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Intracellular receptor for somatostatin in gastric mucosal cells: Decomposition and reconstitution of somatostatin-stimulated phosphoprotein phosphatases

(somatostatin receptor/Sephadex chromatography/histone/[Tyr^1]somatostatin ^{125}I-labeled on the Tyr/rat)

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ABSTRACT **Using ^{32}P-labeled histone as exogenous substrate, we showed a potent stimulatory effect of somatostatin on cytosolic phosphoprotein phosphatases (PPPases; phosphoprotein phosphohydrolase, EC 3.1.3.16) in rat gastric mucosal cells. Partial purification of cytosolic fraction in DEAE-Sephadex ion-exchange chromatography and further gel filtration on Sephadex G-75 and Sephadex G-100 separated somatostatin-dependent PPPases into three distinct molecular species. One corresponding to M_r 130,000 was devoid of any PPPase activity but specifically bound [Tyr^1]somatostatin ^{125}I-labeled on the Tyr ([^{125}I-Tyr^1]somatostatin) with an apparent equilibrium dissociation constant of 3×10^{-10} M. The two other molecular species corresponded to M_rs 64,000 and 13,000. They produced catalytic dephosphorylation of ^{32}P-labeled histone, but they were not sensitive to somatostatin and did not show any specific binding to radiolabeled hormone. Mixing of the larger with either of the two smaller molecular species resulted in concentration-dependent inhibition of PPPase activity. However this inhibition was reversed by increased concentrations of somatostatin, with the concentration for half-maximal reactivation being close to 0.1 nM. Furthermore, somatostatin stimulation in reconstituted materials developed according to a rapid time course ($t_{1/2}$, <5 sec), consistent with that observed for binding of [^{125}I-Tyr^1]somatostatin. These results strongly argue for the presence of an intracellular somatostatin receptor in gastric mucosal cells and characterize this receptor as a PPPase regulatory subunit. Thus, substrate dephosphorylation could be the primary event triggering physiological effects of somatostatin in stomach and perhaps other organs of the digestive tract [Reyl, F. & Lewin, M. J. M. (1981) *Biochim. Biophys. Acta* 675, 297–300].**

In addition to its early recognized effect on pituitary cells as growth hormone release-inhibiting factor (1, 2), the tetradecapeptide somatostatin inhibits a variety of cellular functions in the digestive tract (3); however, the molecular mechanisms involved in this effect are not known. Recent experiments from this laboratory have suggested a potent stimulatory effect of somatostatin on cytosolic phosphoprotein phosphatase (PPPase; phosphoprotein phosphohydrolase, EC 3.1.3.16) activity in pancreas, liver, and intestinal as well as gastric mucosa (4). This finding along with previously reported evidence for somatostatin binding to cytosolic protein (5, 6) prompted us to document the possible existence of an intracellular receptor for somatostatin in the digestive cells.

MATERIAL AND METHODS

Subcellular Fractionation and Preparation of Cytosolic Extract. Unstarved male Wistar rats weighing 200–300 g were killed by a blow on the neck and laparotomized. Gastric fundic mucosa was scraped and homogenized as described (7) in ice-cold 100 mM Tris·HCl, pH 7.4/2 mM dithiothreitol (buffer A) containing 5% sucrose. Subcellular fractionation was performed by submitting the homogenate to differential centrifugation (7, 8). Supernatant used for reconstitution studies was directly obtained from homogenate by 1-hr centrifugation at high speed (105,000 × *g*) (Spinco L50 centrifuge, Beckmann). Biochemical markers cytochrome *c* oxidase, 5′-AMPase, DNA, RNA, and proteins were estimated as reported (8).

Determination of Phosphatase Activities. PPPase activities were determined as described by Khandelwal *et al.* (9) with ^{32}P-labeled calf thymus type II-S histone (^{32}P-histone) as the substrate. The reaction medium contained 50 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 100 mM KCl, ^{32}P-histone (10,000 cpm), and a 50-μl sample of homogenate, supernatant, or subcellular fraction in a total volume of 100 μl. Reaction was initiated by the addition of enzyme and stopped by the addition of 0.4 ml of 5% trichloroacetic acid containing 0.25% sodium tungstate.

After 10 min of centrifugation at 1000 × *g*, radioactivity was estimated in the supernatant by liquid scintigraphy (spectrometric counter SL30, Intertechnique, Plaisir, France).

One unit of PPPase was defined as the amount of enzyme that releases 1 nmol of P_i from the phosphorylated substrate per min.

Purification of PPPases. Cytosol (15 ml) was mixed 1:1 (vol/vol) with DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden) that had been equilibrated for 48 hr in buffer A. After 30 min at 4°C, the DEAE-Sephadex was filtered, washed with 100 ml of buffer A, and introduced into a 13 cm × 1.5 cm column. PPPases were eluted from the column at 4°C with a linear NaCl gradient from 0 to 0.5 M in buffer A.

For further purification steps, fractions 19–24 containing the majority of ^{32}P-histone phosphatase activity were pooled, added to 100 mM 2-mercaptoethanol, and submitted to two successive freeze/thaw cycles. This procedure was suggested to promote dissociation of PPPase subunits (9). Pooled fractions were then mixed 1:1 (vol/vol) with neutral saturated ammonium sulfate solution and incubated for 30 min at 4°C. The mixture was centrifuged at 15,000 × *g* for 30 min, and the pellet containing the precipitated proteins was diluted in buffer A and applied to a 40 cm × 1.5 cm Sephadex G-75 column (Pharmacia). After elution with buffer A, the retarded fractions were pooled and processed. The material eluted in the void volume was concentrated by lyophilization and submitted to further gel filtration on Sephadex G-100 in a 13 cm × 1.5 cm column.

Binding Studies. Specific binding of somatostatin was characterized by using [Tyr^1]somatostatin ^{125}I-labeled on the Tyr

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Abbreviation: phosphoprotein phosphatase(s), PPPase(s).

([^{125}I-Tyr^{1}]somatostatin) as a tracer (6). This was introduced in 100-μl samples (total radioactivity, 150,000 cpm) either alone for determination of "total" binding or in the presence of 0.1 μM unlabeled somatostatin for determination of "nonspecific" binding. After 15 min of incubation at 22°C, the reaction was stopped by addition of 50 mM Tris·HCl (pH 7.4) containing 0.25% activated charcoal, 0.5% bovine serum albumin, and 0.025% T70 dextran (5). The reaction mixture was centrifuged for 10 min at 1000 × g, and radioactivity in the supernatant was estimated with a gamma spectrometric counter (Intertechnique CG 4000, Plaisir, France). "Specific" binding was calculated as the difference between "total" and "nonspecific" binding. Specific and nonspecific bindings, respectively, ranged from 3% to 5% and from 0.5% to 1% of total radioactivity.

RESULTS

Demonstration and Subcellular Distribution of Somatostatin-Stimulated Phosphohistone Phosphatase Activity. Somatostatin (0.1 nM) markedly stimulated phosphohistone phosphatase activity of gastric cell homogenate with both basal and somatostatin-stimulated activities being enriched in the cytosolic fraction (Table 1). This fraction contained no detectable amount of the mitochondrial and nuclear markers cytochrome *c* oxidase and DNA. Moreover, the relative percentages (percent over percent of protein) of 5′-AMPase (0.35 ± 0.04) and RNA (0.69 ± 0.03) (as compared, respectively, with 2.80 ± 0.23 and 2.53 ± 0.15 in the microsomal fraction) indicated a significant purification from plasmic and reticulum membranes. Accordingly, the following studies were carried out on supernatant obtained at high speed. In this material, basal and somatostatin-stimulated activity were respectively 0.85 ± 0.06 and 1.80 ± 0.28 units per mg of protein (mean values ± SEM from five experiments; P, <0.05).

Under the experimental conditions used, enzyme kinetics were linear up to 60 sec (Fig. 1).

Purification of Basal and Somatostatin-Stimulated Activities and Decomposition into Separate Units. Cytosolic phosphohistone phosphatase activity eluted from the DEAE-Sephadex column as a broad peak. Somatostatin-stimulated activity was still present and eluted according to a unimodal profile paralleling that of basal activity (Fig. 2). In one representative experiment, maximal specific activities (fraction 20) corresponded to 36 (basal) and 63 (stimulated) units per mg of protein. This represented an ≈40-fold purification over the initial homogenate.

Fractions 19–23 from the ion-exchange chromatography column were pooled and used as starting material for further purification. In one typical experiment, this material represented 3.7 mg of proteins and 49 units of phosphohistone phosphatase activity (basal)—i.e., only 2.5% of proteins but 36% of enzyme activity initially present in the homogenate.

After freeze/thaw treatment and protein precipitation by ammonium sulfate, the pooled fractions were submitted to Sephadex G-75 gel filtration. From this, phosphohistone phosphatase activity eluted as two distinct peaks. One corresponded to proteins of M_r close to 64,000, the other to M_r between 12,000 and 16,000 (Fig. 3). In these two peaks, maximal specific activity was respectively 14.6 units (fraction 17) and 66 units (fraction 33) per mg of protein. This corresponded to enrichments of 16-fold and 73-fold as compared to enzyme activity of the initial homogenate. Fractions 17–20 (first peak) on the one hand and 30–34 (second peak) on the other hand were pooled

Table 1. Specific activity of basal and 0.1 nM somatostatin-stimulated phosphohistone phosphatase (Histone PPase) in homogenate and subcellular fractions of rat fundic mucosa

Fraction	Proteins, %	Histone PPase, units/mg of protein	
		Basal	Stimulated
Nuclear	38.5 ± 4.5	0.33 ± 0.05	0.48 ± 0.06
Mitochondrial	17.9 ± 0.9	0.22 ± 0.02	0.22 ± 0.03
Microsomal	4.9 ± 1	0.13 ± 0.05	0.18 ± 0.09
Cytosolic	33.7 ± 1.4	0.85 ± 0.18	1.50 ± 0.22*
Homogenate	100	0.54 ± 0.07	1.09 ± 0.23*

Mean values ± SEM from three experiments.
* Significant difference at the level of 0.05 (Student's *t* test for paired data).

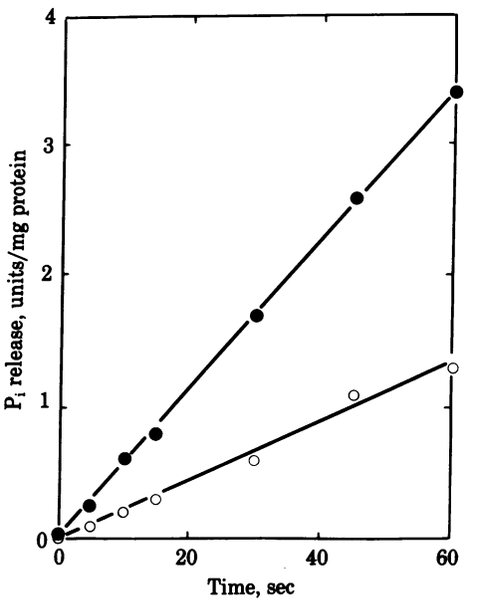

FIG. 1. Time dependence of P_i release from phosphohistone upon incubation with gastric cell homogenate (1 mg/ml) at 30°C, pH 7.4. ○, Basal conditions; ●, effect of 10-min preincubation with 0.1 nM somatostatin.

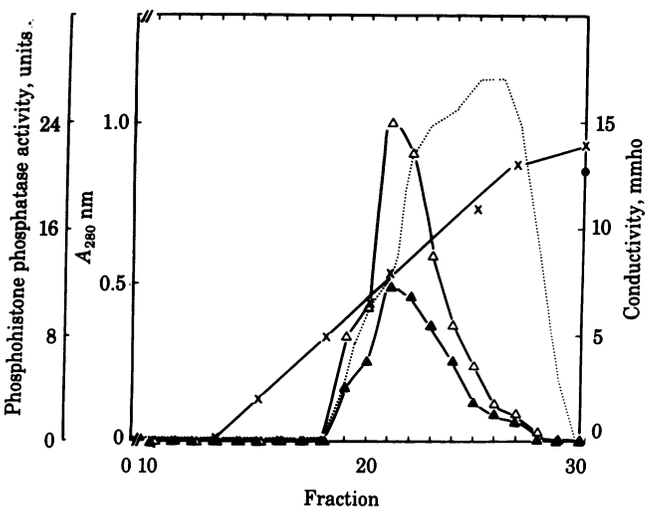

FIG. 2. Ion-exchange chromatography of rat gastric PPPase(s) using DEAE-Sephadex. Chromatographic fractions from the supernatant obtained at high speed were assayed for PPPase activity with ^{32}P-histone as the substrate (▲). △, Stimulated activity; ····, $A_{280\ nm}$ readings; ×, NaCl gradient. Fractions of 1 ml were collected. One typical experiment out of five is shown.

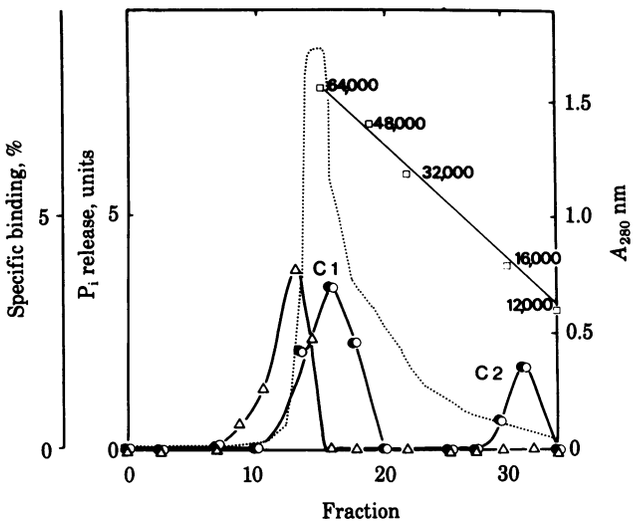

FIG. 3. Gel filtration of rat gastric PPPase(s) using Sephadex G-75. Pooled fractions from the DEAE-Sephadex column, after freeze/thaw and precipitation by ammonium sulfate, were chromatographed. PPPase activity (peaks C1 and C2): ●, basal; ○, 0.1 nM somatostatin stimulated. △, Specific binding of somatostatin expressed as percentage of bound over free hormone; ····, OD at 280 nm. The column was calibrated (□) with hemoglobin (M_r 64,000, 48,000, 32,000, and 16,000) and cytochrome *c* (M_r 12,000). Fractions of 1.5 ml were collected. One typical experiment out of three is shown.

and used afterwards with the respective designations "C1" and "C2."

Noteworthy, the stimulatory effect of somatostatin on phosphohistone phosphatases was apparently lost.

On the other hand, a specific binding of [^{125}I-Tyr^1]somatostatin was shown. This was associated with a nonretarded high M_r material clearly distinct from C1 and C2. Further chromatography of this material on a Sephadex C-100 column, suggested a M_r of ≈130,000 for the somatostatin-binding sites (Fig. 4). The

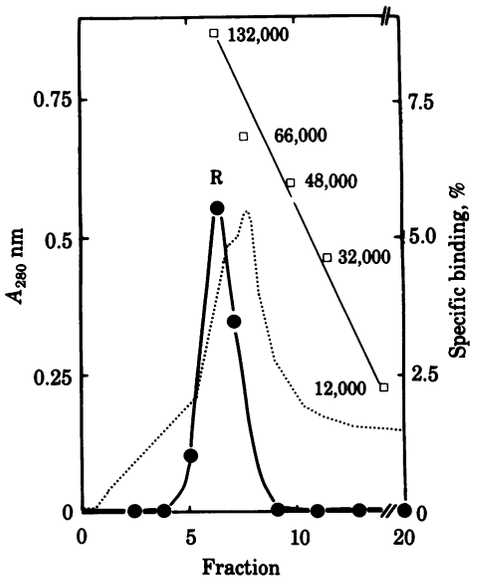

FIG. 4. Gel filtration on Sephadex G-100 of rat gastric mucosal cells cytosolic binding sites. Fractions 9–12 from Fig. 3 were pooled and processed. Fractions of 1 ml were collected. ●, Specific binding as percentage of bound over free hormone (peak R); ····, $A_{280\ nm}$ readings. The column was calibrated with albumin (M_r 132,000), hemoglobin (M_r 64,000, 48,000, 32,000, and 16,000), and cytochrome *c* (M_r 12,000).

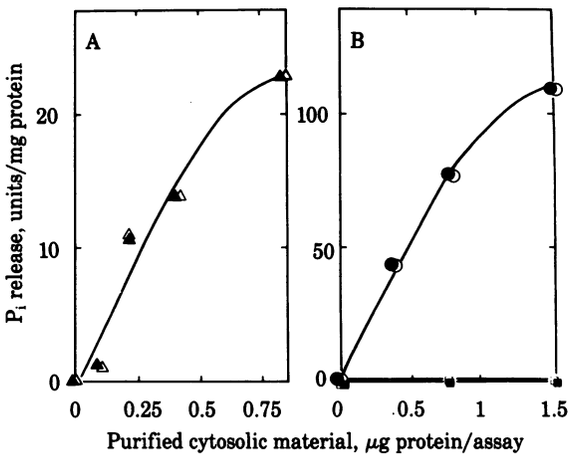

FIG. 5. Phosphohistone phosphatase activity from material purified by Sephadex chromatography. (*A*) Basal (▲) and 10 nM somatostatin-stimulated (△) activity of peak C1 (pooled fractions 17–20). (*B*) Basal (●) and 10 nM somatostatin-stimulated (○) activity of peak C2 (pooled fractions 30–34). Activity of peak R (Fig. 4): ■, basal; □, 10 nM somatostatin stimulated.

corresponding peak was collected and used afterwards under the designation "R."

No detectable phosphohistone phosphatase activity was found in R for protein concentrations up to 3.4 μg per assay, and the additional presence of 10 nM somatostatin in the reaction mixture did not result in unmasking of activity. In contrast with this finding, phosphohistone phosphatases were constantly present in C1 and C2, with activities being proportional to protein concentration (Fig. 5). However, on this material as well, somatostatin did not produce any detectable stimulation.

Reconstitution of Somatostatin Stimulation. Addition of increased concentrations of R to a fixed amount of C1 or C2 resulted in a progessive inhibition of enzyme activity down to residual levels, representing respectively some 26% and 15% of the initial activity (Fig. 6). However, full initial activity was rapidly restored ($t_{1/2}$, <2 s) upon 90% dilution of the medium. Therefore, the inhibitory effect of R on phosphohistone phosphatase activities of C1 and C2 was suggested to account for the

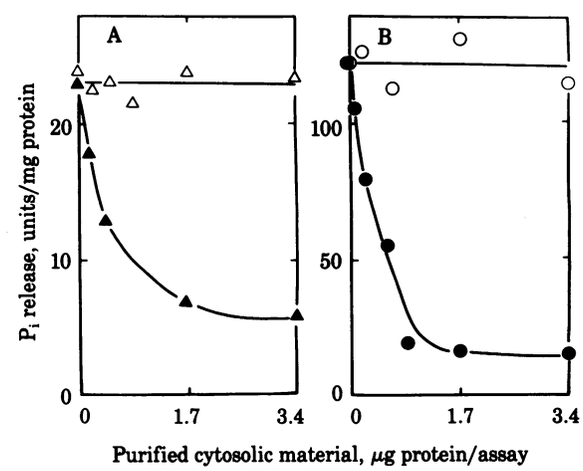

FIG. 6. Effect of chromatographically purified material R on phosphohistone phosphatase activity of cytosolic fractions C1 and C2 from rat gastric mucosa. (*A*) Activity corresponding to peak C1 (0.85 μg per assay of pooled fractions 17–20) in absence (▲) or in the presence (△) of 100 nM somatostatin. (*B*) Activity corresponding to peak C2 (1.5 μg per assay of pooled fractions 30–34) in the absence (●) or the presence (○) of 100 nM somatostatin.

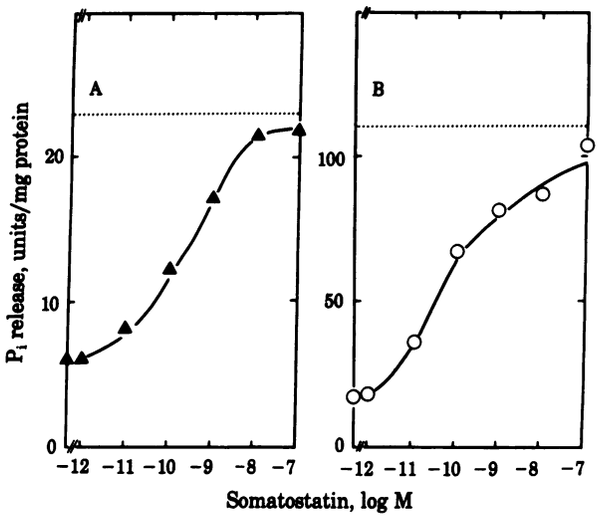

FIG. 7. Stimulation by somatostatin of cytosolic PPPases in rat gastric mucosal cells. Assays were performed with ^{32}P-histone as the substrate. (*A*) Reconstituted material "R–C1" containing per assay 0.85 μg of C1 (pooled fractions 17–20) together with 3.4 μg of R (pooled fractions 5–9). (*B*) Reconstituted material "R–C2" containing per assay 1.5 μg of C2 (pooled fractions 30–34) together with 3.4 μg of R (pooled fractions 5–9). Dotted lines indicate activities corresponding either to C1 or C2 in the absence of added R material.

reversible formation of inactive binary complexes R–C1 and R–C2. This inhibitory effect was prevented by the presence of a high concentration of somatostatin (Fig. 6). Furthermore, subsequent addition of increased concentrations of somatostatin to reconstituted materials R–C1 or R–C2 resulted in a progressive restoration of enzyme activities up to maximal levels that were similar to the initial activities in C1 and C2 in the absence of R, respectively (Fig. 7). This suggested that somatostatin stimulation of phosphohistone phosphatase activities in reconstituted materials was actually due to reversion of the inhibitory effect of R. Furthermore, that this reversion was itself a consequence of somatostatin binding to R was supported by the good agreement between somatostatin concentrations for half-maximal stimulation of enzyme activity on the one hand (Fig. 7) and half-maximal displacement of radiolabeled somatostatin binding to R on the other (Fig. 8). Moreover, time dependence of radiolabeled somatostatin binding to R–C1 and R–C2 (Fig.

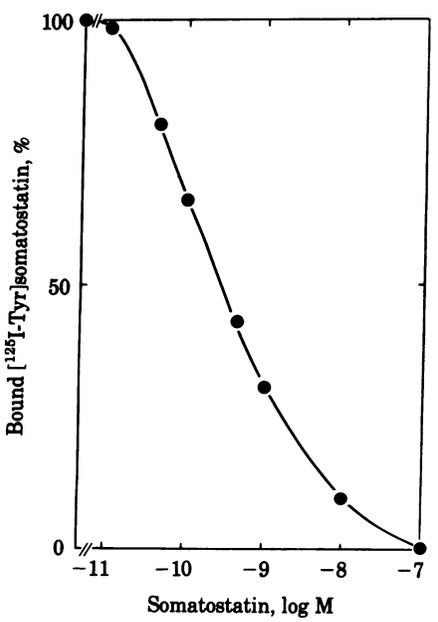

FIG. 8. Displacement of [^{125}I-Tyr^1]somatostatin binding to cytosolic material R (Fig. 4) by native somatostatin. A fixed tracer amount of radiolabeled somatostatin (100,000 cpm per assay) was incubated with R material (pooled fractions 5–9; 17 μg per assay) together with increased concentration of unlabeled hormone; the bound radioactivity was determined and expressed as percentage of radioactivity remaining bound at equilibrium, with 100% corresponding to the amount bound in the absence of somatostatin.

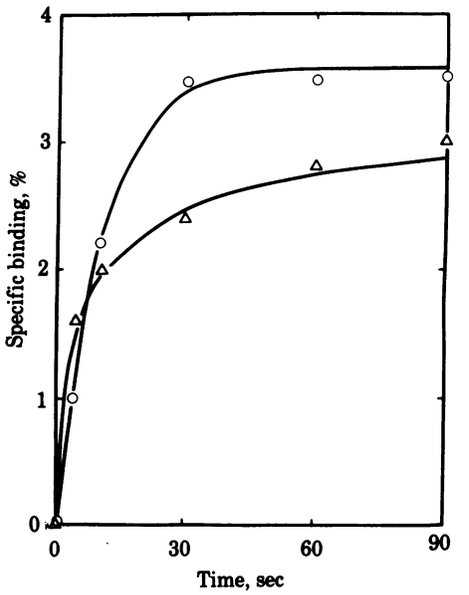

FIG. 9. Time course of [^{125}I-Tyr^1]somatostatin binding to R–C1 (△) and R–C2 (○). Reconstituted materials were prepared as described in the legend of Fig. 7. Each assay tube contained 3.4 μg of R with 0.85 μg of C1 or 1.5 μg of C2. Reaction was started by addition at 0 time of [^{125}I-Tyr^1]somatostatin in native somatostatin (final concentration, 10 nM). Specifically bound radioactivity was estimated as described in the text.

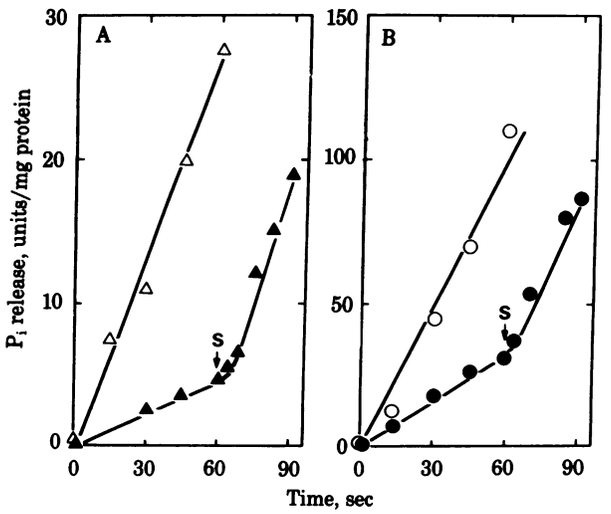

FIG. 10. Time course of stimulation by somatostatin of cytosolic PPPase activity in rat gastric mucosal cells. Assays were performed with ^{32}P-histones as the substrate. (*A*) △, Enzyme activity of chromatographically purified material C1 (pooled fractions 17–20); ▲, enzyme activity of reconstituted material R–C1 (Fig. 7); (*B*) ○, Enzyme activity of chromatographically purified material C2 (pooled fractions 30–34); ●, enzyme activity of partially reconstituted material R–C2 (Fig. 7). The arrows indicate the addition of 10 nM somatostatin (S) to the reaction mixtures.

9) was consistent with the time dependence of phosphohistone phosphatase stimulations in these materials (Fig. 10).

DISCUSSION

The present results strongly support the contention that somatostatin is a potent activator of PPPase in gastric mucosal cells. They furthermore suggest that somatostatin stimulation should proceed as follows:

$$R + C \rightleftarrows R\text{–}C \quad [1]$$

$$R\text{–}C + S \rightleftarrows R\text{–}S + C, \quad [2]$$

where R stands for regulatory (somatostatin binding) unit, C for catalytic subunit(s) and S for somatostatin. According to this model, inhibition of PPPase activity in the absence of somatostatin would be due to the binding of R to C (i.e., either to C1 or C2), which results in the formation of the inactive complex R–C (reaction 1), whereas restoration of enzyme activity in the presence of somatostatin would be due to release of active catalytic subunits from the complex R–C as a consequence of hormone binding to R (reaction 2). Thus, the mechanism by which somatostatin stimulates PPPases in gastric cells is suggested to be formally analogous to that by which cyclic AMP stimulates cyclic AMP-dependent protein kinase.

The suggestion that somatostatin binding to R–C does result in release of free catalytic units C is consistent with the observation that maximally stimulated activities were close to catalytic activities in the absence of R and with the previous demonstration that stimulation of PPPase activity is a consequence of increased maximal velocity (4, 10). Moreover, additional studies suggest displacement of somatostatin binding to R and chase of R-bound somatostatin in the presence of C1 or C2 (unpublished observations). Therefore, it appears likely that the ternary complex S–R–C is only transient, this because binding of S and binding of C antagonize one another as a consequence of competition for a common site or of negative heterotropic cooperativity (10).

Preliminary observations suggest that the effects here reported are specific to somatostatin (4). Because the present work was restricted to the study of *in vitro* dephosphorylation of exogenous histone, the existence and the nature of the physiological substrates *in vivo* remain open to speculation. There was no apparent interaction between the two catalytic subunits C1 and C2, but it is not certain whether these could correspond to separate or identical functions. Because of the known involvement of phosphorylation in regulation of protein synthesis, it is possible that they might participate in such a process. On the other hand, there is strong evidence that protein phosphorylation plays a crucial role in gastric secretory functions such as H^+ and Cl^- transports by the parietal cells (11, 12), but putative control of these transports by PPPase has not been yet documented.

The proposal that activation of cytosolic PPPases by somatostatin could have a physiological meaning obviously led to the corollary question of how is this peptide hormone transported into the cell. Evidence that somatostatin can penetrate plasma membrane *in vitro* is supported by previous binding studies on isolated rat gastric mucosal cells with [^{125}I-Tyr^1]somatostatin as a tracer (6). The process by which such a penetration would occur is so far unknown but may be tentatively suggested to involve internalization of surface receptors, as proposed for insulin (13).

The finding that somatostatin stimulation of PPPase also occurs in pancreas, liver, and intestinal cells (4), strongly suggest that a similar mechanism is present in these cells too. Thus, the model proposed here generally could account for a cytosolic somatostatin receptor ubiquitously present in several, if not all, target cells. This view, emphasizing a basic and postmembrane-located mechanism of action of somatostatin, would be consistent with the remarkable plurality of action of this hormone. However, further studies are needed to provide direct evidence for involvement of this receptor in the inhibitory action of somatostatin *in vivo*.

We are very grateful to Dr. Diaz (Centre de Recherches Clin Midy Industrie-Sanofi, Montpellier, France) for the generous gift of somatostatin.

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