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THE PRESENCE OF A SMOOTH MUSCLE CONTRACTING AND BRADYKININ POTENTIATING FACTOR IN PLASMA

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Abstract—Blood free bradykinin determination by bioassay on smooth muscle *in vitro* was attempted. Neither ion exchange chromatography nor butanol extraction at low pH, currently in use for the purification of the blood extracts, resulted in a solution free from bioassay interfering factors. A smooth muscle contracting and bradykinin potentiating factor was invariably present in all human blood and plasma extracts. It differed from bradykinin by showing a shorter delay before contraction, by showing a steeper slope of the dose–response line, and by being resistant to the action of chymotrypsin and total acid hydrolysis. It could be separated from synthetic bradykinin by Sephadex G-25 gel filtration. The factor thus seems not to be a peptide and is of small molecular size. Its more precise identity is so far unknown.

THE RESULT of the activation of the kallikrein–kininogen–kinin system in physiological and pathological conditions is the liberation of free kinins. The demonstration of this is extremely difficult because of the minute quantities to be detected and measured, and because of the short half-life of the kinins *in vivo*. Furthermore, the liberation of additional kinin from kininogens and the destruction of free kinins during the analytical procedure are great potential sources of error.

The current methods for blood free kinin determination include rapid denaturation of the blood proteins (including kallikreins, kininogens and kininases), purification of the extracts to eliminate active substances interfering with the bioassay, concentration of the extracts, and finally the bioassay itself. Smooth muscle *in vitro* is the most sensitive preparation so far described.[1] Methods used for the purification of the crude blood extracts fall principally into two categories: the use of ion exchange chromatography,[2–5] and the extraction of the kinins with butanol at low pH.[6–8] These methods were originally applied to the preparative purification of plasma bradykinin (see Hamberg.[9])

In attempts to find a clinically applicable method for blood free kinin determination, we found that neither of the above procedures yielded an extract from which bradykinin bioassay on smooth muscle was possible. In all cases a smooth muscle contracting and bradykinin potentiating plasma factor, not previously described in this context, was present.

METHODS

After several attempts to adopt the different previously published methods for free kinin bioassay, the following principles were applied.

1. *Preparation of a crude alcoholic extract.*[10] Ten ml of blood was drawn through a siliconed needle into a plastic syringe. In some experiments the syringe contained

1 ml of a solution of 1 % EDTA as a kininase inhibitor[7] and 0·1 per cent soy bean trypsin inhibitor (Sigma) as a plasma kallikrein inhibitor.[3] The blood was rapidly injected into 30 ml of ice-cold ethanol. After centrifugation the supernatant was taken to dryness under reduced pressure at 37° (Rotavapor, Büchi) and stored at −20°. For bioassay it was dissolved in 2–5 ml of distilled water.

2. *Preparation of an extract purified by ion exchange chromatography.* The method was adapted from the methods of Oates *et al.*[2] and Melmon *et al.*[5] Ten ml of blood was drawn as described above, with or without the mentioned inhibitors, and rapidly injected into 20 ml of ice-cold 0·5 M perchloric acid. After centrifugation 10 ml of distilled water was added to the supernatant, and the pH of the mixture was adjusted to 5·0 with KOH. The solution was then applied to a 2 ml column (0·9 × 3·0 cm in a plastic syringe) of ion exchange resin (Amberlite IRC-50, H^+, British Drug Houses), which retains bradykinin below pH 6. The resin had been equilibrated in 0·05 M NH_4–acetate buffer, pH 5·0. Elution from the column was performed by 10 ml of 1 N NH_4OH-solution and subsequently with 20 ml of 0·1 N NH_4OH-solution. The recoveries of 0·2–1·0 μg of synthetic bradykinin (Bradykinin triacetate, Sandoz) from a 0·5 per cent gelatine solution submitted to similar ion exchange chromatography, have consistently been 85–93 per cent. (We have found gelatine to be the best stabilizer for preserving bradykinin activity, which is known to be labile in dilute purified solutions.) The eluate was collected in siliconed, 100 ml, round bottomed flasks containing 2 ml of 2 per cent gelatine solution, taken to dryness under reduced pressure at 37°, and the dry residue stored at −20° until bioassay. For the bioassay the residue was dissolved in 2–5 ml of distilled water.

3. *Preparation of a butanol extract.* The butanol extraction was performed according to Abe *et al.*[7] Ten ml of blood was drawn as described above and rapidly injected into 5 ml of a 0·8 N HCl-solution, mixed, saturated with solid NaCl and mixed vigorously. The mixture was extracted twice with 20 ml of *n*-butanol (Baker, Analytical grade), and the kinin re-extracted to an aqueous phase after addition of petroleum spirit (May & Baker, Analytical grade). This method yields an aqueous solution with high ionic concentration. In our hands the dehydration of the butanol extract with anhydrous sodium sulphate[7] did not lower the ionic concentration of the final extract enough to be non-toxic for the smooth muscle preparation. To get rid of the salt the ion exchange chromatography described above was applied, because it gives a practically salt free eluate with good recovery of bradykinin. In addition a further purification of the extract is thus achieved.

We have also applied butanol extraction to perchloric acid extracts of whole blood or plasma. Before butanol extraction, the pH was adjusted to 2·0 by NaOH.

4. *Preparation of plasma samples.* Ten ml of blood was drawn through a siliconed needle into a plastic syringe containing EDTA and soy bean trypsin inhibitor (see point 1). The blood was transferred to a 12 ml precooled plastic tube and centrifuged without delay in a refrigerated centrifuge. The plasma was treated according to the different methods described under points 1–3. About 10 min elapsed between the blood letting and the denaturation of the plasma sample.

5. *Sephadex G-25 gel filtration.* In order to separate the found uterus contracting and bradykinin potentiating factor from bradykinin we used gel filtration chromatography (Sephadex G-25, superfine grade, 0·9 × 53 cm column). The eluate after ion exchange chromatography (see point 2) was taken to dryness, dissolved into 1–2 ml

of distilled water and applied to the Sephadex column. Elution was performed with distilled water.

6. *Bioassay*. The majority of the bioassays were performed by using a rat uterus in oestrus. The uterus horn was suspended in a 5 ml organ bath of de Jalon's solution at 33° and bubbled by a gas mixture of 95% O_2 + 5% CO_2. The bath solution contained 10^{-6} g/ml of atropine. Methysergide (Sandoz) in a final concentration of 10^{-6} g/ml in the bath was used as an anti-serotonin agent. It was applied only once before the first contraction, as this renders the uterus permanently resistant to the action of serotonin. The uteri were sensitized with five subsequent doses of 250 μg α-chymotrypsin (Sigma) before starting the bioassay.[11] The contractions elicited by different doses of synthetic bradykinin solution and of the blood or plasma extracts were recorded by a frontal writing lever on a cymograph.

In some experiments guinea-pig ileum was used in Tyrode-solution with atropine and methysergide as described above, and an antihistamine, promethazine (Phenergan, May & Baker) 10^{-7} g/ml.

The magnitude of the contractions were graphically plotted against the logarithm of the dose.

RESULTS

Figures 1 and 2 show the behaviour of the crude alcoholic extract of blood in bioassay on rat uterus. It can be seen that the extract contains a uterus contracting factor, which differs from synthetic bradykinin by showing a shorter delay time before contraction, and a steeper dose–response line. Further, the activity cannot be destroyed by incubation with chymotrypsin (500 μg/ml) for 0·5 hr at room temperature, while synthetic bradykinin added to the extract is completely destroyed in a couple of minutes under similar conditions.

The perchloric acid extract of whole blood, purified by ion exchange chromatography, behaves quite similarly, i.e. it contracts the uterus with a shorter delay time, and usually yields a dose response line with a steeper slope than does synthetic

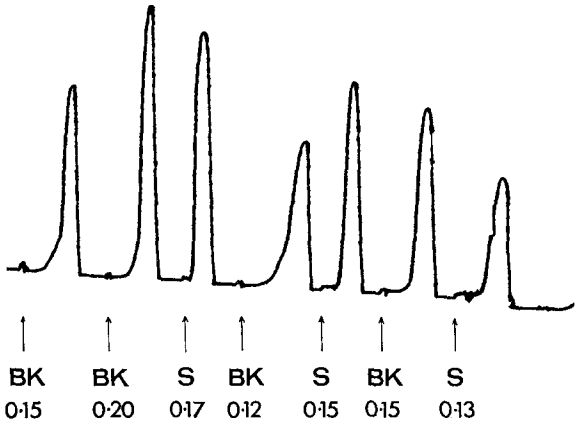

FIG. 1. The contractions of rat uterus *in vitro* elicited by different doses (ml) of synthetic bradykinin solution (BK) 10^{-8} g/ml and by crude alcoholic extract of whole blood(s). The total volume of the extract was 2 ml prepared of 10 ml of blood. Note the shorter delay before contraction obtained by the blood extract.

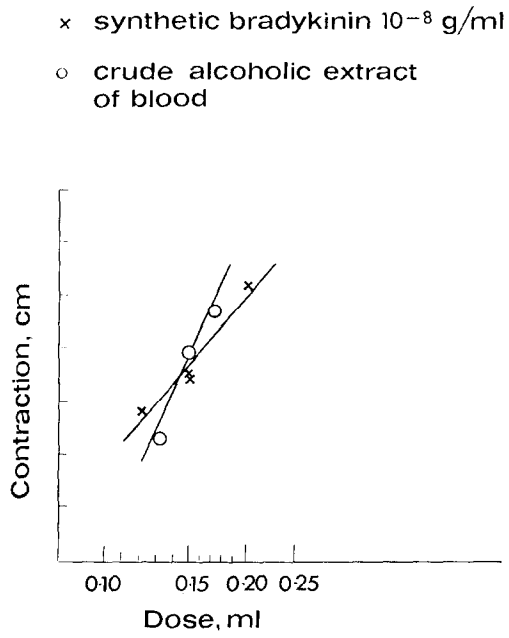

FIG. 2. The contractions shown in Fig. 1 plotted against the dose on a logarithmic scale. Note the steeper dose–response line obtained with the blood extract.

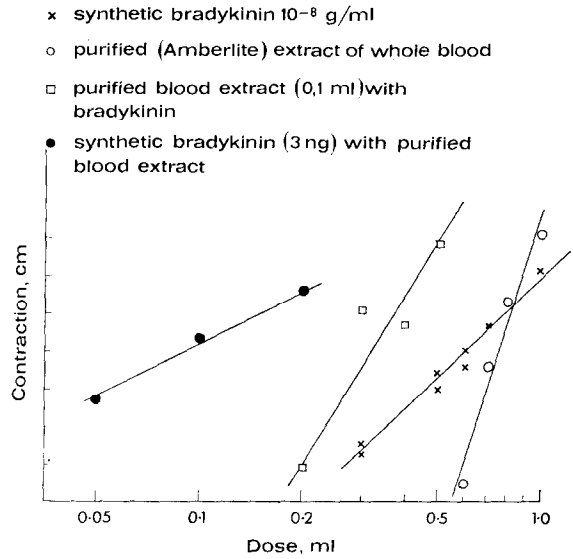

FIG. 3. Dose–response lines obtained in bioassay (rat uterus contraction *in vitro*) of synthetic bradykinin solution 10^{-8} g/ml (×) and of perchloric acid extract of whole blood purified by ion exchange chromatography with Amberlite IRC-50 resin (○). Final total volume of the extract was 5 ml prepared from 10 ml of blood. Note the steeper slope of the blood extract line. When a fixed subcontractile dose (0·1 ml) of the purified blood extract is applied together with different doses of synthetic bradykinin solution (□) a marked potentiation and a steeper dose–response line is obtained. When a fixed dose of bradykinin (0·3 ml = 3 ng) is applied with different doses of the blood extract (●) the potentiation is seen to increase linearily with the logarithm of the dose.

bradykinin. When subcontractile doses of this extract are added together with synthetic bradykinin solution, the result is a remarkable potentiation of the bradykinin activity and a steeper dose–response line. The potentiation is log-linearily dependent on the dose of the extract. These features appear in Fig. 3, which shows the results obtained with a whole blood extract. That the uterus contracting and bradykinin potentiating activity is derived from plasma is shown by the fact that the same results are obtained by a plasma extract. Figure 4 shows the uterus contracting activity of a plasma extract and the effect of chymotrypsin on it. It can be seen that only a small

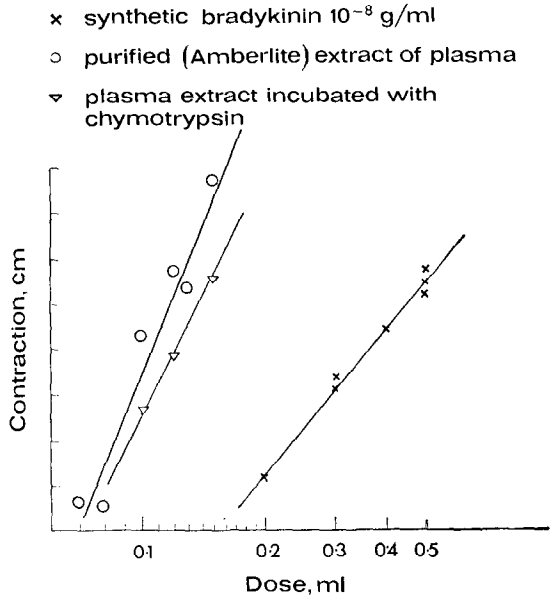

FIG. 4. Dose–response lines obtained in bioassay (rat uterus contraction *in vitro*) of synthetic bradykinin solution 10^{-8} g/ml (×) and of perchloric acid extract of plasma purified by ion exchange chromatography with Amberlite IRC-50 resin (○). Final total volume of the extract was 5 ml prepared from 5 ml of plasma. The result is the same as that obtained by whole blood extracts (Fig. 3), i.e. a steeper dose–response line of the extract as compared to synthetic bradykinin. After chymotrypsin incubation (30 min at room temperature with 500 μg/ml of chymotrypsin) (▽) only a small part of the uterus contracting activity is destroyed, indicating that the major factor in the uterus contracting activity is not bradykinin. The uterus preparation was sensitized with chymotrypsin before starting the assay.

part of the activity could be destroyed by chymotrypsin incubation. The effect of chymotrypsin on different blood or plasma extracts has been somewhat variable. In some cases no effect at all has been seen, and in others a reduction up to 50 per cent of the uterus contracting activity has been demonstrated. (The chymotrypsin sensitive part of the uterus contracting activity might be bradykinin.)

In addition, the following characteristics of this uterus contracting and bradykinin potentiating factor have been observed; it is dialysable through 28 Å pores of a dialysis tube (Union Carbide); it cannot be destroyed by chymotrypsin or trypsin, it is thermostable and even sustains total acid hydrolysis (12 hr at 110° with 6 N HCl). Its *in vitro* effects on guinea-pig ileum do not differ from those shown on rat uterus.

The factor is present whether or not EDTA and/or soy bean trypsin inhibitor are used when taking blood samples. One cannot get rid of the interfering factor by butanol extraction, either, as shown in Fig. 5.

Gel filtration chromatography with Sephadex G-25 resulted in one single uterus contracting faction, which possessed all the characteristics of the previously obtained purified blood extracts. It showed a shorter delay time before contraction than synthetic bradykinin, it potentiated the action of synthetic bradykinin on the uterus, and it was chymotrypsin resistant. When synthetic bradykinin was added before gel

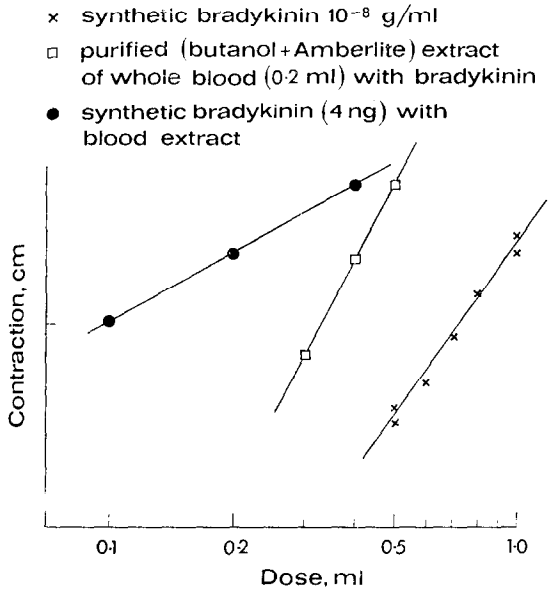

FIG. 5. Dose–response lines obtained in bioassay (rat uterus contraction *in vitro*) of synthetic bradykinin solution 10^{-8} g/ml (×) and of synthetic bradykinin with simultaneously added blood extract (0·2 ml) purified by both butanol extraction and ion exchange chromatography (□). Total volume of the final extract was 2 ml prepared from 10 ml of blood. Note again the marked potentiation with steepening of the slope. This blood extract did not itself contract uterus in doses up to 0·8 ml. When a fixed dose of bradykinin (0·4 ml = 4 ng) was applied with different doses of the blood extract (●) the result is the same as shown in Fig. 3, i.e. a linear increase of the potentiation with the logarithm of the dose.

filtration, there appeared two uterus contracting fractions (Fig. 6), the first of which showed typical bradykinin behaviour in the bioassay, and was chymotrypsin sensitive, thus differing from the second fraction, which again showed all the characteristics described above. It thus could be demonstrated that the uterus contracting and bradykinin potentiating factor interfering with bradykinin bioassay can be separated from synthetic bradykinin by using Sephadex gel filtration. No spontaneous free bradykinin activity could be shown in the blood samples submitted both to ion exchange and gel filtration chromatography.

DISCUSSION

Three principles have been applied for free bradykinin determination in blood and other biological fluids. They include bioassay on smooth muscle *in vitro*, bioassay on

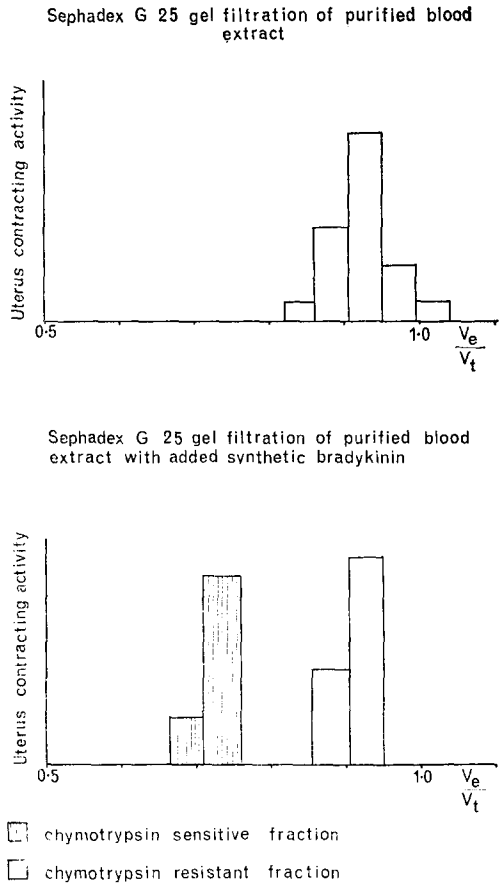

FIG. 6. The presence of uterus contracting activities in the water eluate from a Sephadex G-25 column. The upper figure shows the activity in a blood extract and the lower figure the activity of a similar extract with added synthetic bradykinin.

smooth muscle or on vascular effects *in vivo*, and, more recently, radioimmunoassay (see Trautschold[1]). Bioassay on rat uterus *in vitro* has so far been the most sensitive and most widely used method. Its principal drawback is the lack of specificity. Numerous known substances are able to contract rat uterus. They include acetylcholine, serotonin, prostaglandins, oxytocin, angiotensin, eleodoisin, physelaemin and substance P.[1] There are also several substances able to potentiate the smooth muscle contracting effect of bradykinin.These include chymotrypsin,[11] snake venoms,[12] various kininase inhibitors,[13] plasmic and tryptic peptides from plasma proteins,[14–16] and fibrinopeptides.[17] The specificity of the bioassay procedure thus depends on how efficiently the biological samples are purified, and whether specific inhibitors exist and can be used in the assay.

Extracts prepared according to the principles presented in the literature concerning free bradykinin assay,[2–8] invariably contained in our hands a uterus contracting and bradykinin potentiating factor making free bradykinin bioassay on smooth muscle mpossible. This factor differs from bradykinin in many respects, as described above.

It could be separated from bradykinin only by Sephadex gel filtration chromatography. The more precise identity of this factor, which to our knowledge has not been mentioned previously in the literature concerning blood free bradykinin assay, is so far unknown to us. It seems to be of smaller molecular size than bradykinin, as it moves more slowly in gel filtration, and being resistant to total acid hydrolysis it obviously cannot be a peptide.

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