Biochemical Pharmacology, Vol. 25, pp. 201–203. Pergamon Press, 1976. Printed in Great Britain.

KALLIKREIN-INACTIVATING SUBSTANCE IN HUMAN PLASMA

OLGA B. HENRIQUES, CIRO REYES and RAMON M. ROMERO
Centro Nacional de Investigaciones Cientificas, Havana, Cuba

(*Received* 25 *September* 1974; *accepted* 7 *April* 1975)

Abstract—Some properties of a plasma kallikrein inactivator have been studied. It was shown that this factor is much more active on glass activated plasma kallikrein than on salivary kallikrein and is activated by Cl^- ions. A method for the determination of plasma kallikrein inactivator is described.

Previously [1, 2] we described the disappearance of kallikrein from horse and human plasma, under conditions where activated kallikrein was kept in contact with the plasma from which it had been adsorbed. In human plasma we found that loss of activity is very rapid, a fall of 30 and 50% being observed comparing the levels of kallikrein obtained after 30 sec of contact with glass beads (considering this activity as 100%) with those obtained after 1 and 2 min contact, respectively. This suggested that the inactivation might be due to a kallikrein-inactivating substance described by Werle and Schmal [3] in the serum of rat and which they thought might be a degrading enzyme. These authors also observed its presence in human and horse plasma.

Due to the importance of this inactivator which hinders so much the first step in the preparation of plasma kallikrein activated by glass we decided to study some of its properties.

MATERIAL AND METHODS

Synthetic bradykinin was kindly supplied by Sandoz Products Ltd., London. Loss of bradykinin activity in the diluted solutions was prevented by the addition of oxalic acid in the final concentration of 10^{-3}M, a fresh solution being prepared daily.

Human plasma, for the assays and purification of kallikrein inactivator, was freshly collected and contained 96 ml of a citric acid–citrate–dextrose mixture for each 440 ml of blood. One hundred ml of this solution contained 0·80 g citric acid, 2·2 g sodium citrate and 2·2 g dextrose in bidistilled water. The blood was collected in the Provincial Bank, the whole equipment used for the collection of blood and separation of plasma being silicone-treated.

Heat-treated plasma (HP) was prepared as described previously [2]. Before use this crude substrate was always checked to make sure it contained enough substrate in the volume used for the measurement of kallikrein activity.

The activity of the kallikrein, after incubation with heat-treated human plasma, was measured using isolated guinea-pig ileum suspended in Tyrode solution which contained diphenhydramine and atropine. Its activity was expressed in μg equivalents of bradykinin released by 1 mg of enzyme protein [2].

Protein was determined by the biuret reaction or according to Lowry *et al.*[4].

Glass activated plasma kallikrein (GK), was prepared as described previously [2], by adsorption on and elution from glass beads. For adsorption the plasma was shaken for 30 sec with the beads.

Salivary kallikrein was prepared by centrifugation of freshly collected saliva for 20 min at 2500 rev/min.

Kallikrein inactivator was determined by the amount of kallikrein inactivated by a certain amount of plasma, GK-plasma supernatant or crude kallikrein inactivator preparations, as described in Results.

RESULTS

Preliminary experiments were done to detect the presence of the kallikrein inactivator in plasmas from which kallikrein had been removed by a 30-sec adsorption on a glass surface. These supernatants do not contain either detectable amounts of kininogen I, which is rapidly hydrolysed after the activation of kallikrein, or the released kinins which are consumed by the action of the kininase present in the plasma. The kallikrein-inactivator activity of these supernatants was determined by following the loss of kallikrein activity after exposure of a GK preparation to the supernatants. For this purpose supernatants of GK (plasma) or fractions obtained from them after chromatography on DEAE–cellulose were incubated with GK, at 37°, in the presence of EDTA in the concentration recommended by Henriques [5]. At different periods of time, aliquots of the incubation mixture were transferred to the ileum bath where their GK activity was measured by the amount of kinin released from HP.

The kallikrein inactivator in untreated fresh plasma was more difficult to determine as the plasma had to be pretreated in order to free it from kininogen I. This was done by preincubation with GK. The kinin released from kininogen I was hydrolysed by the kininase present in the plasma, while the kallikrein inactivator contained in the latter inactivated the GK which had been added to comsume the kininogen I. The presence of the kallikrein inactivator in the incubates could then be measured by further addition of known amounts of GK (Fig. 1).

As we found that the kallikrein inactivating substance present in fresh plasma is in an active state and its activity is not changed after treatment of plasma with glass beads for 30 sec, to activate and remove kallikrein [2], this supernatant, rather than fresh plasma, was used for the studies and separation of kallikrein inactivator.

Effect of dialysis on the activity of kallikrein-inactivating substance in plasma. Fresh GK supernatant

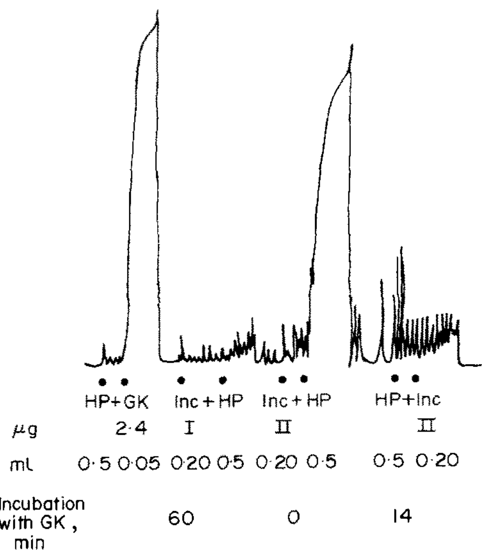
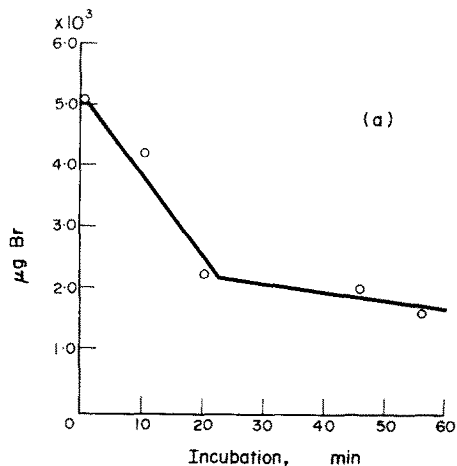


Fig. 1. Kallikrein inactivating substance in fresh plasma. Incubation I. 2 ml of fresh plasma and 1 ml GK (sp. act. 10) were incubated for 60 min at 37°. One ml of 6 × 10^{-3} M EDTA was then added. 0·2 ml of incubate contained 2·4 μg GK (glass activated kallikrein) at 0 min. Incubation II. To one ml of incubate was added more GK. 0·20 ml of this incubate contained 2·4 μg of the newly added GK at 0 min, therefore 'time of incubation' under incubation II refers to the second addition of GK. HP: heat-treated plasma.

plasma was dialysed against 5 × 10^{-3} M sodium phosphate, pH 7·4, and its activity compared with that of the non-dialysed supernatant. Figure 2 shows the loss of activity of the kallikrein inactivator after dialysis and the reversibility of its inactivation by the addition of NaCl to the dialysed sample, in a final concentration of 1% in the incubate.

Separation of kallikrein inactivator by chromatography on DEAE–cellulose. Fresh GK-supernatant plasma was dialysed against 5 × 10^{-3} M phosphate buffer, pH 7·4, at 4°. Ten ml of the dialysed solution was chromatographed on a microgranular DEAE-cellulose column (6 × 2 cm) equilibrated with the same buffer. The flow rate was 10 ml per hr. Fractions of 3 ml were collected. After washing the column with 50 ml of the 5 × 10^{-3} M buffer, a linear gradient was



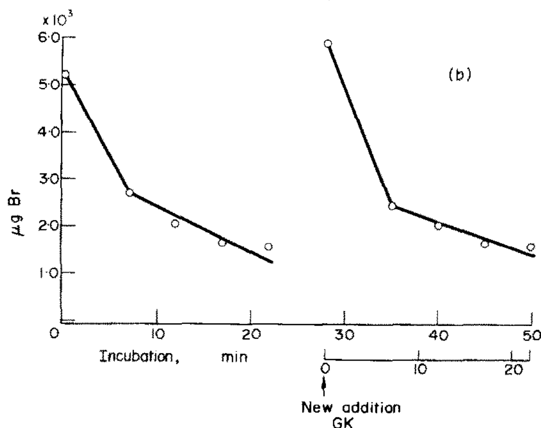


Fig. 3. (a) Time curve inactivation of glass-activated kallikrein (GK). (b) Time curve inactivation of GK after two subsequent additions of GK. The activity is expressed in μg bradykinin released from kininogen in heated plasma (HP).

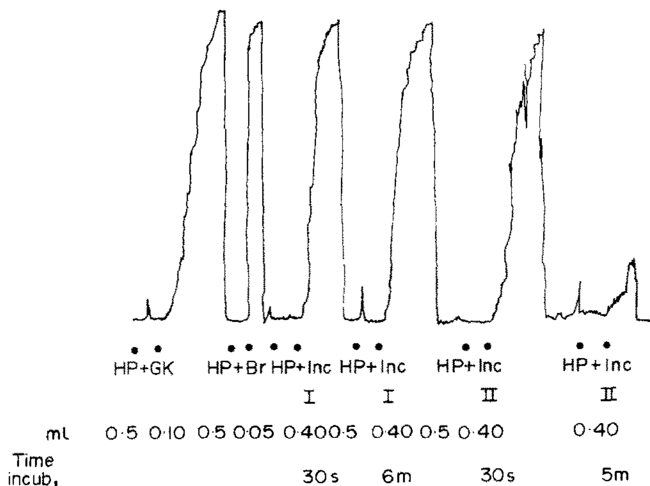


Fig. 2. Effect of dialysis on the plasma kallikrein inactivator. Incubation I. 0·5 ml GK-plasma supernatant dialysed against 5 × 10^{-3} M sodium phosphate buffer, pH 7·4 + 0·5 ml water. Incubation II. 0·5 ml of the same dialysed plasma supernatant + 0·5 ml 4% NaCl. 0·5 ml 6 × 10^{-3} M EDTA and 0·5 ml GK were added to both incubates at zero time. 0·4 ml of the incubates contained 0·1 ml of the GK preparation. Br: bradykinin.

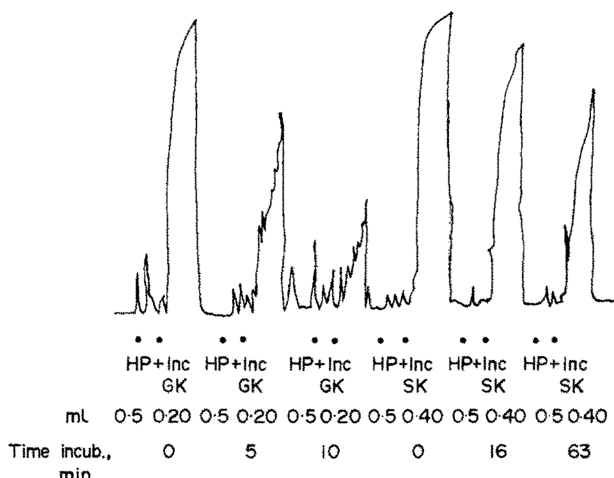


Fig. 4. Inactivation of salivary kallikrein by plasma kallikrein inactivator. SK: salivary kallikrein. Incubation conditions and abbreviations as in previous figures.

established with 50 ml of the same buffer to which 50 ml of 0·3 M NaCl in the initial buffer were added in a LKB gradient mixer. The activity of the kallikrein inactivator started to be eluted when the NaCl concentration reached 0·09 M and the activity appeared in five fractions (15 ml). The most active fraction was found to be purified 8 times and 570 μg of this fraction inactivated 7·2 μg of a GK preparation with a specific activity of 10, within 5 min.

Time curve inactivation of GK. A partially purified preparation of plasma kallikrein inactivator was used in a preliminary experiment designed to study the kinetics of the inactivator. Using a dilute solution it was found that the relation between the time of incubation and the amount of kallikrein which remained active seemed to be linear for up to fifty minutes, but the slope seemed to become far less steep after the incubation time of twenty minutes (Fig. 3a). This decrease in inactivation rate seemed to be due to a decrease in substrate concentration (kallikrein) rather than to loss of activity of the kallikrein inactivator since, at a second addition of kallikrein (Fig. 3b), the same initial velocity and the same pattern of time-kallikrein inactivation was observed. Therefore the change of inactivation rate with time could be due to a change in the order of the kallikrein inactivator reaction due to a decrease in substrate concentration.

It should also be added that kallikrein can be totally and rapidly inactivated if enough kallikrein inactivator is employed; in these experiments no kallikrein activity could be detected even when an ileum preparation sensitive to $5 \times 10^{-3} \mu$g bradykinin was utilized to assay the remaining kallikrein activity.

Inactivation of salivary kallikrein by plasma kallikrein inactivator. One of the active fractions from the column described above was incubated with salivary kallikrein in a parallel experiment in which the same fraction was incubated with glass activated plasma kallikrein. In Fig. 4 it can be seen that half the amount of kallikrein inactivator necessary to inhibit 20% of the salivary kallikrein in 16 min or 50% in 63 min, was able to inhibit 80% of the GK in 10 min.

DISCUSSION

In 1958 Margolis [5] showed that kallikrein is very rapidly inactivated in plasma and presented data indicating the presence of an inactivator of kallikrein in human, dog and rabbit plasma. In 1968 Werle and Schmal [3] described a kallikrein degrading enzyme in rat serum. Our attention to this subject has been called by the finding that the longer the period of shaking of plasma with glass beads for kallikrein activation and adsorption [2], the smaller the specific activity of the kallikrein eluted from the beads. It decreased 10–15 times from respectively 30 sec to 10 min of contact with shaking.

In this preliminary report we present data indicating that the kallikrein inactivator from human plasma may be similar to the kallikrein-degrading enzyme in rat serum described by Werle and Schmal [3]. It is difficult to compare our results with theirs as we determined the kallikrein remaining after incubation with kallikrein inactivator using guinea-pig ileum while they used the blood pressure of anesthetized dogs. Werle and Schmal found that rat kallikrein activator was not affected by dialysis but we found that human plasma inactivator was activated by Cl^- ions, since it lost activity by dialysis but was fully reactivated by the addition of NaCl.

We also found that the inactivation of salivary kallikrein by human plasma kallikrein inactivator was not inhibited by soybean trypsin inhibitor while Werle and Schmal [3] observed the inhibition of rat kallikrein-degrading enzyme by this trypsin inhibitor.

REFERENCES

1. O. B. Henriques, V. Stolpnik, V. Kusnetsova and M. Astrakan, *Biochem. Pharmac.* **19**, 2915 (1970).
2. O. B. Henriques and L. Allan, *Biochem. Pharmac.* **21**, 3163 (1972).
3. E. Werle and A. Schmal, *Z. Physiol. Chem.* **349**, 521 (1968).
4. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
5. O. B. Henriques, *Biochem. Pharmac.* **20**, 2759 (1971).
6. J. Margolis, *J. Physiol., Lond.* **144**, 1 (1958).