A Quick Assay for Na^+-K^+-ATPase Specific Activity

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The method describes a simultaneous determination of inorganic phosphate (Pi) and protein content from a reaction mixture used for assay of adult rat cerebrocortical synaptosomal membrane Na^+-K^+-ATPase specific activity. The present method is more convenient, accurate and quicker compared to the existing methods for the determination of Na^+-K^+-ATPase activity. It also eliminates the possible errors in protein estimation by other classical methods in brain, which have a high lipid content.

Introduction

Measurement of the specific activity of Na^+-K^+-ATPase (nanomol of inorganic phosphate, Pi, liberated per min per milligram of protein) is required to evaluate the purity or to express the actual specific enzyme activity. Accordingly an accurate determination of both inorganic phosphate and protein content is necessary.

Most widely used for the protein assay is the method of Lowry *et al.* (1951), which has been modified for the membranous and detergent-solubilized proteins by Peterson (1979). Other methods used to determine protein concentration more rapidly, involve Coomassie Blue G-250 binding (Bradford, 1976) and a more specific, accurate, and sensitive method as has been described by Vera (1988). In the latter method (Vera, 1988) sodium dodecyl sulfate (SDS) is used for protein solubilization followed by the development of turbidity by the addition of trichloroacetic acid (TCA) to the solution. Methods for the assay of Na^+-K^+-ATPase have also been described (Esmann, 1988; Swann and Steketee, 1989).

The present manuscript describes an assay for Na^+-K^+-ATPase activity by simultaneous determination of the concentration of Pi generated by the hydrolysis of ATP and the corresponding protein used in the same assay mixture tube. This eliminates the error of aliquoting proteins for the enzyme assay in different tubes. This combined method also reduces time and labor to determine the protein content separately from the stock enzyme preparation, which is usually done by most investigators.

Materials and Methods

Preparation of synaptosomal fractions

Cerebral cortices were dissected out from adult male Charles Foster rats of body weight 200–250 g. The synaptosomes were prepared after Sarkar and Ray (1992). Briefly, the cerebral cortex was homogenized (approx. 10% weight/volume) in 0.32 M sucrose and centrifuged at $1000 \times g$ for 10 min to remove cell debris and nuclei. The supernatant was collected and recentrifuged at $1000 \times g$ for another 10 min. The resulting supernatant was layered over 1.2 M sucrose, and centrifuged at $34{,}000 \times g$ for 50 min at 4 °C. The fraction collected between the 0.32 M and 1.2 M sucrose layer was diluted at 1:1.5 with ice-cold bi-distilled water, further layered on 0.8 M sucrose, and again centrifuged at $34{,}000 \times g$ for 30 min. The pellet thus obtained was washed, repelleted at $20{,}000 \times g$ for 20 min, and ruptured with ice-cold 5 mM imidazole-HCl buffer, pH 7.4 and kept in ice for 60 min with occasional vortex at high speed every 5 min and used as synaptosomal fraction for enzyme assay. The synaptosome was lysed hypo-osmotically.

Assay of Na^+-K^+-ATPase specific activity

Synaptosomal Na^+-K^+-ATPase (EC 3.6.1.3) activity was assayed in reaction mixtures of (i) 30 mM imidazole-HCl, 130 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, and (ii) 30 mM imidazole-HCl, 4 mM $MgCl_2$, and 1 mM ouabain, at pH 7.4, each containing 20–50 μg synaptosomal protein. For complete ouabain binding, the tubes were preincubated for 60 min in ice and dark. The reaction was started by adding 4 mM Tris-ATP (Tris hydroxymethylaminomethane salt of adenosine triphosphate, Sigma Co.) and incubated at 37 °C for 10 min. The total volume of the reaction mixture was 1 ml. The enzyme activity was stopped by addition of 100 μl of 20% sodium dodecylsulfate. The Pi formed was

determined from the reaction mixture (Baginski *et al*., 1967). Na^+-K^+-ATPase activity was calculated as difference in the Pi content in nmol/min between media (i) and (ii).

Enzyme protein was solubilized using 100 µl of 20% SDS and from this mixture an aliquot (0.2 ml from a total volume of 1.1 ml) was taken for Pi estimation followed by quick addition of 0.5 ml of reagent A (containing 3% ascorbic acid in 0.5 N HCl and 0.5% ammonium molybdate solution) in a total volume of 1 ml and the tubes were kept in ice for 10 min. Then 1 ml of reagent B (containing 2% sodium meta-arsenite, 2% trisodium citrate and 2% acetic acid) was added. The color developed after 10 min at 37 °C was read at a wavelength 850 nm in a Beckman spectrophotometer DU-70 (Esmann, 1988).

Protein assay

0.3 ml aliquot from the same reaction mixture of Na^+-K^+-ATPase assay after SDS treatment was used directly and 0.3 ml of 30% TCA was added to this solution. The tubes were incubated at room temperature for 30 min to develop the turbidity and the turbidity was read at 340 nm. Bovine serum albumin prepared in 0.2% SDS was used for standard protein assay (Vera, 1988). Protein contents were also determined from the stock solution by the method of Lowry *et al.* (1951).

Results

Figure 1 shows a saturation curve of Na^+-K^+-ATPase activity *versus* protein concentration determined from the same assay mixture after termination of the enzyme reaction following addition of 100 µl of 20% SDS (1.8% final concentration). The protein content shows a linear relationship up to 50 µg of protein and thereafter the activity of Na^+-K^+-ATPase (nmol Pi/min) remained unchanged showing a plateau with increasing concentrations of the protein as estimated.

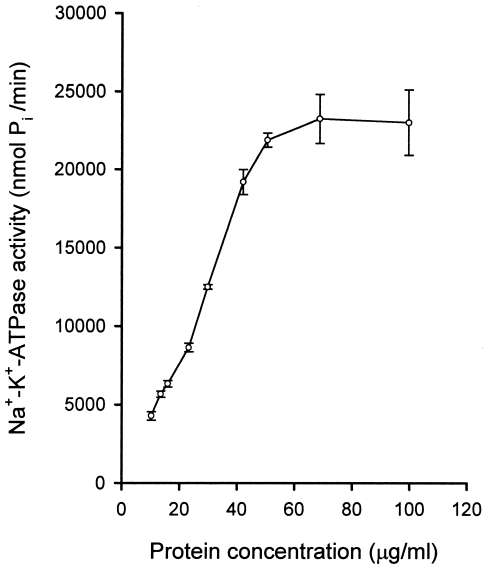

Fig. 1. Saturation of synaptosomal Na^+-K^+-ATPase activity with increasing synaptomal protein concentrations from adult rat brain cerebral cortex. Each point represents mean ± SEM of 15–20 individual data and the vertical lines denote the SEM (Standard error of mean).

The protein content measured from the stock synaptosomal fraction separately also matched with that of measured directly from the Na^+-K^+-ATPase assay mixture.

The protein content measured by the method of Vera (1988) from the reaction mixture that was used to estimate Pi, and from the stock synaptosomal fraction by the method of Lowry *et al.* (1951), showed no significant change in amounts.

The Na^+-K^+-ATPase activity obtained by using rat liver microsomal fraction also showed the same type of reproducible data (data not shown) following the simultaneous assay procedure to determine Pi and respective protein content as described before.

Statistical analysis of the data was made by student's t-test.

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