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Behavioral Effects of Betacarbolines in Pigs: Anxiety or Aversion?

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PARROTT, R. F., S. V. VELLUCCI AND J. A. GOODE. *Behavioral effects of betacarbolines in pigs: anxiety or aversion?* PHARMACOL BIOCHEM BEHAV **66**(4) 713–719, 2000.—Betacarbolines are often considered to be anxiogenic and may, therefore, have similar behavioral effects to those of corticotrophin releasing hormone (CRH); however, their actions have been little studied in pigs. This investigation was concerned with the effects of ethyl-β-carboline-3-carboxylate (BCCE) and noreleagnine (NOR) on operant feeding, cortisol release, and overt behavior in swine, all of which are known to be affected by CRH in this species. Three experiments are described in which BCCE or NOR were given intravenously to prepubertal boars ($n = 7$). In Experiment 1, 400 μg/kg, but not 100 or 200 μg/kg, BCCE produced a rapid inhibition of ingestive activity whereas NOR (100, 200, or 400 μg/kg) was without effect. In Experiment 2, both BCCE and NOR increased plasma cortisol, but not growth hormone, concentrations. In Experiment 3, a high dose of BCCE (2 mg/kg) produced transient arousal and a sustained increase in respiration rate and plasma cortisol. These results indicate that although the responses of pigs to BCCE and CRH are similar in some respects, there are also marked behavioral differences. The possibility that BCCE has aversive rather than anxiogenic actions in this species is discussed. © 2000 Elsevier Science Inc.

Pigs Betacarbolines Feeding Cortisol Behavior CRH

RESEARCH into environmental effects on the behavior and physiology of pigs is driven by the welfare implications of current agricultural practice. In this context, emotional responses generated by uncertainty, i.e., anxiety, and its biological basis, have been little studied in this species.

Although corticotrophin releasing hormone (CRH) is generally considered to be anxiogenic (1,8,13,29), work in rodents suggests that other endogenous neuropeptides may have similar actions. For example, central administration of octadecaneuropeptide (ODN), a ligand for the benzodiazepine (BZ) binding site on the $GABA_A$ receptor, produces anxiogenic responses in rodents (15). Moreover, the reported effects resemble those of betacarboline compounds that act as BZ inverse agonists. However, in a recent study using pigs, intracerebroventricular (ICV) administration of porcine CRH produced anxiety-like responses whereas an equimolar dose of ODN was without effect (26). Given this discrepancy, an examination of the behavioral actions of betacarbolines in swine is indicated.

Some betacarbolines induce seizures (inverse agonists) whereas others are merely pro-convulsant (partial inverse agonists). Amongst the latter, ethyl-beta-carboline-3-carboxylate (BCCE) (7) and its derivative FG7412 have been extensively studied in rodent models of anxiety (33). For example, BCCE, the subject of this report, reduces social interaction (16) and has proconflict activity in tests of punished responding (27). Also, in monkeys, high intravenous (IV) doses of BCCE (2.5 mg/kg) cause behavioral distress and activate the autonomic nervous system and the hypothalamo/pituitary/adrenocortical (HPA) axis (23). All of these effects are prevented by the BZ antagonist flumazenil, indicating action at the BZ binding site. Nevertheless, doubt has to be cast on the view that the effects of BCCE in such tests are actually representative of anxiety (33).

The literature is also unclear regarding the extent to which CRH and betacarbolines may share common neuronal mechanisms mediating anxiety. For example, the effect of CRH on punished responding (5), or acoustic startle (31), is antagonized by BZs possessing anxiolytic activity but the CRH antagonist α-helical $CRF_{9\text{-}41}$ does not modify conflict behavior

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induced by the betacarboline DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; 12). Moreover, flumazenil antagonizes the effect of CRH in the conflict (4,12), but not in the social interaction or elevated plus maze (18), test.

In rodents, CRH inhibits ingestive behavior (22) and also seems to be involved in the stress-induced suppression of eating (20,21). Also, in pigs, CRH (ovine) decreases operant feeding and contemporaneously induces emotional responses but does not affect deprivation-induced drinking (24). Similarly, BCCE produces a dose-related inhibition of operant feeding, but not drinking, in swine (14). However, the latency of this effect (50 min) was surprisingly long, considering that BCCE was given by the IV route. Furthermore, no abnormal behavior was seen and the highest dose had no effect on the HPA axis (Vellucci and Parrott, unpublished).

Owing to the insolubility of betacarbolines, the delayed action of BCCE in the above experiment (14) may have been due to the use of a saline vehicle. Indeed, an earlier study in which FG7142 was given intraperitoneally (IP) to rats reported a reduction in food intake measured after a similar interval (6,10). Therefore, it was hypothesized that a more suitable vehicle (dimethylsulfoxide [DMSO]) and a higher drug dose might reveal more rapid (i.e., CRH-like) effects on feeding in pigs. It might also be anticipated that there would be activation of the HPA axis and that an even greater IV dose of BCCE, similar to that used in the monkey (23), would produce anxiety-like behavioral responses in a nonfeeding situation.

Unusually, noreleagnine (2,3,4,9 tetrahydro-1H-pyrido [3,4-6] indole; tetrahydro norharmane; NOR) is a beta carboline-like compound that is not only soluble in aqueous solution but also affects conflict behavior in the rat in the same way as BCCE when given at similar doses (28). These properties raise the possibility of using NOR for ICV administration, hence, with a view to future studies, a comparison of the peripheral actions of NOR and BCCE in pigs seemed appropriate in the first instance. The objectives of this study, therefore, were, firstly, to examine the effects of BCCE and NOR on operant feeding in growing pigs; secondly, to measure changes in plasma concentrations of cortisol and growth hormone (GH), and, thirdly, to record the behavioral response to a high dose of BCCE under the same test conditions as previously described for porcine CRH (26). In this way, it was hoped to determine whether betacarbolines have CRH-like (anxiogenic) effects in pigs.

METHOD

The animals used were 7 Large White prepubertal boars initially weighing around 25 kg. Each pig was surgically prepared under halothane anesthesia, using sterile precautions, with a catheter in the jugular vein; these were kept patent by daily flushing with sterile heparinized saline and protected by elasticated bandages. Throughout the investigation, the animals lived in individual metabolism cages equipped with operant panels for the delivery of food and water. The pigs rapidly learnt to press the panels with their snouts to obtain reinforcements of pelleted food (approximately 21 g) or water (approximately 20 ml) and were then trained to a fixed ratio of 5 presses per reinforcement. Water was continuously available but food could only be obtained twice daily (1045 to 1130h and 1700 to 1730 h); the start of each feeding period was signaled by a buzzer. The animals were regularly handled so that they became habituated to human contact and were weighed weekly.

All experimental procedures were carried out in accordance with the UK Animal Scientific Procedures Act 1986 (Project Licence No 80/1269) and drugs were obtained from Aldrich Chemical Co (BCCE; Milwaukee, WI, USA) or Sigma UK Ltd (NOR; Dorset, UK). Drug treatments were prepared on an individual animal basis just before administration: both BCCE and NOR were dissolved, or suspended, in 0.8 ml DMSO (Sigma Ltd) and then made up to 2 ml with sterile saline (SAL). Vehicle injections consisted of DMSO + SAL (VEH) and a 2-ml SAL control injection was also used; all injections were flushed in with a further 2 ml SAL.

The effect of BCCE and NOR on operant food intake was examined in Experiment 1. The weight of the animals (mean ± SEM) at the beginning and end of the 4-week experimental period was 30.9 ± 1.5 and 41.9 ± 2.3 kg, respectively. The animals were randomly assigned to the 8 experimental treatments: BCCE, 100, 200, and 400 μg/kg; NOR, 100, 200, and 400 μg/kg; VEH control, and SAL control. Testing was carried out daily (AM) on weekdays, with treatment days alternating with no treatment (NIL) days. The experimental protocol involved injecting the drug or control solution 5 min after the start of feeding; this design was adopted to ensure that only animals that were actively responding for food received injections. Food intake was recorded for the duration of the 45-min period and expressed as the number of reinforcements (mean ± SEM) per 5-min time bin. Results from NIL treatment days were averaged to provide a single data set for each pig. No records were made of operant water intake and no observations were made of the animals' behavior.

In Experiment 2, six of the animals from Experiment 1 that still had patent catheters were used to test the highest (400 μg/kg) doses of BCCE and NOR on hormone release. Pairs of pigs received BCCE, NOR, or VEH injections in a different sequential order each afternoon over a 3 day period. Blood samples were collected between 1410 and 1530 h at 10-min intervals for 30 min before and 60 min after each injection. The samples were held on ice and subsequently centrifuged, with the resultant plasma stored at −30°C pending radioimmunoassay for cortisol and growth hormone (GH), as previously described (9,24). The latter hormone was investigated because a previous study (26) suggested that it might be responsive to anxiogens in this species. No standardized behavioral recordings were made in this experiment.

The behavioral effects of a high IV dose of BCCE (2 mg/kg) were examined in 5 of the pigs in Experiment 3. Three animals received BCCE on the first test day and VEH on the second with the remainder tested in the reverse order. Tests lasted 60 min and were carried out at the same time each day according to a previously devised scheme (25,26). Briefly, extremes of alertness (drowsy or agitated), the pig's posture (standing, sitting or lying), the occurrence of oro-nasal activity (chewing or nosing) and vocalization (regular grunting), and whether there was a high state of activity, was scored for each 5-min period. Thus there was a maximum score of 12 for each of these nonmutually exclusive categories; these scores were then used to derive medians with interquartile ranges. Notes were also made of other activities not included in this list. Blood samples for cortisol analysis also were collected before the treatment injections (time zero) and at 20-min intervals thereafter (i.e., + 20, 40, and 60 min). Plasma was obtained from 4 of 5 pigs with but the samples from one animal, unfortunately, were lost.

The data from Experiment 1 were analyzed in the following manner: the total number of food reinforcements (mean ± SEM) was calculated for each treatment condition in the pretreatment (0 to 5 min) and post-treatment (6 to 45 min) peri-

ods with between-treatment comparisons in both periods carried out using a two-way analysis of variance. Subsequently, VEH x treatment comparisons were made using the two-tailed paired 't' test, with the probability values obtained adjusted for multiple comparisons using Kendall's error correction. In Experiment 2, overall concentrations of cortisol and GH before and after treatment administration were contrasted using the two-tailed paired *t*-test; the same analysis was also carried out for cortisol concentrations in Experiment 3. Finally, differences in behavior between control and treatment effects in Experiment 3 were examined using the sign test. However, because only 5 animals were used, the highest level of significance that could be obtained in a two-tailed test was $p < 0.06$ ($p < 0.03$, one-tailed).

RESULTS

The effects of 3 doses of NOR and BCCE on operant feeding in 17 h food-deprived pigs were examined in Experiment 1. The results (Fig. 1) are presented as graphs showing the change in feeding activity with time for control, NOR, and BCCE treatments, and as histograms indicating the total number of food reinforcements obtained in the postinjection period for each treatment condition. The statistical analysis failed to detect differences between treatments in the first 5 min of feeding, i.e., the preinjection period, whereas treatment effects with respect to total intake in the postinjection period (6 to 45 min) were highly significant ($p < 0.001$). There was a steady decline in reinforcement rate during the test period in the 3 control conditions (VEH, SAL, NIL) and the total intakes were similar; this indicates that neither the use of DMSO as a vehicle (VEH × NIL), nor the injection itself (cf SAL × NIL), affected operant behavior. Moreover, the pattern of feeding was very similar when the animals were given NOR and no dose-related effects on total intake were apparent. By contrast, BCCE reduced operant intake in a dose-dependent fashion, with the highest dose (400 μg/kg) causing a complete cessation of feeding 20 min after administration. Total intake was significantly reduced by both the 200 and 400 μg/kg doses of BCCE; however, after correction for multiple comparisons only the effect of the highest dose remained significant ($p < 0.01$). The data also suggest that there was a partial recovery from the effects of the drug 35 min postinjection.

The results of Experiment 2 are illustrated in Fig. 2 as both time-related changes in cortisol and GH concentrations and histograms showing the change from baseline, taking the preinjection and postinjection periods as a whole. There was a rise in plasma cortisol in the 60-min period following administration of both NOR and BCCE; the overall change was significant for both treatments (NOR, $p < 0.05$; BCCE, $p < 0.01$). However, with respect to GH, there were marked individual variations in the secretory pattern, especially during the pretreatment period. As a consequence, no meaningful treatment effects were found. The behavior of the pigs was not recorded in this experiment although it was noted that arousal, accompanied by vocalization in some animals, occurred in the first few minutes after BCCE administration. However, these effects did not seem to last for more than approximately 10 min.

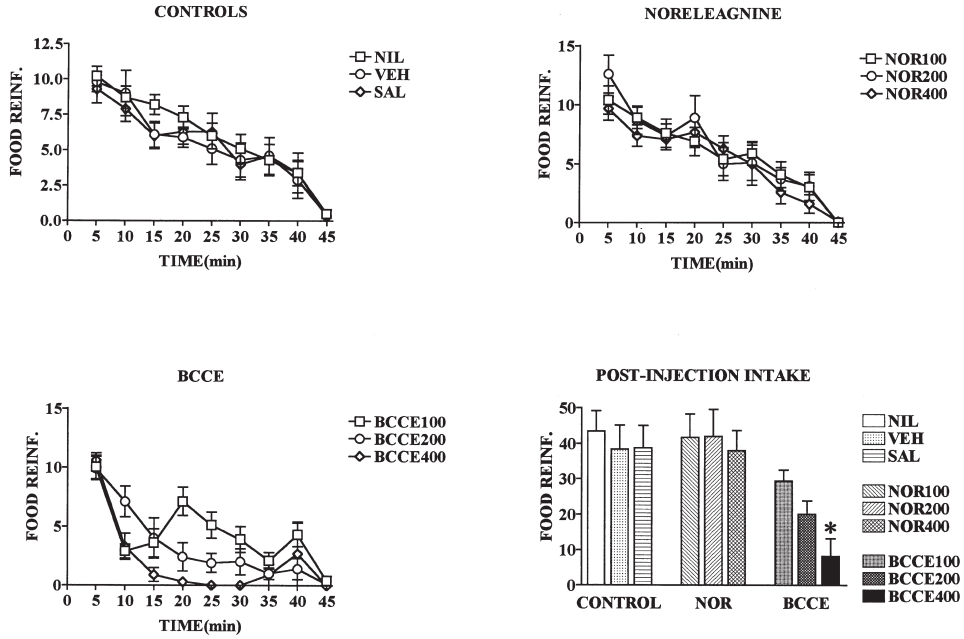

FIG. 1. Effects of control treatments (NIL, VEH, SAL) and 3 doses (100, 200, and 400 μg/kg IV) of NOR and BCCE on operant feeding in pigs ($n = 7$) in Experiment 1. The graphs show the number of reinforcements (mean ± SEM) obtained during each 5-min period of the test; injections were given at the end of the first 5-min period. The histograms show total intake (mean ± SEM) in the postinjection period (6 to 45 min) for each treatment condition. Significant ($p < 0.001$) treatment effects were detected and the highest dose of BCCE produced a significant (*$p < 0.01$ vs. VEH) reduction in responding.

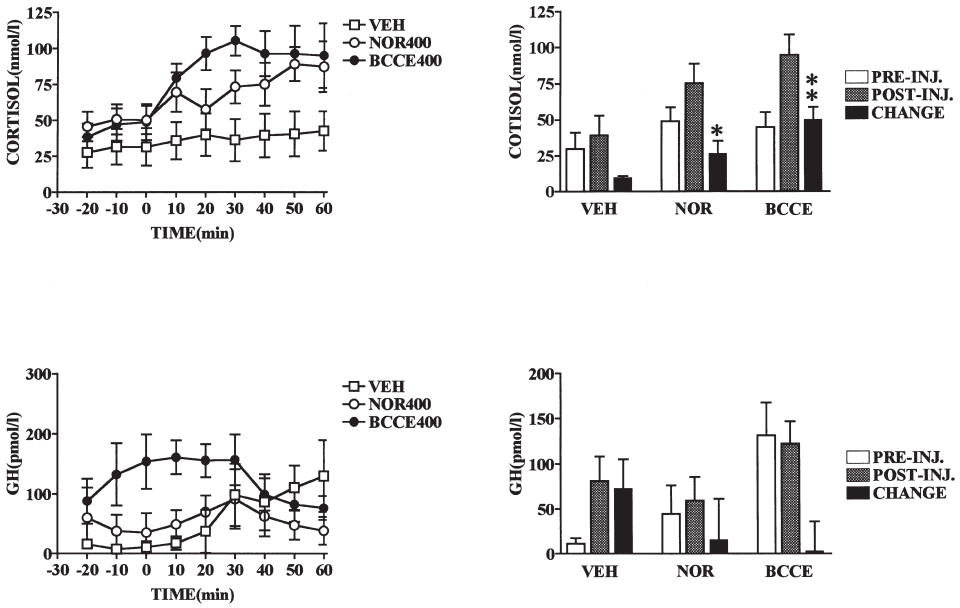

FIG. 2. Effects of NOR and BCCE (400 μg/kg) on plasma cortisol (nmol/l) and GH (pmol/l) concentrations (mean ± SEM; $n = 6$) in Experiment 2. The graphs show the pattern of hormone release before and after injection at time zero. Hormone concentrations (mean ± SEM) averaged over the periods before and after injection, and the mean changes, are shown as histograms. Both NOR and BCCE produced significant increases (*$p < 0.05$, **$p < 0.01$; respectively) in cortisol but not in GH.

The behavioral scores of pigs treated with the high dose (2 mg/kg) of BCCE or VEH in Experiment 3 are presented as histograms in Fig. 3 A–D. Alertness data are shown only for the extremes, i.e., drowsiness, distinguished as resting with the eyes closed, and agitation indicated by a high state of arousal. The reduction in drowsiness caused by BCCE just failed to achieve significance ($p < 0.06$; see Method) and agitation, observed in 4 of 5 pigs after BCCE compared with 0 of 5 receiving VEH, was largely restricted to the 5 min after the injection. The posture of the animals was similar when treated with BCCE or VEH, although BCCE appeared to increase the incidence of standing. Regarding oro-nasal activity, chewing tended to be more frequent following BCCE injection. Finally, in relation movement and vocalization, the intensity of behavior induced by BCCE was not sustained and regular grunting was exhibited by 3 of 5 pigs, but only in the first 10 min of the test.

The high dose of BCCE also produced autonomic and neurologic responses. In particular, two pigs displayed emesis, all 5 exhibited a considerable increase in respiratory rate that lasted for the duration of the test and 4 of 5 animals showed twitches (3 to 4 per test). In addition, administration of this dose of BCCE stimulated cortisol release; this is shown (Fig. 4) graphically as the change in hormone concentrations with time and also as a histogram of the change from baseline. Because data were only available from 3 of the animals, the overall increase in plasma cortisol just failed to achieve significance in a two-tailed test (p approximately equal to 0.05). There was also a tendency for GH concentrations to increase following BCCE injection (Fig. 4).

DISCUSSION

The present results confirm the inhibitory effect of BCCE on operant feeding in pigs; the combination of a higher dose and DMSO vehicle produced a more rapid response than previously reported (14). Unexpectedly, however, the betacarboline-like drug NOR, given over the same dose range, was without effect. Both BCCE and NOR stimulated cortisol release, although the increase induced by NOR achieved a lower level of significance, but neither altered GH concentrations. A high dose of BCCE (2 mg/kg IV) also produced only transient arousal although there was a sustained increase in respiratory rate and cortisol release.

The behavioral effects of BCCE are presumed to be due to an action at the BZ site on the $GABA_A$ receptor and are usually antagonized by flumazenil (17). Recently, however, BCCE has also been shown to be a ligand for the loreclezole site at this receptor (30). Loreclezole is anxiolytic but its actions are unaffected by flumazenil (11). Therefore, it is theoretically possible that some of the behavioral responses to BCCE may be mediated by action at this site. However, the present study did not utilize flumazenil and so this issue cannot be addressed.

When given to pigs in aqueous vehicle, BCCE (30 and 60 μg/kg IV) reduced operant feeding with a latency of approximately 50 min (14). This effect was specific because BCCE did not affect drinking in water-deprived animals and no behavioral or endocrine changes were observed; reduced GABA transmission in hypothalamic neurones mediating feeding may have caused this effect (3). However, although water intake was not investigated in the present study, the fact that inhibition of feeding in Experiment 1 was rapid and associated with activation of the HPA axis is indicative of a nonspecific action. Moreover, this response resembles that induced in pigs by IV administration of the aversive peptide cholecystokinin (2). In contradistinction, the inhibitory action of CRH has a latency of 20 to 30 min and is accompanied by excitation and vocalization (24). Similarly, emotional arousal also appears to be responsi-

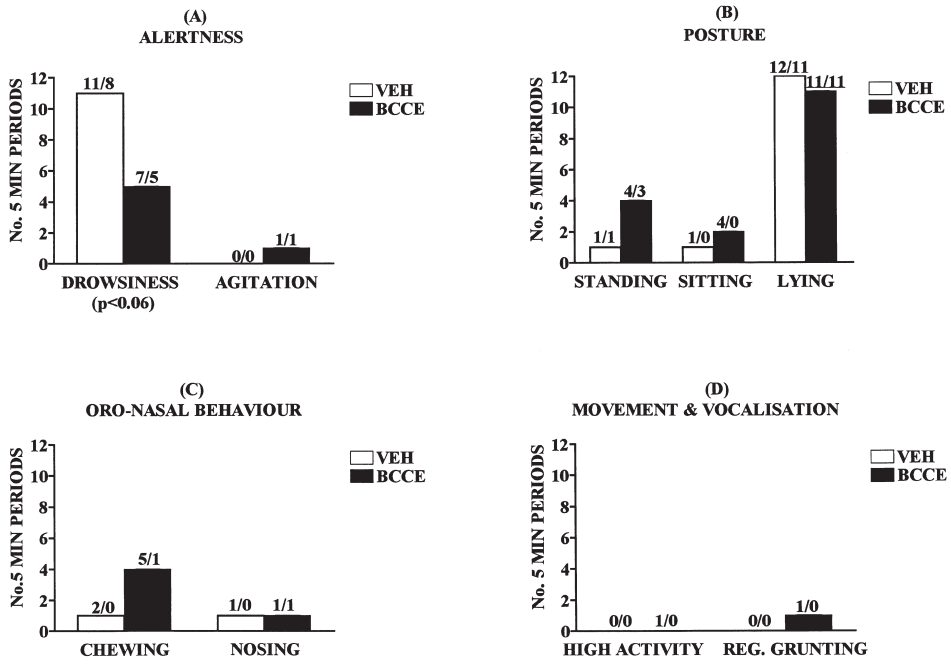

FIG. 3. Behavioral responses (A, Alertness, B, Posture, C, Oro-Nasal Behavior, D, Movement, and Vocalization) of pigs ($n = 5$) to IV injection of VEH or BCCE (2 mg/kg) in 60-min tests in Experiment 3. A behavioral score, i.e., the number of 5-min periods (max = 12) in which a particular activity or state was recorded, was derived for each animal under both experimental conditions. These scores were then used to obtain the median number of responses per treatment, shown as histograms and their respective upper and lower interquartile ranges, given above each column. Statistical comparisons (VEH × BCCE) for a particular behavioral index were made using the sign test (maximum possible significance $p < 0.06$ two-tailed, $p < 0.03$ one-tailed) with the value shown in parenthesis.

ble for the suppression of eating in CRH-treated rats (20,21). Nevertheless, it is possible these dissimilar effects of BCCE and CRH in swine may merely reflect the routes of drug administration used (BCCE, IV; CRH, ICV).

Centrally administered CRH increases plasma cortisol by stimulating adrenocorticotrophic hormone release from the pituitary. This may be caused by diffusion into the portal vessels (neuroendocrine effect) or by activation of hypophysiotrophic neurones (possible psychoneuroendocrine effect). The latter may also occur after IV betacarboline injection, although a peripheral action at the pituitary is also possible. Interestingly, both BCCE and NOR induced cortisol release in Experiment 2 but only BCCE affected behavior in Experiment 1; this may indicate different sites/modes of action in ungulates, as compared with rodents (28). Moreover, in contrast to the trend observed with CRH (26), neither agent altered GH concentrations.

The demonstration of an inhibitory effect of BCCE on operant feeding in Experiment 1 does not, in itself, provide evidence of an anxiogenic action. Similarly, conflict tests, which are commonly used to determine anxiogenic properties of drugs, also rely on inhibition of ingestive activity, the defining factor being a reduction in punished but not unpunished responding. However, such findings are open to question because a mildly aversive agent might not affect unpunished behavior if the motivational state is high whereas, in the punishment condition, aversion and anxiety may summate to reduce responding. Also, a drug that has opposite effects to an anxiolytic BZ in such tests may not necessarily be anxiogenic (33). Furthermore, BCCE can suppress operant responding by acting as a punisher but so also can drugs such as nicotine and histamine (32). Therefore, it is doubtful whether tests in which an anxiogenic response is signaled by a decrement in behavior can truly discriminate between anxiety and aversion. Because of this, it is desirable also to measure activational effects of putative anxiogens. This can be done, for example, by using acoustic startle tests or by making recordings of spontaneous behavior, as in Experiment 3.

There are few observational studies of betacarbolines reported in the literature. However, in chair-restrained monkeys, a high dose of BCCE (2.5 mg/kg) was found to produce extreme and long-lasting (up to 2 h) reactions that included struggling, turning, vocalization, defecation, and increases in heart rate, blood pressure, plasma cortisol, and catecholamines. A subsequent experiment (9) reported similar effects in 3 of 4 animals given 200 μg/kg BCCE IV, but the authors were unable to decide whether the responses represented stress or anxiety. However, in pigs, the high dose of BCCE (Experiment 3) produced less obvious changes in behavior that were of shorter duration, whereas, a long-lasting increase in respiratory rate was noted. The latter may related to a change in body temperature (not measured) because BZs can affect thermoregulation (19).

Comparison of the results from Experiment 3 with those of a previous study (27) in which CRH-treated pigs were tested using the same scoring system indicates major differences in behavior patterns. Statistically significant ($p < 0.03$; 27) responses characteristic of CRH were decreased drowsi-

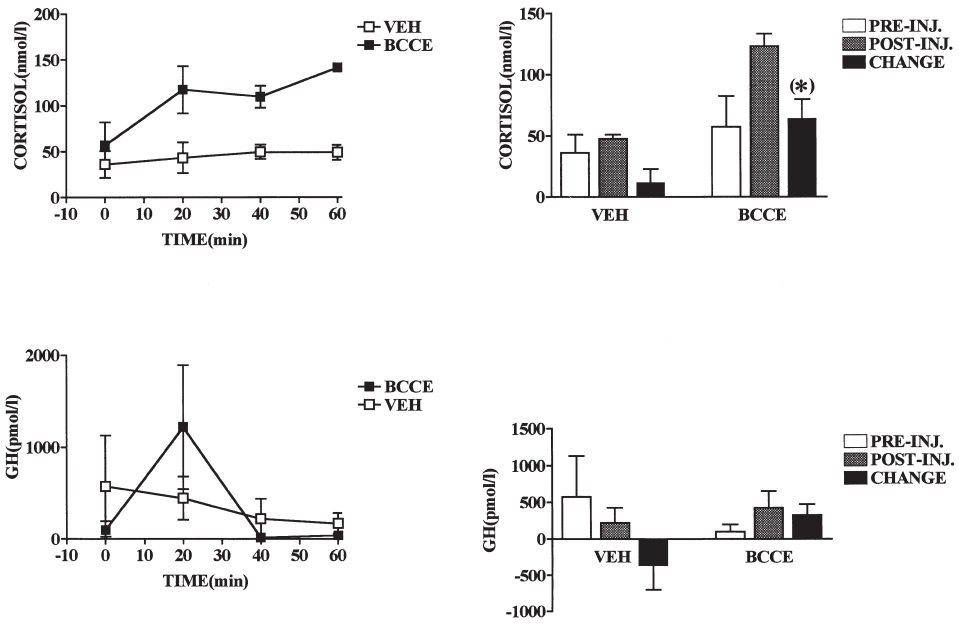

FIG. 4. Effect of BCCE (2 mg/kg) on plasma cortisol (nmol/l) and GH (pmol/l) concentrations (mean ± SEM; $n = 3$) in Experiment 3. The graphs show the pattern of hormone release directly before, and after, the injection at time zero. Cortisol and GH concentrations (mean ± SEM) averaged over the periods before and after injection, and the mean change, are shown as histograms. The increase in cortisol induced by BCCE approached significance, (*)p approximately 0.05.

ness and lying, increased agitation, standing, chewing, nosing, and overall activity level, and the production of regular grunting (27). By contrast, although BCCE-treated pigs tended to be less drowsy ($p < 0.06$; Fig. 4) they showed few other CRH-like responses. It had also previously been noted (26) that CRH induced turning, bowl-play, barking when approached, gagging, scampering, and defecation, none of which were much in evidence in Experiment 3. On the other hand, the pro-convulsant nature of BCCE was revealed by the occasional occurrence of twitching; this also indicates that a sufficiently high dose of the drug was used.

In conclusion, the results of this and the previous (26) experiment suggest that CRH has prolonged activational effects in pigs whereas BCCE is initially arousing and subsequently debilitating. Hence, the decrease in motivated behavior brought about by ICV CRH may be caused by competing emotional responses whereas the inhibition induced by IV BCCE seems to be due to some other factor, possibly aversion associated with malaise. Thus the present findings, together with the nonanxiogenic action of ODN (26), raise questions regarding the role of endogenous betacarbolines as mediators of anxiety in this species.

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