Am J Physiol Renal Physiol 294: F859–F866, 2008.
First published December 19, 2007; doi:10.1152/ajprenal.00089.2007.

Isoform specificity of Na-K-ATPase-mediated ouabain signaling

Sandrine V. Pierre,[1] Yoann Sottejeau,[1] Jean-Michel Gourbeau,[1] Gladis Sánchez,[2] Amjad Shidyak,[1] and Gustavo Blanco[2]

[1]*Department of Physiology and Pharmacology, University of Toledo College of Medicine, Toledo, Ohio; and* [2]*Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, Kansas*

Submitted 20 February 2007; accepted in final form 12 December 2007

Pierre SV, Sottejeau Y, Gourbeau J-M, Sánchez G, Shidyak A, Blanco G. Isoform specificity of Na-K-ATPase-mediated ouabain signaling. *Am J Physiol Renal Physiol* 294: F859–F866, 2008. First published December 19, 2007; doi:10.1152/ajprenal.00089.2007.— The ion transporter Na-K-ATPase functions as a cell signal transducer that mediates ouabain-induced activation of protein kinases, such as ERK. While Na-K-ATPase composed of the α_1-polypeptide is involved in cell signaling, the role of other α-isoforms (α_2, α_3, and α_4) in transmitting ouabain effects is unknown. We have explored this using baculovirus-directed expression of Na-K-ATPase polypeptides in insect cells and ERK phosphorylation as an indicator of ouabain-induced signaling. Ouabain addition to Sf-9 cells coexpressing Na-K-ATPase α_1- and β_1-isoforms stimulated ERK phosphorylation. In contrast, expression of the α_1- and β_1-polypeptides alone resulted in no effect, indicating that the αβ-complex is necessary for Na-K-ATPase signaling. Moreover, the ouabain effect was sensitive to genistein, suggesting that Na-K-ATPase-mediated tyrosine kinase activation is a critical event in the intracellular cascade leading to ERK phosphorylation. In addition, the Na-K-ATPases $\alpha_3\beta_1$- and $\alpha_4\beta_1$-isozymes, but not $\alpha_2\beta_1$, responded to ouabain treatment. In agreement with the differences in ouabain affinity of the α-polypeptides, $\alpha_1\beta_1$ required 100- to 1,000-fold more ouabain to signal than did $\alpha_4\beta_1$ and $\alpha_3\beta_1$, respectively. These results confirm the role of the Na-K-ATPase in ouabain signal transduction, show that there are important isoform-specific differences in Na-K-ATPase signaling, and demonstrate the suitability of the baculovirus expression system for studying Na-K-ATPase-mediated ouabain effects.

α-isoforms; Sf-9; genistein; ERK

THE ENERGY-TRANSDUCING ION pump Na-K-ATPase is an enzyme classically known for its critical role in maintaining the Na^+ and K^+ gradients across the plasma membrane of most eukaryotic cells (27). The Na-K-ATPase is the receptor for cardiotonic steroids, a group of compounds that includes the glycoside ouabain (26). Ouabain binding to the Na-K-ATPase inhibits the catalytic and transport activity of the enzyme (14). In most cell types, ouabain-Na-K-ATPase binding also elicits a cascade of cellular events that include the interaction of the enzyme at the plasma membrane with neighboring proteins and the activation of the tyrosine kinase Src. This initial response subsequently causes the recruitment and phosphorylation of a series of proteins such as the extracellular regulated kinase ERK (22, 30). The process ultimately stimulates transcription and translation of a series of genes that are involved in regulation of cell proliferation and promotes changes in cell migration and metabolism (1, 11, 30).

Structurally, the Na-K-ATPase consists of two major polypeptides, the α- and β-subunits (14). The α-polypeptide is the catalytic subunit that contains the binding sites for Na^+, K^+, ATP, and cardiotonic steroids (14). Different α-subunits (α_1, α_2, α_3, and α_4) have been identified in mammalian cells (3, 19). The α1-isoform is expressed in almost every cell, while the other α-polypeptides exhibit a tissue-specific pattern of expression (3). In addition, Na-K-ATPase isozymes composed of different α-isoforms have unique kinetic properties and a distinctive response to second messengers (3, 9, 23). The characteristic expression, activity, and regulation of the Na-K-ATPase isoforms suggest that the molecular heterogeneity of the Na-K-ATPase catalytic subunit is of physiological relevance. Importantly, evidence for the biological role of the α-isoforms is supported by studies in transgenic animals and through the identification of mutations of the transporter in humans (12, 17, 21, 28, 29, 32, 33).

One of the most conspicuous functional differences among α-isoforms of the Na-K-ATPase in the rat is their reactivity to ouabain, with α_1 having a much lower ouabain sensitivity than α_2, α_3, and α_4 (3). This particular functional property appears to be physiologically important in regulating Na-K-ATPase activity in an isoform-specific manner (3, 12). In support of the biological importance of a modulatory action for ouabain, the cardiotonic steroid has been found to exist as an endogenous substance that circulates in plasma of humans and other mammals (10, 25). In addition to regulating enzymatic and transport activities, the differences in affinity of Na-K-ATPase isoforms for ouabain may translate into differences in the ability of each α-polypeptide to relay ouabain-binding signals into the cell. At present, there is experimental evidence that the signaling function of the Na-K-ATPase is mediated by the α1 polypeptide (22, 30). However, the role of other α-isoforms as transducers of the ouabain message is unknown. Studying the effect of ouabain on the Na-K-ATPase isoforms is a difficult task. A major obstacle is that the various α-polypeptides are coexpressed in cells in different combinations, and with the ubiquitous α_1-polypeptide (3, 19). This problem can be circumvented by using heterologous expression systems. In the past, we have successfully used the baculovirus expression system to study different aspects of the enzymatic and regulatory properties of the Na-K-ATPase isoforms in insect cells (4). Sf-9 insect cells provide several advantages for the study of the Na-K-ATPase, including the production of high amounts of the α- and β-subunits and the proper delivery of catalytically competent Na-K-ATPase isozymes to the plasma membrane (4). Importantly, Sf-9 cells contain negligible levels of endog-

Address for reprint requests and other correspondence: S. V. Pierre, Dept. of Physiology and Pharmacology, Univ. of Toledo College of Medicine, 3035 Arlington Ave., Toledo, OH 43614-5804 (e-mail: sandrine.pierre@utoledo.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

enous Na-K-ATPase, which permits analysis of the baculovirus-produced isoforms in the absence of high background levels of the transporter (4). Finally, and most significantly, the baculovirus expression system preserves the natural intrinsic ouabain binding capabilities of the different α-polypeptides expressed (4). In this work, we have used the baculovirus expression system to explore the ouabain-dependent signaling function of Na-K-ATPase isozymes composed of the different α-isoforms. We show that *1*) Na-K-ATPase-mediated ouabain signaling takes place through a tyrosine kinase-dependent pathway that leads to the activation of ERK in Sf-9 cells; *2*) both the α- and β-subunits of the Na-K-ATPase are required for ouabain signaling by the transporter; *3*) the α_1, α_3, and α_4 isoforms, but not α_2 can transmit ouabain-induced phosphorylation of ERK in the cells; and *4*) Na-K-ATPase isoform-mediated signaling is a ouabain dose-dependent process determined by the particular affinity of each α-polypeptide for the cardiotonic steroid.

MATERIALS AND METHODS

Cell maintenance, baculovirus construction. and viral infections. Sf-9 cells were grown at 27°C in Grace's medium (JRH Biosciences, Lenexa, KS) with 3.3 g/l lactalbumin hydrolysate, 3.3 g/l yeastolate, and supplemented with 10% (vol/vol) fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone (complete medium), as described elsewhere (8). Baculovirus preparation and selection were performed according to the procedures recommended by the supplier (Invitrogen, Carlsbad, CA), as described previously (8). Viral infections were performed in 60-mm petri dishes using a multiplicity of infection of ~5.

Ouabain treatment and processing of cells. Following infection, cells were incubated for 48 h at 27°C, after which complete culture media was changed to serum-free media. After an additional 24-h incubation period, cells were treated with or without different concentrations of ouabain and/or genistein for 15 min unless otherwise stated. Cell treatment was finished by rapidly aspirating the medium and washing the cells once with 5 ml ice-cold PBS. Then, cells were lysed in 1 ml of ice-cold lysis buffer, containing 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, 1 mM NaF, and 50 mM Tris·HCl (pH 7.4). Cell lysates were placed on ice for 15 min and centrifuged at 16,000 *g* for 10 min. The pellets were discarded, and the cleared supernatants were used for immunoblot analysis.

PAGE and immunoblot analysis. Expression of the α- and β-isoforms of the Na-K-ATPase was analyzed by SDS-PAGE (7.5% gel) and immunoblotting. Protein concentration of the cell supernatant was determined using the dye-binding assay based on the method of Bradford from Bio-Rad (Hercules, CA). Samples containing 20 μg of total protein/lane were then separated by SDS-PAGE, transferred onto nitrocellulose membranes (Nitrobind, Osmonics, Minnetonka, MN), and immunoblotted, as described previously (7). Primary antibodies specific for each of the rat Na-K-ATPase isoforms were used to identify the corresponding polypeptides. For α_1, a 1:100 dilution of anti-α_1-synthetic peptide was used (8). The α_2-isoform was identified with MCB2 at a 1:300 dilution (2). For α_3, anti-α_3-synthetic peptide at 1:200 was used (8). The α_4-polypeptide was detected with 1:100 of the anti-α_4-polyclonal antisera (7). The β_1-polypeptide was identified with poly-αA-antiserum at 1:100 (8). Horseradish peroxidase-conjugated goat anti-mouse (1:2,000) and goat anti-rabbit (1:500) secondary antibodies (Santa Cruz Biotechnology, Santa Cruz. CA) and chemiluminescence were used for detection.

Immunoblotting was also used to determine activation of ERK. For this, 60 μg total protein/lane was separated by SDS-PAGE and transferred to nitrocellulose. The monoclonal anti-phospho-ERK (1:500) and polyclonal anti-ERK (1:2,000) antibodies were used to identify the phosphorylated and total forms of ERK, respectively. Immunoblots were first probed with anti-phospho-ERK, then stripped and reprobed with anti-total ERK to control for equal protein loading in the gels, as we have previously reported (13). Horseradish peroxidase and chemiluminescence were used for detection. The intensity of ERK signals was determined using an Imaging Densitometer (GS-670, Bio-Rad). The levels of phosphorylated ERK (p-ERK) were expressed as the ratio between the phosphorylated and nonphosphorylated forms of the protein and were normalized against the corresponding controls. Also, immunoblots were used to explore the presence of the phosphorylated and total forms of c-Src and caveolin-1. Anti-c-Src and anti-caveolin-1 antibodies were purchased from Santa Cruz Biotechnology, anti phospho c-Src was obtained from Invitrogen, and anti-phospho caveolin-1 was acquired from BD Biosciences Pharmingen (San Diego, CA).

Statistical analysis. Statistical significance of the differences in of ERK phosphorylation between ouabain-treated and untreated groups was performed using one-way ANOVA, followed by Dunnett's multiple comparison post hoc test. Statistical significance was defined as $P < 0.05$.

RESULTS

ERK is expressed and phosphorylated in Sf-9 cells. Despite their extensive use as an expression system, the cell biology of Sf-9 cells is still not completely understood. Therefore, initially, we had to establish whether Na-K-ATPase-ouabain signaling could be reconstituted in the cells. ERK is a key kinase in the ouabain-dependent signaling cascade, and its activation is commonly used as an indicator of the intracellular effect of the cardiotonic steroid in mammalian cells (22, 31). Accordingly, to validate the baculovirus expression system as a tool to study Na-K-ATPase isoform signaling, our first goal was to determine the endogenous levels and phosphorylation status of ERK in Sf-9 insect cells. The total and phosphorylated forms of ERK were explored in uninfected Sf-9 cells grown under standard culture conditions by immunoblotting, using antibodies that specifically recognize the nonphosphorylated or the activated forms of the protein. As a control, we used rat neonatal cardiomyocyte lysates, where ERK has been extensively characterized (15, 20). As shown in Fig. 1*A*, ERK is expressed and undergoes phosphorylation in Sf-9 cells. However, the pattern of ERK expression differs between insect cells and rat neonatal cardiomyocytes. While the cardiac cells show the typical bands of ERK, at 42 and 44 kDa, corresponding to ERK1 and ERK2, respectively, Sf-9 cells exhibited a single band, consistent with the total and phosphorylated form of ERK that migrates at 42 kDa. These results suggest that only ERK1 is present in Sf-9 cells. However, the identity of the form of ERK found in the insect cells cannot be unequivocally concluded from the electrophoretic mobility pattern of the proteins. Electrophoretic migration is influenced by protein posttranscriptional modifications, and it is possible that post-translational changes in ERK may differ in insect and mammalian cells, thus affecting the mobility, and precluding identification of the particular ERK isoforms. In any case, these results clearly show that ERK is present in insect cells and that the kinase exists in a phosphorylated state under basal tissue culture conditions of the cells.

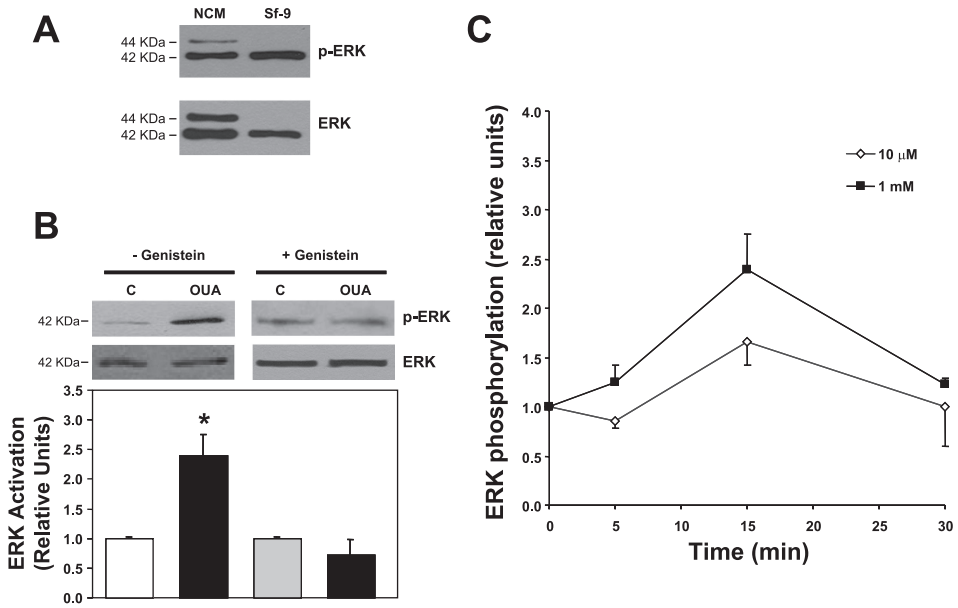

Fig. 1. ERK phosphorylation in insect cells. *A*: characterization of ERK in Sf-9 cells. Proteins from uninfected Sf-9 cells were separated by SDS-PAGE, immunoblotted, and probed with a monoclonal antibody specific for the phosphorylated forms of ERK1 and -2 (*top*). After stripping, blots were reprobed with a polyclonal antibody specific for total ERK1 and -2. Horseradish peroxidase and chemiluminescence were used for detection. Rat neonatal cardiac myocytes (NCM) were included as a control. *B*: ouabain-induced and Na-K-ATPase-mediated ERK phosphorylation in Sf-9 cells involves tyrosine kinases. Sf-9 cells expressing rat Na-K-ATPase-$\alpha_1\beta_1$ were treated in the absence (C) and presence of 1 mM ouabain (OUA) for 15 min, with or without pretreatment for 30 min with 100 μM genistein, a protein tyrosine kinase inhibitor. Phospho- ERK/total ERK ratios were normalized against the corresponding nontreated controls. A representative blot is shown, while bars are means ± SE of 3 independent experiments. *C*: time course of ouabain-induced ERK phosphorylation in insect cells. Sf-9 cells expressing rat Na-K-ATPase $\alpha_1\beta_1$ were treated with either 10 μM or 1 mM ouabain for the indicated times and assayed for ERK phosphorylation as described above. Phospho-ERK/total ERK ratios for each time point were normalized against the corresponding value at *time 0*. Values are means ± SE of 3 independent experiments.

Tyrosine kinase activation is critical for ouabain-induced ERK phosphorylation in Sf-9 cells. We next investigated whether ouabain, acting through the Na-K-ATPase, could induce ERK phosphorylation in insect cells. For this, Sf-9 cells expressing the rat Na-K-ATPase α_1- and β_1-isoforms were exposed to ouabain and the relative levels of ERK1 phosphorylation were determined by immunoblotting. As a first approach, we used a relatively high concentration of ouabain (1 mM) and an incubation time with the cardiotonic steroid of 15 min, which are parameters known to produce maximal ERK phosphorylation in mammalian cells (15). As shown in Fig. 1*B*, compared with the untreated controls, ouabain caused an ~2.5-fold increase in ERK1 phosphorylation. This suggests that ouabain signaling via the Na-K-ATPase can be obtained in Sf-9 cells.

Other proteins that become phosphorylated on ouabain stimulation in myocardial cells are Src and caveolin 1. To determine whether these polypeptides are also involved in Na-K-ATPase signaling in Sf-9 cells, we studied the expression of the total and phosphorylated forms of Src and caveolin 1 in the cells. While we could identify expression of the nonphosphorylated forms of these polypeptides, we were unable to detect the activated Src and caveolin 1 neither in the untreated cells nor in the cells incubated with ouabain (data not shown). This indicated that, in the insect cells, and with the antibodies available, Src and caveolin 1 could not be used as indicators of ouabain-induced Na-K-ATPase-mediated signaling.

In mammalian cells, the Na-K-ATPase-ouabain effect requires the activity of tyrosine kinases (30). Because genistein has been previously used to efficiently inhibit tyrosine kinase activity in insect cells (24), we used the inhibitor to test whether these kinases are involved in the ouabain-induced phosphorylation of ERK1 mediated by the Na-K-ATPase $\alpha_1\beta_1$-isozyme. Figure 1*B* shows that, as has been observed in mammalian cells (13, 16), addition of 100 μM genistein 30 min before treatment with ouabain completely abolished ouabain-induced ERK1 phosphorylation. Altogether, these results suggest that ouabain-Na-K-ATPase signaling can be reconstituted in Sf-9 cells and that the process involves intracellular messengers, such as tyrosine kinases and ERK, which are components of the ouabain-Na-K-ATPase signaling pathway in mammalian cells.

Time course of ouabain-induced ERK phosphorylation in Sf-9 cells. The ouabain-mediated and Na-K-ATPase-dependent increase in ERK phosphorylation in Sf-9 cells occurred relatively quickly and could be observed after 15 min of treatment of the cells with ouabain. However, the time-dependent kinetics of ouabain-induced p-ERK formation in Sf-9 cells was unknown. To determine this, we treated insect cells expressing the Na-K-ATPase $\alpha_1\beta_1$-isozyme with two different concentrations of ouabain, 10 μM or 1 mM, and measured the relative amounts of ERK1 phosphorylation by immunoblotting at various time points after exposure to the cardiotonic steroid. As shown in Fig. 1*C*, ouabain induced p-ERK formation in the cells in a time-dependent manner, with the kinase achieving maximal phosphorylated values 15 min after treatment with ouabain. Although both concentrations of ouabain produced similar temporal patterns of ERK activation, the values of p-ERK obtained with 1 mM ouabain were higher, suggesting that signaling of the Na-K-ATPase $\alpha_1\beta_1$-isozyme is also de-

pendent on the ouabain dose. These results demonstrated the time-dependence of ouabain-Na-K-ATPase signaling in Sf-9 cells and established 15 min as the optimal time to assess ouabain signaling in the cells for all subsequent experiments.

Na-K-ATPase expression is required for ouabain signaling effect. Different experimental approaches have shown that the Na-K-ATPase is the receptor that binds and mediates the effects of cardiotonic steroids in the cell (1, 22, 30). Most recent studies, involving transgenic mice in which ouabain affinity of Na-K-ATPase isoforms was modified, support the physiological relevance of the ouabain binding site in the enzyme and the importance of interaction of ouabain with the transporter (12). Here, we have used the baculovirus expression system to confirm whether the Na-K-ATPase is the receptor and signal transducer for ouabain and to determine which subunits of the transporter are required for mediating signal transduction events. Because Sf-9 cells practically lack endogenous Na-K-ATPase, we were able to directly evaluate this issue in the insect cells. Thus we compared the effect of ouabain on ERK phosphorylation in Sf-9 cells expressing the whole Na-K-ATPase αβ-complex or the individual α- and β-subunits of the enzyme. For this, Sf-9 cells were infected with viruses directing expression of the Na-K-ATPase α_1- and β_1-subunits, together or separately. Cells were then treated with increasing concentrations of ouabain, and ERK phosphorylation was measured. As shown in Fig. 2*A*, cells expressing the Na-K-ATPase α_1-subunit alone exhibited the normal basal levels of ERK phosphorylation, which were not affected by addition of ouabain. Similarly, the Na-K-ATPase β-polypeptide was unable to transmit ouabain effects in the insect cells (Fig. 2*B*). In contrast, coexpression of the α_1- and β_1-polypeptides in Sf-9 cells showed the expected ouabain-dependent phosphorylation of ERK (Fig. 2*C*). p-ERK levels in cells expressing the $\alpha_1\beta_1$-isozyme varied depending on the ouabain dose, with the highest response obtained with ouabain concentrations of 100 and 1,000 μM (Fig. 2*C*). Altogether, these experiments confirm that the baculovirus expression system represents a useful tool for studying Na-K-ATPase signaling, and, most importantly, they show that expression of the Na-K-ATPase αβ-holoenzyme is an absolute requirement for ouabain-induced ERK phosphorylation. This confirms that the Na-K-ATPase is the signal transducer that mediates ouabain effects in the cell.

Ouabain-induced ERK phosphorylation specificity of the Na-K-ATPase α-isoforms. To determine whether Na-K-ATPases containing isoforms different from α_1 could also signal, we explored the effect of increasing concentrations of ouabain on ERK phosphorylation in Sf-9 cells expressing the Na-K-ATPase α_2-, α_3- or α_4-isoforms in combination with the β_1-subunit. The results are shown in Figs. 3, 4, and 5. While Figs. 3*A*, 4*A*, and 5*A* show expression of the corresponding Na-K-ATPase isoforms, Figs. 3*B*, 4*B*, and 5*B* present the amounts of total and phosphorylated forms of ERK. The first striking observation was that the α_2-isoform was not able to elicit the ouabain-dependent activation of ERK in the cells regardless of the amount of ouabain added to the media (Fig. 3*B*). In this manner, cells expressing $\alpha_2\beta_1$ showed comparable levels of p-ERK at all concentrations of ouabain (Fig. 3*B*). The absence of signaling by the α_2-isoform was not dependent on the inability of the cells to produce functionally competent molecules of Na-K-ATPase-$\alpha_2\beta_1$. We measured the ouabain-sensitive hydrolysis of ATP in the cells and determined that the expressed $\alpha_2\beta_1$-enzyme complex had an average maximal activity (V_{max}) of 0.9 ± 0.07 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$, a value that is similar to that previously reported for the baculovirus-generated isozyme (6). Therefore, it appears that, although in the insect cells the Na-K-ATPase α_2-isoform is enzymatically active, it does not function as a signal transduction molecule. In contrast to α_2, when Na-K-ATPase composed of the α_3- and α_4-isoforms were expressed at constant levels (Figs. 4*A* and 5*A*), ouabain-induced ERK phosphorylation was observed in the cells. Signaling by these Na-K-ATPases was dose depen-

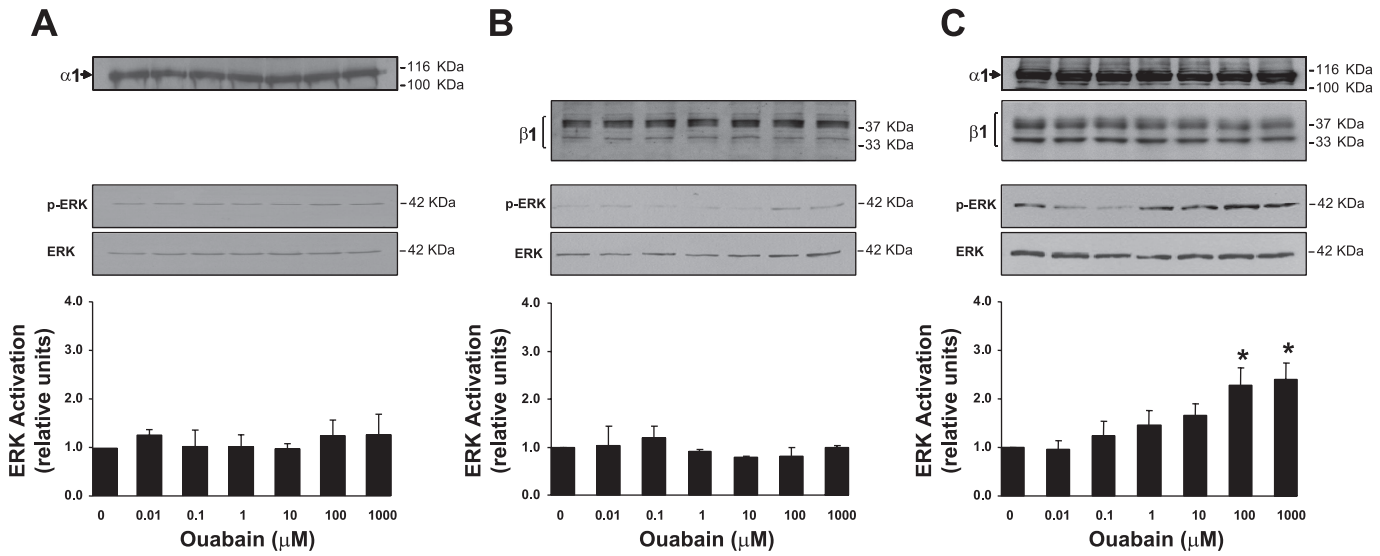

Fig. 2. Expression of a Na-K-ATPase $\alpha_1\beta_1$ complex is required for ouabain-induced ERK phosphorylation. Sf-9 cells expressing the α_1 (*A*)- and the β_1 (*B*)-subunits of the Na-K-ATPase alone, or the α_1- and β_1 polypeptides together (*C*) were treated with the indicated concentrations of ouabain for 15 min, and ERK phosphorylation levels were determined. Representative blots are shown for expression of the corresponding Na-K-ATPase subunits and total and phospho-ERK. The bar graph summarizes data from 3–7 determinations depending on ouabain doses, with bars representing means ± SE. *Statistically different values compared with the control at zero ouabain ($P < 0.05$).

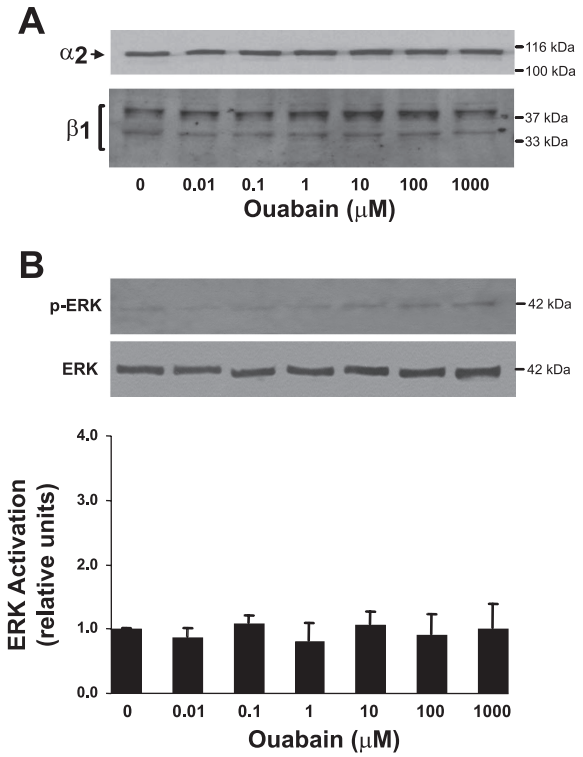

Fig. 3. Na-K-ATPase $\alpha_2\beta_1$ complex is not able to signal ouabain effect in insect cells. *A*: expression of the α_2- and β_1-subunits was confirmed in the insect cells by immunoblotting using antibodies specific to the corresponding Na-K-ATPase polypeptides. *B*: levels of total and phosphorylated (p) ERK were determined in cells expressing $\alpha_2\beta_1$ as explained in MATERIALS AND METHODS after treatment without and with the indicated concentrations of ouabain for 15 min. A representative blot for total and p-ERK is shown, while the bar graph represents data from 3–7 determinations depending on ouabain doses, with bars representing means ± SE.

dent, and the peak of ouabain effect was detected at 0.1 μM for α_3 and 0.1–1 μM for α_4 (Figs. 4*B* and 5*B*, respectively). Although low concentrations of ouabain are able to trigger signaling through both the α_3- and α_4-isoforms, comparison of the dose-response curves for ERK activation suggests that those isoforms respond to the cardiotonic steroid with different kinetics. Thus, while signaling through α_4 remains relatively high on addition of increasing doses of ouabain (Fig. 5*B*), signaling via α_3 is reduced at higher concentrations of the cardiotonic steroid (Fig. 4*B*). In fact, as shown in Fig. 4*B*, in the cells expressing α_3 and treated with 100 and 1,000 μM ouabain, the levels of p-ERK are not significantly different from those of untreated cells.

To better compare the signaling ability of Na-K-ATPases composed of different α-isoforms, the ouabain-dependent ERK phosphorylation values for $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_4\beta_1$, obtained from Figs. 2*B*, 3*B*, 4*B*, and 5*B* were compiled and plotted. As shown in Fig. 6, Na-K-ATPase signaling exhibits a distinct profile depending on the α-isoform considered. Altogether, these results indicate that the α_2-isoform is the only catalytic subunit of the Na-K-ATPase that is not capable of transducing the ouabain effect in the Sf-9 model. In addition, and in agreement with the differences in affinity of the isoforms for ouabain, α_1 requires significantly higher concentrations of the cardiotonic steroid than α_3 and α_4.

DISCUSSION

Molecular heterogeneity of the Na-K-ATPase is important in adjusting the Na^+ and K^+ gradients to the specific needs of each cell (3). In the present work, we have explored whether Na-K-ATPase α-isoform diversity also plays a role in the ability of the enzyme to relay ouabain-induced signaling in the cell. Using heterologous expression in insect cells and ERK phosphorylation as an indicator of ouabain-activated signaling, we confirmed that the Na-K-ATPase is the receptor that mediates ouabain-triggered signaling cascades in the cells. Importantly, the Na-K-ATPase holoenzyme complex is necessary for signaling, and the α- or β-subunits alone cannot mediate ouabain effects in the cells. The inability of the β-subunit to signal agrees with the notion that the β-polypeptide is not directly involved in ouabain binding (14). The lack of reactivity of the α-subunit to ouabain stimulation when it is expressed alone may also depend on the incapacity of the polypeptide to bind ouabain. It is known that to acquire its normal protein conformation, the α-subunit requires the presence of the β-subunit (5, 18). Moreover, in the absence of the β-subunit, the α-polypeptide exhibits properties that are different from those of the Na-K-ATPase catalytic subunit; most noticeably, the isolated α-polypeptide shows insensitivity to ouabain (5). Thus

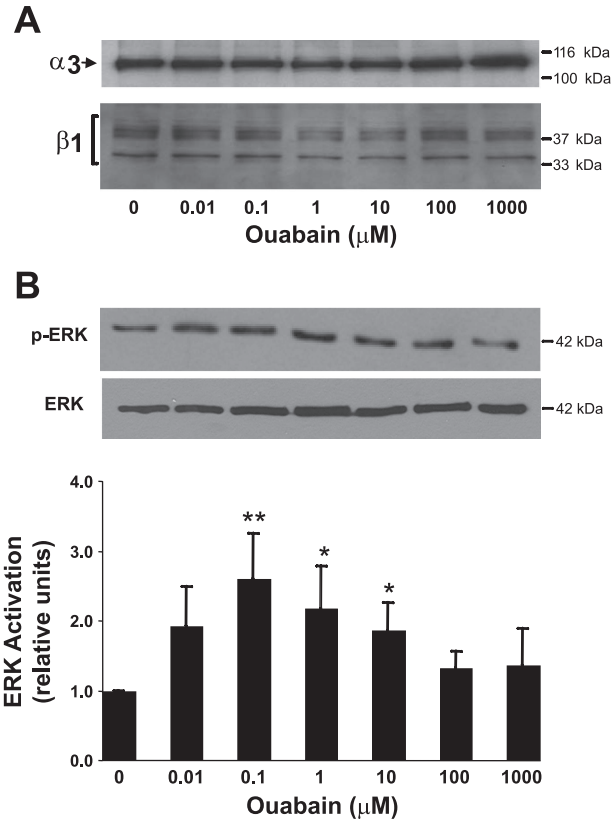

Fig. 4. Expression of $\alpha_3\beta_1$ in Sf9 cells mediates ouabain-induced ERK phosphorylation. *A*: expression of the α_3- and β_1-subunits in the insect cells as confirmed by immunoblot using antibodies specific to the corresponding Na-K-ATPase polypeptides. *B*: levels of total and p-ERK were determined in cells expressing $\alpha_3\beta_1$ as explained in MATERIALS AND METHODS after treatment without and with the indicated concentrations of ouabain for 15 min. A representative gel is shown, and the bars represent means ± SE of 3–5 determinations depending on ouabain doses. Asterisks indicate statistically different values compared with the control at zero ouabain ($P < 0.05$).

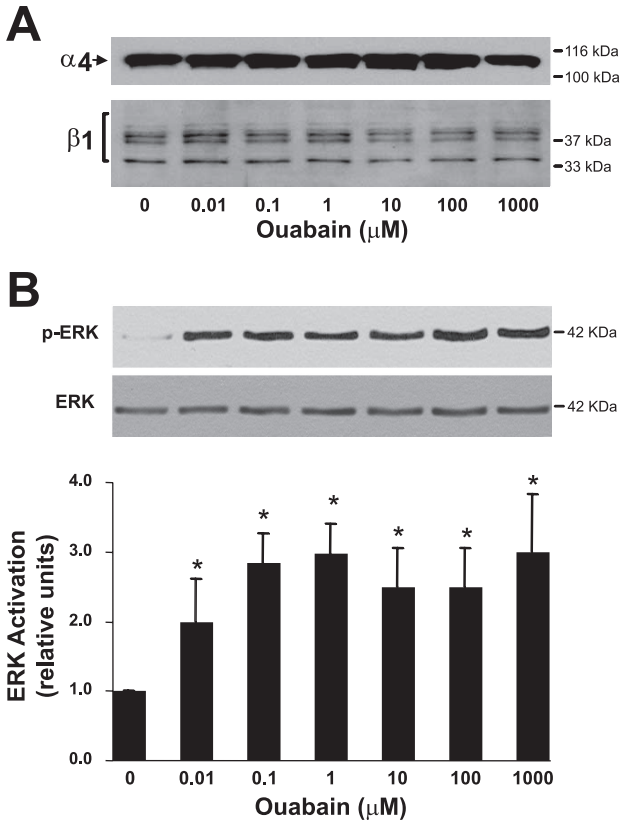

Fig. 5. Expression of $\alpha_4\beta_1$ in Sf9 cells mediates ouabain-induced ERK phosphorylation. *A*: expression of the α_4- and β_1-subunits in the insect cells as confirmed by immunoblot using antibodies specific to the corresponding Na-K-ATPase polypeptides. *B*: levels of total and p-ERK were determined in cells expressing $\alpha_4\beta_1$ as explained in MATERIALS AND METHODS after treatment without and with the indicated concentrations of ouabain for 15 min. A representative gel is shown, and the bars represent means ± SE of 4–6 determinations depending on ouabain doses. *Significantly different from untreated controls ($P < 0.05$).

it appears that the stabilization of the α-protein conformation by the β-polypeptide is necessary for the ouabain-signaling function of the Na-K-ATPase. This requirement of the Na-K-ATPase holoenzyme for signaling confirms previous observations and agrees with the notion that the transporter is the natural receptor for the cardiotonic steroids (10, 26, 30).

Our results also demonstrate that the baculovirus-directed expression of Na-K-ATPase in insect cells is a useful system for studying the signaling function of the enzyme. We have found that ouabain signaling in Sf-9 cells takes place via activation of tyrosine kinases and the phosphorylation of ERK, a key component of the Na-K-ATPase-ouabain cascade in mammalian cells (30). In contrast, we were unable to identify activation of some other messengers described in the ouabain-signaling pathway in mammalian cells, such as the phosphorylated forms of Src and caveolin 1. This observation could be attributed to a lack of cross-reactivity of the anti-phospho Src and caveolin 1 antibodies for mammalian and insect cells, or it may just reflect the absence of ouabain-induced Src and caveolin 1 phosphorylation in the Sf-9 cells. Clearly, a complete understanding of the intracellular pathways responsible for ouabain-mediated ERK activation in insect cells will require further studies. Importantly, our findings of a ouabain-dependent and genistein-sensitive phosphorylation of ERK in Sf-9 cells expressing Na-K-ATPase molecules suggest that the baculovirus expression system represents a valuable system for studying not only the enzymatic properties (4), but also the signaling role of the various isoforms of the transporter.

Interestingly, not all Na-K-ATPase α-isoforms are able to signal, and α_2 is the only catalytic subunit of the enzyme that does not support ouabain-induced ERK activation in the insect cells. Our results show that the α_2-isoform expressed in Sf-9 cells is catalytically competent and has the ability to hydrolyze ATP in a ouabain-sensitive manner. This suggests that the lack of signaling by α_2 is not due to gross alterations in the structure of the expressed isoform but rather to the intrinsic differences in the ability of the α_2-polypeptide to function as a signal transduction molecule. Therefore, expression of a high-affinity receptor for ouabain, such as the α_2-isoform, is not an exclusive factor for ouabain signaling in the cell, and other structural determinants within the α-polypeptide may be important for the signal transduction function of the Na-K-ATPase. However, there are alternative explanations for the inability of α_2 to signal. Thus the β-subunit may influence the signaling ability of the Na-K-ATPase complex, and while α_2 is unable to transmit the ouabain effect when coexpressed with β_1, it may acquire this ability when associated with one of the other β-isoforms. Also, it is conceivable that α_2 may signal through a different intracellular pathway that does not involve activation of ERK. It is possible that signaling by α_2 may require second messengers that are present in mammalian cells but not in Sf-9 cells. Further investigation will be needed in this area to better understand the molecular basis of the peculiar behavior of α_2 in ouabain signaling. In any case, it is clear that among other characteristics, the Na-K-ATPase isoforms can be distinguished by their different ouabain-dependent signaling properties.

For those Na-K-ATPase α-isoforms that do signal a ouabain effect in Sf-9 cells, a dose-dependent response to ouabain was found. According to the intrinsic ability of the isoforms to bind ouabain, the α_1- polypeptide required 100- to 1,000-fold more ouabain to signal than did α_4 and α_3. Therefore, the affinity of

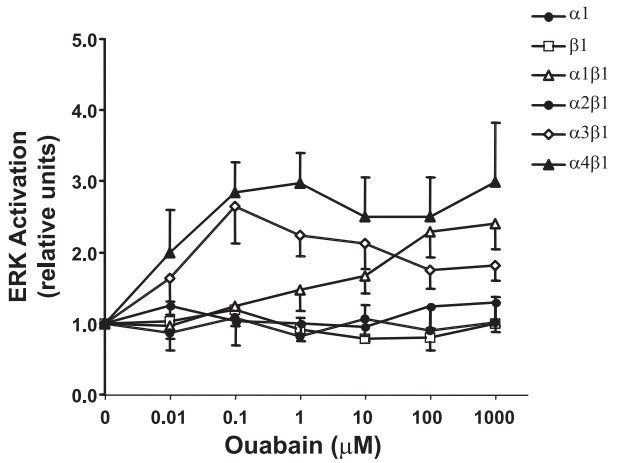

Fig. 6. Summary of ouabain-dependent ERK phosphorylation mediated by different Na-K-ATPase α-isoforms in Sf9 cells. Relative values of ouabain-dependent p-ERK levels in insect cells expressing the various Na-K-ATPase α-isoforms obtained from the data in Figs. 2–5 were plotted for comparison of the signaling properties of Na-K-ATPases composed of different α-isoforms.

these α-isoforms for ouabain is an important property that influences the signaling ability of the Na-K-ATPase. In addition, the ouabain-dependent kinetics of ERK phosphorylation appears to be different between the ouabain-sensitive α_3- and α_4-isoforms. Thus, while both polypeptides start signaling at low ouabain concentrations, α_3 exhibits a narrower ouabain range for optimal signal transduction. Although an explanation for this isoform-specific response is unavailable for the moment, the differences in signal transduction kinetics we observed are suggestive of differences in isoform-specific receptor inactivation rates due, for example, to putative dissimilarities in the rate of internalization of each isoform after ouabain binding. Additional experiments are required to further investigate this particular aspect of Na-K-ATPase isoform signaling. Importantly, the different reactivity of the α-isoforms to cardiotonic steroids may not only be relevant for ouabain-controlled ion transport activity of the Na-K-ATPase but may also represent a mechanism to regulate the ouabain-dependent signaling of the enzyme. This is of physiological relevance, since controlling the expression of the different Na-K-ATPase α-isoforms would confer cells with a distinct susceptibility to the circulating levels of ouabain. In this manner, the response to ouabain can be adapted to the particular needs of each cell type.

In conclusion, we have used the baculovirus expression system to successfully demonstrate Na-K-ATPase-ouabain signaling in insect cells, confirmed the absolute requirement of both subunits of the enzyme in the process, and uncovered important isoform-specific differences in the Na-K-ATPase-mediated cellular response to ouabain.

ACKNOWLEDGMENTS

We thank Kathleen Sweadner for providing the anti-α_2-Na-K-ATPase antibody, Dr. Zi-Jian Xie for helpful discussion and input throughout the study, and Dr. Eric E. Morgan for kind help with the manuscript.

GRANTS

This work was supported by National Institutes of Health Grants HL-36573, HD-043044, HD-055763, and RR-19799. Y. Sottejeau and J.-M. Gourbeau were fellows from the Institut Universitaire Professionnel (IUP) "Genie Physiologique et Informatique," Poitiers, France.

REFERENCES

1. **Aperia A.** New roles for an old enzyme: Na,K-ATPase emerges as an interesting drug target. *J Intern Med* 261: 44–52, 2007.
2. **Arystarkhova E, Sweadner KJ.** Isoform-specific monoclonal antibodies to Na,K-ATPase alpha subunits. Evidence for a tissue-specific post-translational modification of the alpha subunit. *J Biol Chem* 271: 23407–23417, 1996.
3. **Blanco G.** Na,K-ATPase subunit heterogeneity as a mechanism for tissue-specific ion regulation. *Semin Nephrol* 25: 292–303, 2005.
4. **Blanco G.** The NA/K-ATPase and its isozymes: what we have learned using the baculovirus expression system. *Front Biosci* 10: 2397–2411, 2005.
5. **Blanco G, DeTomaso AW, Koster J, Xie ZJ, Mercer RW.** The alpha-subunit of the Na,K-ATPase has catalytic activity independent of the beta-subunit. *J Biol Chem* 269: 23420–23425, 1994.
6. **Blanco G, Koster JC, Sanchez G, Mercer RW.** Kinetic properties of the alpha 2 beta 1 and alpha 2 beta 2 isozymes of the Na,K-ATPase. *Biochemistry* 34: 319–325, 1995.
7. **Blanco G, Melton RJ, Sanchez G, Mercer RW.** Functional characterization of a testes-specific alpha-subunit isoform of the sodium/potassium adenosinetriphosphatase. *Biochemistry* 38: 13661–13669, 1999.
8. **Blanco G, Sanchez G, Mercer RW.** Comparison of the enzymatic properties of the Na,K-ATPase alpha 3 beta 1 and alpha 3 beta 2 isozymes. *Biochemistry* 34: 9897–9903, 1995.
9. **Blanco G, Sanchez G, Mercer RW.** Differential regulation of Na,K-ATPase isozymes by protein kinases and arachidonic acid. *Arch Biochem Biophys* 359: 139–150, 1998.
10. **Blaustein MP.** Endogenous ouabain: physiological activity and pathophysiological implications. *Clin Investig* 72: 706–707, 1994.
11. **Contreras RG, Flores-Beni Tez D, Flores-Maldonado C, Larre I, Shoshani L, Cereijido M.** Na^+,K^+-ATPase and hormone ouabain: new roles for an old enzyme and an old inhibitor. *Cell Mol Biol (Noisy-le-grand)* 52: 31–40, 2006.
12. **Dostanic I, Paul RJ, Lorenz JN, Theriault S, Van Huysse JW, Lingrel JB.** The α_2-isoform of Na-K-ATPase mediates ouabain-induced hypertension in mice and increased vascular contractility in vitro. *Am J Physiol Heart Circ Physiol* 288: H477–H485, 2005.
13. **Haas M, Askari A, Xie Z.** Involvement of Src and epidermal growth factor receptor in the signal-transducing function of Na^+/K^+-ATPase. *J Biol Chem* 275: 27832–27837, 2000.
14. **Kaplan JH.** Biochemistry of Na,K-ATPase. *Annu Rev Biochem* 71: 511–535, 2002.
15. **Kometiani P, Li J, Gnudi L, Kahn BB, Askari A, Xie Z.** Multiple signal transduction pathways link Na^+/K^+-ATPase to growth-related genes in cardiac myocytes. The roles of Ras and mitogen-activated protein kinases. *J Biol Chem* 273: 15249–15256, 1998.
16. **Liu J, Tian J, Haas M, Shapiro JI, Askari A, Xie Z.** Ouabain interaction with cardiac Na^+/K^+-ATPase initiates signal cascades independent of changes in intracellular Na^+ and Ca^{2+} concentrations. *J Biol Chem* 275: 27838–27844, 2000.
17. **Looney MR, Sartori C, Chakraborty S, James PF, Lingrel JB, Matthay MA.** Decreased expression of both the α_1- and α_2-subunits of the Na-K-ATPase reduces maximal alveolar epithelial fluid clearance. *Am J Physiol Lung Cell Mol Physiol* 289: L104–L110, 2005.
18. **McDonough AA, Geering K, Farley RA.** The sodium pump needs its beta subunit. *FASEB J* 4: 1598–1605, 1990.
19. **Mobasheri A, Avila J, Cozar-Castellano I, Brownleader MD, Trevan M, Francis MJ, Lamb JF, Martin-Vasallo P.** Na^+, K^+-ATPase isozyme diversity; comparative biochemistry and physiological implications of novel functional interactions. *Biosci Rep* 20: 51–91, 2000.
20. **Mohammadi K, Kometiani P, Xie Z, Askari A.** Role of protein kinase C in the signal pathways that link Na^+/K^+-ATPase to ERK1/2. *J Biol Chem* 276: 42050–42056, 2001.
21. **Moseley AE, Huddleson JP, Bohanan CS, James PF, Lorenz JN, Aronow BJ, Lingrel JB.** Genetic profiling reveals global changes in multiple biological pathways in the hearts of Na, K-ATPase alpha 1 isoform haploinsufficient mice. *Cell Physiol Biochem* 15: 145–158, 2005.
22. **Pierre SV, Xie Z.** The Na,K-ATPase receptor complex: its organization and membership. *Cell Biochem Biophys* 46: 303–316, 2006.
23. **Pressley TA, Duran MJ, Pierre SV.** Regions conferring isoform-specific function in the catalytic subunit of the Na,K-pump. *Front Biosci* 10: 2018–2026, 2005.
24. **Reyes-Cruz G, Vazquez-Prado J, Muller-Esterl W, Vaca L.** Regulation of the human bradykinin B2 receptor expressed in sf21 insect cells: a possible role for tyrosine kinases. *J Cell Biochem* 76: 658–673, 2000.
25. **Schoner W, Bauer N, Muller-Ehmsen J, Kramer U, Hambarchian N, Schwinger R, Moeller H, Kost H, Weitkamp C, Schweitzer T, Kirch U, Neu H, Grunbaum EG.** Ouabain as a mammalian hormone. *Anne NY Acad Sci* 986: 678–684, 2003.
26. **Schoner W, Scheiner-Bobis G.** Endogenous and exogenous cardiac glycosides and their mechanisms of action. *Am J Cardiovasc Drugs* 7: 173–189, 2007.
27. **Skou JC, Esmann M.** The Na,K-ATPase. *J Bioenerg Biomembr* 24: 249–261, 1992.
28. **Vanmolkot KR, Kors EE, Hottenga JJ, Terwindt GM, Haan J, Hoefnagels WA, Black DF, Sandkuijl LA, Frants RR, Ferrari MD, van den Maagdenberg AM.** Novel mutations in the Na^+, K^+-ATPase pump gene ATP1A2 associated with familial hemiplegic migraine and benign familial infantile convulsions. *Ann Neurol* 54: 360–366, 2003.
29. **Wessman M, Kaunisto MA, Kallela M, Palotie A.** The molecular genetics of migraine. *Ann Med* 36: 462–473, 2004.
30. **Xie Z.** Membrane transporters and signal transduction. *Cell Mol Biol (Noisy-le-grand)* 52: 1–2, 2006.
31. **Xie Z, Cai T.** Na^+-K^+-ATPase-mediated signal transduction: from protein interaction to cellular function. *Mol Interv* 3: 157–168, 2003.

32. **Yamamoto T, Su Z, Moseley AE, Kadono T, Zhang J, Cougnon M, Li F, Lingrel JB, Barry WH.** Relative abundance of alpha2 Na^+ pump isoform influences Na^+-Ca^{2+} exchanger currents and Ca^{2+} transients in mouse ventricular myocytes. *J Mol Cell Cardiol* 39: 113–120, 2005.

33. **Zhang J, Lee MY, Cavalli M, Chen L, Berra-Romani R, Balke CW, Bianchi G, Ferrari P, Hamlyn JM, Iwamoto T, Lingrel JB, Matteson DR, Wier WG, Blaustein MP.** Sodium pump alpha2 subunits control myogenic tone and blood pressure in mice. *J Physiol* 569: 243–256, 2005.