EFFECTS OF BOMSESIN ON PLASMA CORTISOL AND ADRENAL CORTICOTROPIN (ACTH) IN THE DOG

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EFFECTS OF BOMSESIN ON PLASMA CORTISOL AND ADRENAL CORTICOTROPIN (ACTH) IN THE DOG

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Presented to the

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Ronald Marion Thomas

May 1984

To the Graduate and Research Council:

I am submitting herewith a Research Paper written by Ronald Marion Thomas entitled "Effects of Bombesin on Plasma Cortisol and Adrenal Corticotropin (ACTH) in the Dog." I have examined the final copy of this paper for form and content, and I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biology.

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CHAPTER I

INTRODUCTION

Review of the Literature

Bombesin is a tetradecapeptide first isolated from the skin of the amphibian Bombenia bombenia (1). Since its characterization bombesin-like immunoreactive peptides have been isolated in various mammalian tissue. They have been found in evenly distributed, low concentrations (< 0.1-1.0 pmol/g) in the skin of both the feline and procine (2). In the rat concentrations of 18-98 pmol/g have been documented for the whole brain, with 29 pmol/g in the hypothalamus and lesser amounts (3 pmol/g) in the cortex and midbrain, and concentrations of less than 1 pmol/g in the liver, spleen, kidney, pancreas, skeletal muscles, and lungs (3). Bombesin-like peptides in the range of 0.01-0.45 pmol/g have been characterized in the dorsal and ventral horns and white matter of the rat spinal cord (4). Distributions and concentrations similar to those found in the rat have been reported in the dog, although higher concentrations (2-3 pmol/g) are reported in the liver, spleen, and pancreas of the dog (3). In man bombesin-like peptides have been identified in the fetal and neonatal lung (3), in small cell carcinoma of the lung (5, 6), and in the gastrointestinal tract mucosa (7).

Subcutaneous (SUBCU), intraperitoneal (IP), intravenous (IV), and intracerebroventricular (ICV)

administration of bombesin to mammals has shown that it has a wide range of physiological effects. Bombesin caused satiety in both normal and hypothalamically obese rats (2-4 ug/rat SUBCU) (8) and reduced food intake in normal rats when injected ICV into the lateral hypothalamus (5-100 ng) (9). Bombesin (1.0 ug) administered ICV produced hypothermia in hypophysectomized rats (10), and hyperglycemia resulted when 100 ng of bombesin was injected into the rat cisterna magnum (11). Recently, bombesin in the rat has been reported to slow gastric emptying and intestinal transit time (0.01-3.0 ug ICV) (12), to stimulate antral gastrin cell proliferation (10 ug/Kg twice daily for one week IV or SUBCU) (13), and to cause a 3-4 fold increase in plasma corticosterone (1 ug/100 g IP) (14).

In the dog many similar effects have been observed in response to bombesin administration. Gastrin release, bicarbonate (HCO_3^-) production, plasma gastrin and cholecystokinin (CCK) increase, and pancreatic protein production have been reported in response to 0.125-2.0 ug/Kg-hr IV infusions of bombesin (15). Bombesin (500 ng/Kg-hr IV) stimulated pancreatic polypeptide (16) and reduced jejunal Na^+ and Cl^- absorption when infused IV at 0.1-0.5 ug/Kg-hr (17). Significant gastrin, insulin, and glucagon increases are reported in response to 1 ug/Kg-hr IV infusions of bombesin (18). The release of intraduodenal acid and secretin has been observed in the dog when bombesin was infused at a rate of 5-20 ug/Kg-hr IV (19).

In man bombesin (5 ng/Kg-min IV) produced sharp increases in plasma insulin and pancreatic glucagon and decreased blood glucose (20). Intravenous infusion (2.5 ng/Kg-min) resulted in marked increases in human gastrin levels and gastric acid secretions (21), and 10 ng/Kg-min IV infusions elicited significant increases in human prolactin (22). Bombesin (9 ng/Kg-min IV) caused marked and significant increases in the serum trypsin of healthy humans and less drastic but still significant increases in the serum trypsin of patients with chronic pancreatitis (23).

Bombesin, one of the growing list of "gut-brain peptides," is the only peptide known to release endogenous gastrin and has been named as a putative neurotransmitter of gastrin release (24). As a potent mitogen for Swiss 3T3 cells, bombesin has been proposed to play an important role in the pathogenesis of certain cancerous tumors (25), and bombesin has been suggested as an important regulatory agent in the normal and malignant lung (6). It has been proposed that bombesin-like peptides may be released into the portal blood and have a hypophysiotrophic function (26). Recent observations of significant diurnal variations in rat bombesin-like neuropeptides indicate that a circadian rhythm may exist for the mammalian form of this peptide (27).

The pituitary-adrenal hormones function to regulate the metabolism of carbohydrates, proteins, and fats and to help the body deal with stress. The adrenal glucocorticoids are stimulated by pituitary ACTH, which is in turn regulated by

corticotropin releasing factor (CRF) from the hypothalamus. Three major controlling factors work through the system. First, the glucocorticoids exert a negative feedback on both ACTH and CRF, aiding in its own regulation. Second, stress influences pituitary-adrenal secretions. Third, these hormones fluctuate throughout the day in circadian rhythms (28). In most mammals these hormones peak at or near the beginning of the active period—diurnal animals in the early morning hours and nocturnal animals at or near darkness. It is believed that the light-dark (L-D) cycle and other episodic events, such as feeding and temperature and even the glucocorticoids themselves (20), act as zeitgebers of the circadian rhythms through the suprachismatic nuclei (SCN) and ventromedial hypothalamus (VMH).

Figure 1 outlines the generally accepted theory of the relationship between the suprachismatic nuclei, the ventromedial hypothalamus, the hypothalamic media emmience (HME), the pituitary, and the adrenals (30). Imposed upon this is the question of this study—that is, what effect does bombesin have on plasma concentrations of cortisol and ACTH, and how does bombesin work through this system?

Objective of Study

The objective of this study was to determine the influence of bombesin on plasma cortisol and ACTH levels in conscious, awake, fasted dogs by infusing graded doses of bombesin intravenously and measuring plasma cortisol and ACTH by radioimmunoassay (RIA).

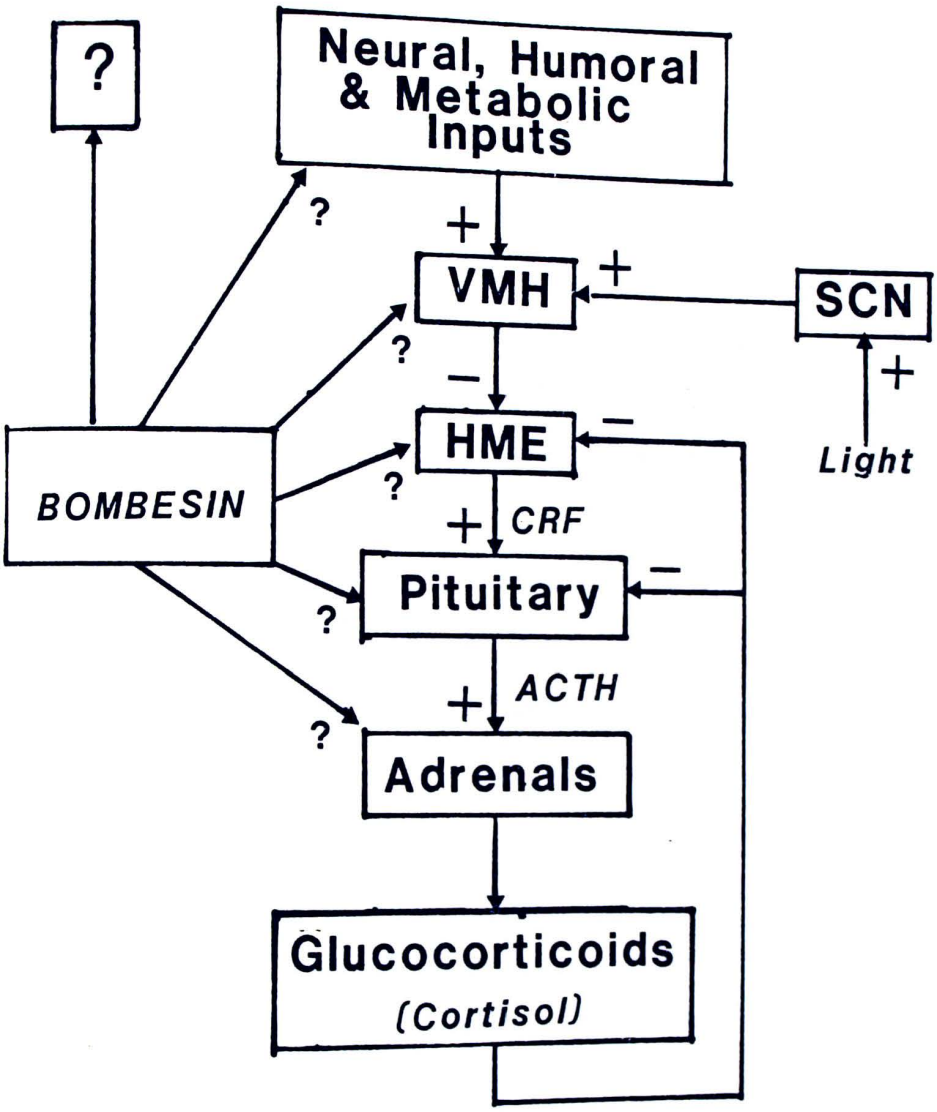

Figure 1. Pituitary-Adrenal Hypothalamic Axis. Supra-chismatic nuclei (SCN), ventromedial hypothalamus (VMH), hypothalamic media emmience (HME).

CHAPTER II

MATERIALS AND METHODS

Materials and Experimental Procedures

Five male mongrel dogs weighing from 15 to 30 Kg were used in the study. All dogs were determined disease and parasite free after an initial three week quarantine. Dogs were housed under climate controlled conditions in 6 by 6 foot floor cages at the Animal Care Facility, Meharry Medical College, Nashville, Tn. with continuous access to water. Dogs were maintained on a 7:00 A.M. to 7:00 P.M. light-7:00 P.M. to 7:00 A.M. dark L-D cycle. Animals were fed one meal daily between 3:00 and 4:00 P.M. consisting of approximately 38 g/Kg of body weight (a standard adult ration) of Purina Dog Chow. Prior to beginning the experiments, all dogs were trained to stand in modified Pavlov slings for periods up to 5 hours.

Bombesin (p-Glu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2) (MW 1619.9) from Calbiochem-Behring was dissolved in 0.01M glacial acetic acid (HAc) for a stock concentration of 100 ug/ml 0.01M HAc. Stock aliquots of 0.5 and 1.0 ml were placed in glass culture tubes, sealed, and frozen at -15 C until used.

Experiments were conducted between 10:00 A.M. and 3:00 P.M. on 16 to 18 hour fasted animals. A catheter was secured in one saphenous vein for both infusion and sampling. A vehicle solution was prepared as follows: a

volume of 0.01M HAc equal to the volume of bombesin stock to be used was dissolved and diluted in sterile 0.9 percent saline to a final volume equal to the final volume of the bombesin dilution. For the first 30 minutes of each experiment, vehicle solution alone was infused at a rate of 1.0 ml/min. The vehicle solution infusion was followed by the infusion of the three graded doses of bombesin. Each concentration was infused at a rate of 1.0 ml/min for 45 minutes. The effective doses were 0.1 ug/Kg-hr, 1.0 ug/Kg-hr, and 2.0 ug/Kg-hr (60, 600, and 1200 pmol/Kg-hr). Blood samples (approximately 5 ml) were collected at 0, 30, 52.5, 75, 97.5, 120, 142.5, and 165 minutes in 0.10 ml of 7.5 percent ethylenediaminetetraacetic acid (EDTA) and maintained on ice until centrifuged at 1000 X g at 0 C. Plasma was collected in 0.5 to 0.75 ml aliquots and frozen at -15 C until assayed by RIA. To prevent enzymatic activity, the protease inhibitor aprotinin (Sigma Chemicals) [0.012 ml aprotinin/0.5 ml plasma = 500 Kallikrein inhibitor units (KIU) or approximately 0.56 trypsin inhibitor units (TIU)/ml plasma] was added to one tube of plasma before it was frozen. This tube was reserved for the ACTH assay.

A series of basal experiments were conducted using pH adjusted vehicle. Vehicle was prepared as above, and pH adjustments were made with 0.1N HCl and 0.1N NaOH until the pH's matched the pH's of the vehicle and graded doses used in the experiments (vehicle, pH 3.3; 0.1 ug/Kg-hr, pH 6.5; 1.0 ug/Kg-hr, pH 6.1; and 2.0 ug/Kg-hr, pH 5.8). The pH

adjusted vehicle infusion and sample times matched experimental ones.

Double antibody ^{125}I RIA's for cortisol (New England Nuclear) and ACTH (Immuno Nuclear Corporation and Diagnostic Systems Laboratories) were conducted according to protocol. Individual samples were assayed in duplicate.

Statistics

All values are expressed in means ± standard error of the means (SEM). Using the APPLE IIe in conjunction with the curve fitter program by Paul K. Warme, Interactive Inc., 1980, Bartlett's test for homogeneity of data was employed to determine whether a one-way analysis of variance (ANOVA) or a logarithmic transformation one-way analysis of variance (LOG ANOVA) would be appropriate. Homogeneous values were processed using ANOVA, and heterogeneous data were analyzed using LOG ANOVA. A difference was considered significant if the resulting F ratio (mean square between groups/mean square within groups—used in one-way ANOVA to evaluate the null hypothesis that population means are equal) gave a probability of <0.05. Differences between the means of pairs of groups were then evaluated using Neuman-Keuls multiple comparison tests.

CHAPTER III

RESULTS

Figure 2 shows the time course results of the pH adjusted vehicle series. While there was a slight tendency for plasma ACTH and cortisol to rise, one-way ANOVA showed no significant differences over the time course for either cortisol [$F(6,31) = 0.6574$] or ACTH [$F(6,31) = 1.0287$].

Plasma cortisol increased drastically in response to bombesin. Figure 3 shows increases of two to two and one-half fold over control values at the middle and high doses (control = 0.399 ± 0.076 ng/ml; 1.0 ug/Kg-hr = 0.950 ± 0.173 ng/ml; and 2.0 ug/Kg-hr = 0.960 ± 0.292 ng/ml). One-way LOG ANOVA revealed a significant change in plasma cortisol at the $p < 0.01$ level [$F(6,114) = 3.9886$]. Plasma cortisol percent of control (Figure 4) shows a maximum increase of 286 percent over control values at the middle dose. LOG ANOVA analysis showed significant changes [$F(6,114) = 9.8527$] at $p < 0.01$. Neuman-Keuls test showed significant differences ($p < 0.01$) between the mean value of the control and the mean values of all doses of bombesin.

Plasma ACTH increases were not as impressive as cortisol increases, however, they were significant at the $p < 0.05$ level. Figure 5 shows that plasma ACTH decreased slightly during the low dose, rose rapidly during the middle dose, and leveled off and started to drop during the high dose. The increases over control values were from 78 ± 11.9

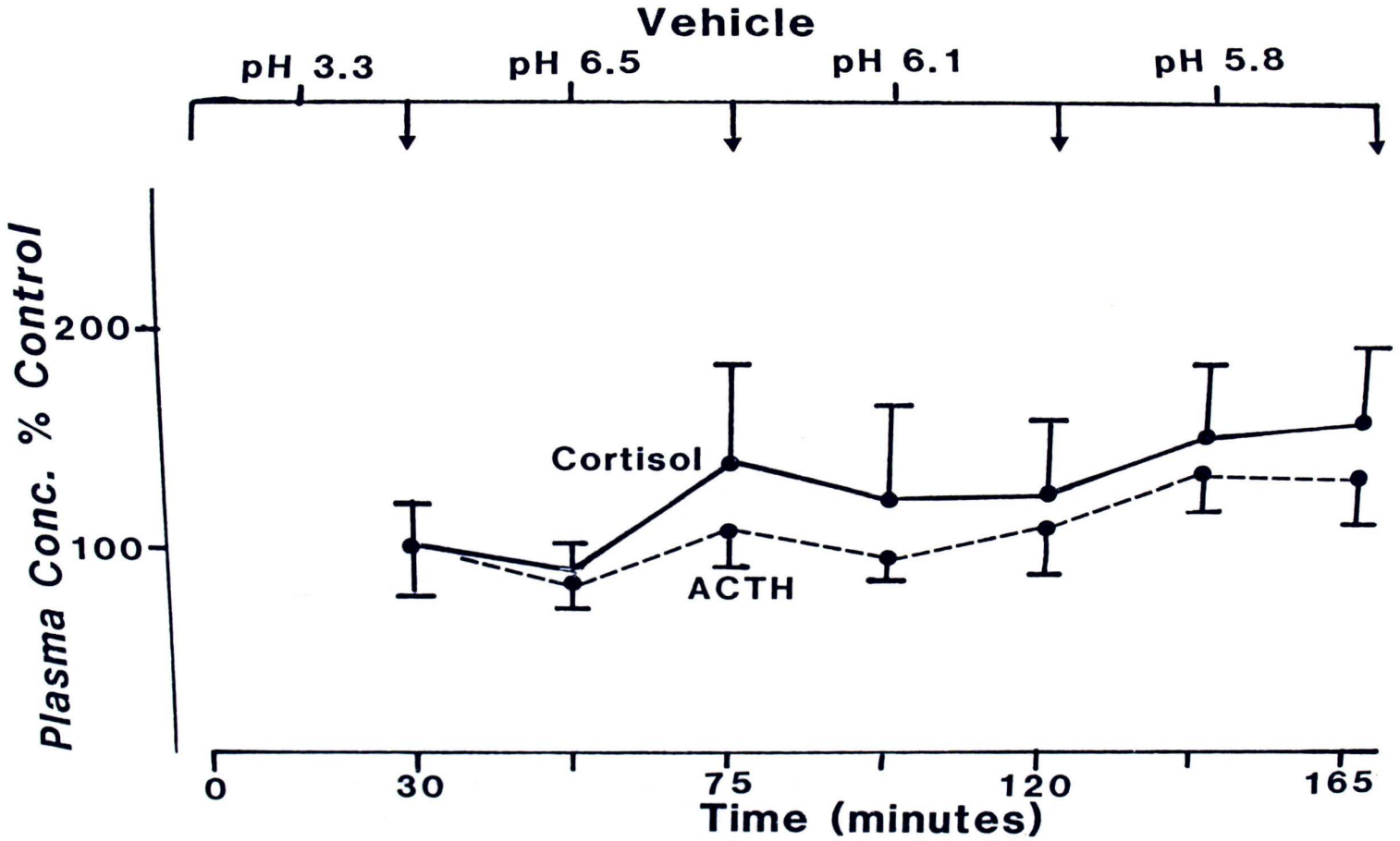

Figure 2. Plasma Cortisol and ACTH Time Course Response to Vehicle. Values means ± SEM. ANOVA F(6,31) = 0.6574 for cortisol. F(6,31) = 1.0287 for ACTH. N = 6.

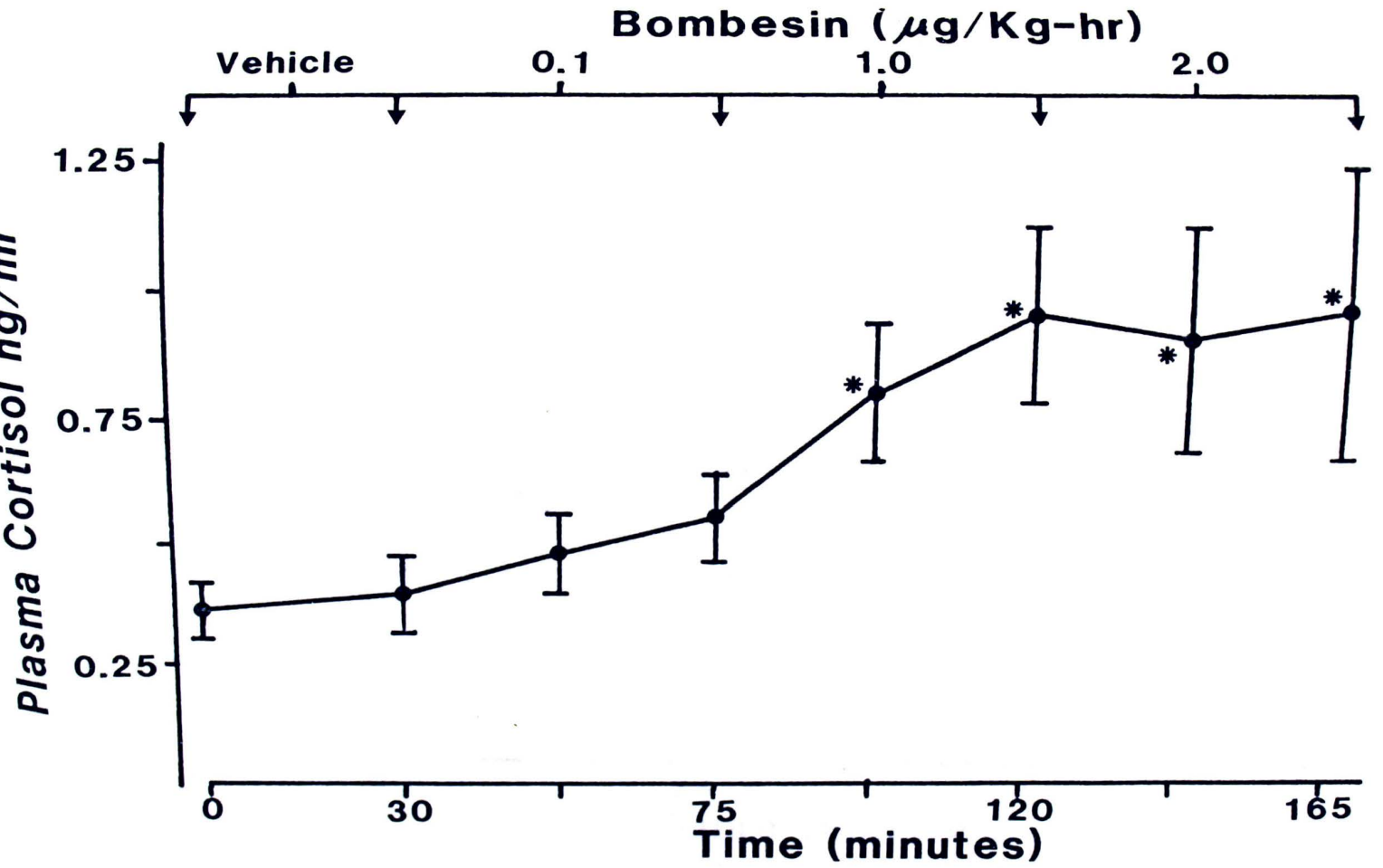

Figure 3. Plasma Cortisol Time Course Response to Bombesin. Values means ± SEM. LOG ANOVA $F(6,114) = 3.9886$. * $p < 0.05$. N=15 to 18.

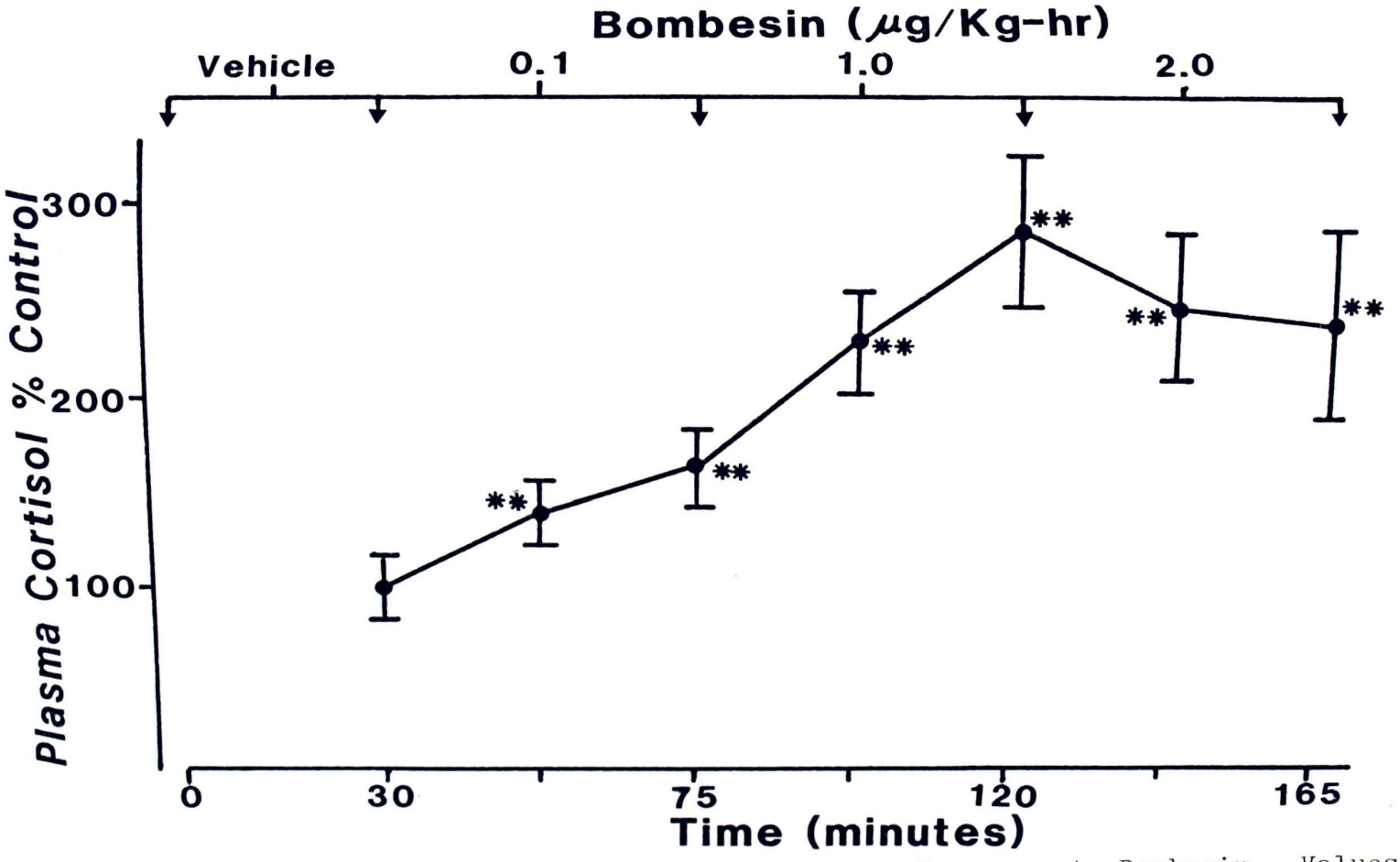

Figure 4. Plasma Cortisol % of Control Time Course Response to Bombesin. Values means ± SEM. LOG ANOVA $F(6,114) = 9.8527$.**$p < 0.01$. N = 15 to 18.

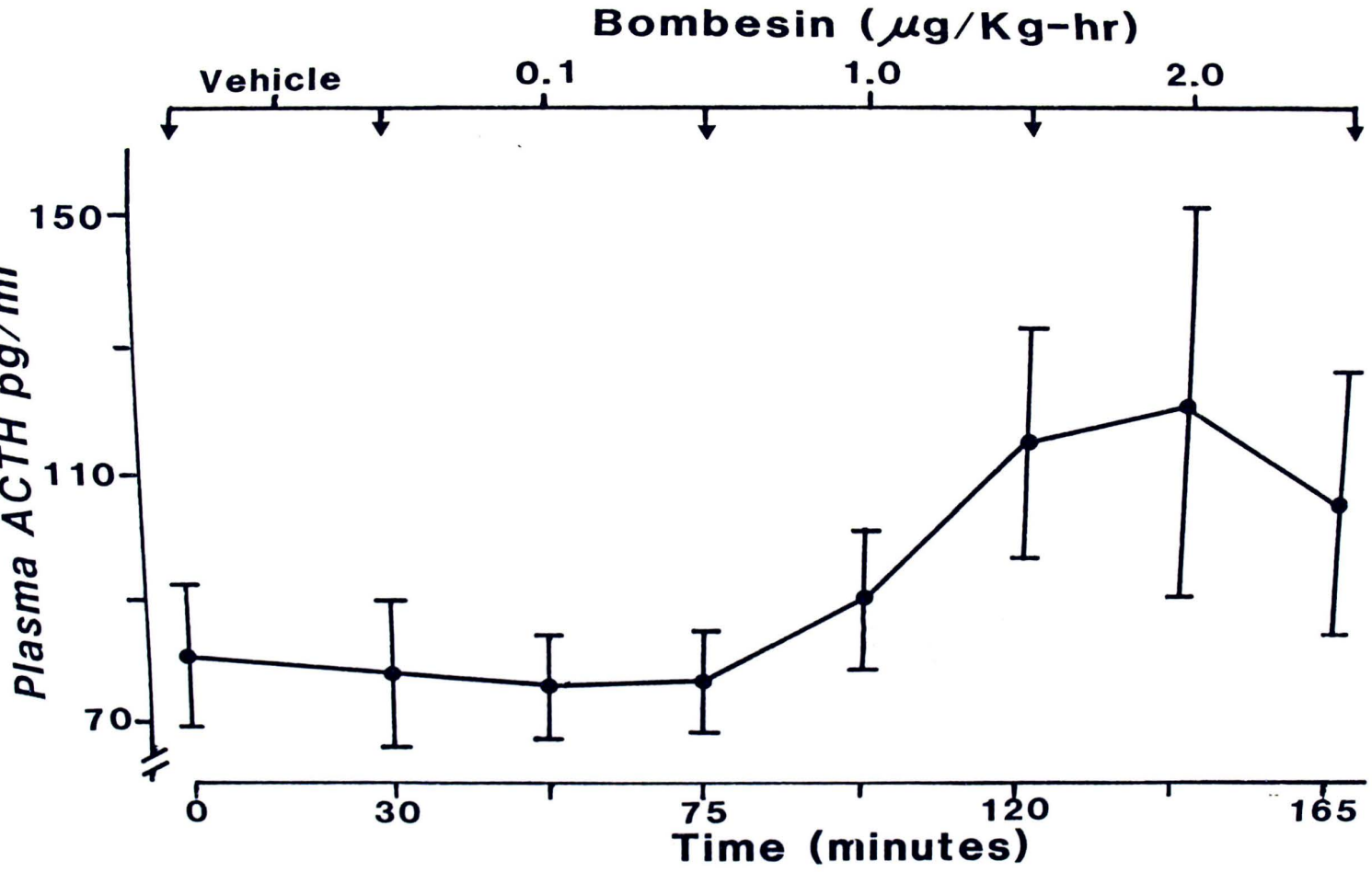

Figure 5. Plasma ACTH Time Course Response to Bombesin. Values means ± SEM. LOG ANOVA F(6,99) = 1.6804. N = 12 to 17.

pg/ml (control) to 115 ± 18 pg/ml at the 1.0 ug/Kg-hr dose and 121 ± 30 pg/ml at the 2.0 ug/Kg-hr dose. LOG ANOVA analysis did not indicate any significant change at $p < 0.05$ [$F(6,99) = 1.6804$] when using pg/ml data, however, when plasma ACTH percent of control values (Figure 6) were evaluated by LOG ANOVA significant increases were indicated [$F(6,99) = 4.5380$] at $p < 0.05$. Neuman-Keuls comparison between means showed significant differences between the means of the middle and high doses of bombesin when compared to the control means ($p < 0.05$). The maximum increase in plasma ACTH was 176 percent of control.

Plasma cortisol and plasma ACTH bombesin dose response curves are seen in Figure 7. Both show a maximum response at the bombesin dose of 1.0 ug/Kg-hr (600 pmol/Kg-hr). Half maximal response for cortisol occurred at about the 0.175 ug/Kg-hr (108 pmol/Kg-hr) dose and for ACTH at about the 0.250 ug/Kg-hr (154 pmol/Kg-hr) dose. Plasma cortisol and plasma ACTH showed similar response to bombesin doses.

There was a moderately high positive linear correlation between plasma ACTH percent of control and plasma cortisol percent of control under bombesin's influence ($r = 0.71$ at $p < 0.005$). The linear correlation and scatter diagram is shown in Figure 8.

ACTH sensitivity, defined as plasma cortisol pmol/ml divided by plasma ACTH pmol/ml, showed a significant change over the control value [one-way ANOVA, $F(6,99) = 2.7334$] at $p < 0.05$ (Figure 9). Neuman-Keuls comparison of means

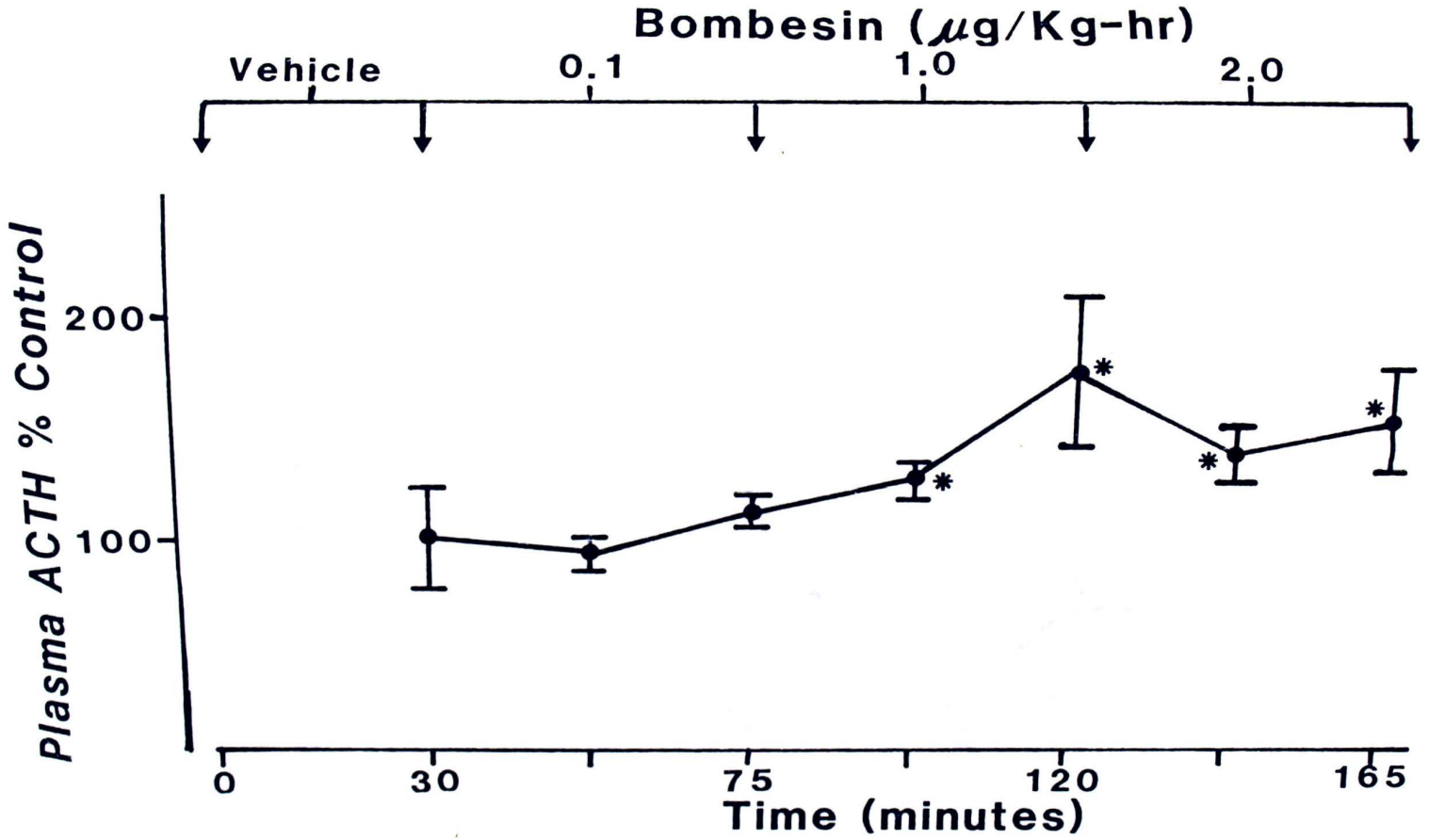

Figure 6. Plasma ACTH % of Control Time Course Response to Bombesin. Values means ± SEM. LOG ANOVA $F(6,99) = 4.5380$. *$p < 0.05$. N = 12 to 17.

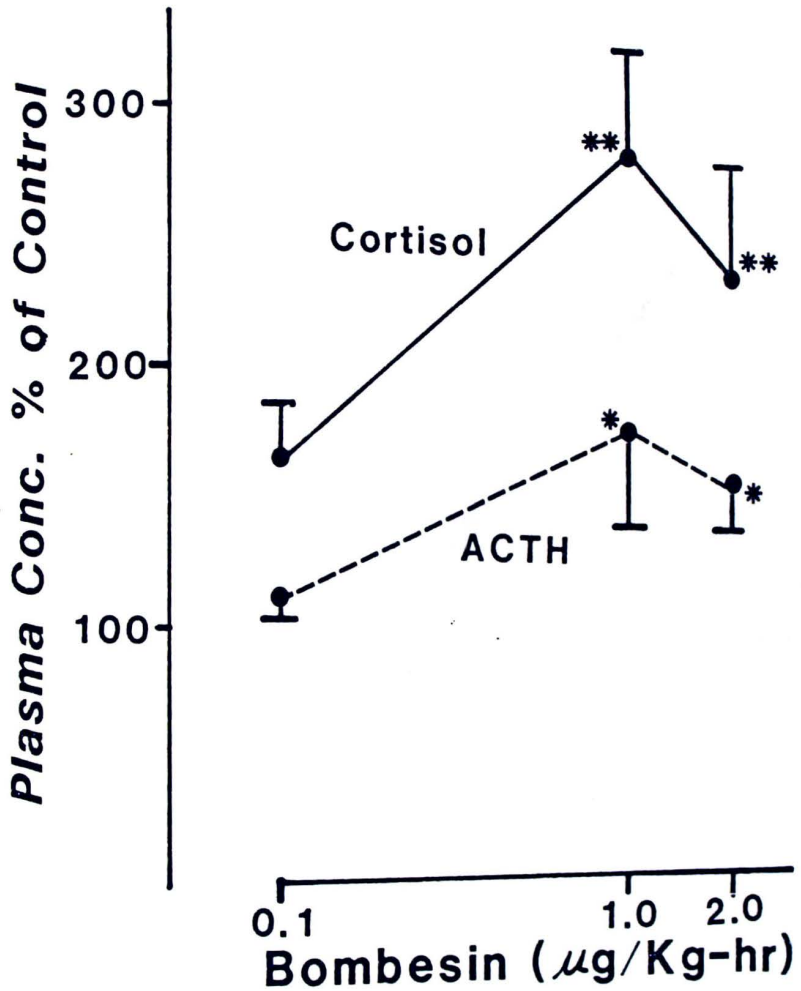

Figure 7. Plasma Cortisol and Plasma ACTH Bombesin Dose Response Curve. Values means ± SEM. *$p < 0.05$. **$p < 0.01$. N (cortisol) = 15 to 18. N (ACTH) = 12 to 17.

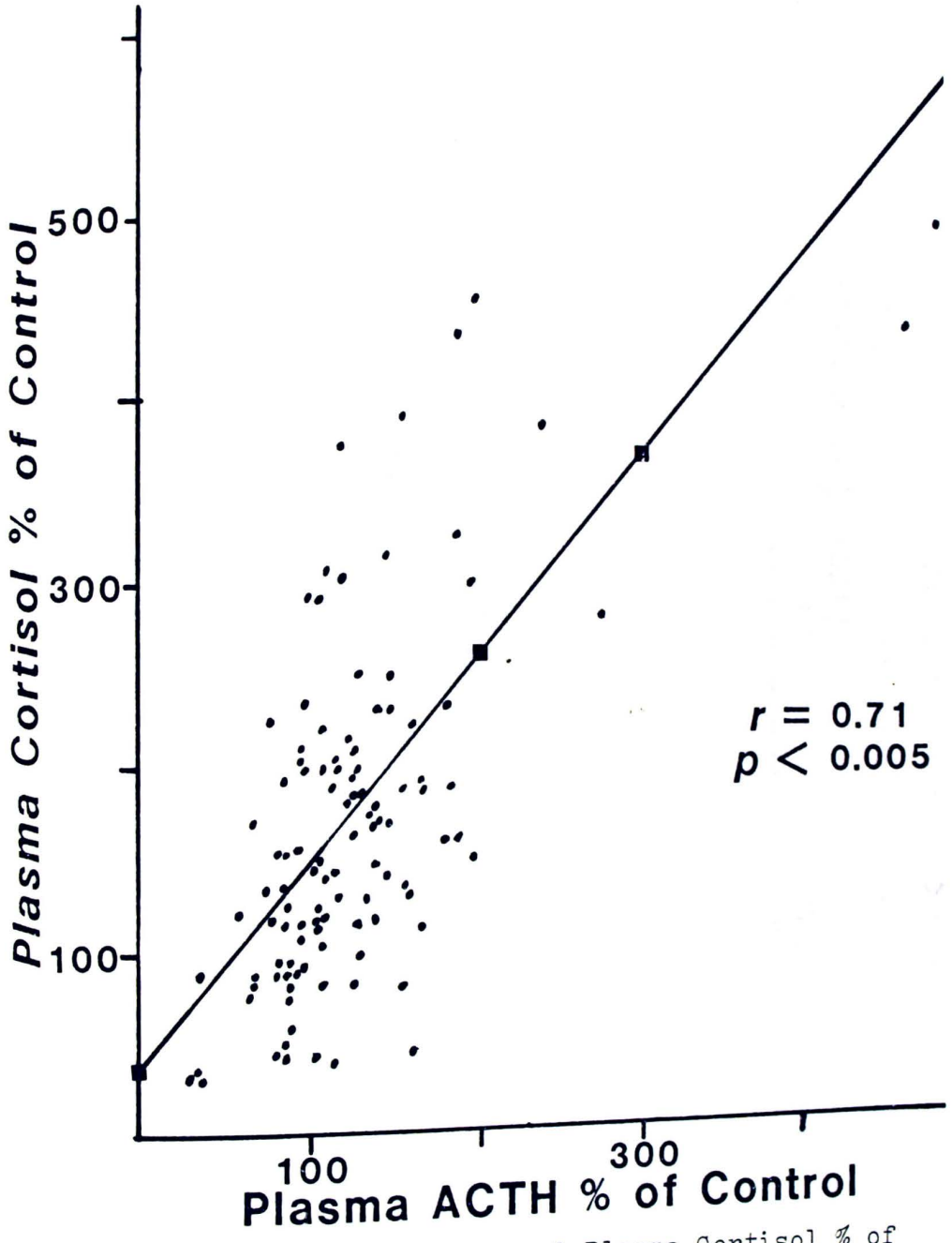

Figure 8. Plasma ACTH % of Control-Plasma Cortisol % of Control Linear Regression and Scatter Diagram. Pairs (x,y) = 108.

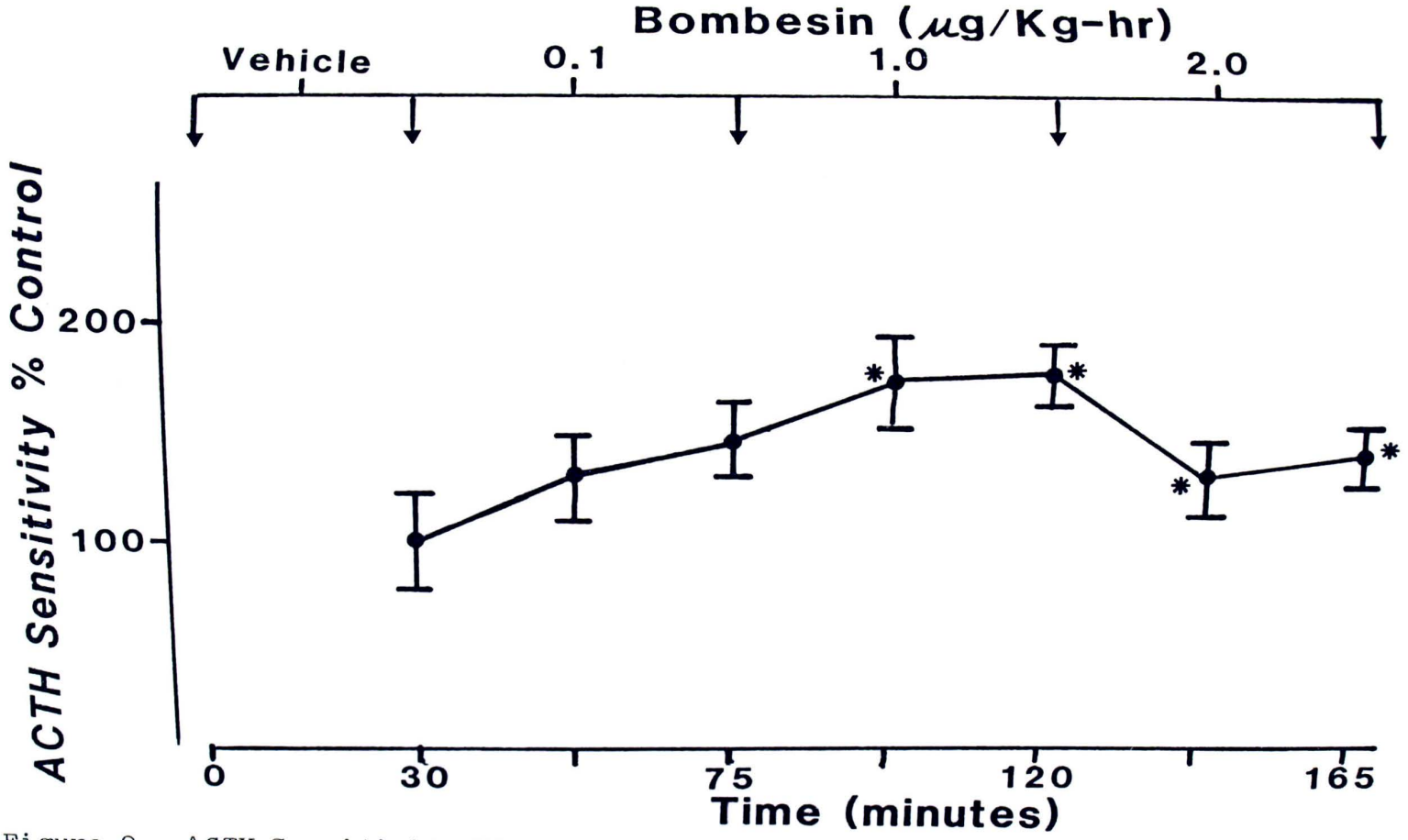

Figure 9. ACTH Sensitivity Time Course Response to Bombesin. Values means ± SEM. ANOVA $F(6,91) = 2.7334$. *$p < 0.05$. N = 12 to 17.

showed significant differences between ACTH sensitivity at the 1.0 ug/Kg-hr dose and control ($p < 0.05$).

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Discussion

The results of this study show that bombesin had a significant influence on plasma cortisol and ACTH levels in the dog. These results are different than those observed in the rat (11, 31), where it was found that bombesin did not stimulate ACTH secretion when administered directly into the brain. It was postulated from those studies that the pituitary adrenocortical system was not involved in mediating the hyperglycemic effect of bombesin. This difference in results may be attributable to species differences or to different methods of injection. It is possible that both factors come into play, since it has been observed that bombesin does cause significant increases in rat corticosterone when injected IP (14). Rasler (10) reported that bombesin's hypothermic effects in the rat probably did not involve the pituitary gland because hypophysectomy did not impair this effect. While the results presented here do not shed any light on the mechanisms of bombesin's hyperglycemic or hypothermic effects in the rat, they do strongly indicate that the pituitary, hypothalamic, or some higher brain level is involved with bombesin's effect in the dog and perhaps other mammals.

It has been proposed that some of bombesin's effects in mammals are mediated through the release of endogenous

CCK (15). Endogenous CCK is believed to be CCK_{39} or CCK_{33}, which is converted to the biologically active form, CCK_8, by the enzymatic action of trypsin and enterokinase (32). One study (33) gave evidence that endogenous CCK release was not involved in bombesin's satiety effects in rats when it demonstrated that vagotomy did not block bombesin satiety but did block CCK_8 induced satiety. Figure 10 shows that CCK_8 had no effect on either plasma cortisol or ACTH in the dog when given IV. These results predict that neither endogenous CCK release nor non-specific protein receptor binding are involved in bombesin's elevation of plasma ACTH and cortisol in the canine.

The first report that CRF caused concurrent increases in human plasma ACTH, cortisol, and aldosterone levels was recently published (34). Similar effects were observed in this study when bombesin was infused IV. While this simultaneous rise in circulating ACTH and cortisol does not allow any conclusions about the exact mechanisms of bombesin's effects, it does strongly suggest a CRF mediated role—thus, hypothalamic or higher brain level involvement.

Conclusions

1. Intravenously administered bombesin stimulates both cortisol and ACTH secretion in awake, fasted dogs. This stimulation is dose-dependent.

   a. The dose of bombesin required for a 50 percent maximal cortisol response is estimated to be 0.175 ug/Kg-hr

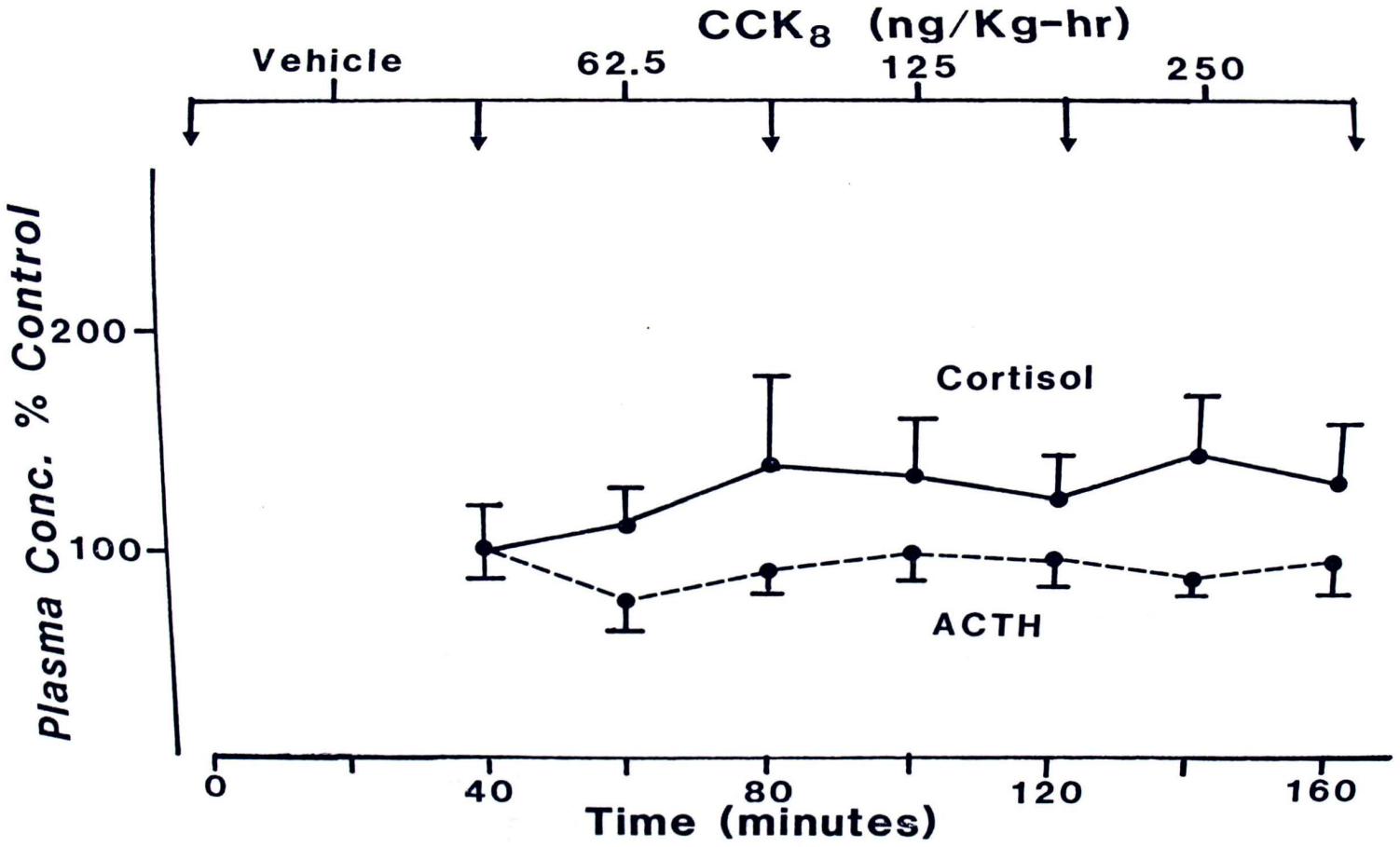

Figure 10. Plasma Cortisol and Plasma ACTH % of Control Time Course Response to CCK_8. Values means ± SEM. N = 10 to 12.

which is similar to the concentration required for 50 percent maximal gastrin, pancreatic polypeptide, and gastrin-releasing peptide release reported by others.

b. There is a direct, positive linear correlation (p 0.005) between bombesin-stimulated ACTH output and cortisol output suggesting that the cortisol effect is mediated by ACTH. Whether bombesin is acting directly on the pituitary or at a higher brain level to release ACTH is unknown.

2. Intravenously administered CCK_8 had no effect on either cortisol or ACTH secretion in awake, fasted dogs. This indicates that although bombesin, in the doses administered, may release endogenous CCK, the pituitary-adrenal secretion observed in response to bombesin is not dependent on endogenous CCK release.

The lack of response to CCK_8 observed in the study conflicts with earlier observations reported by our laboratory (14) indicating that CCK_{33} is a powerful stimulant of corticosterone secretion in the fed rat. This discrepancy may be due to species variation, molecular form of the hormone used, route of administration of the hormone (IV vs IP), and/or the differing fasted/fed state of the animals.

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