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Regulation of Human and Pig Renal Na^+,K^+-ATPase Activity by Tyrosine Phosphorylation of Their α_1-Subunits

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Abstract Modulation of the physiologically influential Na^+,K^+-ATPase is a complex process involving a wide variety of factors. To determine the possible effects of the protein tyrosine phosphatase (PTP) inhibitors dephostatin and Et-3,4-dephostatin on human and pig, renal cells and enzymatic extracts, we treated our samples (15 min–24 h) with those PTP inhibitors (0–100 μM). PTP inhibitors were found to possess a concentration-dependent inhibition of Na^+,K^+-ATPase activity in both human and pig samples. The inhibition was similarly demonstrated on all cellular, microsomal fraction and purified Na^+,K^+-ATPase levels. Despite rigorous activity recovery attempts, the PTP inhibitors' effects were sustained on Na^+,K^+-ATPase activity. Western blotting experiments revealed the expression of both α_1- and β_1-subunits in both human and pig tissues. α_1-Subunits possessed higher tyrosine phosphorylation levels with higher concentrations of PTP inhibitors. Meanwhile, serine/threonine residues of both α_1- and β_1-subunits demonstrated diminished phosphorylation levels upon dephostatin treatment. Accordingly, we provide evidence that Na^+,K^+-ATPase can be regulated through tyrosine phosphorylation of primarily their α_1-subunits, using PTP inhibitors.

Keywords Na^+,K^+-ATPase α_1-subunit · Protein tyrosine phosphatase inhibitor · Tyrosine phosphorylation · Dephostatin · Et-3,4-dephostatin · Renal

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Introduction

Na^+,K^+-ATPase, the ubiquitous ATP-hydrolyzing, active ion-transport protein complex across eukaryotic cell membranes, is the main regulator of cytoplasmic Na^+ and plays a crucial role in maintaining ionic homeostasis (Therien and Blostein 2000; Kaplan 2002; Xie and Cai 2003). Na^+,K^+-ATPase is basically composed of α-, β- and γ-subunits and their isoforms. The α-subunit is a multipass transmembrane protein exhibiting multiple binding sites. The β-subunit is a single crossing transmembrane glycoprotein exhibiting no binding sites, yet it plays a crucial role in the structural and functional biogenesis of the enzyme. Both α- and β-subunits are essential for Na^+,K^+-ATPase activity and cannot act solely (Blanco and Mercer 1998; Kaplan 2002; Scheiner-Bois 2002). The γ-subunit possesses a modulatory role in the affinity of the enzyme to Na^+ and/or K^+ and is not present in all tissues (Blanco and Mercer 1998; Therien and Blostein 2000; Scheiner-Bois 2002; Horisberger 2004).

The functional regulation of Na^+,K^+-ATPase adds to its intricacy, due to the diversity of its regulators. Ouabain was

the first known Na^+,K^+-ATPase inhibitor and is currently the classical inhibitor to which others are compared. Ionic distribution across the plasma membrane seems to be the direct regulatory mechanism (Therien and Blostein 2000; Yu 2003). Other regulatory mechanisms include ATP availability and phosphorylation state as short-term regulators (McDonough and Farely 1993; Lopina 2000; Therien and Blostein 2000), in which different tyrosine kinases have been involved (Narkar, Hussain and Lokhandwala 2002; Wang and Yu 2005), as well as oxidant stress (Kourie 1998). A wide variety of hormones are involved in Na^+,K^+-ATPase regulation (Hernandez 1992; Doris and Bagrov 1998; Ferrandi and Manunta 2000; Therien and Blostein 2000; Yu 2003). Overall, hormones seem to implement short-term effects via translocation of the Na^+,K^+-ATPase between the plasma membrane and intracellular stores, and direct effects on the kinetic behavior of the enzyme, while long-term effects influence enzyme synthesis and degradation (Therien and Blostein 2000).

Phosphorylation has been amply studied as a crucial regulator of a wide variety of cellular mechanisms, including Na^+,K^+-ATPase regulation, and to a much lesser extent protein tyrosine kinases (PTKs) (Wang and Yu 2005) since their discovery in 1980 (Hunter and Sefton 1980). The other face of the dynamic protein tyrosine phosphorylation coin—protein tyrosine phosphatases (PTPs), discovered in 1988 (Tonks et al. 1988)—was almost neglected as a possible Na^+,K^+-ATPase regulator. This might be attributed to its PTK antagonistic role as a phosphate-releasing moiety from protein tyrosine residues; and hence, it might just have been given a reversal role of PTK action (Imoto et al. 1993; Umezawa et al. 2003). In this study we investigated the possible roles of PTP in the regulation of Na^+,K^+-ATPase activity through the use of different PTP inhibitors: dephostatin and Et-3,4-dephostatin (more stable derivative) (Suzuki et al. 2001). Dephostatin (2,5-dihydroxy-*N*-methyl-*N*-nitrosaniline), initially isolated from *Streptomyces* in 1993 and currently synthesized, is a substrate competitive inhibitor of PTP, exhibiting about 10-fold higher potency than the classical nonspecific PTP inhibitor, sodium vanadate, while lacking any inhibitory effect on serine/threonine phosphatases. Et-3,4-dephostatin specifically inhibited PTP-1B and SHPTP-1 but not CD45 or LAR (leukocyte antigen-related tyrosine phosphatase). Meanwhile, the analogue increased the phosphorylation of insulin receptor, insulin receptor substrate-1 and -3 (IRS-1, IRS-3), phospholipase Cγ, c-Cbl, phosphatidylinositol (PI) 3-kinase regulatory subunit and Akt (Imoto et al. 1993; Umezawa et al. 2003). Apart from the reported gradual enhancement of outward Na^+,K^+-ATPase current in mouse cortical neurons by Wang and Yu (2005), we are unaware of other studies reporting any specificity of dephostatin or its analogue to Na^+,K^+-ATPase. Neither PTP inhibitor has been studied beyond the scope of PTP inhibition, yet studies have demonstrated that they possess potential therapeutic effects, especially in diabetes mellitus and neural disease, focusing mainly on their PTP inhibitory effects (Umezawa et al. 2003).

Materials and Methods

Reagents and Antibodies

Dephostatin and Et-3,4-dephostatin were purchased from EMD Chemicals (San Diego, CA). Aliquots were prepared according to the manufacturer's instructions, in which the dimethyl sulfoxide (DMSO) solvent constituted <1% of the final concentration. ATP and ouabain were purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade. Phosflow Fix buffer I and Perm buffer III were purchased from BD Biosciences (San Jose, CA). Anti-Na^+,K^+-ATPase α_1-subunit and its Alexa Fluor 488-conjugated form along with the same conjugate of normal mouse IgG (negative control) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-Na^+,K^+-ATPase β_1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine antibody was purchased from Affinity BioReagents (Golden, CO). Anti-phosphoserine/threonine antibody and phycoerythrin (PE)-conjugated anti-phosphotyrosine antibody were bought from BD Biosciences. Secondary IgG HRP-conjugated antibodies were purchased from Sigma–Aldrich (St. Louis, MO).

Cell Culture

Human renal tumor cells (OS-RC-2) were purchased from Riken BioResource Center, Cell Bank (Ibaraki, Japan), and cultured in RPMI-1640 medium containing L-glutamine and $NaHCO_3$, which was further supplemented with 10% fetal bovine serum. Cells were incubated at 37°C in 5% CO_2 humidified atmosphere.

Protein Assays

Protein assays were performed according to the Folin-phenol method described by Lowry et al. (1951), using the BCA Protein Assay Kit (Pierce, Rockford, IL) employing bovine serum albumin (BSA) standards per the manufacturer's instructions.

Pig Kidney Microsomal Fraction Isolation and Na^+,K^+-ATPase Purification

Membrane-bound Na^+,K^+-ATPase was isolated from the dark red outer medulla of pig kidney as described by

Jorgensen (1988). The microsomal fraction representing the membrane-bound Na^+,K^+-ATPase was resuspended in 20 mM imidazole buffer (pH 6.8), containing 250 mM sucrose, 6 mM EDTA and 6 mM Tris, and stored in 1–10 ml aliquots at −20°C. Further purification of the Na^+,K^+-ATPase enzyme was carried out using the method described by Hayashi et al. (1977), in which sodium dodecyl sulfate treatment was omitted (Taniguchi et al. 1982)—hereafter referred to as purified Na^+,K^+-ATPase.

Na^+,K^+-ATPase Assay

OS-RC-2 cells were grown to almost confluence, treated as indicated (dephostatin, Et-3,4-dephostatin or DMSO), washed twice with PBS and collected in 250 mM sucrose/1 mM EDTA. Collected cells were ultrasonicated thrice for 10 s on ice.

The Na^+,K^+-ATPase activity assay involved 8–15 µl of the investigated sample in 300 µl of assay medium typically composed of (in mM) 25 sucrose, 0.1 EDTA, 50 Tris–HCl (pH 7.4), 160 NaCl, 16 KCl and 5 $MgCl_2$. Each sample was tested in sextuplicates, of which half (triplicates) were treated with ouabain at a final concentration of 1 mM. Reactions were commenced in a water bath at 37°C, initiated by adding 5 mM ATP and stopped by adding 0.3 ml of 12% sodium dodecyl sulfate (SDS) after 30–120 min, in a strict timely fashion (seconds). The amount of released inorganic phosphate (Pi) due to ATP hydrolysis was measured using the colorimetric method described (Chifflet et al. 1988). Na^+,K^+-ATPase activity was the difference between the ATP hydrolysis measured in the presence and absence of ouabain and expressed in terms of micromoles of Pi per milligram of protein per minute.

Recovery of Na^+,K^+-ATPase Activity

Microsomal fractions (50–250 µl) in the above-mentioned assay mixture containing dephostatin, Et-3,4-dephostatin or DMSO were incubated for 30 min at 37°C with frequent vortices. The mixture triplicates were then subjected to ice-cold washes of 250 mM sucrose/1 mM EDTA, followed by 60,000 rpm centrifugations for 15 min at 4°C. The supernatants were discarded; pellets were resuspended in 250 mM sucrose/1 mM EDTA and manually homogenized in a Potter–Elvehjem homogenizer. The washes, centrifugations and homogenizations were repeated thrice in an attempt to recover the inhibited Na^+,K^+-ATPase activity. Finally, the recovered samples were assayed for Na^+,K^+-ATPase activity.

Western Blotting

Cells were grown to almost confluence, treated as indicated, washed twice with PBS and collected in 250 mM sucrose/1 mM EDTA. Collected cells were ultrasonicated thrice for 10 s on ice, clarified by centrifugation at 15,000 rpm for 5 min at 4°C and protein-assayed. Equal protein amounts were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) (7–10% acrylamide) following the method of Laemmli (1970). Proteins were then transferred to polyvinylidine difluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA) according to the manufacturer's instructions. Western blotting was commenced as described by Perkin-Elmer (Boston, MA). Blots were blocked using protein-free blocking buffer (Pierce) for at least 1 h at room temperature. Immunoblots were detected by 4°C overnight incubations with primary antibodies and at least 1 h room temperature incubations in secondary antibody (IgG HRP-conjugated). Luminol light signal was achieved using Supersignal Western detection (Pierce) reagents. The signal was recorded via blot incubations for 1–10 min with Kodak BioMax XAR films (Carestream Health, Rochester, NY) using intensifying screens. Image analysis was commenced using ImageJ (NIH, Bethesda, MD).

FACS Analysis

OS-RC-2 cells of about 80% confluence were serum-starved for 12 h, then treated with PTP inhibitors or their diluting vehicle (DMSO) for time intervals ranging from 15 min to 2 h. Cells were washed twice in ice-cold PBS, trypsinized and divided into equal samples of 1×10^6 to 10^7 cells that were further treated with Phosflow Fix Buffer I and Phosflow Perm Buffer III according to the manufacturer's recommendations. Samples were further blocked in antisera for 30–60 min at room temperature and then probed using fluorescent dye-conjugated antibodies according to the manufacturer's instructions. Samples were analyzed by the BD FACSCalibur system (BD Biosciences). Analysis was repeated with three independent samples. Unstained and untreated cells were used to establish instrument settings for the utilized channels, while stained and untreated cells were used to establish quadrant settings for dot plot analyses. Analyses were performed using FlowJo software (Tree Star, Ashland, OR).

Statistical Analysis

One-way analysis of variance (ANOVA) was performed using the means of at least three independent experiments.

Statistical differences were assessed using the multiple comparative Dunnett test.

Results

Effects of Dephostatin and Et-3,4-Dephostatin on Pig Kidney Extracts

Both dephostatin and Et-3,4-dephostatin inhibited Na^+,K^+-ATPase activity of the extracted microsomal fractions in a concentration-dependent manner (Fig. 1a). Dephostatin demonstrated higher potency, where concentrations starting at 2.5 μM exhibited statistically significant inhibition ($P < 0.05$) and concentrations of 5–100 μM possessed an even more pronounced inhibition ($P < 0.01$) compared to controls. The initially significant inhibitory concentration of Et-3,4-dephostatin was 6.25 μM ($P < 0.05$) and remained significant up to concentrations of 100 μM ($P < 0.01$). Concentrations of 100 μM dephostatin and Et-3,4-dephostatin reduced Na^+,K^+-ATPase activity to 10% and 65%, respectively. Pig kidney microsomal fractions had activities ranging 0.1–1.3 μmol Pi/mg protein/min. Similarly, both PTP inhibitors influenced purified Na^+,K^+-ATPase from pig kidney (Fig. 1b), in which dephostatin also possessed higher inhibitory potency in a concentration-dependent manner in experiments ranging from 30 min to 2 h. Purified Na^+,K^+-ATPase possessed activities of 2–3.4 μmol Pi/mg protein/min.

Activity Following Recovery Attempts

Meticulous attempts to recover Na^+,K^+-ATPase activity following dephostatin (Fig. 2a) and Et-3,4-dephsotatin (Fig. 2b) treatments did not seem to overcome the significant inhibitory effects of the two compounds. Moreover, both PTP inhibitors retained their Na^+,K^+-ATPase inhibitory effects in a concentration-dependent manner following recovery attempts compared to their controls (DMSO). Activity ranged 0.1–0.7 μmol Pi/mg protein/min. Microsomes were used in recovery experiments as the nature of the experiment necessitates the use of large amounts of proteins so that adequate amounts can be retrieved following multiple washes, centrifugations and homogenizations.

Na^+,K^+-ATPase Expression and Activity of Human Renal OS-RC-2 Cells

Western blots demonstrated the expression of Na^+,K^+-ATPase α_1- and β_1-subunits in both human renal OS-RC-2 cells and pig kidney microsomal fractions (Fig. 3a). On the

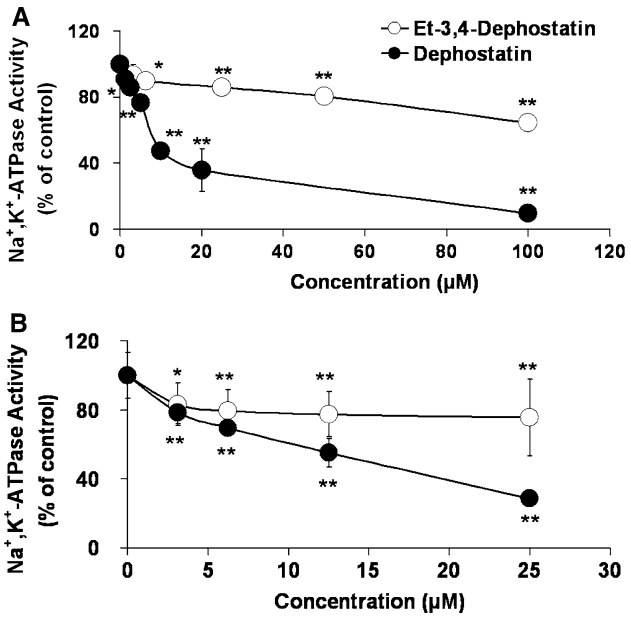


Fig. 1 Inhibitory effects of PTP inhibitors on Na^+,K^+-ATPase activity. Extracted pig kidney **a** microsomal fractions or **b** purified Na^+,K^+-ATPase were treated with different dephostatin (*closed symbols*) or Et-3,4-dephostatin (*open symbols*) concentrations. Changes in Na^+,K^+-ATPase activity compared to controls are presented as percentages of ATP hydrolysis. Data presented as the means of at least three independent experiments ± SD. ANOVA statistical analysis demonstrates statistical significance vs. control (* $P < 0.05$, ** $P < 0.01$)

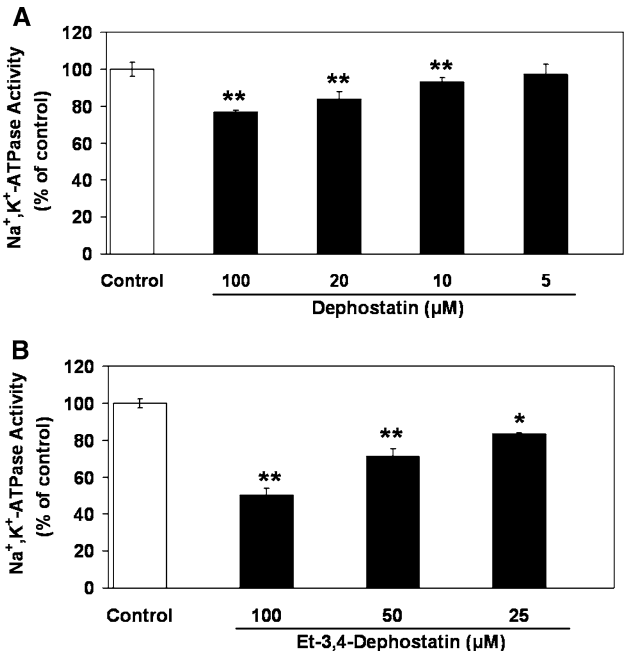


Fig. 2 Na^+,K^+-ATPase activity following recovery attempts. Pig kidney microsomal Na^+,K^+-ATPase activity following activity recovery attempts of **a** dephostatin or **b** Et-3,4-dephostatin treatments (*black bars*) versus controls (*white bars*) are presented. Bars represent the means of at least three independent experiments ± SD. ANOVA statistical analysis demonstrates statistical significance vs. control (* $P < 0.05$, ** $P < 0.01$)

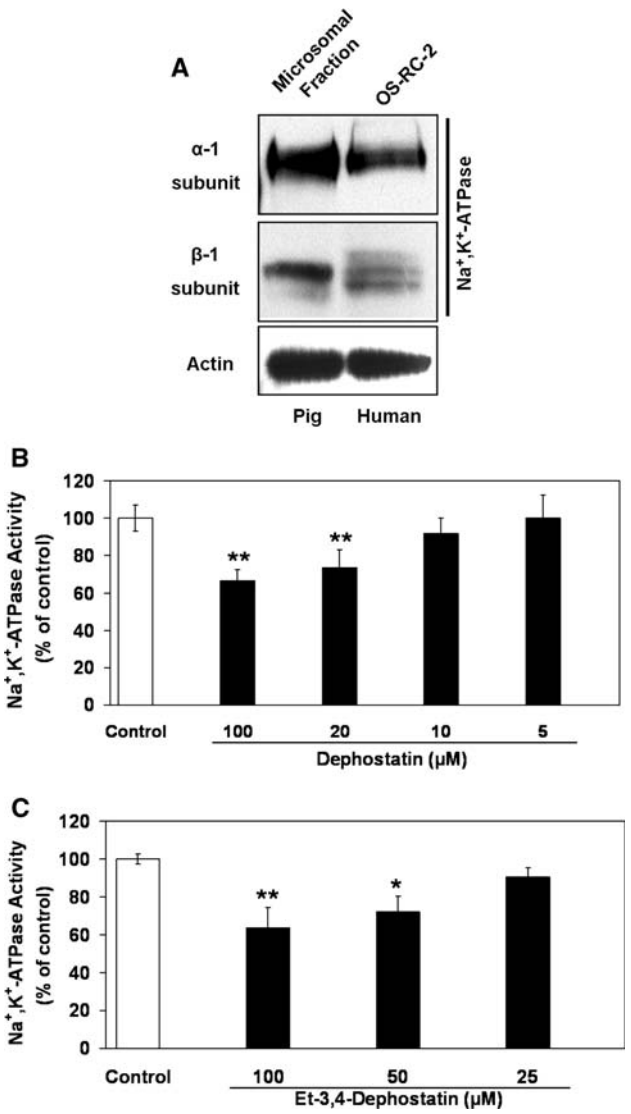


Fig. 3 Na^+,K^+-ATPase expression and activity in human renal OS-RC-2 cells. **a** Western blot of pig kidney microsomal fraction (*left lane*) and human OS-RC-2 cells (*right lane*) probed for Na^+,K^+-ATPase α_1-subunits (*upper*), β_1-subunits (*middle*) and actin (*lower*). Effects of PTP inhibitor, **b** dephostatin, and **c** Et-3,4-dephostatin treatments (*black bars*) vs. controls (*white bars*) are presented. Bars represent the means of at least three independent experiments ± SD. ANOVA statistical analysis demonstrates statistical significance vs. control (* $P < 0.05$, ** $P < 0.01$)

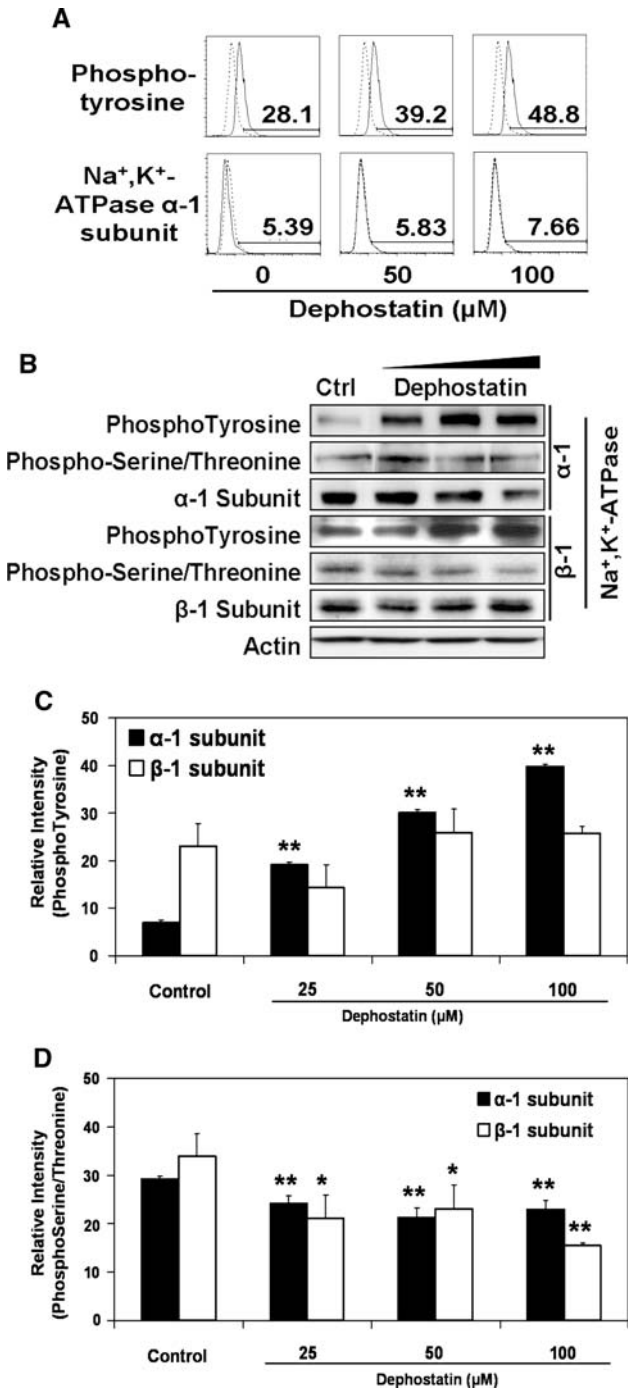
other hand, Na^+,K^+-ATPase α_2- and α_3-subunits were undetectable in both samples (data not shown). In accordance with the pig kidney extract data, human OS-RC-2 cells exhibited a concentration-dependent inhibition of Na^+,K^+-ATPase activity when treated with dephostatin (Fig. 3b) or Et-3,4-dephostatin (Fig. 3c). Moreover, the higher inhibitory potency of dephostatin over Et-3,4-dephostatin in pig kidney extracts was also detected in human OS-RC-2. OS-RC-2 cells exhibited activity of 0.1–3.9 μmol Pi/mg protein/min.

Na^+,K^+-ATPase Phosphorylation in Response to Dephostatin Treatment of Human OS-RC-2 Cells

FACS analysis of dephostatin-treated human OS-RC-2 cells (Fig. 4a) demonstrated an increased Na^+,K^+-ATPase α_1-subunit expression level; meanwhile, the overall cellular tyrosine phosphorylation of dephostatin-treated cells was increased in a concentration-dependent manner. Western blot experiments (Fig. 4b, c) further confirmed the dephostatin concentration-dependent increase in the Na^+,K^+-ATPase α_1-subunit tyrosine phosphorylation level. Meanwhile, the Na^+,K^+-ATPase β_1-subunit tyrosine phosphorylation level was slightly increased with dephostatin treatments (Fig. 4b, c). Conversely, Na^+,K^+-ATPase α_1- and β_1-subunits' serine/threonine phosphoryaltion levels were reduced upon dephostatin treatment (Fig. 4b, d). Despite the clear increase in Na^+,K^+-ATPase α_1-subunit tyrosine phosphorylation, this subunit tends to exhibit reduced expression with increasing dephostatin concentrations, while the Na^+,K^+-ATPase β_1-subunit expression levels seemed unaffected by similar dephostatin concentrations (Fig. 4b). Cells were utilized in FACS experiments to investigate the effects of PTP inhibitors on whole cells and where purified enzymes could not be used.

Discussion

The diverse physiological roles of Na^+,K^+-ATPase in various tissues render the study of its regulators a hot topic for scientists of many disciplines. Major attention was directed to hormonal regulation of Na^+,K^+-ATPase (Hernandez 1992; Doris and Bagrov 1998; Therien and Blostein 2000; Yu 2003), which in turn shed some light on the possible involvement of tyrosine phosphorylation along such hormonal regulation (Feraille et al. 1999; Yingst et al. 2000). PTP as calcineurin (Aperia et al. 1992; Mallick et al. 2000), protein phosphatase type 1 (PP1) (Ragolia et al. 1997) and PP2A (Blot-Chabaud et al. 1996; Lecuona et al. 2000, 2006) have been implicated in the dephosphorylation of the Na^+,K^+-ATPase α_1-subunit. On the other hand, all the reported PTP inhibitors including orthovanadate tend to have other effects, such as inhibiting alkaline phosphatase activity and inhibiting receptor internalization (Hunyady et al. 1991), along with their tyrosine phosphorylation inhibitory effects (Morioka et al. 1998). However, the presented PTP inhibitors (dephostatin and Et-3,4-dephostatin) in this study posses about 10-fold higher PTP inhibitory potency than the nonspecific PTP inhibitor sodium orthovanadate, through substrate-competitive inhibition, while also lacking any effect on serine/threonine phosphatases (Imoto et al. 1993; Umezawa et al. 2003). Moreover, dephostatin and its analogue demonstrated a



◀ **Fig. 4** Na^+,K^+-ATPase tyrosine phosphorylation in human renal OS-RC-2 cells. **a** Direct FACS analysis of OS-RC-2 cells stained for PE-conjugated phosphotyrosine (*upper panels*) and Alexa Fluor 488-conjugated Na^+,K^+-ATPase α_1 (*lower panels*). Plots of OS-RC-2 cells, untreated control (*dotted line*) vs. dephostatin-treated (*solid line*), are presented (0 μM dephostatin denotes vehicle [DMSO] treatments). **b** Western blots of control (DMSO) and dephostatin-treated OS-RC-2 cells probed for phosphotyrosine, phospho-serine/threonine, Na^+,K^+-ATPase α_1- and β_1-subunits and actin. Data demonstrate bands at the Na^+,K^+-ATPase α_1-subunit level (*panels 1–3 from top*), Na^+,K^+-ATPase β_1-subunit level (*panels 4–6 from top*) or actin (*lower panel*). Image analysis of phosphorylated **c** tyrosine or **d** serine/threonine at the Na^+,K^+-ATPase α_1- (*solid bars*) and β_1- (*white bars*) subunit levels in OS-RC-2 cells. All phosphorylated bands were normalized to their corresponding subunits and actin bands. Bars represent the means of at least three independent Western blotting experiments ± SD. ANOVA statistical analysis demonstrates statistical significance versus control (* $P < 0.05$, ** $P < 0.01$)

similar concentration-dependant inhibitory effect in both human and pig renal cells and extracts, which is contradictory to the reported variable vanadate effects on Na^+,K^+-ATPase, from stimulatory in intact cells to inhibitory in purified enzyme preparations (Feraille et al. 1997). Hence, the presented PTP inhibitors are of stable effect on both renal cells and extracts.

Renal pig enzyme extracts were more sensitive to dephostatin than Et-3,4-dephostatin. The presented Na^+,K^+-ATPase in human OS-RC-2 cells was similarly inhibited with both PTP inhibitors in a concentration-dependant manner (Fig. 3). While dephostatin has been reported to possess intrinsic lability and a shorter life in culture medium, the more stable Et-3,4-dephostatin is reported to specifically inhibit cytosolic PTP as PTP-1B and SHPTP-1 (Umezawa et al. 2003). The higher potency of dephostatin might suggest that renal Na^+,K^+-ATPase activity is regulated mainly via transmembrane PTP, rather than cytosolic PTP. The similarity of the human and pig renal extracts' response, despite the species difference, can be attributed to the almost equal coexistence of both Na^+,K^+-ATPase α_1- and β_1-subunits in the two species (Fig. 3a) and the lack of both α_2- and α_3-subunits. The increase in α_1-subunit expression in FACS experiments (Fig. 4a) can be attributed to the shorter intervals of treatment in those experiments, as well as the nature of such experiments, which mainly reflect cell-surface fluorescence; thus, it can be interpreted in terms of the initial response to PTP inhibition that dephostatin increases α_1-subunit presentation on the cellular surface, either by translocation from intracellular compartments or by increased expression. Moreover, even with cell-penetrating antibodies, cytosolic Na^+,K^+-ATPase might be of micelle arrangement, concealing internalized subunits from interacting with antibodies; hence, an overall increase in α_1-subunit cellular content would be inaccurate. On the other hand, the reduced α_1-subunits with higher dephostatin concentrations in Western blotting experiments (Fig. 4b) reflect the overall cellular content of those subunits rather than just the membranous fraction.

The difference in potency of dephostatin and Et-3,4-dephostatin prior to and after recovery experiments (Figs. 1a, 2) might be related to the tyrosine phosphorylation site and that such a site might rather be multiple sites, in which environmental changes in relation to the nature of the experiment might occur. The environment of the tyrosine phosphorylation site affected by dephostatin or Et-3,4-dephostatin might be different; hence, the washing

steps, centrifugation and homogenization incorporated in recovery experiments might result in different responses for the two compounds. Such a difference in response to recovery experiments might also be due to differences in the properties of the two compounds as they have inherent differences in stability and specificity. Future research would help in clarification.

Protein kinases A and C (PKA, PKC) have been reported to phosphorylate Na^+,K^+-ATPase on the serine/threonine residues (Bertorello et al. 1991; Chibalin et al. 1992; Beguin et al. 1994; Fisone et al. 1994), yet failure to fully suppress the α-subunit's basal phosphorylation level with removal of the identified phosphorylation sites (Beguin et al. 1994) or their blockage with specific inhibitors (Beguin et al. 1994; Carranza et al. 1996a, b) led to the search for other regulatory phosphorylation sites. Insulin-induced phosphorylation of the Na^+,K^+-ATPase α_1-subunit on tyrosine residues was hence reported as a possible alternative phosphorylation site of the α_1-subunit, which represented 10–25% of the unstimulated/stimulated studied cells (Feraille et al. 1999). Concordant with those reports, our results demonstrate a concentration-dependent decrease in Na^+,K^+-ATPase activity in all our human and pig cellular extracts and purified enzyme samples, which was similarly parallel to an increase in the Na^+,K^+-ATPase α_1-subunits' tyrosine phosphorylation level (Fig. 4). This led us to suggest a PTP regulatory role on Na^+,K^+-ATPase activity through tyrosine phosphorylation of primarily its α_1-subunit and a possible lower-profile role of its β_1-subunit. Adversely, our data point to a much higher percentage of α_1-subunit tyrosine phosphorylation, where the reduced α_1-subunits at high dephostatin concentrations presented even higher tyrosine phosphorylation levels (Fig. 4). Moreover, the PTP-specific inhibitors (dephostatin and Et-3,4-dephostatin) predominantly increased the tyrosine, but not the serine/threonine, phosphorylation levels of both α_1 and β_1 Na^+,K^+-ATPase subunits (Fig. 4), which is in agreement with the inherent properties of those compounds (Imoto et al. 1993; Umezawa et al. 2003). The dephostatin effects are in opposition to those reported by Wang and Yu (2005) in cortical neurons; however, the two studies are in agreement that regulation by tyrosine phosphorylation may cause a variety of Na^+,K^+-ATPase response patterns in different tissue types, some stimulatory and some inhibitory. Different regulations of the pump activity might be attributed to the tyrosine phosphorylation of a different residue or different subunit (α_1 in the current study and α_3 in theirs), along with the difference in both the tissues and the species studied, which might lead to an opposite effect on the activity of Na^+,K^+-ATPase. Reports covering the contrasting roles (stimulation/inhibition) of Na^+,K^+-ATPase phosphorylation are numerous. While some reports have demonstrated an insulin-induced increase in Na^+,K^+-ATPase α-subunit phosphorylation in the proximal tubules (Feraille et al. 1999), others have reported dephosphorylation in the skeletal muscles (Ragolia et al. 1997). The reported tyrosine phosphorylation implicated in angiotensin II effects on adrenal capsules of rats was thought to be through either the Na^+ pump itself, a protein that interacts with it or part of the signaling pathway (Yingst et al. 2000). Our study demonstrates tyrosine phosphorylation-related regulatory mechanisms, which are independent of serine/threonine phosphorylation; and we further demonstrate tyrosine phosphorylation of Na^+,K^+-ATPase subunits. Similarly, Yingst et al. (2000) reported unaffected angiotensin II Na^+,K^+-ATPase inhibition by PKC inhibitors and serine/threonine phosphatase inhibitors, contrary to other reports of phosphorylative effects of PKA and PKC (Bertorello et al. 1991; Chibalin et al. 1992; Beguin et al. 1994; Fisone et al. 1994). Our findings suggest that the presented dephostatin and analogue are rather tyrosine phosphorylation-targeted regulators of Na^+,K^+-ATPase activity and that the regulatory role of the tyrosine-phosphorylated residues on Na^+,K^+-ATPase is independent of the regulation through serine/threonine phosphorylation. The data also hint at the involvement of β_1-subunits in activity regulation of Na^+,K^+-ATPase, which requires additional research to clarify. In summary, we present dephostatin and the more stable Et-3,4-dephostatin as tyrosine phosphorylation-mediated regulators of Na^+,K^+-ATPase activity.

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