filtration through Celite and the EtOH evaporated to yield an oil (2.6 g). The oil was taken up in $CHCl_3$ (100 ml) and the resulting solution washed with 10% HCl (3 × 30 ml), dried ($MgSO_4$), and evaporated to yield a clear oil which solidified on standing. The solid was recrystallized (C_6H_6–*n*-hexane) to yield white crystals (2.0 g, 83%): mp 108–110°; ir ($CHCl_3$) 3600 (OH), 1660 cm^{-1} (C=O, amide); NMR ($CDCl_3$) δ 1.17 (t, 3, CH_2CH_3), 1.5–3.0 (broad signals, 9, bicyclic envelope and CH_2CH_3), 3.4–3.9 (broad signals, 4, OH, H at C_1 and H at C_3), 7.3–7.9 (m, 5, aromatic). Anal. ($C_{16}H_{21}NO_2$) C, H, N.

The HCl extracts were combined, made basic with K_2CO_3, and extracted with $CHCl_3$ (2 × 40 ml). The $CHCl_3$ was dried ($MgSO_4$) and evaporated to yield no basic products.

2-Methyl-6-*trans*-phenyl-6-*cis*-propionoxy-2-azabicyclo-[2.2.2]octane (3). A solution of **7** (2.0 g, 0.005 mol) in 50 ml of EtOH was added to a Parr flask and the pH adjusted to approximately 2 with concentrated HCl. The solution was then hydrogenated (3.15 kg/cm^2) over 0.3 g of 10% Pd/C for 4 h and filtered through Celite. To the filtrate was added 2 ml of 37% CH_2O and the pH was readjusted to approximately 7 with 10% NaOH. The resulting solution was hydrogenated (3.15 kg/cm^2) over 0.3 g of 10% Pd/C for 4 h and then filtered through Celite. The filtrate was evaporated and the residue taken up in 50 ml of $CHCl_3$. The $CHCl_3$ mixture was extracted with 10% HCl (3 × 30 ml) and the extracts were combined and made basic with K_2CO_3 and extracted with $CHCl_3$ (3 × 50 ml). The $CHCl_3$ was combined, dried ($MgSO_4$), and evaporated to yield a clear oil (1.2 g). The oil was chromatographed on 8.0 g of silica gel using Et_2O–petroleum ether (1:4) as eluent to yield the desired ester (0.9 g, 64%) as a clear oil: ir (CCl_4) 1745 cm^{-1} (C=O, ester); NMR ($CDCl_3$) δ 1.03 (t, 3, CH_2CH_3), 1.2–3.4 [broad signals, 15, bicyclic envelope, CH_2CH_3, and NCH_3 (s, 2.65)], 7.3–7.8 (m, 5, aromatic); *m/e* calculated 273, found 273. Anal. ($C_{17}H_{23}NO_2$) C, H, N.

2-Methyl-5-*cis*-phenyl-5-*trans*-propionoxy-2-azabicyclo-[2.2.2]octane (4). A solution of 2-methyl-5-*cis*-phenyl-5-*trans*-hydroxy-2-azabicyclo[2.2.2]octane[7] (0.8 g, 0.004 mol) and 10 ml of propionic anhydride in 20 ml of dry C_6H_6 was refluxed for 96 h. The C_6H_6 was evaporated and the residue treated with 10% K_2CO_3 and extracted with $CHCl_3$ (3 × 50 ml). The extracts were combined, dried ($MgSO_4$), and evaporated to yield a clear oil (0.6 g, 60%): ir ($CHCl_3$) 1735 cm^{-1} (C=O, ester); NMR ($CDCl_3$) δ 1.0 (t, 3, CH_2CH_3), 1.2–3.1 [broad signals, 15, bicyclic envelope, CH_2CH_3, and NCH_3 (s, 2.28)], 7.3–7.9 (m, 5, aromatic). The picrate was made in the normal manner: mp 171–174°. Anal. ($C_{23}H_{26}N_4O_9$) C, H, N.

Pharmacological Methods. Analgetic potency was determined by the D'Armour–Smith tail-flick method[8] using male albino rats weighing 100–130 g. Each subject served as its own control and was used only once. Three control response times were determined at 15-min intervals. Groups of five rats were used for each dose of drug. After control responses were determined, a solution of the hydrochloride salt of the drug was administered ip using normal saline as the vehicle. The response was determined at 15-min intervals postinjection and a reaction time greater than the average control value was considered to indicate a state of analgesia. The ED_{50} for this study was defined as that dose of the drug which, in 50% of the animals tested, increased the reaction time by 50% at 15 min postinjection. The LD_{50} (ip) of **3** was determined by the method of Litchfield and Wilcoxin.[10] Numbers in parentheses following ED_{50} and LD_{50} data represent 95% confidence limits.

Acknowledgment. This research was supported in part by Grant 1 R01 NS09188 from the National Institute of Neurological Diseases and Stroke and in part by the Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, and is gratefully acknowledged.

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Biological Activity of C-Terminal Partial Sequences of Substance P

Ross W. Bury and Maurice L. Mashford*

Departments of Medicine and Pharmacology, University of Melbourne, St. Vincent's Hospital, Victoria, Australia.
Received October 28, 1975

Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) and the C-terminal partial sequences down to the tripeptide were synthesized by a solid-phase method. These peptides were assayed for vasodilator, spasmogenic, and venoconstrictor properties using three preparations, viz. the hind limb blood flow of the dog, isolated guinea pig ileum, and the isolated rabbit ear vein. The tripeptide and tetrapeptide possessed weak vasodilator properties only but no activity was detected on the other less sensitive preparations. The pentapeptide produced appreciable spasmogenic and vasoactive effects. Sequences of six or more C-terminal amino acids were able to elicit activity at comparable doses to that of the parent endecapeptide; however, the activity did not increase regularly with the chain length. In each assay preparation the octapeptide was the most potent peptide. It was twice as potent as substance P on a molar basis in the guinea pig ileum but the enhancement of activity beyond that of substance P was less pronounced in the vascular preparations.

The amino acid sequence of substance P was determined by Chang et al.[1] to be Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2. This peptide has been synthesized by the solid-phase method[2,3] and was found to produce identical pharmacological effects to the naturally occurring peptide.

In order to study the relationship between the biological activity and chain length, the C-terminal partial sequences of substance P down to the tripeptide were synthesized and assayed for spasmogenic, vasodilator, and venoconstrictor properties.

Synthesis and Purification. Using techniques of a similar nature to those employed by Fisher et al.[3] we have also synthesized substance P on a benzhydrylamine resin[4] (0.06 mmol of amine/g) obtained from I.C.I., Australia. The carboxyl-terminal partial sequences were obtained by

Table I. Thin-Layer Chromatography and Countercurrent Distribution of Substance P and Partial Sequences

Peptide	R_f in solvent[a]					K^b
	1	2	3	4	5	
Substance P (Tregear)	0.00	0.42 (0.03)	0.44 (0.02)	0.59 (0.04)	0.15 (0.01)	
Substance P	0.00	0.42 (0.03)	0.44 (0.02)	0.59 (0.04)	0.15 (0.01)	0.15
Decapeptide	0.00	0.47	0.40			0.15
Nonapeptide	0.00	0.43	0.42			0.18
Octapeptide	0.09	0.79	0.81			5.00
Heptapeptide	0.00	0.61	0.54			1.11
Hexapeptide	0.55	0.87	0.88			1.55
Pentapeptide	0.20	0.86	0.83			1.61

[a] 1, dichloromethane–methanol (9:1); 2, 1-butanol–pyridine–acetic acid–water (30:20:6:24); 3, 1-butanol–pyridine–acetic acid–water (40:10:10:20); 4, ethyl acetate–pyridine–acetic acid–water (5:5:1:3); 5, 1-butanol–ethyl acetate–acetic acid–water (1:1:1:1). Figures in parentheses are standard errors of the means ($N = 6$). [b] K = distribution constant[5] in *sec*-butyl alcohol–1% acetic acid (1:1).

Table II. Amino Acid Analyses of Substance P and Partial Sequences

Peptide	Mole ratio of amino acid								
	Arg	Pro	Lys	Glu	Phe	Gly	Leu	Met	NH_3
Substance P (Tregear)	1.03	1.89	1.00	1.89	2.10	1.13	0.82	1.02	3.24
Substance P	0.97	1.92	1.05	1.94	2.08	1.11	1.00	1.00	3.32
Decapeptide		1.97	1.03	1.88	1.97	1.00	1.00	1.03	*a*
Nonapeptide		1.06	1.02	1.96	2.00	1.02	1.00	1.02	*a*
Octapeptide		0.87		1.86	2.06	1.06	1.00	0.98	*a*
Heptapeptide				1.93	2.00	1.09	1.05	0.92	*a*
Hexapeptide				0.94	2.00	1.12	1.05	0.89	*a*
Pentapeptide					1.96	1.04	1.00	0.96	*a*
Tetrapeptide					0.98	1.01	1.00	1.01	*a*
Tripeptide						1.05	1.00	0.97	*a*

[a] Appreciable quantities of NH_3 in the buffers during amino acid analyses precluded accurate assessment.

removing an aliquot of the peptide-resin from the reaction vessel after the coupling of each amino acid in the developing peptide chain. In this way, the C-terminal penta-, hexa-, hepta-, octa-, nona-, and decapeptide fragments of substance P were obtained during the synthesis of the whole molecule. The purification of the deprotected, liberated peptide differs from those methods previously reported.[2,3] The cleaved peptides were desalted by gel filtration on a column of Sephadex G-15 eluted with 0.5 M acetic acid. These were subjected to 120 transfers of countercurrent extraction in a microautomatic model of countercurrent apparatus (Gallenkamp EV 810/820) using a *sec*-butyl alcohol–1% acetic acid (1:1) solvent pair. The distribution constants[5] for substance P and the partial sequences are shown in Table I. The peptide fractions were finally purified by gel filtration on a Sephadex G-15 (column 0.9 × 60 cm) in 0.2 M acetic acid. The yield of pure substance P after this step was 15.6 mg (12%).

When chromatographed on aluminum sheets coated with a thin layer of silica gel (Merck, 5553) in several solvent systems, the purified substance P and its partial sequences were homogeneous (Table I). Amino acid analyses confirmed their correct mole ratios (kindly performed by Dr. F. Morgan, School of Medical Research, St. Vincent's Hospital).

In a different synthesis, but using similar techniques, the C-terminal tripeptide and tetrapeptide were prepared by the I.C.I. Research Laboratories (Australia) for assessment of vasodilator activity. Although insufficient amounts of these peptides were available for chromatographic characterization, satisfactory amino acid analyses were obtained (Table II).

Substance P, synthesized and purified according to the procedures described above, was chromatographically identical and equiactive with a sample generously provided by Tregear[2] (Tables I and II).

Biological Activity. The relative potencies of the peptides were measured using three preparations, viz. the hind limb of dog, the isolated guinea pig ileum, and the isolated rabbit ear vein.

Table III. Biological Activity of Substance P and Partial Sequences

	Potency[a]		
	Dog femoral artery blood flow ($N = 4$)	Guinea pig ileum ($N = 6$)	Rabbit ear vein ($N = 4$)
Substance P (Tregear)	103 (5)	99 (8)	100 (4)
Substance P	100	100	100
Decapeptide	68 (4)	60 (5)	94 (6)
Nonapeptide	128 (17)	157 (22)	107 (12)
Octapeptide	164 (16)	201 (26)	128 (10)
Heptapeptide	104 (7)	126 (13)	98 (8)
Hexapeptide	65 (10)	108 (14)	48 (15)
Pentapeptide	4.2 (0.8)	2.2 (0.2)	0.9 (0.2)
Tetrapeptide	2.6×10^{-2} (0.8)		
Tripeptide	1.1×10^{-3} (0.4)		

[a] The relative potencies of the C-terminal partial sequences of substance P (substance P = 100). Figures in parentheses are standard errors of the means.

(a) Hind Limb Blood Flow in the Dog. Mongrels (10–25 kg) were given morphine (2 mg/kg iv) and anesthetized with α-chloralose (12 mg/kg iv). Arterial blood pressure was recorded from the brachial artery using a Statham P23dB pressure transducer. Femoral artery blood flow was measured with an electromagnetic flow meter (E.M.I., Australia) and a Gilson recorder. The peptides were injected into the femoral artery 2–3 cm distal to the flow probe. Peak increase in blood flow was the response measured. Full dose–response curves for the peptides were recorded. For the various peptides, the slopes were effectively parallel and the maximal responses identical. The potencies relative to substance P were expressed as the mean ratios of doses for each peptide which produced 25, 50, and 75% of the maximum response (ED_{25}, ED_{50}, and ED_{75}, respectively).

The tripeptide, tetrapeptide, and pentapeptide possessed weak vasodilator properties but the hexapeptide and the larger partial sequences increased vasodilator activity greatly (Table III). However, on a molar basis, the activity did not increase regularly with the chain length; the octapeptide and nonapeptide were considerably more potent than the endecapeptide, substance P.

In this preparation, 10^{-14} mol of substance P could be detected regularly (mean ED_{50} 1.4×10^{-13} mol of substance P). This sensitivity combined with absence of tachyphylaxis aided the evaluation of the activity of relatively small amounts of the fractions with low vasodilator activities. Injections of greater than 5×10^{-12} mol of substance P were required to elicit appreciable hypotensive responses in these animals.

(b) Isolated Guinea Pig Ileum. Segments of distal guinea pig ileum (approximately 4 cm in length) were suspended in 25-ml organ baths containing aerated Tyrode's solution at 32 °C. Contractions were monitored isotonically by Harvard smooth muscle transducers. All of the partial sequences down to the pentapeptide possessed appreciable spasmogenic activity, the octapeptide having the greatest potency. No activity was elicited by the tetrapeptide or tripeptide in concentrations up to 10^{-6} M. The relative spasmogenic potencies of substance P and the partial sequences listed in Table III were calculated from full concentration–response curves as described in the previous section. The mean concentration of substance P required to cause a contraction of 50% of the maximal response was 2.5×10^{-9} M.

(c) Isolated Rabbit Ear Vein. Segments of the central veins of the rabbit ear were perfused with Krebs bicarbonate solution at 37 °C using the techniques described by Horowitz and Mashford.[6] In order to prevent tachyphylaxis, doses below 3.7×10^{-12} mol of substance P were administered. Dose–response curves of the venoconstrictor effects of substance P and the partial sequences were constructed. The mean relative potencies were calculated from the ED_{25} and ED_{50} for each peptide (mean ED_{50} of substance P 3.0×10^{-12} mol). Again, the octapeptide was the most active fragment of the C-terminal partial sequences (Table III). The tetrapeptide and tripeptide were inactive in doses up to 5×10^{-9} mol.

Discussion

The relative potencies of the C-terminal partial sequences of substance P were similar in the vascular and intestinal tissues. Although vasodilator activity of the tripeptide and tetrapeptide could be evaluated in the hind limb blood flow assay, no activity could be detected in the other less sensitive preparations. The pentapeptide had weak spasmogenic and vasoactive properties but sequences of six or more C-terminal amino acids were necessary to elicit activity at comparable doses to that by the parent endecapeptide. The partial sequences of the structurally related endecapeptides, physalaemin and eledoisin, show similar activity profiles in this respect.[7-9] In each assay preparation, the C-terminal octapeptide of substance P was the most potent material but the enhancement of activity beyond that of substance P was less pronounced in the vascular preparations. These results are in close agreement with those of Bergmann et al.[10] in which the guinea pig ileum was used as the tissue for assay.

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