PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 179, 303–308 (1985)

Interaction of Arginine Vasopressin and Corticotropin Releasing Factor Demonstrated with an Improved Bioassay (42101)

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Abstract. We developed an improved *in vivo* bioassay for corticotropin releasing factor (CRF) by modifying the injection schedule in the standard chlorpromazine–morphine–pentobarbital assay procedure. A combined injection of chlorpromazine and morphine followed 75 min later by injection of pentobarbital produced low basal levels of corticosterone and rendered the animals highly sensitive to synthetic CRF but insensitive to the stress of ether or histamine. The lowest dose of CRF that significantly elevated plasma corticosterone levels was 0.01 μg/kg. Using this assay, we studied CRF–arginine vasopressin (AVP) interactions at doses that were expected to raise systemic peptide concentrations to levels measured in hypophysial portal blood. The threshold for a significant corticosterone response was found to be at least 250-fold lower for CRF-41 than for AVP. The order in which CRF and AVP are injected was also found to be important, potentiation being greater if CRF was given first. In addition, rats deprived of water for 24 hr were more sensitive to CRF than normally hydrated animals.

There is currently extensive evidence (1–8) that the 41-residue peptide (CRF-41) recently isolated from ovine hypothalamus (9) is an essential factor in the physiological regulation of the hypothalamic–pituitary–adrenal axis. The overall integrated release of corticotropin (ACTH), however, is most probably controlled by a multifactorial complex (10–12) and not by a single peptide. The role of arginine vasopressin (AVP) as a component of this complex has been the subject of numerous investigations (10–26) and there is now abundant evidence for a physiological role of AVP in ACTH release: (a) AVP appears to stimulate the release of endogenous corticotropin releasing factor (CRF) (27); (b) *in vivo* and *in vitro* synergism has been observed between CRF-41 and vasopressin (16–25); (c) there is coexistence of immunoreactive neurophysin/vasopressin and CRF-41 in nerve terminals of the zona externa of the median eminence (28) and in cell bodies of the paraventricular nucleus (29); (d) hypothalamic content of both AVP and CRF-41 are elevated after adrenalectomy (4, 30, 31); (e) there is a sequence homology between pre pro-CRF and neurophysin II (32); and (f) high levels of both AVP and CRF-41 exist in hypophysial portal blood (3, 33–35). Additional support for a role of vasopressin in the CRF-complex includes potentiation of CRF activity in the Brattleboro rat by addition of synthetic vasopressin (15), diminution of CRF activity in normal rats injected with vasopressin antiserum (17, 35), and decreased plasma ACTH levels in ether-stressed rats pretreated with a vasopressin antagonist (37).

In a series of studies on the effects of delta sleep-inducing peptide (DSIP) on inhibition of CRF-mediated release of corticosterone in rats treated with chlorpromazine–morphine–pentobarbital (Nembutal) (38), we found that a prolonged delay before injection of pentobarbital resulted in dramatically increased sensitivity to CRF-41. In the present study, we optimized this bioassay and used it to study CRF–AVP interactions *in vivo*. Using this assay, we demonstrate that the order in which CRF-41 and AVP are injected is important. Also, we show that rats deprived of water for 24 hr are more sensitive to CRF-41 than normally hydrated animals.

Materials and Methods. *Materials.* Adult male albino Sprague–Dawley rats weighing about 200 g at the time of the experiments

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0037-9727/85 $1.50

were obtained from Charles River Breeding Laboratories, Wilmington, Massachusetts, and maintained on a 12:12 hr light:dark cycle at constant temperature for at least 5 days before use. Laboratory chow and water were freely available except for the animals used in the dehydration experiment which were deprived of water for 24 hr before use. Drugs, peptides, and other reagents were obtained from the following sources: Thorazine (chlorpromazine: CPZ), Smith Kline Beckman Laboratories, Philadelphia, Pennsylvania; morphine sulfate (MS), gift from Penick Laboratories, Lyndhurst, New Jersey; Nembutal (pentobarbital: P), Abbott Laboratories, Chicago, Illinois; histamine chloride, bovine serum albumin (BSA), and ascorbic acid, Sigma Chemical Company, St. Louis, Missouri; RIA-kit for cortisol, New England Nuclear, Boston, Massachusetts; rat CRF, Bachem Laboratories, Torrence, California; AVP was prepared by solid phase methods.

CRF assay. The rats were prepared for all experiments with an initial single subcutaneous injection of a mixture CPZ (10 mg/kg) and morphine sulfate (20 mg/kg) followed after 75 min by pentobarbital (25 mg/kg) ip. Sixty minutes after the injection of pentobarbital, test substances or vehicle (0.9% NaCl, 1% BSA, 0.1% ascorbic acid) were injected into an exposed jugular vein. Blood samples (0.5 ml) were collected in heparinized syringes immediately before and 30 min after injection of the test substances. The blood was transferred to chilled polypropylene tubes containing EDTA (1.0 mg) and aprotinin (Trasylol, 50 μl) and centrifuged (2000*g*, 15 min, 4°C). Plasma samples were stored at −20°C until assay with an RIA-kit for cortisol that showed about 20% cross-reactivity with a corticosterone standard [for details see (4)]. Data were calculated by the log-logit method and further evaluated by analysis of variance or covariance followed by Duncan's multiple range test.

Stress experiment. The procedure was as described above with the exception that after the initial blood collection, the rats were placed in a glass chamber saturated with ether vapors for 30 sec. For histamine stress, the test substance was histamine chloride (20 μg/kg) iv.

CRF–AVP interaction. In this experiment, AVP (0.5 μg/kg) was injected either 1 min before or 1 min after injection of CRF (0.04 μg/kg). Otherwise the procedures were the same as described above.

Results. Injection of CRF into the jugular vein of rats pretreated according to the new experimental schedule produced a dose-dependent increase of corticosterone (Fig. 1). Analysis of variance revealed a highly significant main effect of dose, $F(4, 43) = 23.53$, $P < 0.0001$. Analysis of covariance with preinjection corticosterone levels as the covariate failed to yield a significant effect of the covariate. Multiple range testing showed that the level of corticosterone 30 min after injection of 0.01 μg/kg CRF was significantly ($P < 0.05$) higher than after diluent, and that higher doses reached even more significant probability values (Fig. 1).

Although the rats were highly sensitive to CRF, they did not reliably respond to ether stress or histamine injection. No significant main effect of treatment, $F(2, 17) = 0.18$, NS, was found. The 0 time and 30-min corticosterone levels were diluent, 40.3 ± 10.2 and 77.06 ± 47.5 ng/ml; histamine (20 μg/kg, iv) 33.3 ± 4.3 and 41.7 ± 4.6 ng/ml; and ether inhalation 44.5 ± 7.2 and 77.5 ± 10.4 ng/ml.

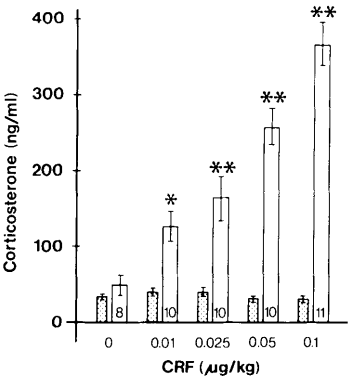

FIG. 1. Effect of increasing dose of CRF on plasma corticosterone levels 30 min later in CPZ/MS-P-treated rats. The dotted bars indicate the means ± SEM of the basal levels (time 0 min), whereas the open bars represent the levels 30 min after iv injection of CRF. Numbers in the bars represent the number of rats used per groups.* $P < 0.05$, ** $P < 0.01$.

In another experiment, one group of rats deprived of water for 24 hr was prepared together with a control group according to the new protocol. Five rats of both groups were injected iv with vehicle and another 10 rats with 0.04 μg CRF/kg. The results are shown in Fig. 2. Both normal and dehydrated rats showed low basal levels of corticosterone that were not affected by saline injections, but the dehydrated animals exhibited significantly higher basal levels by ANOVA $F(1, 26) = 8.62$, $P < 0.01$. Injection of CRF significantly increased the level of corticosterone 30 min later. ANOVA showed a highly significant main effect of treatment, $F(1, 26) = 30.4$, $P < 0.0001$, and of pretreatment (dehydration), $F(1, 26) = 8.781$, $P < 0.01$, and a significant interaction of the presence of dehydration and CRF, $F(1, 26) = 6.05$, $P < 0.05$. Multiple comparison testing demonstrated significant differences (at least $P < 0.05$) between the mean values of CRF-treated rats after stimulation compared to that of the controls. In addition, the dehydrated animals responded significantly ($P < 0.0001$) more to the CRF injection than did the normal rats.

In another experiment, the effect of AVP on the corticosterone response to a low dose of CRF was tested (Fig. 3). Basal levels of corticosterone in the four treatment groups were not significantly different from each other, whereas the main effect of treatment was highly significant, $F(13, 34) = 13.51$, $P < 0.0001$. When injected without CRF, AVP at a dose of 0.5 μg/kg failed to elicit a significant increase in plasma corticosterone levels. By contrast, the increase in plasma corticosterone after low dose CRF treatment (0.04 ng/kg) was highly significant ($P < 0.001$). AVP injected 1 min before CRF did not significantly change the effect of the CRF, but administration of AVP 1 min after CRF increased the concentration of corticosterone significantly ($P < 0.01$) compared with CRF alone.

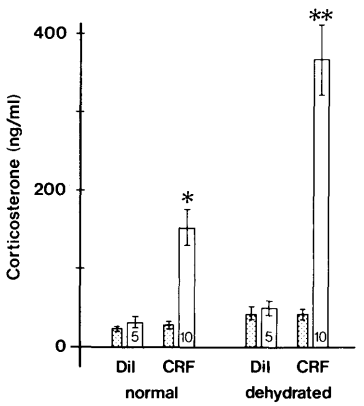

FIG. 2. Effect of CRF (0.04 μg/kg) on normal and dehydrated CPZ/MS-N-treated rats. The dotted bars indicate the means ± SEM of the basal levels (time 0 min) and the open bars represent the levels 30 min after iv injection of CRF. Numbers in the bars are the number of rats per group. $*P < 0.05$, $**P < 0.01$ compared with diluent (Dil).

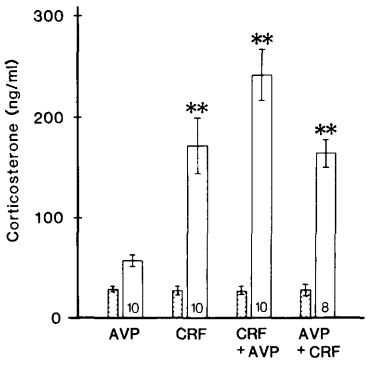

FIG. 3. Interaction of AVP and CRF in CPZ/MS-P-treated rats. The dotted bars indicate the means ± SEM of the basal levels (time 0 min) and the open bars represent the levels 30 min after iv injection of AVP, CRF, CRF followed 1 min later by AVP (third bar) and AVP followed 1 min later by CRF (last bar). $**P < 0.01$ compared with diluent.

Discussion. The CRF bioassay with chlorpromazine-morphine-pentobarbital-treated rats (38) has been extremely useful for evaluation of the biological activity of CRF preparations and for studying various aspects of the physiology of synthetic CRF. Its sensitivity seems to be in a reasonable range for hypothalamic hormones, and satisfactory accuracy and a low mortality rate are characteristic of the assay. In the standard procedure (38), the rats are injected with chlorpromazine (10 mg/kg sc, time 0), morphine sulfate (20 mg/kg, sc, time 3 hr), pentobarbital (25 mg/kg, sc, time 3 hr 15 min) followed after an

additional 45 min by iv injection of the test substance. While using this assay in our laboratory, we determined that higher sensitivities to synthetic CRF were achieved when the time between the injection of morphine and pentobarbital was increased. Since this manipulation increased the total experimental time, we looked for an alternative pretreatment protocol that would require less time. A combined injection of chlorpromazine and morphine followed a little more than 1 hr later by injection of pentobarbital produced low basal levels of corticosterone in plasma of about 30–60 ng/ml and rendered the animals highly sensitive to synthetic CRF but not to stressful agents like ether or histamine.

With the standard assay protocol (38), the minimum dose of synthetic CRF that significantly elevated plasma corticosterone levels was reported to be 6.4×10^{-12} mole/100 g body wt (about 0.3 μg/kg). In our assay system, we found a clear effect of CRF at a dose as low as 0.01 μg/kg, about 30 times lower than that determined previously with the standard procedure (38). These results indicate that when compared on a molar basis, the threshold for a significant corticosterone response is at least 250-fold lower for CRF-41 than for AVP. Furthermore, the improved sensitivity of the modified assay procedure has allowed for the first time the study of CRF–AVP interaction at doses of peptide that are expected to raise peripheral levels to the range of concentrations reported to be present in hypophysial portal blood. For CRF, the reported hypophysial portal level about 0.85 ng/ml (3, 35) and for AVP is 2.0–13.8 ng/ml (33, 34).

In addition to improved sensitivity, the new protocol reduces the time of the assay to less than 3 hr compared to the standard procedure (38) that required at least 4 hr 15 min to prepare the animals and collect the blood samples. Furthermore, an oxygen tent was not required since only two rats died out of a total of more than 200 animals used. The method may have to be altered for other strains of rats because the blockade to stress seemed to be incomplete with Long–Evans rats.

The role of CRF–AVP interaction on the control of pituitary corticotropic function has been the subject of many investigations (10–26). Early studies (13) examining the *in vivo* effect of injection order of crude CRF and lysine vasopressin (LVP) on corticosterone release indicated that potentiation was obtained only when LVP was injected before CRF. The authors explained these results by postulating that pituitary corticotropes synthesize and release ACTH through a series of irreversible steps with vasopressin acting as an early positive modulator and CRF acting as a later step. Recent *in vivo* and *in vitro* studies (11, 18–25) with synthetic CRF-41 have confirmed the CRF-potentiating effect of AVP but no *in vivo* studies with the order of injection as a variable have thus far been reported.

Our results with low doses of synthetic CRF-41 and AVP do not support the previously reported (13) effect of the order of injection. Rather, potentiation of the CRF-mediated corticosterone response was observed only when CRF was injected before AVP. These results are consistent with the known short biological half-life of AVP (39) and the recently reported (40) long half-life of CRF-41. This would imply that if higher doses of AVP were used, potentiation would be expected with both sequences of injection. Another explanation for the observed effect of order of injection relates to the vasoconstrictor activity of AVP. It is possible that prior injection of AVP decreases perfusion to the pituitary and thus decreases exposure to CRF. Although it has been shown (41, 42) that AVP can significantly decrease pituitary blood flow, other experiments have demonstrated that this decreased perfusion has minimal effect on pituitary responsiveness to systemically administered ACTH secretagogs (13, 41).

Possible explanations for this discrepancy of order of injection may be related to the use of hypothalamic extract as a source of CRF in the previous study (13). This material probably contained significant amounts of somatostatin that has been shown to inhibit CRF-mediated ACTH release *in vitro* (12), and perhaps other materials like DSIP that may act similarly (43). If somatostatin more effectively inhibits ACTH release *in vivo* when injected before CRF-41 (possibly by acting at the same site as AVP), the previous results

could be explained. Other possible explanations include the different anesthesia procedures and different times of blood sampling.

Our results with dehydrated rats indicate that this physiological manipulation can potentiate the effect of exogenous CRF on corticosterone release. This observation is consistent with the results of a previous report (13) in which crude hypothalamic extract was used as a source of CRF. In addition, it suggests that while the release of endogenous CRF by stressful stimuli is blocked in our assay system, elevations in vasopressin levels produced by osmotic stimuli probably still occur.

In conclusion, we have shown with an improved *in vivo* bioassay that AVP potentiates the effect of CRF-41 at doses of both peptides that were expected to raise systemic concentrations to levels reported to be present in hypophysial portal blood. Furthermore, we feel that the new protocol represents a distinct improvement in the *in vivo* bioassay for CRF because of its higher sensitivity, shorter experimental time, and easier animal handling.

This work was supported by the VA and ONR. We thank Yolanda Graf for the art work and Linda May for typing the manuscript.

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Received July 2, 1984. P.S.E.B.M. 1985, Vol. 179.
Accepted March 12, 1985.