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Amplitude, but not Frequency, Modulation of Adrenocorticotropin Secretory Bursts Gives Rise to the Nyctohemeral Rhythm of the Corticotropic Axis in Man*

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ABSTRACT. The ACTH-adrenal axis is a critical stress-responsive system with prominent circadian rhythmicity. To test the basis for the circadian ACTH physiology, we have used 1) a sensitive and specific two-site immunoradiometric assay to estimate plasma ACTH-(1–39) concentrations during intensive (every 10 min) and extended (24-h) blood sampling to capture complete diurnal ACTH profiles in eight normal men, and 2) a novel deconvolution model designed to resolve the number, amplitude, and duration of ACTH secretory bursts and simultaneously estimate subject-specific ACTH half-lives under physiological conditions *in vivo*. Deconvolution revealed 40 ± 1.5 significant ACTH secretory bursts/24 h, with a mean interburst interval of 39 ± 2.3 min. ACTH secretory bursts were discrete punctuated events arising without tonic interpulse secretion and had a half-duration (duration at half-maximal amplitude) of 19 ± 2 min. The estimated half-life of *endogenous* ACTH was 15 ± 1.2 min, and its daily production rate was 0.96 ± 0.16 ng/mL (0.21 ± 0.035 nmol/L) distribution volume. Cosinor analysis revealed a significant (3.8-fold) 24-h rhythm in the mass (or rate) of ACTH secreted per burst (maximal at 0818 h), but no nyctohemeral variation in ACTH secretory pulse frequency. The validity of ACTH pulse analysis was supported by the significantly nonrandom associations among ACTH, β-endorphin, and cortisol peaks in the same subjects. Specifically, we found that ACTH and β-endorphin bursts occurred simultaneously ($P < 10^{-4}$) and were both followed in 10 min by a cortisol pulse ($P < 10^{-4}$).

We conclude that 1) selective amplitude control of a punctuated burst-like mode of ACTH secretion can give rise to the nyctohemeral corticotropic rhythm without the need to postulate any tonic (or interpulse basal) component of ACTH release; and 2) there is exquisite 3-fold temporal synchrony among bursts of ACTH, β-endorphin, and cortisol release in normal men. (*J Clin Endocrinol Metab* **71:** 452–463, 1990)

THE CORTICOTROPIC-adrenal axis manifests prominent circadian rhythmicity as well as an episodic or pulsatile mode of hormone release (1–3). The conspicuously pulsatile nature of ACTH release has been recognized in various species, including the rat, dog, and human (1–6). However, exactly how the physiologically circadian variation in plasma ACTH concentration is generated by and/or related to the associated pulses of ACTH release has not yet been clarified. For example, the nyctohemeral rhythm in plasma ACTH concentrations could result from 24-h variations in a tonic (basal) or constitutive mode of ACTH release with superimposed pulsatile episodes. Alternatively, akin to the secretory behavior inferred recently for other anterior pituitary hormones (7), ACTH secretion may occur in an exclusively burst-like pattern, consisting of punctuated release episodes without the need to postulate any interpulse basal (tonic) secretory component. If discrete ACTH bursts suffice, then the 24-h rhythm in the corticotropic axis could be endowed by circadian variations in either the amplitude or frequency (or both) of the individual ACTH secretory events. Such findings would have significant implications to our understanding of corticotropic-axis pathophysiology.

Recently developed deconvolution techniques designed to calculate *in vivo* hormone secretion and clearance rates allow one to investigate further the mechanistic basis for endocrine control systems. For example, one technique referred to as multiple-parameter deconvolution analysis provides quantitative estimates of the number, amplitude, and duration of underlying hormone secretory

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bursts, while simultaneously estimating the endogenous half-time of hormone disappearance in individual subjects (7–9). Here, we have used such a methodology to investigate the following hypothesis: the 24-h rhythm in plasma ACTH concentrations is generated by an exclusively burst-like mode of ACTH secretion, in which amplitude and/or frequency modulation of punctuated secretory events give rise to nyctohemeral variations in mean plasma ACTH concentrations. Deconvolution analysis was combined with a new sensitive and specific two-site immunoradiometric assay (IRMA) of plasma ACTH (1–39) concentrations as well as intensive blood sampling every 10 min for 24 h in normal men. Moreover, to confirm the biological relevance of ACTH pulses, formal statistical analysis of coincident ACTH, β-endorphin, and cortisol peaks was undertaken to test the hypothesis of nonrandom associations among hormone release episodes within the corticotropic axis.

Materials and Methods

Clinical sampling protocols

Eight healthy men [age 30–60 yr; mean, 44 ± 11 (±SD)] participated in this study after provision of written informed consent approved by the Human Investigation Committee of the University of Virginia Health Sciences Center. Each subject had an unremarkable clinical history and physical examination, with normal biochemical tests of renal, hepatic, and hematological function, and normal fasting serum concentrations of T_4, TSH, PRL, estradiol, unbound testosterone, immunoactive LH, FSH, ACTH, and cortisol.

Subjects were admitted to the Clinical Research Center of the University of Virginia the day before study and, after overnight adaptation to an iv catheter, underwent blood sampling (2 cc) every 10 min for 24 h. The men were allowed to drink fluids *ad libitum*, ambulate freely, and eat meals at 0800, 1200, and 1700 h. Lights were extinguished at 2200 h. Plasma samples were collected on ice in chilled EDTA-containing siliconized glass tubes, centrifuged twice at 4 C, frozen, and used for later assays.

Assays

The ACTH IRMA was performed using a two-site sandwich assay designed to detect intact ACTH (1–39) molecules. The IRMA consists of a soluble ^{125}I-labeled (indicator) monoclonal antibody directed to the N-terminus of ACTH as well as a second polyclonal ACTH antibody directed to the C-terminus. The second antibody is covalently conjugated to biotin so as to be reactive with avidin-coated plastic beads. All incubation reagents including antibodies, human ACTH-(1–39) standard, and avidin-coated beads were from Nichols Institute (San Juan Capistrano, CA). Each sample was assayed in duplicate, and all samples (n = 145) from any one subject were assayed in the same run to avoid interassay variations. The sensitivity of the IRMA was 1.0 pg/ml or 0.22 pmol/L (3 SD above the zero dose tube), and the intraassay precision was 3.2 – 5.8% (range of median intrasample coefficients of variation determined from all 145 samples in each of the 8 individual men). The cross-reactivity of this IRMA with β-endorphin, TSH, LH, FSH, GH, or PRL was less than 0.1%. The cortisol and β-endorphin RIA and IRMA, respectively, were described previously (8, 10).

Deconvolution modeling

The aim of our multiple-parameter deconvolution model is the quantitative estimation of various specific measures of hormone secretion and clearance from all plasma hormone concentrations and their intrasample variances considered simultaneously. We calculate secretory patterns, which, when combined with subject-specific clearance kinetics, would give rise to the observed plasma hormone concentrations. Computations are based on a biophysical model of hormone secretion and clearance, in which we assume that the plasma ACTH concentration at any given instant results from the simultaneous operation of four distinct, finite, and determinable parameters of interest: 1) the locations, 2) the amplitudes, and 3) the durations of all prior hormone secretory events (Gaussian bursts), acted upon by 4) endogenous subject-specific clearance kinetics. The mathematical formulation of this model has been summarized previously (7–9). A waveform-independent formulation of our convolution model can also be used to calculate all individual sample secretory rates, while allowing for tonic secretion and/or associated asymmetric or symmetric secretory events of any waveform. In both models, elimination kinetics were defined by a monoexponential function. In the waveform-independent method, the hormone half-life is defined *a priori*, rather than fitted simultaneously with the secretion parameters (see above).

The following individual measures were estimated in each ACTH time series: half-duration of the ACTH secretory bursts (waveform-specific model only), amplitudes of all ACTH secretory events, burst frequency and mean interpulse interval, and subject-specific ACTH half-life (waveform specific model) (7–9).

Investigations of circadian periodicities by cosinor analysis

The deconvolution-resolved ACTH secretory measures (*e.g.* secretory burst mass or frequency) were analyzed for 24-h rhythms by cosinor analysis (nonlinear curve fitting of the data to a cosine function with periodicity of 24 h) (8). The amplitude (half the difference between peak and nadir), acrophase (time of maximal value within the 24-h rhythm), and mesor (mean value about which the cosine rhythm varies) were estimated for the group of eight subjects considered simultaneously. The statistical variance of each of these mean estimates was determined by nonlinear least squares estimation procedures (8).

To determine whether ACTH secretory bursts occurred randomly over time, autocorrelation analysis was carried out on the serial ACTH interpulse intervals, as described previously (8).

Coincident peaks

Individual peak maxima in plasma ACTH, β-endorphin, and cortisol concentration time series were identified objectively by

Cluster analysis (11), using parameters described previously to yield a false positive rate of 5% or less (8, 10).

Definitions of exactly (and lagged) coincident pulses

Exactly coincident hormone pulses were identified when peak maxima occurred simultaneously (*i.e.* in the same blood sample). Double or triple coincidences occur when, respectively, two or three distinct hormone pulse maxima are located in the same sample. Coincidence at a fixed lag was defined by peak maxima in samples separated by a fixed number of time units in any given direction (here, each time unit was 10 min); for example, peak maxima of hormone B could lag those of hormone A by 20 min. In contrast, a "window" of lag allowed for peak maxima in one series to either lead or lag those in the other series by a stated time interval and/or occur simultaneously (*e.g.* a lag of ±10 min allows for three types of coincidences).

The conditional probability of observing on the basis of chance alone any given number of exactly coincident peak maxima in two series of known individual pulse frequencies can be computed from the hypergeometric probability density function (12). Such calculations yield expected means, variances, and cumulative probabilities for purely random peak associations (12, 13). If three contemporaneous pulse trains are considered, corresponding values are calculated from a summed combinatorial equation (13). Probabilities of random peak coincidences in a group of subjects and/or occurring within a particular time window (*e.g.* ±10 min) can also be determined (13).

Cross-correlation analysis and autoregressive modeling

To search for significant auto- and cross-correlations within the β-endorphin, ACTH, or cortisol concentration series and between the β-endorphin, ACTH, and cortisol time series, the data were subjected to auto- and cross-correlation analyses. The auto- or cross-correlation coefficient, r_K, measures the correlation between two values at a distance (lag) k time units apart (10). The corresponding SE of the auto- or cross-correlation coefficient for a series of n samples at lag k can be estimated as $(n - k)^{-1/2}$. Significant auto- and cross-correlations are inferred when r_k values exceed zero by more than twice the SE. Because of sustained autocorrelations within each series, further analysis of the partial auto- and cross-correlation coefficients was undertaken using stepwise autoregressive fitting. The partial correlation coefficient at lag k measures the excess correlation beyond that found at lag (k − 1).

Results

As shown in Fig. 1A, multiple parameter deconvolution analysis of serial plasma ACTH concentrations revealed punctuated discrete bursts of ACTH secretion distributed over 24 h. The calculated curves reconstructing the pulsatile plasma ACTH concentrations over 24 h are shown in the *upper subpanels* and the resolved ACTH secretion profiles in the *lower subpanels*. According to this analysis, the 24-h plasma profiles of pulsatile ACTH release can be accounted for by a purely burst-like mode of ACTH secretion. As illustrated further (Fig. 1B), this inference was confirmed by waveform-independent deconvolution analysis, which also disclosed punctuated episodes of ACTH secretion separated by intervals of secretory quiescence, in which calculated ACTH secretory rates returned to values indistinguishable from zero. Thus, our inference about a burst-like mode of ACTH secretion without a tonic component is not model dependent.

Our estimates of ACTH secretory burst number, amplitude, and duration and the subject-specific ACTH half-lives using the multiple-parameter deconvolution procedure are summarized in Table 1. In the group of eight men, the mean (±SEM) frequency of ACTH secretory bursts was 40 ± 1.5/24 h (range, 33–47). The corresponding mean ACTH intersecretory burst interval was 39 ± 2.3 min. The latter measures the time (minutes) separating the centers of consecutive secretory bursts. The computer-resolved ACTH secretory bursts had a mean half-duration (duration at half-maximal amplitude) of 19 ± 1.8 min. The mean amplitude (maximal secretory rate) of the ACTH secretory bursts was 1.2 pg/mL·min or 0.26 pmol/L·min. (The rate of ACTH release is described in mass units per unit distribution volume/unit time.) Since the area under the computer-resolved ACTH secretory burst is proportionate to the mass of ACTH secreted per burst, we could estimate the amount of ACTH released per pulse. This value averaged 24 ± 3.6 pg/mL or 5.3 ± 0.79 pmol/L.

Based on the above frequency of ACTH secretory bursts and the mean mass of ACTH secreted per burst, one can calculate the daily endogenous ACTH production rate as the product of these two estimates. In these normal men the daily ACTH secretory rate averaged 960 ± 160 pg/mL or 211 ± 35 pmol/L. Assuming a minimal ACTH (plasma) distribution volume of 40 mL/kg, we estimate that at least 60 ng or 13 pmol ACTH are secreted per burst and that the mean daily ACTH secretion rate is 2.4 μg or 0.53 nmol for a 70-kg individual.

The half-life of endogenous ACTH in each of the eight subjects was also estimated simultaneously with the ACTH secretion rates. As shown in Fig. 2, the ACTH half-life in individual normal men averaged 15 ± 1.2 min. The absolute range for the half-life estimates was 10–20 min. Based upon nonlinear least squares error estimation models, we also calculated the statistical confidence limits for the estimates of each subject-specific ACTH half-life, as given by the *vertical bars* in Fig. 2. The ACTH half-life estimates were well determined, since they had coefficients of variation of approximately 4–15%. These deconvolution-based estimates of endogenous ACTH half-life are commensurate with values determined for exogenous ACTH by other investigators studying either

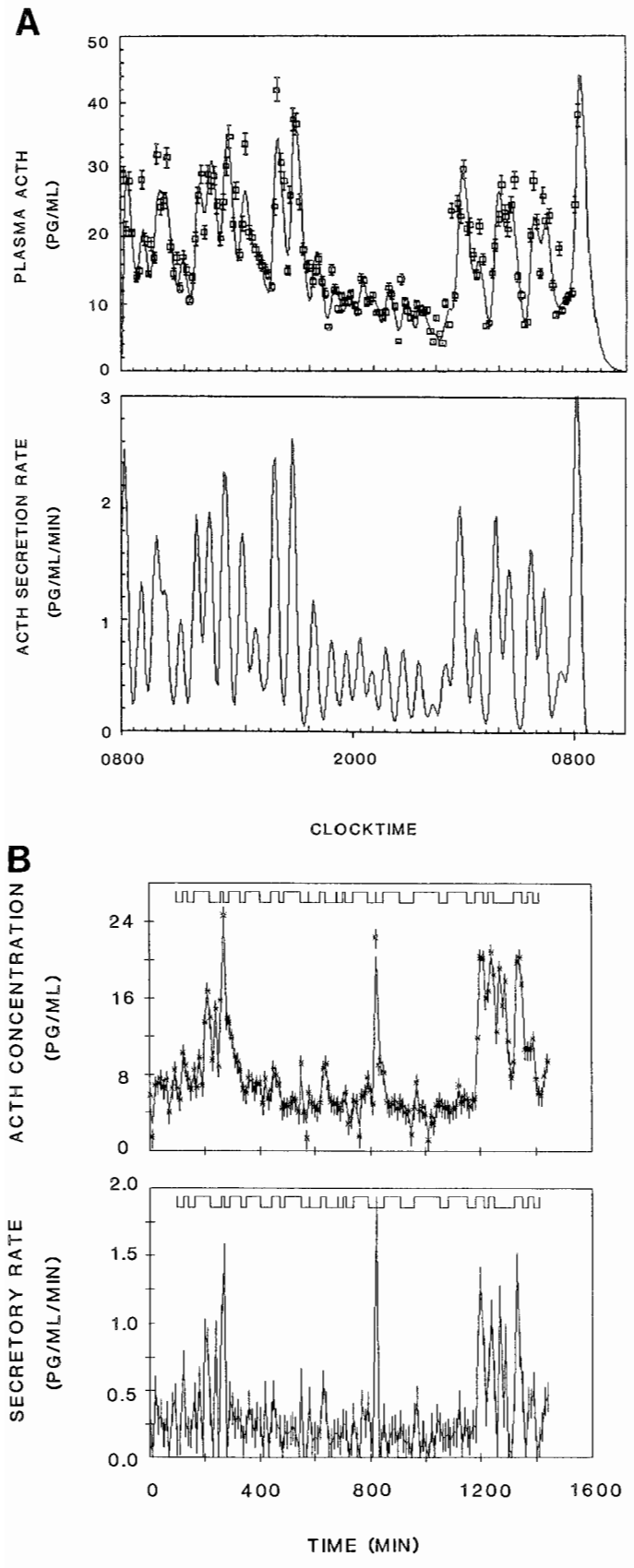


FIG. 1. A, Illustrative plasma ACTH concentration profiles and multiple-parameter deconvolution-resolved ACTH secretion events in a normal man. Subjects underwent blood sampling at 10-min intervals for 24 h. The plasma samples were submitted to a two-site IRMA to determine the concentration of ACTH at each time point. The *upper subpanel* in each figure shows the measured serial plasma ACTH concentrations over 24 h (145 individual samples) as well as the concentration values predicted by the convolution model (*continuous curve*). *Vertical marks* denote the intrasample dose-dependent SDs of the IRMA. The *lower subpanel* depicts the computer-resolved ACTH secretory rates plotted as a function of time. Note that the plasma ACTH concentration profile is built up of delimited ACTH secretory events that are dispersed over the full 24 h of observation and subjected to pronounced amplitude modulation. To convert picograms per mL ACTH to picomoles per L, multiply by 0.22. B, Waveform-independent deconvolution of 24-h profiles of plasma ACTH concentrations in an individual man. Data are presented as described above, except that no specific algebraic waveform was assumed in the convolution algorithm. Rather, applying a nominal ACTH half-life of 14 ± 2 min from pooled literature values, we calculated the sample ACTH secretory rate at each time point by nonlinear least-squares curve fitting (see *Materials and Methods*). The resultant ACTH secretory rates (and the individual SD for each estimate of secretion rate) are given in the *lower panel*. The deconvolution-predicted plasma ACTH concentrations are shown in the *upper panel* by the *continuous line* through the observed data. To convert picograms per mL ACTH to picomoles per L, multiply by 0.22. The deflections above the panels denote pulses.

immunoreactive or bioactive hormones (see *Discussion*) (14–24). Some of these previous estimates are shown for comparison in Fig. 2. Note that such estimates apply to exogenous ACTH, whereas the present estimates are baced on the kinetics of removal of endogenous unmodified hormone.

To evaluate 24-h variations in ACTH secretory measures, we performed cosinor analysis on the computer-resolved maximal ACTH secretory rates (amplitudes) attained in each release episode. The resulting dispersion of ACTH secretory burst amplitudes in all eight men over 24 h is shown in Fig. 3A. There was a significant diurnal rhythm in maximal ACTH secretory rates (burst amplitudes). This 24-h rhythm in maximal ACTH secretory rates had an amplitude of 0.70 (0.48–0.91) pg/mL·min or 0.15 (0.11–0.20) pmol/L·min ($P < 0.01$). The acrophase (time of maximum) of this rhythm in ACTH secretory rates occurred at 0818 h (0711–0924 h), which agrees closely with the acrophase of plasma ACTH concentrations, *viz.* 0853 h (0829–0917 h) in the same subjects. The mesor, or mean value about which the 24-h rhythm in ACTH secretory rates oscillates, was 1.2 (1.0–1.3) pg/mL·min or 0.26 (0.22–0.29) pmol/L·min. Of particular interest, there was no discernible 24-h rhythm in ACTH intersecretory burst interval (the reciprocal of ACTH burst frequency). In particular, as shown in Fig. 3B, the distribution of the ACTH intersecretory burst intervals over 24 h was random about a line of zero slope having a mean value of 39 min.

Typical plasma concentration profiles of pulsatile

TABLE 1. Measures of endogenous ACTH secretion and clearance in normal men

Subject no.	ACTH burst frequency (no./24 h)	Secretory burst half-duration (min)[a]	Amplitude of secretory burst (pg/mL·min)[b]	Mass of ACTH secreted/burst (pg/mL)[c]	Interburst interval (min)	Daily endogenous ACTH production rate (pg/mL)[d]
A	33	27 (25–29)	1.3 ± 0.75	37 ± 21	45 ± 12	1220
B	40	16 (14–18)	1.6 ± 1.2	27 ± 20	36 ± 11	1080
C	39	17 (16–19)	0.72 ± 0.45	13 ± 8.3	52 ± 8	507
D	47	20 (19–22)	1.4 ± 0.79	31 ± 17	31 ± 6	1460
E	44	11 (10–12)	1.5 ± 0.94	18 ± 11	33 ± 9	790
F	43	16 (15–17)	2.2 ± 1.2	38 ± 57	34 ± 9	1630
G	35	26 (24–28)	0.60 ± 0.54	17 ± 15	41 ± 11	595
H	40	16 (15–26)	0.60 ± 0.43	10 ± 7	37 ± 9	400
Group mean ± SEM	40 ± 1.5	19 ± 1.8	1.2 ± 0.19	24 ± 3.6	39 ± 2.3	960 ± 160

Parentheses contain 67% confidence limits for the estimate. Data are otherwise the mean ± SEM.

[a] The half-duration of the secretory burst is the duration of the secretory event at half-maximal amplitude.

[b] Maximal ACTH secretory rate.

[c] The volume term (milliliters) is unit distribution volume for ACTH. To convert picograms per mL ACTH to picomoles per L, multiply by 0.22.

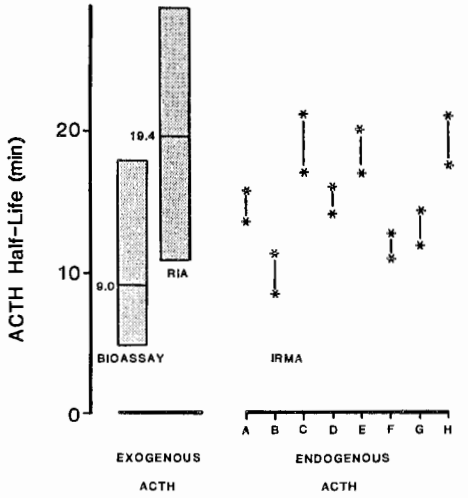


FIG. 2. Comparison of endogenous ACTH half-lives calculated by deconvolution analysis of ACTH time series (determined by IRMA in each of eight normal men; present data) with ACTH half-lives estimated from exogenous ACTH injections and subsequent bioassay or RIA (14). The ranges and mean values for the independent bioassay and immunoassay estimates (14) are shown. The deconvolution estimates of endogenous ACTH half-life in individual men are given with their 67% statistical confidence limits.

ACTH, β-endorphin, and cortisol release are illustrated for two subjects in Fig. 4. As shown in Table 2, we tabulated significant coincidences between individual peaks of ACTH and β-endorphin, ACTH and cortisol, and β-endorphin and cortisol. We counted the number of exactly coincident peaks within each relevant pair of hormones as well as coincident peaks considered at various specific lags (time in minutes separating the coincident peak maxima in the two series of interest). To evaluate the significance of these findings, we calculated the probability of finding at least the observed number of coincident peaks on the basis of chance alone in a group of subjects with the indicated individual pulse frequencies.

As summarized in Fig. 5, we found that the observed numbers of coincident events did not significantly exceed chance expectations (P values ranged from 0.17–0.99) except when 1) β-endorphin and ACTH peak maxima were required to occur in the same sample (zero lag or exact coincidence; $P < 0.0001$), 2) ACTH peak maxima preceded cortisol peak maxima by a fixed lag of 10 min ($P < 0.0001$, 3) simultaneous cortisol and β-endorphin peaks were considered ($P < 0.0001$), and 4) β-endorphin peak maxima were allowed to precede (lead) cortisol peak maxima by 10 min ($P = 0.0017$).

We also used a window of coincidence to evaluate near-synchronous release episodes. A ±10-min window of coincidence was defined as hormone peak maxima in the two series occurring simultaneously or within ±10 min of each other. Using appropriately modified formulations of the hypergeometric probability distribution for a window of coincidence (12, 13), we calculated 1) $P < 0.01$ for observing a total of 86 coincident ACTH and β-endorphin peaks within a ±10-min window (expected value, 64 ± 6.0), 2) $P < 0.01$ for 100 ACTH and cortisol coincidences within a ±10-min window (expected value, 71 ± 6.2), and 3) $P < 0.01$ for 70 β-endorphin and cortisol coincidences within a ±10-min window (expected value, 40 ± 5.0). Note, however, from Table 1 that the significant coincidences within a ±10-min window were due almost exclusively in the case of ACTH and β-endorphin to exactly simultaneous peaks or, for ACTH and cortisol, to cortisol peaks lagging those of ACTH by 10 min and, for β-endorphin and cortisol, simultaneous peaks as well

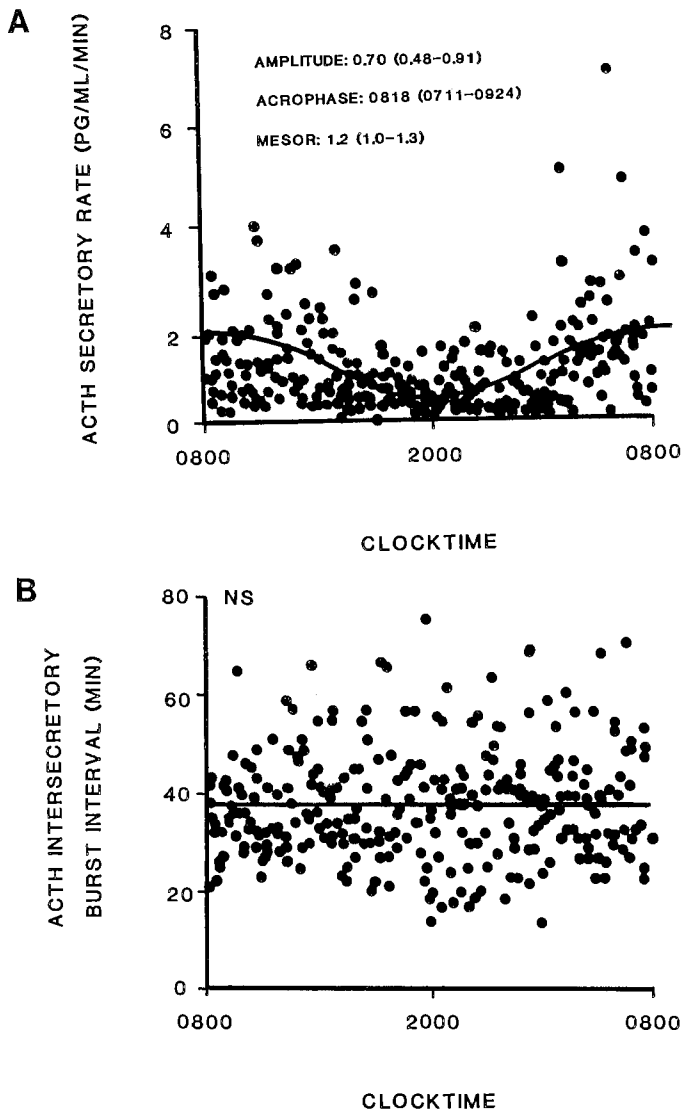


FIG. 3. Twenty-four-hour rhythms in ACTH secretory burst amplitudes (A), but not ACTH intersecretory burst intervals (B). The 24-h rhythms in measures of ACTH secretion were described by plotting the individual secretory burst amplitudes (maximal ACTH secretory rates attained within a release episode) against the corresponding times of the burst centers. Data are from all eight subjects (320 resolved secretory events in total). Cosinor analysis was then used to determine the presence and magnitude of any significant 24-h rhythm in ACTH secretory measures. The amplitude, acrophase, and mesor of the 24-h rhythm in ACTH secretory burst amplitudes were all significant. In contrast, as shown in B, there was no significant 24-h rhythm in ACTH intersecretory burst intervals, plotted as the duration of the interval (minutes) separating consecutive ACTH secretory burst centers against the time of the midpoint of the interval. To convert picograms per mL ACTH to picomoles per L, multiply by 0.22.

as cortisol peaks that lag β-endorphin pulses by 10 min.

To examine exact triple coincidences among ACTH, β-endorphin, and cortisol release episodes, we assessed the numbers of such three-way coincident events at various lags. As summarized in Fig. 6, only certain specific associations among the peaks of these three hormones achieved significance. In particular, we found that ACTH and β-endorphin peak maxima typically occurred simultaneously (exact coincidences), whereas cortisol peaks lagged these concordant ACTH/β-endorphin peaks by 10 min. We enumerated 15 such pattern-specific synchronous events in the group of 8 men ($P < 0.0001$ *vs.* expected number of triple coincidences, 2.4 ± 1.6). In addition, when we allowed cortisol and β-endorphin peak maxima both to lag ACTH pulses by 10 min, there were 9 such triple coincidences (expected, 2.4 ± 1.6; $P < 0.001$). Exact triple coincidences, consisting of simultaneous peak maxima of ACTH, β-endorphin, and cortisol, were observed 9 times ($P < 0.001$), whereas a pattern of simultaneus ACTH and β-endorphin peaks preceded by a cortisol peak 10 min earlier developed 8 times ($P < 0.005$).

To test correlations between pairs of corticotropic hormone concentrations over 24 h (rather than coincidences between pulses *per se*), we carried out cross-correlation analysis. This methodology takes no account of whether or where peaks occur in the data, but, rather, asks whether there are significant correlations between successively paired sample concentrations in the two hormone series. We observed significant cross-correlations between serial plasma concentrations of ACTH and β-endorphin as well as between ACTH and cortisol, and β-endorphin and cortisol. Maximal cross-correlations occurred at zero lag (ACTH and β-endorphin) or at a 10-min lag (ACTH and cortisol, or β-endorphin and cortisol). The cross-correlation coefficients had median r values of 0.63 ± 0.67, which correspond to $P < 10^{-6}$. Autoregressive modeling was used to remove any spurious cross-correlations otherwise introduced by autocorrelations due to the effects of hormone metabolic clearance. This analysis demonstrated that essentially all of the true cross-correlation occurred at 0- to 10-min lags, and that such coupling was expressed for all three possible pairwise relationships among plasma ACTH, β-endorphin, and/or cortisol concentrations.

Discussion

The ACTH-adrenal axis is a critical stress-responsive system with prominent circadian rhythmicity (1–6). The nyctohemeral rhythmicity of the corticotropic axis has been recognized in many species, but the mechanisms that give rise to such 24-h variations in plasma ACTH concentrations have not been delineated rigorously to date. Here, we have applied three distinct strategies to address the basis for the diurnal variations in plasma ACTH concentrations in healthy men. First, we employed a sensitive, reproducible, and specific two-site IRMA of plasma ACTH-(1–39) concentrations, whereby

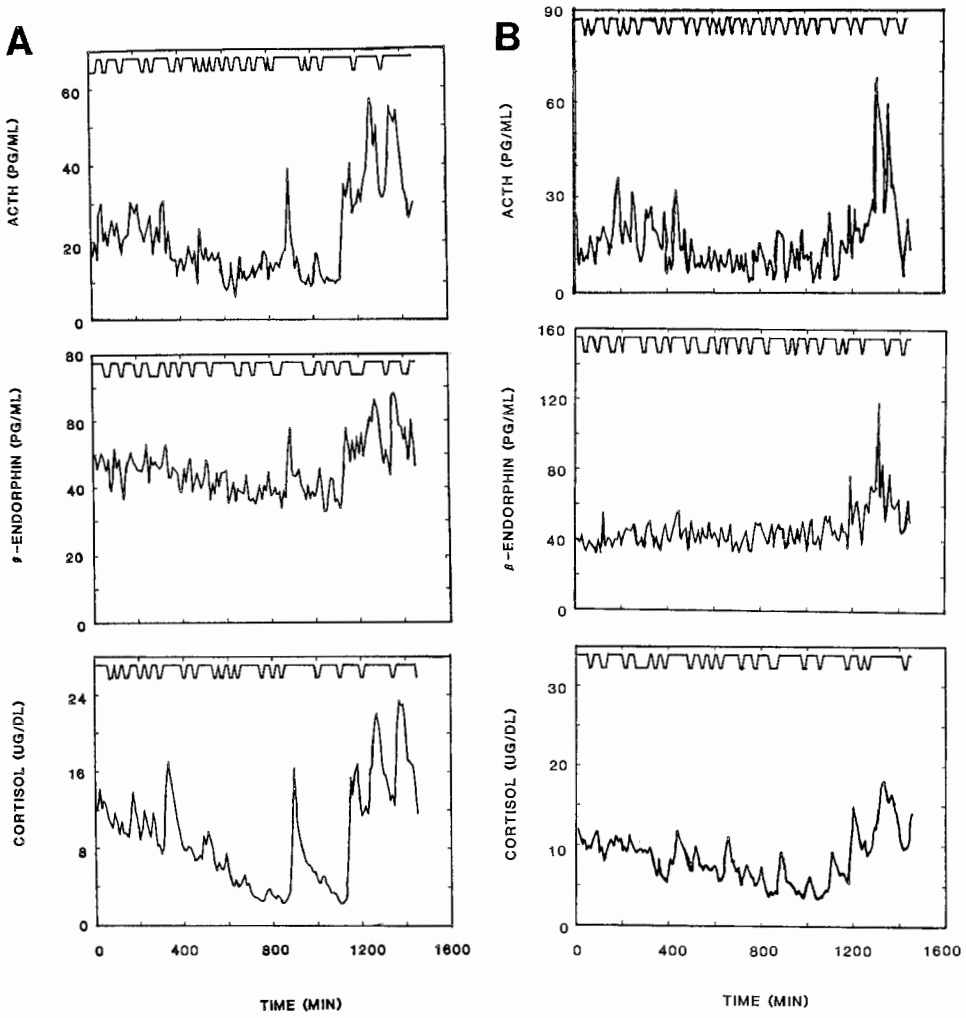


FIG. 4. Illustrative 24-h pulsatile profiles of serial plasma ACTH, β-endorphin, and cortisol concentrations in each of two men, who underwent blood sampling at 10-min intervals for 24 h. The subsequent plasma samples were submitted to IRMA to measure the concentrations of β-endorphin and ACTH, and to RIA to quantitate cortisol concentrations. The resultant hormone time series were analyzed by an objective computerized peak detection algorithm (Cluster) to identify significant hormone concentration peaks, as denoted by the schematized deflections *above each subpanel.* To convert picograms per mL ACTH to picomoles per L, multiply by 0.22; to convert picograms per mL of β-endorphin to picomoles per L, multiply by 0.29; and to convert micrograms per dL cortisol to nanomoles per L, multiply by 27.6.

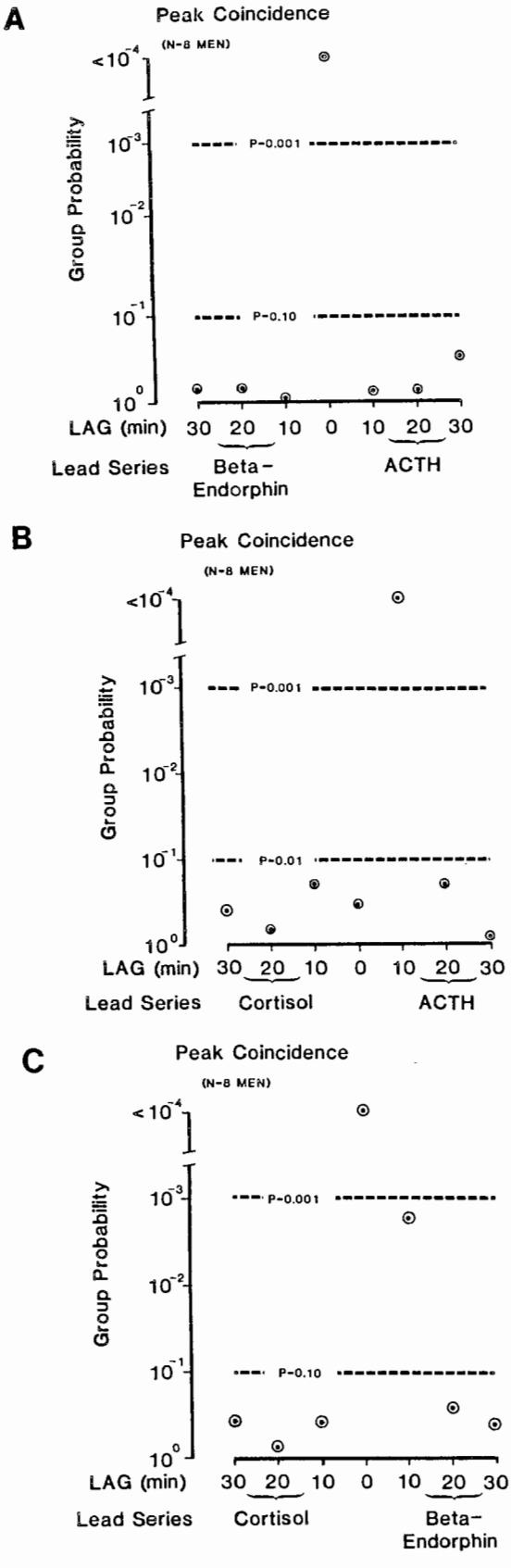
we could measure ACTH concentrations in all plasma samples obtained over the entire course of 24 h. Secondly, we undertook both intensive (every 10 min) and extended (24-h) blood sampling to capture the full 24-h physiological rhythms of ACTH concentrations with high temporal resolution. Thirdly, we used a novel deconvolution model designed to resolve quantitatively the number, amplitude, and duration of ACTH secretory bursts as well as the subject-specific ACTH half-life *in vivo* using all plasma ACTH concentrations and their intrasample variances considered together (7–9). Our results indicate that complete 24-h plasma ACTH concentrations profiles can be accounted for by a burst-like mode of ACTH secretion, which by amplitude modulation (but not frequency control) gives rise to a 24-h rhythm in circulating ACTH concentrations.

Computer-resolved ACTH secretory bursts in normal men occurred at an average interpulse interval of 39 ± 2.3 min in the present study. This periodicity corresponds to 40 ± 1.5 ACTH secretory bursts/24 h, which represents a value considerably higher than that reported using conventional, less frequent sampling paradigms as well as earlier immunoassays of ACTH and discrete peak detection methods (25–29). Several reasons may account for these differences. For example, first, given a median ACTH half-life of 15 min, plasma ACTH concentrations would decay by 50% or more between successive 15- or 20-min samples and thus allow missed peaks. Secondly, in many earlier RIAs, in contrast to the present IRMA, undetectable plasma ACTH concentrations could occur in the late afternoon, which also would lead to underestimates of peak frequency. Thirdly, earlier methods of peak detection did not take into account subject-specific half-lives of ACTH to delineate hormone secretory episodes (9). However, more recently, highly intensive blood sampling schedules in the rat, sheep, and primate have revealed high frequency peaks in plasma ACTH concentrations that can be assumed to correspond to secretory episodes. Thus, pulses of ACTH occurred at average intervals of 17 ± 1.3 min (rat), 15 ± 5 min (sheep), and 17–22 min (rhesus monkey) (30–32). Our results extend these findings by using deconvolution analysis to discern ACTH secretory episodes judged in relation to subject-specific ACTH half-lives, and to identify such ACTH

TABLE 2. Numbers of coincident ACTH, β-endorphin, and/or cortisol peaks in eight normal men

Time (min) by which second series leads or lags first	Subject no. 1	2	3	4	5	6	7	8	Total
ACTH/β-endorphin									
Leads 30	2	1	4	2	0	3	5	1	18
Leads 20	1	3	1	5	3	1	2	2	18
Leads 10	4	3	0	1	2	2	1	0	13
Simultaneous	8	16	6	4	2	4	4	12	56[a]
Lags 10	1	1	3	3	4	4	0	1	17
Lags 20	2	3	1	0	1	3	3	2	18
Lags 30	4	3	4	2	3	3	2	4	25
ACTH/cortisol									
Leads 30	2	4	2	3	3	1	2	5	22
Leads 20	3	3	2	3	3	2	2	2	20
Leads 10	6	4	1	7	1	4	3	2	28
Simultaneous	4	4	1	3	3	4	2	3	24
Lags 10	3	4	5	8	7	9	6	7	48[a]
Lags 20	6	5	5	2	4	2	2	2	28
Lags 30	3	4	0	5	0	0	2	3	17
β-Endorphin/cortisol									
Leads 30	0	4	0	1	3	1	1	4	14
Leads 20	2	3	0	0	1	3	1	0	10
Leads 10	2	3	1	3	1	3	0	1	14
Simultaneous	5	3	4	3	4	6	3	5	33[a]
Lags 10	1	3	3	5	2	1	4	4	23[a]
Lags 20	4	4	2	2	1	2	1	0	16
Lags 30	2	3	0	2	2	2	1	2	14

[a] Statistically exceeds expectations on the basis of random associations alone (see text).



secretory bursts for the first time as high frequency events in the human. Moreover, we have been able to determine how such secretory bursts give rise to the diurnal rhythm in plasma ACTH concentrations and correlate release episodes of ACTH, β-endorphin, and cortisol.

We observed that the half-duration (duration at half-maximal amplitude) of computer-resolved ACTH secretory bursts averaged 19 ± 2 min. This secretion span is intermediate between that estimated recently for immunoactive or bioactive LH (7–14 min) and that inferred for GH and cortisol (8–35 min) (8, 33–35). Importantly, the duration of an ACTH secretory burst is considerable, such that a large proportion of the plasma ACTH concentration peak in men is due to continuing ACTH secretion, rather than being the simple result of instantaneous secretion followed by metabolic clearance. This feature of ACTH release is important, since extrapolation of the ACTH half-life from the descending limb of a plasma ACTH concentration peak alone will yield an

incorrect estimate of hormone half-life in proportion to the extent to which glandular secretion continues at that time (7, 9). The present convolution model of combined ACTH secretion and clearance takes account of noninstantaneous or continued secretion by defining all plasma hormone concentrations as the mathematical consequence of combined secretion and metabolic clearance, with both events contributing nonlinearly over time.

Our estimate of a mean half-life for endogenous ACTH of 15 ± 1.2 min agrees well with independent literature estimates in man that range from 4–40 min based on the injection of various (exogenous) ACTH molecular species measured in different assay systems (14–24, 36). For example, depending upon the use of either a bioassay or RIA, one report gave ranges of exogenous ACTH half-life estimates in men of 4.9–18 min (bioassay) and 11–29 min (RIA) (14). The overall range of previous estimates for the half-life of exogenously injected immunoactive ACTH in the human is 10–40 min, and that of bioassayable ACTH is 4–19 min (14–37). Thus, the present estimate of the endogenous ACTH half-life (15 ± 1.2 min) is consistent with the many available literature values for exogenous ACTH preparations. Moreover, we observed that the variance in half-life estimates was larger between subjects than within a subject. Similar observations have been made recently for LH, in which variable degrees of glycosylation of the polypeptide hormone presumably contribute importantly to the variance in half-life estimates among different healthy subjects (33, 38). In contrast, a narrower range of estimates of endogenous GH half-life prevails, which is consistent with the absence of glycosylation of this protein hormone (or its very sparing glycosylation) (35).

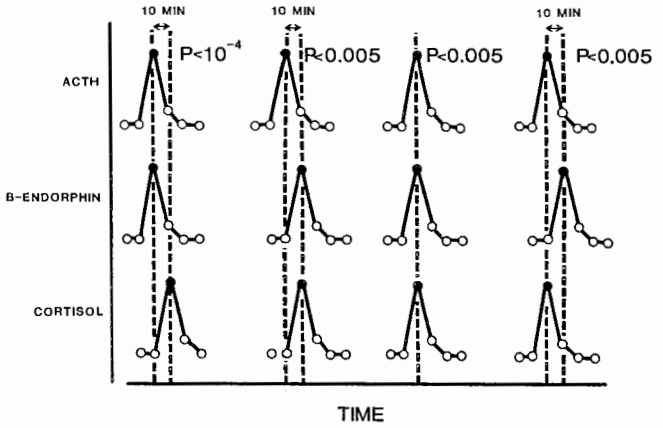


FIG. 6. Schematized depiction of unique and statistically significant patterns of temporally synchronized release bursts of ACTH, β-endorphin, and cortisol in normal men. *P* values denote the probabilities that the observed patterns of coincidence are due to chance (random) peak associations alone.

Based on an average of 40 ACTH secretory bursts/24 h and a mean mass of ACTH secreted per burst of 24 ± 3.6 pg/mL (or 5.3 ± 0.79 pmol/L), we estimate that at least 60 ng (13 pmol) ACTH are secreted/burst and 2.5 μg (0.53 nmol)/24 h in normal men under resting physiological conditions. These values assume a minimal distribution volume for intact ACTH of 40 mL/kg, which is the plasma space as estimated independently by injections of pituitary (glyco)protein hormones (39). The calculated minimal ACTH secretion rate in the present study is in general accord with values inferred from exogenous ACTH infusions and/or other methodologies (21, 24, 37). For example, the earliest estimates of ACTH production rates by Berson and Yalow were 7.2 μg/day, assuming a considerably larger distribution volume (21).

The present analyses demonstrate that a tonic mode of ACTH release in man is not required to provide reasonable estimates of ACTH half-lives and production rates or to obtain a quantitatively precise description of the complex variations in plasma ACTH concentrations over an entire 24 h. Rather, we can infer that a simple burst-like mode of ACTH secretion is sufficient to give rise to both pulsatile and circadian variations in plasma corticotropin concentration profiles. We confirmed this statistically based inference of an exclusively burst-like mode of ACTH secretion by a second waveform-independent deconvolution technique. This basic finding has interesting implications to our understanding of the operating behavior of ACTH target cells as well as the anterior pituitary gland. For example, the intermittency of plasma ACTH concentration peaks may serve to minimize desensitization or down-regulation of adrenal ster-

FIG. 5. Probabilities of observing at least the indicated numbers of coincident cortisol, β-endorphin, and/or ACTH peaks on the basis of chance (random) peak associations alone in a group of eight healthy men, who underwent blood sampling at 10-min intervals for 24 h. The resulting plasma samples were subjected to IRMA to measure concentrations of β-endorphin and ACTH and to RIA to quantitate cortisol. A computerized peak detection method (Cluster) was used to identify significant release bursts (pulses) of these hormones. The temporal locations of the cortisol, β-endorphin, and ACTH peak maxima were compared in each of the eight men, and the total number of coincident events was enumerated in the group of subjects. Exact coincidence was defined as the simultaneous (zero lag) occurrence of peak maxima. In addition, numbers of coincident events were determined at various lags. The total number of coincident events was tabulated for the group of eight subjects, and conditional probability calculations were made (see *Materials and Methods*). The latter values give the group probability that at least the indicated number of peak coincidences could be observed due to chance peak associations alone. The individual data from all eight men are given in Table 1. A, Group probabilities that at least the observed numbers of coincident ACTH and β-endorphin peaks in a group of eight men can be assigned to chance (random) associations. B, Group probabilities that at least the observed numbers of coincident cortisol and ACTH peaks in a group of eight men can be assigned to chance (random) associations. C, Group probabilities that at least the observed numbers of coincident cortisol and β-endorphin peaks in a group of eight men can be attributed to chance (random) associations.

oidogenic responses by providing a poststimulus quiescent interval for target cell recovery. Similarly, the predominantly episodic nature of ACTH secretion inferred here may help to avoid corticotrope cell desensitization that would otherwise be expected if sustained stimulation of the anterior pituitary gland by CRH or other ACTH secretagogues occurred (40). Moreover, given the *in vitro* dependence of acute ACTH release on acute secretagogue exposure (41), the pulsatile mode of ACTH secretion delineated here *in vivo* suggests that physiological stimuli impinge on corticotrope cells in a principally episodic or pulsatile fashion. Such stimuli are believed to include CRF, arginine vasopressin, and other neuropeptides and/or amines (42). In addition, inhibitory signals, such as somatostatin and/or the feedback actions of endogenous glucocorticoids, might modulate the contour, duration, or amplitude of physiological ACTH secretory events (43). Although the exact endogenous patterns of presentation of these stimuli and/or inhibitors under physiological conditions *in vivo* are not known, one can speculate that varying amounts and/or durations of CRF- and arginine vasopressin-pulsed stimuli can act on one or more populations of responsive corticotrope cells to initiate delimited bursts of ACTH secretion when inhibitors are at least partially withdrawn.

Cosinor analysis to detect possible 24-h rhythms in ACTH secretory rates and ACTH secretory burst frequency revealed marked and selective nyctohemeral modulation of ACTH secretory burst amplitude (or mass), but not frequency. In particular, we demonstrated a 3.8-fold variation in the 24-h rhythm of ACTH secretory burst amplitude (maximal rate of ACTH secretion attained per burst) and mass. Spontaneous ACTH secretory burst amplitudes reached a peak at 0818 h, and plasma ACTH concentrations peaked shortly thereafter (0853 h). In contrast, there was no measurable variation in ACTH secretory burst frequency over 24 h. Thus, the circadian variation in plasma ACTH concentrations can arise by way of selective diurnal modulation of ACTH secretory burst amplitude. Exactly how this is accomplished *in vivo* is not known, but it may be due to variable inputs of neurally regulated secretagogues to corticotrope cells and/or diurnally modulated responsiveness of the anterior pituitary gland to available hypothalamic stimuli (42, 43). Of interest, intact adrenal corticosteroid feedback *per se* is not required for the nyctohemeral ACTH rhythm (44). Whatever the mechanism(s), we observed that cascade-like volleys of ACTH secretory events emerged at the time of diurnally maximal ACTH release. This volley pattern is qualitatively similar to the mode of GH secretion recognized in normal men during the nocturnal period of maximal activation of the somatotropic axis (35).

The hypothesis that successive ACTH secretory episodes are independent events (*i.e.* occur without regard to the locations in time of the immediately preceding ACTH secretory bursts) was confirmed by autocorrelation analysis of serial ACTH interpulse intervals. This finding signifies that there is no short term memory in the ACTH burst-generating system(s), *i.e.* bursts are produced randomly over time. The latter inference was also made recently for LH, GH, and cortisol pulse generator mechanisms (8, 33, 35, 45). However, the random nature of consecutive activation of the ACTH burst generator system does not exclude the operation of superimposed deterministic control elements, such as diurnal regulation of ACTH secretory burst amplitude, as shown here.

The validity of the ACTH IRMA, which may not recognize all bioactive ACTH series, was suggested by our finding of co-pulsatility of ACTH, β-endorphin, and cortisol. Formal coincidence analysis allowed us to demonstrate significant nonrandom temporal coupling between spontaneous release episodes (pulses) of β-endorphin and ACTH (near-simultaneous release), ACTH and cortisol (cortisol release bursts lag ACTH peaks by 10 min), and cortisol and β-endorphin (corelease as well as cortisol pulses that lag β-endorphin peaks by 10 min). The close temporal associations among these hormones was corroborated by cross-correlation analysis and autoregressive modeling. In addition, we documented significant triple coincidence among ACTH, β-endorphin, and cortisol pulses, which indicates that exquisite temporal coordination and consistent response times operate within the intact hypothalamo-pituitary-adrenal axis in men. These observations, made under basal physiological conditions, confirm the reliability of our assay procedures and should provide a basis for further investigation of selected derangements in the coordinate regulation of the β-endorphin-corticotropic-adrenal axis in certain clinical pathophysiological states.

In summary, we have used highly resolved ACTH time series and deconvolution procedures to estimate the number, amplitude, and duration of endogenous ACTH secretory bursts and subject-specific ACTH half-lives *in vivo*. These analyses revealed a predominantly burst-like mode of ACTH secretion without the need to postulate any tonic or constitutive ACTH release. We found that a 3.8-fold diurnal variation in the amplitude of ACTH secretory bursts (and, hence, also in the mass of ACTH released per burst) gives rise to the observed 24-h rhythm in plasma ACTH concentrations. In contrast, the frequency of ACTH secretory bursts does not vary over 24 h. We also observed that the hypothalamo-pituitary-adrenal axis maintains close temporal synchrony among discrete release episodes of ACTH, β-endorphin, and cortisol. Thus, we conclude that amplitude, but not frequency, modulation of randomly dispersed ACTH secre-

tory bursts that are closely coupled to β-endorphin and cortisol peaks gives rise to the diurnal rhythm of the corticotropic axis in man.

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