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RELEASE OF KININS BY ACIDIFIED BOVINE PSEUDOGLOBULIN*

J. W. RYAN

Department of Medicine, University of Miami, and the Howard Hughes Medical Institute, Miami, Florida 33136, U.S.A.

and

M. ROCHA E SILVA

Department of Pharmacology, Faculty of Medicine of Ribeirão Prêto, U.S.P., Ribeirão Prêto, São Paulo, Brazil

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Abstract—Acidification of bovine pseudoglobulin followed by neutralization leads to a "spontaneous" release of bradykinin-like activity. The kinin(s) released under these conditions has relatively large effects on arterial blood pressure and small effects on intestinal smooth muscle when compared to bradykinin. However, if pseudoglobulin is heated in a boiling water bath before neutralization, the major kinin released is bradykinin.

THE RELEASE of kinins in plasma or pseudoglobulin acidified to pH 2·0 has been studied in different species of animals and under different experimental conditions. It was shown by Rocha e Silva and Holzhacker[1] that rat plasma acidified to pH 2·0, heated at 90–100° for 3–5 min, cooled and then neutralized released a bradykinin-like activity, due to activation of an enzyme which could be blocked by soya bean trypsin inhibitor. Under such conditions of pH and heating there was a concomitant activation of enzymes capable of hydrolyzing synthetic substrates such as benzoyl-L-arginine ethyl ester (BAEE) or benzoyl-L-arginine methyl ester (BAME).[2] Since it is known that bradykinin is released by enzymes displaying such a specificity,[3] one might assume that heating at pH 2·0 releases bradykinin through the action of such an enzyme. Under similar conditions of pH and heating, Hamberg[4] could isolate from human plasma a polypeptide with the same amino-acid composition as bradykinin. In contrast, Elliott and Lewis[5] isolated the endecapeptide Met-Lys-bradykinin from bovine pseudoglobulin maintained for 3 days in a dialyzing bag against acid solution at pH 2·0, but without heating. They presented evidences of two other compounds with kinin-like activity neither of which appeared to be bradykinin or Lys-bradykinin. Whether those differences are owing to animal species or to the different treatments to which the plasma or the pseudoglobulin was submitted, was not clear.

In the present paper different treatments at pH 2·0 were applied to the same material (bovine pseudoglobulin) with the indication that heating can be critical for a predominant release of bradykinin. On the other hand, the material released by simple acidification, appeared to contain a different kinin, much more active upon the blood

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pressure than bradykinin or even Met-Lys-bradykinin, when compared with its effect upon the guinea pig ileum.

MATERIALS AND METHODS

The material utilized was the pseudoglobulin fraction obtained from fresh bovine plasma by fractional precipitation with ammonium sulfate, redissolved in a small volume of water and then dialyzed for 24 hr against 20 vol. of water. The dialysate was divided into three equal portions labelled A, B and C. Portions A and B were treated with 3 N HCl to give a final pH of 2·0. Sample B then was heated in a boiling water bath (~95°) for 5 min. All three samples were dialyzed against 10 vol. of 0·01 NHCl for 24 hr, and then neutralized with 10 N NaOH to pH 7·6 and incubated for 6 hr at 38°. The reactions were stopped by adding trichloroacetic acid to a final concentration of 5% (w/v), the supernatants collected by centrifugation and then extracted five times with diethyl ether. Each aqueous layer was evaporated to a small volume (~10 ml) and the ether extraction repeated twice more. Aqueous layers were then evaporated to dryness and their residues were dissolved in 10 ml of Tyrode solution.

Samples were assayed on the systemic arterial blood pressure of the rat and on the guinea pig ileum. Synthetic bradykinin (Schwarz Bioresearch, Inc., Orangeburg, N.Y., U.S.A.) was used as the reference standard, and biological activities were expressed as bradykinin-equivalents. Ratios of potency were calculated, the ratio being the activity of a given sample on blood pressure divided by its activity on the guinea pig ileum.

RESULTS AND DISCUSSION

Figure 1 shows the time course of kinin release as assayed on the guinea pig ileum. Under the conditions of these experiments the incubation mixtures were free of kininase activity, making it likely that the apparent rates of kinin release are the true rates.

Ratios of potency of samples A, B and C are shown in Table 1. Met-Lys-bradykinin is included for comparison. These results indicate that samples A and C did not contain bradykinin alone. Indeed, if these samples contained any bradykinin, they

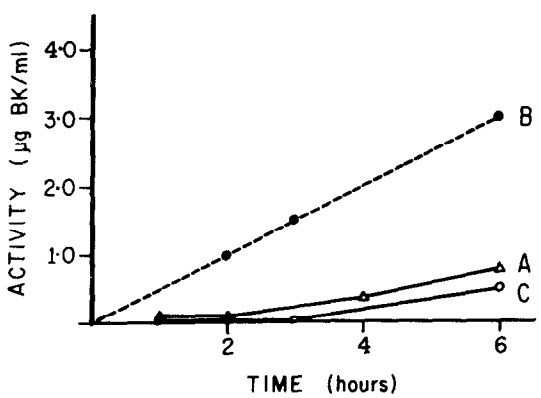

FIG. 1. Time course of "spontaneous" release of kinins by acidified pseudoglobulin. Treatment of samples is described in the text, △——△, sample A; ●- - - -●, sample B; and ○—○, sample C.

also must have contained larger homologs, having a greater effect on arterial blood pressure than on the guinea pig ileum. Using mixtures of synthetic bradykinin and Met-Lys-bradykinin, varying from 1 part of Met-Lys-bradykinin and 2·5 parts of bradykinin to 6 parts of Met-Lys-bradykinin and 1 part of bradykinin, we did not encounter ratios of potency greater than 3. This suggests that bradykinin itself does not make up a significant portion of the kinins in samples A and C. However, we did not rule out the possibility that Met-Lys-bradykinin is the main kinin of those two samples.

TABLE 1. RATIOS OF POTENCY OF KININS RELEASED BY BOVINE PSEUDOGLOBULIN

	Samples (μg of bradykinin-like activity/ml)			
	A	B	C	Met-Lys-bradykinin
Blood pressure, rat	20	15	10	25
Guinea pig ileum	2	10	2	4
Ratio $\frac{\text{B.P.}}{\text{G.p. ileum}}$	10	1·5	5	6·25

Synthetic bradykinin (10 μg/ml) was used as the reference standard. Met-Lys-bradykinin (10 μg/ml) is included for comparison.

The ratio of potency of sample B was close to that expected for bradykinin. We attempted to confirm this identification using separation techniques allowing estimations of molecular size, net charge and solubility. Sample B was mixed with a tracer quantity (0·1 μc) of 2,3-[Pro-^{14}C]-bradykinin (143 μc/μmole, Lot No. 307–087, New England Nuclear Corp., Boston, Mass., U.S.A.). The biological activity of sample B was equivalent to 30 μg of bradykinin, making it 40 times more active than the tracer.

The sample plus tracer was evaporated to dryness, and the residue was resuspended in 1·0 ml of 0·1 M ammonium acetate, pH 6. The solution was chromatographed on Sephadex G-15 (0·9 × 25 cm). Fifty 1·0-ml fractions were collected. Twenty μl of each fraction were dried on 1 × 1 cm pieces of filter paper (Whatman No. 1) and each paper was placed in a vial containing 18 ml of toluene plus Omnifluor® 0·4% w/v (New England Nuclear Corp.) for scintillation counting. The remainder of each fraction was lyophilized. Residues were dissolved in water and were assayed on a guinea pig ileum preparation.

Two peaks of biological activity were found. The first eluted (10–14 ml) just after the void volume (0–10 ml) and corresponded to the elution volume of radioactive bradykinin. The second peak was found in fractions 17–22 and did not correspond or overlap with radioactivity. The second peak was not studied further.

Fractions containing the first peak were combined and lyophilized. The residue was dissolved in 0·1 ml of pyridineacetic acid buffer, pH 5 (0·1 M in respect to acetic acid). The sample was applied to a strip of Whatman No. 1 paper and electrophoresis was carried out at pH 5, at 22 V/cm, 10–20 mA, for 1 hr. Arginine and picric acid were added as reference compounds. Radioactivity of the tracer (R_{arg} 0·68) was located by

strip counting (Actigraph III, Nuclear of Chicago, Inc., Chicago, Ill., U.S.A.). The paper strip was then cut at 1 cm intervals from the origin towards the cathode. Each piece of paper was eluted with 2 ml of 6% acetic acid. Eluates were lyophilized and their residues were dissolved in 1·0 ml of water. Aliquots (0·1 ml) were tested for radioactivity and for effects on a guinea pig ileum. Biological activity corresponded in electrophoretic migration (R_{arg} 0·6–0·7) to that expected of bradykinin. In this electrophoresis system, Lys-bradykinin and Met-Lys-bradykinin migrate faster than bradykinin (R_{arg} 0·8 and 0·75 respectively).

Finally, eluates containing biological activity and radioactivity were combined and lyophilized. The residue was dissolved in 3 ml of trifluoroacetic acid, 1% v/v, previously saturated with butan-1-ol. This solution was mixed with 3 ml of butanol saturated with 1% trifluoroacetic acid. After equilibration the butanol and aqueous layers were dried separately. Their residues were dissolved in water and tested as before for radioactivity and biological activity. Apparent partition coefficients of radioactivity and biological activity were 1·12 and 1·0, values in fair agreement with the partition coefficient (1·2) of authentic bradykinin. For reference, the partition coefficients of Lys-bradykinin and Met-Lys-bradykinin are $<0·5$.

These data are presented as strong evidence that the methods of preparing bovine pseudoglobulin determine the type of kinin formed by endogenous enzymes. Pseudoglobulin acidified but not heated before neutralization releases kinins of considerable activity on arterial blood pressure but of relatively low activity on the guinea pig ileum. The ratios of potency of aliquots taken at different times of incubation do not approach unity, suggesting that bradykinin is not formed in a multi-step procedure such as by conversion of protein substrate to polypeptide substrate to bradykinin.

The simple step of heating acidified pseudoglobulin apparently activates an enzyme capable of catalyzing the release of bradykinin. Identification of bradykinin is based on the findings that the biological activity of sample B cannot be distinguished from 2,3-[Pro-^{14}C]-bradykinin by chromatography on Sephadex G-15, electrophoresis at pH 5 or by partitioning between butan-1-ol and 1% trifluoroacetic acid. The latter two systems allow clear separation of bradykinin from Lys-bradykinin and Met-Lys-bradykinin.

Our results may be of importance to studies of kinins in various inflammatory and shock states. In general kinins are judged to be present or absent in a given biological extract on the basis of the effects of the extract on the guinea pig ileum or the rat uterus. It now appears that the use of either of these preparations as the only screening test carries the risk of missing kinins with relatively large effects on blood pressure and small effects on intestinal or smooth muscle.

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