THE USE OF BACITRACIN AS AN INHIBITOR OF THE DEGRADATION OF THYROTROPIN RELEASING FACTOR AND LUTEINIZING HORMONE RELEASING FACTOR

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Summary: Bacitracin was found to be an effective inhibitor of the in vitro degradation of both thyrotropin releasing factor [1](TRF) and luteinizing hormone releasing factor (LRF) by guinea pig hypothalamic and whole brain homogenates and rat hypothalamic homogenates and subcellular fractions. Bacitracin was effective in inhibiting the degradation of TRF and LRF, as determined by radioimmunoassay, where it exhibited no interference with the assays. Kinetic studies of the degradation of exogenous synthetic [^{3}H]-TRF demonstrated non-competitive inhibition by bacitracin with $K_i = 1.9 \times 10^{-5}$ M, while studies on the degradation of [^{3}H] LRF indicated competitive inhibition with $K_i = 1.7 \times 10^{-5}$ M. Electrophoretic and amino acid analysis revealed that bacitracin itself was not degraded during the course of the in vitro incubation.

Numerous reports have appeared describing the presence of potent peptidase activity in homogenates and fragments of mammalian hypothalamus and other CNS regions, resulting in the rapid degradation of both thyrotropin releasing factor (TRF; 1,2,3,4) and luteinizing hormone releasing factor (LRF; 5,6,7,8). The presence of this peptidase activity has seriously interfered with attempts to study the in vitro biosynthesis and release of hypophysiotropic peptides. While solutions to this problem can be found in the utilization of systems which exhibit minimal or no degradative activity, such as the newt (9), the toadfish (McKelvy, unpublished) or clonal cell lines derived from CNS tumors (10), it is important to study releasing factor metab-

[1]Abbreviations: CNS, central nervous system; DTE, dithioerythritol; EGTA, (ethyleneglycol-bis-ßaminoethyl ether) N,N'-tetraacetic acid; LRF, luteinizing hormone releasing factor; TRF, thyrotropin releasing factor.

olism in the common laboratory rodents for which so much relevant endocrinological and neurobiological information exists. This means that effective inhibitors for these peptidases must be found. The potential usefulness of bacitracin as an inhibitor of releasing factor degradation was brought to our attention by the report of Desbuquois and Cuatrecasas (11) who described its inhibition of glucagon degradation, but did not kinetically characterize the inhibition, nor examine the stability of the bacitracin itself. In the present communication, we report on the effective inhibition of the degradation of endogenous and exogenous TRF and LRF, on the kinetic nature of the latter, and on the lack of cleavage of bacitracin under the conditions used.

MATERIALS AND METHODS

Reagents: pGlu-His-[^{3}H] Pro-NH_2 (40 Ci/mmol) and [^{3}H] pGlu LRF (23.5 Ci/mmol) were from New England Nuclear, Boston, Mass. Synthetic TRF was from Peninsula Laboratories, San Carlos, Cal. Synthetic LRF was obtained from Beckman Bioproducts, Palo Alto, Cal. Reagent grade methanol (Baker) was redistilled and stored in glass bottles prior to use. Dithioerythritol (DTE), (ethyleneglycol-bis-β-aminoethyl ether) N,N'-tetraacetic acid (EGTA) and bacitracin were purchased from Sigma Chemical Corp., St. Louis, Mo.

Radioimmunoassay of Endogenous TRF: Endogenous TRF levels were determined using a ^{125}I radioimmunoassay as previously described (10). The assay was sensitive to 5pg/assay tube with a coefficient of variation of 2.4% and was highly specific for TRF. Guinea pig hypothalami were homogenized at 0° with 3 volumes of 50 mM Tris-Cl (pH 7.4 at 37°) and the homogenate was centrifuged at 27,000 xg for 30 min. The resulting supernatant was divided into 2 equal portions, to one of which bacitracin was added to a final concentration of 1.7×10^{-4}M (0.5 mg/ml). Both portions of the supernatant were then incubated in a shaking water bath at 37°, and at 0, 15 and 30 min., duplicate aliquots of 100 µl each were removed and extracted with 9 volumes of cold absolute methanol. A subsequent radioimmunoassay curve was obtained with bacitracin present at the same concentration as in the experimental samples.

Radioimmunoassay of LRF: LRF levels were determined using a ^{125}I radioimmunoassay as previously described (12). The assay was sensitive to 15pg/assay tube and exhibited a coefficient of variation of 7%. 10 ng of synthetic LRF were added to 0.3M sucrose containing 0.2 hypothalamic equivalent of 13,500 g x 30 min. microsomal supernatant prepared from rat mediobasal hypothalamus, made up to a final volume of 200 µl with Locke's solution. Samples were incubated for 20 min. in the presence of varying amounts of bacitracin, then 20 µl of 1NHCl were added, the tubes centrifuged and the supernatant solutions frozen until radioimmunoassay.

Preparation of Homogenates For Exogenous RF Degradation: Rat (250 g male Sprague-Dawley) and guinea pig (150 g male) hypothalamus and whole brain were homogenized 3:1 (v/w) with buffer A (50 mM Tris-Cl, pH 7.4(0°), 0.1 mM DTE, 1 mM EGTA) at 800 rpm using 10 up and down strokes of a teflon pestle in a glass homogenizer with a clearance of 0.15 mm. Following centrifugation at

27,000 x g for 30 min., the supernatant solution was diluted 6 fold with buffer A prepared at 37°, and used as a peptidase source. Fresh homogenates were used for each experiment.

Assay for Degradation of Exogenous [^{3}H] TRF: From 2-10 µl of peptidase source was incubated with 100 pmoles (2.7×10^4 cpm) of [^{3}H] TRF (prepared by purifying commercial [^{3}H] TRF by the method of McKelvy (13) and diluting with synthetic nonradioactive TRF) in 2 µl buffer A and varying volumes of buffer A or bacitracin (200 pmoles/µl buffer A) to give a final volume of 20 µl. This concentration of TRF approached saturation for the peptidase activity of the homogenate (apparent K_m 2×10^{-5}M). Incubation was carried out for 15 min. at 37° with shaking and the reaction terminated by the addition of 20 µl of absolute methanol. After precipitation at 0° for 15 min., the methanol supernatant was recovered by centrifugation at 12,000 g x 2 min. Five µl of the methanol supernatant was applied to strips (2 x 15 cm) of carboxymethyl cellulose paper (CM-82, Whatman) marked off in 1 cm lengths, and developed in H_2O in ascending fashion to a height of 10 cm. Following drying, each strip was cut into 10 one cm segments which were placed in separate scintillation vials, eluted with 100 µl of 50 mM NaCl, and counted in 10 ml of a mixture of: Omnifluor (New England Nuclear) 17g, BBS-3 (Beckman Bio-Solve) 83 ml and toluene, to 41. Under these conditions, undegraded [^{3}H] TRF was contained in the first 3cm from the origin, and the principle degradation product, pGlu-His-Pro-OH (>90%), and traces of Pro, were contained in the last 3cm. Degradation was assessed as pmoles [^{3}H] TRF degraded by summing the cpm on each strip, computing the ratio of the radioactivity in the first 3 cm to the total radioactivity on the strip and then dividing this ratio by its counterpart for a control incubation in which no enzyme was present (95-97% of total radioactivity in first 3cm). For guinea pig whole brain, which was used in most experiments since it yielded a degradation pattern identical to hypothalamus, and exhibited a greater specific activity, the specific activities of TRF degradation observed under these conditions ranged from 230-290 pmoles TRF degraded min^{-1} mg $protein^{-1}$ expressed as equivalents of bovine serum albumin in a modified Folin procedure (14). Inhibition data was obtained by the method of Dixon (15).

Assay for Degradation of Exogenous [^{3}H] LRF: The assay for degradation of [^{3}H] LRF was carried out identically to that for [^{3}H] TRF degradation except that the specific activity of [^{3}H] LRF was 1 nCi/pmole. The final concentration of LRF in the assay (5 µM) approached the saturation value for LRF (apparent K_m 6.3×10^{-6}M) as determined with this assay. Upon CM-cellulose paper chromatography of [^{3}H] LRF degradation products, 3 peaks of radioactivity were seen, including the one at the origin where intact LRF resided. We did not ascertain the identity of these species, nor that the radioactivity at the origin was exclusively undegraded [^{3}H] LRF. However, the rate of degradation of [^{3}H] LRF, as measured by the loss of radioactivity from the origin, was linear with protein concentration (0.05 - 0.5 µg/ml) and time (up to 15 min.) using guinea pig brain as a source. The specific activity for [^{3}H] LRF degradation by guinea pig brain homogenate, using the above assay, was 1260 pmoles min^{-1}mg $protein^{-1}$. Similar results were obtained using hypothalamic and whole brain tissue in the guinea pig and the rat. It should be noted that, in studies on the degradation of exogenous [^{3}H] TRF and LRF, the contribution of endogenous releasing factor by the extracts was negligible, the exogenous peptide being in excess of a factor of 10^7.

Stability of Bacitracin to the Conditions of In Vitro Incubation: To test whether bacitracin was itself cleaved under the conditions of *in vitro* incubation in which it afforded protection against releasing factor degradation, the assay for the degradation of exogenous [^{3}H] TRF described in (4) was scaled up to 100 µl final volume, using guinea pig whole brain homogenate as

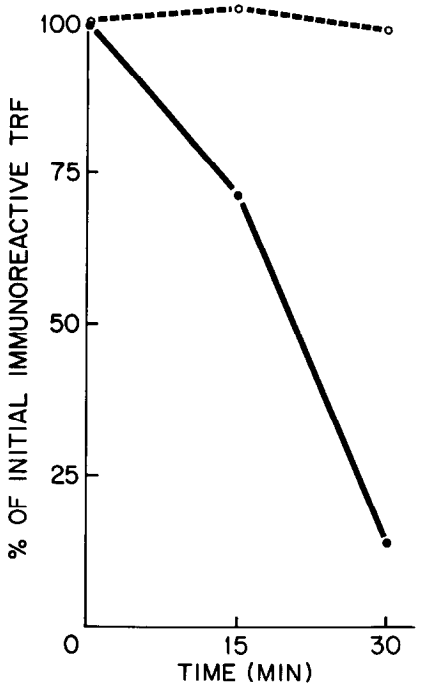

FIGURE 1. Stability of endogenous TRF in a post-microsomal supernatant of guinea pig whole brain at 37° in the presence (0--0) and absence (0 0) of 1.7 x 10^{-4}M bacitracin, as measured by radioimmunoassay. For details, see MATERIALS AND METHODS.

peptidase source, and 4 types of incubations were carried out: (i) buffer, [^{3}H] TRF, bacitracin (3.4 x 10^{-4}M), but no homogenate, incubated at 37° x 15 min.; (ii) buffer, [^{3}H] TRF, bacitracin (3.4 x 10^{-4}M) plus homogenate, incubated at 37° x 15 min; (iii) same as (ii), but with 60 min. incubation and (iv) buffer [^{3}H] TRF, homogenate, but no bacitracin, incubated at 37° x 60 min. The methanol supernatants derived from these incubations were treated as follows: (a) 20 µl were subjected to cellulose acetate electrophoresis at pH 4.2 to assess degradation of [^{3}H] TRF and its inhibition by bacitracin; (b) 70 µl of the same methanol supernatants were removed in duplicate and one aliquot was subjected to automated amino acid analysis (16) without hydrolysis and the other aliquot analysed after hydrolysis in 6N HCl at 108° for 22 hr.; (c) 10 µl of the methanol supernatants were subjected to high voltage paper electrophoresis at pH 1.9 (180 V/cm, 90 min.) in duplicate lanes, one lane being sprayed with Pauly reagent (17) and one with cadmium - ninhydrin reagent (18). The position of migration of native bacitracin standard solution, dissolved in assay incubation buffer-methanol (1:1 v/v) was determined by spraying with Pauly reagent.

Assay for Effect of Bacitracin on TRF Degradation by Serum: The degradation of [^{3}H] TRF by normal rat serum was carried out as described by Bauer (19) in the absence and presence of from 0.5 - 10µM bacitracin, but with analysis of the degradation products as described in this communication.

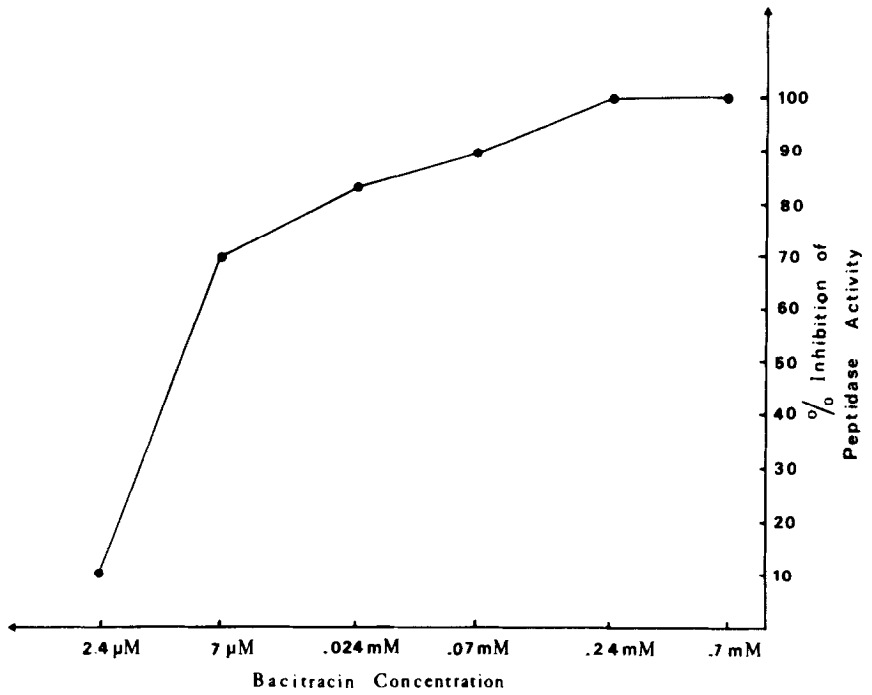

FIGURE 2. Effect of varying concentrations of bacitracin on the degradation of 10 ng of synthetic LRF by a post-microsomal supernatant of rat hypothalamus, as determined by radioimmunoassay. For details, see MATERIALS AND METHODS.

RESULTS AND DISCUSSION

Fig. 1 demonstrates the protective action of bacitracin against the degradation of endogenous TRF in an homogenate of guinea pig hypothalamus. In the absence of bacitracin, about 30% of the zero-time amount of endogenous TRF had been lost by 15 min. of incubation and about 85% by 30 min. In the presence of 1.7×10^{-4}M bacitracin, no significant change in the amount of immunoreactive TRF could be discerned over the entire 30 min. incubation period. Although not shown here, the radioimmunoassay standard curve obtained with the same amount of bacitracin present as in the experimental samples was identical to the standard curve obtained with no bacitracin present.

Fig. 2 shows the protective effect of bacitracin against the degradation of 10 ng LRF added to 0.2 hypothalamic equivalents of microsomal supernatant. After 20 min. of incubation at 37°, 94% of the original amount of LRF was degraded in control tubes; addition of increasing concentrations of baci-

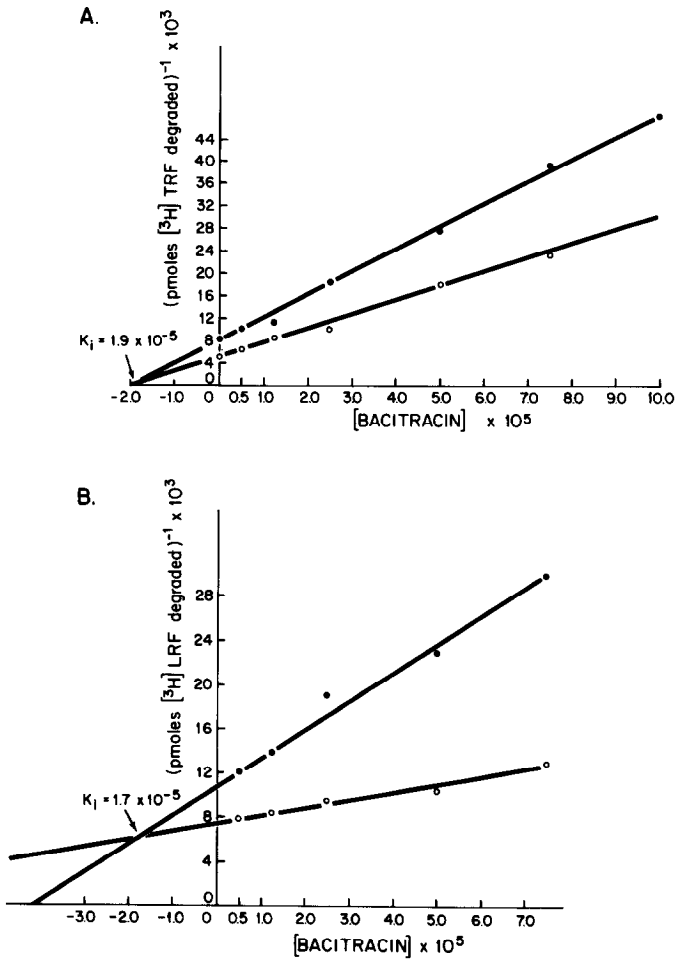

FIGURE 3. A. Dixon plot of kinetics of inhibition of degradation of exogenous [^{3}H] TRF (5 μM 0 0; 2.5 μM 0 0) by varying concentrations of bacitracin; B. Inhibition of degradation of exogenous [^{3}H] LRF (5 μM 0 0; 2.5 μM 0 0) by varying concentrations of bacitracin.

tracin (10^{-7} - 10^{-3}M) induces a dose-dependent inhibition of this degradation, total inhibition being reached at about 10^{-4}M.

The kinetics of bacitracin inhibition of exogenous [^{3}H] TRF and [^{3}H] LRF degradation are shown in Fig. 3. Under the conditions of assay used, bacitracin appeared to act in non-competitive fashion in the inhibition of TRF degradation since extrapolation of the reciprocal of the velocity vs. bacitracin concentration at 2 different TRF concentrations intersected the x-axis at the same point. In contrast, analysis of the inhibition of LRF degrada-

TABLE I

STABILITY OF BACITRACIN TO IN VITRO INCUBATION IN THE PRESENCE OF BRAIN HOMOGENATES

	Incubation Condition							
	A. Homog. No bacitracin 37° x 60 min.		B. Homog. + bacitracin 37° x 15 min.		C. Homog. + bacitracin 37° x 60 min.		D. No homog. + bacitracin 37° x 15 min.	
pmoles [^{3}H] TRF degraded	315		2.9		57		0	
nmoles of	*NoH	**H	NoH	H	NoH	H	NoH	H
Lys	‡ND	ND	ND	45.9	ND	50.9	ND	44.1
His	ND	ND	ND	21.6	ND	28.9	ND	22.2
Asp	ND	6.0	ND	49.4	ND	52.4	ND	43.8
Glu	ND	10.1	ND	34.3	ND	37.3	ND	24.0
Ala	ND	ND	ND	6.5	ND	7.4	ND	7.0
Val	ND	ND	ND	7.4	ND	8.2	ND	5.9
Ileu	ND	ND	ND	43.2	ND	46.8	ND	40.8
Leu	ND	ND	ND	24.0	ND	26.0	ND	21.3
Phe	ND	ND	ND	20.4	ND	21.9	ND	19.2

Conditions as specified in MATERIALS AND METHODS. *No hydrolysis; **hydrolysis in 6N HCl, 108°, 22 hr.; ‡ Not detectable under conditions used (2.4 nmole).

tion in this way provided evidence for a competitive process. It should be emphasized that these results were obtained with crude extracts and that full elucidation of the mode of action of bacitracin in the inhibition of releasing factor degradation awaits studies on the purified enzymes responsible for degradation, work which is presently in progress.

When incubation mixtures containing bacitracin were examined chemically, no evidence for the breakdown of bacitracin was obtained with the methods used. Table 1 shows that no free amino acids were detectable by automated amino acid analysis of unhydrolyzed samples for any of the incubation conditions used, and that the amino acid compositions of the hydrolyzed samples

are identical within experimental error when the contribution by the homogenate is taken into consideration. High voltage paper electrophoresis at pH 1.9 of aliquots of the same sample revealed a single Pauly positive spot, whose migration corresponded exactly to standard bacitracin, at 15 and 60 min. of incubation at 37°. Cadmium-ninhydrin spraying revealed no new ninhydrin spots for 15 and 60 min. incubates. The same incubation mixtures giving rise to samples for the analysis of bacitracin stability exhibited [^{3}H] TRF degradation and the inhibition of this degradation by bacitracin. It should be noted that the concentration of endogenous plus exogenous TRF was below the level of sensitivity of amino acid analysis.

Although not presented here, the results of experiments in which bacitracin inhibition of [^{3}H] TRF degradation by serum was attempted showed no significant inhibition by the antibiotic over a concentration range of 0.5 - 10 μM. The stability of bacitracin in serum was not assessed.

The results of this study provide evidence that bacitracin effectively inhibits the degradation of both endogenous and exogenous TRF and LRF by CNS tissue without itself undergoing degradation. Recent studies in a cell free system (McKelvy, unpublished) indicate that TRF biosynthesis can occur in the presence of bacitracin. We anticipate that this antibiotic will be a useful tool in studies on the metabolism of hypothalamic releasing factors.

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