SYNAPTOSOMAL Na^+,K^+-ATPase IS AN HYSTERETIC ENZYME

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1. Introduction

Maintenance of extracellular K^+ concentrations within relatively narrow limits is important in the regulation of neuronal activity in the brain [1,2]. Alterations of extracellular K^+ may have significant effects on the neuronal function [3]. A very active Na^+,K^+-ATPase (EC 3.6.1.3) is present in isolated nerve endings [4,5].

An impairment in the synaptosome Na^+,K^+ pump is closely associated with the generation of seizure in primary and mirror epileptogenic focus of freezing lesion [6,7]. For these reasons, it seemed very interesting to study the mechanism of activation of the synaptosomal Na^+,K^+-ATPase by K^+ ions.

Using a method of continuous recording of the progress of the reaction, we found that synaptosomal ATPases exhibit hysteretic changes in enzyme activity according to the model in [8].

2. Materials and methods

Synaptosomal fractions were prepared as in [9] from adult rabbit brain. The purity of synaptosome fractions was verified by electron microscopy and by estimation of acetylcholinesterase activity.

Synaptosomal pellets were used directly in 0.32 M sucrose medium or after NaI treatment to eliminate the Mg^{2+}-ATPase activity [10]. The rate of hydrolysis of ATP in the presence of the synaptosomal fraction was measured by following the rate of liberation of H^+ ions [11] (see details in fig.1 legend).

Experimental points were averages of 3 or more experiments. Linear plots were obtained by a linear regression method with a linear correlation coefficient higher than 0.95.

3. Results and discussion

Figure 1 shows typical experimental traces of H^+ production by synaptosomal ATPases. As shown, both activities (Mg^{2+}-dependent as well as Na^+,K^+-dependent ATPases) decreased with time and reached a steady state (or linear progress) only after an initial burst of 3–4 min. To rule out the possibility of an artifact in the measuring procedure, the following controls were carried out:

1. The mixing dead time was determined by addition of small quantities (0.1 μeq.) of standard acid and alkali; a value of 8–10 s was found.

2. Continuous perfusion of an amount of proton similar to that produced by the enzyme reaction yielded a linear trace.

3. The same type of progress curve for the enzymatic reaction was observed:

   (i) Upon modification of volume, viscosity, mixing rate of the reaction medium, as well as of the size or type of the electrode;

   (ii) When the liberation of inorganic phosphate [12] or ADP [13] was monitored instead of the production of proton. This latter method was preferred because of its higher sensibility and greater simplicity;

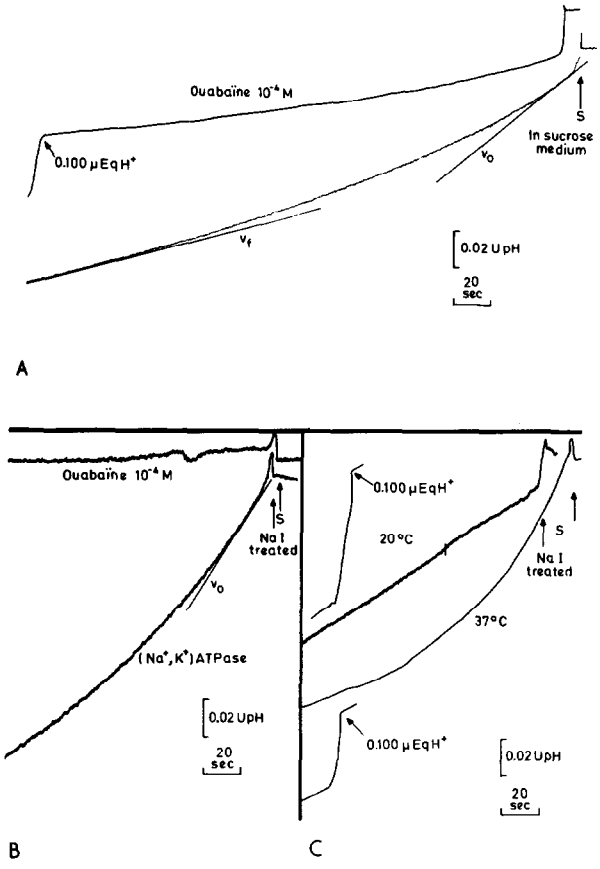

Fig.1. Typical traces of ATPase activities of synaptosomal fractions suspended in 0.32 M sucrose medium (A) or after NaI treatment (B, C). The arrows S indicate the moment of enzyme preparation injection (0.2 ml). The initial velocity is represented here by the tangent (V_0) at t 10 s. This time is necessary for good mixing (see text). In the absence of NaI treatment, ouabaine (10^{-4} M) induces an inhibition of about 50% (A) while this inhibition is clearly complete when a NaI-treatment sample is used (B). The steady state (or linear) velocity, V_f, is also represented by a tangent traced after 3 min. Panel C shows the effect of temperature on the rate of inactivation. Note the change of scale from 37–20°C. The sample (2 ml) was maintained at 37°C (A,B,C) or 20°C (C). The assay mixture contained 0.125 M NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM KCl, 1 mM $MgCl_2$, 1 mM ATP–Na_2 and 2 mM EGTA. All solutions were adjusted at pH 7.42 with NaOH or HCl. The final concentration of tissue was always less than 0.7 mg fresh wt/ml. The pH variations were measured by a combined electrode connected to a M 26C Radiometer pH meter and recorded with the help of a potentiometric tracer (1 mm = 2.10^{-3} pH unit). After 3–6 min the progress curve became linear and the system was calibrated by the addition of 0.1 µeq. H^+.

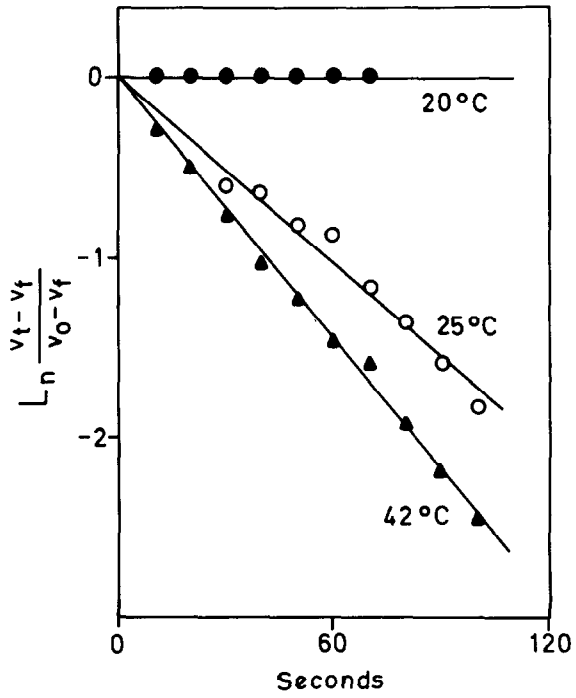

Fig.2. Plot of

$$\ln \frac{V_t - V_f}{V_0 - V_f}$$

as a function of time. The slope of the line is $-k'$. See fig.1 legend for conditions.

(iii) When liberation of protons was assayed using a pH stat system (Radiometer Titrator type TTT); thus the small variations in the pH or the buffer capacity of the system did not modify the activity of the enzyme (optimum pH 7.2–7.8).

Figure 2 shows that the observed time dependence of the reaction rate appears to obey the law [8] for a substrate-induced slow conformation change (hysteretic enzyme):

$$\ln \frac{V_t - V_f}{V_0 - V_f} = k't \qquad (1)$$

where: V_0, the velocity at t 0 (at which time the enzyme and substrate are mixed); V_t, the velocity at time t; V_f, the velocity after the steady-state has been reached; k', a complex rate constant depending upon the substrate concentration. Ouabaine sensitivity of

Table 1
Influence of K^+ concentration on initial velocity (V_0) and steady state velocity (V_f) of Na^+,K^+-ATPase activity of rabbit brain slices and synaptosomal fractions after NaI treatment

K	Slices		Synaptosomes	
(mM)	V_0	V_f	V_0	V_f
0	0.06	0.01	–	–
5	1.66	0.77	21.00	1.91
20	2.02	1.12	29.80	2.40
20+ ouabaine 10^{-4} M	0.00	0.00	0.00	0.00

Values of V_0 are calculated using eq. (1) (see text) and are expressed in neq. $H^+ \times$ mg $prot^{-1} \times s^{-1}$. Medium: ATP_{Na_2} 1 mM; $MgCl_2$ 1 mM; EGTA 2 mM; initial pH 7.42; T 37°C

both velocities (table 1) indicated that V_0 and V_f both represented Na^+,K^+-ATPase activity. Moreover, the synaptosomal fraction was considerably enriched in enzyme activity. The value of k' was strongly dependent upon the $ATP–Mg^{2-}$ complex concentration (fig.3) but appeared to be independent of the concentration of K^+ (0–20 mM). No modification of the inter-conversion phenomenon was observed upon addition of 10^{-3} M *N*-ethylmaleimide (NEM) or utilization of a Na^+-free medium.

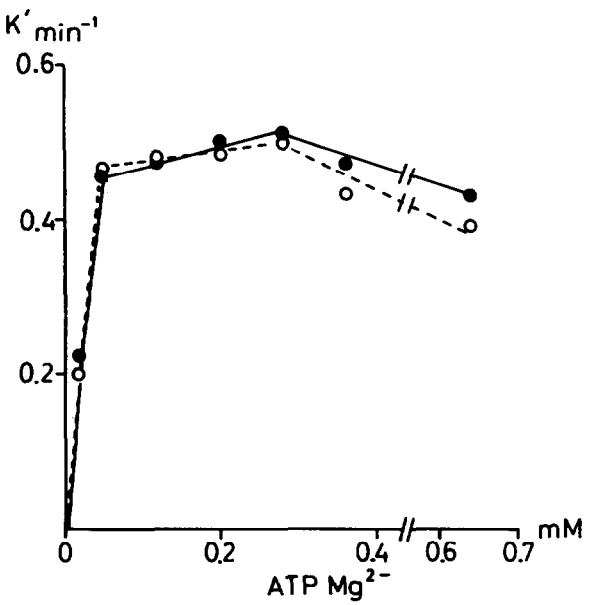

Fig.3. Substrate dependence of rate of enzyme inactivation (k') in 5 mM and 20 mM K^+ medium. Other conditions are as in fig.1,2. Binding constant, calculated from the plot $1/k'$ versus $1/ATP–Mg^{2-}$, is 0.045 mM $ATP–Mg^{2-}$ in presence of 5 mM K^+ and 0.035 mM $ATP–Mg^{2-}$ in 20 mM K^+ medium.

These latter results indicate that the substrate-induced conformation change postulated in this paper must be distinct from the isomerization to E_1P involved in the 'Albers Scheme' [14].

$$E_1 \xrightarrow[Mg,Na]{(ATP)} E_1P \xrightarrow[\text{NEM blocked}]{Mg}$$

$$E_2P \xrightarrow{K} E_2 \longrightarrow E_1 \quad \text{(see [16])}$$

From fig.1C and 2, the interconversion also appears to occur only at a temperature higher than 20°C.

This exponential enzyme inactivation, which is temperature sensitive, also occurs after preincubation of the enzyme, at 37°C or 42°C, for 5–10 min. Therefore elevated temperatures does not appear to be responsible for the observed inactivation.

The reversibility of the phenomenon has been investigated as follows: enzyme was incubated at 37°C for 5 min with 0.64 mM $ATP–Mg^{2-}$ at 37°C; inactivation was observed as usual. The suspension (2 ml) was then dialysed against 500 ml isolation buffer for 24 h at 42°C with two changes of buffer. A control sample was similarly treated, but in the absence of $ATP–Mg^{2-}$. When incubated in the presence of $ATP–Mg^{2-}$ (0.64 mM), both samples produced superimposable kinetics, exhibiting the same 'burst' and inactivation as fresh samples.

This experiment indicated that the elimination of $ATP–Mg^{2-}$ restored the initial behaviour of the preparation. A similar $ATP–Mg^{2-}$-induced interconversion was observed [15] using a purified dog kidney Na^+,K^+-ATPase. However, more recently, the same authors have obtained results indicating that the progressive inactivation that they had observed, could be due to the presence of sodium orthovanadate in commercial ATP preparation obtained from horse muscle ('Sigma grade' ATP) [16,17]. This inactivation by vanadate ions could be suppressed by 2.5 mM norepinephrine. This restriction does not seem to apply to our system since:

1. The ATP preparation that we have utilized is chemically synthetized (Boehringer Mannheim, [18]);

2. It is devoid of inhibitory factor of the Na^+,K^+-ATPase [19];

3. No effect of 2.5 mM norepinephrine was observed, in our experimental conditions, on the inactivation phenomenon.

Consequently, we believe that the results discussed in this paper represent the first demonstration of hysteretic behaviour of a Na^+,K^+-ATPase preparation. The rate of inactivation was, in our case, independent of K^+ concentrations. This could be in relation with the particular physiological functions of the synaptosomal structure and more particularly with the increase in extracellular K^+ concentration after the neuronal firing [1].

This hysteretic nature of the synaptosomal Na^+,K^+-ATPase and of the same enzyme from other brain structures, such as glia cells or perikaria (T.G. et al., in preparation) may account for some of the discrepancies found in the literature concerning K^+-inactivation mechanisms of Na^+,K^+-ATPase in brain (reviewed [20,21]. Indeed the more active enzyme, in this context, must be responsible for the initial velocity (V_0) while the activity measured after 2 min or more reflects the activity of two distinct enzyme forms, which possess, according to our data, very different catalytic properties.

References

[1] Katzman, R. (1976) Fed. Proc. Fed. Am. Soc. Exp. Biol. 35, 1244–1247.
[2] Franck, G., Grisar, T., Moonen, G. and Schoffeniels, E. (1978) in: Dynamic Properties of Glia Cells (Franck, G., Hertz, L., Schoffeniels, E., Tower, D. B. eds) Pergamon, Oxford, in press.
[3] Pedley, T. A., Fisher, R. S., Futamachi, K. J. and Prince, D. A. (1976) Fed. Proc. Fed. Am. Soc. Exp. Biol. 35, 1254–1259.
[4] Hosie, R. J. A. (1965) Biochem. J. 96, 404–412.
[5] Kurokawa, M., Sakamoto, T. and Kato, M. (1965) J. Biochem. 97, 833.
[6] Escueta, A. V., Davidson, D., Hartwig, G. and Reilly, E. (1974) Brain Res. 78, 223–237.
[7] Escueta, A. V., Davidson, D., Hartwig, G. and Reilly, E. (1974) Brain Res. 86, 85–96.
[8] Frieden, C. (1970) J. Biol. Chem. 245, 5788–5799.
[9] Cotman, C. W. and Matthews, D. A. (1971) Biochim. Biophys. Acta 249, 380–394.
[10] Nakao, T., Tashima, Y., Nagano, K. and Nakao, M. (1965) Biochem. Biophys. Res. Commun. 19, 755–758.
[11] Trevor, A. J. and Cummins, J. T. (1969) Biochem. Pharmacol. 18, 1157–1167.
[12] Medzihradsky, F., Nandhasri, P. S., Idoyaga-Vargas, V. and Sellinger, O. Z. (1971) Neurochemistry 18, 1599–1601.
[13] Barnett, R. E. (1970) Biochemistry 9, 4644–4648.
[14] Albers, J. W. (1976) in: The Enzymes of Biological Membranes (Martonosi, A. N. ed) vol. 3, pp. 283–301, Plenum Press, New York.
[15] Cantley, L. C. and Josephson, L. (1976) Biochemistry 15, 5280–5287.
[16] Josephson, L. and Cantley, J. (1977) Biochemistry 16, 4572–4576.
[17] Cantley, L., Josephson, L., Warner, R., Yanagisawa, M., Lechen, C. and Guidotti, G. (1977) J. Biol. Chem. 252, 7421–7423.
[18] Moffatt, J. G. and Khorana, H. G. (1961) J. Am. Chem. Soc. 83, 649–658.
[19] Beauge, L. A. and Glynn, I. M. (1977) Nature 268, 355–356.
[20] Glynn, I. M. and Karlish, S. J. D. (1975) Ann. Rev. Physiol. 37, 13–55.
[21] Stahl, W. L. and Broderson, S. H. (1976) Fed. Proc. Fed. Am. Soc. Exp. Biol. 35, 1260–1265.