Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 7927–7932, July 1996
Medical Sciences

Diazepam binding inhibitor is a potent cholecystokinin-releasing peptide in the intestine

(feedback control/exocrine pancreatic secretion)

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Communicated by Susan E. Leeman, Boston University Medical School, Boston, MA, March 11, 1996 (received for review August 23, 1995)

ABSTRACT **Pancreatic proteases in the duodenum inhibit the release of cholecystokinin (CCK) and thus exert feedback control of pancreatic exocrine secretion. Exclusion of proteases from the duodenum either by the diversion of bile-pancreatic juice or by the addition of protease inhibitors stimulates exocrine pancreatic secretion. The mechanism by which pancreatic proteases in the duodenum regulate CCK secretion is unknown. In this study, we isolated a trypsin-sensitive peptide that is secreted intraduodenally, releases CCK, and stimulates pancreatic enzyme secretion in rats. This peptide was found to be identical to the porcine diazepam binding inhibitor by peptide sequencing and mass spectrometry analysis. Intraduodenal infusion of 200 ng of synthetic porcine diazepam binding inhibitor$_{1-86}$ in rats significantly stimulated pancreatic amylase output. Infusion of the CCK antagonist MK-329 completely blocked the diazepam binding inhibitor-stimulated amylase secretion. Similarly, diazepam binding inhibitor$_{33-50}$ also stimulated CCK release and pancreatic secretion in a dose-dependent manner although it was 100 times less potent than the whole peptide. Using a perfusion system containing isolated mucosal cells from the proximal intestine of rats, porcine diazepam binding inhibitor (10^{-9}–10^{-12} M) dose dependently stimulated CCK secretion. In separate studies, it was demonstrated that luminal secretion of the diazepam binding inhibitor immunoreactivity (7.5×10^{-11} M) could be detected in rat's intestinal washing following the diversion of bile-pancreatic juice. The secretion of this peptide was inhibited by atropine. In conclusion, we have isolated and characterized a CCK-releasing peptide that has a sequence identical to the porcine diazepam binding inhibitor from pig intestinal mucosa and that stimulates CCK release when administered intraduodenally in rat. This peptide may mediate feedback regulation of pancreatic enzyme secretion.**

In animals, it has been shown that proteases such as trypsin in the duodenum exert a negative feedback control for pancreatic enzyme secretion. Exocrine pancreatic secretion is stimulated by feeding raw soy flour containing a potent protease inhibitor (1). In addition, diversion of bile-pancreatic juice also stimulates pancreatic secretion (2). This phenomenon has also been observed in pigs (3), chickens (4), and humans (5). In rats, cholecystokinin (CCK) mediates this feedback mechanism (6, 7). However, the manner in which trypsin regulates CCK release is unknown. We and others have postulated that a trypsin-sensitive CCK-releasing peptide (CCK-RP) is secreted into the lumen of the small bowel and releases CCK (Fig. 1) (8, 9). Rapid perfusion of the duodenum with saline prevented the rise of pancreatic secretion and CCK plasma levels stimulated by the diversion of bile-pancreatic juice. The administration of concentrated perfusate collected from a donor rat into the duodenum of a recipient rat whose bile-pancreatic juice was diverted stimulated pancreatic secretion and CCK plasma levels. Treatment of the perfusate with trypsin, but not with amylase, lipase, or boiling, abolished the stimulatory effect of the perfusate. The secretion of this putative CCK-RP into the lumen appears to be under cholinergic control (8) and can be inhibited by somatostatin (10). A similar mechanism has been postulated for the release of secretin (11). Recently, Iwai *et al.* (12) isolated and sequenced a 61-aa trypsin-sensitive CCK-RP from pancreatic juice that was termed monitor peptide. The peptide is localized in the zymogen granules (13) and stimulates CCK release and pancreatic exocrine secretion. The physiological role of this peptide is unclear, however, because it cannot explain the pancreatic feedback mechanism stimulated by diversion of bile-pancreatic juice.

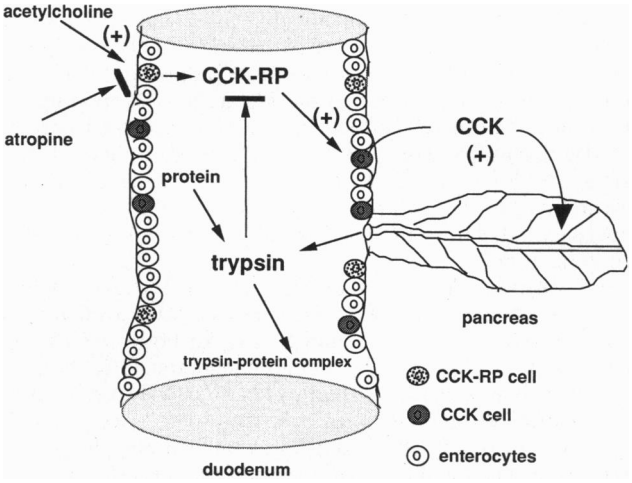

FIG. 1. A schematic representation of the postulated mechanism by which CCK-RP stimulates the secretion of CCK postprandially. CCK-RP is being secreted into the proximal small intestine and inactivated by trypsin. Postprandially, when food enters the duodenum, protein binds to trypsin and prevents CCK-RP from being inactivated. CCK-RP stimulates CCK cells in the duodenum to release CCK into the bloodstream. CCK in turn stimulates pancreatic enzyme secretion.

In this paper, we are reporting the isolation and characterization of a duodenal CCK-RP from pig intestinal mucosa that is secreted into the lumen and may regulate the negative feedback regulation of exocrine pancreatic secretion in rats.

MATERIALS AND METHODS

Materials. Dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), soy bean trypsin inhibitor (SBTI), atropine,

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Abbreviations: DBI, diazepam binding inhibitor; CCK, cholecystokinin; CCK-RP, CCK-releasing peptide.

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tetrodotoxin (TTX), DBI_{33-52}, and alginic acid were purchased from Sigma; *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes) was purchased from Merck. The calcium ionophore A23187 was from Boehringer Mannheim; Sephadex G-50 was from Pharmacia; gastrin-releasing peptide (GRP) and antiserum of diazepam binding $inhibitor_{33-50}$ were from Peninsula Laboratories. The CCK antagonist MK-329 was a gift of Merck Sharp & Dohme.

Preparation of the Intestinal Extract. A crude extract of pig intestinal mucosa was prepared using a modified method as described by Mutt (14). In brief, the mucosa of the upper 30–40 cm of the small bowel distal to the papilla was scraped off and immediately frozen in liquid nitrogen. The mucosa was minced, boiled in 0.5 M acetic acid for 10 min after adding 1 mM DTT and 1 mM PMSF, and then rapidly chilled and homogenized. After centrifugation, peptides in the supernatant were adsorbed on alginic acid at pH 2.7, eluted with 0.2 M HCl, and precipitated by NaCl at saturation. The precipitate was dissolved in 33% ethanol and the pH adjusted to 7.2. After filtration, water was added to the filtrate and the peptides in the solution were readsorbed onto alginic acid and precipitated with NaCl, and the precipitate was dissolved in methanol. After a second filtration, the peptides in the filtrate were precipitated by the addition of ether. About 60 kg of intestinal mucosa yielded 70 g methanol soluble peptide fraction after lyophilization.

***In Vivo* Assay.** Male, unfed, Sprague–Dawley rats weighing between 220 and 270 g were anesthetized with a mixture of xylazine and ketamine (13 and 87 mg/kg body weight i.m., respectively) and prepared with bile-pancreatic, intestinal, and jugular fistulas as described by Lu *et al.* (8). Body temperature was maintained at 37°C with a heating pad. Atropine (150 μg/kg per hr dissolved in saline) was infused into the jugular vein from the beginning of the experiment after a bolus injection (75 μg/kg) to prevent the endogenous release of CCK-RP (8). Bile-pancreatic juice was diverted and collected in small vials for 15-min periods, the volume determined, and amylase measured using an autoanalyzer (Eppendorf ACP 5040, Hamburg) according to the method of Kruse-Jarres *et al.* (15). Bile-pancreatic juice protein was measured spectrophotometrically using the assay method of Bradford (16). SBTI in 0.05 M sodium bicarbonate (pH 7.4) was infused intraduodenally at 1 mg per hr throughout the experiment. After establishing a stable baseline secretion (≈2 hr after the start of the experiment) mucosal extracts or synthetic porcine diazepam binding inhibitor (DBI) dissolved in sodium bicarbonate (pH 7.4) were infused over a 15-min period. Samples that stimulated amylase output were further purified by HPLC. To evaluate whether the CCK antagonist MK-329 could block the stimulatory action of the mucosal extracts or synthetic porcine DBI, MK-329 (1 mg/kg) was given intravenously 30 min before the intraduodenal infusion. MK-329 was dissolved in 1:1 dimethyl sulfoxide (DMSO)/polysorbate 80 and diluted with saline to a final concentration of 1%.

To determine if DBI was released in the duodenum after diversion of bile-pancreatic juice, rats were prepared as described above and then perfused with 0.05 M sodium bicarbonate at a rate of 1 ml/min. The concentrated perfusate was assayed by radioimmunoassay using DBI_{33-52} antibody.

Structural Analysis. The amino acid composition of the purified peptide (0.7 μg) was analyzed with a Hitachi model L-8500 amino acid analyzer after hydrolysis of the sample in 6 M HCl containing 1% phenol at 110°C for 24 hr in an evacuated tube. The intact peptide (≈6 μg) was dissolved in 6 μl of 50 mM sodium bicarbonate (pH 8.3) containing trypsin at 0.2 μg and digested for 18 hr at room temperature. The peptide mixture obtained was separated by reverse-phase HPLC on a YMC-Pack ODS-5 column (4.6 × 250 mm) and eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The amino acid sequences of the peptide following trypsin digestion were determined with an Applied Biosystems model 477A gas-phase protein sequencer equipped with an on-line phenylthiohydantoin amino acid analyzer. Molecular weights of the purified CCK-RP and the synthetic porcine DBI_{1-86} were measured using a matrix-assisted laser desorption ionization time of flight mass spectrometer (Kompact MALD III, Kratos Analytical Instruments). Peptide samples were mixed with 1% sinapinic acid in 50% ethanol and allowed to air dry before loading to the mass spectrometer.

Synthesis of DBI_{1-86}. Porcine DBI was synthesized using an Applied Biosystems model 431A automatic peptide synthesizer on *p*-alkoxybenzyl alcohol resin with a Fmoc protection strategy and a coupling strategy using 2-(1H-benzotriazol-1-yl)-1,1,2,2-tetramethyluronium hexafluorophosphate. The side chains of serine, threonine, and tyrosine were protected with *t*-butyl group; arginine was protected with 4-methyoxyl-2,3,6-trimethylphenylsulfonyl (MTR) group; asparagine and glutamine were protected with trityl group; aspartic acid and glutamic acid were protected with *O*-butyl group; lysine and histidine were protected with *t*-Boc group. The peptide was deprotected and cleaved from the resin by treatment with trifluroacetic acid/thioanisole/ethandithiol/anisole (90:5:3:2) for 6 hr at room temperature. After filtration and removal of the cleavage reagents and precipitation with anhydrous ether, the crude synthetic peptide was purified by HPLC.

***In Vitro* Assay.** Dispersed intestinal mucosa cells were prepared as described by Bouras *et al.* (17). Briefly, 20 cm of the proximal small bowel of male Sprague–Dawley rats, 2 cm distal to the pylorus was removed, washed with saline, and incubated for 10 min in 15 ml of oxygenated calcium-free KHB buffer containing 2.5 mM EDTA. After gentle shaking of the incubation flask, the remaining tissue was removed and the cell suspension was centrifuged. The pellet was rewashed twice with fresh Krebs–Henscleit bicarbonate (KHB) buffer with calcium and filtered through 200 μm gauze (17). The cell suspension (2 ml) was mixed with 2 ml of preswollen Sephadex G-50 medium resin and perfused in a perfusion apparatus at 1 ml/min (18). After a 50-min period, perfusates collected for two 5-min periods were taken as basal level followed by three 5-min periods in which the stimulatory agent was added. The 15-min stimulation period was followed by four additional 5-min periods. CCK released into the medium was assayed as described (19).

Statistics. Results are expressed as mean ± SEM. Data were analyzed using the Student's *t* test for unpaired data. Statistical significance was set at $P < 0.05$.

RESULTS

Extraction, Purification, and Sequencing of CCK-RP from Porcine Intestinal Mucosa. The peptides in the proximal small bowel mucosa were extracted, adsorbed on alginic acid, salt precipitated, and dissolved in methanol. This extraction protocol yielded ≈70 g of crude peptide material, which was further purified by HPLC (Fig. 2 *a–d*). In each purification step, HPLC fractions were assayed for the stimulation of pancreatic enzyme secretion using anesthetized and atropinized rats prepared with jugular, bile-pancreatic, and intestinal fistulas. In the final HPLC purification step (Fig. 2*d*), we obtained a pure peptide that was rich in Asx, Glx, and Lys by amino acid analysis (Table 1). The intact peptide was subjected to amino acid sequence analysis, but we were unable to obtain any sequence data suggesting that the peptide had a blocked N terminus. After digestion of the peptide with trypsin and separation of the tryptic fragments by HPLC, we determined the sequences of eight fragments (Fig. 3), which were identical to the corresponding fragments of porcine DBI originally isolated from rat brain (20). Analysis by mass spectrometer revealed a molecular mass of 9810 Da, which corresponds to

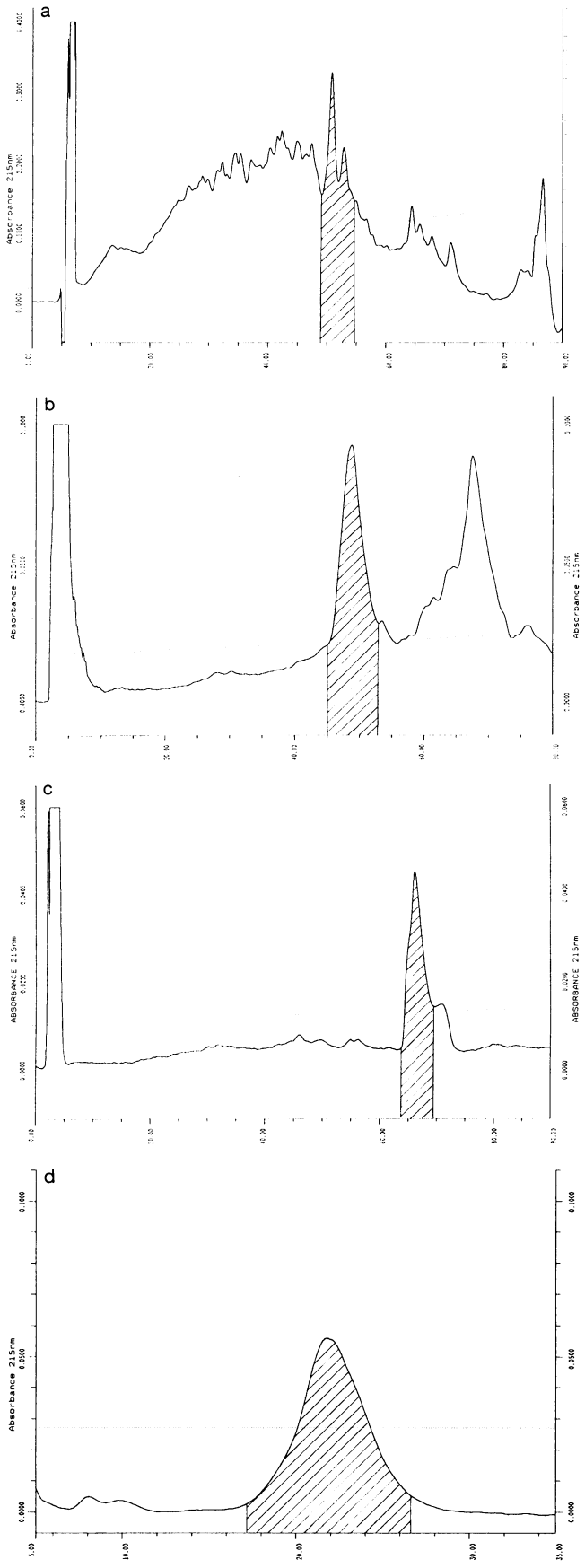

FIG. 2. Isolation of the CCK-RP from porcine intestinal mucosa. (*a*) HPLC profile of porcine mucosa extract dissolved in methanol (60 mg) [Beckman System Gold; C18 Ultrasphere (Beckman) 10 × 250 mm equilibrated with 25% acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA)]; elution by an increasing concentration of ACN to 45% over 90 min at 2 ml/min. The hatched area indicates the biological active fraction. (*b*) HPLC profile of the active fraction of the first HPLC fraction (*a*) [C18 Ultrasphere (Beckman) 4.6 × 250 mm equilibrated with 25% ACN in 5 mM phosphate buffer (pH 5.2)]; elution by an increasing concentration of ACN to 30.5% over 60 min at 1 ml/min. The hatched area indicates the biological active fraction. (*c*) HPLC profile of the active fraction of the second HPLC fraction (*b*) (C18 Macherey & Nagel MPN 4.6 × 250 mm equilibrated with 30% ACN in 0.1% TFA); elution by an increasing concentration of ACN to 33.5% over 70 min at 1 ml/min. The hatched area indicates the biological active fraction. (*d*) HPLC profile of the active fraction of the third HPLC fraction (*c*) (C18 Ultrasphere 4.6 × 250 mm equilibrated with 30.5% ACN in 0.1% TFA); elution by an isocratic gradient of 30.5 ACN at 1 ml/min. The hatched area indicates the biological active fraction.

Table 1. Amino acid analysis of CCK-RP

Amino acid	nmol	Ratio	Integral
Asx	0.68	10.1	10
Thr	0.28	4.1	4
Ser	0.38	5.6	6
Glx	0.87	12.8	13
Pro	0.15	2.2	2
Gly	0.59	8.8	9
Ala	0.60	8.9	9
Cys	0.00	0.0	0
Val	0.23	3.4	3
Met	0.02	0.4	0
Ile	0.32	4.7	5
Leu	0.42	6.3	6
Tyr	0.22	3.2	3
Phe	0.14	2.0	2
Lys	0.88	13.1	13
His	0.11	1.6	2
Arg	0.11	1.7	2
Total	6.00		89

The peptide is rich in Asx, Glx, and Lys.

the molecular mass of porcine intestinal DBI_{1-86} (Fig. 4). Structural analysis showed that we had indeed isolated porcine DBI_{1-86} while in search of a CCK-RP from the proximal small bowel.

Luminal Secretion of DBI-Like Immunoreactivity During Diversion of Bile-Pancreatic Juice. To examine whether DBI_{1-86} is secreted from the duodenal mucosa in the lumen, we used anesthetized rats prepared with bile-pancreatic and intestinal cannula as described above (8). After diversion of bile-pancreatic juice, the duodenum was perfused with sodium bicarbonate-buffered saline (pH 7.4) at 1 ml/min and intestinal perfusates collected every 15 min for 60 min. Using an antibody against a DBI fragment, we were able to detect DBI-like immunoreactivity of 7.5×10^{-11} M in the intestinal washings. Secretion of DBI immunoreactivity was abolished in rats treated with atropine, indicating that DBI secretion is under cholinergic control.

Demonstration that DBI_{1-86} and DBI_{33-52} Stimulate Pancreatic Secretion and CCK Release. Porcine DBI was synthesized using an automatic peptide synthesizer on *p*-alkoxybenzyl alcohol resin with a Fmoc protection strategy. Using our *in vivo* anesthetized rat model (8), intraduodenal infusion of synthetic porcine DBI_{1-86} (200 ng or 0.2 nmol) over 15 min stimulated amylase output by 222 ± 16% over basal (Fig. 5). The stimulation was completely blocked by the CCK-antagonist MK-329 (1 mg/kg), suggesting that DBI-stimulated amylase release is mediated by CCK. In separate studies, we showed that intraduodenal infusion of DBI_{33-52} (62.5–500 μg) caused a dose-dependent increase in pancreatic protein secretion and

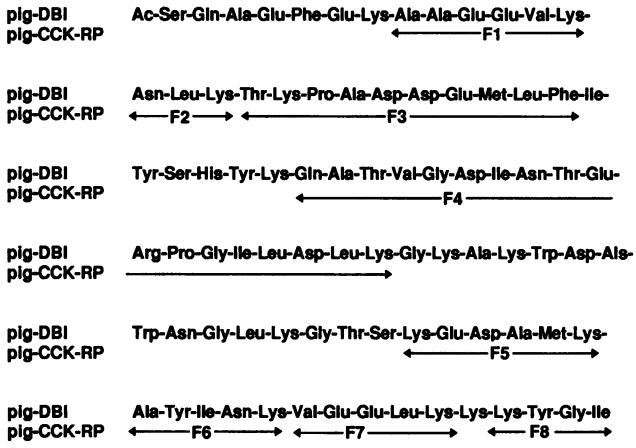

FIG. 3. Amino acid sequence of the CCK-RP. Comparison with porcine intestinal DBI_{1-86} and tryptic fragment (F1-F8) of the porcine CCK-RP. Ac, An acetyl group.

plasma CCK release (Fig. 6). DBI_{1-86} or its fragments had no effect on amylase secretion from isolated acini (data not shown).

***In Vitro* Action of DBI_{1-86} and DBI_{33-52} on CCK Secretion.** To further investigate the mechanism by which DBI stimulates CCK secretion, we used a perfusion system of dispersed intestinal mucosa cells as described by Bouras *et al.* (17). In this perfusion system, we were able to show that KCl, calcium ionophore A 23187, and GRP stimulated CCK release (Fig. 7*a*). DBI_{1-86} (10^{-9}-10^{-13} M) dose-dependently stimulated CCK release with a maximum effect at 10^{-9} M (Fig. 7*b*). Similarly, DBI_{33-52} stimulated CCK release but on a molar basis more than 100 times less potent than DBI_{1-86} (Fig. 7*c*). The CCK release was unaffected by the presence of neurotoxin tetrodotoxin, indicating that DBI directly stimulates CCK release without any neural involvement.

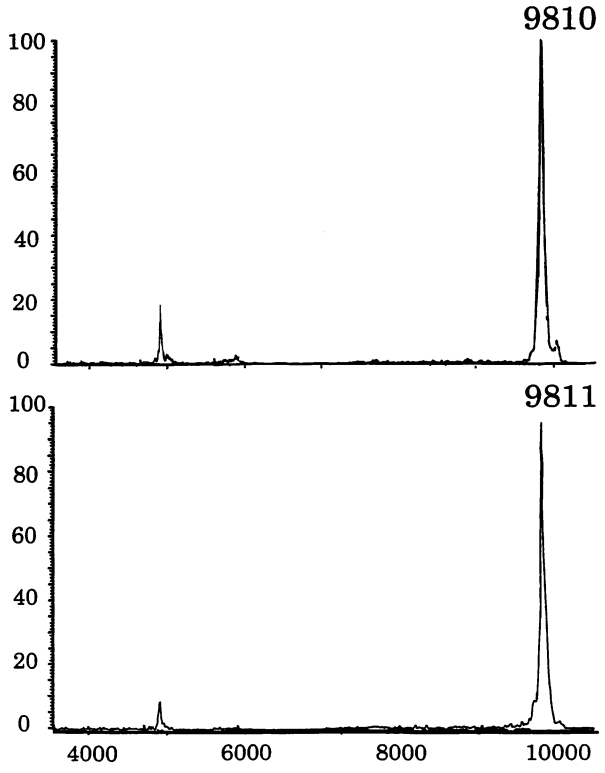

FIG. 4. Molecular mass determination of the purified CCK-RP and the synthetic DBI_{1-86}. Mass spectroscopy indicated a quasimolecular ion at mass/charge 9810, which corresponds to the molecular mass of porcine intestinal DBI (9807 Da).

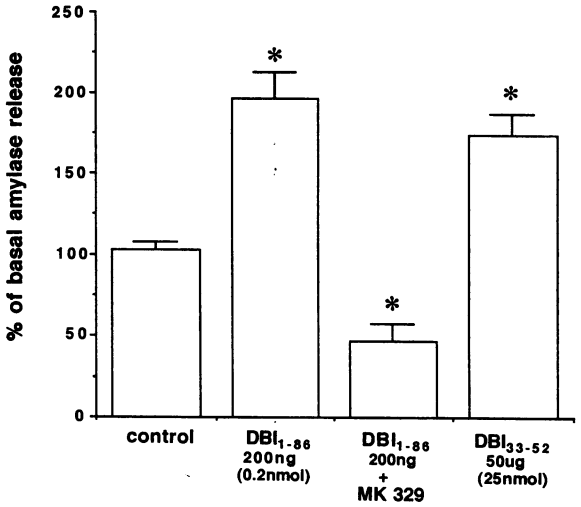

FIG. 5. Effect of intraduodenal DBI_{1-86} and its fragment DBI_{33-52} on amylase output in anesthetized and atropinized rats. Atropine (150 μg/kg/hr) was infused and bile-pancreatic juice diverted throughout the experiment. After a basal period, DBI_{1-86} or DBI_{33-52} dissolved in 0.05 M sodium bicarbonate was infused intraduodenally over 15 min. To evaluate whether the CCK antagonist MK-329 can block the effect of DBI, MK-329 (1 mg/kg) was given intravenously 30 min before the infusion of DBI. MK-329 was dissolved in 1:1 dimethyl sulfoxide (DMSO)/polysorbate 80 and diluted with saline to a final concentration of 1%. Values are means ± SE of seven experiments. *, $P < 0.05$.

DISCUSSION

DBI is a peptide with wide tissue distribution and multiple biological functions (21). It was first isolated from rat brain in 1983 (20) and subsequently found to be present in a number of peripheral organs. The highest concentration of DBI-like immunoreactivity and mRNA levels are found in the cerebral cortex, hypothalamus, liver, duodenum, testis, kidney, and adrenal cortex (21). The first biological action described was its ability to displace diazepam from the GABA (γ-aminobutyric acid)-A receptor (20). This may be responsible for its well-known properties of producing conflict behavior in rats when injected intracerebroventricularly. The action of DBI was blocked by the benzodiazepine antagonist flumazenil (22). In addition, DBI has been reported to function as a high affinity acetyl coenzyme A binding protein (23), as well as to act as an intracellular activator of mitochondrial steroid synthesis (24). Stimulation of steroidgenesis is mediated through a peripheral-type benzodiazepine receptor, which is subcellularly located at mitochondrial membranes in most tissues (21). Recently, cell surface receptors on the plasma membrane have been demonstrated on adrenal cortical cells and Leydig cells (25, 26). Furthermore, DBI secreted by Leydig and Sertoli cells has

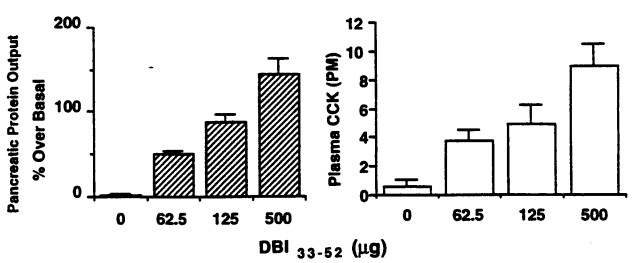

FIG. 6. Effect of intraduodenal administration of DBI_{33-52} on pancreatic protein output and plasma CCK levels in anesthetized and atropinized rats. Values are means ± SE of six experiments.

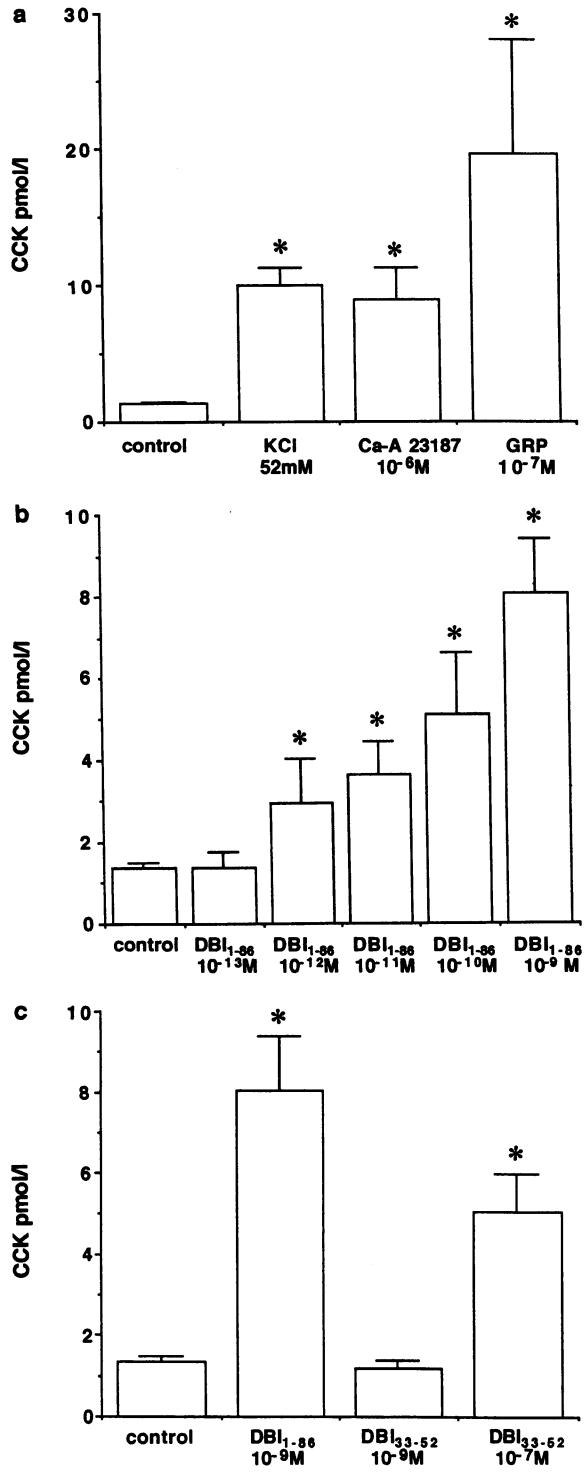

FIG. 7. (*a*) Effects of KCl, the calcium ionophore A 23187, and GRP on CCK release from dispersed intestinal mucosa cells. (*b*) Effects of different concentrations of DBI_{1-86} on CCK release from dispersed intestinal mucosa cells. (*c*) Comparison of DBI_{1-86} and DBI_{33-52} on CCK release from dispersed intestinal mucosa cells. Values are means ± SE of at least four experiments. *, $P < 0.05$.

been identified in rat testicular interstitial fluid by immunoblot analysis, suggesting that DBI may act as an autocrine/paracrine regulator of Leydig cell function (26).

In the gastrointestinal tract, DBI immunoreactivity was detected in the secretory and absorptive epithelial cells of the gut with the highest DBI content in the antrum, duodenum, and colon (27). Endocrine cells of the APUD system, Brunner glands, or neuronal cells in the myenteric plexus were devoid of DBI-immunoreactive material. *In situ* hybridization studies showed high density of DBI mRNA in the epithelial cells of the intestinal villi in the rat duodenum (27). Chen *et al.* (28) isolated the 86 amino acid peptide DBI from porcine intestine. They found that 10 nM DBI inhibited both early and late phases of glucose-induced insulin release from the isolated perfused rat pancreas. In addition, DBI (10^{-8}-10^{-7} M) also significantly inhibited 3-isobutyl-1-methylxanthine (IBMX)- (1 μM) or glibenclamide (1 μg/ml)-stimulated insulin release in the presence of glucose (29). Using an antibody against purified porcine DBI_{1-86}, Johansson *et al.* (30) showed that DBI-like immunoreactivity was present in glucagon-containing cells of rat pancreatic islets, suggesting a possible physiological role in modulating insulin secretion.

During our search for a CCK-RP in the duodenum responsible for the feedback regulation of pancreatic enzyme secretion, we isolated a peptide from the porcine intestinal mucosa that has a sequence identical to DBI. The peptide has a blocked N terminus, which helps to explain our initial difficulty to obtain sequence data. We showed that DBI is released into the intestinal lumen under cholinergic control and that it is capable of releasing CCK *in vivo* and *in vitro*. The ability of DBI to release CCK was not affected by the presence of luminal contents such as casein (unpublished data). Using an isolated mucosa cell system, we demonstrated that the synthetic porcine DBI_{1-86} stimulated CCK secretion at a concentration as low as 10^{-12} M. DBI_{33-52} also stimulated CCK release and pancreatic secretion although it is 100 times less potent than the whole peptide. DBI possesses several tryptic cleavage sites and tryptic digestion results in loss of its ability to stimulate CCK secretion. Therefore, we have isolated a trypsin-sensitive peptide that is secreted intraduodenally, releases CCK, and stimulates pancreatic secretion. This peptide fulfills all the criteria necessary to be the CCK-RP responsible for mediating feedback regulation of pancreatic secretion.

The mechanism(s) by which DBI releases CCK is unclear. Our *in vitro* studies demonstrate that the ability of DBI to release CCK was unaffected by tetrodotoxin, indicating that DBI directly stimulates CCK release without any neural involvement. It has been reported that basic peptides such as spermidine (a polyamine) may release CCK (31). DBI contains 16 basic amino acids and 15 acidic amino acids. Because the N terminus of the peptide is blocked and the C terminus is not, this adds one more acidic group and makes porcine DBI a neutral protein. However, the absence of a net basic charge does not preclude a charge effect of DBI to release CCK. Further studies are needed to examine the cellular mechanism(s) by which DBI releases CCK.

Until now, regulatory peptides were believed to act in an endocrine or paracrine fashion to mediate a physiological process. Recently, gut peptides such as somatostatin (32) and peptide YY (33) have been shown to be released into the bowel lumen, but their physiological functions remain unknown. The pancreatic monitor peptide found exclusively in zymogen granules of pancreatic acinar cells is present in pancreatic juice (34) and can stimulate CCK release when it enters the duodenum (35). This peptide has a partial sequence homology to the 14 residual sequence of epidermal growth factor and has been shown to stimulate growth in Swiss 3T3 cells (36). The physiological function of this peptide is still unclear but because it is only present in the pancreatic juice it is clearly not involved in the feedback regulation of pancreatic secretion first reported by Green and Lyman (2). In contrast, DBI, for all the reasons listed above, is likely to be the peptide responsible for mediating feedback regulation of CCK release by pancreatic proteases. This would be the first demonstration of a complete feedback loop in the gut for maintaining homeostasis during digestion. Our study also demonstrates an important physio-

logical role for a luminal peptide and this may represent a new concept in the regulation of secretion of gut peptides.

We thank Cornelia Wilgus and Gabriele Brunke for their excellent technical assistance. The investigation was supported in part by the Deutsche Forschungsgemeinschaft (Bonn, Germany) (He 1965/1-1 and 1-2), the Ministry of Education, Science and Culture of Japan (Grant 04404084), and by the National Institutes of Health Grants ROI DK-32838 and P30 DK-34933.

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