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Ouabain-Insensitive, Na^+-Stimulated ATPase of Several Rat Tissues: Activity during a 24 h Period

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Summary

Rhythmic daily changes in the Na,K-ATPase activity have been previously described for rat kidney cortex, showing two peaks: at 0900 h and 2100 h, and two valleys: at 1500 h and 0100 h - 0300 h. The oscillations in Na,K-ATPase activity are produced by an inhibitor, which binds the enzyme and is present in the rat blood plasma at valley times and absent or at very low concentrations at peak times. Since it has been demonstrated that active Na^+ extrusion from the cells of several tissues depends not only on the Na,K-ATPase but also on the ouabain-insensitive Na-ATPase, we studied the activity of this latter enzyme of several rat tissues, i.e., kidney cortex, small intestine, liver, heart and red blood cells along the day. None of these tissues showed any variation of their Na-ATPase activity along the day. Preincubation of kidney cortex homogenates obtained at 0900 h, with blood plasma drawn at 0900 h and 1500 h, did not modify the Na-ATPase activity. Our results indicate that the Na-ATPase activity does not oscillate along the day. These results are in agreement with the idea that the Na-ATPase could partially compensate the Na^+ transport affected by oscillations of the Na,K-ATPase activity.

Key words

Na,K-ATPase • Ouabain insensitive Na-ATPase • Circadian rhythm • Inhibitor

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Introduction

Rhythmicity is a widespread and fundamental aspect of life. Endocrine and metabolic rhythms in many tissues have been described to follow ultradian schedules, rhythms with a period of less than 24 h (Hastings *et al.* 2007). The mechanisms underlying the control of such rhythms remain only partially understood. Cell to cell communication has been proposed to explain the nature of ultradian intracellular rhythms (Brodsky 2006).

The Na,K-ATPase, is a very important membrane-bound enzyme, responsible for the active extrusion of Na^+ from the cells in exchange for K^+, in a ratio of 3 Na^+ for 2 K^+ for each ATP hydrolyzed. Consequently, its activity is essential to maintain the Na^+ and K^+ gradients between the cells and their environment. Daily modulation of the Na,K-ATPase activity in both humans and rats has been described (Morise *et al.* 1989, Wang and Huang 2004). On this regard, Segura *et al.* (2004) showed that the activity of the Na,K-ATPase activity from rat kidney cortex shows two peak values along the day: at 0900 h and 2100 h, and two valley values, at 1500 h and 0100 h - 0300 h. Incubations of 0900 h rat kidney cortex slice homogenates with blood plasma obtained at 1500 h, resulted in an important

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inhibition of the Na,K-ATPase activity, which was not seen when the incubations were carried out with 0900 h blood plasma. This was taken as an indication that these oscillations in Na,K-ATPase activity were due to the effect of an inhibitor present at maximal quantity in the blood plasma of the rats at the valley times, and absent or at very low concentrations at the peak times (Segura *et al.* 2004). Since the Na,K-ATPase plays an important role in the renal Na^+ reabsortion, Segura *et al.* (2004) proposed that the oscillations in the activity of this enzyme are associated with the circadian rhythms of urinary sodium excretion and urine flow rates shown to be present in rats (Min *et al.* 1966, Rabinowitz *et al.* 1986, Morise *et al.* 1988), as well as in humans (Mann *et al.* 1976). Further experiments showed that the Na,K-ATPase activity from other tissues, such as small intestine, liver, heart and red blood cells followed the same rhythm shown to be present in the kidney cortex (Proverbio *et al.* 2004).

On the other hand, the ouabain-insensitive, Na^+-stimulated ATPase, also known as Na-ATPase, has been shown to extrude actively Na^+ from the cells accompanied by Cl^- and water in different tissues of many organisms (Caruso-Neves *et al.* 1997, de Almeida-Amaral *et al.* 2008, De Souza *et al.* 2007, Lara *et al.* 2006, Moretti *et al.* 1991, Rangel *et al.* 1999, Whittembury and Proverbio 1970). The fact that the Na-ATPase extrudes Na^+ from the cells accompanied by Cl^- and water, and that its activity is modulated by the cell volume has been utilized to propose an important role of this enzyme in the active regulation of the cell volume (Proverbio *et al.* 1988).

Considering the importance of the regulation of the intracellular Na^+, it becomes very interesting to study the possibility of any variation of the ouabain-insensitive Na-ATPase activity along the day and, if there is any, to determine its pattern of oscillation.

Methods

Experimental animals

Healthy male rats of the Sprague-Dawley strain (body weight 200-300 g, 3 months old) were anesthetized with diethylether and immediately decapitated at the indicated times.

Preparation and homogenization of intestinal scraps and kidney cortex, liver and heart ventricle

The different tissues were removed and collected in a medium containing (mM): sucrose, 250; Tris-HCl (pH 7.2), 20; dithiothreitol (DTT) 0.5; phenylmethyl-sulfonyl fluoride 0.2 (sucrose/Tris/DTT/PMSF solution) at 4 °C. Then, slices of the different tissues and scrapings of the small intestine were weighted and homogenized at 4 °C after adding three volumes of the sucrose/Tris/DTT/PMSF solution. The homogenates were filtered through gauze filters and stored at –70 °C until use.

Preparation of red blood cell ghosts

Blood samples were drawn from rats either at 0900 h or at 1500 h directly by ventricle puncture and collected in heparinized tubes. Blood plasma was removed and kept at –70 °C for further assays. Red blood cell ghosts were prepared as indicated elsewhere (Moretti *et al.* 1991).

SDS pretreatment of the homogenates

In order to avoid the presence of membrane vesicles, before any assay all the samples were pretreated with SDS/BSA/imidazole as previously described (Marín *et al.* 1986, Proverbio *et al.* 1986). In brief, 250 µl of the homogenates (10 mg protein/ml, approx.) were mixed with a solution containing the required amount of SDS, 1 % BSA, 25 mM imidazole pH 7.2 at 37 °C. The optimal SDS/protein ratio was around 0.4 µg SDS/µg protein for the Na-ATPase and around 1.6 µg SDS/µg protein for the Na,K-ATPase. The homogenates were incubated for 20 min at 37 °C, immediately diluted with a solution of 250 mM sucrose, 20 mM Tris-HCl (pH 7.2, 4 °C) to a protein concentration of 0.1-0.2 mg/ml and then used for blood plasma incubations and/or ATPase assays.

Blood plasma preincubations

The preincubations with blood plasma were carried out as follows: 25 µl of either crude homogenates previously treated with SDS or red blood cell ghosts were mixed with 250 µl of plasma (obtained at different times) and $MgCl_2$ to a final concentration of 5 mM, and incubated for 30 min at 37 °C. The whole suspension was diluted by adding the required amount of sucrose/Tris/DTT/PMSF medium, in order to obtain a protein concentration of about 0.2 mg/ml and the ATPase activity was determined immediately.

ATPase assays

Na-ATPase and Na,K-ATPase assays were carried out as previously described (Moretti *et al.* 1991, Proverbio *et al.* 1986). The Na-ATPase activity was assayed as follows. A 180 µl aliquot of the incubation

Table 1. Effect of 5 mM ouabain or 2 mM furosemide on the Na-ATPase and Na,K-ATPase activities of rat kidney cortex homogenates obtained at 0900 h.

Incubation medium	ATPase activity (nmoles Pi / mg protein . min)		
		Additions	
	None	5 mM ouabain	2 mM furosemide
(a) Mg^{2+}	423 ± 14	432 ± 15	402 ± 15
(b) $Mg^{2+}+Na^{+}$	442 ± 12	452 ± 15	403 ± 14
(c) $Mg^{2+}+Na^{+}+K^{+}$	528 ± 18	455 ± 16	476 ± 18
(b-a) Na-ATPase	19 ± 1	20 ± 1*	1 ± 1**
(c-b) Na,K-ATPase	86 ± 4	3 ± 2**	73 ± 4*

The values represent means ± S.E.M. for eight different animals. The Na-ATPase and the Na,K-ATPase activities were calculated by paired data. * p=n.s. vs ATPase activity without any addition, ** p<0.001 vs ATPase activity without any addition.

medium containing (final concentrations): 50 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 2 mM Tris-ATP and 5 mM ouabain, with and without 100 mM NaCl, was preincubated at 37 °C for 2 min. The reaction was started by addition of 20 µl of the homogenates (0.5 mg protein/ml) previously treated with SDS. After 10 min incubation, the reaction was arrested by adding 300 µl of a solution containing (final concentrations): 2.8 % SDS, 0.48 % molybdic acid, 2.8 % ascorbic acid and 0.48 N hydrochloric acid. The test tubes were placed on ice for 10 min, and then mixed with 500 µl of a solution containing (final concentrations), 2 % sodium arsenite; 2 % sodium citrate and 2 % acetic acid. The tubes were rewarmed at 37 °C for 10 min, and the developed color was measured in a Sunrise (Tecan) spectrophotometer at 705 nm. All samples were run in quadruplicate.

The Na-ATPase activity was calculated as the difference between the amount of inorganic phosphate liberated in the presence of Mg^{2+} + Na^+ + 5 mM ouabain minus that liberated in the presence of Mg^{2+} + 5 mM ouabain. The Na,K-ATPase activity was determined by using a similar protocol but with an incubation medium containing (final concentrations): 50 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl and 2 mM Tris-ATP, with and without 5 mM ouabain. The Na,K-ATPase activity was calculated as the difference between the amount of inorganic phosphate liberated in the presence of Mg^{2+} + Na^+ + K^+ minus that liberated in the same medium in the presence of 5 mM ouabain. Activity was expressed as nmoles of P_i liberated per mg of protein per min, after subtraction of a blank run in parallel without the membrane suspension, which was added after the reaction was stopped. In all the cases, the protein content of the samples was estimated according to the Bio-Rad micromethod assay based on Bradford's reaction (Bradford 1976).

Statistical analysis

All the results are expressed as mean ± S.E.M. and (n) represents the number of experiments performed with the different samples. In all the cases, Na-ATPase and Na,K-ATPase activities were calculated by paired data. For the Biological Rhythm Analysis, mean (mesor), amplitude, acrophase and circadian period of Na-ATPase activities during a 24-h period was determined by X^2-periodogram procedure (Refinetti *et al.* 2007, Sokolove and Bushell 1978). The statistical significance was set at $p<0.05$ with 95 % CI. All the other statistical analyses were performed by the Student's *t*-test. $p<0.05$ was considered the level of statistical significance.

Results and Discussion

Table 1 shows the effect of 5 mM ouabain and 2 mM furosemide on the Na-ATPase and the Na,K-ATPase activities of rat kidney cortex homogenates. While ouabain inhibits specifically the Na,K-ATPase activity, furosemide inhibits preferentially the Na-ATPase activity. Similar results were found for small intestine, liver, heart and red blood cells (data not shown).

Figure 1 shows the Na-ATPase activity of kidney cortex homogenates from rats maintained on a schedule of 12 h light - 12 h darkness for 2 weeks. Rats

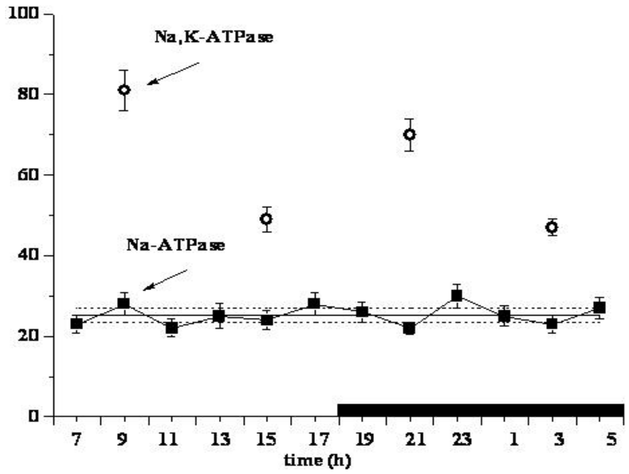

Fig. 1. Enzyme activity over time of Na-ATPase and Na,K-ATPase in homogenates of kidney cortex slices from rats maintained for 2 weeks under the following light-darkness schedule: 12 h light (between 0600-1800 h, 80 lux) and 12 h darkness (between 1800-0600 h). The horizontal black bar represents the darkness period. Before assay, the homogenates were pretreated with SDS as indicated in Methods, at a protein concentration of approximately 10 mg/ml. After treatment, the homogenates were diluted with a cold 250 mM sucrose, 20 mM Tris-HCl (pH 7.2, 4 °C) solution, to a protein concentration of 0.1-0.2 mg/ml and then assayed for Na-ATPase and Na,K-ATPase activity. The values represent means ± S.E.M. for six different animals.

were killed every 2 h, until completion of a 24 h period and kidney cortex homogenates were prepared as indicated. This figure also shows, as a control, the peak values (0900 h and 2100 h) and valley values (1500 h and 0300 h) of the Na,K-ATPase activity of the kidney cortex homogenates, which are in total agreement with previous findings (Segura *et al.* 2004). On the other hand, it can be seen that the Na-ATPase activity does not show a significant oscillation along the 24-h period. In fact, the Biological Rhythm Analysis (Refinetti *et al.* 2007, Sokolove and Bushell 1978), showed that the mean (mesor) of all the activity values during the 24 h period was 25.25 nmoles Pi/mg protein . min. However, the amplitude value of 4.0 nmoles Pi/mg protein . min and acrophase at 15.6 hours (12.93 to 18.27 hours; 95 % CI) were not significant (goodness of fit: 0.679; $p>0.05$). X^2-periodogram shows the best, but not significant, Qp value of 5 at 9.0 hours (d.f.=3.5; $p>0.05$). Similar results were obtained when the Na-ATPase activity was calculated as the difference in activity in the presence and absence of 2 mM furosemide (Moretti *et al.* 1991), which inhibits 100 % of the Na-ATPase activity (data not shown).

The oscillations of the Na,K-ATPase activity shown by Segura *et al.* (2004) in the rat kidney cortex are not exclusive for this tissue, since there are similar oscillations in the heart ventricle, small intestine, liver, and red blood cells (Proverbio *et al.* 2004). Consequently, we studied the Na-ATPase activity of preparations from these tissues either at 0900 h or 1500 h, peak and valley times for the Na,K-ATPase activity (Fig. 1). As shown in Table 2, while the Na,K-ATPase activity of the different tested tissues shows a significant reduction at 1500 h when compared with that at 0900 h, the Na-ATPase activity does not show, for all the studied tissues, any change between 0900 h and 1500 h.

The oscillations of the Na,K-ATPase activity are due to the presence of a putative inhibitor that is circulating in the blood plasma (Segura *et al.* 2004). Therefore, it is important to determine if it has any effect on the activity of the Na-ATPase. To test this possibility, homogenates of rat kidney cortex obtained at 0900 h were preincubated with blood plasma obtained at 0900 h and 1500 h, and then assayed for Na-ATPase and Na,K-ATPase activities. The preincubations were carried-out in the presence of 5 mM $MgCl_2$ since, at least for the Na,K-ATPase, it is required for the binding of the inhibitor to the plasma membranes (Segura *et al.* 2004). As previously shown (Segura *et al.* 2004), preincubation of the rat kidney cortex homogenates with the 1500 h blood plasma produced a significant diminution of the Na,K-ATPase activity, which was not seen when the preincubation was carried out with the 0900 h blood plasma (Fig. 2). On the other hand, preincubation of the homogenates either with the 1500 h or the 0900 h blood plasma did not produce any change in the activity of the Na-ATPase.

The putative inhibitor of the Na,K-ATPase responsible for the daily oscillations of the activity of this enzyme can be released from the membranes if the homogenates are preincubated at 37 °C in the absence or in the presence of low concentrations of K^+ (below 4 mM) (Segura *et al.* 2004). To avoid this possibility and considering the fact that the Na-ATPase is insensitive to K^+ (Moretti *et al.* 1991), we assayed the Na-ATPase activity of rat cortex kidney homogenates along the day, in the presence of 20 mM K^+ and 5 mM ouabain (to inhibit any activity of the Na,K-ATPase). The values of the Na-ATPase along the day, measured under these conditions, came out to be quite similar to those determined in the absence of K^+ in the assay medium (data not shown). The Biological Rhythm Analysis (Refinetti *et al.* 2007, Sokolove and Bushell 1978) of these data, was similar to the one obtained for the data from Fig. 1.

Table 2. Na-ATPase and Na,K ATPase activities of homogenates from kidney, small intestine, liver, heart ventricle and red blood cell ghosts from samples obtained at 0900 h and 1500 h.

Preparations	ATPase activity (nmoles Pi/mg protein . min)					
	Na-ATPase			Na,K-ATPase		
	0900 h	1500 h	p	0900 h	1500 h	p
Kidney cortex	21 ± 1	22 ± 2	ns	82 ± 2	50 ± 2	<0.001
Liver	18 ± 1	16 ± 1	ns	29 ± 2	15 ± 0.7	<0.001
Heart ventricle	12 ± 1	11 ± 1	ns	24 ± 1	12 ± 0.7	<0.001
Small Intestine	21 ± 2	22 ± 1	ns	53 ± 1	29 ± 1	<0.001
Red blood cell	1.9 ± 0.1	1.8 ± 0.1	ns	16 ± 0.4	9.9 ± 0.3	<0.001

The values represent means ± S.E.M. for eight different animals.

The presented results clearly indicate that in contrast to the ouabain-sensitive Na,K-ATPase, the activity of which shows ultradian oscillations along the day, the ouabain-insensitive Na-ATPase from several tissues of the rat does not show any variation in a 24-h period and, besides, it is not inhibited by preincubation of the tissues with aliquots of blood plasma containing the inhibitor of the Na,K-ATPase (Figs 1 and 2, Table 2).

There are several reports showing modulatory effects of different agents on the ouabain-insensitive Na-ATPase activity in kidney proximal tubules, e.g. adenosine (Caruso-Neves *et al.* 1997), angiotensin II (Rangel *et al.* 1999) and angiotensin(1-7) (Lara *et al.* 2006). These effects have been proposed to be mediated through the different cell receptors. Since these agents could oscillate along the day, the possibility of oscillations of the Na-ATPase activity seemed very likely. Our results showed that the Na-ATPase activity does not oscillate in a 24-h period. Therefore the above-mentioned modulators of the Na-ATPase activity are not exerting their effects in a time-dependent manner. The modulation of the Na-ATPase activity may follow the physiological requirements of the cells. Once these requirements are satisfied, the Na-ATPase activity would return to normal values.

Considering that the requirements of energy for the Na,K-ATPase activity are quite high, it has been suggested that the oscillations of the activity of this enzyme along the day, could result in an important reduction in the energy consumption of the organism (Proverbio *et al.* 2004). The fact that the Na-ATPase activity does not oscillate along the day, could be considered of importance for the functioning of the cells as well as for the different tissues and organs. Thus, it is

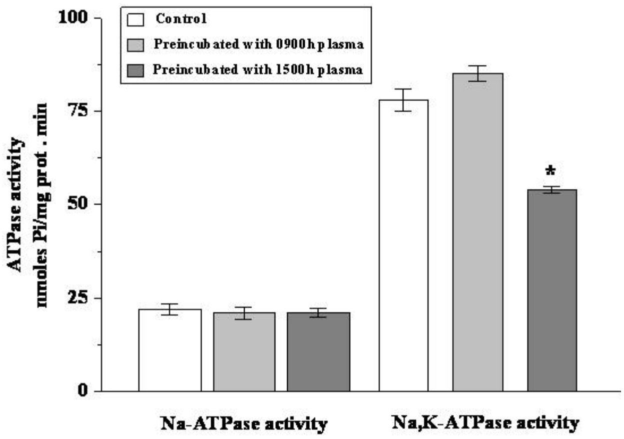

Fig. 2. Na-ATPase and Na,K-ATPase activities of homogenates of kidney cortex slices prepared at 0900 h, from rats maintained for 2 weeks on a 12 h light/12 h dark periods and preincubated or not with blood plasma obtained at either 0900 h or 1500 h from rats under the same schedule, in the presence of 5 mM $MgCl_2$. Before preincubation, the homogenates (10 mg prot/ml) were pretreated with SDS as indicated in Methods and then, 25 µl of the SDS pretreated homogenates were mixed with 250 µl of blood plasma, either drawn at 0900 h or at 1500 h and then preincubated for 30 min at 37 °C. The suspension was then diluted by adding enough sucrose/Tris/DTT/PMSF solution (see Materials and Methods) to obtain a protein concentration of approximately 0.2 mg/ml, and immediately assayed for Na-ATPase and Na,K-ATPase activity. The control assay was carried out in the same way, except for mixing the homogenates with 250 µl of 5 mM $MgCl_2$, 120 mM NaCl, 5.5 mM KCl, 60 mM Tris-HCl (pH 7.4 at 37 °C) solution, instead of blood plasma. The values represent means ± S.E.M. for six different animals. * p<0.001 vs control.

well known that the Na,K-ATPase activity is essential for the regulation of the cellular Na^+/K^+ concentrations and hence, for the maintenance of their gradients across the plasma membranes. These gradients are required for many cell functions such as cell volume regulation, functioning of several membrane exchangers and cotransporters, membrane excitability in neurons and muscles (Glynn and Karlish 1975, Kaplan 1983, Katz 1982). Consequently, changes in the Na,K-ATPase activity might modulate important cell functions for any tissue. Since the Na-ATPase activity does not oscillate along the day, this enzyme could compensate the intracellular concentration of Na^+ during the valley times of the Na,K-ATPase activity.

Conflict of Interest

There is no conflict of interest.

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