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G. L. Haberland, J. W. Rohen and **T. Suzuki**

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F. K. SCHATTAUER VERLAG · STUTTGART – NEW YORK

Development of a Radioimmunoassay for Hog Pancreatic Kallikrein and its Application in Pharmacological Studies

E. FINK

The estimation of hog pancreatic Kallikrein is conventionally performed by proteolytic assays using either synthetic substrates or the natural substrate kininogen. However, these assays are not specific for Kallikrein in that they detect other proteases, too. Since we needed an assay suitable for studies in which the pharmacological effects after administration of Kallikrein are to be associated with the concentration of the enzyme in blood and tissues where other proteases might be present we developed a highly sensitive and specific radioimmunoassay for hog pancreatic Kallikrein.

The radioimmunoassay method is based on the competitive inhibition of the binding of labelled antigen to its specific antibody by the unknown or by the unlabelled antigen used as a standard. The sensitivity of the method results from the use of radioisotopes and its specificity from the immunological reaction between the antigen and its antibody.

Materials and methods

The Kallikrein used for radioiodination and as standard in the RIA was a highly purified neuraminidase treated preparation (1). Rabbit anti-Kallikrein serum was kindly provided by Bayer AG.

As second antiserum donkey anti-rabbit IgG serum, Welcome Company, was used.

Na ^{125}I was purchased from Farbwerke Hoechst AG, Frankfurt/Main.

Buffers: buffer A: 0.015M NaH_2PO_4, 0.15M NaCl, 0.02% Merthiolat, 0.2% bovine serum albumin, 0.01M EDTA, pH 7.4. Buffer B: 0.015M NaH_2PO_4, 0.15M NaCl, 0.02% Merthiolat, 4% bovine serum albumin, pH 7.4. Buffer C: 0.015M Na_2HPO_4, 0.15M NaCl, pH 7.4.

Radioimmunoassay conditions

Anti-Kallikrein serum was diluted with buffer B, dilutions of Kallikrein standard samples, unknowns, anti-rabbit IgG serum and ^{125}I-Kallikrein were prepared with

buffer A, the ^{125}I-Kallikrein dilution (250000 cpm/ml) contained 70 µg/ml rabbit IgG. The incubation mixtures were set up according to the following Table 1:

Table 1. Incubation mixtures.

	Buffer A serum µl	Buffer B serum µl	Sample serum µl	Anti-Kallikrein serum µl	^{125}I-Kallikrein serum µl
N	200	100	–	–	100
Bo	200	–	–	100	100
Samples	100	–	100	100	100

After an incubation period of 24–48 h 100 µl of the dilution of the second antiserum were added followed by 0.5 ml buffer A after 30 min. After 10 min centrifugation at 6000 g the supernatant was removed by aspiration and the precipitates were counted in a well type gamma counter.

Preparation of ^{125}I-Kallikrein

For the labelling of Kallikrein with Na ^{125}I a modification (2) of the chloramine-T method of GREENWOOD and HUNTER (3) was employed. The labelled Kallikrein was separated from free iodine by gel filtration of the reaction mixture through a Sephadex G-75 column (1.3 × 15 cm) equilibrated with buffer C. Fractions of 0.8 ml were collected. The 5 fractions of the Kallikrein peak with the highest radioactivity were combined and diluted with buffer A to a concentration of 250000 cpm/ml. A specific activity between 40 and 130 µCi/µg was achieved. The preparations were used in the RIA for up to 4 weeks.

Results and discussion

Radioimmunoassay

Fig. 1 shows the binding of ^{125}I-Kallikrein (28000 cpm) to antibody after incubation at 21°C for 24 hours using increasing dilutions of the antiserum. More than 80% of the ^{125}I-Kallikrein were bound indicating the high quality of the antiserum.

In order to find the optimal incubation conditions the kinetics of the binding of ^{125}I-Kallikrein to the antibody was studied at 8, 22 and 37°C. The results are summarized in Fig. 2. The lowest reaction rate is observed at 8°C. At 37°C the initial binding rate is insignificantly higher than at 22°C and slows down after 8 h. At

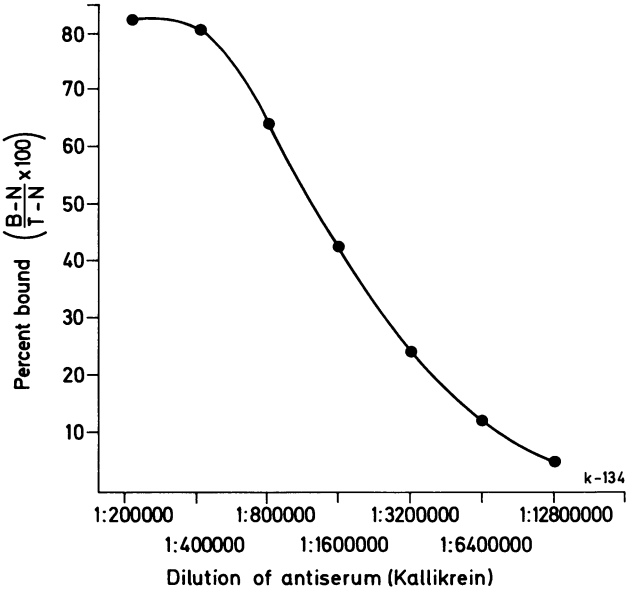

Fig. 1. Antiserum titration curve for ^{125}I-Kallikrein (hog pancreas). For incubation conditions see text.

equilibrium, which is reached after different incubation periods for the different temperatures, the amount of bound radioactivity is highest at 22°C. Incubation periods longer than 72h result in a significant decrease of binding. We interpret this phenomenon by loss of immunologic reactivity of the ^{125}I-Kallikrein and/or the antibody. Based on these studies incubation at 22°C for 24–48h was chosen for the routine RIA.

For the separation of Kallikrein-antibody complex from free Kallikrein by the second antibody method similar studies showed that maximum precipitation was achieved already after 15-min incubation at 22°C.

The evaluation of the RIA was done either manually using logit-log graph paper (4; Fig. 3 shows a typical standard curve) or by means of a computer employing the spline approximation method (5). The lowest level of detection for hog pancreatic Kallikrein in the RIA was between 40 and 300 pg per 100 µl sample volume, depending on the quality of the labelled Kallikrein and the incubation conditions. The coefficient of variation for triplicates was usually between 6 and 14%.

Measurement of Kallikrein in serum

Since in the pharmacological studies it would be necessary to measure the Kallikrein concentration in the serum of dogs we investigated the influence of dog serum

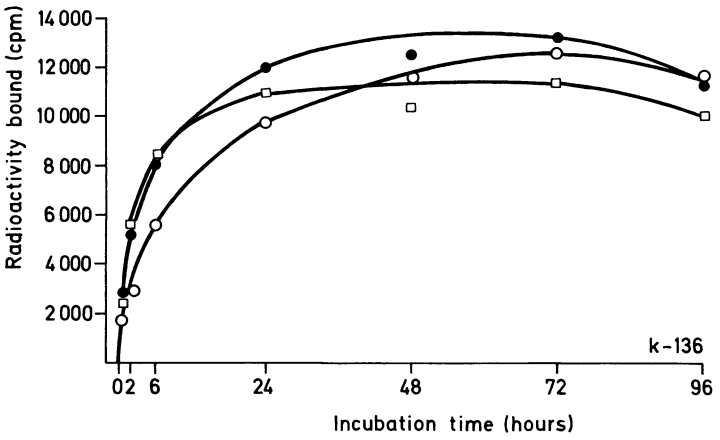

Fig. 2. Time and temperature dependence of the binding of ^{125}I-Kallikrein to antibody. ○—○ 8°C, ●—● 22°C, □—□ 37°C. For incubation conditions see text.

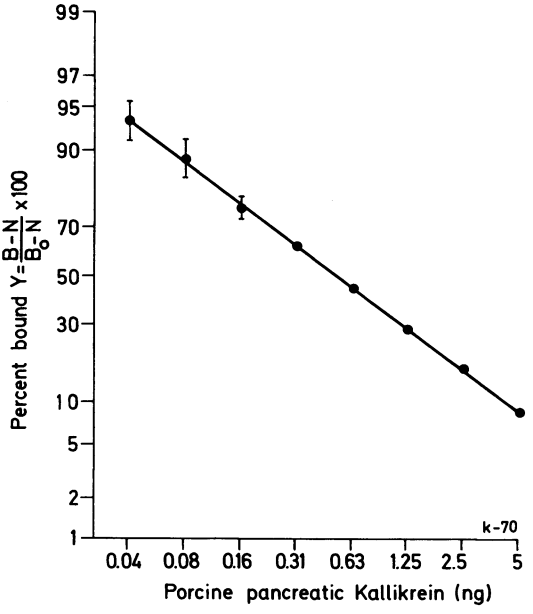

Fig. 3. Standard curve for the radioimmunoassay of hog pancreatic Kallikrein. For incubation conditions see text. The data are presented in the form of the logit-log plot (4).

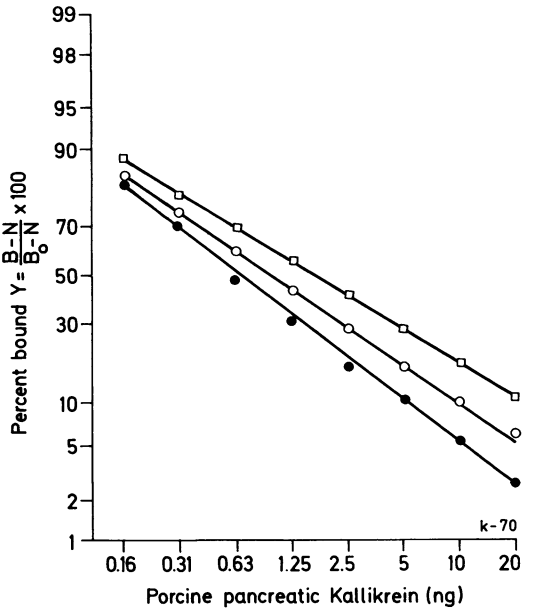

Fig. 4. Standard curve for the radioimmunoassay of hog pancreatic Kallikrein in the presence of dog serum. ●—● without serum, ○—○ and □—□ in the presence of 20 µl and 100 µl serum. For incubation conditions see text.

on the RIA. Fig. 4 demonstrates that the presence of serum has indeed an influence, the slope of the standard curve depends on the amount of serum added to the incubation mixture. Therefore, if serum samples are to be assayed, the standard curve has to be set up under addition of the same volume of serum to the standard incubation mixtures as applied in the unknowns. The influence of serum on the immunoreactivity of Kallikrein in the RIA may be caused by complex formation with serum inhibitors and/or by unspecific interactions of serum components.

The formation of a complex of Kallikrein with (a) serum component(s) was demonstrated by gel filtration experiments. A mixture of Kallikrein and dog serum was applied to a Sephacryl S-200 column and the eluate was tested by RIA. Two peaks were detected, one in the normal elution position of Kallikrein, the other approximately in the position of the albumin peak. This second peak might represent the complex of Kallikrein with α_1-antitrypsin.

Some proteases were checked for cross-reactivity by the RIA; hog-trypsin and bovine trypsin and chymotrypsin showed no cross-reactivity at all whereas hog submandibular and urinary Kallikreins had immunoreactivities comparable to the pancreatic Kallikrein. We have not yet investigated Kallikreins of other species.

Pharmacological studies

The RIA for hog pancreatic Kallikrein offers the possibility to study the long known pharmacological effects of Kallikrein in relation to the Kallikrein concentration in the blood. As already described by FREY and KRAUT (6) and FREY, WERLE and SACKERS (7) intravenous injection of Kallikrein causes an immediate drop of the mean blood-pressure which is due to the release of kinin from kininogen. If Kallikrein is applied intramuscularly the response of the blood-pressure is delayed and is less dramatical.

Fig. 5 summarizes the changes of blood pressure and kininogen concentration in plasma in relation to the Kallikrein concentration in serum after intravenous injec-

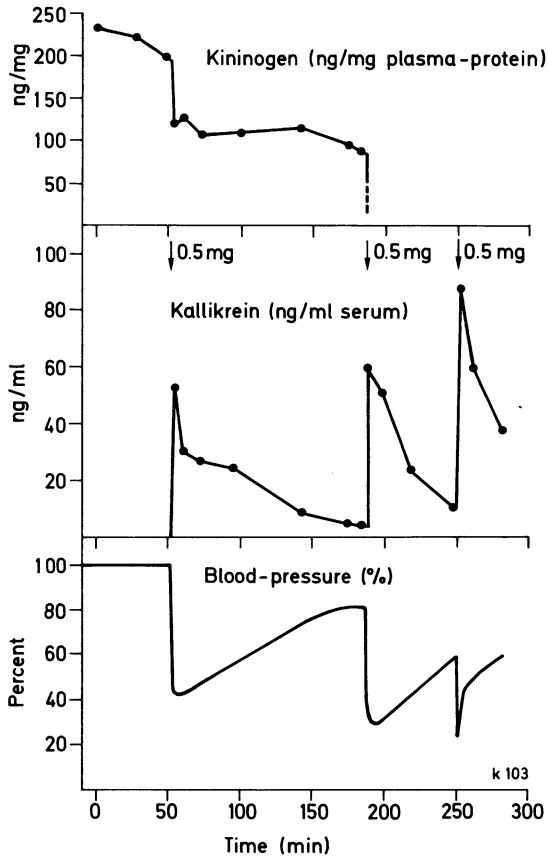

Fig. 5. Changes of mean blood-pressure and plasma kininogen concentration in relation to the Kallikrein concentration after intravenous injection. For details see text.

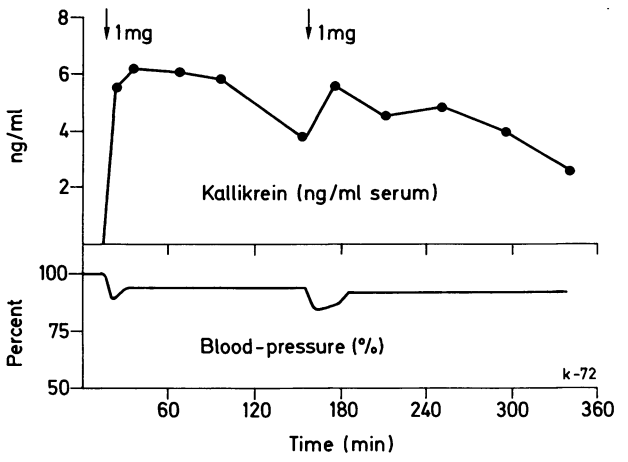

Fig. 6. Response of the mean blood-pressure in relation to the Kallikrein concentration after intramuscular injection. For details see text.

tion. After injection of 0.5mg Kallikrein into the left femoral vein of a dog (15kg body weight, pentobarbital anesthesia) samples of blood were taken from the right femoral vein. The highest concentration of Kallikrein was found in the sample obtained within 1 min after injection. The concentration dropped rapidly within 6 min from 530 ng/ml to 300 ng/ml. After this initial phase the half-life of Kallikrein was approximately 1 hour. After repeated injections of Kallikrein basically identical elimination kinetics were observed. The kininogen concentration on the upper pannel is expressed as ng bradykinin equivalents releasable per mg plasma protein by incubation of plasma samples with trypsin. It was measured in the bioassay with the isolated rat uterus (8) using synthetic bradykinin as reference. Immediately after the Kallikrein injection the kininogen concentration dropped from 600 ng bradykinin equivalents per mg plasma protein to 350 ng. This level was relatively constant for 2h in spite of the presence of relatively high concentrations of immunologically active Kallikrein, an indication that the Kallikrein circulating in the blood must be present mainly as an enzymatically inactive form. A second injection of Kallikrein caused a drop of the kininogen concentration below the level of detection. The response of the mean blood-pressure, recorded from the carotid artery, indicates that at the time of the third injection there was still some kininogen present in the blood.

After intramuscular injection of Kallikrein the maximum of the Kallikrein concentration in the blood is much lower and the time period necessary to reach it is longer than after intravenous injection (Fig. 6). The rate of the decrease of the Kallikrein concentration in the blood is comparable to the slow phase of elimination after intravenous injection. The blood-pressure reached a minimum within 5 min after

injection when the maximum of the Kallikrein concentration in the blood was not yet reached. After the initial phase the blood-pressure remained constant at a level somewhat lower than before injection.

Concluding from the results presented here the radioimmunoassay for porcine pancreatic Kallikrein is a highly suitable tool for the study of the pharmacological effects of this enzyme. It is expected that it will be possible to get new insights into the action and fate of pancreatic Kallikrein in the organism by using this tool.

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