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PHARMACOLOGICAL DATA ON PHYLLOKININ (BRADYKINYL-ISOLEUCYL-TYROSINE *O*-SULPHATE) AND BRADYKINYL-ISOLEUCYL-TYROSINE

BY

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Phyllomedusa rohdei, a small Brazilian amphibian, contains in its skin, in addition to a number of inactive polypeptides, at least three peptides active on plain muscle. The first one (polypeptide *a*) is characterized by a stimulant action on the rat uterus and the rat colon combined with a moderate hypotensive action in the dog; the second (polypeptide *b*) by a typical bradykinin-like activity; the third (polypeptide *c*) by a physalaemin-like activity (cf. Bertaccini, Cei & Erspamer, 1965a, 1965b).

The three polypeptides may be separated from each other by chromatography on alkaline alumina column followed by elution with descending concentrations of ethanol. Polypeptide *a* emerges from the column in the 95% ethanol eluates, polypeptide *b* in the 70% ethanol eluates, and polypeptide *c* in the 60% and 50% ethanol eluates.

Polypeptide *a* is possibly a tryptophan-containing pentapeptide, the synthesis of which is in progress; polypeptide *c* has not yet been obtained in a pure state; polypeptide *b* has been isolated and identified as bradykinyl-isoleucyl-tyrosine *O*-sulphate (Anastasi, Erspamer, Bertaccini & Cei, unpublished). The proposed structure has been confirmed by synthesis (Bernardi, Bosisio, De Castiglione & Goffredo, 1966). For the new naturally occurring bradykinin-like endecapeptide the name of *phyllokinin* has been suggested.

This paper describes some of the pharmacological actions of natural and synthetic phyllokinin in comparison with synthetic bradykinyl-isoleucyl-tyrosine and with synthetic bradykinin.

METHODS

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

(1) One hundred and seventy adult specimens captured near Rio de Janeiro in March 1964. The dried skins weighed together 28.8 g, and the average weight of a skin was 0.17 g.Pool 1964.

(2) Five hundred and eighty adult specimens captured at the same place in January 1965. The dried skins weighed together 83.4 g, and the average weight of a skin was 0.144 g.Pool 1965 A.

(3) Five hundred and ten adult specimens captured at the same place in February and March 1965. The dried skins weighed together 59.6 g, and the average weight of a skin was 0.115 g.Pool 1965 B.

(4) Two thousand two hundred and ninety-five adult specimens captured at the same place in January–March 1966. The dried skins weighed together 283 g, and the average weight of a skin was 0.124 g.Pool 1966.

Soon after their arrival in Italy, by air mail, the dried skins were minced with scissors and then immersed in 20 parts of 80% methanol. The liquid was decanted after a week, and the skins were treated for another week with 15 to 20 parts of the solvent. The methanol extracts were mixed and filtered and then kept in the refrigerator.

The above extracts served, and will serve for the separation and purification of the active and inactive polypeptides, including phyllokinin.

Blood pressure. Dogs were anaesthetized with sodium pentobarbitone (30 mg/kg, intravenously), cats with urethane (1 g/kg, intraperitoneally) followed by chloralose (50 to 70 mg/kg, intravenously), rabbits with urethane (1 to 1.5 g/kg, intravenously).

Smooth muscle preparations. The action of the polypeptides was assayed on the following smooth muscle preparations: guinea-pig ileum, rat duodenum and large intestine, hamster large intestine, and rat and rabbit uterus.

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

Capillary permeability. The injection of polypeptides was made intradermally (0.1 ml.) on the flexor surface of the forearm in five subjects. Attention was paid immediately following the injection to the extension, duration and characteristics of the cutaneous reaction, represented by the typical bradykinin weal. Details of this procedure have been described by De Caro (1963).

Reagents and drugs. Pure natural phyllokinin was obtained by a procedure described elsewhere (Anastasi *et al.*, 1966). Synthetic phyllokinin and bradykinyl-isoleucyl-tyrosine were prepared at the Institute for Basic Research, Farmitalia S.p.A., Milan. The purity of all the above polypeptides was checked by high voltage electrophoresis and paper chromatography.

Synthetic bradykinin was generously put at our disposal by Messrs Sandoz, Basel; crystalline trypsin was obtained from Princeton Lab. Products, Princeton, N.J., U.S.A.

RESULTS

Blood pressure

Dog. The intravenous injection of phyllokinin produced the abrupt, short-lasting blood pressure fall typical for bradykinin. In its intensity of action phyllokinin was 2.5 to 3.5 times more potent than bradykinin on a molar basis; hypotension produced by phyllokinin lasted generally somewhat longer than that caused by equiactive doses of bradykinin (Fig. 1).

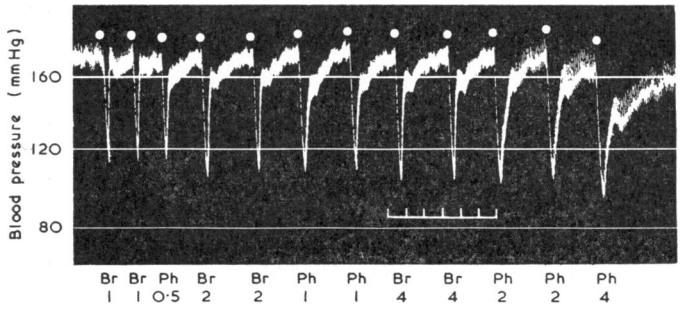

Fig. 1. Blood pressure of a dog anaesthetized with sodium pentobarbitone (30 mg/kg, intravenously) after treatment with 0.2 mg/kg atropine sulphate, intramuscularly. Time in min. The hypotensive effect of different intravenous doses (in μg/kg) of bradykinin (Br) and phyllokinin (Ph) is shown. The hypotensive effect of phyllokinin was, on a weight basis, approximately twice as intense as that caused by bradykinin, and lasted somewhat longer.

Synthetic phyllokinin was indistinguishable from natural phyllokinin.

Bradykinyl-isoleucyl-tyrosine was four to seven times less active than phyllokinin, the duration of its action being generally similar to that of phyllokinin.

It is well known that bradykinin is resistant to trypsin digestion. This was confirmed in the present experiments, in which residual activity after trypsin digestion was found to be 85–95%.

On the contrary, trypsin digestion produced a striking decrease in the activity of phyllokinin, residual activity being barely 30–40%. This corresponded exactly to the activity of the bradykinin originating from phyllokinin following splitting off of the C-terminal dipeptide (Fig. 2).

Anastasi *et al.* (unpublished) have, in fact, observed that phyllokinin rapidly lost its C-terminal dipeptide when digested with trypsin, complete splitting occurring in about 5 min with an enzyme/substrate weight ratio of 1 : 10 at both neutral and alkaline *p*H.

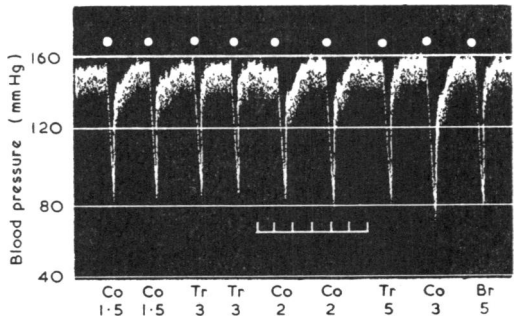

Fig. 2. Blood pressure of a dog anaesthetized with pentobarbitone after treatment with atropine. Time in min. Co, control phyllokinin ; Tr, phyllokinin treated with trypsin ; Br, bradykinin. Doses in μg/kg, intravenously. Note that after trypsin digestion the hypotensive effect of phyllokinin was reduced by 60%, and appeared quite similar in shape to that of bradykinin.

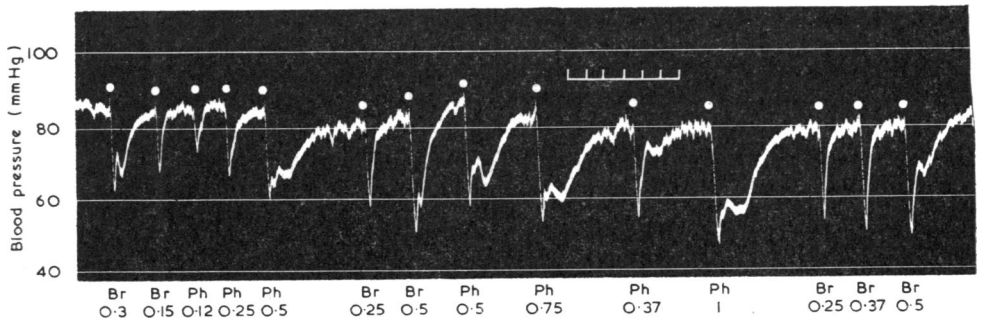

Fig. 3. Blood pressure of a rabbit anaesthetized with urethane (1.2 g/kg, intravenously). Time in min. The effect of different doses (in μg/kg, intravenously) of phyllokinin (Ph) and bradykinin (Br) is shown. The intensity of hypotension caused by phyllokinin was approximately 50–70% of that caused by bradykinin. However, the pressure fall produced by phyllokinin lasted considerably longer.

Rabbit. On the rabbit blood pressure phyllokinin was approximately 2.5 to 3.5 times more potent than bradykinyl-isoleucyl-tyrosine but, as a rule, less potent than bradykinin. However, the pressure fall caused by phyllokinin lasted considerably longer than that caused by bradykinin (Figs. 3 and 4).

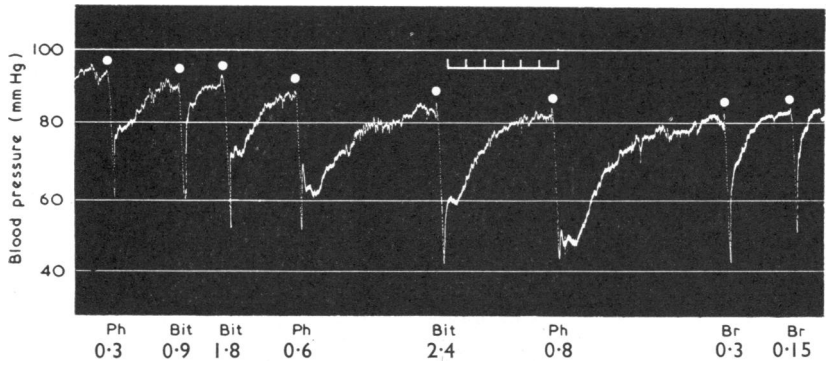

Fig. 4. Blood pressure of a rabbit anaesthetized with urethane. Time in min. Doses in μg/kg, intravenously. In this experiment the intensity of the hypotensive response produced by phyllokinin (Ph) was approximately three times greater than that produced by bradykinyl-isoleucyl-tyrosine (Bit), and three times less than that produced by bradykinin (Br), on a weight basis. However, the pressure fall caused by phyllokinin lasted considerably longer than that caused by the other two polypeptides.

Cat. Owing to the rapid appearance of tachyphylaxis, this preparation was completely unsuitable for the comparison of the three polypeptides.

Extravascular smooth muscle

The relative potencies of the three polypeptides on the guinea-pig ileum, rat uterus and rat duodenum are shown in Table 1, which also summarizes the results of parallel assays on blood pressure and on skin capillaries of the human forearm. The activity of a mole of bradykinin was arbitrarily taken as 100, and that of the other two polypeptides expressed as a percentage of that of bradykinin.

It may be seen that parallel assays, besides having proved the identity of natural and synthetic phyllokinin, showed a remarkable dissociation in potency between bradykinin

TABLE 1

RELATIVE POTENCIES, ON A MOLAR BASIS, OF BRADYKININ (M.W. 1,060), PHYLLOKININ (M.W. 1,417) AND BRADYKINYL-ISOLEUCYL-TYROSINE (M.W. 1,336)

The activities are expressed in relation to that of bradykinin which is given the value of 100. —, not tested

Test preparation	Bradykinin	Phyllokinin		Bradykinyl-isoleucyl-tyrosine
		Natural	Synthetic	
Dog blood pressure	100	270–340	270–340	45–80
Rabbit blood pressure	100	40–90	40–80	15–30
Guinea-pig ileum	100	25–40	25–45	15–20
Rat uterus	100	30–40	30–40	20–25
Rat duodenum	100	6·5–8·5	—	—
Human skin capillaries	100	130	—	—

and phyllokinin. Bradykinyl-isoleucyl-tyrosine was less potent than both bradykinin and phyllokinin on all test preparations.

When the spasmogenic response of the guinea-pig ileum to the three polypeptides was recorded on a drum revolving at sufficiently high speed, it could be clearly observed that the latency, i.e. the time elapsing between the introduction of the peptide into the nutrient bath and the start of contraction of the ileum was longer for phyllokinin (8.8 ± 0.35 sec) than for bradykinin (6.7 ± 0.26 sec), the difference being 2.06 ± 0.31 sec ($P<0.01$). Moreover, the ascent of the lever was somewhat more rapid for bradykinin than for phyllokinin. Doses used were 0.05 to 0.15 μg/ml. nutrient liquid.

The action of bradykinyl-isoleucyl-tyrosine was similar to that of phyllokinin.

Like bradykinin, phyllokinin was virtually inactive on the rabbit uterus and the rat colon. On the hamster colon, phyllokinin was as active as, or somewhat more active (up to 30–40%) than bradykinin.

Capillary permeability

No gross differences could be appreciated between pain produced by intradermal injections of bradykinin and of phyllokinin (50, 100 and 200 ng in 0.1 ml. of physiological NaCl solution). Similarly, cutaneous reaction elicited by phyllokinin was indistinguishable in onset, duration and characteristics from that caused by bradykinin. On the whole, phyllokinin seemed to be as potent as, or a little (30%) more potent than bradykinin.

DISCUSSION

In addition to authentic bradykinin (I), four natural bradykinin-like polypeptides have been so far described: lysyl-bradykinin or kallidin (II), methionyl-lysyl-bradykinin or methionyl-kallidin (III), glycyl-bradykinin (IV) and now bradykinyl-isoleucyl-tyrosine *O*-sulphate or phyllokinin (V).

(I) Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

(II) Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

(III) Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

(IV) Gly-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

(V) Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ile-Tyr (HSO_3 on Tyr)

From the above formulations it may be seen that, in sharp contrast to that of the other bradykinins, the molecule of phyllokinin is lengthened at its carboxyl terminal. This lengthening produces a reinforcement of some biological effects, while decreasing other effects.

Since phyllokinin rapidly loses its C-terminal dipeptide -Ile-Tyr (HSO_3) not only when digested with trypsin, but also when incubated with plasma (Anastasi *et al.*, unpublished), the problem arises as to whether phyllokinin acts on vascular and extravascular smooth muscle as intact molecule or through the release of bradykinin.

All the available evidence suggests that phyllokinin acts as intact molecule. In fact, (a) on the dog blood pressure phyllokinin is three times more active, on a molar basis,

than bradykinin; (b) upon trypsin digestion, i.e. upon splitting off of the C-terminal dipeptide, phyllokinin retains only a third of its original activity. This corresponds exactly to the activity of the bradykinin originating from the phyllokinin molecule; and finally (c) bradykinyl-isoleucyl-tyrosine is four to seven times less active than phyllokinin on the dog blood pressure, three times less active on the rabbit blood pressure and half as active as phyllokinin on the guinea-pig ileum. Yet, bradykinyl-isoleucyl-tyrosine is subjected to the same attack, by trypsin, as is phyllokinin.

In a recent article Stewart & Woolley (1965) emphasize the importance of the carboxyl end of the molecule for the activity of peptides. In particular, they point to the fact that whereas lengthening of the bradykinin molecule at the amino end has only a small effect on potency, lengthening of the same molecule at the carboxyl end produces a reduction of potency. Present results do not support this view. In fact, both the naturally occurring phyllokinin and the synthetic bradykinyl-isoleucyl-tyrosine show a remarkable bradykinin-like potency, which may even exceed that of bradykinin itself.

The functional significance of phyllokinin in the skin of *Phyllomedusa rohdei* is obscure, as it is that of authentic bradykinin in the skin of *Rana temporaria* (Anastasi, Erspamer & Bertaccini, 1965).

The discovery of an increasing number of bradykinin-like polypeptides in different tissues of vertebrates and invertebrates (blood plasma, amphibian skin, wasp venom apparatus) suggests that these polypeptides may display different functions in their different localizations. Exactly the same happens in the case of biogenic amines, especially indolealkylamines and imidazolealkylamines, similarly occurring in blood, amphibian skin and wasp venom apparatus.

SUMMARY

1. The skin of *Phyllomedusa rohdei*, a South-American amphibian, contains several polypeptides active on plain muscle. One of them, *phyllokinin*, has been obtained in a pure form and its amino acid composition and sequence have been elucidated.

2. Phyllokinin is bradykinyl-isoleucyl-tyrosine *O*-sulphate, i.e. bradykinin bearing two additional amino acid residues at its carboxyl end.

3. In its pharmacological actions, phyllokinin greatly resembles bradykinin. On the dog blood pressure phyllokinin is more potent than bradykinin, on extravascular smooth muscles less potent. Upon trypsin digestion phyllokinin is transformed into bradykinin.

4. The actions of phyllokinin are displayed by the intact molecule of the polypeptide, and not by the bradykinin eventually liberated following splitting off of the C-terminal dipeptide. This has been definitely ascertained in the action of phyllokinin on the dog blood pressure.

5. Removing the *O*-sulphate from the molecule of phyllokinin produces a considerable reduction of potency. In fact, bradykinyl-isoleucyl-tyrosine is less potent than phyllokinin on all examined test preparations.

We are indebted to, and wish to thank the Directors of the Proquifar S.A., Duque de Caxias (Rio de Janeiro), for their invaluable collaboration in the collection and preparation of *Phyllomedusa rohdei* skins. This work was in part supported by grants from the Consiglio Nazionale delle Ricerche, Roma, Italy.

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