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Effect of anti-proteases and hexadimethrine bromide on the release of a bradykinin-like substance during heating (46° C) of rat paws

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Summary

1. The conditions in which the release of an active, bradykinin-like agent occurred when rat paws were heated to 46° C were studied by means of the double coaxial perfusion of the subcutaneous spaces.

2. The active material thus released stimulated the isolated rat uterus, produced a relaxing effect on the isolated rat duodenum, was destroyed by incubation with chymotrypsin and was potentiated by bradykinin-potentiating factor. LSD-25, in doses sufficient completely to block 5-hydroxytryptamine, did not affect the responses of the isolated uterus to the active material.

3. The effects on this release of anti-proteases and hexadimethrine bromide, atropine and diphenhydramine were studied.

4. Soy-bean trypsin inhibitor and hexadimethrine bromide added to the perfusion fluid produced a potent and reversible inhibition of the release of the active material ; aprotinin and Kunitz inhibitor caused a temporary block.

5. When administered intravenously, much larger doses of the substances were necessary to produce a similar block.

6. Pretreatment of the animals with atropine plus diphenhydramine did not affect the release of the active kinin(s).

7. Ligature of one iliac artery was followed by disappearance of the active material in the perfusate from the corresponding paw.

8. These facts suggest that heating elicits a process leading to plasma extravasation and that the subcutaneous tissue is the chief site of release of the active material.

Introduction

Direct and indirect evidence that bradykinin participates, as an endogenous mediator, in some acute inflammatory reactions is accumulating (Lewis, 1964 ; Collier, 1965 ; Starr & West, 1967 ; Rocha e Silva, 1968). Moderate or severe heat injury is one of the conditions in which a release of active kinin can constantly be demonstrated. Rocha e Silva & Rosenthal (1961) observed the appearance of histamine and a bradykinin-like agent after scalding the rat's skin and collecting the fluid in an air pouch submitted to a temperature of 96° C for 15 seconds. Using less drastic heat treatment (54° C), Wilhelm & Mason (1960) described a release of histamine together with other possible active substances. If heat treatment is

performed in milder conditions however—at 43° to 46° C for example—histamine is no longer released, but an abundant production of a bradykinin-like substance can be detected, as shown by Rocha e Silva & Antonio (1960) in the so-called " thermic oedema " of the rat's paw. It is known that in such circumstances (heating to 45° C) mast cells are stabilized *in vitro* (Mongar & Schild, 1957) and *in vivo* (Antonio, 1961). Furthermore, no correlation could be found between anti-histamine or anti-5-hydroxytryptamine activities of drugs and their ability to block the development of the heat oedema (Rocha e Silva & Antonio, 1960; Rocha e Silva & Garcia Leme, 1965) but some parallelism existed between the anti-bradykinin activity of some substances *in vitro* (Garcia Leme & Rocha e Silva, 1965) and the inhibition of heat oedema (Rocha e Silva, 1968).

Substances which interfere with the release of bradykinin from its inactive precursor in plasma, such as hexadimethrine bromide (Armstrong & Stewart, 1962), markedly inhibited the development of heat oedema in the rat's paw (Garcia Leme, Schapoval & Rocha e Silva, 1967) or yeast-induced oedema (Kellet, 1965). Depletion of the " labile pool of bradykininogen " caused by chronic treatment with sulphated polysaccharides significantly reduced the intensity of heat oedema (Garcia Leme, Schapoval & Rocha e Silva, 1967).

We have now investigated the conditions in which bradykinin or a bradykinin-like agent is released, by heating the rat's paw at 46° C using a double coaxial perfusion device to estimate the amount of released kinin, as well as the effect of anti-protease agents and the action of inhibitors of the kininogen-kininogenin system, such as hexadimethrine bromide. During these experiments we had the opportunity of submitting the material released in heat oedema to a further characterization, to exclude the possibility of 5-hydroxytryptamine being a contaminating agent present in the perfusates. In parallel assays on the rat uterus and rat duodenum, the material behaved as bradykinin. The presence of LSD-25 in the bath containing the rat uterus did not affect the intensity of the response. Furthermore, the material was quickly destroyed by chymotrypsin and potentiated by bradykinin potentiating factor (BPF).

Methods

Male adult Wistar rats (250–300 g) anaesthetized with pentobarbital sodium (30–40 mg/kg intraperitoneally) were used throughout. Not less than six animals were used in each experimental group.

Double coaxial perfusion. Through a small incision high in the hind limbs, polyethylene tubing (3 mm external and 2 mm internal diameter) was introduced into the subcutaneous space of both paws as far as the most distal portion (Fig. 1). The tubing was sutured to the skin at the level of the incision and narrower tubing, connected to a reservoir containing Tyrode or Tyrode-Tris buffer solutions, was introduced into the larger one, in such a way that the perfusion fluid reached the subcutaneous space through the inner tubing and was collected through the outer one, the flow being adjusted to 1 ml/10 min. The paws were immersed in water which was gradually heated to 46° C.

Assay of the perfusates. Activity of perfusates was assayed on the isolated rat uterus, by comparison with bradykinin standards, using a 5 ml chamber and Jalon's solution at 28° C. The volume of the perfusates added was routinely 0·6 ml,

corresponding to about 6 min of perfusion. When changes of pH were tested, a plastic chamber was used instead of the glass chamber because at acid or alkaline pH there is tendency for bradykinin to be adsorbed on glass. The perfusates were usually collected in test tubes kept on ice, but no essential difference in the estimated activity was observed whether the perfusates were tested immediately after collection or after standing for long or short periods at low temperature. At the normal pH of the Tyrode solution, no difference was found if the perfusates were collected either in glass, in plastic or in siliconized vessels. However, when changes of pH were introduced, plastic test tubes, syringes and so forth were used in order to avoid adsorption of the active material on the glass surface. When the isolated guinea-pig ileum was used, 0·8 ml of the perfusates was added to a chamber of 8 ml capacity.

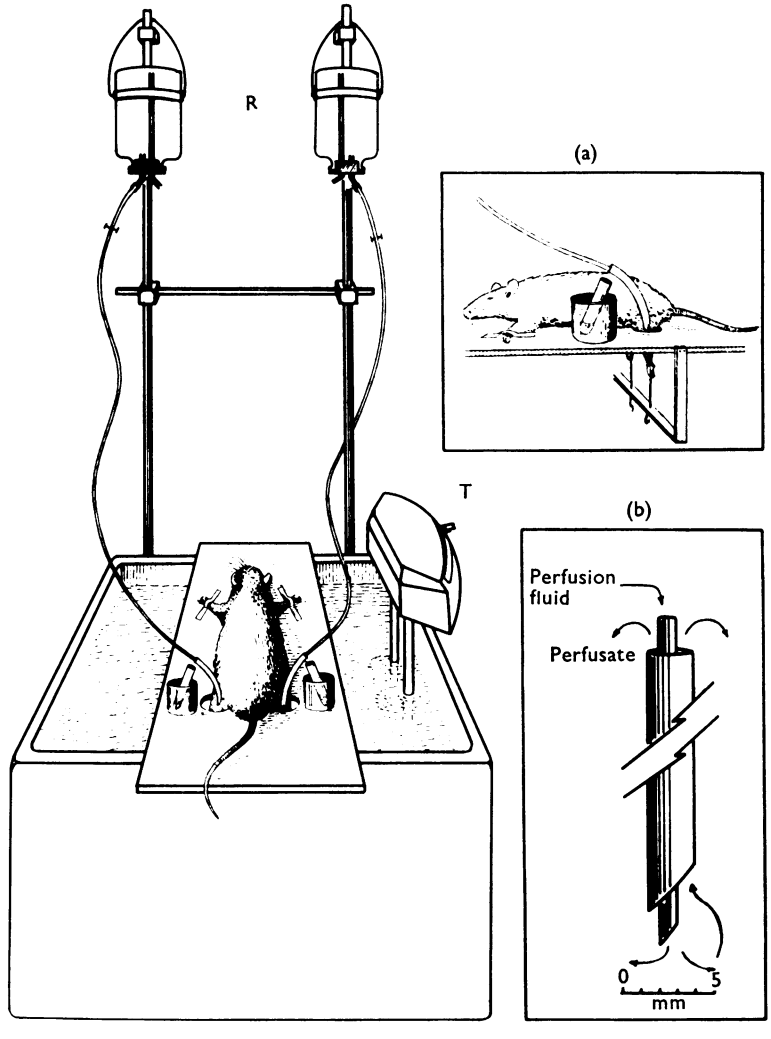

FIG. 1. Device used for double coaxial perfusion of the rat's paw. Fluids kept in the reservoirs (R) reached the subcutaneous tissue of the paws immersed in heated (46° C) water through a polyethylene tubing system shown in detail in (b). T, Thermostat.

In the experiments in which chymotrypsin was employed, the perfusates were collected and divided into two portions of 0·5 ml, one being incubated with 50 μg/ml of the enzyme, the other remaining as control. The perfusates were also divided into two portions in the experiments with BPF. After registering the control response, in the presence of atropine and diphenhydramine, BPF in a concentration of 0·125 μg/ml was added to the chamber containing the isolated ileum and the second sample added immediately afterwards. LSD-25, used as a 5-hydroxytryptamine antagonist, was dissolved in the fluid bathing the preparation, which then remained in continuous contact with the drug.

Identification of the material released. Though a release of histamine has been excluded by previous experiments, a further characterization of the material to exclude the presence of 5-hydroxytryptamine in the perfusates was the object of separate experiments (Figs. 2–6).

Interruption of circulation in one paw. In some animals, one of the iliac arteries was ligated close to the aortic bifurcation through a median incision in the abdominal wall before they were subjected to heat treatment.

Adjustment of pH of the perfusion fluid. When the effect of changes in pH of the perfusion fluid was tested, the composition of the Tyrode solution was modified (Tyrode-Tris buffer solution) as indicated (g/l.): NaCl 8·0, KCl_2 0·2, $MgCl_2$ 0·1, NaH_2PO_4 0·005, $NaHCO_3$ 0·1, Tris buffer 0·6, glucose 1·0. It was then adjusted to the desired pH, from 3 to 10, by adding small volumes of 10 N HCl or 3·5 N NaOH. The perfusates were neutralized before they were assayed on the isolated rat uterus. In such experiments, bradykinin standards were prepared with the corresponding Tyrode-Tris buffer solution and neutralized before testing.

Drugs. Pentobarbital sodium (Nembutal), Abbott; bradykinin triacetate, Cyclochemical; hexadimethrine bromide (Polybrene), Abbott; soy-bean trypsin inhibitor (SI 7CB), Worthington Biochemical; α-chymotrypsin, Worthington Biochemical; LSD-25 (Délyside), Sandoz; 5-hydroxytryptamine, Abbott; bradykinin-potentiating factor (BPF-5a), kindly supplied by Dr. S. H. Ferreira; aprotinin (Trasylol), Bayer; Kunitz inhibitor (Inopol), Choay; diphenhydramine hydrochloride (Benadryl), Parke Davis; atropine sulphate, Merck.

Results

Active material appearing in the perfusates of the heated paws

The active material obtained by perfusion of the rat's paws immersed in heated water usually appeared within a few minutes after the temperature reached 46° C. In a few experiments a delay of no more than 30 min was observed. When assayed in parallel, this material contracted the isolated rat uterus and produced a relaxing effect on the isolated rat duodenum, as seen in Fig. 2.

Table 1 gives the amounts of active material assayed in the perfusates, ranging from 0·5 to 8 ng/ml every 10 min. The total amount appearing in the perfusates can be estimated by considering the rate of release and the duration of the experiments. The amounts released represent the activity appearing in a small area (or volume) of the subcutaneous spaces. A considerable part of it may be destroyed by degrading enzyme systems (kininases) which are either present in the subcutaneous tissue or may be activated by the heat treatment. In the recovery experi-

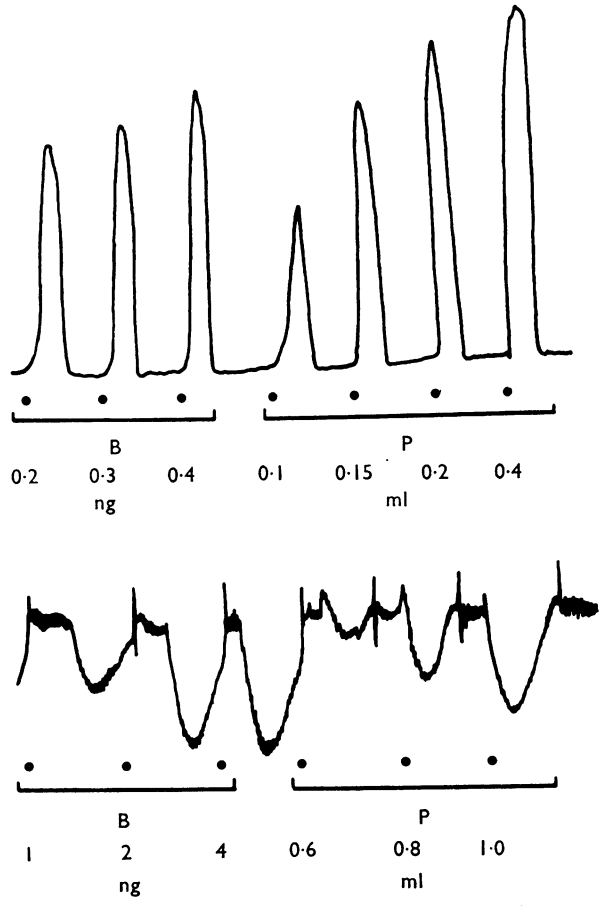

FIG. 2. Parallel assays in which the isolated rat uterus (upper tracings) and the isolated rat duodenum (lower tracings) were used. **B**, Responses of the preparations to bradykinin; **P**, responses to varying volumes of the perfusates collected from the rat's paw immersed in water heated to 46° C.

TABLE 1. *Active material released* by heating the rat's paws to* 46° *C and time of death of animals as compared with a control group (paws heated to* 37° *C)*

Exp. No.	Latency time (min)	Range of active material released ((ng/ml)/10 min)	Duration of experiment (min)
		Paws heated to 46° C†	
1	<20	2–3	86
2	5	2–4	85
3	4	0·5–5	70
4	13	2–5	70
5	<14	2–4	83
6	15	4–8	80
7	5	1–2	90
8	6	1–4	90
9	30	1–4	74
10	6	0·5–1	100
11	5	2–4	72
12	8	1–2	88
		Paws heated to 37° C‡	
13–17	No release	–	133–213 (av. 163)

* Estimated by comparison with responses of the isolated rat uterus to bradykinin.
† Experiments stopped at the death of the animal after the indicated time.
‡ No death occurred.

ments, large amounts of bradykinin perfused through the system disappeared by contact with the subcutaneous tissue. It is to be noted that when the paws were kept at 37° C, the animals stayed alive, the experiments being interrupted after 133–213 min, while heating the paws to 46° C caused the death of the animals within 70–100 minutes. The cause of death was not analysed further.

Influence of α-chymotrypsin on the activity of the perfusates

Incubation of the perfusates with 50 μg/ml α-chymotrypsin resulted in a gradual disappearance of the activity (Fig. 3). About 20% of the activity was destroyed in the first minute of incubation; 50% after 2 min and 100% after 3 min. Higher doses of α-chymotrypsin (100 and 200 μg/ml) caused a more rapid destruction of the activity. The enzyme did not interfere with the responses of the uterus (Fig. 4).

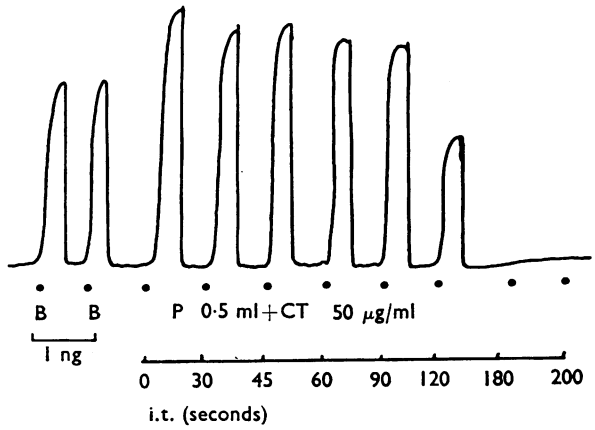

FIG. 3. Responses of the isolated rat uterus to 0·5 ml of the perfusates (P) obtained from the heated (46° C) rat's paw and incubated at 37° C for varying times (i.t., lower scale) with 50 μg/ml α-chymotrypsin (CT). B, Control responses to bradykinin.

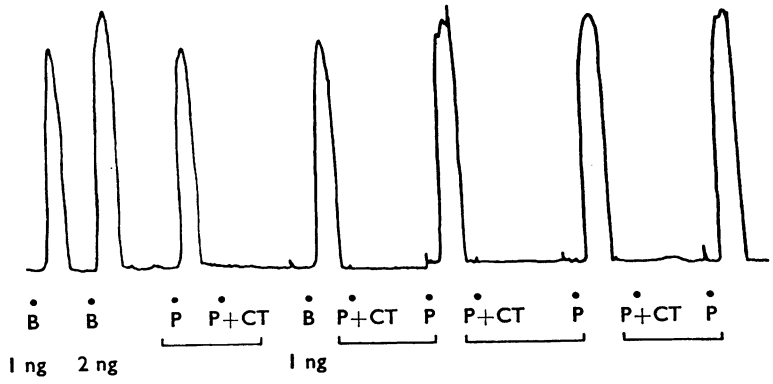

FIG. 4. Addition to the isolated rat uterus of bradykinin (B) and 0·5 ml of the perfusates obtained from the heated (46° C) rat's paw and incubated at 37° C for 3 min in the absence (P) and presence of 200 μg/ml α-chymotrypsin (P+CT). The material incubated with chymotrypsin was kept in the bath for at least 2 min without response of the preparation. A new addition of P while the mixture was in the bath produced a full effect, which shows that the enzyme by itself did not interfere with the muscle response.

Effect of the bradykinin-potentiating factor on the responses of the isolated guinea-pig ileum to the perfusates

In the presence of atropine and diphenhydramine a net potentiation of the activity of the perfusates from heated rat paws was observed by the addition of 0·125 μg/ml BPF. In such conditions the potentiation is practically specific for bradykinin (Ferreira, 1965). Results are presented in Fig. 5.

Influence of LSD-25 on the responses of the isolated rat uterus to the perfusates

While a complete block of the responses of the isolated rat uterus to 5-hydroxytryptamine by the addition of LSD-25, in a concentration of 0·1 μg/ml, to the bathing fluid, was observed, no effect on the activity present in the perfusates was observed. This seems to exclude a major contribution of 5-hydroxytryptamine to the activity (Fig. 6).

Percentage of recovery of bradykinin passing through the system

Destruction of bradykinin, dissolved in Tyrode solution, was calculated by comparing the amounts assayed in the perfusates with those added to the perfusion fluid. The results of such a comparison are given in Table 2. It should be noted

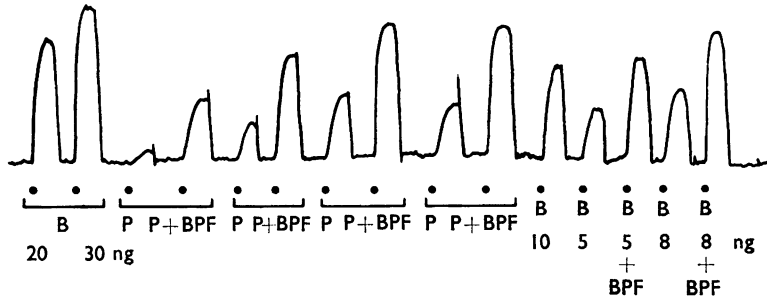

FIG. 5. Responses of the isolated guinea-pig ileum to bradykinin before (B) and immediately after (B+BPF) the addition of 0·125 μg/ml bradykinin-potentiating factor to the chamber containing the ileum. Note that, similarly, the responses of the preparation to 0·8 ml of the perfusates (P) from heated (46° C) rat's paw were potentiated by the presence of the bradykinin-potentiating factor (P+PBF).

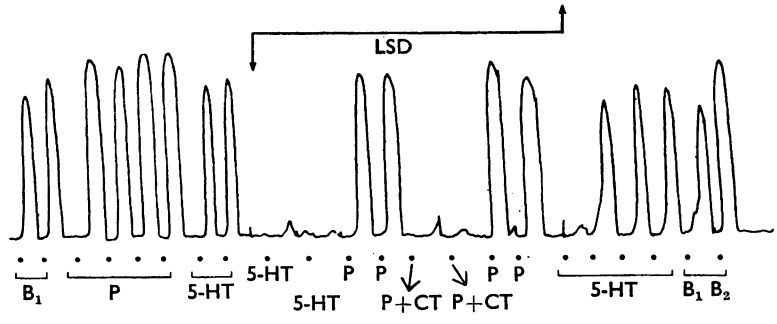

FIG. 6. Responses of the isolated rat uterus to 1 ng (B_1) and 2 ng (B_2) bradykinin, to 40 ng 5-hydroxytryptamine (5-HT) and to 0·5 ml of the perfusates (P) collected from the heated (46° C) rat's paw. The addition of 0·1 μg/ml LSD-25 to the bathing fluid completely blocked the responses to 5-HT, which recovered after withdrawing the inhibitor, while it did not affect the responses to the perfusates. The activity in the perfusates disappeared after incubation (37° C) with 200 μg/ml α-chymotrypsin (P+CT).

that bradykinin was directly injected into the cannula entering the paw and that the temperature of the water in which the paws were immersed was kept at 37° C to avoid release of active material.

About 40% of the injected bradykinin was recovered in most cases. This observation indicates that the amount of active bradykinin-like material released into the perfusion fluid by the heat treatment would be more than twice that actually assayed.

When experiments were carried out using Tyrode-Tris buffer solution adjusted to pH 3, as the perfusion fluid, instead of the usual Tyrode solution, the amount of bradykinin recovered was about 98–100%, as can be seen in Table 3. Edery & Lewis (1962) have shown that kininases are inhibited at slightly acid pH and this fact could explain the improved recovery thus obtained.

TABLE 2. *Recovery of bradykinin in Tyrode solution, pH 8, after perfusion through the subcutaneous spaces of non-heated rat paws*

	Response of rat uterus to bradykinin added:				
	(a) direct		(b) in perfusion fluid		Estimated recovery
Exp. No.	ng	response (mm)	ng	response (mm)	(%)
1	0·8	57	1·0	52	60
	1·0	63	2·0	57	40
	1·2	67	4·0	77	47
	2·0	79			
	4·0	83			
2	0·6	39	2·0	36	25
	1·0	57	4·0	56	25
	2·0	65	8·0	62	25
3	0·8	46	2·0	48	44
	1·0	51	4·0	59	38
	2·0	53	8·0	64	26
4	1·0	9	2·0	9	50
	1·6	18	4·0	22	43
	3·2	33	8·0	35	41
	4·0	47			
5	1·0	45	2·0	44	49
	2·0	52	4·0	55	51
	3·2	60	8·0	58	38

TABLE 3. *Recovery of bradykinin in Tyrode-Tris buffer solution, pH 3, after perfusion through the subcutaneous spaces of non-heated rat paws*

	Response of rat uterus to bradykinin added:				
	(a) direct		(b) in perfusion fluid		Estimated recovery
Exp. No.	ng	response (mm)	ng	response (mm)	(%)
1	2·0	47	2·0	50	106
	4·0	58	4·0	58	100
2	2·0	55	2·0	54	98
	4·0	59	4·0	58	99
3	1·0	59	1·0	61	103
	2·0	66	2·0	65	98
	4·0	70	4·0	70	100
	8·0	74	8·0	74	100
4	1·0	54	1·0	57	105
	2·0	60	2·0	61	101
	4·0	66	4·0	65	98
	8·0	70	8·0	70	100

pH and releasing-capacity

No detectable effect on the release of active material from heated paws was observed when the pH of the perfusion fluid was lowered to 3 or increased to 10. The duration of the perfusion at non-physiological pH was about 50–60 min. When the pH of the perfusion fluid was adjusted to 10, the pH of the collected perfusates, estimated potentiometrically, ranged between 8·9 and 9·2. When the pH was originally adjusted to 3 or 5, the values in the perfusates ranged from 5·7 to 6·0. It is known from the experiments of Edery & Lewis (1962) that while kininases are inhibited at acid pH, the formation of kinins proceeds normally. Our experiments also seem to suggest that high pH values of the perfusion fluid apparently do not interfere with the formation of the bradykinin-like substance released by heat treatment of the rat's paws.

Effect of administration of atropine and diphenhydramine to the animals subjected to heat treatment

A group of rats was injected with atropine (1 mg/kg intraperitoneally) and diphenhydramine (25 mg/kg intraperitoneally), 15 min before the beginning of the experiments, the injection being repeated at 90 min intervals during the experiments. No effect on the activity of the perfusates was observed.

Influence of hexadimethrine bromide on the release of the active material from rat paws submitted to heating

Hexadimethrine bromide added to the perfusion fluid in doses of 0·1–0·5 mg/ml to one of the heated paws inhibited the appearance of active material, the release from the contralateral paw being unaffected. This block could be reversed by omitting the drug from the perfusion fluid (Figs. 7 and 8a).

When hexadimethrine bromide was injected intravenously, much larger doses (4–20 mg/kg) were necessary to produce a similar block (Fig. 8b).

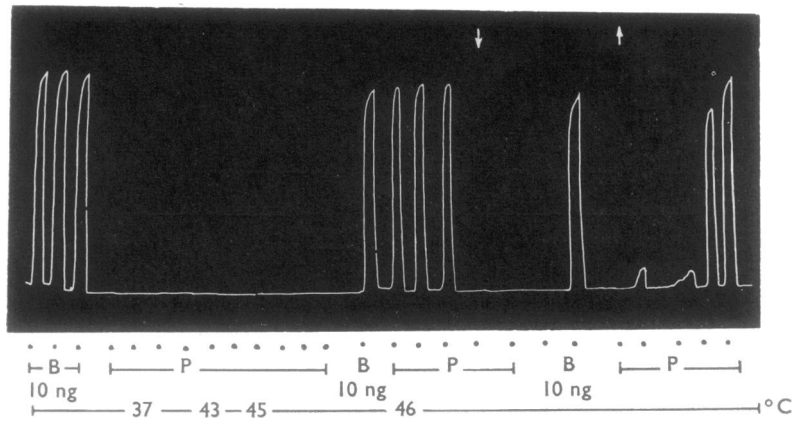

FIG. 7. Temperature dependency and effect of hexadimethrine bromide on the release of the active material from the rat's paw. The material is released when the external (water bath) temperature reached 45°–46° C (lower scale). Hexadimethrine bromide (0·5 mg/ml) added to the perfusion fluid (left arrow) completely blocked the release of such material, which reappeared after omitting the drug from the perfusion liquid (right arrow). B, Standard bradykinin (10 ng); P, perfusate (0·6 ml) from the paw. Test organ: rat uterus.

These facts seem to suggest that the activation of the bradykinin-like material probably occurs in the subcutaneous spaces of the paw, since hexadimethrine bromide, known to affect this process, is very effective when perfused but has little effect when given intravenously.

Influence of soy-bean trypsin inhibitor (SBI) on the release of active material

With concentrations as low as 0·025 mg/ml of SBI in the perfusion fluid results similar to those seen with hexadimethrine bromide could be obtained. The inhibition of the active material released by heating the paws started soon after SBI was added to the perfusion fluid and was reversible. When given intravenously, doses of 4–5 mg/kg were required to produce some inhibition (Fig. 9).

Influence of aprotinin and Kunitz inhibitor on the release of active material

The perfusion of 500 u./ml and upwards of aprotinin produced only a temporary blockade of release of bradykinin-like material (Fig. 10a). When given intravenously, doses had to be increased to 30,000–40,000 u./ml to produce a measurable inhibition of release (Fig. 10b).

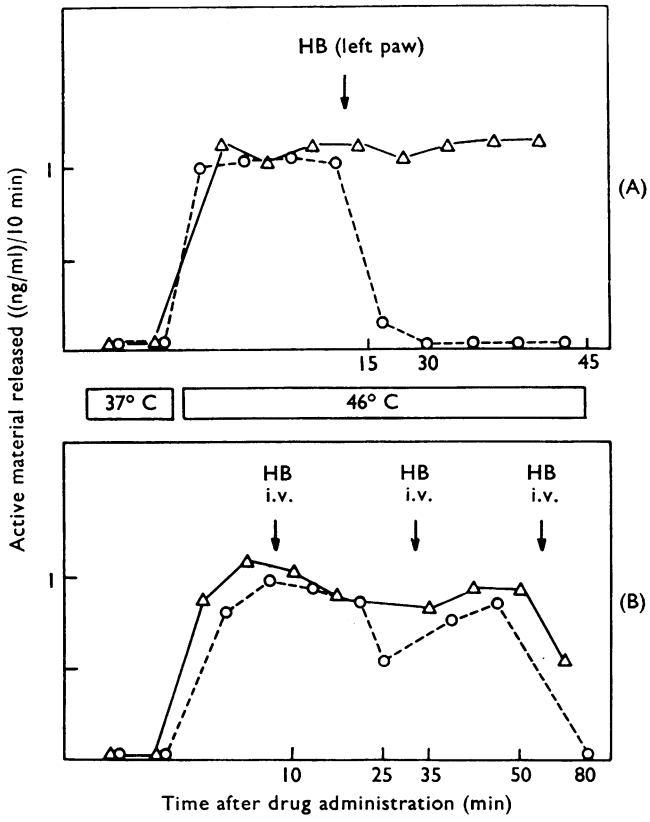

FIG. 8. Effect of hexadimethrine bromide (HB) on the release of active material from heated (46° C) rat paw. (A), 0·1 mg/ml of the drug was added to the perfusion fluid reaching left paw (- - -○- - -). The release of active material from right paw (—△—) remained unchanged. (B), Administration of 4, 20 and 20 mg/kg hexadimethrine bromide intravenously (arrows from left to right, respectively). The amount of active material released was estimated as bradykinin on the isolated rat uterus.

The Kunitz inhibitor was also only temporarily effective as a blocking agent (Fig. 11) and was ineffective by the intravenous route in doses up to 15–20 mg/kg.

With aprotinin and Kunitz inhibitor the inhibition lasted only for a short time, even when the inhibitors were present in the perfusion fluid.

Effect of the ligature of the iliac artery on the swelling produced by heating and upon the release of active material

Ligaturing one of the iliac arteries immediately before starting to heat the paws completely prevented the appearance of active material in the corresponding paw (Fig. 12). This fact seems to exclude the possibility that substances present in the subcutaneous tissues, such as 5-hydroxytryptamine, could easily be released by perfusing the paws and contribute to the activity estimated on the isolated uterus. As expected, in the ligated paw the heat treatment produced no oedema, or only a slight increase in volume, when compared with the paw with intact circulation.

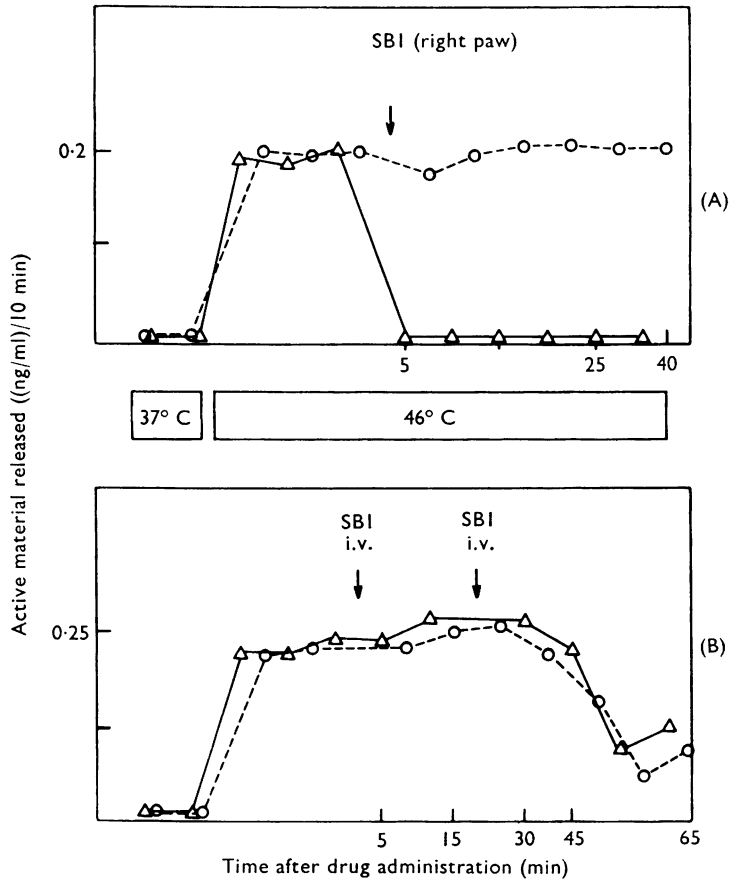

FIG. 9. Effect of soy-bean trypsin inhibitor (SBI) on the release of active material from heated (46° C) rat paw. (A), 0·1 mg/ml of the substance was added to the perfusion fluid reaching the right paw (—△—). The release of active material from left paw (- - -○- - -) remained unchanged. (B), Administration of 2 and 4 mg/kg SBI intravenously (arrows from left to right, respectively). The amount of active material released was estimated as bradykinin on the isolated rat uterus.

An intact circulation of the paw is, therefore, essential for the release of the bradykinin-like material, which would leave the circulation mainly as an inactive form (bradykininogen), being then activated in the subcutaneous spaces.

Discussion

Additional information about the release of a bradykinin-like substance from the paws of the rat submitted to mild heating (46° C) has been obtained using an improvement of the method previously described by Rocha e Silva & Antonio (1960).

By perfusing both hind paws, in strictly comparable conditions, in such a way that one can act as a control of the other, it was possible to investigate the effects of agents acting directly on the site of release of the active material from the heated paw.

The active substance stimulated the isolated rat uterus and produced a relaxing effect on the isolated rat duodenum. It was destroyed by incubation with α-chymo-

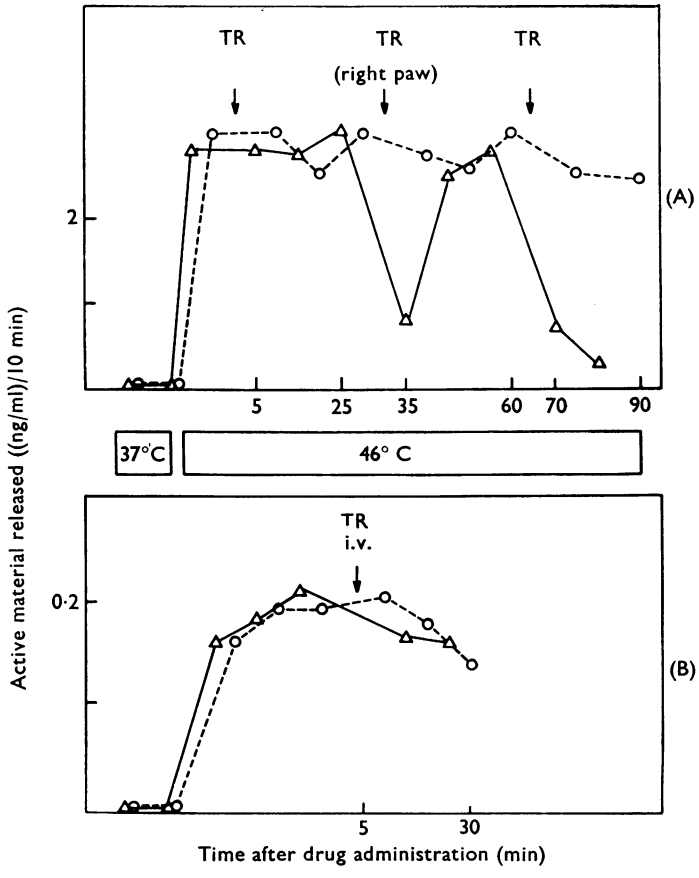

FIG. 10. Partial and temporary blockade of the active material released from the heated (46° C) rat's paw by aprotinin (TR). (A), Addition of 200, 500 and 1,000 u./ml (arrows from left to right, respectively) to the perfusion fluid reaching the right paw (—△—). The release of active material from left paw (- - -○- - -) remained unchanged. (B), Administration of 35,000 u./kg (arrow) aprotinin intravenously. The amount of material released was estimated as bradykinin on the isolated rat uterus.

trypsin and potentiated by the bradykinin-potentiating factor (BPF). The results suggest that the material released from the rat's paw by heating to 46° C is of a bradykinin-like nature. In addition, soy-bean trypsin inhibitor and hexadimethrine bromide, which interfere with the release of bradykinin from its inactive precursor, markedly blocked its release.

A major contribution of 5-hydroxytryptamine to the activity present in the perfusates seems to be excluded, as the activity remained unaffected in the presence of LSD-25 in doses able to block completely the responses of the isolated uterus to 5-hydroxytryptamine.

The total amount of active kinin could be estimated in each paw by the rate of release, which averaged 1·5–4 (ng/ml)/10 min. Since the average time of sur-

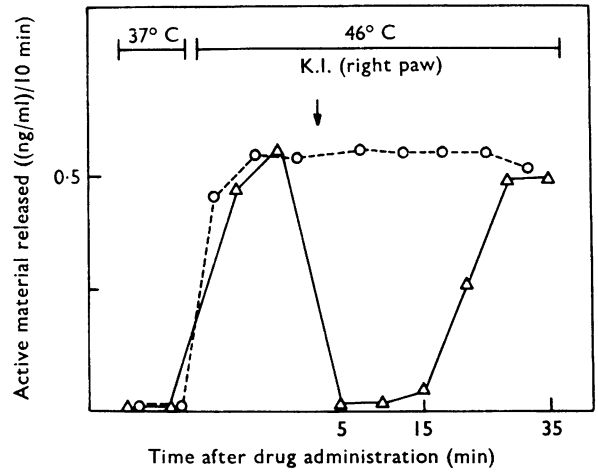

FIG. 11. Evanescent inhibition of the release of active material from heated (46° C) rat paw by addition of 0·1 mg/ml Kunitz inhibitor (K.I.) to the perfusion fluid reaching the right paw (—△—). The release of active material from left paw (- - -○- - -) remained unchanged. The amount of active material released was estimated as bradykinin on the isolated rat uterus.

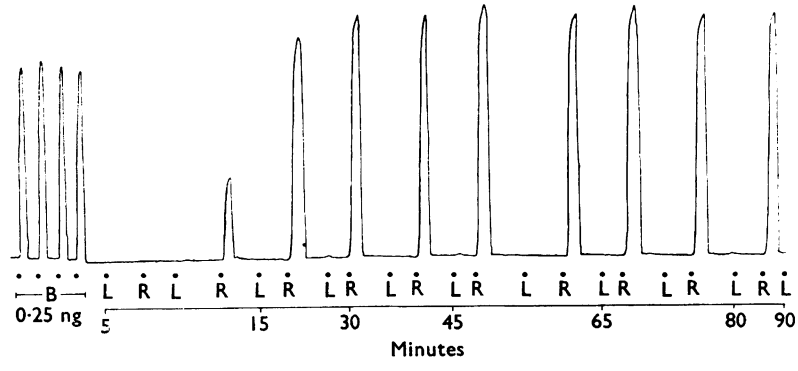

FIG. 12. Effect of ligature of left iliac artery on the release of active material from the heated (46° C) rat's paw. B, Responses of the isolated rat uterus to bradykinin; at L and R, 0·6 ml of the perfusates from left and right paws, respectively, was added. No response was seen to the perfusate from the paw with interrupted circulation. Lower scale indicates time (min) of perfusion.

vival of the animals submitted to the heat treatment was 80–90 min after the temperature of the bath reached 46° C, we might consider 15–30 ng as the total amount collected from each paw. This amount probably represents only a small fraction of the active material actually released. First, as shown by perfusing non-heated paws with known concentrations of bradykinin, the recovery was only 30–40%. Second, the areas (volume) of the whole heated subcutaneous space directly reached by the perfusion fluid are rather small.

The release of the active material was blocked by agents able to interfere with the release of bradykinin from its inactive precursor, as shown in *in vitro* experiments. Thus, soy-bean trypsin inhibitor was one of the most potent substances so far tested. Almost equally potent was the anti-heparin agent, hexadimethrine bromide. In both cases, removal of the inhibitor from the perfusion fluid caused the reappearance of the active material in the perfusates within a few minutes.

A temporary block was observed when aprotinin or Kunitz inhibitor were added to the perfusion fluid. An escape from inhibition was seen after a period of blockade of the release of the active material. This reversal occurred even with high doses of the inhibitors, thus indicating that a somewhat different mechanism could be responsible for the effects of SBI and hexadimethrine bromide when compared with those of aprotinin and Kunitz inhibitor. In a recent report Greenbaum, Freer, Chang, Semente & Yamafuji (1969) emphasized the inefficiency of aprotinin as an anti-inflammatory agent, and since it can block destruction of kinins by kininases it might contribute to increase rather than to decrease the amounts of kinins at the inflammatory site. This fact could explain the escape from inhibition here described when aprotinin was used as a blocking agent. Similarly, Reis & Rocha e Silva (unpublished results) have found that either aprotinin or Kunitz inhibitor are much less effective in blocking the release of bradykinin from acidified and heated fresh plasma *in vitro* than SBI; much higher doses of the former being necessary to give even lesser degrees of inhibition.

As a whole, these results emphasize the bradykinin-like nature of the active material released from the paws by heat treatment.

Recently Starr & West (1967) denied the protective action of most agents mentioned in the present paper against the development of heat oedema. However, this action is quite marked for hexadimethrine bromide, as previously shown in heat (Garcia Leme *et al.*, 1967) or yeast-induced oedema (Kellet, 1965). Similarly, SBI and aprotinin were found to be effective in counteracting experimental inflammatory reactions (Kaller, Hoffmeister & Kroneberg, 1966 ; Hladovec, Mansfeld & Horáková, 1958). Besides the findings of Greenbaum *et al.* (1969), another important factor might explain some of these discrepancies, namely the doses and routes of administration. We have shown that by the intravenous route, much higher doses of the agents have to be used to produce a much smaller inhibition than that obtained by local perfusion of the inhibitors through one of the heated paws.

This fact seems to indicate that the subcutaneous space is the chief site of release of the material thus produced. Irritative stimuli could originate a process leading to minute local plasma extravasations to the subcutaneous spaces where the bradykinin-like material would be formed from its inactive precursor (bradykininogen) in amounts large enough to maintain the vascular alterations responsible for the development of the oedema. Once initiated, the process could also lead to the

release of other endogenous substances such as histamine and/or 5-hydroxytryptamine, which would reinforce the vascular changes. This view agrees with previous experiments of Wilhelm & Mason (1960) and Rocha e Silva & Rosenthal (1961), in which much higher temperatures were used. However, the view sponsored by Edery & Lewis (1963) that histamine might be a decisive factor to start the process of filtration of bradykininogen into the inflamed area could not apply to the release by heat treatment, since anti-histamine (and anti-5-hydroxytryptamine) drugs are ineffective to counteract the whole chain of events (Rocha e Silva & Antonio, 1960). Furthermore, we could observe a continuous production of the bradykinin-like material after treatment of the animals with atropine and diphenhydramine.

Extravasation of plasma to the subcutaneous spaces appears to be a determining factor in the production of heat oedema. When one of the iliac arteries of the animals was ligated, no active material was detected and either no swelling or only slight swelling was observed. This emphasizes the role of vascular reactions to irritative stimuli in inflammatory responses of the type studied to initiate or maintain the activation process of the kininogen-kinin system in the interstitial spaces of the subcutaneous tissues. These observations would indicate that the stores of bradykininogen or the activating enzyme in the extravascular spaces are either too small or not affected by the heat treatment.

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