Ca^{2+}-ACTIVATED ATPase AT THE EXTERNAL SURFACE OF NEUROBLASTOMA CELLS IN CULTURE

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1. Introduction

It has been previously established in erythrocytes that active Ca^{2+} transport is linked to a Ca^{2+}-stimulated Mg^{2+}-dependent ATPase (Mg^{2+} + Ca^{2+})-ATPase [1]. Ohtsuki [2] has suggested that brain cells maintain a low intracellular concentration of Ca^{2+}, by a Ca^{2+} pump. Moreover Mg^{2+}- and/or Ca^{2+}-activated ATPases have been described in the microsomes of the pig and bovine brain [3–6], in synaptosomes of guinea pig and rat brain [7,8], and the electric tissue of eel [9]. The intracortical pattern of distribution of Mg^{2+}- and Ca^{2+}-stimulated ATPase activities in the rat brain [10] and in human frontal cortex [11] has been studied. Novikoff has described the distribution of a Ca^{2+}-stimulated ATPase in dorsal root ganglia and peripheral nerve [12]. A Mg^{2+}- or Ca^{2+}-activated ATPase has been isolated from whole brain of rat or cat with properties similar, in many respects, to those of muscle actomyosin [13].

During our investigation into the cell surface enzyme activities in cultured cells we have observed in neuroblastoma and glial cells the presence of an ouabain sensitive ecto-ATPase [14]. Though derived from neoplastic tissue and cultured through many generations, clonal cell lines from murine neuroblastoma possess many neuronal characteristics [15–20] which indicate their utility as models of neurons. In this paper, we report the existence of a Ca^{2+}-activated ATPase located at the external surface of neuroblastoma cells.

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2. Materials and methods

2.1. *Cell cultivation*

Neuroblastoma clonal cell line N1E-115 was used. The cells were propagated from a culture donated by Dr M. Nirenberg, Laboratory of Biochemical Genetics, National Institute of Health, Bethesda, Md. Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% foetal calf serum and antibiotics. The cultures were incubated at 37°C in Falcon Petri dishes (6 cm ϕ) in a humidified atmosphere of 95% air and 5% CO_2. Cells were grown to stationary phase before use. Cultures were negative for PPLO contamination by bacteriologic criteria.

2.2. *Assay of ATPase*

Growth medium was carefully removed from Petri dishes and discarded and the cell monolayer was gently washed first with a medium containing 0.125 mM EGTA, 0.250 mM EDTA, 30 mM Tris–HCl buffer (pH 7.8), 5.5 mM glucose and 250 mM sucrose and afterwards three times with a total of about 10 ml of incubation solution without ATP. Composition of incubation fluid and other experimental conditions are detailed in legends to table and figures. Incubation was started by adding Tris–ATP and conducted at 37°C for 15 min, in a final volume of 1.5 ml. After this period the medium was transferred to the ice cooled centrifuge tubes. The inorganic phosphate formed was determined colori-

metrically according to the method of Gomori [21]. Appropriate controls without cells were incubated and analysed in order to correct for small amounts of P_i found in commercial preparations of ATP or deriving from its spontaneous hydrolysis. Another control with cells but without substrate was also included.

2.3. *Protein determination*

Protein was determined by a modification of the method of Lowry et al. [22]. Crystalline bovine serum albumin was used as standard.

2.4. *Materials*

Dulbecco's modified Eagle medium and foetal calf serum were obtained from Gibco, Grand Island (N.Y.). Tris–ATP, ouabain, bovine serum albumin, EGTA and EDTA were purchased from Sigma Chemical Co., St. Louis, Mo. Sucrose was from BDH Chemicals, Poole. All other chemicals were from Merck, Darmstadt.

3. Results

3.1. *Cell integrity*

The media which we applied were osmotically and temperature equilibrated, so the cells remained attached to the bottom of the Petri dishes during the entire incubation. During the incubation, the cells were monitored by the phase contrast microscopy and the morphology of cells was found preserved. Cellular integrity was established by the use of enzyme markers as described previously [23].

3.2. *Activation by Mg^{2+} and Ca^{2+}*

Cells were incubated in solutions containing Ca^{2+} or Mg^{2+} from 0.2–5.0 mM. Ca^{2+} or Mg^{2+} alone activated the enzyme. The enzyme was preferentially activated by Ca^{2+} (fig. 1). The apparent dissociation constant for Ca^{2+} was 0.12 mM and for Mg^{2+} was 0.33 mM (table 1).

Activation of enzyme by Ca^{2+} and by Mg^{2+} was further tested by using a constant concentration of Ca^{2+} (5 mM) and adding increasing amounts of Mg^{2+}, as well as by using a constant concentration of Mg^{2+} (5 mM) and adding increasing amounts of Ca^{2+}. The addition of Mg^{2+} to 5 mM Ca^{2+} and vice versa led to inhibition of the enzyme (fig. 1). Equimolar concentrations of both Mg^{2+} and Ca^{2+} were assayed (0.5; 1.0; 2.5; 5.0 mM each) but only at 0.5 and 1.0 mM divalent cation concentration intermediate values between those for Ca^{2+} and Mg^{2+} alone were produced (data are not presented).

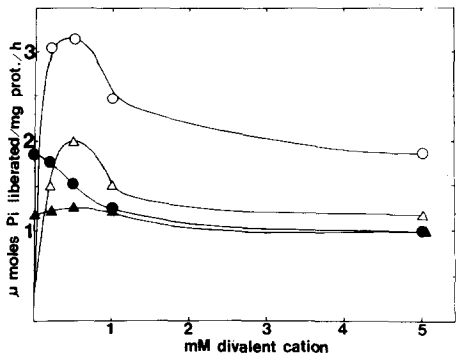

Fig. 1. Stimulation of ATP hydrolysis by divalent cations (present as chlorides). (○–○–○) activation by Ca^{2+}; (△–△–△) Mg^{2+}; (●–●–●) 5 mM Ca^{2+} + Mg^{2+} (varied as indicated); (▲–▲–▲) 5 mM Mg^{2+} + Ca^{2+} (varied as indicated). Unless indicated incubation fluids contained 30 mM Tris–HCl buffer (pH 7.8), 5.5 mM glucose, 2.5 mM ATP (as Tris salt) and sucrose as necessary to obtain an ideal osmolarity of 280 milliosm. Each point is the mean of 3 or 4 determinations. S.E. are less than 5%.

3.3. *Effect of ATP concentration*

The effect of ATP concentration on enzyme

Table 1
Kinetic constants of Mg^{2+}- and Ca^{2+}-activated ATPase activities at the external surface of neuroblastoma cells in culture

Kinetic constant	Mg^{2+}-ATPase	Ca^{2+}-ATPase
K_m (mM)	0.28	0.54
V_{max} (μmoles/mg^{-1}/hr^{-1})	2.17	3.16
$K_{Me^{2+}}$ (mM)	0.33	0.12

K_m, V_{max}: the basic medium and incubation conditions as in fig. 2. ATP varied from 0.1–3.0 mM. $K_{Me^{2+}}$: Incubation medium as in fig. 1, except Ca^{2+} or Mg^{2+} varied from 0.1–1.0 mM.
Michaelis-Menten kinetic constants were calculated by statistical treatment of data plotted in the form of s/v against s according to Wilkinson [24] on an Olivetti Programma 602.

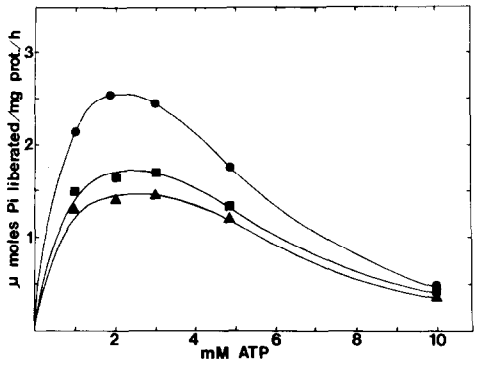

Fig. 2. Effect of ATP concentration on ATPase activity. (●–●–●) 1 mM Ca^{2+}; (■–■–■) 1 mM Ca^{2+} + 1 mM Mg^{2+}; (▲–▲–▲) 1 mM Mg^{2+} 30 mM Tris–HCl buffer (pH 7.8), 5.5 mM glucose and sucrose as necessary to obtain an ideal osmolarity of 280 milliosm.

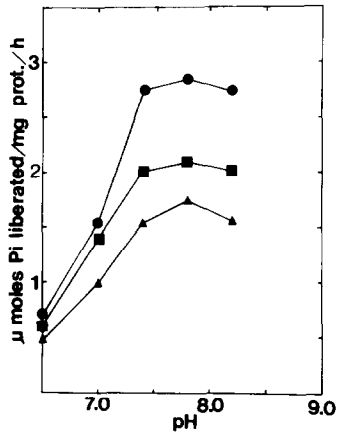

Fig. 3. Effect of pH on enzymic activity. (●–●–●) Ca^{2+}; (■–■–■) Ca^{2+} + Mg^{2+}; (▲–▲–▲) Mg^{2+}. Incubation condition as on fig. 2, except 2.5 mM ATP was present. Each point is the mean of 3 or 4 determinations. S.E. are less than 5%.

activity was examined by incubating the cells at 1 mM divalent cation concentration and various concentrations of ATP (0.5–10 mM). Maximal ATP hydrolysis occurred at 2.5 mM ATP (fig. 2). The apparent K_m value for Ca^{2+}-ATPase was 0.54 mM and for Mg^{2+}-ATPase 0.28 mM (table 1).

Although the enzyme hydrolyses the phosphate groups of ATP and ADP (unpublished), it can be assumed that the initial reaction rates represent the hydrolysis only of the terminal phosphate group of ATP, since under the conditions used in the study the products formed are essentially ADP and P_i.

3.4. *Effect of pH*

As we are dealing with intact cells the effect of pH on enzyme activity was determined in the limited range of 6.5–8.2. The pH optimum was at 7.8 (fig. 3).

3.5. *Effect of ouabain*

Ouabain was added in concentrations of 0.01, 0.1 and 1.0 mM and ATPase activity determined with Ca^{2+}, Mg^{2+} and both Ca^{2+} and Mg^{2+} at 1 mM concentration of each. It has been found that ouabain did not inhibit Mg^{2+}-dependent ATPase nor the Ca^{2+}-dependent ATPase.

4. Discussion

In the present study a Ca^{2+}-activated ATP splitting activity has been demonstrated using whole neuroblastoma cells in culture. This enzyme activity differs from the other Mg^{2+} and/or Ca^{2+}-ATPase activities of brain tissue, in that Ca^{2+} is the preferential cation for stimulation. The preferential activation of neuroblastoma cell ATPase activity by Ca^{2+} is similar to that of Ca^{2+}-ATPases located in plasma membrane of some tissues in which active transport of Ca^{2+} occurs [25,26].

Hydrolysis of extracellular ATP is not a common feature of other Ca^{2+}-ATPases. In erythrocytes the enzymic site faces the cell interior since ATP and Ca^{2+} do not activate the system when present in medium surrounding intact cells or released cells [27]. In a previous work evidence is presented that Mg^{2+}-dependent and K^+-stimulated, ouabain inhibited 'ecto'-ATPase activities in neuroblastoma cells are due neither to leakage of enzyme from the cell nor to broken cells [23]. Applying the same procedure, no leakage of the Ca^{2+}-dependent ATPase activity was found in the present study. Moreover ATP cannot penetrate the cell membrane [28]. Thus the location of the Ca^{2+}-ATPase activity obtained with whole neuroblastoma cells is to be on the plasma membrane

with the active site oriented to the incubation medium. Although the function of the neuroblastoma cell surface Ca^{2+}-dependent ATPase remains to be established, its location and properties would appear to fit it for a role in the surface characteristics of the cell and possibly in the Ca^{2+} transport.

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