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# G Protein $G\alpha_{q/11}$ and $G\alpha_{i1,2}$ Are Activated by Pancreastatin Receptors in Rat Liver: Studies With GTP-$\gamma^{35}$S and Azido-GTP-$\alpha$-$^{32}$P

**José Santos-Álvarez and Víctor Sánchez-Margalet***

Departamento de Bioquímica Médica y Biología Molecular, Facultad de Medicina, Hospital Universitario Virgen Macarena, Sevilla 41009, Spain

**Abstract** In the liver, pancreastatin exerts a glycogenolytic effect through interaction with specific receptors, followed by activation of phospholipase C and guanylate cyclase. Pancreastatin receptor seems to be coupled to two different G protein systems: a pertussis toxin-insensitive G protein that mediates activation of phospholipase C, and a pertussis toxin sensitive G protein that mediates the cyclic GMP production. The aim of this study was to identify the specific G protein subtypes coupling pancreastatin receptors in rat liver membranes. GTP binding was determined by using $\gamma$-$^{35}$S-GTP; specific anti-G protein $\alpha$ subtype sera were used to block the effect of pancreastatin receptor activation. Activation of G proteins was demonstrated by the incorporation of the photoreactive GTP analogue 8-azido-$\alpha$-$^{32}$P-GTP into liver membranes and into specific immunoprecipitates of different G$\alpha$ subunits from soluble rat liver membranes. Pancreastatin stimulation of rat liver membranes increases the binding of $\gamma$-$^{35}$S-GTP in a time- and dose-dependent manner. Activation of the soluble receptors still led to the pancreastatin dose-dependent stimulation of $\gamma$-$^{35}$S-GTP binding. Besides, WGA semipurified receptors also stimulates GTP binding. The binding was inhibited by treatment with anti-$G\alpha_{q/11}$ (85%) and anti-$G\alpha_{i1,2}$ (15%) sera, whereas anti-$G\alpha_{o,i3}$ serum failed to affect the binding. Finally, pancreastatin stimulates GTP photolabeling of particulate membranes. Moreover, it specifically increased the incorporation of 8-azido-$\alpha$-$^{32}$P-GTP into $G\alpha_{q/11}$ and G$\alpha$, but not into $G\alpha_{o,i3}$ from soluble rat liver membranes. In conclusion, pancreastatin stimulation of rat liver membranes led to the activation of $G\alpha_{q/11}$ and $G\alpha_{i1,2}$ proteins. These results suggest that $G\alpha_{q/11}$ and $G\alpha_{i1,2}$ may play a functional role in the signaling of pancreastatin receptor by mediating the production of $IP_3$ and cGMP respectively. J. Cell. Biochem. 73:469–477, 1999. 

**Key words:** chromogranin A; signal transduction; regulatory peptides; phospholipase C; guanylate cyclase; hepatocyte; metabolism

Pancreastatin (PST) is a 49-amino acid peptide that was first isolated from porcine pancreas [Tatemoto et al., 1986]. It arises from proteolytic cleavage of its precursor chromogranin A (CGA), a glycoprotein present in endocrine and neuronal cells [Iancangelo et al., 1986; 1988; Eskeland et al., 1996]. Post-secretory processing of CGA may also occur [Simon et al., 1989]. Rat CGA cDNA showed the existence of a pancreastatin-like sequence, homologous to porcine pancreastatin [Hutton et al. 1988; Abood and Eberwine, 1990; Parmer et al., 1989]. Its role as a regulatory gastroenteropancreatic peptide has been established in the light of a variety of biological effects in a number of tissues that could be assigned to the C-terminal part of

Abbreviations used: PST, pancreastatin; CGA, chromogranin A; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; PMSF, phenylmethanesulphonyl fluoride; TLCK, N-p-tosyl-L-lysine chloromethyl ketone; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; G protein, GTP binding protein; WGA, wheat germ agglutinin; NAG, N-acetyl-β-glucosamine; DTT, dithiothreitol; BSA, bovine serum albumin; cGMP, cyclic guanosine monophosphate; cAMP, cyclic adenosine monophosphate; PLC, polyphosphoinositide-specific phospholipase C; $IP_3$, inositol 1,4,5-triphosphate.

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*Correspondence to: Víctor Sánchez-Margalet, Departamento de Bioquímica Médica y Biología Molecular, Facultad de Medicina de Sevilla. Av. Sánchez Pizjuan 4. Sevilla 41009, Spain. E-mail: vsanchez@cica.es

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the molecule [for review, see Sánchez-Margalet et al., 1996a]. These effects are exerted on endocrine and exocrine pancreatic secretion [Tatemoto et al., 1986; Funakoshi et al., 1989; Sánchez-Margalet et al., 1992a,b; Sánchez-Margalet and Goberna, 1993a], gastric secretion [Hashimoto et al., 1990], parathormone release [Fasciotto et al., 1989], plasma catecholamine levels [Sánchez-Margalet and Goberna, 1993b], and memory retention [Flood and Morley, 1988]. Synthetic rat pancreastatin has also been shown to display biological activity in different tissues [Funakoshi et al., 1989].

In rat liver, we have previously shown that PST has a calcium-dependent glycogenolytic effect [Sánchez et al., 1990, 1992; Sánchez-Margalet et al., 1993] and a counterregulatory effect on the insulin stimulation of glycogen synthesis [Sánchez-Margalet and Goberna, 1994a]. Thereafter, we studied and characterized the PST-specific receptor in rat liver plasma membranes [Sánchez-Margalet et al., 1994a], as well as the specific signal transduction [Sánchez-Margalet and Goberna, 1994b; Sánchez-Margalet et al., 1994b, 1996b]. A pertussis toxin-insensitive G protein leads to the activation of phospholipase C, and therefore mediates the glycogenolytic effect in the liver by increasing cytoplasmic free calcium and stimulating calcium-dependent protein kinases as previously demonstrated for other receptors [Blackmore et al., 1985; Uhing et al., 1986]. Another pathway involves a pertussis toxin-sensitive mechanism that mediates the activation of guanylate cyclase, increasing the production of cGMP, which then may inhibit $IP_3$ synthesis [Sánchez-Margalet et al., 1994b, 1996b]. The effect of pancreastatin in the liver glycogen metabolism, both in vivo and in vitro, suggests a role of pancreastatin as a counterregulatory peptide of insulin action. In fact, high PST levels have been found in insulin-resistant states [Sánchez-Margalet et al., 1995a,b, 1997; Funakoshi et al., 1990].

Very recently, we have solubilized, characterized, and semipurified PST receptors from rat liver plasma membranes in a functional state, still coupled to some G proteins [Sánchez-Margalet and Santos-Alvarez, 1997]. To further assess the nature of this coupling, we sought to study the specific G protein activated by PST receptors. We measured the GTP binding, as well as the photolabeling of G proteins stimulated by PST, using specific anti-Gα antisera to sort out the G protein subtype involved in the signaling. We have found that PST receptor is functionally coupled to G proteins of the $G\alpha_{q/11}$ and $G\alpha_{i1,2}$ families. This association may mediate the activation of PLC and guanylate cyclase by PST in the rat liver.

## MATERIALS AND METHODS

### Materials

Rat pancreastatin was from Peninsula Laboratories Europe (Merseyside, UK). Protein A and WGA coupled to Sepharose 4B were from Pharmacia Biotech (Uppsala, Sweden). Polyethylene glycol 6000 was from Merck (Darmstadt, Germany). Triton X-100, bovine γ-globulin, N-acetyl-β-glucosamine (NAG), bacitracin, leupeptin, TLCK, PMSF, pepstatin, aprotinin, dithiothreitol (DTT), and bovine serum albumin (BSA) (fraction V) were from Sigma Chemical Co. (St. Louis, MO). GTP, GDP, γ-S-GTP and other nucleotides were from Boehringer Mannheim (Barcelona, Spain). Electrophoretic chemicals and molecular-weight standards were from Novex (San Diego, CA). Rabbit antisera against $\alpha_{q/11}$, $\alpha_{i1,2}$, $\alpha_{o,i3}$, and $\beta_{common}$ subunits of G proteins were from DuPont NEN (Du Pont de Nemours, Germany). γ-$^{35}$S-GTP (1000 Ci/mmol) was from Amersham Iberica (Madrid, Spain) and 8-azido-α-$^{32}$P-GTP (10 Ci/mmol) from ICN Iberica (Barcelona, Spain).

### Preparation of Rat Liver Membranes

Animals used were male Wistar rats (150–200 g) fed ad libitum. Rat liver membranes were prepared as previously described [Sánchez-Margalet and Santos-Alvarez, 1997].

### Membrane Solubilization

Under optimal conditions, solubilization was carried out as previously described [Sánchez-Margalet and Santos-Alvarez, 1997]. Briefly, membranes (14 mg/ml) were incubated in Hepes buffer (50 mM, pH 7.4) containing 1% (v/v) Triton X-100, NaCl (100 mM), KCl (1 mM), $MgCl_2$ (2 mM), 10% (v/v) glycerol, bacitracin (200 µg/ml), PMSF (0.1 mM), TLCK (10 µg/ml), leupeptin (10 µg/ml), pepstatin (5 µg/ml), and aprotinin (10 µg/ml) for 1 h on ice. The membrane suspension was then centrifuged for 1 h at 100,000*g* (4°C). The supernatant was then removed and used immediately or stored at −80°C. The yield of protein solubilization was estimated at 50%. Protein concentration was determined by the Bradford procedure [Bradford, 1976] with a Bio-Rad kit, using BSA as the standard.

### Wheat Germ Agglutinin Affinity Chromatography

Solubilized receptors (5 mg protein) were incubated with Sepharose-WGA for 3 h at 4°C rotating. The mixture was then packed into a 5- × 1-cm chromatographic column. The column was then washed with 2 vol of the same buffer used for solubilization but containing 0.1% Triton X-100. The receptor was eluted at 0.3 ml/min with the same buffer containing 0.3 M NAG. Fractions were assayed for PST binding as described previously [Sánchez-Margalet and Santos-Alvarez, 1997]. Fractions containing PST receptors were used for γ-$^{35}$S-GTP binding as described below.

### γ-$^{35}$S-GTP Binding Assay

GTP binding assay was conducted at 23°C in a buffer consisted of 150 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, $10^{-4}$ M GDP, and 10 mM Tris, pH 7.5, containing 0.5 nM γ-$^{35}$S-GTP (0.1 µCi per assay tube) [Smith et al., 1987]. The reaction was started by the addition of liver membranes (100 µg) and stopped at different times by adding 1 ml of cold buffer. After centrifugation at 15,000*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). Nonspecific binding was determined in the presence of $10^{-5}$ M cold γ-S-GTP. All assays were performed in quadruplicate.

For binding assay with solubilized membranes and WGA-semipurified receptors, we employed the following buffer: 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, $10^{-4}$ M GDP, and 25 mM Hepes pH 7.5, containing 0.5 nM γ-$^{35}$S-GTP (0.1 µCi per assay tube). At the end of the incubation, soluble membranes were precipitated at 4°C by addition of bovine γ-globulins (0.4% final concentration) and polyethylene glycol (10% final concentration), pelleted by centrifugation at 4°C and washed two times with cold binding buffer containing polyethylene glycol (PEG) (10%) [Sánchez-Margalet and Santos-Alvarez, 1997]. Specific binding was calculated as the difference between the amount of radioactivity bound in the absence (total binding) or presence (nonspecific binding) of an excess ($10^{-5}$ M) of cold γ-S-GTP. Blocking experiments with antisera were performed by the addition of the specific antisera (1:50 final dilution) to the particulate or solubilized membranes and incubation for 2 h at 4°C with gentle agitation as previously described [Al-Aoukaty et al., 1996].

### Photoaffinity Labeling of GTP Binding Proteins

Membranes (100 µg of protein) were incubated at 23°C in a buffer containing 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, $10^{-4}$ M GDP, 30 mM Hepes (pH 7.4) [Offermanns et al., 1991a]. After 2-min preincubation in the absence or presence of PST, samples were incubated for another 3 min with 300 nM azido-α-$^{32}$P-GTP (0.3 µCi per assay tube). After stopping the reaction by cooling the samples on ice, samples were centrifuged at 4°C for 5 min at 15,000*g*, and pellets were resuspended in 50 µl of photolysis buffer: 30 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 2 mM glutathione, and 50 mM Hepes (pH 7.4) [Offermanns et al., 1991a]. Suspended membranes were then irradiated for 5 min with a 254-nm ultraviolet (UV) lamp (8 W) at 3-cm distance.

Solubilized membranes were incubated at 23°C in the same buffer used for solubilization but supplemented with 0.1 mM EDTA and $10^{-4}$ M GDP. For the photolabeling, glutathione (2 mM final concentration) was added. Samples were denatured in Laemmli buffer and run on an sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8–16%) [Laemmli 1970], or the radioactivity was directly counted. Photolabeled membrane proteins were visualized by autoradiography of the dried gels as previously described [Offermanns et al., 1991b]. The bands obtained in the autoradiograpies were scanned and then analyzed by the PCBAS2.0 program.

### Immunoprecipitation of G Proteins

After the photolabeling, soluble membranes were incubated at 4°C with specific anti-Gα sera for 3 h [Santos-Alvarez et al. 1998]. Then, protein A-Sepharose was added and samples were further incubated for 1 h at 4°C. Immunoprecipitates were washed twice with cold solubilization buffer, denatured in Laemmli buffer, and run on SDS-PAGE (8–16%) [Laemmli 1970]. Photolabeled membrane proteins were then visualized by autoradiography of the dried gels as previously described [Offermanns et al., 1991a,b]. The bands obtained in the autoradiographies were scanned and then analyzed by the PCBAS2.0 program.

### Immunodetection of β and $\alpha_{q/11}$, $\alpha_{i1,2}$, or $\alpha_{o,i3}$ Subunits of GTP-Binding Proteins

Soluble extracts and immunoprecipitates were denatured with Laemmli buffer and run on an SDS-PAGE (8–16%) [Laemmli, 1970]. Proteins were electrophoretically transferred onto nitrocellulose membranes. After blocking with 5% skimmed milk, the membranes were first incubated with anti-$\beta_{common}$, anti-$\alpha_{q/11}$, anti-$\alpha_{i1,2}$, or anti-G$\alpha_{o,i3}$, washed three times, further incubated with second antibody conjugated with horseradish peroxidase (HRP) and developed by the Amersham enhanced chemiluminescence (ECL) detection system [Sánchez-Margalet and Santos-Alvarez, 1997].

## RESULTS

PST receptor-mediated activation of G proteins was determined by measuring the increase in γ-$^{35}$S-GTP binding elicited by PST in rat liver membranes. The signal was time-dependent with maximal response at 3 min and 23°C (Fig. 1A). Under these incubation conditions, PST induced signal was dose-dependent, with half-maximal effect observed at 0.2 nM and maximal effect at $10^{-8}$ M (Fig. 1B).

To further examine the coupling of PST receptors to specific subtypes of G proteins, we incubated rat liver membranes with anti-G protein antibodies before assaying for γ-$^{35}$S-GTP binding (Fig. 1C). Binding of Gα C-terminal antibodies seem to functionally uncouple receptors and G proteins. Thus, anti-G$\alpha_{q/11}$ serum inhibited PST-induced GTP binding about 85%, whereas anti-G$\alpha_{i1,2}$ serum inhibited PST-induced GTP binding about 15%. On the other hand, preimmune serum and anti-G$\alpha_{o,i3}$ were used as controls and they had no effect on PST-stimulated GTP binding (Fig. 1C).

We recently managed to solubilize PST receptors from rat liver membranes in a functional

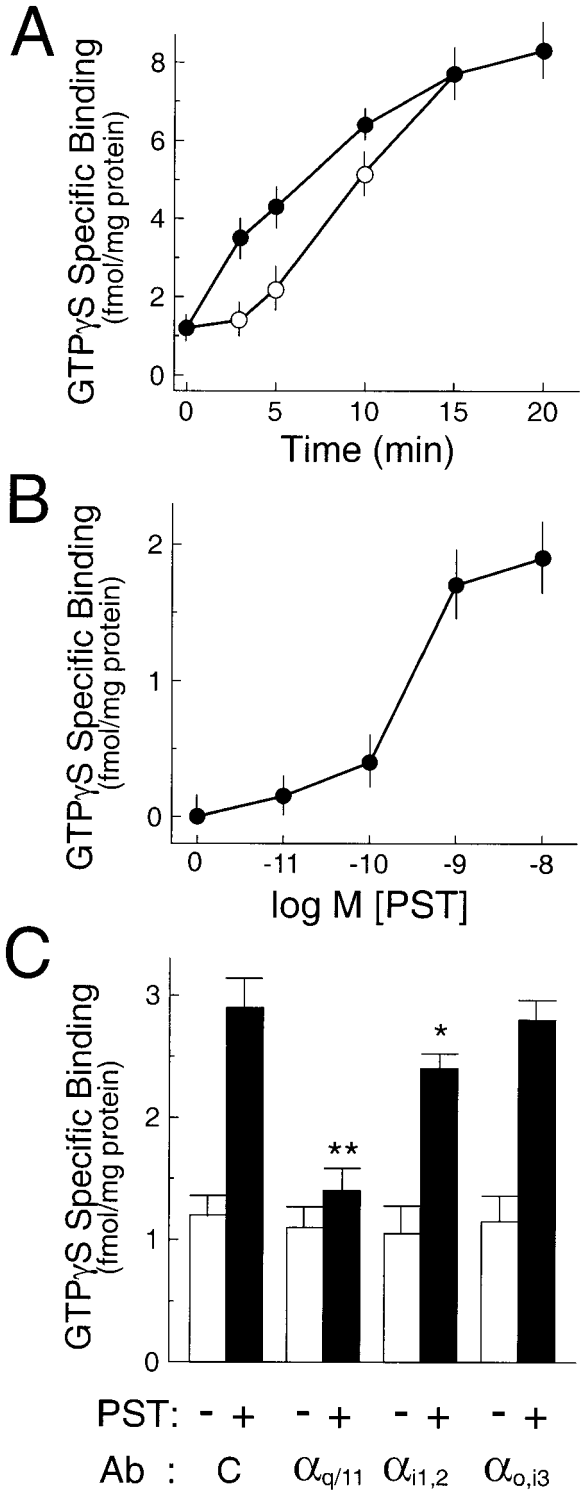


**Fig. 1.** PST stimulates γ-$^{35}$S-GTP binding to rat liver membranes. Blocking by Gα specific antisera. **A:** Rat liver membranes were incubated with γ-$^{35}$S-GTP in the presence (●) or absence (○) of PST ($10^{-8}$ M) and the binding was determined as described under Materials and Methods at different time points. Data are means ± SEM (n = 4) of the GTP binding activity assay run in quadriplicates. **B:** Concentration dependency of the PST-stimulated GTP binding to rat liver membranes. Membranes were incubated at 25°C for 3 min in the presence of increasing concentrations of PST to determine GTP binding activity as described under Materials and Methods. The values of each experiment were based on quadruplicates tubes. Data are means (n = 4) ± SEM of the increase in GTP binding activity above control levels. **C:** Basal (−) and 10 nM PST-stimulated (+) GTP binding to liver membranes was measured in the absence or presence of preincubation for 2 h with different blocking sera: anti-G$\alpha_{q/11}$, G$\alpha_{i1,2}$, G$\alpha_{o,i3}$, or preimmune sera (C, control). Data are means ±SEM of four different experiments run in quadruplicates. *$P < 0.05$, **$P < 0.001$, significant differences versus control.

state [Sánchez-Margalet and Santos-Alvarez, 1997], and we found that PST binding was still sensitive to guanine nucleotides. Therefore, we performed the GTP binding assay using soluble rat liver membranes and precipitating the soluble proteins with polyethylene glycol. Figure 2A shows the time dependency of PST-stimulated GTP binding in soluble rat liver membranes. Maximal response was achieved at 10 min. Under these conditions, the effect of PST was dose dependent (Fig. 2B). Moreover, preincubation of soluble extracts with G protein α-subunits antisera produced results consistent with those observed with particulate membranes (Fig. 1C). Thus, Figure 2C shows the inhibition of PST effect on GTP binding by anti-$G\alpha_{q/11}$ serum, whereas anti-$G\alpha_{i1,2}$ inhibited only 15%. Anti-$G\alpha_{o,i3}$ serum failed to blunt this effect in soluble membranes (Fig. 2C).

Since we have previous data suggesting the association of the PST receptor with G proteins in WGA chromatographic purification [Santos-Alvarez et al., 1998], we tested PST-stimulated GTP binding activity in the fractions of the WGA column. We found that PST induced an increase in GTP binding in the same fractions in which the semipurified receptor was eluted, further suggesting the functional coupling of the soluble PST receptor with G proteins even after WGA semipurification. Figure 3 shows the increase in GTP binding exerted by PST, overlayed with specific PST binding in the WGA fractions.

To confirm the results obtained in GTP binding assay, we studied the coupling of the activated PST receptor to G proteins by photolabeling rat liver membranes with azido-α-$^{32}$P-GTP. As shown in Figure 4, PST stimulated azido-α-$^{32}$P-GTP binding to rat liver membranes in a dose-dependent manner, as assessed by scanning and densitometry of the autoradiogram.

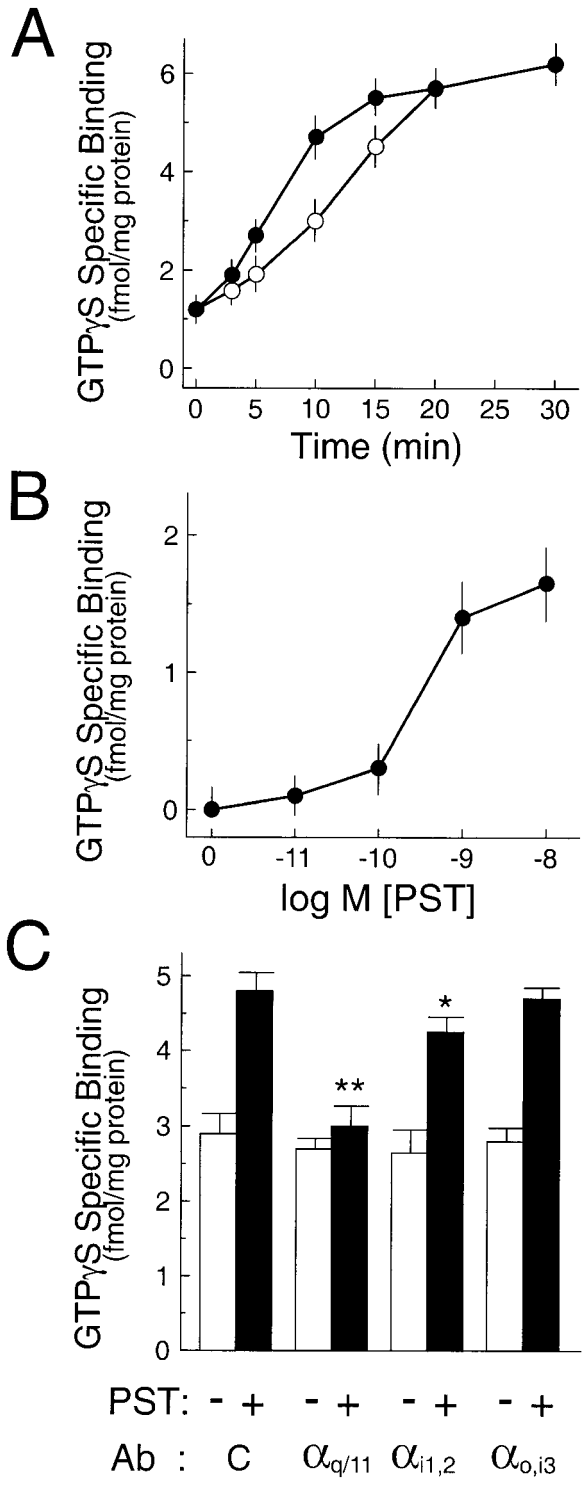


**Fig. 2.** PST stimulates γ-$^{35}$S-GTP binding to solubilized rat liver membranes. Blocking by Gα specific antisera. **A:** Solubilized rat liver membranes were incubated with γ-$^{35}$S-GTP in the presence (●) or absence (○) of PST ($10^{-8}$ M), and the binding was determined as described under Materials and Methods at different time points. Data are means ± SEM (n = 4) of the GTP binding activity assay run in quadriplicate. **B:** Concentration dependency of the PST-stimulated GTP binding to solubilized rat liver membranes. Solubilized membranes were incubated at 25°C for 10 min in the presence of increasing concentrations of PST to determine GTP binding activity, as described under Materials and Methods. The values of each experiments were based on quadruplicates tubes. Data are means ± SEM (n = 4) of the increase in GTP binding activity above control levels. **C:** Basal (−) and 10 nM PST-stimulated (+) GTP binding to soluble liver membranes was measured in the absence or presence of preincubation for 2 h with different blocking sera: anti- $G\alpha_{q/11}$, $G\alpha_{i1,2}$, $G\alpha_{o,i3}$ or preimmune sera (C, control). Data are means ± SEM of four different experiments run in quadruplicates. $^{*}P < 0.05$, $^{**}P < 0.001$, significant differences versus control.

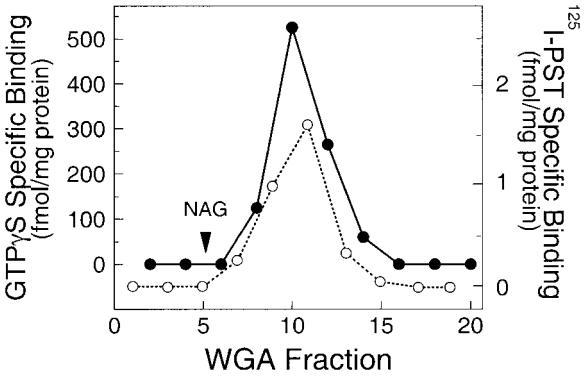


**Fig. 3.** PST stimulates γ-$^{35}$S-GTP binding to WGA purified soluble rat liver membranes. Fractions of the WGA column eluted with NAG were assayed for GTP binding activity (●) and PST binding (○), as described under Materials and Methods (numbers 0–20 refer to the respective fraction from WGA chromatography eluting with NAG). GTP binding refers to the PST-stimulated effect above control (without PST).

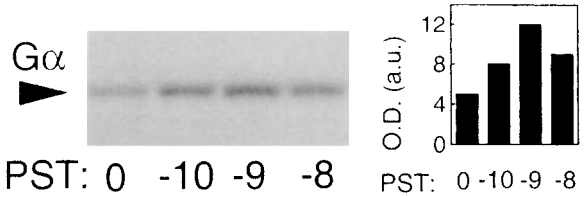


**Fig. 4.** PST stimulates photolabeling of rat liver membranes with 8-azido-α-$^{32}$P-GTP. Concentration dependency of the PST-stimulated GTP binding to rat liver membranes. Membranes were incubated at 25°C for 3 min in the presence of increasing concentrations of PST to determine the photolabeling of G proteins with 8-azido-α-$^{32}$P-GTP, as described under Materials and Methods. Samples were denatured and resolved on SDS-PAGE. A representative autoradiography (of three) with the densitometric analysis is shown.

To further assess the G protein that is associated with pancreastatin receptors, we performed the photolabeling assay in soluble rat liver membranes, followed by immunoprecipitation with specific antisera. Immunoprecipitation with $G\alpha_{q/11}$ antiserum showed increased photolabeling of a 42/43-kDa protein in response to increasing concentrations of PST (Fig. 5), whereas 10 nM PST only slightly increased the incorporation of azido-α-$^{32}$P-GTP into 40/41-kDa proteins immunoprecipitated with anti-$G\alpha_{i1,2}$ serum. On the other hand, $G\alpha_{o,i3}$ immunoprecitation did not show any increase in the incorporation of azido-α-$^{32}$P-GTP (Fig. 5). Control experiments with preimmune serum showed no labeling in the precipitate (not shown). Densitometric analysis (data not shown) revealed similar sensitivity data to those obtained with γ-$^{35}$S-GTP binding in both particulate and soluble membranes.

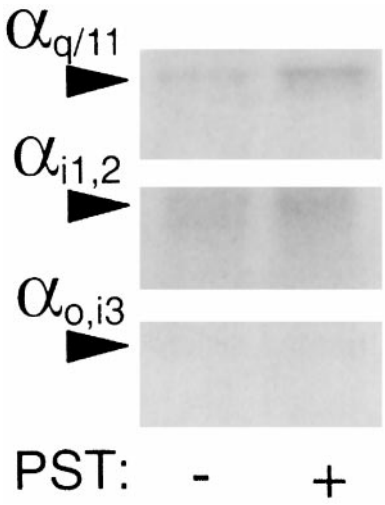


**Fig. 5.** PST stimulates photolabeling of $G\alpha_{q/11}$ and $G\alpha_{i1,2}$ proteins with 8-azido-α-$^{32}$P-GTP. Soluble membranes were incubated at 25°C for 5 min in the absence (−) or presence of 10 nM PST (+) to photolabel G proteins with 8-azido-α-$^{32}$P-GTP, as described under Materials and Methods. $G\alpha_{q/11}$, $G\alpha_{i1,2}$, or $G\alpha_{o,i3}$ were then immunoprecipitated as described under Materials and Methods. Samples were denatured and resolved on SDS-PAGE. Representative autoradiographies of three separate experiments are shown.

Finally, the efficiency of the immunoprecipitation of the different G proteins was assessed by specific immunoblotting by using anti-$G\beta_{common}$ (Fig. 6A) or specific anti-Gα sera (Fig. 6B). The nonspecific bands that appears in Figure 6 (lane b) from $\alpha_{i1,2}$ and $\alpha_{o,i3}$ are probably due to sample overloading, since more immunoprecipitate was loaded to get a better signal.

## DISCUSSION

We have previously characterized PST action in rat liver, as well as PST receptors in rat liver membranes [Sánchez-Margalet et al., 1996a,b]. We recently reported the successful solubilization of PST receptors from rat liver membranes [Sánchez-Margalet and Santos-Alvarez, 1997]. We also reported previous data suggesting that PST receptors are coupled to phospholipase C activation by interacting with some G proteins in rat liver membranes [Sánchez-Margalet and Goberna, 1994b]. Furthermore, we have previously shown indirect evidence of the involvement of a pertussis toxin-insensitive G protein that might mediate the activation of phospholipase C [Sánchez-Margalet and Goberna, 1994b], and subsequently, the increase in cytosolic free calcium [Sánchez-Margalet et al., 1993] and the activation of protein kinase C in the rat liver [Sánchez-Margalet et

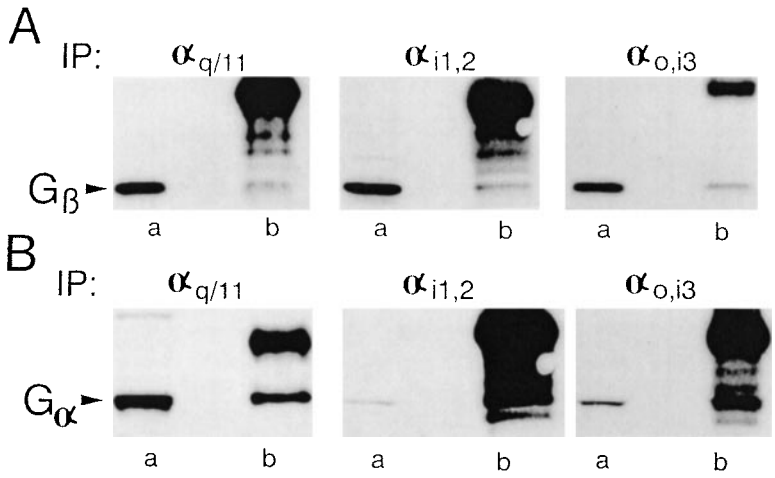


**Fig. 6.** Immunoprecipitation efficiency of different G proteins α-subunits. Soluble rat liver membranes were incubated with the specific antisera to immunoprecipitate each G protein, as described in Materials and Methods. Samples were denatured and resolved on a SDS-PAGE. Immunoprecipitates were then analyzed by Western blotting using anti-$G\beta_{common}$ antibody **(A)** or the specific anti-Gα antibody: anti-$G\alpha_{q/11}$, anti-$G\alpha_{i1,2}$, and anti-$G\alpha_{o,i3}$ **(B)**. **Lane a**, soluble membranes (10 µl, 1 mg/ml); **lane b**, the immunoprecipitate. Equivalent amount of each sample were loaded in the respective lane. In the case of anti-$G\alpha_{i1,2}$ and anti-$G\alpha_{o,i3}$ immunoprecipitation five times more material was loaded to compensate for the lower expression of these G proteins in the rat liver.

al., 1994b]. On the other hand, a pertussis toxin-sensitive mechanism was found to mediate PST stimulation of guanylate cyclase [Sánchez-Margalet and Goberna, 1994b; Sánchez-Margalet et al., 1996b]. Taken together, these data prompted us to look for the specific G proteins that might be involved in the signaling of PST receptors. Moreover, we had previously shown negative data of cyclic adenosime monophospate (cAMP) production stimulated by PST [Sánchez et al., 1992; Sánchez-Margalet and Goberna, 1994b], ruling out the possible role of a $G\alpha_s$ protein in PST receptor signaling and further supporting the possible involvement of G proteins of other families.

In this report, we explored the specific activation of G proteins by PST receptors in rat liver membranes. First, we demonstrated that PST stimulated GTP binding to the membranes in a dose-dependent manner. These results confirm the conclusions raised after indirect data obtained from PST binding inhibition with guanine nucleotides [Sánchez-Margalet et al., 1994a], as well as direct data of GTPase activity stimulated by PST in rat liver membranes [Sánchez-Margalet and Goberna, 1994b]. Moreover, we observed that this direct effect of PST stimulating GTP binding was inhibited mainly by anti-$G\alpha_{q/11}$ serum and slightly by anti-$G\alpha_{i1,2}$ serum, whereas anti-$G\alpha_{o,i3}$ serum had no effect. Similarly, PST still stimulates GTP binding to rat liver membranes after solubilization, although maximal effect was observed at longer time (10 min), suggesting a slower activation under solubilization conditions. These results were in agreement with our previous report showing the sensitivity of PST binding to guanine nucleotides in solubilized membranes [Sánchez-Margalet and Santos-Alvarez, 1997]. Moreover, preincubation with anti-$G\alpha_{q/11}$ and anti-$G\alpha_{i1,2}$, but not with anti-$G\alpha_{o,i3}$ sera, inhibited GTP binding as observed in particulate membranes.

We have also shown that semipurification of PST receptors by WGA chromatography copurified G proteins, suggesting physical association [Santos-Alvarez et al., 1998]. In order to check the functionality of this association we tested the GTP binding activity in the semipurificate. Accordingly, PST increased precipitable GTP binding in the WGA semipurified complexes. These results suggest that semipurified PST receptors are physical and functionally coupled to G proteins. However, it is worth pointing out that other receptors, also bound to WGA, may account for the co-purification. Therefore, even though these data are in line with our hypothesis, it remains to be finally proven.

In order to further address this issue, we next sought to find the specific G protein subunit activated by PST receptors. We employed 8-azido-α-$^{32}$P-GTP to photolabel the α-subunits of the G proteins in response to PST. As expected, there was a dose-dependent effect on the G protein labeling. Thereafter, since we had commercially available antisera to immunoprecipitate different G proteins, we look at the specific α-subunit photolabeled by PST receptors activation. The high basal binding of azido-α-$^{32}$P-GTP to α-subunits of $G_{i1,2}$ proteins that we observed is in agreement with previous work by others [Wange et al., 1991]. On the other hand, the decreased basal labeling of membranes observed in anti-$G\alpha_{q/11}$ immunoprecipitate agrees with previous published data [Wange et al., 1991; Bernstein et al., 1992] and

further support the slower basal GTP binding rate of $G_{q/11}$ α-subunits.

Specific immunoprecipitation of photolabeled G proteins showed that PST could increase the activation of $G\alpha_{q/11}$ and $G\alpha_{i1,2}$ (to a lesser extent), but not $G\alpha_{o,i3}$. The $G\alpha_{q/11}$ and $G\alpha_{i1,2}$ antisera are directed to the C-terminal part of the protein, which is the region necessary for receptor interaction and this way they may block the G protein activation, which may be compatible with immunoprecipitation of the activated α-subunits, as previously shown by others [Georgoussi et al., 1995]. Unfortunately, we could not test the activation immunoprecipitation of G proteins from other families with commercially available anti-$G\alpha_{12}$ and anti-$G\alpha_{13}$ sera because they do not precipitate (data not shown).

In conclusion, PST stimulation activates $G\alpha_{q/11}$ and $G\alpha_{i1,2}$ in the plasma membrane, and these mechanisms may provide the molecular basis for the activation of phospholipase C and guanylate cyclase by PST in the hepatocyte.

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