Pancreastatin activates protein kinase C by stimulating the formation of 1,2-diacylglycerol in rat hepatocytes

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We describe here the stimulation by pancreastatin of 1,2-diacylglycerol production and protein kinase C activity in liver plasma membrane and isolated hepatocytes. The dose-dependency for the stimulation of both processes was similar to the recently described pattern of glucose output and cytosolic Ca^{2+} transients produced by pancreastatin. The time course of diacylglycerol production at 30 °C showed a rapid increase within 5 min, reaching a maximum at 10 min. Protein kinase C from hepatocytes was dependent on Ca^{2+} and phosphatidylserine. Neither the pancreastatin-stimulated diacylglycerol production nor the activation of protein kinase C was affected by pretreatment with pertussis toxin. However, the presence of GTP partially inhibited this pancreastatin stimulation of 1,2-diacylglycerol in a dose-dependent manner, although GTP alone stimulates diacylglycerol accumulation. This inhibitory effect of GTP on pancreastatin stimulation of diacylglycerol synthesis was completely abolished by the pretreatment with pertussis toxin. In conclusion, this study provides evidence that pancreastatin stimulates the formation of 1,2-diacylglycerol by a pertussis-toxin-independent mechanism, which may be responsible for the pancreastatin activation of protein kinase C.

INTRODUCTION

Pancreastatin is a 49-amino-acid peptide, initially isolated from pig pancreatic tissue [1], which was first shown to have an inhibitory effect on insulin secretion [1–3], but whose physiological role, especially in humans, is not completely understood. However, a role as a regulatory pancreatic 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 (for review see [4]). We have reported the glycogenolytic effect of pancreastatin in the rat [5–7]. That effect was found to be dependent on Ca^{2+} and independent of cyclic AMP [7]. The hydrolysis of phosphatidylinositol 4,5-bisphosphate by Ca^{2+}-mobilizing hormones represents a bifurcation in the signal pathway in that it results in the formation of two separate second messengers, i.e. Ins(1,4,5)P_3 and diacylglycerol (DAG) [8]. The primary function of InsP_3 is to mobilize Ca^{2+} from intracellular stores [9,10], whereas DAG stimulates protein kinase C (PKC) [11,12].

We have recently shown the increase in cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) which may be responsible for the triggering of the glycogenolytic response to pancreastatin in isolated hepatocytes by a process which appears to involve both pertussis-toxin-sensitive and -insensitive mechanisms [13]. In the present work, we studied the production of DAG from rat liver membranes in response to pancreastatin and the influence of GTP. We used pertussis toxin to seek a mediation of the effect by a pertussis-toxin-sensitive mechanism. Moreover, the activity of PKC in response to pancreastatin has been determined in isolated rat hepatocytes.

MATERIALS AND METHODS

Chemicals

Rat pancreastatin was provided by Peninsula Laboratories Europe (Merseyside, U.K.). [Arg]-vasopressin, 3-isobutyl-1-methylxanthine, creatine kinase and phosphocreatine were from Boehringer Mannheim (Mannheim, Germany). Pertussis toxin, bacitracin, leupeptin, dithiothreitol, BSA (fraction V), GTP, guanosine 5′-[$\beta\gamma$-imido]triphosphate (p[NH]ppG), EGTA, α-L-phosphatidylserine and lysine-rich histone were from Sigma (St. Louis, MO, U.S.A.). [γ-^{32}P]GTP and [α-^{32}P]NAD^+ were purchased from New England Nuclear (Du Pont de Nemours, Dreieich, Germany).

Isolation of hepatocytes and preparation of membranes

Isolated hepatocytes were prepared from male Wistar rats (180–220 g) fed on a standard diet *ad libitum*. Hepatocytes were prepared by perfusion of the liver with collagenase as described previously [7]. Rat liver membranes were prepared as described by Neville [14] up to step 11. The protein concentrations were determined with a protein assay kit (Bio-Rad) based on the method of Bradford [15], with BSA 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. [16]. The control membranes were treated in an identical manner, except that 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). The ADP-ribosylation was assessed by testing the reverse effect of angiotensin II on glucagon-stimulated cyclic AMP production as previously described [17]. To check whether the ADP-ribosylation of the pertussis-toxin substrate was complete, we performed the radiolabelling by incubating for 1 h at 30 °C with [^{32}P]NAD^+ and preactivated pertussis toxin as previously described [16], after preincubation of the membranes for 4 h with or without pertussis toxin.

Abbreviations used: DAG, diacylglycerol; PKC, protein kinase C; $[Ca^{2+}]_i$, cytosolic free $[Ca^{2+}]$; p[NH]ppG, guanosine 5′-[$\beta\gamma$-imido]triphosphate.

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DAG assay

DAG production was assayed at 30 °C, in a final volume of 0.5 ml, containing 50 mM Hepes, pH 7.4, BSA (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, phosphocreatine (2 mM) and creatine kinase (0.1 mg/ml) were also added. The incubation medium was equilibrated for 3 min at 30 °C. The assay was initiated by addition of membranes (250 μg) and terminated with 1 ml of HCl (0.01 M) and 3 ml of chloroform/methanol (2:1, v/v). Then 0.06 ml of $MgCl_2$ (0.5 %, w/v) was added. After vigorous mixing, the samples were centrifuged at 5000 *g* for 5 min. The aqueous layer was discarded and 1 ml of chloroform was added to the bottom layer, which was then filtered through glass wool in a Pasteur pipette. The solvent was evaporated under N_2 at room temperature and stored at −20 °C for 24 h until the assay for DAG analysis. Samples were assayed by the DAG kinase described by Preiss et al. [18], with a DAG assay reagent system from Amersham International (Amersham, Bucks., U.K.), which only measures *sn*-1,2-diacylglycerol, which is the physiological activator of PKC. γ-[^{32}P]ATP was used at a specific radioactivity of 3000 Ci/mmol. [^{32}P]Phosphatidic acid from the samples was purified by chromatography (Amprep minicolumns, from Amersham International). The final eluate (2 ml of chloroform:methanol:acetic acid, 13:2:2, by vol.) was transferred to scintillation vials and 10 ml of scintillant was added for counting in a liquid-scintillation counter (Wallac 1490).

PKC assay

Hepatocytes were incubated in Krebs–Ringer bicarbonate medium as described previously [7] at 37 °C for 10 min with shaking (60 cycles/min) and gassed with O_2/CO_2 (19:1) throughout the incubation. PKC activity was assayed as phospholipid-sensitive Ca^{2+}-dependent phosphorylation of lysine-rich histone (Sigma) as described previously [19], and 1,2-diolein was omitted to avoid masking of the pancreastatin effect due to the increases in DAG. Basal activity, measured by omission of Ca^{2+} and phosphatidylserine, was subtracted from the activity measured in samples. The enzyme fraction was obtained by sonication of hepatocytes in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution, and centrifugation of the homogenate at 15000 *g* to remove debris, nuclei and mitochondria.

RESULTS

Time- and dose-dependence of pancreastatin and GTP-stimulated DAG production

Figure 1 shows the time course of the production of DAG from rat liver membranes. Pancreastatin (0.1 μM) in the absence of GTP caused a 3-fold stimulation of control DAG production after incubation for 10 min. The degree of stimulation was halved by the presence of 10 μM GTP, although GTP (10 μM) alone increased basal DAG production 1.5-fold. The stimulatory effect of pancreastatin was dose-dependent, as was the partial inhibition of this effect by GTP (Figure 2). Maximal DAG production was obtained with 0.1 μM pancreastatin, and this effect was inhibited by the presence of GTP (0.1–10 μM). GTP did not modify the ED_{50} of pancreastatin (3 nM), but impaired its maximal effect in a dose-dependent manner. Unlike the effect of pancreastatin, the effect of vasopressin was enhanced by the presence of GTP (Table 1). The non-hydrolysable GTP analogue p[NH]ppG produced an increase in both vasopressin- and pancreastatin-stimulated DAG production.

Pertussis-toxin treatment of rat liver membranes

The treatment of liver membranes with pertussis toxin did not affect the ability of either pancreastatin or vasopressin to stimulate the production of DAG (Table 1). However, such treatment completely abolished the inhibitory effect of GTP on pancreastatin-stimulated DAG production. The increase in both pancreastatin- and vasopressin-stimulated DAG production produced by p[NH]ppG was not affected by the pretreatment with pertussis toxin.

Autoradiography after SDS/PAGE of the liver plasma membranes with [^{32}P]NAD^+ and pertussis toxin showed an ADP-ribosylated protein of about 40 kDa (Figure 3). To examine whether the pertussis-toxin substrate was completely ADP-ribosylated, the membranes were pretreated with or without

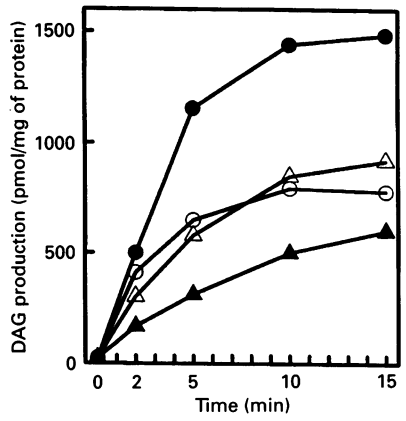

Figure 1 Time course of DAG production: effect of pancreastatin and GTP

Membranes (250 μg) were incubated at 30 °C as described in the Materials and methods section in the presence of 0.5 μM Ca^{2+} to determine the production of DAG. The reaction was started by addition of the membranes to the tubes under the following conditions: ▲, buffer; △, 10 μM GTP; ●, 0.1 μM pancreastatin; ○, 0.1 μM pancreastatin + 10 μM GTP. Results are the values of one experiment representative of three others.

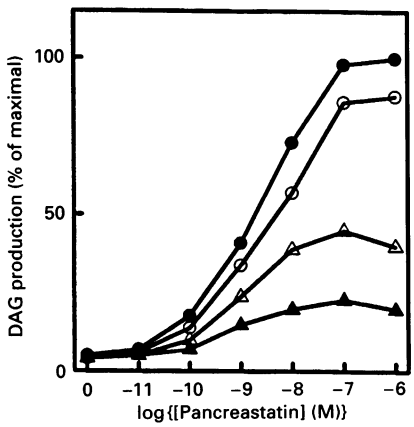

Figure 2 Concentration-dependency of pancreastatin-stimulated DAG production and the inhibition of this effect by GTP

The production of DAG was measured as a function of pancreastatin concentration in the absence (●) or presence of different GTP concentrations: ●, 0; ○, 0.1 μM; △, 1 μM; ▲, 10 μM. Results are given as percentages of the maximal rate of production achieved at 0.1 μM pancreastatin (1440 ± 30 pmol/mg of protein). Data are means of the three separate experiments.

Table 1 Effect of pertussis-toxin pretreatment of liver membranes on pancreastatin-stimulated and/or GTP-stimulated DAG production

Membranes were pretreated with or without pertussis toxin (PT) as described in the Materials and methods section. The incubation medium was equilibrated for 3 min at 30 °C before addition of the agonists (PST, pancreastatin; AVP, vasopressin) and further incubated for 10 min. Data are means ± S.E.M. ($n = 5$): [a] $P < 0.001$ versus control; [b] $P < 0.001$ versus the same condition — PT.

Condition	DAG production (pmol/mg of protein)	
	−PT	+PT
Control	520 ± 16	500 ± 20
GTP (10 μM)	880 ± 18[a]	885 ± 19[a]
PST (0.1 μM)	1440 ± 30[a]	1430 ± 35[a]
PST (0.1 μM) + GTP (10 μM)	200 ± 10[a]	1400 ± 32[ab]
PST (0.1 μM) + p[NH]ppG (10 μM)	1520 ± 38[a]	1511 ± 36[a]
AVP (0.1 μM)	1100 ± 25[a]	1108 ± 24[a]
AVP (0.1 μM) + GTP (10 μM)	1480 ± 32[a]	1471 ± 30[a]
AVP (0.1 μM) + p[NH]ppG (10 μM)	1540 ± 40[a]	1530 ± 35[a]

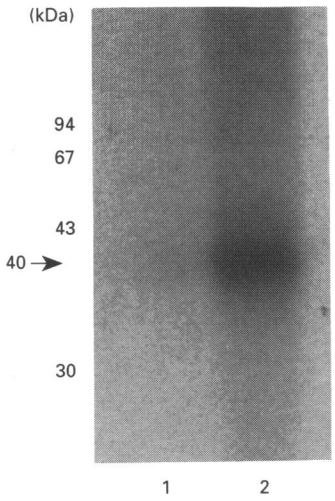

Figure 3 Pertussis-toxin-catalysed ADP-ribosylation of a 40 kDa protein in rat liver plasma membranes

The membranes were pretreated with pertussis toxin (lane 1) or vehicle (lane 2) and then washed and further incubated with pertussis toxin and [^{32}P]NAD$^+$. The proteins were separated by SDS/PAGE and processed by autoradiography.

pertussis toxin for 4 h and then subjected to pertussis-toxin-catalysed ADP-ribosylation in the presence of [^{32}P]NAD$^+$. The incorporation of radiolabel into 40 kDa protein almost completely disappeared after the pretreatment with pertussis toxin (Figure 3).

Pancreastatin stimulation of PKC activity

Basal activity of PKC from incubated hepatocytes was increased up to 10-fold by pancreastatin (Figure 4). This pancreastatin stimulation of PKC activity was dose-dependent, with a significant effect at 0.1 nM pancreastatin (Figure 4). The maximum effect was observed at 10 nM pancreastatin, and half-maximal effect was achieved at about 0.3 nM.

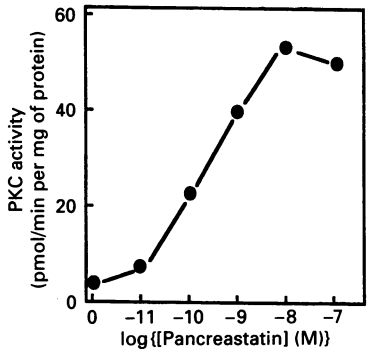

Figure 4 Activation of PKC by pancreastatin

The crude homogenate, prepared as described in the Materials and methods section, were obtained from hepatocytes incubated for 10 min in the absence (0) or the presence of increasing concentrations of pancreastatin (10 pm–0.1 μM). Means ± S.E.M. of four different experiments are shown.

DISCUSSION

DAG activates PKC [12,14] and has been recognized as a second messenger for hormones that cause inositol lipid hydrolysis through the activation of phospholipase C [9]. We have previously demonstrated the role of pancreastatin as a glycogenolytic hormone [5,6], which may exert its action by Ca^{2+} mobilization [7]. In fact, we have described an increase in $[Ca^{2+}]_i$ induced by pancreastatin in isolated hepatocytes which appears to be mediated by pertussis-toxin-sensitive and -insensitive mechanisms [13]. In the present paper, we show the stimulatory effect of pancreastatin on DAG production in rat liver membranes, as well as the activation of PKC. We have observed that the half-maximal concentration of pancreastatin for DAG production is very similar to that for the production of $InsP_3$ [20], as should be expected from the activation of phospholipase C. However, the maximal rate of production of the DAG in response to pancreastatin was achieved at higher concentrations of the hormone than that required for maximal activation of PKC. This may reflect either a biological amplification between the steps of the signalling transduction, or the saturation of PKC by DAG in experiments *in vitro* with intact cells.

Pancreastatin-stimulated production of DAG was not affected by the pretreatment of liver membranes with pertussis toxin. This seems not to be due to an incomplete ADP-ribosylation of the pertussis-toxin substrate, because the membranes pretreated with pertussis toxin could not be further ADP-ribosylated. Moreover, this effect of pancreastatin on DAG production seems to occur in the absence of GTP, as was observed for $InsP_3$ production. In fact, the effect of vasopressin on DAG production was also observed with no GTP added to the incubation mixture. Since GTP is known to be required for phospholipase C activation, we assume that a nanomolar concentration of GTP may be present in membrane preparations. But when GTP was added in the medium, the pancreastatin-stimulated DAG production was inhibited in a dose-dependent manner. A possible explanation is that GTP is impairing the binding of pancreastatin to its receptor by interacting with a G-protein. A similar result should be expected for vasopressin; however, GTP enhanced the DAG production induced by vasopressin. On the other hand, we have observed the pertussis-toxin-sensitive production of cyclic GMP upon pancreastatin stimulation [20], and a role of this cyclic nucleotide is known to be the feedback inhibition of phosphatidyl-

inositol hydrolysis [21]. Therefore, the negative effect of GTP on pancreastatin stimulation of DAG production could be mediated by the formation of cyclic GMP. Thus, after pretreatment of membranes with pertussis toxin, the effect of GTP is abolished. Moreover, the non-hydrolysable analogue of GTP, p[NH]ppG, increases pancreastatin-stimulated DAG production in a similar way as it does the vasopressin-stimulated production of DAG, suggesting a similar pathway for the production of DAG by vasopressin and pancreastatin.

Since the physiological substrate of PKC in the liver has been shown to be glycogen synthase [22,23] by a mechanism that involves phosphorylation of the enzyme [24,25], the inhibition of glycogen synthase may be another expected biological effect of pancreastatin. Thus, studies of the effect of pancreastatin on insulin-stimulated glycogen synthase have shown a potent dose-dependent inhibition over a wide range of insulin concentration [26].

In conclusion, the synergism of both limbs of the phosphatidylinositol 4,5-bisphosphate signalling system, i.e. $Ins P_3/Ca^{2+}$ and DAG/PKC, is confirmed in the transduction of the pancreastatin action in the hepatocyte.

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