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Arginine[8] Vasopressin Potentiates the β-Endorphin-Releasing Activity of Ovine Corticotropin-Releasing Factor (oCRF) *in Vitro* (41642)

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Abstract. The ability of arginine[8] vasopressin (AVP) to potentiate the β-endorphin-releasing activity of synthetic ovine corticotropin releasing factor (oCRF) was examined using an anterior-pituitary quarter assay. Both AVP and oCRF stimulated the release of β-endorphin immunoreactivity (β-END-I) in a dose-dependent manner, with AVP being approximately 10 times less effective than oCRF. Marked potentiation of β-END-I release was observed when pituitary quarters were incubated in the presence of a combination of 0.5 n*M* oCRF and 1.0 n*M* AVP. Further potentiation was not observed when the higher doses of 1.0 n*M* oCRF and 2.0 n*M* AVP were tested in combination; however, maximal β-END-I release may have been attained by the addition of 1.0 n*M* oCRF alone. These results suggest that AVP may play a role in the mediation of β-endorphin release from the adenohypophysis.

Recently, Vale *et al.* (1) reported the structure of a 41-amino acid corticotropin-releasing factor (oCRF) isolated from ovine hypothalamus. Synthetic oCRF possesses potent, dose-related adrenocorticotropin (ACTH) and β-endorphin-releasing activity in monolayer rat (1) and human (2) pituitary cell cultures, rat pituitary quarter assay (3), and *in vivo* (3, 4) at concentrations within the effective range of other hypothalamic releasing hormones. The failure of oCRF to appreciably alter the secretion of other anterior pituitary hormones, both *in vivo* and *in vitro* (3), demonstrates its specificity. In addition, immunoreactive oCRF has been measured in rat hypophysial portal plasma at a concentration comparable to other known releasing factors (5). Although less potent than oCRF, arginine[8] vasopressin (AVP) has also been shown to specifically stimulate release of ACTH and β-endorphin in a dose dependent manner (3, 6).

Early studies to determine the nature of CRF demonstrated that partial purification of hypothalamic extracts resulted in a significant loss of ACTH-releasing activity (7–10). Recombination of gel chromatography fractions possessing weak ACTH-releasing activity with fractions containing immunoreactive AVP (8–10), or the addition of synthetic AVP to those fractions (10), was found to significantly potentiate CRF activity to levels similar to those obtained with crude hypothalamic extracts. Similarly, extracts of hypothalami from the genetically AVP-deficient Brattleboro rat have been demonstrated to contain significantly less CRF activity than extracts from normal rats (11, 12), although conflicting data have been reported (13). Recently, we reported that AVP significantly potentiated the ACTH-releasing activity of the synthetic oCRF in an *in vitro* pituitary assay (14). These results have since been confirmed by others (15). Since it has been established that ACTH and β-endorphin are simultaneously released in response to a number of stressful stimuli (16, 17), pathologic states (16), and secretegogues (4, 6), it seems likely that AVP may also potentiate the β-endorphin releasing activity of oCRF. The present study examines this possibility of synergism between AVP and oCRF in stimulating β-endorphin release.

Materials and Methods. *In vitro quarter pituitary assay.* Adult, male CD strain rats weighing 120–135 g (Charles River Corp., Wilmington, Mass.) were maintained on a 12:12-hr light:dark cycle at constant temperature (24 ± 2°C) and had access to food and water *ad libitum.* Synthetic oCRF and AVP were a gift of Dr. M. Shimizu (Penninsula Laboratories, San Carlos, Calif.) Synthetic luteinizing hormone-releasing hormone

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(LHRH) and thyrotropin-releasing hormone (TRH) were a gift of Takeda Chemical Industries, Ltd. (Osaka, Japan). All peptides were dissolved in Krebs–Ringer bicarbonate buffer containing 200 mg% glucose and 250 mg% bovine serum albumin (KRBG) immediately prior to assay.

β-Endorphin-releasing activity was assessed using the *in vitro* quarter pituitary system as described by Arimura and Schally (18). In brief, rats were decapitated, the neurohypophysis discarded, and the adenohypophysis removed and washed in KRBG. Following quartering, the pituitary fragments were randomly distributed at four quarters/10-ml beaker and preincubated in KRBG for two 1.5-hr periods with the medium changed after each incubation. The quarters were then incubated for 1 hr in 1 ml KRBG to provide basal β-endorphin-release data. The medium from this incubation was collected in test tubes on ice and replaced with 1 ml KRBG containing test substances. After incubation for 1 hr, this medium was also collected. All incubations were conducted in a Dubnoff metabolic incubator at 37°C under an atmosphere of 95% oxygen, 5% carbon dioxide. Due to the inherent variability in the magnitude of response observed between successive bioassays of this type, all treatments within an experiment were examined within the same assay.

Experiment I: release of β-endorphin in response to oCRF or AVP. During the second incubation, pituitary quarters were exposed to KRBG alone or containing 0.1, 1.0, or 10 n*M* oCRF or 0.1, 1.0, or 10 n*M* AVP. One group was incubated with 1.0 n*M* LHRH and TRH to serve as an additional control for specificity. Each treatment group consisted of four replicate beakers.

Experiment II: potentiation of oCRF activity by AVP. Pituitary quarters were incubated with KRBG alone or with 1.0 n*M* AVP, 0.5 n*M* oCRF, 1.0 n*M* AVP + 0.5 n*M* oCRF, 2.0 n*M* AVP, 1.0 n*M* oCRF or 2.0 n*M* AVP + 1.0 n*M* oCRF. Each treatment group consisted of four replicate beakers.

RIA of β-endorphin. Synthetic β-endorphin used for preparation of standards and tracer was a gift of Dr. D. H. Coy (Tulane University School of Medicine and V.A. Hospital, New Orleans, La.). Rabbit anti-β-endorphin serum (gift of Dr. W. Y. Chey, Genesee Hospital, Rochester, N.Y.) was produced by monthly immunizations of 0.5 mg/rabbit of β-endorphin conjugated to bovine serum albumin (BSA) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl. The antiserum shows no cross-reaction with either met- or leu-enkephalin or dymorphin, but does cross-react with β-lipotropin (β-LPH) by 35% (Chang T, Chey WY, Erway B, Coy D. Radioimmunoassay of β-endorphin. Manuscript in preparation). The observed cross-reaction is understandable as β-LPH contains the amino acid sequence of β-endorphin (15). In addition, this antiserum shows no cross-reaction with either $ACTH_{1-24}$ (gift of Ciba–Geigy, Basal, Switzerland) or the NIAMDD rat ACTH reference preparation (gift of NIAMDD, NIH, Bethesda Md.)

Iodination of β-endorphin was accomplished by two consecutive additions of 10 μl chloramine T (0.4 mg/ml 0.01 *M* phosphate, pH 7.5) to 3 μg β-endorphin (1 μg/10 μl 0.001 *M* HCl), 25 μl 0.1 *M* phosphate (pH 7.5), and 1 mCi $Na^{125}I$ (Amersham Corp., Arlington Heights, Ill.), each followed by a 10-sec period of gentle mixing. Following the second 10-sec incubation period, 100 μl sodium metabisulphite (2.0 mg/ml 0.01 *M* phosphate, pH 7.5) was added, followed immediately by the addition of 1 ml 20% human serum albumin and the transfer of the reaction mixture to a tube containing 15 mg Quso G-32 (P Q Corp., P.O. Box 840, Valley Forge, Pa.). The reactant–Quso mixture was centrifuged at 2500 rpm for 10 min, the supernatant discarded, and the pellet washed twice with ice-cold, deionized, distilled water. After the second wash, the Quso was pelleted by centrifugation, the supernatant discarded, and the ^{125}I-β-endorphin eluted by resuspending the pellet in an ice-cold 60:40:1 mixture of water:acetone:glacial acetic acid. Specific activity of the tracer obtained by this procedure was consistently in the range of 38–380 μCi/μg β-endorphin.

The buffer used as diluent for assay and reagents consisted of 0.01 *M* phosphate, 0.14 *M* sodium chloride, 0.025 *M* ethylenediaminetetraacetic acid (EDTA), 0.02% sodium azide, 0.25% BSA, 0.001% bacitracin, and 0.04% β-mercaptoethanol at a pH of 7.4. Rabbit anti-β-endorphin serum was utilized in the

assay at a final dilution of 1:56,000. ^{125}I-β-endorphin was added at a concentration of 11,000–13,000 cpm/tube. All reagents were added simultaneously, vortexed, and incubated at room temperature for 4 hr. To separate bound from free hormone, 1 ml 1.5% charcoal, 1.0% dextran, and 0.25% BSA in 0.01 *M* phosphate, 0.14 *M* sodium chloride, and 0.025 *M* EDTA were added to each tube, vortexed, incubated for 10 min, and centrifuged at 3500 rpm for 30 min. The supernatant was decanted off and counted. Analysis of assay results and interpolation of unknown values was accomplished using a computer program which utilized log-logit transformation to produce a linear dose–response curve. The minimal detectable dose, defined as antigen concentration at the lower 95% confidence limit of the buffer control, was 2.0 pg β-endorphin/tube. The standard curve obtained with synthetic β-endorphin extended from 2.0–1000 pg/tube. The intra-assay coefficient of variation was 1.6% at 45 pg β-endorphin/tube (N = 10) and 8.1% at 600 pg β-endorphin/tube (N = 10). All samples were examined in duplicate within the same assay.

Statistical analysis. The results from each experiment were expressed as percentage basal β-endorphin immunoreactivity (β-END-I) released during the second incubation relative to β-END-I released during the first incubation. Statistical methods consisted of analysis of variance followed by Student–Newman–Keuls multiple range test to determine significant differences between treatment groups. The potentication experiment was further analyzed as a 2 × 2 factorial to test for significant interaction between AVP and oCRF.

Results. *Experiment I: β-END-I release in response to oCRF or AVP.* The mean basal level of β-END-I released during 1 hr in this experiment was 3.86 ± 0.20 ng/ml (mean ± SEM) and did not significantly differ between treatment groups. The results of adding increasing doses of oCRF or AVP to the media during the second incubation are illustrated in Fig. 1. Incubation of pituitary quarters in the presence of diluent alone resulted in a slight but nonsignificant decrease in the release of β-END-I. Similar results were obtained after the addition of 1.0 n*M* LHRH and TRH. Incubation in the presence of oCRF resulted in a dose-related increase in the release of β-END-I. Although 0.1 n*M* oCRF did not appreciably alter β-END-I release, addition of 1.0 and 10 n*M* oCRF significantly increased β-END-I release by approximately four- and ninefold, respectively, over the levels observed with diluent alone.

Incubation of pituitary quarters in the presence of 0.1 and 1.0 n*M* AVP produced results similar to those obtained with diluent controls. Only 10 n*M* AVP significantly stimulated β-END-I secretion, causing an approximately fourfold greater release than diluent

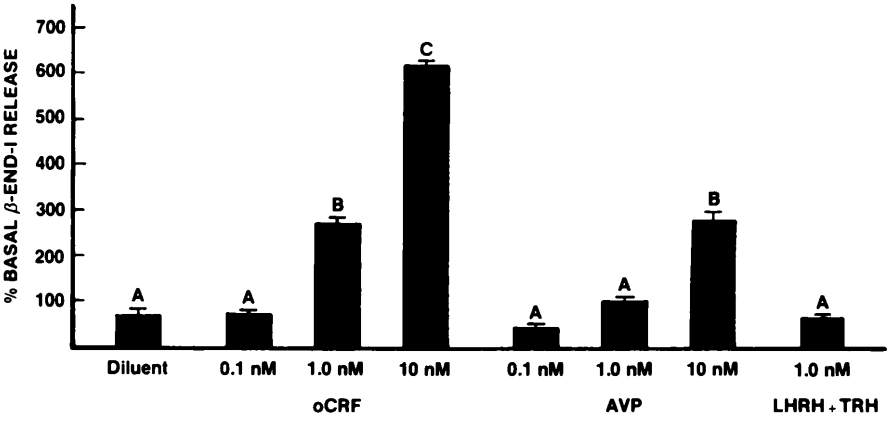

FIG. 1. β-endorphin immunoreactivity (β-END-I) released in response to oCRF and AVP. Results are expressed as percentage basal β-END-I released during a 1-hr incubation in the presence of test substances relative to a prior 1-hr incubation in media alone. Bars and brackets represent the mean of four replicates ± SEM. Values with the same letter superscript are not significantly different ($P < 0.05$).

alone. Thus, while AVP is capable of stimulating β-END-I release, it appears to be approximately 10 times less effective than oCRF in the present assay system.

Experiment II: potentiation of oCRF activity by AVP. The basal level of β-END-I released during the first incubation of this experiment was 3.87 ± 0.33 ng/ml and did not vary significantly between groups. The results of adding AVP and/or oCRF to the second incubation media are illustrated in Fig. 2. As in the first experiment, the addition of diluent alone caused no significant change in β-END-I release as compared to basal values. Incubation of pituitary quarters with 1.0 n*M* AVP or 0.5 n*M* oCRF resulted in statistically similar increases (approximately three- and fourfold, respectively) in β-END-I secretion, although only the increase observed for 0.5 n*M* oCRF was significantly higher than control values. Simultaneous addition of 0.5 n*M* oCRF and 1.0 n*M* AVP to the incubation media resulted in an approximately thirteenfold increase in β-END-I release. The interaction between AVP and oCRF was statistically significant ($P < 0.01$), indicating that the corresponding release of β-END-I was greater than could be attributed to a simple additive effect between the two secretogogues.

Incubation of pituitary quarters in the presence of 2.0 n*M* AVP produced an approximately sixfold greater increase in β-END-I release than diluent alone, whereas 1.0 n*M* oCRF produced a thirteenfold increase. The addition of 1.0 n*M* oCRF and 2.0 n*M* AVP simultaneously, resulted in an approximately 14-fold increase in β-END-I release, however this stimulation was not significantly different than that produced by 1.0 n*M* oCRF alone.

Discussion. In the present study, both synthetic oCRF and AVP stimulated the release of β-END-I from rat pituitary quarters in a dose-dependent fashion. The effective concentrations were within the range previously reported for the ACTH-releasing activity of oCRF and AVP under the same bioassay conditions (3). Simultaneous addition of oCRF (0.5 n*M*) and AVP (1.0 n*M*) to the media, resulted in a significant potentiation of β-END-I release similar to the potentiation of release observed for ACTH (14). The lack of potentiation of β-END-I release by the higher doses of oCRF (1.0 n*M*) and AVP (2.0 n*M*) could be due to maximal β-END-I release having been obtained by the addition of 1.0 n*M* oCRF alone.

β-LPH contains the amino acid sequences of β-melanotropin (β-LPH_{41-58}), met-enke-

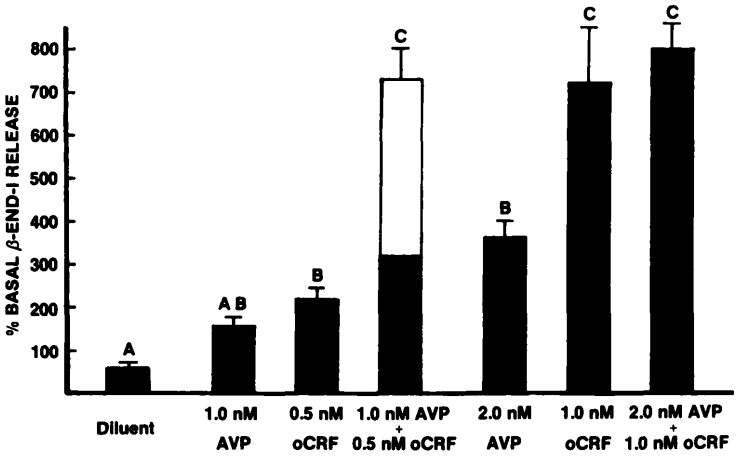

FIG. 2. Potentiation of oCRF activity by AVP. Results are expressed as percentage basal β-endorphin immunoreactivity (β-END-I) released during a 1-hr incubation in the presence of test substances relative to a prior 1-hr incubation in media alone. Bars and brackets represent the mean of four replicates ± SEM. Values with the same letter superscript are not significantly different. Unshaded bar region represents β-END-I release beyond the sum of β-END-I released by 0.5 n*M* oCRF alone and 1 n*M* AVP alone. Analysis as a 2 × 2 factorial revealed significant interaction ($P < 0.01$) between oCRF and AVP in stimulating β-END-I release.

phalin (β-LPH_{61-65}), and β-endorphin (β-LPH_{61-91}) and has been shown to be the immediate precursor for β-endorphin [for review see (19, 20)]. It has been established immunocytochemically that β-endorphin, β-LPH, and ACTH are all present within the same secretory granules and cells of the anterior and intermediate pituitary lobes (21–23) and that all three are probably derived from a common 31-kDa precursor [for review see (19, 20)]. In addition, previous reports have described concomitant alterations of ACTH and β-endorphin as well as β-LPH release in response to stressful stimuli (16, 17, 24), pathologic states (16, 24–27), and various hormonal factors (4, 6, 24, 26). These observations strongly suggest the possibility of common regulatory mechanisms for these pituitary hormones. Therefore, the similarities between the presently observed potentiation of oCRF induced β-END-I release by AVP and that reported for ACTH (14) fit well within these concepts.

The mechanisms by which AVP potentiates the action of oCRF are unknown. However, the weak CRF activity of AVP alone raises the possibility that it binds to CRF receptors and subsequently facilitates the binding of CRF to these receptors. This concept could also account for the common ability of oCRF and AVP to stimulate the concomitant release of ACTH and β-endorphin. Obviously, the possibility of separate AVP and CRF receptors or of intracellular mechanisms which potentiate the releasing activity cannot be excluded.

The concentrations of AVP used in the potentiation study approximate those present in hypophysial portal blood (28). Likewise, oCRF was utilized at concentrations within the physiologic range of other known releasing factors (29). A recent measurement of oCRF immunoreactivity in hypophysial portal blood indicates that tonic levels of immunoreactive oCRF may be somewhat lower (104.0 ± 9.7 p*M*) (5). However, as these levels were determined in anesthetized animals, it is quite possible that the level of oCRF in hypophysial portal blood may increase upon exposure of the conscious animal to stressful stimuli. The simultaneous presence of AVP and CRF in hypophysial portal blood and the demonstration that vasopressin potentiates the ACTH releasing activity of CRF *in vivo* (30), suggest that the currently observed potentiation of oCRF induced β-END-I release by AVP represents a true physiologic phenomenon. It seems likely that AVP and CRF could work together to provide increased control and flexibility in responding to stressful stimuli, such as a rapid increase in the release of corticotropic factors during times of acute stress. It has been demonstrated immunocytochemically, that the majority of immunoreactive oCRF containing perikarya are located within the paraventricular nucleus (PVN) of sheep (31) and rat (32) and, to a lesser extent, in the supraoptic nucleus (SON) (32). The magnocellular neurons which produce AVP are also localized within the PVN and SON (33). Thus, although not demonstrated, the anatomical connections could exist between the AVP and CRF producing neurons such that both hormones could be released in response to common afferent inputs to these hypothalamic regions. Future studies, both immunocytochemical and physiological, will be required in order to elucidate those stimuli and mechanisms which control the individual and coordinated release of CRF and AVP.

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