PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 159, 6–11 (1978)

The Role of Cyclic AMP in CRF-Induced ACTH Secretion[1] (40272)

RONALD PORTANOVA AND W. J. BRATTIN

Department of Physiology, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106

Experiments in this (1) and other (2–4) laboratories have shown that cyclic-3′,5′-adenosine monophosphate (cyclic AMP) and its derivatives stimulate the secretion of ACTH. Recently, we have reported that the stimulation of ACTH secretion by hypothalamic median eminence-corticotrophin releasing factor (HME-CRF) is associated with a concomitant increase in adenylate cyclase activity; however, cordycepin (3′-deoxyadenosine) at sufficient concentration to reduce adenylate cyclase activity to undetectable levels, reduces but does not abolish the HME-CRF induced secretion of the hormone (5). These data suggest that while cyclic AMP may be involved in CRF-stimulated ACTH secretion, the cyclic nucleotide may not act as an obligatory intermediate, but rather may act to potentiate secretion. The experiments described in the present communication were designed to provide further information on this hypothesis.

Materials and methods. The techniques used in the preparation and incubation of isolated pituitary cells have been described in detail elsewhere (6, 7). In brief, anterior pituitary glands were removed from male Sprague-Dawley rats which had been adrenalectomized 14–28 days prior to sacrifice, and were maintained after adrenalectomy on 0.9% saline drinking solution without steroid hormone replacement. Cells were dispersed from the glands by mechanical agitation in Krebs-Ringer bicarbonate (KRB) buffer containing 0.2% glucose and 0.25% trypsin. After dispersion, cells were collected by centrifugation and resuspended in KRB buffer containing 0.2% glucose and 0.5% bovine serum albumin (KRBGA), plus 0.1% lima bean trypsin inhibitor. Aliquots (0.9 ml) of cell suspension were incubated for various times together with appropriate combinations of HME-CRF, N^6,O^2-dibutyryl-cyclic AMP (DBC), corticosterone, or vehicle (controls). At the end of the incubation period, cells were removed by centrifugation, and the incubation medium was acidified, appropriately diluted, and assayed for ACTH. In most cases, the samples were bioassayed according to the isolated adrenal cortex cell technique described by Sayers *et al.* (8), using synthetic ACTH 1-24 (Cortrosyn, Organon) as standard. In one experiment (employing concentrations of DBC greater than 1 m*M*, see Fig. 5), in order to circumvent the problem of direct DBC stimulation of steroidogenesis by isolated adrenal cells, pituitary cell incubation medium was assayed for ACTH by a radioimmunoassay (RIA) technique. Rabbit anti-human ACTH serum was purchased from Burroughs-Wellcome, and ^{125}I-ACTH 1-24 was obtained from Amersham. Samples or standards (ACTH 1-24, Cortrosyn, Organon) were incubated with immune serum in 0.1 *M* sodium phosphate (pH 7.4), for 20 hr (4°), at which time ^{125}I-ACTH was added and the incubation was continued for 6 additional hr. Un-bound ^{125}I-ACTH was adsorbed to charcoal, collected by centrifugation, and counted in a Packard auto-gamma spectrometer. The method appears valid as judged by several criteria: (a) both extracts of pituitary cells and samples of pituitary cell incubation media gave log dose-displacement curves parallel to synthetic ACTH 1-24; (b) a number of polypeptides, including ACTH 5-10, ACTH 5-13, and α-MSH, showed no significant cross-reactivity; and (c) analysis of samples of pituitary cell extracts or incubation media by bioassay and RIA gave essentially identical values. In all experiments, the ACTH content of control incubates was determined and subtracted from that of incubates receiving test substance(s). In each experiment, data obtained from incubates receiving identical treatments were pooled, and means and standard errors of the means (SEM) were calculated. Statistical significance was assessed by means of Student's *t* test.

Extracts of rat hypothalamic median emi-

[1] This work was supported by USPHS Grant No. AM-13820-08.

0037-9727/78/1591-0006$01.00/0

nence tissue (HME-CRF) were prepared by homogenizing freshly excised ventral hypothalamic-median eminence tissue in 0.2 *M* acetic acid. Insoluble material was removed by centrifugation (20,000*g*, 15 min), and was reextracted twice with 0.2 *M* acetic acid. The extracts were combined and stored frozen. For use, a portion of the extract was adjusted to pH 7.0, appropriately diluted (with KRBGA) and added to the incubates in a volume of 0.1 ml. Doses of HME-CRF are expressed as tissue equivalents (i.e., fractions of an HME), which in these experiments had a wet weight of approximately 15 mg. Corticosterone (Sigma) in 0.9% saline plus 2.5% methanol, was added to appropriate incubates in a volume of 10 μl. DBC (Sigma) was added to appropriate incubates in a volume of 0.1 ml of KRBGA.

Results. Both HME-CRF and DBC stimulate the secretion of ACTH by isolated pituitary cells (Fig. 1), and at the concentrations tested (0.2 HME/ml, 1 m*M* DBC) the ACTH secretory responses are nearly identical (150 pg/min/10^5 cells). This concentration of DBC (1 m*M*) in the medium did not interfere in the subsequent steroidogenic bioassay for ACTH, as shown by the fact that addition of DBC at the end of the incubation period with HME-CRF does not significantly alter the response from that of HME-CRF alone. When pituitary cells are exposed to DBC throughout the exposure to HME-CRF, the rate of ACTH secretion is markedly enhanced. The rate of hormone secretion in the presence of HME-CRF plus DBC (575 pg/min/10^5 cells) is almost twice that expected if the response to the two agents were simply additive. As shown in Fig. 2, the potentiating effect of DBC on HME-CRF induced ACTH secretion occurs without an obvious time-lag and persists throughout the duration of a 45 min incubation. The data in Fig. 3 indicate that the exposure of pituitary cells to DBC potentiates HME-CRF induced ACTH secretion, even if the cyclic nucleotide is removed prior to addition of HME-CRF. In these experiments cells were preincubated for 15 min in the presence or absence of DBC (1 m*M*) and then challenged with HME-CRF in the presence or absence of DBC (1 m*M*). HME-CRF induced ACTH secretion by cells exposed to DBC was more than twice that of

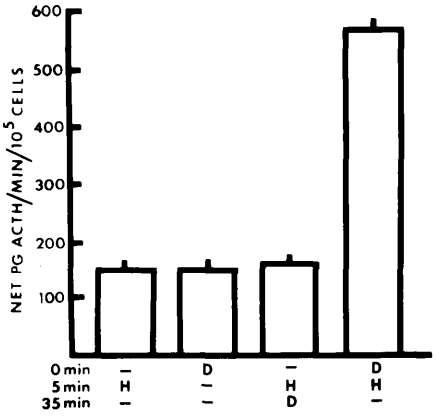

FIG. 1. Interaction of CRF and DBC on ACTH secretion. Isolated pituitary cells prepared from adrenalectomized rats were incubated for 35 min. Substances added, and their time of addition during this interval, are indicated below each bar: H, HME-CRF (.2 HME/ml); D, DBC (1 m*M*). Secretory rates are for the 30 min-period following the addition of HME-CRF; vertical lines represent combined SEM of pituitary and adrenal assays (N = 8).

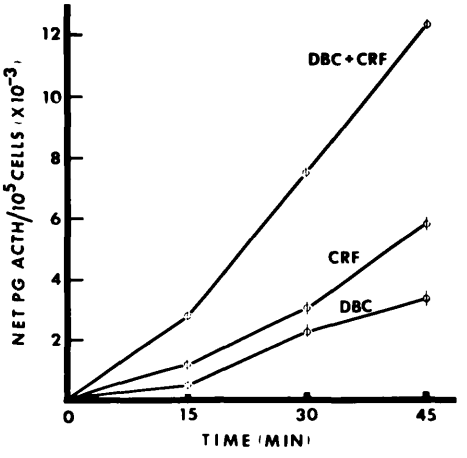

FIG. 2. Interaction of CRF and DBC on ACTH secretion; time course. Pituitary cells were incubated for indicated times in the presence of: DBC (1 m*M*), HME-CRF (.2 HME/ml), or DBC (1 m*M*) plus HME-CRF (.2 HME/ml). The ACTH content of control incubates (920 ± 42 pg/10^5 cells, mean ± SEM, N = 10) did not change from 15 to 45 min, and has been subtracted from the experimental values presented. Vertical lines represent combined SEM of pituitary and adrenal assays (N = 4).

cells which were not exposed to DBC, irrespective of whether the cyclic nucleotide was present during the preincubation only, the incubation with HME-CRF only, or both the

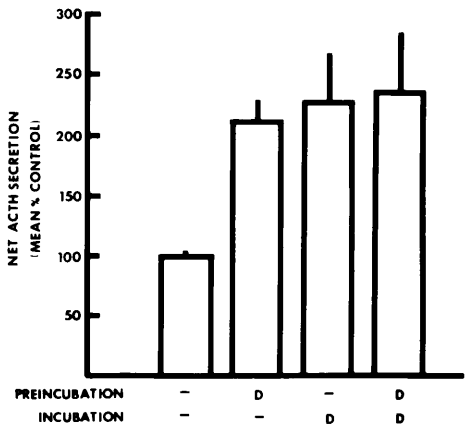

FIG. 3. Effect of time of addition of DBC on CRF induced ACTH secretion. Isolated pituitary cells were preincubated (15 min) in the presence or absence of DBC (1 m*M*); the cells were collected by centrifugation, washed with KRBGA, recollected by centrifugation, and resuspended in KRBGA. Aliquots of both types of cells were then incubated (30 min) with HME-CRF (.2 HME/ml) in the presence or absence of DBC (1 m*M*). The presence of DBC (D) during the preincubation and incubation periods is indicated beneath each bar. Data are expressed as the percentage of the secretory rate of cells which were not exposed to DBC (control); vertical lines represent SEM of the normalized secretory rates (N = 10).

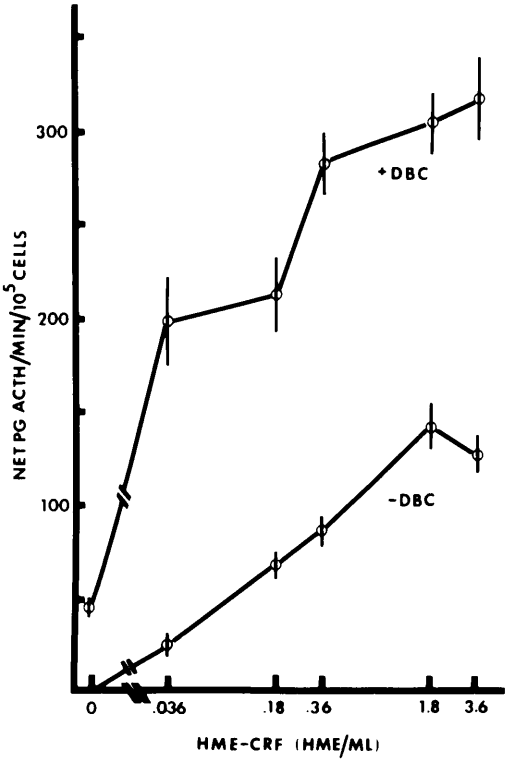

FIG. 4. Effect of DBC on ACTH secretion in response to graded doses of CRF. Isolated pituitary cells were incubated for 10 min in the presence or absence of DBC (1 m*M*); graded doses of HME-CRF were then added and the incubation was continued for an additional 30 min. Data presented are net pg ACTH secreted; vertical lines represent combined SEM of pituitary and adrenal assays (N = 8).

preincubation and the incubation.

The experiments described above demonstrate the interaction of submaximal doses of DBC and HME-CRF. In order to determine whether these secretagogues also interact at maximal dose levels, two experiments were performed. First, isolated pituitary cells were exposed to graded doses of HME-CRF in the presence or absence of DBC (Fig. 4). In the absence of DBC, maximum ACTH secretion is noted at a concentration of about 1.8 HME-CRF/ml. In the presence of DBC (1 m*M*), the secretory response to each dose of HME-CRF is increased more than twofold, even at maximum doses of HME-CRF. In the second experiment, isolated pituitary cells were exposed to graded doses of DBC in the presence or absence of HME-CRF (Fig. 5). In the absence of HME-CRF, maximum ACTH secretion is produced at a concentration of about 10 m*M* DBC. In the presence of HME-CRF (0.4 HME/ml), the secretory response is more than doubled at each dose of DBC, including the maximal doses.

Previous findings in our laboratory have shown that the secretion of ACTH by isolated pituitary cells in response to a variety of secretagogues, including DBC, is inhibited by corticosterone (9). We therefore carried out an experiment to determine if the potentiating effect of DBC on HME-CRF stimulated ACTH secretion is also inhibited by steroid. Pituitary cells were incubated for 30 min in the presence or absence of corticosterone (0.1 μg/ml) and were then stimulated (for 30 additional min) with either HME-CRF (0.2 HME/ml), DBC (1 m*M*), or HME-CRF (0.2 HME/ml) plus DBC (1 m*M*). The data in Fig. 6 show, as expected, that in the absence of exposure to corticosterone, both HME-CRF and DBC stimulate the secretion of ACTH, and HME-CRF stimulated secretion is potentiated by DBC. When the cells are exposed to corticosterone, ACTH secretion

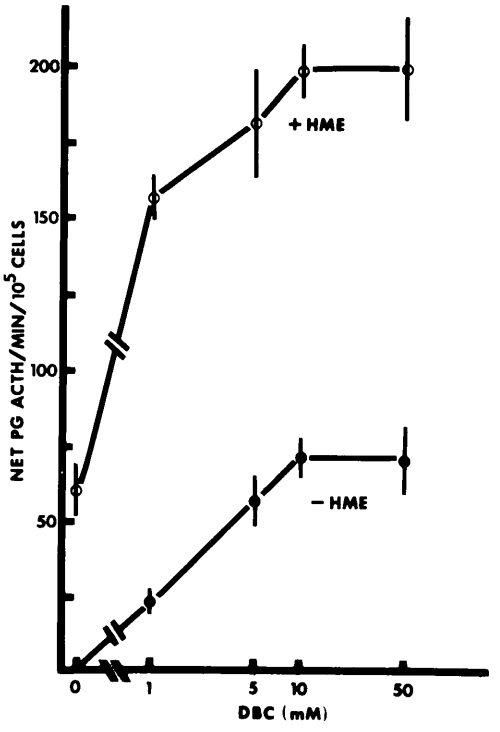

FIG. 5. Effect of HME-CRF on ACTH secretion in response to graded doses of DBC. Isolated pituitary cells were incubated for 30 min with various doses of DBC in the presence or absence of HME-CRF (0.4 HME/ml). ACTH was determined by RIA; data presented are net pg ACTH secreted. Vertical lines represent combined SEM of pituitary and radioimmune assays (N = 6).

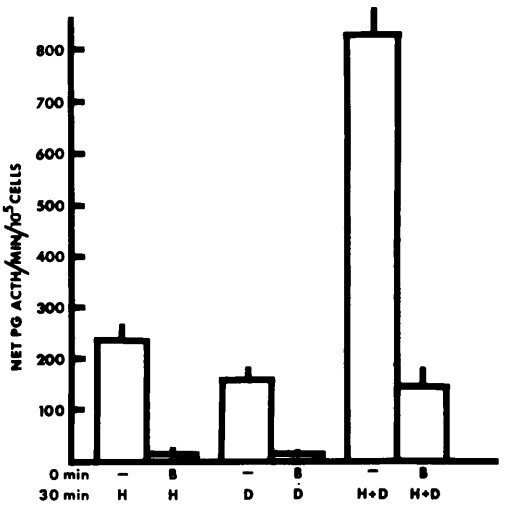

FIG. 6. Interaction of CRF and DBC on ACTH secretion; effect of corticosterone. Isolated pituitary cells were incubated for 60 min. Substances added, and their time of addition during this interval, are indicated below each bar; H, HME-CRF (.2 HME/ml); D, DBC (1 m*M*); B, corticosterone (0.1 μg/ml). Secretory rates are for the 30 min-period following the addition of HME-CRF; vertical lines represent combined SEM of pituitary and adrenal assays (N = 8).

stimulated by either agent acting singly is nearly abolished; ACTH secretion stimulated by HME-CRF and DBC in combination is also markedly inhibited, but is still significantly greater than that induced by HME-CRF ($P < .01$) or DBC ($P < .01$), acting alone.

Discussion. Several lines of evidence suggest an involvement of cyclic AMP in the intracellular mechanisms which regulate ACTH secretion. Cyclic AMP and its derivatives have been found to stimulate the secretion of ACTH both *in vivo* and *in vitro* (1–4). Inhibitors of cyclic nucleotide phosphodiesterase stimulate the secretion of ACTH (4) or act synergistically with other secretagogues of the hormone (2), presumably elevating the intracellular level of cyclic AMP. Recently, we observed that addition of HME-CRF to suspensions of isolated pituitary cells produces an increase in adenylate cyclase activity, concomitant with an increase in the rate of ACTH secretion (5). A stimulation of rat pituitary adenylate cyclase activity has also been reported in response to crude extracts of ovine hypothalamus (10) and vasopressin (4), an agent which is distinct from hypothalamic CRF but nevertheless stimulates the secretion of ACTH (11). These observations are all consistent with the notion that cyclic AMP is involved in the process which mediates ACTH secretion, but they provide no information as to the role of the cyclic nucleotide in this process. In this regard it is well to stress that although a large number of secretagogues of ACTH are known, no "authentic" hypothalamic CRF is yet available in pure form. Experiments employing crude extracts of hypothalamus (including those reported above) are limited in that responses observed may be the consequence of the interaction of several factors. Indeed, it is for this reason that little is known of the cellular and molecular processes which mediate ACTH secretion or the biochemical pathways by which these processes are regulated.

If the role of cyclic AMP in ACTH secretion is that of an obligatory "second messen-

ger", then it would be expected than an agent which inhibits adenylate cyclase activity would interfere with secretion of the hormone. Cordycepin has been found to be an inhibitor of adenylate cyclase activity in fat cell membranes (12) and guinea pig lung (13). Experiments in our laboratory (5) have shown that a dose of cordycepin sufficient to reduce adenylate cyclase activity to undectable levels in isolated pituitary cells only partially reduces the rate of HME-CRF induced ACTH secretion. We interpreted these data to mean that while cyclic AMP may indeed be involved in CRF-stimulated ACTH secretion, the cyclic nucleotide may not act as an olbigatory intermediate but rather may act to potentiate secretion. Sundberg *et al.* (14) have advanced a similar proposal with respect to the role of cyclic AMP in the secretion of several other adenohypophysial hormones.

The data presented in this communication are consistent with this view. DBC potentiates HME-CRF induced ACTH secretion both at submaximal and maximal doses of HME-CRF (Fig. 4), and HME-CRF potentiates DBC induced ACTH secretion both at submaximal and maximal doses of DBC (Fig. 5). The mechanism of the interaction between HME-CRF and DBC is unknown. Potentiation occurs without an apparent lag period and persists for at least 30–45 min (Fig. 2). Significantly, pretreatment of isolated pituitary cells with DBC (followed by removal of the cyclic nucleotide prior to exposure to HME-CRF) potentiates the secretory response to HME-CRF to as great a degree as does exposure to HME-CRF in the presence of DBC (Fig. 3). This finding does not rule out the possibility that cyclic AMP has been sequestered within the cells during the pretreatment period, and subsequently potentiates hormone secretion during exposure to HME-CRF. Alternatively, this finding is consistent with the view that the potentiating effect following DBC pretreatment may represent a physical and/or chemical change in the cell which is exerted after the cyclic nucleotide has been removed.

The data in Fig. 6 dramatically illustrate the potent inhibitory effect of corticosterone on ACTH secretion. At concentrations (0.1 μg/ml) within the physiological range, the steroid markedly suppresses hormone secretion in response to HME-CRF and DBC, acting singly or in combination. These findings indicate that whatever the role of cyclic AMP in ACTH secretion, the site of the inhibitory action of the steroid is distal to the appearance of the cyclic nucleotide.

In conclusion, the data of the present communication support the hypothesis that cyclic AMP acts within corticotrophs to potentiate CRF-induced ACTH secretion. However, our previous findings (5) indicate that an increased level of cyclic AMP is not *required* for ACTH secretion to occur. Taken together these data suggest that CRF has (at least) two actions on the corticotroph: (a) the initiation of the series of events which eventuates in secretory granule exocytosis, and (b) an elevation of cyclic AMP levels within the cell which then facilitates (through unknown mechanisms) the secretory process.

Summary. ACTH secretion by isolated pituitary cells is stimulated both by HME-CRF and DBC, and when given in combination, the two secretagogues interact synergistically. Although the mechanism of this interaction is unknown, the potentiating effect of DBC is displayed without an apparent time lag and persists after removal of the cyclic nucleotide. Corticosterone inhibits ACTH secretion induced by HME-CRF and DBC, acting alone or in combination. The implications of these findings are discussed.

The authors are grateful to Beth Wiblin Portanova and James Roe for their expert technical assistance.

1. Portanova, R., and Sayers, G., *in* "Brain-Pituitary-Adrenal Interrelationships" (A. Brodish and E. S. Redgate, eds.) p. 319. S. Karger, Basel, Switzerland (1973).
2. Fleischer, N., Donald, R. A., and Butcher, R. W., Amer. J. Physiol. **217,** 1287 (1969).
3. Hedge, G. A., Endocrinology **89,** 500 (1971).
4. Vale, W., and Rivier, C., Fed. Proc. **36,** 2094 (1977).
5. Brattin, W. J., and Portanova, R., Mol. Cell. Endo. **9,** 279 (1978).
6. Portanova, R., Smith, D. K., and Sayers, G., Proc. Soc. Exp. Biol. Med. **133,** 573 (1970).
7. Portanova, R., Proc. Soc. Exp. Biol. Med. **140,** 825 (1972).
8. Sayers, G., Swallow, R. L., and Gioradano, N. D., Endocrinology **88,** 1063 (1971).
9. Sayers, G., and Portanova, R., *in* "Handbook of Physiology" (R. O. Greep and E. B. Astwood, eds.), Sect. 7, Vol. 6, p. 41 (1975).

10. Zor, V., Kaneko, T., Schnieder, H. P. G., McCann, S. M., and Field, J. B., J. Biol. Chem. **245,** 2883 (1970).
11. Portanova, R., and Sayers, G., Proc. Soc. Exp. Biol. Med. **143,** 661 (1973).
12. Fain, J. N., Pointer, R. H., and Ward, W. F., J. Biol. Chem. **247,** 6866 (1972).
13. Weinryb, I., and Michel, I. M., Biochem. Biophys. Acta **334,** 218 (1974).
14. Sundberg, D. K., Fawcett, C. P., and McCann, S. M., Proc. Soc. Exp. Biol. Med. **151,** 149 (1976).

Received January 3, 1978. P.S.E.B.M. 1978, Vol. 159.