Effect of Synthetic Ovine Corticotropin-releasing Factor

DOSE RESPONSE OF PLASMA ADRENOCORTICOTROPIN AND CORTISOL

DAVID N. ORTH, RICHARD V. JACKSON, G. STEPHEN DECHERNEY, C. ROWAN DEBOLD, A. NANCYE ALEXANDER, DONALD P. ISLAND, JEAN RIVIER, CATHERINE RIVIER, JOACHIM SPIESS, and WYLIE VALE, *Department of Medicine, Vanderbilt Medical Center, Nashville, Tennessee 37232; Peptide Biology Laboratory, The Salk Institute, San Diego, California 92138*

ABSTRACT Synthetic ovine corticotropin-releasing factor (CRF) was administered to normal male volunteer subjects as an intravenous bolus or 30-s infusion. Doses of CRF ranging from 0.001 to 30 μg/kg body wt were administered, and plasma immunoreactive (IR)-ACTH and IR-cortisol concentrations were measured. The threshold dose appeared to be 0.01–0.03 μg/kg, the half-maximal dose 0.3–1 μg/kg, and the maximally effective dose 3–10 μg/kg. Basal concentrations of IR-ACTH and IR-cortisol were 14±7.6 pg/ml (mean±SD) and 5.6±2.2 μg/dl, respectively. IR-ACTH rose as early as 2 min after CRF injection, reached peak levels in 10–15 min, and declined slowly thereafter. IR-cortisol rose at 10 min or later and reached peak levels in 30–60 min. At a dose of 30 μg/kg, neither IR-ACTH nor IR-cortisol fell from peak levels of 82±21 pg/ml (mean±SE) and 23±1.4 μg/dl, respectively, during the 2-h course of the experiment, indicating that CRF has a sustained effect on ACTH release and/or a prolonged circulating plasma half-life. There was little or no increase in the levels of other anterior pituitary hormones. At doses of 1 μg/kg and higher, facial flushing, tachycardia, and, in some subjects, a 15–29-mmHg decline in systemic arterial blood pressure were observed, even though blood volume was replaced and the subjects remained supine. These data indicate that synthetic ovine CRF is a very potent and specific ACTH secretagogue in man. Administered with caution until its vasomotor effects are more fully defined, CRF promises to be a safe and very useful investigative, diagnostic, and, possibly, therapeutic agent in man.

INTRODUCTION

Corticotropin-releasing factor (CRF)[1] is a hypothalamic peptide whose existence had long been postulated (1, 2). Although it was the first of the releasing factors to be described (3, 4), it has only recently been isolated from ovine hypothalamic extracts (5) and its primary structure, a single chain of 41 amino acid residues, determined (6). Ovine CRF has subsequently been synthesized (5, 7) and shown to be active in vitro and in vivo (5, 8). In addition to its action as an ACTH secretagogue, CRF has been shown to produce a variety of behavioral (9), metabolic (10), and hemodynamic (5, 11, 12) changes in animals, depending upon whether it is administered intracerebroventricularly or intravenously. When administered intravenously to urethane-anesthetized rats (5) or to conscious dogs (12), CRF causes systemic arterial hypotension that is dose dependent and, with large doses of CRF, is profound and prolonged.

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[1] *Abbreviations used in this paper*: CRF, corticotropin-releasing factor; IR, immunoreactive; proOLMC, proopiolipomelanocortin; RIA, radioimmunoassay.

The present study was undertaken to establish the dose-response relationship between synthetic ovine CRF (7), administered as an intravenous bolus, and the release of pituitary ACTH, assessed by measuring concentrations of immunoreactive (IR)-ACTH and IR-cortisol in peripheral plasma in normal human subjects. Because of the possibility that hypotension or other potentially harmful side-effects might be encountered, we started with a dose that was ~0.1% of the expected threshold dose on the basis of studies in rats (8) and subsequently increased it in threefold increments over a 30,000-fold dose range.

METHODS

Subjects. 29 healthy male volunteer subjects, aged 20 to 46 (mean, 29.7) yr, participated in this study. A pertinent medical history and physical examination were recorded for each subject, and an electrocardiogram, urinalysis, complete blood cell count, and serum chemistry profile were obtained. Subjects who had taken any medication, including cannabis, within the previous 6 wk, who had a history of endocrine, central nervous system, cardiac, hepatic, or renal disease, or who had abnormal laboratory findings were excluded from the study. The urinalysis, complete blood cell count and serum chemistry profile were repeated for each subject the day after each injection.

CRF. Synthetic ovine CRF (7) (lot 96-116-20) was dissolved in sterile 0.001 N HCl, 154 mM NaCl, 0.9% (vol/vol) benzyl alcohol at a concentration of 1 or 3 mg/ml. This stock solution was further diluted with sterile 154 mM NaCl containing 10% (wt/vol) mannitol U. S. Pharmacopeia, 0.25% (wt/vol) human serum albumin U. S. Pharmacopeia and 0.9% (vol/vol) benzyl alcohol. CRF concentrations in the solutions were such that, at any dose, the volume administered was calculated as [(0.01 × kg body wt) × 1 ml] and only rarely exceeded 1 ml. Aliquots of the solutions were transferred to empty, sterile 10-ml vials (Gibco/Invenex Div., Dexter Corp., Chagrin Falls, OH) and were either used immediately, were maintained temporarily (<1 h) at 4°C or were stored frozen at −56°C until use. Stored aliquots were thawed only once, immediately prior to use. The vials were prepared and were identified by date and experiment number, but not by the CRF concentration in the contained solution.

Study design. The study was performed in a double-blind fashion: neither the subject nor the attending physician knew whether the vial contained vehicle alone or vehicle-plus-CRF. Each subject was told he would participate in 1 or more individual experiments separated by intervals of at least 1 wk. No subject received more than two injections of CRF sufficient to cause a plasma IR-ACTH and/or IR-cortisol response. The results of ACTH and cortisol radioimmunoassays (RIA) were available to only one of us until the study was completed. The initial overall design, which was known to the attending physician, was one of incremental increases in the CRF dose, randomly interspersed with injections of placebo only. Before beginning each new experiment, one of us reviewed the RIA data from the previous experiment and all of the Vanderbilt Medical Center investigators reviewed the laboratory data obtained before and the day after the previous experiment as well as the blood pressure, pulse rate, electrocardiogram, and subjective and objective clinical observations obtained during the previous experiment. When the efficacy and safety of CRF had been established in at least two subjects at each dose level, additional subjects were studied at all dose levels except 0.001 and 0.003 μg/kg in a double-blind random scheme that included placebos.

Experimental protocol. This study protocol was approved under an Investigational Exemption for a New Drug by the Food and Drug Administration, U. S. Public Health Service, by the Vanderbilt University Committee for the Protection of Human Subjects—Health Sciences and by the Salk Institute Human Investigations Committee. Each subject gave his written informed consent prior to commencing the study. Experiments were performed in the Vanderbilt Clinical Research Center in late afternoon, a time when we anticipated that basal pituitary ACTH secretion and, therefore, plasma ACTH and cortisol concentrations would be relatively low (13). Subjects were fasted after a light lunch, which was consumed before noon to avoid the reported postprandial rise in plasma ACTH and cortisol (14, 15).

The experiment was performed with the subject supine. At about 4:00 p.m., an intravenous infusion line was established in each forearm through which 154 mM NaCl solution was infused at the rate required to replace the volume of blood (~300 ml) withdrawn by the end of the experiment. At least 30 min after the infusion lines were established, the first of two basal blood samples was withdrawn. 15 min later, the second basal blood sample was obtained, and the injection was given in the opposite forearm as an intravenous bolus or, in later experiments, a 30-s infusion. Blood samples were obtained at intervals thereafter. Blood pressure and pulse were recorded automatically every min for the first 15 min after injection, every 2 min for the next 15 min and every 5 min thereafter. In some experiments, a standard 6-lead electrocardiogram was recorded shortly before injection, and a continuous lead 2 rhythm strip was recorded 2 min before, during, and 5 min after the injection.

After the last blood sample was withdrawn, 2 h after the injection, the subject was allowed to sit for 15 min and then to stand for 2 min with frequent monitoring of pulse rate, blood pressure, and symptoms. Subjects who felt lightheaded or had tachycardia lay down, and some were infused with an additional 100–200 ml of saline. Once they were asymptomatic, they were allowed to leave. They returned the next day to have blood and urine collected for repeat analyses.

Hormone assays. Cortisol was measured in unextracted plasma by solid-phase RIA (Micromedic Systems, Horsham, PA). ACTH was measured in unextracted plasma by modifications (16)[2] of a previously published RIA method (17) using antibody IgG-ACTH-1 (IgG Corp., Nashville, TN), which is directed at the ACTH-(5–18) sequence. Serum growth hormone (GH), prolactin, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and thyroid-stimulating hormone (TSH) were measured in the Vanderbilt Medical Center Clinical Endocrinology Laboratories using standard RIA procedures. RIA results were calculated using a standard RIA program and a Wang Series 2200 computer.

CRF assays. To determine the amounts of ovine CRF actually being administered to the subjects, CRF concentration was measured retrospectively in aliquots of stock solutions and in injected solutions by bioassay using rat anterior pituitary cell monolayer cultures (18, 19) and by RIA[3] using antibody IgG-CRF-1 (IgG Corp., Nashville, TN), which was raised in a rabbit to synthetic ovine CRF conjugated to bo-

[2] Nicholson, W. E., B. J. Sherrell, D. R. Davis, and D. N. Orth. Submitted for publication.

[3] Nicholson, W. E., R. V. Jackson, G. S. DeCherney, C. R. DeBold, A. N. Alexander, H. Uderman, J. Rivier, W. Vale, and D. N. Orth. Manuscript in preparation.

vine thyroglobulin. Ovine CRF (7) was used as ^{125}I-labeled tracer and as reference standard. The RIA does not crossreact with a 10^5-fold excess of any human anterior pituitary hormone, nor do extracts of human hypothalamus, posterior pituitary, or anterior pituitary gland equivalent to 0.1 mg of wet tissue cause any displacement of ^{125}I-labeled ovine CRF from the antibodies. Sensitivity (effective dose, ED_{15}) was 10 pg/tube; ED_{50} was 60 pg/tube. Intraassay coefficient of variation was 10.5% (n = 6). Vials containing the remainder of the infused solutions were kept in an ice bath for up to 2 h during the experiments before they were stored frozen at −56°C for subsequent CRF assays. Each of the solutions was thawed, frozen, and thawed again at least once after use and prior to either assay.

Statistical analysis. For the purpose of calculating mean values for and increments in hormone levels, undetectable levels of IR-ACTH (i.e., <8–10 pg/ml) and IR-cortisol (i.e., <1.3–1.7 μg/dl) were assigned values half those of the detection limit (i.e., 4–5 pg/ml and 0.65–0.85 μg/dl, respectively). Responses of groups of subjects that received different doses of CRF were compared to those of the group of subjects that received placebo by analysis of variance (20).

RESULTS

CRF in solutions. The concentration of IR-CRF in solutions supposed to contain 3 mg/ml was 100±0% (mean±SEM) of that expected, and 87±4.9% in 1-mg/ml solutions, both of which were dissolved in 0.001 N HCl and were used both as stock solutions and as injected solutions for the 30- and 10-μg/kg doses, respectively. The IR-CRF concentration was 101±6.2% in solutions of 0.3 mg/ml, 78±6.7% in 0.1-mg/ml solutions, 72±6.8% in 0.03-mg/ml solutions and 48% in a solution supposed to contain 0.01 mg/ml CRF. In infused solutions made up to contain 0.003 mg/ml CRF, solutions administered to achieve a 0.03-μg/kg dose, the concentration of IR-CRF was 63±37% of that expected, and the concentration in a 0.001-mg/ml solution was 30% of that expected. The vehicle alone caused no displacement of ^{125}I-labeled CRF from IgG-oCRF-1 antibody. The concentration of bioactive CRF in a solution supposed to contain 3 mg/ml was 45(10–113)% (mean and 95% confidence limits) of that expected, 156(91–277)% in a 0.3-mg/ml solution, and 80(34–160)% in a 0.002-mg/ml solution; none of these is significantly different from the expected CRF concentration. These results indicate that recovery of CRF from solutions containing <1 mg/ml CRF is variable and may be suboptimal, even when 0.25% albumin and 10% mannitol are included as carriers. The losses are presumably due to adsorption on glass and plasticware. It is possible that some loss of activity occurred after the CRF was injected, due to additional adsorption to glass and plastic surfaces.

Further studies are required to define systems that are more efficient in preventing loss of CRF in solution. For the purposes of the present study, we have presented the results as though the calculated amounts of CRF were actually administered, including at low doses, where CRF recovery was less than optimal.

IR-ACTH and IR-cortisol in plasma. Basal plasma IR-ACTH, calculated as the mean±SD of all of the −15 min and 0 min values, was 14.1±7.6 pg/ml; basal plasma IR-cortisol was 5.6±2.2 μg/dl. Four subjects had basal IR-ACTH levels > 25 pg/ml and basal IR-cortisol levels > 10 μg/dl during one of the experiments in which they participated. The data from those experiments were excluded from further analysis, since the subjects appeared to have been stressed prior to CRF injection.

In subjects who received placebo injections, there was a gradual decline in IR-cortisol (Fig. 1) and, to a lesser extent, IR-ACTH (Fig. 2), reflecting the normal circadian pattern during this 2-h interval. The lowest doses of CRF, 0.001 and 0.003 μg/kg body wt, were administered to only two subjects each. They appeared to have no effect on plasma IR-cortisol (Figs. 1, 3). The apparent effect on IR-ACTH of the 0.001-μg/kg dose was presumably an artifact of the small population sample, since a dose three times greater had no apparent effect (Figs. 2, 3). The threshold dose, reflected in increases in both plasma IR-ACTH and IR-cortisol, appeared to be between 0.01 and 0.03 μg/kg. As the dose of CRF administered was increased by threefold increments, there were progressive increases in the plasma IR-ACTH and IR-cortisol levels, expressed either as a function of time after injection (Figs. 1, 2) or of maximum deviation from the 0 min basal value (Fig. 3). This directly dose-dependent relationship was more obvious for IR-ACTH (Fig. 2) than for IR-cortisol (Fig. 1), suggesting that direct RIA of

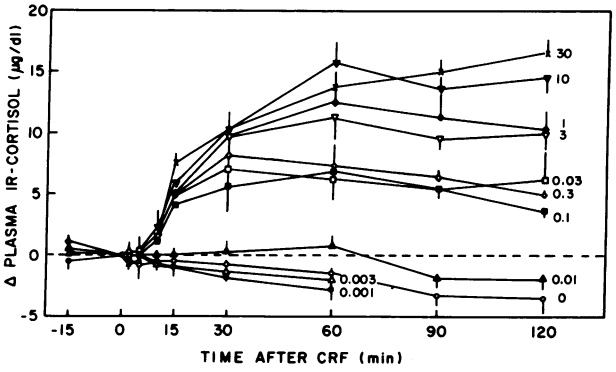

FIGURE 1 Changes in the concentration of plasma IR-cortisol after intravenous administration of synthetic ovine CRF to normal male volunteer subjects. The IR-cortisol concentration for each individual at each time was calculated as the deviation from his IR-cortisol level at zero time, immediately before CRF injection. Each point represents the mean of the data from two to seven individuals (see Fig. 3 for the number of subjects at each dose level); the bracket represents the SEM (the range for the 0.001 and 0.003 μg/kg doses). The CRF dose, in micrograms per kilogram body weight, is indicated to the right of the respective curve. Blood samples were obtained for only 60 min after administration of the 0.001- and 0.003-μg/kg doses.

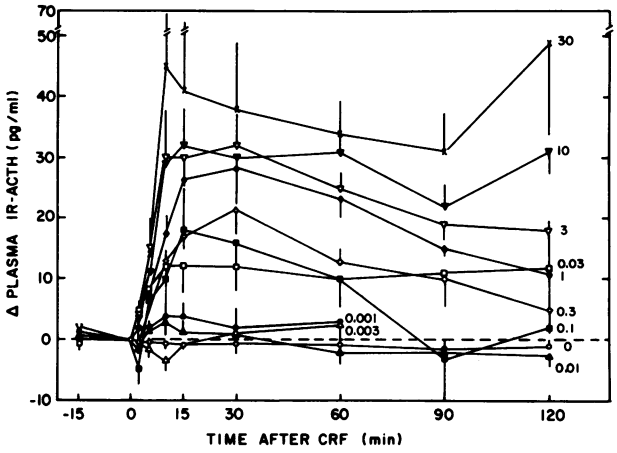

FIGURE 2 Changes in the concentration of plasma IR-ACTH after intravenous administration of synthetic ovine CRF to normal male volunteer subjects. The data were calculated and are plotted in the same manner as those in Fig. 1.

plasma ACTH was a more precise index of CRF action than the indirect "bioassay" provided by measuring plasma cortisol. The increases in plasma IR-ACTH and IR-cortisol concentrations were more sustained, the higher the dose of CRF administered. In fact, at doses

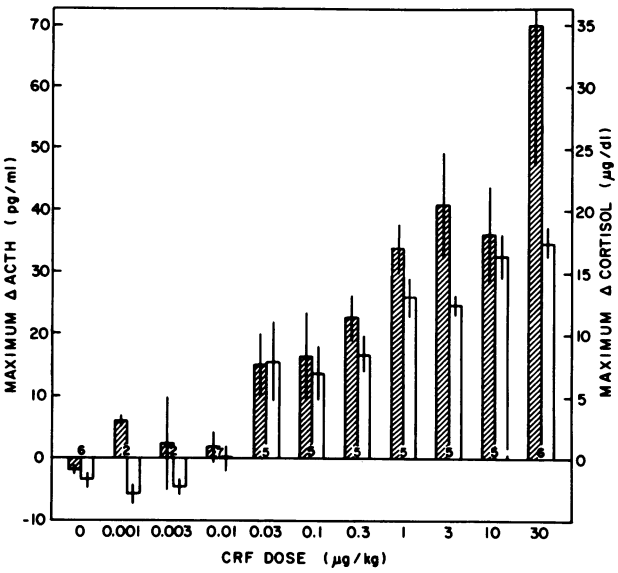

FIGURE 3 Maximum changes in the concentrations of plasma IR-ACTH and IR-cortisol after intravenous administration of synthetic ovine CRF to normal male volunteer subjects. The maximum change in concentration (either decrement or increment, whichever was greater) during the 120 min after CRF injection from the level measured at zero time, immediately before CRF injection, was calculated for each individual. The height of the bar represents the mean of individual values, the bracket represents the SEM. The number of subjects in each group is indicated at the base of each pair of bars. Shaded bars represent IR-ACTH concentrations. Open bars represent IR-cortisol values.

of 1 μg/kg and above, there was no evidence that plasma IR-cortisol was falling by the end of the 2-h experiment; the same was true for IR-ACTH at CRF doses of 10 μg/kg and more.

The levels of IR-ACTH rose earlier than those of IR-cortisol, as might be expected. Increases in IR-ACTH were observed as early as 2 min after injection of CRF doses of 0.3 μg/kg or more; plasma IR-cortisol did not increase until 10 min or later. The rapidity with which plasma IR-ACTH increased did not appear to be a function of CRF dose. Rather, the maximum rate of increase (~5 pg/ml·min) was sustained for a longer time, resulting in higher peak levels. In no case were the maximum rates of increase sustained for >10 min, however. Rates of increase declined thereafter until peak levels were attained, within 10–15 min of CRF injection in virtually every case (Fig. 2). The maximum rate of increase in plasma IR-cortisol was ~1 μg/dl·min. Peak levels were reached later, 30 to 60 min after injection, except after the 30-μg/kg CRF dose, when there was a gradual, sustained rise for as long as samples were obtained (Fig. 1), apparently reflecting continued adrenal stimulation by the high circulating levels of ACTH (Fig. 2). There was a suggestion of a second increase in plasma IR-ACTH between 90 and 120 min after the two highest doses of CRF.

Based on these results, it is not clear whether or not the upper limit of the CRF dose-response curve has been defined. Although there were no significant differences ($P > 0.05$) between the IR-ACTH or IR-cortisol levels at any time after the 10- and 30-μg/kg injections, the mean values for IR-ACTH, expressed either as a function of time or as maximum increment, were consistently higher for the 30-μg/kg than for the 10-μg/kg dose of CRF (Figs. 2, 3).

Other hormones. We measured serum levels of all other anterior pituitary hormones in the serum of subjects who received the two highest doses of CRF. Serum IR-follicle-stimulating hormone increased only minimally, by 0.7±1 IU/ml, in the first 60 min after 30 μg/kg CRF and not at all after the 10-μg/kg dose. Serum IR-luteinizing hormone increased by only 1.6±1.3 IU/ml during the same interval after the larger dose and not at all after the smaller dose of CRF. The maximum increases above basal levels observed, 1.2 IU/ml for IR-follicle-stimulating hormone and 5.9 IU/ml for IR-luteinizing hormone, were well within the range of spontaneous variation observed in normal men (21). There was no increase in serum IR-thyroid-stimulating hormone or IR-growth hormone and only a slight increase in IR-prolactin (maximum change from base line, 0.8±0.2 U/ml, −3.7±3.1 ng/ml, and 3.0±0.55 ng/ml, respectively) after 30 μg/kg CRF.

Blood cell count, urinalysis, and serum chemistry. There were no changes in complete blood cell count,

urinalysis, or serum chemistry that could be attributed to CRF.

Signs and symptoms. There were no objective signs or subjective symptoms in any subject who received <1 μg/kg CRF. Even at doses of 30 μg/kg, all signs and symptoms were markedly reduced by administering the CRF as a 30-s infusion, rather than as a bolus injection.

Flushing. Two of five subjects who were given 1 μg/kg, four of five subjects who were given 3 μg/kg, and all of the subjects who received higher doses of CRF had objective facial, neck, and upper chest flushing. This was first observed 30 s to 2 min after injection. Both its severity and its duration seemed dose dependent. At the lowest dose at which it was observed, it lasted ~30 min. At the 30-μg/kg dose, it was diminished after ~1 h, but was still obvious at the conclusion of the experiment and may have lasted as long as 4 h in some subjects. In the more severe flushing reactions, the subjects' faces appeared edematous. Most of these subjects also felt flushed and warm within 30 s to 1 min after CRF injection. This sensation lasted only 1 to 5 min, even though objective flushing persisted much longer, except in three subjects who received 10 or 30 μg/kg CRF, in whom this sensation persisted up to 1 h.

Shortness of breath. One of five subjects who received 1 μg/kg CRF, two of five subjects given 3 μg/kg CRF, one of five subjects who received 10 μg/kg CRF and all six subjects who were given 30 μg/kg CRF complained of a transient sensation of shortness of breath. This was sometimes described as tightness in the chest, but more often as a sensation of needing to take deeper breaths. This symptom occurred within 1 min of CRF injection in all subjects and lasted 30 s or less. Some of these subjects appeared to breathe more deeply and rapidly during this time. There was no obvious dyspnea, respiratory wheezing, or stridor. There were no ST-T wave changes suggestive of myocardial ischemia during these symptoms in the seven subjects who had this symptom and for whom electrocardiograph records were obtained immediately before CRF injection and during this symptom, and the serum creatine phosphokinase level was actually lower in all of these subjects the day after CRF injection than immediately before.

Tachycardia and hypotension. Intravenous injection of CRF in rats at a dose of 8 μg/kg (5, 11) and in dogs at a dose of 1.4 μg/kg (12) caused systemic arterial hypotension. The degree of hypotension was dose-dependent in both species. In dogs, doubling the dose of CRF lowered mean arterial pressure by ~5 mmHg. With very large doses of CRF, 80 μg/kg and more, the hypotension became progressively severe and prolonged, but was not fatal. The hypotension in dogs appeared to be due to increased mesenteric blood flow and resultant decreased peripheral circulating blood volume (12).

Because we were aware of the hypotensive effects in animals prior to beginning this study, several precautions were taken. We began with very low doses of CRF, less than we anticipated would have ACTH-releasing activity, let alone hypotensive effects. The dose was increased by threefold increments, and the effects on at least two subjects were analyzed before proceeding to the next highest dose. The subjects remained supine throughout the study to eliminate postural effects, their blood pressure and pulse rate were monitored and recorded automatically at frequent intervals, and the volume of blood removed was replaced with normal saline solution. Thus, we attempted to minimize whatever hypotensive effect CRF might have had, since this was not the object of our investigation and since we did not want to complicate the study by introducing a potential stress.

Nevertheless, some subjects did develop tachycardia and transient hypotension. The changes in mean pulse rate and systolic and diastolic blood pressure for subjects receiving each dose of CRF are shown in Fig. 4. Pulse rate increased transiently but significantly ($P < 0.05$) at a CRF dose of 3 μg/kg or more. At a CRF dose of 30 μg/kg, the pulse rate increased markedly within 2 min and remained elevated for 30 min or more. In contrast, mean systolic blood pressure never fell significantly below that of subjects who received placebo injections at any time after injection of any dose of CRF, and mean diastolic blood pressure was significantly less ($P < 0.05$) only at 30 and 60 min after the largest dose of CRF. The blood pressure of the placebo group fell during the first 30 to 60 min of the experiment, presumably the effect of supine posture and inactivity. Despite the fact that group mean pressures did not, in general, fall significantly lower than those of subjects who received placebo injections, however, some individual subjects had transient, but significant falls in blood pressure. One subject who received a dose of 10 μg/kg had a fall from 115/56 to 95/46 mmHg at 7 min with an accompanying pulse rise of 28 beats/min. In another who was given the same dose, blood pressure fell from 119/54 to 94/47 mmHg, accompanied by a rise in pulse rate of 24 beats/min, 17 min after injection. One subject who received 30 μg/kg CRF had a fall from 121/73 to 108/49 mmHg and a pulse increase of 17 beats/min. Another subject who received the same dose had a fall from 127/68 to 98/50 mmHg at 15 min, with a 12-beat/min increase in pulse rate, and a third had a blood pressure fall from 116/67 to 91/54 mmHg at 54 min, accompanied by 41 beat/min pulse increase. In general, the pulse rate and blood pressure returned to normal within 30 min. Other subjects receiving the same doses had less than a 15-mmHg fall in blood

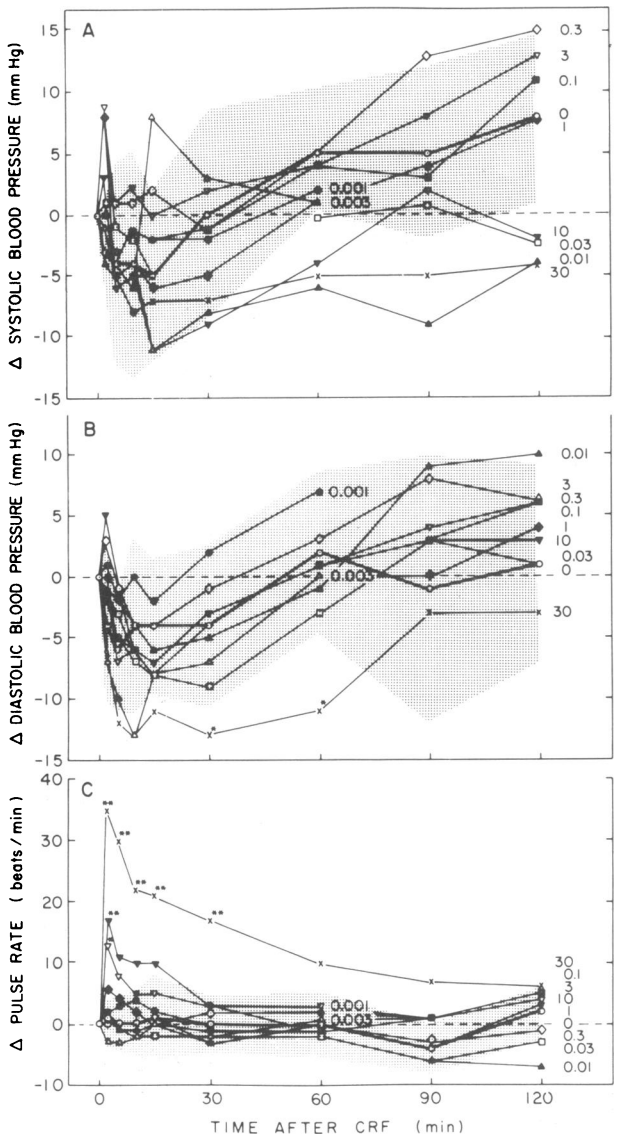

FIGURE 4 Changes in systolic and diastolic blood pressure and pulse rate after intravenous administration of synthetic ovine CRF to normal male volunteer subjects. The changes were calculated as the deviation from the mean of two basal measurements made 15 min and immediately before CRF injection. Each point represents the mean of the measurements for two to seven individuals (see Fig. 3 for the number of subjects at each dose level). The shaded areas indicate the range±1 SD from the mean for subjects who received placebo injections. Asterisks indicate values that are significantly different from placebo controls (*, $P < 0.05$; **, $P < 0.01$). The CRF dose, in micrograms per kilogram body weight, is indicated to the right of the respective curve.

pressure, and three of six subjects who received placebo injections had falls in blood pressure of 15 mmHg or more.

Some of the subjects felt faint and one actually experienced brief syncope shortly after standing at the completion of the experiment. This was not dose-related (the syncopal episode occurred in a subject who received 0.01 μg/kg), was usually not accompanied by hypotension or tachycardia, and was successfully treated by having the subject lie down for a few minutes and, occasionally, by brisk infusion of an additional 100–200-ml saline solution. Furthermore, it was subsequently successfully prevented by having the subjects flex their leg muscles during the 15-min period of sitting and the 2-min period of standing while vital signs were monitored. All of the subjects felt well when they left the Clinical Research Center and when seen again the next day to have blood drawn for posttreatment analyses.

Other symptoms. Four of the subjects receiving CRF doses of 10 or 30 μg/kg reported a cool, "menthol-like" sensation on their tongues and/or palates, and one of them described a similar, "cool breeze" sensation in his ears. On two occasions, during which he received injections of 3 and 10 μg/kg CRF, one subject who had chronic allergic rhinitis reported sudden, transient reduction in nasal stuffiness. In every instance, these sensations were reported 30 s to 2 min after CRF injection and lasted only 1 to 2 min. Two subjects who received a 30-μg/kg dose of CRF had increased peristalsis for ~1 h, felt they needed to have a bowel movement, but produced only explosive flatus. One of these subjects also had increased peristalsis of a milder degree after receiving a placebo injection, however. One of these subjects and a subject who received 0.3 μg/kg CRF experienced transient nausea within 1 min of injection. One of the subjects who received a bolus injection of 30 μg/kg CRF felt a sense of impending doom in the first few minutes after the drug was given. He volunteered immediately thereafter, however, for a second experiment. Except for this, none of the subjects reported any unusual thoughts or emotions, and none exhibited any behavioral changes suggestive of a stress response (9). In fact, some of them dozed intermittently or constantly throughout the experiment.

DISCUSSION

Secretion by the anterior pituitary gland of ACTH and other peptides derived from proopiolipomelanocortin (proOLMC) [i.e., γ-melanocyte-stimulating hormone (γMSH), β-lipotropin (βLPH), γLPH, and β-endorphin] is thought to be controlled by a number of factors, the most important of which are CRF and glucocorticoids. CRF is secreted by cells in the parvicellular portion of the paraventricular nucleus of the hypothalamus (22) and is released into the hypothalamic-hypophysial portal venous system. Its presumed role is one of stimulating proOLMC peptide secretion in a variety of physiological situations, including maintaining the circadian rhythm and responding to stimuli

that are in one way or another perceived as stressful to the organism, such as hypotension, hypoglycemia, and physical or psychological trauma. The major effect of glucocorticoids, cortisol, in the case of man, appears to be to inhibit proOLMC secretion by blocking the action of CRF (8) and, possibly, other secretagogues, such as vasopressin (23), on the pituitary corticotroph; glucocorticoids may also inhibit secretion of CRF by the hypothalamus (2).

Although this overall schema has generally been accepted for more than 25 years and although CRF was the first of the putative hypothalamic releasing factors to be looked for, CRF eluded final purification and structural analysis until 1981, when it was isolated from ovine hypothalamic extract (5). It has since been synthesized (5, 7) and shown to be active in vitro (5) and in vivo (8). The action of CRF, which apparently involves generation of intracellular adenosine 3',5'-monophosphate (24), can be inhibited by prior exposure to glucocorticoids, such as dexamethasone (8).

In this study, we examined the dose-response relationship of administered synthetic ovine CRF to pituitary ACTH release, as manifested by changes in concentrations of plasma IR-ACTH and IR-cortisol. We chose late afternoon, rather than morning (25), as the time for conducting these studies in order to take advantage of the relatively low, stable basal secretion of ACTH and cortisol that could be expected at this point in the normal circadian rhythm. Results in normal subjects given placebo injections confirmed this expectation.

Previous studies had indicated that the threshold intravenous dose of CRF was ~0.04 μg/kg body wt in unanesthetized, chronically cannulated rats (8) and ~0.5 μg/kg in stalk-sectioned macaques (26). These doses can be estimated to have resulted in initial plasma levels of ~400 pg/ml and 5 ng/ml, or ~100 pM and 1 nM, respectively. The minimally effective dose of CRF in vitro had been found to be <10 pM in rat anterior pituitary cell cultures (5) and ~2 nM in mouse AtT20/D16-16 anterior pituitary tumor cells (27). Our results indicate that the minimum effective dose in man was between 0.01 and 0.03 μg/kg body wt (as little as ~0.01 μg/kg, when corrected for losses incurred prior to injection). Thus, this dose is less than that found to be effective in animals. It can be estimated to have resulted in initial plasma CRF concentrations of ~200 pg/ml, or ~90 pM (~100 pg/ml, or 45 pM, when corrected for possible preinjection losses), a level which is comparable to threshold levels previously observed in vitro.

Our data have not unequivocally established the maximally stimulating dose of CRF in man, since the greatest increment in mean IR-ACTH was observed after the largest dose of CRF, 30 μg/kg body wt. Psychological or physiological stress cannot be excluded as contributing to the total IR-ACTH response after high doses of CRF. However; there was no apparent correlation between the magnitude of the IR-ACTH increment and subjective or objective manifestations of stress in individual subjects, no apparent difference in IR-ACTH increments after bolus injections vs. 30-s infusions of CRF, despite the fact that the latter produced less symptoms, and no increase in serum IR-GH, which is generally thought to be a sensitive index of stress, in any of the subjects receiving the highest two doses of CRF. We elected not to administer CRF in doses >30 μg/kg (~2.25 mg total dose) because of the risk of hypotension. Although this is a large mass of peptide to administer, it represents only 0.48 mmol of CRF. For comparison, standard 200-μg diagnostic doses of thyrotropin-releasing hormone and luteinizing hormone-releasing hormone represent 0.55 and 0.17 mmol, respectively, of those peptides.

Establishing the exact dose-response curve was further complicated by the fact that CRF appeared to exert its effects for a prolonged period of time. Thus, the area under the curve of IR-ACTH level vs. time (Fig. 2) continued to increase with higher CRF doses, although the initial peak level of IR-ACTH no longer increased. Based on the maximum level of IR-ACTH attained within the first 15 min, it appears that 0.01–0.03 μg/kg was the minimally effective dose, 0.3–1 μg/kg was the half-maximal dose, and 3–10 μg/kg was the maximally effective dose of synthetic ovine CRF. The dose-response curve appeared to cover a 20-fold range in macaques (26) and at least a 100-fold range in rats (8). Our results suggest that the range is at least 300- to 1,000-fold in man.

The maximum rate of increase in plasma IR-ACTH during the first 5 to 10 min appeared to be independent of the CRF dose. This suggests that any effective dose of CRF stimulates release of ACTH at a maximum, very rapid rate. Larger doses of CRF sustained this maximum ACTH release rate for longer periods of time. After peak IR-ACTH levels were achieved, they declined at a fairly constant rate of ~ −15 pg/ml·h for CRF doses of 3 μg/kg or less. The decline in IR-ACTH was slower than can be accounted for by the plasma disappearance half-time of ACTH itself, which is ~20 to 40 min (13, 28), depending upon the specific RIA. Thus, the data indicate that additional ACTH was being secreted even as plasma IR-ACTH levels were falling. They further suggest that ovine CRF has a sustained effect on ACTH release and/or a prolonged biologic half-life in human circulating plasma, as has been described in macaques (29). In these animals, the plasma half-life was found to be ~200 min. Our indirect evidence suggests that it is ~60 min in man.

Peak levels of IR-ACTH (82±21 pg/ml) achieved within 120 min after injection of the 30-μg/kg dose of CRF were not as high as those (131±14 pg/ml) we

recently observed within 40 min after insulin-induced hypoglycemia (30). The peak IR-cortisol values, 23±1.4 and 25±1.2 μg/dl, respectively, were similar, presumably reflecting maximum ACTH stimulation of the adrenal cortex in both situations. It is well known that other agents, such as arginine vasopressin, antidiuretic hormone (2) and α-adrenergic agonists (31), stimulate anterior pituitary ACTH release. Furthermore, it has been found that CRF may act additively or synergistically with arginine vasopressin in stimulating ACTH secretion (32, 33). Thus, it would appear that CRF alone, at least when given by the intravenous route in the doses reported here, does not maximally stimulate pituitary ACTH release. This suggests that other secretagogues may act, alone or in concert with CRF, to stimulate the degree of ACTH secretion observed in response to stimuli such as hypoglycemic stress.

At near maximally effective doses in terms of ACTH release, CRF had little or no effect on plasma levels of other anterior pituitary hormones, in contrast to what has been reported in cynomolgus macaques (26). Our data are in agreement with preliminary results in six normal subjects given smaller doses of CRF (25). The reason for the difference between humans and monkeys is not clear, but may represent species specificity of certain actions of CRF, species differences in response to CRF, or differences in the model system (i.e., stalk-sectioned, restrained macaques vs. intact, unrestrained humans). These results indicate that in man, CRF is a highly specific secretagogue that acts only on the corticotrophs to release ACTH. However, other effects on the pituitary of males, such as inhibiting basal or stimulated release of other hormones, or different effects on female pituitaries have not been excluded by this study.

At these same doses, CRF did produce other effects. The most prominent and consistent of these was facial flushing. This has been observed after administration of a variety of other peptides (34), so that it seems unlikely that it is a specific effect of CRF. The mechanism by which the flush is produced by any of these agents is unknown, however. In the case of CRF, it is interesting to speculate on whether this effect might be mediated by the same mechanism that causes the increase in mesenteric blood flow and, thus, results in systemic arterial hypotension. Ovine CRF has strong sequence homology (5) with sauvagine, a 40-residue peptide recently isolated from the skin of the South American frog, *Phyllomedusa sauvagei* (35). Sauvagine not only releases ACTH and β-endorphin in vitro and in vivo, but is an even more potent hypotensive agent, also via the mechanism of enhanced mesenteric blood flow (36). CRF, on the other hand, is ~100 times more potent as an ACTH secretagogue than as a hypotensive agent in the rat (5). Despite our concern that ovine CRF might act as sauvagine in man, hypotension was not an important problem in these subjects or in two patients with Cushing's disease (37). When examined as groups, the only significant difference between the post-CRF blood pressures in subjects who were given high doses of CRF and those who received placebo injections was in the diastolic pressure 30 and 60 min after the highest CRF dose. There was significant tachycardia, however, which may have represented a compensatory response to decreased atrial filling. Furthermore, individual subjects did have falls in systolic and diastolic blood pressure of as much as 29 and 24 mmHg, respectively. Since we used an experimental protocol designed to minimize the possibility of significant hypotension, we conclude that caution must be taken when administering CRF to human subjects, until its hemodynamic effects are more fully defined.

In summary, synthetic ovine CRF appears to be a potent and specific ACTH secretagogue in man. It seems to have a prolonged half-life in circulating plasma and a sustained stimulatory effect on ACTH secretion. It produces vasomotor effects, including systemic arterial hypotension, at higher doses. Administered with proper precautions, however, CRF promises to be a safe and very useful investigative, diagnostic and, possibly, therapeutic agent in man.

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