Identification of nonsulfated cholecystokinin-58 in canine intestinal extracts and its biological properties

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Reeve, Joseph R., Jr., Rodger A. Liddle, Douglas C. McVey, Steven R. Vigna, Travis E. Solomon, David A. Keire, Grace Rosenquist, John E. Shively, Terry D. Lee, Peter Chew, Gary M. Green, and Tamer Coskun. Identification of nonsulfated cholecystokinin-58 in canine intestinal extracts and its biological properties. Am J Physiol Gastrointest Liver Physiol 287: G326–G333, 2004. First published April 2, 2004; 10.1152/ajpgi.00520.2003.—Nonsulfated CCK58 [CCK58(ns)] has not been considered to be of biological importance because CCK58(ns) binds poorly to the CCKA receptor and has only been identified once in intestinal extracts. In this work, a radioimmunoassay specific for the COOH-terminal region of gastrin and CCK (antibody 5135) was used to monitor the purification of CCK molecular forms from canine intestinal extracts. A minor immunoreactive peak was associated with a major absorbance peak during an ion-exchange, HPLC step. Characterization of this minor immunoreactive peak demonstrated that it was CCK58(ns). CCK58(ns) is 14% as immunoreactive as sulfated CCK8 [CCK8(s)]. Amino acid analysis demonstrated that CCK58(ns) was present at 50% the amount of CCK58(s). In addition, we found that CCK58(ns) does not potentially displace an 125I-labeled CCK8 analog from the CCKA receptor in mouse pancreatic membranes and does not stimulate amylase release from isolated pancreatic acini, or stimulate pancreatic secretion in an anesthetized rat model. By contrast, CCK58(ns) does bind to CCKB receptors and stimulates gastric acid secretion via this receptor. The presence of CCK58(ns) and its ability to selectively stimulate the CCKB receptor without stimulation of the CCKA receptor suggest that CCK58(ns) may have unique physiological properties, especially tissues where the nonsulfated peptide can act as a paracrine or neurotransmitter.

NO BIOLOGICAL RELEVANCE HAS been attributed to nonsulfated molecular forms of CCK because 1) nonsulfated forms have not been routinely detected in brain or intestinal extracts and 2) nonsulfated CCK forms cannot bind and activate the CCKA receptor. However, if a nonsulfated molecular form of CCK is present in tissue, this form could act at CCKB receptors through endocrine, neurocrine, or paracrine pathways.

Recently, a report (2), contrary to the conventional idea that all molecular forms of CCK are fully sulfated (22), described the purification and characterization of nonsulfated CCK58 [CCK58(ns)] from porcine intestinal extracts. Sulfated CCK8 [CCK8(s)] was 35 times more potent than CCK58(ns) for contraction of the guinea-pig gallbladder, whereas sulfated CCK8 [CCK8(s)] was 150 times more potent than nonsulfated CCK8 [CCK8(ns)] for contraction of gallbladder (28). This led the authors to conclude that “the NH2-terminal end of CCK8(ns) partially compensates for the decrease in activity arising from the lack of sulphated tyrosine” (2).

CCK binds to two receptors. The CCKA (CCK-1) receptor regulates pancreatic secretion (44), gallbladder contraction (8), gastric motility (25), and is one component of satiety (19). The CCKB (CCK2 or gastrin/CCK B) receptor regulates gastric acid secretion (42). We have studied the binding of CCK8(s) to these receptors (34), but we are unaware of any other binding studies with CCK58(ns).

Sulfation of prohormones (e.g., pro-CCK) could influence the expression of their physiological activities at several levels. The sulfate moiety could influence processing of the prohormone as shown for progastrin, where the extent of proteolytic processing has been correlated with sulfation (3, 35, 41). In addition, sulfation could also alter the bioavailability of a hormone by changing its circulating half-life (29) or the susceptibility of the hormone to degradation after leaving the circulation (5, 6). Furthermore, sulfation has been shown to greatly increase the potency of CCKs for binding and activation of the CCKA receptor (21).

If the amino terminus of CCK58(ns) is able to partially compensate for the lack of the tyrosine sulfate on its COOH terminus [as suggested by its actions on the gallbladder (2)], then binding and activation of the rat pancreatic CCKA receptor may be influenced by the NH2 terminus of CCK58(ns). We have previously shown that the NH2 terminus of CCK58(s) decreases the ability of this molecular form to stimulate release of amylase compared with CCK8(s) in a purified acinar cell preparation (32). However, CCK8(s) and CCK58(s) were equipotent for stimulation of amylase release in an in vivo anesthetized rat model (30), suggesting that the greater bioavailability (20) of CCK58(s) offsets its lower intrinsic potency to release amylase from purified acinar cells.

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In this report, we demonstrated that CCK_{58}(ns) is present in the intestine of dog and that CCK_{58}(ns) binds and activates the CCK_{A} receptor on rat pancreas in vivo or in vitro only at high doses. Therefore, if CCK_{58}(ns) is of physiological relevance at CCK_{A} receptors, it must act in a neurocrine or paracrine manner to achieve the concentrations necessary for activation of the CCK_{A} receptor. However, we also demonstrate that CCK_{58}(ns) potently binds to mouse brain CCK_{B} receptors. Furthermore, CCK_{58}(ns) stimulates gastric acid in an anesthetized rat model, demonstrating its ability to stimulate CCK_{B} receptor in vivo. The presence of CCK_{58}(ns) in intestinal extracts and its ability to bind and activate CCK_{A} receptors suggest that CCK_{58}(ns) may be of physiological relevance.

METHODS

Radioimmunoassay. A radioimmunoassay based on antibody 5135 was performed as previously described (36). Tracer was radioiodinated gastrin 17 (I) purified by reverse-phase HPLC. This antisem reacts equally with CCK_{A} and CCK_{33} (13), but about 50 and 20% with canine CCK_{58} (31) and rat CCK_{58} (33, respectively. Purification of nonsulfated canine [CCK_{58}(ns)]. Canine intestines from dogs used for other purposes were kindly provided by Dr. A. Soll. Briefly, canine intestinal mucosa was obtained after boiling unopened, freshly dissected intestine in water for 5–10 min. The cooled intestine was cut open, and the mucosa was scraped away from the muscle. The mucosa was then stored at −70°C until extraction. The frozen mucosa (140 g) was blended with 1.4 liters of 2% trifluoroacetic acid in a Waring blender and then stirred for 30 min. The extract was centrifuged at 3,000 g for 30 min and filtered through an Amicon HIL10,000 filter (10,000 MW cut off). The filtrate was loaded directly onto a Rainin C_{18} preparative HPLC column (42 mm × 25 cm). After rinsing with 1 liter of 0.1% trifluoroacetate CCK, immunoreactivity was eluted with increasing concentrations of acetonitrile (see Table 1 for gradients used in all HPLC purification steps). All purification eluates were monitored by absorbance at 220 and 280 nm and CCK radioimmunoassay. Fractions containing CCK immunoreactivity were pooled and diluted threefold with 0.1% trifluoroacetate (TFA) and loaded onto a Rainin C_{8} preparative HPLC column (21.4 mm × 25 cm). The pooled immunoreactive fractions were diluted threefold with 0.1% TFA and loaded onto a Rainin C_{4} preparative HPLC column (21.4 mm × 25 cm). The immunoreactive fractions were loaded directly onto an Aspartamide anion-exchange HPLC column (Nest Group) equilibrated in potassium phosphate (0.01 M, pH 3, containing 25% acetonitrile). The column was eluted with increasing concentrations of KCl. The pooled CCK immunoreactivity from this column was diluted threefold with 0.1% TFA and loaded onto an analytical C_{4} reverse-phase HPLC column (Vydac) equilibrated in aqueous 0.1% TFA. The column was eluted with increasing concentrations of acetonitrile. Finally, the peptide was stored at 4°C for 10 wk before dilution and repurification on the same C_{4} column with a slightly different gradient 1 wk before commencing the bioassays.

Amino acid analysis of sulfated and canine CCK_{58}(ns). A small aliquot of the purified peptides corresponding to ~5 nmol was placed into a pyrolyzed hydrolysis tube. The solvent was removed under vacuum, 6 N HCl was added to the container holding the tubes, the container was sealed under a vacuum, and the mixture was hydrolyzed for 20 h at 110°C. The resulting amino acids were dried, dissolved, and analyzed on a Beckman 126 amino acid analyzer by manufacturer recommended procedures.

Mass spectral analysis. Mass spectrometric analyses were performed at the Beckman Research Institute of the City of Hope (Duarte, CA) on a TSQ-700 triple-sector quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA) equipped with an electrospray ion source. Online separations were performed using a 90°C-Hope-built capillary LC system described previously (10). Mass assignments were made using the Finnigan MAT BIOMASS data-reduction software.

Tryptic mapping of canine sulfated and CCK_{58}(ns). Approximately 200 pmol of the peptides were vacuum centrifuged until the volume was ~50% of the original sample volume to remove acetonitrile. The volume was adjusted to 50 μl with 1 M ammonium bicarbonate. Trypsin (5 pmol in 50 μl of 1 M ammonium bicarbonate) was added, and the mixture was incubated at 37°C for 18 h. A portion (5 μl) of the digestion mixture was loaded onto the liquid chromatography-mass spectrometer.

Membrane preparation for radioreceptor binding assays. Mouse brain membranes were used as a source of CCK_{A} receptors and mouse pancreatic membranes as a source of CCK_{B} receptors as previously described (32). Pancreatic acini were prepared as previously described in response to natural canine CCK58 (ns), synthetic CCK 8 (ns), and synthetic CCK 8 (s). Pancreatic acini were prepared and assayed in response to natural canine CCK_{58}(ns), synthetic CCK_{58}(ns), and synthetic CCK_{8}(s). Pancreatic acini were prepared as previously described (23, 44) from Sprague-Dawley rats weighing 250–280 g, according to the method of Williams et al. (44). Krebs-Henseleit bicarbonate buffer containing 0.1 mg/ml soybean trypsin inhibitor and 0.1 mg/ml purified collagenase was injected into the pancreatic parenchyma, and the tissue was dissociated at 37°C and 50 min. The acini were dissociated by passage through pipettes with restrictive orifices and purified by centrifugation through step gradients of buffer containing 4% BSA. Acini were then incubated for 30 min at 37°C in

<table>
<thead>
<tr>
<th>Table 1. Purification of CCK_{58}(ns)</th>
<th>Step</th>
<th>Column Size, mm</th>
<th>Elution Gradient, % buffer B/time</th>
<th>Recovery, nmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Extract</td>
<td>42×250</td>
<td>0–100% B/100 min</td>
<td>11*</td>
<td>9.6</td>
</tr>
<tr>
<td>2) Rainin C-18(prep)</td>
<td>21.4×250</td>
<td>0–100% B/25 min</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>3) Rainin C-8 (prep)</td>
<td>21.4×250</td>
<td>40–80% B/80 min</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>4) Rainin C-4 (prep)</td>
<td>21.4×250</td>
<td>40–80% B/80 min</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>5) Polysulfopropyl (Nest)</td>
<td>4.6×250</td>
<td>40–100% B/50 min</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>6) Purification of nonsulfated canine CCK_{58}(ns)</td>
<td>4.6×250</td>
<td>40–70% B/60 min</td>
<td>0.073</td>
<td></td>
</tr>
<tr>
<td>7) Vydac C-4</td>
<td>4.6×250</td>
<td>45–70% B/65 min</td>
<td>0.032</td>
<td></td>
</tr>
</tbody>
</table>

*In the first chromatography step, a total of 33 nmol of CCK immunoreactivity were observed in 3 immunoreactive peaks. Only the latest immunoreactive peak (11 nmol) was used in the purification of nonsulfated CCK_{58} [CCK_{58}(ns)]. Buffer A for the reverse-phase HPLC steps was 0.1% trifluoroacetic acid. Buffer B was 50% acetonitrile containing 0.1% trifluoroacetic acid. The major loss between steps 2 and 3 was due to multiple molecular forms of CCK being present at step 2, totaling 6.5 nmol, and using only the latest eluting form (1.5 nmol) for further purification. Buffer A for the ion-exchange HPLC step was 5 mM phosphate adjusted to pH 3.0 and then diluted with acetonitrile to 75% aqueous buffer. Buffer B was buffer A containing 0.4 M KCl.
40 mM Tris-Ringer buffer with (in mM) 103 NaCl, 1 NaH2PO4, 4.7 KCl, 1.28 CaCl2, 0.56 MgCl2, and 11.1 glucose with 0.1 mg/ml soybean trypsin inhibitor enriched with Eagle’s medium amino acid supplement, and 5 mg/ml BSA with various concentrations of the CCK peptides.

In vivo studies: animals. Male Sprague-Dawley albino rats weighing 280–350 g (Harlan Laboratory, San Diego, CA) were housed in group cages under conditions of controlled temperature (22–24°C) and illumination (12:12-h light-dark cycle starting at 06:00 AM) for at least 7 days before experiments. Animals are maintained on Purina Laboratory Chow (Ralston Purina, St. Louis, MO) ad libitum and tapwater. Collections were performed in urethane-anesthetized rats deprived of food for 18 h but given free access to water up to the beginning of the study. Experimental protocols were approved by the Animal Research Committee of the Veteran Affairs Greater Los Angeles Healthcare System.

Surgery and comparison of in vivo stimulation of pancreatic amylase secretion. Exocrine pancreatic secretory responses to canine CCK58(ns) and CCK58(s) were determined by previously described methods (37). Briefly, the right jugular vein of anesthetized rats (280–400 g body wt, fasted for 20–24 h) was catheterized; intravenous fluids were delivered at an infusion rate of 1.08 ml/h. The trachea was intubated to facilitate breathing and to separately collect bile-pancreatic juice diluted 1:1 with saline was infused into the stomach lumen cannula was inserted through a small incision into the non-glandular forestomach. The plastic gastric cannula was secured in the stomach and exited the abdomen through the midline incision. The surgical wounds were sutured. The abdomen was opened through a right jugular vein. After the completion of the neck surgery, the cervical esophagus was ligated. For CCK4(s) or CCK4(ns) or gastrin-17(ns) infusion, an intravenous line (PE-50, IntraMedic) was placed into the right jugular vein of Oddi (close to duodenum) and 2) distal to the pancreas (close to the liver) to collect bile and pancreatic juice separately. Collections were begun after a 2 h postsurgical period of stabilization. Collections were made at 30-min intervals. Previously collected bile-pancreatic juice diluted 1:1 with saline was infused into the duodenum at 1.08 ml/h to prevent the release of endogenous CCK.

After unstimulated pancreatic secretion was collected for 1 h, either CCK4(s) or CCK4(ns) or CCK58(ns) was administered intravenously for 2 h at a dose of 1 nmol·kg⁻¹·h⁻¹. Only one dose was given to each rat. The volume of collected pancreatic juice was measured, and amylase concentration in each sample was determined.

Surgery and gastric acid secretion measurement. Rats were anesthetized with urethane (1.25 g/kg ip). The trachea was cannulated (PE-240, IntraMedic) to facilitate the breathing, and the cervical esophagus was ligated. For CCK4(ns) or CCK58(ns) or gastrin-17(ns) infusion, an intravenous line (PE-50, IntraMedic) was placed into the right jugular vein. After the completion of the neck surgery, the surgical wounds were sutured. The abdomen was opened through a ventral median celiotomy. The pylorus was ligated, and a double-lumen cannula was inserted through a small incision into the non-glandular forestomach. The plastic gastric cannula was secured in the stomach and exited the abdomen through the midline incision. The surgical wound was covered by cotton soaked in saline. The body temperature of the animal was kept at 37°C by a heating pad. The experiments were started 2–3 h after completion of the surgery. During this stabilization period, the stomach lumen was flushed with pH 7.0 saline at room temperature.

Gastric effluent was collected every 10 min by flushing the gastric lumen through the gastric cannula twice with 3-ml boluses of saline under gravity drainage and followed once with 3-ml boluses of air at 10-min intervals. Gastric samples were back-titrated to pH 7.0 with 0.01 N NaOH using an automatic titrator (Radiometer, Copenhagen, Denmark). After a 30-min basal period, CCK4(s) or CCK58(ns) at 0.1, 0.3, 1, 3, 10, or 30 nmol·kg⁻¹·h⁻¹, or gastrin-17 at 10 nmol·kg⁻¹·h⁻¹ was infused for 2 h. Intravenous infusions were performed in a volume of 1 ml/h.

RESULTS

Purification of canine CCK58(s) and -ns. Three peaks of immunoreactivity (a total of 33 nmol) eluted during the first reverse-phase HPLC step (results not shown). The immunoreactivity in the first peak tube eluted in a similar position to sulfated CCKs during analytical reverse-phase chromatography (results not shown). No nonsulfated CCKs was observed in this series of experiments or in previous evaluations of CCK forms from canine intestinal extracts (15, 31). The immunoreactivity in the second peak was not characterized, because it did not bind to the ion-exchange column used in a subsequent step of purification. The elution position of this CCK immunoreactivity during reverse-phase chromatography suggested that it may be CCK22. The third peak contained 11 nmol of CCK immunoreactivity and was purified as shown in Table 1. There was no clear resolution of two CCK immunoreactive peaks until the ion-exchange step (Fig. 1). The ion-exchange step contained two immunoreactive CCK peaks (7.1 and 1.4 nmol of CCK-like immunoreactivity) that were associated with absorbance peaks of similar heights. Further purification of the two peaks resulted in the purification of CCK58(s) (results not shown) and a new CCK molecular form. The final step (Table 1) in the purification of the new molecular form of CCK is shown in Fig. 2.

Chemical characterization of the new molecular form of CCK. The amino acid composition of the new molecular form of CCK was the same as that determined for CCK58(s) (Table 2). The new molecular form was cleaved by trypsin, dried, and characterized by liquid chromatography coupled to a mass spectrometer. Mass spectral analysis of the tryptic fragments of the two purified peptides showed that the earliest eluting, major peak from the ion-exchange column was CCK58(s) (Fig. 1), whereas that of the latter eluting peak was CCK58(ns) (Fig. 3, Table 3).

Fig. 1. Ion-exchange separation of canine sulfated [CCK58(s)] and nonsulfated CCK58 [CCK58(ns)]. The CCK immunoreactivity from the C4 reverse-phase chromatography (step 4, Table 1) was loaded and eluted as described in the text. The absorbance at 220 nm and CCK immunoreactivity are plotted against fraction number.

![Sulfated CCK-58](image1.png)

![New Molecular form of cholecystokinin](image2.png)
**Immunoreactivity of CCK**<sub>58</sub>(s) and -(ns). Amino acid analysis and radioimmunoassay of the purified peaks demonstrated that the sulfated and nonsulfated peptides were 59 and 14% as immunoreactive as CCK<sub>58</sub>(s), respectively, showing that CCK<sub>58</sub>(s) is four times more cross-reactive than CCK<sub>58</sub>(ns) (Table 4). Because the immunoreactivity of antisera 5135 is based on a shared homology between CCK and gastrin, factors other than primary sequence must account for the decreased immunoreactivity of the CCK<sub>58</sub>(s) and -(ns) molecular forms. All data presented here did not adjust for the lower immunoreactivity of canine CCK<sub>58</sub>(ns) or -(s) compared with CCK<sub>8</sub>. Figure 1 shows that the absorbance at 280 nm for CCK<sub>58</sub>(ns) was about one-half the height of CCK<sub>58</sub>(s), but the peak for when their amounts were determined by amino acid analysis (Table 4).

**Table 2. Amino acid analysis of canine CCK**<sub>58</sub>(s) and -(ns)**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Observed CCK&lt;sub&gt;58&lt;/sub&gt;(s)</th>
<th>Observed CCK&lt;sub&gt;58&lt;/sub&gt;(ns)</th>
<th>Theoretical CCK&lt;sub&gt;58&lt;/sub&gt;, nmol acid/nmol peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>7.2</td>
<td>6.8</td>
<td>7</td>
</tr>
<tr>
<td>Thr</td>
<td>0.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ser</td>
<td>3.3</td>
<td>3.3</td>
<td>4</td>
</tr>
<tr>
<td>Glu</td>
<td>5.4</td>
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<td>Gly</td>
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<td>Ala</td>
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<td>Val</td>
<td>2.9</td>
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<tr>
<td>Met</td>
<td>1.4</td>
<td>1.2</td>
<td>3</td>
</tr>
<tr>
<td>Ile</td>
<td>2.7</td>
<td>2.7</td>
<td>3</td>
</tr>
<tr>
<td>Leu</td>
<td>4.9</td>
<td>4.9</td>
<td>5</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.8</td>
<td>0.6</td>
<td>2</td>
</tr>
<tr>
<td>Phe</td>
<td>1.0</td>
<td>1.1</td>
<td>1</td>
</tr>
<tr>
<td>His</td>
<td>1.9</td>
<td>2.2</td>
<td>2</td>
</tr>
<tr>
<td>Lys</td>
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</tr>
<tr>
<td>Trp</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1</td>
</tr>
<tr>
<td>Arg</td>
<td>5.4</td>
<td>5.0</td>
<td>6</td>
</tr>
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</table>

Tryptophan is destroyed during acid hydrolysis and is not detected during amino acid analysis. CCK<sub>58</sub>(s), sulfated CCK<sub>58</sub>; CCK<sub>58</sub>(ns), nonsulfated CCK<sub>58</sub>.

**Figure 2. Final step in purification of CCK<sub>58</sub>(ns).** Ion-exchange purified CCK from the minor immunoreactive peak (Fig. 1) was loaded onto a reverse-phase C-18 column and eluted with increasing concentrations of acetonitrile.

**Figure 3. Structural analysis of CCK<sub>58</sub>(ns).** The new molecular form of CCK (Figs. 1 and 2) was digested by trypsin, dried, and chromatographed by HPLC. The effluent from the HPLC was introduced into a mass spectrometer by electrospray ionization. All anticipated tryptic peptides of CCK<sub>58</sub>(s) and CCK<sub>58</sub>(ns) were observed except for T4, which was not detected from either peptide. T7 is the COOH-terminal dodecapeptide of CCK<sub>8</sub>. The masses of this peptide from CCK<sub>58</sub>(s) and CCK<sub>58</sub>(ns) are shown in Table 3. These masses identified the intact peptides as CCK<sub>58</sub>(s) and CCK<sub>58</sub>(ns).

**Table 3. Mass spectral analysis of the COOH-terminal peptide of canine CCK<sub>58</sub>(s) and -(ns) after tryptic digestion**

<table>
<thead>
<tr>
<th>Figure 1 Peak</th>
<th>Observed MW, Da</th>
<th>Calculated MW, Da</th>
<th>Intact Peptide Identity</th>
</tr>
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<tbody>
<tr>
<td>Peak 1</td>
<td>1,614.78</td>
<td>1,614.60</td>
<td>CCK&lt;sub&gt;58&lt;/sub&gt;(s)</td>
</tr>
<tr>
<td>Peak 2 (new molecular form of CCK)</td>
<td>1,534.96*</td>
<td>1,534.65</td>
<td>CCK&lt;sub&gt;58&lt;/sub&gt;(ns)</td>
</tr>
</tbody>
</table>

*The second mass in the CCK<sub>58</sub>(s) analysis corresponded to nonsulfated COOH-terminal dodecapeptide of CCK<sub>8</sub>. Nonsulfated tyrosine is produced from sulfated tyrosines during mass spectral analysis.

**Binding to CCK<sub>A</sub> and CCK<sub>B</sub> receptors.** Both sulfated and nonsulfated peptides were potent at CCK<sub>A</sub> receptors (Fig. 4A). CCK<sub>8</sub>(s) was about fivefold more potent than CCK<sub>58</sub>(ns) and ~20-fold more potent than CCK<sub>58</sub>(ns) at mouse brain CCK<sub>B</sub> receptors (Table 5). Only sulfated CCK<sub>58</sub> exhibited high affinity binding at CCK<sub>A</sub> receptors in mouse pancreatic membranes, but some displacement was observed at the highest concentrations of CCK<sub>58</sub>(ns) (Fig. 4B). CCK<sub>58</sub>(s) was ~30-fold more potent than CCK<sub>58</sub>(ns) for displacement of the label at CCK<sub>A</sub> receptor. We anticipate that if higher concentrations of CCK<sub>58</sub>(ns) had been available for testing, then some label would have been displaced as observed for CCK<sub>58</sub>(ns).

**In vitro stimulation of pancreatic secretion.** CCK<sub>58</sub>(ns) was a poor stimulant of amylase release from isolated pancreatic acini. The potency of CCK<sub>58</sub>(ns) was <1% of CCK<sub>8</sub>(s) and ~1% that of CCK<sub>58</sub>(s) (Fig. 5). There was not enough of the natural peptide for evaluation of amylase secretion at higher doses.

**In vivo stimulation of pancreatic secretion.** The basal in vivo amylase output was 399.7 ± 64.6 U/30 min. CCK<sub>8</sub>(s) was a potent stimulant of amylase (Δincrease = 6,559.7 ± 680.0 U/30 min; P < 0.05 vs. basal) at 1 nmol·kg<sup>−1</sup>·h<sup>−1</sup> iv. However, the same intravenous dose of nonsulfated CCK<sub>8</sub> did not cause any stimulation in amylase output (32.2 ± 114.1 U/30 min). CCK<sub>58</sub>(ns) (1 nmol·kg<sup>−1</sup>·h<sup>−1</sup> iv) caused a slight but significant increase in amylase output (Δincrease = 480.6 ± 148.4 U/30 min; P < 0.05 vs. basal; Fig. 6). The ratio of activity between canine CCK<sub>58</sub>(ns) and -(s) (0.02) for pancreatic output was similar to the ratio of activity between porcine CCK<sub>58</sub>(ns) and -(s) (0.03) for gallbladder contraction (2).
In vivo stimulation of gastric acid secretion. Basal gastric acid output was 1.88 ± 0.16 μmol/10 min in urethane-anesthetized rats. Both CCK₈(ns) and CCK₅₈(ns) were able to stimulate gastric acid (Fig. 7) starting from a dose of 3 nmol·kg⁻¹·h⁻¹ iv. Gastric acid stimulation with either CCK₈(ns) or CCK₅₈(ns) at a dose of 10 nmol·kg⁻¹·h⁻¹ iv reached plateau level in the first hour (13.0 ± 1.5 μmol/10 min or 16.6 ± 2.0 μmol/10 min; P < 0.05 vs. basal), and the level of stimulation remained the same for both peptides as in the first hour. CCK₈(ns) and CCK₅₈(ns) were equipotent (change from the basal values 17.6 ± 5.6 μmol/10 min and 14.0 ± 1.6 μmol/10 min, respectively) but not as potent as gastrin (change from the basal values 39.7 ± 4.0 μmol/10 min). The level of gastric acid stimulation by CCK₈(ns) or CCK₅₈(ns) at 10 nmol·kg⁻¹·h⁻¹ iv was 47.8 ± 15.1 or 38.1 ± 4.4%, respectively, of the gastric acid output caused by gastrin-17(ns) stimulation.

**Table 4. Immunoreactivity of synthetic canine CCK₅₈(ns) and -(ns)**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IR amount, nmol</th>
<th>AAA amount, nmol</th>
<th>Ratio of IR to AAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCK₅₈(ns)</td>
<td>4.2</td>
<td>7.1</td>
<td>0.59</td>
</tr>
<tr>
<td>CCK₅₈(ns)</td>
<td>1.1</td>
<td>8.0</td>
<td>0.14</td>
</tr>
<tr>
<td>CCK₈(ns)</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AAA, amino acid analysis; IR, immunoreactive; CCK₈(s), sulfated CCK₈.

**DISCUSSION**

This work reports the presence of major amounts of CCK₅₈(ns) in canine small intestinal extracts, that CCK₅₈(ns) weakly displaced CCK label from mouse pancreatic CCKₐ receptors, and that CCK₅₈(ns) is a very weak stimulant of amylase from isolated pancreatic acini or from the in vivo pancreas. In contrast, CCK₅₈(ns) is active at CCKₐ receptors as shown by mouse brain CCKₐ receptor binding and its stimulation of gastric acid secretion in vivo. The presence of CCK₅₈(ns) in canine intestinal extracts and its ability to stimulate gastric acid suggests that it may be of physiological relevance, especially for neurocrine and paracrine actions mediated by CCKₐ receptors.

Importantly, CCK₅₈(ns) has now been characterized from two species using two different methods of extraction and purification. In pig, the CCK₅₈(s) to CCK₅₈(ns) ratio is 1:1 (2), and for dogs, this ratio is 2:1. The absence of detectable CCK₅₈(ns) in other studies may be due to either the poor sensitivity of the assay used to detect CCK forms or the difficulty of separating CCK₅₈(s) and -(ns). The presence of CCK₅₈(ns) in pig and dog suggests that this peptide may be characterized from other species if appropriate methods are used for its detection, which include chromatographic separation from sulfated CCK₅₈ and appropriate measurement with a CCK₅₈(ns) standard. CCK₅₈(ns) has been detected in extracts of dog (17), pig (39), and cow (12) brain, but the ratio of CCK₅₈(ns) to -(ns) in brain is not known. Likewise, CCK₅₈(s) is a major endocrine form of CCK in the blood of human (11), dog (14), and rat (33), but it is not known whether CCK₅₈(ns) is present in the blood of any of these species.

The extent of gastrin sulfation in mammals varies from 24–80% (1). For example, in dogs and pigs, the percentage of gastrin that is sulfated is 24 and 57%, respectively (1). Of note, this work shows that 67% of canine CCK is sulfated. Sulfation of propeptides occurs in the trans-golgi network (40), and the...

**Table 5. Relative affinities expressed as Kᵢ (nM) of CCK₈(s), CCK₈(ns), CCK₅₈(s), and CCK₅₈(ns) at CCKₐ and CCKₐ receptors in mouse pancreas and brain membranes**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>CCK₈(s)</th>
<th>CCK₈(ns)</th>
<th>CCK₅₈(s)</th>
<th>CCK₅₈(ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCKₐ</td>
<td>1.41±0.11</td>
<td>0.34±0.11</td>
<td>1.34±0.50</td>
<td>0.28±0.07</td>
</tr>
<tr>
<td>CCKₐ</td>
<td>0.5±0.15</td>
<td>0.98±0.53</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CCK₅₈(ns), nonsulfated CCK₈.

**Fig. 4. Relative affinities of CCK₈(s), CCK₈(ns), and CCK₅₈(ns) at mouse CCKₐ and CCKₐ receptors. A: competitive inhibition of saturable CCK label binding to mouse brain membranes (CCKₐ receptors). B: competitive inhibition of saturable CCK label binding to mouse pancreas membranes (CCKₐ receptors).**

**Fig. 5. Stimulation of amylase release from isolated rat pancreatic acini by CCK₄(ns), CCK₆(s), CCK₅₈(s), and CCK₅₈(ns).**
Similarly, other studies (2) have shown that porcine CCK58 (ns) vivo, although at greatly diminished potency (Figs. 5 and 6). The presence of CCK58 (ns) in tissue and its presumed ability to reach neurocrine, paracrine, and endocrine targets suggest that regulation of processing can cause pro-CCK gene products to act exclusively at CCKB receptors (nonsulfated peptide) or at both CCKA and CCKB receptors (sulfated peptide) or at both CCKA and CCKB receptors (sulfated peptide). Natural canine CCK58 (ns) does not potently displace CCK label from mouse CCKA receptors at the concentrations available (Fig. 4). Similarly, CCK58 (ns) does cause stimulation of amylase secretion (CCKA receptor mediated) in vitro or in vivo, although at greatly diminished potency (Figs. 5 and 6). Similarly, other studies (2) have shown that porcine CCK58 (ns) contracted guinea pig gallbladder (CCKA receptor mediated). The concentrations of CCK58 (ns) required for minimal actions at the CCKA receptor suggest that the peptide does not express its endocrine activity through the CCKA receptor. We cannot evaluate whether CCK58 (ns) acts in an endocrine fashion to stimulate the CCKB receptor because the circulating levels of the peptide are not known. However, the presence of CCKB receptors in vagal afferent neurons suggests that CCK58 (ns) may act in a paracrine or neurocrine manner, in which much higher concentrations of the peptide are possible (27).

CCK58 (ns) and CCK8 (ns) have similar potencies for binding to CCKA receptors and stimulation of gastric acid secretion. In this work CCK8 (ns) and CCK58 (ns) were about one-tenth as potent as gastrin for stimulation of gastric acid. The greater potency of gastrin-17 (ns) is most likely due to its higher potency at the CCKB (21) receptor relative to the nonsulfated CCK peptides and the longer circulating half-life of gastrin (16). The similarity of CCK58 (ns) and CCK8 (ns) for in vivo gastric acid stimulation is unexpected because this action is an integration of several components including circulating half-life, resistance to degradation after leaving the circulation, receptor binding, and receptor activation. Therefore, CCK8 (ns) could be a valuable reagent for the evaluation of how each of these components contributes to the integrated response for stimulation of CCKB receptors.

The presence of CCK58 (ns) solves an apparent inconsistency between processing of progastrin and pro-CCK. The processing of progastrin results in both sulfated and nonsulfated peptides (1). Processing of pro-CCK was formerly thought to result in only sulfated peptides (22). This work and the results from Bonetto et al. (2) demonstrate that CCK, similar to gastrin, exists as sulfated as well as nonsulfated peptides in pigs and dogs (present work). The degree of sulfation of CCK in other tissues, such as brain and blood, in other species and in varied physiological states will aid our understanding of the physiological relevance of sulfation for pro-CCK.

The existence of CCK58 (ns) in tissue and its presumed physiological relevance of sulfation for pro-CCK.
tide). Maeda et al. (24) demonstrated that CCK9 (ns) is a potent stimulant of gastric acid, whereas CCK8 (s) did not stimulate gastric acid. The difference between CCK8 (s) and (ns) effects is that the nonsulfated peptide can only act at CCK receptors on gastric enterochromaffin-like and parietal cells to stimulate gastric acid, whereas the CCK8 (s) reacts at these receptors and also at the CCKB receptor on gastric D cells to release somatostatin. Somatostatin inhibits the release of acid through actions on the parietal cell (18). Thus, for CCK8 (ns), the stimulatory effects at CCKB receptors and inhibitory actions at CCKA receptors result in no net stimulation of gastric acid (24). Of note, a similar pattern of concurrent stimulation and inhibition of gastric acid secretion probably explains the fact that CCK-33(s) stimulates much less gastric acid than gastrin (9).

In this work, 10 nmol·kg⁻¹·h⁻¹ gastrin stimulated about threefold more gastric acid secretion than CCK58 (ns) infused at the same rate (Fig. 7B). This increased response could result from gastrin’s higher affinity binding at the CCKB receptor and/or a lower metabolic clearance rate. In this work, studies of the pharmacokinetics of CCK8 (ns) were not performed because of the limited amounts of natural peptide available. However, previously reported data for sulfated and nonsulfated gastrins show conflicting conclusions for the effect of sulfation on peptide half-lives. Cantor et al. (7) showed that there is little difference in the rates of metabolism for sulfated and nonsulfated forms of gastrin-17. By contrast, Pauwels et al. (29) have reported a two- to fivefold longer half-life for sulfated gastrin-17 compared with the nonsulfated form. Therefore, because of the limited amounts of natural peptide available and the difficulty of performing half-life studies in the rat (due to limited blood volumes), we have not evaluated whether the decreased potency of CCK58 (ns) resulted from lower affinity binding or lower plasma concentrations.

Tertiary structural differences between CCK58 (s) and CCK58 (ns) may influence how they express their biological activity by altering their metabolism in the circulation and interstitial fluid. We have previously demonstrated (34) that CCK58 (s) is much more stable than CCK8 (s) to digestion by neural endopeptidase. Similar differences in stability may exist for CCK58 (ns) and CCK8 (ns). This could account for the equal potency of the nonsulfated peptides for gastric acid secretion, even though CCK8 (ns) binds more potently to the CCKB receptor than CCK8 (s) (Table 5).

Regulation of sulfation alters the pattern of physiological activity of CCK by changing an exclusive CCKB agonist into an agonist for both CCKA and CCKB receptors. Therefore, the physiological relevance of CCK58 (ns) can be evaluated by determining 1) its existence in blood, brain, and peripheral nerves, 2) whether there are physiological states that alter the ratio of sulfated and nonsulfated forms of CCK in these tissues, and 3) whether the concentration of CCK58 (ns) is sufficient to activate CCKB receptors in an endocrine, paracrine, or neurokinine manner.

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