METHODS

ENZYMIC MICROMETHOD OF DETERMINING MEMBRANE Na,K-ATPase ACTIVITY IN THE RAT CEREBRAL CORTEX

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A method of determining activity of Na,K-ATPase in the fraction of unpurified synaptosomes isolated from weighed microsamples (2-3 mg wet weight of tissue) of rat cerebral cortex is described. The method is based on fluorometric measurement of ADP formed in the course of the ATPase reaction; it is highly sensitive and can be used to determine the Na,K-ATPase activity of membrane preparations with a protein content of 0.5-1.0 μg per sample.

KEY WORDS: Na,K-ATPase — determination of activity; brain menbranes; fluorescence micromethod.

An urgent problem in functional neurochemistry is the study of relations between electrophysiological and biochemical processes in the brain. Recently attention has been specially concentrated on the explanation of the role of Na,K-ATPase in various neurophysiological processes. However, the solution to this problem requires the use of highly sensitive methods of determination of ATPase activity in synaptic structures isolated from weighed microsamples of nerve tissue. The generally accepted colorimetric methods of measurement of ATPase activity, based on the quantity of orthophosphate formed [1] are unsuitable in this case because of their low sensitivity. The highly sensitive method of enzymic determination of orthophosphate [2] is very complicated and requires reagents in short supply. In 1960 [3] a method of measuring ATPase activity was suggested, based on the quantity of ADP formed in the course of the ATPase reaction, and using two coupled enzymic reactions:

$$\mathrm{ADP + PEP \xrightarrow[PK]{K^+, Mg^{2+}} ATP + pyruvate} \quad (1)$$

$$\mathrm{Pyruvate + NADH + H^+ \xrightarrow{LD} lactate + NAD^+} \quad (2)$$

where PEP stands for phosphoenolpyruvate, PK for pyruvate kinase, and LD for lactate dehydrogenase.

The quantity of NAD^+ formed during a definite time was measured by a spectrophotometer, and this lowers the sensitivity of the method by comparison with fluorometric measurement. However, the main defect of this method, as also of its more recent modification [4], is the presence of an ATP-regenerating system actually in the medium for determination of ATPase, for under these conditions the presence of adenylate kinase [5] in the ATPase preparations studied can distort the results substantially. It must be remembered that real preparations of synaptic structures are usually contaminated with mitochondria, i.e., besides adenylate kinase they also contain AMP. Moreover a certain amount of AMP is always present in ATP preparations or may be formed as a result of the enzymic hydrolysis of ATP and ADP. As a result of all these factors, equilibrium of the adenylate kinase reaction

$$\mathrm{ATP + AMP \rightleftharpoons 2ADP} \quad (3)$$

is shifted in the presence of an ATP-regenerating system (reaction 1) toward ADP formation. Under our experimental conditions, involving the use of a fraction of unpurified synaptosomes

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(FUS) from the rat cerebral cortex, this method does not enable the ouabain-sensitive Na,K-ATPase of synaptic structures to be detected.

The method suggested by the writers is based on fluorometric determination of ADP formed in the course of the ATPase reaction. For this purpose, to reduce the effect of adenylate kinase to the minimum, reagents necessary for the determination of ADP are added to the incubation medium after inactivation of the enzymes of the FUS by means of HCl and subsequent utilization with KOH.

To isolate the FUS a micromodification of the standard method [6] was used. A weighed sample (2-3 mg) of tissue from the rat cerebral cortex was disintegrated in 0.5 ml of isolation medium (0.32 M sucrose, 0.001 M EDTA-Na_2, 0.01 M Tris-HCl, pH 7.4) manually in a microhomogenizer with a glass pestle. The nuclear fraction and fraction of unpurified nerve endings were sedimented in special plastic tubes with a capacity of 2 ml. Under these conditions the yield of FUS, as protein, was 10% of the weight of the original tissue. The FUS thus obtained was suspended in isolation medium.

The ATPase reaction was carried out in 260-280 µl of incubation medium of the following composition (in mM): NaCl 100, KCl 20, $MgCl_2$ 5, Tris-HCl 50, pH 7.6 (25°C), ouabain (from Sigma, USA), 0.8, FUS from 0.05 to 10 µg protein. After preincubation (37°C) for 10 min the reaction was started by the addition of ATP (in a final concentration of 4.7 mM) and it continued for 5-40 min at 37°C. The reaction was stopped by adding 10 µl 1 N HCl to each tube, after which the tubes were placed in an icebath (incubation time 15 min) and neutralized with 10 µl 1 N KOH. Next, 50 µl of a 10 mM solution of PEP (from Sigma, USA) and 50 µl of a 0.8-2.5 mM solution of NADH were added in succession to the samples. After incubation for 20 min at 20°C, 5 µl of a suspension of LD and 5 µl of PK with an activity of about 320 milliunits were added successively to the samples (all reagents from Boehringer, West Germany). The final volume of the samples was 410 µl. The reaction of determination of ADP under these conditions took 2-3 min. The quantity of NADH oxidized was determined by means of a Hitachi-204 (Japan) spectrofluorometer, with the following parameters: wavelength of excitation 388 nm, wavelength of fluorescence 450 nm. The accuracy of determination of 10^{-9} M ADP was ±1%. The most stable results for measurement of Na,K-ATPase activity, determined as the difference between activity of total Na,K,Mg-ATPase and Mg-ATPase, were obtained if the protein content of the FUS was 0.5-10 µg per sample. It was shown that with a protein content of FUS of 5 µg per sample the rate of accumulation of ADP at 37°C was constant for 30 min. Activity of Mg- and Na,K-ATPases in the FUS preparation in these experiments averaged 7.5 and 5.0 µmoles ADP/mg protein/h respectively, in agreement with results obtained for this fraction by a modified method of Lowry and Lopez. The suggested highly sensitive micromethod is thus capable of adequately estimating Na,K-ATPase activity in an FUS preparation isolated from definite areas of the rat cerebral cortex.

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