Medical Sciences. In the article “Diazepam binding inhibitor is a potent cholecystokinin-releasing peptide in the intestine,” by Karl-Heinz Herzig, Ilona Schön, Kazuhiko Tatemoto, Yoshihide Ohe, Ying Li, Ulrich R. Fölisch, and Chung Owyang, which appeared in number 15, July 23, 1996, of Proc. Natl. Acad. Sci. USA (93, 7927–7932), the authors request that the following correction be noted. On page 7927 in the Abstract, diazepam binding inhibitor 33-50 is mentioned incorrectly and should read: “Similarly, diazepam binding inhibitor 33-52 also stimulated CCK release and pancreatic secretion in a dose-dependent manner although it was 100 times less potent than the whole peptide.” Also, due to a printer’s error, the corresponding author’s address should read D-24105 Kiel.
Diazepam binding inhibitor is a potent cholecystokinin-releasing peptide
(feedback control/exocrine pancreatic secretion)

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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 inhibitor1-88 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 inhibitor32-99 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.

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,

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

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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.

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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<sub>1-86</sub> 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<sub>1-86</sub>**. 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 butyl group; arginine was protected with 4-methoxyethyl-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 R-Boc group. The peptide was deprotected and cleaved from the resin by treatment with trifluoroacetic acid/thioanisole/ethanethiol/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.

**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 \( \sim 70 \) 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. 2d), we obtained a pure peptide that was rich in Asx, Gln, 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
Table 1. Amino acid analysis of CCK-RP

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>nmol</th>
<th>Ratio</th>
<th>Integral</th>
</tr>
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<tbody>
<tr>
<td>Ass</td>
<td>0.68</td>
<td>10.1</td>
<td>10</td>
</tr>
<tr>
<td>Thr</td>
<td>0.28</td>
<td>4.1</td>
<td>4</td>
</tr>
<tr>
<td>Ser</td>
<td>0.38</td>
<td>5.6</td>
<td>6</td>
</tr>
<tr>
<td>Glx</td>
<td>0.87</td>
<td>12.8</td>
<td>13</td>
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<tr>
<td>Pro</td>
<td>0.15</td>
<td>2.2</td>
<td>2</td>
</tr>
<tr>
<td>Gly</td>
<td>0.59</td>
<td>8.8</td>
<td>9</td>
</tr>
<tr>
<td>Ala</td>
<td>0.60</td>
<td>8.9</td>
<td>9</td>
</tr>
<tr>
<td>Cys</td>
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<td>Val</td>
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<td>3.4</td>
<td>3</td>
</tr>
<tr>
<td>Met</td>
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<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td>Ile</td>
<td>0.32</td>
<td>4.7</td>
<td>5</td>
</tr>
<tr>
<td>Leu</td>
<td>0.42</td>
<td>6.3</td>
<td>6</td>
</tr>
<tr>
<td>Tyr</td>
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<tr>
<td>Phe</td>
<td>0.14</td>
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<tr>
<td>Lys</td>
<td>0.88</td>
<td>13.1</td>
<td>13</td>
</tr>
<tr>
<td>His</td>
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<td>2</td>
</tr>
<tr>
<td>Arg</td>
<td>0.11</td>
<td>1.7</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>6.00</td>
<td>89</td>
<td></td>
</tr>
</tbody>
</table>

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

the molecular mass of porcine intestinal DBI₁₋₈₆ (Fig. 4). Structural analysis showed that we had indeed isolated porcine DBI₁₋₈₆ 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 DBI₁₋₈₆ 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 DBI₁₋₈₆ and DBI₃₃₋₅₂ 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 DBI₁₋₈₆ (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 DBI₃₃₋₅₂ (62.5–500 μg) caused a dose-dependent increase in pancreatic protein secretion and

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% acetonitrile (ACN) in 0.1% trifluoroacetic

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
plasma CCK release (Fig. 6). DBI1-86 or its fragments had no effect on amylase secretion from isolated acini (data not shown).

**In Vitro Action of DBI1-86 and DBI33-52 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. 7a). DBI1-86 (10⁻⁹-10⁻¹⁳ M) dose-dependently stimulated CCK release with a maximum effect at 10⁻⁹ M (Fig. 7b). Similarly, DBI33-52 stimulated CCK release but on a molar basis more than 100 times less potent than DBI1-86 (Fig. 7c). The CCK release was unaffected by the presence of neurotoxin tetrodotoxin, indicating that DBI directly stimulates CCK release without any neural involvement.

**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 steroidogenesis 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 been proposed to act as an autocrine/paracrine signal.
been identified with paracrine means. Values DBI3152 on dispersed effects of different factors on CCK release from dispersed intestinal mucosa cells. 

Values of DBI33-52 on CCK release from dispersed intestinal mucosa cells. 

Values of DBI10-8 and DBI33-52 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.

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. 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-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 endocrine 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 DBI1-86 stimulated CCK secretion at a concentration as low as 10^{-12} M. DBI33-52 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 trypsin 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 polypeptide) 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-residue 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.

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