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Acta Medica Okayama

Volume 33, *Issue* 2 1979 *Article* 2

APRIL 1979

Specificity of cultured anterior pituitary cells in detecting corticotropin releasing factor(s): the effect of biologically active peptides and neurotransmitter substances on ACTH release in pituitary cell cultures.

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Abstract

Biologically active peptides and neurotransmitter substances were added to anterior pituitary cell cultures to examine the presence of corticotropin releasing factor (CRF)-like activity. Hypothalamic extract (HE) induced significant dose-related increase of ACTH, and the lowest effective dose was 0.01 HE/ml. Other tested substances including luteinizing hormone-releasing hormone, thyrotropin releasing hormone, melanocyte stimulating hormone release inhibiting factor, somatostatin, substance P, neurotensin, beta-endorphin. leu-enkephalin, met-enkephalin, bradykinin, norepinephrine, dopamine, serotonin, acetylcholine, histamine, gamma-amino butyric acid or gamma-hydroxy butyric acid showed no CRF-like activity. Relatively high doses of lysine vasopressin, arginine vasopressin and angiotensin II increased the release of ACTH in pituitary cell cultures, but the maximal ACTH response was markedly less than with HE. These results indicate that cultured anterior pituitary cells are sensitive and fairly specific in detecting CRF(s) comparing with other detecting procedures.

KEYWORDS: pituitary cell cultures, corticotropin releasing factor, ACTH, neuropeptides, neurotransmitter substances

*PMID: 38634 [PubMed - indexed for MEDLINE]
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Acta Med. Okayama 33, (1), 81—90 (1979)

SPECIFICITY OF CULTURED ANTERIOR PITUITARY CELLS IN DETECTING CORTICOTROPIN RELEASING FACTOR(S): THE EFFECT OF BIOLOGICALLY ACTIVE PEPTIDES AND NEUROTRANSMITTER SUBSTANCES ON ACTH RELEASE IN PITUITARY CELL CULTURES

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Received October 12, 1978

Abstract. Biologically active peptides and neurotransmitter substances were added to anterior pituitary cell cultures to examine the presence of corticotropin releasing factor (CRF)-like activity. Hypothalamic extract (HE) induced significant dose-related increase of ACTH, and the lowest effective dose was 0.01 HE/ml. Other tested substances including luteinizing hormone-releasing hormone, thyrotropin releasing hormone, melanocyte stimulating hormone release inhibiting factor, somatostatin, substance P, neurotensin, β-endorphin, leu-enkephalin, met-enkephalin, bradykinin, norepinephrine, dopamine, serotonin, acetylcholine, histamine, γ-amino butyric acid or γ-hydroxy butyric acid showed no CRF-like activity. Relatively high doses of lysine vasopressin, arginine vasopressin and angiotensin II increased the release of ACTH in pituitary cell cultures, but the maximal ACTH response was markedly less than with HE. These results indicate that cultured anterior pituitary cells are sensitive and fairly specific in detecting CRF(s) comparing with other detecting procedures.

Key words: pituitary cell cultures, corticotropin releasing factor, ACTH, neuropeptides, neurotransmitter substances

Little is known about the chemical structure and nature of corticotropin releasing factor (CRF), and no one has succeeded in developing a radioimmunoassay procedure for CRF. Therefore, many *in vivo* and *in vitro* assay procedures have been developed for CRF following the classical method of Saffran and Schally (1). For sensitivity and precision, the procedure utilizing monolayer cultured anterior pituitary cells seems to be one of the most useful methods (2), but little information is available on the specificity of cultured pituitary cells. The sensitivity and specificity of the bioassay are both important in studying CRF activity, because a lack of sensitivity results in a failure to detect real CRF

activity and a lack of specificity may produce a false positive CRF activity. In this report, many kinds of biologically active peptides and neurotransmitter substances were tested in pituitary cell cultures to investigate CRF activity and to examine the specificity of monolayer cultured anterior pituitary cells for CRF assay.

MATERIALS AND METHODS

Preparation of cultured pituitary cells. Charles River SD strain male rats, weighing 200-300 g, were decapitated. The anterior pituitaries were immediately removed, minced into small pieces and placed in Hank's-HEPES buffer. The pituitaries were then dispersed with enzyme using the procedure reported by Vale *et al.* (3) with modifications. The pituitaries were shaken at 37°C for 45 min with 10 ml Hank's-HEPES buffer containing 0.35% collagenase (GIBCO) and 1% bovine serum albumin (BSA). They were then dispersed with 10 ml Hank's-HEPES buffer containing 0.25% pancreatin and 1% BSA for 10 min. The dispersed pituitary cells were washed 5 times with 3 ml of sterile Dulbecco modified Eagle's medium (DMEM) (GIBCO) containing 10% horse serum, 2.5% fetal calf serum, 1% non-essential amino acid (GIBCO), fungisone (2.5 μg/ml), penicillin (50 U/ml) and streptomycin (50 μg/ml). The dispersed cells were resuspended in an appropriate volume of medium and were placed in sterile plastic Petri dishes (Falcon, 35×10 mm) containing serum-supplemented DMEM. Usually $2\text{-}5\times10^5$ cells per 1.5 ml medium were placed in a single dish, and 2-3 dishes were usually cultured from one pituitary. The dishes were cultured at 37°C for 4 days in an incubator with 95% air and 5% CO^2 and continuously supplied with water vapor.

Experiments using cultured cells. After 4 days of culture, cells were washed twice with fresh DMEM, and then DMEM containing the test substances was added for incubation. The test substances and their sources were: luteinizing hormone-releasing hormone (LH-RH), thyrotropin releasing hormone (TRH) and γ-amino butyric acid (GABA) (Daiichi Pharmaceutical Co.); melanocyte stimulating hormone release inhibiting factor (MSH-IF), substance P, neurotensin, β-endorphin, leu^5-enkephalin, met^5-enkephalin and bradykinin (Protein Research Foundation in Osaka); norepinephrine (NE), serotonin (5-HT), acetylcholine (ACh) and histamine (Wako Pure Chemical Industries, Ltd.); protaglandin E^1 (PGE^1), PGE^2 and γ-hydroxy butyric acid (GHB) (Ono Pharmaceutical Co); lysine vasopressin (LVP) (Sandoz); arginine vasopressin (AVP) (Sigma Chemical Co.); dopamine (DA) (Nakarai Chemicals, Ltd.) and angiotensin II (CIBA Ltd.). Somatostain was a gift from Prof. N. Yanaihara (Shizuoka Pharmaceutical College). Rat hypothalamic extract (HE) was obtained from NIH (NIAMDD-Rat-HE-RP-1). The HE was dissolved in 0.01 N-HCl, divided into small aliquots (5 HE/100 μl) and frozen until used. One HE means one median eminence extract (0.8 mg). All test substances were dissolved and diluted with DMEM immediately before use. Trasylol (1,000 U/dish) was added to all dishes to protect the test substance and ACTH from degradation. Test incubations were carried out in a total volume of 2.0 ml/dish for 3-4 h. Cells were incubated with DMEM alone

to determine the control level of ACTH release into the medium. In each assay, DMEM containing each dose of the test substance was incubated in a dish without cells to determine the amount of ACTH present in the test substance. After incubation, the medium from each dish was centrifuged for 5 min at 470 g to remove cell contamination, and then stored at −20°C for subsequent ACTH assay. The amount of ACTH contamination in the HE was substracted from the total ACTH released to determine the net quantity of ACTH released.

ACTH radioimmunoassay. ACTH in the culture medium was assayed by a sensitive radioimmunoassay employing an anti-porcine ACTH which reacted with 1-39 ACTH but did not cross react significantly with other pituitary hormones (4). The ACTH assay was performed directly on 50–100 μl of culture medium without prior extraction. Synthetic human 1-39 ACTH was used as a reference standard. Sample, buffer (0.05 M phosphate buffer containing 0.5% BSA, trasylol 400 U/ml and 0.4% mercaptoethanol, pH 7.6), antiserum and ^{125}I-ACTH (10,000 cpm) were mixed and incubated at 4°C for 72–96 h. Dextran coated charcoal was employed to separate bound and free ^{125}I-ACTH.

Statistical analysis was conducted with Duncan's new multiple range test.

RESULTS

Effect of hypothalamic extract (HE) on ACTH release. Fig. 1 shows the dose-response effect of HE on ACTH release from pituitary cells in culture. The lowest HE dose producing statistically significant ACTH release was usually 0.01 HE/ml, and a positive dose-response relationship was obtained between 0.01 and 0.5 HE/ml. We had observed that 0.2 HE evoked a similar amount of ACTH release on 4 through 13 days of culture (unpublished data).

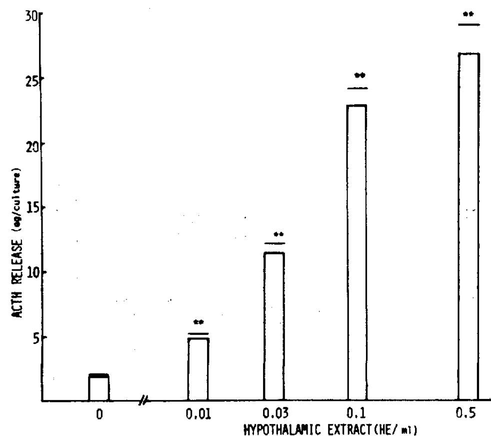

Fig. 1. Effect of hypothalamic extract (HE: NIAMDD-Rat-HE-1) on ACTH release in pituitary cell cultures. Three or four dishes were employed in each group. Histogram and bar represent the mean+SEM. All tested concentration of HE (0.01–0.5 HE/ml) evoked significant (**P<0.01) increase of ACTH.

Effects of biologically active peptides on ACTH release. TRH at a concentration of 100 pg/ml, 1 ng/ml and 10 ng/ml did not stimulate the release of ACTH. The same concentration of LH-RH did not stimulate ACTH release. TRH and LH-RH at a dose of 10 ng/ml evoked the maximal TSH and LH secretion, respectively, from cultured pituitary cells. Somatostatin (1 and 10 ng/ml) did not increase the release of ACTH (Fig. 2). LVP and AVP (1, 10 and 100m U/ml) evoked a statistically significant release of ACTH, but these induced increases were less than the increase induced by 0.1 HE/ml (Fig. 3). Neither β-endorphin, leuenkephalin nor met-enkephalin (1, 10 and 100 ng/ml) had corticotropin releasing activity in pituitary cell cultures (Fig. 4). Substance P (1, 10 and 100 ng/ml) did not stimulate ACTH release. Angiotensin II (10 ng/ml) showed a slight corticotropin releasing activity (Fig. 5). MSH-IF (1, 10 and

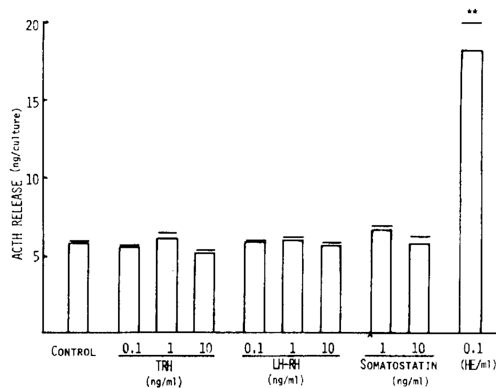

Fig. 2. Effect of TRH, LH-RH, and somatostatin on ACTH release in pituitary cell cultures. The HE-induced response was significantly higher compared with the control group (**P<0.01).

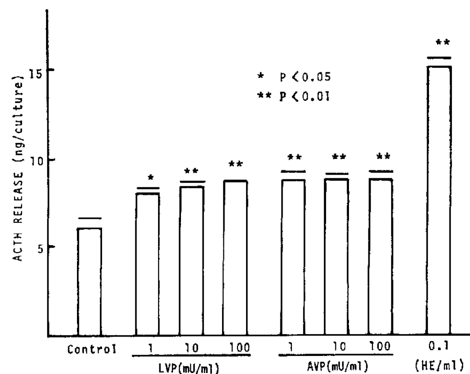

Fig. 3. Effect of LVP and AVP on ACTH release in pituitary cell cultures.

100 ng/ml) showed neither corticotropin releasing nor inhibiting activity. Neither neurotensin nor bradykinin had corticotropin releasing activity (Fig. 6).

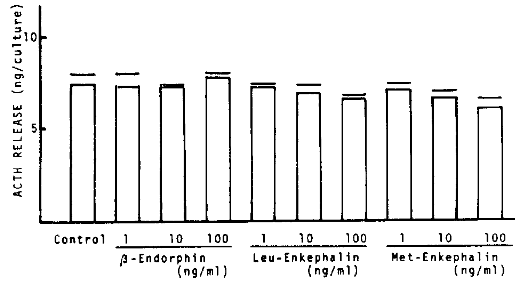

Fig. 4. Effect of β-endorphin, leu-enkephalin and met-enkephalin on ACTH release in pituitary cell cultures.

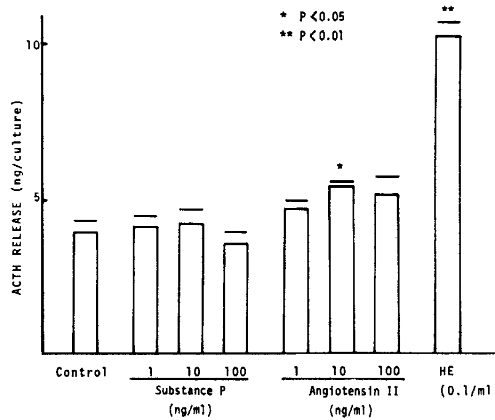

Fig. 5. Effect of substance P and angiotensin II on ACTH release in pituitary cell cultures.

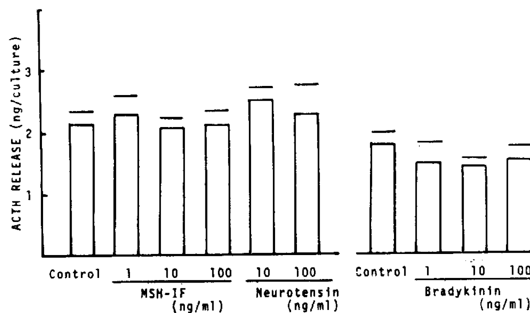

Fig. 6, Effect of MSH-IF, neurotensin and bradykinin on ACTH release in pituitary cell cultures.

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Effects of neurotransmitter substances on ACTH release. GABA and GHB at concentrations of 10 ng/ml, 100 ng/ml and 1 μg/ml stimulated the release of ACTH but the increases were not statistically significant. NE also had similar

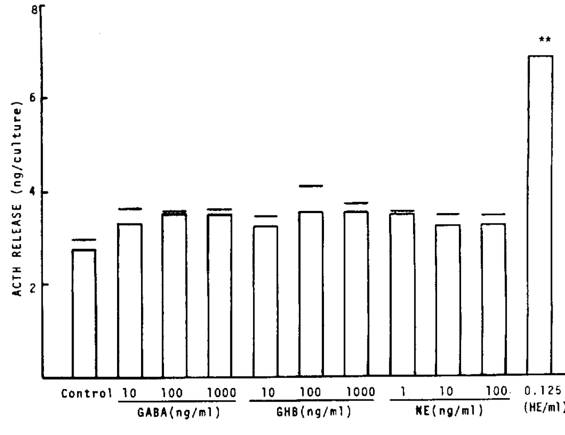

Fig. 7. Effect of GABA, GHB and norepinephrine (NE) on ACTH release in pituitary cell cultures. HE-induced response was markedly higher than the control group (**P<0.01).

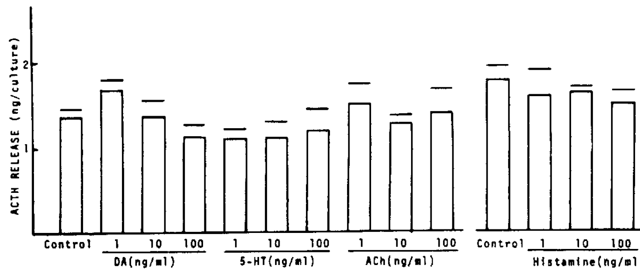

Fig. 8. Effect of dopamine (DA), serotonin (5-HT), acetylcholine (ACh) and histamine on ACTH release in pituitary cell cultures.

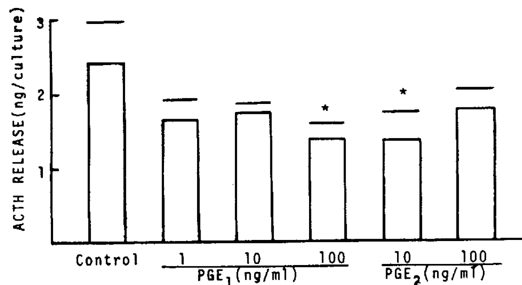

Fig. 9. Effect of PGE_1 and PGE_2 on ACTH release in pituitary cell cultures. PGE_1 at 100 ng/ml and PGE_2 at 10 ng/ml produce lower concentration of ACTH than in the control group (*P<0.05)

effects (Fig. 7). Neither DA, 5-HT, ACh nor histamine (1, 10 and 100 ng/ml) showed corticotropin releasing activity in pituitary cell cultures (Fig. 8). PGE[1] at 1, 10 and 100 ng/ml evoked decreased ACTH release (Fig. 9). PGE[2] had similar effects.

DISCUSSION

According to previous reports (5–7), vasopressin is the most potent substance with corticotropin releasing activity except for genuine CRF. However, Yasuda and Greer (8), using pituitary cell culture, reported that synthetic AVP or LVP were devoid of CRF activity, even with the largest tested dose. Our data here show that vasopressin induced a slight ACTH increase, and the maximal release of ACTH by vasopressin was markedly less than that induced by HE. This finding confirms some other reports (9–12). We observed using pituitary halves that LVP at 10 mU/ml evoked similar ACTH release to that induced by 0.1 HE/ml (4). The discrepancy between present and previous results may be explained by the better specificity of cultured pituitary cells. In our present study, LH-RH or TRH at 10 ng/ml which respectively stimulated maximal LH and TSH release were not effective in stimulating ACTH secretion. Somatostatin and MSH-IF which suppress growth hormone (GH) and MSH, respectively, had neither a stimulatory nor suppressive effect on ACTH secretion. These results indicate that this method is specific for detecting CRF activity.

Recently, other biologically active hypothalamic peptides have been found to affect the secretion of pituitary hormones. β-Endorphin administered intravenously or intracisternally stimulate GH and prolactin (PRL) secretion, but this agent did not stimulate GH and PRL secretion from the pituitary glands *in vitro* (13). Our study shows that β-endorphin and enkephalins do not stimulate ACTH secretion in pituitary cell cultures. Substance P which stimulates GH secretion in systemic administration (14, 15) did not stimulate ACTH release in our pituitary cell cultures. Vasoactive peptides which also exist in the hypothalamus were reported to increase ACTH secretion when administered *in vivo* (16). Angiotensin II showed a slight CRF-like activity but bradykinin did not show CRF-like activity in pituitary cell cultures. Maran and Yates (17) observed that intrapituitary infusion of angiotensin II caused the increase of cortisol in dogs, and concluded that it can be classified as a CRF. But our data show that CRF-like activity of angiotensin II is markedly less than that of hypothalmic CRF.

Jones *et al.* (18) reported that ACh and 5-HT induced a significant CRF release from hypothalamic fragments and that NE inhibited ACh and 5-HT -induced CRF release. Our study shows that neither ACh, 5-HT, NE nor DA showed significant corticotropin releasing activity in pituitary cell cultures.

Uemura *et al.* (19) observed using acutely dispersed cells that NE (4×10^{-4}M), 5-HT (3.3×10^{-5}M) and DA (2×10^{-4}M) caused a ACTH release. However, their findings might be non-physiological because the concentrations used were very high. We previously observed using pituitary halves that NE at 100–200 ng/ml stimulated ACTH release (4). The discrepancy between previous and present data might be also explained by the better specificity of cultured pituitary cells than pituitary halves. Histamine which evokes significant adrenal 17-OHCS increase in systemic administration (20) did not stimulate the release of ACTH in pituitary cell cultures. Other investigators have already examined the CRF-like activity of several substances using other CRF assay methods. De Wied *et al.* (21) observed that PGE^1 and PGE^2, angiotensin II, AVP and carbacoal stimulated the release of ACTH in some *in vivo* CRF assay methods, but these agents did not stimulate ACTH release by the *in vitro* assay method utilizing anterior pituitary glands. Buckingham and Hodges (22) examined the corticotropin releasing activity of various neurotransmitter substances using adenohypophyseal segments *in vitro*. In these *in vitro* studies, however, they tested only high doses of these substances. In our present study, we used concentrations nearer the physiological level of the test substances in sensitive cultured pituitary cells, and some substances showed CRF-like activity, but further investigation is necessary to determine whether these substances really possess corticotropin releasing activity or that the results merely reflect the low specificity of this method. Hiroshige and Abc (23) reported using the sensitive *in vivo* assay system of intrapituitary injection that NE, epinephrine, DA and 5-HT did not show CRF-like activity, but that vasopressin, histamine and spermidine stimulated ACTH release.

Sensitive bioassay methods usually have low specificity. But the above results show that the method utilizing cultured anterior pituitary cells is not only sensitive but specific enough for detecting CRF compared with other methods (9, 10, 21, 22).

Many of our tested substances did not show corticotropin releasing activity in pituitary cell cultures. But this does not necessarily mean that these substances have no effect on the hypothalamo-hypophyseo-adrenocortical axis. They could have an effect on hypothalamic CRF release or on CRF-induced release of ACTH from the pituitary.

The use of crude HE for the reference preparation rather than pure CRH has the disadvantage that such an extract may contain vasopressin and other substances which possess CRF-like activity. However, 0.01–0.1 HE probably dose not contain enough of these substances to stimulate the release of ACTH.

Acknowledgment: The authors are grateful to Professor Noboru Yanaihara for the generous gift of somatostatin.

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