# Synthesis and Biological Activity of C-Terminally Truncated Fragments of Human α-Calcitonin Gene-Related Peptide

D. David Smith,*,† Jianzhong Li,† Qiming Wang,† Richard F. Murphy,† Thomas E. Adrian,† Yvonne Elias,‡ Charles S Bockman,‡ and Peter W. Abel‡

*Department of Biomedical Sciences and Department of Pharmacology, Creighton University School of Medicine, 2500 California Plaza, Omaha, Nebraska, 68178*

*Received July 27, 1992*

C-terminally truncated fragments of human α-calcitonin gene-related peptide (h-α-CGRP) were tested for their ability to stimulate amylase secretion from pancreatic acinar cells and relax precontracted mesenteric arteries. h-α-CGRP, h-α-CGRP (1-36), h-α-CGRP (1-35), and h-α-CGRP (1-34) were made by Merrifield's solid-phase peptide synthesis methodology. Peptides were purified by gel filtration, cation-exchange chromatography, and semipreparative reversed-phase high-performance liquid chromatography. The products were characterized by amino acid analysis, mass spectrometry, and tryptic digestion. h-α-CGRP stimulated amylase secretion from dispersed guinea pig pancreatic acini in a biphasic concentration-dependent manner. The initial increase in amylase secretion reached 8% of total cellular amylase content with an $ED_{50}$ value of 7.7 nM, and the second increase reached 11% of total cellular amylase content at a concentration of h-α-CGRP of $10^{-4}$M. h-α-CGRP (1-36) caused a small, significant increase in amylase release. C-terminally truncated fragments h-α-CGRP (1-35) and h-α-CGRP (1-34) did not increase amylase release at concentrations $<10^{-5}$ M. At concentrations $>10^{-5}$ M the fragments h-α-CGRP (1-35) and h-α-CGRP (1-34) caused a smaller increase in amylase release than that caused by h-α-CGRP whereas h-α-CGRP (1-36) caused the same increase. h-α-CGRP caused a concentration-dependent relaxation of rat mesenteric artery, precontracted with prostaglandin $F_{2\alpha}$, with an $EC_{50}$ of 2.9 nM and a maximal relaxation that was 60% of the prostaglandin $F_{2\alpha}$-induced tone. h-α-CGRP (1-35) also relaxed the mesenteric artery in a concentration-dependent manner with a maximum response that was 40% of the prostaglandin $F_{2\alpha}$-induced tone. The remaining fragments did not relax rat mesenteric arteries. Additionally, h-α-CGRP (1-36) and h-α-CGRP (1-34) did not block h-α-CGRP-induced relaxation of the mesenteric artery. An intact C-terminus is required for h-α-CGRP to cause potent biological effects in pancreatic acini and mesenteric artery. The different effects of h-α-CGRP (1-35) in mesenteric artery compared with those in pancreatic acini suggest that the CGRP receptors in these two tissues may be different.

## Introduction

Calcitonin gene-related peptide (CGRP)[1] is a 37 residue peptide (Figure 1) first discovered as an alternative splicing product of the rat calcitonin gene.[2] Similarly, the strucure of hCGRP was elucidated from the human calcitonin gene[3] and confirmed by its isolation from medullary thyroid carcinoma tissue and subsequent sequence determination.[4] A second gene has been identified in both rat and human[5,6] coding for a similar CGRP sequence. This has been designated the β-form of CGRP while the original sequences are the α-forms of CGRP. Recently, h-β-CGRP was isolated from the spinal cord and sequenced.[7] CGRP is widely distributed in the central nervous system[8,9] and in the peripheral nerves associated with the vascular system.[10] It has several biological actions including inhibition of gastric acid secretion,[11] relaxation of vascular smooth muscle,[12,13] and stimulation of pancreatic amylase secretion.[14] CGRP acts in various tissues by stimulating cAMP formation.[15-18]

On the basis of circular dichroism studies[19,20] and nuclear magnetic resonance spectroscopy,[21] the structure of CGRP is thought to be an N-terminal loop bridged by a disulfide bond, followed by an α-helical portion between position 8 and positions 18-22. Structure-activity studies with

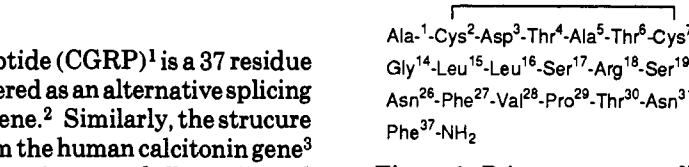


**Figure 1.** Primary sequence of human α-calcitonin gene-related peptide.

bioactive analogues of rCGRP, containing idealized amphiphilic α-helices of varying lengths, suggest that the helix ends at position 18.[19] The remainder of the peptide chain apparently does not have predominant secondary structure. Since all of the conformational studies had trifluoroethanol, which is known to stabilize α-helical structures, as a cosolvent the exact relevance of the predicted structures of CGRP to the *in vivo* structure is unclear. In all experiments that had water as the sole solvent, CGRP had no regular secondary structure.

Structure-activity studies of the naturally occurring variants, h-α-CGRP and h-β-CGRP, show that residues in positions 3, 22, and 25 are not involved in triggering the biological response.[22] All species variants of CGRP have 37 residues, a C-terminal (phenylalanyl)amide, and a disulfide bridge between positions 2 and 7.[23-26] The minor amino acid differences between species appears to be of little biological significance. It has been shown,[22] using a variety of assays for CGRP biological activity, that h-α-CGRP fragments (1-18) and (19-37) are biologically inactive.[22] The analogue [Cys(ACM)$^{2,7}$] h-α-CGRP, in

* Author to whom reprint requests and correspondence should be addressed.
† Department of Biomedical Sciences.
‡ Department of Pharmacology.

0022-2623/93/1836-2536$04.00/0 

which the disulfide bridge is not present, has no chronotropic or ionotropic activities on the rat atria[22,27] and is unable to inhibit gastric acid secretion.[12] It does, however, inhibit the twitch response of isolated rat vas deferens[27] at a potency 50-fold lower than that of h-α-CGRP. Prevention of protonation of the α-amino group of CGRP, by acetylation, results in a less potent but full agonist on rat atria.[22] Two C-terminal fragments of h-α-CGRP possess antagonist activity. [Tyr$^0$] r-CGRP (28–37) inhibits r-α-CGRP-stimulated secretion of amylase from pancreatic acini[28] and inhibits the increase in cAMP in gastric smooth muscle cells caused by r-α-CGRP.[29] h-α-CGRP (8–37) is an antagonist in guinea pig right atrium and guinea pig ileum. Also, h-α-CGRP (8–37) blocks the inhibitory effect of h-α-CGRP on food intake.[30] It does not, however, antagonize h-α-CGRP-induced hyperthermia.

The presence of a C-terminal phenylalanyl residue in all known variants of CGRP suggests that it has a role in determining CGRP biological activity. This study focuses on the contribution of the CGRP C-terminal region to biological activity. We now describe the synthesis and biological actions of a series of C-terminally truncated analogues of h-α-CGRP.

## Results and Discussion

All peptides were made by Merrifield's solid-phase peptide synthesis methodology.[31] *N*-α-Boc amino acid derivatives were coupled to the *p*-methylbenzhydrylamine resin as preformed hydroxybenzotriazole active esters in *N*-methylpyrrolidinone. Each coupling was monitored by the quantitative ninhydrin test.[32] When coupling yields were greater than 99%, unreacted amino groups were acetylated with acetic anhydride. In the syntheses of all peptides, the last 15 residues, Leu$^{15}$ to Ala$^1$ of the native h-α-CGRP sequence, had coupling yields less than 99%. The reduced efficiency of these couplings is attributed to conformations of the growing peptide chain in which the α-amino group is less accessible. Residues were then double coupled, and the resin was capped with acetic anhydride. The bulk of the resin became too great for the reaction vessel after the twentieth coupling and was halved before continuing the synthesis. The low–high trifluoromethanesulfonic acid method of Tam[33] was used to cleave the peptides from the resin and remove the side-chain blocking groups. The crude products were then treated with dithiothreitol overnight before being oxidized in high dilution to form the corresponding disulfide compound. Pretreatment with dithiothreitol was essential to reduce amounts of polymeric material in the crude product resulting from disulfide bond formation between the peptide and thiol scavengers employed in the cleavage mixture. Peptides were purified by gel filtration on Bio-Gel P-6, cation-exchange chromatography on CM-Sephadex C25, and semipreparative RP-HPLC on Vydac C18 silica. The first two chromatographic steps were necessary to remove impurities which coeluted with the products from the C18 reversed-phase column. Purity of the peptides was assessed by analytical RP-HPLC. Structures were confirmed by amino acid analysis and FAB-MS (Tables I and II) of the intact peptides and their tryptic fragments (Figure 2). The fragments were numbered in order of elution from the C18 column.

The ability of human-α-CGRP and C-terminally truncated fragments of human-α-CGRP to stimulate amylase secretion from dispersed guinea pig pancreatic acini and relax isolated rat mesenteric artery was determined.

**Table I.** Analytical Data of h-α-CGRP and C-Terminally Truncated Fragments of h-α-CGRP

| | HPLC $k'$ values | | | FAB-MS $[M+H]^+$ | |
|---|---|---|---|---|---|
| peptide | system 1[a] | system 2[b] | system 3[c] | calcd | obsd |
| 1 | 17.13 | 3.31 | 26.41 | 3790 | 3790 |
| 2 | 7.40 | 1.60 | 11.59 | 3643 | 3643 |
| 3 | 7.27 | 1.53 | 11.12 | 3572 | 3571 |
| 4 | 8.77 | 2.06 | 13.06 | 3444 | 3445 |

[a] Vydac 218TP54 $C_{18}$ column (250 × 4.6 mm), water/acetonitrile (73:27), 0.1% TFA. [b] Vydac 214TP54 $C_4$ column (250 × 4.6 mm), water/acetonitrile (73:27), 0.1% TFA. [c] Waters Delta Pak HPI $C_{18}$ column (150 × 3.9 mm), water/acetonitrile (73:27), 0.1% TFA.

Figure 3 shows the effects of h-α-CGRP and C-terminally truncated fragments of h-α-CGRP on amylase secretion from pancreatic acinar cells. All h-α-CGRP fragments contained a C-terminal carboxamide functionality to mimic the neutral peptide bond in the native sequence. When used over the concentration range $10^{-11}$–$10^{-4}$ M, h-α-CGRP increased amylase secretion in a biphasic dose–response manner. Initial amylase secretion increased to a plateau of 7–8% of total cellular amylase content with a corresponding $ED_{50}$ value of 7.7 ± 1.8 nM. In the absence of the phosphodiesterase inhibitor, IBMX, the maximal response to h-α-CGRP was only 5% of total amylase content. All studies in the present report were therefore carried out in the presence of 1 mM IBMX. A second increase in amylase secretion was seen in response to very high concentrations of h-α-CGRP. Amylase output rose to 11% of total cellular amylase content in response to a h-α-CGRP concentration of $10^{-4}$ M but did not plateau, thereby preventing determination of an $ED_{50}$ value for the latter response.

h-α-CGRP (1–36) **2** caused a small but significant increase in amylase secretion at a concentration of $10^{-8}$ M. Peptides h-α-CGRP (1–35) **3** and h-α-CGRP (1–34) **4** did not stimulate amylase release on pancreatic acinar cells at concentrations $\leq 10^{-5}$ M. At concentrations above $10^{-5}$ M, all of the above peptide fragments stimulated amylase secretion. Fragment **2**, at the highest concentration, caused an increase in amylase secretion of 11% of total cell content which is identical with that caused by h-α-CGRP at the same concentration. The increase in amylase secretion caused by fragments **3** and **4** was less than that of h-α-CGRP, the largest response being 9% of total amylase content.

These results suggest that h-α-CGRP may bind at two different sites on pancreatic acinar cells to stimulate amylase secretion. At the high affinity site, h-α-CGRP has an $ED_{50}$ value of 7.7 nM which is in close agreement with literature reports for responses mediated by a CGRP receptor using cAMP as a second messenger.[14,28] The amylase secretion caused by high concentrations of h-α-CGRP may be the result of one of the following possibilities: (i) activation of a hitherto unknown, CGRP receptor, (ii) activation of a receptor for a structurally similar endogenous ligand such as the islet amyloid polypeptide receptor, or (iii) nonspecific interactions with receptors capable of stimulating amylase release, such as the cholecystokinin receptor.

h-α-CGRP relaxed rat superior mesenteric arteries, precontracted with prostaglandin $F_{2\alpha}$, in a concentration-dependant manner (Figure 4) with an $EC_{50}$ of 2.9 ± 0.31 nM and a maximal relaxation that was 60% of the

**Table II.** Amino Acid Compositions[a] of h-α-CGRP and C-Terminally Truncated Fragments of h-α-CGRP

| peptide | Cys | Asp | Thr | Ser | Pro | Gly | Ala | Val | Leu | Phe | His | Lys | Arg |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.83(2) | 4.17(4) | 3.80(4) | 2.44(3) | 0.97(1) | 4.05(4) | 4.06(4) | 4.79(5) | 3.23(3) | 2.07(2) | 0.95(1) | 1.96(2) | 2.09(2) |
| 2 | 1.81(2) | 4.06(4) | 3.42(4) | 2.26(3) | 1.07(1) | 4.06(4) | 3.98(4) | 4.71(5) | 3.24(3) | 0.99(1) | 0.95(1) | 1.98(2) | 2.05(2) |
| 3 | 1.76(2) | 4.11(4) | 3.48(4) | 2.21(3) | 1.05(1) | 4.00(4) | 2.85(3) | 4.69(5) | 3.20(3) | 1.00(1) | 0.96(1) | 2.15(2) | 2.04(2) |
| 4 | 1.90(2) | 4.03(4) | 3.34(4) | 2.30(3) | 1.08(1) | 3.99(4) | 2.99(3) | 4.73(5) | 3.17(3) | 1.04(1) | 0.95(1) | 1.06(1) | 2.03(2) |

[a] Theoretical values in parentheses.

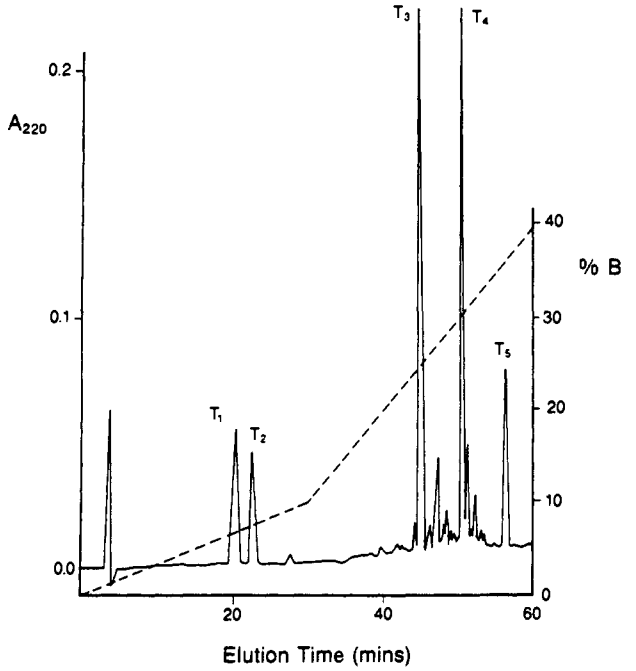


**Figure 2.** RP-HPLC of h-α-CGRP tryptic digest mixture.

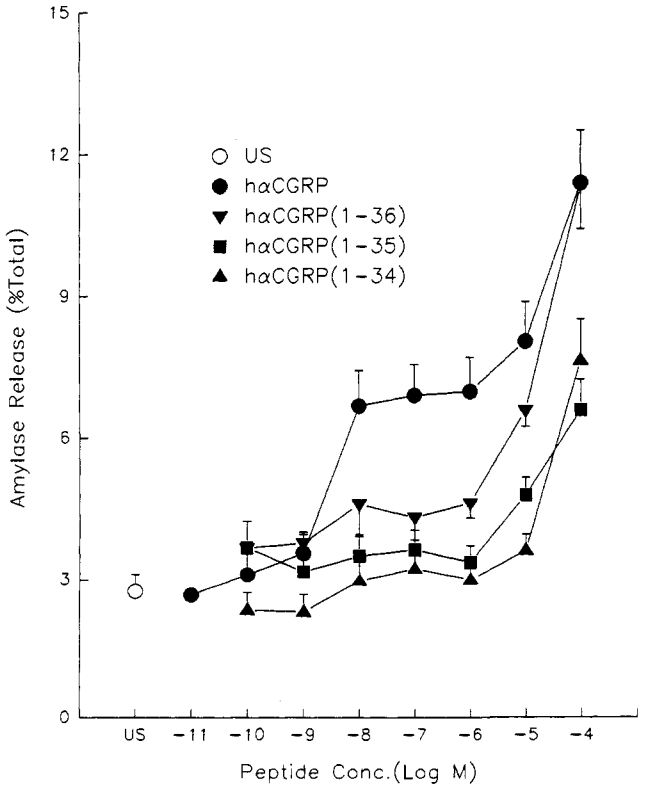


**Figure 3.** Effect of h-α-CGRP and C-terminally truncated fragments of h-α-CGRP on amylase secretion from pancreatic acini, $n = 7$–9. $P < 0.01$.

prostaglandin $F_{2\alpha}$–induced tone. Fragments **2** and **4** did not relax mesenteric arteries. Fragment **3** relaxed mesenteric arteries with a maximal response that was approximately 40% of active tone at the highest concentration tested. The potency of fragment **3** was at least 100-fold lower than that of h-α-CGRP (Figure 4).

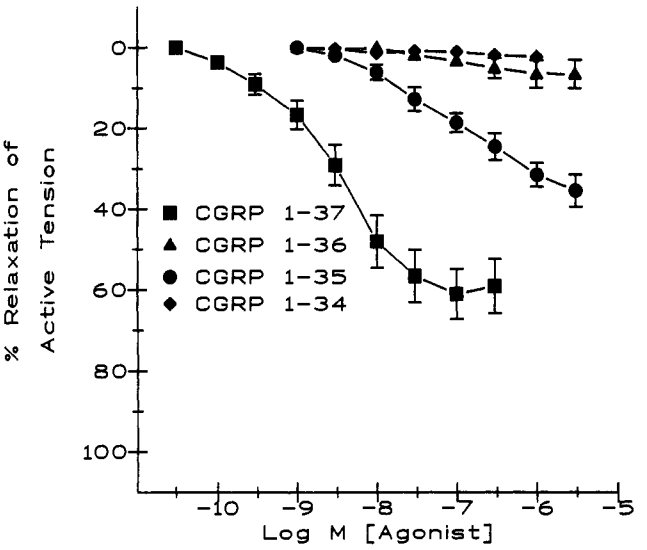


**Figure 4.** Relaxant effects of h-α-CGRP and C-terminally truncated fragments of h-α-CGRP on rat mesenteric arteries, $n = 4$–7.

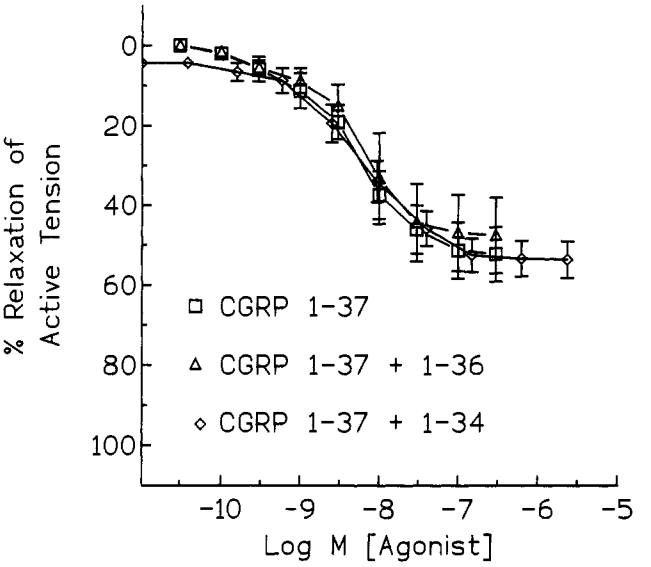


**Figure 5.** Relaxant effects of h-α-CGRP in the absence and presence of 1 μM peptide **2** and 1 μM peptide **4**.

Inactive fragments **2** and **4** were also tested as antagonists of h-α-CGRP on rat mesenteric arteries. Concentration, relaxation response curves for h-α-CGRP, in the absence and presence of 1 μM **2** and **4**, are shown in Figure 5. $EC_{50}$ values were 3.22 ± 0.80 nM for h-α-CGRP alone, 5.18 ± 1.78 nM for h-α-CGRP in the presence of 1 μM **2** and 2.03 ± 0.96 nM for h-α-CGRP in the presence of 1 μM **4**. These values are not significantly different, indicating that the fragments do not act as antagonists at CGRP receptors.

It is not clear why fragment **3** is a less potent agonist than h-α-CGRP, whereas fragment **2** is inactive as an

agonist or an antagonist. Presumably, the absence of a C-terminal Phe residue in fragment **2** causes a conformational change in the C-terminal region and potentially of the whole peptide, which prevents the fragment from interacting with the CGRP receptor. Removal of the Ala allows the conformation to return to a bioactive state allowing fragment **3** to function as an agonist. However, the much lower potency of fragment **3** suggests that it is lacking some important structural features that either are involved in binding to the CGRP receptor or contribute to intrinsic efficacy.

The relaxant effects of fragment **3** on rat mesenteric arteries and its inability to stimulate amylase secretion from guinea pig pancreatic acinar cells suggests that the CGRP receptor in these tissues may be different. This interpretation is in agreement with other reports[27,34] of two CGRP receptors, called $CGRP_1$ and $CGRP_2$. $CGRP_1$ receptors have been found in guinea pig atria and are characterized by high affinity for the antagonists h-α-CGRP (8–37) and h-α-CGRP (9–37). $CGRP_2$ receptors have been identified in rat vas deferens and have a lower affinity for the above fragments but are sensitive to the agonist [Cys(ACM)$^{2,7}$]-h-α-CGRP, which is inactive in guinea pig atria. Further work is required to classify the CGRP receptors in rat mesenteric arteries and guinea pig pancreatic acini.

In conclusion, this study has shown that h-α-CGRP requires its C-terminal portion to elicit a biological response at the CGRP receptors on guinea pig pancreatic acinar cells and rat mesenteric artery. Specifically, the Phe$^{37}$ residue is involved in the binding of h-α-CGRP to its receptor and may play a role in triggering its biological response. This conclusion is in complete agreement with the recent results of O'Connell *et al.*[35] and Poyner *et al.*[36] The differential agonist effects of fragment **3** on acinar cells and mesenteric artery may be indicative of receptor heterogeneity among CGRP receptors.

## Experimental Section

*N*-α-Boc amino acid derivatives were purchased from Bachem (Torrence, CA) and ABI (Foster City, CA). All solvents and reagents were peptide synthesis grade and obtained from ABI and Fisher Biotec (Pittsburgh, PA). Water for chromatography was purified on a Barnstead Nanopure system, and solvents for HPLC were from Fisher (Optima grade). TFA for HPLC was supplied by Pierce (Rockford, IL). Young guinea pigs (100–200 g) were obtained from the breeding colony at Creighton University. HEPES was from Research Organics (Cleveland, OH), purified collagenase (type CLSPA, 1489 u/mg) was from Worthington Biochemical (Freehold, NJ), MEM (50x), amino acids, essential vitamin mixture, nonessential amino acids, and BSA were all from Sigma Chemical Co. (St. Louis, MO). The Phadebas amylase test kit was purchased from Pharmacia Diagnostics (Fairfield, NJ). Peptide syntheses were performed on an ABI automated peptide synthesizer, Model 430A. Reactive side chains were protected as follows: Cys, *p*-methoxybenzyl; Asp, benzyl ester; Thr, benzyl ether; His, benzyloxymethyl; Arg, mesitylene-2-sulfonyl; Ser, benzyl ether; Lys, 2-chlorocarbobenzoxy. Gel filtration was performed using Bio-Gel P6 from Bio Rad (Richmond, CA) packed in a glass column (2.5 × 90 cm). Cation-exchange chromatography was performed on CM Sephadex C25 from Pharmacia (Piscataway, NJ) packed in a glass column (1.6 × 15 cm). HPLC was performed on a Waters (Milford, MA) Model 600E instrument which included a U6K injector, a Model 484 UV detector, and a Model 745B integrator. Solvent A was 0.1% TFA in water, and solvent B was a mixture of acetonitrile and water (60:40) containing 0.09% TFA. Analytical RP-HPLC was performed on a Vydac 218TP54 C18 column (4.6 × 250 mm), a Vydac 214TP54 C4 column (4.6 × 250 mm), and a Waters Delta Pak HPI C18, 300 Å column (3.9 × 150 mm). Semipreparative HPLC was performed on a Vydac 218TP510 Column (10 × 250 mm). $k'$ values were obtained under isocratic conditions. For amino acid analysis, samples were hydrolyzed in 6 M constant boiling hydrochloric acid in sealed evacuated tubes for 24 h at 110 °C. Amino acid analysis was performed on a Beckman instrument, Model 116, modified to a one-column system. Cysteine was determined as cysteic acid in the full length peptides. Incomplete hydrolysis of the Val$^{22}$–Val$^{23}$ peptide bond was sometimes observed.

**Tryptic Digestion.** Peptides (1 mg) were digested with trypsin (5 μg) in 50 mM ammonium bicarbonate buffer (1 mL) at pH 8.5 for 24 h at 37 °C. The digestion was stopped by freezing, and the mixture was stored at −20 °C. Tryptic fragments were purified by RP-HPLC using the elution conditions described for each peptide. Tryptic fragments are listed in order of elution from the column.

**Human α-Calcitonin Gene-Related Peptide, h-α-CGRP 1.** The hydrochloride salt of *p*-MBHA resin (0.65 g, 0.4 mmol) was treated with a 5% solution of DIEA in DCM (1 min) and a 5% solution of DIEA in NMP (1 min) before being washed with NMP (5 × 1 min). Boc-Phe (0.53 g, 2 mmol) and a 1 M solution of HOBt in NMP (2 mL, 2 mmol) were added to NMP (5 mL) before a 1 M solution of DCCI in NMP (2 mL, 2 mmol) was added. The reaction mixture was left for 40 min with occasional mixing. The resulting DCU was removed by filtration, and the filtrate was transferred immediately to the reaction vessel containing the neutralized *p*-MBHA resin. The coupling mixture was vortexed for 30 min, and DMSO was added to a final concentration of 20%. The mixture was vortexed for a further 16 min, 3.8 equiv of DIEA was added, and vortexing was continued for 7 min. The resin was washed with DCM (3 × 1 min) to yield Boc-Phe-*p*-MBHA resin. A sample of resin was removed for the quantitative ninhydrin test. To cap the remainder, the peptide resin was treated with a mixture of acetic anhydride, DIEA, and DCM (10:5:85, vol/vol/vol) followed by acetic anhydride in DCM (1:9, vol/vol) and then washed with DCM (4 × 1 min). To remove the *N*-α-Boc group the resin was mixed with a 30% solution of TFA in DCM for 3 min and a 50% solution of TFA in DCM for 17 min and further washed with DCM (5 × 1 min).

This procedure was repeated for the coupling of the next 21 residues. After the twentieth coupling, the peptide–resin was dried, and half was used for the rest of the synthesis. Due to poor coupling yields, double couplings were used for the twenty-third and all subsequent couplings. Once the desired sequence was assembled, the final Boc group was removed, and the peptide–resin was dried under reduced pressure.

DMS (1.62 mL), *m*-cresol (0.54 mL), TFA (2.7 mL), and TFMSA (0.54 mL) was added to the peptide–resin (539 mg, 0.074 mmol) in a 50-mL round-bottomed flask immersed in an ice–salt–water bath. The reaction mixture was stirred for 3 h at −5 to 0 °C. The peptide resin was collected by filtration, washed with anhydrous ether, and dried under reduced pressure for 30 min. It was then mixed with ethanedithiol (0.27 mL) and thioanisole (0.54 mL) for 10 min, TFA (5.4 mL) was added, and stirring was continued for a further 10 min. TFMSA (0.54 mL) was added dropwise, and the mixture was stirred for 2 h at ambient temperature. Anhydrous ether was added to the reaction mixture, and the resin and precipitated peptide were collected by filtration and washed with more ether. The peptide was dissolved in TFA, and the suspended resin was removed by filtration and washed with more TFA. The combined TFA filtrates were concentrated under reduced pressure at room temperature and triturated with anhydrous ether. The resulting white precipitate was collected by filtration, washed with ether, and dried to yield the crude linear peptide.

DTT (0.78 g, 5 mmol) and the crude linear peptide were dissolved in a solution of ammonium acetate (50 mM, pH 8.5, 100 mL) and left to stir overnight. The solution was diluted to 1 L with more of the ammonium acetate solution (50 mM, pH 8.5), and 0.01 M $K_3Fe(CN)_6$ solution was added until the yellow color persisted. The solution was stirred for 30 min before the pH was lowered to approximately 4.0 with glacial acetic acid. Amberlite IRA68 ($Cl^-$ form, 30 mL settled volume) was added, and the mixture was stirred for 30 min. The ion-exchange resin was collected by filtration and washed with water (3 × 50 mL).

The combined filtrates, containing the crude cyclized peptide, were concentrated under reduced pressure to approximately 100 mL and lyophilized.

The crude cyclized peptide was subjected to gel filtration on Bio-Gel P6 using 5% acetic $acid_{aq}$ as the eluent, at a flow rate of 18 mL/h. The effluent was continuously monitored at 254 nm and collected at a rate of four fractions per hour. Fractions 40–71 were pooled and lyophilized. The freeze-dried material was dissolved in 10 mM ammonium acetate buffer, pH 5.5 (30 mL), and loaded onto a column of CM-Sephadex C25, previously equilibrated with the same solution. The peptide was eluted using a linear gradient of 10–800 mM ammonium acetate, pH 5.5 (300 mL of each buffer in each chamber) at a flow rate of 50 mL/h. The eluant was continuously monitored at 254 nm and collected in 12 fractions every hour. Fractions 85–119 were pooled and lyophilized. The peptide was dissolved in 0.1% $TFA_{aq}$ and loaded on to a Vydac C18 semipreparative column previously equilibrated with 0.1% $TFA_{aq}$ at a flow rate of 4 mL/h. The effluent was continuously monitored at 220 nm and collected in 4-mL fractions. The peptide was eluted from the column using the following gradient conditions: 0% B–36% B in 10 min followed by 36% B–50% B in 60 min. Fractions 41–53 were pooled and freeze-dried to yield h-α-CGRP 1, 23 mg (4.4% based on coupling yields and original resin substitution). Table I shows the analytical data, and Table II shows the amino acid composition. Following tryptic digestion the tryptic fragments were eluted from an analytical Vydac C18 column using the gradient conditions: 0–10% B in 30 min and 10–40% B in 30 min to yield the following peptides: **T1** (fragment 36–37), AAA, Ala 1.02 (1), Phe 0.98 (1); FAB-MS $MH^+$ calcd 236, found 236; **T2** (fragment 19–24), AAA, Ser 0.74 (1), Gly 2.07 (2), Val 1.80 (2), Lys 1.14 (1); FAB-MS $MH^+$ calcd 546, found 546; **T3** (fragment 1–11), AAA, Asx 1.04 (1), Thr 2.35 (3), Ala 1.92 (2), Cys n.d., Val 0.97 (1), His 0.99 (1), Arg 1.08 (1); FAB-MS $MH^+$ calcd 1175, found 1175; **T4** (fragment 25–35), AAA, Asx 3.26 (3), Thr 0.90 (1), Ser 1.08 (1), Pro 1.05 (1), Gly 1.01 (1), Val 1.80 (2), Phe 0.90 (1), Lys 1.04 (1); FAB-MS $MH^+$ calcd 1176, found 1177; **T5** (fragment 12–18), AAA, Ser 0.81 (1), Gly 1.01 (1), Ala 0.97 (1), Leu 3.05 (3), Arg 0.97 (1); FAB-MS $MH^+$ calcd 729, found 729.

**h-α-CGRP (1-36) 2.** h-α-CGRP (1–36) was synthesized and purified to apparent homogeneity according to the methods outlined in the synthesis of compound 1. Table I shows the analytical data, and Table II shows the amino acid composition. After tryptic digestion the tryptic fragments were purified on an analytical Vydac C18 column using the gradient conditions 0–50% B for 30 min using concave gradient number 7 followed by 50–100% B in 30 min to yield tryptic peptides **T1–T4**. **T1** (fragment 19–24): AAA, Ser 0.94 (1), Gly 2.11 (2), Val 0.97 (2), Lys 1.00 (1); FAB-MS $MH^+$ calcd 546, found 546. **T2** (fragment 1–11): AAA, Asx 1.13 (1), Thr 2.05 (3), Ala 1.98 (2), Cys n.d., Val 0.91 (1), His 0.88 (1), Arg 0.98 (1); FAB-MS $MH^+$ calcd 1175, found 1175. **T3** (fragment 25–35): AAA, Asx 2.99(3), Thr 0.93 (1), Ser 0.90 (1), Pro 1.01 (1), Gly 1.00 (1), Val 1.97 (2), Phe 0.98 (1), Lys 1.02 (1); FAB-MS $MH^+$ calcd 1176, found 1176. **T4** (fragment 12–18): AAA, Ser 0.79 (1), Gly 1.00 (1), Ala 1.00 (1), Leu 3.03 (3), Arg 0.98 (1); FAB-MS $MH^+$ calcd 729, found 729. The C-terminal alanylamide was not detected.

**h-α-CGRP (1-35) 3.** The title peptide was made and purified by the procedure outlined for the synthesis of compound 1. Table I shows the analytical data, and Table II shows the amino acid composition. The peptide was subjected to tryptic digestion, and the tryptic fragments were purified using an analytical Vydac C18 column and the solvent gradient 0–50% B over 50 min to yield the peptides **T1–T4**. **T1** (fragment 19–24): AAA, Ser 0.82 (1), Gly 2.01 (2), Val 0.59 (2), Lys 0.99 (1); FAB-MS $MH^+$ calcd 546, found 546. **T2** (fragment 1–11): AAA, Asx 1.02 (1), Thr 2.49 (3), Ala 2.02 (2), Cys n.d., Val 0.99 (1), His 0.91 (1), Arg 1.06 (1); FAB-MS $MH^+$ calcd 1175, found 1175. **T3** (fragment 25–35): AAA, Asx 3.01 (3), Thr 0.96 (1), Ser 0.98 (1), Pro 1.09 (1), Gly 0.89 (1), Val 1.43 (2), Phe 0.71 (1), Lys 1.07 (1); FAB-MS $MH^+$ calcd 1176, found 1177. **T4** (fragment 12–18): AAA, Ser 0.81 (1), Gly 1.02 (1), Ala 1.01 (1), Leu 2.98 (3), Arg 1.02 (1); FAB-MS $MH^+$ calcd 729, found 730.

**h-α-CGRP (1-34) 4.** h-α-CGRP (1–34) was prepared and purified using the procedure described for the synthesis of compound 1. Table I shows the analytical data, and Table II shows the amino acid composition. After tryptic digestion of the peptide the tryptic fragments were isolated from an analytical Vydac C18 column using the gradient conditions of 0–50% B in 50 minutes to yield following peptides **T1–T4**. **T1** (fragment 19–24): AAA, Ser 0.86 (1), Gly 2.04 (2), Val 0.62 (2), Lys 0.96 (1); FAB-MS $MH^+$ calcd 546, found 546. **T2** (fragment 1–11): AAA, Asx 1.01 (1), Thr 2.41 (3), Ala 2.06 (2), Cys n.d., Val 0.98 (1), His 0.98 (1), Arg 0.98 (1); FAB-MS $MH^+$ calcd 1175, found 1176. **T3** (fragment 25–34): AAA, Asx 3.03 (3), Thr 0.89 (1), Ser 1.07 (1), Pro 1.09 (1), Gly 1.11 (1), Val 1.52 (2), Phe 0.71 (1); FAB-MS $MH^+$ calcd 1047, found 1048. **T4** (fragment 12–18): AAA, Ser 0.81 (1), Gly 1.01 (1), Ala 0.98 (1), Leu 3.02 (3), Arg 0.99 (1); FAB-MS $MH^+$ calcd 729, found 730.

**Bioassay. Preparation of Pancreatic Acini.** Guinea pigs (100–200 g) were fasted overnight before sacrifice by decapitation. Dispersed pancreatic acini were prepared as described previously.[37] The composition of the incubation solution was the same as that used in previous studies.[38] Briefly, the pancreas was removed and trimmed of excess fat and mesentery. Isolated pancreatic acini were obtained by collagenase digestion for 30 min at 37 °C, followed by gentle mechanical disruption through a series of needles of decreasing diameter (15, 17, and 18 gauge). The dispersed acini were then washed four times with incubation medium, filtered through two nylon meshes of decreasing pore size (500 and 350 μm), and resuspended in 40–45 mL of incubation solution. A 25% aqueous BSA solution (6–8 mL) was added to give a final concentration of approximately 3–4%. The incubation of pancreatic acini with h-α-CGRP and its fragments was carried out in a microtitre plate (millititer SV, Millipore Inc., Bedford, MA) according to the procedure published previously.[38]

**Amylase Assay.** Amylase release was determined using the Phadebas reagent, employing a modification of the method of Ceska.[39] Amylase release into the medium was calculated as a percentage of the total enzyme activity present in the acini at the beginning of the incubation period. Statistical significance was determined by analysis of variance using the Bonferroni Post test for repeated measures.

**Mesenteric Artery Relaxation.** Male albino Sprague–Dawley rats (270–325 g) were anesthetized with $CO_2$ and sacrificed by decapitation. The method for measurement of superior mesenteric artery relaxation was the same as that used in previous studies.[40] The superior mesenteric artery was cut into 3-mm-long ring segments, and two stainless steel pins were placed through the lumen of the ring for tension measurement. The rings were placed in glass muscle chambers containing Krebs solution gassed with 95% $O_2$–5% $CO_2$ at 37 °C. The rings were equilibrated at an optimal resting tension of 0.4 g for 1 h, maximally contracted with 10 μM norepinephrine, and then relaxed with 1 μM acetylcholine to confirm that endothelium-mediated relaxation was intact. Arteries were washed with Krebs solution for 1 h and contracted to one-half of maximal norepinephrine contraction with prostaglandin $F_{2\alpha}$, followed by relaxation concentration–response curves for CGRP or fragments. Each analogue was tested four to seven times on tissues from separate animals.

**Acknowledgment.** This work was funded, in part, by the Pharmaceutical Manufacturers' Association Foundation, American Heart Association–Nebraska Affiliate, and the Health Future Foundation of Creighton University. The authors wish to thank Donald Babin for the amino acid analyses. Mass spectral determinations were made at the Midwest Center for Mass Spectrometry with partial support by the National Science Foundation, Biology Division (Grant No. DIR9017262).

## References

(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977). All optically active amino acids are of the L variety unless otherwise stated. Other abbreviations include the following: AAA, amino acid analysis; *N*-α-Boc, *N*-α-(*tert*-butyloxycarbonyl); BSA, bovine serum albumin; CGRP, calcitonin gene-related peptide; DCCI, dicyclohexylcarbodiimide; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DIEA, diisopro-

pylethylamine; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; FAB-MS, fast atom bombardment mass spectrometry; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; HOBt, N-hydroxybenzotriazole; IBMX, 3-isobutyl-1-methylxanthine: p-MBHA, p-methylbenzhydrylamine; nd, not determined; NMP, N-methylpyrrolidinone; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; h, human; r, rat.

(2) Amara, S. G.; Jones, V.; Rosenfeld, M. G.; Ong, E. S.; Evans, R. M. Alternative RNA Processing in Calcitonin Gene Expression Generates mRNAs Encoding Different Polypeptide Products. *Nature* **1982**, *298*, 240–244.

(3) Steenbergh, P. H.; Hoppener, J. W. M.; Zandberg, J.; Van de Van, W. J. M.; Jansz, H. S.; Lips, C. J. M. Calcitonin Gene-Related Peptide Coding Sequence is Conserved in the Human Genome and is Expressed in Medullary Thyroid Carcinoma. *J. Clin. Endocrinol. Metab.* **1984**, *59*, 358–360.

(4) Morris, H. R.; Panico, M.; Etienne, T.; Tippens, J.; Girgis, S. I.; MacIntyre, I. Isolation and Characterization of Human Calcitonin Gene-Related Peptide. *Nature* **1984**, *308*, 746–748.

(5) Steenbergh, P. H.; Hoppener, J. W. M.; Zandberg, J.; Lips, C. J. M.; Jansz, H. S. A Second Human Calcitonin/CGRP Gene. *FEBS Lett.* **1985**, *183*, 403–407.

(6) Amara, S. G.; Arriza, J. L.; Jeff, S. E.; Swanson, L. W.; Evans, R. M.; Rosenfeld, M. G. Expression in Brain of a Messenger RNA Encoding a Novel Neuropeptide Homologous to Calcitonin Gene-Related Peptide. *Science* **1985**, *229*, 1094–1097.

(7) Wimalawansa, S. J.; Morris, H. R.; Etienne, A.; Blench, I.; Panico, M.; MacIntyre, I. Isolation, Purification and Characterization of β-hCGRP from Human Spinal Cord. *Biochem. Biophys. Res. Commun.* **1990**, *167*, 993–1000.

(8) Gibson, S. J.; Polack, J. M.; Bloom, S. R.; Sabate, I. M.; Mulderry, P. M.; Ghatei, M. A.; McGregor, G. P.; Morrison, J. F. B.; Kelly, J. S.; Evans, R. M.; Rosenfeld, M. G. Calcitonin Gene-Related Peptide Immunoreactivity in the Spinal Cord of Man and of Eight Other Species. *J. Neurosci.* **1984**, *4*, 3101–3111.

(9) Skofitsch, G.; Jacobowitz, D. M. Calcitonin Gene-Related Peptide: Detailed Immunohistochemical Distribution in the Central Nervous System. *Peptides* **1988**, *6*, 721–745.

(10) Mulderry, P. K.; Ghatei, M. A.; Rodrigo, J.; Allen, J. M.; Rosenfeld, M. G.; Polak, J. M.; Bloom, S. R. Calcintonin Gene–Related Peptide in Cardiovascular Tissue of the Rat. *Neuroscience* **1985**, *14*, 947–954.

(11) Lenz, H. J.; Mortrud, M. T.; Rivier, J. E.; Brown, M. R. Calcitonin Gene-Related Peptide Inhibits Basal, Pentagastrin, Histamine and Bethanecol Stimulated Gastric Acid Secretion. *Gut* **1985**, *26*, 550–555.

(12) Brain, S. D.; Williams, T. J.; Tippens, J. R.; Morris, H. R.; MacIntyre, I. Calcitonin Gene-Related Peptide is a Potent Vasodilator. *Nature* **1985**, *313*, 54–56.

(13) Tornebrandt, K.; Nobin, A.; Owman, C. Contractile and Dilatory Actions of Neuropeptides of Isolated Human Mesenteric Blood Vessels. *Peptides* **1987**, *8*, 251–256.

(14) Seifert, H.; Sawchenko, P.; Chesnut, J.; Rivier, J.; Vale, W.; Pandol, S. J. Receptor for Calcitonin Gene-Related Peptide: Binding to Exocrine Pancreas Mediates Biological Actions. *Am. J. Physiol.* **1985**, *249*, G147–G151.

(15) McEwan, J. R.; Ritter, J. M.; MacDermot, J. Calcitonin Gene-Related Peptide (CGRP) Activates Adenylate Cyclase of Bovine Aortic Endothelial Cells: Guanosine 5′Triphosphate Dependence and Partial Agonist Activity of a Tyrosinated Analogue. *Cardiovasc. Res.* **1989**, *23*, 921–927.

(16) Fouchereau-Peron, M.; Arlot-Bonnemanis, Y.; Milhaud, G.; Moukhtar, M. S. Calcitonin Gene-Related Peptide Stimulates Adenylate Cyclase Activity in Trout Gill Membranes. *Biochem. Biophys. Res. Commun.* **1990**, *172*, 582–587.

(17) Edwards, R. M.; Stack, E. J.; Trizna, W. Calcitonin Gene-Related Peptide Stimulates Adenylate Cyclase and Relaxed Intracerebral Arterioles. *J. Pharm. Exp. Ther.* **1990**, *257*, 1020–1024.

(18) Kubota, M.; Moseley, J. M.; Butera, L.; Dusting, G. J.; MacDonald, P. S.; Martin, T. J. Calcitonin Gene-Related Peptide Stimulates Cyclic AMP Formation in Rat Aortic Smooth Muscle Cells. *Biochem. Biophys. Res. Comm.* **1985**, *132*, 88–94.

(19) Lynch, B.; Kaiser, E. T. Biological Properties of Two Models of Calcitonin Gene-Related Peptide with Idealized Amphiphilic α-Helices of Different Lengths. *Biochemistry* **1988**, *27*, 7600–7607.

(20) Manning, M. C. Conformation of the Alpha Form of Human Calcitonin Gene-Related Peptide (CGRP) in a Aqueous Solution as Determined by Circular Dichroism Spectroscopy. *Biochem. Biophys. Res. Commun.* **1991**, *160*, 388–392.

(21) Breeze, A. L.; Harvey, T. S.; Bazzo, R.; Campbell, I. D. Solution Structure of Human Calcitonin Gene-Related Peptide by $^1$H NMR and Distance Geometry with Restrained Molecular Dynamics. *Biochemistry* **1991**, *30*, 575–582.

(22) Tippens, J. R.; DiManzo, V.; Panico, M.; Morris, H. R.; MacIntyre, I. Investigation of the Structure/Activity Relationship of Human Calcitonin Gene-Related Peptide (CGRP). *Biochem. Biophys. Res. Commun.* **1986**, *134*, 1306–1311.

(23) Minvielle, S.; Cressent, M.; Lasmoles, F.; Jullienne, A.; Milhaud, G.; Mouktar, M. S. Isolation and Partial Characterization of the Calcitonin Gene in a Lower Vertebrate; Predicted Structure of Avian Calcitonin Gene-Related Peptide. *FEBS Lett.* **1986**, *203*, 7–10.

(24) Minvielle, S.; Cressent, M.; Delehaye, M. C.; Segond, N.; Milhaud, G.; Jullienne, A.; Moukhtar, M. S.; Lasmoles, F. Sequence and Expression of the Chicken Calcitonin Gene. *FEBS Lett.* **1987**, *223*, 63–68.

(25) Kimura, S.; Sugita, Y.; Kanazawa, I.; Saito, A.; Goto, K. Isolation and Amino Acid Sequence of Calcitonin Gene-Related Peptide from Porcine Spinal Cord. *Neuropeptides* **1987**, *9*, 75–82.

(26) Eysselein, V. E.; Reeve, J. R., Jr.; Sternini, C.; Cominelli, F.; Davis, W. M.; Davis, M. T.; Lee, T. D.; Ho, F.-J.; Ridout, D.; Shiveley, J. E. Structural Characterization of Calcitonin Gene-Related Peptide Purified from Rabbit Intestine. *Peptides* **1991**, *12*, 289–295.

(27) Dennis, T.; Fournier, A.; St-Pierre, S.; Quirion, R. Structure–Activity Profile of Calcitonin Gene-Related Peptide (CGRP) in Peripheral and Brain Tissue. Evidence for Receptor Multiplicity. *J. Pharmacol. Exp. Ther.* **1989**, *251*, 718–725.

(28) Maton, P. N.; Pradhan, T.; Zhou, Z.-C.; Gardner, J. D.; Jensen, R. T. Activities of Calcitonin Gene-Related Peptide (CGRP) and Related Peptides at the CGRP Receptor. *Peptides* **1990**, *11*, 485–489.

(29) Maton, P. N.; Webber, P.; Zhou, Z.-C.; Gardner, J. D.; Jensen, R. T. Discovery of a Peptide that acts as an Antagonist of Calcitonin Gene-Related Peptide. *Gastroenterology* **1988**, *94*, A289.

(30) Dennis, T.; Fournier, A.; Cadieux, A.; Pomerleau, F.; Jolicoeur, F. B.; St-Pierre, S.; Quirion, R. hCGRP$_{8-37}$, a Calcitonin Gene-Related Peptide Antagonist Revealing Calcitonin Gene-Related Peptide Receptor Heterogeneity in Brain and Periphery. *J. Pharmacol. Exp. Ther.* **1990**, *254*, 123–128.

(31) Merrifield, R. B. Solid Phase Peptide Synthesis I. The Synthesis of a Tetrapeptide. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154.

(32) Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. Quantitative Monitoring of Solid Phase Peptide Synthesis by the Ninhydrin Reaction. *Anal. Biochem.* **1981**, *117*, 147–157.

(33) Tam, J. P.; Heath, W. F.; Merrifield, R. B. Mechanisms for Removal of Benzyl Protecting Groups in Synthetic Peptides by Trifluoromethanesulfonic Acid–Trifluoroacetic Acid–Dimethyl Sulfide. *J. Am. Chem. Soc.* **1986**, *108*, 5242–5251.

(34) Mimeault, M.; Fournier, A.; Dumont, Y.; St-Pierre, S.; Quirion, R. Comparative Affinities and Antagonistic Potencies of Various Human Calcitonin Gene-Related Peptide Fragments on Calcitonin Gene-Related Peptide Receptors in Brain and Periphery. *J. Pharmacol. Exp. Ther.* **1991**, *258*, 1084–1090.

(35) O'Connell, J. P.; Kelly, S. M.; Raleigh, D. P.; Hubbard, J. A. M.; Price, N. C.; Dobson, C. M.; Smith, B. J. On the Role of the C-Terminus of α-Calcitonin Gene-Related Peptide (αCGRP). *Biochem. J.* **1993**, *291*, 205–210.

(36) Poyner, D. R.; Andrew, D. P.; Brown, D.; Bose, C.; Hanley, M. R. Pharmacological Characterization of a Receptor for Calcitonin Gene-Related Peptide on Rat, L6 Myocytes. *Brit. J. Pharmacol.* **1992**, *105*, 441–447.

(37) Schulz, G. S.; Sarras, M. P.; Gunther, G. R.; Hull, B. E.; Alicea, H. A.; Gorelick, F. S.; Jamieson, J. D. Guinea Pig Pancreatic Acini Prepared with Purified Collagenase. *Exp. Cell Res.* **1980**, *134*, 49–62.

(38) Adrian, T. E.; Zucker, K. A.; Bilchik, A. J.; Modlin, J. M. A Novel Micromethod for Pancreatic Secretion. *Int. J. Pancreatol.* **1990**, *6*, 61–69.

(39) Ceska, M.; Birath, K.; Brown, B. A New and Rapid Method for the Clinical Determination of Amylase Activities in Human Serum and Urine. *Clin. Chim. Acta* **1969**, *26*, 437–444.

(40) Bockman, C. S; Jefferies, W. B.; Pettinger, W. A.; Abel, P. W.; Enhanced Release of Endothelium-Derived Relaxing Factor in Mineralocorticoid Hypertension. *Hypertension* **1992**, *20*, 304–313.