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JORGE D. WILLIAMSY BIBLIOTECA
Dr. José Miguel CeíSeparatum EXPERIENTIA 20, 489 (1964)
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Structure and Pharmacological Actions of Physalaemin, the Main Active Polypeptide of the Skin of *Physalaemus fuscumaculatus*¹

It was shown² that methanol extracts of the skin of the South American amphibian *Physalaemus fuscumaculatus* contain an eledoisin-like polypeptide which, like eledoisin, exerts a powerful hypotensive action and stimulates extravascular smooth muscle. This polypeptide, called *physalaemin*, has now been isolated in pure form and identified as the endcapeptide Pyr-Ala-Asp(OH)-Pro-Asp(NH₂)-Lys-Phe-Tyr-Gly-Leu-Met-NH₂.

Synthesis confirmed the above constitution³, closely resembling that of eledoisin^{4,5}.

Isolation procedure and determination of structure. 471 fresh skins of *Physalaemus fuscumaculatus* weighing 206 g and 323 dried skins weighing 32.8 g (physalaemin withstands drying perfectly) were extracted twice with 80% methanol. The combined filtered extracts were kept in a refrigerator and served as standard crude extract for the isolation of physalaemin.

The first step in the purification of physalaemin was the absorption of the crude polypeptide dissolved in 95% ethanol on alkaline alumina and subsequent elution with decreasing concentrations of ethanol. Physalaemin was eluted by 60% ethanol, whilst one or two minor active polypeptides were eluted with 80 and 70% ethanol.

Final purification was achieved by submitting the partially purified material to a double 40 transfers counter-current distribution using two *n*-butanol water systems with pH 2 and 9 respectively in the water phase. The yield was 70 to 80%.

Pure physalaemin was homogeneous in paper chromatography and electrophoresis, showing a single spot with ninhydrin, chlorine, the iodoplatinate reagent of sulphur amino acids⁶ and the α -nitroso- β -naphthol reagent of tyrosine⁷. In ascending paper chromatograms with the system *n*-butanol:acetic acid:water (4:1:1), physalaemin had R_f 0.41. On electropherograms it migrated towards the cathode at acid pH and showed no mobility at neutral pH. Acid hydrolysis yielded glutamic

and aspartic acids, alanine, proline, lysine, phenylalanine, tyrosine, glycine, leucine and methionine. Ten out of eleven residues were present in a 1:1 mole ratio, and only the aspartyl residue was found to be present in a 2:1 ratio with respect to the other amino acids.

The sequence was elucidated through chemical and enzymatic degradation. Physalaemin, like eledoisin, was resistant to enzymatic attack at both the C and N terminus. The Sanger and Edman techniques failed to reveal a free N-terminal residue. Upon digestion with chymotrypsin, the consecutive appearance of the following fragments was observed in the reaction mixtures:

C ₁ [Glu, Ala, 2 Asp, Pro, Lys, Phe, Tyr],	C ₂ [Gly, Leu, Met],
C ₃ [Glu, Ala, 2 Asp, Pro],	C ₄ [Lys, Phe, Tyr],
C ₅ [Lys, Phe],	C ₆ H-Tyr-OH,
C ₇ [Gly, Leu]	and C ₈ H-Met-NH ₂

It followed that chymotrypsin caused a rapid cleavage at the carboxyl bond of tyrosine with release of the C-terminal tripeptide C₂, then a bond was split between the C end of the N-terminal pentapeptide and the amino group of lysine. Upon further action of the enzyme, tyrosine was slowly released from C₄ and finally a very slow and incomplete splitting of methionamide from C₂ was observed.

Tryptic digestion was anomalous, since two bonds were split, one of which was a lysyl and the other a tyrosyl bond, and the rapid and simultaneous formation of the following three peptides was observed:

T₁ [Glu, Ala, 2 Asp, Pro, Lys], T₂ [Phe, Tyr] and T₃ [Gly, Leu, Met]

From the above results the sequence of the C-terminal hexapeptide was deduced, the position of the single residues being established by the specificity of the enzymes and by the composition of the fragments. The sequence Gly-Leu was determined by the fluorodinitrobenzene procedure on the tripeptide Gly-Leu-Met-NH₂.

Under the action of carboxypeptidase lysine was slowly split from the tryptic hexapeptide T₁, thus confirming the position of lysine already deduced by the specificity of trypsin cleavage. The sequence of the five N-terminal amino acids was found by submitting T₁ to partial acid hydrolysis with 0.5 N acetic acid. The presence of the following fragments was consecutively observed in the acid hydrolysates:

Ac₁ [Glu, Ala], Ac₂ [H-Asp-(OH)₂], Ac₃ [Pro, Asp(NH₂)]-Lys-OH,
Ac₄ Pyr-OH, Ac₅ H-Ala-OH, Ac₆ [Pro, Asp(NH₂)] and H-Lys-OH

The sequence -Pro-Asp-NH₂ was deduced by the positive reaction to isatine of the spots Ac₃ and Ac₆. The status of the glutamyl and aspartyl residues was established by the relative electrophoretic mobilities of the fragments in which they occurred.

Pharmacological actions. Physalaemin is a powerful vasodilator and hypotensive agent in dog and rabbit. It is 3 to 4 times as active as eledoisin. In the dog the i.v. threshold hypotensive dose is approximately 1 ng/kg. The pressure fall is proportional, both in intensity and even more in duration, to the dose of the polypeptide, and tachyphylaxis is completely lacking. A persistent, controllable lowering of blood pressure may be obtained with i.v. infusion of physalaemin, the threshold being 2-5 ng/kg/min. An appreciable lowering of blood pressure is also produced by subcutaneous doses of 1 μg/kg physalaemin. In the majority of experiments, hypotension produced by medium i.v. doses of physalaemin is of shorter duration than that produced by equiactive doses of eledoisin.

1 μg physalaemin is capable of counteracting completely the hypertensive action of 50-100 μg L-adrenaline or L-noradrenaline, 10-15 μg angiotensin, 30-50 μg Lys-8-vasopressin, or 3 mg nicotine. On a weight basis physalaemin is 100-700 times more potent than bradykinin, and 200-400 times more than histamine. The effect on the blood pressure of the dog and rabbit of 1 μg physalaemin is equivalent to 100-200 units of substance P.

Both in the intact and in the decapitated chicken, physalaemin is predominantly hypertensive. Pressure rise seems to be mainly due to liberation of catecholamines.

In increasing the permeability of the skin vessels of the human forearm, physalaemin is slightly more active than eledoisin⁸.

Generally, physalaemin stimulates the same extravascular smooth muscles which are stimulated by eledoisin but, with the exception of the rabbit large intestine and the guinea-pig ileum, the effect is weaker than with eledoisin and tachyphylaxis is not infrequent. The rat uterus is poorly sensitive to physalaemin, and even the rabbit uterus is several times less responsive to physalaemin than to eledoisin.

Large doses of physalaemin stimulate salivary secretion in the dog, but this effect seems less intense compared with eledoisin.

Whole blood slowly inactivates physalaemin, homogenates of liver and kidney far more quickly. Generally, physalaemin is inactivated more rapidly than eledoisin⁹.

In parallel assays physalaemin may clearly be distinguished from known natural polypeptides active on plain muscle, including substance P and eledoisin, which in most of their biological properties are very similar to physalaemin.

Physalaemin or strictly related polypeptides are also contained in the skin extracts of *Physalaemus centralis*, *Physalaemus bresslaui* and *Physalaemus cuvieri*.

Full reports of the experiments and results described in this paper will be published elsewhere.

Riassunto. Viene descritto il procedimento che ha permesso l'isolamento e il chiarimento della struttura chimica della *fisalemina*, il principale dei polipeptidi attivi della pelle di *Physalaemus fuscumaculatus*, anfibio dell'Argentina. La *fisalemina*, strettamente vicina all'eledoisina anche da un punto di vista chimico è, come questa, dotata di potente azione sulla muscolatura liscia vasale ed extravasale. Essa è nettamente distinguibile, mediante saggi paralleli, da tutti gli altri polipeptidi biogeni finora noti.

V. ERSPAMER, A. ANASTASI,
G. BERTACCINI, and J. M. CEI

Istituto di Farmacologia, Università di Parma, Laboratori Ricerche Farmitalia, Milano (Italia), and Instituto de Biologia, Universidad Nacional de Cuyo, Mendoza (Argentina), May 28, 1964.

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³ L. BERNARDI, G. BOSISIO, O. GOFFREDO, and R. DE CASTIGLIONE, *Exper.* **20**, 491 (1964).

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⁵ Pyr = Pyroglutamyl-.

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⁹ M. B. NOBILI, to be published.