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The Effect of the Gamma Modulator on Na/K Pump Activity of Intact Mammalian Cells

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Abstract. This study concerns the modulatory effects of the gamma modulator of the Na/K pump, in particular whether the effects seen in previous experiments with isolated membranes are relevant to Na/K pump behavior in intact mammalian cells. For this purpose, HeLa cells previously transfected with the rat Na/K catalytic subunit were used. The results show that both variants of the regulator, γa and γb, decrease the apparent affinity of the pump for Na^+ and cause a modest increase in apparent ATP affinity as seen in measurements of ouabain-sensitive $^{86}Rb(K^+)$ influx into cells in which ATP was varied using antimycin A and glucose. Equivalent results had been obtained previously in our analyses of Na,K-ATPase activity of membrane fragments, i.e., an increase in $K_{0.5(Na)}$ at high K^+ concentration and a decrease in K'_{ATP}. Comparison of clones of γ-transfected and mock-transfected cells (with similar V_{max} values) indicated that γ causes a modest ≈30% increase in the steady-state concentration of intracellular Na^+. Furthermore, for both γa and γb, values of intracellular Na^+ were similar to those predicted from the kinetic constants, $K_{0.5(Na)}$ and V_{max}. Finally, there was a γ-mediated increase in apparent affinity for extracellular K^+, which had not been detected in assays of permeabilized membranes.

Key words: Na/K pump — γ Modulator — Transport — Na,K-ATPase

Introduction

The Na/K pump, or Na,K-ATPase, is an integral membrane protein found in virtually all cells of higher eukaryotes. It comprises two subunits, a large catalytic α subunit and a smaller highly glycosylated β subunit necessary for the proper folding, insertion and maturation of α in the plasma membrane. The αβ dimer is the minimal structural unit required for Na/K pump activity.

The Na,K-ATPase catalyzes the ATP-driven electrogenic exchange of three intracellular Na^+ ions for two extracellular K^+ ions coupled to the hydrolysis of one molecule of ATP. It plays a key role in chemiosmotic balance and in maintenance of the resting membrane potential. Furthermore, the Na^+ gradient generated by this pump has a critical role in regulating intracellular pH and Ca^{++} via Na^+/H^+ and Na^+/Ca^{++} exchange systems, and in the uptake of many solutes via Na^+-coupled co-transport processes.

Some of the main factors contributing to the modulation of the Na/K pump are the enzyme's substrates (Na^+, K^+ and ATP), cytoskeletal components, endogenous digitalis-like inhibitors and hormones and the signalling cascades that hormones activate (Therien & Blostein, 2000). Regulation may also occur through the association of the Na/K pump with other intrinsic membrane proteins such as members of the FXYD family. This family comprises small Type I membrane proteins comprising a single transmembrane domain in which a PFXYD motif in the extramembranous N-terminus is invariant in cells of all mammals (Sweadner & Rael, 2000). To date, seven FXYD proteins have been identified. Several have been shown to modulate the kinetic behavior of the pump in a tissue-specific manner (*see* refs. Cornelius, Mahmmoud & Christensen, 2001; Crambert & Geering, 2003; Geering et al., 2003).

The γ modulator (FXYD2) of the renal Na,K-ATPase was discovered over 25 years ago (Forbush, Kaplan & Hoffman, 1978), and the cDNA cloned later by Mercer et al. (1993). It is the best char-

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Table 1. Effects of γ on apparent cation affinities

	α1-C	α1-γa	α1-γb	α1-γbG41R
$K_{0.5(Na)app}$ mM	8.7 ± 0.4 (24)	14.9 ± 1.2*** (12)	17.2 ± 0.8***† (14)	8.0 ± 0.5* (6)
[a]$K_{0.5(K)}$ mM	0.62 ± 0.02 (14)	0.40 ± 0.02*** (7)	0.40 ± 0.02*** (7)	0.58 ± 0.01* (3)

Numbers in brackets indicate the number of separate experiments. V_{max} values are shown in Table 2.
[a]$K_{0.5(K)}$ was determined with cells equilibrated with 20 mM Na^+ as described in Materials and Methods.
***$p < 0.001$ compared to control, *$p > 0.05$ compared to control, † $p > 0.05$ compared to γa

acterized member of the FXYD family. It exists as two main variants, γa and γb. Mass spectrometry indicated that γa and γb differ only in the N-terminus (Kuster et al., 2000). In rat kidney, γa has TELSANH and γb has Ac-MDRWYL. Some regions of the renal tubule have one or the other variant and other regions have both (Pu et al., 2001; Wetzel & Sweadner, 2001; Arystarkhova, Wetzel & Sweadner, 2002). Both variants are particularly abundant in the medullary thick ascending limb.

Previous studies from our laboratory as well as others, using membrane fragments isolated from γ-transfected rat α1-HeLa or rat NRK cells, have shown that γ serves at least two distinct functions in regulating Na,K-ATPase activity. These are (i) an increase in the apparent affinity for ATP (Therien et al., 1997; Therien, Karlish & Blostein, 1999) and (ii) a decrease in apparent affinity for Na^+ (Arystarkhova et al., 1999; Pu et al., 2001). The latter was shown to be due primarily to an increase in apparent K^+ affinity at cytoplasmic Na^+ activation sites (Pu et al., 2001).

A point mutation, G41→R, in the transmembrane region of γ has been associated with autosomal dominant renal magnesium wasting (Meij et al., 2000). Studies have shown that this mutation alters γ association with the Na/K pump through abnormal trafficking of γ (Meij et al., 2000). Trafficking of αβ pumps to the cell surface is not altered (Pu, Scanzano & Blostein, 2002).

The present study addresses the question of how the effects of γa and γb inferred from the experiments on unsided membrane preparations are manifested in the intact cell.

Materials and Methods

Mutagenesis and Transfection

Rat γa and γb cDNA's were subcloned into the pIRES expression vector and transfected into HeLa cells stably expressing the rat α1 subunit of the Na,K-ATPase kindly provided by Dr. J.B Lingrel as previously described (Pu et al., 2001). The point mutation G41R was introduced into γb-pIRES using the QuikChange Site Directed Mutagenesis Kit (Stratagene). Control rat α1-HeLa cells were also transfected with the pIRES empty vector.

Transport Assays

Na/K pump activity was measured by determining unidirectional $^{86}Rb(K^+)$ influxes. Assays were carried out in the presence of a low (10 μM) ouabain concentration to inhibit endogenous human pump activity and ± a high (5 mM) ouabain concentration to inhibit activity of pumps containing the rat α1 subunit and determine baseline activity (rat Na/K pumps have a very low sensitivity to ouabain). To determine the apparent affinity for intracellular Na^+, $^{86}Rb(K^+)$ influxes (nmoles/mg/min) were determined in cells grown to 70–80% confluence on 24-well plates and assayed with various Na^+ concentrations and constant (4 mM) K^+ concentration in media containing monensin (to raise Na^+ permeability) as described previously (Munzer et al., 1994) with minor modifications (Garty et al., 2002). Briefly, to vary intracellular Na^+, cells were preincubated for 15 minutes at 37 °C with 12 μM monensin and 10 μM bumetanide added to solutions with various Na^+ concentrations. For the determination of the kinetic constants for Na^+ activation, values of $^{86}Rb(K^+)$ influxes expressed as functions of added extracellular Na^+ concentration were fitted to a simple one-site Michaelis-Menten model (Garty et al., 2002) since it was not technically feasible to determine intracellular Na^+ at each concentration of medium Na^+. The apparent affinity constant thus obtained is only a measure of the apparent affinity for Na^+ and is therefore represented as $K_{0.5(Na)app}$ (*see* Table 1), whereas the apparent affinity for Na^+ calculated from previous experiments with membrane fragments and precisely known Na^+ concentrations (Na,K-ATPase assays) is expressed as $K_{0.5(Na)}$ (*see* Table 2).

For the determination of $K_{0.5(K)}$ and V_{max} for K^+ activation, extracellular K^+ activation of $^{86}Rb(K^+)$ influxes was analyzed with cells equilibrated with monensin and the indicated Na^+ concentration and varying K^+ concentrations. The data were fitted to a cooperative model (c.f. Jewell & Lingrel, 1991):

$$V = V_{max}[cat]^n / (K_{0.5(cat)} + [cat]^n) \quad (1)$$

where "cat" is either Na^+ or K^+. An average value for the Hill coefficient ($n = 1.3$) was used for all experiments with varying extracellular K^+ since no significant difference between n values of control and γ-transfected cells was seen ($1 > p > 0.1$). Assays were carried out with cells transfected with γ (γa and/or γb or γbG41R) and control mock-transfected cells. All assays were carried out on two clones of each type of transfected cell line. Data for the Na^+ and K^+ activation profiles were expressed as percentages of V_{max}.

Determination of Intracellular Cation Concentrations

Cells were grown in triplicate to 70–80% confluence on 100 mm tissue culture dishes and washed as described previously (Munzer et al., 1994). Following treatment with 3 mL 0.05% Triton X-100, the extract was analyzed for Na^+ and K^+ content by atomic absorption and for protein by the Lowry method. Determinations were carried out on two clones of each type of transfected cell line.

Table 2. Effect of γ on steady-state parameters of Na^+ transport

Transfected α1-HeLa cells	[a]$K_{0.5(Na)}$mM	V_{max} nmoles/mg/min	$[Na]_{in}$ (measured) mM	$[Na]_{in}$ (calculated) mM
Mock	16.5 ± 0.1	37.8 ± 0.9 (24)	10.4 ± 0.6 (11)	–
γa	20.7 ± 0.3***	42.2 ± 1.9* (12)	11.7 ± 0.6* (9)	12.2
γb	21.9 ± 0.5***[§]	36.5 ± 1.7* (14)	14.5 ± 0.5***[†] (7)	13.9
γb-G41R	16.2 ± 0.3*	30.7 ± 0.8** (6)	10.7 ± 0.6* (7)	11.4

Numbers in parentheses refer to the number of separate $[Na]_{in}$ (each in triplicate) and V_{max} determinations. The measured value of $[Na]_{in}$ for mock cells was 10.4 mM and this value was used to calculate the leak constant needed for estimation of intracellular Na^+ concentration (*see* Materials and Methods Eq. 2).
[a]Calculated from data obtained by Pu et al. (2001) using Eq. 1 and $n = 3$, and are based on assays carried out at 100 mM K^+.
***$p < 0.001$ compared to control, ** $p < 0.01$ compared to control, *$p > 0.05$ compared to control, [§]$p > 0.05$ compared to γa, [†]$p < 0.05$ compared to γa

Procedures for Varying Intracellular ATP and Measuring $^{86}Rb(K^+)$ Influx Concurrently

To reduce intracellular ATP concentration in a graded and reproducible manner, cells cultured in 24-well plates were incubated for 2 h at 37 °C with gentle agitation in medium containing (in mM) 40 NaCl, 2 KC1, 108 choline chloride, 5 HEPES-Tris, 1.5 PO_4-Tris, 0.5 $MgCl_2$, 1 $CaCl_2$, pH 7.4, 1 μM ouabain, 0.1 μM antimycin *A* and D-glucose ranging from 0.05–1 mg/mL. The medium was aspirated and replaced by the same medium containing 12 μM monensin, 10 μM bumetanide (to inhibit $NaKCl_2$ cotransport) and 10 μM ouabain (as in flux assays). Following a preincubation of 15 min, one set of triplicate wells was taken for the determination of ATP and protein and a second set was taken for the determination of transport activity as described above. For each experiment, assays were carried out in triplicate, with either γa-, γb -, or γbG41R-transfected rat α1-HeLa together with control mock-transfected cells assayed concurrently.

Measurement of ATP Concentration

Following preincubation as described above, the medium from each well was aspirated and the cells were immediately chilled on ice and lysed with 0.5 mL 5% TCA. Following gentle agitation for 10 min on ice, the 24-well plate was centrifuged and the supernatant adjusted to pH 7.6 with 2 M Tris. To each assay vial were added 2.5 mL diluent (in mM: 50 glycine, 1 Tris, 5 $MgSO_4$, 0.5 EDTA, 0.1% BSA, 0.1% NaN_3), 200 μL of sample supernatant or standards (solutions containing 0.001 nmol–1.2 nmol ATP in 5% TCA neutralized to pH 7.6), 50 μL luciferin (357 μM) and 10 μL luciferase ($1.5–3 \times 10^6$ units/mL). The light emissions were measured by liquid scintillation spectrometry using a wide channel of the single-photon monitor of the Beckman Coulter LS 6500 Multi-Purpose liquid scintillation counter. Protein remaining in the wells was solubilized with 0.5 mL 0.5 M NaOH and measured by the Lowry method. The concentration (mM) of intracellular ATP was estimated by assuming 8 μL water/mg protein (Munzer et al., 1994).

Estimation of Intracellular Na^+ Concentration

To estimate intracellular Na^+ concentration in γ- and γG41R-cells, we used the following relationship (based on Hoffman et al., 2002):

$$L \cdot ([Na]_{out} - [Na]_{in}) = V_{max} \cdot [Na]_{in}^3 / (K_{0.5(Na)} + [Na]_{in})^3 \quad (2)$$

where L is the rate constant of the Na^+ leak in s^{-1}, $[Na]_{out}$ and $[Na]_{in}$ represent extracellular and intracellular Na^+ concentrations, respectively, and the other parameters have their usual meaning. L was first calculated using the kinetic parameters of V_{max} and $K_{0.5(Na)}$ of the Na/K pump and the measured $[Na]_{in}$ of the control (α1-C) mock-transfected cells. This value of L thus calculated was then used to estimate $[Na]_{in}$ in γ- and γG41R-transfected cells using values of V_{max} of the same cells. Since the Na^+ concentrations are precisely known in experiments carried out with membrane fragments, $K_{0.5(Na)}$ values used were calculated from earlier Na^+-activation profiles of membrane Na,K-ATPase carried out at high (100 mM) K^+ concentration (Pu et al., 2001) using Eq. 1. with $n = 3$. Iterative techniques using Excel software were used to solve the above non-linear Eq. 2.

Data Analysis

Curve fitting was carried out using the KaleidaGraph computer program (Synergy software). Data are presented as mean ± SEM of the number of experiments shown in parenthesis. One-way ANOVA, or, where required, a nonparametric ANOVA, followed by appropriate pair-wise comparisons were carried out with GraphPad Instat software.

Results

The effect of the two γ variants on Na/K pump behavior of intact cells was assessed by comparing the kinetics of Na^+ and K^+ activation of ouabain-sensitive $^{86}Rb(K^+)$ influx into γa- and γb-transfected α1-HeLa cells assayed concurrently with control mock-transfected HeLa cells. γbG41R-transfected α1-HeLa cells were similarly assayed. As described in our earlier studies (Munzer et al., 1994), intracellular Na^+ was fixed at a range of concentrations by preincubation with monensin.

Effect of Varying Na^+

In the representative experiment depicted in Fig. 1, cells were equilibrated with varying concentrations of Na^+ and extracellular K^+ concentration was kept constant at 4 mM. Figure 1 as well as the results of replicate experiments summarized in Table 1 show that both γ variants decrease the apparent affinity for Na^+, whereas a difference between γbG41R and control could not be detected. This is consistent with

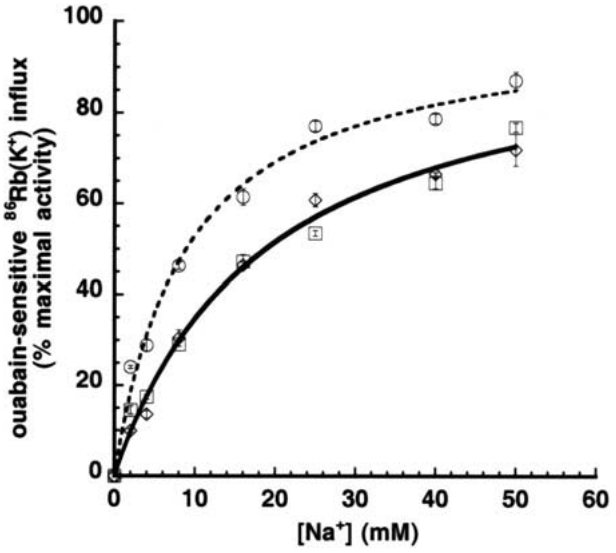

Fig. 1. Activation of ouabain-sensitive $^{86}Rb(K^+)$ influx by intracellular Na^+. Ouabain-sensitive $^{86}Rb(K^+)$ influxes were measured in cells equilibrated with the indicated concentrations of Na^+ as described in Material and Methods and with 4 mM extracellular K^+. Data are expressed as percentages of V_{max} calculated as described in Materials and Methods. In the representative experiment shown, $K_{0.5(Na)app}$ (mM) was determined as described in Materials and Methods. Values for control- (○), γa- (□) and γb- (◇) transfected cells were 8.9, 18.9 and 18.7, respectively.

the results of earlier biochemical assays of Na,K-ATPase activity using permeabilized membranes. In those experiments (Pu et al., 2001), the precise Na^+ concentrations were known and the data were thus fitted to a 3-site cooperative model (Eq. 1) (*see* Table 2). The differences in kinetic constants seen with intact cells ($K_{0.5(Na)app}$) compared to membranes ($K_{0.5(Na)}$) are due presumably to deviations of intracellular Na^+ from equilibrium values, particularly at low concentrations of added Na^+ (Munzer et al., 1994). V_{max} values of the cells used for the determination of $[Na]_{in}$ are shown in Table 2. However, the variations between them are not considered meaningful since they probably reflect differences in transfection efficiency of the different cDNAs.

A key question is whether the decrease in apparent affinity for Na^+ seen with both γ variants in intact cells results in an increase in the steady-state intracellular Na^+ concentration ($[Na]_{in}$) of the cultured HeLa cells. To address this issue, we measured $[Na]_{in}$ in γa- and γb- as well as γbG41R-and mock-transfected rat α1-HeLa cells. The results shown in Table 2 indicate that, for all γ's (γa, γb and γbG41R), the measured $[Na]_{in}$ values are similar to those calculated from V_{max} of the same cells and $K_{0.5(Na)}$ obtained from our earlier Na,K-ATPase assays of permeable membranes (Pu et al., 2001) for which Na^+ concentrations are known precisely.[1] As evident in equation 2, V_{max} and $K_{0.5}$ have opposite effects on $[Na]_{in}$. Therefore, in γb cells which had a V_{max} similar to the control, $[Na]_{in}$ is ≈ 30% higher than the mock control. In contrast, in γa, the higher V_{max} compared to control offsets a significant effect of $K_{0.5(Na)}$ on $[Na]_{in}$.

Effect of Varying Extracellular K^+ Concentration

In previous studies using unsided membrane fragments, we failed to detect a significant effect of γ on K^+-activation kinetics of Na,K-ATPase of rat α1-transfected HeLa cells (ref. Pu et al., 2001 and experiments not shown). In contrast, Arystarkhova et al. (1999) reported a γ-mediated decrease in K^+ affinity in their experiments with γ-transfected NRK52E cells. Similarly, treatment of rat renal membranes (αβγ pumps) with anti-γ (C-terminal) antibodies increased K'_K (Therien et al., 1997). In the latter study, the increase in K'_K diminished with increase in ATP concentration, consistent with the consecutive model of ATP-accelerated K^+ deocclusion following K^+-activated dephosphorylation of E_2P (c.f. Eisner & Richards, 1981) whereby an increase in ATP affinity should decrease apparent K^+ affinity and vice versa.

Although a statistically significant effect of γ on $K_{0.5(K)}$ was not detected in our initial flux studies with intact cells (Pu et al., 2001), this issue was addressed further by performing a large number of experiments with cells equilibrated with Na^+ at various concentrations. The results of these flux studies comprising control versus γa and γb assayed concurrently (summarized in Table 1; typical experiment shown in Fig. 2) indicate that both variants cause a modest but significant increase in apparent affinity for K^+. Similar percentage decreases (≈ 30%) in $K_{0.5(K)}$ were observed over a range of Na^+ concentrations tested as shown in Table 3. As in the experiments carried out at 20 mM Na^+ and shown in Table 1, a difference between the effects of γa and γb was not detected.

Does γ Affect the ATP-dependence of Na/K Pump Activity?

To determine whether the γ-mediated increase in ATP affinity seen in biochemical Na,K-ATPase assays carried out with fragmented membranes is evident in intact cells, the relationship between pump

[1]Appropriateness of using $K_{0.5(Na)}$ from ATPase assays carried out at 100 mM K^+ is indicated by data showing that $K_{0.5(Na)}$ values for at least the two isoforms, α1 and α2, are 16.5 ± 0.1 (Table 2) and 21.9 ± 2.25 (A. Zouzoulas and R. Blostein, *unpublished data*), respectively, which are notably similar to $K_{0.5(Na)}$ determined in flux experiments, i.e., 17.2 ± 1.12 and 19.68 ± 0.96, respectively (values from Munzer et al., 1994).

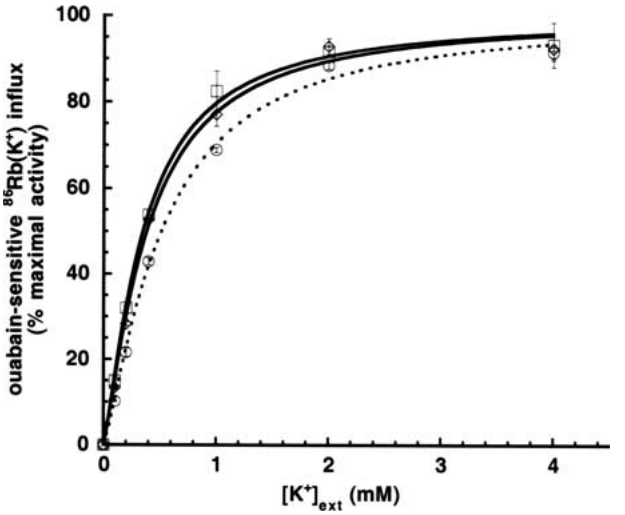

Fig. 2. Activation of ouabain-sensitive $^{86}Rb(K^+)$ influx by extracellular K^+. Ouabain-sensitive $^{86}Rb(K^+)$ influx was carried out as described in Materials and Methods. Data are expressed as percentages of V_{max}. In the representative experiment shown, the cells were equilibrated with 20 mM Na^+. $K_{0.5(K)}$ (mM) for control- (○), γa- (□) and γb- (◇) transfected cells were 0.51, 0.35 and 0.38, respectively.

Table 3. Effects of γ on $K_{0.5(K)}$ *as a function of varying extracellular* Na^+

	$K_{0.5(K)}$ (mM)	
Na^+ (mM)	Mock	γa and γb
10	0.34 ± 0.03 (4)	0.25 ± 0.02** (7)
20[a]	0.62 ± 0.02 (14)	0.40 ± 0.01** (14)
40	1.01 ± 0.12 (7)	0.73 ± 0.04* (6)
80	2.90 ± 0.02 (3)	1.87 ± 0.05** (4)

Numbers in brackets indicate the number of separate experiments.
[a]Data taken from Table 1
**$p < 0.01$, *$p < 0.05$ compared to control (unpaired *t*-test)

flux and cellular ATP concentration was compared in γ-transfected and mock-transfected HeLa cells. In preliminary experiments, we used various strategies to deplete intracellular ATP and found that preincubation of cells with antimycin A and varying the concentration of glucose prior to the transport assay provided an optimal means of varying intracellular ATP in a graded fashion. Fig. 3 depicts a typical range of intracellular ATP concentrations obtained following a 2 h depletion. The data show similar depletion patterns for control and γ cells.

It was shown previously (Ikehara et al., 1984) that incubation of HeLa cells with metabolic inhibitors causes the cells to gain Na^+ to different extents depending on the intracellular ATP content. To avoid changes in $[Na]_{in}$ as the ATP is altered, cells were equilibrated with monensin and 40 mM Na^+ after

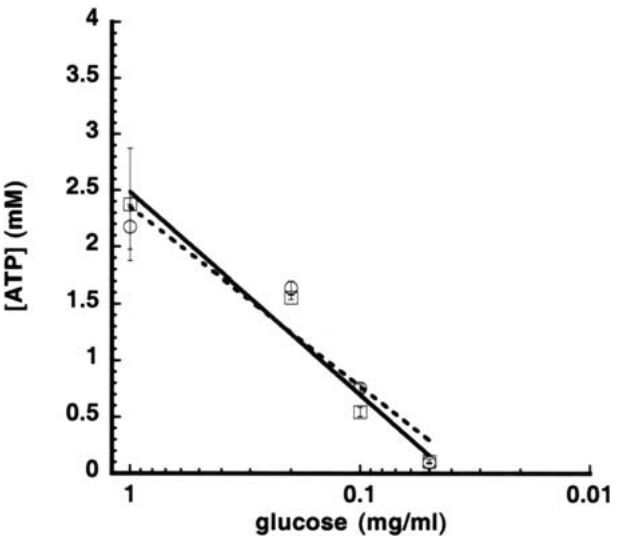

Fig. 3. ATP concentration in cells varied by incubation for 2 h with antimycin A and varying concentrations of glucose. *See* Materials and Methods. Values for control- (○), γ- (□).

ATP depletion as described in Materials and Methods. Figure. 4*A* shows a representative experiment in which pump activity was measured in mock- and γb-transfected cells with varying ATP concentration. In all experiments, the flux showed no saturation up to the highest ATP concentration measured. In each paired experiment, the slope of the relationship between flux and ATP concentration was greater for γ-compared to mock-transfected cells. Thus, for 8 paired experiments (3 with γa- and 5 with γb- versus the mock-transfected control cells) a modest 22.7 ± 4.5% increase in pump rate as a function of ATP concentration in γ-transfected compared to control mock-transfected cells was seen. When γbG41R-transfected and mock-transfected cells were compared (2 experiments), a difference was not detected (representative experiment shown in Fig. 4*B*), which is consistent with results that this mutant lacks any of the functional effects of wild-type γ (Pu et al., 2002), thus behaving like mock-transfected cells.

Discussion

The present study with intact cells confirms our earlier conclusion from experiments with membrane fragments (Na,K-ATPase assays; *see* Pu et al., 2001), that both variants of the γ modulator of the sodium pump lower the apparent affinity for Na^+. We showed further that the γ-mediated decrease in apparent Na^+ affinity is secondary to an increase in K^+ affinity at intracellular Na^+-activation sites (K^+/Na^+ antagonism) (Therien & Blostein, 1999). Thus, an earlier extensive kinetic analysis showed that a γ-mediated decrease in apparent Na^+ affinity seen at high K^+ concentrations is due to a higher apparent

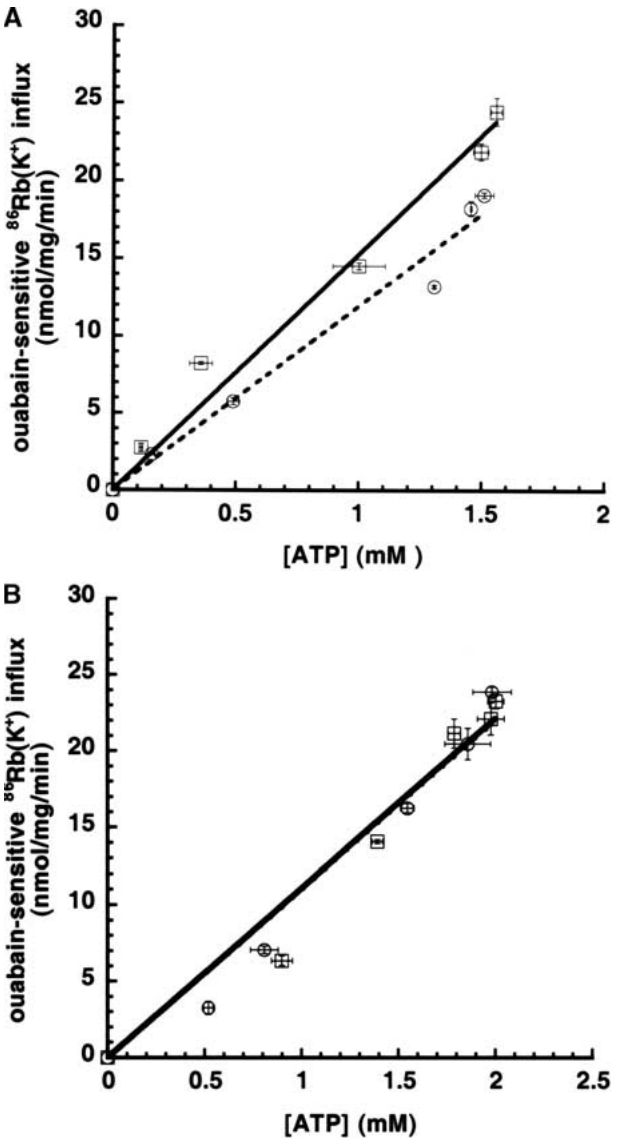

Fig. 4. Effect of ATP depletion on ouabain-sensitive ^{86}Rb(K) influx. Following ATP depletion, ^{86}Rb(K^+) influx was measured as described in Materials and Methods. (*A*) Representative experiment of control- (○) vs γb- (□) transfected α1-HeLa cells. (*B*) Representative experiment of control-(○) vs γbG41R- (□) transfected α1-HeLa cells.

affinity for K^+ as an antagonist at Na^+ activation sites rather than a difference in Na^+ affinity per se. Consistent with those experiments, the γ-mediated increase in $K_{0.5(Na)app}$ is seen here in intact cells in which the intracellular K^+/Na^+ ratio is high. A similar result was reported by Garty et al. (2002), although the variant analyzed was not specified. The present study also shows that the lowered apparent affinity for Na^+ is associated with an ≈30% increase in the steady-state Na^+ content of our cultured γb-transfected HeLa cells (Table 2). Despite the similar increase in $K_{0.5(Na)app}$ seen with both γ variants, a significant increase in $[Na]_{in}$ is not seen in our γa clones; presumably the effect of a higher $K_{0.5(Na)}$ on $[Na]_{in}$ of those cells is offset by their higher V_{max}. It is noteworthy that in both variants, $[Na]_{in}$ values agree with those predicted from the relationship between $K_{0.5(Na)}$ and V_{max} values assuming that all cells have the same leak constant as control mock-transfected cells (see Eq. 2).

The γ-stimulated increase (≈20%) in pump flux as a function of varying intracellular ATP concentration is notably lower than the increase (≈50%) in ATP affinity observed in membrane Na,K-ATPase assays (Pu et al., 2002). This discrepancy probably reflects the two distinct and opposite effects of γ on apparent Na^+ and ATP affinities. Thus, if one takes into account the lower apparent Na^+ affinity caused by γ as depicted in Fig. 1 and Table 1, an ≈ 18% decrease in flux rate due to the change in $K_{0.5(Na)app}$ is predicted under the condition used in Fig. 4*A* (cells equilibrated with 40 mM Na^+). Therefore, the expected γ-mediated increase in flux over the range of ATP concentrations of the experiments in Fig. 4*A* should be reduced from 50% (due to the effect on K'_{ATP}) to 23% (due to the additional effect on $K_{0.5(Na)app}$; Fig. 1), which is similar to that observed (22.7 ± 4.5%; *see* Results). It is also clear that the γ modulator decreases the apparent affinity for Na^+ at high K^+ in both cells (this study) and membranes (Pu et al., 2001; Pu et al., 2002), without a significant difference in the effect of γa versus γb. However, the absolute values of $K_{0.5(Na)app}$ and $K_{0.5(Na)}$ (Na,K-ATPase assays) cannot be quantitatively compared since, despite the presence of monensin, chemical equilibrium is not attained. The physiological relevance of these opposing effects of gamma is discussed below.

Na,K-ATPase assays of isolated membrane preparations measured as a function of ATP concentration indicate that the Na/K pump has a $K_{0.5}$ for ATP of approximately 400 μM, whereas experiments with intact cells reveal that the ouabain-sensitive flux does not appear to saturate with ATP up to at least 2 mM. The near linear relationship between ^{86}Rb(K^+) influx and ATP seen in this study is consistent with previous results obtained with HeLa cells (Ikehara et al., 1984). This behavior is also reminiscent of the findings with intact renal tubules in which a linear dependence of the Na/K pump on ATP up to about 3 mM was observed (Soltoff & Mandel, 1984). As these authors point out, the products of ATP hydrolysis inhibit the forward pump reaction. Thus, increased formation of products, particularly ADP, as ATP is lowered would inhibit the pump, thereby linearizing the normally hyperbolic curve relating flux to ATP concentration.

Overall, the increase in flux as a function of ATP concentration as well as the increase in steady-state intracellular Na^+ concentration are relatively modest. Nevertheless, considering the major role of the

pump in salt and water balance and as we have discussed elsewhere (Therien et al., 2000; Therien et al., 2001), the γ modulator may serve to maintain pump activity under energy-deprived conditions such as may occur in the anoxic mTAL. At the same time, by lowering apparent Na^+ affinity, an appropriate intracellular Na^+ level for Na^+-coupled transport processes is maintained.

The increase in apparent K^+ affinity of Na/K pumps seen in the present study with intact cells was not seen in experiments with permeable membrane preparations (Pu et al., 2001). We considered the possibility that this difference is a consequence of the high intracellular K^+/Na^+ concentration ratio of intact cells since we showed previously that the γ modulator increases the apparent affinity for K^+ as an antagonist of cytoplasmic Na^+ activation (Therien & Blostein, 1999; Pu et al., 2001). Accordingly, γ may increase the backward rate of the $E_2(K) \leftarrow \rightarrow E_1 + K^+_{cyt}$ reaction step and thus increase the apparent affinity for extracellular K^+ in the preceding $E_2P + K^+_{ext} \rightarrow E_2(K) + P_i$ step. This is an unlikely explanation since the γ-mediated decrease in $K_{0.5(K)}$ was similar in cells equilibrated over a range of Na^+ concentrations (*see* Table 3) and hence changing intracellular K^+/Na^+ ratios. Alternatively, it is notable that in experiments with oocytes, the apparent affinity for extracellular K^+ was increased by both γ variants provided that the membrane potential was clamped at values more negative than ≈−50 mV (Beguin et al., 2001; Li et al., 2004). Therefore it is plausible that in HeLa cells as in oocytes, there is a membrane potential-sensitive γ-mediated increase in the intrinsic affinity of the pump for extracellular K^+.

What is the physiological significance of the increase in apparent K^+ affinity caused by γ seen in experiments with intact cells? Under physiological conditions the normal serum K^+ concentration ranges from 3.7 to 5.2 mM and thus, extracellular K^+ activation sites would not be completely saturated, particularly in cells without γ. Thus, at the highest concentration of Na^+ tested (80 mM), $K_{0.5(K)}$ values were 2.90 mM and 1.87 mM for control and γ-transfected cells, respectively (*see* Table 3). These values would be even higher at physiological concentrations (≈140 mM) of extracellular Na^+ due to the well-documented Na^+/K^+ competition at extracellular K^+-activation sites (Balshaw et al., 2000). Accordingly, the increase in apparent affinity caused by γ is another means of maintaining pump activity under energy-compromised conditions.

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