Circadian Relationships Between Circulating Atrial Natriuretic Peptides and Serum Calcium and Phosphate in Healthy Humans

D.L. Vesely, R.B. Sothern, L.E. Scheving, F.W. Bremner, J.L.H.C. Third, J.B. McCormick, S. Dawson, S. Kahn, G. Augustine, M. Ryan, J. Greco, B.A. Nemchausky, P. Shirazi, and E.L. Kanabrocki

Long-acting natriuretic peptide (LANP), vessel dilator (VSDL), and atrial natriuretic factor (ANF) consisting of amino acids (aa) 1 to 30, 31 to 67, and 99 to 126, respectively, of the 126-aa ANF prohormone circulate in humans. Among the biologic properties of these peptides is the ability of ANF to decrease intracellular calcium concentrations. To determine if atrial natriuretic peptides are directly related to serum calcium and/or phosphate in healthy normocalcemic humans, we examined 21 24-hour profiles of VSDL, LANP, ANF, and serum calcium and phosphate in 14 healthy humans. VSDL, LANP, and ANF each had significant ($P < .001$) circadian rhythms, with peak concentrations late during sleep (at 4:00 AM) being nearly twice the concentrations in the afternoon and evening. Serum calcium and phosphate also had significant circadian rhythms ($P < .001$) with troughs nearly opposite to those of the atrial natriuretic peptides, suggesting that atrial peptides may be important in the modulation of the circadian rhythms of calcium and phosphate. The nearly identical circadian rhythms of the atrial natriuretic peptides and of parathyroid hormone (PTH) reported by others, along with evidence that PTH may increase atrial peptide release, suggest that some of the effects attributed to PTH may be mediated by atrial natriuretic peptides.

CALCIUM is thought to be one of the regulators of atrial natriuretic factor (ANF) synthesis and secretion.[1] When primary cultures of neonatal rat cardiocytes are exposed for 24 hours to 2 mmol/L $CaCl_2$ in the culture media, ANF mRNA increases threefold.[1] Addition of calcium-channel–blocking agents to these cardiocytes results in a 25% to 40% decrease in the synthesis and secretion of ANF.[1] Both oral and intravenous calcium have been reported to increase ANF release.[2-4] Calcium infusions that increase serum calcium to within the normal physiologic range will also increase circulating ANF levels.[5] Some of the effects of ANF, on the other hand, are calcium-dependent.[6,7] The calcium-dependent effects do not appear to be due to calcium uptake, since ANF does not increase $^{45}Ca^{2+}$ uptake.[7] Rather, ANF can also decrease free Ca^{2+} within the cell[8,9] which, in part, appears to be due to its enhancing calcium extrusion from the cell.[7,10]

Stimulation of the ANF gene by calcium results in increased synthesis of the 126–amino acid (aa) ANF prohormone.[1] This 126-aa prohormone is processed by proteases to produce several atrial natriuretic peptide hormones[11] (Fig 1). These peptides consisting of aa 1 to 30 (long-acting natriuretic peptide [LANP]), aa 31 to 67 (vessel dilator [VSDL]), and aa 99 to 126 (ANF) of the 126-aa ANF prohormone circulate[12,13] and have known biologic effects (blood pressure decrease, water, sodium, and/or potassium excretion) in animals[14,15] and humans.[16,17]

These atrial natriuretic peptides (VSDL, LANP, and ANF) have a circadian rhythm in healthy diurnally active individuals, whose peak concentrations in the middle of the night[18,19] are near the 24-hour nadir of serum calcium.[20,21] These circadian studies suggest that serum calcium may be regulated by atrial natriuretic peptides in healthy humans in an inverse relationship, whereby normal circadian increases in atrial natriuretic peptides may result in a decrease in serum calcium. The present investigation was designed to determine at each time point throughout the day whether one or more of the atrial peptides have a temporal (circadian) and/or functional (overall) relationship to calcium and/or phosphate.

SUBJECTS AND METHODS

Subjects

Ten clinically healthy men were studied in 1988 (mean age, 46.5 years; range, 41 to 60) and 11 men were evaluated in 1993 (mean age, 55.2 years; range, 46 to 72). Clinical characteristics and 24-hour mean blood values of these men are listed in Table 1. On each occasion, the volunteers were admitted to the clinical research unit of the Hines Veterans Affairs Hospital for one 24-hour period on May 15, 1988, and 5 years later on May 14, 1993. Each subject received a complete physical, and none were found to have any predisposing condition or were on any prescribed or over-the-counter medication. Nutritional assessments, including anthropometric measurements, were made for each subject, and all were found to have a good nutritional status. Meals prepared by the dietetic service of the hospital consisted of a general hospital diet totaling 2,500 cal and were served at 7:30 AM, 1:30 PM, and 4:30 PM. Mean calcium and phosphate intakes during this investigation were 952 mg (range, 737 to 1,068) and 1,452 mg (range, 1,292 to 1,508), respectively. Calcium and phosphate intakes were not restricted before this investigation. Water intake was not restricted except for

From the Department of Internal Medicine, University of South Florida Health Sciences Center, Tampa; James A. Haley Veterans Administration Hospital, Tampa, FL; The Rhythmometry Laboratory, St. Paul-Ramsey Medical Center, St. Paul; and the University of Minnesota, Minneapolis, MN; the University of Arkansas for Medical Sciences, Little Rock; and John L. McClellan Memorial Veterans Administration Hospital, Little Rock, AR; the Cardiovascular Institute, Chicago; and the Pathology Department, Foster G. McGraw Hospital, Loyola University Medical Center, Chicago; Pathology Medicine, Swedish Covenant Hospital, Chicago; and the Nursing Service, Nuclear Medicine Service, and Spinal Cord Injury Service, Edward J. Hines, Jr., Veterans Affairs Hospital, Hines, IL.

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Address reprint requests to D.L. Vesely, MD, PhD, James A. Haley Veterans Administration Hospital-151, 13000 Bruce B. Downs Blvd, Tampa, FL 33612.

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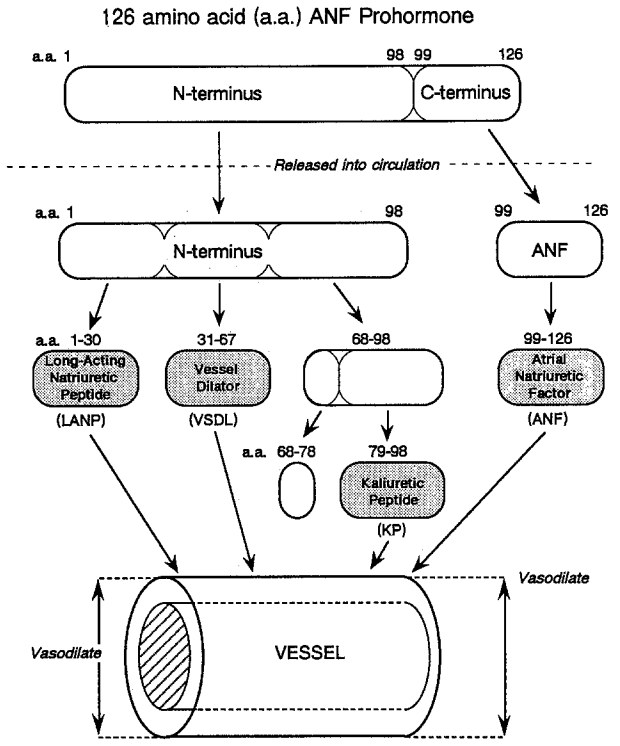

Fig 1. Origination of LANP, VSDL, and ANF from the ANF prohormone. LANP consisting of aa 1 to 30 and VSDL composed of aa 31 to 67 originate from the *N*-terminus and ANF consisting of aa 99 to 126 originates from the C-terminus of this 126-aa prohormone. Each of these peptide hormones circulate as distinct entities.[13,16,17]

Table 1. Clinical Characteristics and 24-Hour Mean Values for Subjects Studied in 1988 and/or 1993

Subject No.	Study Year	Age (yr)	Weight (lb)	Height (in)	Plasma (pg/mL)			Serum (mg/dL)	
					LANP	VSDL	ANF	Ca	P
1	1988	45	277	74.00	1,869	1,708	75.1	9.71	4.10
2	1988	46	207	68.50	2,104	1,877	78.6	9.85	3.90
3	1988	47	194	68.00	2,366	2,344	81.4	9.04	3.11
4	1988	41	179	74.00	2,137	2,074	74.1	9.59	4.32
	1993	46	185	74.00	1,905	1,996	67.2	9.25	4.11
5	1988	43	179	72.00	2,244	2,115	74.6	9.35	3.46
	1993	48	180	72.00	1,929	1,956	68.7	9.16	3.38
6	1988	45	164	67.00	2,361	2,279	73.2	9.47	3.83
	1993	50	144	67.50	1,851	2,268	87.0	9.13	3.33
7	1988	45	165	66.00	2,231	2,026	79.9	9.39	4.05
	1993	50	175	67.00	1,823	2,192	82.8	9.06	3.71
8	1988	45	212	74.50	1,878	1,744	72.1	9.64	3.88
	1993	50	214	72.75	1,472	1,527	72.8	9.23	4.06
9	1988	53	229	67.75	2,094	2,009	77.9	9.32	4.07
	1993	58	250	68.00	2,391	1,495	62.4	8.98	3.92
10	1988	60	175	68.00	2,209	2,159	70.6	9.09	3.17
	1993	65	182	67.75	2,604	2,007	68.6	9.04	3.78
11	1993	47	220	66.00	2,015	1,419	66.2	8.94	3.78
12	1993	50	162	66.50	2,021	1,475	73.6	9.04	3.63
13	1993	71	175	66.00	1,605	1,356	73.5	9.36	3.51
14	1993	72	180	68.50	2,191	1,685	65.3	9.19	3.49

NOTE. Mean values for blood variables represent 24-hour rhythm-adjusted means (MESORs) from 8 3-hour values obtained during each study.

the half-hour before sampling, but subjects were required to abstain from other liquids and food between meals. All subjects had maintained their regular diurnal activity schedule with sleep at night for at least 2 weeks before study. During the study, participants were ambulatory by day and slept at night, being briefly awakened for the 1:00 and 4:00 AM sampling. Lights were turned off at 10:45 PM and on at 6:45 AM. Informed consent was obtained from each of the volunteers after the nature and possible consequences of the study were fully explained. This study was approved by the Institutional Review Board of the Edward Hines Veterans Affairs Hospital.

Materials

^{125}I-labeled VSDL, LANP, and ANF and unlabeled pure human sequences of VSDL, LANP, and ANF were obtained from Peninsula Laboratories (Belmont, CA). Specific activities of ^{125}I-labeled VSDL, LANP, and ANF were 1,500, 1,100, and 2,000 Ci/mmol, respectively. Other reagents and supplies were obtained from sources previously described.[18,22]

Extraction of VSDL, LANP, and ANF From Plasma

Blood samples were collected at 3-hour intervals beginning at 7:00 PM, with subsequent sampling beginning at 10:00 PM, 1:00, 4:00, 7:00, and 10:00 AM, and 1:00 and 4:00 PM following a protocol described previously in detail.[23] Blood was collected into chilled 5 ml EDTA tubes to prevent proteolytic breakdown of any peptides that might be present. These samples were transported on ice and immediately centrifuged at 3,000 *g* for 15 minutes. After centrifugation, each sample was extracted with 100% ethanol (1:1 dilution), vortexed, and allowed to stand at 4°C for 30 minutes.[12,16]

Measurement of VSDL, LANP, and ANF

Radioimmunoassays to measure LANP and VSDL levels were devised to aa 1 to 30 and 31 to 67 of the 120-aa ANF prohormone, whereas our ANF assay measures aa 99 to 126 of this prohormone as described in detail previously.[12,13,17] The VSDL assay immunologically recognizes only VSDL in plasma and does not recognize ANF or any other portion of the ANF prohormone.[13,17] Likewise, the ANF assay immunologically recognizes only ANF in plasma.[12,13] The ANF radioimmunoassay does not recognize VSDL or any other portion of the ANF prohormone in plasma. The LANP assay immunologically recognizes LANP (50%) and the 98-aa *N*-terminus of the ANF prohormone (50%) in plasma.[13,17] All determinations were performed in triplicate. Intraassay coefficients of variation for LANP, VSDL, and ANF radioimmunoassays were 4.8%, 5.3%, and 5.7%, respectively. Interassay coefficients of variation were 8% for both LANP and VSDL, and ANF interassay variation was 6.9%.

Recovery was examined by adding synthetic unlabeled VSDL, LANP, and ANF at 100, 200, and 400 pg/mL to pooled plasma. Recovery of VSDL was 100.9% ± 11% and of ANF 92% ± 11%. Recovery of LANP was 83.5% ± 13.2%. The lowest detectable concentrations were 40 fmol (140 pg/mL), 35 fmol (136 pg/mL), and 1.4 fmol (4.3 pg/mL), and nonspecific binding was 2.1%, 2.5%, and 2.8% for LANP, VSDL, and ANF radioimmunoassays, respectively. Serial dilution of pooled plasma has shown excellent parallelism of standards and unknowns in these assays.[12,22]

Serum Calcium and Phosphate

Blood samples for calcium and phosphate determinations were obtained at the same time points throughout the day as the samples used to measure circulating concentrations of atrial natriuretic peptides. Serum calcium and phosphate concentrations were measured using a CHEM-1 Random Excess Continuous Chemistry

Analyzer (Technicon, Terrytown, NJ). Intraassay coefficients of variation were 1.8% and 2.1% for calcium and phosphate, respectively.

Statistics

All the data are expressed as the group mean ± SEM at each time point using original units and after normalization to percent of individual mean. Each variable was analyzed for circadian time effect by one-way ANOVA. Individual time series were analyzed for circadian rhythm by a computerized inferential statistical method involving the fit of a 24-hour cosine curve by the method of least squares.[24] A *P* value for the rejection of the zero-amplitude assumption, the acrophase (timing of peak), the amplitude (half the peak-trough difference), and the MESOR (a rhythm-adjusted average) was determined for each individual. Figure 2 illustrates the rhythm characteristics derived from the least-squares fit of a cosine used to validate a rhythm as circadian (ie, 24 hours). Individual rhythm characteristics for each variable were summarized for the group by population mean cosinor summary.[25] To test for a functional relationship, individual values and overall 24-hour mean values for each ANF peptide were correlated with mean serum calcium and phosphate concentrations by simple linear regression.[19]

RESULTS

Circadian Rhythm of LANP, VSDL, and ANF

VSDL, LANP, and ANF each showed a high-amplitude, significant ($P < .001$) circadian rhythm (Fig 3). The group mean range of change (ROC) from the lowest to the highest values for LANP in the 24-hour period was 106% (1,554 pg/mL). The ROC for VSDL was 82% (1,210 pg/mL) and for ANF 41% (68 pg/mL). A marked increase occurred in each of the atrial peptides at 4:00 AM, and these levels remained elevated at 7:00 AM (Fig 3). Each atrial peptide's circulating concentration decreased by half by 10:00 AM and then remained fairly stable the rest of the day. The mean amplitude (which measures the predictable variability of a given rhythm) was 30% for LANP, 25% for VSDL, and 23% for ANF. The acrophase, which is an objectively determined time of the crest of a validated rhythm, occurred between 5:00 and 6:00 AM for each of the atrial peptides (see Table 2 for 95% confidence limits). The high-amplitude circadian rhythms of VSDL, LANP, and ANF were highly significant ($P < .001$) by both ANOVA and cosinor techniques using original units or percent of the mean (Table 2).

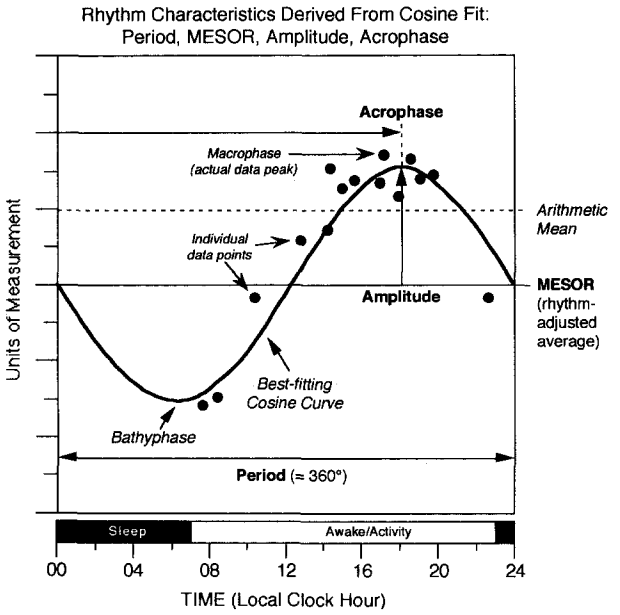

Fig 2. Characteristics (MESOR, amplitude, and acrophase) used to objectively describe a circadian rhythm. MESOR (middle estimated statistic of rhythm) is a rhythm-adjusted mean. Amplitude is equal to half the extent of rhythmic changes for the period considered (ie, half peak-trough difference of fitted cosine). The amplitude must be significantly greater than zero ($P \leq .05$ from a non-zero amplitude test) for a rhythm to be significant (ie, circadian). Acrophase is the time to reach crest of validated rhythm typically referenced to midnight, whereas bathyphase is time of lowest point of the curve. Period, length of fitted cosine (ie, 24 hours).

Circadian Rhythm of Serum Calcium

Serum calcium also had a circadian rhythm in these healthy human subjects that was significant at a *P* value of .001 (Fig 4). The mean ROC within 24 hours was 0.57 mg/dL (6.4%) and ranged from 0.4 to 0.9 (4.3% to 10.2%). The MESOR for calcium for all individuals investigated was 9.28 ± 0.06 mg/dL (range, 8.94 to 9.85), the group amplitude being 0.92% (95% confidence limit, 0.41% to 1.44%). The acrophase, ie, crest, of the serum calcium rhythm was at 6:32 PM (95% confidence limit, 3:28 to 9:40 PM). Thus, serum calcium had a significant low-amplitude rhythm within its physiologic range, with an early morning low between 4:00 and 7:00 AM (Fig 4), a minor peak at 1:00 PM, and a major peak at 7:00 PM (Fig 4). The bathyphase, or lowest point, of the calcium rhythm in the circulation between 4:00 and 7:00 AM coincided with the peak values for the atrial peptides (Fig 3), suggesting an inverse temporal relationship of their circadian rhythms. This relationship is illustrated in Fig 5, wherein the superimposed patterns (using data as percent of the mean for comparison) for serum calcium and atrial natriuretic peptides at each time point are shown.

Circadian Rhythm of Serum Phosphate

Phosphate also exhibited a prominent, albeit low-amplitude, circadian rhythm that was significant at *P* less than .001 (Fig 4). The mean ROC within 24 hours was 1.25 mg/dL (42%) and ranged from 0.6 to 2.5 (18% to 104%). The mean MESOR for phosphate for all the individuals investigated was 3.74 ± 0.07 mg/dL (range, 3.11 to 4.32), with the group amplitude being 7.4% (Table 2). The waveform for phosphate showed a minor peak at 1:00 PM and a major peak between 10:00 PM and 1:00 AM. The acrophase for phosphate was at 10:12 PM (95% confidence limit, 7:52 PM to 12:16 AM). The bathyphase of the phosphate rhythm in the circulation (Fig 4) coincided with the peak values for each of the atrial natriuretic peptides (Fig 3), indicating an inverse temporal relationship of their circadian rhythms. This relationship is illustrated in Fig 5, wherein the superimposed patterns (using data as percent

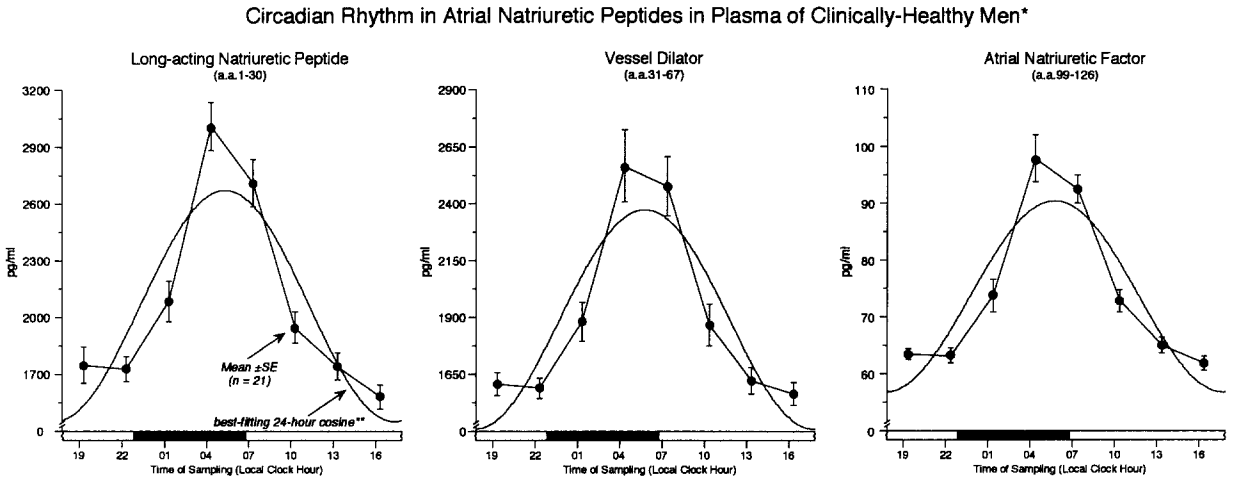

Fig 3. Circadian rhythms of LANP, VSDL, and ANF in the circulation of normocalcemic men. A circadian time effect was significant at $P < .001$ for LANP, VSDL, and ANF by ANOVA and the least-squares fit of a 24-hour cosine (shown). Highest values are found during sleep.

of the mean for comparison) for serum phosphate and each of the peptides are compared.

Correlation of Serum Calcium and Phosphate with Atrial Natriuretic Peptides Throughout the 24-Hour Period

Correlation analyses between individual values for serum calcium, phosphate, and atrial natriuretic peptides by simple linear regression resulted in a significant inverse correlation for calcium and LANP ($P = .039$) and significant inverse correlations between serum phosphate and VSDL ($P = .003$), ANF ($P = .022$), and LANP ($P = .028$; Table 3). These correlations between plasma atrial peptides and calcium and phosphate throughout the 24 hours suggest functional and temporal relationships (Table 3).

DISCUSSION

In the present investigation, LANP, VSDL, and ANF had significant circadian variations that were similar, with an approximate doubling at 4:00 AM compared with the values 12 hours distant at 4:00 PM. Figure 2 illustrates rhythm parameters derived from the single-cosinor method used in the description of rhythm characteristics to help define whether a rhythm is significant. According to the cosinor method, which takes into consideration the period, MESOR (ie, rhythm-adjusted mean), amplitude, and acrophase of a rhythm, it was found that each of the atrial peptides have significant circadian rhythms. These data compliment and confirm the nearly identical rhythms of atrial natriuretic peptides observed in 20-year-old women and men[18] and middle-aged men.[19] In addition to the similar circadian rhythms in women and men, it has been found that when medical staff were evaluated over a 24-hour period in which half of the staff were awake and working all night while the other half were able to sleep in the hospital, they have similar circadian rhythms of these peptides.[18] Thus, the fact that the peak was at 4:00 AM for each of these atrial natriuretic peptides in both upright and supine subjects indicates that although posture has an effect on circulating atrial natriuretic peptides, there is also an intrinsic 24-hour circadian rhythm in persons who perform their normal daily routines that cannot be ascribed to positional change.[18]

Table 2. Statistical Evaluation of Circadian Characteristics for Plasma Atrial Natriuretic Peptides and Serum Calcium and Phosphate in Healthy Humans

		ANOVA*				Population Mean Cosinor Summary (period = 24.0 hours; acrophase (∅) reference = 12 midnight)				
		Original Units		% of Mean						
Variable	No. of Data	F	P	F	P	No. of Series	P	MESOR ± SE	%Amp (95% confidence limits)	∅ (95% confidence limits)
LANP (pg/mL)	168	29.9	<.001	52.0	<.001	21	<.001	2,062 ± 59	29.8 (23.2, 36.5)	5:28 AM (4:36 AM, 06:12 AM)
VSDL (pg/mL)	168	19.3	<.001	43.4	<.001	21	<.001	1,891 ± 67	24.6 (18.1, 31.1)	5:52 AM (05:00 AM, 6:36 AM)
ANF (pg/mL)	168	37.7	<.001	62.7	<.001	21	<.001	73.6 ± 1.3	22.6 (18.5, 26.8)	5:52 AM (05:12 AM, 06:28 AM)
Ca (mg/dL)	168	1.7	.115	5.3	<.001	21	.001	9.28 ± 0.06	0.92 (0.41, 1.44)	6:32 PM (3:28 PM, 9:40 PM)
P (mg/dL)	168	9.2	<.001	19.3	<.001	21	<.001	3.74 ± 0.07	7.41 (5.16, 9.96)	10:12 PM (7:52 PM, 12:16 AM)

NOTE. Results are from 21 series in 14 men aged 41 to 72 years sampled every 3 hours for 24 hours in May 1988 and/or May 1993 (7 men studied twice).

*Analyses for time effect: ANOVA, across 8 time points using all data in original units and as % of mean; cosinor, summary of individual 24-hour rhythm characteristics by population mean cosinor using amplitude (Amp) as % of MESOR (24-hour rhythm-adjusted mean); acrophase (∅) in h/min from reference, local midnight. 95% limits for Amp and ∅ given if $P \leq .05$ from cosinor analysis.

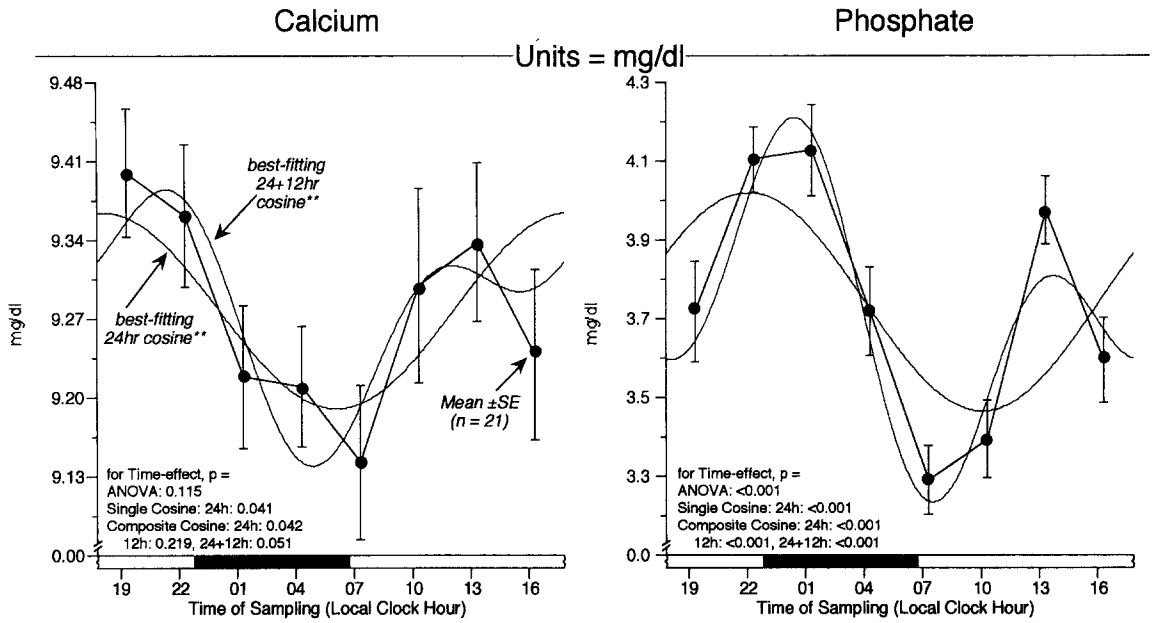

Fig 4. Circadian rhythm of serum calcium and phosphate in healthy humans. Samples taken at the same time points as in Fig 3 showed a circadian time effect that was significant at $P < .001$ for calcium and phosphate when evaluated by ANOVA and at $P = .041$ for calcium and $P < .001$ for phosphate from the least-squares fit of a 24-hour cosine (shown). Both calcium and phosphate had a circadian rhythm, but the waveform for both could be more accurately described by addition of a 12-hour (ultradian) component to the cosine model (shown as the lighter curve).

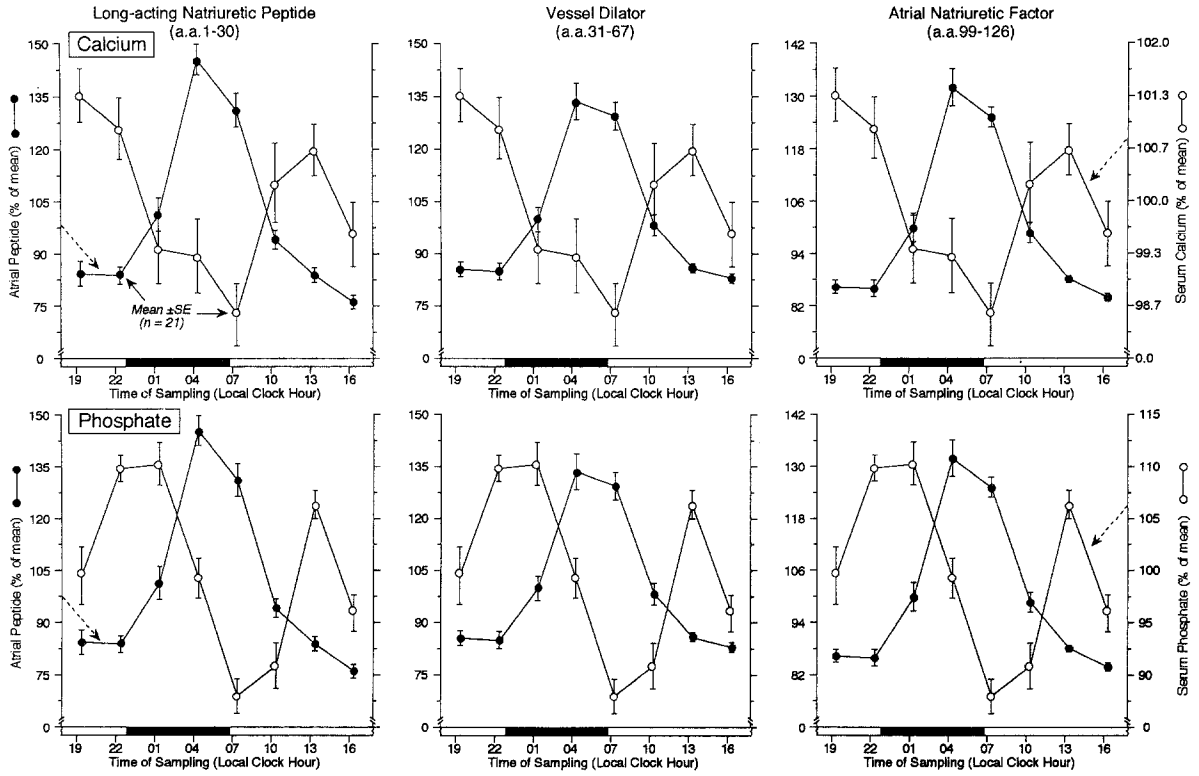

Fig 5. Temporal relationships between circadian rhythms of atrial natriuretic peptides and minerals (calcium and phosphate) in the circulation. Both serum calcium and phosphate had lowest values in the 24-hour period following the peak of each of the respective atrial natriuretic peptides. To compare each variable (calcium, phosphate, LANP, VSDL, and ANF) on the same graph, the concentrations at each time point are expressed as % of the mean.

Table 3. Correlations Between Plasma Atrial Natriuretic Peptides and Serum Calcium and Phosphate Imply Temporal and Functional Relationships

		Atrial Natriuretic Peptides					
		LANP		VSDL		ANF	
Mineral	No.	*r*	*P*	*r*	*P*	*r*	*P*
Ca	168	−.16	.039	−.08	.298	−.08	.285
P	168	−.17	.028	−.23	.003	−.18	.022

NOTE. Individual values for Ca and P were positively correlated ($r = .33, P = .0001$).

Serum calcium also had significant circadian variation (Fig 4). The rhythm for serum calcium showed that its nadir was nearly opposite the peak of the atrial natriuretic peptides (Fig 5), suggesting a temporal relationship. Thus, when LANP, VSDL, and ANF were highest in the circulation, serum calcium was lowest. In addition, the correlation of each individual calcium value throughout the 24-hour period with LANP ($P = .039$) implies a functional relationship between LANP and overall calcium regulation. Previous studies have demonstrated that ANF does affect free calcium concentration within the cells,[8,9] which, in part, appears to be due to its enhancing calcium excretion from the cell.[7,10] The present investigation would suggest that atrial peptides may be important for maintaining calcium within a narrow range in healthy humans. Parathyroid hormone (PTH), known to be important in calcium balance, has a circadian rhythm nearly identical to that of atrial natriuretic peptides, with the PTH peak concentration being at 4:00 AM also.[20] These circadian studies would suggest that the regulation of calcium within its narrow normal range is more intricate than previously thought, with atrial peptides helping PTH maintain this balance by their effects on intracellular calcium metabolism. In addition to the nadir in serum calcium (which correlated with the respective atrial peptides' peak concentrations in the circulation), serum calcium appeared to have two peaks (at 1:00 and 7:00 PM). Since these peaks were distant in time from the peak release of the atrial peptides, it would appear from the present study that the circadian variation of serum calcium does not regulate the amount of atrial peptides released into the circulation under physiological conditions, even though oral and intravenous calcium can increase ANF levels.[5] The stimulus to PTH release is, on the other hand, hypocalcemia rather than increasing calcium concentrations, and the data from the present investigation in which serum calcium was found to be the lowest between 4:00 and 7:00 AM are compatible with low calcium's being important for release of PTH, with an increased calcium known to inhibit PTH release.[20]

With respect to PTH and atrial natriuretic peptides having nearly identical circadian rhythms, there is some evidence to suggest that PTH may release atrial natriuretic peptides.[2,26,27] Thus, in parathyroidectomized rats that were hypocalcemic, ANF in response to a saline load was only one third of the level in rats with intact parathyroid glands.[26] When oral calcium supplementation was added to render the parathyroidectomized rats normocalcemic, the ANF response from volume expansion increased from 271 to 402 pg/mL but did not equal the response (702 pg/mL) observed in rats with intact parathyroids, suggesting that PTH is important in modulating the release of ANF in response to a saline load.[26] Further, several investigations have shown that with removal of the parathyroid glands, the circulating level of ANF significantly decreases.[3,27] The present investigation is compatible with a PTH influence on the release of atrial natriuretic peptides, since each of the atrial peptides increased at the identical time points that PTH increases in its circadian rhythm.[20,21]

The nocturnal increase in PTH has been associated with bone resorption, since urinary deoxypyridinoline excretion, a sensitive marker of bone resorption, increases 48% at night.[28] The demonstration that atrial peptides increase at the same time as PTH would likewise suggest a possible role for atrial peptides in bone resorption. Prostaglandin E_2 is one mediator of bone resorption,[29] and both LANP and VSDL are potent stimulators of prostaglandin E_2 production.[30,31] Their circadian increase in the middle of the night, which would stimulate prostaglandin E_2 synthesis, could mediate the increased bone resorption in the middle of the night. Further support that LANP and VSDL rather than PTH itself may be important for the circadian pattern of bone resorption comes from a study where neutralizing the effect of PTH did not affect the unknown substance(s) in serum with bone resorption activity.[32] The circadian rhythm of this substance(s) was highest in serum at 3:00 AM[32] which is nearly identical to the circadian peak of LANP and VSDL. ANF does not increase prostaglandin E_2,[29-31] and therefore would not cause bone resorption. ANF, on the other hand, partially inhibits bone resorption stimulated by prostaglandin E_2.[29] ANF has been found to have specific receptors on osteoblasts and to increase cyclic GMP in osteoblastic cultures,[33] suggesting that it may enhance new bone formation as well. Serum osteocalcin (also called bone Gla protein) is secreted by osteoblasts and used as a marker of bone formation.[29] Osteocalcin increases in the middle of the night[29] when ANF does. Urinary calcium excretion, which increases in the middle of the night in a circadian fashion, has been examined using 2% saline infusion, which caused an increase in urinary calcium excretion with a simultaneous increase in ANF but no change in plasma PTH levels, suggesting that PTH does not mediate this urinary calcium excretion.[34] Infusion of 2% saline simultaneously increases each of the atrial natriuretic peptides,[22] suggesting that they may be important for the increased calcium excretion in addition to their well-documented increase in sodium excretion.[15,17] These studies, taken together, would suggest a unifying hypothesis that atrial natriuretic peptides may help to regulate bone resorption, with LANP and VSDL stimulating bone resorption and ANF modifying the extent of this resorption of bone and possibly enhancing new bone formation at the same time.

Serum phosphate had a circadian rhythm similar to the rhythm of calcium in the circulation, suggesting that the rhythms may be linked. The individual values for serum

phosphate correlated best with VSDL ($P = .003$), but LANP ($P = .028$) and ANF ($P = .022$) also had significant 24-hour correlations with serum phosphate. These correlations suggest that VSDL and, to a lesser extent, ANF and LANP have a functional relationship with phosphate regulation. The peaks of phosphate in the serum, on the other hand, were distant in time from the peak release of atrial peptides into the circulation, suggesting that phosphate is probably not an important physiologic regulator of the release of atrial natriuretic peptides.

The present circadian studies of calcium, phosphate, and atrial natriuretic peptides also demonstrate that the time of day (circadian stage) of sampling is important for defining what is "normal." When blood samples are obtained at 7:00 AM (ie, near the time [8:00 AM] when most normal ranges are determined), calcium is a mean of 0.26 mg/dL (2.8%) and phosphate 0.84 mg/dL (21.9%) lower than their peak concentrations (at 7:00 PM for calcium and between 10:00 PM and 1:00 AM for phosphate) in the same individuals (Fig 4). Each of the atrial natriuretic peptide concentrations were almost twice as high at 4:00 AM as at 4:00 PM. Thus, in defining the normal circulating concentrations of atrial natriuretic peptides, calcium, or phosphate, the present investigation demonstrates the importance of specifying the time of day of sampling. This is especially true since most investigations in biology and clinical laboratory measurements rely on single samples.

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