Modulation of Na\(^+\)-K\(^+\)-ATPase cell surface abundance through structural
determinants on the \(\alpha_1\)-subunit

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Pierre SV, Belliard A, Sottejeau Y. Modulation of Na\(^+\)-K\(^+\)-ATPase cell surface abundance through structural determinants on the \(\alpha_1\)-subunit. Am J Physiol Cell Physiol 300: C42–C48, 2011. First published November 3, 2010; doi:10.1152/ajpcell.00386.2010.—Through their ion-pumping and non-ion-pumping functions, Na\(^+\)-K\(^+\)-ATPase protein complexes at the plasma membrane are critical to intracellular homeostasis and to the physiological and pharmacological actions of cardiotonic steroids. Alteration of the abundance of Na\(^+\)-K\(^+\)-ATPase units at the cell surface is one of the mechanisms for Na\(^+\)-K\(^+\)-ATPase regulation in health and diseases that has been closely examined over the past few decades. We here summarize these findings, with emphasis on studies that explicitly tested the involvement of defined regions or residues on the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) polypeptide. We also report new findings on the effect of manipulating Na\(^+\)-K\(^+\)-ATPase membrane abundance by targeting one of these defined regions: a dileucine motif of the form [D/E][X]XXL[LI]. In this study, opossum kidney cells stably expressing rat \(\alpha_1\) Na\(^+\)-K\(^+\)-ATPase or a mutant where the motif was disrupted (\(\alpha_1\)-L499V) were exposed to 30 min of substrate/coverslip-induced-ischemia followed by reperfusion (I-R). Biotinylation studies suggested that I-R itself acted as an inducer of Na\(^+\)-K\(^+\)-ATPase internalization and that expression of the mutant was higher than the native Na\(^+\)-K\(^+\)-ATPase before and after ischemia. Annexin V/propidium iodide staining and lactate dehydrogenase release suggested that I-R injury was reduced in \(\alpha_1\)-L499V-expressing cells compared with \(\alpha_1\)-expressing cells. Hence, modulation of Na\(^+\)-K\(^+\)-ATPase cell surface abundance through structural determinants on the \(\alpha_1\)-subunit is an important mechanism of regulation of cellular Na\(^+\)-K\(^+\)-ATPase in various physiological and pathophysiological conditions, with a significant impact on cell survival in face of an ischemic stress.

dileucine motif; ischemia-reperfusion injury; opossum kidney cells; cardiotonic steroids

The Na\(^+\)-K\(^+\)-ATPase is the membrane-spanning enzyme that both establishes and maintains the electrochemical gradient across the plasma membrane of animal cells by coupling the hydrolysis of ATP to the transport of Na\(^+\) and K\(^+\) (23, 43). The Na\(^+\)-K\(^+\)-ATPase complex consists of two dissimilar \(\alpha\)- and \(\beta\)-subunits, which exist as multiple isoforms. The \(\alpha\)-subunit is the primary contributor to overall catalysis and contains the binding sites for the substrates required by the enzyme. Expression of the \(\alpha\)-isoform is apparently ubiquitous, while the three others (\(\alpha_2\)–4) have increasingly restricted expression patterns (5, 6). Three distinct isoforms of the \(\beta\)-subunit, which is critical to the structural and functional maturation of Na\(^+\)-K\(^+\)-ATPase and regulates its transport properties, have been identified (21). In addition, several members of the FXYD family of accessory proteins have been shown to bind to and regulate Na\(^+\)-K\(^+\)-ATPase function in a tissue-specific manner (19, 20).

The Na\(^+\)-K\(^+\)-ATPase is also the pharmacological target of endogenous and exogenous cardiotonic steroids (CTS). CTS have long been known as potent inhibitors of Na\(^+\)-K\(^+\)-ATPase ion-pumping function, which is critical to their effect on Na\(^+\)-coupled influx of ions, amino acids, or glucose. This inhibitory action on Na\(^+\)-K\(^+\)-ATPase ion-pumping function and subsequent modulation of the Na\(^+\)/Ca\(^2+\) exchange has been extensively studied in the cardiac positive inotropic action of CTS. In addition, CTS such as ouabain, digoxin, or marinobufogenin, initiate intracellular signaling cascades via stimulation of the Na\(^+\)-K\(^+\)-ATPase receptor function (30, 36, 47, 48). The role of this more recently discovered property in the hormone-like function of endogenous CTS and in the therapeutic effect of exogenous CTS in health and diseases is being increasingly recognized. Progress in the understanding of CTS action in the cardiovascular and nervous systems, metabolism, or cell growth and differentiation has been emphasized in recent reviews (1, 2, 40, 42).

Regulation of Na\(^+\)-K\(^+\)-ATPase Cell Surface Abundance and Known Structural Determinants on the Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-Polypeptide

Localization of Na\(^+\)-K\(^+\)-ATPase at the cell surface is important to both ion-pumping and receptor functions, and modulation of cellular Na\(^+\)-K\(^+\)-ATPase activity through changes in cell surface expression has been reported in response to major physiological or pathophysiological stimuli. Such stimuli include CTS themselves (32, 45), the parathyroid hormone (24), dopamine (4), insulin (3, 12, 18), hypoxia (14), and hypercapnia (46). Over the past 15 years, investigations using heterologous expression systems have focused on the identification of key structural determinants along the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) polypeptide that influence its expression at the cell surface under basal conditions or in response to specific stimuli. Data from such studies are compiled in Table 1. We have recently examined one of these molecular determinants, a dileucine-based motif for recognition by clathrin-coated vesicle (CCV) adaptor proteins of the structure n(p)_2..LL, where n is a negatively charged residue and p is a polar residue (26). The sequence is well conserved among all the known mammalian \(\alpha_1\) sequences (Table 2), and our studies revealed that mutations targeting this motif such as L499V or E495S resulted in an increased abundance of Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)-units at the cell surface (44).

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**Using a Na⁺-K⁺-ATPase α1 Structural Determinant of Surface Abundance as a Target for Protection Against Ischemia-Reperfusion Injury**

We reckoned that an increased abundance of Na⁺-K⁺-ATPase pump units at the cell surface could be salutary to cells with critically high levels of intracellular Na⁺ such as those reported during ischemia-reperfusion (I-R) injury and may result in protection against I-R-induced cell death (34, 35). This hypothesis was tested in opossum kidney (OK) cells stably expressing native and L499V-mutated forms of Na⁺-K⁺-ATPase α1 polypeptide exposed to substrate/coverslip-induced I-R.

**METHODS**

**Cell Lines**

OK cells stably expressing native and L499V-mutated forms of Na⁺-K⁺-ATPase α1 were used. Details on the experimental procedures related to expression vectors and site-directed mutagenesis, heterologous expression, and initial characterization of Na⁺-K⁺-ATPase enzyme properties in these cells can be found in Sottejeau et al. (44).

**Substrate and Coverslip-Induced Ischemia-Reperfusion**

Ischemia was induced by removal of the substrate and placement of a glass coverslip over a portion of the OK cell monolayers, as described previously (38). Briefly, 70% confluent OK cells grown in 100-mm dishes were rinsed once with PBS and incubated in Krebs-Henseleit (KH) buffer containing (in mmol/l) 118.0 NaCl, 4.0 KCl, 1.8 CaCl₂, 1.3 KH₂PO₄, 1.2 MgSO₄, 0.3 EGTA, 25 NaHCO₃, and 37 D-glucose for 20 min at 37°C. Ischemia was then simulated by gentle removal of the LifterSlips and returned to KH buffer at 37°C. For confocal imaging studies, OK cells were grown on square coverslip 22 × 22 mm (Fisher) in six-well plates, and I-R was induced as described above using 18-mm diameter round glass coverslips (Fisher).

**Annexin V/Propidium Iodide Staining**

At the end of the experimental protocol, OK cells were fixed in 2% paraformaldehyde for 10 min at room temperature after a wash in PBS 1X. Cells were then incubated with Alexa Fluor 488 annexin V and red fluorescent propidium iodide (PI) (Vybrant Apoptosis Assay Kit no. 2, Invitrogen) according to the manufacturer’s recommendations. Confocal images were captured by sequential scanning with no overlap using a Leica TCS SP5 broadband confocal microscope system coupled to a DMI 6000CS inverted microscope equipped with multiple continuous wave lasers and a 63×/1.3 oil objective.

**Measurement of Lactate Dehydrogenase Activity**

At the end of a 60-min long reperfusion period, the cell incubation buffer was collected and lactate dehydrogenase (LDH) activity was determined colorimetrically using a standard assay (Cytotoxicity Detection Kit, Roche Applied Science), according to the manufacturer recommendations.

**Assessment of Na⁺-K⁺-ATPase α1 Total Protein Abundance and Surface Expression**

Total abundance of the introduced rat α1 constructs was determined by electrophoresis and immunoblotting of proteins from cell lysates using anti-NASE antibody as described (44). For total expression, equal loading of the samples among the lanes of the gel was confirmed by staining the membranes with a Ponceau red solution.

**Table 1. Summary of domains and sites of posttranslational modifications involved in the regulation of rat Na⁺-K⁺-ATPase α1 surface expression**

<table>
<thead>
<tr>
<th>Structural Determinant</th>
<th>Trigger/Signal Cascade</th>
<th>α1 Surface Expression</th>
<th>Ref. No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine-based domain for AP-binding IVYY-255</td>
<td>ANG II/AT1/1Dopamine</td>
<td>Up</td>
<td>17</td>
</tr>
<tr>
<td>Tyrosine-based domain for AP-binding 537-YLEL</td>
<td>Dopamine/DR1/1AP2</td>
<td>Down</td>
<td>9, 13</td>
</tr>
<tr>
<td>Dileucine-based motif for AP-binding EPKHL-499L</td>
<td>Hypoxia</td>
<td>Up</td>
<td>44</td>
</tr>
<tr>
<td>Phosphorylation/S-18</td>
<td>Dopamine</td>
<td>Down</td>
<td>10</td>
</tr>
<tr>
<td>Phosphorylation/S-18</td>
<td>Hypoxia/ PKC</td>
<td>Down</td>
<td>14</td>
</tr>
<tr>
<td>Ubiquitination/K-16/K-17/K-19/K-20</td>
<td>PTH/ PKC/ERK/CCV</td>
<td>Down</td>
<td>24</td>
</tr>
<tr>
<td>Proline-rich domain TPPPTPT-87</td>
<td>Hypoxia/ubiquitination</td>
<td>Down</td>
<td>15</td>
</tr>
</tbody>
</table>

**Table 2. Conserved dileucine motif of the form [D/E][XXX][L/I] motif in Na⁺-K⁺-ATPase α1 sequences in various species**

<table>
<thead>
<tr>
<th>Species</th>
<th>NCBI Access No.</th>
<th>[D/E][XXX][L/I] Motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rattus norvegicus (rat)</td>
<td>NM_0012504</td>
<td>SIHK497NPNASEPKHL497LVMK</td>
</tr>
<tr>
<td>Homo sapiens (human)</td>
<td>NM_000701</td>
<td>SIHK497NPNASEPKHL497LVMK</td>
</tr>
<tr>
<td>Dario rerio (zebrafish)</td>
<td>NM_131686</td>
<td>SIHK497NPNASEPKHL497LVMK</td>
</tr>
<tr>
<td>Ovis aries (sheep)</td>
<td>NM_00199360</td>
<td>SIHK497NPNASEPKHL497LVMK</td>
</tr>
<tr>
<td>Mus musculus (mouse)</td>
<td>NM_144900</td>
<td>SIHK497NPNASEPKHL497LVMK</td>
</tr>
<tr>
<td>Sus scrofa (pig)</td>
<td>NM_214249</td>
<td>SIHK497NPNASEPKHL497LVMK</td>
</tr>
<tr>
<td>Bos taurus (cattle)</td>
<td>BC123864</td>
<td>SIHK497NPNASEPKHL497LVMK</td>
</tr>
<tr>
<td>Canis familiaris (dog)</td>
<td>NM_001003306</td>
<td>SIHK497NPNASEPKHL497LVMK</td>
</tr>
<tr>
<td>Gallus gallus (chicken)</td>
<td>NM_205521</td>
<td>SIHK497NPNASEPKHL497LVMK</td>
</tr>
</tbody>
</table>

The conserved sequence of amino acid residues that form the dileucine motif appears in boldface.
confirmed by probing with a commercial antibody against actin (Santa Cruz Biotechnology, Santa Cruz, CA). Introduced a1 expressed at the cell surface was detected by biotinylation following the recommended procedures of Gottardi et al. (22), as we have recently reported in detail (44). I-R-induced endocytosis of Na1-K1-ATPase a1 units was tested with a pulse-chase strategy as slightly modified from previous studies (27, 44). Briefly, proteins expressed at the cell surface were first biotinylated as described above, quenched with PBS–glucose buffer, and rinsed twice with saline solution. The cells were then incubated in DMEM medium for 20 min at 37°C in 10% CO2 followed by 30 min of ischemia and 30 min of reperfusion. The remaining surface-bound biotin was then cleaved by treatment with 50 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) reducing agent for 15 min at 4°C.

Immunocytochemistry and Fluorescence Imaging

At the end of the experimental protocol, cells were fixed by 20 min incubation with ice-cold methanol, washed with PBS, and blocked with Signal Enhancer (Invitrogen). The cells were then incubated with a mouse anti-Na1-K1-ATPase a1 monoclonal antibody (clone C464.6, Upstate) in PBS containing 1% bovine serum albumin for 2 h at room temperature. After three washes with PBS, cells were exposed to AlexaFluor 488-conjugated anti-mouse secondary antibody for 2 h at room temperature, washed, and mounted onto slides. Image visualization was performed using a Leica TCS SP5 broadband confocal microscope system coupled to a DMI 6000CS inverted microscope.

Statistical Analysis

Statistical analysis was conducted using one-way ANOVA followed by Tukey’s multiple comparison post hoc test. P < 0.05 was considered statistically significant.

RESULTS

Decreased Post-I-R Cell Death in Cells Expressing the Na1-K1-ATPase a1-L499V Mutant

In vitro I-R was induced in OK cells by removing the metabolic substrates from the culture medium and by placing coverslips over the monolayer, according to the protocol of Pitts and Tombs (38). Whereas all cells were exposed to substrate depletion for 30 min, it is important to note that the three LifterSlips represented about 57% of the surface of the 100-mm diameter dishes and hence did not cover the entire monolayer. As shown in Fig. 1, this resulted in a significant increase in LDH release in the media over the course of 60 min of reperfusion (IR60), indicative of cell injury. The LDH release was measured as an index of cell injury in the media of a1- and a1-L499V-expressing cells after 110 min Krebs-Henseleit (KH) buffer (C, control, n = 6) or after 20 min KH/30 min substrate/coverslip-induced ischemia/60 min KH (IR60: ischemia-reperfusion 60, n = 6). Values are expressed as means ± SE. ***P < 0.001 and **P < 0.01 vs. respective controls; and #P < 0.05 vs. IR60 a1.

Fig. 1. Lactate dehydrogenase (LDH) released by a1- and a1-L499V-expressing opossum kidney (OK) cells exposed to ischemia followed by reperfusion. LDH release was measured as an index of cell injury in the media of a1- and a1-L499V-expressing cells after 110 min Krebs-Henseleit (KH) buffer (C, control, n = 6) or after 20 min KH/30 min substrate/coverslip-induced ischemia/60 min KH (IR60: ischemia-reperfusion 60, n = 6). Values are expressed as means ± SE. ***P < 0.001 and **P < 0.01 vs. respective controls; and #P < 0.05 vs. IR60 a1.

Increased Post-I-R Surface Abundance in Cells Expressing the Na1-K1-ATPase a1L449V Mutant

With the use of biotinylation techniques, Na1-K1-ATPase surface expression was compared in OK cells stably expressing native and L499V Na1-K1-ATPase a1 with or without exposure to 30 min ischemia and 5 min reperfusion (IR5). The data presented in Fig. 3 confirmed our previously reported finding that basal surface expression of Na1-K1-ATPase a1-units is significantly higher in the mutant group without change in total expression (44). After 5 min of reperfusion, total expression of Na1-K1-ATPase a1-was unchanged (3B), but its surface expression was significantly decreased in both a1- and a1-L499V Na1-K1-ATPase-expressing cells compared with their respective controls. The I-R-induced decrease was about 25–30% for both groups. As a result, the post-I-R surface expression in the a1-L499V-expressing cells was comparable to the pre-I-R level in the a1-expressing group.

I-R Induces Internalization of Na1-K1-ATPase Units

The data collected from the biotinylation studies presented in Fig. 3 suggested that I-R results in decreased abundance of Na1-K1-ATPase a1 units at the cell surface. To test whether this was due to an increased internalization of Na1-K1-ATPase a1 units during I-R, we compared Na1-K1-ATPase removal from the cell surface using a cell surface biotinylation and TCEP treatment (see METHODS) in a1-expressing cells in control conditions (80 min pulse-chase aerobic buffer) or exposed to the IR30 protocol (20 min aerobic buffer, 30 min
As shown in Fig. 4A, the amount of Na\(^{+}\)-K\(^{+}\)-ATPase internalized in 80 min was significantly increased in the IR30 group compared with the control \((P < 0.01)\). Immunofluorescent labeling of Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha 1\) units before and after I-R was consistent with increased intracellular signal after 30 min of reperfusion (Fig. 4B).

**DISCUSSION**

In this article, we review the growing list of physiological and pathological regulators of Na\(^{+}\)-K\(^{+}\)-ATPase surface abundance with emphasis on studies that explicitly tested the involvement of defined regions or residues on the Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha 1\) polypeptide. We also report new findings on the potential protective effect of manipulating Na\(^{+}\)-K\(^{+}\)-ATPase surface abundance during I-R injury by targeting one of these defined regions.

Regulation of Na\(^{+}\)-K\(^{+}\)-ATPase Cell Surface Abundance and Structural Determinants on the \(\alpha 1\)-Subunit

The concept that membrane trafficking is an important regulator of Na\(^{+}\)-K\(^{+}\)-ATPase is not a new one. In fact, early studies like those of Lamb and Ogden in HeLa cells pointed to CTS-induced changes in surface expression more than 30 years ago (28). Over the past two decades, this phenomenon has been observed in many other models, and a considerable amount of knowledge has been accumulated on the multiple physiological and pathological regulators of Na\(^{+}\)-K\(^{+}\)-ATPase surface abundance. Based on the results of our in vitro study in OK cells presented in Figs. 3 and 4, we propose that I-R be added to the growing list of those regulators. Studies that identified regulators also provided insights into the cellular pathways and compartments involved, but we are just beginning to understand the role of structural determinants on Na\(^{+}\)-K\(^{+}\)-ATPase enzyme complex in the integrated response to a given stimulus. As shown in Table 1, most of the determinants identified on the \(\alpha 1\) polypeptide are located within the amino terminal part or the large cytoplasmic loop of the molecule. Additional determinants and mechanisms of regulation of surface abundance remain to be identified, and studies like those by Kimura et al. on the regulation of Na\(^{+}\)-K\(^{+}\)-ATPase trafficking by arrestins and spinophilin (25) point to additional roles for the large intracellular loop in particular.

I-R-Induced Decrease of Na\(^{+}\)-K\(^{+}\)-ATPase Cell Surface Abundance

The results from this study are consistent with an I-R-induced internalization of Na\(^{+}\)-K\(^{+}\)-ATPase units in OK cells. As shown in Fig. 3, the extent of I-R-induced internalization is...
Mechanism of Protection Against I-R-Induced Injury

According to the data presented in Figs. 1 and 2, an increased number of Na\(^+\)-K\(^+\)-ATPase units at the cell surface correlates with an increased tolerance to I-R in α1-L449V-expressing cells. However, these studies do not reveal the underlying mechanism of protection. Additional Na\(^+\)-K\(^+\)-ATPase ion-pumping capacity at the cell surface may help preserve intracellular ion homeostasis during I-R, but studies like our initial characterization of the α1-L449V mutant itself (44) or the graded knockdown of α1 subunit (31) have shown that surface expression does not necessarily correlate with increased ion-pumping function. In fact, other non-ion-pumping functions of Na\(^+\)-K\(^+\)-ATPase may be involved, such as survival signaling or preservation of the integrity of intracellular structures (7, 8, 45). Clearly, further investigation in cells exposed to I-R is needed to clarify the relative contribution of Na\(^+\)-K\(^+\)-ATPase ion-pumping and non-ion-pumping functions in the protection afforded by increased cell surface expression.

In conclusion, a substantial number of studies have underscored the importance of surface abundance modulation in the comparable in α1- and α1-L449V-expressing cells, suggesting that the particular dileucine motif that we chose to mutate to increase surface expression is not involved in I-R-induced internalization itself, at least in this model. Although beyond the scope of this study, an investigation of the role of intracellular mediators such as ROS and PKCs, as well as the clathrin-coated pits network and the ubiquitin system in I-R-induced internalization of Na\(^+\)-K\(^+\)-ATPases may reveal a great deal about the underlying mechanism. Indeed, several of these machineries and mediators have been shown to regulate hypoxia-induced Na\(^+\)-K\(^+\)-ATPase internalization as part of a “phosphorylation-ubiquitination-recognition-endocytosis-degradation” (PURED) pathway (14, 15, 29) and are also important components of the cellular response during exposure to I-R, especially in the heart (11, 16, 33, 39). In addition, studies in cells expressing Na\(^+\)-K\(^+\)-ATPase α1 mutated on Ser-18 or one of the surrounding Lys (involved in hypoxia-induced internalization as shown in Table 1) may prove useful in future attempts to characterize the mechanism of I-R-induced internalization. Structural determinants may hence be instrumental to future studies on the mechanism underlying I-R-induced Na\(^+\)-K\(^+\)-ATPase internalization and its importance in I-R-induced cell death.
regulation of \(\text{Na}^+\text{-K}^+\)-ATPase function. In addition, a number of structural determinants have been identified on the \(\alpha\)-polypeptide, with variable degree of divergence among various \(\alpha\)-isoforms and between different species. The exact role of these variations in tissue- and species-specific response to various stimuli and diseases remains to be established. The data presented here suggest that modulation of \(\text{Na}^+\text{-K}^+\)-ATPase cell surface abundance by targeting structural determinants on the \(\alpha\)-subunit has a significant impact on I-R-induced cell injury.

ACKNOWLEDGMENTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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