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COMPARATIVE PHARMACOLOGICAL ACTIONS OF BRADYKININ AND RELATED KININS OF LARGER MOLECULAR WEIGHTS*

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Abstract—Three polypeptides with the terminal sequence of bradykinin: Gly-Arg-Met-Lys-Bk (GAML-Bk), Met-Lys-Bk, Lys-Bk (kallidin) and bradykinin itself were assayed for biological activity on: guinea pig ileum, rat uterus, rat duodenum, arterial blood pressure of the rat by venous and arterial routes, and vascular permeability (blue test). The pharmacological actions were qualitatively similar but differed quantitatively according to the molecular weights of the peptides tested. Those with the highest molecular weights were much less active upon the different smooth muscle preparations, while 10–12 times more potent in the vascular permeability test. The effects on the rat duodenum and rat uterus ran parallel, but again were discrepant when compared with the guinea pig ileum. The nature of the receptors for bradykinin is discussed.

THERE is considerable experimental evidence to show that the pharmacological actions of bradykinin (Bk) and its natural derivatives (Lys-Bk and Met-Lys-Bk) change with their molecular size according to the biological preparations utilized for the assays, as well as to their resistance to destruction by kininases.[1–4] In the present study we had the opportunity of assaying a new synthetic peptide with 13 amino acid residues responding to the formula: Gly-Arg-Met-Lys-Bk. In preliminary experiments we could find this compound to behave qualitatively like a kinin, producing fall in blood pressure, stimulation of the guinea pig ileum and rat uterus, relaxation of the rat's duodenum and increased vascular (capillary) permeability. This spectrum of activities seemed enough to characterize the new kinin as belonging to the group of *Kinin Hormones* related to bradykinin, see Ref. 5. Furthermore, its potency when compared with bradykinin shifts in the same direction as that of the other larger kinins, when assayed by different bio-assays. This circumstance afforded a clear cut characterization of kinins of different molecular weights displaying, however, the structure of bradykinin as the main pharmacologically active sequence.

In a previous paper[6] one of us with J. W. Ryan has shown that according to the preliminary treatment to which the acidified ox pseudoglobulin fraction was submitted, a different material was released, bradykinin being apparently the main substance released if the acidified precursor was heated in a boiling water bath (for 5 min) and then dialyzed against pH 2·0, though Met-Lys-Bk, as shown previously by

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Elliott and Lewis,[7] and apparently a larger peptide, were released if the heating was avoided before dialysis.[6]

These findings are of importance to identify the nature of biologically active peptides of the bradykinin group, released under physiological conditions, and having predominant hypotensive and vascular permeability activity.

MATERIALS AND METHODS

Smooth muscle preparations. The assays on the guinea pig ileum were performed as described in other papers of this laboratory and are discussed in Ref. 5. For the registration of the responses we have used a two channel Beckmann Dynograph. For convenience we have adapted an isotonic lever to allow an ampler contraction of the muscle strip, instead of the usual isometric registration afforded by a direct connection of the muscle to the Statham transducer. This device was especially useful for the registration of the contractions of the guinea pig ileum. The experiments with the rat uterus and rat duodenum were performed by using the conventional smoked drum and an isotonic lever. All measurements of potency were derived from bio-assays in 4-point designs, using at least four groups of four doses each, for the calculation of the potency ratio. See Ref. 5.

Rat blood pressure. Experiments of measuring rat blood pressure (arterial) were performed by registration in one of the channels of the Beckmann Dynograph through a polyethylene cannula inserted into one of the carotid arteries. The other artery received a long polyethylene tube (2 mm dia.) to reach the aorta arch through which the arterial injections were done. A similar cannulation of the jugular vein was reserved for the intravenous injections. To establish the ratios of activity artery/vein, a random design was (previously) adhered to by using two doses of the material in the ratio 1:2 alternating the routes of administration according to a sequence previously established. As the dose–response curves were not strictly parallel, the ratio was calculated from the dilutions utilized to produce approximately the same effects in either route. The animals were anesthetized with sodium pentobarbital (25–30 mg/kg) given intraperitoneally. If further anesthesia was required during the assay, a fraction of that dose was given intravenously.

Blue test. To test the activity of the kinins upon vascular (capillary) permeability, the conventional blue test was performed in the abdominal wall of rats, injecting intravenously 1 ml of a 1 % solution of Evans blue in saline and not more than 0·1 ml of the kinin solution intradermally. The results were assessed after 10 min of the intradermal injection, at the inner face of the excised skin. The method described by Steele and Wilhelm[8] to quantitate the blue color was utilized in some preliminary experiments. Control rats received increasing concentrations of an Evans blue solution by the intradermal route and the intensity of the color served to match the colors of the experimental animals receiving the blue intravenously and the permeability increasing agent intradermally. As indicated by Steele and Wilhelm[8] the curve relating the amount of blue and the usual scoring in $\pm$, $+$, $++$, $+++$ and $++++$, is not a straight line but an S-shaped curve, when the intensity is matched against the logarithm of the dye concentration. Though some degree of correction is introduced by such a procedure, as regards the low concentrations of dye, permitting a better quantification for the smaller effects (up to $++$) for higher concentrations of the dye the readings tend to lower down and give a distorted dose–response curve. To avoid

such a distortion we have used the method described by Frimmer and Müller[9] with modifications. Pieces of skin including the blue spot were suspended in 3 ml of formamide and homogenized with an Ultra-Turrax (Janke & Kunkel KG.) and 3·0 ml of ethyl ether were added and the mixture thoroughly shaken before centrifugation; the lowest phase containing the blue pigment was separated and submitted to a short heating at 45° for removal of the ether, and the volume completed to 5·0 ml for reading in a Coleman Jr. at a 600 nm wavelength. The total amount of blue dye was calculated against a standard curve of increasing concentrations of the Evans Blue solution in formamide. The recovery of the blue pigment in control experiments varied from 100 to 84 per cent from skin spots obtained in the living animal by intradermal injections of the indicated amounts of the blue pigment, as seen in Fig. 1.

By using this method, satisfactory log dose–response curves were obtained that followed the general trend of S-shaped curves, as indicated in Results.

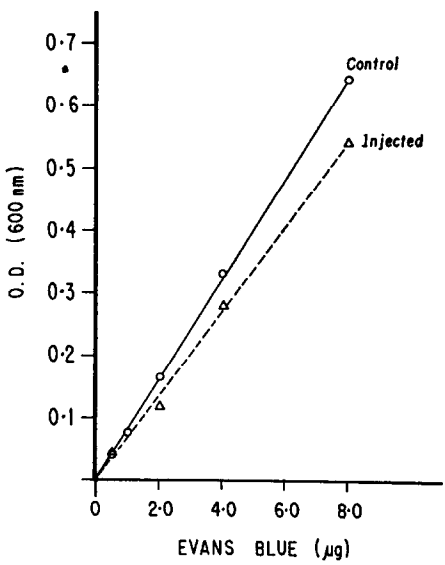


FIG. 1. Recovery of Evans blue from areas of the skin *injected* with increasing amounts of dye, as compared with a direct estimation of blue in *control* solutions.

Discrimination ratios. The indexes of discrimination were calculated according to Gaddum[10] as the quotient of potencies related to a common standard (bradykinin) upon two different biological preparations. For instance, if the potency of bradykinin is taken as 1·0, that of Met-Lys-Bk being 0·09 on the guinea pig ileum and 0·36 on rat uterus, the discrimination index rat uterus/guinea pig ileum = 4·0. Again if the potency of Bk is taken as 1·0 on the vascular permeability (capillary permeability), and the potency of Gly-Arg-Met-Lys-Bk (GAML-Bk) is measured as 10–12 times larger on the blue test, and 16 times less on the guinea pig ileum, the discrimination index vascular permeability/guinea pig ileum $\simeq$ 180.

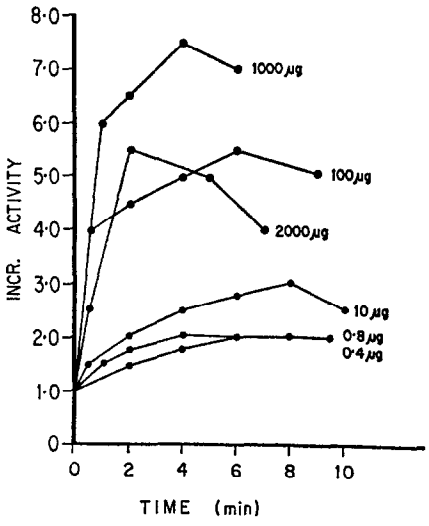
Activation of the peptides by trypsin. Incubation of the larger peptides (Met-Lys-Bk and GAML-Bk) with trypsin produced conspicuous increases in activity when tested

upon the guinea pig ileum. The enzymatic experiments were done in incubates containing 10 μg of peptide + trypsin solution + 0·02 M tris buffer (pH = 7·8), the volume completed to 1 ml. Aliquots of the incubate were applied to the guinea pig ileum in a bath of 8 ml capacity at the indicated intervals of time. The contractions were registered in a Beckmann Dynograph, as indicated above. The increases in activity refer to the control activity at 0 incubation time. Previously to the additions, the ileum was desensitized to the amounts of trypsin to be added, but sometimes a potentiation of the preparation to the larger doses of the enzyme could be observed. This effect was corrected in the design used since the first control addition contained already the concentration of the enzyme utilized in the incubation experiments.

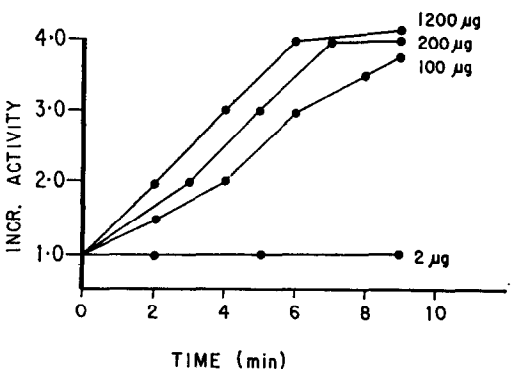
Materials. The polypeptides bradykinin and Met-Lys-Bk were purchased from Schwarz & Co. Lys-Bk (kallidin) was a gift from Sandoz (Basel). The new kinin Gly-Arg-Met-Lys-Bk (GAML-Bk) was supplied by Schwarz & Co., through the courtesy of Dr. J. W. Ryan, from the Department of Medicine of the University of Miami, Florida. This material was supplied with the indication that it might be 70–80 per cent pure. The impurity is mainly free arginine. From our assays there was no evidence of contamination with lower kinins, as indicated by the ratio 1:1 (artery/vein) in the rat's blood pressure. If any contamination with lower kinins were present the discrimination ratio vascular permeability/guinea pig ileum would be even greater than that found below. Additional experiments were done with samples of pure Met-Lys-Bk and GAML-Bk kindly supplied by Dr. L. J. Greene of Brookhaven National Laboratories, L.I., N.Y. The discrimination ratios were essentially the same as those obtained with the Schwarz material. The trypsin utilized in the experiments of activation of the compounds (ML-Bk and GAML-Bk) was a brand from Worthington Biochemical Corp. (lyophylized TRL) free from chymotrypsin. The other materials utilized had the following origins: sodium pentobarbital (Abbott, North Chicago), Evans Blue (K & K Laboratories, Inc.), formamide (Merck Co.), BAL (California Corporation).

RESULTS

Release of bradykinin by trypsin. It has been reported that Met-Lys-Bk yields bradykinin on incubation with trypsin.[11] When the assay was performed upon the guinea pig ileum, the treatment with trypsin increased four times the activity of the peptide. The same would be expected with the 13-peptide GAML-Bk with the difference that instead of a 4-fold increase in activity, a 6- to 7-fold increase in activity was observed. Figure 2(a) shows the result of an experiment of activation of GAML-Bk by increasing concentrations of trypsin. It is to be noted that the reaction proceeds clearly in two steps, since with small concentrations of trypsin a plateau was attained at an increase of two to three times activity level, and only with a much larger concentration of trypsin, the full seven time increase of activity was observed. A first explanation for this phenomenon might be found in the ratios of activity indicated in the next section, for Met-Lys-Bk and GAML-Bk, since the former peptide is about 1·5–1·8 times more active than the latter. It appears that in a first step GAML-Bk is transformed into Met-Lys-Bk, and the splitting of the latter requires much higher concentrations of the enzyme. This is clearly shown in the graphs of Fig. 2 (b) in which not less than 200 μg of trypsin were required to produce a full activation of Met-Lys-Bk, though as shown in Fig. 2(a) already 0·4–0·8 μg of trypsin was enough



(a)



(b)

FIG. 2 (a). Increase in activity on the guinea pig ileum of GAML-Bk when incubated with the indicated amounts of trypsin; (b) A similar experiment with Met-Lys-Bk.

to double the activity of GAML-Bk. However, there is still a point requiring further investigation, namely the fact that the full activity of the bradykinin moiety contained in both Met-Lys-Bk and GAML-Bk (11× and 20× respectively) could not be recovered. In our experiments, even with an excess of trypsin, Lys-Bk was not split into bradykinin. It might occur that the transformation of the larger kinins has as end-point the decapeptide Lys-Bk, although this appears improbable since it has been previously shown that Met-Lys-Bk can be transformed into bradykinin.[11]

Figure 3 gives a picture of a typical assay on the guinea pig ileum of the activation of GAML-Bk and Met-Lys-Bk after treatment with trypsin.

Comparative effects on smooth muscle preparations. The comparative potencies of the four peptides upon the guinea pig ileum, rat uterus and rat duodenum (relaxation)

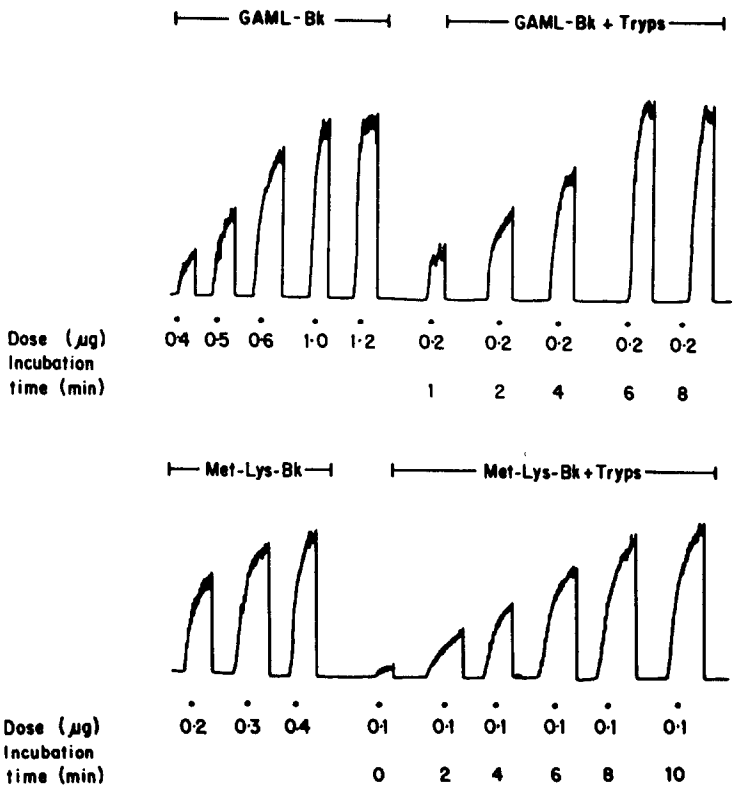


FIG. 3. Activity of GAML-Bk as assayed on the guinea pig ileum after incubation without and with trypsin (upper tracings); similar experiment with Met-Lys-Bk (lower tracings). In both cases, the concentration of trypsin was 200 μg/ml.

are indicated in Table 1. The following order of activity was found in the three structures:

Guinea pig ileum: Bk > Lys-Bk > Met-Lys-Bk > GAML-Bk
Rat uterus: Bk > Lys-Bk > GAML-Bk > Met-Lys-Bk
Rat duodenum: Lys-Bk > Bk > GAML-Bk > Met-Lys-Bk

The greatest discrepancy appeared to be obtained with the relaxation effect upon the rat duodenum. Lys-Bk was about 1·98 times more potent than Bk itself, though upon the other smooth muscle structures Bk was definitely more potent than the larger kinins, the maximal discrimination being observed in relation to the guinea pig ileum with a ratio of 0·09 and 0·05 for Met-Lys-Bk and GAML-Bk. But again, in this case Lys-Bk was more active than the larger kinins, being only three times less potent than Bk.

Rat blood pressure (*B.P.*). By comparing the effects of the kinins when given by the arterial route, the kinins were about equipotent with somewhat stronger effects of Lys-Bk (0·91) and Met-Lys-Bk (0·87) as indicated in Tables 1 and 2, and in Fig. 4. However, if BAL was given before, the effect of Bk upon the B.P. by the intravenous route almost equalled that by the arterial route, as indicated in Table 2 and Fig. 5.

TABLE 1. EQUIPOTENT CONCENTRATIONS (ng/ml OR TOTAL) OF THE DIFFERENT POLYPEPTIDES IN PRODUCING EFFECTS ON VARIOUS BIOLOGICAL PREPARATIONS

Preparation	Peptides			
	Bk	Lys-Bk	Met-Lys-Bk	GAML-Bk
Guinea pig ileum	6·25	18·75	66·0	103·00
Rat duodenum	0·62	0·28	1·25	0·86
Rat uterus	0·25	0·33	0·70	0·43
Artery B.P.*	100·00	91·00	80·64	100·00
Vein B.P.*	1000·00	272·70	161·28	100·00
Vascular permeability*	100·00	100·00	12·00	10·00

* The doses (ng) refer to the total amounts injected.
Note: The doses presented in this table were calculated from ratios obtained in the biological assays presented in Table 2.

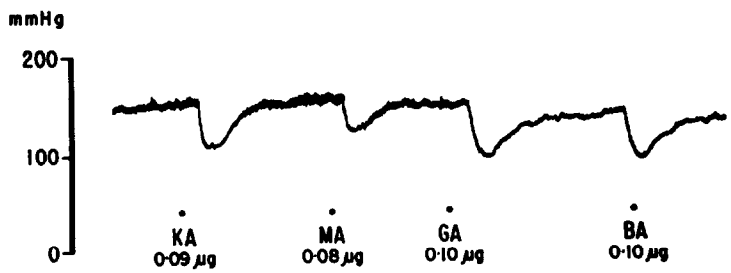


FIG. 4. Effect of Lys-Bk (KA), Met-Lys-Bk (MA) and GAML-Bk (GA) given through the artery, on the rat's systemic blood pressure. BA = Bk given intra-arterially.

The artery/vein ratios of the other kinins were altered according to the sequence Bk > Lys-Bk > Met-Lys-Bk. The ratio corresponding to GAML-Bk was virtually not altered by the BAL treatment as shown in Fig. 6. According to Ryan *et al.*[13] the value of such a ratio would indicate the rate of inactivation of the peptides by the lung of the rat. The small value of the artery/vein ratio for the larger kinins Met-Lys-Bk and GAML-Bk would indicate that they are less susceptible or not at all to the inactivating action of kininases which are able to destroy bradykinin in a single passage through the lungs.[12,13]

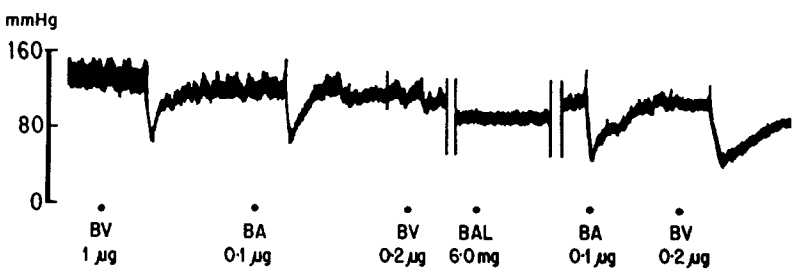


FIG. 5. Rat systemic blood pressure. BV, BA indicate bradykinin by venous or arterial route respectively, before and after the injection of 6·0 mg of BAL.

TABLE 2. RATIOS OF POTENCY* OF THE INDICATED PEPTIDES TAKING BRADYKININ (= 1·0) AS STANDARD

Peptides	Guinea pig ileum	Rat uterus	Rat duodenum	Rat's blood pressure via Artery	Rat's blood pressure via Vein	Ratio B.P. Artery/vein	Vascular permeability
Bradykinin	1·00	1·00	1·00	1·00	0·12	8·4 **1·0**‡	1·00
Lys-Bk	0·30 (0·29–0·33)	0·88 (0·76–1·00)	1·98 (1·47–2·60)	0·91 ± 0·14 (9)	0·30 ± 0·1 (4)	3·0 **1·0**	1·00 ± 0·5
Met-Lys-Bk	0·09 (0·08–0·10)	0·30 (0·25–0·36)	0·49 (0·46–0·52)	0·87 ± 0·13 (9)	0·43 ± 0·08 (7)	2·0 **1·1**	10·00 ± 0·73 (6)
GAML-Bk†	0·063 (0·06–0·067)	0·60 (0·50–0·70)	0·72 (0·67–0·76)	1·00 ± 0·18 (7)	1·00 ± 0·05 (7)	1·0 **1·0**	11·00 ± 0·6 (4)

* The ratios were calculated on molar concentrations. The pairs of figures between brackets are confidence limits obtained in 4 point assays for the 5 per cent level of significance. For the assays on blood pressure and vascular permeability the means ± S.E. are given, and the number of experiments indicated between brackets.

† Corrected for 30 per cent impurities (GAML-Bk = Gly-Arg-Met-Lys-Bk).

‡ The values in bold figures were obtained by a previous treatment of the animal with BAL 20 mg/kg, i.v.

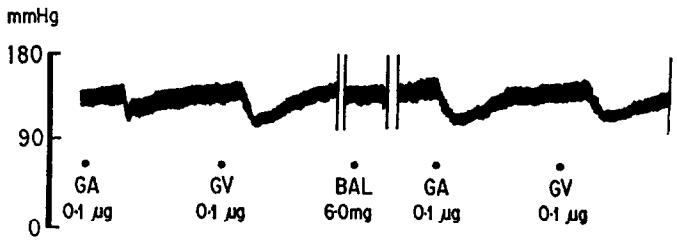


FIG. 6. Rat's systemic blood pressure. GA, GV, indicate GAML-Bk by venous or arterial route respectively, before and after the injection of 6·0 mg of BAL.

This conclusion is further strengthened by the fact that *in vitro* GAML-Bk could not be potentiated by the Bradykinin Potentiating Factor (BPF) extracted from *Bothrops jararaca* venom and one of its recently synthetized active fraction.[12]

Vascular (capillary) permeability. The permeability increasing activity of the four kinins in the rat skin, is presented in the diagrams of Fig. 7. The tendency of the smaller kinins is to give shallower log dose–response curves. This could be understood by the more rapid destruction of Bk and Lys-Bk by kininases as indicated in the previous experiments of the artery/vein ratio in the rat's B.P. In these experiments Met-Lys-Bk was intermediary between the smaller kinins and the 13-peptide GAML-Bk. This again can be understood by the rate of breakdown of Met-Lys-Bk by kininases, as shown by the ratio artery/vein (Table 1). The steepest log dose–response curve was obtained with GAML-Bk which was also the least affected as to the artery/vein ratio.

The derivation of ratios of potency from such log dose–response curves is somewhat difficult because they are not parallel. If the curves are compared at their lowest level, the ratio tends to approach 10–12 for GAML-Bk/Bk, or slightly less for Met-Lys-Bk/Bk. However, if the comparison is done at higher levels of activity of GAML-Bk

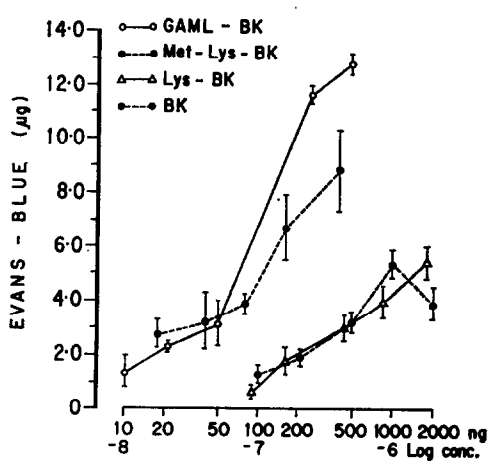


FIG. 7. Dose–response curves of the different kinins by vascular permeability test (Blue test). The total amounts of Evans blue recovered from the skin spots are indicated in ordinates against the total amounts of the kinins given in ng or Log concentration.

and Met-Lys-Bk toward Bk, the ratios will be much greater approaching 1·3 log units (20×). The comparison between Lys-Bk and Bk did not offer any difficulty since they were approximately equipotent when tested by the Blue test, as shown in Fig. 7.

For the general purpose of comparing activities upon different biological preparations, even the more conservative ratio of 10–12 for the larger kinins, is already very conspicuous, as it will be shown below.

Discrimination indexes. Table 3 indicates the discrimination indexes of the four peptides on the different biological preparations, by comparing with the guinea pig ileum or the rat duodenum. A few features of such a comparison are striking at a first glance. As far as smooth muscles are concerned it seems clear that the sensitivity

TABLE 3. DISCRIMINATION INDEXES OF THE DIFFERENT PEPTIDES IN RELATION TO PAIRS OF BIOLOGICAL STRUCTURE

	Indexes of discrimination*				
	Uterus Rat	Duodenum Rat	Duodenum Rat	B.P. (vein) Rat	Vasc. Permeability
Peptides	Ileum G.P.	Uterus Rat	Ileum G.P.	Ileum G.P.	Ileum G.P.
Bradykinin	1·00	1·00	1·00	1·00	1·00
Lys-Bk	2·00	2·50	5·20	2·60	2·65
Met-Lys-Bk	4·00	1·36	5·40	41·00	111·00
GAML-Bk	9·10	1·30	11·80	142·00	180·00

* The indexes of discrimination were calculated by dividing the ratios of potency indicated in Table 2, for each pair of biological structures.

of the uterus and the duodenum of the rat run parallel, giving a ratio approaching unity, if Bk, Met-Lys-Bk and GAML-Bk are considered. The decapeptide Lys-Bk (kallidin) appeared to be discrepant with a higher ratio for the duodenum than for the uterus. Furthermore, both structures behave similarly in relation to the guinea pig ileum giving discrimination indexes that increase proportionally to the molecular weight of the peptides. Another striking parallelism is that existing between blood pressure (intravenous route) and vascular permeability (blue test). It is to be noted that such a parallelism cannot be explained only by rates of destruction, but indicates a possible parallelism in sensitivity of the vascular structures for the four kinins.

However, such differences might be even more conspicuous if we consider the higher levels of comparison in the Blue test as indicated in the preceding section, when the discrimination index "vasc. perm./guinea pig ileum" can have values approaching 300, as will be commented in the discussion.

DISCUSSION

The facts presented above enlarge considerably the kinin field, since we can assume that larger peptides, with the bradykinin sequence attached to them, might have elusive pharmacological properties. It was known that lengthening the sequence

attached to the NH_2-terminal group of bradykinin (Lys-Bk and Met-Lys-Bk) accentuates the effect upon the blood pressure, though the effects upon the smooth muscles of the guinea pig ileum, rat uterus and rat duodenum were reduced. The observations presented in this paper on the effects produced by a still larger kinin, having four extra amino acid residues attached to bradykinin, namely glycyl-arginyl-methionyl-lysyl-Bk (GAML-Bk) indicate that a polypeptide of the bradykinin family, may display potencies from 100 to 300 times greater when tested upon the vascular permeability, when compared to the potency upon the guinea pig ileum. A situation might arise in which a natural product, derived from bradykininogen, might be classified as a predominantly "permeability factor" with considerably less activity upon the guinea pig ileum. One can think immediately of the many permeability factors described in the literature, that have been obtained from the globulin fraction, by simple dilution, complement action and by the action of kininogenins derived from white blood cells.[15–18] That the mechanism of release of kinins can depend upon the conditions and the nature of the enzymes *in vivo* and *in vitro*, has been shown in a recent publication by Ryan and Rocha e Silva[6] using the precursor contained in the ox pseudoglobulin fraction. If the pseudoglobulin was heated to 90° for 5 min, at pH = 2·0, dialysed for 3 days against this low pH and then incubated at pH = 7·4 at 37°, a rapid release of bradykinin took place, although if the heating step was omitted, following the technique described by Elliott and Lewis,[7] larger peptides were released including the known undecapeptide Met-Lys-Bk. There was also evidence that in such a material a still larger peptide was present giving a high ratio of discrimination, between the effects on rat blood pressure and the guinea pig's ileum.[6] Since bradykininogen can be a large globular protein, there is no limit to the assumption that the bond split by endogenously activated enzymes might release a larger peptide than the already identified Met-Lys-Bk or even larger than the 13-peptide GAML-Bk studied in the present paper.

Another characteristic of the larger kinins is that they become more resistant to destruction by kininases, indicated by their smaller potentiation by BAL or BPF, or by a ratio (approaching 1·00) of the effects upon B.P. when given through artery or vein. In the above experiments, GAML-Bk was by far the most stable when compared with Bk or Lys-Bk.

It would be premature to draw conclusions on the identity of receptors for bradykinin in the different smooth muscle structures, as well as in the vascular beds. In Ref. 5, these receptors have been tentatively called S-receptors, with the possibility of distinguishing: S_1 for vascular permeability, S_2 for the guinea pig ileum, S_3 for the fall in blood pressure and S_4 for the relaxation of the rat duodenum. From the above indexes of discrimination one might assume a probable correlation between S_1 and S_3, though S_4 seems to be a different entity to denote common receptors in the rat duodenum and the rat uterus. However, no correlation could be found in the sensitivities of the guinea pig ileum and the rat vascular bed, including the increase of vascular permeability. If such a difference in sensitivity means difference in receptors S_1 and S_2 or the presence of difference kininases in relation with the tested structures is still a matter of speculation. To decide these possibilities longer peptides should be tried and we might think of a kinin practically inactive upon the guinea pig ileum and very potent upon the blood pressure (by the venous route) and the vascular permeability.

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