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Cholinergic Modulation of Spontaneous Hypothalamic-Pituitary-Adrenal Activity and Its Circadian Variation in Man*

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ABSTRACT

Controversy still exists regarding the role of cholinergic pathways in the regulation of the hypothalamic-pituitary-adrenal axis in man. We studied the effects of the administration of placebo, pyridostigmine (PD; 120 mg, orally), and the combination of PD and pirenzepine (PZP; 100 mg, orally) on ACTH, cortisol, and GH secretion at 0730 and 2230 h in seven normal males. PD induced a clear decrease in ACTH levels at both times of the day compared to treatment with placebo, producing higher suppression in the nocturnal period (34.4 ± 5.8% *vs.* 21.8 ± 10.7%). The combination PD and PZP prevented the inhibitory action of PD on ACTH secretion in the morning, but not in the evening, when ACTH values showed a decrease similar to that seen after giving PD alone (38.1 ± 5.6% *vs.* 34.4 ± 5.8%, respectively). Cortisol values declined only when the association PD plus PZP was given in the evening. GH levels had a significant increase after PD administration in the morning (4.1 ± 1.2 ng/mL) and in the evening (10.2 ± 1.6 ng/mL), confirming that cholinergic stimulation was taking place, whereas the addition of PZP to PD induced a significant attenuation of these responses. It is concluded that cholinergic pathways have a inhibitory role in ACTH secretion in man. M1 muscarinic receptors seem to be involved in the diurnal inhibition of PD, whereas our observations are consistent with the mediation of another type of cholinergic receptors as an explanation for the nocturnal effect of PD on ACTH secretion. PD did not alter the circadian variation in the hypothalamic-pituitary-adrenal axis, whereas the association of PD and PZP increased the differences between diurnal and nocturnal ACTH values, suggesting a modulatory effect of the cholinergic system on the circadian rhythm of ACTH secretion. (*J Clin Endocrinol Metab* **81:** 2902–2907, 1996)

SEVERAL LINES of experimental evidence suggest that the cholinergic system exerts a stimulatory influence on hypothalamic-pituitary-adrenal (HPA) function. In 1967, Naumenko (1) showed that administration of anticholinesterase drugs to experimental animals was followed by an increase in glucocorticoid levels. Subsequent studies gave more support to the stimulatory role played by cholinergic pathways by demonstrating that implantation of atropine in the hypothalamic paraventricular nucleus inhibited the ACTH response to surgical stress (2). Other studies confirmed that acetylcholine or its agonists are able to stimulate hypothalamic CRH secretion, an effect that can be blocked by muscarinic and nicotinic antagonists, suggesting the involvement of both types of cholinergic receptors (3, 4). According to this, Calogero *et al.* (5) showed that either atropine or CRH antiserum administration prevented the stimulating effect of the muscarinic agonist arecoline on ACTH and corticosterone secretion in rats, providing further information about the preferential involvement of muscarinic receptors in the cholinergic modulation of the HPA axis.

However, evidence of the role of cholinergic pathways in the control of the HPA system in humans is scanty and controversial. Risch *et al.* (6, 7) reported a stimulatory effect of the cholinergic agonist physostigmine on ACTH, β-endorphin, and cortisol levels when given to normal subjects. Conversely, other researchers failed to show any influence of pyridostigmine administration on basal ACTH or cortisol levels in normal subjects (8, 9). Also, Raskind *et al.* (10) found no changes in β-endorphin or cortisol levels in normal young individuals treated with physostigmine, in contrast to elderly people who exhibited a stimulatory response. Recently, Murialdo *et al.* (11) demonstrated that pyridostigmine increases the pituitary-adrenal response to CRH in normal subjects, but not in patients with dementia.

The relationships between cholinergic pathways and the HPA axis are of interest because of the participation of both hormonal systems in stress activation and behavior control (12, 13). Our aim was to investigate the modulatory influence of cholinergic tone on spontaneous HPA activity and its circadian variation by looking at the changes in ACTH and cortisol secretion induced by administration of the anticholinesterase agent pyridostigmine and the muscarinic antagonist pirenzepine to a group of normal men in the morning and the evening. GH levels were also measured to ensure that effective cholinergic manipulation was taking place.

Subjects and Methods

Subjects

Seven healthy male volunteers, aged 27–38 yr (mean, 29.7 ± 1.42 yr), were studied. All subjects were nonsmokers and had a normal physical

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examination and a body mass index within the normal range (24.5 ± 0.66 kg/m^2). Screening test results, including complete blood counts and standard biochemical profile, were normal. All of the subjects had been free of any medical treatment for at least 3 months before starting the study. Subjects were submitted to the Hamilton test to rule out depression. Informed consent for the study was obtained in all cases, and the protocol was approved by the local ethical committee.

Methods

Study design. All tests were performed in the clinical research unit. Subjects were fasted from 2200 h the night before each test. At 0730 h, an iv catheter was placed in an antecubital vein, which was kept patent by slow 0.9% saline infusion. Blood samples were taken every 15 min from 0800–1030 h for ACTH, cortisol, and GH estimations. At 0730 h, placebo, pyridostigmine (PD; 120 mg; Mestinon, Roche, Madrid, Spain), or a combination of PD (120 mg) and pirenzepine (PZP; 100 mg; Gastrozepin, Boehringer Ingelheim, Barcelona, Spain) were given orally in random order on different days. All tests were repeated in the evening in each subject; drugs were administered at 2230 h, and blood samples were taken from 2300–0100 h. Tests were separated by at least 4 days. Subjects were not allowed to sleep during the tests and remained in the recumbent position until the end of the study. Blood samples for ACTH were collected in prechilled tubes containing ethylenediamine tetraacetate, placed immediately on ice, cold-centrifuged within 30 min, and stored at −70 C until assayed.

Hormone assays. ACTH was measured by immunoradiometric assay (Nichols Institute, San Juan Capistrano, CA). The sensitivity of the assay was 1 pg/mL. Inter- and intraassay coefficients of variation were 3% and 3.2%, and 7.8% and 6.8%, respectively, for ACTH concentrations of 7.8 and 81 pmol/L. Cortisol levels were estimated by RIA (Serono Diagnostics, Chavannes-de-Bogis, Switzerland). The sensitivity of the assay was 7.4 nmol/L. Intra- and interassay coefficients of variation were 4.05% and 4.64%, respectively. GH was measured by immunoradiometric assay (Biomerieux, Marcy-L'Etoile, France). The sensitivity of the assay was 0.2 ng/mL. Intra- and interassay coefficients of variation were between 2–2.2% and between 3.7–6%, respectively, for GH concentrations ranging from 2.1–80.2 ng/mL.

Data analysis. Integrated secretion corresponding to different time intervals (initial, 0800–0830 h and 2300–2330 h; middle, 0830–0930 h and 2330–0030 h; final, 0930–1030 h and 0030–0130 h) as well as the area under the curve (AUC) of the full sampling period (total AUC, 0800–1030 h and 2300–0130 h) of ACTH, cortisol, and GH were calculated by trapezoidal solution to compare the results obtained under each condition. The percent variation in integrated hormonal secretion after the administration of cholinergic drugs with respect to that observed after placebo treatment was estimated. Quantitative assessment of ACTH and cortisol circadian rhythms was calculated by measuring the percent reduction of integrated nocturnal values compared to morning levels. Statistical analysis was carried out by Wilcoxon's rank sum test. Data are presented as the mean ± SEM.

Results

All subjects had mild transient abdominal pain after PD administration when given alone or in combination with PZP. PZP caused transient dry mouth in all individuals. In no case were tests stopped or medications given.

ACTH

In the morning, ACTH levels remained stable following placebo administration. Maximum values were attained at 0800 h (7.49 ± 1.09 pmol/L), declining slowly thereafter and reaching a nadir at 0915 h (4.50 ± 0.7 pmol/L) to rise progressively until the end of the sampling period (Fig. 1). As expected, nocturnal ACTH levels after placebo treatment were significantly lower, as assessed by total AUC values (Table 1), according to a normal circadian variation. Thus, integrated ACTH secretion decreased to 45.5 ± 4.8% with reference to morning values when placebo was given.

Pretreatment with PD led to a significant decrease in ACTH levels (Fig. 1) and total AUC values (Table 1) in the morning as well as in the evening period compared with respective values after placebo treatment. The percent reduction in total AUC values for ACTH in the evening was higher than that estimated in the morning test (34.4 ± 5.8% *vs.* 21.8 ± 10.7%). Although the inhibitory action of PD on ACTH secretion became evident from the beginning and was uniform over the duration of the evening test, during the morning the effect took place mainly in the final phase of the sampling period (Fig. 1). PD administration did not change the circadian pattern of ACTH, as assessed by estimation of the percent variation in integrated ACTH secretion between morning and evening values (53.6 ± 3%).

The administration of PD plus PZP in the morning prevented the inhibitory effect of PD on ACTH secretion, resulting in a secretory pattern identical to that found when subjects were pretreated with placebo (Fig. 1). Thus, ACTH total AUC values were reduced by only 6 ± 1.3% with respect to those after placebo treatment. In contrast, pretreatment with both drugs in the evening did not modify the decreasing effect on ACTH levels induced by exclusive PD administration. In fact, total AUC values (Table 1) and integrated ACTH levels corresponding to partial time intervals were similar under both sets of conditions (Fig. 1). The percent reduction in ACTH total AUC with respect to that during the placebo test was identical to that calculated after exclusive PD treatment (38.1 ± 5.6%). However, the ACTH area corresponding to the initial interval after PD plus PZP was slightly lower than that measured after exclusive administration of PD (6.7 ± 1.1 *vs.* 8.1 ± 1.4 pmol/L·30 min; P = NS; Fig. 1). Consequently, the combined treatment with PD and PZP induced an amplification of circadian ACTH variation (62.6 ± 5.5%; $P < 0.05$ *vs.* placebo).

Cortisol

Morning basal cortisol levels after placebo treatment were maximal at 0800 h (516.9 ± 33.9 nmol/L) and declined progressively throughout the sampling period (nadir, 1015 h; 264.1 ± 26 nmol/L; Fig. 2). According to the circadian variation displayed by ACTH values, cortisol concentrations were significantly lower in the evening, as demonstrated by the comparison between total AUC values (Table 1). Pretreatment with PD did not induce any change in the cortisol secretory pattern in either the morning or the evening despite the reduction in ACTH concentrations induced by the cholinergic agonist in both situations. Combined administration of PD and PZP led to a significant reduction in evening cortisol levels compared to values after either placebo or PD treatment, whereas the same treatment was devoid of any effect when given in the morning (Fig. 2). The percent variation in nocturnal cortisol levels with respect to morning values was similar when subjects received placebo, PD, or the combination of PD and PZP (71.4 ± 5.3%, 75.2 ± 4.4%, and 82.1 ± 1%, respectively).

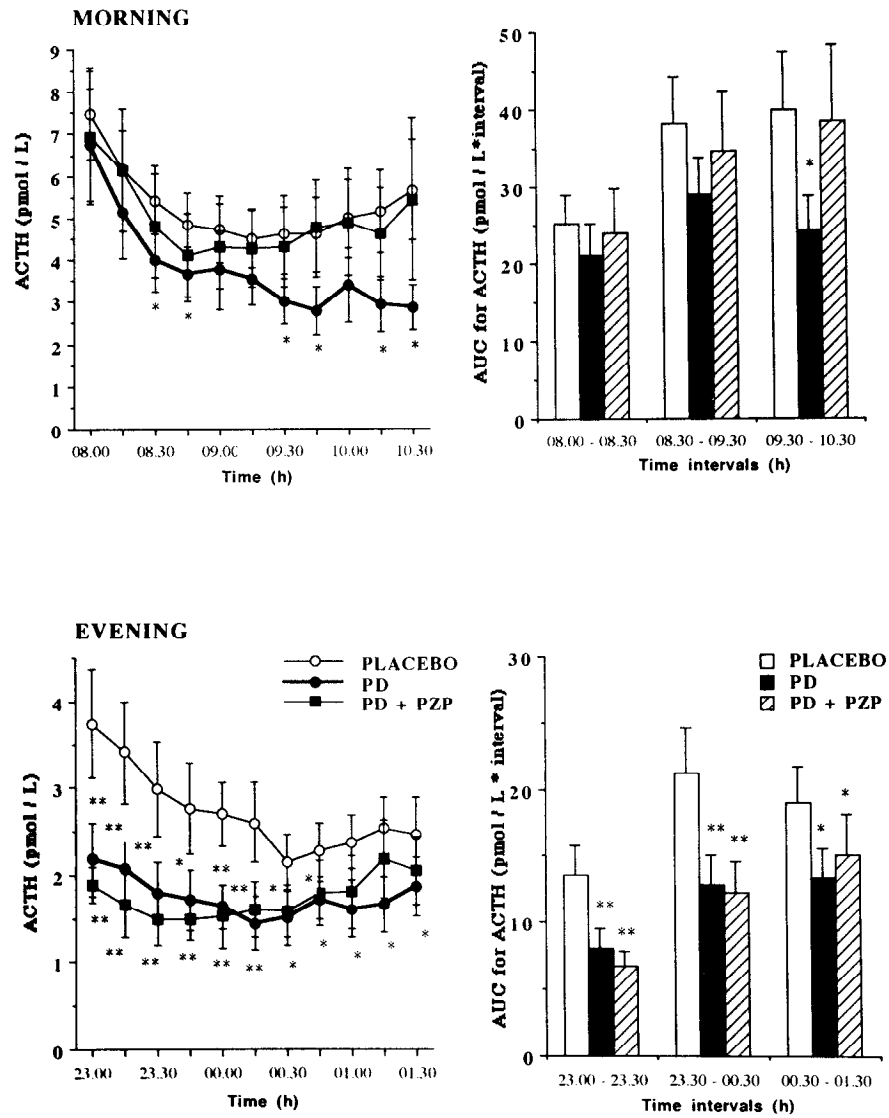

FIG. 1. Mean values and partial integrated areas of secretion of ACTH after placebo (*open circles and open bars*), PD (*closed circles and black bars*), and PD plus PZP (*closed squares and dashed bars*) administration. *Top panel*, Morning studies. *Bottom panel*, Evening studies. *, $P < 0.05$; **, $P < 0.01$.

TABLE 1. Total AUC values of ACTH, cortisol, and GH after placebo, PD, and PD plus PZP in the morning and evening

	Placebo	PD	PD + PZP
ACTH			
Morning	103.1 ± 16.9	74 ± 11.9[a]	96.8 ± 21
Evening	53.8 ± 7.9[b]	34.3 ± 5.5[a,b]	34.2 ± 6.2[a,b]
Cortisol			
Morning	6876 ± 479	7254 ± 809	7137 ± 442
Evening	1895 ± 326[b]	1725 ± 320[b]	1277 ± 113[b]
GH			
Morning	2.7 ± 0.9	45.2 ± 13.7[a]	7.57 ± 3.2[a]
Evening	76.3 ± 33.7[b]	130.4 ± 24.3[b]	32.3 ± 19.1[b]

Values shown are the mean ± SEM.
[a] Significantly different from placebo values ($P < 0.05$).
[b] Significantly different from morning values ($P < 0.05$).

GH

Morning GH levels after placebo treatment were near the lower detection limit of the assay, remaining unchanged over the duration of the study. Evening GH concentrations were higher than morning levels, as assessed by total AUC values (Table 1). As expected, PD administration elicited a clear GH response in the morning as well as the evening (mean GH peak, 4.1 ± 1.2 and 10.2 ± 1.6 ng/mL, respectively). Thus, GH AUC values after PD treatment were superior to those found after placebo treatment at both times of the day (Table 1), confirming that cholinergic stimulation was taking place. The percent GH stimulation over basal values after PD treatment was higher in the morning (1574 ± 214% *vs.* 70.9 ± 15%). The addition of PZP to PD led to a marked reduction of the GH response to PD in the morning (GH peak, 0.73 ± 0.4 *vs.* 4.1 ± 1.2 ng/mL; $P < 0.01$) as well as in the evening (3.66 ± 2.34 *vs.* 10.2 ± 1.6 ng/mL; $P < 0.05$). Estimations of GH total AUC values also suggested that cholinergic GH stimulation was significantly attenuated when both drugs were given (Table 1).

Discussion

Although animal experiments have provided data supporting a positive influence of cholinergic pathways on the HPA axis, studies on the effect of cholinergic agents on ACTH release in man have produced conflicting results. Investigations carried out by Risch *et al.* (6, 7) and Lewis *et*

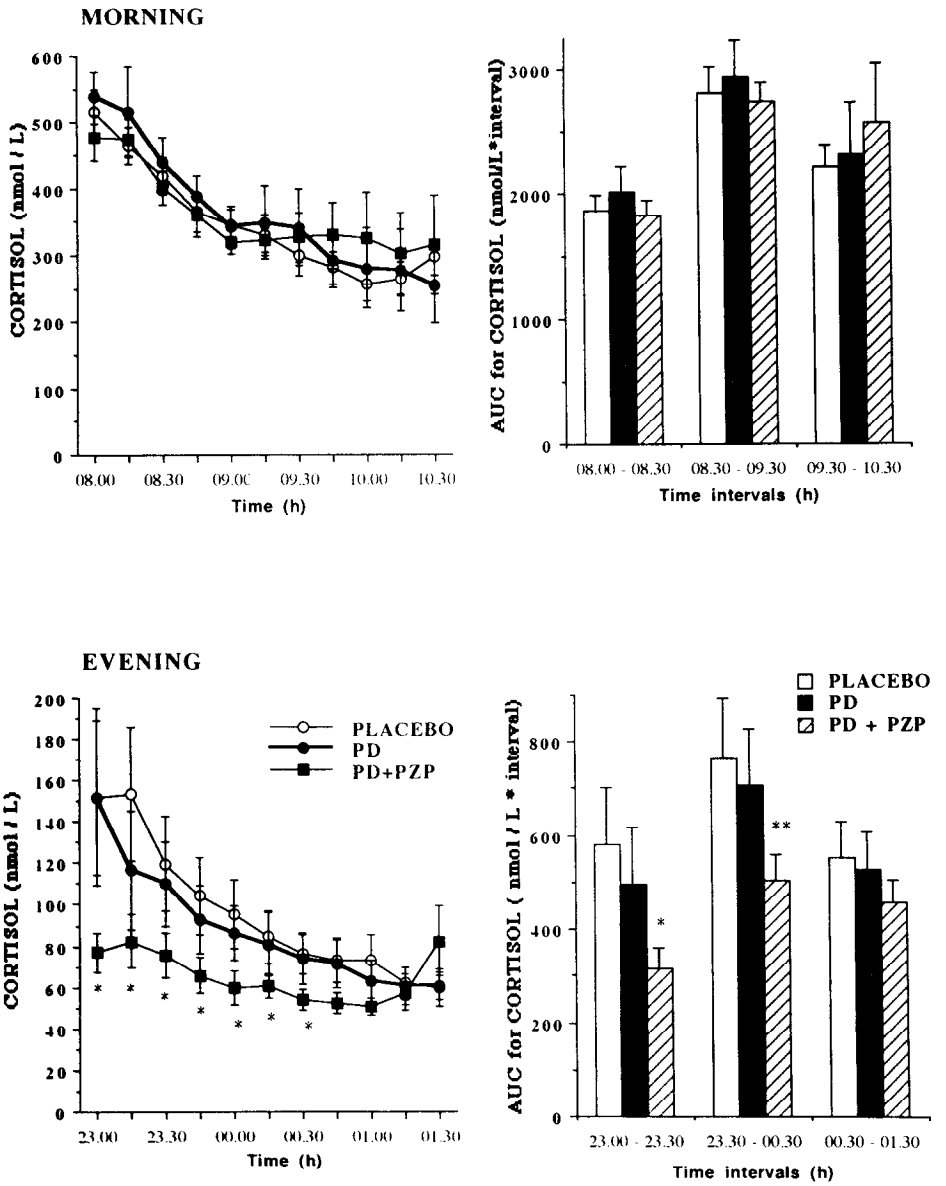

FIG. 2. Mean values and partial integrated areas of secretion of cortisol after placebo (*open circles and open bars*), PD (*closed circles and black bars*), and PD plus PZP (*closed squares and dashed bars*) administration. *Top panel*, Morning studies. *Bottom panel*, Evening studies. *, $P < 0.05$; **, $P < 0.01$.

al. (14) showed that physostigmine administration to normal volunteers increased basal ACTH and cortisol levels. On the contrary, other researchers (8–10) failed to demonstrate a significant variation in ACTH or cortisol concentrations after treatment with anticholinesterase agents. More recently, new data have been presented showing that PD or physostigmine increases the pituitary-adrenal responses to CRH (11) and meal intake (15) in normal individuals, which argues in favor of a stimulatory influence exerted by the cholinergic system on HPA function in these conditions.

Our results show that nonspecific cholinergic activation by PD inhibits spontaneous ACTH secretion during both the morning and evening periods. Some methodological factors may help to explain the apparent discrepancies between previous studies and our results. In contrast to other reports, normal function of the HPA axis was documented in our group of volunteers, who exhibited a normal circadian variation of ACTH and cortisol levels after placebo administration. On the other hand, side-effects induced by the administration of high doses of physostigmine, such as those used by Risch *et al.* (6, 7) and Lewis *et al.* (14), led to a classic symptomatic and hormonal stress responses, with participation of PRL, cortisol, and ACTH (12). Both the simultaneous rise in PRL and the prevention of cortisol and ACTH responses by pretreatment with the peripheral cholinergic antagonist glycopyrrolate (14) support the hypothesis that physostigmine-induced HPA axis stimulation is mediated by mechanisms independent of specific cholinergic activation. Our data do not agree with those reported by Freeman *et al.* (8), who did not find any change in ACTH levels after the administration of PD to normal subjects in the morning. One possible explanation is that stressful effects due to PD administration could lead to HPA axis activation in some individuals, thus contributing to mask the real cholinergic effect on morning ACTH secretion just when the inhibitory effect of PD on ACTH secretion seems to be weaker. The stimulation of cortisol and β-endorphin secretion induced by low doses of physostigmine in old men could be due to

disturbances in the cholinergic system related to aging (10). The impairment of GH responses to the cholinergic agonist exhibited by the same individuals supports that hypothesis. Our study demonstrates that the ACTH inhibition induced by PD administration in the morning is completely abolished by the addition of PZP, suggesting that the reduction in ACTH concentrations observed at that time of day requires the participation of M1 muscarinic receptors. Stimulation of GH secretion by PD and its inhibition by concurrent PZP treatment support the view that cholinergic tone manipulation was, in fact, taking place as expected. Finally, the inhibitory effect of PD on ACTH secretion cannot be ascribed to stress, a condition that promotes ACTH and cortisol release. There is a genuine possibility that doses of PD superior to those given in this study have a different effect on ACTH secretion. However, this is unlikely because administration of doses higher than 120 mg has been reported to cause similar GH release (16). In addition, high doses of PD may produce significant side-effects and stress system activation, leading to HPA axis stimulation by noncholinergic mechanisms.

Our data agree with those reported by Evans *et al.* (17), who showed that iv administration of atropine in the morning increased the ACTH and β-endorphin responses to insulin-induced hypoglycemia in normal subjects, attributing to cholinergic muscarinic receptors an inhibitory influence on stimulated ACTH secretion. Unfortunately, no information was available on the effect of muscarinic receptor blockade on spontaneous HPA activity in this study.

To date, no studies have been conducted to investigate the effect of cholinergic stimulation on nocturnal HPA activity. Our data show that the inhibitory effect of PD on ACTH secretion is also operative at night, despite the fact that absolute concentrations and pulsatility of ACTH secretion differ from those observed during the morning period. ACTH inhibition during the initial and middle phases of the sampling period was greater in the evening as was the overall suppression of ACTH levels compared to those found in the morning, suggesting that corticotroph sensitivity to the inhibitory effect of PD is higher during the nocturnal period. However, the addition of PZP does not reverse the PD-induced reduction of ACTH secretion, suggesting that M1 muscarinic receptors are not involved in the nocturnal effect of PD on ACTH secretion. Thus, in contrast to results obtained in morning experiments, the association of PD and PZP led to a decrease in ACTH levels comparable to that seen after PD alone. These results are consistent with the participation of cholinergic receptors different from those of the M1 type in the nocturnal effect of PD on ACTH levels, raising the possibility that modulation of ACTH secretion by the cholinergic system involves a different type of receptor depending on the time of the day.

PD administration induced a substantial GH response during both study periods as a consequence of somatostatin inhibition (18), thus corroborating an effective stimulation of cholinergic tone. Relative GH increase after pretreatment with PD in the morning was greater than that found in the evening, suggesting a low somatostatinergic tone during the nocturnal period, as previously proposed (19). This circadian variation in the GH response to PD is not paralleled by that of ACTH, which suggests that the mechanisms mediating GH stimulation after cholinergic agonist administration may be different from those involved in ACTH suppression, as has been suggested in elderly people (10).

Regarding the mechanisms by which PD inhibits ACTH secretion, a suprapituitary site of action should be considered, as there is no proof of the existence of cholinergic receptors on corticotroph cells, and according to this, addition of the cholinergic agonist arecoline to dispersed anterior pituitary cells in culture does not have any effect on ACTH secretion (5). The fact that PZP, due to its hydrophilic properties, does not penetrate the blood-brain barrier to a great extent (20) points to the median eminence as the most likely site of action for this drug to antagonize the effects of PD on ACTH release. However, effects on other hypothalamic structures leading to changes in CRH or AVP secretion cannot be ruled out, as experimental evidence suggests that cholinergic pathways have a significant influence on the synthesis of both peptides (21, 22).

Interestingly, cortisol levels remained unchanged despite the fact that ACTH concentrations were reduced after pretreatment with PD. This suggests that the sensitivity of adrenal glands to small changes in ACTH levels is not enough to induce significant variations in cortisol concentrations. Alternatively, the sampling period might be too short to detect a reduction of cortisol concentrations that may occur later. Furthermore, a direct adrenal effect of cholinergic drugs cannot be ruled out. In this context, a stimulatory effect of acetylcholine infusions on adrenal cortisol output in hypophysectomized animals has been described (23). Thus, PD administration may counteract the effects of ACTH reduction by promoting direct adrenal cortisol release. These results are in agreement with other studies (8–10) that failed to find any cortisol variation when giving PD to normal subjects in the morning, and with that of Evans *et al.* (17), who observed no changes in hypoglycemia-stimulated cortisol levels after atropine administration to normal individuals despite achieving a significant rise in ACTH concentrations. The addition of PZP induced a significant decrease in cortisol values in the initial phase of the nocturnal test, just when maximum ACTH suppression was attained. A superior reduction of ACTH levels in the early part of the experiment or a direct effect of PZP on adrenal cortisol release represent possible mechanisms to explain the different cortisol patterns seen after the administration of PD and PZP. It seems that both factors, a decrease in ACTH levels and simultaneous administration of PZP, are required to induce a significant reduction in cortisol levels. Taken together, these data indicate that estimation of cortisol levels is not a reliable index to assess the effects of cholinergic manipulation on corticotroph cell function.

When assessing the relationship between diurnal and nocturnal HPA activities, it becomes evident that the percent variation in ACTH or cortisol observed after placebo administration remained constant after pretreatment with PD. However, the differences seen in the cortisol and ACTH responses to combined PD and PZP treatment at both times of the day give support to the possibility of a modulatory influence of cholinergic tone on the circadian rhythm of the HPA axis.

In summary, this study reveals that, in contrast to experimental animals, cholinergic activation by PD inhibits ACTH secretion in man, suggesting the involvement of a different type of cholinergic receptors depending on the time of day at which the test is carried out. This functional relationship between the cholinergic system and the HPA axis provides further insight into the regulation of HPA function and opens new perspectives in the pathophysiology of behavior disorders and stress conditions.

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