NOTE

ACTH RELEASE BY PURIFIED HYPOTHALAMIC CRF PREPARATION AS ASSAYED BY INTRAPITUITARY MICROINJECTION

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SYNOPSIS

ACTH-releasing potency of a purified hypothalamic CRF of pig origin was examined by use of the intrapituitary microinjection method. A good log dose-response relationship was obtained in the dosage range from 12.5 to 50 ng injected per one anterior pituitary lobe. Two dose factorial analysis showed that the difference in response between purified CRF and median eminence extract of the rat at corresponding dosage levels was not significant and that parallelism was well maintained with a highly significant combined slope. Relative potency of the median eminence extract was calculated to be 102%. From these findings it was estimated that the median eminence of the rat would contain the CRF of the order of 600 ng.

The modern concept on hypothalamic neurohumoral integration of adenohypophysial functions postulates the existence in the hypothalamus of several specific substances related to the secretion of corresponding pituitary tropic hormones. Among these postulated substances, corticotropin-releasing factor (CRF) was claimed for the first time by Saffran et al. (1955) and independently by Guillemin and Rosenberg (1955) to exist, as distinct from neurohypophysial hormones, in the posterior pituitary lobe. Although CRF in the neurohypophysis was proposed to be a peptide (Schally et al., 1960, 1962; Schally and Guillemin, 1963), the chemical nature of the hypothalamic CRF has not been clear yet (Guillemin, 1967), and therefore determination of dynamics of CRF activity in the hypothalamic tissue under varying conditions may chiefly depend upon the adequacy of an assay system employed for detecting this elusive activity. Several criteria necessary for the determination of CRF activity have been extensively discussed (Leeman et al., 1962: Guillemin, 1964) and Guillemin recommended strongly an adoption of an in vitro system along with an in vivo method for the validation of CRF, because it is well known that many non-specific stimuli may affect the intrinsic CRF activity of the assay animal.

In a previous paper (Hiroshige et al., 1968a), we offered a new assay method for CRF activity, i.e. intrapituitary microinjection of test materials into the adenohypophysis, exposed parapharyngeally, of dexamethasone-Nembutal blocked rats. This method is a modification of the original method of Hedge et al. (1966), to observe the direct effect of releasing substances on the anterior pituitary tissue as in the case of in vitro methods, yet to enable the gland respond in situ to test materials, thus avoiding artifacts due to the incubation in artificial media. With this assay method, we have tested in the present experiment ACTH-releasing potency of a puri-
Y = 28.3 log X - 10.0

b = 28.34

P < 0.001

Fig. 1. ACTH release following direct microinjection of pig hypothalamic CRF into the adenohypophysial tissue of dexamethasone-Nembutal blocked female rats. Change in plasma Cpd. B level during 20 min. period after the start of microinjection was considered as a measure for endogenous ACTH secretion and was given as Δ20. Numbers in parentheses indicate the number of animals. Bars at each point denote standard errors of the mean.

A purified pig hypothalamic CRF preparation. Comparison was also made of the potency between the purified CRF and crude acetic acid saline extracts of the median eminence of the rat.

MATERIALS AND METHODS

Female rats of Wistar strain, weighing between 170 and 220 g, were used in all experiments. They were housed at a constant ambient temperature of 20 ± 2°C and fed rat’s biscuit, with water given ad libitum.

In the microinjection assay method, use was made of direct injection of test materials in an amount of 0.4 μl into the adenohypophysis, exposed parapharyngeally, of dexamethasone-Nembutal blocked rats. Increases in plasma Cpd. B level (μg/100 ml) 20 mins. after the start of microinjection were considered as a measure for endogenous ACTH secretion and given as Δ20 in the text. The plasma corticosterone level was determined according to the method of Zenker and Bernstein (1958) with minor modifications. Details and validation of this assay method were given elsewhere (Hiroshige et al., 1968a).

A purified pig hypothalamic CRF preparation was kindly provided by Dr. A. V. Schally, VA Hospital, New Orleans, U.S.A. Crude acetic acid saline extracts of the median eminence were prepared in the following way: one median eminence was homogenized and extracted with 10 μl of ice-cold 0.01 N acetic acid saline solution for 1 hr. After centrifugation at 3000 rpm for 10 mins., an aliquot of 0.4 μl of the supernatant was used for direct microinjection. When
n necessary, two median eminences were used for each 10 µl extraction.

Statistical analysis was performed according to Bliss (1952).

RESULTS AND DISCUSSION

As shown in Figure 1, doses of the CRF ranging from 0.25 to 1 µg per one anterior pituitary lobe elicited a maximum response. A good log dose-response relationship was obtained in the dosage range from 12.5 to 50 ng injected per anterior pituitary lobe. As controls, acetic acid saline in the same amount was given. The relation of log doses of CRF (log X) to responses (Y) in terms of \( \Delta_{20} \) was given by an equation, \( Y = 28.3 \log X - 10.0, b = 28.3 \) (P<0.001). Since a significant CRF activity was demonstrated by this microinjection method in crude acetic acid extracts of the median eminence of the rat (Hiroshige et al., 1968a), the activity was then compared with that of the purified hypothalamic CRF at two dose levels as shown in Figure 2. Two dose factorial analysis showed that the difference in response between the purified CRF and median eminence extract at corresponding dosage levels was not significant and that parallelism was well maintained with a highly significant combined slope (P<0.001). Relative potency of the extract was calculated to be 102%. From these results it was estimated that the median eminence of the rat would contain the CRF of the order of 600 ng, an amount several times larger than that of vasopressin present in the same tissue, as McCann and Haberland (1959) reported the existence of 34 mU of vasopressin, i.e. around 100 ng in absolute amount. Although it is conceivable that hypothalamic tissue contains relatively large amount of CRF in view of its importance in the regulation of ACTH release, the above estimation remains essentially tentative, since the value may vary depending upon the purity of standard CRF preparation used.

Among several advantages of the present microinjection method for CRF activity, emphasis should be laid on the specificity as well as sensitivity. Schally et al. (1965) reported that in the morphine-Nembutal blocked rats CRF activity of vasopressin, on a weight basis, was similar to that of \( \beta \)-CRF, the most potent CRF preparation ever known. This was also shown by Arimura et al. (1967) in chlorpromazine-morphine-Nembutal blocked rats. Consequently, main problem in in vivo systems for assaying CRF activity in crude extracts appears to lie in the difficulties in separating the potency of CRF from that of vasopressin. Since we have already shown (Hiroshige et al., 1968b) that either of the posterior pituitary hormones, when placed directly into the adenohypophysis, is virtually devoid of CRF activity, an advantage of the direct microinjection method is apparent. As to the sensitivity of this method, comparison

\[ \text{Crude MEE (U)} \]

\[ \begin{align*}
1/25 & \quad 1/12.5 \\
1 & \quad 1 & \quad 1 & \quad 1 & \quad 1 & \quad 1 & \quad 1 \\
\text{S1} & \quad \triangle & \quad \circ & \quad \circ & \quad \triangle & \quad \circ & \quad \circ & \quad \triangle & \quad \circ & \quad \circ \\
\text{U1} & \quad \circ & \quad \triangle & \quad \circ & \quad \circ & \quad \triangle & \quad \circ & \quad \circ & \quad \triangle & \quad \circ & \quad \circ \\
\text{S2} & \quad \circ & \quad \triangle & \quad \circ & \quad \circ & \quad \triangle & \quad \circ & \quad \circ & \quad \triangle & \quad \circ & \quad \circ \\
\text{U2} & \quad \circ & \quad \triangle & \quad \circ & \quad \circ & \quad \triangle & \quad \circ & \quad \circ & \quad \triangle & \quad \circ & \quad \circ \\
\end{align*} \]

Fig. 2. Comparison of the potency between the purified CRF preparation (S) and crude acetic acid saline extracts (U) of the median eminence of the rat. Each point represents the mean value of six experiments.
of the minimum effective doses is relevant. Earlier, Guillemin et al. (1959) reported the minimum effective dose of their CRF fraction D, when given intravenously, to be of the order of 10 µg in morphine-Nembutal blocked rats and of 20 µg in hypothalamic lesioned animals. Later, Guillemin and Schally (1963) in an extensive review gave the minimum effective dose of their β-CRF to be 0.05 µg in in vitro and 0.1 µg in in vivo assay systems. Recently, Arimura et al. (1967) reported a significant increase in ACTH release following intravenous injection of 4 µg of pig hypothalamic CRF in their assay system using chlorpromazine-morphine-Nembutal blocked rats. The present results that direct placement of approximately 10 ng of similar preparation into the adenohypophysis elicited a significant ACTH release are therefore taken as evidence for much higher sensitivity of our intrapituitary microinjection method for assessing CRF activity.

REFERENCES