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EFFECT OF 1,10-PHENANTROLINE AND EDTA ON BRADYKININ ASSAY ON THE GUINEA-PIG ILEUM

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Abstract—It is shown that 1,10-phenantroline, currently used as kininase inhibitor in plasma incubates, cannot be used for the inhibition of human plasma kininase in kinin bioassay on the guinea-pig ileum as, in the concentration needed to inhibit kininase, it decreases the response of the ileum to bradykinin to levels which are not proportional to the doses of standard run comparatively. The use of EDTA is recommended as inhibitor for human plasma kininase in the kinin assay on the guinea-pig ileum. However, the final concentration of EDTA in the ileum bath should not exceed 6×10^{-5} M, otherwise the bioassay of kinins is affected.

COMPLETE inhibition of kininase I and II in human plasma was observed by Yang and Erdös,[1] using 1×10^{-3} M concentration of 1,10-phenantroline or 3×10^{-3} M concentration of EDTA in their incubates. Since then these inhibitors, mainly 1,10-phenantroline, are currently used by most of the authors who measure kinin in media containing kininase, including ourselves.[2] Recently however, using fresh human plasma as substrate for glass activated human plasma kallikrein,[3] we tried various concentrations of 1,10-phenantroline in our incubates and noticed that the ileum is affected by this kininase inhibitor, even in a concentration as low as 5×10^{-6} M in the ileum bath.

MATERIAL AND METHODS

Bradykinin, synthetic, was kindly supplied by Sandoz Ltd., Basle. Loss of bradykinin activity in diluted solutions was prevented by the addition of oxalic acid,[4] in the final concentration of 10^{-3} M.

Plasma. Citrated human plasma was obtained from the Blood Bank of the Western General Hospital.

1,10-*phenantroline* and *EDTA disodium salt* were both BDH "Analar".

Bioassay of bradykinin. The kinin was assayed on the guinea-pig ileum.[5] Previous to the addition of bradykinin to 0·4 ml samples of plasma in siliconized tubes, various amounts of 1,10-phenantroline (6×10^{-3} M) were added and the volumes made up to 1·2 ml with Tyrode solution. These mixtures were incubated for 10 min at 37° and then 0·4 ml of bradykinin solution (1 and 2 μg/ml respectively, or 2 and 4 μg/ml, for each concentration of phenantroline) were added and the mixtures incubated another 20 min. As controls a parallel series of incubates were run using the same plasma previously heated at 60° for 1 hr and in which no kininase could be detected after this period of incubation. The addition of heat-treated plasma was made in order to

protect bradykinin decay which is known to occur in aqueous media during incubation and can be prevented by the addition of protein.

Another experiment was run without previous incubation with plasma, using various amounts of phenantroline added to bradykinin solution, to measure roughly the degree of inhibition of the response of the ileum to bradykinin.

The same experiments were repeated using various concentrations of EDTA di-sodium salt as kininase inhibitor.

The pH of the various incubates was always controlled.

RESULTS AND DISCUSSION

Effect of 1,10-*phenantroline on the guinea-pig ileum response to bradykinin*

The following experiments were performed.

(1) Fresh plasma was incubated with phenantroline and bradykinin as described above, so that the final concentration of phenantroline in the incubates ranged from $6{\cdot}25 \times 10^{-5}$ to 1×10^{-3} M, in geometrical progression. The final concentration of phenantroline in the ileum bath, after pipetting in 0·2 ml of each sample, ranged from $2{\cdot}5 \times 10^{-6}$ M to 4×10^{-5} M. Figure 1 shows an experiment where it can be seen that the response to the same dose of bradykinin diminished with an increase in the concentration of 1,10-phenantroline. It can also be seen that previous exposure of the ileum to phenantroline diminishes its sensitivity to further additions of bradykinin, in the absence of phenantroline.

(2) The experiment above was repeated using the same plasma heated for 1 hr at 60°. Figure 2 shows the degree of inhibition of the response of the ileum to bradykinin

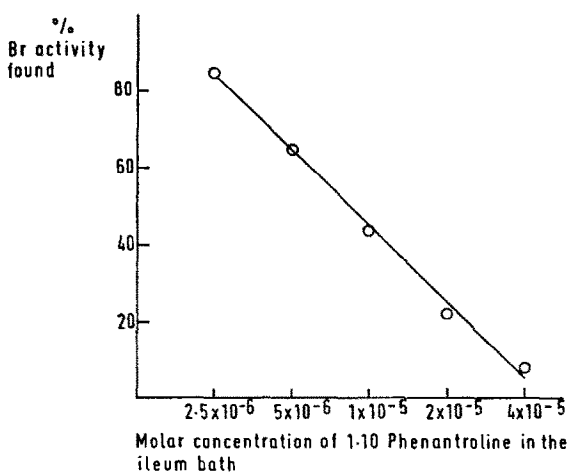

FIG. 2. Inhibition of the guinea-pig ileum response to bradykinin in the presence of 1,10-phenantroline in plasma incubates.

in presence of increasing concentrations of phenantroline in the ileum bath. Each point is the average of two doses of bradykinin incubate with the same concentration of phenantroline compared to two doses of standard bradykinin. The doses of bradykinin added were made to match approximately the response given to the plasma-bradykinin-phenantroline incubates. Bradykinin was added always after the incubates, followed by the incubate containing a double concentration of phenantroline and so on.

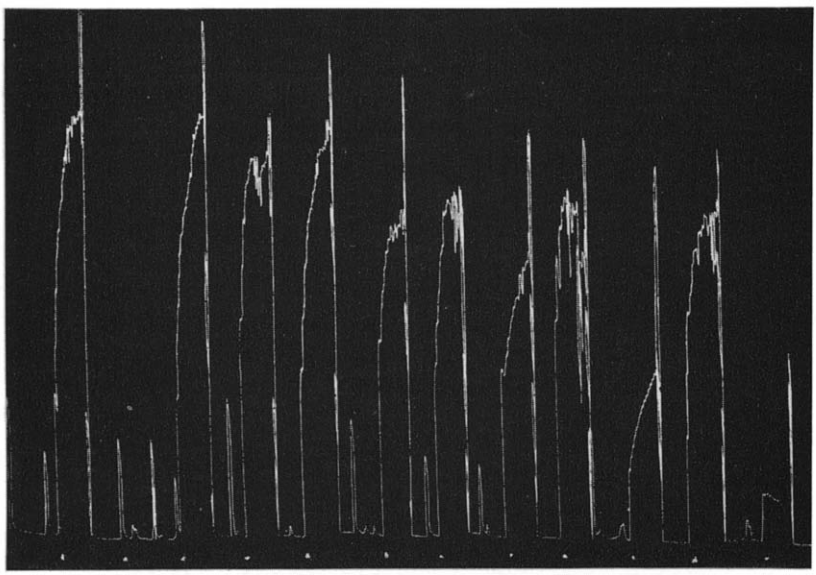

FIG. 1. Incubates of 0·4 ml plasma + 1,10-phenantroline + 1·6 μg Br + Tyrode to complete the volume to 1·6 ml. From the incubates 0·15 ml were pipetted into the ileum bath. 1: incubate without phenantroline. The final concentrations of phenantroline in the other incubates were respectively $2{\cdot}5 \times 10^{-6}$ M in 2, increasing in geometrical progression to 4×10^{-5} M in 6. Br: 0·15 μg.

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Therefore the results in Fig. 2 are given in comparison to the contraction caused by samples of bradykinin which followed each incubate of bradykinin + phenantroline. Figure 3 shows the curve of the ileum response to bradykinin, before the ileum has been exposed to 1,10-phenantroline, compared to a similar curve obtained after exposure of the ileum to various concentrations of 1,10-phenantroline. It can be seen

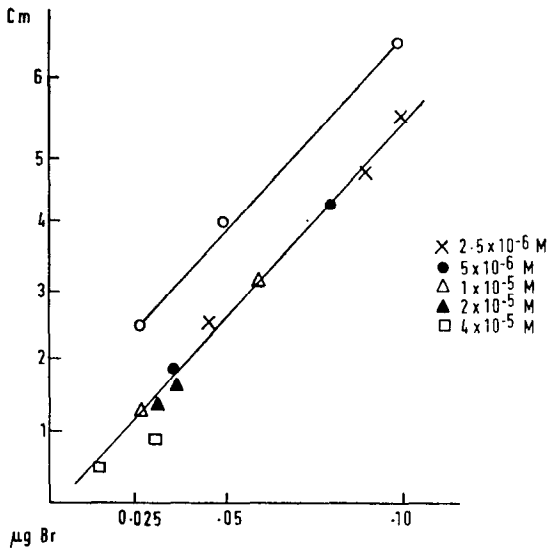

FIG. 3. Guinea-pig ileum response to bradykinin, before the ileum has been exposed to phenantroline (○) and after exposure to increasing concentrations of 1,10-phenantroline (×, ●, △, ▲, □) when this had been washed out.

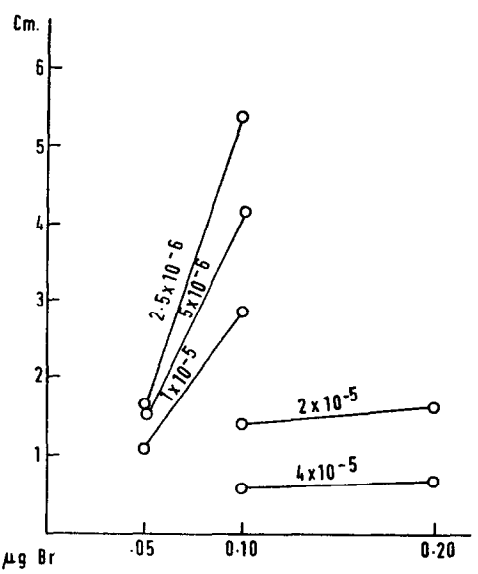

FIG. 4. Response of the guinea-pig ileum to bradykinin added to heated plasma incubates containing the indicated molar concentrations of 1,10-phenantroline.

that after phenantroline a permanent decrease of the sensitivity of the ileum to bradykinin is observed but the slopes of the two curves are still the same. Figure 4 shows the slopes observed with two doses of bradykinin (each point is the average of two readings) in presence of various concentrations of 1,10-phenantroline in incubates of heated plasma and it can be seen that the slope changes with each new concentration of phenantroline at the same time as the response decreases for the same dose of bradykinin. Therefore no estimate of potency of kinin can be made in the presence of 1,10-phenantroline in the concentrations reported here.

(3) Mixtures of bradykinin + Tyrode with increasing amounts of phenantroline tested on the ileum caused the same degree of inhibition in the response of the ileum to bradykinin as in the previous experiments.

Effect of EDTA on the ileum response to bradykinin

The same series of experiments were repeated, using EDTA as kininase inhibitor, in incubates of human plasma, heat-treated plasma and mixtures of bradykinin with Tyrode, containing different concentrations of EDTA: $3{\cdot}75 \times 10^{-4}$, $7{\cdot}5 \times 10^{-4}$, $1{\cdot}5 \times 10^{-3}$ and 3×10^{-3} M, giving a 25 times lower concentration in the ileum bath. In all these concentrations EDTA completely prevented the kininase activity of human plasma on bradykinin. In the highest concentration used, $1{\cdot}2 \times 10^{-4}$ M in the ileum bath, it reduced the response to bradykinin to about 50 per cent; the response was, however, not affected by any of the lower concentrations used.

In separate experiments we observed that a concentration as low as $1{\cdot}5 \times 10^{-4}$ M EDTA in human plasma incubates completely inhibited its kininase activity, the final concentration in the ileum bath being in this case 6×10^{-6} M. Therefore four-point assays were performed, comparing the responses to incubates of two doses of bradykinin added to fresh plasma previously incubated with EDTA with the same doses of bradykinin added to heated plasma, using a latin square each time. The result of one such experiment gave the best estimate of the potency ratio T/S as 107 per cent, with the fiducial limits of 87 and 118 per cent.

In a similar experiment where incubates of EDTA with fresh plasma plus bradykinin were compared to the corresponding doses of bradykinin directly added to the ileum bath, the best estimate of the potency ratio T/S was 104 per cent, with fiducial limits 92 and 109 per cent.

The results presented in this paper show that 1,10-phenantroline cannot be used as plasma kininase inhibitor in the bioassay of kinin on the guinea-pig ileum.

In contrast to that, EDTA does not interfere with the guinea-pig ileum response to bradykinin up to a concentration of 6×10^{-5} M in the ileum bath and inhibits completely the kininase activity of human plasma in a concentration as low as $1{\cdot}5 \times 10^{-4}$ M in the incubate, under the conditions described in this work. Therefore various doses of plasma incubate can be used for a quantitative assay in comparison to the standard, as long as the final concentration in the ileum bath does not exceed 6×10^{-5} M.

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REFERENCES

1. H. Y. T. YANG and E. G. ERDÖS, *Nature, Lond.* **215,** 1402 (1967).
2. P. BUDNITSKAYA, E. GAPANHUK and O. B. HENRIQUES, *Biochem. Pharmac.* **19,** 2083 (1970).

3. O. B. HENRIQUES, V. STOLPNIK, V. KUSNETSOVA and M. ASTRAKAN, *Biochem. Pharmac.* **19,** 2915 (1970).
4. L. M. GREENBAUM, K. YAMAFUJI and T. HOSODA, *Biochem. Pharmac.* **14,** 411 (1965).
5. O. B. HENRIQUES, Z. P. PICARELLI and M. C. FERRAZ DE OLIVEIRA, *Biochem. Pharmac.* **11,** 707 (1962).