HYPOTHALAMIC PEPTIDES INFLUENCING SECRETION OF ACTH BY ISOLATED ADENOHYPOPHYSIAL CELLS

Two corticotrophin releasing factors and a potentiator

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1. Introduction

Bovine hypothalamic tissue contains two low molecular weight corticotrophin releasing factors, resolved by gel filtration and designated CRF_1(G-15) and CRF_2(G-15). CRF_1(G-15) contains an inhibitor as well as a stimulator of ACTH secretion [1]. We now report the separation of a stimulator and a potentiator present in the CRF_2(G-15) fraction, utilizing counter current distribution and the isolated pituitary cell system of bioassay [2]. The observations suggest that the hypothalamus elaborates a complex of oligopeptides regulating ACTH secretion which complex includes two secretagogues, an inhibitor and a potentiator.

2. Materials and methods

Fractions were assayed for corticotrophin releasing activity by the isolated adenohypophysial cell technique [2]. Routinely 24, but occasionally as many as 48, aliquots of cell suspension ($1.0–1.5 \times 10^5$ cells/0.9 ml aliquot) were pipetted into 10 ml teflon beakers. Test substances in solution, or vehicle (controls), were added in 0.1 ml to the teflon beakers the day before the assay and desiccated overnight. The cells were incubated at 37°C for 60 min in a Dubnoff shaker, under an atmosphere of 95% O_2, 5% CO_2. The media were separated by centrifugation ($800 \times g$ for 10 min), acidified to pH 3.5 with HCl and assayed for ACTH by the isolated adrenal cortex cell technique [3]. Fraction CRF_2(G-15) is our interim house standard; one corticotrophin unit is the activity exhibited by 5 μg of this material. Comparison of unknowns with standard for each assay eliminates interassay variation. We recognize that the response to CRF_2(G-15) is the result of the combined actions of at least two peptides. Decision on a permanent standard must await further purification, perhaps isolation of the involved peptides.

One hundred pieces of bovine hypothalamic tissue (one batch) were carried through extraction and gel filtration, first on Sephadex G-25 then on Sephadex G-15 as follows: A batch of frozen (dry ice) tissue (~800 g) was pulverized and defatted with acetone. The powder (200 g) was extracted with 1600 ml 30% acetic acid and the mixture centrifuged. The supernatant was lyophilized, the syrup taken up in 200 ml 0.01 M ammonium acetate and adjusted to pH 7.0 with concentrated ammonium hydroxide. The mixture was centrifuged, the supernatant lyophilized and the concentrate extracted with 0.01 M ammonium acetate. The extract (~20 g Folin-Lowry protein) was applied to a Sephadex G-25 column and eluted with 0.01 M ammonium acetate. CRF activity eluted between 1200 and 1800 ml; the contents of the tubes in this region were pooled, lyophilized and the dried material extracted with 6 ml 0.2 N acetic acid. The mixture was centrifuged and the supernatant applied to a Sephadex G-15 column (2.2×100 cm; bed vol. 450 ml; void vol. 100 ml; eluted with 0.2 N acetic acid at 20 ml/h, 4 ml/tube). CRF activity located in two regions, tubes 75–81 [CRF_1(G-15)] and tubes 90–105 [CRF_2(G-15)].

Ten batches of hypothalamic tissue were carried through extraction, and gel filtration, first on Sephadex G-25 then Sephadex G-15, as above. The

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contents of the tubes of the CRF_2(G-15) region were lyophilized to dryness, pooled and taken up in 6.0 ml 1.0 N acetic acid. A sample, 4.2 ml, representing a quantity of CRF_2(G-15) derived from 700 pieces of bovine hypothalamic tissue, was applied to a Craig counter current apparatus (520 tubes, 3 ml phases of a system of 1-butanol–1.0 N acetic acid) and distributed through 520 transfers.

3. Results

To estimate recovery of the CRF activity that had been applied to counter current distribution, small aliquots (100 μl) of the aqueous phase from each of the 520 tubes were combined; similarly, aliquots of the butanol phase were combined. Assay of these combined aliquots indicated that 70% of the applied activity was present in the aqueous phase, ~5%, in the butanol phase. This experiment was followed by one designed to localize CRF activity. The applied CRF was distributed as follows: in tubes 56–82, 6 ± 2%; 83–109, 5 ± 2%; 110–140, <1%; in the regions 1–55, 141–190, 191–244, 245–298, 299–406, 407–520, no significant activity was detected. In the butanol phase, significant activity was found only in the regions 56–82 and 83–109; the estimated recovery was <1% for each of these regions. The marked differences in the recovery between that estimated for a pool of all 520 tubes vs that estimated for regions 56–82 plus 83–109 plus 110–140 prompted us to survey certain regions for potentiator activity. The total CRF units in the aqueous phase of regions 56–82 and of 83–109 increased ~10-fold when aliquots were added to the cell suspension together with an aliquot of the region 110–140. The aliquot of region 110–140 (10 μl of 75 ml total vol.) induced no increase in secretion of ACTH when added alone to the isolated cells. No other region of the aqueous phase exhibited potentiator activity when tested in 50 μl doses. Potentiator activity was present in the butanol phase of region 110–140 and exhibited by a dose of 50, but not 10 μl. CRF activity in individual tubes of the counter current distribution instrument were then assayed. In fig.1, the CRF units present in selected tubes of the region 50–140 are presented. The total CRF units under the curve of best fit equalled 32, a recovery of 9% of the applied CRF units.

When it became apparent that CRF_1 and potentiator overlapped, a pool of the contents of tubes in the region 120–140 (presumed to be free from CRF) was selected for a quantitative estimate of the influence of potentiator upon the activity of CRF. Assay of the CRF activity of individual tubes in the region 50–110 were conducted as described for the experiment presented in fig.2, but in this case a 10 μl aliquot of a pool of the contents of tubes 120–140 was added to each sample of isolated pituitary cells. The addition of potentiator enhanced CRF activity as follows: tube 67, 24-fold; 71, 15-fold; 78, 10-fold; 85, 11-fold; 91, 8-fold; 96, 2-fold. The observation points are presented in fig.2. The curve was calculated on the basis of a normal distribution centered about tube 78. We have located potentiator on the basis of assays of various regions; no quantitative estimates of the relative quantities in individual tubes have been made. The skewness of the curve of CRF activity when potentiator was not added (fig.1) is attributed to overlap of the two activities in the region 95–110.

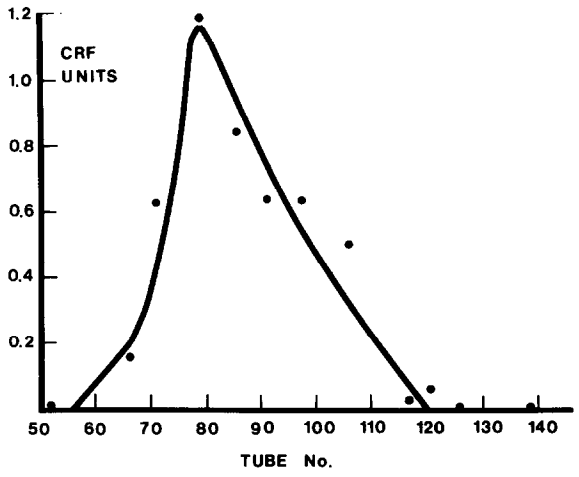

Fig.1. Counter current distribution of fraction CRF_2(G-15), 520 transfers, 1.0 N acetic acid (3 ml) vs 1-butanol (3 ml). CRF activity in the aqueous phase of tubes 50–140. The points represent estimates of total CRF units in a given tube; 1, 10 and 100 μl aliquots in duplicate for each of two assays conducted on different days; error for estimate based on the combination of the two assays approximately ±15%. The curve through the experimental points was fitted by eye.

4. Discussion

The asymmetry of distribution of CRF activity in the instance in which aliquots of the contents of the tubes were added alone to suspensions of isolated

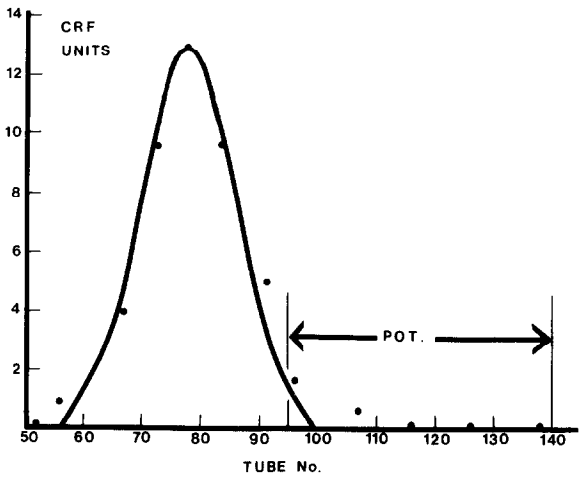

Fig.2. Counter current distribution of fraction CRF_2(G-15), 520 transfers, 1.0 N acetic acid–1-butanol, 3 ml in both phases. Assay protocol as described in legend to fig.2 but in this instance 10 μl of a combination of the contents of tubes 120–140 (aqueous phase) was added to each aliquot of the contents of individual tubes in the CRF region. The 10 μl aliquot of the combination of tubes 120–140 when added alone induced no increase in ACTH secretion. Aliquots (100 μl) of tubes 126 and 138 added alone induced no response. Note the 10-fold difference in the scale of the ordinate as compared to that of fig.1. The continuous line is the curve calculated for a distribution coeficient $K = 0.177$, with r_{max} = 13 CRF units in tube 78. The experimentally determined values are represented by solid circles. For the location of potentiator (POT.) consult the text.

pituitary cells (fig.1) is consistent with the conclusion that even after 520 transfers separation of CRF and potentiator activities is still incomplete. The good agreement between calculated and found values in the potencies determined for individual tubes to which potentiator had been added indicates the presence of a single stimulatory entity in the region centered around tube 78.

The biological activities of CRF and of potentiator are lost upon treatment with 6 N HCl at 110°C for 18 h or with protease, indicating that the active materials are peptides. CRF and a cofactor have been prepared as distinct fractions from an extract of rat hypothalamic tissue [4]. The cofactor, unlike potentiator, retains activity after heat (150°C) in 6 N HCl for 18 h. The CRF activity of a crude stalk median eminence extract has been ascribed to a multi-factor system [5]. Vasopressin is the major component whose potency is 'modulated' by synergistic factors which have weak inherent CRF activity. CRF_1(G-15) and CRF_2(G-15) are chromatographically [6] and biologically [7] distinct from vasopressin. Despite differences as to the exact nature of stimulators, cofactors , modulators, potentiators, the evidence cited, together with these findings, should alert one to the possibility that loss of CRF activity following a purification step may be apparent rather than real.

The physiological significance of the observations reported here needs some comment. The increase in rate of secretion of ACTH by the isolated cells in response to CRF_1(G-15) and to CRF_2(G-15) is energy-dependent (blunted in the absence of glucose from the medium), Ca^{2+}-dependent, and inhibited by physiological concentrations of corticosterone (0.1 μg/ml). These facts rule out a toxic, lytic action on the plasma membrane resulting in increased permeability, with non-specific release of ACTH. The response of the cells appears to be quite specific; a number of substances including luteinizing hormone releasing hormone (LHRH), thyrotrophin releasing hormone (TRH), somatostatin, angiotensin II, serotonin, glucagon, insulin, pressinoic acid, histamine, tryptophan, arginine, polyarginine and $ACTH_{4-10}$ do not stimulate ACTH secretion. The material in tubes corresponding to the peak of biological activity in the counter current distribution (fig.2) induces a significant increase in ACTH secretion at a dose of ~100 pg. The circumstantial evidences, taken together, suggest that we are dealing with compounds of physiological significance.

Acknowledgements

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