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STUDIES ON KALLIKREIN: FAILURE OF SOME ANTI-INFLAMMATORY DRUGS TO AFFECT RELEASE OF KININ

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Several authors have implicated the kinins and kinin-liberating enzymes in the inflammatory process (Hilton & Lewis, 1955; Edery & Lewis, 1962; Spector, Westall & Willoughby, 1962; Davies & Lowe, 1963). The present study was undertaken to see if known anti-inflammatory drugs owe any of their biological activity to an ability to inhibit the release of kinin by kallikrein. This property has already been attributed to one such drug, indomethacin (Walters & Willoughby, 1965).

Northover & Subramanian (1961) claimed that anti-inflammatory compounds, including aspirin and phenylbutazone, inhibited the release of kinin formed by dilution of guinea-pig or ox serum, but Hebborn & Shaw (1963) failed to confirm that sodium salicylate and aspirin had any noteworthy effect on kallikrein. Lewis (1963) showed that a low pH markedly reduced the activity of kallikrein and that aspirin was not inhibitory when the pH was maintained at 7.4.

METHODS

Preparation of kallikrein

The 7S γ-globulin fraction was isolated from fresh guinea-pig serum by chromatography on diethylaminoethyl cellulose, as described by Davies & Lowe (1961), activated with glass (Davies & Lowe, 1963), and freeze-dried. This material was used as the source of kallikrein.

Preparation of kininogen

The method used was a modification of part of the procedure of Suzuki, Mizushima, Sato & Iwanaga (1965). Bovine plasma was used as the source of kininogen and all manipulations were performed at 2° C. To 500 ml. of plasma was added, with stirring, an equal volume of saturated ammonium sulphate solution over a period of 30 min. After stirring for a further 60 min the suspension was centrifuged (4,000 g for 10 min). The precipitate was redissolved in distilled water and applied to a column of Sephadex G25 (50×3.5 cm) equilibrated with 0.85% (w/v) saline. The protein was eluted with further quantities of saline. To the eluate, saturated ammonium sulphate was added to 25% saturation over a period of 30 min. After a further 45 min stirring the suspension was centrifuged (4,000 g for 10 min) and the precipitate discarded. The saturation was increased to 40% over a period of 30 min and, after a further 45 min the precipitate was separated by centrifugation (4,000 g for 10 min). It was redissolved in distilled water and applied to a Sephadex G25 column (equilibrated with distilled water) and eluted. The eluate, free from ammonium sulphate,

was heated to 75° C in boiling water for 90 sec, cooled in an ice bath to 2° C and centrifuged at 25,000 g for 20 min. The opalescent supernatant fluid was freeze-dried.

The product released kinin (50–100 ng of bradykinin-equivalent/mg kininogen protein), when incubated with either kallikrein or trypsin, contained no free kinin or kinin destroying enzymes and appeared to be stable indefinitely when stored at −20° C over calcium chloride.

Incubation of kallikrein with kininogen

In preliminary experiments the incubation of kallikrein with kininogen was performed in glass vessels and the resulting kinin extracted by pouring the mixture into boiling ethanol (2 vol.). After boiling for a further 2–3 min it was cooled, centrifuged to remove precipitated protein and evaporated under reduced pressure in the Rotavapor (Büchi). The residue was dissolved in water and assayed for kinin on the isolated rat uterus. However, this procedure was found to be unreliable for a number of reasons (see Discussion) and the method described below was developed.

Kininogen and kallikrein preparations were dissolved in 0.05M sodium phospate buffer, pH 8. Kininogen solution (0.5 ml. containing usually 4 mg protein), plus 1 ml. of either water or an aqueous solution, at pH 8, of the compound under test, was incubated in polypropylene tubes at 37° C in a water bath. Kallikrein solution at 37° C (0.5 ml., containing usually 1 mg protein) was then added, and the reactants incubated for 30 min. The reaction was terminated by the addition of soya-bean trypsin inhibitor (0.2 ml. of 1 mg/ml. solution) (T. J. Sas & Son Ltd.).

Immediately after termination of the reaction, the kinin released was assayed as described below. Each control and experimental group contained samples in triplicate, and to enable the assay of every sample to be performed immediately on termination kallikrein was added to individual tubes at 15-min intervals from the start of the experiment.

Assay of kinin

This was performed on the isolated rat uterus suspended in a 5 ml. bath of oxygenated de Jalon solution at 30° C. On each of the two days immediately before the assay the rat received a subcutaneous injection of 0.1 mg/kg stilboestrol. After removal, the uteri could be used either the same day, or stored at 2° C in de Jalon solution for a few days with no deleterious effect.

After the preparation had been set up, the uterus was allowed 1 hr to obtain an equilibrium before the start of the experiment. The dose-response was then determined with a solution of synthetic bradykinin (Sandoz Ltd.) diluted in polypropylene tubes to 100 ng/ml. All additions to the bath were made using a calibrated 1 ml. nylon syringe. The bath was washed out several times immediately after the contraction had reached its maximum height as recorded on a kymograph tracing. Exactly 5 min were allowed between additions of either kinin standard or experimental sample. This allowed for three tests in the 15-min period between assays of successive samples, the second two tests being done with synthetic kinin.

The kinin in an experimental sample was determined by comparison of the induced contraction with those of standard amounts of synthetic bradykinin.

A typical experiment with three groups (nine samples) extended over a period of nearly 3 hr and it was usually found necessary to make fresh dilutions (to 100 ng/ml.) of the bradykinin standard every hour as some instability in this very dilute solution was observed.

Compounds

The following compounds were used: indomethacin (Merck, Sharp & Dohme), flufenamic acid (Parke Davis), phenylbutazone (Geigy), ibufenac (Boots), chloroquine phosphate B.P., aspirin B.P., paramethasone (I.C.I.), and Trasylol (Bayer A.G., 100 kallikrein inactivator units/ml.).

RESULTS

Quantitative assay of kinin

A linear dose-response curve was obtained when log weight of kinin added (usually between 2–15 ng) was plotted against height of contraction (Fig. 1). A linear relationship

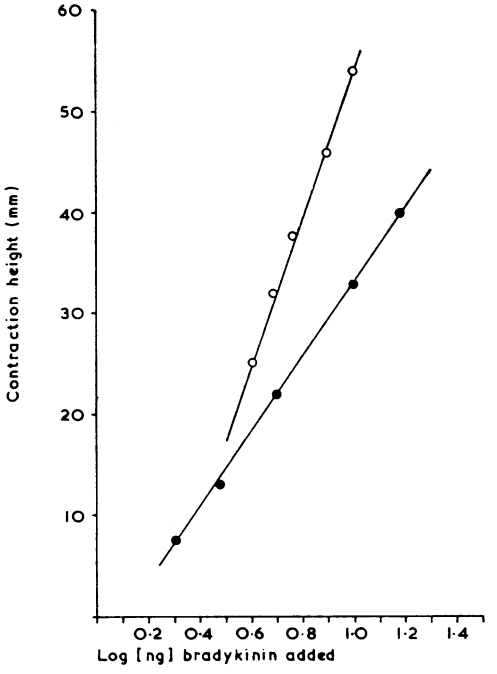

Fig. 1. Dose response curves obtained for two typical rat uterus preparations on addition of standard amounts of synthetic bradykinin in terms of contraction height.

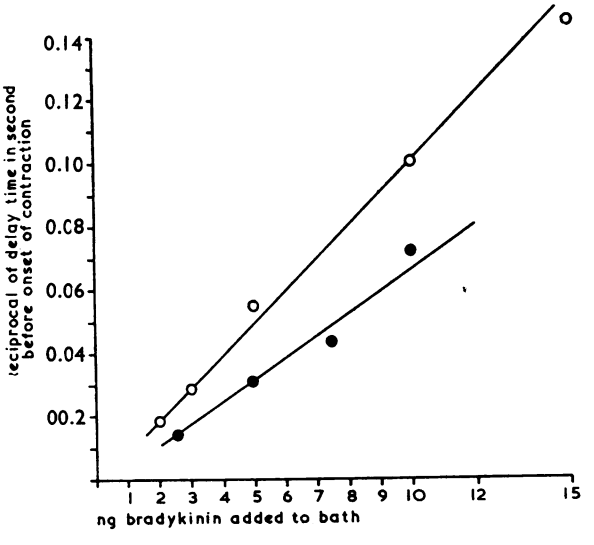

Fig. 2. Dose response curves obtained for two typical rat uterus preparations on addition of standard amounts of synthetic bradykinin in terms of reciprocal of contraction time.

was also obtained when the amount of kinin added was plotted against the reciprocal of the delay time before contraction (Fig. 2). As can be seen, the slopes of these curves varied from preparation to preparation. These two relationships were used in conjunction to measure the amounts of kinin in a sample.

Individual experimental samples could be assayed to within ±20%, and a high degree of reproducibility within a group was obtained. The kymograph tracing from a typical experiment is shown in Fig. 3, and the results derived from the tracing are summarized in Table 1. In this particular experiment the concentration of Trasylol was varied in order to demonstrate that different degrees of inhibition could be quantitated. The degree of reproducibility is illustrated by both control and chloroquine groups, and it is apparent that, while chloroquine has no effect at 100 μg/ml., Trasylol exhibits marked inhibition.

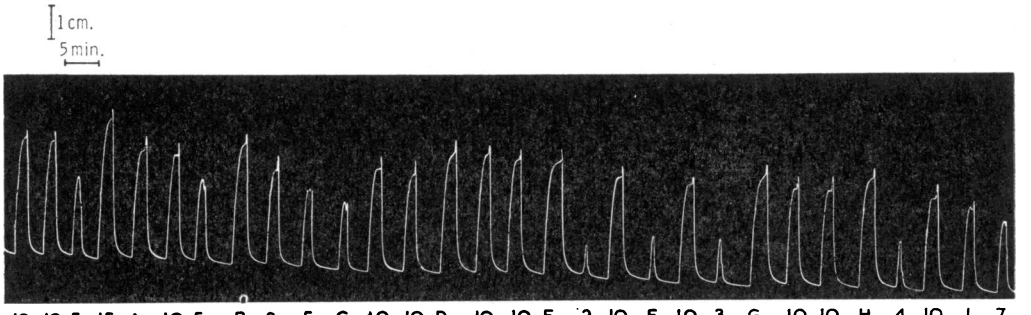

Fig. 3. Kymograph tracing from a typical experiment to determine the effect of chloroquine and Trasylol on release of kinin from kininogen. Thirty minutes incubation at 37° C of 8 mg kininogen with 2 mg kallikrein in 2 ml. 0.025M phosphate pH 8.0. A, D, G, are control samples; B, E, H, contain 100 μg chloroquine/ml.; C, F, I, contain respectively 50, 100, 250 "kallikrein inactivator units" of Trasylol/ml. Contractions of isolated rat uterus in 5 ml. de Jalon solution on addition of either synthetic bradykinin, or samples of the incubated mixtures after addition of 0.2 ml. soya-bean trypsin inhibitor (1 mg/ml.). The upper row of figures indicates the addition of either standard amounts of bradykinin (ng), or aliquots of experimental samples (A, B, C, etc.). 0.1 ml. amounts of experimental sample were added, except in the case of I, where 0.3 ml. was added. A fresh dilution to 100 ng/ml. of the synthetic bradykinin was prepared and used after the addition of sample D. The lower row of figures indicates the delay in sec between the addition (of standard kinin or sample) and the onset of contraction.

TABLE 1

EFFECT OF CHLOROQUINE AND TRASYLOL ON KALLIKREIN-MEDIATED KININ RELEASE

Results derived from the kymograph tracing shown in Fig. 2. Experimental details as described under Fig. 2 and in text. K.i.u.=kallikrein inactivator units (Bayer)

Group	Sample	Compound (concn.)	Kinin released (ng/ml.)	Inhibition (%)
Control 1	A	—	100	—
2	D	—	100	—
3	G	—	100	—
Chloroquine 1	B	100 μg/ml.	100	0
2	E	100 μg/ml.	100	0
3	H	100 μg/ml.	100	0
Trasylol 1	C	50 K.i.u./ml.	40	60
2	F	100 K.i.u./ml.	30	70
3	I	250 K.i.u./ml.	20–25	75–80

Effect of compounds on kinin release

Indomethacin, flufenamic acid, phenylbutazone, ibufenac, chloroquine, aspirin and paramethasone were all inactive in this system at 100 μg/ml., whereas Trasylol and soya-bean trypsin inhibitor were shown to exhibit marked inhibitory properties. The results are summarized in Table 2.

TABLE 2

EFFECT OF COMPOUNDS ON KALLIKREIN MEDIATED KININ RELEASE

Compound	Concentration	Inhibition (%)
Chloroquine	100 μg/ml.	0
Indomethacin	100 μg/ml.	0
Flufenamic acid	100 μg/ml.	0
Phenylbutazone	100 μg/ml.	0
Ibufenac	100 μg/ml.	0
Aspirin	100 μg/ml.	0
Paramethasone	100 μg/ml.	0
Soya-bean trypsin inhibitor	100 μg/ml.	ca. 100%
Trasylol	100 K.i.u./ml.	70%

DISCUSSION

The isolation of purified kininogen has now been achieved by several groups (Habermann, Klett & Rosenbusch, 1963 ; Suzuki *et al.*, 1965). In our view it is not necessary to use pure kininogen in studies on the action *in vitro* of drugs on the kallikrein-mediated release of kinn from kininogen. However, as pointed out by Lewis (1960) the crude kininogen must be stable, free from kinin-forming or destroying enzymes and contain no kinin. This problem has also been discussed by Amundsen, Nustad & Waaler (1963), who described the preparation of a substrate-plasma. However the stability of their preparation was quoted only in hours, and it is obviously preferable to have a single batch of substrate which can be used over a period of months. The preparation described by Davies & Lowe (1963) fulfils all the requirements, but has the disadvantage that the procedure is lengthy, and that only relatively small amounts of serum (50–100 ml.) can be processed at one time. Suzuki *et al.* (1965) prepared highly purified kininogen, but in so doing lost a large amount of the available substrate. By adopting, in a modified form, the first part only of their procedure we have obtained a very stable substrate in reasonable yield.

One of the major difficulties encountered in any study of the kallikrein/kinin system is the accurate measurement of the small amounts of kinin formed. Aspects of the problem which have been found to be very troublesome are the apparent instability of the kinins in dilute solutions (ca. 100 ng/ml.) and a strong tendency of the peptides to adsorb on glass surfaces. In our earlier experiments these properties caused a total lack of reproducibility between samples within a group, and provided contradictory results from day to day. When the causes were ascertained they were overcome by the use of plastic vessels, syringes, etc., and by designing the experiments so that the assay of each sample could be performed within seconds of the reaction being terminated. Previously, kinin had been extracted from a sample with boiling ethanol and evaporated to dryness, involving both glass vessels and a substantial delay before drying which was not constant for all samples. In addition, an unidentified residue from the analytical grade ethanol was shown to cause a non-specific suppression of the contractions.

The technique described in this paper appears to have resolved the problems, and has shown that the anti-inflammatory drugs used have no effect on kallikrein-mediated kinin formation *in vitro*. The possibility that false negative results were being obtained was excluded by demonstrating a positive inhibition by the known kallkrein inhibitors Trasylol and soya-bean trypsin inhibitor. The inactivity of all the anti-inflammatory drugs was a somewhat surprsing finding, particularly in view of the fact that indomethacin has already been reported as being inhibitory (Walters & Willoughby, 1965) and has had its effect in suppressing non-immune inflammation attributed to this property. An explanation for these discrepant results may lie in the different materials and techniques used in the two cases, but in our view none of the drugs examined is acting by a suppression of kallikrein activity. Our results do not preclude the possibility that these drugs may have an action on the kallikrein system *in vivo*. It is still possible that metabolites of the drugs could be antagonists of kallikrein. On the other hand, the drugs or their metabolites could inhibit some other stage of the system—for example, activation of Hageman factor or prekallikrein.

SUMMARY

1. A reliable method is described for the quantitative assay of kinin released by the action of guinea-pig serum kallikrein on crude bovine kininogen. This involved the use of plastic vessels, termination of the reaction with soya-bean trypsin inhibitor and the immediate assay of the released kinin on an isolated rat uterus.

2. The anti-inflammatory drugs indomethacin, flufenamic acid, phenylbutazone, ibufenac, chloroquine, aspirin and paramethasone at a concentration of 100 μg/ml. did not inhibit the release of kinin, whereas Trasylol and soya-bean trypsin inhibitor were markedly active.

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