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Expression of sodium pump activities in BALB/c 3T3 cells transfected with cDNA encoding α_3-subunits of rat brain Na^+,K^+-ATPase

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The cDNAs encoding α_3-subunits of rat brain Na^+,K^+-ATPase and the neomycin resistance gene were incorporated into BALB/c 3T3 cells by the co-transfection method. Stably transformed cells were selected with 300 μg/ml of neomycin (G-418) for 6 weeks. Northern blot analysis using the 3'-non-translated region of the cDNA as a probe revealed that the α_3 mRNA appeared in transfected cells. Na^+,K^+-ATPase activity of the transfected cells was twice that of wild-type cells. Regarding ouabain sensitivity, the Na^+,K^+-ATPase showed two K_i values for ouabain (8×10^{-8} and 4.5×10^{-5} M) in transfected cells while wild-type cells displayed only the higher value. Ouabain sensitivity of Rb^+ uptake also demonstrated two K_i values in the transfected cells (8×10^{-8} and 4×10^{-5} M) and a K_i in wild-type cells of 4×10^{-5} M. It is concluded that α_3 is a highly ouabain-sensitive catalytic subunit of Na^+,K^+-ATPase. It is also suggested that ouabain sensitivity is exclusively determined by the properties of the α-subunit rather than the β-subunit. This is the first report on the catalytic charateristics of the α_3 isoform of Na^+,K^+-ATPase.

Na^+,K^+-ATPase; Gene transfection; Subunit isoform; Ouabain sensitivity; Na^+ pump

1. INTRODUCTION

Na^+,K^+-ATPase is known to exist in the plasma membranes of all higher animal cells and to maintain the Na^+ and K^+ gradients across the membrane [1]. This enzyme consists of two kinds of subunit, α and β. The α-subunit is catalytic and carries sites for ATP-binding, phosphorylation and ouabain binding. The β-subunit is a glycoprotein with a lower molecular mass than the α-subunit, and its function in the enzymatic activity remains unclear. Two isoforms of the α-subunit have been found. One is a kidney-type α, designated α_1, having a relatively low sensitivity to ouabain ($K_i = 1 \times 10^{-4}$ M) in rodents, and it is found in almost all cells in animals. The other is a brain-type isoform, known as α_+ or α_2, and occurs in central nervous tissues, cardiac muscles, spinal cord and adipocytes [2]. It has a high sensitivity to ouabain in rodents ($K_i = 1 \times 10^{-6}$–10^{-7} M). Recently, the existence of the third isoform, called α_3 or rat UID, was suggested on the basis of cDNA cloning in rat brain [3–5], and the mRNA was found in central nervous, spinal cord and neonatal cardiac tissues [5–7]. However, neither the translated product of α_3 mRNA nor its enzymatic activity has yet been detected. On the other hand, the ouabain sensitivity of the rat α_3-subunit was estimated to be higher than that of the kidney-type form on the basis of the amino acid sequences deduced from the cDNAs [5]. In order to confirm that α_3 is a catalytic subunit of Na^+,K^+-ATPase and to determine its ouabain sensitivity, expression of active α_3-subunit from the cDNA is necessary.

Expression of α- or β-subunit from cDNA

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[8–11] and of both subunits from mRNAs [12] has been reported. Here, the cDNA encoding α_3 was incorporated into cultured mouse cells having a low sensitivity to ouabain and the expressed Na^+,K^+-ATPase and sodium pump activities were studied. It was confirmed that rat brain α_3 has a high sensitivity to ouabain.

2. MATERIALS AND METHODS

The recombinant plasmid of Okayama-Berg vectors containing SV40 promoter with the cDNA encoding rat brain α_3-subunit (pNAK131) was cloned as described [5]. An SV40-based expression vector carrying the neomycin resistance gene (pCDNeo) was a generous gift from Dr H. Okayama [13]. The plasmids were amplified in HB101 cells, extracted and purified by Sephacryl S-1000 chromatography.

Introduction of DNAs into BALB/c 3T3 cells was performed as described by Chen and Okayama [13]. pNAK131 (10 μg) and pCDNeo (1 μg) were mixed with 0.125 M $CaCl_2$. The mixture was added to plates of the cultured cells at the density of $2 \times 10^5/27$ cm^2. After cells had been incubated at 35°C under 4% CO_2 for 20 h, the plate was rinsed twice with Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal calf serum and cells were cultured for 2 days in the medium at 37°C under 5% CO_2. Cells were again spread at a density of $1 \times 10^5/75$ cm^2 and cultured in the same medium with 300 μg/ml of G-418 (100% potency) for 6 weeks. The medium was changed at intervals of 3–4 days.

2.1. *RNA blot hybridization*

Total RNA was isolated from cultured cells by the guanidinium thiocyanate/CsTFA method [14]. Total RNA was denatured with glyoxal and fractionated by electrophoresis in a 1% agarose gel as described by Maniatis et al. [15]. RNA was transferred to Hybond-N membrane (Amersham). The blot was prehybridized in 10 ml of 5 × SSPE, 50% formamide, 5 × Denhardt's solution, 10% dextran sulfate and 20 μg/ml of heat-denatured salmon sperm DNA for 6 h at 45°C. It was then hybridized for 20 h at 45°C in 10 ml of the solution to which 5×10^6 dpm boiled ^{32}P-labeled cDNA probe was added. The probe was a multiprimed *DdeI*(3044)/*ApaI*(3379) fragment excised from pNAK131. The filters were washed twice with 5 × SSPE at 45°C for 15 min each and once with 1 × SSPE and 0.1% SDS for 30 min at 45°C followed by a wash with 0.1 × SSPE and 0.1% SDS for 15 min at room temperature.

2.2. *Ouabain-sensitive Rb^+ uptake by cultured cells*

The cells were washed twice with 154 mM NaCl and resuspended in it. The reaction was initiated with the addition of 5 mM radioactive RbCl to around 1×10^5 cells in a final volume of 100 μl. Uptake was carried out at 37°C for 10 min with shaking. The time course of transport was linear up to at least 15 min.

2.3. *NaI treatment of cultured cell homogenate*

Pelletted BALB/c 3T3 cells were homogenized in 20 vols of 5 mM EDTA, 20 mM Tris-HCl (pH 8.0) with a glass/Teflon homogenizer. The homogenate was centrifuged at 3100 × g for 10 min. The supernatant was treated with 2 M NaI according to Nakao et al. [16].

ATPase activity was assayed at 37°C in a reaction medium containing 140 mM NaCl, 14 mM KCl, 5 mM $MgCl_2$, 3 mM ATP (imidazole), 0.5 mM EDTA (imidazole), pH 7.4, and 10–20 μg NaI-treated homogenate in a final volume of 0.5 ml. Ouabain-insensitive activity was measured in the presence of 2 mM ouabain.

Protein concentration was determined by the method of Lowry et al. [17].

3. RESULTS

3.1. *Incorporation of rat α_3 cDNA into 3T3 cells*

SV40-based expression vectors (Okayama-Berg vectors) carrying the cDNA encoding the α_3-subunit of rat Na^+,K^+-ATPase were introduced into BALB/c 3T3 cells by the calcium phosphate coprecipitation method as described in section 2. Cells were cotransfected with 10 μg pNKA131 DNA and 1 μg pcDNeo DNA. We obtained 48 neomycin-resistant clones from 2×10^5 3T3 cells initially. From RNA blot analysis using a 3′-untranslated region of the α_3 cDNA as a probe, 17 positive clones were selected. An autoradiogram of Northern blot analysis of total RNA from a positive clone (T3-3-3), wild-type BALB/c 3T3 and rat brain is shown in fig.1. Positive bands were detected in lane 1 (rat brain) and lane 2 (T3-3-3 cells), but not in lane 3 (untransfected BALB/c 3T3 cells). The size of the mRNA from transfected cells was larger than that from rat brain.

3.2. *Effect of ouabain concentration on Na^+,K^+-ATPases from 3T3 and T3-3-3 cells*

Cell homogenates were treated with NaI in order to reduce the ouabain-insensitive ATPase fraction. Na^+,K^+-ATPase activities in NaI-treated homogenates from 3T3 and T3-3-3 cells were 2.3 and 4.1 μmol P_i/mg per h, respectively. Ouabain sensitivity was 78.1% in 3T3 cells and 87.6% in T3-3-3 cells. The NaI-treated 3T3 homogenate showed a monophasic response to ouabain (fig.2). The calculated K_i for ouabain was 4.5×10^{-5} M. On the other hand, the NaI-treated T3-3-3 cell homogenate showed at least two phases in its response to ouabain, as shown in fig.2. The calculated K_i values were 4.5×10^{-5} and 8.0×10^{-8} M.

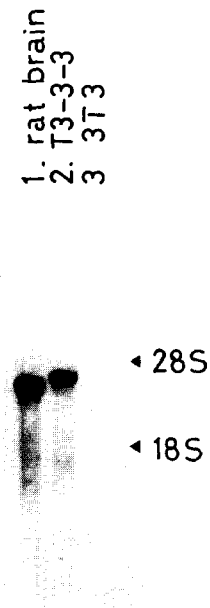

Fig.1. Northern blot analysis of mRNA encoding the α_3-subunit of rat Na^+,K^+-ATPase. 10 μg total RNAs from rat brain (lane 1), transfected cell clone T3-3-3 (lane 2) and wild-type 3T3 (lane 3) were hybridized with an α_3-specific probe isolated from the 3'-untranslated region of α_3 cDNA.

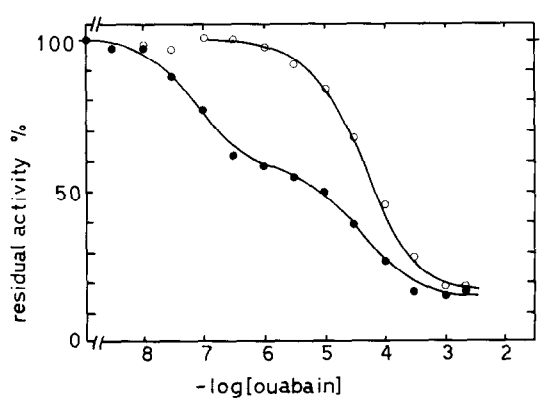

Fig.2. High ouabain sensitivity of Na^+,K^+-ATPase from transfected cells. Cells were homogenized and treated with NaI as described in section 2. Calculated K_i values were 4.5×10^{-5} M in control cells (○—○) and 4.5×10^{-5} and 8×10^{-8} M in transfected cells (●—●). The curves were drawn based on the K_i values.

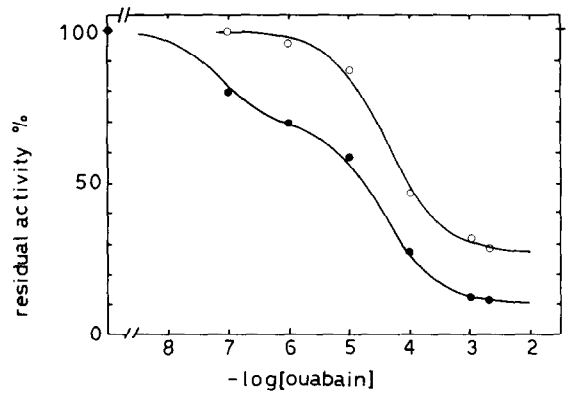

Fig.3. High ouabain sensitivity of Rb^+ uptake by mouse 3T3 cells expressing rat Na^+,K^+-ATPase α_3-subunit. Rb uptake was measured as described in section 2. Calculated K_i values were 4×10^{-5} M in control cells (○—○), and 4×10^{-5} and 8×10^{-8} M in transfected cells (●—●). The curves were drawn based on the K_i values.

3.3. *Ouabain sensitivity of Rb^+ uptake by cultured cells*

The effect of ouabain concentration on transport was measured using a transfected cell clone, T3-3-3, and wild-type cells. The transfected cells showed a biphasic response to ouabain, as shown in fig.3. The K_i values were calculated to be 4×10^{-5} and 8×10^{-8} M. On the other hand, wild-type cells showed a monophasic response to ouabain with a K_i of 4×10^{-5} M.

4. DISCUSSION

Mouse 3T3 cells were transfected with SV40-based plasmid vector carrying cDNA encoding the α_3-subunit of rat Na^+,K^+-ATPase. In Northern blot analysis, 17 positive clones were obtained from 2×10^5 cells. A positive clone, T3-3-3, was used for analysis of the ouabain sensitivity of Na^+,K^+-ATPase and sodium pump activity. (i) Transfected cells had almost twice as much Na^+,K^+-ATPase activity as wild-type cells. (ii) Na^+,K^+-ATPase in transfected cells showed an at least biphasic response to ouabain ($K_i = 8 \times 10^{-8}$, 4.5×10^{-5} M), while that in wild-type cells was monophasic ($K_i = 4.5 \times 10^{-5}$ M). (iii) From Rb^+ transport rates, wild-type cells had only a weakly ouabain-sensitive fraction but T3-3-3 cells showed both weakly and highly sensitive fractions. These results indicate that the increased Na^+,K^+-ATPase

in transfected cells should be highly sensitive to ouabain ($K_i = 8 \times 10^{-8}$ M). As T3-3-3 cells contained α_3 mRNA, it is likely that the highly sensitive fraction is the expressed activity from α_3 cDNA.

The size of mRNA in transfected cells was larger than that in rat brain, as shown in fig.1. This indicates that the mRNA in transfected cells is initiated upstream of the original transcriptional site, probably at the early region promoter sites [18].

A common structure for the highly ouabain-sensitive α-subunit was proposed and high ouabain sensitivity of the α_3-subunit was suggested from its amino acid sequence [5]. The present results confirmed the proposal that the presence of residues Arg 113 and/or Asp 124 in rat α_1 instead of Gln 113 and/or Asn 124 in rat α_2, rat α_3 and α_1 of other species is important for high ouabain sensitivity. On the other hand, rat α_1 contained Pro 120-Pro 121 in the M1-M2 junction. As proline is a well-known helix breaker and the Pro-Pro structure is expected to be far from α-helical [19], the Pro-Pro structure in rat α_1 may play some role in the low ouabain sensitivity.

The localization of α_3 in rat tissues was extensively studied. The α_3 mRNA has been reported to occur in brain, neonatal heart, gastric mucosa and lung [7]. From the present results, α_3 is strongly suggested to be a highly ouabain-sensitive catalytic subunit of Na^+,K^+-ATPase. Thus, a part of the highly ouabain-sensitive Na^+,K^+-ATPase activity in rat brain or cardiac tissue might be due to the α_3-subunit as well as the α_2-subunit.

Highly ouabain-sensitive ATPase activity was expressed from α_3 cDNA without addition of rat β cDNA. This indicates that a hybrid promoter of Na^+,K^+-ATPase between rat α_3 and mouse (3T3 cells) β-subunit was formed, as suggested by Takeyasu et al. [9,10]. It is therefore suggested that the ouabain sensitivity is determined by the properties of the α-subunit and not by those of the β-subunit.

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