GASTROENTEROLOGY 1988;94:284–93

Inhibitory Effect of Calcitonin Gene-Related Peptide and Calcitonin on Opossum Esophageal Smooth Muscle

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Calcitonin gene-related peptide (CGRP) is widely distributed in the gastrointestinal nerves, including those of the esophagus. The present investigation was undertaken to examine the effect and the mechanism of action of CGRP on the lower esophageal sphincter and esophageal contractions. This peptide caused dose-dependent relaxation of the lower esophageal sphincter. The D_{50} for inhibitory effect of intraarterial CGRP on the sphincter was 5.0×10^{-13} mol/kg. Calcitonin gene-related peptide is 3000 times more potent than calcitonin. The effect of CGRP on the lower esophageal sphincter was partially antagonized by tetrodotoxin or black widow spider venom. The inhibitory effect of CGRP on the sphincter appears to be exerted at two levels: (a) at the sphincteric smooth muscle, and (b) at the noncholinergic, nonadrenergic inhibitory neurons. Calcitonin gene-related peptide also exerts a potent inhibitory effect on the peristaltic contraction of the esophageal body in response to swallowing and vagal efferent stimulation. Using immunohistochemical studies we also showed the presence of CGRP-immunoreactive neurons within the myenteric ganglia of the esophagus. These studies suggest that CGRP may play an important role as an inhibitory neurotransmitter in the esophagus.

Calcitonin gene-related peptide (CGRP), a 37-residue peptide, has been shown immunocytochemically to be widely distributed in the central and peripheral nervous system in pathways subserving sensory, motor, and autonomic functions (1–8). In the esophagus, CGRP immunoreactivity has been localized to (a) sensory nerves in the mucosa forming the subepithelial plexus, (b) terminal motor nerves to smooth muscle and motor endplates of the striated muscle, (c) nerves to intramural ganglia, and (d) nerves to blood vessels (6). Calcitonin gene-related peptide has been localized to myenteric neurons in the small bowel but not in the esophagus (4,8). The effect of CGRP and calcitonin on the esophagus is not known.

The purpose of the present investigation was to examine the actions of CGRP, a newly identified neuropeptide, on the esophageal smooth muscle. This report (a) describes the effect of CGRP on the lower esophageal sphincter (LES) and esophageal body contractions, (b) compares the effect of CGRP with that of calcitonin, and (c) investigates the sites of actions of CGRP. We also performed immunohistochemical studies and showed that CGRP is localized to myenteric neurons in the opossum esophagus. The results of the present studies suggest that CGRP is a potential inhibitory neurotransmitter in the esophagus.

Materials and Methods

Experimental Animals and General Techniques

These studies were performed in 25 adult opossums (*Didelphis virginiana*) of either sex weighing between 2 and 4 kg. The animals were anesthetized using pentobarbital sodium (40 mg/kg, intraperitoneally). After anesthesia the animals were strapped supine on the animal board. The brachial vein was cannulated for administration of various agents. One of the brachial arteries was cannulated for direct monitoring of the blood pressure.

The respiration of the animals was assisted with an artificial respirator through endotracheal tubing. The rate of the respirator was set at 20 strokes/min, and the stroke volume was determined following the manufacturer's guidelines (Harvard Apparatus Co., Millis, Mass.). The

Abbreviations used in this paper: CGRP, calcitonin gene-related peptide; D_{50}, dose causing 50% of the maximal fall in sphincter pressure; LESP, lower esophageal sphincter pressure; PBS, phosphate-buffered saline; TTX, tetrodotoxin.

0016-5085/88/$3.50

experimental protocol and care and treatment of the animals before, during, and at the conclusion of the experiment were approved by the Institutional Animal Research Committee and were in accordance with the NIH Guide published in 1980.

Recording of Intraluminal Pressures

A specially designed catheter assembly was employed to record pressures from the LES as described earlier (9). The assembly consisted of six polyvinyl catheters, each 50 cm long (ID 0.86 mm and OD 1.16 mm). Each catheter had a sidehole. The three distal sideholes were situated 1 cm apart and the three proximal ones were spaced 4 cm apart. Each catheter was continuously perfused with bubble-free water through a side opening using a low-compliance pneumohydraulic capillary system as described elsewhere (9). Sudden occlusion of the catheter tip produced a pressure rise of 200 mmHg in 0.1 s. The maximum outside diameter of the assembly was 5 mm. The baseline of the recordings was set at atmospheric pressure. The pressures were recorded on a Beckman dynograph recorder (model R612, Beckman Instruments, Schiller Park, Ill.) using Statham transducers.

The catheter assembly was introduced orally and was positioned so that recordings were made from the stomach, LES, and the esophageal body 1, 5, 9, and 13 cm above the LES. The catheter assembly was then anchored in the sphincter under direct vision after laparotomy (9). Gastric contents were drained with an intragastric catheter. The intragastric catheter was passed through a small gastrostomy incision that was closed using the purse-string suture around the catheter.

Drug Administration

The branch of the left gastric artery leading to the LES was cannulated for close intraarterial administration of agents. The following agents were used: atropine sulfate (muscarinic antagonist) (Eli Lilly and Co., Indianapolis, Ind.); bulbocapnine hydrochloride (dopaminergic antagonist) (ICN Pharmaceuticals, Inc., Life Sciences Group, Plainview, N.Y.); calcitonin and CGRP (Peninsula Laboratories, Inc., Belmont, Calif.); haloperidol hydrochloride (dopaminergic antagonist) (McNeil Laboratories, Inc., Fort Washington, Pa.); hexamethonium hydrochloride (nicotinic antagonist) (City Chemical Corporation, New York, N.Y.); indomethacin (prostaglandin synthesis inhibitor) (Sigma Chemical Co., St. Louis, Mo.); 5-methoxy-N,N-dimethyltryptamine (5-hydroxytryptamine antagonist at the neural level) (Aldrich Chemical Co., Milwalkee, Wis.); metiamide (histaminergic H_2-receptor antagonist) (a gift from Dr. W. W. Brimblecombe of Smith Kline & French Laboratories, Ltd., Welyn Garden City, Hertz, U.K.); naloxone hydrochloride (opioid antagonist) (a gift from C. A. Segretta of Endo Laboratories, Inc., Garden City, N.Y.); phentolamine mesylate (α-adrenergic antagonist) (Ciba Pharmaceutical Co., Summit, N.J.); propranolol hydrochloride (β-adrenergic antagonist) (Ayerst Laboratories, New York, N.Y.); pyrilamine maleate (histaminergic H_1-antagonist) (a gift from Dr. M. E. Garabedian of Alcon Laboratories, Inc., Fort Worth, Tex.); and tetrodotoxin (TTX) (a neurotoxin that blocks sodium-mediated axonal conduction) (Calbiochem, San Diego, Calif.). All agents were dissolved or diluted in 0.9% sodium chloride except indomethacin, which was dissolved in 50 mM Tris-HCl buffer (pH 8). The doses of all agents were expressed on the basis of their salts.

The agents were administered intraarterially into the left gastric artery or intravenously into the brachial vein as a single 20-s bolus. The volumes of intraarterial boluses ranged from 0.15 to 0.5 ml, whereas those given intravenously varied from 0.5 to 2 ml. The administration of physiologic saline alone in these volumes by any of the routes had no effect on the LES pressure (LESP). In the animals used to determine the site of action of CGRP on LESP, the vagi were identified in the cervical region and sectioned. The response to CGRP was tested before and after the antagonists in the same animals. Only one antagonist was used in each animal. Thus, each animal served as its own control. The length of the experiment varied from 4 to 8 h depending on the type of experimental protocol. The results are expressed as percent and absolute changes in LESPs.

Calcitonin gene-related peptide and calcitonin were administered close intraarterially as a single bolus at random in doses ranging from 6.2×10^{-14} to 4.1×10^{-8} mol/kg. No tachyphylaxis to the response of CGRP and calcitonin was observed. The doses of different antagonists (administered intravenously) were selected on the basis of previously published studies, where the antagonists given in the doses used here nearly abolished the effect of maximally effective doses of respective agonists (10).

Tetrodotoxin was administered intraarterially in doses of 5 μg/kg at a time at 30-min intervals until the responses to esophageal distention and vagal stimulation were abolished. The effect of CGRP or calcitonin was examined during the time when complete obliteration of the responses to esophageal distention and vagal stimulation was present.

Swallowing Reflex and Vagal Stimulation

To examine the influence of CGRP on the esophageal peristaltic contractions, the responses to swallowing and vagal stimulation were examined. Swallowing was induced by electrical stimulation of the superior laryngeal nerve (5 mA; 0.5-ms pulse duration; 20–30 Hz for 1-s train). The onset of swallow-induced esophageal contractions was determined in relation to the onset of a swallow-induced spike burst of the mylohyoid muscle. The mylohyoid electromyography was recorded using conventional bipolar electrodes as described previously (11). The response of the esophagus to electrical stimulation of the vagus nerve (5 mA; 0.5 ms; 10 Hz for 1-s train) was determined in relation to the onset of electrical vagal stimulation. The responses to swallowing and vagal stimulation were examined before and after two doses of CGRP (1.0×10^{-9} mol/kg and 4.0×10^{-9} mol/kg) administered intravenously every 15 min.

Data Analysis

The values are expressed as mean ± SE. The statistical analysis was performed using Student's un-

paired t-test or paired t-test where applicable. The significance of the shift in the dose-response curve before and after different treatments was examined using linear regression analysis (12).

Immunocytochemistry Studies

Immunocytochemistry studies were performed as follows: fresh esophagi were excised, cut longitudinally, and pinned, slightly stretched, to cork board. The tissue was fixed for 16–24 h at 4°C in 15% saturated picric acid and 2% formaldehyde in 0.1 M phosphate buffer (pH 7.3). After fixation the tissue was removed from the cork board, dehydrated through a series of graded alcohols, cleared in xylene, and rehydrated according to the method of Costa et al. (13). Subsequently, the tissue was washed overnight in phosphate-buffered saline (PBS) at 4°C. This was followed by dissection of the mucosal, smooth muscle, and longitudinal muscle layers.

Lyophilized antisera to CGRP were obtained from Peninsula Laboratories and reconstituted in sterile distilled water. The antiserum is very specific for CGRP, having no cross-reactivity with other neuropeptides (5). The cross-reactivity for the most closely related peptide, rat calcitonin, is <0.001%.

The smooth muscle layer with overlying myenteric plexus was immersed in 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase. The tissue was rinsed in PBS and incubated in primary antisera (dilution 1:400 in PBS) for 16 h at room temperature. Subsequently, the specimens were rinsed in PBS and sheep-antirabbit immunoglobulin G (Cappel Laboratories, Malvern, Pa.) at room temperature for 1 h (dilution 1:40). The tissue was then rinsed in PBS and incubated in rabbit peroxidase-antiperoxidase immune complex (Cooper Biomedical Malvern, Pa.) for 30 min. After it was rinsed in PBS, the tissue was finally incubated for 40 min in 3,3-diaminobenzidine tetrahydrochloride-H_2O_2 mixture, rinsed, and examined with an Olympus BH-2 light microscope (Olympus Corp., Lake Success, N.Y.).

Specificity of staining was established by incubating tissues in antigen-inactivated antiserum before application of a second-layer antiserum.

Results

Studies on the Lower Esophageal Sphincter

Effect of calcitonin gene-related peptide and calcitonin on the lower esophageal sphincter. The administration of calcitonin or CGRP in the arterial supply of the LES caused a fall in the sphincter pressure as shown in Figure 1. Intravenous administration of these agents also caused a fall in the sphincter pressures, but only with very large doses.

Figure 2 summarizes the dose-response curve of intraarterially and intravenously administered calcitonin and CGRP on the relaxation of the sphincter. The threshold doses were 1.0×10^{-14} mol/kg for intraarterial CGRP, 1.6×10^{-11} mol/kg for intravenous CGRP, 1.0×10^{-11} mol/kg for intraarterial calcitonin, and 1.0×10^{-7} mol/kg for intravenous calcitonin. The D_{50} (dose causing 50% of the maximal fall in the sphincter pressure) values were 5.0×10^{-13} mol/kg for intraarterial CGRP, 1.0×10^{-10} mol/kg (0.38 μg/kg) for intravenous CGRP, and 1.7×10^{-9} mol/kg for intraarterial calcitonin. Complete dose-response curves for intravenous calcitonin were not made because of the very large doses of peptide required. However, the maximal relaxation caused by intraarterial calcitonin was smaller than that caused by CGRP.

Based on D_{50} values of intraarterially administered agents, CGRP was 3000 times more potent than calcitonin as a relaxant of the LES. It should also be noted that the maximal effective dose of CGRP when injected intraarterially (1.6×10^{-11} mol/kg) was a

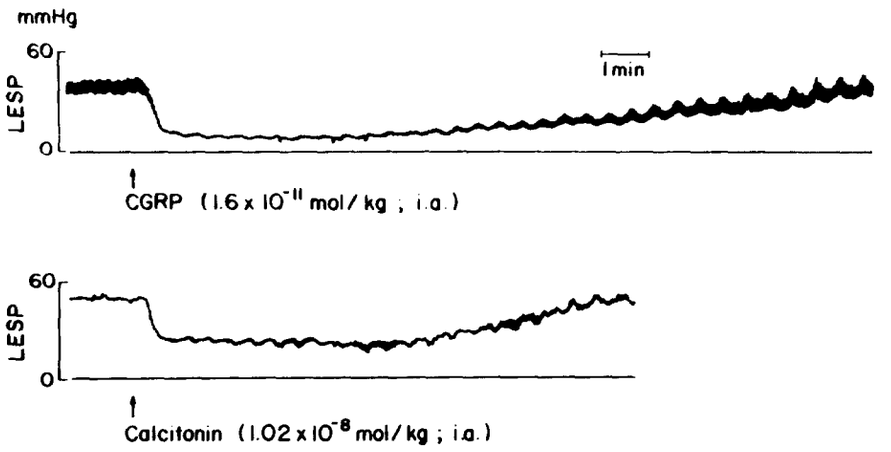

Figure 1. Representative response showing the effects of calcitonin gene-related peptide (CGRP) and calcitonin on the lower esophageal sphincter pressures (LESP). In these experiments these peptides were administered in the esophageal branch of the left gastric artery in their maximal effective doses. Both CGRP and calcitonin caused a fall in the sphincter pressure. The inhibitory responses of these peptides on the LES began within 20 s and lasted for 10–15 min.

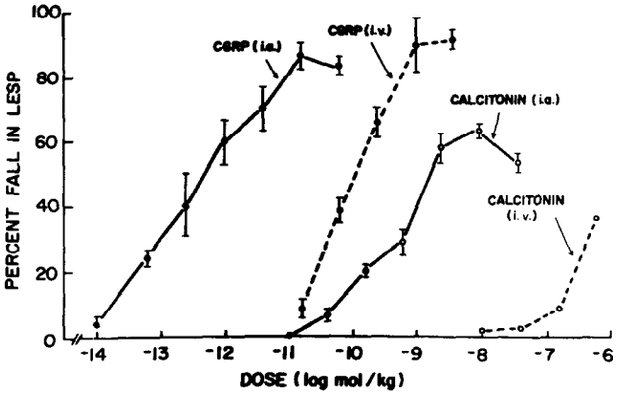

Figure 2. Dose-response curves showing the inhibitory effects of calcitonin gene-related peptide (CGRP) and calcitonin on the lower esophageal sphincter pressure (LESP; n = 5 in 5 animals, one observation each). Note the comparison of dose-response curves showing the fall in the sphincter pressures when CGRP and calcitonin were administered intraarterially versus intravenously. The influence of intravenous calcitonin in doses higher than 1.0×10^{-6} mol/kg was not examined because of insufficient amounts of the peptide available. Note that when given intraarterially, CGRP was ~3000 times more potent that calcitonin. Calcitonin gene-related peptide was ~200 times more potent when given intraarterially as compared with intravenously.

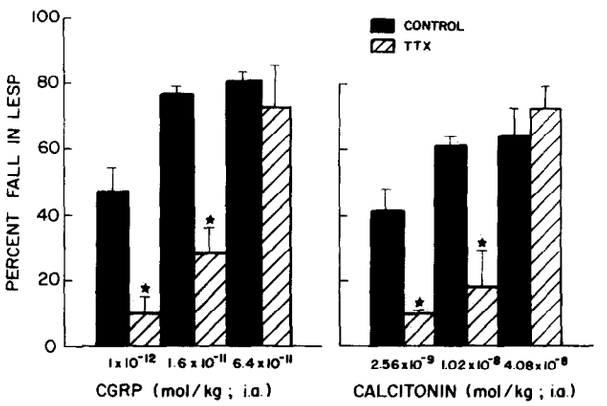

Figure 3. Bar graphs showing percent fall in the lower esophageal sphincter pressure (LESP) before (*solid bars*) and after (*hatched bars*) tetrodotoxin (TTX). Note that the inhibitory effect of calcitonin gene-related peptide (CGRP) and calcitonin in the lower dose was significantly antagonized by TTX ($p < 0.05$), whereas the responses to higher doses were not significantly modified ($p > 0.05$; n = 4 in 4 animals).

threshold dose when administered intravenously. The latency of onset and duration of fall in LESP with different doses of CGRP administered intravenously and intraarterially are given in Table 1.

Influence of tetrodotoxin. Tetrodotoxin antagonized but did not completely block the inhibitory effect of calcitonin or CGRP on the LES. The doses of TTX used were such that they completely blocked the LES relaxation caused by esophageal balloon distention or vagal efferent stimulation. Therefore, we examined the influence of increasing doses of calcitonin on the sphincter pressure in the presence of neural block with TTX. As shown in Figure 3, the effect of calcitonin or CGRP in the doses that caused maximal fall in the sphincter pressure was markedly antagonized by TTX. Increasing the doses of these substances at supramaximal levels caused near-maximal relaxation of the sphincter pressure in the presence of TTX. The shift in the dose-response curve in the presence of TTX was significant when compared with that of the control (Figure 4; $p < 0.05$; n = 4 in 4 animals).

The rightward shift in the dose-response curve with lower doses of calcitonin and CGRP in the presence of TTX and the inability of TTX to modify the inhibitory response at the high doses may be explained by a number of possibilities. The fall in LESP with the lower doses of calcitonin and CGRP is due to combined actions of the peptides at the inhibitory neurons as well as at the LES smooth

Table 1. Latency of Onset and Duration of Fall in the Lower Esophageal Sphincter Pressure With Different Doses of Calcitonin Gene-Related Peptide Given Intravenously and Intraarterially

Intravenous administration			Intraarterial administration		
Dose (*mol*/kg)	Latency of onset of fall in LESP[a](s)	Duration of fall in LESP[a] (*min*)	Dose (*mol*/kg)	Latency of onset of fall in LESP[a](s)	Duration of fall in LESP[a] (*min*)
1.6×10^{-11}	44 ± 6	5.0 ± 1.0	2.5×10^{-13}	8 ± 6	2.3 ± 0.8
6.4×10^{-11}	34 ± 4	9.0 ± 0.5[b]	1.0×10^{-12}	13 ± 3	5.3 ± 1.8[b]
2.5×10^{-10}	26 ± 8[b]	10.0 ± 1.0[b]	4.0×10^{-12}	16 ± 5	5.3 ± 1.0[b]
1.0×10^{-9}	39 ± 9	13.3 ± 3.9[b]	1.6×10^{-11}	16 ± 4	11.5 ± 4.1[b]
4.0×10^{-9}	21 ± 6[b]	36.7 ± 5.5[b]	6.4×10^{-11}	11 ± 2	15.0 ± 3.2[b]

LESP, lower esophageal sphincter pressure. [a] Each value represents mean ± SE of five observations in 5 animals. [b] The values are significantly different from those of the smallest dose tested ($p < 0.05$).

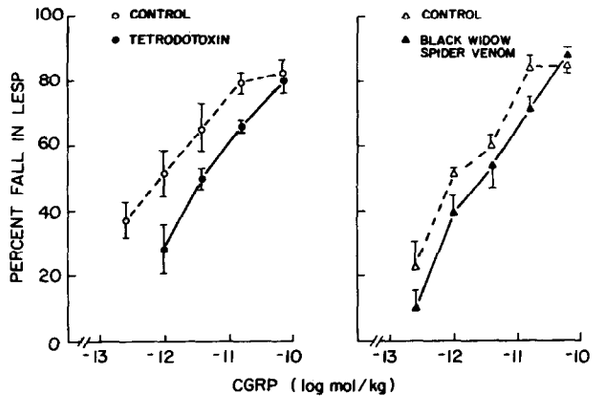

Figure 4. The influence of TTX and black widow spider venom on the inhibitory response of calcitonin gene-related peptide (CGRP). Note that both TTX and black widow spider venom caused significant shifts in the dose-response curves of the CGRP toward the right ($p < 0.05$; $n = 4$ in 4 animals).

muscle. However, at the higher doses both types of receptors (neural and myogenic) can be fully activated. In addition, calcitonin and CGRP in maximal and supramaximal doses may overcome the neural block caused by TTX, or they may act via TTX-resistant mechanisms to cause sphincter relaxation.

Influence of black widow spider venom. Black widow spider venom is known to destroy nerve endings and cause denervation (14). After neural block with black widow spider venom, CGRP caused dose-dependent relaxation of the sphincter (Figure 4). The neural block with the venom was tested by examining the effect of vagal stimulation after the administration of black widow spider venom. In control experiments, vagal stimulation (5 mA; 0.5 ms; 10 Hz for 2-s train) caused a fall in the LESP of 78.6% ± 2.9%; from 43.0 ± 4.7 mmHg to 9.3 ± 2.3 mmHg. After the administration of the venom, the response of vagal stimulation was antagonized to 9.2% ± 5.2%; from 45.5 ± 2.8 mmHg to 40.1 ± 2.8 mmHg ($p < 0.05$; $n = 12$ in 4 animals, three observations each). Moreover, 0.1 nmol/kg of intraarterial nicotine in control experiments produced a fall in the LESP of 77.1% ± 3.1%, which was antagonized to 19.2% ± 5.3% in the presence of the venom ($p < 0.05$; $n = 4$ in 4 animals). The dose-response curve obtained with CGRP in the presence of black widow spider venom showed a significant shift toward the right when compared with the one obtained before the venom was given (Figure 4; $p < 0.05$; $n = 4$ in 4 animals).

Influence of indomethacin pretreatment. In-

Table 2. Influence of Different Antagonists on the Fall in Lower Esophageal Sphincter Pressure Caused by Calcitonin Gene-Related Peptide at a Dose of 1.6×10^{-11} mol/kg Given Intraarterially

	No. of observations	Basal LESP[a] (mmHg)	Final LESP[a] (mmHg)	Fall[a] (mmHg)	% Fall	
Control	4	46 ± 9	8 ± 4	37 ± 6	84 ± 5	
Indomethacin (10 mg/kg)	4	53 ± 13	9 ± 3	45 ± 2	85 ± 3	NS
Control	4	48 ± 7	14 ± 3	33 ± 5	74 ± 3	
Atropine (30μg/kg) + hexamethonium	4	44 ± 5	9 ± 2	34 ± 4	81 ± 3	NS
Control	4	41 ± 5	7 ± 2	34 ± 4	84 ± 5	
Propranolol (1 mg/kg)	4	47 ± 10	4 ± 1	42 ± 9	91 ± 3	NS
Control	4	68 ± 15	21 ± 6	47 ± 9	71 ± 3	
Haloperidol (2 mg/kg)	4	79 ± 14	25 ± 8	52 ± 11	68 ± 10	NS
Control	4	40 ± 1	4 ± 2	36 ± 3	90 ± 7	
Bulbocapnine (5 mg/kg)	4	28 ± 1	3 ± 2	24 ± 1	90 ± 7	NS
Control	6	46 ± 9	8 ± 4	37 ± 6	84 ± 5	
5-Methoxy-N,N-dimethltryptamine (200 μg/kg)	6	80 ± 17	14 ± 5	66 ± 13	85 ± 3	NS
Control	4	51 ± 12	12 ± 4	39 ± 8	78 ± 3	
Pyrilamine (5 mg/kg) + Metiamide (2 mg/kg)	4	50 ± 5	6 ± 2	44 ± 5	87 ± 4	NS
Control	4	51 ± 11	12 ± 4	39 ± 7	78 ± 3	
Naloxone (4 mg/kg)	4	53 ± 2	5 ± 1	48 ± 5	91 ± 10	NS

LESP, lower esophageal sphincter pressure. [a] The values represent mean ± SE. NS, not significantly different from control ($p > 0.05$).

domethacin pretreatment failed to modify the effect of CGRP in causing a fall in sphincter pressure (Table 2). The percent fall in sphincter pressure in control experiments was 84.0% ± 5.0%; after indomethacin pretreatment, it was 85.0% ± 3.0%. These values were not significantly different from controls (Table 2; $p > 0.05$; n = 4 in 4 animals).

Influence of hexamethonium and atropine. The combination of hexamethonium and atropine did not significantly modify the fall in sphincter pressure caused by CGRP. The fall in sphincter pressure before and after the administration of the combination was 74.0% ± 3.0% and 81.0% ± 3.0%, respectively (Table 2; $p > 0.05$; n = 4 in 4 animals).

Influence of adrenergic, serotonergic, histaminergic, and opioid antagonists. β-Adrenergic receptor activation caused a fall in sphincter pressure. However, propranolol administered in doses that blocked the inhibitory effect of isoproterenol (β-adrenergic agonist) did not modify the inhibitory response of CGRP on the LES. Dopamine receptor antagonists, haloperidol, and bulbocapnine also failed to significantly alter the fall in sphincter pressure caused by CGRP (Table 2). Table 2 also shows that 5-hydroxytryptamine, histamine, and opioid antagonists failed to modify the inhibitory effect of CGRP on the LES.

Influence of Calcitonin Gene-Related Peptide on Blood Pressure

Intravenously administered CGRP in the doses of 6.4×10^{-11}, 2.5×10^{-10}, and 1.0×10^{-9} mol/kg caused no significant effect on the systolic and diastolic blood pressure. A control reading of blood pressure taken 1 min before CGRP administration was compared with one taken 1 min after CGRP administration. Blood pressure was 95 ± 18/65 ± 15 mmHg (systolic/diasystolic) before CGRP and 87 ± 15/61 ± 12 mmHg after CGRP (1.0×10^{-9} mol/kg) ($p > 0.05$; n = 5 in 5 animals). However, a significant fall in blood pressure was observed with the supramaximal dose of CGRP, 4.0×10^{-9} mol/kg (109 ± 17/79 ± 14 mmHg vs. 54 ± 5/33 ± 4 mmHg; $p < 0.05$ for both systolic and diastolic; n = 5 in 5 animals).

When CGRP was administered intraarterially up to the dose of 6.4×10^{-11} mol/kg, which was the supramaximal dose in causing a fall in LESP, it had no significant effect on the blood pressure. The blood pressure with this dose was 104 ± 9/79 ± 5 mmHg, compared with the control value of 102 ± 8/77 ± 4 mmHg ($p > 0.05$; n = 5 in 5 animals).

Studies on the Esophageal Body

The effect of CGRP on the esophageal body was examined with intravenous administration of the peptide. Intraluminal pressures were recorded from the esophageal body at four sites: 13, 9, 5, and 1 cm above the LES.

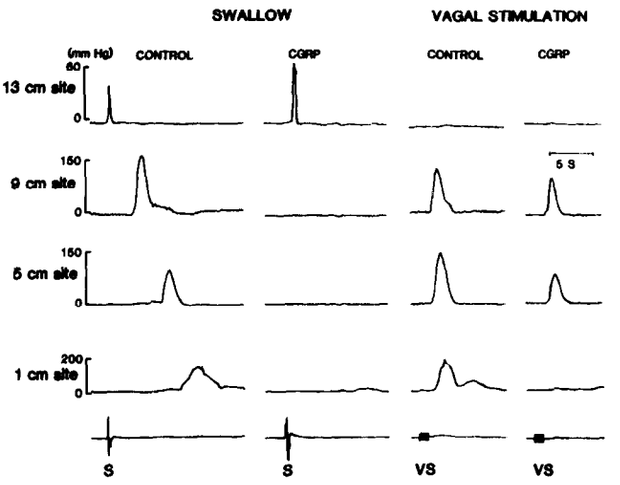

Figure 5. Typical examples showing the influence of calcitonin gene-related peptide (CGRP; 1.0×10^{-9} mol/kg; i.v.) on the esophageal contractions produced by swallowing and vagal stimulation. The sites refer to centimeters above the lower esophageal sphincter. The site 13 cm above the lower esophageal sphincter represents the response of the striated portion of the esophageal body. Note that swallowing produced peristaltic contractions of all regions of the esophagus, whereas vagal stimulation caused contractions only in the lower portions of the esophagus. Calcitonin gene-related peptide caused obliteration of swallow-induced esophageal contractions in different regions of the esophagus, but not in the striated portion of the esophagus. Vagal-stimulated esophageal contractions at the 9- and 5-cm sites were significantly suppressed but not obliterated by CGRP.

Effect on swallow-induced peristalsis. Swallowing induced by superior laryngeal nerve stimulation caused peristaltic contraction in the esophageal body and relaxation of the LES. Calcitonin gene-related peptide administration inhibited the resting sphincter pressure and swallow-induced peristaltic contraction in the lower (smooth muscle) portion, but the upper (striated muscle) portion of the esophagus was resistant to the inhibitory effect of CGRP (Figure 5; Table 3).

Table 3 summarizes the information on the effect of two doses of CGRP on esophageal peristaltic contractions. Note that the smaller dose of CGRP markedly antagonized, and the larger doses almost completely abolished, the swallow-induced contractions in the smooth muscle portion. However, the contractions in the striated muscle portion were not affected by CGRP. In the higher dose CGRP mildly antagonized the esophageal contraction in the striated portion of the esophagus, but it abolished the contractions in the lower portion of the esophagus.

Table 3. Influence of Calcitonin Gene-Related Peptide on Esophageal Contractions Induced by Swallowing and Vagal Efferent Stimulation

		CGRP (mol/kg)	
	Control	1.0×10^{-9}	4.0×10^{-9}
Swallowing			
13-cm site[a]	47.2 ± 3.3[b]	56.1 ± 4.2	37.3 ± 33[c]
9-cm site	71.7 ± 10.2	15.8 ± 4.9[c]	7.9 ± 2.5[c]
5-cm site	67.4 ± 6.5	7.5 ± 2.7[c]	0[c]
1-cm site	61.2 ± 7.2	0.8 ± 0.6[c]	0[c]
Vagal Stimulation			
13-cm site	0	0	0
9-cm site	75.3 ± 8.3	41.2 ± 5.7[c]	4.9 ± 1.8[c]
5-cm site	82.0 ± 8.0	40.9 ± 4.7[c]	16.2 ± 5.2[c]
1-cm site	83.6 ± 13.2	26.6 ± 4.2[c]	0[c]

[a] The sites refer to centimeters above the lower esophageal sphincter. [b] The values represent mean amplitude of esophageal contractions (mmHg) ± SE for 21 observations in 3 animals (seven observations each). [c] The values are significantly different from those of controls ($p < 0.05$).

Effect on esophageal contractions due to vagal stimulation. Vagal efferent stimulation causes peristaltic contractions in the esophageal body and relaxation of the LES. Calcitonin gene-related peptide antagonized vagal-stimulated esophageal contractions. Interestingly, however, CGRP was less effective in antagonizing the vagal-stimulated esophageal contractions (Table 3), compared with esophageal contractions mediated by swallowing. The lower dose of CGRP, 1.0×10^{-9} mol/kg, was more discriminatory in this regard than the higher dose (Figure 5, Table 3). Moreover, the inhibitory effect of CGRP was more marked in the lower segments of the esophagus than in the upper smooth muscle segments. The percent inhibition of esophageal contraction with CGRP in response to vagal stimulation at 9, 5, and 1 cm above the LES was 45%, 50%, and 68%, respectively, as compared with the swallow-mediated inhibition of contractions at these levels of 79%, 86%, and 98%. These differences at different levels between vagal stimulation and swallowing responses after administration of CGRP were significant ($p < 0.05$; $n = 21$ in 7 animals in each case).

Localization of Calcitonin Gene-Related Peptide

Abundant immunoreactive fibers were found throughout the myenteric plexus lying between circular and longitudinal muscle layers. Calcitonin gene-related peptide-containing fibers were detected in the skeletal and smooth muscle portion of the

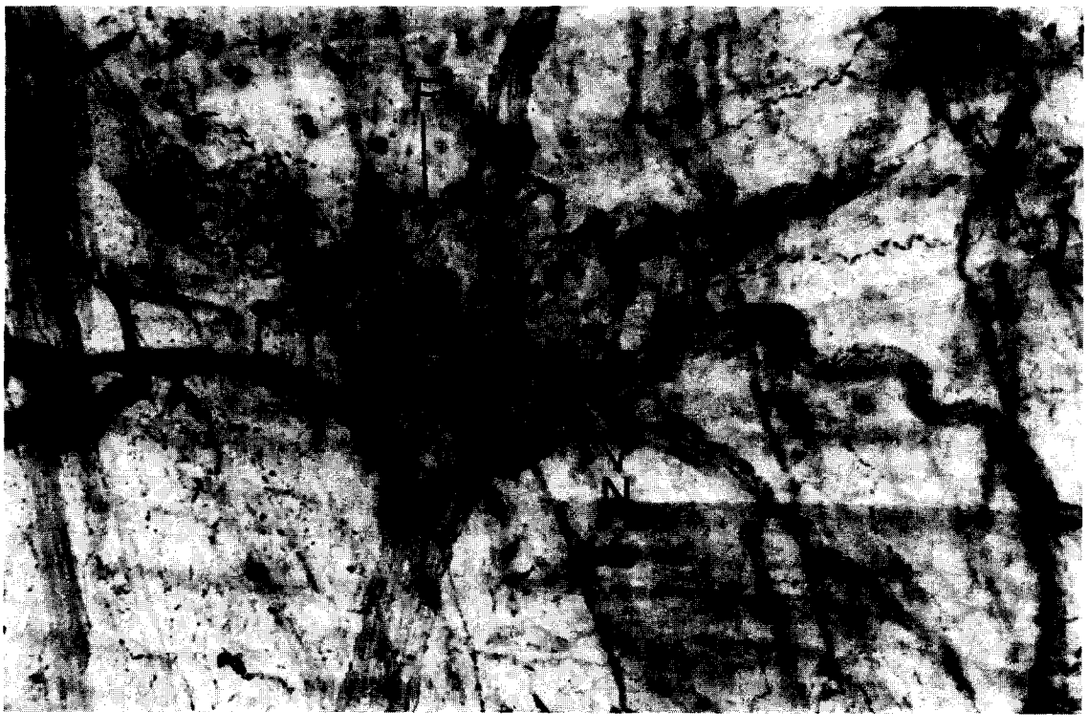

Figure 6. Calcitonin gene-related peptide-containing nerve fibers (*F*) surround an intramural ganglion of the opossum esophagus. Calcitonin gene-related peptide-immunoreactive neurons (*N*) are present within the ganglion. Nonimmunoreactive neurons remain unstained (*arrow*). Calcitonin gene-related peptide and immunoreactive fibers were distributed in both circular and longitudinal muscle layers. (Peroxidase-antiperoxidase method, ×375.)

esophagus, including the LES. Both smooth and varicose fibers were seen in the nerve bundles that connect the myenteric ganglia. A network of varicose CGRP-positive fibers surrounded the nerve cell bodies of some intramural ganglia (Figure 6). Both immunoreactive and nonimmunoreactive neurons were detected. Some myenteric ganglia were devoid of CGRP-containing neurons. Stained varicosities were found along smooth muscle and longitudinal muscle bundles.

Discussion

There is limited information on the biological effects of CGRP. However, this peptide has been shown to exert a positive inotropic and chronotropic effect on the heart in vivo (15,16) and in vitro (17,18). It also causes hypotension secondary to generalized vasodilation when injected intracerebroventricularly, due to central stimulation of sympathetic pathways (15). Calcitonin gene-related peptide is also an inhibitor of acid secretion (19–21) and a stimulant of pancreatic secretion (8,22,23). However, the mechanisms of actions of CGRP are not fully understood. Our studies show that CGRP causes dose-dependent relaxation of the opossum LES and inhibition of peristaltic contraction of the esophagus. Calcitonin has been reported to abolish pentagastrin-induced contraction of the LES (24,25), prevent gallbladder contraction evoked by cholecystokinin or a test meal (26), and delay gastric emptying (27). The present studies show that calcitonin also exerts an inhibitory effect on the LES; however, CGRP is ~3000 times more potent than calcitonin.

The inhibitory effect of CGRP on the LES is mediated by neural and nonneural mechanisms. The neurally mediated effect is observed in smaller doses than those that produce nonneural inhibition of the sphincter tone. This is evidenced by the fact that after TTX treatment larger doses of the peptide were needed to cause relaxation of the sphincter.

The neural inhibitory effect of CGRP on the LES was not due to central stimulation of the vagal pathways, as the animals in these experiments had prior bilateral vagotomies. According to previous studies (15,16), CGRP is also known to cause a fall in blood pressure. The hypotensive effect of CGRP may not be responsible for the fall in LESP, as the latter was observed in doses that caused no significant effect on the blood pressure. It is most likely that the neurally mediated effect of CGRP was due to stimulation of noncholinergic, nonadrenergic inhibitory neurons in the sphincter (28). It has been shown that CGRP can act to stimulate adrenergic nerves by a central action (15). The participation of adrenergic nerves in the inhibitory action of CGRP on LES is unlikely, as intraarterial CGRP (in the doses used here) may not reach the central nervous system via circulation in amounts sufficient to produce its effect by a central activation of adrenergic nerves. The possibility can be further excluded as adrenergic nerve stimulation causes contraction and not inhibition of the sphincter (29). Moreover, the inhibitory effect of CGRP was not antagonized by propranolol, phentolamine, or haloperidol. Histamine or 5-hydroxytryptamine receptor antagonism also failed to modify the effect of CGRP. The ineffectiveness of hexamethonium and atropine in blocking the action of CGRP suggests that CGRP is not acting by stimulating a cholinergic preganglionic nerve or an interneuron. These results are consistent with the view that CGRP causes inhibition of the LES by stimulating noncholinergic, nonadrenergic inhibitory neurons (28).

It has been shown that certain TTX-resistant actions in the LES may be mediated by local release of autacoids such as prostaglandins (14). A suggestion has been made that some effects of CGRP may be mediated by prostaglandin E (19). However, the inhibitory effect of CGRP on the LES was not modified by the prostaglandin synthesis inhibitor indomethacin. These studies support the view that CGRP may also act directly on the sphincter smooth muscle.

The possibility that CGRP may act to stimulate intramural inhibitory neurons is interesting in view of the fact that CGRP has been localized in cells in the dorsal motor nucleus of the vagus and presumably in preganglionic vagal fibers that synapse around the intramural ganglia (6,28). It is not known if CGRP plays any role in the vagal or intramural inhibitory pathway.

Calcitonin gene-related peptide is also localized in the sensory (afferent) fibers (6,30). Intramural sensory CGRP-containing fibers may impinge on intramural inhibitory neurons, and thus CGRP may play a role in the modulation of descending inhibition. This is suggested by our data when one examines the influence of CGRP on esophageal peristalsis as follows: Calcitonin gene-related peptide exerted marked inhibitory effects on swallow-induced peristaltic contractions of the esophagus in its smooth muscle portion. The site of action of intravenous CGRP in causing inhibition of esophageal contractions is not known. It appears, however, that CGRP exerts its effects by central as well as peripheral mechanisms. Calcitonin gene-related peptide could cause inhibition of vagal nuclei in the central nervous system that mediate swallow-induced peristalsis. Inhibitory CGRP receptors have been described in several regions of the central nervous system (30–32). The fact that peristaltic contractions in the

striated muscle portion of the esophagus were not affected by CGRP argues against the possibility of a generalized inhibitory effect of CGRP on the swallowing center. It is possible, however, that CGRP may exert an inhibitory effect selectively on the dorsal motor nucleus of the vagus, which innervates the smooth muscle portion of the esophagus.

Calcitonin gene-related peptide also inhibited esophageal peristalsis caused by vagal efferent stimulation, suggesting that at least a large portion of CGRP inhibition of swallow-induced peristaltic contraction was due to inhibition at the peripheral neuromuscular sites. This peripheral inhibitory effect of CGRP may be due to inhibition of intramural neurons that participate in peristaltic contractions or to direct inhibition of the esophageal muscle. Further studies are needed to distinguish between different possibilities. A central action of CGRP was suggested, as CGRP was found to be more effective in inhibiting swallow-mediated peristalsis than vagal efferent stimulation-mediated peristalsis. The experiments dealing with the swallowing reflex were performed with intact vagi and those dealing with vagal efferent stimulation were performed after bilateral cervical vagotomy.

In conclusion, the present study shows that CGRP exerts a potent inhibitory effect on the LES and the esophageal body smooth muscle. Calcitonin gene-related peptide is ~400 times more potent than vasoactive intestinal polypeptide (33) in inhibiting the LES. The potent inhibitory effect of CGRP at the neuromuscular site, along with the observation that CGRP-immunoreactive nerves innervate the esophageal circular muscle, suggests that CGRP may also be a potential inhibitory neurotransmitter candidate.

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Received March 20, 1987. Accepted August 18, 1987.

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This work was supported by grants DK 31092, DK 35385 and Electrophysiology Core No. 49915 from the National Institutes of Health. The statistical analysis was supported by grant RR 01032 from the General Clinical Research Center Program of the Division of Research Resources, National Institutes of Health.

The authors thank Dr. Jeffrey Crist and Dr. Ashok Sengupta for helpful suggestions, Dr. Bernard Ransil for assistance in statistical analysis, John Kaczmarek for technical assistance, and Peggy Prahl for secretarial assistance.