Br. J. Pharmac. (1975), 55, 221-227

RELATIVE POTENCY OF BOMBESIN-LIKE PEPTIDES

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1 The pharmacological activity of two natural bombesin-like peptides, alytesin and litorin, and 25 related synthetic peptides has been compared to that of bombesin.

2 The minimum length of the amino acid chain required for the first appearance of bombesin-like effects was represented by the C-terminal heptapeptide, and the minimum length for maximal effects by the C-terminal nonapeptide. The latter possessed approximately the same activity as bombesin and may be considered a good substitute.

3 Both the tryptophan and histidine residues seemed to be essential for bombesin-like activity.

4 The C-terminal octapeptide was less active than either bombesin or the C-terminal nonapeptide and its action was more rapid in onset and less sustained.

5 Litorin apparently has an intermediate position between bombesin octapeptide and bombesin nonapeptide in the speed and duration of its effects. The relationship between structure and activity is discussed.

Introduction

Four natural bombesin-like peptides have been isolated from methanol extracts of the amphibian skin: the tetradecapeptides bombesin from *Bombina bombina* and *Bombina variegata variegata* and alytesin from *Alytes obstetricans* (Anastasi, Erspamer & Bucci, 1971), the endecapeptide ranatesin from *Rana pipiens* (Nakajima, Tanimura & Pisano, 1970), and the nonapeptide litorin from *Litoria (Hyla) aurea* (Anastasi, Erspamer & Endean, 1975). All these peptides have in common the C-terminal octapeptide, with the only difference that in bombesin and alytesin the amino acid residue at position 2 from the C-terminus is leucine, and in ranatesin and litorin it is phenylalanine.

In order to determine the minimum length of the amino acid chain required for the first appearance of bombesin activity, and the minimum length necessary for maximal effects, 25 bombesin-like peptides have been synthesized and compared with bombesin. Alytesin and litorin have been included in this comparison but ranatensin was not available. For a few peptides, and especially for the C-terminal nonapeptide of bombesin, not only were effects on smooth muscle preparations investigated, but also effects on blood pressure, gastric acid secretion, and intestinal myo-electric activity.

Methods

The methods employed were identical to those described in detail in earlier papers (Erspamer, Falconieri Erspamer, Inselvini & Negri, 1972a; Erspamer, Melchiorri & Sopranzi, 1972b; Bertaccini, Erspamer, Melchiorri & Sopranzi, 1974; Caprilli, Melchiorri, Improta, Vernia & Frieri, 1975),

Alytesin was the natural peptide prepared by Anastasi *et al.* (1971); all other peptides, including caerulein, were synthesized at the Farmitalia S.p.A., Research Laboratories, Milan.

Results

Isolated smooth muscle preparations

In the six isolated smooth muscle preparations examined in this study, only stimulant actions and

Table 1 Relative potency of bombesin-like peptides, on a weight basis (bombesin = 100)

Peptide	*Test preparations*					
	Rat uterus	*Rat urinary bladder*	*Guinea-pig urinary bladder*	*Guinea-pig large intestine*	*Kitten small intestine*	*Rat large intestine*
Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 Bombesin	100	100	100	100	100	100
Pyr-Gly-Arg-Leu-Gly-Thr-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 Alytesin	70-100 (5)	80-120 (3)	–	75-125 (3)	70-200 (7)	75-100 (4)
Pyr-Gln-Trp-Ala-Val-Gly-His-Phe-Leu-Met-NH_2 Litorin	200-600 (10)	150-500 (8)	150-250 (7)	200-500 (6)	150-350 (20)	300-800 (5)
His-Leu-Met-HN_2	< 0.1 (2)	< 0.1 (3)	–	< 0.1 (2)	0.2 (2)	–
Ala-Val-Gly-His-Leu-Met-HN_2	< 0.05 (5)	< 0.01 (2)	0.1-0.2 (2)	< 0.1 (7)	< 0.1 (5)	< 0.1 (3)
BOC-Ala-Val-Gly-His-Leu-Met-NH_2	< 0.1 (3)	< 0.1 (3)	–	< 0.1 (3)	< 0.1 (3)	< 0.1 (3)
Trp-Ala-Val-Gly-His-Leu-Met-NH_2	2.5-10 (4)	0.2-0.5 (4)	0.2-0.4 (3)	0.1-0.5 (7)	< 0.1-3 (10)	0.5-1 (3)
BOC-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	30-60 (7)	6-14 (3)	1-3 (3)	1-3 (10)	0.5-1 (10)	3-6 (3)
Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	20-30 (6)	2-10 (4)	2-4 (4)	2-10 (7)	1-4 (17)	10-20 (3)
BOC-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	40-75 (9)	30-60 (4)	–	25-60 (10)	40-100 (6)	50-80 (3)
Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	130-300 (7)	150-300 (4)	200-350 (3)	100-120 (7)	100-150 (11)	90-130 (3)
BOC-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	160-500 (7)	300-500 (3)	–	150-180 (3)	110-160 (7)	100-130 (3)
Thr-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	150-400 (5)	150-350 (3)	–	100-140 (7)	100-150 (5)	–
Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	120-200 (5)	100-150 (3)	150-200 (3)	110-200 (5)	90-200 (8)	70-90 (3)
BOC-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	30-60 (5)	25-40 (3)	–	30-60 (4)	30-110 (7)	–
Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	50-200 (8)	100-170 (5)	200-400 (5)	100-160 (6)	80-150 (8)	90-100 (3)
BOC-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-NH_2	35-130 (5)	70-120 (3)	–	120-210 (5)	60-120 (6)	35-80 (3)
Asn-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	70-100 (5)	4-10 (3)	4-7 (3)	3-12 (5)	1-4 (5)	20-40 (3)
Phe-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	150-220 (7)	6-20 (3)	1-2 (3)	7-35 (6)	4-13 (5)	60-120 (3)
BOC-Phe-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	250-350 (3)	20-60 (3)	–	5-25 (3)	1.5-5 (4)	–
Val-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	55-85 (5)	1-5 (3)	1-2 (3)	2-8 (4)	1.5-5 (6)	35-70 (3)
BOC-Val-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	200-320 (4)	2-8 (6)	–	–	5-10 (7)	–
Tyr-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	150-250 (3)	150-200 (3)	–	80-120 (3)	100-150 (3)	–
BOC-Asn-Gln-Trp-Ala-Val-Gly-His(DNP)-Leu-Met-NH_2	5-12 (3)	6-13 (3)	—	1-2 (3)	1-1.5 (3)	4-6 (3)
Pyr-Gly-Arg-Leu-Gly-Thr-Gln-Trp	< 0.01 (2)	< 0.1 (2)	–	< 0.1 (2)	< 0.1 (2)	< 0.1 (2)
Pyr-Gly-Arg	< 0.01 (2)	< 0.1 (2)	–	< 0.01 (2)	< 0.1 (2)	< 0.1 (2)

Pyr, pyroglutamic acid; BOC, *ter*-butyloxycarbonyl; DNP, 2,4-dinitrophenyl; –, not tested. The number of experiments is given in parentheses.

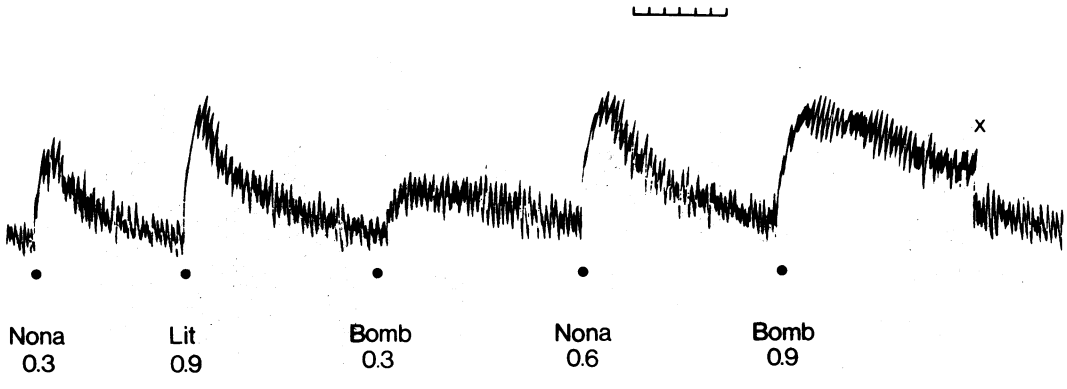

Figure 1 Rat anaesthetized with urethane. Responses of the urinary bladder *in situ* to different doses of bombesin (Bomb), litorin (Lit) and bombesin nonapeptide (Nona). Time marks, 1 minute. At x, drum stopped for 10 minutes. Duration of effect was greatest for bombesin, least for litorin.

never inhibitory effects were observed. Results are summarized in Table 1 in which the activity of bombesin was taken as 100, and that of the bombesin-like peptides was expressed as a percentage.

The stimulant effects displayed on movements and tone by all members possessing a sequence of more than eight amino acid residues were (with the exception of litorin) almost indistinguishable from those of bombesin. However, litorin and to a greater extent the octapeptides frequently caused a stimulation which was more rapid in onset and in disappearance, on washing with fresh physiological solution, than that produced by bombesin.

Results shown in Table 1 were essentially confirmed on isolated preparations of human gastrointestinal tract by Bertaccini, Impicciatore, Molina & Zappia (1974): bombesin 100, C-terminal nonapeptide 100-120, octapeptide 10-30; heptapeptide 1-5, hexapeptide < 1.

Rat and guinea-pig urinary bladder in situ

Relative potencies (bombesin = 100) on the rat and guinea-pig urinary bladder *in situ* are shown in Table 2. The figures in the table refer only to the intensity of action. For the duration of response which they produced the peptides may be ranged in the following order: bombesin = endecapeptide and decapeptide > nonapeptide > litorin > octapeptide and Asn^1-octapeptide > Phe^1-octapeptide and Val^1-octapeptide (Figure 1).

Guinea-pig gall bladder in situ

Bombesin, litorin and the bombesin nonapeptide were potent stimulants of the guinea-pig gall bladder *in situ*. In the experiment illustrated in Figure 2 the relative potency of the three above peptides and of caerulein was as follows: bombesin 100; litorin 200-250; nonapeptide 100-125; caerulein 2000.

Whereas the shapes of the contractions elicited by litorin, bombesin nonapeptide and caerulein were very similar, the contraction produced by bombesin was less rapid in onset and more sustained.

Systemic blood pressure

The relative potency of bombesin-like peptides on the blood pressure could be evaluated only approximately because of the development of tachyphylaxis.

Table 2 Potencies of bombesin-like peptides relative to bombesin (100) on the rat and guinea-pig urinary bladder *in situ*

	Urinary bladder in situ	
Peptide	***Rat***	***Guinea-pig***
C-terminal		
heptapeptide	0.1 (3)	0.2 (3)
octapeptide	2.5 (3)	5-6 (3)
Asn^1-octapeptide	4.0 (3)	
Phe^1-octapeptide	2.0 (3)	
Val^1-octapeptide	1.2 (3)	
nonapeptide	100-200 (5)	150-250 (3)
decapeptide	75 (3)	100-150 (3)
endecapeptide	75 (3)	100-150 (3)
Litorin	100-200 (5)	100-200 (3)

Number of preparations given in parentheses.

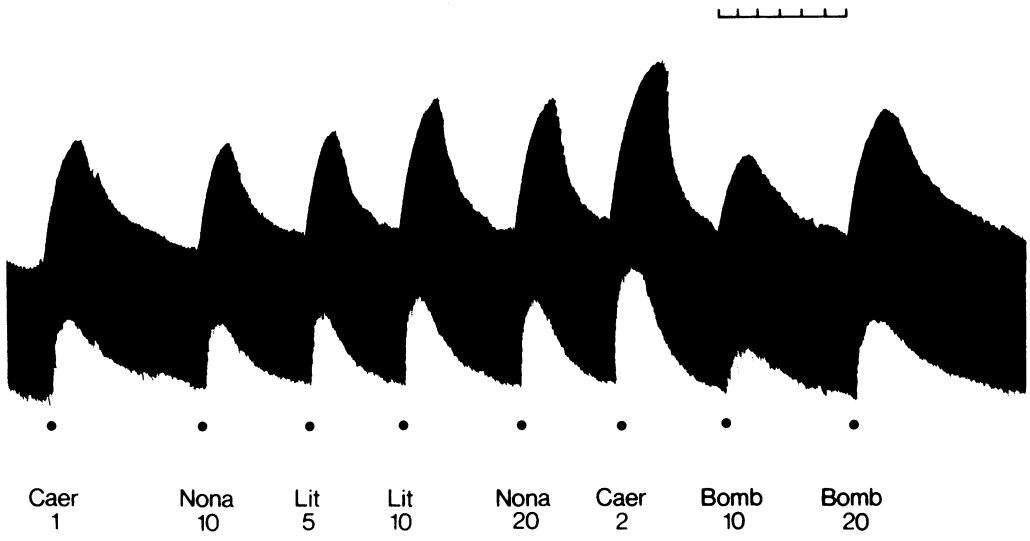

Figure 2 Guinea-pig anaesthetized with urethane. Responses of the gall bladder *in situ* to different intravenous doses (all in ng/kg) of bombesin (Bomb), litorin (Lit), bombesin nonapeptide (Nona) and caerulein (Caer). Time marks, 1 minute.

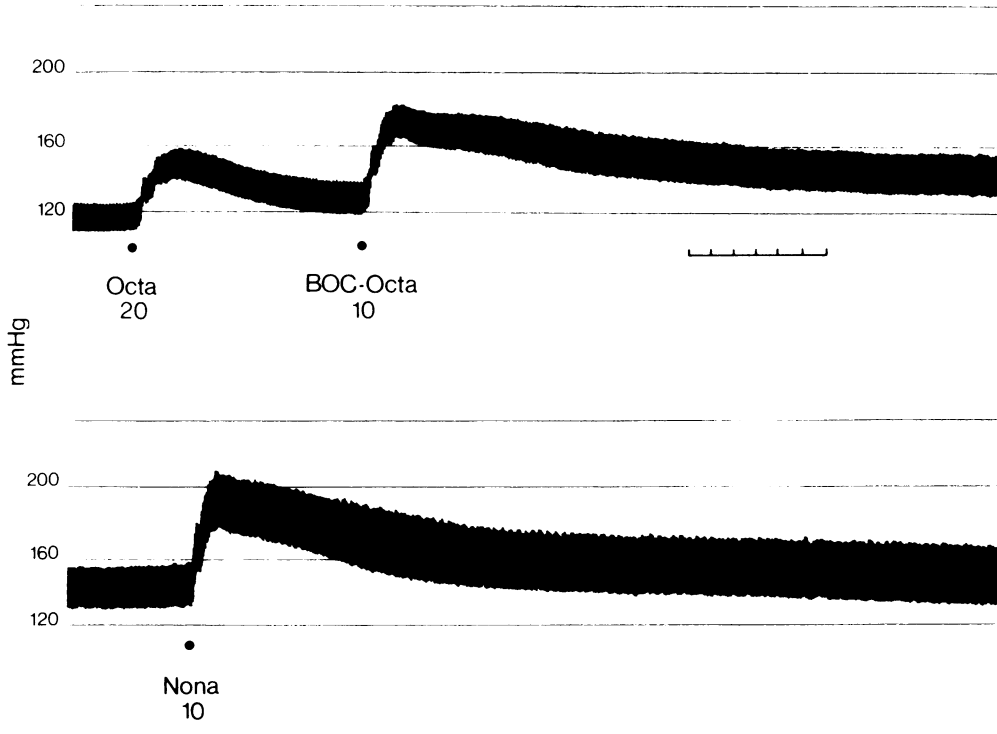

Figure 3 Blood pressure of a dog anaesthetized with sodium pentobarbitone (40 mg/kg, i.v.). Time marks, 1 minute. The effects of intravenous doses (in μg/kg) of octapeptide (Octa), BOC-octapeptide (BOC-Octa) and nonapeptide (Nona) of bombesin are shown. Note the increase in both intensity and duration of action produced by the passage from octapeptide to BOC-octapeptide and then to nonapeptide.

Dog Hexapeptide, BOC-hexapeptide, heptapeptide and BOC-heptapeptide were inactive up to 25 μg/kg given by rapid intravenous injection. Threshold doses for the octapeptide were 10 to 20 μg/kg. The relative potencies of the active peptides examined were as follows: bombesin = alytesin = nonapeptide > BOC-octapeptide > octapeptide (Figure 3). Equiactive doses of bombesin, nonapeptide and octapeptide produced effects of decreasing duration, in that order. Litorin was considerably less potent than bombesin, and its action less sustained.

Cat In two experiments the BOC-octapeptide showed 10-20% of the hypertensive activity of bombesin, and the nonapeptide 60%.

Rat The octapeptide possessed barely 3 to 5% of the hypertensive activity of bombesin, the nonapeptide 50%. Litorin was generally more potent than bombesin, but its action was less sustained.

Gastrin release and gastric acid secretion

The effects of the C-terminal nonapeptide of bombesin, infused at a rate of 10 ng kg^{-1} min^{-1} over a 60 min period were studied in gastric fistula dogs provided with Heidenhain pouches. Results are shown in Figure 4. On the whole, the nonapeptide produced the same release of gastrin as bombesin, at comparable infusion rates. However, gastric acid secretion was stimulated somewhat less by the nonapeptide, both in regard to peak response and to duration of effect.

Litorin was half as active as bombesin, and effects elicited by the peptide sharply decreased before the infusion of the peptide was stopped (Endean, Erspamer, Falconieri Erspamer, Improta, Melchiorri, Negri & Sopranzi, 1975).

Myo-electric activity of the dog duodenum

The C-terminal nonapeptide of bombesin showed approximately 75% of the activity of the parent peptide, and litorin 50% on the electric activity of the dog duodenum *in situ* (Caprilli *et al.*). The hepta- and the octapeptide were inactive at infusion rates of up to 50 ng kg^{-1} min^{-1}.

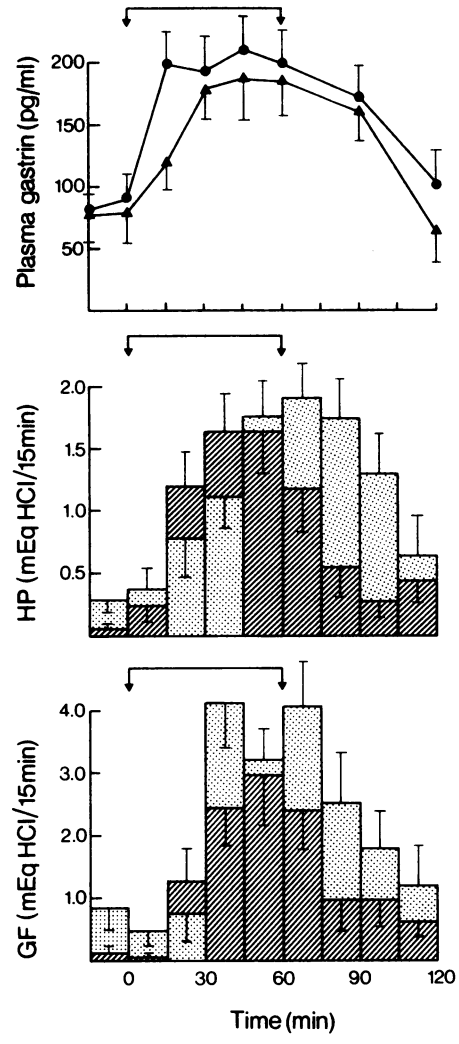

Figure 4 Gastric fistula dogs provided with Heidenhain pouches. Plasma gastrin levels and acid outputs, in the Heidenhain pouch (HP) and the main stomach (GF) following intravenous infusion, for 60 min, of 10 ng kg^{-1} min^{-1} of bombesin (▲, stippled areas) and bombesin nonapeptide (●, cross-hatched areas). Columns represent the mean of 2-3 measurements in each of 2-3 dogs. Vertical bars show s.e. mean.

Discussion

From the comparative bioassay of the 27 bombesin-like peptides examined in this study the following conclusions may be drawn:

(a) A minimum C-terminal sequence of seven amino acid residues was required for the appearance of bombesin-like effects. The C-terminal hexapeptide was quite inactive and similarly the N-terminal octapeptide (< 0.1%). The preparation most sensitive to the heptapeptide was the rat uterus.

(b) Addition of the glutamine residue to the N-terminus of the heptapeptide produced a 5- to 20-fold increase of activity on all preparations

tested. Addition of an asparagine residue to the N-terminus of the octapeptide also produced a striking increase of activity on all preparations tested. The C-terminal nonapeptide of bombesin was as active as, or even more active, than bombesin on all isolated smooth muscle preparations examined. On *in vivo* test objects the nonapeptide had on a weight basis, 70 to 200% of the activity of bombesin.

(c) Protection of the N-terminal alanine residue of the hexapeptide with a *ter*-butyloxycarbonyl group (BOC-hexapeptide) did not cause the appearance of any activity. However, protection with BOC of the N-terminal tryptophan residue of the heptapeptide (BOC-heptapeptide) produced a 5- to 20-fold increase of activity, yielding a compound which was as active as the octapeptide itself; similarly the BOC-octapeptide was several times more potent than the octapeptide, approaching the activity of the nonapeptide. The BOC-nona-, deca- and endecapeptides, on the contrary, did not possess greater activity than the corresponding peptides lacking the BOC group.

Thus, the presence of the tryptophan residue seemed necessary for the appearance of the bombesin-like activity. Tryptophan could not be substituted by the BOC group. However, further lengthening of the peptide chain from the hepta- to the nona-peptide, which caused striking increase in activity up to an optimum in the nonapeptide, could be obtained not only by addition of amino acid residues, but also by addition of the BOC group.

(d) No appreciable differences in activity could be seen between the nona- and the decapeptide, and between the deca- and the endecapeptide. These peptides were also hardly distinguishable from each other from a qualitative point of view, for example in the appearance of tachyphylaxis and in the shape of the response.

(e) Replacement in the C-terminal octapeptide of bombesin of glutamine by asparagine or valine produced no change in activity, except perhaps in the stimulant action on the rat uterus, which appeared to be increased. Replacement of glutamine by phenylalanine, on the contrary, increased the activity on nearly all preparations tested.

It should be stressed that all the octapeptides examined differed from peptides having a longer chain of amino acid residues, in that their action was more rapid but less sustained, denoting either a more rapid inactivation or a less tenacious binding to the receptor sites.

(f) Addition of a 2,4-dinitrophenyl group (DNP) to the histidine residue of the BOC-nonapeptide drastically reduced the activity of the peptide, demonstrating the importance for bombesin-like activity of an intact histidine residue.

All conclusions listed under (c), (d), (e) and (f) were drawn from experiments on isolated smooth muscle preparations.

Parallel bioassay showed that the most simple bombesin-like peptide closely mimicking bombesin was the C-terminal nonapeptide of bombesin itself. Qualitatively its spectrum of activity was very similar to that of bombesin, except for the somewhat shorter duration of the effects *in vivo*; quantitatively its potency was, as already stated, equal to or greater than that of bombesin on isolated preparations, and more than half that of bombesin on gastrin release and electrical activity of the gut. Thus, it appears that the nonapeptide possesses all the prerequisites of an excellent substitute for bombesin in experimental as well as clinical investigation.

Two other natural bombesin-like peptides, in addition to bombesin, have been isolated by our group from the amphibian skin; the tetradecapeptide alytesin and the nonapaptide litorin, the smallest natural bombesin-like peptide so far found. Alytesin was indistinguishable from bombesin on all smooth muscle preparations tested. Litorin, differing from the C-terminal nonapeptide of bombesin in having the pyroglutamic acid[1] and phenylalanine[8] residues substituted for the asparagine[1] and leucine[8] residues, showed consistent differences from bombesin in its spectrum of biological activity. They have been described in detail in a preceding paper (Endean *et al.*, 1975).

This work was supported by grants from the Consiglio Nazionale delle Ricerche, Rome.

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Received April 10, 1975.
Revised May 27, 1975.)