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Presence of caerulein in extracts of the skin of *Leptodactylus pentadactylus labyrinthicus* and of *Xenopus laevis*

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Summary

1. The South American amphibian *Leptodactylus pentadactylus labyrinthicus* and the South African amphibian *Xenopus laevis* contain in their skin a polypeptide indistinguishable from caerulein prepared from the Australian amphibian *Hyla caerulea*.

2. The caerulein content of different batches of *Leptodactylus pentadactylus labyrinthicus* skins varies from 10 to 500–600 μg/g tissue. Drying of the skin causes either a moderate decrease or a slight increase in the caerulein content. Methanol extraction gives considerably higher yields of caerulein than acetone extraction.

3. Caerulein or caerulein-like polypeptides also occur in the skin of several other species of *Leptodactylus* together with 5-hydroxyindole alkylamines and imidazole alkylamines. Yet other species of *Leptodactylus* lack caerulein-like polypeptides and 5-hydroxyindole alkylamines.

4. It is suggested that caerulein and caerulein-like polypeptides may have some function either in the regulation of secretory processes of the skin or in the exchange of water and electrolytes through the skin, or in both.

Introduction

In a preceding paper (Anastasi, Bertaccini, Cei, De Caro, Erspamer & Impicciatore, 1969) the isolation and identification of phyllocaerulein, a caerulein-like nonapeptide, in extracts of the skin of *Phyllomedusa sauvagei* was described, as well as the occurrence of similar peptides in skin extracts of other *Phyllomedusa* species of South and Central America.

This paper presents evidence for the occurrence of caerulein-like polypeptides in skin extracts of seven *Leptodactylus* species from South and Central America and in *Xenopus laevis* from Africa. It also describes the isolation of caerulein from extracts of the skin of *Leptodactylus pentadactylus labyrinthicus* and of *Xenopus laevis* and its identification with the caerulein of *Hyla caerulea*.

Methods

Amphibian material

The amphibians used are summarized in Table 1.

Extracts of fresh skins were prepared as soon as possible after capture of the animals. The skins were removed from the animals after killing and extracted twice with five parts (w/v) of methanol. 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 cut into small pieces with scissors and immersed in twenty times their weight of 80% methanol or 70% acetone. The liquid was decanted after a week and the skins were treated for another week with a further fifteen to twenty parts of the solvent. The first and second extracts were combined and filtered. When kept in dark bottles at 4° C the extracts can be stored for years without appreciable loss of activity.

Bioassay

Biological assay of caerulein-like polypeptides was carried out, in parallel, by the following methods: the blood pressure of the dog, the contraction of the guinea-pig gall bladder *in situ*, the secretion of the denervated gastric pouch of the dog and of the perfused stomach preparation of the rat, the pancreatic secretion in the dog

TABLE 1. *Amphibian material*

Batch	Species	Number of animals	Average weight of a skin (g)		Source	Date of capture
1	*Leptodactylus pentadactylus labyrinthicus*	7	10·4	D	Misiones, Argentina	Feb. 1961
2	,, ,,	5	6·3	D	Misiones, Argentina	Sept. 1961
3	,, ,,	2	17·0	D	Misiones, Argentina	Dec. 1963
4	,, ,,	5	34·3	F	Misiones, Argentina	Sept. 1966
5	,, ,,	3	11·9	D	Minas Gerais, Brazil	Nov. 1966
6	,, ,,	10	35·8	F	Minas Gerais, Brazil	Nov. 1966
7	,, ,,	12	23·3	F	Minas Gerais, Brazil	Dec. 1966
8	,, ,,	4	27·5	F	Misiones, Argentina	Apr. 1967
9	*Leptodactylus pentadactylus pentadactylus*	5	10·4	D	Iquitos, Peru	Sept. 1962
10	,, ,,	2	43·8	F	Amazonas, Brazil	Jan. 1967
11	,, ,,	6	17·0	D	Iquitos, Peru	May 1967
12	*Leptodactylus pentadactylus dengleri*	19	6·0	D	Costa Rica	Sept. 1964
13	,, ,,	1	21·2	D	Costa Rica	Aug. 1964
14	*Leptodactylus laticeps*	1	21·2	F	Formosa, Argentina	Jan. 1966
15	,, ,,	2	18·5	F	Formosa, Argentina	Apr. 1966
16	,, ,,	2	17·5	F	Formosa, Argentina	May 1966
17	,, ,,	2	19·5	F	Formosa, Argentina	June 1966
18	*Leptodactylus vilarsi*	1	10·1	D	Ecuador	Feb. 1968
19	*Leptodactylus curtus*	31	0·3	D	Tumbes, Peru	Jan. 1965
20	*Leptodactylus rubido*	2	0·3	D	Tingo Maria, Peru	Jan. 1965
21	*Leptodactylus ocellatus*	100	2·1	D	Argentina	1961–1963
22	*Leptodact. chaquensis*	200	1·17	D	Chaco, Argentina	1961–1963
23	*Leptodact. bolivianus*	18	0·52	D	Iquitos, Peru	Jan. 1965
24	*Leptodact. macrosternum*	11	0·73	D	São Paulo, Brazil	Apr. 1968
25	*Leptodactylus bufonius*	11	0·72	D	Tucuman, Argentina	Dec. 1961
26	*Leptodactylus prognatus*	48	1·1	D	Tucuman, Argentina	Dec. 1961
27	*Leptodactylus gracilis*	3	0·27	D	Cordoba, Argentina	Dec. 1964
28	*Xenopus laevis*	10	3·0	F	Netherlands	May 1967
29	,, ,,	139	3·0	F	Netherlands	Sept. 1967
30	,, ,,	102	5·4	F	Netherlands	Sept. 1967
31	*Xenopus mülleri*	6	0·32	D	Uganda	Jan. 1964

D, Dried skin; F, fresh skin.

(Bertaccini, De Caro, Endean, Erspamer & Impicciatore, 1968a, b, 1969 ; Bertaccini, Endean, Erspamer & Impicciatore, 1968).

Caerulein caused a fall in the blood pressure of the dog, contraction of the guinea-pig gall bladder, stimulated acid secretion by the denervated gastric pouch of the dog and the perfused stomach preparation of the rat, and increased the flow of pancreatic juice in the dog.

Paper chromatography

Because of the presence in their molecules of a tryptophanyl residue, caerulein-like polypeptides could be visualized on paper chromatograms by means of 2-chloro-4-nitrobenzenediazoniumnaphthalene (NNCD), which gave a yellow-orange colour, and *p*-dimethylaminobenzaldehyde, which gave a violet colour slowly turning into blue. Threshold amounts were of the order of 10–20 μg. The qualitative detection and semiquantitative estimation of aromatic amines by means of paper chromatograms has been described previously (Erspamer, Vitali, Roseghini & Cei, 1964, 1967 ; Erspamer, Roseghini & Cei, 1964).

Chromatography on alumina column

In order to obtain a better separation of the biogenic amines and facilitate their detection when occurring in small amounts, crude skin extracts were passed through a column of alkaline alumina which was then eluted with descending concentrations of ethanol (Erspamer, Vitali, Roseghini & Cei, 1967). An alumina column was unsuitable for purification of caerulein-like polypeptides.

Reagents and drugs

Analytical grade reagents and solvents were used throughout the investigation. Caerulein was the pure natural polypeptide prepared at the Farmitalia S.p.A. Laboratories for Basic Research, Milan. Synthetic biogenic amines used for paper chromatographic comparison were as follows: 5-hydroxytryptamine creatinine sulphate (5-HT, 43% base), bufotenine base, bufotenidine iodide (66% base), histamine dihydrochloride (60% base), N-methylhistamine dihydrochloride (63% base), N,N-dimethylhistamine dihydrochloride (66% base), leptodactyline picrate (44% base). 2-Chloro-4-nitrobenzenediazoniumnaphthalene-2-sulphonate (NNCD) was obtained from Hopkin & Williams Ltd.

Results

Identification of caerulein in skin extracts of Leptodactylus pentadactylus labyrinthicus *and* Xenopus laevis

The starting material for the isolation of the caerulein-like peptides was the 80% methanol extract of the fourteen fresh skins of *Leptodactylus pentadactylus labyrinthicus* (batches 6 and 8) and the methanol extract of the 241 fresh skins of *Xenopus laevis* (batches 29 and 30).

The isolation of the caerulein-like peptides from the crude skin extracts of the two species was carried out on the lines used for the isolation of caerulein and, except for some minor modifications, consisted of two steps of counter-current distribution followed by ion-exchange chromatography on a column of DEAE-Sephadex and a

final step of desalting on a column of Amberlite CG-50. The sequential analysis was based, again like that of caerulein, on the enzymatic degradation with chymotrypsin and subtilisin (Anastasi, Erspamer & Endean, 1968; Anastasi, 1969). The resulting fragments were carefully examined and compared, in parallel experiments, with the fragments obtained from caerulein of *Hyla caerulea*.

It was demonstrated that fragments obtained from the caerulein-like peptide of *Leptodactylus pentadactylus labyrinthicus*, from the caerulein-like peptide of *Xenopus laevis* and from caerulein of *Hyla caerulea* showed exactly the same chromatographic and electrophoretic mobility and had exactly the same amino-acid composition. End group determinations with leucine amino peptidase, carboxypeptidase, partial acid hydrolysis, dinitrophenylation and hydrazinolysis indicated that they had identical amino-acid sequences.

These results permit the unequivocal conclusion that the caerulein-like peptides occurring in the skin of *Leptodactylus pentadactylus labyrinthicus* and in the skin of *Xenopus laevis* are identical with each other and with caerulein obtained from *Hyla caerulea* skin.

Caerulein content in different batches of Leptodactylus pentadactylus labyrinthicus *skin*

The content of caerulein in different batches of *Leptodactylus pentadactylus labyrinthicus* skin is shown in Table 2, which also gives the content of biogenic amines; some of the latter data have already been reported by Cei, Erspamer & Roseghini (1967).

It may be seen that the polypeptide contents of the different batches of skin differed considerably. Moreover, the variations in the caerulein concentration did not parallel those of the biogenic amines. Caerulein values assessed by the different

TABLE 2. *Content of caerulein and biogenic amines in different batches of* Leptodactylus pentadactylus labyrinthicus *skin*

(*a*) Caerulein (μg/g) determined by:

Batch	Blood pressure of dog	Guinea-pig gall bladder	Gastric acid secretion		Pancreatic secretion in dog
			Dog	Rat	
1 D,Acet	8–15	13–20	—	13	13–25
2 D,Acet	640	390	390–450	260–320	390–450
3 D,Acet	30	20	—	25	20
4 F,Met	250–300	350	190–260	190–260	200–250
5 D,Met	750–800	390	400	400–500	400
6 F,Met	600–650	400	300	390	400–450
7 F,Met	10	10	10	10	10
8 F,Met	800	520	520	660	700

(*b*) Amines (μg base/g)

Batch	5-HT	Histamine	N-Methylhistamine	N,N-Dimethylhistamine	Spinaceamine	6-Methyl spinaceamine	Leptodactyline
1	220	100	110	35	20	90	1·5
2	1,900	740	670	230	120	410	12·5
3	1,500	50	215	85	15	75	—
4	70	35	13	6·5	23	26	< 1
5	1,500	720	740	80	23	50	30
6	200	90	95	5	3·5	6·5	< 1
7	50	36	38	6·5	5·5	3	1·3
8	225	90	230	50	—	—	—

D, Dried skin; F, fresh skin; Met, methanol extract; Acet, acetone extract; —, not investigated.

assay methods were in fairly good agreement, with the exception of the usually higher values obtained by the blood pressure assay. The discrepancy may be explained by the presence in the crude extracts of large amounts of 5-HT and histamines having a hypotensive effect, like caerulein.

A few experiments were carried out in order to obtain some information on the effect of drying of the skin on the caerulein content. The skins of four specimens of *Leptodactylus pentadactylus labyrinthicus* obtained from Misiones in April 1967 were divided longitudinally into two parts, immediately after the animals had been killed. One part was extracted at once with methanol, the other was dried and extracted with methanol 10 days later. The caerulein content was determined on the guinea-pig gall bladder *in situ*. Values were as follows: fresh skin, 45, 115, 85 and 390 μg/g fresh tissue ; dried skin, 60, 115, 60 and 520 μg/g fresh tissue. These findings suggested that biosynthesis of caerulein might take place during the process of drying of the skin. It is obvious, however, that more experiments are necessary to solve this interesting problem.

Caerulein content in different batches of skin of Xenopus laevis

The contents of caerulein and indole alkylamines of three batches of fresh skin of *Xenopus laevis* are given in Table 3.

In addition to caerulein and 5-hydroxyindole alkylamines, skin extracts of *Xenopus laevis* contain unknown compounds, probably of indolic and phenolic nature. Their identification is in progress.

Experiments on samples of fresh and dried skin exactly similar to those described for *Leptodactylus pentadactylus labyrinthicus* were carried out on eight specimens of *Xenopus laevis*. The pooled fresh skins contained, per g fresh tissue, 950 μg caerulein, 140 μg 5-HT and 250 μg bufotenidine ; the pooled dried skins contained, per g fresh tissue, 650 μg caerulein, 140 μg 5-HT and 260 μg bufotenidine. In this experiment, therefore, drying of the skin produced no change in the amine content, but caused a loss of 30% in the caerulein content.

The skins of *Xenopus mülleri* contained 30 μg 5-HT per g of dried tissue and 50 μg bufotenidine, but no detectable amounts of caerulein.

Occurrence of caerulein-like polypeptides in the skin of Leptodactylus *species other than* Leptodactylus pentadactylus labyrinthicus

The content of caerulein in extracts of the skin of other *Leptodactylus* species is shown in Table 4, which also gives the content of biogenic amines ; some of the latter data have already been reported by Cei *et al.* (1967) and Falconieri Erspamer & Cei (1969).

TABLE 3. *Content of caerulein and biogenic amines in different batches of the fresh skin of* Xenopus laevis

	Caerulein (μg/g) determined by:						
			Gastric acid secretion				
Batch	Blood pressure of dog	Guinea-pig gall bladder	Dog	Rat	Pancreatic secretion in dog	5-HT (μg base/g)	Bufotenidine (μg base/g)
28	650	250–350	300–350	200–250	250–300	190	1,800
29	650–700	350–450	600	300–400	350–450	80	335
30	750–800	500–800	600	400–600	500–750	75	530

Caerulein-like polypeptides were lacking in the skin of *Leptodactylus ocellatus, Leptodactylus chaquensis, Leptodactylus bolivianus, Leptodactylus macrosternum, Leptodactylus bufonius, Leptodactylus prognatus* and *Leptodactylus gracilis.*

For the *Leptodactylus* species containing caerulein in their skin, the results of the parallel assays may be considered satisfactory. Discrepancies among the values obtained with the different assay methods can be explained by the fact that caerulein is not the only active constituent of the crude skin extracts. Apart from the amines, it is well possible that other unknown constituents may influence in some way the effects of caerulein-like polypeptides.

TABLE 4. *Content of caerulein-like polypeptides and of biogenic amines in the skin of* Leptodactylus *species other than* Leptodactylus pentadactylus labyrinthicus

(*a*) Caerulein-like polypeptides (μg/g) determined by:

Species and batch	Blood pressure of dog	Guinea-pig gall bladder	Gastric acid secretion		Pancreatic secretion of dog
			Dog	Rat	
Lept. pentadactylus pentadactylus					
9 D,Met	520–580	500	—	—	—
10 F,Met	35	25–40	—	—	40–65
11 D,Met	50–60	25–40	40–60	65	20–25
Lept. pentadactylus dengleri					
12 D,Met	45–50	105–130	110	50–60	80–90
13 D,Acet	90–115	105–130	—	—	—
Lept. laticeps					
14 F,Met	850	900–1,300	900–1,050	900–1,200	850–980
15 F,Met	620	400–700	650	430	620
16 F,Met	670	650–780	400–450	390–520	390–520
17 F,Met	1,500	900–1,200	900–1,200	1,500–1,700	1,400–1,550
Lept. vilarsi					
18 D,Met	510	780	860	910	760
Lept. curtus					
19 D,Met	40–50	35–50	—	40–50	25–40
Lept. rubido cope					
20 D,Met	60	65–80	—	80–105	105–130

(*b*) Amines (μg base/g)

Species and batch	5-HT	N-Methyl-5-HT	Bufotenidine	Histamine	Leptodactyline
Lept. pentadactylus pentadactylus					
9	230	0	0	150	13
10	35–40	0	0	7–8	—
11	50	0	0	20	2
Lept. pentadactylus dengleri					
12	200	1	550	25	11
13	150	3–4	65	60	40
Lept. laticeps					
14	340	0	0	100	0·5
15	100	0	0	150	0·5
16	90	0	0	100	0·5
17	240	0	0	240	0·5
Lept. vilarsi					
18	295	10	1,440	155	57
Lept. curtus					
19	70–80	0	15	0	1,200
Lept. rubido cope					
20	7	0	40–45	0	200

D, Dried skin; F, fresh skin; Met, methanol extract; Acet, acetone extract; 0, not detectable (< 1 μg/g); —, not investigated.

Although the tabulated data suggest that the caerulein-like peptides found in other *Leptodactylus* species might be identical with caerulein from *Hyla caerulea,* it is obvious that only isolation and analysis of these peptides will permit definite conclusions.

The skin of *Leptodactylus ocellatus, Leptodactylus chaquensis, Leptodactylus bolivianus* and *Leptodactylus macrosternum* contained large amounts of leptodactyline, but that of *Leptodactylus bufonius, Leptodactylus prognatus* and *Leptodactylus gracilis* only minute amounts. Indole alkylamines and imidazole alkylamines were completely lacking. It is interesting to note that caerulein was not found in the *Leptodactylus* species lacking indole alkylamines in their skin, although tryptophan was present in all skin extracts.

From a single experiment it would appear that methanol is a better solvent than acetone for the extraction of caerulein-like polypeptides from the *Leptodactylus* skin. Immediately after death, the skin of a large specimen of *Leptodactylus laticeps* was divided by a longitudinal cut into two symmetrical pieces, each weighing 10·6 g. One piece was extracted with 4 parts of acetone, the other with 4 parts of methanol, and extraction was then repeated with the same quantity of 70% acetone and 80% methanol. The caerulein content of the methanol extract was 850 μg/g fresh skin, whereas that of the acetone extract was only 280 μg/g fresh skin, approximately 32%. This confirms a similar observation made in *Hyla caerulea* (De Caro, Endean, Erspamer & Roseghini, 1968).

Discussion

Present results show that the caerulein-like polypeptide occurring in skin extracts of *Leptodactylus pentadactylus labyrinthicus* and of *Xenopus laevis* is identical with caerulein from *Hyla caerulea.* It is possible that the same conclusion is valid also for the caerulein-like peptides occurring in the skin of the other *Leptodactylus* species examined.

The content of caerulein may vary considerably among the different *Leptodactylus* and *Xenopus* species and, for a given species, among the different batches of skin. In *Xenopus laevis, Leptodactylus pentadactylus labyrinthicus* and *Leptodactylus laticeps,* however, it may attain values of more than 500 μg/g fresh skin, values similar to those observed for *Hyla caerulea.*

Caerulein-like peptides are usually accompanied by large amounts of 5-hydroxyindole alkylamines, imidazole alkylamines and possibly hydroxyphenylalkylamines. It is possible that this simultaneous occurrence of amines and peptides is not accidental, but is due to some biochemical or functional correlations between the two groups of compounds.

From this study and the work on phyllocaerulein (Anastasi *et al.,* 1969) it clearly emerges that caerulein or caerulein-like peptides occur in the skin of amphibia from Central and South America, South Africa and Australia. Hitherto no caerulein-like peptides have been traced in amphibia from North America, Europe and Asia.

Xenopus laevis and leptodactylid frogs are taxonomically not closely related, as they belong to the different suborders Pipidae and Leptodactylidae. On the other hand, the problems of the evolutionary and taxonomic relationships between the leptodactylid frogs from South America and the hylid frogs from Australia still present many points of debate. It cannot be ignored, for instance, that the spectrum

of biogenic amines and polypeptides found in the skin of *Leptodactylus laticeps* from Formosa, Argentina, is virtually indistinguishable from that of *Hyla caerulea* from Queensland, Australia.

The presence of caerulein-like polypeptides in the skin of amphibia living under considerably different ecological conditions does not facilitate the understanding of the biological significance of these peptides. The only hypothesis which may be put forward at present is that caerulein-like polypeptides, in common with bradykinin-like and physalaemin-like polypeptides, have something to do with the regulation of the external secretion of the skin or the exchange of electrolytes and water through the skin, or both.

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