

Hormonal Stimulation in the Exocrine Pancreas Results in Coordinate and Anticoordinate Regulation of Protein Synthesis

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ABSTRACT 24-h intravenous caerulein infusion studies in the rat were combined with in vitro amino acid incorporation studies followed by high-resolution separation of proteins by two-dimensional isoelectric focusing and SDS gel electrophoresis to study the extent to which persistent changes in the biosynthesis of exocrine pancreatic proteins are regulated by cholecystokinin-like peptides. Beginning in the third hour of optimal hormone infusion at $0.25 \mu\text{g kg}^{-1} \text{h}^{-1}$, changes were observed in the synthetic rates of 12 proteins, which progressed over the course of the 24-h study. Based on coordinate response patterns, exocrine proteins could be classified into four distinct groups. Group I (trypsinogen forms 1 and 2) showed progressive increases in synthetic rates reaching a combined 4.3-fold increase over control levels. Group II (amylase forms 1 and 2) showed progressive decreases in synthesis to levels 7.1- and 14.3-fold lower than control levels, respectively. Group III proteins (ribonuclease, chymotrypsinogen forms 1 and 2, procarboxypeptidase forms A and B, and proelastase 1) showed moderate increases in synthesis, 1.4–2.8-fold, and group IV proteins (trypsinogen 3, lipase, proelastase 2, and unidentified proteins 1–4) did not show changes in synthesis with hormone stimulation. Regulation of protein synthesis in response to caerulein infusion was specific for individual isoenzymic forms in the case of both trypsinogen and proelastase. The ratio of biosynthetic rates of trypsinogen forms 1 + 2 to amylase forms 1 + 2 increased from a control value of 0.56 to 24.4 after 24 h of hormonal stimulation (43.5-fold increase). Biosynthetic rates for an unidentified protein (P23) with an $M_r = 23,000$ and isoelectric point of 6.2 increased 14.2-fold, and the ratio of synthesis of P23 to amylase 2 increased 200-fold during caerulein infusion. During hormone stimulation the anticoordinate response in the synthesis of pancreatic glycosidases (decreased synthesis) and serine protease zymogens (increased synthesis) explain previous observations that showed little change in rates of total protein synthesis under similar conditions.

Previous studies have shown dramatic effects of cholecystokinin-like peptides on protein secretion (1–3), amino acid uptake (4, 5), glucose uptake (6), and growth and cell proliferation (7–9) in the exocrine pancreas. In marked contrast, little change in exocrine pancreatic function at the level of protein synthesis has been observed under similar conditions. Earlier studies showed conflicting results with modest increases in total protein synthesis in vivo after bolus injections of cholecystokinin (10–13) and modest decreases in total synthesis during cholecystokinin stimulation in vitro (14). Since the exocrine pancreas synthesizes and secretes approx-

imately 20 exportable proteins (15), anticoordinate changes in individual biosynthetic rates could explain the modest changes observed at the level of total protein synthesis. In preliminary studies which analyzed biosynthetic rates of rat exocrine pancreatic proteins separated in one dimension by isoelectric focusing (IEF),¹ rates for chymotrypsinogen increased and amylase decreased following bolus hormone injection (16).

¹ *Abbreviations used in this paper:* IEF, isoelectric focusing; IEP, isoelectric point.

In vivo studies allow for the continuous intravenous infusion of hormone in the intact organism. Previous dose-response studies in the rat indicate that infusion of caerulein, a cholecystokinin-like hormone, at $0.25 \mu\text{g}, \text{kg}^{-1} \text{h}^{-1}$ for periods up to 24 h resulted in optimal pancreatic ductal secretion of (pro)enzymes without morphological evidence of acinar cell injury (17). To look for both coordinate and anticonordinate changes in protein synthesis in the exocrine pancreas during in vivo caerulein stimulation, we studied persistent changes in protein synthesis by following the incorporation of amino acids into proteins contained in pancreatic lobules incubated in vitro. Subsequent separation of proteins by two-dimensional IEF/SDS gel electrophoresis allowed us to follow the response observed in the protein synthetic rates of 20 discrete pancreatic gene products (18). We have previously shown that changes in protein synthesis measured in this manner reflect, in part, changes in levels of functional mRNA (19).

MATERIALS AND METHODS

In Vivo Hormone Infusion: Male Wistar rats weighing ~200–250 g obtained from Hoechst, AG (Frankfurt, Federal Republic of Germany) were prepared for infusion studies by surgical cannulation either of the jugular vein or the tail vein as previously described (20, 21). Cannulated rats were maintained unanesthetized in cages with unrestricted movement and free access to food and water. Infusion studies were begun at 9:00 a.m. when alimentary and metabolic functions in the rat exist at minimal levels. Rats were infused intravenously with caerulein at $0.25 \mu\text{g} \text{kg}^{-1} \text{h}^{-1}$ for various periods over 24 h.

Measurement of Protein Synthesis: Animals were killed by exsanguination during light ether anesthesia. The pancreas was surgically removed and lobules were prepared as described by Scheele and Palade (22). Lobules were incubated at 37°C for 120 min in a Krebs-Ringer HEPES solution, pH 7.4, containing a mixture of $15 \text{ }^{14}\text{C}$ -amino acids (algal hydrolyzate, Amersham CFB25, 0.25 Ci/ml, Amersham Corp., Arlington Heights, IL) and physiological concentrations of all amino acids (23). Under these conditions protein synthesis was linear over the 2-h period tested, levels of trichloroacetic acid-soluble radioactivity achieved steady state by 30 min, and discharge of labeled proteins from lobules occurred at insignificant levels (3% at 120 min). Protein synthesis was terminated by homogenization of lobules (~90 mg wet wt) in 2.5 ml lysis buffer (25 mM Tris HCl, pH 8.9, 0.01% Triton X-100, 20 $\mu\text{g}/\text{ml}$ soybean

trypsin inhibitor, 100 KIU/ml Trasylol, and 1 mM diisopropyl fluorophosphate). Insoluble membranous material was sedimented in an Eppendorf microfuge and samples were stored at -70°C before separation by two-dimensional gel electrophoresis.

Total protein synthesis was measured by quantitation of trichloroacetic acid-insoluble radioactivity and expressed per microgram of DNA as determined by the method of Richards (24). Rates of synthesis of individual exocrine proteins were measured by following the incorporation of radiolabeled amino acids into proteins separated by two-dimensional gel electrophoresis as follows. Nonreduced proteins were separated in two dimensions as described by Scheele (25) using IEF in the presence of 0.1 mM diisopropyl fluorophosphate, 0.33% pH 3.5–10, 0.33% pH 5–8, 0.5% pH 4–6, and 0.4% pH 9–11 Ampholine (LKB Instruments, Inc., Gaithersburg, MD) in the first dimension and SDS gel electrophoresis in a 10–20% acrylamide gradient containing 0.2% SDS in the second dimension. Coomassie Blue spots were removed from two-dimensional gels by scalpel, acrylamide pieces were homogenized in 2 ml of distilled water, and radioactivity contained in homogenized acrylamide was suspended in Packard Instagel (Packard Instrument Co., Inc., Downers Grove, IL) in a volume ratio of 1:5, respectively. Radioactivity contained in the resulting gel phase was counted in a liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). Using labeled secreted products, we recovered 90–91% of the radioactive proteins in the two-dimensional gel spots.

Fractional rates of protein synthesis were determined by following the incorporation of radioactive amino acids into individual proteins separated by the two-dimensional gel procedure and relating individual values to the total incorporation value obtained for the complete set of two-dimensional gel spots. In association with measurements of total protein synthesis per unit DNA, fractional rates can be expressed as absolute synthetic rates.

RESULTS

Fig. 1 shows the Coomassie Blue staining pattern of Wistar rat exocrine pancreatic proteins separated by two-dimensional IEF/SDS gel electrophoresis. Biological activities of these proteins were determined by measurement of enzymic and proenzymic activities of proteins separated in the first dimension IEF gel after elution from gel slices and by co-migration with commercially available pancreatic proteins separated in the second dimension SDS gel as previously described (25). Multiple forms of individual enzymes were found including three forms of trypsinogen, two forms each for chymotrypsinogen, proelastase, and amylase, and single forms for lipase

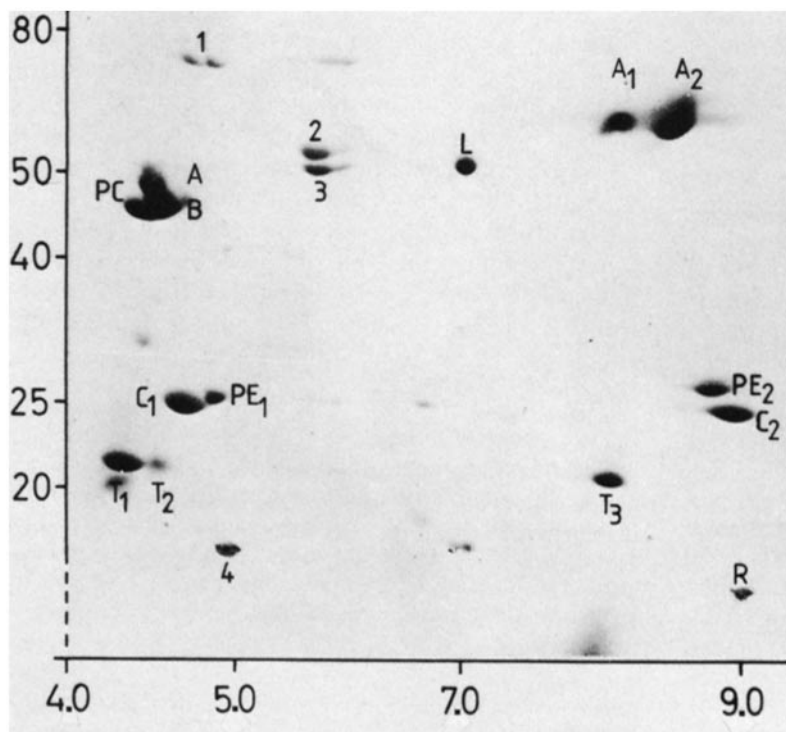


FIGURE 1 Separation of Wistar rat exocrine pancreatic proteins by two-dimensional IEF/SDS gel electrophoresis. Soluble proteins extracted with alkaline buffer from a purified zymogen granule fraction were separated in the first dimension by IEF and in the second dimension by SDS gel electrophoresis using a 10–20% acrylamide gradient as described by Scheele (25). Protein spots were stained with Coomassie Blue and are identified in the figure by abbreviations described in Table I. Numbers on the abscissa indicate isoelectric values. Numbers on the ordinate indicate apparent molecular weight values $\times 10^{-3}$.

and ribonuclease. Several forms of procarboxypeptidase A and B (at least four) showed similar isoelectric points (IEPs) and M_r values and could not be completely separated by the two-dimensional gel procedure. They were accordingly analyzed as a group. Four proteins, labeled 1–4, could not be identified by enzymic or proenzymic activity. Abbreviations are given in Table I along with the isoelectric points, apparent molecular weights, and quantitative distribution as judged by the percent incorporation of radioactive amino acids into individual proteins relative to the incorporation into all exocrine proteins.

Data presented in Fig. 2 show the persistent effects of optimal caerulein infusion over 24 h on the synthesis of the major groups of functional enzymes and zymogens in the rat pancreas. Little change was observed over the first 2 h of stimulation. From 3 to 24 h we observed changes in the synthesis of each of these groups of proteins, as shown in Fig. 2.

To compare the changes in synthetic rates of individual isoenzymic forms within each of the groups shown in Fig. 2, we determined the pattern of response of individual enzymes and zymogens to caerulein stimulation (Fig. 3). The findings varied. Whereas the fractional percentage response of chymotrypsinogen forms 1 and 2 appeared similar, the response of the three trypsinogen forms were dissimilar. Trypsinogen forms 1 and 2 both showed progressive increases in fractional percent synthesis with time. Synthesis of trypsinogen 3, the major cationic form of this proenzyme, did not show significant changes. Amylase forms 1 and 2 showed similar re-

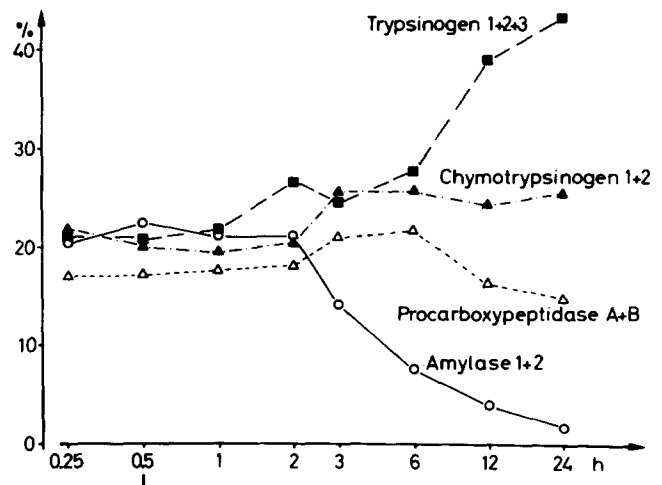


FIGURE 2 Fractional synthetic rates of exocrine pancreatic proteins as a function of time during caerulein stimulation. Rats were infused intravenously with caerulein for various periods of time, as described in Materials and Methods. Animals were killed and pancreatic lobules were prepared and incubated in the presence of radioactive amino acids for 120 min *in vitro* as described in Materials and Methods. Radioactive proteins contained in cell homogenates were separated by two-dimensional IEF/SDS gel electrophoresis, and radioactivity was quantified in individual protein spots and expressed as a fractional percent of radioactivity incorporated into all exocrine proteins. Data are given for groups of isoenzyme forms representing individual functional enzymes and zymogens. At each time point, the data represent average values from four animals.

sponses to caerulein stimulation, a progressive decrease in fractional percentage synthesis from 3 to 24 h.

Total protein synthesis remained unchanged for the first 2–3 h of caerulein stimulation. At later time points a progressive but modest increase in protein synthesis was observed. Average incorporation rates representing four experiments each at 0.25, 0.5, 1, 2, 3, 6, 12, and 24 h were 525, 610, 560, 560, 650, 715, 750, and 930 dpm/ μ g DNA, respectively. At 24 h rates of protein synthesis were increased 60% compared with those from control animals.

Table II presents data that correct the fractional percentage synthesis values for the progressive increase in total protein synthesis observed at the later time points. These figures represent absolute values expressed in disintegrations per minute per microgram of DNA.

Fig. 4 shows the appearance of a protein with $M_r = 23,000$ and IEP = 6.2 during caerulein infusion which was not observed among Coomassie Blue-stained exocrine proteins derived from the pancreas of control animals (cf. Fig. 1).

DISCUSSION

Caerulein, a synthetic decapeptide originally obtained from the skin of the frog *Hyla caerulea* (27), was chosen for these studies since it shares an identical C-terminal pentapeptide amide with cholecystokinin-pancreozymin, the major peptidergic stimulant for protein secretion in the exocrine pancreas. No attempt was made to mimic serum levels of cholecystokinin-pancreozymin during normal alimentation. Instead, infusions were conducted with optimal doses of caerulein in order to observe the limits of biochemical response to hormonal stimulation. Caerulein infusions at $0.25 \mu\text{g kg}^{-1} \text{h}^{-1}$ result in maximal secretory responses without pathological effects in the morphological appearance of the gland or in the

TABLE I
Wistar Rat Exocrine Pancreatic Proteins

Label	(Pro)enzyme	IEP	M_r^*	Distribution [†]	
				Mean	SD
%					
A1	Amylase 1	8.6	55,000	10.6	1.5
A2	Amylase 2	8.9	53,000	15.9	2.4
L	Lipase	6.8	50,000	4.5	0.5
T1	Trypsinogen 1	4.3	21,000	13.2	1.4
T2	Trypsinogen 2	4.4	21,000	1.6	0.3
T3	Trypsinogen 3	8.0	22,500	6.9	1.0
C1	Chymotrypsinogen 1	4.8	25,000	11.9	1.5
C2	Chymotrypsinogen 2	9.0	25,000	4.4	0.7
PE1	Proelastase 1	4.9	26,000	2.5	0.4
PE2	Proelastase 2	9.2	28,500	3.6	0.6
PCA	Procarboxypeptidase A	4.4	49,000	5.8	0.4
PCB1	Procarboxypeptidase B1	4.3	47,000	5.2	0.4
PCB2	Procarboxypeptidase B2	4.5	47,000	3.7	0.3
PCB3	Procarboxypeptidase B3	4.6	47,000	1.7	0.3
R	Ribonuclease	9.2	14,000	0.6	0.2
1	Unidentified	4.7	77,500	2.4	0.6
2	Unidentified	5.4	56,000	1.6	0.5
3	Unidentified	5.4	54,000	0.9	0.2
4	Unidentified	5.0	13,000	1.8	0.7

Isoenzyme forms are labeled numerically with increasing isoelectric points in agreement with the IUPAC-IUB Commission on Biochemical Nomenclature for the identification of multiple forms of enzymes separated by gel electrophoresis.

* Apparent molecular weight of nonreduced proteins.

[†] Distribution of proteins as quantified after incorporation of a mixture of ^{14}C -amino acids into pancreatic lobules incubated *in vitro* for 2 h and separation of proteins by two-dimensional IEF/SDS gel electrophoresis. Data are expressed as percent relative to radioactivity contained in all exocrine protein spots. Mean and standard deviation values were derived from 20 animals fed a diet containing 22% protein, 63% carbohydrate, and 3% fat.

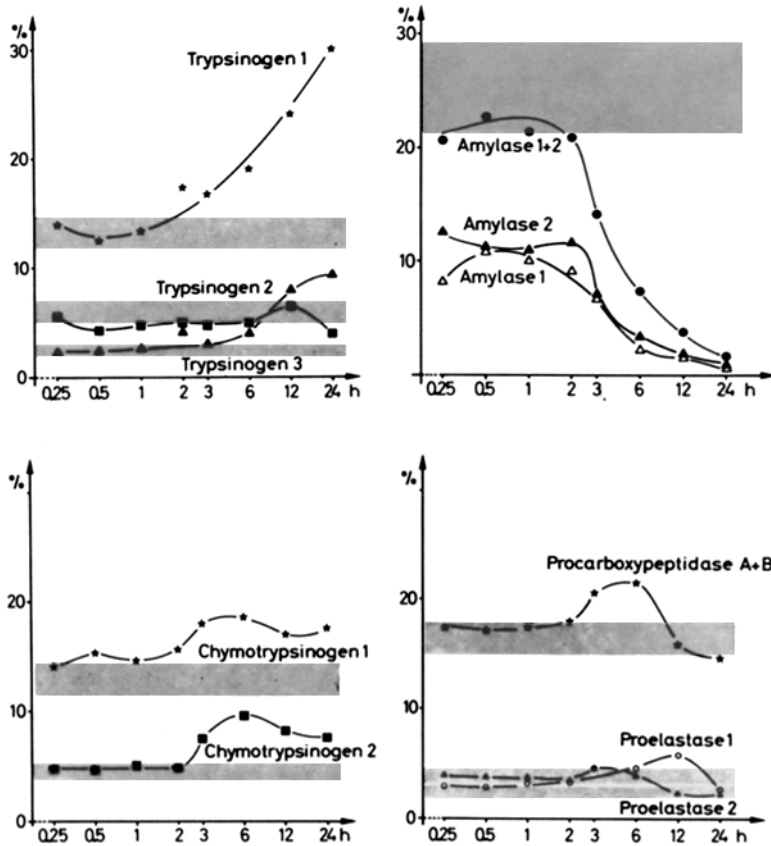


FIGURE 3 Fractional synthetic rates for isoenzyme and isozymogen forms of exocrine proteins as a function of time during caerulein stimulation. Methods of procedure are summarized in the legend to Fig. 2. Protein synthetic rates for individual forms are presented as fractional percents of radioactivity incorporated during a 120-min *in vitro* incubation into individual proteins relative to radioactivity incorporated into all exocrine proteins. Shaded areas indicate the normal range of values for individual proteins (or amylase 1 + 2 combined) ± 1 SD. The identities of individual proteins are indicated. The data given at each time point represent average values from four animals.

TABLE II
Absolute Changes in Rates of Protein Synthesis during Caerulein Stimulation

Group*	Identity†	Time of caerulein infusion, h					Fold change with time‡
		0	3	6	12	24	
		<i>dpm/μg DNA</i>					
I	P23	2.3	10.5	20.2	24.3	32.7	14.2
	T1	76.9	107.3	139.9	193.1	282.2	4.3
	T2	9.3	18.3	22.4	60.7	89.1	—
II	A1	61.8	46.5	18.4	13.3	8.6	0.14
	A2	92.7	44.9	21.0	14.2	6.6	0.07
III	R	3.5	5.7	7.5	13.0	11.5	3.3
	C1	69.4	118.6	134.2	121.4	166.3	2.4
	C2	25.6	50.1	61.7	61.3	72.9	2.8
	PE1	14.6	31.3	27.8	46.9	27.7	1.9
	PCA + B	95.6	136.5	155.2	119.4	138.0	1.4
	PE2	21.0	30.8	33.8	16.2	23.1	1.1
IV	T3	40.2	28.3	35.3	45.9	35.3	0.88
	L	26.2	23.5	23.9	14.0	22.2	0.85
	1	14.0	20.4	18.3	9.5	17.9	1.3
	2	9.3	15.3	19.4	8.7	9.3	1.0
	3	5.2	12.5	8.7	3.7	4.3	0.83
	4	10.5	4.3	8.1	8.6	10.1	0.97
	T1 + 2/A1 + 2 [§]	0.56	1.4	4.1	9.3	24.4	43.5
	P23/A2 [¶]	0.0248	0.23	0.96	1.71	4.95	199.5

* Groups of proteins that show coordinated changes in synthesis rates in response to caerulein infusion.

† Abbreviations described in Table I.

‡ 24-h value divided by 0 h value.

§ Ratios of protein synthetic rates.

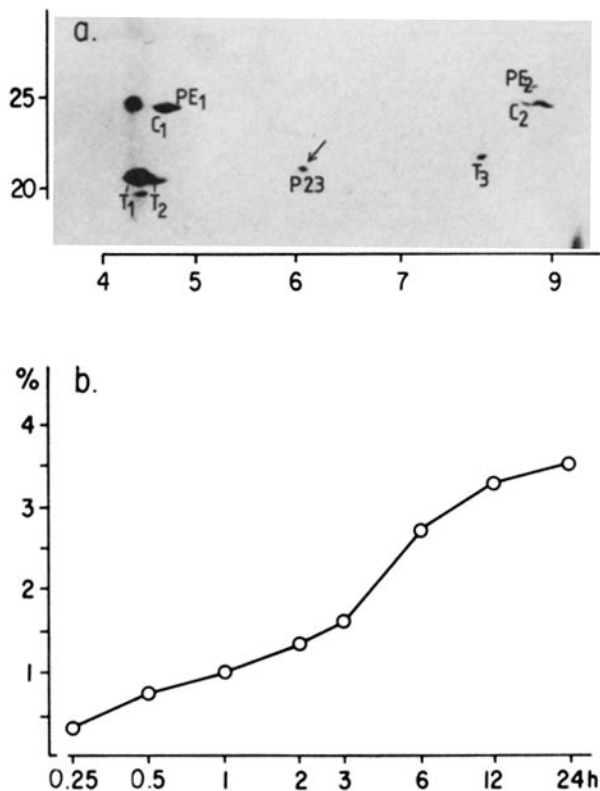


FIGURE 4 Hormone induction of P23. a shows the presence of P23 among serine protease zymogens obtained in a zymogen granule fraction isolated from rats infused with $0.25 \mu\text{g kg}^{-1} \text{h}^{-1}$ caerulein for 24 h. P23, indicated by the arrow, shows an IEP of 6.2 and an M_r of 22,500. b shows the increase in biosynthetic rate of P23 relative to the entire mixture of exocrine proteins as a function of time during caerulein ($0.25 \mu\text{g kg}^{-1} \text{h}^{-1}$) stimulation.

extracellular compartmentation of pancreatic proteins (17, 27). Two-dimensional IEF/SDS gel electrophoresis was used to follow individual rates of synthesis for all the major pancreatic exocrine proteins. 15 of the 20 proteins analyzed by this procedure were identified according to their biological activity. Pulse-chase studies suggest that each of the proteins shown in Fig. 1 are coded by separate cytoplasmic mRNA species since the appearance of none of the spots can be attributed to polyprotein structures or posttranslational modifications (data not shown).

Several new findings emerged from this study. In sharp contrast with earlier findings, dramatic changes were observed in the synthesis of exocrine proteins during hormone stimulation. Large changes in the protein synthetic rates were observed for forms of amylase and serine protease zymogens during caerulein infusion and such changes showed a similar period of latency, 2 h. From the response patterns at the level of absolute changes in rates of protein synthesis (cf. Table II), four patterns of response can be defined for the forms of exocrine proteins that are observed in the two-dimensional gel presented in Fig. 1. Trypsinogens 1 and 2, two closely related acidic forms, each showed progressive and significant increases with hormone stimulation. Together they showed a 4.3-fold increase with 24 h of caerulein infusion and their response patterns appeared to be coordinate in time and direction. Synthesis rates of amylase forms 1 and 2 decreased progressively and dramatically with hormonal stimulation to levels 0.14 and 0.07, respectively, of control values. The

response pattern of each of these separate amylase forms also appeared to be coordinate, but in a direction opposite to that of the response of trypsinogen forms 1 and 2. The response patterns of proteins in group III (ribonuclease, chymotrypsinogen forms 1 and 2, proelastase 1, procarboxypeptidase forms A and B) showed moderate increases, 1.4–3.3-fold, during caerulein infusion. Proteins contained in group IV (trypsinogen 3, proelastase 2, lipase, and the four unidentified proteins labeled 1–4 in Fig. 1) showed insignificant changes in protein synthetic rates during caerulein infusion.

A further finding was the appearance of a protein (P23) during hormonal stimulation that was previously unobserved among the rat exocrine pancreatic proteins. This protein, with an $M_r = 23,000$ and IEP = 6.2 was easily detected by Coomassie Blue stain after hormonal stimulation. Within the 24-h period of caerulein infusion the biosynthetic rate of P23 increased from control levels of 2.3 to 32.7 dpm/ μg cellular DNA. The 14.2-fold increase in its synthesis rate and the appearance of P23 in Coomassie Blue-stained gels during caerulein infusion indicate that the synthesis of P23 is dramatically increased in the exocrine pancreas during hormonal stimulation. A protein with similar M_r and IEP was recently observed in an extract of secretory granules from a transplantable rat pancreatic acinar cell carcinoma but not in extracts obtained from normal rat pancreas (28). P23 has not been identified according to biological activity. It could represent an additional pancreatic hydrolase or, alternatively, a growth factor specific for pancreatic or small intestinal epithelial tissue. P23 and amylase 2 show the greatest changes in synthetic rates during hormonal stimulation. Since these two proteins show anticominate regulation, the ratio of synthesis of P23 to amylase 2 increased 200-fold during caerulein stimulation.

The observations reported here indicate dramatic changes in the synthesis of pancreatic glycosidases, certain of the serine protease zymogens, ribonuclease, and an unidentified protein with an $M_r = 23,000$. In some instances coordinate changes were observed among isoenzymic forms, e.g., trypsinogen forms 1 and 2, chymotrypsinogen forms 1 and 2, and amylase forms 1 and 2. In other instances hormone-induced changes appeared to be isoenzyme specific, e.g., the synthesis rate of trypsinogen forms 1 and 2 increased 4.3-fold in response to caerulein infusion while the synthesis rate of trypsinogen 3 under the same conditions showed no change. Also synthesis of proelastase 1 but not proelastase 2 showed a response to hormone stimulation. Finally, the anticominate changes in protein synthesis observed during caerulein stimulation leading to a decrease in the synthesis of amylase forms 1 and 2 and an increase in the synthesis of trypsinogen forms 1 and 2, chymotrypsinogen forms 1 and 2, proelastase 1, and ribonuclease explain the inability of previous investigators to demonstrate significant changes in levels of total protein synthesis in response to hormone stimulation.

The observed changes in rates of protein synthesis reflect persistent effects of caerulein stimulation. The *in vitro* assay needed to study the incorporation of amino acids into 20 discrete protein products in the presence of physiological concentrations of amino acids required surgical removal of the pancreas, preparation of lobules, and a 2-h incubation for incorporation of radioactive amino acids. Short-lived effects of the hormone, such as those that mediate stimulus-secretion coupling, were not measured since the *in vitro* conditions used for protein synthesis contained no hormone. Under these

conditions the persistent effects of hormonal stimulation on protein synthesis in the rat pancreas are believed to represent, in part, changes in levels of mRNA. Using a similar protocol for the study of changes in protein synthesis in response to alterations in nutritional substrates in the diet, we have directly demonstrated that persistent changes in biosynthetic rates are mediated by changes in mRNA levels (19). While further experiments are needed to measure directly the effect of hormone stimulation on transcription of individual genes representing exocrine proteins, the coordinated time course in the response of synthetic rates of exocrine proteins to caerulein stimulation, the appearance of coordinate patterns of response (individual proteins within groups I, II, and III), and the appearance of anticonordinate responses (compare the progressive increase in trypsinogen forms 1 and 2 with the progressive decrease in amylase forms 1 and 2) suggest that specific mechanisms may exist for hormonal modulation of gene expression in the exocrine pancreas.

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