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SHORT COMMUNICATIONS

Circadian rhythm in peripheral type benzodiazepine binding sites in human platelets

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Benzodiazepine binding sites which mediate the pharmacological effects of such medications in the brain, have also been found in peripheral tissues [1, 2]. Such peripheral binding sites can be differentiated from central ones by the relative displacing potencies of agents such as RO5-4864, diazepam and clonazepam [3, 4]. PK 11195, a potent and selective ligand for peripheral benzodiazepine binding sites was recently characterized as chemically unrelated to benzodiazepines [5, 6]. Thermodynamic analysis of the binding properties of this compound [7] as well as electrophysiologic studies [8, 9] have led us to consider PK 11195 as an antagonist at the peripheral level and RO5-4864 as an agonist [10, 11]. Peripheral benzodiazepine binding sites have recently been characterized in human and rat platelets by using ^{3}H-PK 11195 as a ligand [12]. Since extensive work in rats [13–16] and limited data in humans [17] have documented circadian rhythms in the number of binding sites for several specific agents, we examined whether this would also be the case for peripheral benzodiazepine binding sites receptors in the membrane of human platelets. Platelets have a very high density of binding sites for benzodiazepines on their membrane [10]. Moreover, they are easily obtainable from human beings, and are easily separable.

Subjects and methods

Seven healthy young adults, aged 21–39 years (median: 31 years) volunteered for this study. Three of them were heavy smokers (>20 cigarettes per day), with minimal smoking during the study (<5 cigarettes). All were synchronized with a diurnal activity from 0730 until 2300 hr. During the study meals were taken at 0730, 1300 and 2000 hr. None of the subjects were taking any medicine or alcoholic beverage before and during the study. Venous blood (total of 45 ml) was sampled from each subject at 0600, 1200, 1800 and 2400 hr on the same day. All subjects were studied in February 1984. Thirty millilitres of blood were gently aspirated on sodium citrate buffer as described by Rossi [18] for the determination of platelet binding sites. Fifteen millilitres of blood were collected on EDTA: 3 ml were processed for automatic determinations of platelet count (Ortho ELT8) and 12 ml were centrifuged for plasma separation, which was frozen for subsequent electrolyte and hormonal determinations. Platelets were prepared according to Rossi [18], and resuspended in Tris buffered saline (140 mM NaCl, 5 mM KCl, 5 mM glucose, 0.38 mM sodium citrate and 25 mM Tris–HCl at pH 7.4). Platelet membrane fraction was prepared by suspending the platelet pellet in Tris–HCl (pH = 7.4) at 10^7 cells/ml, followed by 20 sec ultrasonic disruption in a Branson sonifier-cell disruptor B-15 set at power 7. Homogenates were then centrifuged at 46,000 *g* for 10 min and the pellet washed twice with 50 mM Tris–HCl buffer (pH = 7.4). Protein content was measured by the method of Lowry *et al.* [19]. Binding experiments were carried out in 50 mM Tris–HCl (pH = 7.4) buffer. The platelet membrane fraction (10 μg/ml) was incubated in the presence of ^{3}H-PK 11195 for 15 min at 25°. Binding was stopped by vacuum filtration through GF/C filters which were subsequently washed four times with 4 ml incubation buffer. Retained radioactivity was measured by liquid scintillation spectrometry.

Binding experiments were carried out in triplicate and non-specific binding was defined by the radioactivity bound in the presence of 10^{-5} M of RO5-4864. Binding parameters were computed from data at six different concentrations of ^{3}H-PK 11195 (60, 30, 15, 7, 3, and 1 nM) by a linear transformation [20].

Data were analysed by conventional analysis of variance and by cosinor [21]. Because of marked interindividual differences in 24-hr mean values, analyses were performed both on raw data and on data expressed as percentages of each subject's 24-hr mean.

A rhythm was characterized by parameters of the best fitting cosine function approximating all data with a period, $\tau = 24$ hr. The rhythm characteristics estimated by this linear least squares method include the mesor (M; rhythm-adjusted mean), the double-amplitude (2A; difference between minimum and maximum of fitted cosine function) and the acrophase (ϕ; time of maximum in fitted cosine function, with local midnight as ϕ reference). They are given with their 95% confidence limits. A circadian rhythm was detected when A differed from zero (non-null amplitude test) with $P < 0.05$.

Results and discussion

Peripheral benzodiazepine binding sites were found in platelet membranes from all subjects and at all time-points. Such sites demonstrated a high affinity (K_D) for ^{3}H-PK 11195 (group 24-hr mean ± 1 S.E.M.: 3.4 ± 0.2 nM) and a high capacity (B_{max}, 4.1 ± 0.1 pmol/mg protein). Nonetheless large interindividual differences in 24-hr mean values of B_{max}, K_D and platelet count were documented and statistically validated (Table 1). A circadian rhythm was found and statistically validated for both B_{max} and platelet count, but not for K_D (Fig. 1, Table 2). The maximum of the binding capacity was estimated to occur at 0350 hr, and the difference between maximum values (double-amplitude) was ~20% of the 24-hr mean. Circadian changes in binding capacity were not related to either platelet count (differences in peak times of respective circadian rhythms) or the affinity (no circadian rhythm in K_D). As a result, the circadian change in the binding capacity most likely reflects that in the number of binding site per platelet. The present results demonstrate that the expression of benzodiazepine

Table 1. Interindividual differences in 24-hr means of B_{max}, K_D and platelet count

Subject	B_{max} (fM/mg protein)	K_D (nM)	Platelet number ($\times 10^3$ el/mm^3)
1	3980 ± 342*	3.19 ± 0.35	277 ± 4
2	4653 ± 152	5.00 ± 0.78	349 ± 6
3	3808 ± 110	2.57 ± 0.02	198 ± 12
4	4062 ± 62	3.98 ± 0.13	249 ± 3
5	3194 ± 195	2.68 ± 0.02	190 ± 8
6	4664 ± 29	3.79 ± 0.15	255 ± 17
7	4402 ± 453	2.53 ± 0.18	234 ± 2
1–7	4112 ± 131	3.39 ± 0.21	250 ± 11

* 24-hr mean ± S.E.M.

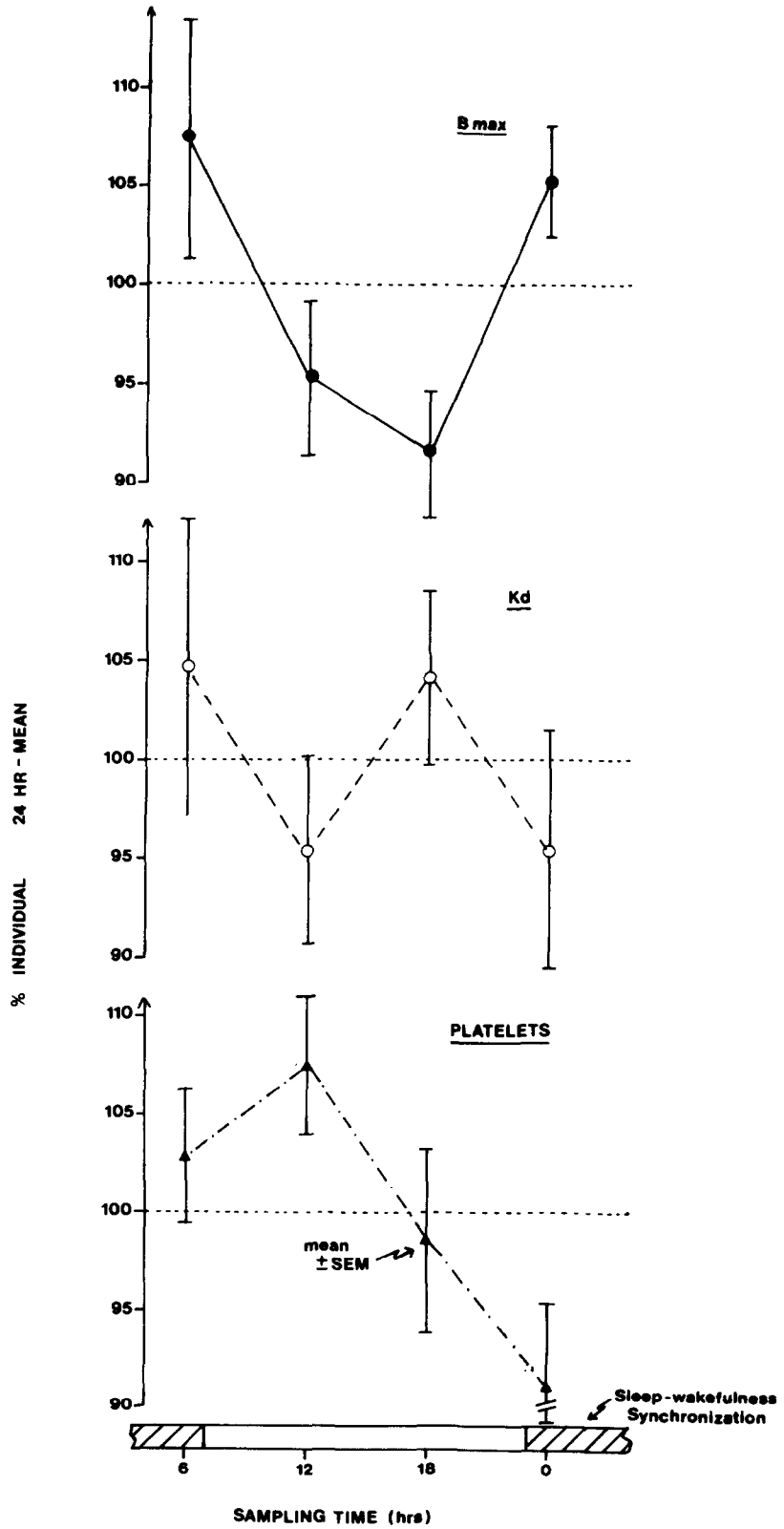

Fig. 1. Circadian rhythm in the number of binding sites for PK 111. Group 24-hr mean ± S.E.M., respectively equals 4112 ± 131 fmol/mg protein for B_{max}, 3.39 ± 0.21 nM for K_D and 250.10 ± 11.10 elements/mm^3 for the platelet count. Because of large interindividual variations in 24-hr means (see Table 1), time-qualified values are expressed as percentages of the individual 24-hr mean.

Table 2. Circadian variation in B_{max}, K_D and platelet count. Results from cosinor analysis with a period $\tau = 24$ hr, and data expressed as % of individual 24-hr mean (M) in order to minimize interindividual differences in 24-hr means

Variable	No. of data	P*	Double-amplitude (% M)†	Acrophase (hr., min)‡ (95% confidence limits)
B_{max}	28	0.01	19 (3; 34)	0350 (0015; 0800)
K_D	28	0.99	—	—
Platelet number	28	0.003	17 (5; 29)	1100 (0800; 1410)

* P-value from an F-test of the rejection of the null-amplitude hypothesis.
† Difference between values at maximum and minimum in fitted cosine function with $\tau = 24$ hr.
‡ Location of the maximum in fitted cosine function, referred to midnight.

binding sites in human platelets exhibits a circadian rhythm in Man. Such a predictable variation along the 24-hr time scale must be taken into account in further investigations of the physiological role and regulation of such peripheral benzodiazepine binding sites.

In summary, a circadian rhythm in peripheral type benzodiazepine binding sites is described and statistically validated in platelets from peripheral blood of healthy human volunteers. Maximum values in B_{max} are observed near the middle of the night and the peak–trough difference equals 20% of the 24-hr mean.

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REFERENCES

1. C. Baestrup and R. F. Squires, *Proc. natn. Acad. Sci. U.S.A.* 3805 (1977).
2. J. W. Regan, H. J. Yamamura, S. Yamada and W. R. Roeske, *Life Sci.* **30**, 991, (1981).
3. H. Schoemaker, R. G. Boles, W. O. Horst and H. I. Yamamura, *J. Pharmac. exp. Ther.* **225**, 61 (1983).
4. J. K. T. Wang, T. Taniguchi and S. Spector, *Molec. Pharmac.* **25**, 349 (1984).
5. G. Le Fur, M. L. Perrier, N. Vaucker, F. Imbault, A. Flamier, J. Benavides, A. Uzan, C. Renault, M. C. Dubroeucq, and C. Guerremy, *Life Sci.* **32**, 1839 (1983).
6. G. Le Fur, F. Guilloux, P. Rufat, J. Benavides, A. Uzan, C. Renault, M. C. Dubroeucq and C. Gueremy, *Life Sci.* **32**, 1849 (1983).
7. C. Le Fur, N. Vaucher, M. L. Perrier, A. Flamier, J. Benavides, C. Renault, M. C. Dubroeucq, C. Gueremy and A. Uzan, *Life Sci.* **33**, 449 (1983).
8. M. Mestre, T. Carriot, C. Belin, A. Uzan, C. Renault, M. C. Dubroeucq, C. Gueremy and G. Le Fur, *Life Sci.* **35**, 953 (1984).
9. M. Mestre, T. Carriot, C. Belin, A. Uzan, C. Renault, M. C. Dubroeucq, C. Guérémy, A. Doble and G. Le Fur, *Life Sci.* **36**, 391 (1985).
10. J. Benavides, F. Guilloux, D. E. Allam, A. Uzan, J. Mizoule, C. Renault, M. C. Dubrouecq, C. Gueremy and G. Le Fur, *Life Sci.* **34**, 2613 (1984).
11. J. Mizoule, A. Gauthier, A. Uzan, C. Renault, M. C. Dubroeucq, C. Gueremy and G. Le Fur, *Life Sci.* (in press).
12. J. Benavides, P. Quarteronet, P. F. Plouin, F. Imbault, T. Phan, A. Uzan, C. Renault, M. C. Dubrouecq, C. Gueremy and G. Le Fur, *Biochem. Pharmac.* **33**, 2467 (1984).
13. M. S. Kafka, A. Wirz-Justice and D. Naber, *Brain Res.* **207**, 409 (1981).
14. A. Wirz-Justice, I. Tobler, M. S. Kafka, D. Naber, P. Marangos, A. Borbelly and T. Wehr, *Psychiatry Res.* **5**, 67 (1981).
15. D. Naber, A. Wirz-Justice and M. S. Kafka, *Neurosci. Lett.* **2**, 45 (1981).
16. A. Wirz-Justice, K. Kräuchi, T. Morimasa, R. Willever and H. Feer, *Eur. J. Pharmac.* **87**, 331 (1983).
17. E. K. Perry, R. H. Perry and B. E. Tomlinson, *Neurosci. Lett.* **4**, 185 (1977).
18. E. C. Rossi, *J. Lab. clin. Med.* **78**, 483 (1971).
19. O. H. Lowry, N. J. Rosebrough, A. C. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
20. J. A. Zivin and D. R. Waud, *Life Sci.* **30**, 1407 (1982).
21. W. Nelson, Y. L. Tong and F. Halberg, *Chronobiologia* **6**, 305 (1979).

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Bile flow decrease and altered bile composition in streptozotocin-treated rats

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Streptozotocin (SZ), an antibiotic produced by *Streptomyces achromogenes*, is a potent inducer of diabetes in laboratory animals, attributable to the irreversible damage which it exerts, especially on the β-cells [1]. SZ distribution in tissues indicates the important role played by the liver in the metabolism and excretion of this compound [2]. Previous studies from this laboratory demonstrated that SZ administration to rats induced a transient impairment of hepatobiliary function [3]. In the present study, we investigated the effect of SZ treatment of rats on the bile-acid-dependent flow (BADF) and the bile-acid-independent flow (BAIF) of bile. In addition, the permeability of the biliary system to sucrose and the biliary secretion of some endogenous bile components were also investigated.