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A Microtiter Plate Assay for the Detection of Inhibitors of the Na^+, K^+-ATPase

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ABSTRACT

A rapid microtiter plate assay for the detection of inhibitors of the Na^+, K^+-ATPase has been developed. The assay is based on the measurement of inorganic phosphate released from the substrate, ATP, and has been designed to be carried out in the individual wells of a microtiter plate. Since the production of inorganic phosphate is determined colorimetrically, multiple samples can be tested simultaneously using a microtiter plate reader. This microtiter plate assay is particularly useful for screening large numbers of samples, such as microbial culture supernatants.

Index Entries: ATPase inhibitors; enzyme inhibitors; screening.

INTRODUCTION

The Na^+, K^+-ATPase is a membrane-bound enzyme found in animal cells that is responsible for the coupling of the free energy stored within the ATP molecule to the translocation of Na^+ ions outward and K^+ ions inward across the plasma membrane (*1,2*). The continuous operation of this macromolecular machine ensures the generation and maintenance of steep gradients of Na^+ ions and K^+ ions across the cell membrane that are essential to many cellular functions. For example, these gradients are responsible for the maintenance of the osmotic stability of the cell despite large variations in salt and water intake, and also control the generation and maintenance of a resting membrane potential, which provides the

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driving force for the action potential in excitable cells and tissues (3). The involvement of this ubiquitous enzyme in many diverse physiological functions suggests an alteration in its activity may have a key role in many pathological processes, e.g., modulation of cardiac contractility.

The classical therapeutic regulators of the Na^+, K^+-ATPase are the cardiac glycosides, which are very specific and effective in their action on this enzyme. However, these drugs are potentially hazardous because of their very narrow safety margin, and there is, therefore, a need to identify new compounds with the therapeutic efficacy of the cardiac glycosides, but without their toxic effects.

The microtiter plate assay described here has, therefore, been developed to enable the screening of large numbers of compounds for inhibitory activity of this nature with relative ease and efficiency. In our own work, we have routinely used the assay to screen microbial culture filtrates for Na^+, K^+-ATPase inhibitors.

MATERIALS AND METHODS

Materials

Sodium-potassium-activated adenosine-5′-triphosphatase, disodium adenosine triphosphate, ouabain, ammonium molybdate, and sodium dodecyl sulfate were obtained from Sigma Chemical Company. Other chemicals used in buffer and reagent preparation were from BDH Chemicals Ltd. and were of "Analar" grade, unless otherwise stated. All water used was distilled deionized, and all glassware was pretreated with "Decon 90" to remove all traces of phosphate.

Assay Principle

Inorganic phosphate released from the substrate, ATP, is detected in a one-step reaction using a colorimetric method for phosphate determination first described by Lin and Morales (4). Addition of the color reagent both terminates the enzyme reaction and enables a colorimetric measurement of the released inorganic phosphate, which in turn allows the determination of the enzyme rate.

Assay Reagents

Substrate Preparation

Adenosine-5′-triphosphate was dissolved in substrate buffer (133.4 m*M* Tris-HCl, pH 7.4, 266 m*M* NaCl, 8 m*M* $MgCl_2$, and 2.66 m*M* Na_2 EDTA) at a concentration of 2.1 mg/mL to provide a stock substrate solution.

Enzyme Preparation

Sodium and potassium-activated adenosine-5′-triphosphate was suspended in enzyme buffer (133.4 m*M* Tris-HCl, pH 7.4, 266 m*M* NaCl, 8 m*M* $MgCl_2$, 2.66 m*M* Na_2EDTA, and 20 m*M* KCl) at a concentration of 1 mg/mL to provide a stock enzyme suspension.

Reagent for Phosphate Determination (4)

The following three solutions were prepared:

1. Ammonium molybdate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$): 10 g ammonium molybdate was dissolved in 100 mL water containing 1 mL ammonium hydroxide solution (specific gravity 0.91).
2. Ammonium metavanadate (NH_4VO_3): 0.235 g ammonium metavanadate was dissolved in 40 mL hot water and cooled as soon as it had dissolved: 0.6 mL concentrated nitric acid (specific gravity 1.42) diluted with 1.4 mL of water was added and the volume adjusted to 100 mL with water.
3. Sodium dodecyl sulfate solution: 20 g of sodium dodecyl sulfate was dissolved in 100 mL water with warming.

The final reagent was prepared by mixing the three above solutions together with 18.5 mL concentrated nitric acid (specific gravity 1.42), and the volume adjusted to 500 mL with water. This reagent was stable for several weeks and, following preparation, was stored at +4°C until required.

Reagents for Phosphate Calibration Curve

Standards of di-potassium hydrogen orthophosphate were diluted with distilled water to give a range of concentrations from 50 μM to 3 m*M* in the final assay solution.

Standard Inhibitor

Ouabain was dissolved in water and diluted to provide standards in the concentration range of 50 n*M* to 10 μM.

RESULTS

Phosphate Estimation

A phosphate calibration curve was plotted to determine the linearity of the color reaction (Fig. 1). The absorbance change effected by a series of phosphate standard solutions was determined by mixing 175 μL of each phosphate standard with 50 μL of phosphate reagent. This was carried out in triplicate in the individual wells of a microtiter plate. In accordance with the assay procedure, the plate was left to stand for 15 min to allow

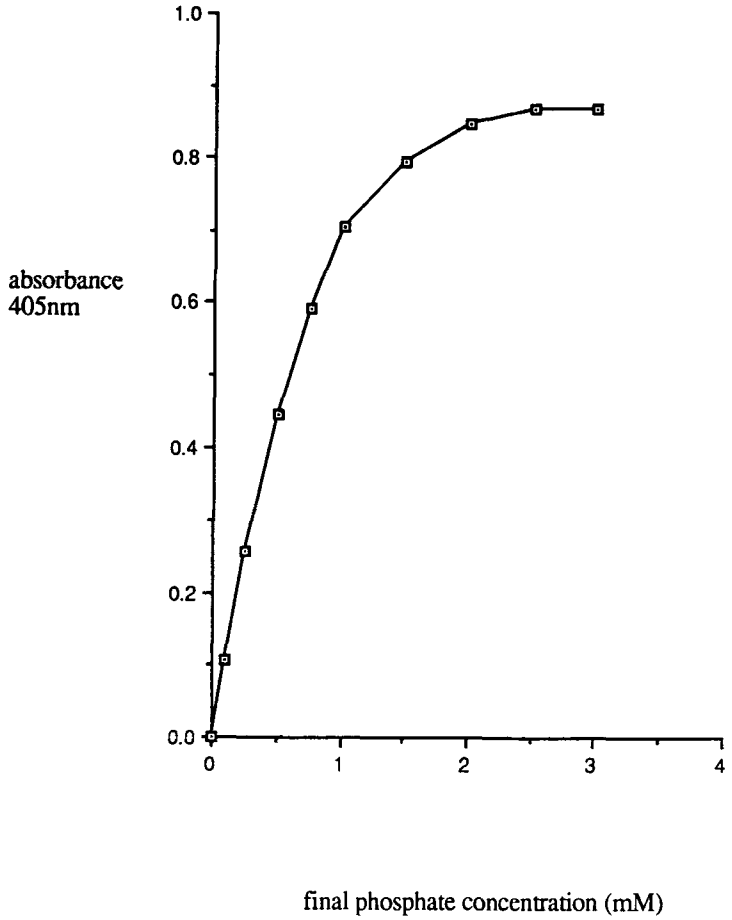

Fig. 1. Graph showing the relationship between color development at 405 nm and phosphate concentration.

for the development of the yellow chromophore, and the absorbance then read at 405 nm. This standard curve was linear up to a phosphate concentration of 1 m*M*, which translated into an absorbance change of 0.65. When designing the enzyme assay, therefore, care was taken to ensure that absorbance changes of no more than 0.6 (equivalent to a phosphate concentration of approx 800 μM) occurred.

Determination of K_m

In order to determine the substrate concentration to be used in the assay, the K_m of the reaction was determined using an enzyme concentration of 0.29 mg/mL (Fig. 2). The value of K_m was determined as 0.6 mg/mL. In order to optimize the detection of competitive inhibitors, it is necessary to use a low substrate concentration for inhibitor assays (normally in the region of 0.5 K_m). However, this must be balanced against the need to generate a significantly measurable amount of product from the substrate. It was, therefore, decided to use a substrate concentration that

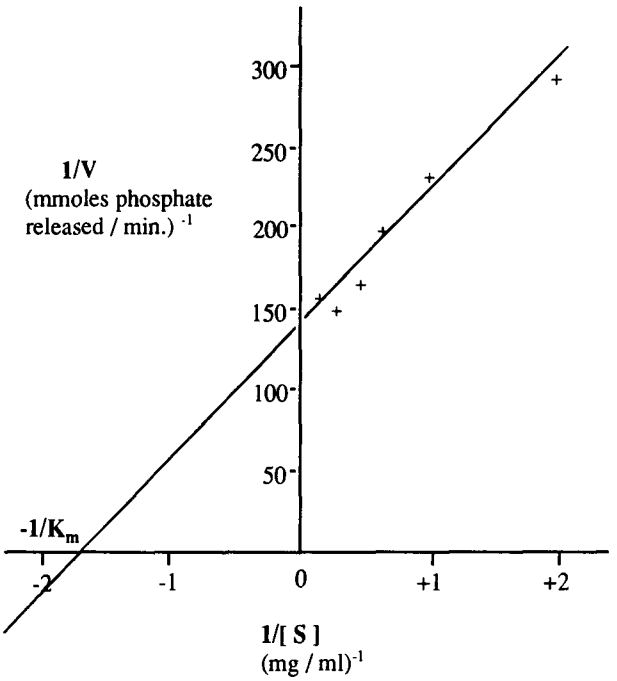

Fig. 2. Lineweaver-Burk plot for the determination of K_m for the hydrolysis of ATP by the Na^+, K^+-ATPase.

would allow an absorbance change of approx 0.3 over a 30-min period. In order to accommodate this compromise, it was found necessary to use a substrate concentration of 1 × K_m, i.e., 0.6 mg/mL. Under these conditions, color development was linear over approx 40 min, with an absorbance change of approx 0.25 being reached at 30 min (Fig. 3).

Final Assay Procedure

Duplicate microtiter plates (A+B) were prepared with 75 μL of test solution plus 50 μL of enzyme suspension in each well. Following preincubation at 37°C for 15 min, 50 μL of substrate solution were added to each well on both plates. The reaction in plate A was then immediately terminated by the addition of 50 μL of phosphate reagent, and the plate was left to stand at room temperature for 15 min before reading the absorbance at 405 nm.

Meanwhile, plate B was returned to the 37°C incubator for a further 30 min, after which time 50 μL phosphate reagent were added. As for plate A, plate B was left to stand for 15 min before reading the absorbance of the reaction mixture at 405 nm. The activity of the enzyme in the individual wells was estimated by subtracting the absorbance reading of the reaction mixtures in plate A from those in plate B.

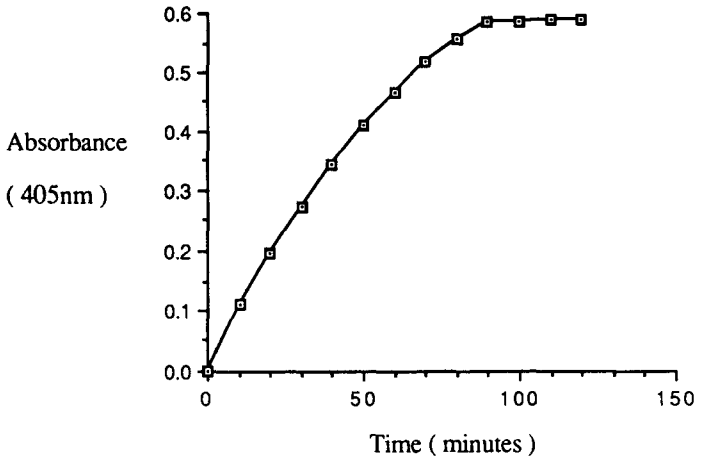

Fig. 3. Graph showing the relationship between absorbance change (405 nm) and time, at a substrate concentration of 0.6 mg/mL and an enzyme concentration of 0.25 mg/mL.

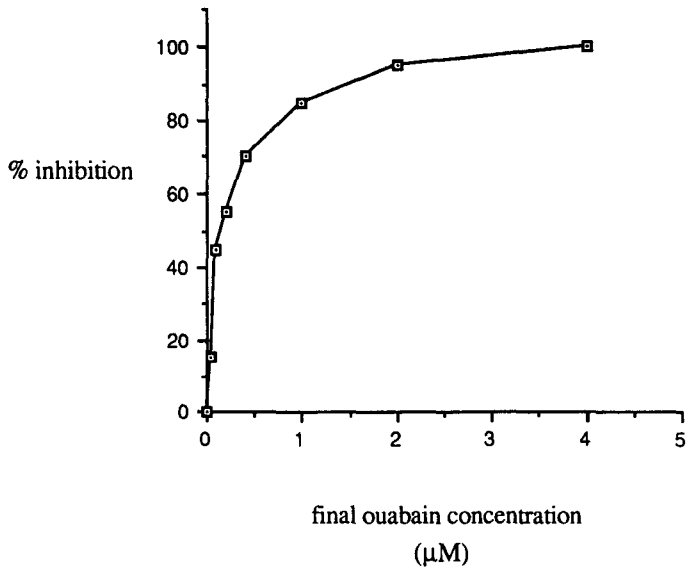

Fig. 4. Graph showing the inhibitory effect of ouabain on the Na^+, K^+-ATPase assay.

DISCUSSION

We have used the assay described here to screen algal culture supernatants routinely for the presence of Na^+, K^+-ATPase inhibitors, although there is no reason why the assay should not be used to screen any extract or solution as long as the free phosphate concentration is not too high. Using this assay, up to approx 500 samples can be screened in a normal

working day. Methanol extracts of microbial pellets have also been screened for inhibitory activity, but in this case, in order to avoid denaturation of the enzyme, samples must first be diluted 1:5 with deionized water before taking a 75 μL aliquot for screening in the assay.

In order to test the sensitivity of the assay, the effect of the cardiac glycoside inhibitor, ouabain, was observed by substituting a 75-μL aliquot of inhibitor solution for sample in the assay procedure and performing the assay as previously indicated. The results (Fig. 4) showed the assay to be capable of detecting the presence of ouabain at concentrations as low as 100 n*M* (7.29×10^{-5} mg/mL).

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