ELSEVIER

European Journal of Pharmacology 397 (2000) 229–235

ejp

www.elsevier.nl/locate/ejphar

Characterization of pancreastatin receptor and signaling in rat HTC hepatoma cells

Víctor Sánchez-Margalet *, Carmen González-Yanes, José Santos-Alvarez, Souad Najib

Departamento de Bioquímica Médica y Biología Molecular, Facultad de Medicina, Unidad de Investigación, Hospital Universitario Virgen Macarena, Universidad de Sevilla, Av. Sánchez Pizjuan 4, 41009 Sevilla, Spain

Received 19 August 1999; received in revised form 20 March 2000; accepted 24 March 2000

Abstract

Pancreastatin, a chromogranin A-derived peptide widely distributed throughout the neuroendocrine system, has a general inhibitory effect on endocrine secretion and a counterregulatory effect on insulin action. We have recently described the cross-talk of pancreastatin with insulin signaling in rat hepatoma cells (HTC), where it inhibits insulin action and signaling through the serine phosphorylation of the insulin receptor, thereby impairing tyrosine kinase activity. Here, we have characterized pancreastatin receptors and signaling in HTC cells. The pancreastatin effector systems were studied by determining phospholipase C activity in HTC membranes and mitogen-activated protein kinase (MAPK) phosphorylation activity in HTC cells. Binding studies with radiolabeled pancreastatin showed a population of high affinity binding sites, with a B_{max} of 8 fmol/mg protein and a K_d of 0.6 nM. Moreover, we assessed the coupling of the receptor with a G protein system by inhibiting the binding with guanine nucleotide and by measuring the GTP binding to HTC membranes. We found that pancreastatin receptor was coupled with a $G\alpha_{q/11}$ protein which activates phospholipase C-β_1 and phospholipase C-β_3, in addition to MAPK via both $\beta\gamma$ and $\alpha_{q/11}$.

Keywords: Pancreastatin receptor; Signaling; G protein; Phospholipase C; MAPK; HTC cell; Insulin resistance

1. Introduction

Pancreastatin, a chromogranin A-derived peptide, is widely distributed throughout the neuroendocrine system (Tatemoto et al., 1986; Iacangelo et al., 1986, 1988a,b). Pancreastatin arises from proteolytic cleavage of chromogranin A either intracellularly in the neuroendocrine cell or by post-secretory processing of chromogranin A (Eskeland et al., 1996; Parmer et al., 1993; Simon et al., 1989; Watkinson et al., 1990). A pancreastatin-like sequence has been found in different species including the rat (Abood and Eberwine, 1990; Iacangelo et al., 1988b; Parmer et al., 1989). Pancreastatin was named after its first described effect, namely, inhibition of insulin secretion (Tatemoto et al., 1986). Thereafter, many different effects have been described in a variety of cell targets (Sánchez-Margalet et al., 1996a). However, the mechanisms of these pancreastatin effects are not well understood. The best characterized effect of pancreastatin has been studied in the rat liver (Sánchez-Margalet et al., 1996a,b, 1997), where we have found a calcium-dependent glycogenolytic effect (Sánchez et al., 1990, 1992; Sánchez-Margalet et al., 1992, 1993) and a counterregulatory effect on insulin-stimulated glycogen synthesis (Sánchez-Margalet and Goberna, 1994a). We have also shown that these effects are mediated by a specific receptor coupled to G proteins in the plasma membrane (Sánchez-Margalet et al., 1994a; Sánchez-Margalet and Santos-Alvarez, 1997). Pancreastatin activates phospholipase C (PLC) activity, and therefore increases $[Ca^{2+}]_i$ and activates protein Kinase C (PKC) in rat hepatocytes (Sánchez-Margalet and Goberna, 1994b; Sánchez-Margalet et al., 1993, 1994b), through the activation of $G\alpha_{q/11}$ (Santos-Alvarez et al., 1998; Santos-Alvarez and Sánchez-Margalet, 1998, 1999).

Recently, we described the counterregulatory effect of pancreastatin in rat hepatoma cells (HTC), and studied the mechanisms of cross-talk with the insulin signaling system (Sánchez-Margalet, 1999). The aim of the present work

* Corresponding author. Tel.: +34-95-455-7356; fax: +34-95-455-7481.

E-mail addresses: vsanchez@cica.es, vsanchez@asterix.cica.es (V. Sánchez-Margalet).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00253-3

was to further elucidate the mechanism of pancreastatin action in HTC cells by studying the pharmacology of pancreastatin receptors and the signaling pathways activated in this cell line.

2. Materials and methods

2.1. Materials

Rat [Tyr^0]-Pancreastatin was synthetized by Chiron Technologies (Clayton Vic, Australia) and radioiodinated with ^{125}I (Amersham Radiochemicals, UK) by the chloramine T method (Hunter and Greenwood, 1962) to a specific activity of 1500 Ci/mmol and was purified by gel filtration (Sephadex G-50) (Sánchez-Margalet et al., 1994a). Bacitracin, leupeptin, phenylmethylsulfonylfluoride, *N-p*-tosyl-L-lysine chloromethyl ketone, pepstatin, aprotinin, dithiothreitol, and bovine serum albumin (fraction V) were from Sigma (Alcobendas, Madrid). GTP, GDP, γ-S-GTP, GMP-P(NH)P and other nucleotides were from Boehringer Mannheim (Barcelona, Spain). Electrophoretic chemicals and molecular weight standards were from Novex (San Diego, CA). Rabbit antisera against β_{common}, $\alpha_{i1,2}$, and $\alpha_{q/11}$ subunits of G proteins were from DuPont NEN (Du Pont de Nemours, Germany). Antisera against the β1, β2, β3 and β4 isoforms of phospholipase C were from Santa Cruz (Santa Cruz, CA). Monoclonal antibody against phosphoMAPK and polyclonal antibody against MAPK were purchased from New England Biolabs (Beverly, MA). γ-^{35}S-GTP (1000 Ci/mmol) was from Amersham Iberica (Madrid, Spain).

2.2. Cells and preparation of membranes

Rat HTC hepatoma cells were kindly provided by Dr. Ira D. Goldfine (UCSF, San Francisco, CA). Cells were prepared and maintained in Dulbecco's modified Eagle's medium (DMEM) as previously described (Sung et al., 1994; Sánchez-Margalet et al., 1995a). For membrane preparation, cells were washed and harvested in phosphate buffered saline (pH 7.4). The cell suspension was next centrifuged at 4°C ($300 \times g$) for 10 min. The supernatant was discarded and the pellet resuspended in homogenation buffer (Sánchez-Margalet et al., 1999): 20 mM HEPES (pH 7.4), 0.5 mM EDTA, supplemented with bacitracin (200 μg/ml), phenylmethylsulfonylfluoride (0.1 mM), *N-p*-tosyl-L-lysine chloromethyl ketone (10 μg/ml), leupeptin (10 μg/ml), pepstatin (5 μg/ml) and aprotinin (10 μg/ml). Cells were homogenized using a glass Potter homogenizer (10 strokes) and the homogenate was centrifuged at 4°C ($1000 \times g$) for 10 min. The supernatant was then centrifuged at 4°C ($45{,}000 \times g$) for 30 min, and the pellet was washed once with the same buffer and repelleted to collect crude plasma membranes, which were stored at −80°C. Protein concentration was determined by the Bradford procedure (Bradford, 1976), with a kit from Bio-Rad (Richmond, CA), using bovine serum albumin as standard.

2.3. Binding of ^{125}I-pancreastatin to HTC membranes

Binding to the membrane receptors was performed with the radiolabeled rat pancreastatin analog [^{125}I]-[Tyr^0-Pancreastatin] (1500 Ci/mmol specific activity) as previously described (Sánchez-Margalet et al., 1994a). Specific binding represented about 2.5% of total radioactivity, and non-specific binding represented 30–40% of total binding. Non-specific binding, obtained with an excess of rat pancreastatin (10^{-7} M) was substracted from the total binding to calculate the specific binding. Scatchard analysis of binding data from radioligand displacement experiments was performed with the LIGAND software (Sánchez-Margalet et al., 1994a; Sánchez-Margalet and Santos-Alvarez, 1997).

2.4. GTP-γ-^{35}S binding assay

GTP binding assay was conducted at 23°C in a buffer consisting of 150 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 10^{-4} M GDP, and 10 mM Tris, pH 7.5, containing 0.5 nM GTP-γ-^{35}S (0.1 μCi per assay tube) (Smith et al., 1987; Sánchez-Margalet et al., 1999; Santos-Alvarez and Sánchez-Margalet, 1999). The reaction was started by the addition of HTC cell membranes (100 μg) and stopped after 10 min of incubation by adding 1 ml of cold buffer. After centrifugation at 15,000 $\times g$ at 4°C and two washes with cold buffer, the pellet was dissolved in scintillation cocktail and counted in a scintillation counter (Wallac 1409, Turku, Finland). Non-specific binding was determined in the presence of 10^{-5} M cold GTP-γ-S, and was 25–35% of total binding. Specific binding represented about 2% of total radioactivity. All assays were performed in quadruplicate.

2.5. Phospholipase C assay

Phospholipase C activity from rat HTC membranes was assayed as previously described for rat liver membranes (Sánchez-Margalet and Goberna, 1994b). IP_3 (inositol 1,4,5-triphosphate) was determined by a radioreceptor assay from Amersham Pharmacia Biotech (Buckinghamshire, UK), using D-*myo*-inositol 1,4,5-triphosphate[^{3}H] as a tracer. Pretreatment with G protein and phospholipase C antibodies was performed by adding the specific antisera (1:100 final dilution) to the membranes and incubating them for 60 min at 23°C with gentle agitation as previously described for liver membranes (Santos-Alvarez et al., 1998; Santos-Alvarez and Sánchez-Margalet, 1998).

2.6. Immunodetection of α subunits of GTP-binding proteins and phospholipase C-β isoforms

HTC cell membranes were denatured with Laemmli buffer (Laemmli, 1970) and run on SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) (8–16%). Proteins were electrophoretically transferred onto nitrocellulose membranes. The membranes were first incubated with anti-α_{i1-2}, anti-$\alpha_{q/11}$, or anti-phospholipase C-β isoforms, further incubated with second antibody conjugated with horseradish peroxidase and developed with a highly sensitive chemiluminescence system (SuperSignal from Pierce, Rockfold, IL).

2.7. MAPK (mitogen-activated protein kinase) activation

HTC cells were grown in 100 mm dishes to 90% confluence and starved of serum for 16 h. Cells were incubated in culture medium without serum and with 0.5% bovine serum albumin, and stimulated with or without 10 nM insulin or 10 nM pancreastatin for 10 min. Next, cells were solubilized as described above. Precleared cell lysates were then denatured and separated by SDS-PAGE. MAPK activation was analysed by Western blotting with anti-phosphoMAPK monoclonal antibody (Sánchez-Margalet, 2000) which specifically recognises the Tyr/Thr phosphorylated form of MAPK, since MEK (MAP kinase kinase), the upstream kinase is known to activate MAPK through phosphorylation of threonine and tyrosine residues (Payne et al., 1991). The bands obtained in the Western blots were scanned and then analysed by using the PCBAS2.0 program.

3. Results

3.1. Binding properties of pancreastatin in HTC membranes

Using ^{125}I-labeled rat pancreastatin, we studied the binding characteristics of pancreastatin in rat hepatoma HTC membranes. The specific high-affinity binding of ^{125}I-pancreastatin was dependent on temperature, pH (not shown) and protein concentration (Fig. 1A), reaching a maximum at 20°C, 90 min and pH 7.4, and an optimal protein concentration of 300 μg/ml (Fig. 1). The association–dissociation study showed that pancreastatin binding was a slowly reversible process at 20°C (Fig. 1B). Under these conditions, the binding of pancreastatin was saturable. As shown in Fig. 2A, rat pancreastatin in the concentration range between 10^{-10} and 10^{-8} M competitively inhibited the binding of ^{125}I-pancreastatin to HTC cell membranes. Scatchard analysis of the data gave a straight line, indicating the presence of a single site with a dissociation constant (K_d) of 0.6 nM and a maximum concentration of binding sites at 8 fmol/mg protein. Taking into account the amount of membrane protein obtained from 10^6 cells, which ranged between 0.11 and 0.14 mg, the estimated number of binding sites averaged about 700/cell.

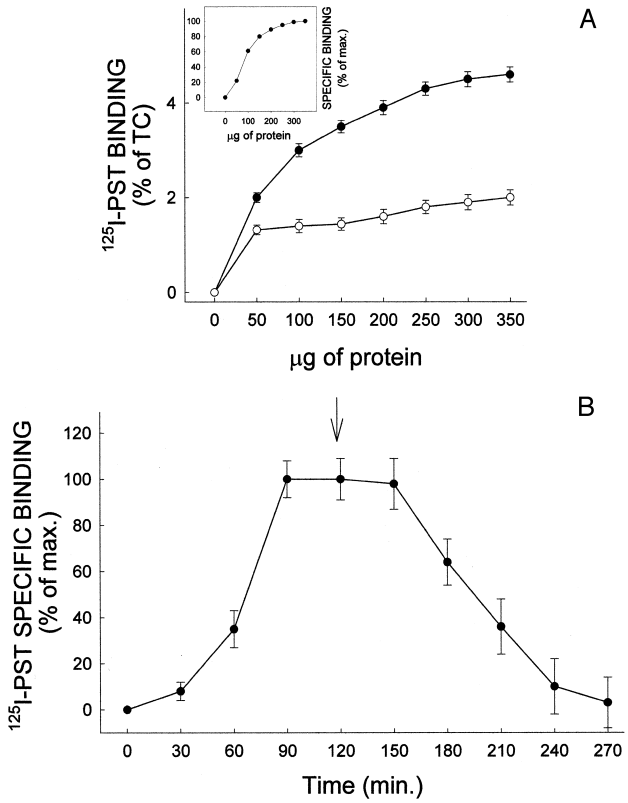

Fig. 1. Time and protein concentration dependence of pancreastatin binding in HTC membranes. (A) Total (closed circles) and non-specific binding (open circles) with increasing amount of HTC membranes during 90 min at 20°C. Inset shows relative specific binding of ^{125}I-pancreastatin (PST). Data are means ± SEM from four separate experiments run in triplicate. (B) Time course of association and dissociation of specific binding of ^{125}I-pancreastatin (PST) to HTC membranes (300 μg/500 μl). Association was performed at 25°C for 120 min. Thereafter, dissociation was measured after the addition of unlabeled PST (10^{-7} M) (arrow). Data are shown as means ± S.E.M. from four independent experiments run in triplicate.

When membranes were incubated with increasing concentrations of the non-hydrolyzable nucleotide GMP-P(NH)P, binding of the tracer was reduced in a dose-dependent manner (Fig. 2B), suggesting that pancreastatin receptors are coupled to G proteins in the plasma membrane of HTC cells.

3.2. Pancreastatin stimulation of G protein activation

We next determined pancreastatin receptor-mediated activation of G proteins by measuring the increase in γ-^{35}S-GTP binding elicited by pancreastatin in HTC membranes. The signal was time-dependent, and the maximal response was achieved at 10 min and 23°C (not shown). Under these incubation conditions, the pancreastatin-induced signal was

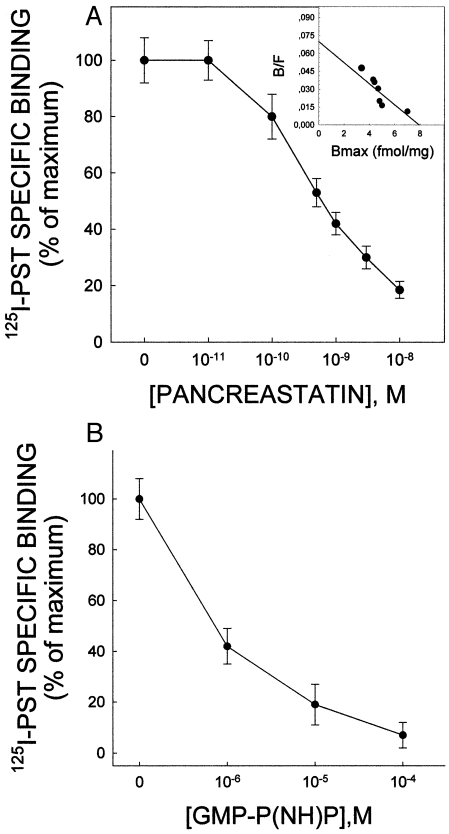

Fig. 2. (A) Inhibition of ^{125}I-pancreastatin (PST) binding to HTC membranes by unlabeled pancreastatin. Membranes were incubated with ^{125}I-pancreastatin and increasing concentrations of unlabeled PST under standard conditions. Data are means ± SEM from four independent experiments run in triplicate. Inset, Scatchard analysis of the PST binding data. (B) Inhibition of binding of ^{125}I-pancreastatin by the nucleotide analog GMP-P(NH)P. HTC membranes were incubated with the tracer and increasing concentrations of GMP-P(NH)P. Data are means ± S.E.M. from four separate experiments run in triplicate.

dose dependent, with a half-maximal effect observed at 0.3 nM and a maximal effect at 10^{-8} M (Fig. 3). These data agree with those of the binding data and suggest the specific coupling of pancreastatin receptors with a G protein system in the membrane of HTC cells.

3.3. Pancreastatin stimulation of phospholipase C in HTC membranes

We previously showed that pancreastatin stimulates phospholipase C activity in plasma liver membranes, increasing the production of IP_3 and diacylglycerol (Sánchez-Margalet and Goberna, 1994b; Sánchez-Margalet et al., 1994b). Similarly, pancreastatin stimulated phospholipase C activity in HTC membranes, as assessed by measuring IP_3 production (Fig. 4). The previous data for GTP binding suggested the functional association of pancreastatin receptors with some G protein. Since the $G_{q/11}\alpha$ family is the best known activator of phospholipase C (Exton, 1996), we checked whether this G protein coupled the pancreastatin receptor to the activation of phospholipase C in HTC membranes. We found that this stimulatory effect of pancreastatin on phospholipase C activity was completely inhibited by anti $G_{q/11}\alpha$ serum, whereas preimmune serum had no effect (Fig. 4). Anti $G_{i1,2}\alpha$ and anti $G\beta_{common}$ sera failed to block pancreastatin stimulated phospholipase C activity (not shown), even though the $\beta\gamma$ dimer may also activate phospholipase C after being released from $G\alpha_{i1,2}$ (Exton, 1996). This result demonstrates that the activation of the effector phospholipase C by the pancreastatin receptor is mediated by $G_{q/11}\alpha$ protein rather than by $\beta\gamma$ dimer released upon $G_{i1,2}\alpha$ activation. The presence of these $G\alpha$ subunits in HTC membranes was checked by Western blotting using specific antibodies (Fig. 5A).

We studied previously the specific phospholipase C-β isoenzymes involved in the pancreastatin response and the specific $G\alpha$ subunit that mediated the phospholipase C-β activation in rat liver membranes (Santos-Alvarez and Sánchez-Margalet, 1998). In the present work, we also used antibodies raised against the C-terminal specific sequences of each phospholipase C-β isoform to block the agonist-mediated activation of phospholipaseC in HTC membranes, as previously described in rat liver membranes (Santos-Alvarez et al., 1998, Santos-Alvarez and Sánchez-Margalet, 1998), to sort out the specific phospholipase C-β isoform activated by pancreastatin stimulation. As shown in Fig. 4 only anti-phospholipase C-β1 serum was able to block the pancreastatin (10 nM) response in HTC membranes, whereas anti-phospholipase C-β3 only partially blocked the response, and anti-phospholipase C-β2 had no significant effect on pancreastatin stimulated phospholipase C activity. These results demonstrate the preferential stimulation of phospholipase C-β1 by pancreastatin receptor in spite of the fact that the three isoforms are present in

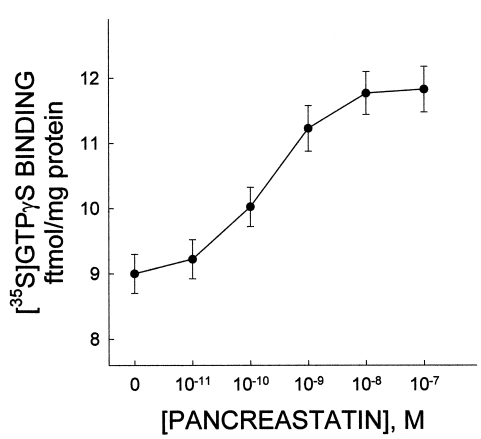

Fig. 3. Pancreastatin dose dependently stimulates γ-^{35}S-GTP binding to HTC membranes. Membranes were incubated at 23°C for 10 min in the presence of increasing concentrations of pancreastatin to determine GTP binding activity as described under Section 2. The values of each experiment were based on quadruplicate tubes. Data are means ($n = 4$) ± S.E.M.

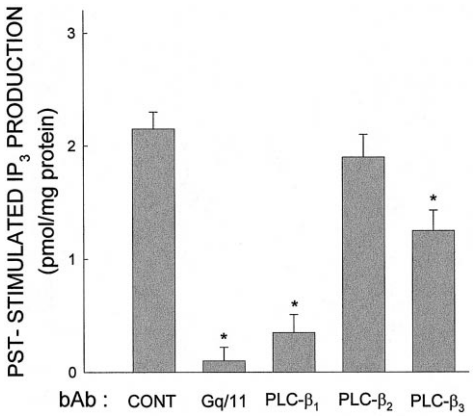

Fig. 4. Coupling of pancreastatin receptors to phospholipase C-β via $G\alpha_{q/11}$ protein in HTC membranes. Phospholipase C activity was measured with or without pancreastatin (10 nM) in the presence of Gα and phospholipase C-β antibodies. HTC cells were incubated with 10 nM pancreastatin after pretreatment with anti-$G\alpha_{q/11}$ or anti-phospholipase C-β isoforms (β1, β2, β3). Control experiments were performed in the presence of preimmune sera. Basal IP_3 production was 2.3 ± 0.1 pmol/mg of protein. Data are means ± S.E.M. ($n = 4$). $^{*}P < 0.001$ compared with control.

HTC membranes, as assessed by specific immunoblots (Fig. 5B), and that phospholipase C-β3 is the isoform activated by pancreastatin in rat liver membranes (Santos-Alvarez and Sánchez-Margalet, 1998). Phospholipase C-β4 was not found in HTC membranes (data not shown).

3.4. Pancreastatin stimulates MAPK pathway in HTC hepatoma cells

Since the $G\alpha_{q/11}$ activation may lead to the stimulation of the MAPK pathway (Gutkind, 1998), we checked whether MAPK is activated by pancreastatin in HTC cells, by studying its tyrosine/threonine phosphorylation level, which reflects the activation of MEK and, indirectly, all the MAPK pathway. As shown in Fig. 6, pancreastatin (10 nM) stimulated tyrosine/threonine phosphorylation of MAPK as assessed by specific immunoblot. As expected,

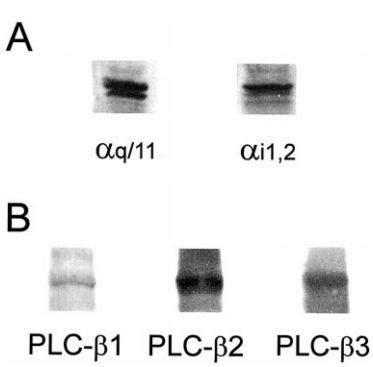

Fig. 5. Expression of $\alpha_{q/11}$ and $\alpha_{i1,2}$ subunits of G proteins (A) and phospholipase C-β isoforms (B) in rat hepatoma HTC membranes. HTC membranes were denatured and subjected to SDS-PAGE (80 μg/lane) and immunoblotted as described under Section 2.

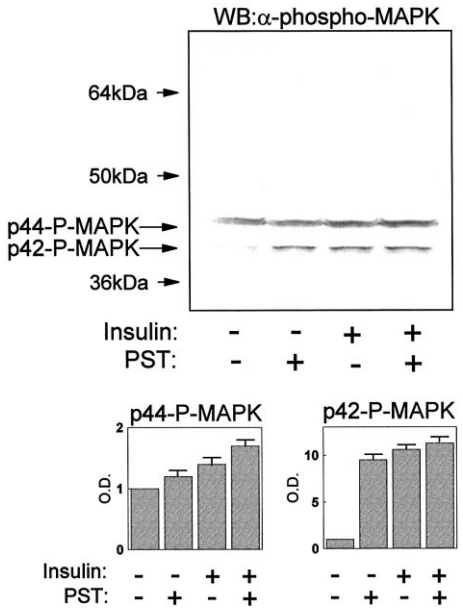

Fig. 6. Pancreastatin activates MAPK pathway in HTC cells. Cells were incubated for 10 min with 10 nM pancreastatin. Insulin stimulation (10 nM) was used as a positive control. Cells were next solubilized, and cell lysates were analyzed by Western blotting with α-phosphoMAPK. The immunoblot of one experiment out of three is shown. Means ± S.E.M. ($n = 3$) of densitometric analysis of p44 and p42 bands from scanned blots are also shown.

insulin (10 nM) stimulated MAPK phosphorylation in HTC cells (Sánchez-Margalet, 2000). As previously observed in HTC cells (Sánchez-Margalet, 2000), p44 MAPK was phosphorylated under basal conditions and was not strongly activated by agonist challenge. On the other hand, p42 MAPK was found to be activated upon stimulation with either pancreastatin or insulin. The effect of pancreastatin was about 85% of that elicited by insulin stimulation in HTC cells. Simultaneous challenge with pancreastatin and insulin had no synergistic, but a mild additive, effect on p42 MAPK phosphorylation.

4. Discussion

We have previously found that treating HTC cells with pancreastatin inhibits both insulin action and signaling (Sánchez-Margalet, 1999). Moreover, we observed that this effect of pancreastatin was mediated by protein kinase C activation and phosphorylation of the insulin receptor at serine and threonine residues. Other agonists are known to cause insulin resistance by activating protein kinase C (Häring and Mehnert, 1993; Velloso et al., 1996), and increased serine kinase activity has been observed in insulin resistance (Quiao et al., 1999). Since pancreastatin inhibits the action of insulin in hepatocytes and adipocytes (Sánchez-Margalet and Goberna 1994a; Sánchez-Margalet and González-Yanes, 1998) and levels of pancreastatin-like immunoreactivity are increased in insulin resistance syndromes (Funakoshi et al., 1990; Sánchez-Margalet et al.,

1995b,c, 1998), correlating with catecholamine levels, pancreastatin could play a role in the pathophysiology of insulin resistance along with other counterregulatory hormones (Sánchez-Margalet and Goberna, 1993).

In the present study, we characterized the pancreastatin receptor and signaling in HTC rat hepatoma cells to further investigate the mechanisms of pancreastatin action. We previously characterized pancreastatin receptors in rat liver membranes (Sánchez-Margalet et al., 1994a; Sánchez-Margalet and Santos-Alvarez, 1997). Here, we found similar characteristics of affinity and sensitivity to guanine nucleotides in HTC membranes. Thus, the K_d was also in the subnanomolar range (0.6, versus 0.3 nM in liver membranes). The binding capacity was lower in HTC than in liver membranes (8 fmol/mg protein, versus 15 fmol/mg protein in liver membranes), yielding a total number of binding sites (700/cell) about five to six times lower than that observed in liver membranes. The sensitivity of pancreastatin binding to guanine nucleotides was also comparable to that observed in rat liver membranes. Moreover, pancreastatin activated specific GTP binding to HTC membranes in a dose dependent manner, similar to that observed in rat liver membranes, further suggesting the coupling of the pancreastatin receptor with G proteins. Even though the amount of receptors was low in these cells, the similar characteristics of the binding data compared to hepatocytes may validate the use of this cell line in any receptor purification approach.

The phospholipase C-β signaling pathway was activated by pancreastatin in HTC cells, as observed in liver membranes (Santos-Alvarez et al., 1998, Santos-Alvarez and Sánchez-Margalet, 1998). As previously shown in rat liver membranes, $G\alpha_{q/11}$ is the specific G protein that links the pancreastatin receptor with the phospholipase C effector in the plasma membrane. However, unlike the situation in liver membranes, phospholipase C-β1 rather than phospholipase C-β3 seems to be preferentially activated in HTC cells. In fact, both phospholipase C-β1 and phospholipase C-β3 have been found to respond equally to α_q and α_{11}, using purified phospholipase C-β isozymes (Hepler et al., 1993). However, it should be pointed out that phospholipase C signaling was quantitatively lower in HTC cells than that observed in rat liver membranes upon pancreastatin stimulation. This discrepancy may be explained by the lower number of pancreastatin receptors found in HTC cells compared to those found in rat hepatocytes, by the light differences in affinity, and by the amount of phopholipase C-β isoforms in the plasma membrane.

G-protein-coupled receptors are known to signal to MAPK through the $G_{\beta\gamma}$ dimer of the heterotrimeric G protein, by activating the $p21^{ras}$–$p74^{raf}$ pathway, independently of protein kinase C (Gutkind, 1998). Besides, Gq-coupled receptors have an alternative pathway to connect with MAPK by activating protein kinase C (Gutkind, 1998; Schonwasser et al., 1998). Therefore, the pancreastatin activation of the MAPK pathway should not be striking, since pancreastatin signaling is mediated by $G\alpha_{q/11}$-phospholipase C-β-PKC. The activation of the MAPK pathway is known to be a signal for growth in many cell systems. Therefore, a possible role of pancreastatin in protein and DNA synthesis is hypothesized, and these data open a new line of investigation. Nevertheless, this effect of pancreastatin on the MAPK pathway should be confirmed in normal hepatocytes. Simultaneous stimulation of HTC cells with pancreastatin and insulin resulted in only a mild increase in the phosphorylation of MAPK. This result may be explained by the previously reported inhibition of insulin signaling by pancreastatin in this system (Sánchez-Margalet, 1999).

In conclusion, these results may give some clues to the mechanism of the observed effect of pancreastatin in HTC rat hepatoma cells and therefore provide a better understanding of the mechanism of action of pancreastatin.

Acknowledgements

We are grateful to Dr. I.D. Goldfine (UCSF, San Francisco) for providing the HTC cells. This work was supported by grants from the Fondo de Investigación Sanitaria (FIS 96/1411; FIS 00/0311), Ministerio de Sanidad (Spain). Carmen González-Yanes is a recipient of a fellowship from the Fondo de Investigación Sanitaria, Ministerio de Sanidad (Spain) (BEFI 98/9158).

References

Abood, M.E., Eberwine, J.H., 1990. Characterization and regulation of a cDNA clone for rat pancreastatin. Biochem. Biophys. Res. Commun. 167, 1079–1085.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Eskeland, N.L., Zhou, A., Dihn, T.Q., Wu, H., Parmer, R.J., Mains, E., O'Connor, D.T., 1996. Chromogranin A processing and secretion. Specific role of endogenous and exogenous prohormone convertases in the regulated secretory pathway. J. Clin. Invest. 98, 148–156.

Exton, J.H., 1996. Regulation of phosphoinositide phospholipases by hormones, neurotransmitters, and other agonists linked to G proteins. Annu. Rev. Pharmacol. Toxicol. 36, 481–509.

Funakoshi, A., Tateishi, K., Shinozaki, H., Matsumoto, M., Wakasugi, H., 1990. Elevated plasma levels of pancreastatin (PST) in patients with non-insulin-dependent diabetes mellitus (NIDDM). Regul. Pept. 30, 159–164.

Gutkind, J.S., 1998. The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. J. Biol. Chem. 273, 1839–1842.

Häring, H.U., Mehnert, H., 1993. Pathogenesis of type 2 (non-insulin-dependent) diabetes mellitus: candidates for signal transmitter defect causing insulin resistance of the skeletal muscle. Diabetologia 36, 176–182.

Hepler, J.R., Kozasa, T., Smrcka, A.V., Simon, M.I., Rhee, S.G., Sternweis, P.C., Gilman, A.G., 1993. Purification from Sf9 cells and characterization of recombinant $G_{q\alpha}$ and $G_{11\alpha}$. Activation of phospholipase C isoenzymes by Gα subunits. J. Biol. Chem. 268, 14367–14375.

Hunter, W.M., Greenwood, F.C., 1962. The preparation of 125I-labeled human growth hormone of high specific radioactivity. Nature 19, 495–496.

Iacangelo, A.L., Affolter, H.U., Eiden, L.E., Herbert, E., Grimes, M., 1986. Bovine chromogranin A sequence and distribution of its messenger RNA in endocrine tissues. Nature 323, 82–86.

Iacangelo, A.L., Fischer-Colbrie, R., Koller, K.J., Brownstein, M.J., Eiden, L.E., 1988a. The sequence of porcine chromogranin A messenger RNA demonstrates chromogranin A can serve as the precursor for the biologically active hormone, pancreastatin. Endocrinology 15, 2339–2341.

Iacangelo, A.L., Okayama, H., Eiden, L.E., 1988b. Primary structure of rat chromogranin A and distribution of its mRNA. FEBS Lett. 227, 115–121.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227, 680–685.

Parmer, R.J., Koop, A.H., Handa, M.T., O'Connor, D.T., 1989. Molecular cloning of chromogranin A from rat pheochromocytoma cells. Hypertension 14, 435–444.

Parmer, R.J., Xi, X.-P., Wu, H.-J., Helman, L.J., Petz, L.N., 1993. Secretory protein traffic. Chromogranin A contains a dominant targeting signal for the regulated pathway. J. Clin. Invest. 92, 1042–1054.

Payne, D.M., Rossomando, A.J., Martino, P., Erickson, A.K., Her, J.H., Shabanowitz, J., Hunt, D.F., Weber, M.J., Sturgill, T.W., 1991. Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). EMBO J. 10, 885–892.

Quiao, L.Y., Goldberg, J.L., Russell, J.C., Sun, X.J., 1999. Identification of enhanced serine kinase activity in insulin resistance. J. Biol. Chem. 274, 10625–10632.

Sánchez, V., Calvo, J.R., Goberna, R., 1990. Glycogenolytic effect of pancreastatin in the rat. Biosci. Rep. 10, 87–91.

Sánchez, V., Lucas, M., Calvo, J.R., Goberna, R., 1992. Glycogenolytic effect of pancreastatin in isolated rat hepatocytes is mediated by a cyclic-AMP-independent Ca^{2+}-dependent mechanism. Biochem. J. 284, 659–662.

Sánchez-Margalet, V., 1999. Modulation of insulin receptor signalling by pancreastatin in HTC hepatoma cells. Diabetologia 42, 317–325.

Sánchez-Margalet, V., 2000. Insulin-stimulated glycogen synthesis requires S6 kinase and phosphatidylinositol-3-kinase in HTC-IR cells. J. Cell. Physiol. 182, 182–188.

Sánchez-Margalet, V., Goberna, R., 1993. Pancreastatin, a new peptide associated with essential hypertension and hyperinsulinemia. In: Galteau, M.M., Henny, J., Siest, G. (Eds.), Biologie Prospective. Libbey, Paris, pp. 575–580.

Sánchez-Margalet, V., Goberna, R., 1994a. Pancreastatin inhibits insulin-stimulated glycogen synthesis but not glycolysis in rat hepatocytes. Regul. Pept. 51, 215–220.

Sánchez-Margalet, V., Goberna, R., 1994b. Pancreastatin activates pertussis toxin-sensitive guanylate cyclase and pertusis toxin-insensitive phospholipase C in rat liver membranes. J. Cell. Biochem. 55, 173–181.

Sánchez-Margalet, V., González-Yanes, C., 1998. Pancreastatin inhibits insulin action in rat adipocytes. Am. J. Physiol. 275, E1055–E1060.

Sánchez-Margalet, V., Santos-Alvarez, J., 1997. Solubilization and molecular characterization of pancreastatin receptors from rat liver membranes. Endocrinology 138, 1712–1718.

Sánchez-Margalet, V., Calvo, J.R., Goberna, R., 1992. Glycogenolytic and hyperglycemic effect of 33–49 C-terminal fragment of pancreastatin in the rat in vivo. Horm. Metab. Res. 34, 455–457.

Sánchez-Margalet, V., Lucas, M., Goberna, R., 1993. Pancreastatin increases free cytosolic Ca^{2+} in rat hepatocytes involving both pertussis-toxin-sensitive and insensitive mechanisms. Biochem. J. 294, 439–442.

Sánchez-Margalet, V., Valle, M., Goberna, R., 1994a. Receptors for pancreastatin in rat liver membranes: molecular identification and characterization by covalent cross-linking. Mol. Pharmacol. 46, 24–29.

Sánchez-Margalet, V., Lucas, M., Goberna, R., 1994b. Pancreastatin activates protein kinase C by stimulating the formation of 1,2-diacylglycerol in rat hepatocytes. Biochem. J. 303, 51–54.

Sánchez-Margalet, V., Zoratti, R., Sung, C.K., 1995a. Insulin-like growth factor-1 stimulation of cells induces formation of complexes containing phosphatidylinositol-3-kinase, guanosine triphosphatase-activating protein (GAP), and p62 GAP-associated protein. Endocrinology 136, 316–321.

Sánchez-Margalet, V., Valle, M., Lobón, J.A., Maldonado, A., Escobar, F., Oliván, J., Perez-Cano, R., Goberna, R., 1995b. Increased plasma pancreastatin-like immunoreactivity levels in non-obese patients with essential hypertension. J. Hypertens. 13, 251–258.

Sánchez-Margalet, V., Valle, M., Lobón, J.A., Escobar-Jimenez, F., Perez-Cano, R., Goberna, R., 1995c. Plasma pancreastatin-like immunoreactivity correlates with plasma norepinephrine levels in essential hypertension. Neuropeptides 29, 97–101.

Sánchez-Margalet, V., Lucas, M., Goberna, R., 1996a. Pancreastatin: further evidence for its consideration as a regulatory peptide. J. Mol. Endocrinol. 16, 1–8.

Sánchez-Margalet, V., Lucas, M., Goberna, R., 1996b. Pancreastatin action in the liver: dual coupling to different G proteins. Cell. Signal. 8, 9–12.

Sánchez-Margalet, V., Santos-Alvarez, J., Goberna, R., 1997. Pancreastatin signaling in the liver. In: Teelken, A., Korpf, J. (Eds.), Neurochemistry: Cellular, Molecular and Clinical Aspects. Plenum, New York, pp. 589–593.

Sánchez-Margalet, V., Lobón, J.A., Gonzalez, A., Escobar-Jimenez, F., Goberna, R., 1998. Increased plasma pancreastatin-like levels in gestational diabetes subjects. Correlation with plasma catecholamine levels. Diabetes Care 21, 951–954.

Sánchez-Margalet, V., González-Yanes, C., Santos-Alvarez, J., Najib, S., 1999. Insulin activates $G\alpha_{i1,2}$ protein in rat hepatoma (HTC) plasma membranes. Cell. Mol. Life Sci. 55, 142–147.

Santos-Alvarez, J., Sánchez-Margalet, V., 1998. Pancreastatin activates $\beta 3$ isoform of phospholipase C via $G\alpha_{11}$ protein stimulation in rat liver membranes. Mol. Cell. Endocrinol. 143, 101–106.

Santos-Alvarez, J., Sánchez-Margalet, V., 1999. G protein $G\alpha_{q/11}$ and $G\alpha_{i1,2}$ are activated by pancreastatin receptors in rat liver. Studies with GTP-γ-^{35}S and azido-GTP-α-^{32}P. J. Cell. Biochem. 73, 469–477.

Santos-Alvarez, J., González-Yanes, C., Sánchez-Margalet, V., 1998. Pancreastatin receptor is coupled to a GTP binding protein of the $G_{q/11}\alpha$ in rat liver membranes. Hepatology 27, 608–614.

Schonwasser, D.C., Marais, R.M., Marshall, C.J., Parker, P.J., 1998. Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. Mol. Cell. Biol. 18, 790–798.

Simon, J.P., Bader, M.F., Aunis, D., 1989. Proteolytic processing of CGA in cultured chromaffin cells. Biochem. Biophys. Acta 1051, 123–130.

Smith, C.D., Uhing, R.J., Snyderman, R., 1987. Nucleotide regulatory protein-mediated activation of phospholipase C in human polymorfonuclear leukocytes is disrupted by phorbol esters. J. Biol. Chem. 262, 6121–6127.

Sung, C.K., Sánchez-Margalet, V., Goldfine, I.D., 1994. Role of p85 subunit of phosphatidylinositol-3-kinase as an adaptor molecule linking the insulin receptor, p62 and GTPase-activating protein. J. Biol. Chem. 269, 12503–12507.

Tatemoto, K., Efendic, S., Mutt, V., Makk, G., Feistner, G.J., Barchas, J.C., 1986. Pancreastatin, a novel pancreatic peptide that inhibits insulin secretion. Nature 324, 476–478.

Velloso, L.A., Folli, F., Sun, X.J., White, M.F., Saad, M.J.A., Kahn, C.R., 1996. Cross-talk between the insulin and angiotensin signaling systems. Proc. Natl. Acad. Sci. U. S. A. 93, 12490–12495.

Watkinson, A., O'Sullivan, A., Burgoyne, R., Dockray, G., 1990. Differential accumulation of catecholamines, proenkephalin-and CGA-derived peptides in the medium after chronic nicotine stimulation of cultured bovine adrenal chromaffin cells. Peptides 11, 435–441.