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OUABAIN-INDUCED ENDOCYTOSIS AND SIGNAL TRANSDUCTION OF THE NA/K-ATPASE

Jiang Liu

Department of Medicine, Medical College of Ohio, 3120 Glendale Avenue, Toledo, Ohio 43614-5809

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1. ABSTRACT

Na/K-ATPase can function as a signal transducer as well as an energy transducing ion pump. Cardiac glycosides (including ouabain and marinobufagin, MBG) are a new class of steroid hormones. Ouabain-activated signaling pathways lead to the induction of some early response proto-oncogenes, activation of transcription factors, and cardiac hypertrophy. Low concentration of ouabain also induced endocytosis of the Na/K-ATPase and compartmentalization of some signaling molecules (e.g. c-Src, EGFR, and p42/44 MAPKs) into clathrin-coated pits, early and late endosomes. Ouabain-induced endocytosis of the Na/K-ATPase depends on the activation of Src kinase, clathrin-coated pits formation, and caveolin-1 (the major component of caveolae). Moreover, low concentration ouabain significantly reduced transcellular Na^+ transport. The data also show a srtonge interplay of ouabain-induced endocytosis of the Na/K-ATPase and signaling transduction.

2. INTRODUCTION

Na/K-ATPase (EC 3.6.3.9), or sodium pump, is a ubiquitous transmembrane enzyme that transports Na and K across the plasma membrane. The enzyme also functions as a signal transducer for ouabain (1-7). Low concentration of ouabain (50nM, ~$1/20^{th}$ of acute IC_{50}) induces marked decreases in the activity and quantity of plasmalemmal Na/K-ATPase, and marked decrease in transcellular sodium transport in LLC-PK1 cells, a pig renal proximal tubule cell line (8, 9). Significantly, low concentration of ouabain also activated Src and MAPK signal pathways (1, 2) and induced endocytosis of the enzyme in a clathrin-mediated manner (9) in LLC-PK1 cells. The purpose of this review is to provide a broad overview of the interplay between ouabain-induced endocytosis and signal transduction of the Na/K-ATPase

3. NA/K-ATPASE

Na/K-ATPase was first described by Skou in 1957 (10). Na/K-ATPase belongs to the family of P-type ATPases and consists of two non-covalently linked α and β subunits (11-13). The catalytic α subunit (about 112 kDa) contains the ATP, digitalis, and other ligand binding sites. The α subunit, in which both N- and C-termini are localized on the cytosolic side, is essential for the functional enzyme. Several α and β subunits have been identified and functionally characterized (11-13). The isoforms are expressed in a tissue-specific manner; the $\alpha 1$ isoform is found in all cells whereas the $\alpha 2$ and $\alpha 3$ isoforms are expressed in skeletal muscle, neuronal tissue, and cardiac myocytes (14, 15). Both SERCA and the Na/K-

ATPase belong to the type-II class of P-type ATPases, and both contain four distinct functional domains, in which both the A and N domains are well isolated and exposed (16) (17). The efforts of numerous laboratories (11, 13, 18) have led to the following conclusions: (a) This enzyme is the molecular machine for the ATP-dependent and -coupled transports of Na^+ and K^+ across the plasma membranes of all cells; (b) Cardiac Na/K-ATPase is the functional receptor for the inotropic effects of digitalis; and (c) The enzyme is also a signal transducer involved in regulation of multiple genes and pathways including activation of Src, EGFR, Ras, PKC, MAPKs, and intracellular ROS production (1-4, 6, 19-27).

4. ENDOGENOUS DIGITALIS-LIKE SUBSTANCES

It is now accepted that endogenous cardiac glycosides (e.g. ouabain and marinobufagin, MBG) are a new class of steroid hormones (for review, see (28)). The search for endogenous cardiac glycosides has led to the isolation of ouabain as well as several other additional cardiotonic steroids from blood plasma, adrenal glands, and hypothalamus of mammals (28-31). Hamlyn et al. were the first to demonstrate that the concentration of a circulating factor in blood plasma inhibiting purified Na/K-ATPase correlated with the blood pressure of the donors(32), and Schneider et al. were the first to show that ouabain is in fact a constituent of the adrenals(33). Elevated endogenous ouabain levels have been found under a number of conditions such as sodium imbalance, chronic renal failure, hyperaldosteronism, hypertension, congestive heart failure, and preeclampsia (30, 34-36). It appears that ouabain and MBG (which displays greater affinity for the ouabain-resistant rodent α1 subunit of the Na/K-ATPase), are the major endogenous cardiac glycosides that produce effects on the kidney (37-42). MBG concentrations in the plasma increase in a variety of experimental and clinical settings associated with volume expansion and hypertension (38, 39, 43, 44). Recently, it has been demonstrated that synthesis of MBG occurs in mammalian adrenal cells and its effect on Na/K-ATPase is PKC-dependent (45, 46). However, we would stress that although MBG can be shown to inhibit the renal Na/K-ATPase at physiological concentrations, there is still a considerable discrepancy in the magnitude of this inhibition from what is seen *in vivo.* Specifically, increased MBG excretion was associated with a 40% inhibition of the renal Na/K-ATPase whereas nM concentrations of MBG acutely inhibit the Na/K-ATPase of rats by only about 15% (37). This suggests that additional mechanisms may be involved to sensitize the renal Na/K-ATPase.

5. NA/K-ATPASE AS A SIGNAL TRANSDUCER

The ouabain-induced signaling pathways, which through the partial inhibition of Na/K-ATPase and independent of changes in intracellular ion concentrations and contractility, have also been reported recently in cells other than cardiac myocytes, including smooth muscle cells and kidney proximal tubular cells (1-3, 24, 25, 27) . It has been proposed that the ouabain-bound (activated) Na/K-ATPase is capable of recruiting and activating protein tyrosine kinases through specific protein-protein interactions (6). A database search identified several potential protein binding motifs in the α1 subunit of Na/K-ATPase from rat, pig, and human (e.g. in pig α1: Ankyrin: **A^{453}LLK**; AP-2: **Y^{540}**LEL; caveolin: **W^{985}**W**F**CAFP**Y,** also see below; PRD: TP**P^{81}**PTTP; and endocytic sorting motif: tyrosine-based **Y**XXΦ and di-leucine based **LL**). Phosphorylation of Ser-18 by PKC increases the interaction of PI-3K SH3 domain with the rat a1 subunit conserved proline-rich domain (PRD, at the N-terminus) (47), and tyrosine phosphorylation of the α1 subunit (Tyr-10) has been reported in response to insulin stimulation in rat kidney proximal tubule cells (48). Both a and ß subunits of mammalian Na/K-ATPase contain conserved caveolin-binding motif (CBM, e.g., ΦXXΦXXXXΦ and ΦXΦXXXXΦ, where Φ represents an aromatic amino acid residue, Tyr, Phe, or Trp). In a subunit, two binding motifs have been identified. One of them resides at the border of the cytoplasmic side of M1 and the other is at the extracellular side of M10. The fact that ouabain stimulated the association of caveolin-1 (Cav-1) to the Na/K-ATPase, and the Na/K-ATPase is able to bind to the Cav-1 scaffolding domain directly, indicates a potential role of these caveolin-binding motifs in the interaction of the enzyme with caveolins, which is important to assembly the caveolar signaling complex through the Na/K-ATPase (49). In short, there is sufficient evidence that Na/K-ATPase is involved in protein-protein interactions that are important for the function of the enzyme as well as those that are regulated by the enzyme.

6. ENDOCYTOSIS AND SIGNAL TRANSDUCTION

The clathrin-dependent endocytosis is the main endocytosis pathway for many membrane proteins in mammalian cells and extensively reviewed (50-54). Caveolae/lipid rafts are also believed to play a central role in transcytosis and endocytosis (55-59). Caveolae were first identified as flask shaped, non-coated membrane vesicular invagination and are enriched in cholesterol, glycosphingolipids, and sphingomyelin (56, 59-61). Caveolins are 21-24 KDa membrane-associated scaffold proteins (a substrate of v-Src (59)) and the major structural components of caveolae (55, 56, 59). Many signaling molecules and membrane receptors are dynamically associated with caveolae, such as the Src-family kinases, Ras, PKC, ERK, insulin receptor, PDGFR (platelet-derived growth factor receptor), EGFR, and some entire signaling modules like PDGFR-Ras-ERK, mainly through their interactions with caveolins (58, 62, 63). Caveolins stabilized caveolae and modulated signal transduction by attracting signaling molecules to caveolae and regulating their activities (63). There is also evidence that caveolins may modulate endocytosis through their interactions with clathrin (64-67). Moreover, free cholesterol is also essential for maintaining the shape of caveolae and clathrin-coated pit, because depletion of cholesterol correlated directly with the flattening of caveolae and clathrin-coated pits, indicating that cholesterol affects the morphology and curvature of the plasma membrane (68).

Apart from its endocytic function, the clathrin-coated pits may also represent a specialized microdomain,

like caveolae, where proteins are assembled into active signaling complexes before internalization of some or all of their components (69). Several molecules involved in transmembrane signaling, such as β-arrestin, RGS-GAIP (a GTPase-activating Protein for Gαi heterotrimeric G Proteins) (70), GIPC (a PDZ domain containing protein) (71), and Src family kinases (72), have been localized to clathrin-coated pits, suggesting that interaction with the components of the pit machinery may facilitate some signaling functions of transmembrane receptors. Both caveolin and clathrin heavy chain are substrates of Src kinase (59, 73). Strikingly, recycling endosomes (in MDCK cells) are also enriched in the raft lipids sphingomyelin and cholesterol as well as in caveolin-1(74).

Significantly, the Na/K-ATPase α2 subunit is concentrated in caveolae isolated from the heart using detergent-free method, and ouabain activated caveolar ERKs in the isolated heart preparation (75). Furthermore, several signaling molecules, such as EGFR and Src, are also concentrated in clathrin-coated pits in response to ouabain (9), suggesting that both clathrin-coated pits and caveolae may be involved in ouabain-mediated Na/K-ATPase signal transduction and endocytosis.

While receptor-mediated endocytosis has been traditionally considered an effective mechanism to attenuate ligand-activated responses, it is becoming clear that signaling continues on the endocytic pathway, including from endosomes (50, 52, 76, 77). Endocytosis plays an important role in the activation and propagation of signaling pathways (78-80), and signal transduction can also regulate endocytosis (73, 81). Furthermore, it was also suggested that receptor uptake may be regulated not only by the interplay between components of signaling and endocytic pathways but also by their relative spatial organization in membrane microdomains, like the clathrin-coated pits and caveolae/lipid rafts (67). As discussed above, ouabain inhibits the Na/K-ATPase activity and induces endocytosis of the enzyme; ouabain also activates Src and PI-3K (both involved in ouabain-induced endocytosis of the Na/K-ATPase (9)), and transactivates EGFR, leading to the activation of MAPKs. Moreover, caveolae may also be involved in signal transduction resulting in changes in cytosolic calcium and inotropy (7, 75). This raises the possibilities of the interactions among the Na/K-ATPase, caveolins, and proteins important in clathrin-dependent endocytosis, and of the cross talk between ouabain-induced Na/K-ATPase endocytosis and signal transduction.

7. ENDOCYTOSIS OF THE NA/K-ATPASE

In renal tubule epithelium, endocytosis of the rat Na/K-ATPase, especially in response to dopamine, has been clearly demonstrated (82-85). Endocytosis of the Na/K-ATPase in response to dopamine is triggered by the phosphorylation of Ser-18 of rat α1-subunit and activation of PI-3K (47). The activation of PI-3K facilitates the binding of the α1-subunit with adaptor protein-2 (AP-2), providing the inclusion of the Na/K-ATPase into clathrin-coated pits. However, Ser-18 is found only in rat α1-subunit and is not present in pig and dog α1-subunit (86). Depending on the type of renal tubular epithelium, dopamine-induced internalization of the Na/K-ATPase may be mediated through PKC or PKA dependent mechanisms (84, 87-89). More recently, parathyroid hormone (PTH)-induced inhibition and endocytosis of the Na/K-ATPase were also demonstrated in OK cells, which is clathrin-mediated and requires ERK-dependent phosphorylation of Ser-11 within the α1-subunit (90). In heart and other cells, early studies from the laboratories of Cook and Lamb, demonstrated that [^{3}H]-ouabain (bound to the Na/K-ATPase) was translocated from the plasmalemmal to intracellular compartments (lysosomes) in HeLa cells, chick embryo heart cells, and Girardi heart cells (91-95). In isolated guinea-pig heart, blocking clathrin-dependent endocytosis pathway significantly inhibited ouabain toxicity (96). In mouse cardiac cells, receptor-mediated endocytosis was also demonstrated (97), and over-expression of Rab1 GTPase in myocardium distorts the subcellular localization of proteins and is sufficient to cause cardiac hypertrophy and failure (98). Caveolin-3 is the only caveolin family member expressed in striated muscle cell types (cardiac and skeletal), caveolin-3 knock-out mice showed hyperactivation of p42/44 MAPKs cascade, and loss of caveolin-3 expression is sufficient to induce a molecular program leading to cardiac myocyte hypertrophy and cardiomyopathy (99).

It has demonstrated that non-toxic ouabain induced similar signal transduction both in rat neonatal myocytes (leading to cardiac hypertrophy) and LLC-PK1 cells (leading to endocytosis of the Na/K-ATPase (9)), and ouabain causes dose and time dependent decreases in ^{86}Rb uptake in LLC-PK1 cells. To understand the molecular mechanisms involved in this process, studies were performed with cultured LLC-PK1 and SYF cells (9). Low concentration of ouabain was applied to the basal, but not apical, aspect for 12 hrs, which caused decreases in the plasmalemmal Na/K-ATPase. This loss of the plasmalemmal Na/K-ATPase could be reversed completely within 12-24 hrs following removal of ouabain. Ouabain also increased the Na/K-ATPase content in both early and late endosomes, activated PI-3K, and caused a translocation of some Na/K-ATPase to the nucleus. Immunofluorescence demonstrated that the Na/K-ATPase co-localized with clathrin both before and following exposure to ouabain, and immunoprecipitation experiments confirmed that ouabain stimulated interactions amongst the Na/K-ATPase, AP-2 and clathrin. Potassium (K) depletion, chlorpromazine, or PI-3K inhibition all significantly attenuated this ouabain-induced endocytosis. Inhibition of the ouabain-activated signaling process through Src by PP2 (a specific Src kinase inhibitor) significantly attenuated ouabain-induced endocytosis. Experiments performed in SYF cells demonstrated that ouabain induced endocytosis of the Na/K-ATPase in SYF+c-Src cells (c-Src was reconstituted into the cell), but not in the Src deficient (SYF-c-Src) cells. Moreover, depletion of cholesterol (by Mß-CD) or caveolin-1 (by siRNA) blocked ouabain-induced endocytosis of the Na/K-ATPase, compartmentalization of signaling molecules in clathrin-coated pits and early endosome. In addition, depletion of caveolin-1 also

significantly reduced the protein-protein interactions among α-1 subunit, AP-2, PI-3K, and clathrin heavy chain, suggesting that caveolin-1 is involved in both ouabain-induced endocytosis of the Na/K-ATPase and ouabain-induced signal transduction (100). These data demonstrate that ouabain stimulates a clathrin- and caveolin-1-dependent endocytosis pathway that translocate the Na/K-ATPase to intracellular compartments, which also requires the ouabain-induced signal transduction, thus suggesting a potential role of endocytosis in ouabain-induced signal transduction, as well as proximal tubule sodium handling. Taking these together, it most likely that clathrin- and/or caveolae/rafts-mediated endocytosis of the Na/K-ATPase is a common phenomena, but the mechanism and the relationship of the endocytosis of the Na/K-ATPase and signal transduction are not fully understood.

8. THE ROLE OF THE ENDOCYTOSIS OF THE NA/K-ATPASE IN THE REGULATION OF RENAL SODIUM EXCRETION

The regulation of renal tubule epithelial cell sodium transport by endocytosis has been extensively studied, especially in G protein receptor mediated signal transduction induced by dopamine (101). Dopamine alters the Na/K-ATPase trafficking and alters renal tubular epithelial sodium handling by decreasing plasmalemmal pump expression. Recently, Bertorello and colleagues have identified that Tyr^{537} on the α1 subunit is essential for AP-2 binding and clathrin-dependent endocytosis of the Na/K-ATPase in OK cells expressing the rodent α-1 isoform (84) whereas Ser^{18} phosphorylation (also on α1) is essential for dopamine-induced endocytosis in primary culture of rat proximal tubules cells (82, 85). Although the binding of radio-labeled ouabain or digoxin to the Na/K-ATPase has been utilized as a way to follow the trafficking of the Na/K-ATPase through the different cell compartments (93, 94, 102, 103), it is the first time to demonstrate that ligand-modulated internalization of the Na/K-ATPase as a mechanism by which sodium transport by proximal tubular epithelium is altered in a physiologically meaningful manner (8, 9).

As discussed above, elevated endogenous ouabain levels have been found under a number of conditions such as sodium imbalance, chronic renal failure, hyperaldosteronism, hypertension, congestive heart failure, and preeclampsia (30, 34-36). The endocytosis of the Na/K-ATPase in proximal tubule cells (but not distal tubule cells) was demonstrated both *in vitro* and *in vivo* (unpublished data). This also supports a recent *in vivo* (rat) study, that demonstrated that very low concentration of ouabain may concentrate the Na/K-ATPase, Src, EGFR, and MAPKs within rat caveolae membrane subdomain, activate the Na/K-ATPase/Src/MAPKs signaling pathway, leading to the hypertrophic response both in heart and kidney(104). The sodium-hydrogen exchangers (NHEs) are present in all mammalian cells to regulate intracellular pH, cell growth, cellular volume, and transepithelial Na^+ absorption (105, 106). To date, eight NHE isoforms have been identified from mammalian cells (107). Specifically, NHE3 is expressed in the apical membrane in the proximal tubule (S1 and S2 segments) and in the cortical thick ascending limb of the loop of Hendle (108-112), mediating transcellular reabsorption of Na^+ and HCO_3^- in the proximal tubules and reabsorption of HCO_3^- in the thick ascending limb (108, 113, 114). In LLC-PK1 cells, our results showed that ouabain treatment decreased apical NHE3-mediated Na^+ absorption, apical NHE3 protein and mRNA abundance. Our results also suggested that c-Src, PI-3K, and caveolin might be involved in ouabain-induced downregulation of NHE3 activity (unpublished data). Although the mechanisms that initial the endocytosis of the Na/K-ATPase and inhibit the NHE3 activity (and expression) are not fully understood, endocytosis of the Na/K-ATPase may play an important role in renal sodium handling. This is because if digitalis-like substances induce a significant depletion of plasmalemmal Na/K-ATPase in proximal tubule type cells (rat proximal tubule primary culture, LLC-PK1), but not distal tubule type cells (rat distal tubule primary culture, MDCK), it will make perfect physiological "sense" in terms of allowing bulk sodium transport (primarily in the proximal tubule) to be altered and leaving fine tuning (distal tubule) sodium handling intact.

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Abbreviations: AP-2, adaptor protein-2; CHC, clathrin heavy chain; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; MAPK(s), p42/44 mitogen-activated protein kinase(s); NHE3, sodium-hydrogen exchanger, isoform 3; PI-3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; ROS, reactive oxygen species; SERCA, sarcoplasmic reticulum Ca-ATPase

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Send Correspondence to: Dr. Jiang Liu, Department of Medicine, Medical College of Ohio, 3120 Glendale Avenue, Toledo, Ohio 43614-5809. Tel: 419-383-3923, Fax: 419-383-6244, Email: jiliu@mco.edu

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