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OCCURRENCE OF PHYSALAEMIN IN EXTRACTS OF THE SKIN OF *PHYSALAEMUS FUSCUMACULATUS* AND ITS PHARMACOLOGICAL ACTIONS ON EXTRAVASCULAR SMOOTH MUSCLE

BY

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Methanol extracts of the skin of *Physalaemus fuscumaculatus*, a South American amphibian, contain a principle which potently stimulates extravascular smooth muscle and possesses a powerful vasodilator and hypotensive action in the dog and other mammals (Erspamer, Bertaccini & Cei, 1962). This principle, called physalaemin, has now been isolated in a pure form and its amino acid composition and sequence have been fully elucidated (Erspamer, Anastasi, Bertaccini & Cei, 1964; Anastasi, Erspamer & Cei, 1964). Physalaemin is an endecapeptide having the following amino acid sequence (I), which closely resembles that of eledoisin (II):

(I) Pyr–Ala–Asp(OH)–Pro–Asp(NH_2)–Lys–Phe–Tyr–Gly–Leu–Met–NH_2

(II) Pyr–Pro–Ser–Lys–Asp(OH)–Ala–Phe–Ile–Gly–Leu–Met–NH_2

(Pyr = pyroglutamyl)

The structure proposed has been confirmed by synthesis (Bernardi, Bosisio, Goffredo & De Castiglione, 1964).

This paper describes: the occurrence of physalaemin in the skin of different *Physalaemus fuscumaculatus* batches and of different *Physalaemus* species; the occurrence of physalaemin-like polypeptides in the skin of other amphibians; a simple method for obtaining physalaemin in a biologically pure form and for separating it from other active polypeptides occurring in the *Physalaemus* skin; and the main pharmacological actions of physalaemin on extravascular smooth muscle.

As just stated, the skin of *Physalaemus fuscumaculatus* contains, in addition to physalaemin, at least two other peptides active on smooth muscle. A preliminary description of these peptides will also be given.

METHODS

Amphibian material. The *Physalaemus* material used in this study was as follows:

(1) Fifty adult specimens captured near Tucuman (Argentina) in January 1959. The dried skins weighed together 4.8 g, and the average weight of a skin was 0.1 g. Thirty skins were extracted with 80% methanol,

as usual, and twenty skins with 70% acetone. This was done in order to check the effectiveness of the two solvents in the extraction of physalaemin.

(2) 323 adult specimens captured near Tucuman in January and February 1963. The dried skins weighed together 32.8 g, and the average weight of a skin was 0.1 g.

(3) 471 adult specimens captured at the same place and time as for (2). The fresh skins weighed together 206 g, and the average weight of a skin was 0.43 g.

(5) Ninety-seven adult specimens captured near Tucuman at the end of January 1964. The total weight of the dry skins was 10.35 g, and the average weight of a skin was 0.11 g.

(4) 275 adult specimens captured in part near Tucuman and in part near Cordoba in December 1963. The total weight of the dried skins was 43 g; the average weight of the Cordoba specimens was 0.19 g, and of the Tucuman specimens was 0.094 g.

(6) Fifty-three adult specimens captured near Cordoba in December 1963. The total weight of the fresh skins was 35 g, and the average weight of a skin was 0.66 g. Separately, the whole bodies of twenty-five animals carefully deprived of their skins (142 g) were also extracted with methanol.

Batches (1) to (6) all refer to *Physalaemus fuscumaculatus*.

(7) Ten adult specimens of *Physalaemus centralis*, captured in Rondonia (Brazil) in November 1962. The total weight of the dried skins was 1.26 g, and the average weight of a skin was 0.126 g.

(8) Ninety-seven adult specimens of *Physalaemus bresslaui* captured near Rio de Janeiro in December 1963. The total weight of the dried skins was 4.1 g, and the average weight of a skin was 0.042 g.

(9) Two adult specimens of *Physalaemus cuvieri*, captured in Formosa (Argentina) in December 1963. The two fresh skins weighed 0.33 g.

Approximately 150 other amphibian species were investigated in regard to their content of physalaemin-like polypeptides. It will be seen that positive results were obtained only in several *Phyllomedusa* species and in one *Telmatobius* species.

Extracts of fresh skins were prepared in Argentina by extracting twice with five parts (w/v) of methanol the skins removed from the animals immediately after killing them. The skins destined to be dried were carefully spread out and dried in the shade. Soon after their arrival in Italy, by air mail, they were minced with scissors and then immersed in twenty parts of 80% methanol. Only twenty skins of batch (1) were extracted with 70% acetone. The liquid was decanted after a week, and the skins were treated for another week with fifteen to twenty parts of the solvent. The methanol extracts, yellow in colour, were mixed and filtered. Kept in dark bottles and refrigerated, they could be stored for months or even years without appreciable loss of activity.

Smooth muscle preparations. The action of physalaemin was assayed on the following smooth muscle preparations: rabbit large intestine, duodenum and ileum, guinea-pig ileum and colon, rat stomach, duodenum and large intestine, dog duodenum and large intestine, cat small and large intestine, pigeon duodenum, fowl rectal caecum, frog stomach, rat, rabbit and guinea-pig uterus, and rat and guinea-pig seminal vesicles.

These smooth muscle preparations were prepared exactly as described in a previous paper (Erspamer & Falconieri Erspamer, 1962), and the bath fluids had the same composition.

Collection of saliva. In dogs and cats one submaxillary duct was cannulated using a fine polyethylene tube, either after exposure in the neck as described by Sherrington (1919) or directly from the mouth at about 10 to 20 mm from their termination. The drops secreted were recorded with a Palmer electromagnetic drop-timer on a smoked drum. In rats the saliva, withdrawn directly from the mouth, was allowed to fall into small glass tubes for 5 min after each administration of physalaemin and was then measured by weighing.

Reagents and drugs. Reagents and solvents used in this investigation were of the analytical grade.

Pure natural physalaemin was obtained by a procedure described in detail elsewhere (Anastasi *et al.*, 1964). Synthetic physalaemin was prepared at the Farmitalia Research Laboratories, Milan, and the synthetic product was submitted by Dr Anastasi to two additional counter-current distributions in parallel with pure natural physalaemin. After this treatment, solutions of synthetic and natural physalaemin having

the same concentration, as determined by quantitative colorimetric estimation of leucine, possessed the same biological activity.

Physalaemin used in the present experiments was either the pure natural or synthetic polypeptide or a partially purified extract of *Physalaemus fuscumaculatus* skin containing 180 to 200 μg of physalaemin per mg.

We are grateful to Messrs Hoffmann-La Roche, Basel, for a preparation of substance P from horse serum (Ro 1-9256/7), containing 150 units per mg, as estimated by Dr Haefeli; to Messrs Sandoz, Basel, for samples of synthetic bradykinin, (+)-lysergic acid diethylamide and 2-bromolysergic acid diethylamide; and to Farmitalia S.p.A., Milan, for samples of synthetic eledoisin, 5-hydroxytryptamine creatinine sulphate and leptodactyline (*m*-hydroxyphenylethyltrimethylammonium). Other drugs were obtained from the following sources: crystalline trypsin and chymotrypsin from Princeton Lab. Products, Princeton, N.J., U.S.A.; and carboxypeptidase from Fluka A.G., Buchs, Switzerland.

For the detection and semiquantitative estimation of 5-hydroxytryptamine and leptodactyline on paper chromatograms the following developing reagents were respectively used: NNCD reagent (2-chloro-4-nitro-1-diazobenzene-α-naphthalene sulphuric acid in 0.1 M-hydrochloric acid) and Gibbs reagent (dichloroquinonechlorimide alcoholic solution followed by sodium carbonate).

RESULTS

Physalaemin content of different batches of Physalaemus fuscumaculatus *skin*

Table 1 shows the physalaemin content of the six examined batches of *Physalaemus fuscumaculatus* skin.

TABLE 1

THE CONTENT OF PHYSALAEMIN IN DIFFERENT BATCHES OF *PHYSALAEMUS FUSCUMACULATUS* SKIN

Skin			Physalaemin content (μg/g tissue)
Patch	Condition	Extract	
1	Dried	Acetone	280
1	Dried	Methanol	570
2	Dried	Methanol	650
3	Fresh	Methanol	150
4	Dried	Methanol	700
5	Dried	Methanol	370
6	Fresh	Methanol	75
Total body (fresh) minus the skin			<0·2

It may be seen that, exactly as with eledoisin (Anastasi & Erspamer, 1963), acetone is not a suitable solvent for the extraction of physalaemin. In fact, the amount of polypeptide extracted from the dried skins of batch (1) by acetone was barely 50% of that extracted by methanol.

With methanol extracts, the physalaemin content was fairly even in the different batches of skin. Fresh tissue contained 4.5-times less physalaemin than dry tissue (compare batches 2 and 3 collected at the same place and at the same time), but 4.3 g of fresh skin were required to make up 1 g of dried skin. This means that the physalaemin content of a skin is the same before and after drying, and therefore that physalaemin withstands the drying process perfectly (Fig. 1,*b*).

The only site of physalaemin in the *Physalaemus* body is the skin. The body deprived of the skin does not contain detectable amounts of the polypeptide.

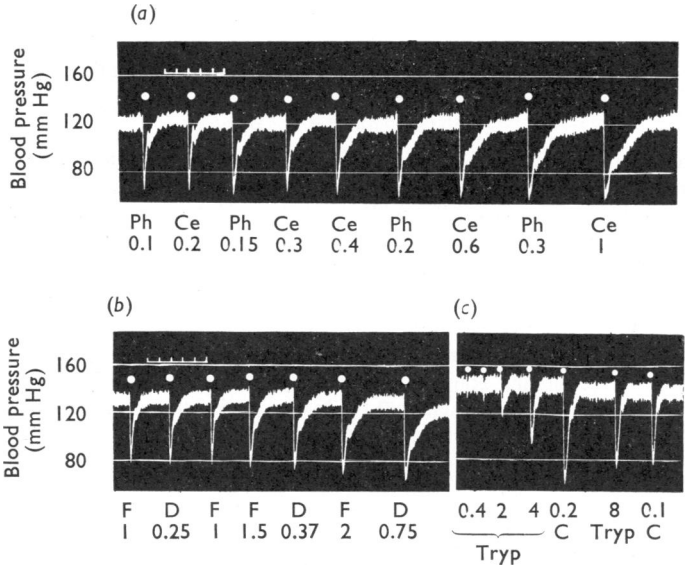

Fig. 1. Blood pressure records from two dogs anaesthetized with pentobarbitone (30 mg/kg, intravenously) and previously treated with 0.2 mg/kg of atropine sulphate, intramuscularly. Time marks, 1 min.

(*a*) Quantitative estimation of physalaemin (Ph, synthetic physalaemin in μg) in the crude methanol extract of the dried skin of *Physalaemus centralis* (Ce, dried skin in mg). The extract corresponding to 0.6 mg of skin showed the same activity as 0.25 μg of physalaemin.

(*b*) The same dog as for (*a*). The relative activity of extracts of dried (D) and fresh (F) skin of *Physalaemus fuscumaculatus* (doses in mg). Dried skin was approximately 4.5-times as active as fresh skin.

(*c*) A different dog. The action of trypsin on physalaemin (doses in μg). C, Control physalaemin; Tryp, physalaemin incubated with trypsin. Trypsin produced a 98% inactivation of physalaemin.

Occurrence of physalaemin-like polypeptides in the skin of other Physalaemus *species and of amphibians belonging to other genera*

Table 2 shows the content of physalaemin or physalaemin-like substances in the skin of three additional *Physalaemus* species, as estimated in parallel, against physalaemin, on seven test systems.

TABLE 2

THE CONTENT OF PHYSALAEMIN IN THE SKIN OF THREE OTHER *PHYSALAEMUS* SPECIES DETERMINED IN PARALLEL ASSAY

Contents are expressed as μg of physalaemin per g of skin. —, not tested

	Content of physalaemin-like substances (μg/g) in skin of		
Test preparation	*Phys. centralis*	*Phys. bresslaui*	*Phys. cuvieri*
Rabbit large intestine	400	34	0·1
Guinea-pig ileum	380	32	—
Guinea-pig colon	400	—	—
Dog colon	410	—	—
Dog blood pressure	380	14	0·1
Chicken blood pressure	300	45	—
Rabbit blood pressure	430	—	—

While it is highly probable that the active polypeptide occurring in the skin of *Physalaemus centralis* is authentic physalaemin (see also Fig. 1,*a*), the same cannot be said for the polypeptide present in the skin of *Physalameus bresslaui* which, compared with physalaemin, seems more active on intestinal smooth muscle than on the dog blood pressure. It appears therefore prudent to speak in this case of a physalaemin-like polypeptide.

Approximately 150 other amphibian species were examined with regard to their content of physalaemin-like polypeptides, that is of polypeptides which, beside possessing, like physalaemin, a potent stimulant action on the rabbit colon and a potent hypotensive action in the dog, are completely inactivated when incubated with either chymotrypsin or trypsin.

Positive results were obtained only in several *Phyllomedusa* species and in one not exactly determined *Telmatobius* species (Table 3). For all other examined amphibians the stimulant action on the rabbit colon and the hypotensive action in the anaesthetized dog displayed by the methanol extract corresponding to 1 g skin were always less than, and qualitatively different from, those produced by 0.1 to 1 μg of physalaemin.

TABLE 3

OCCURRENCE OF PHYSALAEMIN-LIKE POLYPEPTIDES IN THE SKIN OF AMPHIBIAN SPECIES OTHER THAN *PHYSALAEMUS*

Contents are expressed as μg of physalaemin per g of skin. —, not tested

Species and preparation	Content of physalaemin-like polypeptides (μg/g) in skin determined on		
	Dog blood pressure	Rabbit colon	Guinea-pig ileum
Phyllomedusa annae			
(Costarica) dry skin	60–70	100	50
Phyllomedusa helenae			
(Costarica) dry skin	300	300	160
Phyllomedusa callidryas			
(Panama) dry skin	330–350	300–350	230–250
(Mexico) dry skin	270	280	160–200
Phyllomedusa dacnicolor			
(Mexico) fresh skin	120–150	160	—
(Mexico) dry skin	500	450–500	250–280
Phyllomedusa hypochondrialis			
(Rondonia) dry skin	270	150	—
(Chaco, Argentina) fresh skin	2–3	2–4	2–2·5
Phyllomedusa rohdei			
(Brazil) dry skin	15	20–25	30
Telmatobius sp. (jelskii)	20	15–18	—

On the basis of its behaviour in high-voltage electrophoresis it is certain that the physalaemin-like polypeptide of *Phyllomedusa hypocondrialis* is different from physalaemin, but we do not know whether the different *Phyllomedusa* species contain the same physalaemin-like polypeptide or different polypeptides. The problem must be left open until the isolation or a sufficient purification of the single active polypeptides is attained. This is particularly true for the *Phyllomedusae* which, in addition to physalaemin-like polypeptide(s), contain other polypeptides, for example bradykinin-like ones.

Partial purification of crude extracts of Physalaemus fuscumaculatus *skin on an alumina column; separation of physalaemin from other active polypeptides*

The first step in the purification and isolation procedure of physalaemin was passage through an alumina column (Anastasi *et al.*, 1964). It was carried out in order to obtain

large amounts of biologically pure physalaemin and to separate from physalaemin the minor active polypeptides occurring in the *Physalaemus* skin.

In a typical experiment, 300 ml. of methanol extract, corresponding to 27.5 g of fresh skin (batch 6), was evaporated, at 45 to 50° C under reduced pressure, to 30 to 40 ml. and the remaining aqueous liquid was extracted repeatedly with petroleum ether to remove fats. The distillation was then continued until the residue was of syrupy consistency.

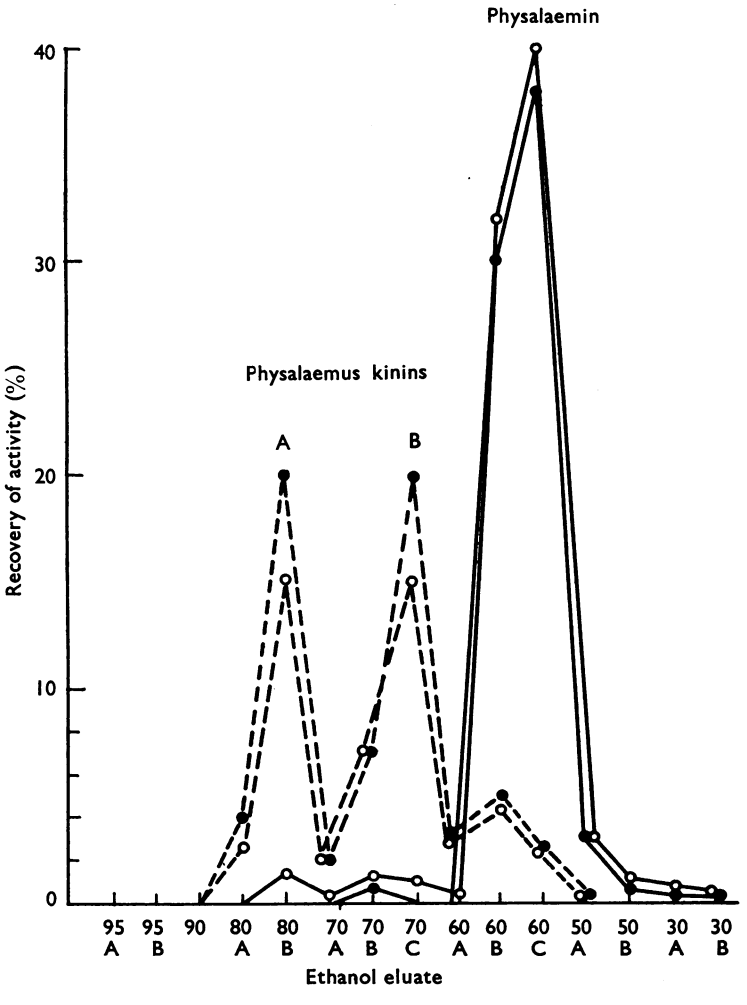

Fig. 2. Elution of physalaemin and *Physalaemus* kinins from an alkaline alumina column with descending concentrations of ethanol. The column was loaded with a crude extract of fresh skin of *Physalaemus fuscumaculatus*. The activity of eluates was assayed on the rat uterus (●---●), rat colon (○---○), rabbit large intestine (○——○) and dog blood pressure (●——●). Physalaemin appeared in ethanol eluates 60B and 60C, *Physalaemus* kinin A in eluate 80B, and *Physalaemus* kinin B in ethanol eluates 70B and 70C. Numbers at bottom indicate the ethanol concentrations of the eluates; capital letters indicate the eluate fractions obtained successively with ethanol of the same concentration.

The residue was taken up, in a warm-water bath, by stirring in 100 ml. of 96% ethanol. The precipitate found after storing overnight was discarded and the liquid was passed through a column of 140 g (21 × 3.3 cm) of alkaline alumina. Elution was performed at room temperature with descending concentrations of ethanol and the eluate was collected in fractions of 100 ml. each.

Each fraction was assayed in parallel on four test objects and the activity was expressed as a percentage of the activity of the crude methanol extract. Furthermore, the eluates were chromatographed on paper after having been suitably concentrated (1 ml. of liquid derived from 10 g of fresh skin) in order to check the eventual occurrence of biogenic amines. Essential results are shown in Fig. 2. Eluates from the alumina column show three main peaks of activity, two of which were evident on the rat large intestine and the rat uterus, and one on the dog blood pressure and the rabbit large intestine. The major activity displayed by eluates 60B+60C was entirely due to physalaemin, as definitely demonstrated by the isolation of the polypeptide. The two peaks of activity shown by eluates 80B and 70C, respectively, were due to at least two other polypeptides of unknown nature, provisionally called *Physalaemus* kinins A and B.

The dry residue left by an amount of ethanol eluates 60A+60B possessing the hypotensive activity of 1 g of fresh skin was approximately 1 mg, compared to 25 mg of residue left by the crude methanol extract of 1 g of fresh skin. In four separate experiments of chromatography on alumina, recovery of physalaemin in the 60% ethanol eluates varied between 60 and 70%.

In order to check whether *Physalaemus* kinins A and B were really distinct compounds, and not artefacts due to possible overloading of the column, eluates 90, 80A, 80B, 70A, 70B and 70C were mixed together and evaporated to dryness and the residue was taken up in 100 ml. of 96% ethanol. This was then passed through another alumina column. Elution was carried out as described above. Again two distinct peaks of activity on rat uterus and rat colon appeared in 80A+80B eluates (*Physalaemus* kinin A) and in 70B eluate (*Physalaemus* kinin B), respectively.

The only biogenic amine occurring in minute amounts (2.5 μg/g of fresh tissue) in the skin of *Physalaemus fuscumaculatus* was leptodactyline. It was eluted by 96% ethanol.

On paper chromatograms run with the *n*-butanol : acetic acid : water mixture (4 : 1 : 5), the spot containing the physalaemin activity gave sharp positive reactions not only to ninhydrin and chlorine, but also to the iodoplatinate reagent for sulphur amino acids and the α-nitroso-β-naphthol reagent for tyrosine (Anastasi *et al.*, 1964).

Effect on physalaemin of proteolytic enzymes and of treatment with diazonium salts

Chymotrypsin. 10 to 50 μg/kg of pure physalaemin or an equivalent amount of the semipurified *Physalaemus* extract were brought to *p*H 7.4 to 7.6 with sodium carbonate and then, after addition of 100 μg of crystalline chymotrypsin, incubated in a water-bath at 37° C for 30 min. Chymotrypsin action was arrested by immersion of the incubation flasks in boiling water for 3 to 5 min. Inactivation of physalaemin was always greater than 98%.

Trypsin. A similar experiment was carried out using 1 mg of crystalline trypsin. Inactivation was again complete (over 97%) (Fig. 1,*c*).

Carboxypeptidase. 100 μg of pure physalaemin were incubated for 12 hr at 37° C and at *p*H 8.2 with 10 μg carboxypeptidase. No loss of activity occurred.

It may be seen that the behaviour of physalaemin towards proteolytic enzymes was similar to that of eledoisin, with the remarkable difference that, whereas digestion with trypsin was complete for physalaemin, it was incomplete for eledoisin (remaining activity, 10 to 15%). This depends upon the nature of the split products after trypsin has acted upon the two polypeptides (Anastasi & Erspamer, 1963; Anastasi *et al.*, 1964).

The less-active polypeptides, *Physalaemus* kinins A and B, were completely inactivated by chymotrypsin, but after digestion with trypsin inactivation was partial.

Treatment with diazonium salts. Adding diazotized *p*-nitroaniline or diazotized sulphanilic acid plus sodium carbonate to crude *Physalaemus* extracts or to pure physalaemin produced a 90 to 95% decay of the hypotensive action of physalaemin in the dog. Sodium nitrite plus sodium carbonate were ineffective. No inactivation of eledoisin could be observed under the same conditions.

It is highly probable that inactivation of physalaemin is due to coupling of the diazonium salt with the tyrosine residue in the molecule of the polypeptide.

General effects in the unanaesthetized animal

Dog. The general effects of subcutaneous administration of physalaemin were studied in nine mongrel dogs, weighing 7 to 18 kg.

Dog 1 received, in the inguinal region, a subcutaneous injection of 300 μg/kg of synthetic physalaemin. After a few minutes vomiting occurred, accompanied by profuse salivation, and soon followed by discharges of formed stools and then of watery stools. In all, three episodes of vomiting, of moderate severity, and fourteen discharges were counted during the first hour. Recovery was rapid. Dogs 2 and 3 each received 125 μg/kg of physalaemin. Phenomena were similar to those observed in dog 1, but less intense.

Physalaemin in doses of 60 μg/kg caused moderate salivation and diarrhoea in both dogs 4 and 5, and vomiting in dog 4; doses of 30 μg/kg caused moderate salivation in dogs 6 and 7, and one evacuation of formed stools in dog 6. No evident alimentary effects, except slight salivation, appeared in dogs 8 and 9 given 15 μg/kg of physalaemin.

It may be seen that general effects produced by subcutaneous physalaemin were remarkably less intense than those produced by similar subcutaneous doses of eledoisin. In fact, the tremendous gastro-intestinal stimulation, joined to profound depression of the animal, produced by 25 to 50 μg/kg of eledoisin (Erspamer & Falconieri Erspamer, 1962) was never observed even with 125 to 300 μg/kg of physalaemin.

The general effects of rapid intravenous injection of physalaemin into eighteen dogs weighing 12 to 19 kg were studied by Bertazzoli & Cheli (personal communication). 0.15 μg/kg of physalaemin (four dogs) produced no obvious effects; after 0.375 μg/kg (four dogs) the only appreciable effect was that the dog had difficulty in sitting (tenesmus ?), and this only for a few minutes; 0.75 μg/kg (four dogs) produced the same effect and in addition one or more evacuations of the bowel during the first 5 min; 3 μg/kg (four dogs) caused not only diarrhoea but also salivation, lasting 5 to 10 min. Increased salivation and diarrhoea were accompanied by vomiting in the two dogs treated with 15 μg/kg of phy-

salaemin. All symptoms disappeared within 15 to 20 min. Similar doses of physalaemin (3 μg/kg), given intravenously every day for ten successive days, elicited the same phenomena.

Rat. Groups of three to eight rats weighing 150 to 200 g were given, subcutaneously, doses of 6, 15, 30, 60, 120, 300, 600 and 1,800 μg/kg of physalaemin, respectively per group. 6 μg/kg produced no appreciable effect, and 15 to 30 μg/kg slight flushing of the ears, snout and paws. Flushing increased in intensity and duration with the dose, and was sometimes accompanied by palpebral oedema (300 μg/kg and more). Increased salivation was first detected with 30- to 60-μg/kg doses and then, starting from 300 μg/kg, increased nasal and lacrimal secretion was also seen, together with some respiratory distress. Nasal secretion was occasionally slightly haemorrhagic. There was no chromodacryorrhoea, diarrhoea or other signs of gastro-intestinal stimulation. Full recovery took, even for the largest doses, not more than 30 to 45 min.

Action on isolated preparations of gastro-intestinal smooth muscle

Rabbit. Isolated loops from all sections of the rabbit intestine responded to physalaemin with contraction. The most suitable segment was the large intestine, and particularly its terminal 20 to 30 cm.

The response to physalaemin was indistinguishable from that to eledoisin and to substance P. Like eledoisin, physalaemin caused the appearance or the reinforcement of rhythmic movements and an increase in tone. The effect was immediate with high doses of the polypeptide, and preceded by a short latency period with small doses. Stimulation was never preceded by depression. High doses of physalaemin produced an intense spasm of the intestinal loop followed, as spastic contraction slowly subsided, by rhythmic movements of increasing amplitude lasting for hours (Fig. 3). There was neither tachyphylaxis nor sensitization.

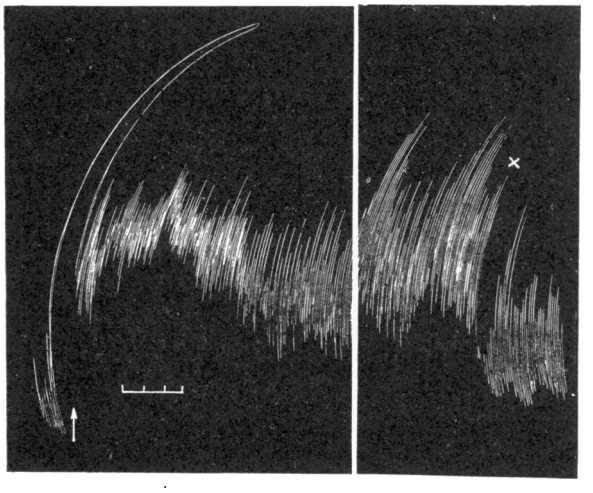

Fig. 3. Rabbit large intestine preparation suspended in 10 ml. of Tyrode solution at 37° C. At the arrow 5 μg of physalaemin; at ×, washing, 4 hr later. Time marks, 10 min. Physalaemin produced a formidable increase in tone and rhythmic activity which lasted until washing, 4 hr later.

The stimulant action due to 1 to 3 ng/ml. of physalaemin was unaffected by atropine or hexamethonium, in concentrations up to 1 and 100 μg/ml., respectively. Similarly, nicotine (1 to 100 μg/ml.) and lysergic acid diethylamide (0.01 to 10 μg/ml.) failed to produce any change in the response to physalaemin.

Morphine had no action at concentrations of 1 to 10 μg/ml., but at higher concentrations (100 to 200 μg/ml.) somewhat reinforced the action of physalaemin. Papaverine was ineffective up to 1 μg/ml.; concentrations of 3 μg/ml. reduced by 50 to 75% the stimulant action of 1.5 ng/ml. of physalaemin (Fig. 4), and concentrations of 10 μg/ml. by 80 to 90% the action of 5 ng/ml. physalaemin. The effect of papaverine was reversible. Chlorpromazine behaved like papaverine.

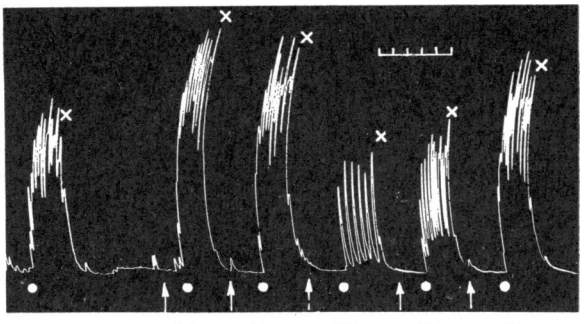

Fig. 4. Rabbit large intestine preparation suspended in 10 ml. of Tyrode solution at 37° C. At the white dots, physalaemin (doses in ng); at the arrows, papaverine (doses in μg); at ×, washing. Time marks, 1 min. 30 μg of papaverine clearly depressed the spasmogenic action of physalaemin.

0.5 μg/ml. of (−)-noradrenaline reduced by 50%, and 1 μg/ml. by 60 to 70% the stimulation elicited by 2 ng/ml. of physalaemin.

As with eledoisin, the rabbit large intestine is one of the best preparations for the qualitative detection and quantitative estimation of physalaemin in tissue extracts. It is extremely sensitive to the polypeptide (threshold concentration 0.2 to 1 ng/ml.) and shows a very satisfactory dose/response relationship. In addition, the preparation is rather insensitive to most biogenic substances known to stimulate smooth muscle (5-hydroxytryptamine, tryptamine, histamine, darmstoff and bradykinin). Among the hypotensive polypeptides, practically the only group of compounds which so far has been found to be highly active in stimulating the rabbit colon is that of eledoisin-like polypeptides, including in this group substance P.

An amount of 1 μg of physalaemin corresponds to approximately 1.3 to 1.6 μg of eledoisin and to 180 to 200 units of substance P.

The large intestine was most suitable for the bioassay of physalaemin after it had been kept for 12 to 36 hr in Tyrode solution at 3 to 5° C. However, intestines stored in cold Tyrode solution for a considerably longer time also gave excellent responses.

Guinea-pig. Like the rabbit intestine, that of guinea-pig, especially the ileum, responded to physalaemin with only a contraction, the effect produced by the polypeptide again

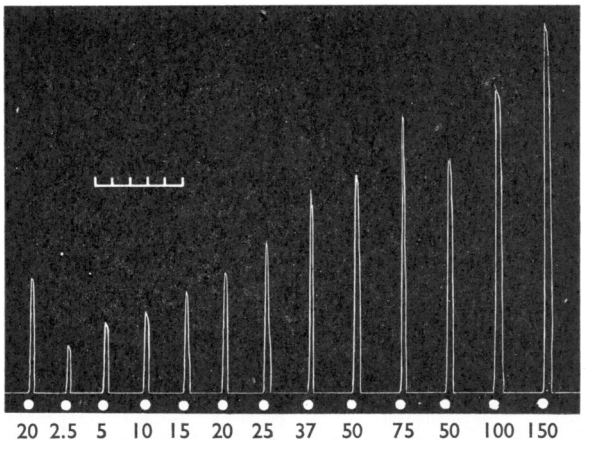

Fig. 5. Guinea-pig ileum preparation suspended in 10 ml. of Krebs solution at 32° C. Contractions produced by increasing doses of physalaemin (doses in ng). Time marks, 1 min. Note the excellent dose/response relationship.

closely resembling that elicited by eledoisin or substance P. The threshold concentration was 0.2 to 0.5 ng/ml. and there was again a good dose/response relationship (Fig. 5).

Atropine and mepyramine in doses (0.1 to 0.2 μg/ml.) that completely abolished the action of acetylcholine and histamine did not affect the spasmogenic action of physalaemin. The same was true for hexamethonium (20 μg/ml.). Morphine (2 μg/ml.) produced only a slight decrease in the response to physalaemin, but a considerable decrease in the response to nicotine. Similarly, lowering the temperature of the bath from 32 to 15° C for 30 min did not appreciably affect the height of contraction elicited by physalaemin, while producing a sixfold reduction of the response to nicotine. The above results point to a predominantly direct effect of physalaemin on intestinal smooth muscle.

The guinea-pig large intestine was less sensitive to physalaemin than was the ileum. On the latter preparation 1 μg of physalaemin was equiactive to 1.5 to 1.6 μg of eledoisin, 250 units of substance P, 5 to 40 μg of bradykinin, and 5 to 15 μg of histamine.

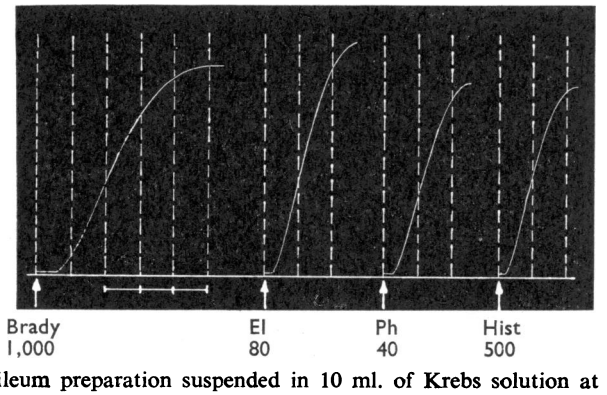

Fig. 6. Guinea-pig ileum preparation suspended in 10 ml. of Krebs solution at 32° C. Contractions produced by bradykinin (Brady), eledoisin (El), physalaemin (Ph) and histamine (Hist). All doses in ng. Time marks, 5 sec.

Fig. 6 shows that, whereas physalaemin and eledoisin are indistinguishable from each other and from histamine in the rapidity of onset of the stimulant action, the onset of action of bradykinin is considerably more delayed and the ascent of the contraction curve less steep.

Rat. Both rat intestines (duodenum and large intestine) and stomach were contracted by physalaemin and the contraction, which was never preceded by depression, showed a good correlation with dose both for the stomach (Fig. 7) and for the large intestine (Fig. 8). However, whereas rat gastro-intestinal smooth muscle was not very sensitive to physalaemin

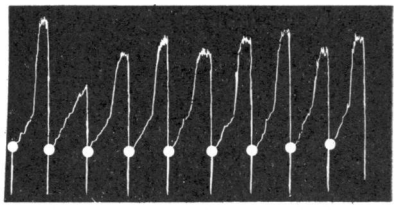

Fig. 7. Rat stomach preparation suspended in Krebs solution at 37° C. Contractions produced by different doses (in μg) of physalaemin (Ph) and eledoisin (El). In this experiment 0.4 μg of physalaemin was equiactive to 0.06 μg of eledoisin.

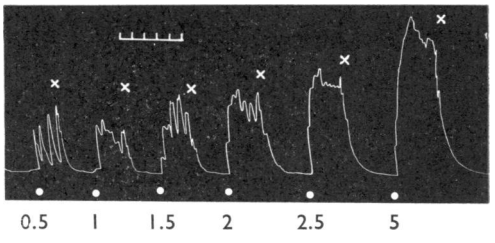

Fig. 8. Rat large intestine preparation suspended in 10 ml. of Tyrode solution at 30° C. Contractions produced by increasing doses (in μg) of physalaemin. At ×, washing. Time marks, 1 min. The dose/response relationship was satisfactory.

(threshold 0.05 to 0.2 μg/ml.), it showed considerable sensitivity towards some other polypeptides of the amphibian skin, among which were the *Physalaemus* kinins. This makes these preparations unsuitable for the study of physalaemin in crude tissue extracts. The approximate equivalents to 1 μg of synthetic physalaemin were as follows: stomach, 0.1 to 0.2 μg of eledoisin; duodenum, 0.1 μg of eledoisin; large intestine, 0.03 to 0.04 μg of eledoisin, 10 to 100 μg of bradykinin and 2.5 to 3.5 units of substance P.

In crude *Physalaemus* extracts as much as 90 to 95% of the stimulant action on rat large intestine was due to *Physalaemus* kinins, and only 5 to 10% to physalaemin.

Dog. Both duodenum and large intestine responded to physalaemin with a reinforcement of the rhythmic activity and an increase in tone which was proportional to the dose. However, in a number of experiments the preparations did not give satisfactory results and in no instance did they offer any advantage over the rabbit large intestine or the guinea-pig ileum preparation.

In the dog large intestine preparation, the threshold dose of physalaemin was 1 to 10 ng/ml. and 1 μg of the polypeptide was equivalent to 1.2 to 1.6 μg of eledoisin, 0.2 to 0.4 μg of bradykinin and 250 to 300 units of substance P (Fig. 9).

Pigeon. The duodenum was not very sensitive to physalaemin (threshold 5 to 30 ng/ml.), but an increase in tone, eventually accompanied by reinforcement of rhythmic activity, was proportional to the dose. This renders the preparation a useful subsidiary test object in the bioassay of physalaemin. 1 μg of physalaemin was equiactive to approximately 0.5 to 0.7 μg of eledoisin, more than 200 μg of bradykinin and 40 to 50 units of substance P.

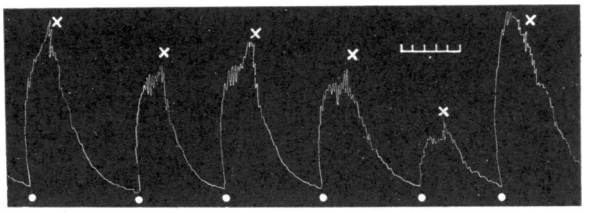

Fig. 9. Dog large intestine preparation suspended in 10 ml. of Tyrode solution at 37° C. Responses to physalaemin (Ph), eledoisin (El) and bradykinin (Brady). At ×, washing. Time marks, 1 min. All doses in ng. Note the similarity of contractions produced by the three polypeptides. In this preparation bradykinin was the most active polypeptide, and eledoisin was the least active.

Other intestinal preparations. Cat small and large intestine, fowl rectal caecum and frog stomach responded to physalaemin with an increase in tone and an enhancement of spontaneous motility. However, none of these preparations is recommended in the routine bioassay of physalaemin.

Action on other smooth muscle preparations

Rabbit uterus. Physalaemin stimulated the rabbit uterus in concentrations of more than 10 ng/ml., and the increase in tone was proportional to the dose. Since the preparation is poorly sensitive to 5-hydroxytryptamine, histamine and bradykinin but, at the same time, about ten-times more sensitive to eledoisin than to physalaemin, it may be used in the distinction of these two polypeptides.

Rat, cat and guinea-pig uterus. Uterine strips from these three species were poorly sensitive to physalaemin and the response was irregular.

For the rat uterus the threshold concentration of physalaemin varied between 0.5 and 2 μg/ml. The approximate equivalents to 1 μg of physalaemin were 0.10 to 0.15 μg of eledoisin, 0.1 ng of bradykinin, and 3 to 10 units of substance P.

The rat uterus is extremely sensitive not only to bradykinin but also to bradykinin-like polypeptides, as well as to 5-hydroxytryptamine. This makes the preparation useless in the bioassay of physalaemin, but useful in the detection of other active constituents eventually accompanying physalaemin in crude tissue extracts. Even with extracts of *Physalaemus* skin as much as 95% of the stimulant activity on the rat uterus was due to *Physalaemus* kinins, and barely 5% to physalaemin.

Guinea-pig and rat seminal vesicles. The first preparation responded with contraction to concentrations of physalaemin above 0.05 to 0.15 μg/ml.; the second was virtually insensitive to the polypeptide.

At usual doses, atropine did not affect the action of physalaemin on any of the above preparations.

Action on salivary secretion

Salivary secretion was studied in anaesthetized dogs and rats. Details on methods and results will be published elsewhere (Bertaccini & De Caro, 1965).

The threshold intravenous dose of physalaemin in the dog was 0.5 to 0.75 μg/kg, and in the rat 0.15 to 0.3 μg/kg. There was always a clear dose/response relationship, and in both animal species physalaemin was three-times as active as eledoisin. Neither atropine (0.2 mg/kg in the dog, up to 20 mg/kg in the rat, intravenously) nor 2-diethylaminomethylbenzo-1,4-dioxan (prosympal; 2 mg/kg in the dog, up to 50 mg/kg in the rat, intravenously) altered the effect of physalaemin on the salivary gland.

Distinction of physalaemin from eledoisin, substance P and other active tissue constituents

Criteria for the distinction, in crude tissue extracts, of physalaemin from biogenic amines, lipid-soluble organic acids and bradykinin-like polypeptides capable of stimulating extravascular smooth muscles and of lowering the dog and rabbit blood pressure are exactly the same as those already described for eledoisin in a previous paper (Erspamer & Falconieri Erspamer, 1962). This distinction never presented a problem.

Considerably more difficult, on the contrary, is the distinction of physalaemin from eledoisin, substance P and strictly related polypeptides. In fact, not only are the actions of physalaemin on all tested extravascular smooth muscles and on blood pressure of all experimented animals indistinguishable, from a qualitative point of view, from those of the other two polypeptides, but even in parallel assays indices of discrimination are rarely high, as is clearly apparent from Table 4.

TABLE 4

APPROXIMATE EQUIVALENTS TO 1 μG PHYSALAEMIN

—, not tested

Test preparation	Eledoisin (μg)	Substance P (units)	Bradykinin (μg)	Histamine (μg)
Dog blood pressure	3·5	100–140	200–1,000	200–800
Rabbit blood pressure	2·8–3·0	280–380	15–25	—
Chicken blood pressure	2·8–3·6	300	100	—
Rabbit large intestine	1·3–1·6	180–200	75–500	1,000
Guinea-pig ileum	1·5–1·6	250	5–40	5–15
Dog large intestine	1·2–1·6	250–300	0·2–0·4	—
Pigeon duodenum	0·5–0·7	40–50	>200	—
Rat colon	0·03–0·04	2·5–3·5	10–100	—
Rat uterus	0·11–0·15	3–10	0·0001	Inhibition
Rabbit uterus	0·09–0·12	—	1,000	—

In addition, it should be stressed that results for substance P must be considered provisional, because the available substance P sample was a very crude preparation, and Haefeli & Hürlimann (1962) and Cleugh & Gaddum (1963) have shown that pure substance P is considerably less potent than crude substance P on several smooth muscle preparations, and therefore that crude substance P must contain active contaminants. Hence the validity of indexes of discrimination for physalaemin against substance P using rabbit large intestine (or guinea-pig ileum or dog blood pressure) and rat uterus (or rat colon or pigeon duodenum) is questionable.

Table 4 shows that physalaemin is more potent than or as potent as eledoisin on a number of preparations, but consistently less potent on other preparations, especially on rat smooth muscles and on the rabbit uterus. This should help in the distinction of the two polypeptides.

Other additional criteria for the discrimination of the different eledoisin-like polypeptides may be found in their behaviour towards proteolytic enzymes and towards diazonium salts.

Whereas, for example, physalaemin is totally inactivated by trypsin, this does not occur with eledoisin and, similarly, whereas activity of physalaemin is reduced by 95% after coupling with diazonium salts, activity of eledoisin remains practically unchanged.

Amphibian skin contains, beside physalaemin- and bradykinin-like polypeptides, other active polypeptides. Although their study is still in progress, it has already been firmly established that they may be sharply and easily distinguished from eledoisin-like polypeptides in parallel assays.

DISCUSSION

Methanol extracts of the skin of *Physalaemus fuscumaculatus* contain several active polypeptides, the most potent and important of them being *physalaemin*. The polypeptide is lacking in all other tissues of the amphibian. Physalaemin or physalaemin-like polypeptides are present also in the skin of other *Physalaemus* species and in several *Phyllomedusae*. The polypeptide withstands drying very well, so that dried skins may be used as profitably as fresh skins for the preparation of highly active methanol extracts.

In its pharmacological actions on extravascular smooth muscle and on secretions, physalaemin strongly resembles eledoisin, and appropriate parallel assays are necessary to distinguish the two polypeptides from each other.

Physalaemin possesses a more-or-less intense stimulant action, which is never preceded by depression, on all examined preparations of gastro-intestinal tract. The threshold dose is sometimes less than 1 ng/ml. of nutrient liquid. On extravascular smooth muscles other than the gastro-intestinal tract, physalaemin is considerably less potent, with the possible exception of the bronchial musculature of the guinea-pig (Berretta & Nobili, unpublished). Among all tested preparations, the rabbit large intestine and the guinea-pig ileum are most suitable for the qualitative demonstration and the quantitative estimation of physalaemin. The rabbit large intestine is particularly recommended owing to its low sensitivity to indolealkylamines, histamine and bradykinin, and to its exceptionally long responsiveness when stored in cold Tyrode solution. It will be seen that the blood pressure of the dog and, subordinately, that of the rabbit and the chicken, are other excellent test objects for the bioassay of physalaemin.

The mechanism of action of physalaemin on the intestinal smooth muscle is not completely clear, like that of eledoisin and substance P. While results obtained in the rabbit large intestine and guinea-pig ileum point to a predominantly direct stimulation of the smooth muscle fibres, other results, which will be discussed in a later paper (hypertensive action of physalaemin in fowls and rats), indicate that the polypeptide may also act through stimulation of nervous structures.

With the aid of parallel assays on different smooth muscle preparations and on the blood pressure of different experimental animals, and by the use of proteolytic enzymes, physalaemin may easily be distinguished from biogenic amines, lipid-soluble acids and bradykinin-like polypeptides. However, it is considerably more difficult to distinguish physalaemin from eledoisin and substance P. Often the index of discrimination for physalaemin against the two other polypeptides is rather low. The possibility that the chemical structure of substance P closely resembles physalaemin and especially eledoisin will be discussed in detail elsewhere.

The significance of physalaemin and related peptides in the amphibian skin is obscure, as is that of bradykinin (Anastasi, Erspamer & Bertaccini, 1965) and bradykinin-like polypeptides and that of the several other polypeptides we have traced in the same tissue. Eledoisin-like and bradykinin-like polypeptides strongly increase capillary permeability in man and other mammals (De Caro, 1963). One would be tempted to suggest that these polypeptides display a similar action in the amphibian skin which is known to be of exceptional importance in the exchange of water and electrolytes.

It is possible that the function of a given polypeptide in the skin of a group of amphibians, or even of a single amphibian species, is taken over by another polypeptide in the skin of other amphibian species. Available results show that active polypeptides are mainly localized, often together with biogenic amines, in the cutaneous glands, the secretion of which is external. This circumstance should not be overlooked in the interpretation of the biological significance of amines and polypeptides in the amphibian skin.

SUMMARY

1. The skin of *Physalaemus fuscumaculatus*, alone among the tissues of this South-American amphibian, contains *physalaemin*, an endecapeptide strictly related to eledoisin, and also two or more minor polypeptides provisionally called *Physalaemus* kinins.

2. Physalaemin or physalaemin-like polypeptides are also present in methanol extracts of the skin of other *Physalaemus* species, as well as of several *Phyllomedusa* species.

3. Physalaemin is unaffected when the skin is dried. It is completely inactivated both by chymotrypsin and trypsin, while it is resistant to carboxypeptidase. Owing to the occurrence of tyrosine in its molecule, the polypeptide is largely inactivated by coupling with diazonium salts in an alkaline medium.

4. Passage of crude skin extracts of *Physalaemus* through an alumina column yields preparations of physalaemin which may be considered pure from a biological point of view and which are suitable for a complete chemical purification of the polypeptide.

5. In the unanaesthetized dog physalaemin has a considerable stimulant effect on gastro-intestinal motility, as shown by diarrhoea and vomiting, whereas the rat gut shows no signs of obvious stimulation. Salivary secretion is stimulated both in the dog and in the rat.

6. All the preparations of gastro-intestinal smooth muscle tested are rather strongly stimulated by physalaemin. Preparations of other smooth muscles are less sensitive.

7. Exactly as with eledoisin, the rabbit colon, the guinea-pig ileum and, to a lesser degree, the pigeon duodenum, may be profitably used, owing to their sensitivity and the clear dose/response relationship, for the quantitative bioassay of physalaemin in crude or pure preparations of the peptide.

8. In parallel assays physalaemin may be easily distinguished from the biogenic amines and from all known naturally occurring hypotensive polypeptides. However, the strict biological resemblance of physalaemin with eledoisin and substance P is emphasized.

9. The biological significance of physalaemin and physalaemin-like polypeptides in the amphibian skin is obscure. It may be tentatively suggested that they have something to do with regulation of the permeability of the skin to water and electrolytes.

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