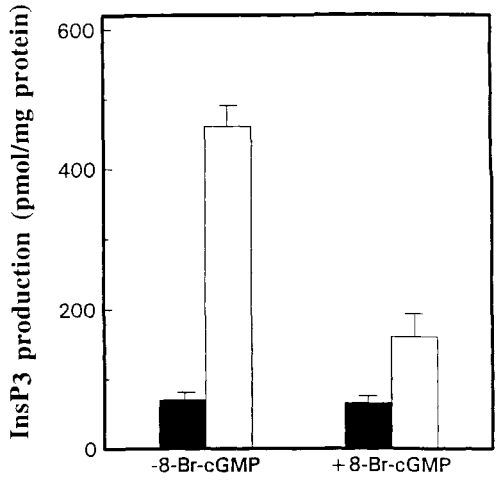

Fig. 6. Effect of 8-Br-cGMP on pancreastatin-stimulated $InsP_3$ production. The phospholipase C assay was conducted as described in the legend to Figure 4 and under Materials and Methods, except that buffer or 1 mM 8-Br-cGMP was added to the membranes 2 min before starting the reaction. Open bars refer to controls, and closed bars are the pancreastatin-stimulated liver membranes. Data are means of three separate experiments.

Effect of 8-Br-cGMP on Pancreastatin-Stimulated Phospholipase C Activity

To elucidate the hypothesis that cGMP could be implicated in the inhibition of pancreastatin-stimulated $InsP_3$ production by GTP, we investigated the effect of the analog 8-Br-cGMP (1 mM) on the pancreastatin-stimulated phospholipase C activity. As shown in Figure 6, 8-Br-cGMP inhibited the pancreastatin-stimulated $InsP_3$ production to half values, i.e., the same extent as that produced by GTP, whereas it had no effect on basal phospholipase C activity. However, 8-Br-cGMP did not affect the vasopressin-stimulated phospholipase C activity of the membranes (not shown).

DISCUSSION

We have recently evidentiated the glycogenolytic effect of pancreastatin in the rat liver [Sánchez et al., 1990; Sánchez-Margalet et al., 1992b, Sánchez et al., 1992]. This effect of pancreastatin in isolated hepatocytes was shown to be independent of the production of cAMP and dependent of calcium [Sánchez et al., 1992]. Moreover, we further studied the pancreastatin effect on calcium mobilization, demonstrating that two separate GTP binding proteins should be involved in the mechanism of action of pancreastatin [Sánchez-Margalet et al., 1993]. In fact, pretreatment of hepatocytes with pertussis toxin only affected partially the pancreastatin-stimulated intracellular Ca^{2+} increase in the presence of extracellular calcium. However, such a treatment did not affect calcium mobilization when calcium was omitted from the incubation medium. We have just found the presence of pancreastatin receptors in rat liver membranes, and its binding to the hormone was very dependent on the presence of guanine nucleotides [unpublished data]. The inhibition of the binding by GTP suggested that pancreastatin receptor may be coupled to a G protein.

Hormone stimulation of GTPase activity has been used as evidence for the involvement of a GTP-binding protein in hormone responses. Thus, low K_m GTPase activity has been demonstrated to be stimulated in different membranes preparations by numerous agonists. In this context, we assayed specific GTPase activity of rat plasma liver membranes and we found a dose-dependent stimulation by pancreastatin (Fig. 1). The per cent stimulation over the basal GTPase activity was of the same magnitude as previously reported in liver membranes [Fain et al., 1985]. ADP-ribosylation of the membranes with pertussis toxin did not abolished the pancreastatin-stimulated GTPase activity, but was partially inhibited. It seems that two distinct mechanisms (G protein) are involved in the stimulation of GTPase activity by pancreastatin, and it is consistent with the previous observation that pancreastatin-mobilization of calcium seems to be mediated by both pertussis toxin-sensitive and -insensitive mechanisms.

The lack of effect of pancreastatin on adenylate cyclase activity in rat liver plasma membranes is in accordance with the previously described lack of effect of pancreastatin on basal and glucagon-stimulated cyclic AMP production in isolated hepatocytes. The present study demonstrates that the recently known calcium-mobilizing hormone pancreastatin stimulates the synthesis of $InsP_3$ in rat liver plasma membranes. There is now enough provided experimental evidence to indicate a role for $InsP_3$ as a second-messenger for receptor-mediated, intracellular Ca^{2+} mobilization in liver [see Exton, 1988 for review], although a role for inositol 1,3,4,5-tetrakisphosphate [Hansen et al., 1986] allowing the influx of extracellular Ca^{2+} has been suggested [Irvine and Moor, 1986]. The concentration dependency of the pancreastatin effect is very similar to that observed in hepatocytes for the calcium-dependent stimulation of glycogenolysis [Sánchez et al., 1992] and the increase in intracellular free Ca^{2+} concentration [Sánchez-Margalet et al., 1993]. However the EC_{50} of pancreastatin to increase the synthesis of $InsP_3$ (3 nM) is higher than that observed in the increase in intracellular free Ca^{2+} [Sánchez-Margalet et al., 1993] as well as the K_d found for the pancreastatin receptor in rat liver membranes (0.2 nM) (unpublished data). This apparent disparity could be explained by a mechanism of amplification of the signaling transduction system, as previously suggested for agonists that mobilize Ca^{2+} [Creba et al., 1983].

The data presented here show that pancreastatin stimulation of phospholipase C activity is unaffected by treatment of the plasma membrane with pertussis toxin plus NAD as it occurs for vasopressin. These results could suggest that the putative guanine nucleotide binding protein involved in pancreastatin-stimulated calcium mobilization may be the same from that coupled to vasopressin. Moreover, the pancreastatin-stimulated synthesis of $InsP_3$ seems to occur in the absence of GTP as observed for vasopressin, although nanomolar concentration of GTP could be present in membrane preparations, since it has been demonstrated the complete dependency of the $InsP_3$ release induced by calcium-dependent hormones upon the presence of Ca^{2+} and GTP [Uhing et al., 1986]. However, the presence of added GTP seems to partially negatively modulate this effect of pancreastatin, in spite of the fact that GTP can by itself activate plasma membrane-bound phospholipase C (Fig. 4, Table III). A competitive inhibition at the receptor level should be ruled out, since the inhibition turned out to be non-competitive as shown in Figure 5. It has been previously suggested that cyclic GMP may be involved in a feedback inhibition of phosphatidyl inositol hydrolysis in other systems [Hirata et al., 1990;

Takai et al., 1981; Nakashima et al., 1986]. The fact that pancreastatin stimulates the plasma membrane-bound guanylate cyclase in the presence of GTP and the GTP generating system could point a role to the cyclic GMP synthesized as a terminate signal to reduce $InsP_3$ levels and thus acting as a feed-back regulator of the metabolism of myoinositol in plasma liver membranes. This effect could be useful to restore the system to a state that could produce a maximal response soon after the hormone challenge. This hypothesis is further supported by the abolishment of this GTP inhibition of pancreastatin-stimulated $InsP_3$ formation when the membranes are treated with pertussis toxin, at the same time as the production of cyclic GMP is completely abolished. Moreover, the non-hydrolyzable GTP analog GMP-PNP not only failed to inhibit the stimulatory effect of pancreastatin on $InsP_3$ production, but, even enhanced this effect. A similar enhancement by GMP-PNP was observed in vasopressin-stimulated $InsP_3$ production in liver plasma membranes.

To finally seek the possible role of the cGMP produced by pancreastatin stimulation, we checked the effect of the analog 8-Br-cGMP on basal and pancreastatin-stimulated $InsP_3$ production. Recent work [Ruth et al., 1993] shows that cGMP acts by interfering with $InsP_3$ synthesis through the stimulation of the kinase dependent of cGMP, which interacts with phospholipase C. These findings differ from earlier studies, where it was reported that 8-Br-cGMP did not affect the vasopressin-mediated stimulation of inositol phosphate accumulation in hepatocytes [Pittner and Fain, 1989]. In fact, we have not observed any effect of cGMP on the vasopressin-stimulated $InsP_3$ production in plasma liver membranes (data not shown). Besides, the kinase sensitive to cGMP seems to be cytosolic [Ruth et al., 1993]. Thus, these observations may suggest that the possible interaction between the pancreastatin-stimulated increase in cGMP and the inhibition of pancreastatin-stimulated synthesis of $InsP_3$ is not mediated by the kinase and may not be directly exerted at the phospholipase C level. Rather, it suggests an indirect effect of cGMP, which may interfere by itself in the pertussis toxin-insensitive transduction system of the pancreastatin receptor that leads to the activation of phospholipase C, which therefore seems to be different from that coupled to vasopressin-receptor.

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