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Adrenomedullin, amylin, calcitonin gene-related peptide and their fragments are potent inhibitors of gastric acid secretion in rats

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Abstract

Adrenomedullin, amylin and calcitonin gene-related peptides (CGRP) share close sequence homology and have overlapping spectra of biological activities, particularly with respect to cardiovascular and gastrointestinal functions. Comparisons of the effects of these three peptides on gastric acid release have been made by i.v. infusions in conscious rats equipped with gastric fistulae. All peptides were extremely potent inhibitors of basal, pentagastrin- and 2-deoxy-D-glucose-stimulated gastric acid secretion with IC_{50} values in the subnanomolar to nanomolar range. These effects were not inhibited by C-terminal extra-cyclic fragments of the peptides which often act as competitive receptor antagonists in other biological systems. At high concentrations C-terminal fragments of human adrenomedullin and rat α-calcitonin gene-related peptide displayed some receptor agonist activity. Furthermore, the N-terminally situated disulfide-bridged ring fragments, human adrenomedullin-(15–22), rat amylin-(1–8) and rat α-calcitonin gene-related peptide-(1–8), retained significant gastric acid inhibitory potencies thus suggesting involvement of receptor(s) with significantly differing ligand binding profiles than those characterized previously. © 1997 Elsevier Science B.V.

Keywords: Adrenomedullin; Amylin; α-Calcitonin gene-related peptide; (*N*-terminal cyclic octa-peptides); (C-terminal fragments); Gastric acid; (Chronic fistulae equipped rat); (Rat); (Human)

1. Introduction

Adrenomedullin is a new member of the calcitonin gene-related (CGRP) peptide family and possesses some sequence homology with both calcitonin gene-related peptide and amylin. Common structural characteristics also include six amino-acid-containing N-terminal rings with Cys^2–Cys^7 disulfide bonds and a C-terminal amide (Poyner, 1995). The full human adrenomedullin sequence, however, contains an additional 15 amino acids on the N-terminal side of Cys^{16}, although thus far full biological activity and potency appears to reside in the (16–52) C-terminal fragment (Heaton et al., 1995; Santiago et al., 1995) which is over 20% homologous with calcitonin gene-related peptide and amylin and much easier to synthesize than the full 1–52 sequence. The three peptides also demonstrate physiological and pharmacological similarities, including depressor effects on the vasculature (Sakata et al., 1993). Calcitonin gene-related peptide and amylin were found to inhibit basal and insulin-stimulated glucose uptake (Molina et al., 1990) and to inhibit glycogen synthesis (Leighon and Cooper, 1988). Both peptides were potent inhibitors of gastric acid secretion in different species (Taché et al., 1984; Helton et al., 1989; Guidobono et al., 1994; Kato et al., 1995) and adrenomedullin- and amylin-containing cells were found in the gastric mucosa (Ferrier et al., 1989; Miyazato et al., 1991; Mulder et al., 1996; Kaneko et al., 1996). Calcitonin gene-related peptide was present in the afferent, capsaicin-sensitive nerve fibers innervating the rat gastric mucosa (Sternini et al., 1987). Adrenomedullin receptors have been cloned and the receptor gene was found to be widely expressed in different tissues including: lung, adrenals, ovary, heart (Kapas et al., 1995) and duodenum (Sakata et al., 1993). In the target cells, adrenomedullin acts through specific, plasma membrane bound receptors to activate adenylate cyclase activity and modulate intracellular Ca^{2+} flux (Shimekake et al., 1995). These signal transduction pathways are known to be involved in the regulation of many physiological processes, including hormone secretion in the gastric mucosa (Hirschowitz et al., 1995). Recently, adrenomedullin has been reported to influence the secretion of several hor-

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mones including pituitary adrenocorticotropin (Samson et al., 1995), aldosterone (Yamaguchi et al., 1995) and insulin (Martinez et al., 1996). A calcitonin gene-related peptide receptor has also been cloned (Aiyar et al., 1996) with similarities in tissue expression to adrenomedullin. This receptor had high affinity for the calcitonin gene-related peptide antagonist, human calcitonin gene-related peptide-(8–37), and appeared to be the postulated type 1 receptor. A proposed α-calcitonin gene-related peptide type 2 receptor which apparently only binds with high affinity to full sequence calcitonin gene-related peptides (Muff et al., 1995) remains to be identified. It appears likely that amylin and adrenomedullin also possess multiple, as yet uncharacterized, receptors. This, together with probable overlapping receptor affinities and biological properties, make it necessary to rationalize the complex pharmacology of these peptides by examining them and their analogs side-by-side in as many biological systems as possible. In the present study we have investigated the potential role of human adrenomedullin and some of its fragments in regulating basal and stimulated gastric acid secretion in conscious rats in comparison to the effect of rat α-calcitonin gene-related peptide (r α-CGRP) and rat amylin and several of their fragments. We have used infusion protocols to minimize pharmacokinetic differences between peptides of greatly differing sequence lengths. Special attention was given to the functional roles of the N-terminal ring sequences present in human adrenomedullin, rat α-calcitonin gene-related peptide and rat amylin, and the N-terminal extra-cyclic fragments.

2. Materials and methods

2.1. Peptides

Human adrenomedullin-(1–52); human adrenomedullin-(15–52); human adrenomedullin-(15–22); human adrenomedullin-(22–52); human adrenomedullin-(40–52) fragments; rat amylin-(1–37), rat amylin-(1–8) and rat amylin-(8–37) fragments; rat α-calcitonin gene-related peptide-(1–37); rat α-calcitonin gene-related peptide-(1–8) and rat α-calcitonin gene-related peptide-(8–37) fragments were synthesized by standard solid-phase methodologies and purified by reverse-phase high-pressure liquid chromatography. They were characterized by amino acid analysis and molecular weight determination using matrix-assisted laser desorption mass spectrometry (Finnegan). Rat adrenomedullin-(1–50) was purchased from Bachem (Torrance, CA, USA). Peptides, depending upon their biological activity, were studied in doses ranging from 0.01 to 1500 nmol/kg per h and were intravenously (i.v.) infused in sterile 0.9% saline solution (Sodium Chloride Injection USP, Baxter Healthcare, Deerfield, IL, USA) containing 0.1% bovine serum albumin (BSA, Fraction V, Sigma, St. Louis, MO, USA).

2.2. Animals and experimental procedures

About 300 adult male Charles River CD rats weighing 250–350 g were used for all experiments. They were housed under standard conditions and kept in an artificial 12 h light cycle while receiving standard Purina rat chow between experiments. Tap water was given ad libitum. The rats were trained in Bollman cages (Plas Labs., Lansing, MI, USA) for 1 week prior to surgery, when they were equipped with chronic gastric fistulas and jugular venous cannulae after an intraperitoneal (i.p.) injection of pentobarbital (50 mg/kg; Nembutal Sodium Solution, Abbott Labs, North Chicago, IL, USA), using completely sterile surgical procedures (Rossowski et al., 1994). After surgery, rats were allowed to recover for 7 days and experiments were then performed twice a week. Rats were deprived of food but not water for 18 h prior to an experiment. They were placed in Bollman cages and the plastic screw caps were removed from the gastric fistulae. Extension tubes were then attached to the fistulae and the stomach was cleansed with warm saline. At this time i.v. infusion of sterile 0.9% sodium chloride was started at constant infusion rate 1 ml/h. After the initial 30 min collection had been discarded, three 30 min basal samples of gastric juice were collected. All gastric juice samples were collected by gravity drainage and all experiments were started at 8.30 a.m. and were completed at 4.00 p.m. To study effects of human adrenomedullin, rat α-calcitonin gene-related peptide and rat amylin and their fragments on resting (basal) gastric acid secretion, after 3 basal collections i.v. infusion of peptide was started at constant infusion rate (1 ml/h) and was continued for three consecutive collection periods followed by i.v. infusion of saline. Samples of gastric juice were collected every 30 min, the volume and pH measured. Samples were titrated with 0.01 M NaOH to pH 7.0 using an autotitrator (Radiometer, Copenhagen, Denmark). Results were expressed in μmol H^+ and presented as 30 min outputs.

To study the possibility that the C-terminal fragments human adrenomedullin-(22–52), rat amylin-(8–37) and rat α-calcitonin gene-related peptide-(8–37) might antagonize the effect of full sequence peptides, after 3 basal collection periods the fragments were i.v. injected at a dose of 500 nmol/kg as an i.v. bolus and after 10 min the full sequence peptide (10 nmol/kg per h) with a second dose of truncated peptide (500 nmol/kg per h) administered as an i.v. continuous infusion for 90 min.

The effects of peptides on mucosal-stimulated gastric acid secretion by pentagastrin (Peptavlon, Ayerst Laboratories, New York, NY, USA) (24 μg/kg/ml per h in sterile 0.9% sodium chloride solution containing 0.1% bovine serum albumin) were examined during continuous i.v. infusion for the following nine collection periods. After pentagastrin-stimulated gastric acid secretion reached a plateau (3 collection periods), peptides were added into the infusion solution for the next three collection periods.

Control rats were infused with 0.9% saline solution containing 0.1% bovine serum albumin. Gastric juice samples were collected every 30 min and processed as described above. In some experiments pentagastrin was replaced by bombesin (0.3 mg/kg per h) or histamine (1 mg/kg per h) and experiments were processed as described for pentagastrin. With respect to testing human adrenomedullin-(22–52), rat amylin-(8–37) and rat α-calcitonin gene-related peptide-(8–37) as possible receptor antagonists, after pentagastrin stimulation of gastric acid had reached a plateau, the individual fragment (500 nmol/kg) in 0.1% bovine serum albumin-saline was injected into the jugular vein as a bolus and, after 10 min, the full sequence peptide (10 nmol/kg per h) with a second dose of truncated peptide (500 nmol/kg per h) was administered as i.v. continuous infusion for the following 90 min. Control rats were identically treated with solution without peptide/s. Gastric acid secretion was monitored as described above.

Peptides effects on central vagal stimulation of gastric acid secretion were studied in chronic gastric fistulae equipped rats and three 30 min basal samples of gastric juice were collected followed by single subcutaneous (s.c.) injection of 2-deoxy-D-glucose (2-DG, Grade III, Sigma) (200 mg/kg). Two 30 min 2-deoxy-D-glucose-stimulated samples of gastric juice were collected and peptides in doses ranging from 0.01 to 250 nmol/kg in 0.9% saline solution containing 0.1% bovine serum albumin were s.c. injected in total volumes of 200–300 μl. Control rats were s.c. injected with sterile 0.9% saline solution containing 0.1% bovine serum albumin. Gastric juice samples were collected and processed as described above. All experiments on living animals were approved by the Advisory Committee for Animal Resources, Tulane University School of Medicine.

2.3. Statistical analysis

Results of the effects of rat and human adrenomedullin, rat amylin, rat α-calcitonin gene-related peptide and its fragments, on gastric acid secretion were analysed using one-way analysis of variance (ANOVA), with Bonferroni multiple comparisons tests, if applicable (InStat Biostatistics, GraphPad Software). Student's paired *t*-test was used to compare means between two groups. Results are presented as means ($n = 6–12$) ± S.E. A probability level of random difference $P < 0.05$ was considered significant. IC_{50} values were calculated by means of non-linear regression analysis of the concentration response curves using the GraphPad computer program.

3. Results

3.1. Effect of human adrenomedullin-(1–52), rat adrenomedullin-(1–50), rat amylin and rat α-calcitonin gene-related peptide on basal gastric acid secretion

Basal gastric acid output measured during three subsequent collection periods was calculated to be between 45–85 μmol of H^+/30 min with small fluctuations during the observation period. Continuous i.v. infusion of human adrenomedullin-(1–52), human adrenomedullin-(15–52) and rat adrenomedullin-(1–50) administered at a dose of 1 nmol/kg per h significantly inhibited basal gastric acid secretion (*P*-values = 0.002, 0.01 and 0.0001, respectively) and at 10 nmol/kg per h completely inhibited gastric acid secretion throughout the entire infusion period (Fig. 1A). There was no statistically significant difference between the inhibitory activities of human and rat adrenomedullin and human adrenomedullin-(15–52) when tested at doses of 0.3, 1 and 10 nmol/kg per h (*P*-values = 0.3, 0.2 and 0.6, respectively) (Fig. 1A). The IC_{50} values calculated from dose–response curves were in the range of 0.95–1.25 nmol/kg (Table 1). The N-terminal ring fragment, human adrenomedullin-(15–22), was significantly less active, decreasing resting gastric acid secretion maximally by 60% ($P = 0.0006$) at a concentration of 10 nmol/kg per h and at higher doses was even less effective (Fig. 1A). The C-terminal fragment human adrenomedullin-(22–52) at 100 and 500 nmol/kg per h doses inhibited basal gastric acid secretion by about 30 and 60% ($P = 0.03$ and 0.0001, respectively) and by about 90% at 1500 nmol/kg per h dose (Fig. 1A). The human adrenomedullin fragment-(40–52) significantly inhibited basal gastric acid secretion ($P = 0.01$) only at the highest, studied dose of 1500 nmol/kg per h (Fig. 1A).

Inhibition of basal gastric acid secretion by i.v. continuous infusion of human adrenomedullin-(15–52) was dose- and time-dependent and was completely reversed after cessation of i.v. peptide infusion (Fig. 2A).

Rat amylin-(1–37) administered i.v. significantly inhibited resting gastric acid secretion at a dose of 0.01 nmol/kg per h ($P = 0.03$) and maximally at a dose of 3 nmol/kg per h ($P < 0.0001$) (Fig. 1B) with an IC_{50} value of 0.21 nmol/kg (Table 1). The N-terminal amylin ring fragment rat amylin-(1–8) was about 100 fold less active and maximally inhibited basal gastric acid secretion by approximately 60% ($P = 0.0007$) (Fig. 1B). The C-terminal fragment rat amylin-(8–37) when tested at doses of 10, 100 and 500 nmol/kg per h did not significantly alter basal gastric acid secretion ($P = 0.06$) (Fig. 1B). Rat amylin-induced inhibition of gastric acid secretion was dose- and time-dependent and returned to the basal values approximately 90 min after completion of i.v. peptide infusion (Fig. 2B).

Intravenous continuous infusion of rat α-calcitonin gene-related peptide-(1–37) administered at doses ranging from 0.1 to 10 nmol/kg per h strongly inhibited basal gastric acid secretion (Fig. 1C). At a concentration 0.1 nmol/kg per h inhibition was approximately 30% and was statistically significant ($P = 0.005$) and full inhibition was achieved at 10 nmol/kg per h ($P < 0.0001$) with an IC_{50} value of 0.24 nmol/kg (Table 1). The N-terminal fragment, rat α-calcitonin gene-related peptide-(1–8), retained roughly 100–1000 times less biological activity in inhibiting basal gastric acid secretion with inhibition reaching

30% at concentration of 50 nmol/kg per h ($P = 0.02$) (Fig. 1C). The C-terminal fragment rat α-calcitonin gene-related peptide-(8–37), displayed weak agonist activity in inhibiting basal gastric acid secretion at doses 500 and 1500 nmol/kg per h ($P = 0.01$ and 0.005, respectively). Similarly to human adrenomedullin and rat amylin, rat α-calcitonin gene-related peptide-(1–37)-induced inhibition of basal gastric acid secretion was dose- and time-dependent and returned to the basal values after i.v. peptide infusion was completed (Fig. 2C).

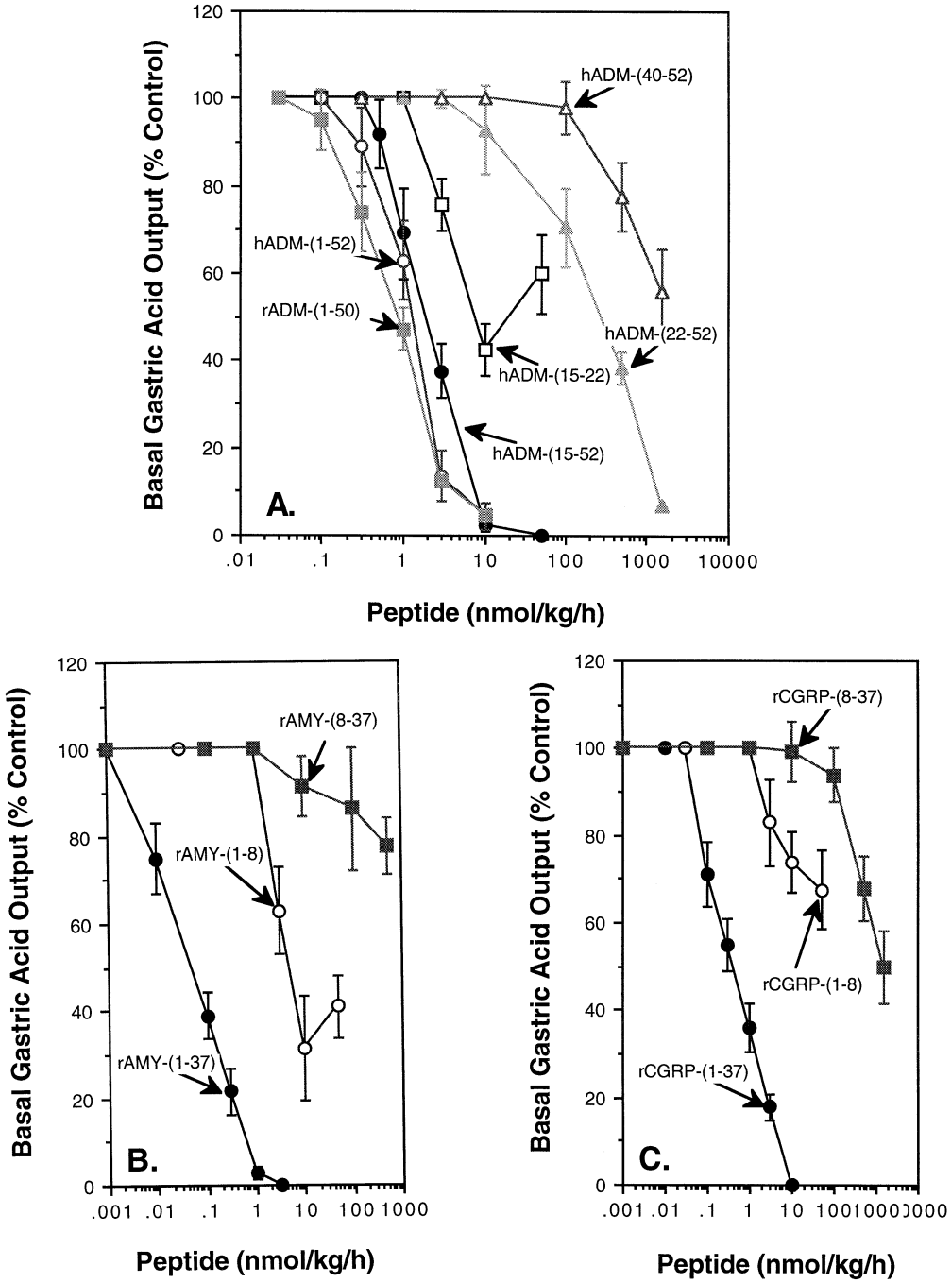

Fig. 1. Dose-dependent inhibition of basal gastric acid secretion by continuous i.v. infusion of: (A) human adrenomedullin-(1–52) (○-), rat adrenomedullin-(1–50) (-■-), human adrenomedullin-(15–52) (-●-), N-terminal fragment human adrenomedullin-(15–22) (-□-) and C-terminal fragments human adrenomedullin-(22–52) (-▲-) and human adrenomedullin-(40–52) (-△-); (B) rat amylin-(1–37) (-●-) and rat amylin-(1–8) (-○-) and rat amylin-(8–37) (-■-); (C) rat α-calcitonin gene-related peptide-(1–37) (-●-) and rat α-calcitonin gene-related peptide-(1–8) (-○-) and rat α-calcitonin gene-related peptide-(8–37) (-■-). After 3 basal gastric acid collection periods (30 min each), peptides at concentrations ranging from 0.03 to 1500 nmol/kg per h depending from biological activity were intravenously infused at 1 ml/h for 3 successive collection periods. Gastric acid volume and pH were measured and expressed as gastric acid output/30 min. Peptide-induced gastric acid inhibition was calculated as percent of control. Each point is the mean of 6–10 individual experiments ± S.E.

Table 1
Ability of human and rat adrenomedullin, rat amylin and rat α-calcitonin gene-related peptide to inhibit basal and stimulated gastric acid secretion in conscious rats

Peptide	Inhibition of gastric acid secretion (IC_{50} nmol/kg)		
	Basal	Pentagastrin-stimulated PG	2-Deoxy-D-glucose stimulated 2DG
hADM-(1–52)	1.25 ± 0.37	1.67 ± 0.10	6.84 ± 0.91
rADM-(1–50)	0.95 ± 0.40	1.85 ± 0.26	N.D.
hADM-(15–52)	1.95 ± 0.62	2.22 ± 0.26	6.57 ± 0.80
hADM-(22–52)	251.3 ± 49.1	N.D.	N.D.
hADM-(40–52)	> 1000	N.D.	N.D.
rAMY-(1–37)	0.048 ± 0.003	0.088 ± 0.023	0.36 ± 0.06
r α-CGRP-(1–37)	0.24 ± 0.04	1.14 ± 0.38	10.80 ± 1.70
r α-CGRP-(8–37)	> 1000	> 1000	N.D.

Data are means ± S.E.
N.D. = not determined.

3.2. Effect of human and rat adrenomedullin, rat amylin and rat α-calcitonin gene-related peptide on stimulated gastric acid secretion

The continuous i.v. infusion of pentagastrin (24 μg/kg per h) after 3 basal collection periods resulted in about a 5-fold increase in gastric acid secretion (Fig. 3A) which reached a plateau after ninety minutes and remained elevated for about 3 h (Fig. 3A). After the stimulatory effect of pentagastrin had reached a plateau, continuous i.v. infusion of human adrenomedullin-(15–52) at doses of 1, 3 and 10 nmol/kg per h resulted in dose-and time-dependent inhibition of pentagastrin-stimulated-gastric acid secretion ($P = 0.05$, 0.001 and 0.001, respectively) (Fig. 3A). Adrenomedullin-induced inhibition of pentagastrin-stimulated gastric acid secretion was dose-, and time-dependent and reversible upon completion of i.v. peptide infusion (Fig. 3A). There was no statistically significant difference between the inhibitory activities of human adrenomedullin-(1–52), human adrenomedullin-(15–52) and rat adrenomedullin-(1–50) at all tested doses ($P > 0.05$), with the only exception for human adrenomedullin-(1–52) and human adrenomedullin-(15–52) fragment at the 3 nmol/kg per h dose ($P < 0.05$), (Fig. 4A). The IC_{50} values of human adrenomedullin-(1–52) and -(15–52) and rat adrenomedullin-(1–50) were very similar in the range of 1.67–2.22 nmol/kg (Table 1). The N-terminal ring fragment, human adrenomedullin-(15–22), was approximately 10 times less effective with the maximal inhibition of about 50% at 10 nmol/kg per h ($P = 0.001$) and lower inhibition at higher concentrations (U shape curve profile) (Fig. 4A). The C-terminal fragments human adrenomedullin-(22–52) and -(40–52) were not significantly active in inhibiting pentagastrin-stimulated gastric acid secretion when tested at doses 10, 100, 500 and 1500 nmol/kg per h (Fig. 4A). Human adrenomedullin-(15–52) i.v. infused at a dose of 10 nmol/kg per h also strongly inhibited bombesin- and histamine-stimulated gastric acid secretion (Table 2).

Rat amylin-(1–37) administered as a continuous intra-

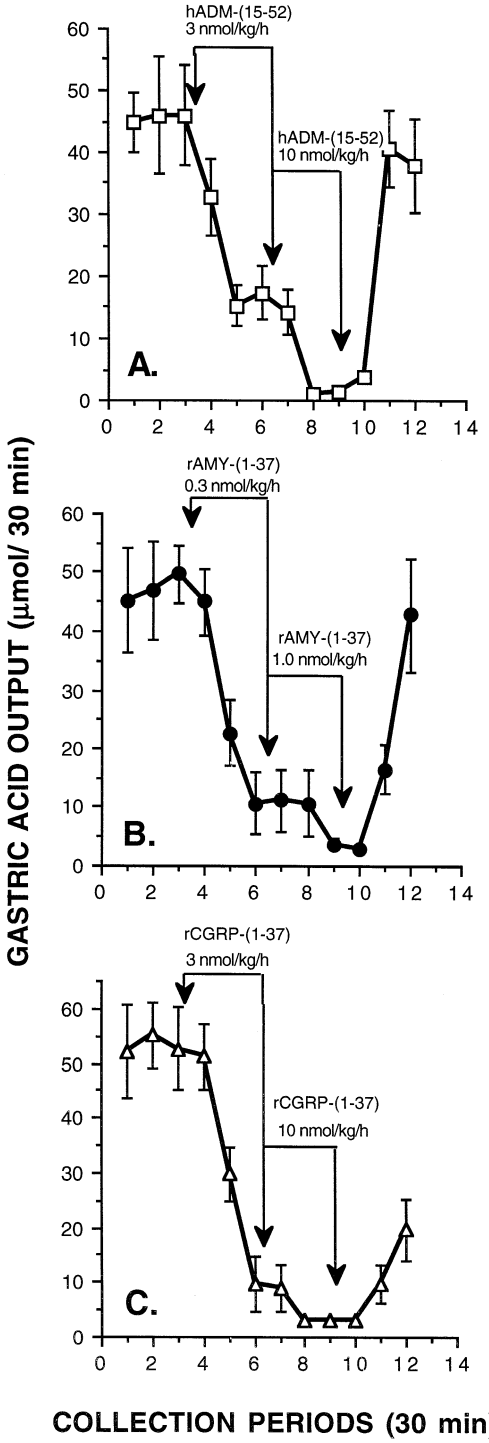

Fig. 2. Dose- and time-dependent inhibition of basal gastric acid secretion by continuous i.v. infusion of: (A) human adrenomedullin-(15–52) at doses of 3 and 10 nmol/kg per h (-□-) ($P < 0.05$ and < 0.01, respectively); (B) rat amylin-(1–37) at doses of 0.3 and 1 nmol/kg per h (-●-) ($P < 0.001$) and (C) rat α-calcitonin gene-related peptide-(1–37) at dose of 3 and 10 nmol/kg per h ($P < 0.001$) (-△-); in conscious, gastric fistulae equipped rats. Each point is the mean of 8–10 separate experiments ± S.E.

venous infusion at doses of 0.3, 1 and 3 nmol/kg per h potently inhibited pentagastrin-stimulated gastric acid secretion ($P < 0.001$), (Fig. 3B and Fig. 4B) with an IC_{50} value less than 0.1 nmol/kg (Table 1). Rat amylin-induced inhibition of pentagastrin-stimulated gastric acid secretion was dose- and time-dependent and returned to the stimulated level of secretion 90 min after peptide infusion was completed (Fig. 3B). The N-terminal ring fragment, rat amylin-(1–8), was less active with the maximal inhibition reaching approximately 50% at dose of 30 nmol/kg per h (Fig. 4B). Pentagastrin-stimulated gastric acid secretion was not affected by continuous i. v. infusion of rat amylin-(8–37) at doses of 10, 100 or 500 nmol/kg per h (Fig. 4 B).

Rat α-calcitonin gene-related peptide administered as a continuous i.v. infusion into conscious, chronic gastric fistulae equipped rats potently inhibited pentagastrin-stimulated gastric acid secretion at doses of 0.5, 3 and 10 nmol/kg per h ($P < 0.001$), (Fig. 3C, Fig. 4C and Table 1). At the maximal tested dose level of 10 nmol/kg per h, rat α-calcitonin gene-related peptide (like rat and human adrenomedulin and rat amylin) inhibited pentagastrin-stimulated gastric acid secretion below the basal secretion level. Rat α-calcitonin gene-related peptide-induced inhibition of pentagastrin-stimulated gastric acid secretion was dose- and time-dependent and completely reversible after completion of i.v. peptide infusion (Fig. 3C). The N-termi-

Table 2
Effect of human adrenomedullin-(15–52) on pentagastrin-, bombesin- and histamine-stimulated gastric acid secretion

Treatment	Gastric acid output	
	μmol H^+/30 min	(%)
Pentagastrin	261.6 ± 16.6	100
Pentagastrin + hADM-(15–52)	20.9 ± 10.9	8.0 ± 4.2
Bombesin	195.3 ± 16.2	100
Bombesin + hADM-(15–52)	3.0 ± 1.0	1.5 ± 0.5
Histamine	137.3 ± 15.4	100
Histamine + hADM-(15–52)	35.8 ± 6.8	26.1 ± 4.9

After three basal gastric acid collections, pentagastrin (24 μg/kg per h), bombesin (0.3 mg/kg per h), or histamine (1 mg/kg per h) in sterile 0.9% sodium chloride containing 0.1% BSA was continuously infused into the jugular vein and, after stimulation have reached plateau, human adrenomedullin-(15–52) (10 nmol/kg per h) was continuously i.v. infused for the next three collection periods. Data are means ± S.E. ($n = 6$–8).

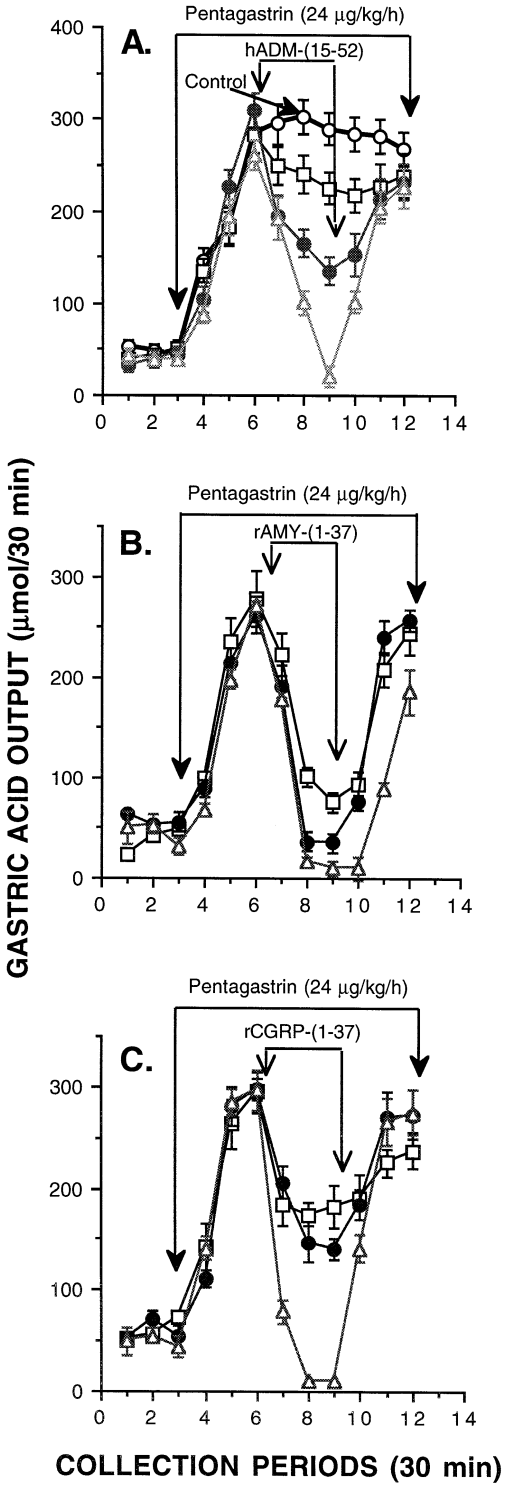

Fig. 3. Dose- and time-dependent inhibition of pentagastrin-stimulated gastric acid secretion by human adrenomedullin, rat amylin and rat α-calcitonin gene-related peptide. After baseline collections, pentagastrin at the concentration of 24 μg/kg per h was i.v. infused and continued until the end of the experiment. 90 min after pentagastrin infusion was started, i.v. infusion of human adrenomedullin-(15–52), rat amylin-(1–37), or rat α-calcitonin gene-related peptide at a different concentrations was added and continued for another 90 min. (A) human adrenomedullin-(15–52) at concentrations of 1 nmol/kg per h (-□-), 3 nmol/kg per h (-●-) and 10 nmol/kg per h (-△-) significantly inhibited pentagastrin-stimulated gastric acid secretion ($P < 0.05$, 0.001 and 0.001, respectively); (B) rat amylin-(1–37) at dose of 0.3 nmol/kg per h -□-), 1 nmol/kg per h (-●-) and 3 nmol/kg per h (-△-) potently inhibited pentagastrin-stimulated gastric acid secretion ($P < 0.001$ for all three doses); (C) rat α-calcitonin gene-related peptide-(1–37) at dose of 0.5 nmol/kg per h (-□-), 3 nmol/kg per h (-●-) and 10 nmol/kg per h (-△-)significantly inhibited pentagastrin-stimulated gastric acid secretion ($P < 0.001$ for all three doses). Inhibition of gastric acid secretion was reversible, and time- and concentration-dependent. Values are the means of 8–10 experiments ± S.E.

nal ring fragment of rat α-calcitonin gene-related peptide-(1–8) still retained some gastric acid inhibitory activity with inhibition approaching 50% at concentration of 50 nmol/kg per h ($P = 0.0005$) (Fig. 4C). Pentagastrin-stimulated gastric acid secretion was not modified by continuous i.v. infusion of rat α-calcitonin gene-related peptide-(8–37) at doses of 10 and 100 nmol/kg per h, but was slightly decreased when rat α-calcitonin gene-related peptide-(8–37) was i.v. infused at dose of 500 nmol/kg per h ($P = 0.01$) (Fig. 4C).

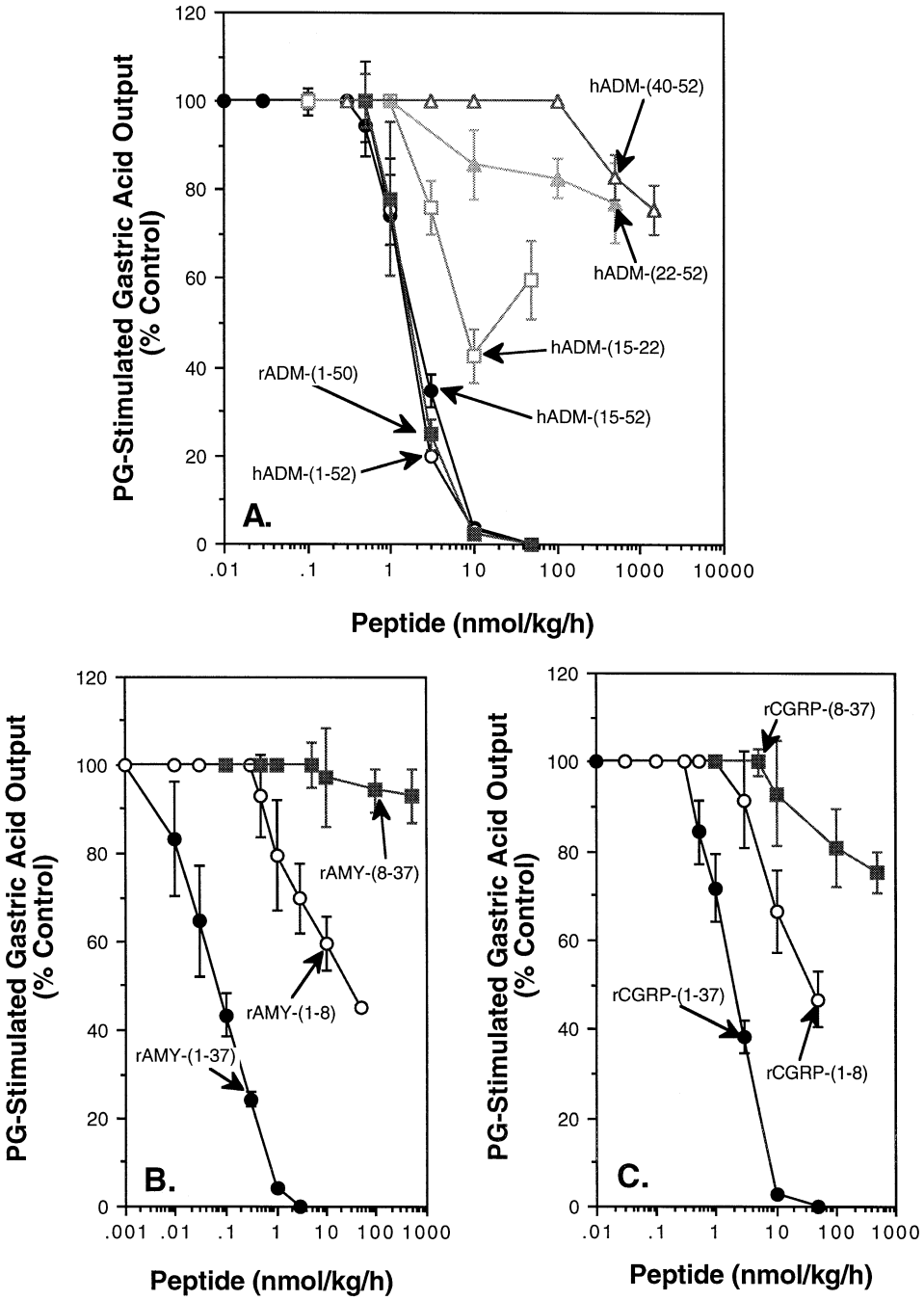

Fig. 4. Dose-dependent inhibition of pentagastrin-stimulated gastric acid secretion by continuous i.v. infusion of: (A) human adrenomedullin-(1–52) (-○-), rat adrenomedullin-(1–50) (-■-), human adrenomedullin-(15–52) (-●-), human adrenomedullin-(15–22) (-□-), human adrenomedullin-(22–52) (-▲-) and human adrenomedullin-(40–52) (-△-); (B) rat amylin-(1–37) (-●-), rat amylin-(1–8) (-○-) and rat amylin-(8–37) (-■-); (C) rat α-calcitonin gene-related peptide-(1–37) (-●-), rat α-calcitonin gene-related peptide-(1–8) (-○-) and rat α-calcitonin gene-related peptide-(8–37) (-■-). After 3 basal gastric acid collection periods, pentagastrin (24 μg/kg per h) was i.v. infused for the whole time of experiment. After the stimulatory effect of pentagastrin had reached a plateau, continuous i.v. infusion of peptide at doses ranging from 0.01 to 1500 nmol/kg per h was started at a constant rate of 1 ml/h and continued for the next 3 collection periods. Peptide-induced gastric acid inhibition was calculated as a percent of control. Results are the means from at least 6–8 separate experiments ± S.E.

Human adrenomedullin-(15–52), rat amylin-(1–37) and rat α-calcitonin gene-related peptide-induced inhibition of pentagastrin-stimulated gastric acid secretion could not be inhibited by pre- and co-infusion of the C-terminal fragments of human adrenomedullin-(22–52), rat amylin-(8–37), and rat α-calcitonin gene-related peptide-(8–37) at a dose 500 nmol/kg per h (Fig. 5A, B and C).

Subcutaneous administration of 2-deoxy-D-glucose produced a significant increase in gastric acid secretion above the basal output value of 57.3 ± 11.7 μmol/30 min to 205 ± 29.1 μmol/30 min after 60 min ($P = 0.0002$) and stayed at a plateau for another 60 min before steadily declining (Fig. 6). For this reason, we chose the second plateau period to compare with the second collection period-after peptide injection. Human adrenomedullin-(1–52), human adrenomedullin-(15–52), rat amylin-(1–37) and rat α-calcitonin gene-related peptide, time- and dose-dependently inhibited 2-deoxy-D-glucose-stimulated gastric acid secretion. At a dose of 10 nmol/kg per h the most active was rat amylin ($P < 0.001$), followed by human adrenomedullin-(15–52) ($P < 0.001$), human adrenomedullin-(1–52) ($P < 0.001$) and rat α-calcitonin gene-related peptide ($P < 0.01$), (Fig. 6). There were no statistically significant differences in inhibitory activity of 2-deoxy-D-glucose-stimulated gastric acid secretion between human adrenomedullin-(1–52) and the human adrenomedullin-(15–52) fragment at doses of 3, 10 and 30 nmol/kg ($P > 0.05$). IC_{50} values for human adrenomedullin-(1–52) and human adrenomedullin-(15–52) were 6.8 and 6.6, respectively (Table 1). The N-terminal cyclic peptide human adrenomedullin-(15–22) retained about 40 and 50% of gastric acid inhibitory activity when tested at doses 10 and 50 nmol/kg, however, at a higher dose (250 nmol/kg) was less active, also displaying a U shape curve (Fig. 7A). Rat amylin-(1–37) (Fig. 7B), was potent inhibitor of 2-deoxy-D-glucose-stimulated gastric acid secretion at concentration of 1 nmol/kg ($P = 0.001$) and rat α-calcitonin gene-related peptide-(1–37) (Fig. 7C)

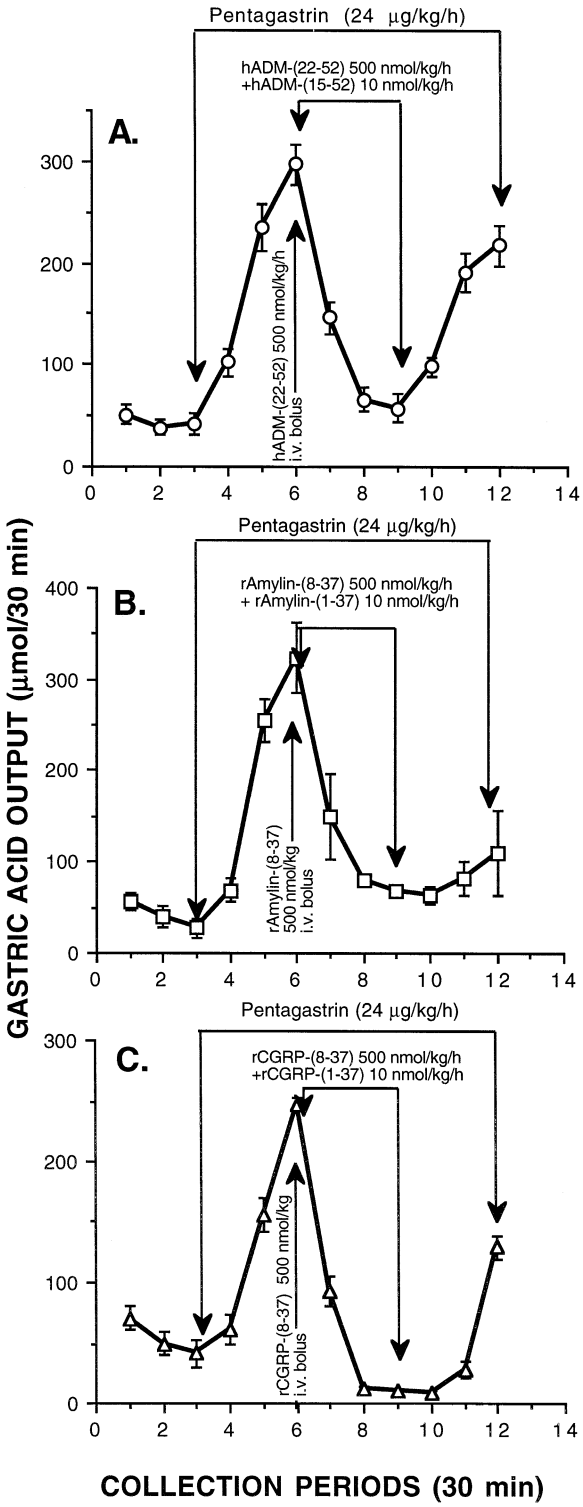

Fig. 5. Effect of the C-terminal fragments of human adrenomedullin-(22–52); rat amylin-(8–37) and rat α-calcitonin gene-related peptide-(8–37) on human adrenomedullin-(15–52), rat amylin-(1–37) and rat α-calcitonin gene-related peptide-(1–37)-induced inhibition of pentragastrin-stimulated gastric acid secretion. (A) After 3 basal collections of gastric juice, pentagastrin (24 μg/kg per h) was i.v. infused for the following 4.5 h. After maximal stimulation was reached (3 collection periods), i.v. bolus injection of human adrenomedullin-(22–52) at dose of 500 nmol/kg was, followed by continuous i.v. infusion of human adrenomedullin-(15–52) (10 nmol/kg per h) + human adrenomedullin-(22–52) (500 nmol/kg per h) for the next 3 collection periods. Gastric acid volume and pH were measured and results expressed as gastric acid output/30 min. (B) After 3 basal collections of gastric acid, pentagastrin (24 μg/kg per h) was i.v. infused for the following 4.5 h. After maximal stimulation was reached, i.v. bolus injection of rat amylin-(8–37) (500 nmol/kg) was administered followed by continuous i.v. infusion of rat amylin-(1–37) (10 nmol/kg per h) + rat amylin-(8–37) (500 nmol/kg per h) for the next 3 collection periods. (C) After 3 basal collections of gastric acid, pentagastrin (24 μg/kg per h) was i.v. infused for the following 4.5 h. After maximal stimulation of gastric acid was reached, bolus i.v. injection of rat α-calcitonin gene-related peptide-(8–37) (500 nmol/kg) was administered followed by continuous i.v. infusion of rat α-calcitonin gene-related peptide-(1–37) (10 nmol/kg per h) + rat α-calcitonin gene-related peptide-(8–37) (500 nmol/kg per h) for the next 3 collection periods. Values are the means of 6–8 experiments ± S.E.

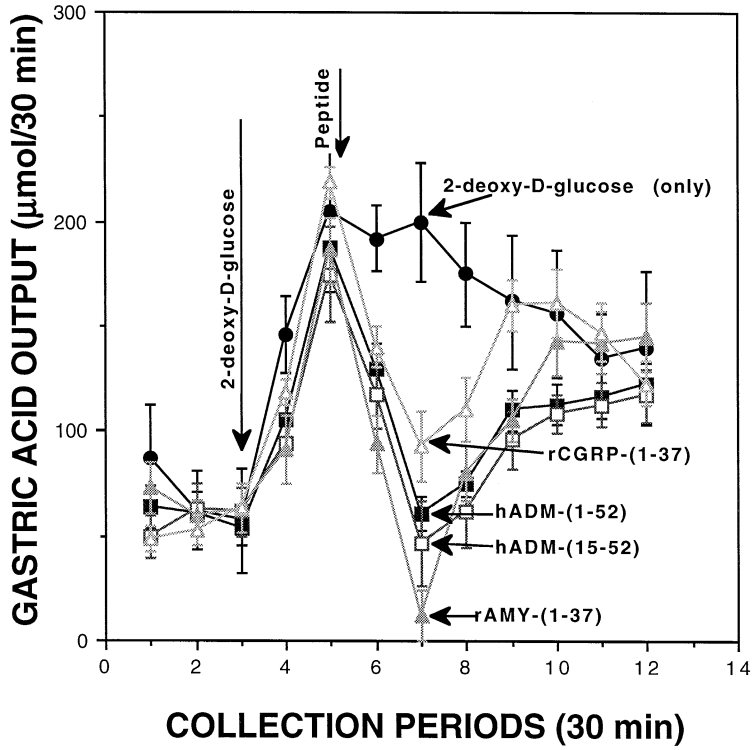

Fig. 6. Time-dependent inhibition of 2-deoxy-D-glucose-stimulated gastric acid secretion by human adrenomedullin-(1–52) (-■-) human adrenomedullin-(15–52) (-□-), rat amylin-(1–37) (-▲-) and rat α-calcitonin gene-related peptide-(1–37) (-△-), at comparative dose of 10 nmol/kg. After three basal collection of gastric juice, 2-deoxy-D-glucose at dose 200 mg/kg was s.c. injected. After maximal stimulation of gastric acid was reached, human adrenomedullin-(1–52), human adrenomedullin-(15–52), rat amylin-(1–37) or rat α-calcitonin gene-related peptide at dose of 10 nmol/kg was s.c. injected and gastric juice samples were collected for the next seven collection periods. Human adrenomedullin-(1–52), human adrenomedullin-(15–52), rat amylin-(1–37) and rat α-calcitonin gene-related peptide-(1–37) at dose of 10 nmol/kg significantly inhibited 2-deoxy-D-glucose-stimulated gastric acid secretion. Values are the means of 10–12 experiments ± S.E.

significantly inhibited 2-deoxy-D-glucose-stimulated gastric acid secretion at concentration of 3 nmol/kg ($P = 0.03$). Rat amylin-(1–8) at a concentration of 3 nmol/kg and rat α-calcitonin gene-related peptide-(1–8) at a concentration of 10 nmol/kg were effective inhibitors of 2-deoxy-D-glucose-stimulated gastric acid secretion ($P = 0.01$ and 0.04, respectively) with the tendency of demonstrating lower activity at higher doses (Fig. 7B and C).

4. Discussion

The major findings in the present study concern the ability of human and rat adrenomedullin to dose and time-dependently inhibit resting and stimulated gastric acid secretion and the indication that this property is related to the presence of the N-terminal disulfide ring structure. These activities of adrenomedullin were compared with the gastric acid inhibitory profiles of rat amylin and rat α-calcitonin gene-related peptide in both resting and stimulated conditions and indeed, human and rat adrenomedullin display similar inhibitory activities on both resting and stimulated gastric acid secretion. Also, we found that truncated human adrenomedullin-(15–52) preserved full gastric acid inhibitory activity. In addition, human adrenomedullin-(15–52) also potently inhibited bombesin-, histamine- and 2-deoxy-D-glucose-stimulated gastric acid secretion. Thus, the peptide can inhibit either unstimulated or stimulated gastric acid secretion by the described gastric mucosal stimulants or central vagal stimulant. These observations are in agreement with previously published data indicating that human adrenomedullin-(13–52) and human adrenomedullin-(15–52) retained full biological activity in the pulmonary vascular bed in rats (Heaton et al., 1995) and in the mesenteric vascular bed of the cat (Santiago et al., 1995). However, further shortening of adrenomedullin with elimination of disulfide ring structure in human adrenomedullin-(22–52) and human adrenomedullin-(40–52) generates fragments which are very weak agonist when tested in resting or pentagastrin-stimulated gastric acid secretion model. Surprisingly, none of the adrenomedullin fragments studied were antagonists in our experimental model. The inhibitory potency of human and rat adrenomedullin on basal and stimulated gastric acid secretion was comparable to that displayed by rat α-calcitonin gene-related peptide, but was significantly less than rat amylin. It should be mentioned that although these quantitative potency comparisons between peptides have not

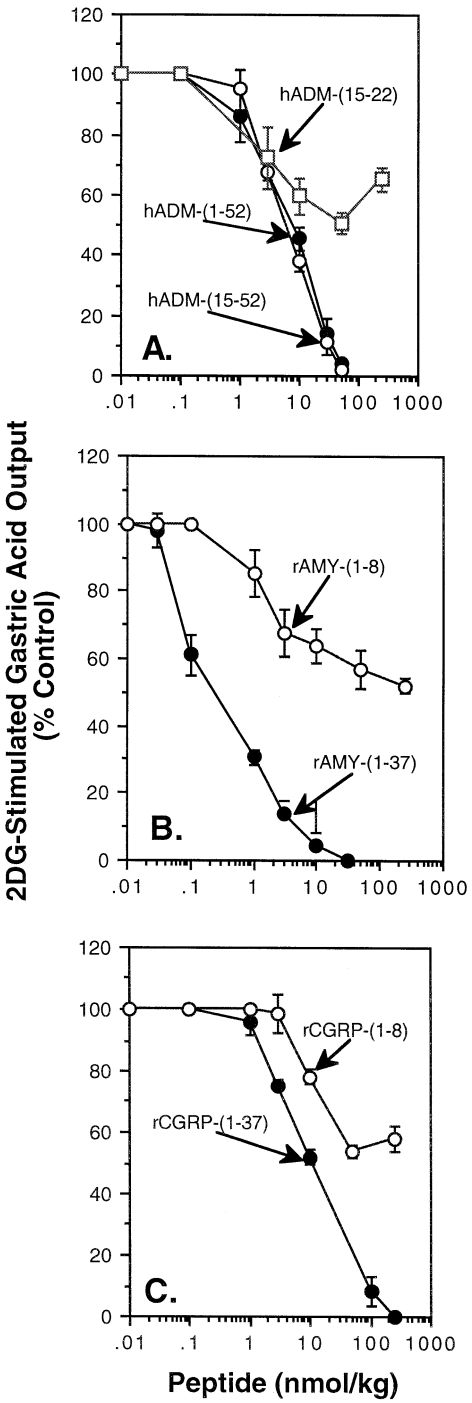

Fig. 7. Dose-dependent inhibition of 2-deoxy-D-glucose-stimulated gastric acid secretion by human adrenomedullin, rat amylin, rat α-calcitonin gene-related peptide and their N-terminal cyclic fragments. After 3 basal collections of gastric juice, 2-deoxy-D-glucose (200 mg/kg) in deionized water was s.c. injected. After maximal stimulation of gastric acid was reached, peptide was administered as a bolus s.c. injection (0.01 to 250 nmol/kg, depending from the peptide biological activity). (A) Inhibition of 2-deoxy-D-glucose-stimulated gastric acid secretion by human adrenomedullin-(1–52) (-●-), human adrenomedullin-(15–52) (-○-) and by human adrenomedullin-(15–22) (-□-). (B) Inhibition of 2-deoxy-D-glucose-stimulated gastric acid secretion by rat amylin-(1–37) (-●-) and rat amylin-(1–8) (-○-). (C) Inhibition of 2-deoxy-D-glucose-stimulated gastric acid secretion by rat α-calcitonin gene-related peptide-(1–37) (-●-) and by rat α-calcitonin gene-related peptide-(1–8) (-○-). Values are the means of 10–12 experiments ± S.E.

previously been described, inhibition of gastric acid secretion has been reported using rat calcitonin gene-related peptide and rat amylin. Thus, calcitonin gene-related peptide was shown to inhibit pentagastrin-stimulated and histamine-stimulated gastric acid secretion in rats or dogs (Taché et al., 1984; Helton et al., 1989) and amylin suppressed basal and insulin-stimulated gastric acid secretion in pylorus-ligated rats (Guidobono et al., 1994). We have also found that the N-terminal eight amino acid ring structures human adrenomedullin-(15–22), rat α-calcitonin gene-related peptide-(1–8) and rat amylin-(1–8), also significantly inhibited gastric acid secretion in conscious rats at about 10–1000-fold higher concentrations than the full sequences. Although we could never achieve full inhibition (maximal inhibition was approximately 60–70%) of basal and stimulated gastric acid secretion, this significant effect demonstrates that the cyclic octa-peptide part of adrenomedullin, amylin, and calcitonin gene-related peptide molecule is an essential component of the active site responsible for gastric acid inhibitory effects. The ring octapeptides appear to function only as partial agonists with the remaining C-terminal sequences being required for full agonist characteristics. These observations might be supported by reports of Tippins et al. (1989) that chemical destruction of the disulfide bridge between Cys^2 and Cys^7 abolished biological activity of calcitonin gene-related peptide, however, the N-terminal human α-calcitonin gene-related peptide enzymic digest fragments calcitonin gene-related peptide-(1–11) and calcitonin gene-related peptide-(1–18) were not biologically active when tested using the rat isolated atrial preparations. In the studies reported by Dennis et al. (1989) N-terminal fragments $cyclo^{2-7}$ human α-calcitonin gene-related peptide-(1–7) and $cyclo^{2-7}$ human α-calcitonin gene-related peptide-(1–8) retained little or no affinity for ^{125}I-labeled human α-calcitonin gene-related peptide binding sites in both rat brain and spleen membrane preparations when tested in μM concentrations, however, when used at similar concentrations, $cyclo^{2-7}$ human α-calcitonin gene-related peptide-(1–7) displayed significant antagonist properties towards the positive chronotropic effect of human α-calcitonin gene-related peptide on isolated guinea pig right atrium. Also the chronotropic and inotropic activities of human α-calcitonin gene-related peptide were altered markedly when the N-terminal disulfide bridge was ruptured (Dennis et al., 1989). The N-terminal fragments calcitonin gene-related peptide-(1–12), -(1–15) and -(1–22) have also been found to be biologically active, causing hypotension and tachycardia in rats (Maggi et al., 1990). An important function for the N-terminal ring structure in regulation of gastric acid secretion fully agrees also with the recently published studies on inhibition of [^{125}I] human α-calcitonin gene-related peptide binding to cultured human neuroblastoma SK-N-MC cells (Zimmermann et al., 1995). These authors found that human calcitonin gene-related peptide-(1–37), human adrenomedullin-(1–52) and -(13–52) were the most potent blockers of [^{125}I]human

α-calcitonin gene-related peptide binding, while human adrenomedullin-(22–52), which lacks the N-terminal ring structure, very slightly inhibited [^{125}I] human α-calcitonin gene-related peptide binding with an $IC_{50} > 1000$ nM. Human adrenomedullin-(26–52) and -(29–52) were completely inactive at the doses tested (Zimmermann et al., 1995). The ring structures of human adrenomedullin and human calcitonin gene-related peptide were also required for stimulation of cAMP production and the full sequence of both peptides was necessary for the expression of full activity (Zimmermann et al., 1995). Finally, the importance of the N-terminal ring structure for human adrenomedullin bioactivity was also recently shown by Watanabe et al. (1996) on the rat cardiovascular system. They found that a ring structure analog, *N*-acetyl-human adrenomedullin-(16–21), had surprisingly strong vasopressor activity in rats (Watanabe et al., 1996).

Various C-terminal fragments of calcitonin gene-related peptide and amylin have been studied extensively in many different biological systems. In displacement studies of radio-iodinated calcitonin gene-related peptide and amylin from the lung membrane receptors, the C-terminal fragments calcitonin gene-related peptide-(8–37) and amylin-(8–37) were significantly less potent than full sequence peptides. C-terminal amylin fragments-(12–37), -(25–37) and -(28–37) could displace both radioligands when present at high concentrations and with affinities which decreased with shortening of the amino acid chain (Bhogal et al., 1993). C-terminal fragments of human α-calcitonin gene-related peptide-(8–37), -(19–37) and -(23–37) could antagonize human α-calcitonin gene-related peptide-mediated positive inotropic effect in guinea pig left atria at micro-molar concentrations (Rovero et al., 1992). Other studies have shown that micro-molar concentrations of the C-terminal fragments calcitonin gene-related peptide-(8–37), amylin-(8–37) and amylin-(10–37) antagonize the amylin-induced inhibition of glycogen accumulation (Deams et al., 1991) and human calcitonin gene-related peptide-(8–37) reversed the inhibitory effects of rat α-calcitonin gene-related peptide-induced inhibition of pentagastrin-stimulated gastric acid secretion in urethane-anesthetized rats, but did not modified basal gastric acid secretion in conscious rats with chronic gastric fistula (Kato et al., 1995). In conscious dogs, human calcitonin gene-related peptide-(8–37) blocked native calcitonin gene-related peptide inhibitory effects on gastric acid secretion (Lawson et al., 1994), however, responses to rat α-calcitonin gene-related peptide in rat descending colon mucosa and in the adenocarcinoma cell line Colony-29 were insensitive to the inhibitory effects of the C-terminal fragment human calcitonin gene-related peptide-(8–37) (Cox, 1995). It was previously suggested that the loss of residues 1–7 from the calcitonin gene-related peptide molecule may result in a loss of its recognition for high affinity calcitonin gene-related peptide receptors, or may induce a conformational change that favors conversion of the receptor into its lower affinity form (Bhogal et al., 1993). In generally accepted terms, it could indicate that in dogs inhibition of gastric acid was mediated through α-calcitonin gene-related peptide-1 (α-CGRP 1) receptor subset, whereas in rats gastrointestinal mucosa responses to calcitonin gene-related peptide were mediated through α-calcitonin gene-related peptide-2 (α-CGRP 2) receptor subset. However, there are a growing numbers of new data indicating that the situation is much more complicated due to the existence of tissue and species specific differences and that even more receptor subtypes might be discovered (Bhogal et al., 1993; Tomlinson and Poyner, 1996; Nandha et al., 1996).

Our present observations demonstrate quite clearly the inability of human adrenomedullin-(22–52) and -(40–52), rat α-calcitonin gene-related peptide-(8–37) and rat amylin-(8–37) to antagonize gastric acid inhibition induced by the parental full sequence peptides in conscious rats and might indicate that the receptors involved in mediating gastric acid inhibition are not of the amylin or calcitonin gene-related peptide 1 (CGRP1) variety but have properties more readily attributable to calcitonin gene-related peptide 2 (CGRP2) or another receptor. In fact, when tested at high doses, human adrenomedullin-(22–52) and -(40–52), rat α-calcitonin gene-related peptide-(8–37) and rat amylin-(8–37) displayed more potent agonist activities when tested on basal gastric acid secretion than in pentagastrin-stimulated gastric acid secretion.

Although the precise structure/conformation features of this group of peptides responsible for receptor binding and activation are far from being fully elucidated, some molecular modeling studies on calcitonin gene-related peptide are of relevance. Hakala and Vihinen (1994), using energy minimization and molecular dynamics computer techniques, have proposed that calcitonin gene-related peptide consists of three principal structural domains: the disulfide bridged (1–8) sequence which adopts a type II β-bend conformation, a long α-helical central region from residues 8–30, also confirmed by protein secondary structure predictive methods (Coy, unpublished observations) and another type II folded motif from residues 30–37 centered around Gly^{28}. Their calculations were supported by the subsequent synthesis of several cyclic, constrained analogs which retained high binding affinities as predicted by their model (Hakala et al., 1994). They also proposed that a principal function of the 2 folded domains was to stabilize the α-helical spacer through side-chain interactions, with the active site of the molecule being present in the N-terminal 2–7 ring. Thus, the active site would be brought into tight contact with the receptor through binding of the α-helical region. Our results appear to support this model up to a point, particularly in relation to the active site assignment. However, the retained biological activity of the ring portions of all 3 peptides indicates that these small sequences alone can retain affinity for some receptor(s) even in the absence of the α-helix. It will be interesting to

see if this new putative receptor(s) can be further characterized, perhaps using simpler isolated organ bioassay techniques which have historically been useful in elucidating multiple receptor types with this family of peptides (Tomlinson and Poyner, 1996).

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