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Does Binding of Ouabain to Human α1-Subunit of Na^+,K^+-ATPase Affect the ATPase Activity of Adjacent Rat α1-Subunit?

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ABSTRACT—To ascertain whether ouabain binding to human α1-subunit influences coexpression of rat α1-subunit, the ouabain-sensitive profiles of Na^+,K^+-ATPase activity and $^{86}Rb^+$ uptake activity and ouabain binding capacity were measured in HeLa cells stably expressing rat α1-subunit. The ouabain-sensitive profile of ATPase and $^{86}Rb^+$ uptake activity seemed to be the sum of two components, one with high and one with low apparent affinity to ouabain, which were similar to that observed in HeLa and NRK-52E cells derived from human and rat, respectively. The ATPase activity with low sensitivity to ouabain increased in simple proportion to the amount of the rat α1 mRNA derived from transfected cDNA, which was determined by the reverse transcription-polymerase chain reaction method. The turnover number of the human Na^+,K^+-ATPase activity obtained from the ratio of the Na^+,K^+-ATPase activity to the ouabain binding capacity is about 150/sec. The expression of the rat α1-subunit had no effect on the turnover numbers of the Na^+,K^+-ATPase activity with high affinity to ouabain estimated from the ouabain binding capacity as the active site concentration. These results suggested that the ouabain bound to human α1-subunit did not inhibit the ATPase activity of the coexpressing rat α1 in these cells.

Keywords: HeLa cell, Na^+,K^+-ATPase, Ouabain binding, Phosphorylation, Rb^+ uptake

The sodium pump ATPase is an ubiquitous membrane protein that couples the free energy from the hydrolysis of ATP to the countertransport of Na^+ and K^+ across the plasma membrane. The enzyme is composed of a catalytic α-subunit and a glycosylated β-subunit. The α-subunit is a 100-kDa multispanning membrane protein and contains the binding sites for ATP and the specific inhibitor ouabain. The function of the β-subunit remains unknown, but it is required for functional expression of normal enzymatic activity (1–3).

Previous investigations (4, 5) support an $\alpha\beta$ ratio of 1 : 1 for the functional units. Several studies (6–10) have suggested that the sodium pump ATPase exists as an oligomer of $\alpha\beta$ protomers within the plasma membrane. The functional role of the oligomeric structure is still under debate (11–14). It has been reported that the solubilized Na^+,K^+-ATPase remains protomeric during turnover and has the same catalytic property as the membrane-bound enzyme (15) or both $\alpha\beta$ and $(\alpha\beta)_2$ are active and under dynamic equilibrium (16).

The α- and β-subunits of Na^+,K^+-ATPase are encoded by multigene families. Three isoforms for the α- (α1, α2 and α3) and β- (β1, β2 and β3) subunits have been identified (17–24), and these subunits are expressed in a tissue-specific manner (25, 26). Each isoform shows distinct kinetic behavior (27). The heterogenous isoforms of the α- and β-subunits are expressed in many cells. It was reported that heterogenous α-subunits associate into oligomers (28, 29). However, it is not known whether there is functional interaction between heterogenous α isoforms or not.

The amino acid sequence of rat α1-subunit (18) shows 96.8% identity to that of human α1 (30), but the former has a three order of magnitudes higher to ouabain than the latter (31), which may make it a very useful tool for studying the functional interaction between heterogenous α-subunits.

In this study, we stably expressed rat α1-subunit in HeLa cells derived from a human cancer and measured Na^+,K^+-ATPase activity, $^{86}Rb^+$ uptake and the amount of ouabain binding. The linear relationship between the Na^+,K^+-ATPase activity with low sensitivity to ouabain and the expression level of rat α1-subunit was observed in the stable cell lines expressing rat α1-subunit. The turnover number obtained from the ATPase activity with high sensitivity to ouabain and the ouabain binding capacity with high affinity was not changed in the

presence of rat α1-subunit. These results suggested that the interaction between human and rat α1-subunits could not be detected through differences in ouabain sensitivity of ATPase activity in HeLa cell lines stably expressing rat α1-subunit.

MATERIALS AND METHODS

Cloning of the rat Na^+,K^+-ATPase α1-subunit

Total RNA was extracted from rat kidney with TRIzol reagent (Gibco BRL Life Technologies, Gaithersburg, MD, USA), and then first strand cDNA was synthesized with a random primer using a SuperScript preamplification system from Gibco BRL Life Technologies. The DNA fragments encoding a rat Na^+,K^+-ATPase α1-subunit were amplified by PCR using three pairs of sense and antisense oligonucleotide primers: for sense, 5′-CTTTCTAGTCTCCAGCC-3′, 5′-GTGGGGTCTCCTTTGAC-3′ and 5′-CTGAGGAGCTGGATGA-3′ corresponding to nucleotides 189–205, 1460–1476 and 2264–2280, respectively, and for antisense, 5′-ACAGAGCGAACCAGGTG-3′, 5′-CAAAGACAATCTCCGTG-3′ and 5′-CTACCAGGGTAGAGTTC-3′ corresponding to nucleotides 1488–1504, 2295–2311 and 3400–3416, respectively (18). The PCR products were inserted in the pGEM-T vector (Promega, Madison, WI, USA). The sequence of the cDNA inserts was confirmed by the dideoxy-mediated chain-termination method (32).There was one base substitution at position 1683 from T to C, but the amino acid did not change. The full length cDNA (223 (*Apa* I)–3395 (*Sac* I)) was constructed from these plasmids and inserted down stream of the SRα promoter in pCDL-SRα296 vector (33) to obtain the expression plasmid pCDL-NaK.

Cell culture and transfection

HeLa cells, expressing only human α1-subunit, and NRK-52E cells, expressing only rat α1-subunit, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol./vol.) fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). HeLa cells were transfected with pCDL-NaK by the calcium phosphate precipitation method (34) with a CellPhect Transfection Kit (Pharmacia, Uppsala, Sweden) and then subjected to selection in 1 μM ouabain. The colonies of ouabain-resistant HeLa cells were picked using cloning cylinders and expanded into stable cell lines.

Determination of expression level of rat Na^+,K^+-ATPase α1-subunit by a reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from HeLa, NRK-52E or ouabain-resistant cell lines with TRIzol reagent, treated

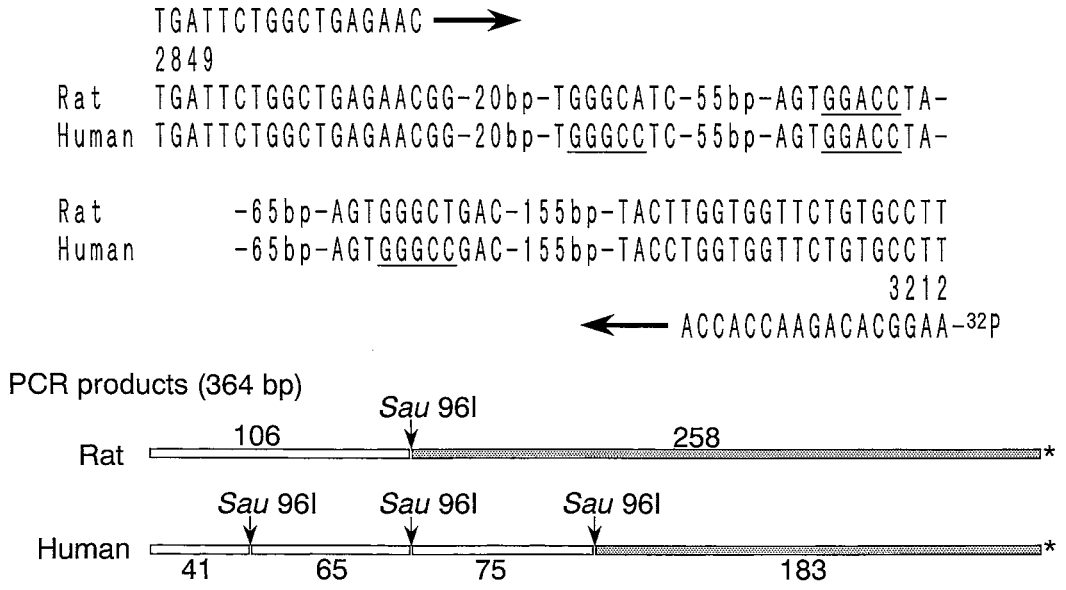

Fig. 1. Strategy for the determination of the expression ratio of human to rat Na^+,K^+-ATPase α1-subunit by RT-PCR. The nucleotide sequences of rat (2849–3212) and human (2930–3293) α1-subunits were taken from refs. 18 and 30, respectively. A pair of PCR primers (shown at the top and bottom lines, respectively) corresponding to nucleotides 2849–2865 and 3196–3212 of rat α1-subunit cDNA were designed (18). These regions were the same DNA sequences in both rat and human α1-subunit (30). By using these PCR primers, the 364-bp DNA fragments were amplified from both rat and human cDNA. The DNA fragment derived from rat and human cDNA contains one and three recognition sites (underlined) of restriction enzyme *Sau* 96I, respectively. When antisense primer is labeled by ^{32}P (asterisks), the 258-bp DNA fragment derived from rat cDNA is radioactive, and the 183-bp DNA fragment from human cDNA is ^{32}P-labeled (shaded bars).

with DNase I (Amp grade, Gibco BRL), and then first strand cDNA was synthesized with a oligo dT primer as described above. The DNA fragments (364 base pairs (bp)) corresponding to nucleotides 2849–3212 of rat α1-subunit (18) and 2930–3293 of human α1-subunit (30) were amplified by PCR using a pair of sense and antisense oligonucleotide primers: for sense, 5′-TGATTCTGGCT GAGAAC-3′, corresponding to nucleotides 2849–2865, and for antisense, 5′-AAGGCACAGAACCACCA-3′, corresponding to nucleotides 3196–3212 (18) (Fig. 1). The antisense primer was pre-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. After the PCR reaction, aliquots (10 μl) were incubated with 10 μl of restriction enzyme solution containing 2–4 units of *Sau* 96I for 2 hr at 37℃ and then separated on 5% polyacrylamide gel by electrophoresis. After drying the gels, the radioactivity of ^{32}P-labeled DNA fragments (258 and 183 bp fragments derived from rat and human mRNA, respectively) was quantitated with a BAS 2000 system (Fuji, Tokyo). The expression level of rat α1-subunit was calculated by the amount of radioactivity in the 258-bp fragment divided by total radioactivity. All determinations were performed in triplicate.

Isolation of leaky membrane vesicles

Crude plasma membranes were prepared essentially as described (35). To obtain the leaky vesicles, crude plasma membranes at 0.4 mg/ml were incubated with 0.65 mg/ml of sodium deoxycholate in the presence of 2 mM EDTA-Tris and 10 mM imidazole-HCl, pH 7.4 for 15 min on ice. The samples were used for characterization directly or after centrifugation at 130,000×*g* for 10 min at 4℃ followed by a sixfold dilution.

Preparation of $C_{12}E_8$-permealized cells

Cells were washed twice in phosphate-buffered saline (PBS) and detached with the Ca^{2+}- and Mg^{2+}-free Hank's solution containing 0.25% trypsin and 1 mM EDTA. The cells were washed three times by centrifugation at 300×*g* for 1 min with HEPES-buffers saline (HBS) containing 146 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES-Tris, pH 7.4. The cells were suspended in 4–6 mg/ml with HBS in the presence of 5 μg/ml trypsin inhibitor. The cells at 2 mg/ml were incubated with 0.32 mg/ml of $C_{12}E_8$ for 10 min at room temperature and then diluted fourfold with HBS.

Na^+,K^+-ATPase activity

The ATPase activity of leaky vesicles and $C_{12}E_8$-permealized cells was measured at 37℃ for 30 min in a reaction medium containing 40 mM NaCl, 16 mM KCl, 5 mM $MgCl_2$, 12.5 mM sucrose, 1 mM EDTA and 20 mM Tris-HCl, pH 7.4 in the absence or presence of various concentrations of ouabain. The reactions were stopped by adding an equal volume of 12% SDS. The colorimetric determination of inorganic phosphate with ammonium molybdate complexes was performed as described by Chifflet et al. (36). Na^+,K^+-ATPase activity was determined as the activity inhibited in the presence of 5 mM ouabain. All determinations were performed in triplicate.

Estimation of the activities with low and high sensitivity to ouabain

The activities with low (Low) and high (High) sensitivity to ouabain in stable cell lines were estimated from the Na^+,K^+-ATPase activity in the absence or presence of 5 μM or 5 mM ouabain by solving the following simultaneous equations:

$$\text{Activity (0)} = \text{High} + \text{Low} \quad \text{(Equation 1)}$$

$$\text{Activity (5 } \mu\text{M)} = 0.14 \times \text{High} + 0.96 \times \text{Low} \quad \text{(Equation 2)}$$

where Activity (0) or Activity (5 μM) is the Na^+,K^+-ATPase activity which was obtained from the difference of the activity in the absence or presence of 5 μM ouabain and that in the presence of 5 mM ouabain, respectively.

[^{3}H]Ouabain binding

Leaky vesicles (10 μg) were incubated with ouabain binding solution containing 150 nM [^{3}H]ouabain, 5 mM $MgCl_2$, 5 mM Tris-phosphate, and 40 mM Tris-HCl, pH 7.4, in the absence (total binding) or presence (nonspecific binding) of 250 μM non-radioactive ouabain for 30 min at 37℃. The samples were centrifugated at 400,000×*g* for 10 min at 4℃. The precipitates were washed with 0.5 ml of washing solution containing 5 mM $MgCl_2$, 5 mM Tris-phosphate, and 40 mM Tris-HCl, pH 7.4, and suspended in 100 μl of 1% SDS and 10 mM $Na_2B_4O_7$.

Cells were washed twice with PBS, detached with trypsin, and washed three times by centrifugation at 300×*g* for 1 min with K^+,Ca^{2+}-free solution containing 130 mM NaCl, 0.5 mM $MgCl_2$, 0.6 mM sodium phosphate, and 20 mM HEPES-Tris, pH 7.4. The cells (500 μg) were suspended with 50 μl of 500 nM [^{3}H]ouabain in K^+,Ca^{2+}-free solution in the absence (total binding) or presence (nonspecific binding) of 0.5 mM non-radioactive ouabain and incubated for 30 min at 37℃. The mixtures were washed three times by centrifugation at 2000×*g* for 1 min with K^+,Ca^{2+}-free solution, and then the precipitates were suspended with 450 μl of 1% SDS and 10 mM $Na_2B_4O_7$.

The radioactivity incorporated into the precipitates was measured by a liquid scintillation counter. Specific binding was calculated by subtracting the nonspecific binding from the total binding. All determinations were performed in triplicate.

^{86}Rb uptake

^{86}Rb uptake was measured essentially as described (37, 38). Briefly, 140 μl of cell suspension (2–2.5 mg protein /ml) was incubated with DMEM/5 mM HEPES, pH 7.4, which contains 155 mM Na^+, 5.37 mM K^+, 1.8 mM Ca^{2+}, 0.81 mM Mg^{2+} and 0.25 mM Fe^{3+}, in the presence of various concentrations of ouabain for 10 min at 37℃, and then the reaction was initiated by the addition of 7 μl of ^{86}RbCl solution (50–100 KBq, about 1 mM) in DMEM/5 mM HEPES, pH 7.4. After 5 min, the samples were centrifuged through 400 μl of the mixture containing *n*-butyl phthalate / dioctyl phthalate (2 : 1). The amount of ^{86}Rb incorporated into the pellet was counted with a liquid scintillation counter. The activity in the presence of 10 mM ouabain was subtracted from all data. All determinations were performed in triplicate.

Others

Protein concentration was determined by the method of Lowry et al. (39) with bovine serum albumin as a standard. All other chemicals were of the highest grade commercially available.

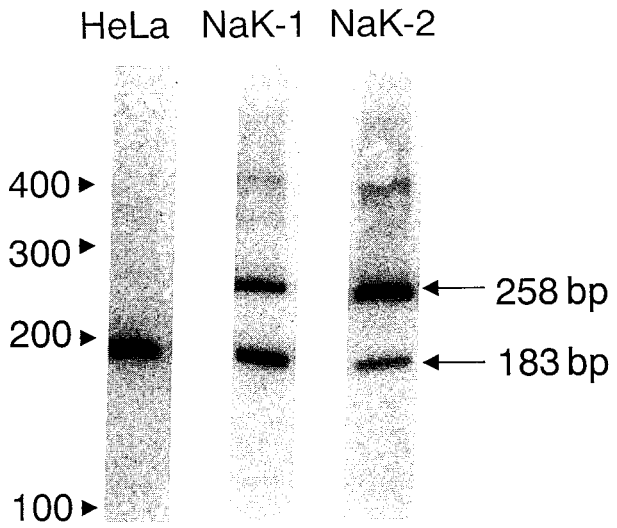

Fig. 2. Difference of *Sau* 96I site in RT-PCR products from rat and human Na^+,K^+-ATPase α1-subunits. Total RNA was prepared from HeLa cells and the ouabain-resistant cell line NaK-1 and -2, the first strand cDNA was synthesized, and then PCR amplification was carried out as described under "Materials and Methods". After PCR, the PCR products were digested with *Sau* 96I. The DNA fragments were separated on 5% polyacrylamide gel by electrophoresis. After drying the gels, the radioactivity of ^{32}P-labeled DNA fragments (258- and 183-bp fragments derived from rat and human mRNA, respectively) were detected with a BAS 2000 system.

RESULTS

Stable expression of rat α1-subunit in HeLa cells

HeLa cells were transfected with an expression plasmid pCDL-NAK, which contains the entire coding region of rat Na^+,K^+-ATPase α1-subunit. The cells were cultured in the presence of 1 μM ouabain, and sixteen stable cell lines (NaK-1–NaK-16) expressing rat Na^+,K^+-ATPase α1-subunit were obtained.

The amounts of mRNAs derived from transfected cDNA and HeLa genomic DNA were estimated by RT-PCR (Fig. 1). Total RNA was extracted from the cells and treated with DNase I. We confirmed that there is no contamination of genomic DNA, because no DNA fragment was amplified by PCR without synthesis of the first strand cDNA (data not shown). After the first strand cDNA synthesis, the specific amplification of the 364-bp DNA fragment by the PCR reaction was observed. After treatment with *Sau* 96I, a single radioactive band at position 183 bp in HeLa cells and two radioactive bands at

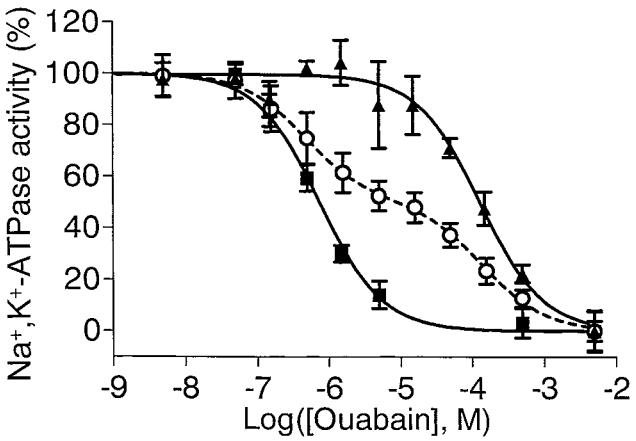

Fig. 3. Ouabain-sensitive profile of Na^+,K^+-ATPase activity. The ATPase activity in the leaky membrane vesicles from HeLa (■), NRK-52E (▲) and NaK-1 (○) cells were measured in the absence or presence of various concentrations of ouabain as described under "Materials and Methods". The results have been normalized so that the activity in the absence of ouabain or presence of 5 mM ouabain was set at 100% or 0%, respectively. Each point represents the mean±S.D. of three separate experiments, each performed in triplicate. Data were fit to the Equation 3 by computer analysis using nonlinear least squares fitting procedures.

$$\text{Activity} = (100 - \text{Low}) \times (1 - C / (IC_{50,H} + C)) + \text{Low} \times (1 - C / (IC_{50,L} + C)) \quad \text{(Equation 3)}$$

where C is the concentration of ouabain; Low is the fraction of the low sensitivity component (%); and $IC_{50,H}$ and $IC_{50,L}$ are the apparent affinity for the high and low sensitivity component, respectively. In HeLa or NRK-52E cells, Low is 0% or 100% and $IC_{50,H}$ or $IC_{50,L}$ was allowed to vary to obtain the best fit, respectively. The best fits are represented by the solid lines; and $IC_{50,H}$ and $IC_{50,L}$ are 0.84 μM and 130 μM, respectively. In NaK-1 cells, the best fit is represented by the dotted line; and Low, $IC_{50,H}$ and $IC_{50,L}$ are 50%, 0.47 μM and 142 μM, respectively.

positions 258 and 183 bp in the stable cell lines NaK-1 and NaK-2 (Fig. 2) were detected. By dividing the radioactivity of the 258-bp fragment by the total radioactivity of the 258- and 183-bp fragments, the expression level of rat α1-subunit was determined as 43.7±3.2% and 79.4±1.2% in NaK-1 and -2 cells, respectively. The expression levels of rat α1-subunit in the 16 stable cell lines ranged from 12.9% to 79.4%.

Ouabain-sensitive profile of Na^+,K^+-ATPase activity in HeLa cells, NRK-52E cells and stable cell lines

Crude membrane fraction was prepared from each cell line. The effect of varying ouabain concentration on ouabain-sensitive ATPase activity was determined. In HeLa or NRK-52E cells, the ouabain-sensitive ATPase activity seemed to comprise one component (Fig. 3, closed squares or closed triangles) with an apparent affinity of 0.84 or 130 μM, respectively. The ouabain-sensitive ATPase activity of stable cell line NaK-1 is also shown (open circles); the data fit the sum of the activities with low and high sensitivity to ouabain, and the apparent affinities were 0.47 and 142 μM. The fraction of the component with low sensitivity to ouabain was estimated to be approximately 50% of the total ouabain-sensitive activity.

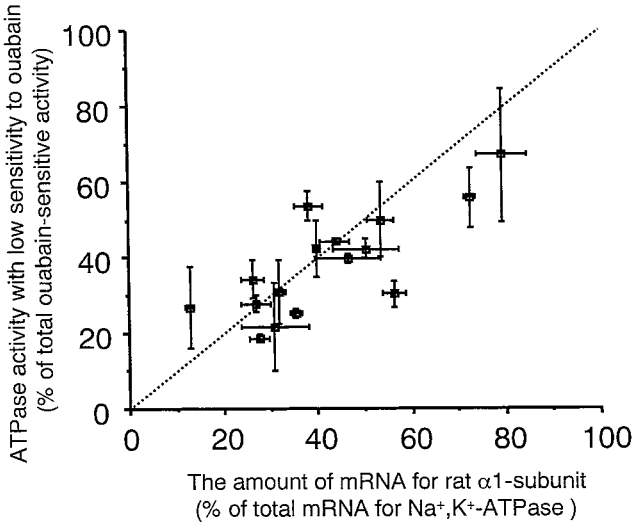

Fig. 4. The relationship between the ATPase activity with low sensitivity to ouabain and the expression level of mRNA for rat α1-subunit. We measured the expression level of mRNA for rat α1-subunit and the ATPase activity in the absence or presence of 5 μM or 5 mM ouabain as described under "Materials and Methods". We estimated the activities with low and high sensitivity to ouabain in stable cell lines from the ouabain-sensitive ATPase activities in the absence or presence of 5 μM ouabain as described under "Materials and Methods". Each point represents the mean±S.D. from three separate experiments, each done in triplicate. Correlation coefficient was calculated as 0.76 (95% confidence interval; 0.43 to 0.91, R squared; 0.58).

The fraction of the component with low sensitivity to ouabain was estimated in another way. In the presence of 5 μM ouabain, the Na^+,K^+-ATPase activity was inhibited 86% and 4% in HeLa and NRK-52E cells, respectively (Fig. 3). By the estimation described under "Materials and Methods", the ATPase activity with high and low sensitivity to ouabain in the NaK-1 cells was estimated to be 6.69±1.54 and 5.85±1.49 μmol/mg/hr (the mean± S.D., n=3), respectively. The fraction of the low sensitivity component was calculated to be 46.1±3.5% (the mean± S.D., n=3). This was consistent with the value obtained from Fig. 3. The content of the low sensitivity component in the sixteen stable cell lines was estimated to range from 18.6% to 66.9% of the total Na^+,K^+-ATPase activity.

In Fig. 4, we plotted the content of the low sensitivity component against the expression levels of mRNA for rat α1-subunit in each cell line. The former increased in simple proportion to the latter.

Turnover number of Na^+,K^+-ATPase activity with high sensitivity to ouabain estimated from the amount of high affinity ouabain binding

Little cooperativity of the ouabain-dependent inhibition of Na^+,K^+-ATPase activity is consistent with the idea that each subunit hydrolyzed ATP independent of adjacent ouabain bound. To investigate this point further, turnover numbers of Na^+,K^+-ATPase activity were estimated from the ATPase activity with high sensitivity to ouabain and the high affinity ouabain binding capacity

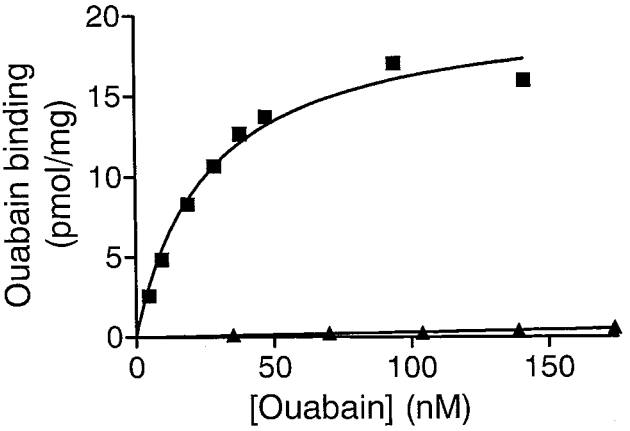

Fig. 5. Ouabain binding to crude membrane vesicles from HeLa and NRK-52E cells. Crude membrane vesicles were prepared from HeLa and NRK-52E cells and incubated with various concentrations of [^{3}H]ouabain in the absence (total binding) or presence (nonspecific binding) of 250 μM nonradioactive ouabain for 30 min at 37℃. After washing by ultracentrifugation, the radioactivity incorporated into the pellet was measured by a liquid scintillation counter. Specific binding was calculated by subtracting the nonspecific binding.

in HeLa cells and the cell lines stably expressing rat α1-subunit. If heterogenous α1-subunits of Na^+,K^+-ATPase form oligomers in HeLa cells expressing rat α1-subunit and ouabain bound to the human α1-subunit inhibits activity of the adjacent rat α1-subunit, the turnover number in HeLa cells expressing rat α1-subunit would be larger compared with that in HeLa cells, because the ATPase activity of the heterogenous complex would be inhibited at low ouabain concentrations, where only human α1-subunit bound ouabain.

Figure 5 shows the [^{3}H]ouabain binding to crude membrane preparations from HeLa and NRK-52E cells. In HeLa cells, we observed single saturable binding kinetics with an apparent dissociation constant (K_d) of 20 nM as determined by computer analysis by nonlinear least squares fitting procedures (Fig. 5). The affinity of rat α1-subunit to ouabain was too low to measure. Therefore, we measured the high affinity ouabain binding capacity at 150 nM ouabain.

The turnover number calculated from the ATPase activity with high sensitivity to ouabain and high affinity ouabain binding was about 150/sec in HeLa cells, which was similar to that in two different stable cell lines: the values were obtained from DOC-treated leaky membranes and from $C_{12}E_8$-permealized cells (Table 1). These results suggested that rat α1-subunit in the heterogenous complex was not inhibited by the ouabain bound to adjacent human α1-subunit.

Turnover number of Na^+,K^+-ATPase activity estimated from the amount of $^{86}Rb^+$ uptake

The Na^+,K^+-ATPase activity described above was measured with the detergent-treated samples. Detergent might influence subunit interaction between heterogenous α-subunits. To investigate this point further, ouabain-sensitive profiles of $^{86}Rb^+$ uptake activity in intact cells were measured. In HeLa or NRK-52E cells (Fig. 6, closed squares or close triangles), $^{86}Rb^+$ uptake activity fit to the cooperative model (Equation 4 in Fig. 6), in which the fraction of low sensitivity component is 0% or 100%, and the apparent affinity and Hill coefficient were 0.25 μM and 1.69 or 109 μM and 1.14, respectively. In the stable cell line expressing rat α1-subunit (NaK-1), the ouabain-sensitive profile was also shown to fit to the sum of the activities with low and high sensitivity to ouabain, and the apparent affinity for the high or low sensitivity component was 0.22 or 154 μM, respectively, assuming that n_H and n_L are 1.69 and 1.14, respectively.

In the presence of 3 μM ouabain, $^{86}Rb^+$ uptake was completely inhibited in HeLa cells, but not in NRK-52E cells. The $^{86}Rb^+$ uptake activity with high sensitivity to ouabain in stable cell lines was determined as the activity inhibited in the presence of 3 μM ouabain.

Turnover numbers, the ratio of $^{86}Rb^+$ uptake activity which was inhibited by 3 μM ouabain to the ouabain binding capacity in the presence of 500 nM ouabain, were smaller in the stable cell lines than in HeLa cells (Table 2).

Table 1. Turnover number of ATPase activity with high sensitivity to ouabain estimated from the ouabain binding capacity in leaky membrane vesicles and $C_{12}E_8$-permealized cells

	Na^+,K^+-ATPase activity			
	Low (μmol/mg/hr)	High (μmol/mg/hr)	Ouabain binding (pmol/mg)	Turnover No. (sec^{-1})
Leaky membrane vesicles				
HeLa		9.28±1.78	15.9±5.1	153± 2
NaK-1	3.88±0.47	6.56±0.29	11.5±0.6	161±17
NaK-2	6.90±1.91	8.57±2.35	14.0±4.3	170±31
$C_{12}E_8$-permealized cells				
HeLa		1.26±0.12	2.31±0.38	154±14
NaK-1	0.90±0.06	1.11±0.12	2.04±0.24	151± 6
NaK-2	1.33±0.14	1.07±0.04	1.88±0.17	159±12

The ATPase activities in the absence or presence of 5 μM or 5 mM ouabain were measured by using leaky membrane vesicles and $C_{12}E_8$-permealized cells, and then used to calculate the Na^+,K^+-ATPase activity with high and low sensitivity to ouabain by solving the simultaneous Equations (1 and 2) as described under "Materials and Methods". The ouabain binding capacity was measured at 150 nM ouabain as described under "Materials and Methods". Turnover number was the ratio of the ATPase activity with high sensitivity to ouabain to the ouabain binding capacity. Mean±S.D., n=3.

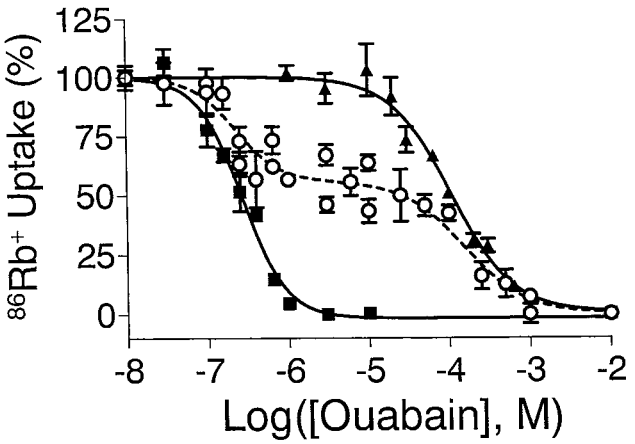

Fig. 6. Ouabain-sensitive profile of $^{86}Rb^+$ uptake activity. The $^{86}Rb^+$ uptake activities in intact HeLa (■), NRK-52E (▲) and ouabain-resistant cell line NaK-1 (○) cells were measured in the presence of various concentrations of ouabain as described under "Materials and Methods". The activities have been normalized so that the activity in the absence of ouabain or presence of 10 mM ouabain was 100% or 0%, respectively. Each point represents the mean±S.D. of experiments performed in triplicate. Data were fit to Equation 4 by computer analysis using nonlinear least squares fitting procedures.

$$\text{Activity} = (100 - \text{Low}) \times (1 - C^{nH} / (IC_{50,H}{}^{nH} + C^{nH})) + \text{Low} \times (1 - C^{nL} / (IC_{50,L}{}^{nL} + C^{nL})) \quad \text{(Equation 4)}$$

where C is the concentration of ouabain; Low is the fraction of the low sensitivity component (%); and $IC_{50,H}$ or $IC_{50,L}$ and n_H or n_L are the apparent affinity and Hill coefficient for the high or low sensitivity component, respectively. In HeLa or NRK-52E cells, Low is 0% or 100%, respectively, and then $IC_{50,H}$, $IC_{50,L}$, n_H and n_L were allowed to vary to obtain the best fit. The best fits are represented by the solid lines; and $IC_{50,H}$, $IC_{50,L}$, n_H and n_L are 0.25 μM, 109 μM, 1.69 and 1.14, respectively. In NaK-1 cells, n_H and n_L are fixed to 1.69 and 1.14, respectively, and then Low, $IC_{50,H}$ and $IC_{50,L}$ were allowed to vary to obtain the best fit. The dotted line represents the best fit; and Low, $IC_{50,H}$ and $IC_{50,L}$ are 56%, 0.22 μM and 154 μM, respectively.

DISCUSSION

To estimate the expression level of the rat α1-subunit accurately, we used a method combining the RT-PCR with restriction enzyme digestion using mRNA for the endogenous human α1-subunit as a standard. The efficiency of the PCR-amplification was the same, because the DNA fragments from mRNAs for human and rat α1-subunits were amplified with the same primers. The DNA fragments derived from human and rat mRNAs were clearly separated on acrylamide gel electrophoresis after the digestion with *Sau* 96I (Fig. 2).

The sequences flanking the initiation ATG codon are very important for translational efficiency (40). The sequences of the rat α1-subunit mRNA are the same as those for the human one in this region (from −8 to +20) (18, 30). Therefore, we assumed that the efficiency of these mRNAs in the translation step was the same and the ratio of the mRNAs reflected the ratio of the active ATPase proteins.

The ouabain-sensitive profile of ATPase (Fig. 3) and $^{86}Rb^+$ uptake activity (Fig. 6) in the cells stably expressing rat α1-subunit fit to the sum of two components with high and low sensitivity to ouabain, which were defined from HeLa or NRK-52E cells, respectively. These results suggested that the affinity to ouabain did not change on coexpression of heterogenous α1-subunits.

Cooperativity in ouabain binding on the inhibition of Na^+,K^+-ATPase activity was not observed (Fig. 3). Figure 4 shows that the Na^+,K^+-ATPase activity with a low sensitivity to ouabain increased in simple proportion to the expression level of rat α1-subunit. In Table 1, the turnover number of the ATPase activity estimated from ouabain binding capacity did not change on coexpression of human and rat α1-subunits. These data indicate that

Table 2. Turnover number of $^{86}Rb^+$ uptake activity with high sensitivity to ouabain estimated from the ouabain binding capacity in intact cells

	$^{86}Rb^+$ uptake activity			
	Low (nmol/mg/hr)	High (nmol/mg/hr)	Ouabain binding (pmol/mg)	Turnover No. (sec^{-1})
HeLa		16.2±4.6	2.19±0.43	2.33±0.74
NaK-1	11.2±1.93	10.2±2.9	1.58±0.59	1.88±0.34
NaK-2	15.1±1.80	4.3±1.5	1.47±0.25	0.85±0.32

The ouabain binding capacity at 500 nM ouabain and the $^{86}Rb^+$ uptake activity in the absence or presence of 3 μM or 10 mM ouabain were measured as described under "Materials and Methods". The high sensitivity component of the $^{86}Rb^+$ uptake activity was the activity inhibited in the presence of 3 μM ouabain. The low sensitivity component was calculated by subtracting the amount of high sensitivity component from the activity inhibited in the presence of 10 mM ouabain. The turnover number was estimated from the ratio of the high sensitivity component of $^{86}Rb^+$ uptake activity to the ouabain binding capacity. Mean±S.D., n=4.

ouabain bound to human α1-subunit did not inhibit the ATPase activity of rat α1-subunit in these cells.

A stable association between rat α1- and α2- or α3-subunits was demonstrated in rat brain and baculovirus expression system by coimmunoprecipitation (28, 29). The amino acid identity between rat α1 and α2 or α3 is 86.3% or 85.2%, respectively (18). The amino acid sequence of the rat α1-subunit (19) shows 96.8% identity to that of the human α1-subunit (30). Koster et al. (10) have reported that a cytoplasmic region in the rat α1-subunit (Gly^{554}-Pro^{785}) is necessary for α/α association. The amino acid identity in this region between rat and human α1-subunits is 97.0% and higher than that between rat α1 and α2 or α3 (91.4% or 91.8%, respectively). Therefore, it seemed likely that the oligomeric complexes consisting of heterogenous α1-subunits are formed in the stable cell lines. These considerations suggested that rat α1-subunit hydrolyzed ATP independent of adjacent ouabain bound human α1-subunit.

The apparent turnover numbers of $^{86}Rb^+$ flux 0.9–2.3/sec (Table 2) were two orders of magnitude smaller than those of Na^+,K^+-ATPase activity, 151–170/sec (Table 1), and that of the theoretical value of K^+ flux of HeLa cell, 306/sec (2 mol K^+/mol ATP hydrolysis (153/sec)). This was due to the fact that Rb^+ flux was measured in the presence of 5.37 mM K^+ and 0.047 mM Rb^+. For simplicity, we assumed that the enzymes of HeLa cell transported K^+ and Rb^+ with similar affinity, although Rb^+ has a 1.5-fold higher affinity than K^+ to dephosphorylate K^+-sensitive-phosphoenzyme (41). The apparent turnover number of ($K^+ + Rb^+$) flux should increase by 115- (5.37/0.047 + 1) fold, namely to 268, 216 and 98/sec for HeLa, NaK-1 and NaK-2 cells, respectively. The results suggested that the apparent efficiency of cation transport activity with high affinity to ouabain was reduced especially in NaK-2 cells.

It is possible to estimate the active fraction or the efficiency of Na^+,K^+-ATPase during ($K^+ + Rb^+$) flux in another way. The total amount of K^+ transport supported by Na^+,K^+-ATPase under V_{max} conditions would be twice that of ATP hydrolysis. The ratio of 115-fold of total $^{86}Rb^+$ uptake activity (Table 2) to 2-fold of total Na^+,K^+-ATPase activity of permeabilized cells (Table 1) was 0.74, 0.61 and 0.46, for HeLa, NaK-1 and NaK-2 cells, respectively. These results suggest the possibility that the increase in the amount of Na^+,K^+-ATPase induced changes in ionic balances in the cell and/or in the rate determining step of ($Rb^+ + K^+$) transport, causing decrease in the apparent turnover number of Rb^+ flux.

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