J. Steroid Biochem. Molec. Biol. Vol. 43, No. 4, pp. 351–358, 1992
Printed in Great Britain

0960-0760/92 $5.00 + 0.00
Pergamon Press Ltd

ACTIONS OF ACETYL-L-CARNITINE ON THE HYPOTHALAMO–PITUITARY–GONADAL SYSTEM IN FEMALE RATS

LAZAR Z. KRSMANOVIĆ,[1]* MOHAMED A. VIRMANI,[2] STANKO S. STOJILKOVIĆ[1] and KEVIN J. CATT[1]†

[1]Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, NIH, Bethesda, MD 20892, U.S.A. and [2]Research and Development, Department of Pharmacology, Sigma-Tau S.p.A. 00040 Pomezia, Italy

(Received 25 March 1992)

Summary—Acetyl-L-carnitine (ALC) is known to affect several aspects of neuronal activity. To evaluate the neuroendocrine actions of this compound, several endocrinological parameters were followed in ALC-treated and control animals during recovery from dark-induced anestrus. In treated animals, serum luteinizing hormone (LH) and prolactin levels were higher than those of controls during the proestrous and estrous phases of the cycle, and serum estradiol levels were higher during estrus. No significant changes were observed in serum levels of follicle-stimulating hormone and progesterone. Uterine weight was increased in ALC-treated rats during proestrus and estrus, but not in diestrus. The basal release of gonadotropin-releasing hormone (GnRH) from perifused hypothalamic slices of ALC-treated animals was elevated at proestrus and diestrus, and GnRH release elicited by high K^+ was higher during all three phases of the cycle. The basal release of LH from perifused pituitaries of treated animals was elevated in diestrus, and the LH response to GnRH was higher in estrus and diestrus I. Depolarization with K^+ caused increased LH secretion during proestrus and estrus in treated animals. In contrast to these effects of ALC treatment *in vivo*, no direct effects of ALC were observed during short- or long-term treatment of cultured pituitary cells. These results indicate that ALC treatment influences hypothalamo–pituitary function in a cycle stage-dependent manner, and increases the secretory activity of gonadotrophs and lactotrophs. Since no effects of ALC on basal and agonist-induced secretory responses of gonadotrophs were observed *in vitro*, it is probable that its effects on gonadotropin release are related to enhancement of GnRH neuronal function in the hypothalamus.

INTRODUCTION

Acetyl-L-carnitine (ALC) and carnitine are involved in mitochondrial energy metabolism and are found throughout the body [1]. These compounds are distributed unevenly in the brain, with the highest concentration in the hypothalamus [2]. Carnitine levels in the hypothalamus are increased after 24 h treatment with hydrocortisone [2], and carnitine transport into CCL 27 heart cells is increased by prednisolone treatment [3]. In the brain, both ALC and carnitine have been reported to reduce neuronal death and damage associated with aging, or caused by drugs such as methylazoxymethanol [4, 5]. These compounds bear a structural resemblance to acetylcholine, and ALC has been reported to exert cholinomimetic effects [e.g. 6, 7]. Carnitine is present in cerebrospinal fluid at higher concentrations than gamma-amino butyric acid (GABA), and has been proposed to modulate the GABA system via an effect on the GABA carrier [8, 9]. Since both compounds are abundant in the hypothalamus and can affect neuronal activity, either directly or by influencing the energy status of the cells, it was of interest to evaluate their actions on neuroendocrine function.

In preliminary studies we observed that under normal lighting conditions (14 h light, 10 h dark), 4-day estrous cycles occurred more frequently in ALC-treated rats than control females. To evaluate the effects of ALC on the establishment of the cycle, and the possible endocrine actions of ALC, experiments were performed on dark-induced anestrus rats that were treated with ALC during darkness and recovery under normal lighting conditions. Several changes in

*On leave from Institute of Biology, University of Novi Sad, Yugoslavia.
†To whom correspondence should be addressed.

pituitary and gonadal hormone levels were observed in ALC-treated females, indicating that the neural actions of the drug include effects on neuroendocrine function.

EXPERIMENTAL

Animals

Three-month-old female Sprague–Dawley rats were treated for 1 month with ALC by addition of the compound (50 mg/kg/day) to their drinking water. Estrous cycles were followed by vaginal smears taken daily at 8 a.m. and animals showing 2 consecutive 4-day estrous cycles were selected for experiments. Controls and ALC-treated rats were kept under constant dark conditions to induce anestrus. After return to normal lighting, rats in early proestrus, estrus, and diestrus I were sacrificed between 8 and 9 a.m. and their blood, pituitary glands, hypothalami and reproductive organs were collected for analysis. The blood collected from the trunk was allowed to clot for 20 min at 4°C and centrifuged, and serum was stored at −20°C for subsequent hormone analyses.

Tissue preparation

The borders of the excised hypothalami were delineated by the anterior margin of the optic chiasm, the posterior margins of the mammillary bodies, and laterally by the hypothalamic sulci. Hypothalami were taken from animals in the early proestrous, estrous, or diestrous I phases of the reproductive cycle, cross-chopped into 0.5 mm slices, and kept in cold medium 199 (M199) containing 25 mM Hepes and 0.1% bovine serum albumin (BSA). The pituitary glands were chopped into 0.8 × 0.8 mm cubes, and placed into collecting medium before perifusion. Anterior pituitary glands from adult female rats at random stages of the cycle were dispersed into single cells by controlled trypsinization [10]. Static cultures were performed in M199, 10% horse serum at 37°C under 5% CO_2/air and saturated humidity for 3–5 days with or without ALC. Wet weights of endocrine organs (pituitary, ovary and uterus) were measured immediately after decapitation.

Measurement of hormone release

The hypothalamic slices were transferred into chambers and perifused with prewarmed (37°C) M199 at a flow rate of 0.2 ml/min. After 2 h of perifusion to allow baseline gonadotropin-releasing hormone (GnRH) release to stabilize, fractions were collected every 5 min and the hypothalami were stimulated by pulses of medium containing high K^+ (60 mM, 30 min). Aliquots of each sample were stored at −20°C prior to radioimmunoassay (RIA) of GnRH. Assays were performed as described previously [11], using [^{125}I]GnRH from Amersham (Chicago, IL) unlabeled GnRH from Peninsula, (Belmont, CA), and primary antibody donated by Dr V. D. Ramirez (Urbana, IL). The intra- and inter-assay coefficients of variation at 80% of binding of standard sample (15 pg/ml) were 12 and 14%, respectively. Concentrations of luteinizing hormone (LH) in serum and perifusion medium were measured by double antibody RIA, using the RP-3 rat LH standard provided by the National Pituitary Agency (Baltimore, MD). The sensitivity of the assay was 2.5 ng/ml, and intra- and inter-assay coefficients of variation were 5.3 and 8.8%, respectively. Plasma prolactin (PRL) was determined by RIA using kits from NIADDK. The results were expressed in terms of ng/ml of rPRL-RP-3 standard. The intra- and inter-assay coefficients of variation were 5.4 to 11.4%, respectively. Plasma follicle-stimulating hormone (FSH) was determined by RIA using kits from NIADDK. The results were expressed in terms of ng/ml of rFSH-RP-2 standard. The intra- and inter-assay coefficients of variation were 8.3 and 10.2%, respectively. Serum estradiol (E_2) levels were measured by solid-phase RIA, using kits from Diagnostic Products Corporation (DPC; Los Angeles, CA). The sensitivity of the assay was 8 pg/ml. The intra- and inter-assay coefficients of variation were 5.3 and 8.7%, respectively. Serum progesterone (P_4) was measured with kit reagents from DPC. The sensitivity of the method was 0.05 ng/ml, and the intra- and inter-assay coefficients of variation were 5 and 12%, respectively.

Materials

ALC was provided by Sigma-Tau (Rome, Italy). GnRH was purchased from Peninsula Labs and ^{125}I-labeled GnRH from Amersham. Perifusion media (M199 + 25 mM Hepes + 0.1% BSA pH 7.4) were obtained from the NIH Media Unit (Bethesda, MD).

Data calculations

All results are expressed as mean ± SEM, and statistical tests were performed on a MacIntosh IIci computer using the StatView II Statistical Package. Significance levels were determined by the Mann–Whitney nonparametric test.

RESULTS

Effects of constant dark on the estrous cycle

After 3 weeks of exposure to constant dark conditions, rats with regular 4-day cycles, both controls and ALC-treated, entered into constant diestrus (85%) or showed irregular cycles in which diestrus was occasionally interrupted by proestrus (15%). On return to normal lighting conditions (14 h light/10 h dark) the control and ALC-treated groups usually resumed their normal estrous cycles at about the same time, within 2 weeks. However, in an initial study, we observed an accelerated return to normal cycling activity in the ALC-treated animals.

In vitro release of GnRH and LH

Release of GnRH from hypothalamic slices was detectable under basal conditions in tissue from controls and ALC-treated animals. Hypothalami from both control and ALC-treated animals showed higher basal release of GnRH during proestrus and estrus than in diestrus I. However, the basal GnRH release from hypothalami of ALC-treated rats was significantly increased in proestrus and diestrus I [Fig. 1(A)]. In control animals, exposure of hypothalamic tissue to high K^+ (60 mM) caused significant increases in GnRH release during proestrus (54%), estrus (26%) and diestrus I (87%) [Fig. 1(B)]. In ALC-treated animals, significant increases in GnRH release were observed in tissue obtained during proestrus (154%), diestrus I (96%), and estrus (92%). The K^+-induced release of GnRH was significantly increased above controls at all three phases of the estrous cycle: proestrus 130% ($P < 0.01$), estrus 54% ($P < 0.01$) and diestrus I 141% ($P < 0.01$) [Fig. 1(B)]. The largest percent increase of GnRH release in control animals occurred during diestrus (153%), and in ALC-treated animals during proestrus [409%; Fig. 1(C)].

The *in vitro* release of LH under basal conditions was similar in pituitaries from untreated and treated animals in proestrus (7.1 ± 0.6 vs 8.8 ± 0.6 ng/ml) and estrus (14.7 ± 0.8 vs 18.3 ± 2.1 ng/ml), but was higher in ALC-treated rats during diestrus I [17.9 ± 0.6 vs 29.8 ± 1.4 ng/ml, $P < 0.01$; Fig. 2(A)]. High K^+ (60 mM)-stimulated LH release was higher in glands from ALC-treated animals during proestrus [45% increase, $P < 0.01$; Fig. 2(B)] and estrus [53% increase, $P < 0.01$; Fig. 2(B)], and showed a slight increase (20%) during diestrus. The relative increase in LH release (basal to K^+-stimulated) was highest in both groups during proestrus and estrus, and was much lower in diestrus I [Fig. 2(C)]. An increase in LH release, expressed as a percentage above the basal level, was evident during all three phases of the estrous cycle in both controls and treated animals: proestrus controls, 172%; ALC, 218%; estrus controls, 156%; ALC, 215%; diestrus I controls, 91%, ALC 38% [Fig. 2(C)]. GnRH (100 nM)-stimulated LH release was significantly higher in pituitaries from ALC-treated rats during estrus and diestrus I (57 ± 2.8 vs 34.9 ± 3.4 ng/ml, $P < 0.01$, and 75.5 ± 15.1 vs 35.4 ± 6.9 ng/ml, $P < 0.01$, respectively; Fig. 3).

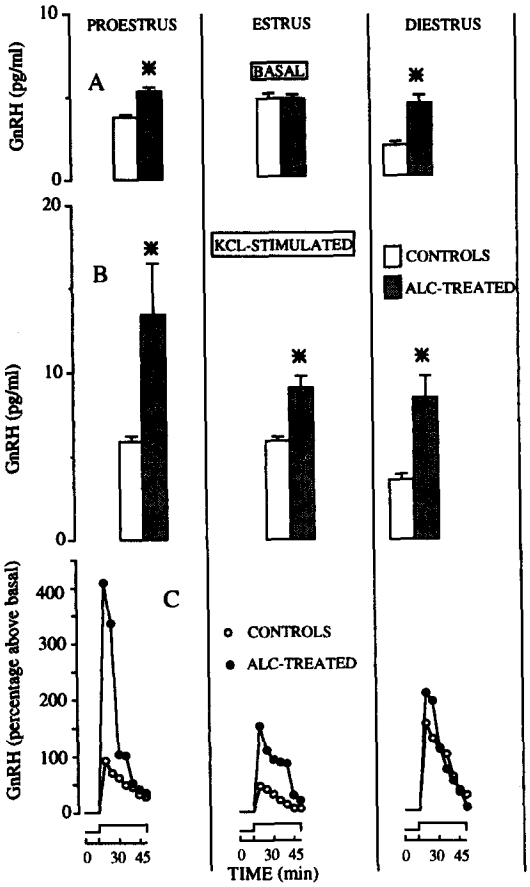

Fig. 1. GnRH release from hypothalamic tissue. (A) Basal GnRH release expressed as the mean ± SEM of 16 time points from 4 different hypothalami. (B) Responsiveness of control and ALC-treated pituitaries to depolarization by 60 mM K^+; mean ± SEM from 6 time points from 4 different animals. Open columns, controls; shaded columns, ALC-treated. (C) Time-course of GnRH secretion after 30 min pulse of 60 mM K^+, expressed as a percent increase over the basal level. Open circles, controls; closed circles, ALC-treated. $^*P < 0.01$.

Serum hormone levels

Serum LH levels were significantly increased in ALC-treated rats during the proestrous phase

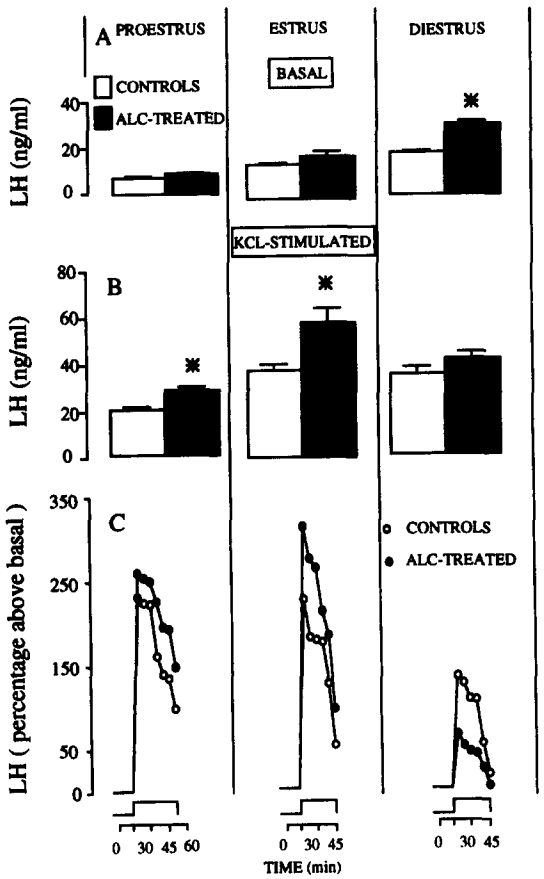

Fig. 2. LH release from perifused pituitary slices. (A) Basal LH release expressed as the mean ± SEM of 16 time points from 4 different pituitaries. (B) Responsiveness of control and ALC-treated pituitaries to depolarization by 60 mM K^+; mean ± SEM of 6 time points from 4 different animals. Open columns, controls; shaded columns, ALC-treated. (C) Time-course of LH secretion during 30 min depolarization by 60 mM K^+, expressed as the percent increase relative to the basal level. Open circles, controls; closed circles, ALC-treated. *$P < 0.01$.

(0.71 ± 0.25 vs 0.35 ± 0.02 ng/ml, $P < 0.05$; Fig. 4) and also during estrus (6.3 ± 3.4 vs 0.29 ± 0.02 ng/ml, $P < 0.05$; Fig. 4). There was a wide variation in the serum LH concentrations, with large increases in some rats and smaller changes in others. LH levels were not affected during diestrus I (0.4 ± 0.04 vs 0.43 ± 0.08 ng/ml).

Serum PRL levels showed a similar trend, with statistically higher levels in proestrus (22.1 ± 11 vs 5.3 ± 1 ng/ml, $P < 0.05$; Fig. 4). Large and statistically significant increases in PRL secretion also occurred during the estrous phase (122 ± 71 vs 7.9 ± 1.1 pg/ml, $P < 0.05$), but no change in PRL levels was observed during diestrus (Fig. 4). In contrast, the levels of serum FSH were not affected by ALC treatment at any stage of the cycle (Fig. 4).

In control animals, serum E_2 levels were elevated during early diestrus I (135.3 ± 29.3 vs 28.8 ± 5.7 pg/ml in estrus, $P < 0.01$; and proestrus 51.8 ± 6.1, $P < 0.05$; Fig. 5). In ALC-treated animals, serum E_2 levels were significantly higher during estrus (28.8 ± 5.7 vs 51.8 ± 6.1, $P < 0.05$; Fig. 5), and significantly lower in diestrus I (75.7 ± 21.3 vs 135.3 ± 29.3, $P < 0.05$; Fig. 5) compared to untreated animals. Serum P_4 levels were not affected by ALC treatment (Fig. 5).

Endocrine organ weight

No differences in organ weight were found in animals after 3 weeks in the dark. However, ALC-treated animals which were sacrificed after return to normal cycling showed an increase

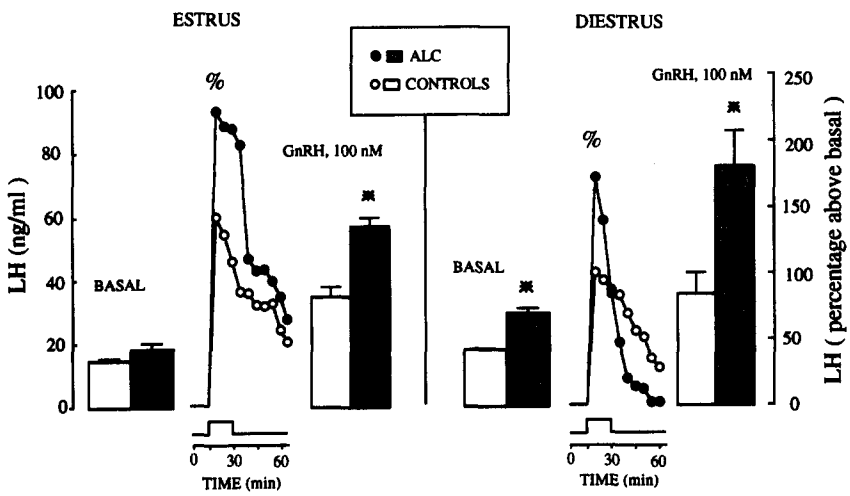

Fig. 3. LH release from GnRH-stimulated pituitary slices (10 min pulse of 100 nM GnRH) expressed as the mean ± SEM of 8 time points from 4 different pituitaries. Basal LH secretion is represented as the mean ± SEM of 16 time points from 4 different animals. The line-graph indicates percent increase of LH during GnRH stimulation and the subsequent washing period. *$P < 0.01$.

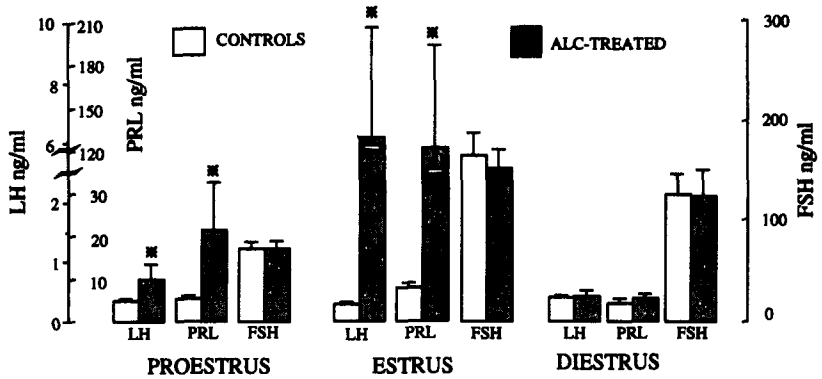

Fig. 4. Serum levels of LH, PRL, and FSH in control and ALC-treated animals during the estrous cycle. *$P < 0.05$.

in uterine weight in the proestrous (799 ± 51 vs 636 ± 27 mg; $P < 0.05$) and estrous phases (565 ± 7 vs 506 ± 18 mg; $P < 0.05$). No difference was found in adrenal, ovary and pituitary weights, or in whole body weights, between the different treatment groups.

DISCUSSION

These findings provide evidence that ALC exerts significant neuroendocrine actions in adult female rats. The presence of ALC in the brain, with highest concentration in the hypothalamus [2], its active transport by a plasma membrane carrier [12–14], the enhancement of long-chain fatty acid oxidation by carnitine in rat brain homogenate [15, 16], and the sensitivity of carnitine acetyl transferase to steroid hormone treatment [17], suggest a potential role of the compound in hypothalamic function. In the present study, basal GnRH release from hypothalamic tissue was higher in proestrus than in estrus and diestrus I in both control and ALC-treated rats. Those results were in agreement with data obtained by push–pull perifusion studies in normal 4-day cycling rats [18]. A significant increase in basal GnRH release was observed in hypothalami from ALC-treated rats, especially during the proestrous and diestrous I phases of the ovarian cycle. Those results suggest that ALC increases the secretory activity of GnRH-producing neurons in the hypothalamus.

Further evidence for such an increase in secretory activity was provided by the effects of depolarization with high K^+, which promotes the release of GnRH from the rat mediobasal hypothalamus [19, 20]. The GnRH response to K^+ depolarization was significantly elevated in hypothalamic tissue taken from ALC-treated animals during the proestrous, estrous and diestrous I phases of the cycle. The highest percent increase (basal to K^+-stimulated) occurred in diestrus I in control animals, and in proestrus in ALC-treated animals, perhaps reflecting more rapid recovery of the treated animals to normal cycling activity.

Under our experimental conditions, basal LH release from pituitary slices taken at 8 a.m. was higher during estrus and diestrus I than at proestrus in both control and experimental

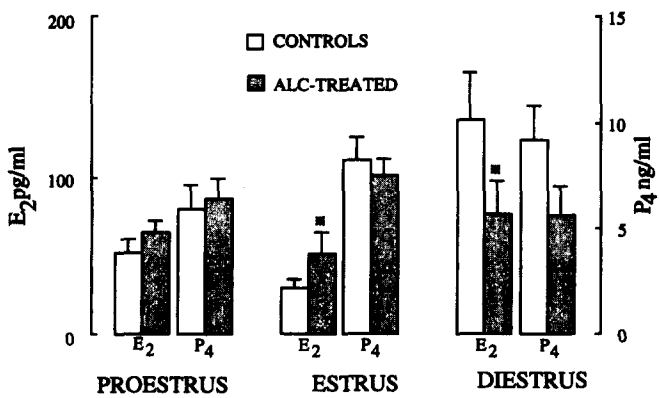

Fig. 5. Serum levels of E_2 and P_4 in control and ALC-treated animals during the estrous cycle. *$P < 0.05$.

animals. These findings may result from a shift in the LH release profile during recovery from dark-induced anestrus, since basal LH release is highest during proestrus in the normal cycle [21–23]. GnRH has been shown in numerous reports to stimulate LH release from pituitary slices [24–27] and dispersed pituitary cells [28–31]. In our study the percent increase from basal to GnRH-stimulated LH release in both groups was higher in estrus then in diestrus I. Larger amounts of LH were released from the pituitaries of ALC-treated animals after GnRH stimulation, providing evidence for increased capacity or sensitivity of these glands to GnRH stimulation. The latter is more probable, since treatment with ALC *in vitro* did not affect the release of LH and PRL from cultured pituitary cells. The absence of direct stimulation by ALC of LH secretion from cultured pituitary cells indicates that its effects are probably exerted at a central level.

The higher levels of LH in perifusion medium from pituitaries of ALC-treated animals after K^+ depolarization is consistent with the increased response to GnRH, and provides further evidence for a permissive effect of ALC on LH secretion. Previous *in vitro* studies have shown that treatment with ALC in doses (50 mg/kg/day) that attenuated the symptoms of senility and aging [32] caused increases in serum LH, PRL and E_2 levels, as well as an increase in uterine weight [33].

The pattern of LH secretion during the specific stages of the rat estrous cycle has been well defined [34]. In normal 4-day cycles, the proestrous surge of LH occurs in the afternoon and declines to baseline on the morning of estrus. Low LH levels are found during diestrus I, II and on the morning of proestrus [35, 36]. The serum LH profile measured during recovery from dark-induced anestrus was low on the mornings of proestrus, estrus and diestrus I, but was statistically higher during proestrus and estrus in ALC-treated animals. These findings may reflect an increased capacity of the hypothalamus and pituitary of such animals to secrete larger amounts of GnRH and LH *in vitro*. It is likely that GnRH-producing neurones in the hypothalamus [37] are the primary targets for ALC action. This would be consistent with the higher secretory activity of pituitaries from ALC-treated animals *in vivo* and *in vitro*, and with a central action of the drug on LH secretion. A surge in serum PRL normally occurs in the afternoon of proestrus, and is followed by a decline to baseline [38]. A similar trend of serum PRL was found in our experiments, with statistically higher levels during proestrus and estrus in ALC-treated animals.

Serum E_2 values during recovery from dark-induced anestrus were typical of the normal 4-day cycle, with highest levels on the morning of diestrus I and lowest levels on the morning of estrus [35]. In ALC-treated rats, serum E_2 levels were increased during early estrus, consistent with the higher sensitivity of such pituitaries to GnRH stimulation *in vitro*. The decreased responsiveness to K^+ stimulation of pituitaries taken at diestrus I from ALC-treated animals is consistent with the statistically lower level of E_2 found in early diestrus I. A similar trend, but without statistically different changes, was found for serum P_4 levels. These changes in steroid hormone levels correlated with those in uterine weight, which was statistically higher in ALC-treated animals during the proestrous and estrous phases of the cycle.

These results are in agreement with the known hormonal events in 4-day cycling rats, in which uterine weight increases at proestrus, the pituitary LH content falls, serum LH increases (5 p.m.) and ovulation occurs [39, 40]. The hormonal profiles observed in ALC-treated rats during the estrous cycle, with statistically higher levels of LH and PRL during proestrus and estrus, indicates that the secretory responsiveness of the pituitary gland is increased in these animals. Further experiments are in progress to determine the mechanisms by which ALC promotes the secretion of LH and PRL. These actions of ALC are analogous to its recently described effects on β-endorphin and corticosteroid levels in rats and human subjects [41, 42], and may reflect a more general action of the compound on hypothalamic function.

Acknowledgements—LZK was supported by a grant from Sigma-Tau, Rome, Italy. We thank Dr Sylvie Dufour for helpful discussions.

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