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The A2 isoform of rat Na^+,K^+-adenosine triphosphatase is active and exhibits high ouabain affinity when expressed in transfected fibroblasts

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The α isoforms of the Na^+,K^+-ATPase (Na^+ pump) are expressed with developmental and tissue heterogeneity in rodents and possess different sensitivity to inhibition by ouabain. We directly characterized the ouabain sensitivity of the rat A2 (α2) isoform by transfecting NIH 3T3 cells with rat A2. The treated cells exhibit high affinity (40 nM) ouabain binding with a density of 2 pmol/mg protein. $^{86}Rb^+$ flux studies confirm that A2 is functional in this system and that A2 is inhibited by submicromolar concentrations of ouabain. These findings are consistent with measurements of ouabain affinity in tissues which express the A2 isoform.

Na^+,K^+-ATPase; $^{86}Rb^+$ flux; Cardiac glycoside; α2 Isoform

1. INTRODUCTION

The Na^+,K^+-ATPase (Na^+ pump) is a multi-subunit protein essential for the maintenance of the transmembrane ionic gradient in cells, via exchange of three Na^+ ions for two K^+ ions. this enzyme is composed of a catalytic α subunit (~112,000 Da) and a smaller β subunit (~35,000 Da). Currently three α-subunit isoforms are recognized, A1 (α1), A2 (α2), and A3 (α3), and the results of recent work indicate a remarkable heterogeneity of tissue and developmental expression of these isoforms [1,2]. A1 is ubiquitous and is especially abundant in transport epithelia such as kidney; A2 is found predominantly in muscle and brain, as well as in adipose tissue. A3 is appreciably expressed only in neural tissue, as well as in fetal heart [3–7]. Because the marked variation in tissue distribution may be related to functional differences among the isoforms, characterization of their individual transport properties remains an important goal of ongoing studies [8].

The α subunit of the Na^+ pump contains an extracellular binding site for ouabain and other cardiac glycosides, which inhibit enzymatic activity [1,2]. One functional aspect in which the isoforms markedly differ is in a species-dependent difference of inhibition by cardiac glycosides. Initial studies using gel electrophoresis identified two molecular forms in rat brain characterized by either a high affinity ('α+') or low affinity ('α') for ouabain [9]. Following the molecular cloning of the Na^+,K^+-ATPase and the identification of three discrete α isoforms, it became apparent that the previously identified 'α' isoform was distinct (A1), while the 'α+' isoform represented both the A2 and A3 isoforms [10]. These two isoforms are considered to have high affinity for ouabain, although direct determination of the ouabain sensitivity of each isoform has been difficult since A2 and A3 are generally co-expressed in the same cells. The A3 isoform has been demonstrated to have a high affinity for ouabain by selective trypsinization of A2 [11] as well as through transfection studies [12]. Several lines of evidence have suggested that the rat A2 isoform is also highly ouabain sensitive. First, murine C2C12 myotubes express A2 and bind ouabain with high affinity [13]. Second, A2 can be made ouabain resistant by chimera construction or site-directed mutagenesis, and expressed in mammalian cells [8,14]. Third, previous work has indicated that A2 is a major isoform in skeletal muscle [4,5] and a correlation exists between the amount of A2 present in this tissue and high affinity binding of ouabain [15]. Further, Kjeldsen [16] has shown that there is a single component of high affinity binding in rat skeletal muscle, which is likely to be due to the presence of A2.

To examine further the ouabain sensitivity of rat A2 we transfected the A2 gene into a cell line which normally expresses only A1, and pharmacologically assessed the resultant Na^+ pump expression. Here we provide additional evidence that the rat A2 isoform is a distinct Na^+,K^+ pump, and that it is highly sensitive to ouabain with an affinity similar to the A3 isoform.

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2. MATERIALS AND METHODS

2.1. *Construction of expression vector*

An expression vector encoding rat A2 isoform of Na^+,K^+-ATPase was constructed as follows. A Bluescript (KS+) (Stratagene, La Jolla, CA) plasmid containing the cDNA encoding the rat A2 isoform was linearized with *Hin*dIII, and the 3' polyA tail of the cDNA was removed using BAL-31 nuclease (New England Biolabs, Beverly, MA). *Hin*dIII linkers were added and the plasmid was re-ligated and used to transform DH5α competent cells (BRL, Gaithersburg, MD); oligonucleotide sequencing (Sequenase; USB, Cleveland OH) confirmed that the 3' end of the A2 coding region still contained the region coding for the protein stop site. The 3.1 kb coding region was released by *Eco*RI digestion.

The plasmid vector pMT-21 (kindly supplied by Genetics Institute, Cambridge, MA) which contains a single *Eco*RI restriction site was linearized by *Eco*RI digestion, treated with calf intestinal alkaline phosphatase, and ligated to the A2 cDNA fragment. This was propagated in DH5α competent cells to generate the 7.8 kb plasmid pMT21-A2 (Fig. 1). Restriction analysis confirmed that the clone contained the A2 cDNA in the proper orientation.

2.2. *Cell culture and DNA transfection*

NIH 3T3 cells (obtained from ATCC, Rockville, MD) were grown in Dulbecco's modified Eagle medium (DME) supplemented with glutamine, penicillin, streptomycin, and 10% fetal calf serum (Gibco, Grand Island, NY). For transfection pMT21-A2 was linearized with *Nde* and co-transfected at a 20:1 molar ratio with circular pSV2neo by the modified calcium phosphate precipitation technique described by Chen and Okayama [17], using a commercial transfection kit (Stratagene). After 48 h cells were split 1:6 into 150 mm dishes, and after an additional 24 h the medium was replaced with medium containing the antibiotic G-418 (Geneticin; Gibco) at 400 μg/ml to select stable transfectants. Individual G-418-resistant colonies were subsequently isolated and maintained in G-418 containing medium.

2.3. *RNA isolation and blot analysis*

Total RNA was prepared from confluent cell cultures by the guanidinium isothiocyanate CsCl gradient method [18]. RNA was separated by 1% agarose/formaldehyde gel electrophoresis, transferred to Gene Screen Plus nylon membranes (New England Nuclear, Boston, MA), and hybridized as recommended by the membrane supplier. cDNA probes were labeled with [α-^{32}P]dCTP using a multipriming kit (Amersham Corp., Arlington Heights, IL). After initial hybridization, blots were re-probed with [α-^{32}P]human γ-actin cDNA to assure equivalency of RNA loading.

2.4. *Membrane preparation and Western blot analysis*

To identify expression of A2 protein by transfectants crude microsomal preparations were prepared in a simplified method analogous to that of Jørgensen and Skou [19]. Cells were grown to confluence, washed twice with phosphate-buffered saline (PBS) at 4°C, and collected using a rubber policeman in 4 ml of an isotonic buffer [20] (100 mM KCl, 3 mM NaCl, 1 mM ATP(Na_2), 3.5 mM $MgCl_2$, 10 mM PIPES, 1 mM EGTA, pH 7.3) containing protease inhibitors (aprotinin 1 μg/ml, leupeptin 2 μg/ml, PMSF 0.5 mM). They were pelleted in a micro-centrifuge (RCF 82 × g, 5 min), resuspended in a small volume and then disrupted in a dounce B homogenizer. The nuclear material was pelleted (RCF 735 × g, 5 min), and the supernatant recentrifuged (RCF 16000 × g, 15 min) to yield a crude microsomal pellet which was resuspended in a small volume. Aliquots of this preparation were separated by electrophoresis on a 6.25% SDS-polyacrylamide gel in a Laemmli buffer system [21]. Proteins were electrophoretically transferred to a nitrocellulose membrane and reacted with the monoclonal antibody McB2 (kindly supplied by K. Sweadner) which is specific for the A2 isoform of the Na^+,K^+-ATPase [10]. Colorimetric development of the Western blot by alkaline phosphatase was carried out by standard procedures using a commercial kit (Promega, Madison, WI).

2.5. *Ouabain binding studies*

To assess ouabain affinity and functional characteristics of the A2 isoform, both intact cell ouabain binding studies and $^{86}Rb^+$ flux studies were performed.

Binding of ouabain to intact cells was accomplished using a technique modified from previously published methods [13,22]. Briefly, 1×10^6 cells were seeded per 35 mm well on multi-well tissue culture plates and grown to near confluence overnight at 37°C, 5% CO_2, in DME-10% FCS. The culture medium was removed and the cells washed twice with 2.5 ml of K^+- and Ca^{2+}-free HEPES-NaOH-buffered saline solution at 37°C (HBSS: 20 mM HEPES-NaOH, 130 mM NaCl, 0.5 mM $MgCl_2$, 0.2 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, pH 7.4). The cells were then incubated with 0.5 ml of HBSS containing 25 nM to 400 nM [^{3}H]ouabain (18.0 Ci/mmol ouabain; Amersham) in the absence (total binding) or presence (non-specific binding) of 400 μM unlabeled ouabain for 30 min at 37°C, since preliminary experiments established that specific binding saturated by 30 min and remained stable for at least 90 min under the K^+-free conditions employed. Following incubation, the [^{3}H]ouabain HBSS was removed, and the cells washed twice with 2.5 ml of HBSS at 4°C for 10 min each. The cells were removed from the wells with a rubber policeman in 0.4 ml HBSS, and radioactivity was determined by liquid scintillation counting. Specific [^{3}H]ouabain binding was calculated by subtracting non-specific binding from total binding. Parallel wells were washed and incubated identically with HBSS, and aliquots were used for protein determination using the BCA protein assay (Pierce, Rockford, IL). Binding analysis was performed using the EBDA program adapted by McPherson [23] from Munson and Rodbard [24].

For dissociation experiments, multiple sets of plates were incubated with 200 nM [^{3}H]ouabain for total binding or non-specific binding as above, and individual wells analyzed at time points prior to and subsequent to addition of 200 μM unlabeled ouabain.

2.6. *Transport studies*

K^+ uptake into stable transfectants and control cells was assessed using $^{86}RB^+$ as a tracer, as modified from published methods [25]. Briefly, $0.3–0.6 \times 10^6$ cells were seeded 24–48 h prior to study onto 35 mm tissue culture dishes, and maintained at 37°C, 5% CO_2. The cells were washed twice and then pre-incubated for 60 min at 37°C, 5% CO_2, with 1 ml of K^+-free Earle's solution with choline chloride substituting for KCl, in order to facilitate internal Na^+ loading and subsequent stimulation of the Na^+,K^+-ATPase ('preload solution'): 140 mM NaCl, 25 mM HEPES, 5.4 mM choline chloride, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5 mM dextrose, buffered to pH 7.4 with 1 M Tris-HCl). At 60 min the preload solution was aspirated and 1 ml of K^+-containing Earle's solution (5.4 mM K^+) also containing 1–2 μCi/ml of $^{86}RB^+$ (Amersham) and varying concentrations of ouabain, was added to the cells ('uptake solution'). Preliminary studies indicated that as much as 70% of K^+ uptake was not inhibited by ouabain, but was virtually abolished by the addition of bumetanide (100 μM in DMSO; Leo Pharmaceutical Products, Denmark), which inhibits the $Na^+/K^+/2Cl^-$ cotransporter [26]. Therefore all subsequent studies were carried out in the presence of 100 μM bumetanide to allow specific assessment of the Na^+ pump. Uptake proceeded for 2–6 min at 37°C and was terminated by aspirating the uptake buffer and rinsing quickly 3 times with 0.1 M $MgCl_2$ at 4°C. After the cultures were air-dried 1 ml of 10% TCA was added and aliquots removed for radioactivity determination by liquid scintillation counting. Uptake was expressed as nmol of K^+ per 10^6 cells based on the known K^+ concentration of the uptake buffer. Parallel plates were handled in the same manner and cell number determined using a Coulter counter (Coulter Electronics Inc., Hialeah, FL).

3. RESULTS

3.1. *Expression of A2 Isoform RNA in transfected cells*

The plasmid vector pMT21 is an adeno-driven vector capable of high expression in mammalian cells [27], and

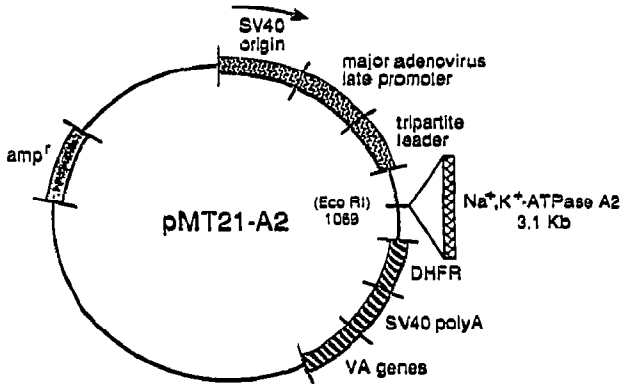

Fig. 1. Structure of expression vector pMT21-A2. A 3.1 kb cDNA fragment encoding rat Na^+,K^+-ATPase A2 isoform was inserted at the *Eco*RI site of the vector pMT21. The plasmid contains an SV40 origin and enhancer segment, the adenovirus major late promotor, and other eukaryotic regulatory elements designed to enhance expression in mammalian cells [23].

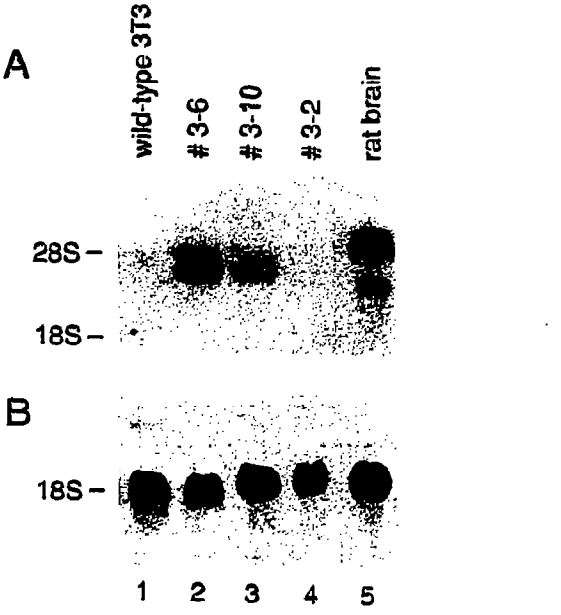

Fig. 2. Northern blot hybridization. Total RNA (10 μg/lane) prepared from wild-type NIH 3T3 cells, from stable clones derived from NIH 3T3 cells transfected with pMT21-A2, and from rat brain was separated by gel electrophoresis and transferred to a nylon membrane. (A) Hybridization with rat Na^+,K^+-ATPase A2 isoform cDNA. A2 RNA is not expressed in either wild-type 3T3 cells or clone no. 3-2. A2 RNA is expressed with a single transcript at ~4 kb in clones no. 3-6 and no. 3-10. Specificity is confirmed by the presence of signal in rat brain (with 2 characteristic transcripts at ~3.4 and ~5.3 kb). (B) Hybridization with human γ-actin cDNA. A single band of approximately equal intensity is seen in each lane. The positions of the 28 S and 18 S rRNAs are indicated.

we have used it to develop a stable expression line in NIH 3T3 cells. A new expression vector encoding the rat A2 isoform of Na^+,K^+-ATPase was constructed and designated pMT21-A2, and was used for transfection (Fig. 1). As described above, in NIH 3T3 cells which were co-transfected with pSV2neo, we obtained 18 G-418-resistant clones after 4–6 weeks and prepared RNA from them at that time. Fig. 2A demonstrates a Northern blot of RNA from transfected and control cell lines, probed with rat A2 cDNA, under conditions which eliminate cross-hybridization with other highly homologous Na^+,K^+-ATPase isoforms [6]. Lane 1 shows the absence of A2 RNA in wild-type NIH 3T3 cells, while lanes 2 and 3 indicate the presence of A2 RNA in two stable clones (clones no. 3-6 and no. 3-10). Lane 4 demonstrates that A2 is not expressed in another of the selected clones (clone no. 3-2). Lane 5 shows RNA from rat brain which is known to express A2 [4]. Note that the A2-expressing transfected cells all have a single A2 transcript (~4 kb) resulting from expression of the transfected plasmid, while brain has two characteristic A2 transcripts at ~3.4 and ~5.3 kb. Of note, Western analysis (see below), showed corresponding presence of protein in clones no. 3-6 and no. 3-10, and absence in no. 3-2.

3.2. *Protein expression in transfected cells*

To demonstrate that A2 mRNA presence was associated with appearance of mature A2 protein at the cell membrane, Western blot analysis was performed on crude microsomes prepared from a series of the stable cell clones. To accomplish this, we used rat monoclonal antibody McB2, which reacts specifically with A2 isoform protein [10]. As noted above, 18 independent G-418-resistant clones were obtained 4–6 weeks after co-transfection; all were analyzed for presence of A2 protein, and 7 clones were positive. Fig. 3 shows a composite of Western blots examining 5 of the clones. Lanes 1, 3, 4 and 5 show a band at about 112,000 M_r consistent with the A2 isoform; this band is similarly present in lane 8 which contains microsomes from rat brain. Note the absence of reactive protein in lane 2 (clone no. 3-2), lane 6 (wild-type NIH 3T3 cells) and lane 7 (rat kidney). Similar results were obtained with analysis of the transient transfection assays (data not shown).

Based on the protein and RNA presence, subsequent studies of Na^+,K^+-ATPase activity were performed mainly using positive clone no. 3-6. Control studies were performed with both the transfected but non-expressing clone no. 3-2, as well as with wild-type NIH 3T3 cells.

3.3. *Measurement of [^{3}H]ouabain binding in transfected and control cells*

Clone no. 3-2, which did not express A2, exhibited virtually no specific binding during incubation with [^{3}H]ouabain at concentrations ranging from 0–400 nM ouabain, as expected (Fig. 4). Similar results were observed using wild-type NIH 3T3 cells (data not shown). In contrast, transfected clone no. 3-6 showed significant ouabain binding even at low concentrations of ouabain (25 nM). There was a progressive increase in specific binding with increasing ouabain concentrations, which

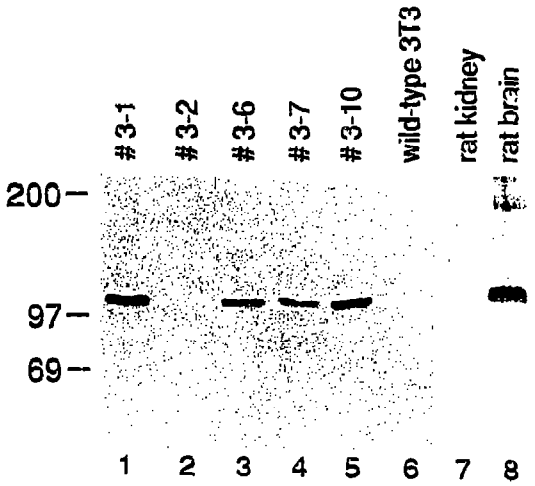

Fig. 3. Protein expression of transfected and wild-type cells. Crude microsomes were prepared from stable cell clones derived from NIH 3T3 cells transfected with pMT21-A2, wild-type 3T3 cells, and rat kidney and brain. Proteins were separated on a 6.25% SDS-polyacrylamide gel, transferred to a nitrocellulose filter, and stained with monoclonal antibody to A2 isoform (McB2) at 1:750. A band at about 112,000 M_r consistent with A2 isoform is present in microsomes from four clones, as well as from rat brain. This protein is absent in preparations from one clone, wild-type cels, and rat kidney. Subsequent studies on A2 binding and transport activity used mainly positive clone no. 3-6, and used clone no. 3-2 as a non-expressing control. The positions of the molecular mass markers are indicated.

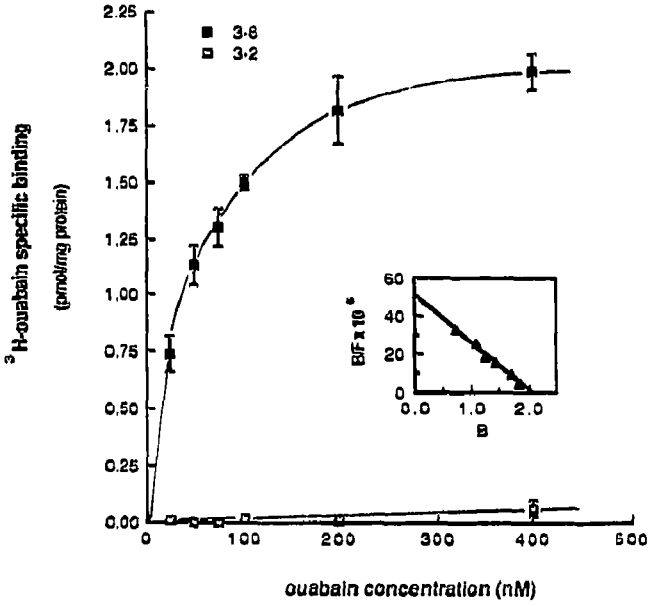

Fig. 4. Comparison of [^{3}H]ouabain binding in transfected and control cells. Non-A2 expressing clone no. 3-2 cells and A2-expressing clone no. 3-6 cells were incubated for 30 min at 37°C with varying concentrations of [^{3}H]ouabain, then washed twice for 10 min at 4°C with ouabain-free buffer. Cells were collected and radioactivity determined by scintillation counting. Specific binding is total minus non-specific binding, per mg protein. Clone no. 3-6 cells (closed squares) show progressive increase in binding with increase in ouabain concentration. Clone no. 3-2 cells (open squares) show <2% of maximal binding of A2 transfected cells (clone no. 3-6). Each curve is the mean ± S.D. of two experiments. Inset: Scatchard analysis (B, bound; F, free) of [^{3}H]ouabain binding, normalized per mg protein (mean 0.73 mg/well). The K_d is 40 nM, and the B_{max} is 2 pmol/mg protein.

approached saturation at 400 nM ouabain. This specific binding, attributed to the presence of A2 isoform protein, was characterized by a K_d of 40 nM (Fig. 4, insert) and a B_{max} of 2 pmol/mg protein.

To confirm that the binding was reversible as well as specific, dissociation of bound [^{3}H]ouabain by unlabeled ouabain was measured. After 30 min of incubation with 200 nM [^{3}H]ouabain, the medium was replaced by medium containing 200 μM non-radiolabeled ouabain. This resulted in a rapid decrease in bound [^{3}H]ouabain, of ~50% after 15 min, and of >90% after 60 min (data not shown).

3.4. *Na^+,K^+-ATPase activity in transfected and control cells*

We found that pre-incubation of the cells with a K^+-free buffer ('pre-load solution') for 60 min markedly stimulated K^+ uptake in either transfected or control cells (e.g. increasing pre-load time from 15 to 60 min increased uptake by ~75–90%; data not shown). Subsequent studies were performed with a 60 min pre-incubation time. K^+ uptake (in the presence of bumetanide) was linear in both A2-expressing clone no. 3-6 cells and non-A2-expressing clone no. 3-2 cells for at least 6 min, and was markedly inhibited (~90% of control) by 1 mM ouabain (Fig. 5). Total uptake was similar for transfected or control cels, although there was a tendency for slightly greater uptake in the control cells.

Next, the effect of ouabain concentration on K^+ uptake was studied in transfected and control cells. Fig. 6 demonstrates that in control cells (clone no. 3-2) there was no effect on K^+ uptake until at least 50 μM ouabain was added. In contrast, A2-expressing transfected cells (clone no. 3-6) were significantly inhibited at concentrations as low as 0.1 μM ouabain, with an IC_{50} of ~5 μM. These findings were substantiated by additional study of two other transfected clones which were positive for A2 protein by Western analysis (clones no. 3-4 and no. 3-10). Interestingly, clone no. 3-10 showed less inhibition of K^+ uptake by low ouabain concentration than no. 3-6 (10% vs. ~30% inhibition at 0.5 μM ouabain, data not shown), and correspondingly expressed less A2 RNA (Fig. 2). In contrast, both wild-type NIH 3T3 cells and non-A2-expressing transfectants which were negative for protein by Western blot showed no effect at submicromolar ouabain concentrations.

4. DISCUSSION

Prior work has clearly shown that rodents are unique in having Na^+,K^+-ATPase which displays two markedly different affinities for ouabain, attributed to the pres-

ence of different α-subunit isoforms. More recently, studies have indicated that both A2 and A3 are apparently ouabain sensitive. Our goal was to use a transfection model to further examine the A2 isoform. We therefore first determined whether A2 was functional in our system. Second, we asked whether A2 was inhibitable by ouabain, and if so, whether it displayed a low ouabain sensitivity comparable to A1, or a high sensitivity.

Early work by Sweadner in rat brain tissue identified two distinct classes of ouabain sensitivity associated with two molecular forms of the Na^+,K^+-ATPase as resolved by gel electrophoresis, with a ouabain-resistant form (α) and a ouabain-sensitive form (α+) [9]. More recently there has been characterization of these high affinity binding sites in specific locations of the rat and rabbit central nervous system [28,29], as well as from adipose cells [30], and rat soleus muscle [16]. However, the molecular cloning of three distinct α isoforms led to the understanding that the earlier described 'α+' isoform likely comprised both A2 and A3 isoforms, with the relative amounts of each dependent on the tissue studied [10]. Additionally, a recent finding of two separate high affinity sites in rat brain is consistent with this concept and implies that A2 and A3 have similar but unique affinities [31]. Confirmation that A3 was indeed a high affinity form was reported by Hara et al. via transfection studies of A3 cDNA [12]. Recently, data has accumulated regarding the characteristics of the A2 protein as well. Orlowski and Lingrel, studying a murine myogenic cell line, found a marked induction of A2 mRNA during fusion of proliferating myoblasts to form myotubes, accompanied by an increase in high affinity ouabain sites and in ouabain-inhibitable Na^+,K^+-ATPase activity [13]. Also Hsu and Guidotti [15] studied Na^+,K^+-ATPase in skeletal muscle from hypokalemic rats and found a correlation between levels of A2 isoform and the ouabain sensitive fraction of the pump. Kjeldsen has demonstrated a single component of high affinity ouabain binding in rat skeletal muscle [16] which is likely A2 based on prior studies [4,5]. Additionally it has been found that specific amino acid residues within the H1-H2 extracellular domain of the alpha subunit are critical for ouabain resistance [32], and several groups have altered A2 cDNA in these regions to produce ouabain resistant forms of A2 [8,14,33].

We approached the issue of ouabain sensitivity with the specific aim of expressing A2 in a cell which did not express endogenous A2 or A3, to allow for a direct assessment of ouabain sensitivity. We transfected a plasmid construct containing A2 into ouabain-resistant NIH 3T3 cells and confirmed high levels of A2 expression by Northern and Western blots (Figs. 2 and 3). Previous work has shown two RNA transcripts for A2 in rat brain, of about 3.4 and 5.3 kb, as a result of multiple polyadenylation sites [3,4]. The size of the single RNA transcript seen in the transfected cells is slightly larger than the 3.4 kb transcript, with the slight increase in size accounted for by the transcription of additional plasmid RNA including a DHFR region and an SV40 polyadenylation site at the 3' end (Fig. 1). Studies of ouabain binding in these transfected cells indicate that the A2 isoform is indeed highly ouabain-sensitive, with a K_d of 40 nM (Fig. 4).

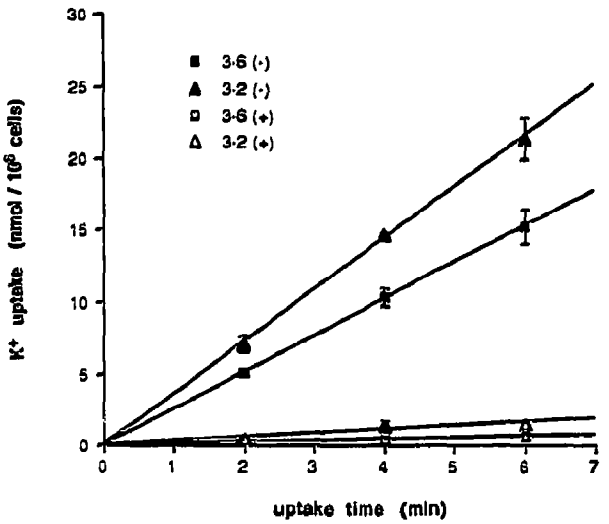

Fig. 5. Effect of uptake time on K^+ uptake by Na^+,K^+-ATPase. A2-expressing clone no. 3-6 and non-A2-expressing clone no. 3-2 cells were rinsed and then preincubated for 60 min at 37°C with K^+-free EBSS. Then $^{86}Rb^+$-EBSS buffer containing bumetanide ('uptake buffer') was added and uptake proceeded for 2, 4, or 6 min in absence (−) or presence (+) of 1 mM ouabain. Uptake was stopped, cells precipitated, and $^{86}Rb^+$ uptake determined. $^{86}Rb^+$ uptake was converted to K^+ uptake/10^6 cells based on known K^+ concentration of the medium. Uptake was linear for at least 6 min in both clones (closed symbols), and was inhibited by ~90% of control at all uptake times by 1 mM ouabain in both clones (open symbols).

This dissociation constant for ouabain determined for A2 is comparable to reports of high affinity binding obtained in several other tissues. In rat soleus muscle, which expresses predominantly A2 [4,5], and therefore should be similar to our model, Kjeldsen reported an apparent K_d for ouabain binding of ~40–70 nM [16]. We found rat A2 characterized by a B_{max} for ouabain of ~2 pmol/mg protein, results which compare well to reports for high affinity binding in rat brain (7.6 pmol/mg protein) [28]. Kjeldsen reported from rat soleus muscle a B_{max} of ~0.3 pmol/mg wet wt; [16] this would be higher and perhaps closer to the value we found if expressed per mg protein. In rat brain, which expresses all three α isoforms, Berrebi-Bertrand et al. recently described finding three distinct ouabain affinities: two of high affinity with K_d values of 17 nM ('very high') and 80 nM ('high'), and one of low affinity [31]. Each of these high affinity sites represented about one-third of the total ouabain binding sites. Comparing this study with our findings that rat A2 has a K_d of 40 nM, along with

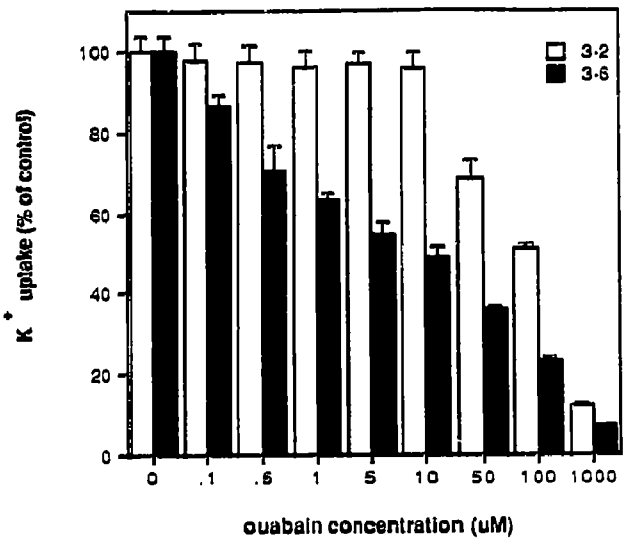

Fig. 6. Ouabain inhibition of K^+ uptake by Na^+,K^+-ATPase in transfected and control cells: effects of varying ouabain concentration. Cells were rinsed and preincubated for 60 min at 37°C with K^+-free EBSS, and then incubated with uptake buffer for 6 min in the presence of varying concentrations of ouabain. Uptake was stopped, cells precipitated, and $^{86}Rb^+$ uptake determined. $^{86}Rb^+$ uptake was converted to K^+ uptake/10^6 cells based on known K^+ concentration of the medium, and expressed as % of uptake occurring in each cell clone in the absence of ouabain. (Maximal uptake in the absence of ouabain was 17.4 ± 0.6 nmol $K^+/10^6$ cells for the no. 3-6 cells, and 22.5 ± 0.8 nmol $K^+/10^6$ for the no. 3-2 cells.) Non-A2-expressing control cells (clone no. 3-2, open bars) showed no inhibition of uptake until at least 50 μM ouabain was added. A2-expressing transfectants (clone no. 3-6, solid bars) showed significant inhibition at ouabain concentrations as low as 0.1 μM; the IC_{50} was ~5 μM. Each curve is the mean ± S.D. of triplicate determinations from a representative experiment, repeated three times.

evidence that rat A3 has a K_d of 80 nM [12], it is possible that A2 accounts for the 'very high' and A3 for the 'high' affinity sites. This would require direct comparison in a transfection model for confirmation. Thus the A2 and A3 isoforms together likely account for the high affinity binding noted in earlier studies, and the relative amounts of these two isoforms expressed in individual tissues will determine the overall sensitivity for ouabain inhibition in that tissue. Because of their similarity in ouabain binding affinity, it is important that studies of high affinity binding in tissue preparations be correlated with either RNA analysis or Western blotting using antibodies which do not show cross-hybridization between A2 and A3.

The binding curve for A2 in the transfected cells shows that binding is saturable. Additionally, binding is reversible and dissociation requires many minutes (data not shown); this long dissociation time is also consistent with binding of high affinity. Further, these data indicate that the bound ouabain is not internalized by the cells in significant amounts, since >90% of binding is displaced after 60 min. This is consistent with the finding that ouabain resistance is related to one of the extracellular domains of the α subunit [8,14,33], and that ouabain may not require internalization for its effect on enzyme activity.

The finding of high affinity ouabain binding in the transfected cells is complemented by studies using ouabain inhibition of $^{86}Rb^+$ flux. $^{86}RB^+$ is a valid tracer for K^+ uptake [25,34] and has been frequently used to demonstrate enzyme activity. Our studies show that the A2 protein in transfected cells is functional and that low concentrations of ouabain (0.1–10 μM) inhibit $^{86}Rb^+$ flux only in the transfected cells (Fig. 6). With increasing ouabain concentrations, increasing amounts of A2 protein bind ouabain with additional inhibition of activity, and only at still higher concentrations ($\geq$50 μM) is the endogenous A1 activity inhibited.

These flux studies were performed in the presence of bumetanide to inhibit the $Na^+/K^+/2Cl^-$ cotransporter, since our preliminary experiments indicated up to 70% of $^{86}Rb^+$ uptake was inhibited by addition of the specific antagonist bumetanide. This is a higher level of ouabain-resistant K^+ uptake than is often seen, but high levels of ouabain-resistant K^+ uptake have previously been reported in fibroblasts, particularly after serum-stimulation [35]. In general, levels of $Na^+/K^+/2Cl^-$ cotransporter activity (total $^{86}Rb^+$ uptake minus ouabain-inhibitable uptake) were comparable in the transfected and control cells (data not shown). The remainder of the flux in our experiments was sensitive to inhibition by ouabain at high concentration (1 mM), as would be expected for both ouabain-sensitive and -resistant isoforms of the Na^+,K^+-ATPase. Further, the ouabain-inhibitable flux was comparable between transfected and control lines, although there was typically a slightly greater level in the non-A2-expressing clone (Fig. 5).

We chose to carry out the flux studies after stimulation of the Na^+ pump such that K^+ uptake would occur at a rapid and constant rate. Several methods of stimulation of the Na^+ pump have been described including use of a low K^+ bath, use of agents which affect transmembrane potentials, and temperature effects [36]. We found that pre-incubation with a K^+-free medium markedly stimulated subsequent flux occurring in a physiologic K^+ concentration. Flux was linear under these conditions for both control and transfected cells (Fig. 5). Thus, we do not ascribe differences in $^{86}Rb^+$ flux observed at low ouabain concentrations to differences in V_{max} of the enzymes.

Both the ouabain binding and $^{86}Rb^+$ flux studies were performed on stable clones isolated by selection in G-418. Because transfection itself could conceivably induce changes in ouabain binding or Na^+ pump activity, as control cells we examined both wild-type 3T3 cells which do not express A2 isoform, as well as cells transfected and selected in G-418 but which did not achieve expression of A2 protein. These latter cells were thus exposed to identical conditions as the A2-expressing

clones throughout the time period of the experiments. These non-A2-expressing cells and the wild-type 3T3 cells were indistinguishable.

It is interesting that total Na^+ pump activity in the transfected and control cells was comparable (Fig. 5). This finding suggests that total pump activity is regulated in the transfected cells, with a decrease in the amount of endogenous A1 pump activity as a function of the presence of A2. In addition, study of two different A2-expressing transfected clones (no. 3-6 and no. 3-10) revealed that a higher level of A2 RNA expression was associated with a greater percentage of inhibition of Na^+,K^+-ATPase at low ouabain concentrations. This is consistent with regulation of A1 either at the transcriptional or post-translational level. Alternatively, limiting amounts of β subunit protein may be present and be preferentially complexed with A2 in these cells. Further experiments are required to evaluate and confirm these possibilities.

In summary, we have used a mammalian cell transfection model to directly study the ouabain sensitivity of the rat A2 isoform. The results demonstrate that A2 binds ouabain with high affinity and that its transport activity is inhibited by ouabain at submicromolar concentrations. This confirms that each of the three known α isoforms have distinct ouabain affinities, with A2 and A3 having very similar binding equilibrium constants. This model may also be useful in further studies on Na^+,K^+-ATPase isoform regulation and in analyses of specific coding regions of the isoforms.

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