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PARALLEL BIOASSAY OF BOMBESIN AND LITORIN, A BOMBESIN-LIKE PEPTIDE FROM THE SKIN OF *Litoria aurea*

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1 The spectrum of biological activity exhibited by litorin, a bombesin-like nonapeptide found in extracts of the skin of the Australian leptodactylid frog *Litoria aurea* was compared with that exhibited by the tetradecapeptide bombesin.

2 Litorin proved to be more potent than bombesin on isolated smooth muscle preparations and on the urinary bladder *in situ*. However, it was less potent on dog systemic blood pressure and kidney vasculature, activation of the renin-angiotensin system being slight or lacking.

3 Gastrin release and acid secretion produced by litorin was more rapid in onset but less intense and less sustained than that elicited by bombesin. The same could be observed for pancreatic secretion.

4 Gall bladder contraction stimulated by litorin was probably caused by a double action of the peptide, directly on the bladder smooth muscle, and indirectly by cholecystokinin release.

5 In its effects on the myo-electric activity of the dog duodenum (inhibition of spikes and increase in frequency of pacesetter potentials leading to the appearance of a sequence of slow and small potentials) litorin possessed approximately 50 to 70% of the activity of bombesin.

Introduction

After the isolation of bombesin from methanol extracts of the skin of the two European discoglossid frogs *Bombina bombina* and *Bombina variegata variegata* and of alytesin from skin extracts of another European discoglossid frog, *Alytes obstetricans* (Anastasi, Erspamer & Bucci, 1971), a third bombesin-like peptide was isolated by our research group from methanol extracts of the skin of the Australian leptodactylid frog *Litoria (Hyla) aurea*. It is presumably present in the skin of other *Litoria* species and of other Australian amphibian genera as well (Anastasi, Erspamer & Endean, 1973; Erspamer, Negri, Falconieri Erspamer & Endean, 1975). Litorin has been reproduced by synthesis (Angelucci & De Castiglione, 1975). Its amino acid composition and sequence are shown below, together with those of bombesin and alytesin:

Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 bombesin

Pyr-Gly-Arg-Leu-Gly-Thr-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 alytesin

Pyr--------Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2 litorin.

It may be seen that whereas only minor differences exist between the tetradecapeptides bombesin and alytesin, the latter being Gly^2-Thr^6-bombesin, litorin differs conspicuously from the other two members of the family, being a simple nonapeptide, lacking the entire amino acid sequence Gln-Arg-Leu-Gly-Asn and having a phenylalanine residue substituted for the leucine residue at position 2 from the C-terminus.

This paper describes the results of a parallel bioassay of bombesin and litorin on a number of *in vitro* and *in vivo* test objects, virtually covering the whole spectrum of biological activity of bombesin. It will be seen that litorin closely mimics bombesin in its pharmacological effects although there are some notable differences.

Methods

The methods employed were identical to those described in detail in earlier papers (Erspamer, Falconieri Erspamer, Inselvini & Negri, 1972a;

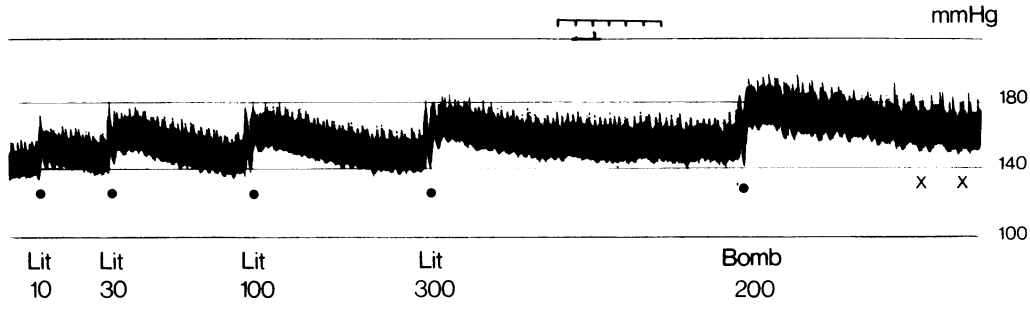

Figure 1 Blood pressure of a dog anaesthetized with sodium pentobarbitone (40 mg/kg, i.v.). Time marks, 1 minute. The effects of increasing intravenous doses (ng/kg) of litorin (Lit) and of one dose of bombesin (Bomb) are shown. Note the moderate dose-response relationship for litorin, especially in the duration of pressure rise. Bombesin was more potent than litorin both in intensity and duration of action. At X drum stopped for 10 minutes.

Erspamer, Melchiorri & Sopranzi, 1972b; Bertaccini, Erspamer & Impicciatore, 1973; Bertaccini, Erspamer, Melchiorri & Sopranzi, 1974; Erspamer, Improta, Melchiorri & Sopranzi, 1974).

To measure the flow of gall bladder bile, and hence indirectly gall bladder contraction, dogs provided with a Gregory cannula implanted in the duodenum behind the orifice of the choledochus, were injected intravenously 8 h before the experiment, with a total dose of 50 μCi of ^{125}I-biligraphin. Bile was collected from the cannula and the radioactivity of the bile was counted in a well type scintillator (Italelettronica S.p.A., Rome). During the interval between biligraphin administration and the start of the experiment, all biligraphin had been eliminated except that stored in the gall bladder and thus only gall bladder bile was labelled.

The samples of bombesin and litorin used were prepared by synthesis at the Farmitalia S.p.A. Research Laboratories, Milan; ^{125}I-biligraphin was obtained from Schering A.G., Berlin.

Results

Isolated smooth muscle preparations

Table 1 shows the activity of litorin on various smooth muscle preparations expressed as a percentage of that of bombesin (taken as 100). On the urinary bladder *in situ* of the rat and the guinea-pig, the activity of litorin was 100-300 (3 preparations), and 100-200 (3 preparations), respectively. On all these preparations bombesin produced stimulation, involving the appearance and reinforcement of movements and increase of tone.

In general the response to litorin in isolated preparations was more rapid in onset than that to bombesin and, upon washing with fresh physiological solution, relaxation was more rapid. The same rapid onset and disappearance of the activity of litorin could also be clearly seen in the rat urinary bladder *in situ*. Tachyphylaxis frequently occurred, but it was less intense for litorin than for bombesin.

Systemic blood pressure

Owing to the rather rapid appearance of tachyphylaxis, data on blood pressure can only be approximate.

Dog Like bombesin, litorin elicited a moderate, prolonged rise in systemic blood pressure, rarely exceeding 30 to 40 mmHg. The threshold dose by rapid intravenous injection was of the order of 10-30 ng/kg. The effect showed little relationship to dose in its intensity although a relationship between dose and duration of effect was more evident. Bombesin was more active than litorin, especially in the duration of the pressure rise (Figure 1).

Table 1 Activity of litorin expressed as a percentage of that of bombesin (taken as 100) on isolated smooth muscle preparations

Smooth muscle	*No. of preparations*	*Activity of litorin*
Rat uterus	10	200-600
colon	5	300-800
stomach	4	200-300
urinary bladder	8	150-500
Guinea-pig large intestine	6	200-300
urinary bladder	7	150-250
gall bladder	5	50-100
Kitten small intestine	20	150-350

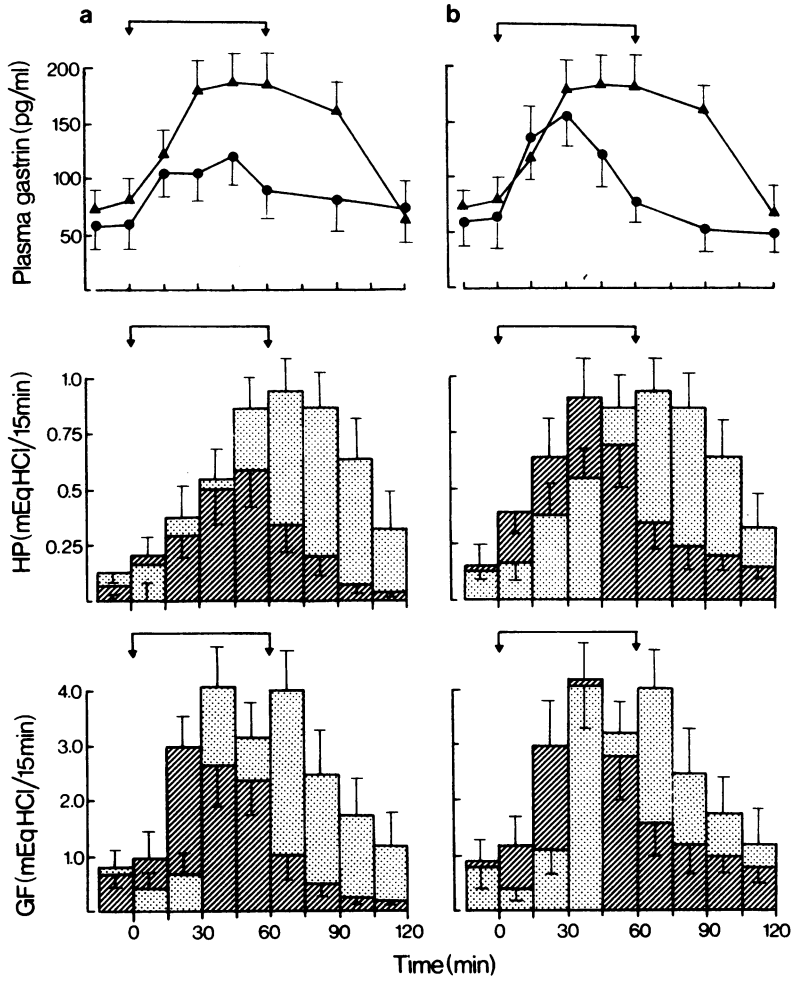

Figure 2 Gastric fistula dogs provided with Heidenhain pouches. Plasma gastrin levels and acid output, in the Heidenhain pouch (HP) and in the main stomach (GF), following intravenous infusions, for 60 min, of bombesin (▲ and stippled areas) and of litorin (● and hatched areas). Doses of bombesin were 10 ng kg^{-1} min^{-1} both in (a) and (b), doses of litorin were 10 ng kg^{-1} min^{-1} in (a) and 20 ng kg^{-1} min^{-1} in (b). Columns represent the mean of 3 measurements in each of 3 dogs. Vertical bars show s.e. mean.

Rabbit In this species litorin was generally hypotensive at 10 to 100 ng/kg doses, given intravenously. Higher doses produced unpredictable results (hypertension followed by hypotension or *vice versa*), with evident tachyphylaxis. Comparison with bombesin was impossible.

Rat The rapid hypertensive effect of litorin (threshold 3-10 ng/kg, by rapid i.v. injection) was not sustained and was sometimes followed by a fall of blood pressure. Tachyphylaxis was evident, especially at high dose levels. Bombesin was often, but not always, less potent than litorin, but its effect was more prolonged.

Gastrin release and acid gastric secretion

The effects of intravenous infusions of 10 and 20 ng kg^{-1} min^{-1} of litorin were compared, in dogs provided with gastric fistulae and Heidenhain pouches, with the effects of 10 ng kg^{-1} min^{-1} of bombesin. Results are shown in Figure 2.

It may be seen that litorin was less effective than bombesin in releasing gastrin and stimulating

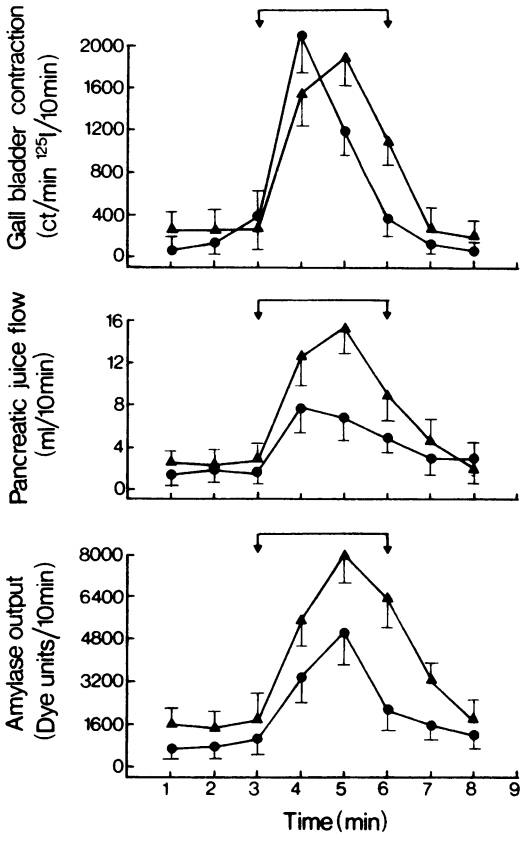

Figure 3 Conscious dogs provided with chronically implanted cannulae for collection of bile and pancreatic juice. Gall bladder contraction (as inferred from flow of radioactive bile), pancreatic juice flow and amylase output following intravenous infusion, for 60 min, of 10 ng kg^{-1} min^{-1} of bombesin (▲) and litorin (●). Each point represents the mean of 2 measurements in each of 2 dogs. Vertical bars show s.e. mean.

gastric acid secretion, both in the main stomach and in the Heidenhain pouch. In producing a maximum response, litorin was approximately half as active as bombesin on a weight basis, and one third as active on a molar basis. However, responses to litorin were more rapid and after having reached their maximum, less sustained, declining even during the infusion period. Fifteen min after the infusion of litorin 20 ng kg^{-1} min^{-1} had been stopped, gastrin levels had returned to basal values and gastric acid output was similarly greatly reduced. This is in sharp contrast to bombesin, the maximum effects of which persisted unchanged for 15-30 min after the infusion had been discontinued.

The threshold dose of litorin affecting gastrin release was 5 ng kg^{-1} min^{-1}. Plasma levels of gastrin rose from 66 ± 18 pg/ml to 81 ± 17 pg/ml.

Pancreatic secretion

Like bombesin, litorin produced in the dog the secretion of a pancreatic juice rich in amylase. However, the effect of 10 ng kg^{-1} min^{-1} litorin, infused over a 30 min period, was considerably less intense than that produced by the same dose of bombesin. Moreover, pancreatic response declined earlier for litorin than for bombesin, during the infusion period (Figure 3).

Gall bladder

Dog The motility of the gall bladder, as recorded in anaesthetized animals by measuring the bile flow through a duodenal cannula, was stimulated by bombesin and litorin as shown in Figure 3.

In this preparation litorin was as potent as bombesin, but its effects were more rapid in onset and disappearance. A peak effect with litorin was obtained after 10 min and with bombesin after 20 minutes. At the end of the 30 min infusion period, the gall bladder tone had returned to pre-infusion levels in the case of litorin, while remaining elevated for bombesin.

Guinea-pig Rapid intravenous injection of litorin caused a prompt contraction of the gall bladder, followed by rapid relaxation. The threshold dose was of the order of 2-10 ng/kg and there was often a definite dose-response relationship. Litorin was 2 to 3 times more potent than bombesin in the intensity of the response produced, on a weight basis, but equal to bombesin in the duration of the response produced.

It should be noted that the action of litorin on the guinea-pig gall bladder may well be compared with caerulein, the most potent stimulant so far known. In fact, in 14 experiments the effect of litorin was 5 to 20% that of caerulein (Figure 4).

Myo-electric activity of the gut

Caprilli, Melchiorri, Improta, Vernia & Frieri (1974) have shown that bombesin is capable of abolishing spikes and of increasing the frequency of pacesetter potentials in the duodenal and jejunal musculature of conscious dogs, which leads to disappearance of rhythmicity and appearance of an irregular sequence of slow and small potentials (uncoupling of local oscillators). Litorin had a similar effect (Figure 5). As in the case of bombesin, disappearance of spikes elicited by litorin was accompanied by complete arrest of

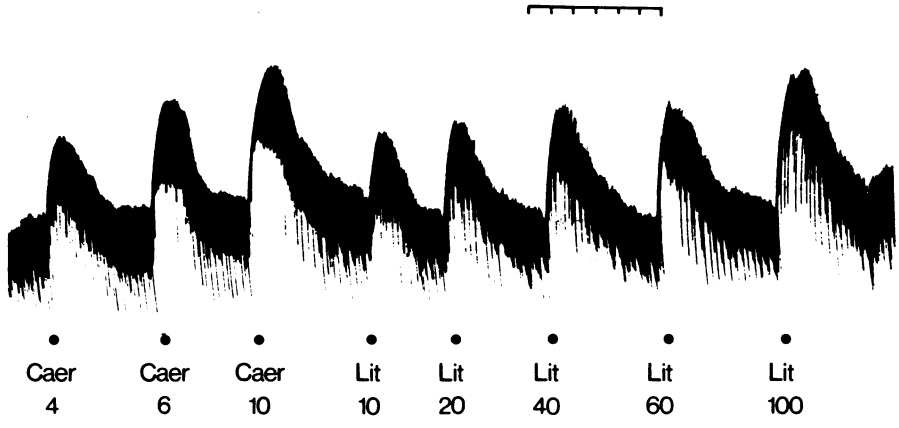

Figure 4 Guinea-pig anaesthetized with urethane. Responses of the gall bladder *in situ* to different intravenous doses (ng/kg) of caerulein (Caer) and litorin (Lit) are shown. Time marks, 1 minute. Although responses to caerulein and litorin were very similar increases in response produced by increasing the dose were more striking for caerulein than for litorin.

mechanical activity of the upper small intestine. The secondary hypermotility which followed the interruption of the peptide infusion was more evident for litorin than for bombesin.

Kidney function and renin release

In the dog bombesin caused a striking antidiuretic effect due to reduction of the glomerular filtration rate caused by a fall in intraglomerular hydrostatic pressure. This, in its turn, was provoked by vasoconstriction, invariably leading also to an activation of the renin angiotensin system.

Litorin was considerably less active than bombesin on the kidney. In fact, following intravenous infusion over a 20 min period of as much as 20 ng kg^{-1} min^{-1} of litorin, the glomerular filtration rate fell (not significantly) from 115 ± 25 to 76 ± 11 ml/min, and renin activity in arterial blood remained unchanged (1.9 ng/ml in control-blood and 2.2 ng/ml in litorin-blood). As bombesin produced significant effects on these parameters at infusion rates ranging between 1 and 3 ng kg^{-1} min^{-1} it is evident that, on a weight basis, litorin was at least 7 to 20 times less potent than bombesin.

Discussion

The nonapeptide bombesin is the smallest bombesin-like peptide found in amphibian skin. It differs from the C-terminal nonapeptide of the tetradecapeptide bombesin in having the pyroglutamyl[1] and the phenylalanyl[8] residues substituted for the threonyl[1] and the leucyl[8] residues, respectively.

It will be shown in a forthcoming paper that the activity spectrum of the C-terminal nonapeptide of bombesin closely resembles that of bombesin, with only minor quantitative differences. Litorin, on the other hand, is definitely, although not markedly different from bombesin.

Apparently the most striking diversity lies in the fact that the actions of litorin are more rapid in onset and in disappearance than those of bombesin, both in *in vitro* and *in vivo*. This could signify that it occupies its receptor sites in the target cells more rapidly but less tenaciously than bombesin. It is probable that the rapidity of action accounts for the more intense effects on isolated smooth muscle preparations and on the gall bladder *in situ*; the less tenacious binding to the receptors may account for the less intense and the less prolonged effects on blood pressure, on intrarenal vessels, on electrical activity of the gut, and finally on release of gastrin and cholecystokinin.

The gastrin-releasing activity of litorin is unequivocally demonstrated by the increase in plasma levels of immunoreactive gastrin, observed during the infusion of the peptide. However, cholecystokinin release is only presumptive, although strongly supported by the effects of litorin on pancreatic secretion and on gall bladder motility.

It seems probable that contraction of the gall

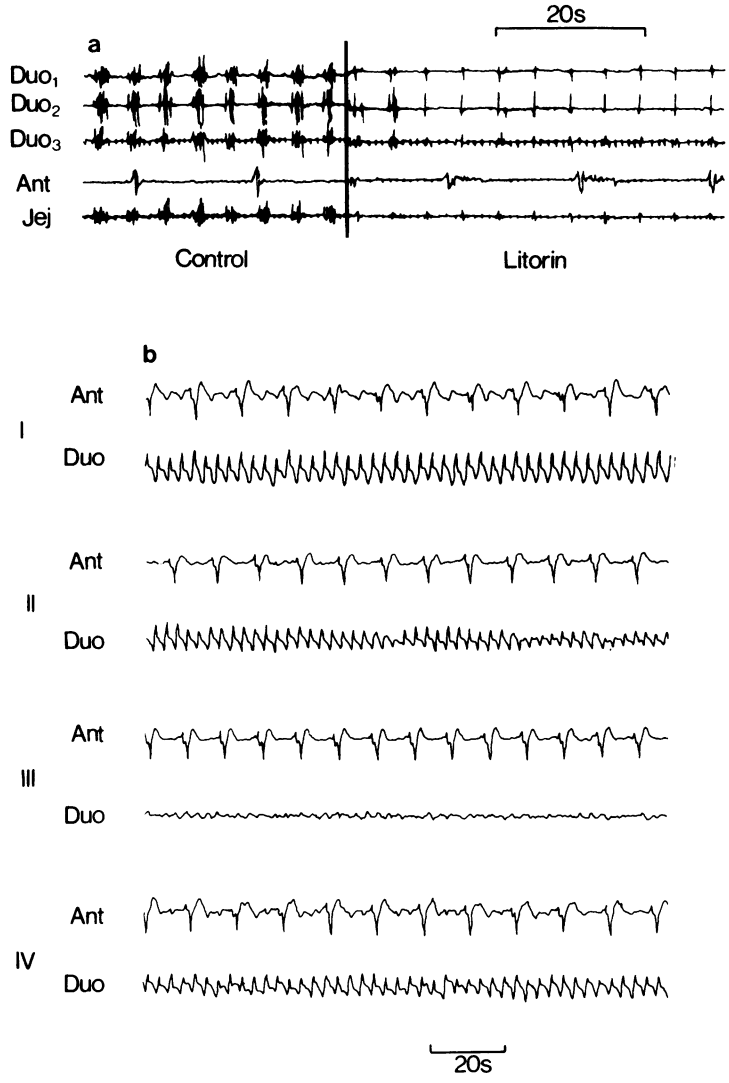

Figure 5 Conscious dog provided with electrodes chronically implanted on the serosal surface of different gastrointestinal segments. (a) Fast activity (spikes) evoked by a meal in the 1st, 2nd and 3rd duodenal portion (Duo_1, Duo_2, Duo_3), the antrum (Ant) and the jejunum (Jej), before (Control) and 10 min after start of an intravenous infusion of 2 ng kg^{-1} min^{-1} of litorin (Litorin). (b) Pacesetter potentials of the antrum (Ant) and the duodenum (Duo) before litorin administration (I), during an intravenous infusion of 10 ng kg^{-1} min^{-1} of litorin (II and III, at 10 and 30 min of infusion, respectively), and 20 min after discontinuing litorin infusion (IV).

bladder *in vivo* is the result of a twofold action of litorin, one direct on the bladder smooth muscle, and the other indirect, secondary to release of cholecystokinin. Evidence in favour of a direct mechanism is provided by the rapidity in onset and disappearance of litorin stimulation, particularly well shown by the gall bladder of the guinea-pig, as well as by the dissociation between activity ratios of litorin to bombesin on gall bladder and on pancreas. Although litorin was as active as bombesin on gall bladder motility in the dog, it was much less active on pancreatic secretion. Only measurements of cholecystokinin levels in plasma following bombesin and litorin

administration will elucidate the relative importance of the two mechanisms of action.

The most striking quantitative difference in activity between litorin and bombesin probably lies in the renal effect of the peptides. It appears that litorin has very little action on the afferent glomerular arteries.

The possibility cannot be excluded that the less sustained effects of litorin *in vivo* stem partially from the fact that, in contrast to bombesin, litorin lacks renal actions. As a consequence, both gut hormones released by litorin and litorin itself could be more easily excreted and/or inactivated by the kidney.

Litorin offers a natural example of how relatively small changes in the amino acid composition of a peptide may bring about considerable changes in its biological activity. Synthesis of bombesin and litorin analogues seem to offer interesting possibilities.

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