Primary structure, distribution, and effects on motility of CGRP in the intestine of the cod Gadus morhua

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Shahbazi, Fatemeh, Paul Karila, Catharina Olsson, Susanne Holmgren, J. Michael Conlon, and Jörgen Jensen. Primary structure, distribution, and effects on motility of CGRP in the intestine of the cod Gadus morhua. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R19–R28, 1998.—Calcitonin gene-related peptide (CGRP) was isolated from an extract of the intestine of the cod Gadus morhua. The primary structure of this 37-amino acid peptide was established as follows: ACNTA TCVTH RLADF LSRSG GIGNS NFVPT NVGSK AF-NH2. The peptide shows close structural similarities to other nonmammalian (3–4 amino acid substitutions) and mammalian (5–8 amino acid substitutions) CGRPs, and it contains the two residues Asp13 and Phe5 that seem to be characteristic for CGRP in nonmammalian vertebrates. Cod CGRP (10−9–10−10 M) inhibited the motility of spontaneously active ring preparations from the cod intestine and was significantly (P < 0.05) more potent than rat α-CGRP. Neither prostaglandins nor nitric oxide is involved in the inhibitory response produced by cod CGRP, and the lack of effect of tetrodotoxin suggests an action of CGRP on receptors on the intestinal smooth muscle cells. The competitive CGRP antagonist human α-CGRP-(8–37) significantly (P < 0.05) reduced the response to cod CGRP. Immunohistochemistry demonstrated CGRP-immunoreactive neurons intrinsic to the intestine, and a dense innervation with immunoreactive nerve fibers was observed in the myenteric plexus and the circular muscle layer. Myotomy studies show that CGRP-containing nerves project orally and anally in the myenteric plexus, whereas nerve fibers in the circular muscle layer project mainly anally, indicating a role for CGRP in descending inhibitory pathways of the cod intestine.

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide widely distributed in nerves of the gastrointestinal canal of mammals. The CGRP-containing nerves of the gastrointestinal tract have a dual origin, being present in extrinsic sensory and intrinsic neurons, and CGRP and substance P (SP) or other tachykinins are extensively colocalized in the sensory nerves (6, 11, 35, 37, 38). Two forms of CGRP (α- and β-CGRP) are expressed in rats and humans, and they differ by only one and three amino acid residues in rats and humans, respectively (1, 34). In rat, α-CGRP is preferentially present in extrinsic sensory neurons, whereas β-CGRP is predominant in nerves intrinsic to the intestine (28, 36). Nerves containing CGRP-like immunoreactivity have also been described in the gastrointestinal canal of birds, reptiles, amphibians, and fish, although the origin of these nerve fibers is unknown (14, 29, 31).

Isolation and structural characterization of CGRP from a nonmammalian species has been accomplished for the frog Rana ridibunda (7), and the amino acid sequence of CGRP may be deduced from the nucleotide sequence of cDNA from chicken and salmon genomic libraries (16, 25). These studies indicate that the structure of CGRP has been strongly conserved during evolution of the vertebrates.

The predominant action of CGRP on gastrointestinal motility is inhibitory, and CGRP has been proposed as a sensory transmitter in the peristaltic reflex (12). In the rat duodenum the relaxation produced by CGRP is partly reduced by tetrodotoxin (TTX), suggesting a direct and an indirect effect on the smooth muscle (23). However, in most tissues studied the relaxation produced by CGRP is unaffected by TTX (3, 5, 21, 30), and receptors for CGRP have been demonstrated on the muscle cells of the guinea pig stomach (24).

Almost nothing is known about the function of CGRP in the gastrointestinal canal of nonmammalian vertebrates. In the rainbow trout, human CGRP is without effect on unstimulated or electrically stimulated intestinal preparations (4).

The present study was initiated to isolate and structurally characterize CGRP from the cod intestine and to investigate the effect of this peptide on the intestinal motility and the distribution and projection of CGRP-containing nerves in the intestine to increase the knowledge of the molecular and functional evolution of CGRP.

MATERIALS AND METHODS

Atlantic cod (Gadus morhua) of either sex, with a body mass of 250–700 g, were captured off the Swedish west coast and kept in aerated, recirculating seawater at 10°C. The fish were killed by a sharp blow to the head, and the intestine was dissected out and used according to the methods described below.

Tissue extraction. Intestinal tissue (720 g) was taken from 286 specimens and immediately frozen on dry ice. The tissue was homogenized with six volumes of 3.1 ethanol-0.7 M HCl (by vol). The homogenate was stirred for 2 h at 0°C and centrifuged (4,000 g, 25 min, 4°C). The ethanol was removed under reduced pressure, and after further centrifugation (4,000 g, 20 min, 4°C) the extract was pumped onto 10 Sep-Pak C18 cartridges (Waters Associates, Milford, MA) connected in series. Bound material was eluted with 70:29:0.1 acetonitrile-water-trifluoroacetic acid (by vol) and lyophilized.

Purification. The extract was redissolved in 12 ml of 0.1% (by vol) trifluoroacetic acid in water and chromatographed on a 100 × 2.5-cm Sephadex G-50 (fine) column equilibrated...
with 1 M acetic acid at a flow rate of 18 ml/h. Fractions (6 ml) were collected, and the presence of CGRP-like immunoreactivity was determined by RIA. The fractions containing the immunoreactive material were pooled (54 ml), and the volume was reduced and injected onto a 1 x 25-cm Vydac 218TP510 (C18) column equilibrated with 0.1% (by vol) trifluoroacetic acid at a flow rate of 2 ml/min. The concentration of acetonitrile was raised to 21% over 10 min, held at this concentration for 30 min, and further raised to 49% over 60 min. Absorbance was measured at 214 and 280 nm, and 2-ml fractions were collected. The fraction containing the majority of the immunoreactive material was rechromatographed on a 0.46 x 25-cm Vydac 214TP54 (C4) column equilibrated with 17.5:82.4:0.1 acetonitrile-water-trifluoroacetic acid (by vol) at a flow rate of 1.5 ml/min. The concentration of acetonitrile was raised to 35% over 50 min, and individual peaks were collected by hand. The fraction containing CGRP-like immunoreactivity (arrow in Fig. 1B) was chromatographed on a 0.46 x 25-cm Vydac 219TP54 (phenyl) column under the conditions of chromatography used with the C4 column. The immunoreactive peak denoted by the arrow in Fig. 1C was purified to apparent homogeneity by chromatography on a 0.46 x 25-cm Vydac 218TP54 (C18) column with the same elution conditions used with the C4 and phenyl columns.

Structural characterization. The primary structure of cod CGRP (~500 pmol) was determined by automated Edman degradation with use of a sequenator (model 471A, Applied Biosystems) modified for on-line detection of phenylthiohydantoin-coupled amino acids under gradient elution conditions. Standard operating procedures were used, and the detection limit for phenylthiohydantoin-coupled amino acids was 0.5 pmol. Hydrolysis (110°C, 5.7 M HCl, 24 h) of ~500 pmol of peptide was carried out, and amino acid composition was determined by precolumn derivatization with phenylisothiocyanate with use of a derivatizer (model 420A, Applied Biosystems) and a separation system (model 130A, Applied Biosystems). The detection limit for phenylthiocarbamyl-labeled acids was 1 pmol. Cysteine was not determined.

Fig. 1. Successive reverse-phase chromatography on a semipreparative Vydac C18 column (A), an analytic Vydac C4 column (B), and an analytic Vydac phenyl column (C) of an extract of cod intestine after partial purification by gel permeation chromatography. Arrows, fractions containing calcitonin gene-related peptide (CGRP)-like immunoreactive material; dashed lines, concentration of acetonitrile in eluting solvent.
Mass spectrometry was performed on a Voyager RP MALDI-TOF instrument (Perspective Biosystems, Framingham, MA) equipped with a nitrogen laser (337 nm). The instrument was operated in linear mode with delayed extraction, and the accelerating voltage in the ion source was 25 kV. Sample solution (1 µl, 50 pmol) was mixed with 10 µl of matrix solution (α-cyano-4-hydroxycinnamic acid dissolved in 5:4:1 acetonitrile-water-3% trifluoroacetic acid), and 1 µl was deposited on the sample plate and allowed to dry. Calibration was performed using internal standards (luteinizing hormone-releasing hormone and insulin), and the accuracy of the mass determination was within 0.1%.

RIA. CGRP-like immunoreactivity was measured using antiserum 6012 (Peninsula Laboratories, Belmont, CA). The antiserum (diluted 1:100,000), rat α-CGRP standards, or test samples and tracer (2-[125I]iodohistidyl CGRP; Amersham) were incubated in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.2% BSA for 48 h at 4°C. Antibody-bound radioactivity was precipitated by addition of γ-globulin (100 µl, 10 mg/ml) and polyethylene glycol (1 ml, 20% wt/vol) followed by centrifugation (3,000 g, 20 min). The supernatant was decanted, and radioactivity was measured in the pellet and supernatant.

Peptide synthesis. Cod CGRP was synthesized by solid-phase methodology on a 0.025-mmol scale with a synthesizer (model 432, Applied Biosystems). Fluorenlymethoxy carbonyl-labeled amino acids were coupled as their hydroxybenzotriazole active esters following the manufacturer's standard protocols. The peptide was deaved from the resin by use of 90:30:30:40 trifluoroacetic acid-water-ethanedithiolethioanisol (by vol). Formation of the intramolecular disulfide bridge was carried out as previously described (33), and the peptide was purified to near homogeneity by reverse-phase HPLC. Identity of the peptide was confirmed by automated Edman degradation, amino acid analysis, and electrospray mass spectrometry.

In vitro studies. The intestine was placed in cold cod Ringer solution (composition in mM: 150.1 NaCl, 5.2 KCl, 1.9 CaCl₂, 1.8 MgSO₄, 1.9 NaH₂PO₄, 7.0 NaHCO₃, 5.6 glucose, pH 7.8–7.9). Ring preparations (3–5 mm wide) were cut from the proximal part of the intestine and mounted on silver wires in organ baths containing 10 ml of cod Ringer solution at 10°C bubbled with 0.3% CO₂ in air. The tension of the circular smooth muscle was measured isometrically using a force transducer (model FT03, Grass) connected to a polygraph (model 7, Grass). An initial tension of 8 mN was applied, and ~1 h was allowed for the preparations to obtain a steady baseline tension and to develop spontaneous rhythmic contractions before any drugs were added. A few experiments were also made on preparations, ~2 × 10 mm, cut along the axis of the longitudinal smooth muscle layer, with use of the experimental setup described above.

Cod CGRP was added in single concentrations to the preparations, inasmuch as initial experiments showed that cumulative concentration-response curves could not be established because of tachyphylaxis. After they were washed and reequilibrated for ~1 h, the preparations were subjected to a second addition of the same concentration of cod CGRP or, in one group of experiments, the same concentration of rat α-CGRP. In another series of experiments, different antagonists and inhibitors were added 10–35 min before the second addition of cod CGRP. To be able to study the effect of TTX, which abolished the spontaneous contractions of the preparations, ACh was added before the addition of cod CGRP to stimulate the contractile activity.

Myotomy operations. The method has been described extensively by Karila and Holmgren (19). The fish were anesthetized in MS-222 (3-aminobenzoic acid ethyl ester, 100 mg/l; Sigma Chemical, St. Louis, MO), and anesthesia was maintained during the operation. A 20-mm incision was made ventrally, and a loop of the intestine was pulled out. About 50% of the circumference of the proximal intestine (n = 7) was cut to sever the myenteric plexus and the muscle layers. The incision in the abdominal wall was sutured, and the fish were left for 6 days. The fish were killed by a blow to the head, and pieces of the intestine, including the myotomy, were taken for immunohistochemistry. The pieces were pinned flat to dental wax, without being stretched, and fixed in 4% formaldehyde in phosphate buffer for 4 h at 4°C. The tissue was rinsed in hypotonic PBS (0.1 M, pH 7.4, 2% NaCl) containing 0.2% Triton X-100 (vol/vol), 0.2% sodium azide (wt/vol), and 0.1% BSA (wt/vol). The tissue pieces were transferred to PBS containing 30% sucrose (wt/vol) and sectioned on a cryostat at −20°C. In control animals (n = 6) the myotomies were performed immediately before fixation, with no time allowed for accumulation or degeneration of immunoreactive material. In addition, adjacent tissue pieces were taken for whole-mount immunohistochemistry of the myenteric plexus and stretched onto dental wax before fixation.

Immunohistochemistry. The slides were preincubated with normal nonimmune donkey serum (1:10; Jackson ImmunoResearch Labs, West Grove, PA) for 1 h. The tissues (sections and whole mounts) were incubated in a moist chamber at room temperature with one or two of the primary antisera for 20–72 h. The preparations were rinsed in PBS three times for 10 min each and incubated with secondary antibodies for 1–2 h. The secondary antibodies (affinity-purified donkey IgGs, all from Jackson ImmunoResearch Labs) were conjugated to dichlorotriazinyl aminofluorescein (1:1000), indocarbocyanine (1:800), or Texas red (1:100). The preparations were rinsed three times in PBS before they were mounted in carbonate-buffered glycerol. In addition to being examined under a Vanox fluorescence microscope (Olympus, Tokyo, Japan), the preparations were scanned with a Multiprobe 2001 confocal laser scanning microscope (CLSM; Molecular Dynamics, Sunnyvale, CA) with suitable laser wavelength and power with use of a ×20/0.75 Nikon fluorescence lens. To enable comparison of the images, the settings were not altered during the acquisition of images from the set of slides incubated with the same antibody. Optical sections of the myenteric plexus and surrounding muscle layers were stored on the CLSM workstation (Indigo, Silicon Graphics Computer Systems, Mountain View, CA), and the images that were acquired at a certain threshold value were calculated oral and anal to the myotomy operation (19). The threshold value was set to exclude background from the area occupied by immunoreactive material. First, the areas immediately oral and anal to the myotomy were compared and, subsequently, also the areas immediately oral and anal to the myotomy were compared with locations 2.1 mm oral or anal to the myotomy, respectively. Also in the control group (where no time was allowed for accumulation or degeneration) the areas immediately oral and anal to the myotomy were compared.

Primary antisera. CGRP antisera 6006 and 6012 (both from Peninsula Laboratories; diluted 1:400), raised in rabbits, were used for single labeling or in combination with antisera against SP (B-GP 450-1) or vasoactive intestinal peptide (B-GP 340-X), both raised in guinea pigs (Euro-Diagnostica; diluted 1:400). A CGRP antiserum raised in guinea pig (B-GP 470-1, Euro-Diagnostica; diluted 1:100) was also used in combination with the cod tachykinin antiserum NKA 2809 raised in rabbit (J. Jensen; diluted 1:6,400).
Preabsorptions with CGRP were made, and no spurious binding was detected with 10 μM antigen and with the concentrations of antisera 6006 and 6012 used in the study.

Drugs. The following drugs were used: ACh hydrochloride, indomethacin, Nω-nitro-L-arginine methyl ester, and TTX (all from Sigma Chemical, St. Louis, MO), rat α-CGRP (Auspep, Melbourne, Victoria, Australia), human α-CGRP-(8—37) (Neo-system, Strasbourg, France), and cod CGRP (Peptide Chemistry Core Facility, Creighton University). Indomethacin was dissolved in DMSO and diluted in phosphate buffer. DMSO (0.1%, the highest concentration used in the study) had no effect on the response studied. All other drugs were dissolved in distilled water and further diluted in 0.9% NaCl.

Data analysis and statistics. Changes in tension recorded on the Grass polygraph were also sampled on data acquisition software (AD/DATA, P. Thorén, Dept. of Physiology, Karolinska Institute, Stockholm, Sweden).

The mean tension of a 5-min period starting 2 min after addition of CGRP was compared with a 5-min control period just before the addition of CGRP. The response was calculated by subtracting the basal tension from the mean tension in the control period and in the period after addition of CGRP and is expressed as percent inhibition of tension.

For calculations of the effect of TTX on the response to CGRP, the difference between the mean tension in a 1-min period starting 3 min after addition of CGRP and a 1-min control period was compared on the same preparation before and after TTX treatment.

Wilcoxon matched-pairs signed ranks test was used for statistical evaluation of the results, and the Bonferroni procedure (13) was used to eliminate the risk of discarding a true null hypothesis when repeated tests were made. P ≤ 0.05 was regarded as statistically significant.

RESULTS

Peptide purification. After partial purification of the cod intestinal extract on Sep-Pak cartridges and by gel chromatography, the fractions containing CGRP-like immunoreactivity were injected onto a semipreparative C18 reverse-phase HPLC column. The majority of the immunoreactive material eluted in the fraction denoted by the arrow in Fig. 1A. This fraction was rechromatographed on an analytic C4 column and eluted as a peak indicated by the arrow in Fig. 1B. After further chromatography on an analytic phenyl column the CGRP-like material appeared as a single peak (arrow in Fig. 1C), which was purified to apparent homogeneity on an analytic C18 column.

Peptide characterization. The primary structure of the cod CGRP was determined by automated Edman degradation (Table 1). Unambiguous assignment of amino acid phenylthiodyantoin derivatives was possible for 37 cycles of operation of the sequencer. No phenylthiodyantoin derivatives were detected during cycles 2 and 7 of the sequence analysis, which is consistent with the presence of a cysteine bridge in cod CGRP. In analogy to all other CGRPs, the COOH-terminus of cod CGRP is probably α-amidated, although this was not experimentally confirmed. The proposed primary structure of cod CGRP was confirmed by mass spectrometry. The observed molecular mass of the peptide was 3,813.2 ± 3.8 compared with the calculated mass of 3,811.2 for the proposed structure; the presence of a cysteine bridge is assumed. The amino acid composition of the peptide was established as follows: Asx 5.0 (5), Ser 3.8 (4), Gly 4.0 (4), His 0.8 (1), Arg 2.2 (2), Thr 4.3 (4), Ala 4.1 (4), Pro 1.4 (1), Val 2.9 (3), Ile 1.2 (1), Leu 2.4 (2), Phe 2.6 (3), and Lys 1.2 (1) residues/mol peptide; numbers in parentheses represent values predicted from the proposed structure.

In vitro studies. Cod CGRP (10−8 and 10−7 M) produced an inhibition of the spontaneous rhythmic activity of the circular muscle from the distal intestine, and in more than one-half of the preparations an additional reduction of the basal tension was observed (Fig. 2A). The lowest concentration tested (10−9 M) only occasionally produced a reduction of the rhythmic contractions. No significant difference in the response was found between two consecutive exposures to the same concentration of cod CGRP (10−8 and 10−7 M; Fig. 3A). Similar to our finding in circular muscle, the spontaneous activity of the longitudinal muscle strip preparations of the cod intestine was reduced or abolished by cod CGRP (10−8 M (n = 10) and 10−7 M (n = 7)).

The CGRP receptor antagonist human α-CGRP-(8—37) (3 × 10−6 M) added before cod CGRP significantly reduced the magnitude of the response to cod CGRP

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CGRP, calcitonin gene-related peptide; ND, not determined.
Projections of enteric neurons. Accumulations of CGRP-immunoreactive material were seen on both sides of the myotomy in the proximal intestine in two animals, whereas accumulations of immunoreactive material were seen on only the oral \((n = 2)\) or anal \((n = 2)\) side in other animals. In one fish no accumulation was observed. Consequently, quantification (using the CLSM) of the CGRP-immunoreactive material in the areas immediately on the oral and anal sides of the myotomy did not reveal any significant differences, although a marked (and significant) reduction in the amount of immunoreactive material was seen 2.1 mm anal to the cut (Fig. 5). However, examination of the circular muscle layer showed a reduced density of CGRP-immunoreactive fibers on the anal side in some preparations \((n = 4)\). In control animals, where no time was allowed for accumulation or degeneration, no differences were found in the amount of immunoreactive material between the oral and the anal side of the cut.

**DISCUSSION**

The present study describes the isolation and structural characterization of CGRP from a teleost fish and demonstrates for the first time an inhibitory effect of CGRP on the intestinal motility in a nonmammalian vertebrate.

The primary structure of cod CGRP is compared with CGRPs from other vertebrates in Fig. 6, and the data indicate a strong preservation of the CGRP structure during evolution. One novel amino acid substitution is found in the cod CGRP, an isoleucine at position 22, that has not been described in CGRPs from any other species. In common with the predicted sequence of CGRP from salmon, the cod peptide has an asparagine at position 24, instead of the lysine in other vertebrate CGRPs. The presence of aspartic acid and phenylalanine at positions 14 and 15, respectively, seems to be characteristic for the nonmammalian CGRPs. The aspartic acid at position 14 appears to be important for the biologic activity of CGRP. Thus chicken CGRP is more effective than human CGRP in lowering serum calcium and phosphate in rats, and the substitution of Asp\(^{14}\) with Gly, as in humans, reduces the activity, whereas the replacement of Gly\(^{14}\) in human CGRP with Asp enhances the activity. None of the other amino acid differences between chicken and human CGRP affects the potency of the peptide in this system (26). Similarly, chicken CGRP is more potent than human CGRP in raising intracellular cAMP levels in a preosteoblast cell line, whereas Asp\(^{14}\) human CGRP is equipotent (40). In the present study, cod CGRP was significantly more potent than rat CGRP in reducing the activity of the intestinal strip preparations. Although there are several differences in amino acid sequence between the peptides, it is possible that the presence of Asp\(^{14}\) in cod CGRP is responsible for the difference in potency.

There were no indications of the presence of more than one form of CGRP in cod intestine in the present study. In rat, \(\alpha\)-CGRP and \(\beta\)-CGRP are preferentially expressed by sensory neurons and enteric neurons,
respectively (28), and it cannot be ruled out that an additional form of CGRP is present in other parts of the nervous system of the cod.

The inhibition of the spontaneous activity produced by CGRP on the cod intestine is consistent with the inhibitory effect of CGRP demonstrated in the intestine of several mammalian species (2, 3, 23, 32). In gastric smooth muscle of rat and guinea pig the CGRP-induced inhibition is reduced by the cyclooxygenase inhibitor indomethacin, and the effect is restored by exogenous prostaglandins (21). However, similar to what has been shown in the mouse colon (5), prostaglandins are probably not involved in the mediation of the response to CGRP in the cod intestine, since indomethacin is without effect on the relaxation. In addition, the present results show that the effect of CGRP does not involve a release of nitric oxide. Nitric oxide is suggested as a mediator of the vasodilatory effect of CGRP in the gastric circulation (15), whereas the nitric oxide synthase inhibitor N-nitro-L-arginine is ineffective on
the relaxation produced by CGRP in the guinea pig colon (22). TTX did not affect the response to CGRP on precontracted preparations, suggesting that CGRP is acting on receptors situated on the circular smooth muscle cells in the cod intestine. This agrees with findings in most mammalian studies, although there are examples of direct and indirect effects of CGRP in gastrointestinal tissues (30).
Human α-CGRP-(8—37), which acts as a competitive antagonist on the CGRP type 1 receptor in mammals (42), reduced the inhibition produced by cod CGRP on the intestine. Although the antagonistic properties of CGRP-(8—37) in fish need to be investigated in more detail, the results clearly indicate that this CGRP fragment may be a useful tool in studies of the importance of CGRP in the control of gastrointestinal function also in nonmammalian vertebrates.

The immunohistochemical work provides, for the first time, evidence for the presence of CGRP-immunoreactive neurons intrinsic to the intestine in teleost fish. No CGRP-SP coexistence was revealed in neuronal cell bodies, and only few fibers showed coexisting immunoreactivities. This, in addition to the different distributions of the immunoreactivities to the two peptides (20), indicates that CGRP and tachykinins are mainly present in separate populations of enteric neurons. The CGRP-SP-immunoreactive fibers observed in the circular muscle layer may derive from neurons extrinsic to the intestine. In the sensory ganglion of the vagus outflow, the nodose ganglion, a portion of the neurons is CGRP immunoreactive, whereas no SP immunoreactivity has been found there (P. Karila, J. Messenger, and S. Holmgren, unpublished observations). Coexistence of CGRP and SP immunoreactivity usually indicates extrinsic sensory neurons (11, 41) and is found in a subpopulation of fibers in peripheral organs of other animals studied, including lungfish, amphibians, and reptiles (8, 14, 18, 27). In other teleost fish species, CGRP-SP fibers have been observed in cardiac tissue and in blood vessels (9; P. Karila, unpublished observations).

The delineation of projections in the enteric nervous system of teleost fish is problematic, since there is little or no distal loss of peptide immunoreactivity over a period of 4 wk after a nerve lesion is made (17, 19). This is in strong contrast to the situation in mammals, where peptide immunoreactivity is lost after ~2 days and up to 20 mm from the cut (10). Swellings of nerve fibers and aggregations of peptide-immunoreactive material have, however, been seen on the proximal side of myotomy operations in the myenteric plexus of the cod stomach and intestine (17, 19, 20). The accumulations observed on both sides of the myotomy in the present study suggest that the CGRP-immunoreactive neurons project orally and anally. Also, in the rat small intestine, CGRP-immunoreactive neurons project orally and anally (39), and CGRP has been proposed as a transmitter in intrinsic sensory pathways involved in the peristaltic response produced by mucosal stimulation (12). The reduced fiber density observed in the circular muscle on the anal side of the myotomies in the cod intestine indicates a predominating anal projection. This observation, together with the direct inhibitory effect produced by CGRP on the intestine, is in agreement with the role of CGRP as a transmitter in

![Fig. 5. Areas occupied with material immunoreactive (IR) to CGRP in Atlantic cod proximal intestine from 2 regions oral to and 2 regions anal to myotomy operation (n = 7). Ordinate, distance from myotomy operation. Values (means ± SE) represent areas of IR material within a circle of 100 µm diameter. No significant differences were found in areas covered with CGRP-IR material between 2 sides of myotomy after immunohistochemistry. However, when areas adjacent to and 2.1 mm from myotomy on oral and anal sides, respectively, were compared, a reduction in amount of IR material was seen 2.1 mm anal to cut. *Statistically significant difference (P < 0.05).](http://ajpregu.physiology.org/)

![Fig. 6. Comparison of primary structure of CGRP from different vertebrates. Residue identity to cod CGRP.](http://ajpregu.physiology.org/)
descending inhibitory nerve pathways, although it is evident that CGRP is present in additional pathways.

Perspectives

Knowledge about differences and similarities in the gastrointestinal control between mammalian and non-mammalian species is important for an understanding of the evolution of the complex control system of the gastrointestinal canal. Comparison of the structure of cod CGRP with mammalian CGRPs indicates that the primary structure has been well conserved during evolution. Nevertheless, comparison of the potency of cod and rat CGRP shows that cod CGRP is more effective in causing contractions of the fish intestinal preparations, which points to the importance of using the endogenous peptides. Interestingly, previous studies have indicated that nonmammalian CGRP is more potent also on mammalian receptors, demonstrating the potential use of nonmammalian peptides as agonists in mammalian physiological studies.

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