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A BRADYKININ-POTENTIATING FACTOR (BPF) PRESENT IN THE VENOM OF *BOTHROPS JARARACA*

BY

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In previous papers from this laboratory (Ferreira & Rocha e Silva, 1962; Ferreira, Corrado & Rocha e Silva, 1962) it was shown that some metal-binding agents, like dimercaprol (BAL) and thioglycollic acid, were able to potentiate *in vitro* and *in vivo* some of the pharmacological actions of synthetic bradykinin. While determining whether dimercaprol could potentiate *in situ* the slowly reacting substances released by the venom of *Bothrops jararaca* when acting on the guinea-pig isolated ileum, it was found that the venom itself had a strong potentiating action upon the concentrations elicited by bradykinin. The present report deals with the chemical nature and biological actions of this bradykinin-potentiating factor (BPF).

METHODS

Partial purification of BPF

Preliminary experiments (Ferreira & Rocha e Silva, 1963) led to the following partial purification of BPF: 2 g of *Bothrops* venom were suspended in 200 ml. of distilled water and heated for 5 min in a boiling-water bath. Absolute ethanol (1.5 l.) was added. The mixture was centrifuged (2,000 revs/min) during 60 min and the supernatant fluid was evaporated under reduced pressure. The factor was extracted from dry powder with three successive additions of 20 ml. of 90% ethanol. To the alcoholic solution four volumes of ethyl ether were added. The precipitate was separated by centrifugation and dissolved in 50 ml. of distilled water. The aqueous solution was then lyophilized. The resulting powders will be referred to in this paper as " bradykinin-potentiating factor " (BPF).

Physicochemical properties of BPF

Solubility tests. These were performed by shaking for 1 hr, 100 μg of BPF (previously dried in glass tubes) with 2 ml. of the following solvents: absolute ethanol and acetone, ethyl ether, chloroform and water-saturated *n*-butanol. The activity of the supernatant fluid collected by centrifugation and dried under reduced pressure was estimated as a percentage of a control value determined with distilled water.

Dialysis. BPF (1.5 mg) was dissolved in 1.5 ml. of distilled water and dialysed (dialyser tubing $\frac{1}{4}$ to $\frac{3}{8}$ in., Thomas Co., U.S.A.) against thirty volumes of distilled water with constant agitation by a magneto-stir system. After 20 hr the potentiating activity was estimated inside and outside the dialysis bag.

Acid hydrolysis. This was performed at 100° C in sealed tubes by incubating 250 μg of BPF with 6 N-hydrochloric acid or 200 μg of BPF with 1.5 N-hydrochloric acid. After a definite time interval the hydrolysis was stopped in the first experiment by evaporation under reduced pressure or in the latter by neutralization with a sufficient amount of N-sodium hydroxide solution. One sample was set apart for ninhydrin reaction (Troll & Cannan, 1963) and another, to which tris buffer, *p*H 7.8, was added, for biological estimation.

Enzymatic hydrolysis. This was tested by incubating BPF with trypsin or chymotrypsin at 37° C. The concentrations of the reagents in the incubation mixtures were: (1) Trypsin, 100 μg/ml.; BPF, 500 μg/ml.;

the 2-ml. volume was completed with tris buffer, *p*H 7.8. (2) Chymotrypsin, 1 mg/ml.; BPF, 100, 200 or 400 μg/ml.; up to 2 ml. with tris buffer, *p*H 8.6. The hydrolysis was stopped by boiling the incubation mixtures for 3 min in a water-bath. A solution of the boiled enzyme was added to control samples.

Paper electrophoresis. This was performed on Whatman Paper No. 1 with sodium phosphate buffer, *p*H 7.7, μ=0.1, 30 V/cm, for 9 to 15 hr. Activity of the fractions was assayed either in eluates from cut paper strips or by adding the paper directly to the organ-bath. Spot reactions (Smith, 1963) were performed concomitantly on control strips.

In vitro *experiments*

The activity of BPF was tested on the guinea-pig ileum, using arbitrary " potentiating units," one unit corresponding to the dose of BPF which increases the effect of a single dose of bradykinin to that of a double dose (Fig. 3). The doses of synthetic bradykinin added to a 5-ml. guinea-pig organ-bath varied from 0.2 to 0.05 μg. BPF was added 5 to 10 sec before the addition of bradykinin.

Although the absolute values of the " potentiating unit " (U) may vary from one gut to the other, it remains constant upon the same preparation.

In vivo *experiments*

These were done upon the arterial blood pressure and the vascular (capillary) permeability. Arterial blood pressure was measured through a cannula inserted into the carotid artery of dogs and cats anaesthetized with pentobarbitone sodium (30 mg/kg). Drugs were administered through a polyethylene tube into a saphenous vein. Hypotension was measured by the size of the fall and by calculating the area above the isometric tracing obtained during the 5 min following administration of bradykinin.

Capillary permeability was tested in rats weighing 200 to 250 g, anaesthetized with pentobarbitone sodium (40 mg/kg, intraperitoneally). Trypan blue (200 mg) was injected into a jugular vein 5 min before the intradermal injection (0.1 ml.) of bradykinin (doses from 0.5 to 1 μg), BPF (1 or 2 μg) or a mixture of both in saline. The animals were killed after 30 min by exsanguination.

The following drugs and reagents were used: synthetic bradykinin (BRS 640, Sandoz, Basel); synthetic oxytocin (Sandoz, Basel); angiotensin amide (Ciba, Basel); histamine diphosphate (Abbott); acetylcholine (Roche); chymotrypsin (Armour); and acetylated trypsin (Mann Research Laboratories).

RESULTS

Partially purified BPF. In relation to the activity of the crude venom, the purified preparation showed an eight- to tenfold increase of activity. Although this purification ratio was small, the BPF so obtained was no longer contaminated with pharmacologically active amines and did not release bradykinin when incubated with plasma. When injected intravenously into cats and dogs it no longer produced shock and was unable to clot heparinized plasma, like the original venom. The " potentiating unit " upon the guinea-pig ileum ranged from 0.5 to 0.3 μg/ml. final concentration in the bath of the isolated preparation.

Characteristics of the BPF. Solubility tests showed that the factor was soluble in 5% trichloracetic acid and in absolute ethanol; partially soluble in water saturated with *n*-butanol, and insoluble in absolute acetone, chloroform or ether. BPF was thermostable (at 110° C during 5 hr) and was dialysable. Acid hydrolysis under 6 N-hydrochloric acid completely inactivated BPF after 5 hr incubation, as shown in the upper section of Table 1. It was observed that the simple incubation of BPF with 6 N-hydrochloric acid and immediate evaporation under reduced pressure was enough to inactivate 67% of the original activity. It must be pointed out that in equal conditions of time and temperature without acid all activity of BPF could be recovered. Incubation of BPF with 1.5 N-hydrochloric acid, as

TABLE 1

ACID HYDROLYSIS OF BRADYKININ POTENTIATING FACTOR (BFP)

The recovery is expressed in percentage of initial activity of BPF

Expt.	Sample	Time of hydrolysis (min)	Hydrochloric acid strength (N)	Total BPF added (μg)	Ninhydrin (Klett units)	Activity recovered (%)
1	Control	0	—	250	50	100
	I	0	6	250	143	33
	II	300	6	250	590	0
2	Control	0	—	200	45	100
	I	15	1·5	200	60	33
	II	30	1·5	200	97	28
	III	60	1·5	200	106	6·6

Table 1 indicates, showed a parallelism between the progression of the hydrolysis and the increase in the ninhydrin reaction. The results of two experiments in which BPF was incubated with trypsin are shown in Fig. 1. A slow, but progressive destruction of BPF activity was observed. On incubation with chymotrypsin for 24 hr no inactivation of BPF was observed.

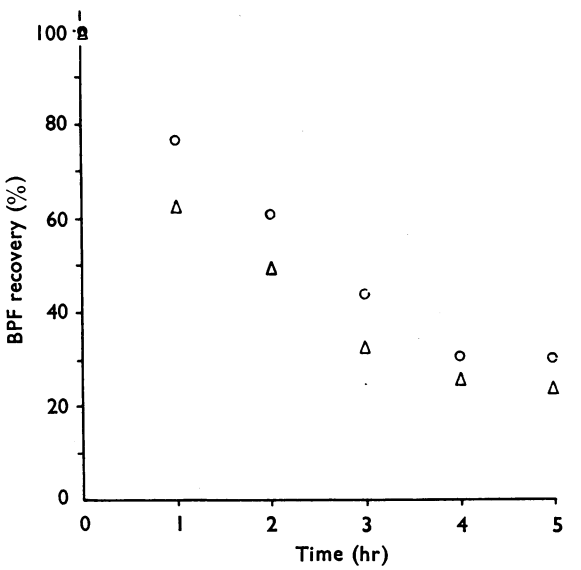

Fig. 1. Recovery of BPF after incubation with trypsin at 37° C. The points represent the values found in two different experiments. The reagents in the incubating mixture were: trypsin (100 μg), BPF (500 μg/ml.) and tris buffer, *p*H 7.8, up to 2 ml.

As shown in Fig. 2, on paper electrophoresis the active material migrated to the anode in two distinct bands both fluorescing in the ultra-violet and giving positive Sakaguchi and ninhydrin tests.

In vitro *potentiation.* In isolated preparations BPF also potentiated the stimulating effect of angiotensin upon the guinea-pig ileum (Table 2), and oxytocin upon the rat uterus (Table 3), though bradykinin appeared to be more sensitive to its effects. Our results show

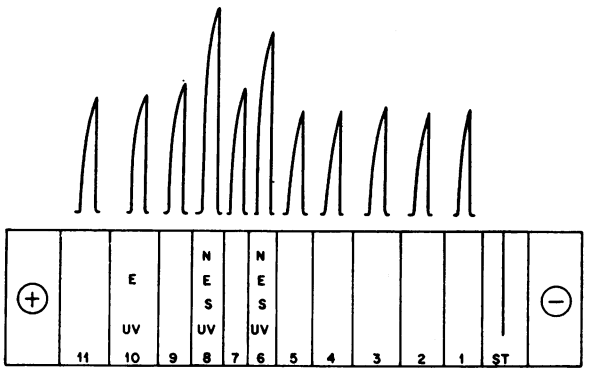

Fig. 2. Paper electrophoresis performed in phosphate buffer, *p*H 7.7, $\mu=0.1$ during 12 hr. Control strips were developed with ninhydrin (N), Sakaguchi (S) and Erlich (E) reagents. The limits of the bands were determined by fluorescence in the ultra-violet. Upper section shows the bioassay of BPF upon a guinea-pig isolated ileum. Before a standard dose of bradykinin an aliquot of the eluate of the electrophoresis fraction was added. A bradykinin-potentiating activity could be detected in bands Nos. 6 and 8.

TABLE 2

BRADYKININ AND ANGIOTENSIN POTENTIATION BY BPF UPON GUINEA-PIG ILEUM

Each experiment was performed upon guts from different animals. The final concentration of bradykinin (Brady) and angiotensin (Angio) was 0.01 μg/ml.

Expt.	Final concentration of BPF (μg/ml.)	Height of contraction: Brady (mm)	Brady+BPF (mm)	Increase (%)	Height of contraction: Angio (mm)	Angio+BPF (mm)	Increase (%)
1	0·2	33	37	12	27	26	—
2	0·2	19	30	57	19	21	10
3	0·2	36	47	30	37	41	10
4	0·2	27	40	48	24	29	20
5	2	26	68	161	20	20	0
6	2	42	87	107	36	44	22
7	2	20	61	205	21	22	5
8	2	21	64	204	24	35	4
9	20	35	72	105	35	35	0
10	20	38	74	100	30	45	50
11	20	22	71	238	20	28	40
12	20	31	130	319	22	27	22

TABLE 3

BRADYKININ AND OXYTOCIN POTENTIATION BY BPF UPON RAT ISOLATED UTERUS

Each experiment was performed upon the isolated organ from different animals. Results show the height of contraction measured in mm and the potentiation is expressed as percentage increase of a control response. The doses of bradykinin and oxytocin added to a 5 ml. bath were respectively 0·003 μg and 0·005 U. BPF concentrations refer to the organ-bath

	Contraction heights for bradykinin and BPF					Contraction heights for oxytocin and BPF				
Expt.	Bradykinin (mm)	BPF 2 μg/ml. (mm)	Increase (%)	BPF 20 μg/ml. (mm)	Increase (%)	Oxytocin (mm)	BPF 2 μg/ml. (mm)	Increase (%)	BPF 20 μg/ml. (mm)	Increase (%)
1	27	44	63	47	74	17	—	—	19	11
2	39	40	2	49	26	32	40	25	46	44
3	29	29	0	33	37	30	30	0	35	17
4	24	34	41	41	71	13	19	46	44	234
5	24	26	8	32	33	27	31	15	34	26
6	15	19	26	23	53	30	33	10	35	17

that guinea-pig gut is also more sensitive to bradykinin potentiation by BPF than is rat uterus. As shown in Fig. 3, neither histamine nor acetylcholine could be potentiated by BPF to any considerable extent.

In vivo *potentiation.* When given intravenously in potentiating doses (0.2 to 2 mg/kg) BPF presented only small and transient hypotensive effects.

Some experiments in cats are listed in Table 4, showing the effects of injections of BPF (2 mg/kg) at various intervals before administration of bradykinin (1 μg/kg). The sensitivity to bradykinin remained increased after 30 min from BPF administration, as shown in Fig. 4. Similar results were obtained in dogs. A fact to be stressed is that neither histamine nor acetylcholine were potentiated by BPF in such *in vivo* preparations.

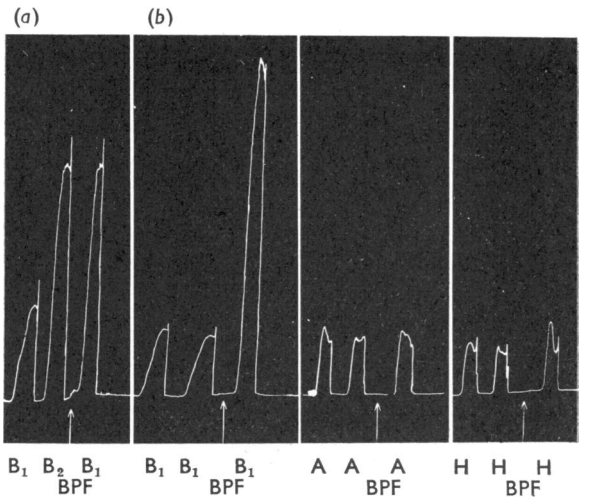

Fig. 3. Action of BPF upon contractions elicited by bradykinin, acetylcholine and histamine in the guinea-pig ileum. (*a*) " One potentiating unit," the dose of BPF (2 μg, at the arrow) which increases the effect of a single dose of bradykinin (B_1=0.05 μg) to match that of a double dose (B_2=0.1 μg). (*b*) 10 μg of BPF was added (at the arrows) before bradykinin (B_1) acetylcholine (A_1=0.01 μg) and histamine (H=0.02 μg). The volume of the perfusion bath was 5 ml.

TABLE 4

ACTION OF BPF (2 MG/KG) UPON THE HYPOTENSIVE EFFECT OF 1μG/KG OF SYNTHETIC BRADYKININ IN CATS

Hypotension area was measured by calculating the area between the original base line and the recorded tracing during 5 min after bradykinin injection

	Before BPF			After BPF					
				Within 5 min			After 30 min		
Expt.	Blood pressure (mm Hg)	Initial fall (mm)	Hypo-tension area (sq. mm)	Blood pressure (mm Hg)	Initial fall (mm)	Hypo-tension area (sq mm)	Blood pressure (mm Hg)	Initial fall (mm)	Hypo-tension area (sq mm)
1	150	10	30	150	74	375	150	56	262
2	160	30	67	160	60	275	—	—	—
3	140	20	50	140	86	360	130	60	275
4	170	20	55	170	80	315	130	50	130
5	140	18	40	150	58	340	130	48	260
Mean±s.e.		19·6±3·3	48±7·0		72±5·5	333±18·0		53·5±2·7	232±3·41

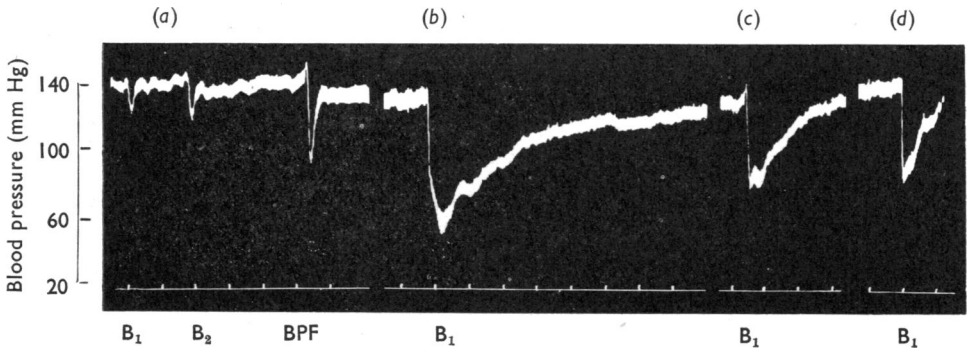

Fig. 4. The effect of bradykinin potentiating factor (BPF) upon the arterial hypotension produced in a cat by bradykinin. (*a*) Before; (*b*), (*c*) and (*d*) 3, 15 and 30 min after 2 mg/kg of BPF. B_1, 1 μg/kg and B_2, 2 μg/kg of synthetic bradykinin. Drugs were injected intravenously. Time marks, 1 min.

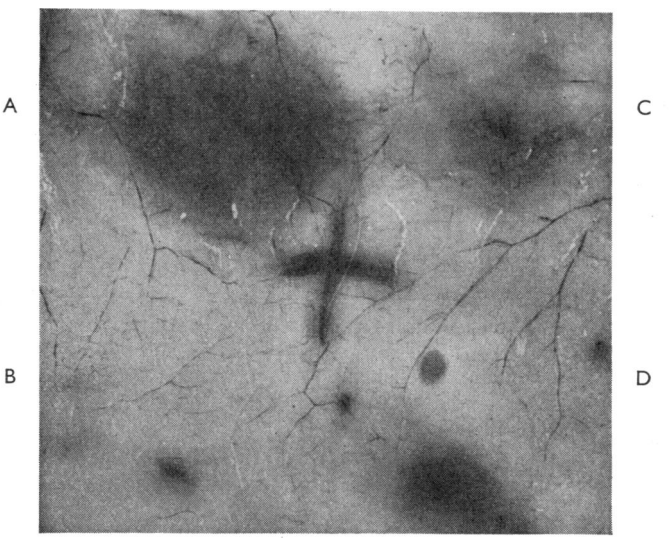

Fig. 5. Potentiation of bradykinin by BPF in capillary permeability test. The photograph shows the areas of blueing in the skin of a rat which had received trypan blue intravenously followed by intradermal injections of 0.1 ml. of the following solutions: A, bradykinin (1 μg) and BPF (2 μg); B, bradykinin (1 μg); C, saline; and D, BPF (1 μg).

In vivo experiments were also performed upon vascular (capillary) permeability and a typical potentiation of the bradykinin effect (1 μg) by BPF (2 μg) is shown in Fig. 5; only higher doses of BPF (10 μg) produced by themselves some leakage of the blue dye.

DISCUSSION

The findings described in the present paper indicate that *Bothrops jararaca* venom contains a factor that potentiates *in vivo* and *in vitro* some of the pharmacological actions of bradykinin.

BPF activity could be destroyed by long incubation with trypsin and by acid hydrolysis, which suggests a polypeptide structure. BPF was unable to potentiate histamine or acetylcholine upon the guinea-pig ileum. This fact suggests that the potentiation observed with bradykinin depends on a mechanism which differs from a " sensitization " of the smooth muscle preparations. BPF *per se* did not elicit responses of the isolated preparations used. BPF action could be explained by inhibition of enzymes which break down polypeptides (peptidases) possibly present in the muscle. Preliminary experiments showed that BPF may inhibit enzymes from plasma and haemolysed red cells of the guinea-pig. An inhibitory effect was obtained with doses of 40 μg/ml. of BPF, but doses 200-times greater did not stop bradykinin destruction as effectively as did 250 μg/ml. of dimercaprol.

In some papers (Ferreira & Rocha e Silva, 1962; Ferreira *et al.*, 1962; Corrado, 1963; Rocha e Silva, 1963) we have described how some thio-compounds such as dimercaprol and thioglycollic acid potentiated the hypotensive actions of bradykinin in the rabbit and the dog. The effective potentiating doses of dimercaprol varied from 3 to 10 mg/kg. However, in the cat the results were disappointing because the drug produced only a small and transient potentiation of the vasodilatation produced by bradykinin. Other thio-compounds were assayed by Erdös & Wohler (1963) as bradykinin potentiators upon the arterial pressure of the guinea-pig. The findings of Erdös & Wohler (1963) showed that dimercaprol was among the most potent substances used, so confirming our previous results. Considering that BPF is more active *in vivo* than is dimercaprol, it becomes the most effective bradykinin potentiator so far described. BPF is not limited to *Bothrops jararaca* venom, a similar activity being found in other *Bothrops* species such as *jararacussu*, *neuwiedii* and *atrox*. No potentiating activity was found in the venom of *Crotalus*.

SUMMARY

1. *Bothrops jararaca* venom contains a factor that potentiates some of the pharmacological actions of bradykinin.

2. This bradykinin-potentiating factor (BPF) seems to constitute a new principle, the most effective potentiator of bradykinin *in vivo* that we know.

3. The purification, physicochemical nature and pharmacological actions of BPF are described.

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