Br. J. Pharmac. (1972), **45**, 333–348.

Occurrence of bombesin and alytesin in extracts of the skin of three European discoglossid frogs and pharmacological actions of bombesin on extravascular smooth muscle

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Summary

1. Methanol extracts of the skin of *Bombina bombina* and *Bombina variegata variegata*, two European discoglossid frogs, contain an active tetradecapeptide, bombesin. Alytesin, a tetradecapeptide strictly related to bombesin is present in extracts of the skin of *Alytes obstetricans*, another European discoglossid frog. The American frog *Rana pipiens*, contains in its skin ranatensin, an endecapeptide related to bombesin and alytesin.

2. Passage of crude skin extracts of *Bombina* through a column of alumina yields eluates which may be considered free of other peptide contaminants and are suitable for the isolation of bombesin in a pure form.

3. Bombesin has a stimulant action on several preparations of intestinal, uterine and urinary tract smooth muscle. Sometimes the effect is easily repeatable and shows a fair proportionality to the dose, but at other times a prompt and intense tachyphylaxis is observed. Other smooth muscle preparations are poorly sensitive or insensitive to bombesin. The rat uterus, the kitten small intestine, the guinea-pig colon and the rat urinary bladder may be used for the quantitative bioassay of bombesin.

4. Bombesin-like peptides may easily be distinguished from all other naturally occurring peptides by parallel assay. They constitute a new group of active peptides possessing a peculiar spectrum of activity.

Introduction

Methanol extracts of the skin of the two European discoglossid frogs *Bombina bombina* and *Bombina variegata variegata* contain a principle which displays a number of pharmacological actions on vascular and extravascular smooth muscle and on gastric secretion (Erspamer, Falconieri Erspamer & Inselvini, 1970; Melchiorri, Sopranzi & Erspamer, 1971). This principle, called bombesin, has been isolated in a pure form and its amino acid composition and sequence have been elucidated (Anastasi, Erspamer & Bucci, 1971). Bombesin is a tetradecapeptide having the amino acid sequence shown below (I), which closely resembles that of alytesin (II), a substance found in extracts of the skin of *Alytes obstetricans*, another European discoglossid frog (Anastasi *et al.*, 1971), and of ranatensin (III) isolated from methanol extracts of the skin of the American frog *Rana pipiens* (Nakajima, Tanimura & Pisano, 1970).

(I) Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

(II) Pyr-Gly-Arg-Leu-Gly-Thr-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

(III) Pyr-Val-Pro-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2
(Pyr=pyroglutamyl)

The structures of bombesin and ranatensin have been confirmed by synthesis (Bernardi, De Castiglione, Goffredo & Angelucci, 1971 ; Nakajima *et al.*, 1970).

This paper describes the occurrence of bombesin and alytesin in the skin of different batches of *Bombina bombina, Bombina variegata variegata* and *Alytes obstetricans*, respectively and the occurrence of bombesin-like polypeptides in the skin of other amphibians. A simple method is given for obtaining bombesin-like polypeptides in a biologically pure form and for separating them from other active peptides and the main pharmacological actions of bombesin on extravascular smooth muscle are described.

Methods

Amphibian material

The amphibians used are shown in Table 1. Extracts of fresh skins were prepared as soon as possible after capture of the animals. The skins were removed from the frogs immediately after killing and extracted twice with a volume of methanol five times the weight of the tissue. Skins that could not be treated immediately were spread out and dried in the shade. Soon after their arrival in the laboratory, they were cut into small pieces with scissors and immersed in a volume of 80% methanol twenty times their weight. The liquid was decanted after a week and the skins were treated with a further fifteen to twenty volumes of the solvent. The first and second extracts of the dried skins were combined and filtered. When kept in dark bottles at 4° C the extracts could be stored for a long time (months or even years) without appreciable loss of activity.

TABLE 1. *Amphibian material*

Batch	Species	Number of animals	Average weight of skin (g)	Source	Date of capture
1	*Bombina bombina*	500	0·64 F	Germany	June 1968
2	,, ,,	833	0·48 F	,,	June 1969
3	*Bombina variegata variegata*	10	1·35 F	France	June 1968
4	,, ,, ,,	50	1·31 F	,,	June 1968
5	,, ,, ,,	403	1·25 F	,,	May–Sept. 1969
6	*Bombina variegata pachypus*	65	2·4 F	South Italy	April 1953
7	*Alytes obstetricans*	30	0·58 F	France	Sept. 1968
8	,, ,,	71	0·48 F	,,	Sept. 1968
9	,, ,,	186	0·81 F	,,	April 1969
10	,, ,,	526	0·66 F	,,	May–July 1969
11	*Rana pipiens*	22	0·5 D	Mexico	October 1963
12	,, ,,	9	0·6 D	Costa Rica	August 1964
13	,, ,,	1	1·2 D	Nicaragua	Sept. 1964
14	,, ,,	5	1·3 D	Kansas, U.S.A.	Sept. 1964
15	,, ,,	9	1·14 D	,, ,,	June 1968
16	,, ,,	24	1·08 D	,, ,,	July 1969
17	,, ,,	38	1·3 D	,, ,,	July 1970
18	*Discoglossus pictus*	20	1·6 F	Sicily	June 1963

F, fresh skin; D, dried skin.

Paper chromatography

Because of the presence in their molecules of reactive amino acid residues, bombesin and alytesin could be visualized on paper chromatograms by means of (a) NNCD reagent which gave a yellow-orange colour, and *p*-dimethylaminobenzaldehyde reagent which gave a violet colour, slowly turning to blue (tryptophan), (b) Pauly reagent which produced a pink-red colour (histidine), and (c) Sakaguchi reagent, which produced a lobster red colour (arginine). Threshold amounts of the polypeptides were of the order of 20–30 μg. The qualitative detection and semiquantitative estimation of aromatic amines by paper chromatography has been described previously (Erspamer, Vitali, Roseghini & Cei, 1967 ; Erspamer, Roseghini & Cei, 1964).

Chromatography on alumina column

Crude skin extracts were passed through a column of alkaline alumina which was then eluted with decreasing concentrations of ethanol (cf. Anastasi, Erspamer & Cei, 1964). By this simple procedure it was often possible to obtain bombesin in a biologically pure form, and to trace even small amounts of bombesin-like peptides in a mixture of active compounds.

Smooth muscle preparations

Bombesin was assayed on the following isolated smooth muscle preparations, the bathing solution and temperature are given in parentheses: guinea-pig ileum and colon, hamster ileum and large intestine (Krebs solution at 32° C), rat duodenum and colon (Krebs and Tyrode solutions at 30–32° C), rabbit ileum and colon (Tyrode and Krebs solutions at 37° C), kitten small intestine (Tyrode solution at 30–32° C), fowl rectal caecum and terminal ileum (Tyrode solution at 35–37° C), tortoise ileum (Tyrode solution for cold-blooded animals), rat, cat, hamster and guinea-pig uterus (Tyrode solution at 30° C), guinea-pig, rat, dog and monkey (*Macacus rhesus*) urinary bladder, guinea-pig, rat and dog ureter (Krebs solution at 37° C), guinea-pig tracheal chain (Krebs solution at 37° C equilibrated with 95% O_2–5% CO_2). The motility of the isolated ureters, the isolated guinea-pig and rat urinary bladder and the guinea-pig tracheal chain was recorded on a smoked drum by means of an isometric microdynamometer (U. Basile, Milan).

Unless otherwise stated, the above preparations were prepared as described by Erspamer & Falconieri Erspamer (1962) and the bath fluids had the same composition.

Movements of the rat urinary bladder *in vivo* were recorded by connecting the free end of the bladder, by means of a thin thread sewn in it, to the isometric transducer of the microdynamometer. Rats were anaesthetized with ethyl urethane (1·3 g/kg, intraperitoneally or subcutaneously).

The guinea-pig gall bladder was prepared as described by Bertaccini, De Caro, Endean, Erspamer & Impicciatore (1968).

In the intact animals intravenous injections or infusions were given via a thin polythene tube (PG 50) inserted into the jugular vein.

Reagents and drugs

Analytical grade reagents and solvents (Merck, Darmstadt and B.D.H.) were used throughout the investigation.

Synthetic bombesin, eledoisin, physalaemin, phyllokinin, caerulein, 5-hydroxytryptamine and creatinine sulphate as well as natural alytesin were prepared at the Farmitalia Laboratories for Basic Research, Milan.

Other drugs and reagents used were as follows: synthetic bradykinin, oxytocin, Lys^8-vasopressin, Val^5-angiotensin II-Asp-β-amide, prostaglandins E_1 and $F_{1\alpha}$, histamine dihydrochloride, (±)-propranolol hydrochloride, phenoxybenzamine hydrochloride, hexamethonium bromide, atropine sulphate, nicotine tartrate, mepyramine maleate, methysergide, crystalline trypsin and chymotrypsin (Princeton Lab. Products, Princeton, N.J.) and 2-chloro-4-nitrobenzenediazoniumnaphthalene-2-sulphonate (NNCD reagent, Hopkins & Williams).

Results

Bombesin content of different batches of Bombina *skin*

Table 2 shows the bombesin content of six batches of *Bombina* skin, as determined by bioassay on the rat uterus and the kitten small intestine preparations. The 5-hydroxytryptamine content, shown in the table, was determined semiquantitatively on paper chromatograms.

Attempts to confirm the results of bioassay by semiquantitative estimation of bombesin on paper chromatograms with the aid of colour reactions were unsuccessful, owing to the occurrence of inactive or poorly active bombesin-like peptides, or peptide fragments, and to the possible superimposition of reacting contaminants on the spot of bombesin. This occurred especially for *Bombina variegata variegata*, even on chromatograms of ethanol eluates from alumina columns.

It may be seen from Table 2 that results obtained with the two biological preparations were in satisfactory accordance and that the bombesin content ranged between 200 and 600 μg/g fresh skin.

Data concerning the bombesin content of *Bombina variegata pachypus* are only indicative because of the age of the extract and the unsatisfactory nature of the solvent acetone, used in this instance.

TABLE 2. *Content of bombesin and 5-hydroxytryptamine (5-HT) in different batches of* Bombina *skin*

Species and batch	Bombesin (μg/g) determined on: Rat uterus	Bombesin (μg/g) determined on: Kitten intestine	5-HT (μg base/g)
Bombina bombina			
1	200–300	200–300	1,100
2	250–500	250–400	1,200
Bombina variegata variegata			
3	200–400	200	520
4	500–650	350–500	600
5	400–700	400–600	500
Bombina variegata pachypus			
6	30–50	20–40	150

Content of bombesin-like peptides in different batches of skins of Alytes obstetricans, Rana pipiens *and* Discoglossus pictus

The content of bombesin-like peptides shown in Table 3, together with that of 5-hydroxytryptamine, was always expressed in terms of synthetic bombesin. Natural alytesin had an activity on the rat uterus preparation that was 70 to 200% of the activity of synthetic bombesin ; on the cat small intestine the activity was 70 to 300% as compared with synthetic bombesin. No data on the relative activity of ranatensin are available.

The content of bombesin-like peptides was often very different in the different batches of skins from the same species (Table 3) and quantitative data obtained with the rat uterus were at variance with those obtained with the cat small intestine. This is not surprising in the case of *Rana pipiens*, because in parallel assay bombesin is expected to behave differently from ranatensin.

Discoglossus pictus, although belonging to the same family as *Bombina* and *Alytes*, did not contain detectable amounts of bombesin-like peptides.

Partial purification of bombesin by passage through alumina column

Chromatography of crude tissue extracts on alumina followed by elution with decreasing concentrations of ethanol was very effective in the isolation of eledoisin, physalaemin and phyllokinin. It gave excellent results also for bombesin, alytesin and related peptides. They were eluted from the column with 80–85% ethanol. The only biologically active contaminant was 5-HT which, however, generally had a peak of elution with 70–80% ethanol.

Physalaemin, eledoisin, bradykinin, phyllokinin, phyllomedusin, caerulein, phyllocaerulein and other active peptides from the amphibian skin were all eluted by lower concentrations of ethanol. Thus, in skin extracts containing a mixture of active peptides, bombesin-like peptides may be easily separated and characterized, even if they are minor constituents of the mixture.

TABLE 3. *Bombesin-like activity (expressed as bombesin) and 5-hydroxytryptamine (5-HT) content of different batches of skins of* Alytes obstetricans, Rana pipiens *and* Discoglossus pictus

Species and batch	Bombesin-like activity (in μg bombesin per g skin) determined on: Rat uterus	Bombesin-like activity (in μg bombesin per g skin) determined on: Kitten intestine	5-HT (μg base/g)
Alytes obstetricans			
7	900–1,000	700–1,000	330
8	600–750	650–800	330
9	1,200–1,300	1,000–1,300	300
10	50	30–50	20
Rana pipiens			
11	0·5	0·7	6–8
12	1·5	0·6	2–3
13	4	1	7
14	75–100	50	60–70
15	70–90	40	60
16	100–120	60	30
17	16	7	40
Discoglossus pictus			
18	<1	<1	225–250

Action on isolated preparations of gastrointestinal smooth muscle

Cat. The isolated loop of the kitten small intestine proved to be one of the most suitable preparations for the bioassay of bombesin-like peptides. The best results were obtained with 10–30-day-old kittens. The response of the gut of adult animals was often irregular and bioassay more tiresome. Bombesin caused the appearance or reinforcement of rhythmic movements and an increase in tone. With small or moderate doses both the contraction and return to the base line, upon washing, were generally gradual. The threshold dose of bombesin ranged, in more than thirty experiments, between 0·1 and 0·5 ng/ml. There was generally a fair dose-response relationship for concentrations up to 3–5 ng/ml. With larger doses the difference in response lay in the rapidity of the response rather than in the size of the contraction (Fig. 1).

Tachyphylaxis was either lacking or moderate. However, sensitivity to bombesin often declined during the course of an experiment. Good responses were obtained not only with fresh loops, but also with intestines kept for 24–72 h in Krebs solution at 3–5° C.

It may be seen from Table 4 that another important advantage of the kitten intestine in the bioassay of bombesin is that it is poorly sensitive to most biogenic

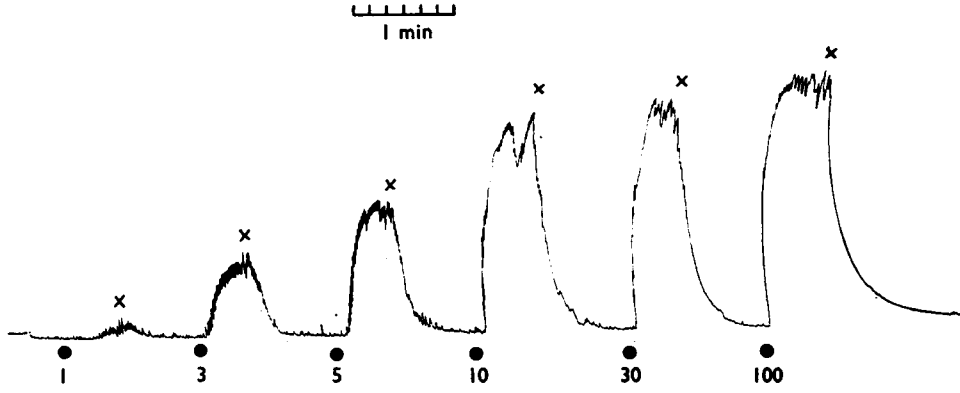

FIG. 1. Kitten small intestine preparation suspended in 10 ml of Tyrode solution at 32° C. Response produced by increasing doses of bombesin (doses in ng). At ×, washing. Time marks, 1 minute. Note the good dose-response relationship.

TABLE 4. *The relative potency on the kitten intestine, of amines, peptides and prostaglandins*

Alytesin	100, 75, 70, 300, 250, 200[1]
5-HT base	0·5, <0·2, <0·1[1]
Histamine base	0·3, 0·1, 0·2, 0·05[1]
Physalaemin	8–10, 2, 5, 10, 7–20, 4, 5, 10, 7–10, 10, 20, 5–7[1], 3[1]
Eledoisin	8–10, 5–7, 3–4, 2–3, 30, 6, 1·5–2[1]
Bradykinin	50, 100, 50–80, 200, 60, 5–20, 15, 10, 100[1], 100[1], 125[1]
Phyllokinin	10, 20, 35
Caerulein	200, 50, 200–300, 70–150, 30, <1[1], <0·5[1], 1[1], <0·5[1]
Oxytocin	2–5, 0·2, 0·2, 0·5, 0·5[1]
Lys[8]-vasopressin	0·2, 0·3–0·5, <0·1, <0·2, 2, 3–5[1]
Val[5]-angiotensin	0·05, <0·05, <0·1, 2, 1, <0·2, <0·1, 1, 2, 2, <0·3[1], 0·1[1], <0·3[1]
Prostaglandin E_1	<1, <0·5, 0·1, 2, 1[1], <0·02[1]
Prostaglandin $F_{1\alpha}$	1, 0·5, 0·3, 2, 1, <0·3[1]

[1] Intestines kept in cold nutrient liquid for more than 24 hours.

The activity of bombesin was considered equal to 100 and that of the other compounds was expressed in terms of this activity.

substances known to stimulate smooth muscle. Among them Val[5]-angiotensin possessed barely <0·1 to 2% of the activity of bombesin (Fig. 2). Physalaemin, eledoisin and phyllomedusin showed 1 to 10% of the activity of bombesin; caerulein was very active on fresh intestinal loops, but had little activity on intestines kept in cold Krebs solution for 24–48 hours.

Atropine (0·5–1 μg/ml) did not affect the response to bombesin. After nicotine (10 μg/ml) or hexamethonium (100 μg/ml) the response was unchanged or slightly increased.

Guinea-pig. The guinea-pig ileum responded to bombesin with repeated spike contractions, eventually accompanied by moderate increase in tone. The threshold dose was of the order of 1 to 5 ng/ml, and the sensitivity varied markedly from one preparation to another. Tachyphylaxis was prompt and intense and although it could be diminished to some extent by prolonging the interval between the doses, the preparation proved unsuitable for the bioassay of bombesin. With doses of 10–100 ng/ml of bombesin spike contractions sometimes persisted for hours, but were promptly abolished by washing. It should be added that the guinea-pig ileum is highly sensitive to a number of other compounds active on smooth muscle (5-hydroxytryptamine, histamine, eledoisin, physalaemin, bradykinin, caerulein, prostaglandin E_1, etc.).

Atropine (1 μg/ml) regularly reduced or abolished the response to bombesin (Fig. 3). In some cases the blockade was complete but transitory and spike contractions of lesser intensity appeared spontaneously after a few minutes of total lack of activity. The introduction into the bath of a second dose of atropine was then ineffective. Mepyramine (0·1 μg/ml), nicotine (10 μg/ml), or methysergide (0·2 μg/ml) did not affect the response to bombesin.

In contrast to the ileum, the guinea-pig colon was a fairly useful preparation in the bioassay of bombesin, because of its considerable sensitivity and the satisfactory dose-response relationship. In more than 30 experiments the threshold dose for bombesin ranged from 0·03 to 0·5 ng/ml and response was proportional to the dose up to 2–10 ng/ml. However, moderate tachyphylaxis often developed, especially with high doses.

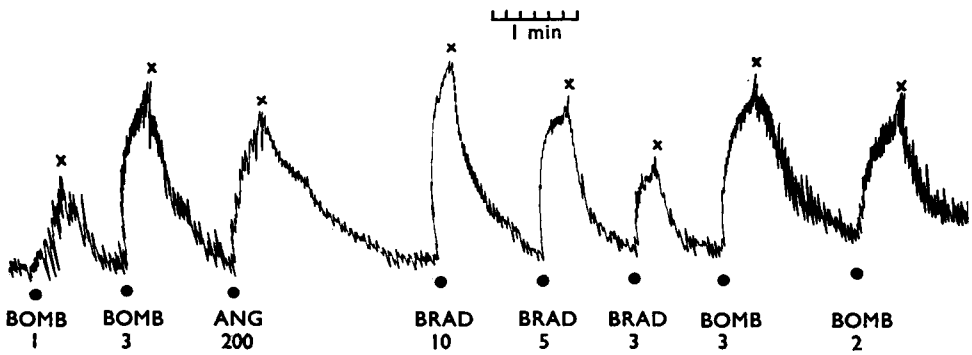

FIG. 2. Small intestine preparation from a 27-day-old kitten, suspended in 10 ml of Tyrode solution at 32° C. BOMB, bombesin; ANG, angiotensin; BRAD, bradykinin. All doses in ng. At ×, washing. Time marks, 1 minute. In this preparation angiotensin showed 1% and bradykinin 60% of the activity of bombesin.

Atropine introduced during the bombesin contraction caused an abrupt but transient fall of tone; given prior to bombesin it caused a moderate reduction of the response to the first dose of bombesin. Then, in spite of the continuous presence of atropine, the effect of the polypeptide returned to normal. Nicotine, hexamethonium (both 100 μg/ml) and phenoxybenzamine (0·1 μg/ml) did not appreciably affect the response to bombesin; propranolol (2 μg/ml) reduced it slightly.

The stimulant action of bombesin on the guinea-pig colon was by no means specific, as it was shared by several other biogenic active compounds. If the activity of bombesin is taken as 100, that of other substances was as follows: alytesin 50–150, 5-HT<1–5, histamine 3–20, bradykinin 50–200, phyllokinin 10–50, eledoisin 50–200, physalaemin 40–100, caerulein<5, Val^5-angiotensin 20–300, oxytocin 0·5–10, vasopressin 3–50, prostaglandin E_1<0·1, prostaglandin $F_{1\alpha}$<0·2.

Rat. The rat large intestine responded to bombesin with an increase in tone which, unlike that produced by bradykinin, was never preceded by relaxation. The threshold dose was 0·03–0·1 ng/ml. The preparation gave variable results: sometimes an acceptable dose-response relationship was obtained, but in other experiments different doses of the polypeptide produced hardly distinguishable responses. The percent activity of other polypeptides, in comparison to that of bombesin (taking the activity of bombesin as 100) was as follows: alytesin 50–100, bradykinin 2–10, eledoisin 1–10, physalaemin 0·2–3, caerulein <1.

It is well known that bradykinin causes relaxation of the rat duodenum. Response of the preparation to bombesin was variable. Low doses (0·2–2 ng/ml) sometimes caused increase in rhythmic movements; large doses (5–50 ng/ml)

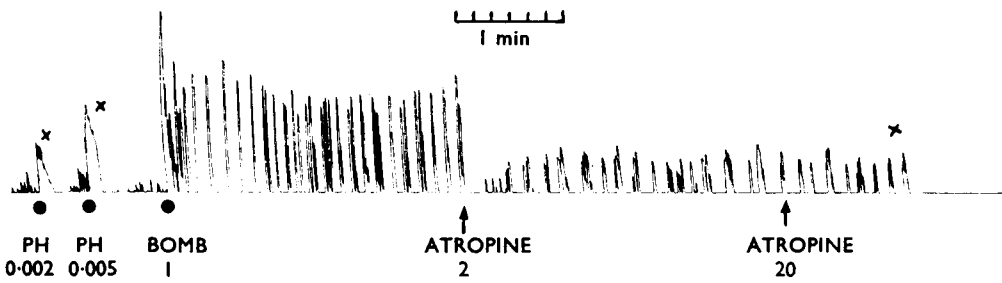

FIG. 3. Guinea-pig ileum preparation suspended in 10 ml of Krebs solution at 32° C. At ×, washing. Time marks, 1 minute. PH, physalaemin; BOMB, bombesin. Doses in μg. Atropine produced inhibition of the response to bombesin.

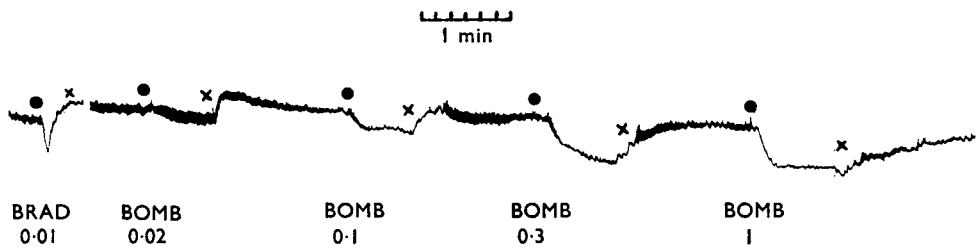

FIG. 4. Rat duodenum preparation suspended in 10 ml of Krebs solution at 32° C. BRAD, bradykinin; BOMB, bombesin. All doses in μg. At ×, washing. Time marks, 1 minute. Note the slow, long lasting relaxation elicited by bombesin.

generally produced a gradual decrease of tone with reduction of movements (Fig. 4). No acceptable dose-response relationship could be observed.

Neither rat large intestine nor duodenum was affected by atropine (1 μg/ml) in its response to bombesin.

Hamster. Both the small and the large intestine were poorly sensitive to bombesin (threshold 2–4 ng/ml), which always produced an increase in tone, occasionally accompanied by reinforcement of rhythmic movements. No dose-response relationship could be observed.

Rabbit. Small and large intestine were often very sensitive to bombesin at the beginning of the experiment (threshold concentration 0·01–0·3 ng/ml), but tachyphylaxis was prompt and intense. The large intestine responded with a gradual increase in tone and intensification of spontaneous movements. Return to the base line on washing was sometimes difficult. In certain preparations of small intestine the initial strong contraction was followed by spontaneous relaxation with reduction of the amplitude of movements. Washing produced complete inhibition of activity and decrease of tone below the base line, followed by gradual increase of tone and reappearance of rhythmic activity. This sequence was very similar to that produced by catecholamines.

Fowl. Both the terminal ileum and the rectal caecum were stimulated by bombesin (threshold dose 0·1–1 ng/ml), but the response was irregular and not proportional to the dose.

Tortoise. Bombesin produced an increase in tone which, at the beginning of the experiment, showed some proportionality to the dose (threshold, 1–2 ng/ml). Later on, tachyphylaxis occurred.

Guinea-pig gall bladder in situ. Bombesin, given intravenously, produced a contraction of the guinea-pig gall bladder. The response varied strikingly from one preparation to another in intensity and repeatability, and was 0·5 to 5% of that elicited by caerulein.

Action on isolated preparations of uterine muscle

Rat. The isolated oestrous uterus of the rat is a suitable preparation for the bioassay of bombesin. The peptide caused appearance or intensification of rhythmic activity and increase in tone, which was proportional to the dose. Response was prompt and its peak was reached rapidly. For high doses an authentic spasm could be observed (Figs. 5 and 6). The effect lasted as long as bombesin remained in the bath ; upon washing, return to the base line was rapid for low concentrations of the polypeptide, but took a long time, even hours, for high concentrations. In this case the slow decrease of tone was often interrupted by a series of strong contractions. Fresh uterine horns were more sensitive and suitable than those kept in cold Tyrode solution for 24 hours. The threshold dose ranged, in more than forty experiments, between 5 and 50 pg/ml. Taking the activity of bombesin as 100, the activity of other substances known to stimulate smooth muscle was as follows: alytesin 70–200, physalaemin 0·1–2, eledoisin 0·1–2, caerulein 0·1–2, bradykinin 50–200, Lys^8-vasopressin 10–20, Val^5-angiotensin II 20–30, oxytocin 30–120, 5-HT 0·2–2, acetylcholine 2–3, prostaglandin E_1 1–2, prostaglandin $F_{1\alpha}$ <1.

Whereas increase in tone produced by 5-HT and bradykinin was promptly abolished by washing with fresh Tyrode solution, that produced by bombesin was

much more resistant to washing. The effect of bombesin lasted longer than that of oxytocin (Fig. 6).

The action of bombesin was completely abolished by digestion with chymotrypsin (0·1 mg chymotrypsin+5 μg bombesin, pH 7·6, 1 h at 37° C) but only slightly reduced by digestion with trypsin (1 mg trypsin+5 μg bombesin, pH 7·6,

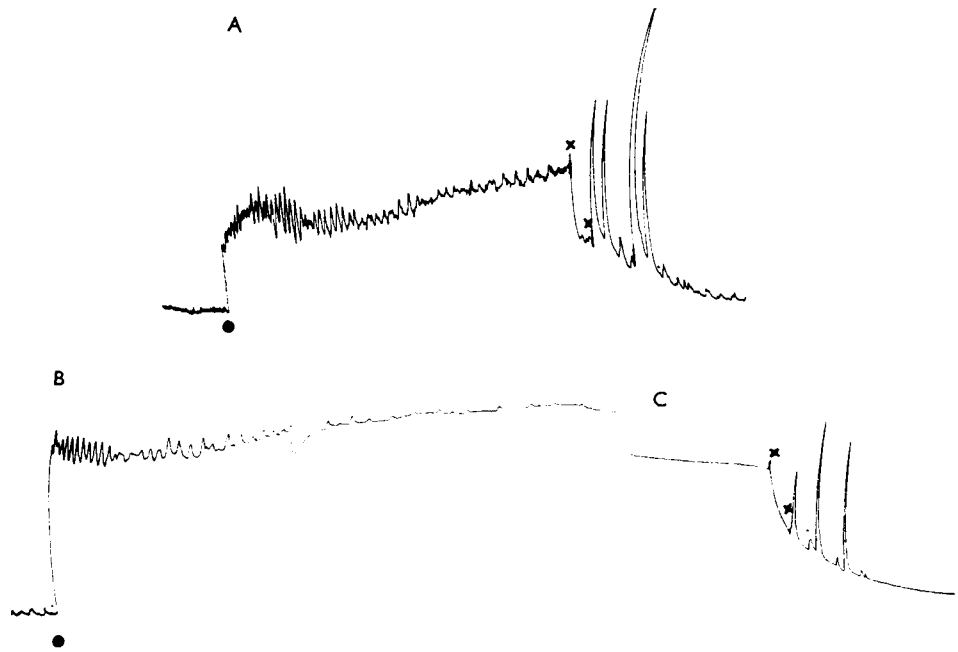

FIG. 5. Rat oestrous-uterus preparation suspended in 10 ml of Tyrode solution at 32° C. The effect of 0·1 μg (A) and 1 μg (B–C) of bombesin. At ×, washing. The first dose was left in the bath for 1 h, the second for 4 hours. Between B and C the drum was stopped for 1½ hours. Note the conspicuous and persistent increase of tone which, however, subsided quite rapidly after washing.

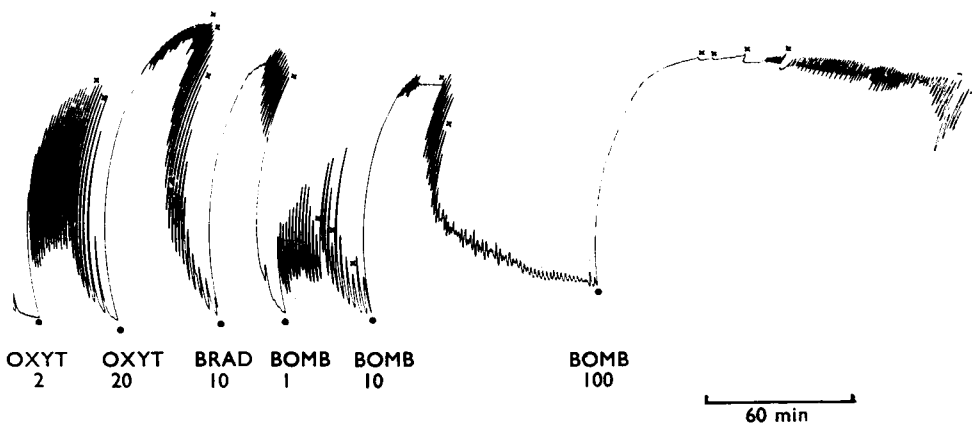

FIG. 6. Rat oestrous-uterus preparation suspended in 10 ml of Tyrode solution at 32° C. The effect of different doses of oxytocin (OXYT), bradykinin (BRAD) and bombesin (BOMB). All doses in ng. On washing (×) return to the base line was prompt for bradykinin and took the longest time for bombesin. Following 100 ng bombesin the spasm was very persistent and the uterus did not relax even with repeated washing.

1 h at 37° C). Similarly, the response of the uterus to the polypeptide was not affected by atropine (1 μg/ml), mepyramine (0·1 μg/ml) or methysergide (0·2 μg/ml).

Cat. The post-partum uterus (6–8 h) of the cat was insensitive to bombesin concentrations of up to 500 ng/ml. The threshold dose for oxytocin was 2 ng/ml and for bradykinin 0·1–0·5 ng/ml.

Guinea-pig. The response of the uterine horn of non-ovariectomized animals pretreated with folliculin was difficult to interpret, owing to the occurrence of spontaneous rhythmic activity. A moderate response was sometimes seen with 10–100 ng/ml of bombesin, whilst the threshold dose for oxytocin was 0·02–0·07 ng/ml.

Hamster. The oestrous-uterus of non-ovariectomized animals was insensitive to bombesin in concentrations up to 100 ng/ml. The threshold concentration for oxytocin was 0·05–0·1 ng/ml.

Rabbit. The non-pregnant uterus was not affected either by bombesin or oxytocin, up to 100 ng/ml.

Guinea-pig tracheal chain

The preparation was insensitive to bombesin in concentrations up to 2–5 μg/ml. The polypeptide was at least 500–1,000 times less potent than physalaemin, eledoisin or caerulein.

Action on the smooth muscle of the urinary tract

Rat urinary bladder. Both *in situ* and isolated preparations were extremely sensitive to bombesin. The threshold concentration for isolated preparations was of the order of 0·2–1 ng/ml. The response consisted of an increase in tone with eventual reinforcement of movements. The increase of tone was gradual or rapid, depending on the dose. Relaxation upon washing was usually gradual. A satisfactory dose-response relationship was obtained with small or medium doses; high doses, however, produced tachyphylaxis (Fig. 7). Atropine (0·1–0·5 μg/ml) did not alter the response to bombesin. The isolated rat urinary bladder was also sensitive to eledoisin, physalaemin and bradykinin, but poorly sensitive to other compounds known to stimulate smooth muscle. If the activity of bombesin is taken as 100 the activity of other compounds was as follows: eledoisin 30–80, physalaemin 40–100, bradykinin 15–40, caerulein <0·1–2, oxytocin <1, vasopressin <3–7, Val^5-angiotensin 3–15, 5-hydroxytryptamine <0·1–2, histamine <0·1, prostaglandin E_1 <1–3, prostaglandin $F_{1\alpha}$ <1.

The urinary bladder *in situ* responded to bombesin with a long lasting increase of tone, often accompanied by reinforcement of movements. Within certain limits response was again proportional to the dose.

The threshold dose of bombesin by rapid intravenous injection was 50–100 ng/kg. In one experiment the effect produced by 100, 200, 500 and 1,000 ng/kg bombesin lasted 15, 20, 25 and 45 minutes, respectively (Fig. 8). Physalaemin and eledoisin were approximately as active as bombesin, but increase in tone was short-lived. Even more fleeting was the effect of intravenous bradykinin, which possessed less than 10% of the activity of bombesin. Angiotensin was virtually inactive up to 5–10 μg/kg.

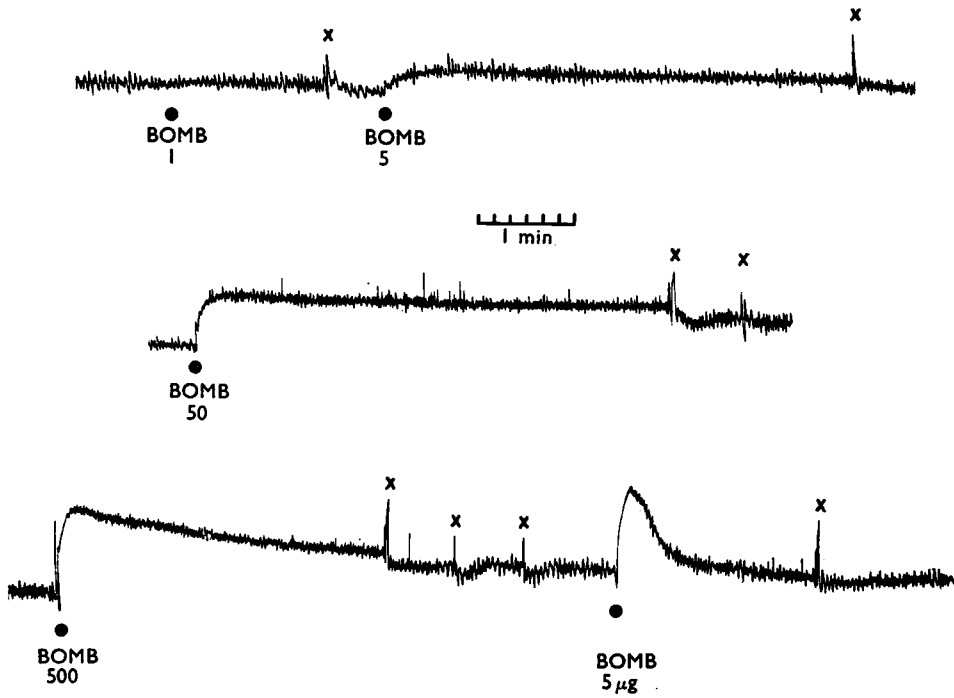

FIG. 7. Rat urinary bladder preparation suspended in 10 ml of Krebs solution at 37° C. Response produced by increasing doses of bombesin: 1 and 5 ng (top tracing), 50 ng (middle tracing), 500 ng and 5 μg (bottom tracing). At ×, washing. Time marks, 1 minute. Note the sustained contraction produced by all doses of bombesin, except the 5 μg dose, which caused an abrupt but short-lived increase in tone.

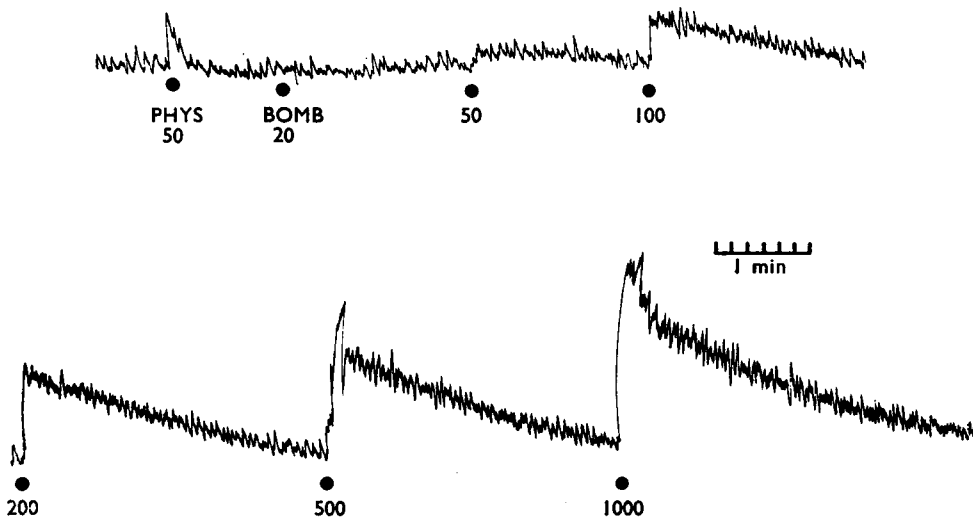

FIG. 8. Rat anaesthetized with urethane. Response of the urinary bladder *in situ* to a single dose of physalaemin (PHYS) and to increasing doses of bombesin (BOMB). All doses, in ng/kg, were given intravenously. Note the good dose-response relationship for bombesin and the short-lived effect of physalaemin. Time marks, 1 minute.

The intravenous infusion of bombesin produced similar effects. The threshold infusion rate was (30–50 ng/kg)/minute. In one experiment three doses of bombesin were infused successively, for 30 min, at rates of (50, 125 and 250 ng/kg)/minute. The tone increase was proportional to the dose. After discontinuing the infusion, return of the tone to pre-infusion levels took 15–20, 60–80 and 100–120 min, respectively.

The threshold subcutaneous dose of bombesin capable of causing an increase in tone of the urinary bladder was 3–10 μg/kg. The effect was always long lasting, even for threshold doses. With 100 μg/kg spasm of the urinary bladder could be observed, with temporary suppression of movements. After 3–4 h the tone was still above the base line.

Rat ureter. The isolated ureter was poorly sensitive to bombesin. Concentrations of 0·3–1 μg/ml were required to elicit a series of spike contractions. Physalaemin and eledoisin were at least 20–30 times as potent as bombesin.

Dog urinary bladder. Isolated longitudinal strips of the dog urinary bladder were insensitive to bombesin. The threshold dose was of the order of 0·5–1 μg/ml. In contrast, the preparation was extremely sensitive to bradykinin (threshold 0·05–0·2 ng/ml) and to eledoisin (threshold 0·03–1 ng/ml).

Dog ureter. No response could be elicited, in the isolated ureter, by bombesin concentrations up to 1 μg/ml. Physalaemin and eledoisin, in contrast, produced a series of spike contractions, starting from threshold concentrations of 5–20 ng/ml. Bradykinin showed less than 5–10% of the activity of physalaemin.

Guinea-pig urinary bladder. The isolated preparation was sensitive to bombesin, the threshold concentration ranging from 0·3 to 3 ng/ml. However, there was usually marked tachyphylaxis. The effect of bombesin, which was atropine-resistant, was 3–5 times that of eledoisin and physalaemin, 10–40 times that of bradykinin, and more than 100 times that of histamine, 5-hydroxytryptamine, prostaglandin E_1, prostaglandin $F_{1\alpha}$ and caerulein.

Guinea-pig ureter. The isolated preparation responded to bombesin, from threshold doses of 1–3 ng/ml, with spike contractions (Fig. 9). Tachyphylaxis was frequent. Angiotensin, bradykinin and 5-hydroxytryptamine possessed little activity, less than 2% of that shown by bombesin.

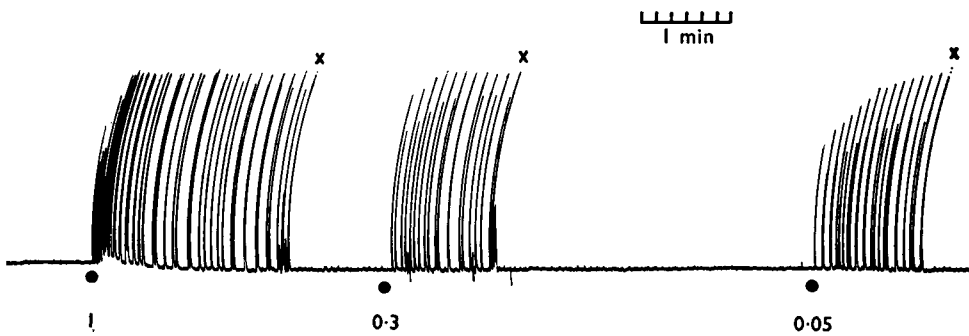

FIG. 9. Guinea-pig ureter preparation suspended in 10 ml of Krebs solution at 37° C. Response produced by three decreasing doses of bombesin (in μg). Time marks, 1 minute. No obvious difference may be seen between the responses produced by 0·3 and 0·05 μg of bombesin.

Monkey urinary bladder. Like that of the dog, the isolated monkey urinary bladder was poorly sensitive to bombesin ; the threshold dose was of the order of 1 μg/ml or more. The preparation was poorly sensitive also to physalaemin.

Discussion

To the three polypeptide groups hitherto known to occur in the amphibian skin (physalaemin-like peptides, bradykinin-like peptides, caerulein-like peptides) another should now be added, that of bombesin-like peptides, at present represented by bombesin, alytesin and ranatensin. Bombesin has been isolated from the skin of *Bombina bombina* and *Bombina variegata variegata*, alytesin from the skin of *Alytes obstetricans*, and ranatensin from the skin of *Rana pipiens*.

However, bombesin-like peptides have already been traced in this laboratory not only in the skin of other species of *Rana* but also in that of amphibian species belonging to genera and families other than Ranidae and Discoglossidae. It is also possible that the skin of a single species contains more than one bombesin-like peptide. Our present efforts are directed to the collection of enough material to isolate the new peptides.

The bombesin and alytesin content of the skin varied considerably from one batch to another, although the reason for this was uncertain. Peptides were always accompanied by large amounts of 5-hydroxytryptamine. One of the *Alytes* batches which had an exceptionally low content of alytesin also had a very poor content of 5-hydroxytryptamine.

Bombesin-like peptides displayed a stimulant action on a number of smooth muscle preparations of the gut, uterus and urinary tract. However, the effect on a given preparation varied greatly in intensity not only in different species, but even in different experiments. Moreover, initial strong stimulation was often followed by intense and prompt tachyphylaxis. Among all preparations tested, the rat uterus, the kitten small intestine, the guinea-pig colon and the rat urinary bladder were the most suitable for the qualitative demonstration and the quantitative estimation of bombesin-like peptides. They showed a conspicuous sensitivity and a satisfactory dose-response relationship.

The mechanism of action of bombesin on smooth muscle has not been elucidated. A cholinergic mechanism is certainly responsible, at least in part, for the spike contractions produced by the polypeptide in the guinea-pig ileum, as shown by their partial inhibition by atropine, and it is quite possible that in other preparations bombesin may also act partially through stimulation of nervous structures. However, the most important mechanism of action seems to be a direct one on the smooth muscle. In accordance with this assumption is the ineffectiveness of pretreatment with autonomic blocking drugs, and the persistence of the effects of bombesin after keeping the smooth muscle preparations in cold physiological solutions for a long time.

Bombesin-like peptides may easily be distinguished from the other peptides occurring in amphibian skin and, more generally, from all other compounds known to stimulate smooth muscle. In the present study distinctive criteria emerged from (a) parallel bioassay on different smooth muscle preparations, (b) digestion with proteolytic enzymes, (c) use of specific inhibitors, and finally (d) column chromatography.

Physalaemin-like peptides, unlike bombesin peptides, were very active on the rabbit large intestine, the guinea-pig ileum and the dog urinary bladder, and were poorly active on the rat uterus and the kitten intestine. They rarely produced tachyphylaxis and were more or less completely digested by trypsin. Bradykinin-like peptides, like bombesin peptides, were very active on the rat uterus, the guinea-pig colon and, in part, the kitten intestine, but they were also extremely active, unlike bombesin, on the dog urinary bladder. Moreover, bradykinin-like peptides elicited effects on the guinea-pig ileum and the rat duodenum which were strikingly different from those produced by bombesin peptides. Caerulein-like peptides were poorly active on the rat uterus, the rat and guinea-pig urinary bladder and on preparations of intestinal smooth muscle kept in cold physiological solutions for more than 24 hours. They were, in contrast, extremely active on the guinea-pig gall bladder *in situ*.

Bombesin and alytesin were easily eluted from an alumina column with 80–90% ethanol, bradykinin-like and physalaemin-like peptides with 50–70% ethanol, and caerulein-like peptides were eluted with difficulty with 20–40% ethanol.

Ranatensin has frequently been compared with angiotensin. Bombesin resembled angiotensin in its potent stimulant action on the rat uterus and the guinea-pig colon, but differed strikingly from angiotensin in its far greater activity on the rat urinary bladder and the kitten small intestine.

Studies are now in progress on the action of bombesin-like peptides on systemic blood pressure, renal circulation and gastric acid secretion which will offer additional criteria for their distinction from all other active peptides.

Clineschmidt, Geller, Govier, Pisano & Tanimura (1971) have described in detail the action of ranatensin on the rat uterus, the rat duodenum and the guinea-pig ileum. Most results were similar although not identical to those obtained in this study with bombesin. For example, in our experiments bombesin produced a contraction of the rat uterus which, unlike that elicited by ranatensin, was very much more prolonged than that caused by bradykinin or by oxytocin. It is quite possible that the tetradecapeptide bombesin occupies the receptor sites in the uterine smooth muscle more tenaciously than the endecapeptide, ranatensin.

It has been pointed out that alytesin could not be distinguished from bombesin in any of the tested preparations. Hence, the description of the pharmacological effects of bombesin reported here is valid also for alytesin.

This work was supported by grants from the Consiglio Nazionale delle Ricerche, Rome. The following gifts are gratefully acknowledged: synthetic bombesin, eledoisin, physalaemin, phyllokinin, caerulein and natural alytesin (Farmitalia S.p.A., Milan), synthetic bradykinin, oxytocin, Lys^8-vasopressin and methysergide (Sandoz, Basel), Val^5-angiotensin II-Asp-β-amide (Ciba Ltd., Basel), prostaglandins E_1 and $F_{1\alpha}$ (The Upjohn Co., Kalamazoo, Michigan), phenoxybenzamine hydrochloride (Smith, Kline & French Labs., Philadelphia, Pa.) and (±)-propranolol hydrochloride (I.C.I.).

REFERENCES

ANASTASI, A., ERSPAMER, V. & CEI, J. M. (1964). Isolation and amino acid sequence of physalaemin, the main active polypeptide of the skin of *Physalaemus fuscumaculatus*. *Arch. Biochem. Biophys.*, **108**, 341–348.

ANASTASI, A., ERSPAMER, V. & BUCCI, M. (1971). Isolation and structure of bombesin and alytesin, two analogous active peptides from the skin of the European amphibians Bombina and Alytes. *Experientia (Basel)*, **27**, 166–167.

BERNARDI, L., DE CASTIGLIONE, R., GOFFREDO, O. & ANGELUCCI, F. (1971). Synthesis of bombesin. *Experientia (Basel)*, **27**, 873–874.

BERTACCINI, G., DE CARO, G., ENDEAN, R., ERSPAMER, V. & IMPICCIATORE, M. (1968). The actions of caerulein on the smooth muscle of the gastrointestinal tract and the gall bladder. *Br. J. Pharmac.*, **34**, 291–310.

CLINESCHMIDT, B. V., GELLER, R. G., GOVIER, W. C., PISANO, J. J. & TANIMURA, T. (1971). Effects of ranatensin, a polypeptide from frog skin, on isolated smooth muscle. *Br. J. Pharmac.*, **41**, 622–628.

ERSPAMER, V. & FALCONIERI ERSPAMER, G. (1962). Pharmacological actions of eledoisin on extravascular smooth muscle. *Br. J. Pharmac. Chemother.*, **19**, 337–354.

ERSPAMER, V., ROSEGHINI, M. & CEI, J. M. (1964). Indole-, imidazole- and phenyl-alkyl-amines in the skin of thirteen *Leptodactylus* species. *Biochem. Pharmac.*, **13**, 1083–1093.

ERSPAMER, V., VITALI, T., ROSEGHINI, M. & CEI, J. M. (1967). 5-Methoxy- and 5-hydroxyindoles in the skin of *Bufo alvarius*. *Biochem. Pharmac.*, **16**, 1149–1164.

ERSPAMER, V., FALCONIERI ERSPAMER, G. & INSELVINI, M. (1970). Some pharmacological actions of alytesin and bombesin. *J. Pharm. Pharmac.*, **22**, 875–876.

MELCHIORRI, P., SOPRANZI, N. & ERSPAMER, V. (1971). On the action of bombesin on the kidney of the rat and the dog. *J. Pharm. Pharmac.*, **23**, 981–982.

NAKAJIMA, T., TANIMURA, T. & PISANO, J. J. (1970). Isolation and structure of a new vasoactive peptide. *Fedn Proc.*, **29**, 282 Abs.

(*Received November* 8, 1971)