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Intravenous Calcitonin Gene-Related Peptide Stimulates Net Water Secretion in Rat Colon *in Vivo*

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We have studied the effect of exogenous calcitonin gene-related peptide on net fluxes of water and electrolytes in the rat small and large intestine. In ligated intestinal loops, intravenous calcitonin gene-related peptide (CGRP) induced colonic fluid secretion but had no effect on the small intestine. Subsequently, using a single-pass perfusion technique, we observed an immediate dose-dependent secretion of water by the rat colon upon intravenous administration of CGRP. Net secretion of sodium, potassium, and chloride were also raised. The implications of these observations for the possible involvement of high circulation concentrations of CGRP in the watery diarrhea syndrome accompanying medullary thyroid carcinoma are discussed.

KEY WORDS: CGRP; colonic secretion; perfusion.

Immunoreactive calcitonin gene-related peptide (CGRP) is present in nerves throughout the rat gastrointestinal tract (1–5). CGRP exerts several potent actions on gastrointestinal function including stimulation of mesenteric blood flow (6), inhibition of gastric acid secretion (7, 8), and stimulation of gastrointestinal somatostatin release (9, 10). The peptide also causes relaxation of rat intestinal smooth muscle (11). These observations suggest that CGRP may act physiologically as a neurotransmitter within the gut.

Elevated circulating concentrations of CGRP have been reported in patients with medullary thyroid carcinoma (12–15). Since such patients often experience severe watery diarrhea syndrome due to impaired absorption of fluid in the intestine, we have investigated the ability of CGRP to influence water secretion in the rat to see whether high circulating levels of CGRP could be a contributing cause of watery diarrhea syndrome in medullary thyroid carcinoma.

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MATERIALS AND METHODS

Intestinal fluid and electrolyte movement was determined in male Wistar rats, weighing 200–250 g, using two standard techniques: a closed loop and a single-pass open-perfusion technique.

All animals were housed under temperature- and light-regulated conditions. Body temperature during the experiments was maintained by using heating pads.

Closed Loops. The model used was that described by Mitchenere et al (16). Rats were starved for 24 hr but with free access to water. They were anesthetised with fentanyl citrate 315 mg/kg and fluanisone 10/kg intramuscularly (Hypnorm, Janssen Pharmaceutical) and diazepam 5 mg/kg intraperitoneally (Valium, Roche Products).

The jugular vein was cannulated and isotonic saline infused by means of a syringe ram pump (Harvard Apparatus, Millis, Massachusetts) at 0.041 ml/min. The abdo-

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men was opened by a midline incision and closed loops constructed, one in the duodenum distal to and excluding the bile duct, three each in the jejunum and ileum, and one in the first part of the colon just distal to the cecum. Each loop was injected with 0.3 ml of a modified Krebs solution (305 ± mosm/kg) containing (in millimoles per liter): sodium chloride 118.5; potassium chloride 4.7; calcium chloride 2.5; potassium dihydrogen phosphate 1.2; magnesium sulfate 1.2; sodium bicarbonate 25.0.

The abdomen was closed and pure synthetic rat CGRP (Peninsula Laboratories), dissolved in normal saline containing 1% bovine serum albumin (BSA) in the study group, or vehicle alone in the control group was infused for 45 min. CGRP was infused in two doses, 50 pmol/kg/min ($N = 4$) and 500 pmol/kg/min ($N = 4$), at a flow rate of 0.041 ml/min. After 45 min the animal was sacrificed and the loops of intestine carefully removed and weighed before and after being opened, dried, and blotted. The differences in weight indicated fluid accumulation (net secretion) or loss (net absorption) in the segments, expressed as milliliters per gram of blotted tissue weight, assuming that 1 ml of fluid weighs 1 g.

Perfusion Studies. In the rat colon, water electrolyte movement was determined using a single-pass perfusion technique (17). Rats that had been maintained on a standard low-residue diet were starved for 24 hr prior to the study with free access to water. Under anesthesia with sodium pentobarbital, 60 mg/kg intraperitoneally, a jugular vein was cannulated, and a constant saline infusion (0.041 ml/min) was begun as in the closed loop studies. The colon was cannulated at the cecocolonic junction and rinsed with the same modified Krebs solution described above, which in these experiments contained in addition 2.5 g/liter labeled polyethylene glycol 4000 (PEG) and 5.0 μCi [^{14}C] PEG (Amersham International plc, Amersham, U.K.). A collection catheter was placed in the rectum. The colon was perfused with the Krebs solution at a constant temperature (37°C) and rate (0.5 ml/min). After an equilibration period of 30 min, colonic effluent was collected for 15 consecutive 10-min periods in preweighed test tubes to facilitate estimation of [^{14}C] PEG recovery.

During the first hour (pre-CGRP) and third hour (post-CGRP), the jugular vein was infused with normal saline containing 1% BSA. During the second hour, CGRP dissolved in saline containing 1% BSA was infused at either 50 pmol/kg/min, 250 pmol/kg/min, or 1000 pmol/kg/min. In a separate series of control experiments, a group of seven rats were infused normal saline containing 1% BSA instead of CGRP throughout the second hour as well.

At the completion of the perfusion, a sample of blood from the heart was collected in heparinized tubes containing 400 KIU aprotinin (Trasylol, Bayer) and the plasma was separated and stored at −20° C until assay. The animals were killed and the perfused segments of colon removed. The colon was carefully cut open, blotted, weighed, and its length measured under stretch with a standard weight.

The concentrations of [^{14}C] PEG and electrolytes in each 10-min effluent collection were determined. [^{14}C] PEG was measured in an LKB 1210 Ultrobeta liquid scintillation counter, sodium and potassium concentrations with a Corning 439 flame photometer, and chloride concentration using an EE1 921 chloridometer.

Net water electrolyte fluxes were calculated using standard formulas (18) and expressed as microliters per centimeter per hour for water and micromoles per centimeter per liter for electrolytes. Net absorption from the lumen was expressed as a positive value, net secretion into the lumen as a negative value. Mean control values were obtained for each animal by averaging the results of the three collection periods before CGRP infusion.

cAMP Determination. Colonic cAMP levels were determined in a separate series of single-pass perfusion experiments. These were identical to the perfusion studies described except that the colonic tissue was obtained by freeze-clamping at the end of the 1 hr of CGRP (50 pmol/kg/min) infusion. These were compared with colonic tissue similarly obtained at the end of the second hour in control animals infused with the BSA–saline vehicle alone throughout the 2-hr perfusion period. cAMP levels were determined using the protein binding assay (19).

Radioimmunoassay. Ten-microliter and one 100-μl aliquots of plasma were assayed for CGRP in duplicate in 0.8 ml phosphate buffer, 0.06 M, pH 7.4, containing 0.01 mol/liter ethylenediaminetetraacetate, 0.05% (w/v) sodium azide, and 3% (w/v) BSA. The assay used an antiserum, previously raised in rabbits immunized with synthetic rCGRP glutaraldehyde-conjungated to BSA, at a final dilution of 1:400,000. The antiserum showed no cross-reactivity (less than 0.2%) with calcitonin, somatostatin-28, substance P, VIP, or gastrin-releasing peptide. Radioiodination of the histidine residue in synthetic rCGRP (Peninsula Laboratories) was carried out using the chloramine-T method (20). [^{125}I]CGRP was purified by reverse-phase high-performance liquid chromatography on a μBondapak C-18 column (Waters Associates).

Following five-day incubation at 4° C, bound and free fractions of the peptide were separated by charcoal adsorption of the free fraction using 8 mg [Norit GSX suspended in 250 μl phosphate buffer containing 0.25% (w/v)] gelatine added to each assay tube. The tubes were then centrifugated at 4° C and the supernatant immediately separated from the charcoal pellet. CGRP concentrations were measured against a synthetic rCGRP standard (Peninsula Laboratories), and the assay could detect changes of 1 fmol with 95% confidence.

Statistics. Data were expressed as the mean ± SEM and analyzed using the Student's t test.

RESULTS

Closed Loops. In this preliminary experiment, intravenous CGRP in doses of 50 and 500 pmol/kg/min reversed the normal pattern of fluid absorption in the colon to that of secretion, but this did not achieve statistical significance (control +0.20 ± 0.12, 50 pmol/kg/min −0.12 ± 0.10; 500 pmol/kg/min −0.11 ± 0.10 ml/g respectively). There was no effect on net fluid movement in the small bowel.

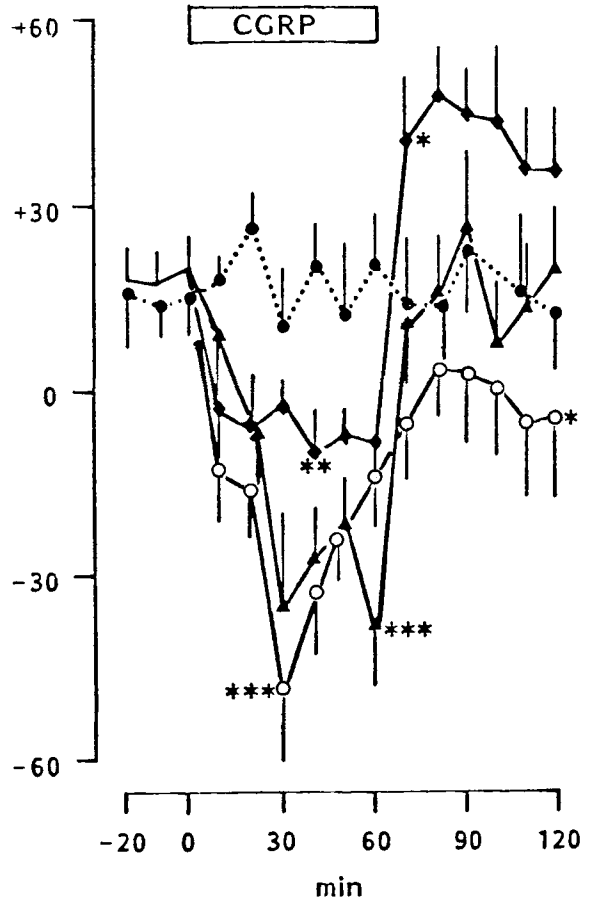

Fig 1. Effect of intravenous rCGRP on net water flux across the rat colon *in vivo* as determined by a single-pass perfusion technique. The colon was perfused continuously for 190 min. After a 70-min equilibration period of colonic perfusion during which normal saline was infused intravenously, rCGRP was infused for 1 hr followed by 1 hr of normal saline (see Materials and Methods). Basal values were derived from colonic effluent collected during the last three 10-min intervals of the equilibration period. As there was no significant difference between mean basal values for the three experimental groups (intravenous CGRP 50, ◆—◆; 250, ▲—▲; and 1000, pmol/kg/min ○—○, these were pooled to give the mean control values during this period. Results are expressed as the mean + SEM. There was a dramatic, dose-dependent increase in net water secretion into the colon in all three groups during the rCGRP infusion. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ compared to mean control value. In a separate group of rats infused with 1% BSA saline instead of CGRP, net water flux remained unchanged throughout the 190 min of perfusion (●—●).

Perfusion Experiments. Results of the closed loop study led to experiments using a single-pass perfusion technique for evaluating water and electrolyte movement in the colon. In this group of experiments, intravenous CGRP at infusion rates of 50, 250, and 1000 pmol/kg/min produced a dramatic, dose-dependent secretion of water into the perfused rat colon (Figure 1). Water flux values during the last half hour of equilibration were similar in all three groups and were used to derive a mean control value (+18.67 ± 2.81 μl/cm/hr; positive values indicate net water absorption). A maximum secretory response was observed between 30 and 60 min and was (mean ± SEM) −9.56 ± 7.4; −39.2 ± 9.3 and −49.37 ± 11.2 μl/cm/hr in the three groups, respectively ($P < 0.01$ vs control period). The secretory effect of CGRP was immediate in all three groups and immediately reversed on discontinuation of CGRP in those animals infused 50 and 250 pmol/kg/min.

TABLE 1. PLASMA CGRP LEVELS*

CGRP Infusion Dose (pmol/kg/min)		
50 (N = 6)	*250 (N = 7)*	*1000 (N = 7)*
76.36 ± 5.28	371.43 ± 24.2	1113.5 ± 45.7

*Values are picomoles per liter (mean+SEM) at 190 min in three groups of rats infused with CGRP.

In the 50 pmol/kg/min group, the net secretion rapidly converted to net absorption showing a rebound phenomenon with values significantly greater than those during the control period ($P < 0.05$). With the 1000 pmol/kg/min dose, the inhibitory effect on water absorption was sustained at statistically significant levels for the greater part of the last hour of saline infusion.

In the separate series of experiments, a group of seven rats were infused with 1% BSA-saline instead of CGRP. No change in water flux across the colon was observed throughout the 190-min period in this group (Figure 1).

Plasma levels of CGRP in blood taken at the end of the experiment are shown in Table 1. The plasma levels in the 1000 pmol/kg/min group remained elevated, and this probably explains the continued effect on net water flux even after CGRP infusion was discontinued (Figure 1).

Secretions of sodium, potassium, and chloride were also raised to some degree during CGRP infusion, but no clear dose–response relationship could be established (Figure 2).

Recovery of [^{14}C]PEG during these experiments was 101.61 ± 2.4%. Recovery of CGRP from the infusates was 148 ± 6.1%.

cAMP. In order to determine whether the observed secretory response to CGRP is mediated via cAMP, colonic cAMP levels were measured. There was no significant difference in tissue cAMP levels in rats perfused CGRP 50 pmol/kg/min for 1 hr as

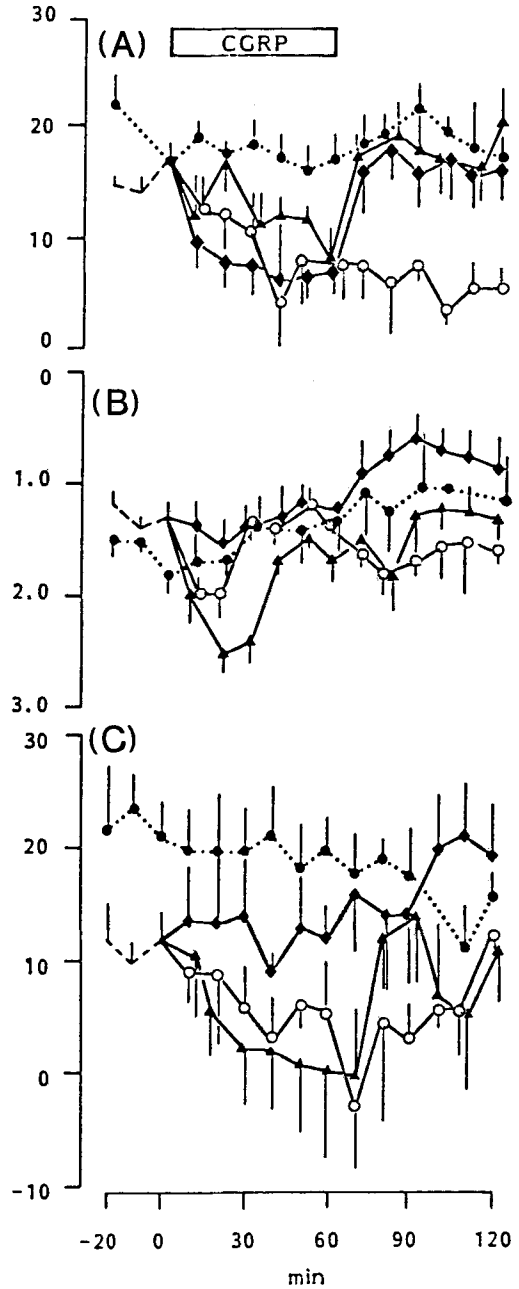

Fig 2. New sodium (A), potassium (B), and chloride (C) fluxes across the rat colon *in vivo* in response to intravenous rCGRP infusion. Experimental conditions are the same as described for Figure 1. Results are expressed as mean ± SEM. The control values represent the mean values for rats in the three experimental groups (intravenous CGRP 50, ◆—◆; 250, ▲—▲; and 1000 pmol/kg/min, ○—○ during the control period of intravenous saline before CGRP infusion ●- - -●. Net sodium flux (A) parallels net water flux (Figure 1). Potassium secretion occurred during both saline and rCGRP infusion (B). Impaired chloride absorption was observed with CGRP 250 and 1000 pmol/kg/min but not with the 50 pmol/kg/min infusion (C).

compared to 1% BSA saline controls, the values being 221 ± 34 and 207 ± 23 pmol/g wet weight.

DISCUSSION

Our investigation has demonstrated that exogenous synthetic rat CGRP stimulates net water secretion in the rat colon *in vivo*. The mechanism by which CGRP influences the movement of water and electrolytes across the colonic mucosa remains unknown. It also remains to be established how this phenomenon relates to the previously described stimulatory effects of salmon calcitonin on water and electrolyte secretion in the small bowel (22, 23).

Recent studies have shown that CGRP-like immunoreactivity present within the rat gut is associated with both extrinsic sensory nerve fibers and intrinsic enteric neuron (4, 5) and, furthermore, that CGRP-like immunoreactivity associated with the enteric nervous system is actually a peptide closely homologous to CGRP and encoded by a different gene (5). Our findings suggest that physiologically CGRP or a similar peptide may act as an intestinal secretagogue following stimulation of intramural sensory or enteric nerves. It is not known whether high circulating concentrations of CGRP in man will result in stimulation of colonic secretion, but the gastric and vascular effects of the peptide appear to be common to several mammalian species (7–10, 21). It is therefore feasible that high circulating concentrations of CGRP could contribute to the watery diarrhea syndrome associated with medullary thyroid carcinoma.

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